

**VALORIZATION OF RICE HUSK FOR CITRIC ACID
PRODUCTION USING ASPERGILLUS NIGER BY SOLID
STATE FERMENTATION**

BY

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**A DISSERTATION SUBMITTED TO THE SCHOOL OF
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**DEPARTMENT OF BIOCHEMISTRY,
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ZARIA, NIGERIA**

OCTOBER, 2017

DECLARATION

I declare that the work in this dissertation entitled **VALORIZATION OF RICE HUSK FOR CITRIC ACID PRODUCTION USING ASPERGILLUS NIGER IN SOLID STATE FERMENTATION** has been carried out by me in the Department of Biochemistry, Faculty of Life Sciences, Ahmadu Bello University, Zaria under the supervision of Dr. A.B. Sallau and Prof S. Ibrahim. The information derived from the literature has been duly acknowledged in the text and the list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other university.

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Name of student

Signature

Date

CERTIFICATION

This dissertation entitled **VALORIZATION OF RICE HUSK FOR CITRIC ACID PRODUCTION USING ASPERGILLUS NIGER IN SOLID STATE FERMENTATION** by Jamila TANIMU meets the regulations governing the award of the degree of Master of Science of the Ahmadu Bello University, Zaria, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This dissertation is dedicated to my parents Alhaji Tanimu Uthman and Hajiya Aisha. Your love has being my driving force up till now, and to my children Ameerah, Humairah, Muhammad and Ahmad.

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ABSTRACT

The utilization of agro-residues as substrate to generate new products of commercial interest through a bioprocess is considered as an important strategy for the development of sustainable technologies. Citric acid production was carried out from various pretreated (acid, alkaline, hydrogen peroxide and un-pretreated) rice husk by solid state fermentation (SSF) using *Aspergillus niger*. Pyridine-acetic acid anhydride method was used for quantitative analysis of the citric acid produced. Plackett Burman Design (PBD) was used to screen ten media components ((NH₄)₂SO₄, MgSO₄.7H₂O, NH₄NO₃, KH₂PO₄, FeCl₃.6H₂O, CuSO₄, FeSO₄, ZnSO₄, MnSO₄, and Molasses) required by *Aspergillus niger* (*A. niger*) to enhance citric acid production. Face Centered Central Composite Design (FCCCD) was used to select the most contributing nutrients for the production. Thin layer chromatography, Fourier Transform Infra-Red (FTIR) analysis and melting point determination of both standard and citric acid produced were also carried out. The result obtained from PBD indicated that hydrogen peroxide pretreated rice husk was the best pretreatment method with citric acid yield of 3.65 ± 0.26 g/kg RH which is statistically higher ($p < 0.05$) than that of acid, alkaline and un-pretreated rice husk whose yields were 2.94 ± 0.83 , 1.97 ± 1.24 and 2.34 ± 0.13 g/kg RH, respectively. Among the media components NH₄NO₃, KH₂PO₄, and molasses were found to be vital in citric acid production using rice husk as a substrate with the optimum concentrations of 2.5g/L, 3.25g/L and 0.2275g/L, respectively with citric acid yield of 4.765 ± 0.001 . The coefficient of determination "R²" was found to be 0.9790 and is in reasonable agreement with the "Adjusted R²" of 0.9601 at $p < 0.05$. Both the standard and *A. niger* produced citric acid gave similar R_f value of 0.242 and melting point of 153 ± 0.00 °C and 153 ± 0.02 °C, respectively, wavenumbers corresponding to O-H and C=O of 3338.7 cm^{-1} and 1565.5 cm^{-1} and 3324.8 cm^{-1} and 1625.1 cm^{-1}

for standard and citric acid produced, respectively. The result of this study has therefore, revealed the potential of rice husk to produce citric acid with similar characteristic to that of standard citric acid. It also showed the importance of using a statistical design like Face Centred Composite Design (FCCCD) of Response surface methodology (RSM) to optimize the production of citric acid using rice husk as a substrate by *A niger* as it is proved to be a useful tool in establishing optimum conditions for citric acid production.

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T OF ABBREVIATIONS

ACO	Aconitase.
ANOVA	Analysis of Variance
BAW	Butanol Acetic Acid and Water
FCCCD	Faced Centered Central Composite Design
RSM	Response Surface Methodology
GRAS	generally recognized as safe
FTIR	Fourier Transform Infra-Red
SSF	Solid State Fermentation
PFK	Phosphofructokinase
PC	Pyruvate carboxylase
Rf	Retention factor
SPSS	Statistic package for social sciences
FAO	Food and agricultural Organization
RH	Rice Husk
SD	Standard Deviation
TLC	Thin Layer Chromatography

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of Study

The amount of wastes produced from agro-processing industries is continuously increasing and poses serious environmental problems. This reason leads to many research groups to be investigating the opportunities for the valorization of such residue (Tobias *et. al.*, 2014).

Agricultural residue is any unwanted or unsalable material produced wholly from agricultural operations which is directly related to the growing of crops or raising of animals for the primary purpose of making a profit or for a livelihood (Tobias *et. al.*, 2014). The term “agricultural residue” is used to describe all the organic materials which are produced as by-products from harvesting and processing of agricultural crops. Agricultural residues, which are generated in the field at the time of harvest, are known as primary or field based residues whereas those that are co-produced during processing are called secondary or processing based residues (Zafar, 2013). Many agricultural residues such as apple pomace (Hang and Woodams, 1984), kiwifruit peel (Hang and Woodams, 1987), orange waste (Aravantinos-Zafiridis *et. al.*, 1994), sugar cane bagasse (Shankaranand and Lonsane, 1993), coffee husk (Shankaranand and Lonsane, 1994), carrot waste (Garg and Hang, 1995), pineapple waste (Tran *et. al.*, 1998), cassava bagasse, (Vandenberghe *et. al.*, 1999), pumpkin (Majumdar, *et. al.*, 2010), date syrup (Mostafa and Alamri, 2012), rice straw (Ali *et. al.*, 2012), and oat bran (Raja and Kruthi 2013) were tried successfully as substrates for citric acid formation and under optimum conditions are known to produce good quantities of citric acid.

Rice husk (or hull) is the outermost layer of the paddy grain that is separated from the rice grains during the milling process (Alaric 2013). Rice husk is one of the most widely available agricultural

residues in many rice producing countries around the world (Ajay *et al.*, 2012). It has potential application in different field of endeavour which includes; preparation of activated carbons, insulating board material, chopstick production, fuel in power plant, source of silica and silicon compound, etc. (Ajay *et al.*, 2012). The global rice production in **2015/2016** was 472.04 million tons by the United States Department of Agriculture (**USDA**), and estimates that the **world rice production 2016/2017** will be 480.13 million metric tons. The estimated 480.13 million tons could represent an increase of 8.09 million tons or a 1.71% in **rice production** around the globe. Paddy, (rice before processing) on an average, consists of about 72 percent of rice, 5-8 percent of bran, and 20-22 percent of husk (Muhammad, 2015). In Nigeria the Federal Ministry of Agriculture and Rural Development (April, 2017) reported that in 2016, total paddy production estimate is put at 17.5 million tons equivalent to 5.7 million tons milled rice.

Citric acid (2- hydroxypropane-1, 2, 3-tricarboxylic acid), is an essential multifunctional weak organic acid, which has wide range of household and industrial applications (Selvankumar, 2014). It is a common metabolite of plants and animals. It exists widely in nature and is one of the organic acids found in juice of lemon, orange, pineapple, plum, peas, and peach. It is also found in animal bones, muscles and blood (Pratiti, 2013). Microbial citric acid is globally used in most of the food and pharmaceutical industries because of its ecofriendliness, biodegradability, and cost-effectiveness (Angumeenal and Venkappayya, 2013). It has many applications in food, pharmaceutical and cosmetic industries as an acidulant, flavour enhancer, preservative, antioxidant, emulsifier and chelating agent (Soccol *et al.*, 2006). Citric acid is mainly used in food industry because of its pleasant acid taste and its high solubility in water. It is worldwide accepted as “GRAS” (generally recognized as safe), based on approval by the Joint FAO/WHO Expert Committee on Food Additives (Pandey *et al.*, 2001).Global citric acid production at

industrial-scale processes was reported to be 1.7×10^6 tons annually (Dhillon *et al.*, 2011), and its demand is continuously increasing.

Several microorganisms belonging to the genera *Aspergillus* sp., *Bacillus* sp., *Penicillium* sp., *Candida* sp., *Arthrobacter* sp., *Corynebacterium* sp., have been reported as efficient citric acid producers (Grewal and Kalra, 1995; Schuster *et al.*, 2002 and Betiku *et al.*, 2013). Fungus *Aspergillus niger* has received much attention recently as a potential candidate for large-scale production of citric acid. The rapid growth rate, ability to utilize wide substrates as carbon and nitrogen source, high yield and secretion of different metabolites enhanced the application of *Aspergillus niger* in fermentation studies (Selvankumar *et al.*, 2014).

The primary uses of *Aspergillus niger* are for the production of enzymes and organic acids such as citric acid and gluconic acid by fermentation. Perhaps the largest application of *Aspergillus niger* is described as the major source of citric acid as this organism accounts for over 99% of global citric acid production (Mohsen and Alireza, 2013).

Studies by Micheal *et al.*, (2013), revealed that all the *Aspergillus niger* strains studied have the potential for citric acid production. Citric acid productivity further increased after strain improvement studies.

Solid state fermentation (SSF) is a fermentation process which is carried out in solid matrix with limited volume of water. Microorganisms especially fungi, can favourably grow in the solid substrate as a carbon source and growth carrier. Solid state fermentation is economical, low water consuming and eco-friendly. It plays an important role and has a great perspective for bioconversion of different agro-industrial residues (Selvankumar *et al.*, 2014).

1.2 Statement of Research Problem of the Study

Most of the citric acid that is being used in this country today is imported (Betiku *et al.*, 2013), and therefore there is a need for indigenously produced citric acid for domestic and industrial use.

The global demand for citric acid is growing faster than its production, which implies that more economical process is required (Adeoye *et. al.*, 2015). Many residues of agriculture and agro industries can be used for production of citric acid (Pandey *et. al.*, 2000). Therefore, reduction in production cost can be achieved by using less expensive substrates. In addition, the use of agro industrial residues as support materials in fermentation processes is not only economically important, but will also minimize environmental problems arising from improper disposal and handling of agro wastes (Adeoye *et. al.*, 2015).

The current method of producing citric acid via fermentation requires the use of glucose and other soluble sugars which are costly, hence there is need to explore relatively low cost raw material for citric acid production.

1.3 Justification of the Study

Both the continuous growing demand for citric acid and the economics of fermentation encourage the exploration of different technical approaches to use cheap raw materials for citric acid production (Parekh *et. al.*, 2000; Haq *et. al.*, 2003; Husseiny *et. al.*, 2010).

Non-conventional raw materials like rice husk could be used for production of several value added products including citric acid.

This research could serve as source of effective utilization of low cost raw materials (rice husk) as well as using non-conventional substrate for citric acid production.

1.4 Aim of the Study

The aim of this work is to produce citric acid from rice husk as a carbon source using *Aspergillus niger* by solid state fermentation under optimized media conditions.

1.5 Objectives of the study

- I. To determine the best pretreatment method(s) for rice husk to be used as carbon source for the production of citric acid.
- II. To optimize the medium components required for citric acid production by *Aspergillus niger* using rice husk as a carbon source.
- III. To characterize the citric acid produced from rice husk.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Chemistry and Occurrence of Citric Acid

Citric acid or 2-hydroxypropane 1, 2, 3-tricarboxylic acid is an alpha-hydroxyl acid with a three carbon skeleton, which has three carboxylic acid groups (COOH), and one hydroxyl group (Max, *et al.*, 2010), with molecular formula of $C_6H_8O_7$ and molar mass of 192.12 g/mol., it's also known as *p*-hydroxyl tricarboxylic acid is a weak organic acid occurring in high concentrations in citrus fruits (Anastassiadis and Rehm, 2006). It is ubiquitous in nature as it serves as an intermediate in citric acid cycle, where by carbohydrates are oxidized to CO_2 . The widespread presence of citric acid in animal and plant kingdom is an assurance of its non- toxic nature and it has been used as an acidulant in manufacture of soft drinks, jams and confectioneries (Anastassiadis and Rehm, 2006). Citric acid is found as colorless translucent crystals, odorless, with strongly acid taste. The solid has density of 1.66 g/mL, melting point of $153^\circ C$ and boiling point of $175^\circ C$. It is highly soluble in water to give an acidic, sour tasting solution (Pratiti, 2013). Citric acid is found in large quantities in citrus fruits with lime having the highest concentration of the acid (Pratiti, 2013). In addition to fruits, citric acid is found in all animal species. The citric acid cycle is vital in the oxidation of sugars and acetate to CO_2 and water, releasing energy for physiological functions (Pratiti, 2013). The chemical structure of citric acid is presented in Figure 2.1

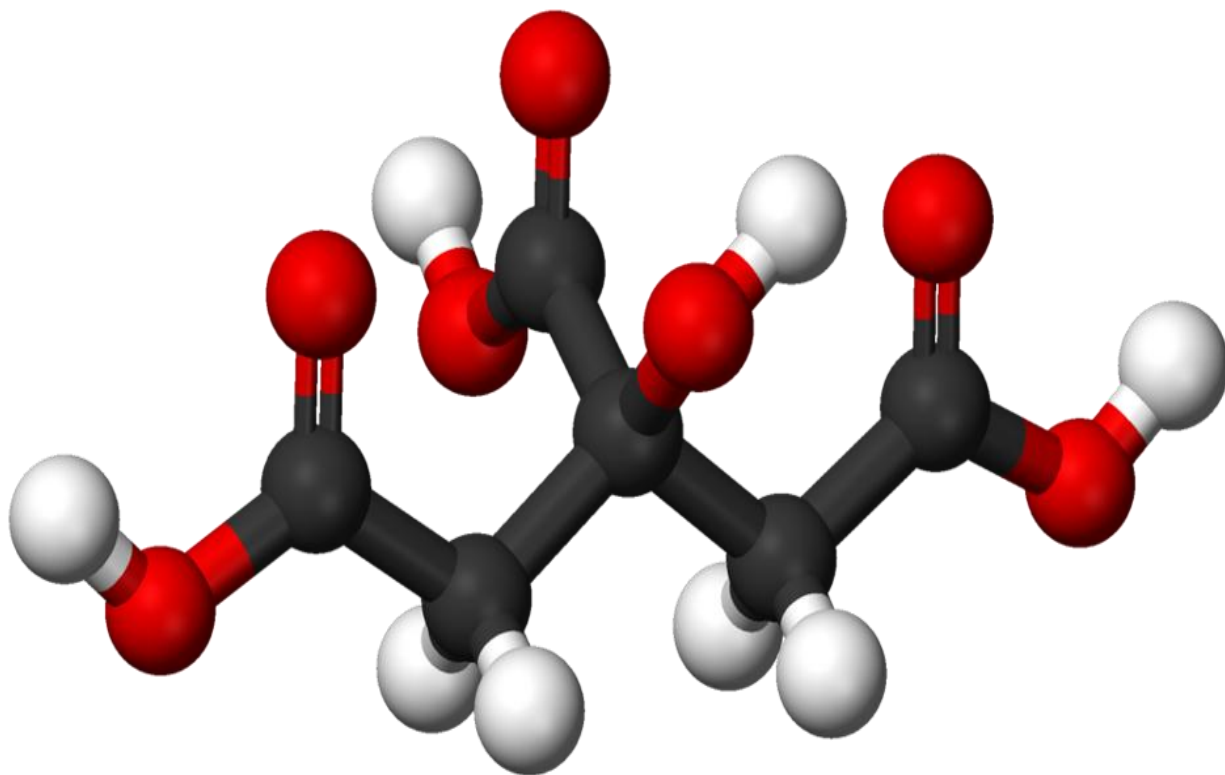


Figure 2.1: Chemical Structure of Citric Acid ([www. pubchem.ncbi.nlm.nih.gov/compound/citric acids](http://www.pubchem.ncbi.nlm.nih.gov/compound/citric%20acids))

Among the different organic acids, citric acid is used massively worldwide because of its low toxicity when compared with other acidulant (Radwan *et. al.*, 2010). In 2008, the reported estimated world production of citric acid was over 1.6 million tons and this trend is expected to increase in future (Husseiny *et. al.*, 2010).

Citric acid is used by the beverage industry as a flavoring and preservative agent (Jianlong, 2000). In the field of pharmaceutical sciences, it is used with bicarbonate to produce carbon dioxide in cosmetics and effervescent medicines. The cleansing characteristics of citric acid make it ideal for use in industrial cleaners (Dhillon *et. al.*, 2011). As a result of the great world demand for citric acid, there has been an increasing trend toward efficient use of agro industrial residues and by-products in its production (Husseiny *et. al.*, 2010).

The production of citric acid using agro industrial residues and by-products offers the important combined advantage of waste material management and reduced cost (Khosravi-Darani *et. al.*, 2008; Husseiny *et. al.*, 2010). There is a very limited natural supply of citric acid and the high demand for citric acid can be achieved only by biotechnological fermentation processes. It was reported that over 90% of the world production of citric acid is obtained by fermentation (Khosravi-Darani *et. al.*, 2008).

Many microorganisms can be used to produce citric acid; however, *Aspergillus niger* remains the main industrial producer (Alagarsamy and Nallusamy, 2010). The main benefits of using *Aspergillus niger* are its ease of handling, its ability to ferment a wide range of inexpensive raw materials and high yields (Alagarsamy and Nallusamy, 2010). The industrial production of citric acid can be carried out in three different fermentation methods; i.e. submerged fermentation, surface fermentation, and solid-state fermentation (Khosravi-Darani *et al.*, 2008). However,

these classical fermentation methods are facing various constraints such as low cell density, nutritional limitations and batch-mode operations with high down time (Al-Shehri and Mostafa, 2006).

2.2. History and Discovery of Citric Acid

Citric acid was first commercially-produced in England around 1826 from imported Italian lemons (lemons contain 7-9% citric acid). Lemon juice remained the commercial source of citric acid until 1919, when the first industrial process using *Aspergillus niger* started in Belgium. Citric acid was synthesized from glycerol by Grimoux and Adams (Max *et al.*, 2010) and later from symmetrical dichloroacetone. Other routes have been published from various synthetic materials since then, but chemical methods have so far proved uncompetitive. Currie (1917) found that some strains of *A. niger* were able to grow in a medium containing sugars and salts at an initial pH of 2.5-3.5. Throughout their growth, these strains excreted large amounts of citric acid, which established the basis for industrial production (Max *et al.*, 2010). In this production technique, which is still the major industrial route of citric acid production today, culture of *Aspergillus niger* are fed on a sucrose or glucose-containing medium to produce citric acid (Max *et al.*, 2010). Despite a long and successful history of producing citric acid, there is no unanimous explanation of the biochemical basis of the process (Soccol *et al.*, 2006).

2.3 Raw Materials (Substrates) for Citric Acid Production

Several raw materials such as hydrocarbons, starchy materials and molasses, have been employed as substrates for commercial submerged citric acid production (Grewal and Kalra, 1995), although citric acid is mostly produced from starch or sucrose based medium using submerged fermentation. Generally, citric acid is produced by fermentation using inexpensive

raw material including crude natural products, such as hydrolysate starch, sugar cane broth and by-products like sugar cane and beet molasses (Yokoya, 1992). Molasses is preferably used as the source of sugar for microbial production of citric acid due to its relatively low cost and high sugar content (40–55 %) (Grewal and Kalra, 1995). Several agro-industrial residues and by-products have also been investigated with solid-state fermentation techniques for their potential to be used as substrates for citric acid production such as cassava bagasse, coffee husk, wheat bran, apple pomace, pineapple waste, kiwi fruit peel, grape pomace, citrus waste, *etc.* (Vandenberghé *et al.*, 1999). It has been an increasing trend towards efficient utilization of and value-addition to these residues, apart from being a form of reducing environmental problems. These residues are very well adapted to solid-state cultures due to their cellulosic and starchy nature. A cost reduction in citric acid production can be achieved by using less expensive substrates, such as waste products mentioned (Soccol *et al.*, 2006).

2.4 Biochemistry of Citric Acid Production

Over production of citric acid requires a unique combination of unusual nutritional conditions (excess of carbon source, hydrogen ions and dissolved oxygen and suboptimal concentrations of some trace metals and phosphate), which synergistically influence the fermentation performance (Kristiansen and Sinclair, 1978). According to Rohr and Kubicek, (1981) and Habison *et al.*, (1983), a deficiency of manganese, or phosphate and nitrogen limitation, inhibits the *A. niger* anabolism, and the resulting degradation of proteins lead to increased ammonium ion concentration. This increase is able to counterbalance the inhibition exerted by citric acid on phosphofructokinase (Habison *et al.*, 1983), being a positive end-effector (Arts *et al.*, 1987). High concentrations of NH_4^+ and glucose also repress the synthesis of α -ketoglutarate

dehydrogenase, inhibiting the citric acid catabolism via the Krebs cycle, leading to its accumulation (Rohr and Kubicek, 1981). An important aspect concerns the need that the Krebs cycle can be completed to support the continuous production of citric acid. Thus, in order to address the lack of cycle intermediates consequent to the metabolic dysfunction responsible for the accumulation of citric acid (Kubicek, and Rohr 1980), pyruvic acid produced from glucose is not only decarboxylated to acetyl-CoA by the pyruvate dehydrogenase complex, but it is also partially carboxylated to oxaloacetic acid during the idiophase by the action of pyruvate carboxylase (Woronick *et. al.*, 1960; Wongchai and Jefferson, 1974). This reaction, which requires ATP and is dependent on K^+ and Mg^{2+} , is not the only anaplerotic reaction used to replenish the Krebs cycle. Depending on the organism, more oxaloacetic acid can be produced from phosphoenolpyruvic acid and CO_2 by phosphoenolpyruvate carboxykinase, phosphoenolpyruvate carboxytransphosphorylase, and phosphoenolpyruvate carboxylase, and from pyruvic acid by the NADPH-dependent malic enzyme (Marzona, 1996).

It has been proposed that the accumulation of citric acid requires deactivation of the Krebs cycle enzymes responsible for its degradation, aconitase and/or isocitrate dehydrogenase, but there are evidences that during the production of citric acid, the Krebs cycle is active in the production of intermediates required for biomass formation (Jernejc *et. al.*, 1992). Therefore, as stressed by Kubicek, (2001), citric acid accumulation may likely be the result of enhanced (deregulated) biosynthesis rather than inhibited degradation. Another alternative hypothesis to explain the accumulation of citric acid is associated to tricarboxylate transporter activity (Kubicek, 1987), which competes with aconitase for citric acid. Under conditions in which its affinity for citric acid is greater than that of aconitase, this enzyme ejects citric acid out of the mitochondria without inhibition of enzymes of the cycle. Finally, there are three different isozymes of

isocitrate dehydrogenase in *A. niger*, a NAD^+ -dependent form present in mitochondria at low concentrations, and two NADP^+ -dependent forms, one in the mitochondria and the other in the cytoplasm (LaNauze, 1966; Kubicek, and Röhr, 1977). All the enzymes require Mg^{2+} or Mn^{2+} and the NADP^+ -dependent form is inhibited by citric and α -ketoglutaric acids (Marzona, 1996), which ensures the accumulation of citric acid.

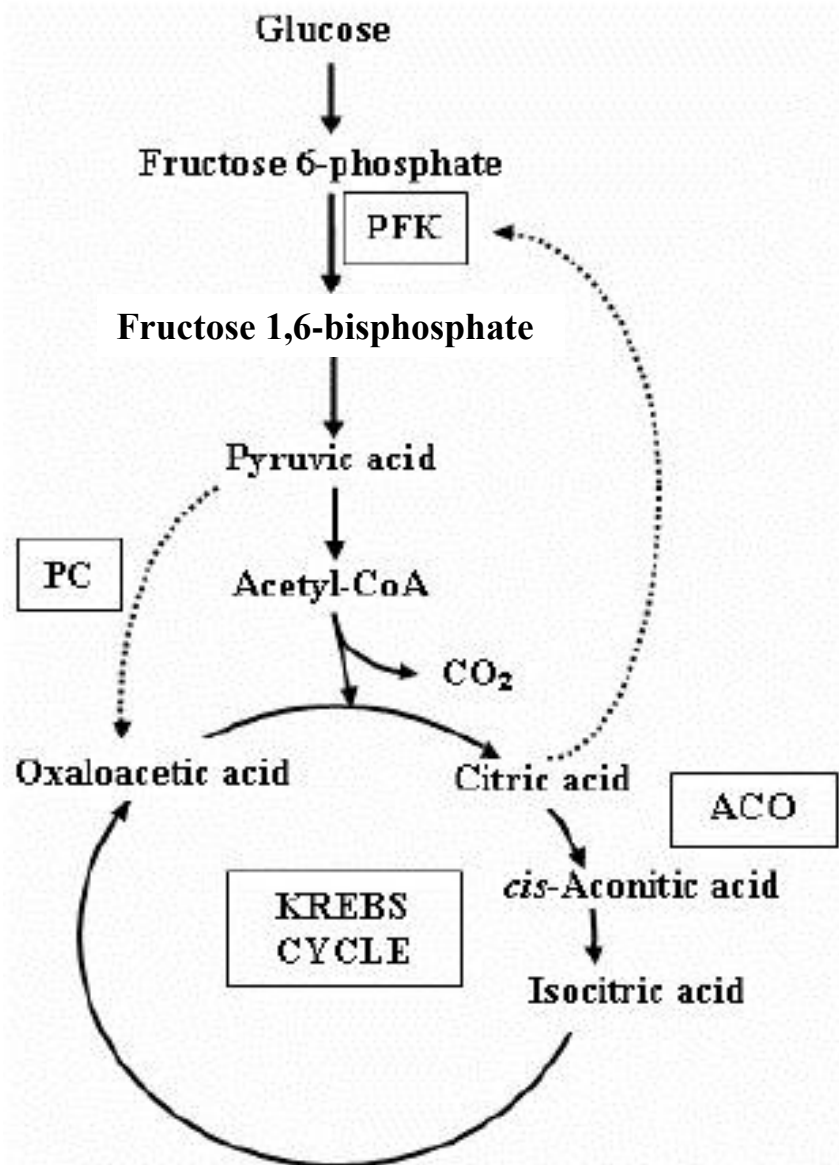


Figure 2.2: Schematic Representation of the Main Metabolic Reactions Involved in the Production of Citric Acid by *A. niger* (Manzoni, 2006).

Key;

PFK = phosphofructokinase, PC = pyruvate carboxylase, ACO = aconitase

2.5 Citric Acid Production Techniques

Citric acid production by fermentation is the most economical and widely used way of obtaining this product. More than 90 % of the citric acid produced in the world is obtained by fermentation, which has its own advantages: operations are simple and stable, the plant is generally less complicated and needs less sophisticated control systems, while technical skills required are lower, energy consumption is lower and frequent power failures do not critically affect the functioning of the plant.

Citric acid production by fermentation can be divided in three phases, which include preparation and inoculation of the raw material, fermentation, and recovery of the product. The industrial citric acid production can be carried in three different ways; by submerged fermentation, surface fermentation and solid-state fermentation or Koji process (Yokoya, 1992). All of these methods require raw materials and inoculum preparation. In industrial citric fermentation, the large-scale spore production is made by using appropriate means and conditions such as direct inoculation in the production fermenter. Sometimes it is necessary to remove the remaining mineral of the raw material and add other nutrients such as phosphorus, magnesium and nitrogen for development of the mycelium and good production of the citric acid. Several types of fermenters have been used for citric acid production in solid-state fermentation such as Erlenmeyer conical flasks, glass incubators, trays, rotating and horizontal drum bioreactors, packed-bed column bioreactor, single-layer packed-bed, multi-layer packed-bed, *etc.* (Grewal and Kalra, 1995; Vandenberghe *et al.*, 1999; Pandey *et al.*, 2000).

2.6 Factors Affecting Citric Acid Production

Citric acid accumulation is strongly influenced by the type and concentration of carbon source (Soccol *et al.*, 2006) and other component of the medium. The presence of carbohydrates which are rapidly taken up by microorganisms has been found to be essential for a good production of citric acid (Yokoya, 1992). The accumulation of citric acid is strongly influenced by the composition of the medium, especially in submerged fermentation processes. It was found that the factors mainly affecting the citric fermentation are the type and concentration of carbon source, nitrogen and phosphate limitation, pH, aeration, oligoelements concentration, and morphology of the producing microorganism. Certain nutrients have to be in excess (such as sugars, protons or oxygen), others at limiting levels (such as nitrogen and phosphate) and others below well-established threshold values (such as trace metals, particularly manganese) (Max *et al.*, 2010).

2.6.1 Carbon source

The concentration of carbon source is very crucial for citric acid fermentation. The final yield of citric acid increases with initial sugar concentration in batch processes or glucose feeding rate in chemostat, while the specific growth rate has an opposite behaviour (Papagianni *et al.*, 1999). The highest productivities are usually achieved using 14 - 22% sugar, because such high concentrations of the carbon source lead to suppression of α -ketoglutarate dehydrogenase (Hossain *et al.*, 1984).

2.6.2 Nitrogen and phosphate limitations

Some complex media (such as molasses) are rich in nitrogen and rarely need to be supplemented with a nitrogen source. The highly-pure media used in laboratory scale-research are usually

supplemented with ammonium salts, particularly ammonium nitrate and sulfate, which in turn leads to a decrease in pH that favors fermentation (Mattey *et. al.*, 1992).

2.6.3 pH of culture medium

The pH of the medium is important in two stages of the process. All fermentations start from spores and their germination requires $\text{pH} > 5$. The absorption of ammonia by germinating spores causes release of protons, thus lowering the pH and improving the production of citric acid. The low pH value during the production phase ($\text{pH} \leq 2$) reduces the risk of contamination by other microorganisms and inhibits the production of unwanted organic acids (gluconic and oxalic acids), which makes the product recovery easier (Max *et al.*, 2010).

2.6.4 Trace elements

Aspergillus niger requires certain trace metals for growth (Mattey *et. al.*, 1992). However, a limitation by other trace elements is necessary for citric acid production (Max *et. al.*, 2010), especially in the submerged fermentation. The metals that should be in limiting concentrations are Zn, Mn, Fe, Cu and heavy metals. Manganese has also been proven to be important in many cell functions, particularly in the cell wall synthesis, sporulation and production of secondary metabolites (Max *et. al.*, 2010).

2.7 Citric Acid Product Recovery

The recovery of citric acid from liquid fermentation is generally accomplished by three basic procedures: precipitation, extraction, and adsorption and absorption (mainly using ion exchange

resins). Citric acid obtained using extraction method has been recommended suitable for use in food and drugs by the United State (US) Food and Drug Administration (1975). Precipitation is the classical method and it is performed by the addition of calcium oxide hydrate (milk of lime) to form the slightly soluble tri-calcium citrate tetrahydrate. The precipitated tri calcium citrate is removed by filtration and washed several times with water. It is then treated with sulphuric acid to form calcium sulphate, which is filtered off. Mother liquor containing citric acid is treated with active carbon and passed through cation and anion exchangers (Vandenberghe *et al.*, 1999). Several anion-exchange resins are commercially available. Finally, the liquor is concentrated in vacuum crystallizers at 20 – 25 °C, forming citric acid monohydrate. Crystallization at temperatures higher to this is used to prepare anhydrous citric acid (Vandenberghe *et al.*, 1999).

2.8 Uses and Importance of Citric Acid

Citric acid has many uses. In beverages, it is used as flavor enhancer and as preservative. It has been used most frequently, and for hundreds of years, as a preservative for meat, it increases the acidity of the bacterial environment that may develop, making it difficult for the bacteria to survive (Vandenberghe *et al.*, 1999). It eliminates haze due to trace metals, prevents color and flavor deterioration. It prevents turbidity in wine and when used in wines; it eliminates low acidity and improves taste. When used in beers, it reduces sugar loss and helps the process of turning sugar into beer. Citric acid inhibits oxidation and adjusts pH. In soft drinks, it gives the cool taste maintaining carbonation. In food and candy, it inverts sucrose, prevents oxidation and produces darker color in candies, jams and jellies. Citric acid neutralizes residual dye in frozen fruits, protects ascorbic acid from oxidation, and inactivates trace metals and oxidative enzymes by lowering pH (Vandenberghe *et al.*, 1999). In dairy products it acts as an antioxidant and as emulsifier in cheese, cream etc. In pharmaceutical industry it acts as a solvent and flavoring

agent, producing effervescence when combined with bicarbonate (Vandenberghe *et al.*, 1999). Citric acid destroys bacteria, fungi, mold, and viruses, hence it is used in insecticides, fungicides, and disinfectants. Citric acid acts as antioxidant and as synergist in cosmetics. It is good for skin care, skin color, and mineral absorption. It is also used for treatment of boiler water, in metal plating, in detergents as builder, in tanning and in textiles. Due to its biodegradability, this organic acid has also found acceptance in detergent industry in place of phosphates and in the removal of sulfur in stack gases in power stations and elsewhere where sulfur is to be removed. (Soccol *et al.*, 2006)

The multifunctional uses of citric acid obligate its demand, which exceeds the production rate.

2.9 Microorganisms Used For Citric Acid Production

Wehmer was the first to demonstrate that *Citromyces* (now *Penicillium*) accumulated citric acid in a medium containing sugar and inorganic salts. Since then, many organisms have been found to accumulate citric acid: *A. niger*, *Aspergillus awamori*, *Aspergillus nidulans*, *Aspergillus fonsecaeus*, *Aspergillus luchensis*, *Aspergillus phoenicus*, *Aspergillus wentii*, *Aspergillus saitoi*, *Aspergillus flavus*, *Absidia* sp., *Acremonium* sp., *Botrytis* sp., *Eupenicillium* sp., *Mucor piriformis*, *Penicillium janthinellum*, *Penicillium restrictum*, *Talaromyces* sp., *Trichoderma viride* and *Ustilina vulgaris* (Papagianni, 2007). Besides fungi, it is known that several yeasts produce citric acid from *n*-alkanes and carbohydrates (Mattey, 1999), especially species belonging to the genera *Candida*, *Hansenula*, *Pichia*, *Debaromyces*, *Torula*, *Torulopsis*, *Kloekera*, *Saccharomyces*, *Zygosaccharomyces* and *Yarrowia*. During the '60s and '70s oil was cheap, and citric acid was produced industrially from this source by *Candida* sp., including *C. tropicalis*, *C. catenula*, *C. guilliermondii* and *C. intermediate* (Papagianni, 2007.) Today, this production is not economical due to fact that the fermentation by yeasts led to the formation of

large quantities of isocitric acid as an unwanted byproduct, so mutant strains with low aconitase activity should be used. Although many microorganisms can be employed to produce citric acid, *Aspergillus niger* is still the main industrial producer (Schuster *et. al.*, 2002).

2.10. *Aspergillus niger*

Aspergillus niger belongs to the genus *Aspergillus* which include a set of fungi that are generally considered asexual, although forms that reproduce sexually have been found. They are ubiquitous in nature and can be found widely distributed in a broad range of habitats because they can grow on a wide variety of substrates (Polacheck *et. al.*, 1989). The versatility of *Aspergillus niger* have been exploited as an expression platform for large-scale production of proteins, enzymes and organic acids (Meyer, 2008). The variation in conditions of growth can result in altered expression of several genes, which in turn may affect various phenotypes, such as growth, development, mycotoxin and enzyme production (Iwashita, 2002). *Aspergillus niger* is a common storage mold that influence the spoilage of agricultural goods and contamination of food and feedstock with mycotoxins (Mogensen *et. al.*, 2010).

2.11 Physiology and Ecology of *Aspergillus niger*

Aspergillus spores are drift by air current, dispersing both at short and long distances depending on the conditions of the environment. The spores are deposited on a solid or liquid surface if the moisture content is right and they germinate (Kanaani *et. al.*, 2008). *Aspergillus* is often found in terrestrial habitats and can be isolated from soil and plant decay. They decompose complex organic compounds which are important in driving natural carbon cycle process and contribute to

replenishment of carbon dioxide and other inorganic compounds (Carroll and Wicklow, 1992). *Aspergillus*, in conjunction with other organisms, play an important role in recycling starches, hemicelluloses, pectin and other sugar polymers. The degradation of refractive compounds such as fats, oils, chitin and keratin by some *Aspergilli* with maximum decomposition is achieved in the presence of appreciable nitrogen, phosphorus and other essential inorganic nutrients (Carroll and Wicklow, 1992). *Aspergillus niger* is exclusively known to reproduce through an asexual life cycle, which finally leads to the formation of black airborne mitotic spores (Adams *et. al.*, 1998). The gene expression is auto-regulated, resulting in accumulation of its mRNA during asexual development (Adams *et. al.*, 1998).

2.12. Economic Importance of *Aspergillus niger*

Aspergillus niger is the major organism used in citric acid production by fermentation (Currie, 1917). Citric acid is widely used in the food industry as ingredients. The pharmaceutical and cosmetic industries use citric acid as an acidulant and also in the process of dissolving active ingredients. Citric acid is a true bulk chemical with an estimated production approximately more than 1.6 million tons each year (Dodds and Gross, 2007). *Aspergillus niger* has been used industrially in the production of gluconic acid which serves as additives in some metal cleaning application and also in the therapy of calcium and iron deficiencies (Ruijter *et. al.*, 2002).

Aspergillus niger and *Aspergillus oryzae* are among the major producers of hydrolytic enzymes which have been adopted as bio-based modern commercialization of enzymology business. The extracellular enzymes can be easily exploited in the enzyme production used for baking, beverage and brewing industries; in making of animal feeds; and in paper pulping industry due to the high secretion capacity of the enzymes. The genus *Aspergillus* has a huge potential

industrially for finding new enzymes that could be used to convert plant biomass into fuels and other useful products (Baker *et. al.*, 2008).

2.13. Solid State Fermentation.

Solid-state fermentation (SSF) consists of the microbial growth and product formation on solid particles in the absence (or near absence) of water; however, the substrate contains sufficient moisture to allow the microbial growth and metabolism (Pandey *et. al.*, 2000). This bioprocess has been subject of several studies and it has been proved that SSF has the advantages of leading to higher yields and productivities or better product characteristic (Mienda *et. al.*, 2011).

Solid state fermentation (SSF) has been recently considered as the cheapest and more environmental friendly relative to submerged fermentation (SmF) in the production of value added industrial based products such as organic acids, enzymes, biofuels and the likes (Mienda *et. al.*, 2011).

The advantage of solid state fermentation include the following:

1. The low availability of water reduces the possibilities of contamination by bacteria and yeast. This allows working in aseptic conditions in some cases.
2. Higher levels of aeration, especially adequate in those processes demanding an intensive oxidative metabolism.
3. Similar environmental conditions to those of the natural habitats for fungi, which constitute the main group of microorganisms used in SSF.
4. The inoculation with spores (in those processes that involve fungi) facilitates its uniform dispersion through the medium.

5. The substrate usually provides all the nutrients necessary for growth. Therefore, culture medium composition is often quite simple.
6. Reactors with simple design and few spatial requirements can be used due to the concentrated nature of the substrates.
7. Polluting effluents volumes are generally small. Fewer requirements of dissolvent are evident for product extraction due to their high concentration.
8. The peculiar feature of low moisture availability may facilitate the production of specific compounds that may not likely be produced or poorly produced in SmF
9. The product obtained in SSF have slightly different desired properties i.e. more thermotolerance relative to their counterparts obtained in SmF.

Despite the aforementioned advantages of SSF over SmF, SSF is beset with following disadvantages (Hesseltine, 1972; Cannel and Moo-Young, 1980; Steinkraus, 1984; Kumar and Lonsane, 1989; Raimbault, 1998):

1. The substrate in most cases requires pretreatment which include size reduction by grinding, physical or chemical and enzymatic hydrolysis, and cooking or vapour treatment.
2. Microorganisms like bacteria which may require high moisture levels can perform poorly in SSF. Therefore fungi perform better than bacteria, because of its low moisture requirement.
3. Difficulties are usually encountered in biomass determination.
4. Monitoring of process parameters such as pH, moisture content, substrate, oxygen and biomass concentration becomes a problem because of solid nature of the substrate.
5. Static condition is mostly preferred as agitation most often proved to be very difficult.

6. The engineering and some scientific characterization of SSF bioreactors is not yet fully matured as such there are scarcity of information about the design and operation of reactors on a large scale.
7. There is possibility of contamination with unwanted fungal species.
8. Aeration may be difficult sometimes due to high solid concentrations (Mienda *et al.*, 2011).

Despite the disadvantages of SSF, scientists still believe that problems associated with present industrial production and some of the environmental plights can be solved (Mienda *et al.*, 2011).

2.14 Optimization of Fermentation Conditions for Citric Acid Production

In order to maximize the profits from fermentation process, it is important to consider the optimization of fermentation medium and process conditions. For an industrial fermentation process, medium and fermentation process conditions play critical roles because they effect the formation, concentration and yield of a particular fermentation end product thus, effecting the overall process economics (Schmidt, 2005).

There are many challenges associated with optimization of fermentation process; it is laborious, expensive, open ended and time consuming process involving many experiments. In bioprocessing industry, there is a need to conduct optimization experiments because new mutants and strains are continuously being introduced. In fermentation process, optimization of different combinations and sequence of process conditions and medium components are needed to determine the growth conditions that produce the biomass with the physiological state best constituted for product formation (Stanbury *et. al.*, 1997). The different methods involved in fermentation process optimization include:

2.14.1 Borrowing

This is an open-ended system for process optimization. The medium components and process conditions are obtained from the literatures and what other workers were used to grow the same genus, species or strains are analyzed. The problem with this method is that there are too many options for a given fermentation process. Therefore, short listing is necessary and advantage of this method is that it is simple, easy and requires no mathematical skill (Kennedy and Krouse, 1999).

2.14.2 Component replacement

This is an open-ended system for process optimization and only used to compare the component of one type in a fermentation medium (Bibhu *et. al.*, 2007). In this method, one of component of the medium is replaced by a new one at same incorporation level. However, this method does not consider the components interactions. This method can be useful for screening different carbon, nitrogen and other sources for improving the medium utilization (Kennedy and Krouse, 1999; Tavares *et. al.*, 2005; Jatinder *et. al.*, 2006). Screening of suitable carbon source for citric acid production by solid-state fermentation was carried out by component replacing techniques (Kumar and satyanarayana, 2004; Ahamad *et. al.*, 2006).

2.14.3 Biological Mimicry

Biological mimicry is a close-ended system for fermentation process optimization. This method is useful for optimization of various components of fermentation media and based on concept that cell grow well in a medium that contains every things it needs in right proportion (mass balance strategy). The medium is optimized based on elemental composition of microorganisms

and growth yield. The limitation of this method is measuring elemental composition of microorganisms is expensive, laborious and time consuming. Moreover it does not consider the component interaction. However, this method gives an idea about different micro and macro elements level require in the media for optimal growth of microorganisms (Kennedy and Krouse, 1999).

2.14.4 One-factor-at-a-time

One-factor-at-a-time is a close-ended system for fermentation process optimization. This method can be applied for optimization of medium components as well as for process conditions and it is based on the classical method of changing one independent variable while fixing all other at a certain level (Alexeeva *et. al.*, 2002; Patidar *et. al.*, 2005; Ahamad *et. al.*, 2006). This strategy has the advantage that it is simple, easy and the individual effects of medium components and process condition can be seen on graphs (Kumar and Jain, 2003) but the limitations of this method are interaction between the components are ignored, extremely time consuming, expensive for large number of variable as it involves a relatively large number of experiments (Bibhu, *et. al.*, 2007).

2.14.5 Factorial design

Factorial design is a close-ended system for process optimization. In this method, level of factors/parameters are independently varied, each factor at two or more levels. This effects that can be attributed to the factors and their interactions are assessed with maximum efficiency in factorial design. Moreover it allow for the estimation of the effects of each factor and interaction (Bibhu, *et. al.*, 2007). The optimization procedure is facilitated by construction of an equation

that describes the experimental results as a function of the factor level. A polynomial equation can be constructed in the case of a factorial design where the co-efficient in the equation are related to the effects and interactions of the factors. In a full factorial (complete factorial) design, every combination of factor level is tested (Xie *et al.*, 2003). Typical factors are microbial strain, medium components, temperature, humidity, initial pH and inoculum volume. The most commonly used full factorials in medium improvement experiments are two factorial designs (denoted by 2^n when there are n factors). These designs are the smallest capable of providing detailed information on factor interaction (i.e., antagonistic or synergistic effects) (Xie *et al.*, 2003).

A partial factorial design provides a compromise when the number of runs required in full factorials is impracticable. These are usually two-level factorial design. Two level fractional factorial are denoted by 2^{n-k} , where n is the number of factors and $\frac{1}{2} k$ is the fraction of the complete factorial used. This notation gives an immediate idea of the number of runs required (Rajendhran *et al.*, 2002). In most case factorial design were combined with other different optimization techniques such as central composite design (Park *et al.*, 2005) and evolutionary operation (Tunga *et al.*, 1999) to optimize fermentation process.

2.14.6 Plackett Burman Design

Plackett and Burman's design may be useful to find out the important variable in a system. This design is suitable when more than five independent variables are to be investigated. Plackett and Burman's design are useful to screen out important factors, which influence the fermentation process (Naveena *et al.*, 2005). These are optimized by response surface methodology in further studies (Sayyad *et al.*, 2006). This technique allows for evaluation of n variables by $n+1$

experiments. $n+1$ must be multiple of 4 e.g., 8, 12, 16, 24, etc. therefore, the number of independent variables which can be investigated by this method are 7, 11, 15, 19, 23, etc. Any factors not assigned to a variable can be designated as a dummy variable. The incorporation of dummy variable into an experiment makes it possible to estimate the variance of effects (Plackett and Burman, 1946).

2.14.7 Response Surface Methodology

Statistical experiment design is a powerful method for accumulating information about a process rapidly and efficiently from a small number of experiments, thereby minimizing experimental costs. Box and Wilson introduced response surface methodology (RSM). Response surface methodology seeks to identify and optimize significant factors with the purpose of determining what levels of factors maximize the response (Sayyad *et. al.*, 2006). Response surface methodology uses statistical experimental design such as Central Composite Design (Chakravarti and Sahai, 2002; Dahiya *et. al.*, 2005), Box-Behnken Design (Sayyad *et. al.*, 2006) etc.

The purpose of response surface methodology was to obtain a predicted model and this model can be useful for optimizing the fermentation media formulation or for optimization of fermentation process condition, to carry out simulation with model equation and for better understanding the fermentation process (Kumar and satynarayana, 2004). Central composite design (CCD) was introduced by Box and Wilson; CCDs are formed from two level factorials by addition of just enough points to estimate curvature and interaction effects. The design can be viewed as partial factorials with factors at five levels. The number of runs in CCD increases exponentially with number of factors (Kennedy and Krouse, 1999).

2.14.8 Genetic Algorithms

Non-statistical optimization techniques such as genetic algorithms are used in fermentation technology. This technique can be used to optimize fermentation process without need of statistical designs and empirical models, and based on the principle that after a continuous process of mutation only best individual exist (Bibhu *et. al.*, 2007). In fermentation media or fermentation process optimization, rules of genetic algorithms can be applied successfully where the set of one experiment i.e. medium composition are coded in one chromosome and each medium constituent level represents one gene after completing the first generation of experiments chromosome with highest productivity are selected and replicated proportionally to the productivity then crossover of chromosome and mutation of some randomly chosen genes are performed. In such a way, new generations of experiments are obtained. The main disadvantage of genetic algorithms is that it does not store the information generated at each stage of the optimization process (Zuzek *et. al.*, 1996).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Materials

Chemicals

H₂SO₄, NaOH, H₂O₂, Potato Dextrose Agar (PDA), (NH₄)₂SO₄, MgSO₄.7H₂O, NH₄NO₃, KH₂PO₄, FeCl₃.6H₂O, CuSO₄, FeSO₄, ZnSO₄, MnSO₄, Molasses, pyridine and acetic anhydride.

All chemicals and reagents used were purchased from B D H chemical limited Poole England (AnalaR).

Equipment

Water bath (Grant OLS 200), Orbital shaker (IKA-Labortechnik. KS 501), Haemocytometer, Melting point analyzer (MEL-TEMP), Incubator (Gen lab.Limited), Weighing balance (Sartorius Entris124-1S), Autoclave (Omega prestige medical model 220220), FTIR Analyzer (Agilent technologies).

3.2 Methods

3.2.1 Sample collection

Rice husk was obtained from rice milling Centre in Samaru, sabon Gari, Kaduna state. The husk was sieved to obtain a particle size of 1 - 2 mm and dried at 70⁰C in an oven for 48 hours. To ensure uniform particle, a sieve was used to remove the larger particles.

3.2.2 Pretreatment of Rice Husk

Acid, alkali and hydrogen peroxide pretreatment of sample was carried out according to the methods of Khosravi-Darani *et. al.*, (2008) with slight modification. Twenty (20) grams of the substrate was added each to 400 ml of 1N H₂SO₄, 1N NaOH, and 1N H₂O₂, respectively, in a 500 ml conical flask each, and was cotton plugged and allowed to stand at room temperature (25-28⁰C) for 2-3 hours. After pretreatment the substrate was rinsed thoroughly with distilled water and filtered through a muslin cloth. The process was repeated several times to ensure complete removal of traces of the solvent. Finally the substrate was dried at 70⁰C in an incubator for 48 hours and sealed to avoid moisture until needed.

3.2.3 Collection and Identification of Organism

A citric acid producing strain of *Aspergillus niger* was obtained from Department of Microbiology Ahmadu Bello University, Zaria Nigeria.

3.2.4 Inoculum and Spore Suspension Preparation

Seven-day-old potato dextrose agar plate [Composition of Potato Dextrose Agar (PDA) (Potato, 100 g; Dextrose, 10 g; Agar, 10 g and distilled water, 500 ml. pH: 5.6 ± 0.2 at 25°C)] containing *Aspergillus niger* was used to prepare the inoculum culture as described by Alam *et al.*, (2004). Spores were harvested by flooding the plates with sterile distilled water containing 0.5 % Tween 80 as a wetting agent, after which spores were scrapped from the surface of the colonies with a sterile spatula. The resulting suspension was shaken in a 100 ml Erlenmeyer flask to break up the spore chain. The concentration of spores was determined using a haemocytometer (under $400 \times$ magnification), after which the suspension was further diluted in sterile Tween 80 solution until the desired concentration (10^7 spores per ml) was achieved.

3.2.5 Citric Acid Production by Solid State Fermentation

Eight grams of rice husk (1-2 mm particle size) was weighed into a 250 mL Erlenmeyer flask, and the medium components of varying concentrations were added into the flask based on the Plackett- Burman design matrix to achieve a desired moisture level of 60 %. The contents were thoroughly mixed and autoclaved at 121°C for 20 min. The medium was inoculated with 2 ml of spore suspension containing about 10^7 spores per ml of *Aspergillus niger* inoculum and incubated at 30°C for 5 days (Dhandayuthapani, 2009).

3.2.6 Fermentation Media Preparation Based on Plackett- Burman Design

Twelve experimental runs were generated using Plackett- Burman Design for ten media components; these are $(\text{NH}_4)_2\text{SO}_4$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, NH_4NO_3 , KH_2PO_4 , $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, CuSO_4 , FeSO_4 , ZnSO_4 , MnSO_4 , and Molasses. Each component was tested at two concentration levels, low (-1) and high (+1). The experiment was carried out in triplicate according to the design matrix in 250 ml Erlenmeyer flasks.

Table 3.1: Twelve-trial Plackett Burman Design to Study Ten Factors in Citric Acid Production from Rice Husk in Solid State Fermentation.

Runs	(NH ₄) ₂ SO ₄ (g/L)	MgSO ₄ (g/L)	NH ₄ NO ₃ (g/L)	KH ₂ PO ₄ (g/L)	FeCl ₃ (×10 ⁻³ g/L)	CuSO ₄ (×10 ⁻³ g/L)	FeSO ₄ (×10 ⁻³ g/L)	ZnSO ₄ (×10 ⁻³ g/L)	MnSO ₄ (×10 ⁻³ g/L)	Molasses (×10 ⁻³ g/L)	Citric acid yield g/Kg RH
1	0.00	0.00	2.50	1.00	1.00	0.00	0.22	0.38	0.00	0.140	
2	3.00	0.25	2.50	0.00	1.00	0.48	0.00	0.38	0.00	0.00	
3	0.00	0.25	2.50	0.00	1.00	0.00	0.00	0.00	1.40	0.140	
4	0.00	0.25	0.00	0.00	0.00	0.48	0.22	0.38	0.00	0.140	
5	3.00	0.25	0.00	1.00	1.00	0.00	0.22	0.00	0.00	0.00	
6	3.00	0.25	0.00	1.00	0.00	0.00	0.00	0.38	1.40	0.140	
7	3.00	0.00	2.50	0.00	0.00	0.00	0.22	0.38	1.40	0.00	
8	3.00	0.00	0.00	0.00	1.00	0.48	0.22	0.00	1.40	0.140	
9	0.00	0.00	0.00	1.00	1.00	0.48	0.00	0.38	1.40	0.00	
10	3.00	0.00	2.50	1.00	0.00	0.48	0.00	0.00	0.00	0.140	
11	0.00	0.25	2.50	1.00	0.00	0.48	0.22	0.00	1.40	0.00	
12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	

3.2.7 Optimization of Citric Acid Production

Three media components (NH_4NO_3 , KH_2PO_4 and molasses) were subjected to optimization by Face Centered Central Composite Design (FCCCD) of Response Surface Methodology (RSM) to consider the effect of individual variables and relationship between media components in obtaining optimum conditions for an efficient multivariable system (Bandaru *et al.*, 2006; Kar and Ray, 2007). A Face Centered central composite design was used to generate twenty experimental runs based on the design expert software with concentration range of low (-1), medium (0) and high (+1). Thus six center points and fourteen axial points were generated making up to twenty experiment to be conducted. All experiments were carried out in triplicate and the mean for each was calculated.

Table 3.2: Design Matrix for Faced Centered Central Compose Design (FCCCD) to Optimize the Three Factors Affecting Citric Acid Production Using Hydrogen Peroxide Pretreated Rice Husk by Solid State Fermentation

Runs	NH ₄ NO ₃ g/L	KH ₂ PO ₄ g/L	MOLASSES g/L	Citric Acid Yield g/Kg RH	
				Experimental	Predicted
1	2.50	3.0	0.215		
2	1.75	3.5	0.200		
3	1.75	2.5	0.200		
4	2.13	3.0	0.215		
5	2.13	2.5	0.215		
6	2.13	3.0	0.215		
7	2.50	3.5	0.230		
8	2.13	3.0	0.215		
9	1.75	3.5	0.230		
10	2.50	2.5	0.200		
11	2.13	3.5	0.215		
12	2.13	3.0	0.230		
13	2.13	3.0	0.215		
14	2.13	3.0	0.215		
15	1.75	2.5	0.230		
16	2.13	3.0	0.215		
17	2.13	3.0	0.200		
18	2.50	2.5	0.230		
19	2.50	3.5	0.200		
20	1.75	3.0	0.215		

Developed model equation for optimization of medium component for citric acid production was also generated as;

$$\text{Citric acid yield} = +0.99 + 0.090 A + 0.047 B + 0.071 C + 0.098 A^2 - 0.076 B^2 - 0.070 C^2 - 0.039 AB + 0.032 AC + 0.039 BC.$$

3.2.8 Extraction of Citric Acid from Fermented Substrate

Recovery of citric acid was done after 5 days of fermentation as describe by Xu *et al.*, (1989). To 8 g of fermented product, 80 ml distilled water was added. After loosening the impregnated mycelium with the substrate using a glass rod, the flask containing spent media was shaken on an orbital shaker at 121 rpm for 1 hr. The supernatant was filtered using Whatman No.1 filter paper. The supernatant was tested for the pH and stored at 4°C for citric acid analysis.

3.2.9. Quantitative Analysis of Citric Acid

Quantitative analysis of citric acid produced was carried out by the method of Marrier and Boulet (1958), which they (Marrier and Boulet) modified in 1983. Briefly: 1 ml of the sample was added to a test tube containing 1.3 ml pyridine, followed by the addition of 5.7 ml acetic anhydride. The contents of the test tube were mixed manually by swirling and were immediately placed in a constant temperature (22 °C) water bath. Colour development was allowed for 30 min, followed by reading the colour intensity at 420 nm with the blank set on 100 % transmission. The citric acid concentration was determined by referring to a standard curve for citric acid concentration. A citric acid concentration was expressed per kilogram of dried rice husk for solid state fermentation.

3.2.10 Validation of Model for Citric Acid Production

Three media components (NH₄NO₃, KH₂PO₄ and molasses) were subjected to validation to consider the effect of individual variables and relationship between media components in

obtaining optimum conditions for an efficient multivariable system. Based on the design expert software experimental runs with concentration range of low (-1), medium (0) and high (+1) were generated.

Table 3.3 Validation of Model for Citric Acid Production using Hydrogen Peroxide Pretreated Rice Husk as a Substrate in Solid State Fermentation.

Runs	NH₄NO₃g/L	KH₂PO₄ g/L	Molasses g/L	Citric Acid Yield g/kg RH
1	2.3	3.2	0.225	
2	2.5	3.25	0.2275	
3	2.4	3.0	0.215	
4	2.5	3.5	0.230	

3.2.11 Characterization of Citric Acid

Qualitative analysis of the chemical structure and physical properties of the citric acid produced were carried out using Thin Layer Chromatography and Infrared Spectroscopy methods as described by Marjadi and Dharaiya, (2014).

Thin Layer Chromatography (TLC)

Thin Layer Chromatography was carried out in a glass plate coated with silica gel prepared using a spreader. Both extracted and standard citric acid (10mg) were dissolved in 1ml of methanol each. The sample (50 μ l) was loaded on the TLC plate and allowed to run in the solvent system consisting of n- Butanol, acetic acid and water (BAW) mixture at ratio 3:1:1 for 30-40 minutes. The plate was left to air-dry and sprayed with p-anisaldehyde and allowed to air dry. The TLC plate was placed in an oven and heated at 140⁰ C for 2 minutes and the migrating spots were observed. The Rf values of the spots were calculated using standard formula below.

$$\text{Retention factor (Rf)} = \frac{\text{Distance Travel by Solute}}{\text{Distance Travel by Solvent}}$$

Spectroscopic Analysis of Citric Acid Produced

Infra-Red absorption spectrum of both produced citric acid and standard citric acid was determined. Five milligram of each (produced and standard citric acid) was separately picked and emulsified. It was then placed in the FTIR machine and allowed to read. The relative intensity of the transmitted light was measured against the wavelength of absorption on the region 400 to 4000 cm^{-1} using IR double beam spectrophotometer. The procedure was carried out at room temperature (According to the manufacturer instruction).

The principle of FTIR relies on the fact that most molecules absorb light in the infra-red region of the electromagnetic spectrum. This absorption corresponds specifically to the bonds present in the molecule. The frequency ranges are measured as wave numbers typically over the range 4000 – 600 cm^{-1} . The background emission spectrum of the IR source is first recorded, followed by the emission spectrum of the IR source with the sample in place. The ratio of the sample spectrum to the background spectrum is directly related to the sample's absorption spectrum. The resultant absorption spectrum from the bond natural vibration frequencies indicates the presence of various chemical bonds and functional groups present in the sample. FTIR is particularly useful for identification of organic molecular groups and compounds due to the range of functional groups, side chains and cross-links involved, all of which will have characteristic vibration frequencies in the infra-red range (Saravanan and Vijayakumar, 2012).

Melting Point Determination

Melting point of the standard and produced citric acids were determined using melting point analyzer. Capillary tube were Filled with crystals up to about 3 mm high and placed in the MEL-TEMP melting point apparatus (According to the manufacturer instruction). The melting process was observed through the magnifying lens. The test was done in triplicate and the results were recorded.

CHAPTER FOUR

4.0 RESULTS

Different pretreatments were made to the substrate and using Placket Burman design, twelve set of experiments were generated which comprise of different nutrient combinations and the result obtained were presented in Tables 4.1. For acid pretreatment, run eight produced the best yield of 3.9424 g/kg rice husk, while the least production was observed at run twelve of 1.8944 g/kg rice husk. For 1N alkaline pretreatment, the best yield of 3.4868 g/kg rice husk was obtained at run three, while the least production was observed at run five of 0.5112 g/kg rice husk. In case of oxidative pretreatment, run ten produced the best yield of 4.1212 g/kg rice husk, while the least production was observed at run four of 3.2032 g/kg rice husk. Similarly, for unpretreated substrate the best yield of 2.5496 g/kg rice husk was obtained in run one, while the least production of 2.1476 g/kg rice husk was observed at run four. Duncan multiple range test was conducted following one way ANOVA to select the best pretreatment method(s) to be used for citric acid production using rice husk as a substrate and the results were presented in Table 4.2. Hydrogen peroxide pretreated substrate gave the best yield statistically with 3.65 ± 0.26 g/kg rice husk and Alkaline pretreated substrate gave the least of 1.97 ± 1.24 g/kg rice husk.

Table 4.1: Citric Acid Produced using Various Pretreated Rice Husk (Acid, Alkaline, Hydrogen Peroxide and Unpretreated Substrate) According to Placket Burman Design and their Responses

Runs	(NH ₄) ₂ SO ₄ (g/L)	MgSO ₄ (g/L)	NH ₄ NO ₃ (g/L)	KH ₂ PO ₄ (g/L)	FeCl ₃ (×10 ⁻³ g/ml)	CuSO ₄ (×10 ⁻³ g/ml)	ZnSO ₄ (×10 ⁻³ g/ml)	MnSO ₄ (×10 ⁻³ g/ml)	Molasses (×10 ⁻³ g/ml)	citric acid produced (g/Kg RH)±SD			
										Acid unpretreated	alkaline	H ₂ O ₂	
1	0.00	0.00	2.50	1.00	1.00	0.00	0.38	0.00	140.00	3.24±0.02	2.70±0.01	3.61±0.01	2.55±0.05
2	3.00	0.25	2.50	0.00	1.00	0.48	0.38	0.00	0.00	2.07±0.04	0.67±0.01	3.58±0.01	2.35±0.06
3	0.00	0.25	2.50	0.00	1.00	0.00	0.00	1.40	140.00	3.93±0.01	3.49±0.01	3.49±0.01	2.44±0.03
4	0.00	0.25	0.00	0.00	0.00	0.48	0.38	0.00	140.00	3.12±0.06	1.06±0.02	3.20±0.03	2.15±0.02
5	3.00	0.25	0.00	1.00	1.00	0.00	0.00	0.00	0.00	1.90±0.02	0.51±0.02	3.89±0.01	2.26±0.03
6	3.00	0.25	0.00	1.00	0.00	0.00	0.38	1.40	140.00	3.80±0.02	2.23±0.03	3.98±0.02	2.26±0.03
7	3.00	0.00	2.50	0.00	0.00	0.00	0.38	1.40	0.00	2.15±0.02	0.64±0.01	3.40±0.01	2.43±0.01
8	3.00	0.00	0.00	0.00	1.00	0.48	0.00	1.40	140.00	3.94±0.04	3.48±0.02	3.48±0.01	2.25±0.01
9	0.00	0.00	0.00	1.00	1.00	0.48	0.38	1.40	0.00	2.47±0.04	1.35±0.04	3.75±0.04	2.42±0.01
10	3.00	0.00	2.50	1.00	0.00	0.48	0.00	0.00	140.00	3.93±0.02	3.46±0.02	4.12±0.02	2.24±0.01
11	0.00	0.25	2.50	1.00	0.00	0.48	0.00	1.40	0.00	2.79±0.06	3.19±0.04	3.78±0.01	2.20±0.02
12	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.89±0.02	0.90±0.02	3.57±0.03	2.50±0.03

Table 4.2: Comparison of Citric Acid Produced Under Different Pretreatments

Pretreatment.	Citric acid yield (g/kg) of rice husk
Acid pretreated substrate	2.94±0.83 ^b
Alkaline pretreated substrate	1.97±1.24 ^a
H ₂ O ₂ pretreated substrate	3.65±0.26 ^c
Un-pretreated substrate	2.34±0.13 ^{ab}

Values are Mean ± SD; Values with the same superscript within the column are not significantly different (P>0.05) by Duncan multiple range test.

Effect of individual nutrient component on the citric acid production with respect to the pretreatment methods was presented in Figure 4.1. $(\text{NH}_4)_2\text{SO}_4$, NH_4NO_3 , KH_2PO_4 , CuSO_4 , MnSO_4 , and Molasses contributed positively in various proportion in acid pretreated substrate while $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, FeSO_4 , and ZnSO_4 , contributed negatively in various proportion. In alkaline pretreated substrate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, NH_4NO_3 , KH_2PO_4 , $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, CuSO_4 , MnSO_4 , and molasses contributed positively in various proportion, while $(\text{NH}_4)_2\text{SO}_4$, FeSO_4 , and ZnSO_4 , contributed negatively in various proportion. In case of oxidative pretreated substrate $(\text{NH}_4)_2\text{SO}_4$, NH_4NO_3 , KH_2PO_4 , CuSO_4 , and Molasses contributed positively in various proportion while $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, FeSO_4 , MnSO_4 , and ZnSO_4 , contributed negatively in various proportion. Similarly in unpretreated substrate, $(\text{NH}_4)_2\text{SO}_4$, NH_4NO_3 , KH_2PO_4 , CuSO_4 , MnSO_4 , and Molasses contributed positively in various proportion while $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, FeSO_4 , and ZnSO_4 , contributed negatively in various proportion.

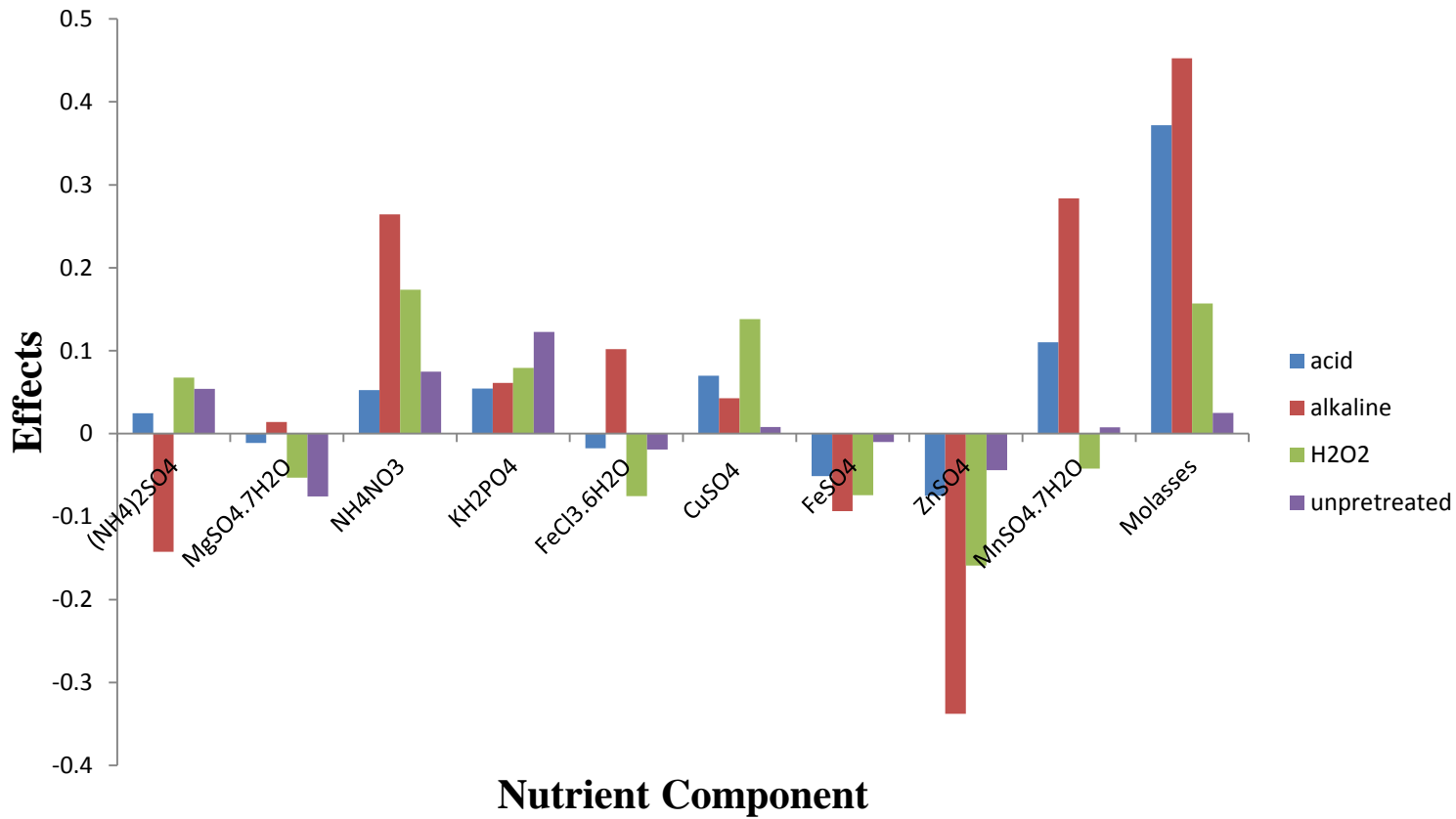


Figure 4.1: Effect of Various Nutrients on Citric Acid Production by *A. niger* using Different Pretreated Rice Husk (Acid, Alkaline, Oxidative and Unpretreated Substrate) Via Solid State Fermentation

In order to determine the optimum concentration of each contributing nutrient, one factor at a time experiment was done where the concentration of the contributing nutrients were varied, to enhanced citric acid production.

The optimum concentration of $(\text{NH}_4)_2\text{SO}_4$ for citric acid production from rice husk was found to be 2.0 g/L, with citric acid yield of 1.832 ± 0.007 g/kg rice husk, and the result obtained was presented in Figure 4.2. In the case of NH_4NO_3 , the optimum concentration for citric acid production from rice husk was found to be 2.0 g/L, with citric acid yield of 2.841 ± 0.003 g/kg rice husk, and the result obtained was presented in Figure 4.3. Similarly, optimum concentration of KH_2PO_4 for citric acid production from rice husk was found to be 3.0 g/L, with citric acid yield of 3.731 ± 0.027 g/kg rice husk, also the result obtained was presented in Figure 4.4 The optimum concentration of molasses for citric acid production from rice husk was found to be 220.0 mg/L, with citric acid yield of 6.051 ± 0.010 g/kg rice husk, and the result obtained was presented in Figure 4.5 The optimum concentration of CuSO_4 for citric acid production from rice husk was found to be 0.7 mg/L, with citric acid yield of 4.384 ± 0.010 g/kg rice husk, and the result obtained was presented in Figure 4.6.

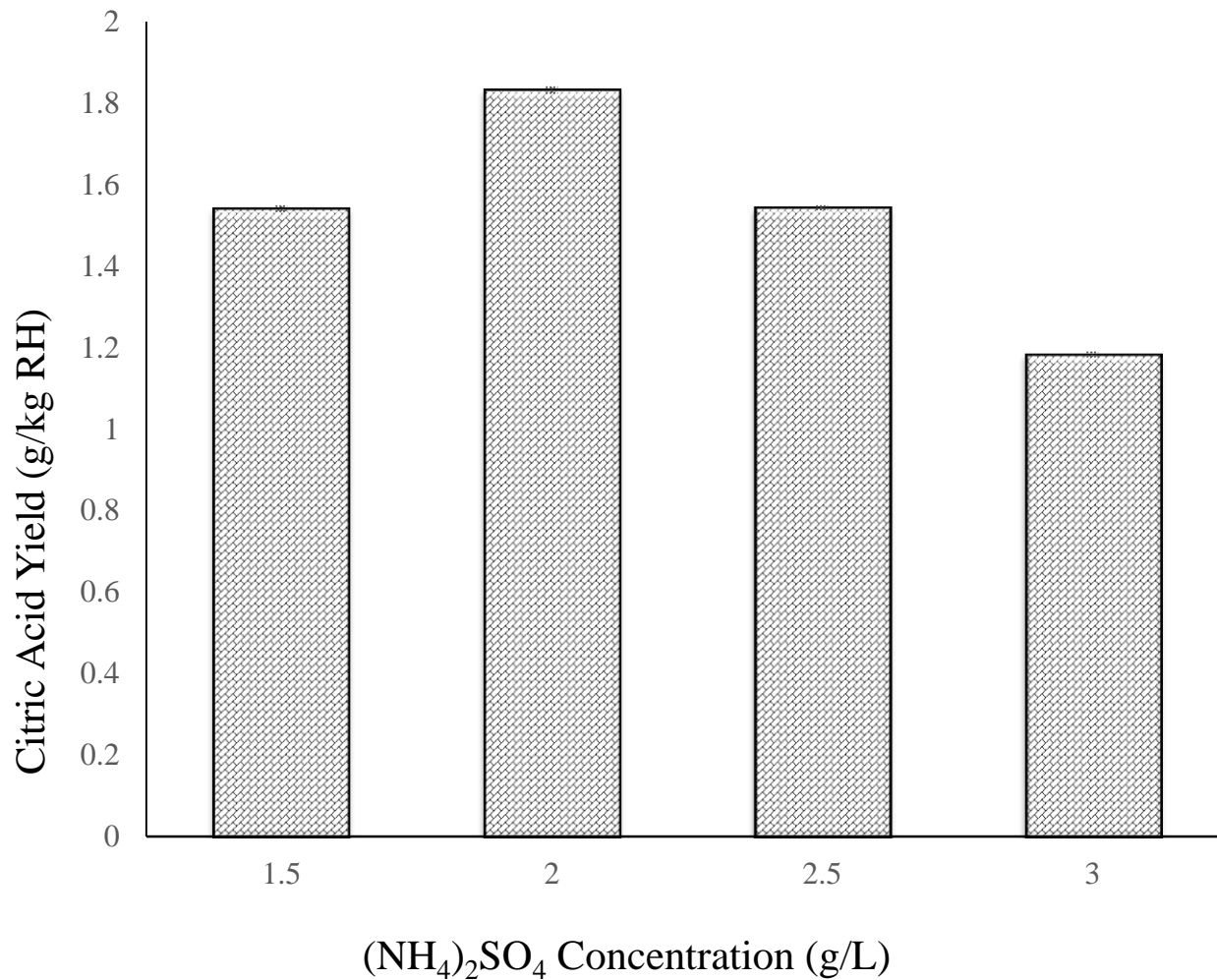


Figure 4.2: Effect of $(\text{NH}_4)_2\text{SO}_4$ Concentrations on Citric Acid Production using Hydrogen Peroxide Pretreated Rice Husk in Solid State Fermentation.

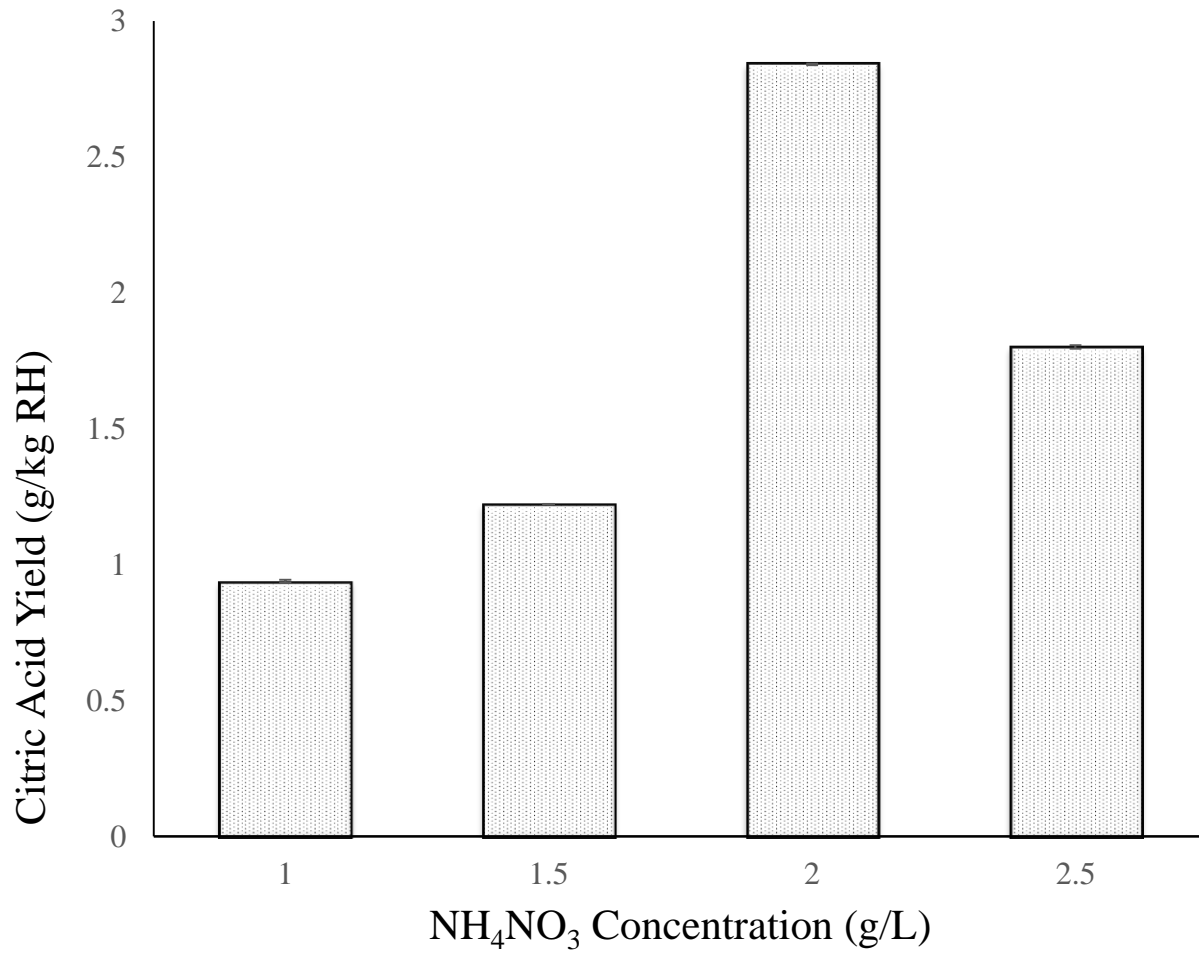


Figure 4.3: Effect of NH_4NO_3 Concentrations on Citric Acid Production using Hydrogen Peroxide Pretreated Rice Husk in Solid State Fermentation.

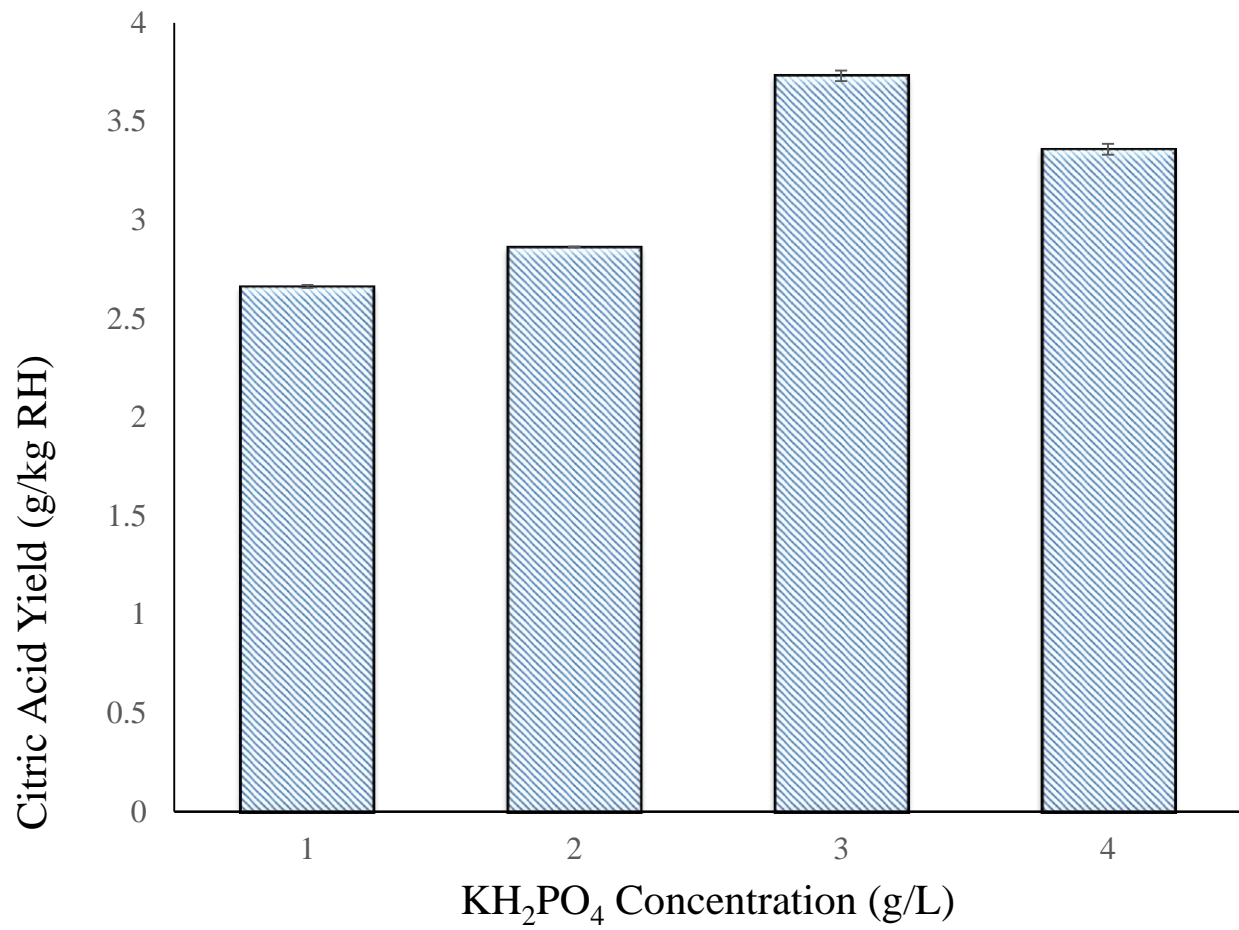


Figure 4.4: Effect of KH_2PO_4 Concentrations On Citric Acid Production Using Hydrogen Peroxide Pretreated Rice Husk In Solid State Fermentation.

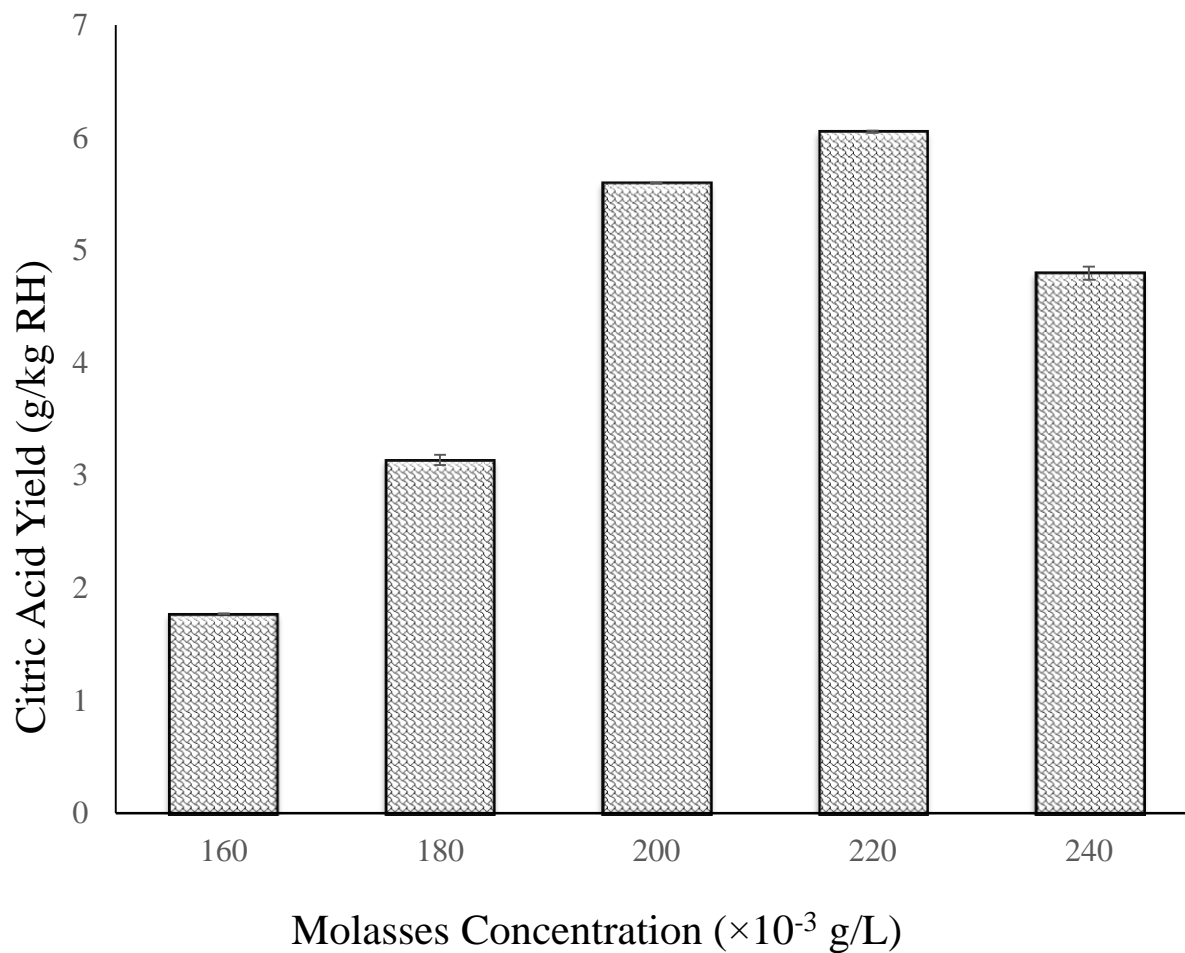


Figure 5.5: Effect of Molasses Concentrations on Citric Acid Production using Hydrogen Peroxide Pretreated Rice Husk in Solid State Fermentation.

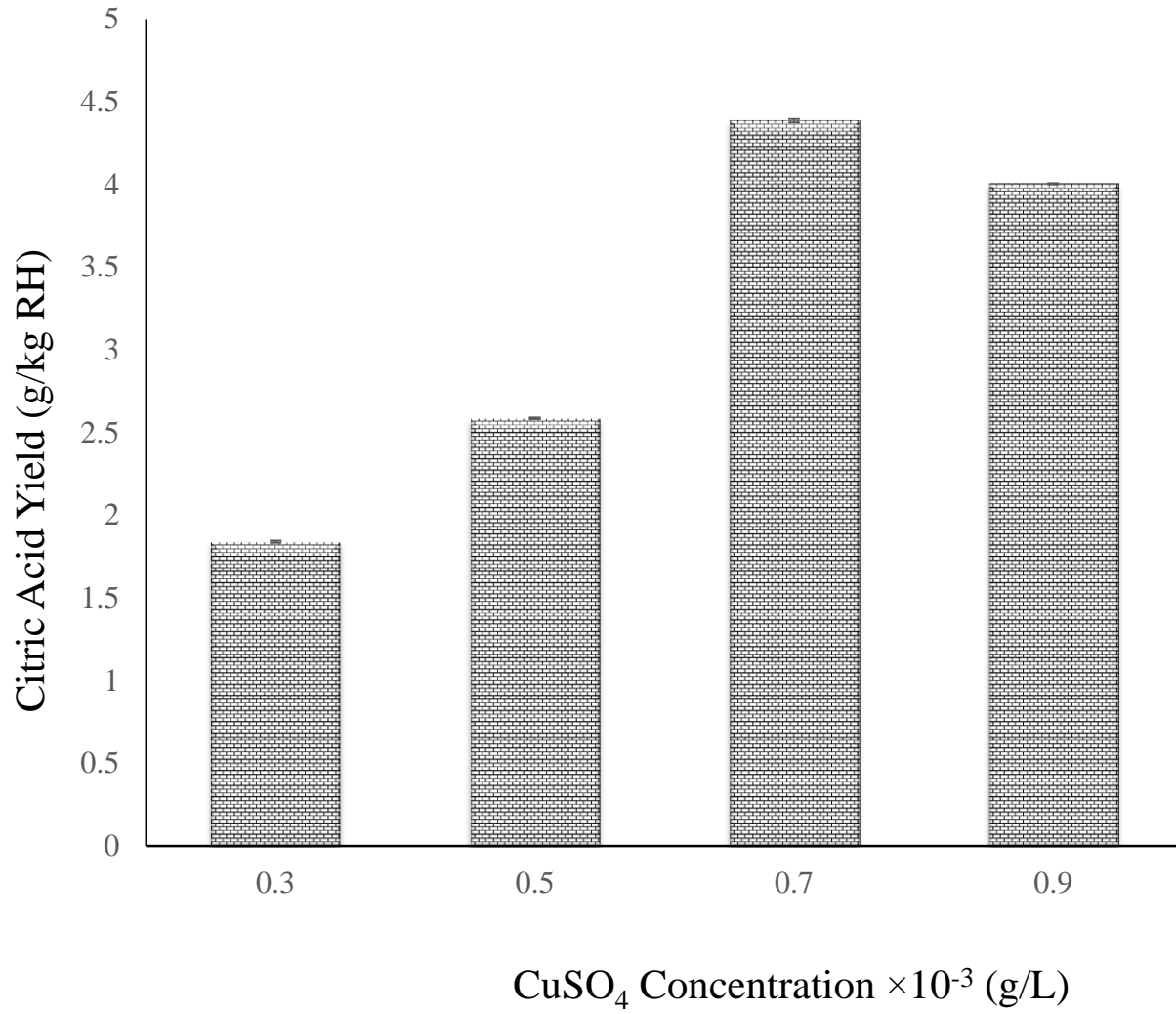


Figure 4.6: Effect of CuSO₄ Concentrations on Citric Acid Production using Hydrogen Peroxide Pretreated Rice Husk in Solid State Fermentation.

Optimization of nutrient component for citric acid production using Faced Centered Central Compose Design (FCCCD) was done using the Design expert software (version 6.0.8, Stat Ease Corporation, USA). The ranges of contributing nutrient were presented in Table 4.3. The response showed close relationship between the experimental and predicted values which were presented in Table 4.4. ANOVA Table was generated and presented in Tables 4.5. Coefficient of determination R^2 was found to be 0.9790 with Adjusted R^2 of 0.9601 and Predicted R^2 of 0.8888 with Adequate Precision of 26.289 which measures signal to noise ratio.

The plot of predicted and actual (experimental) values indicating the closeness of the values obtained from experimental and the predicted runs was presented in Figure 4.7

Table 4.3 Concentration Ranges of Factors used for Optimization Using Faced Centered Central Composite Design for Citric Acid Production from Oxidative Pretreated Rice Husk.

Factors	Ranges g/L
NH ₄ NO ₃	1.75-2.50
KH ₂ PO ₄	2.50-3.50
Molasses	0.200-0.230

Table 4.4: Citric Acid Produced using Hydrogen Peroxide Pretreated Rice Husk in Response Surface Methodology using Central Composite Design to Optimize Media Component and their Responses in Production.

Runs	NH ₄ NO ₃ g/L	KH ₂ PO ₄ g/L	MOLASSES g/L	Citric Acid Yield g/Kg RH	
				Experimental	Predicted
1	2.50	3.0	0.215	4.76	4.72
2	1.75	3.5	0.200	3.42	3.44
3	1.75	2.5	0.200	3.06	3.04
4	2.13	3.0	0.215	3.97	3.96
5	2.13	2.5	0.215	3.42	3.44
6	2.13	3.0	0.215	3.93	3.96
7	2.50	3.5	0.230	4.69	4.72
8	2.13	3.0	0.215	3.96	3.96
9	1.75	3.5	0.230	4.00	4.08
10	2.50	2.5	0.200	3.87	3.84
11	2.13	3.5	0.215	4.00	3.84
12	2.13	3.0	0.230	4.06	3.96
13	2.13	3.0	0.215	3.85	3.96
14	2.13	3.0	0.215	3.93	3.96
15	1.75	2.5	0.230	3.07	3.04
16	2.13	3.0	0.215	3.84	3.96
17	2.13	3.0	0.200	3.41	3.40
18	2.50	2.5	0.230	4.33	4.36

19	2.50	3.5	0.200	3.55	3.60
20	1.75	3.0	0.215	4.06	4.00

Table 4.5: Analysis of Variance for Citric Acid Production using Hydrogen Peroxide Pretreated Rice Husk via Solid State Fermentation ($p < 0.05$).

Source	Sum of squares	Mean square	F-value	P-Value	
Model	0.23	0.026	51.74	< 0.0001	significant
A(NH ₄ NO ₃)	0.081	0.081	160.71	< 0.0001	
B(KH ₂ PO ₄)	0.022	0.022	44.69	< 0.0001	
C(Molasses)	0.050	0.050	100.60	< 0.0001	
A ²	0.026	0.026	52.79	< 0.0001	
B ²	0.016	0.016	31.88	0.0002	
C ²	0.013	0.013	26.90	0.0004	
AB	0.012	0.012	24.23	0.0006	
AC	8.017×10^{-3}	8.017×10^{-3}	16.00	0.0025	
BC	0.012	0.012	24.41	0.0006	
Lack of Fit	4.061×10^{-3}	8.123×10^{-4}	4.28	0.0682	not significant
R ²	0.9790				
Adjusted R ²	0.9601				
Predicted R ²	0.8888				
Adequate Precision	26.289				

The " Predicted R²" of 0.8888 is in reasonable agreement with the " Adjusted R²" of 0.9601.

Response surface plots showing the interaction between the contributing nutrients were generated and presented in Figures 4.8-4.10. The validation experiment using NH_4NO_3 , KH_2PO_4 , and molasses were considered at various combination of concentrations as generated by the software. The result is presented in Table 4.6. Thin Layer Chromatograph gave the Rf value of 0.242 for both standard citric acid and microbial produced citric acid. The photograph of TLC plate aligning the standard and the produced citric acid was shown in Plate I. Citric acid obtained from TLC was subjected to FTIR analyses. The wave numbers corresponding to O - H and C = O of standard citric acid was found to be 3338.7 cm^{-1} , 1565.5 cm^{-1} while that of rice husk produced citric acid was 3324.8 cm^{-1} , 1625.1 cm^{-1} respectively as presented in Table 4.7.

The melting point determination of citric acid produced in comparison with that of the standard was presented in Table 4.8. The mean \pm SD melting temperatures of standard and *A. niger* produced citric was found to be $153 \pm 0.00^\circ\text{C}$ and $153 \pm 0.02^\circ\text{C}$, respectively.

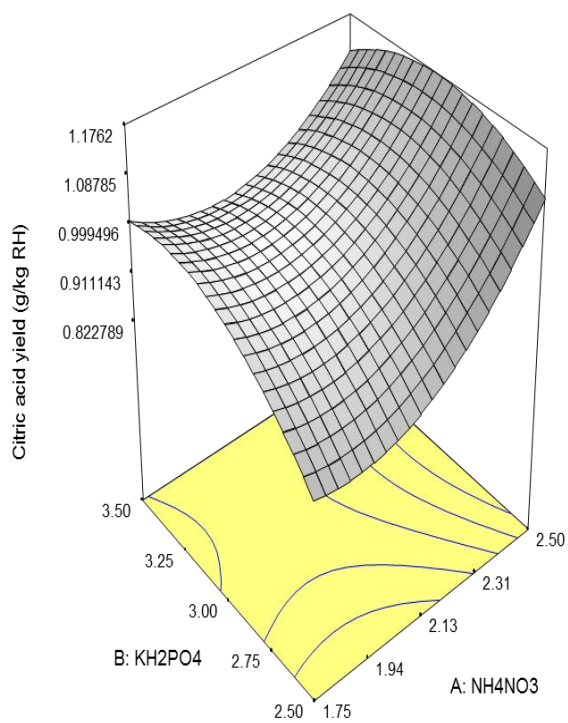


Figure 4.8: Response Surface (3D) Plot Showing the Effect of NH_4NO_3 and KH_2PO_4 at Fixed Concentration of Molasses (215.00mg/L) on Citric Acid Production by Hydrogen Peroxide Pretreated Rice Husk by Solid State Fermentation.

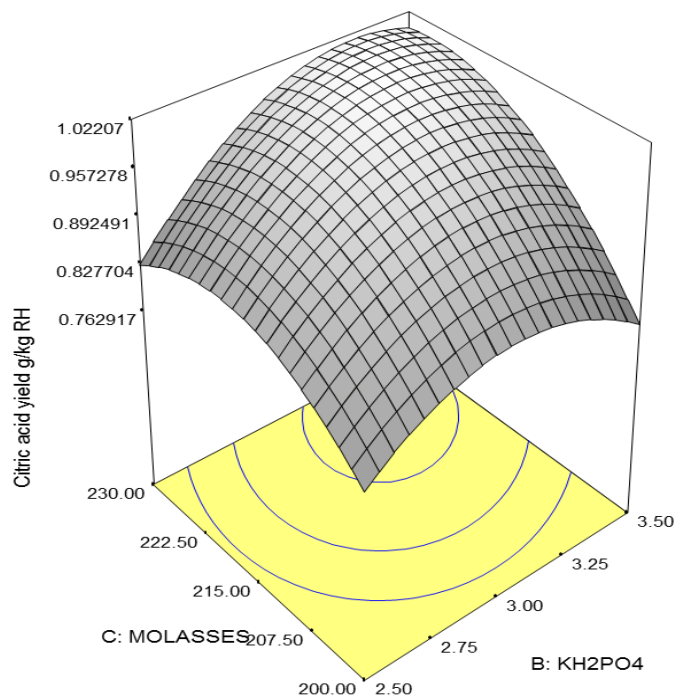


Figure 4.9: Response Surface (3D) Plot Showing the Effect of Molasses and KH_2PO_4 at Fixed Concentration of NH_4NO_3 (2.13g/L) on Citric Acid Production by Hydrogen Peroxide Pretreated Rice Husk by Solid State Fermentation.

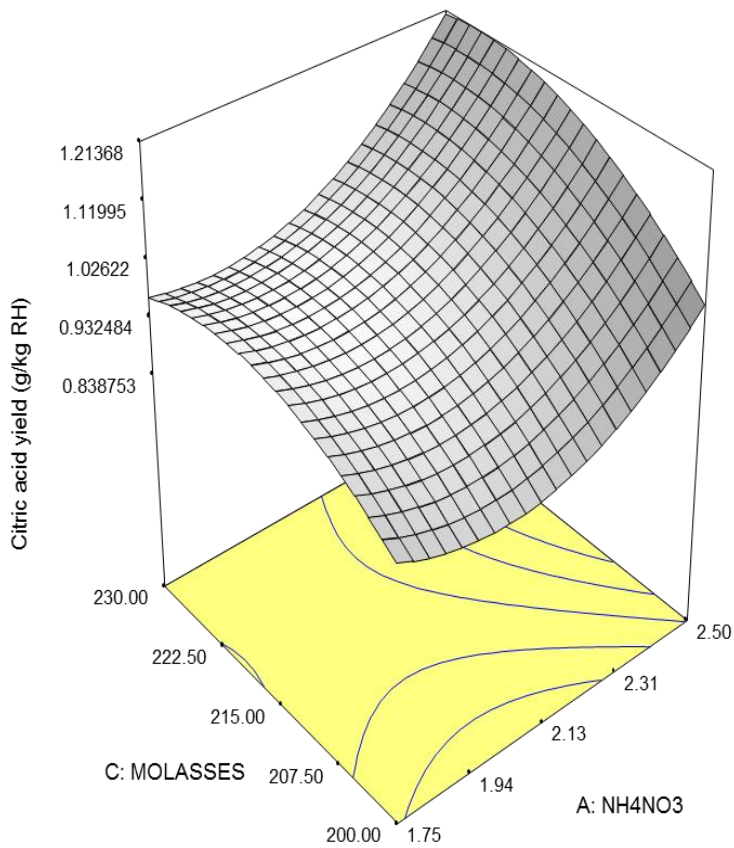


Figure 4.10: Response Surface (3D) Plot Showing the Effect of Molasses and NH_4NO_3 at fixed concentration of KH_2PO_4 (3.00) on Citric Acid Production by Hydrogen Peroxide Pretreated Rice Husk by Solid State Fermentation.

Table 4.6: Validation of Model for Citric Acid Production using Hydrogen Peroxide Pretreated Rice Husk as a Substrate in Solid State Fermentation.

Runs	NH ₄ NO ₃ g/L	KH ₂ PO ₄ g/L	Molasses g/L	Citric Acid Yield g/kg RH	
				Experimental	Predicted
1	2.3	3.2	0.225	4.330±0.002	4.5772
2	2.5	3.25	0.2275	4.765±0.001	4.85788
3	2.4	3	0.215	4.754±0.001	4.70452
4	2.5	3.5	0.230	4.733±0.002	4.72008

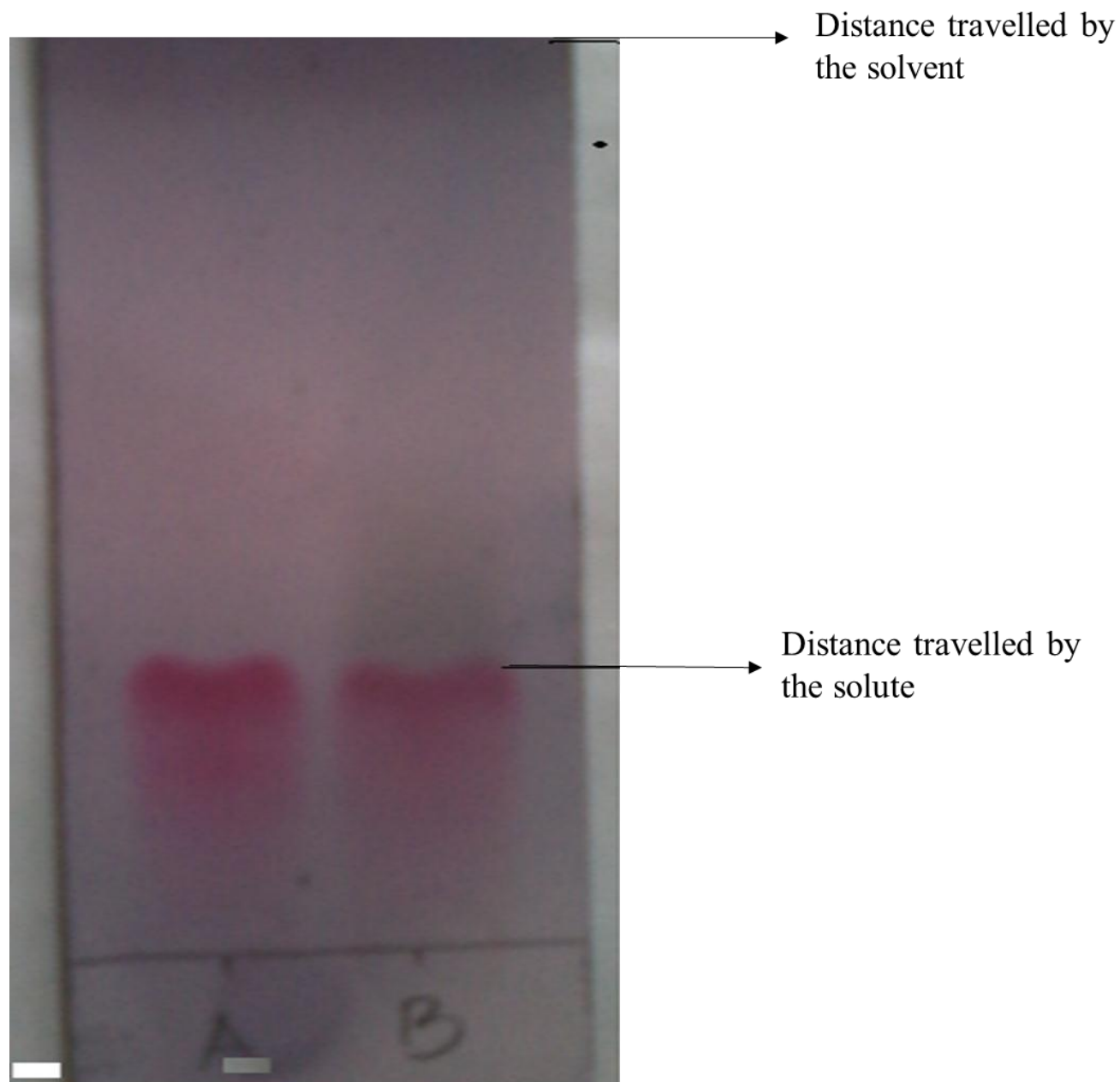


Plate I: Thin Layer Chromatography Plate of Standard and Produced Citric Acid from Hydrogen Peroxide Pretreated Rice Husk by Solid State Fermentation in 3:1:1 n-Butanol Acetic Acid and Water (BAW) as Mobile Phase.

Where A is a Standard citric acid and B is a Citric acid produced from rice husk.

The Rf value = 0.242.

Table 4.7: The Wave Number of Standard and Produced Citric Acid.

Sample	Wavenumbers cm^{-1}	
	O-H	C=O
Standard citric acid	3338.7	1565.5
Produced citric acid	3324.8	1625.1
Ranges (in a carboxylic acid).	2500-3300	1680-1750

Table 4.8: Melting Point of Standard and Citric Acid Produced from H₂O₂ Pretreated Rice Husk.

Sample	Melting point (°C) ± SD
Standard citric acid	153±0.00
Produced citric acid	153±0.02

CHAPTER FIVE

5.0 DISCUSSION

Various pretreatment methods were employed in the present study which includes acid, alkaline and hydrogen peroxide. Among which, hydrogen peroxide pretreatment was found to be the best pretreatment method for rice husk to produced citric acid by *A. niger* in solid state fermentation (Table 4.2). Dilute acid hydrolysis can be an effective pretreatment to recover the sugars from the hemicellulosic part of the material, to improve further lignin separation, and to produce partially pure cellulose (Soccol *et. al.*, 2011). Dilute alkaline treatment of lignocellulosic material causes rupture of structural linkages between lignin and carbohydrates of hemicelluloses and disruption of the lignin structure, leading to a decrease in the lignin polymerization degree, a decrease in cellulose crystallinity, and a separation of the hemicellulose sugars (Soccol *et. al.*, 2011).

Delignification of lignocellulose can also be achieved by treatment with an oxidizing agent such as hydrogen peroxide. The effectiveness in delignification can be attributed, to the high reactivity of oxidizing chemicals with the aromatic ring. Thus, the lignin polymer will be converted into e.g. carboxylic acids. Since these acids formed will act as inhibitors in the fermentation step, they have to be neutralized or removed. In addition to an effect on lignin, oxidative treatment also affects the hemicellulose fraction of the lignocellulose complex. A substantial part of the hemicellulose might be degraded and can no longer be used for sugar production (Soccol *et. al.*, 2011).

The work of Khosravi-Darani, and Zoghi, (2008) reported that, urea pretreatment was the most influential support for citric acid productivity from sugarcane bagasse, which under optimum growth condition yield of 137.6 g citric acid/kg urea-pretreated dry bagasse (96% yield based on the amount of sugar consumed) was obtained.

Masoud *et. al.*,(2013) reported that among the pretreatments implemented on sugarcane bagasse, pretreatment with sodium hydroxide produced the best result in such manner that citric acid production increased from 75.45 g/kg to 97.81 g/kg.

Mostafa and Alamri (2012) reported that maximum production of citric acid (56.7g/l) was obtained from date syrup treated with 1.5% tricalcium phosphate. Roukas and Kotzekidou (1997), Mehyar *et. al.*, (2005) and Lotfy *et. al.*, (2007) reported similar findings. They found that the pre-treatment of date syrup with tricalcium phosphate reduced significantly the concentrations of a number of heavy metal ions which might retard the use of molasses or affect the formation of citric acid by fungal cells.

The Plackett-Burman design (PBD) is a statistical method used for screening the media components and finds the most significant influence on factors in a minimum number of experiments. This design only considers on the main effect of these variables, but not their interacting effects (Plackett and Burman, 1946).

In the present study, the nutrient contents of the media are shown to have an important role in the fermentation of citric acid (Figure4.1). Plackett-Burman design (PBD) was used for screening the parameters (nutrients) (Table 4.1). The *p*-value was considered as a tool for evaluating the significance of each of the coefficients. The parameters with confidence levels greater than 95 % were considered as influencing the response significantly. It was found that $(\text{NH}_4)_2\text{SO}_4$, NH_4NO_3 ,

KH_2PO_4 , CuSO_4 , and Molasses contributed positively in various proportion while $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, FeSO_4 , MnSO_4 , and ZnSO_4 , contributed negatively in various proportion for oxidative pretreated rice husk. Similarly, NH_4NO_3 , KH_2PO_4 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were found to be the most significant nutrient for citric acid production at $p < 0.05$ using paddy straw as substrate (Ali and Zulkali 2011).

One-factor-at-a time (OFAT) approach was used to examine one parameter while holding all other parameter constant.

Nitrogen source either in form of organic (e.g. Yeast extract, Peptone, Urea) or inorganic (NH_4NO_3 , NaNO_3 , NH_4SO_4 etc.) are required for enhanced citric acid production. Generally, microorganism need nitrogen for growth and metabolism of most primary metabolites. Raja, and Kruthi (2013) reported that Nitrogen constituent has a profound effect on citric acid production because nitrogen is not only important for metabolic rates in the cells but it is also a basic part of cell proteins. Thus limitation of nitrogen was necessary to produce citric acid by *A. niger* which produced citrate from carbohydrates only under nitrogen deficient conditions. Also, they found that a higher concentration of nitrogen led to the formation of oxalic acid which, ultimately, reduced the yield of citric acid as reported by Maddox and Brooks (1995).

Presence of NH_4NO_3 concentration at 0.20% (w/v) gave maximum production of citric acid in the present study (Figure 4.3). A gradual increase in NH_4NO_3 concentration in the fermentation medium led to a fall in citric acid levels. Less than 0.20% (w/v) of NH_4NO_3 also led to a decline in citric acid production, the optimum concentration of 0.20% (w/v) gave the citric acid yield of 2.841 ± 0.003 g/Kg RH.

Similarly, Raja and Kruthi (2013) reported the concentration of 0.25% (w/v) NH_4NO_3 as maximum for production of citric acid. A gradual increase in its concentration in the

fermentation medium led to a fall in citric acid levels. Supplementing less than 0.25% (w/v) of NH_4NO_3 also led to a decline in citric acid production, which finally reached the control value (30g CA per Kg of Oat bran). Masoud, *et. al.*, (2013) reported that 0.1% ammonium nitrate was the optimal concentration for the production of citric acid (sugarcane baggase) which was 67.1 g/l CA under optimal condition. Increasing the nitrogen concentration over 0.1% resulted in a decrease in the production of citric acid production but increased the sugar consumption which could have been as a result of fungal growth. These result was in agreement with the findings of Demirel *et al.* (2005); Ganne *et al.* (2008) and Kareem *et al.*, (2010). Bayraktar and Mehmetoglu, (2000) reported that reduced production of citric acid at higher concentrations of nitrogen might have been due to the extensive mycelium formation on the surface of the pellets, which might have clogged the pores and then, decreased the production rate of citric acid.

Potassium and phosphate in KH_2PO_4 were considered to be growth-enhancing nutrients and buffering agents which maintained the desired values of pH. Therefore, a reasonable amount should be used in order to keep the pH of the substrate within the desired ranges (Imandi *et. al.*, 2008; Ali *et. al.*, 2012). On the other hand, Mourya and Jauhri (2000) and Zhang and Roehr (2002) reported that the limitation of phosphorus could have a beneficial effect on the yield of citric acid, whilst the high concentrations of phosphate led to a decrease in the fixation of carbon dioxide which, in turn, increased the formation of certain sugar acids.

From the present study the highest production of citric acid was obtained by the addition of 3.0 g/L of KH_2PO_4 to the production medium (Figure 4.4). This is in contrast with the findings of some researchers such as Haq *et. al.*, (2001) who reported that maximum production of citric acid was achieved at 0.1% KH_2PO_4 , when rice straw was used as a carbon source while Mostafa and Alamri (2012) reported that only 0.001% KH_2PO_4 , when date syrup was used as a carbon

source. This variation in concentration of KH_2PO_4 indicated that if the substrate is rich in phosphorus containing compounds and needed rarely to be supplemented with a source of phosphorus.

Molasses concentration of 0.220 g/L was found to be the optimum concentration for citric acid production in the present study. Grewal, and Kalra, (1995) reported that Molasses is preferably used as the source of sugar for microbial production of citric acid due to its relatively low cost and high sugar content (40 – 55 %). Kristiansen *et. al.*, (1999) reported that heavy metals such as iron, zinc and copper in high concentrations, can cause critical problems during the fermentation of citric acid. They inhibit the growth of microorganisms, influence the ionic strength, and the pH of the medium involved in the inactivation of the enzymes associated with citric acid metabolism in the citric acid cycle.

The face centered central composite design (FCCCD) under the response surface methodology (RSM) was employed in order to illustrate the nature of the response surface in the experimental region and elucidate the optimal conditions of the most significant independent variables (Rashid *et. al.*, 2009). Three major variables namely NH_4NO_3 , KH_2PO_4 and molasses concentrations were included in this model. The factors were examined at three different levels (low, basal, high) coded (-1, 0, +1). The Coefficient of determination R^2 was found to be 0.9790 with Adjusted R^2 of 0.9601 and Predicted R^2 of 0.8888 with Adequate Precision of 26.289 which measures signal to noise ratio. The analysis of variance (ANOVA) for citric acid after fermentation was used to estimate the citric acid response as a function of NH_4NO_3 , KH_2PO_4 and molasses. Mathematically, when the Prob > F values greater than 0.05, those factors are considered statistically insignificant. Meanwhile when Prob > F values less than 0.05, it could be defined as a significant factor (Rashid *et. al.*, 2009). In present study, the quadratic model with 0.0001 was

significant. The “lack-of-fit” F-value of 4.28 implies that the “lack-of-fit” was insignificant relative to the pure error. In general, the non-significant lack-of fit was good and implied that all data in this experiment were adequate as well as good predictability of the model (Rashid *et. al.*, 2009).

The highest production of citric acid from lignocellulosic substrates which have been obtained by different researchers were 259 g/kg from corn husk after 5 days of fermentation (Hang and Woodams, 2000), 264 g /kg from carob pod after 12 days of fermentation (Roukas, 1999), 150 g/ kg from coffee husk after 3 days of fermentation (Shankaranand and Lonsane, 1994), 206 g/ kg from sugarcane bagasse after 9 days of fermentation (Kumar and Jain, 2003), 62 g /kg after 9 days of fermentation (Kumar and Jain, 2003) and 49.21 g/ kg after 2 days of fermentation from wheat bran (Shankaranand and Lonsane, 1994). All these observations were obtained by the solid state fermentation with optimum media and process conditions. Therefore, the present study shows the production of citric acid of 4.765g/ kg RH obtained from Hydrogen peroxide pretreated rice husk after 5 days of incubation and optimize growth conditions.

A set of experiment was performed to verify the optimization result to validate the developed model. Medium was composed with the parameters of the independent variables (Table 4.6). The highest citric acid of 4.765g/ kg RH, was obtained in the optimum condition, which is slightly lower than the predicted value, 4.856g/ kg RH It is evident from this study that the production of citric acid was optimized at around 4.765 ± 0.001 g/Kg RH with the medium constituent concentration of NH_4NO_3 , KH_2PO_4 and molasses of 2.5g/L, 3.25g/L and 0.2275g/L respectively.

The citric acid produced in the present study has similar physicochemical properties (retention factor, FTIR peaks, and melting points) with standard citric acid, this indicates that the citric acid

produced from H₂O₂ pretreated rice husk could be up to standard in usage after finishing polishing and formulation.

The melting points of the samples were determined in relation to the melting temperatures. The standard citric acid has melting temperature of 153±0.00°C while the produced citric acid melting temperature was 153±0.02°C (Table 4.8).

The R_f value of both standard and produced citric acid was found to be 0.242 in 3:1:1 *n*-Butanol Acetic Acid and Water (BAW) as Mobile Phase. Similarly Patit and Patit (2014) reported the R_f value of standard citric acid as 0.35 and produced citric acid using corn starch as substrate to be 0.36 in 1:1 *n*-Butanol water as mobile phase.

FTIR is particularly useful for identification of organic molecular groups and compounds due to the range of functional groups, side chains and cross-links involved, all of which will have characteristic vibration frequencies in the infra-red range (Saravanan and Vijayakumar, 2012). The wave numbers corresponding to O - H and C = O of standard citric acid was found to be 3338.7 cm⁻¹, 1565.5 cm⁻¹ while that of rice husk produced citric acid was 3324.8 cm⁻¹, 1625.1 cm⁻¹ respectively (Table 4.7).

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1 Summary

The major findings of this work can be summarized as follows;

1. The result obtained from this study indicated that rice husk can produce citric acid under optimum nutrients condition.
2. hydrogen peroxide pretreated rice husk was the best pretreatment method for rice husk with citric acid yield of 3.65 ± 0.26 g/kg RH which is statistically higher ($p < 0.05$) than that of acid, alkaline and un-pretreated rice husk whose yields were 2.94 ± 0.83 , 1.97 ± 1.24 and 2.34 ± 0.13 g/kg RH respectively..
3. Optimum concentrations of nutrient component (NH_4NO_3 , KH_2PO_4 , and Molasses) required for citric acid production from rice husk using Central Composite Design of

Response Surface Methodology were found to be 2.5g/L, 3.25g/L and 0.2275g/L respectively with citric acid yield of 4.765 ± 0.001 g/Kg RH

4. Model of the optimized conditions for rice husk in the production of citric acid was developed as Citric acid yield = $+ 0.99 + 0.090 A + 0.047 B + 0.071 C + 0.098 A^2 - 0.076 B^2 - 0.070 C^2 - 0.039 AB + 0.032AC + 0.039 BC$.
5. Both the standard and produced citric acid by *A. niger* gave similar Rf value of 0.242 and Melting point of 153 ± 0.00 °C and 153 ± 0.02 °C, respectively. Also wave numbers corresponding to O-H and C=O of 3338.7 cm^{-1} and 1565.5 cm^{-1} and 3324.8 cm^{-1} and 1625.1 cm^{-1} for standard and produced citric acid, respectively.

6.2 Conclusion

The present study has shown a great potential for utilizing rice husk as a substrate for the production of citric acid by *A. niger*. Rice husk is an abundant agricultural waste material that could be used as a cheap alternative substrate for the production of citric acid due to its availability, affordability and citric acid production potential. Hydrogen peroxide pretreatment was found to be the best pretreatment method for rice husk to produce citric acid. Optimization studies revealed that NH_4NO_3 , KH_2PO_4 , and Molasses were the main nutrient component required for citric acid production using rice husk at the concentrations of 2.5g/L, 3.25g/L and 0.2275g/L, respectively. Polynomial equation was derived. Thin Layer chromatography, melting point determination and FTIR analysis were carried out to characterize citric acid produced.

6.3 Recommendations

1. Since Rice husk can served as a substrate for citric acid production as revealed by the present study, it was recommended therefore, that an improved strain of *A. niger* should be used to enhanced a better citric acid yield.
2. Upscaling of production of citric acid in a bioreactor using rice husk need to be explored which will help in developing a cost effective process for wider utilization of citric acid in various industrial application.
3. Other process parameters including temperature, pH, aeration, O₂ uptake ratio etc. should also be optimized to enhance better citric acid production.

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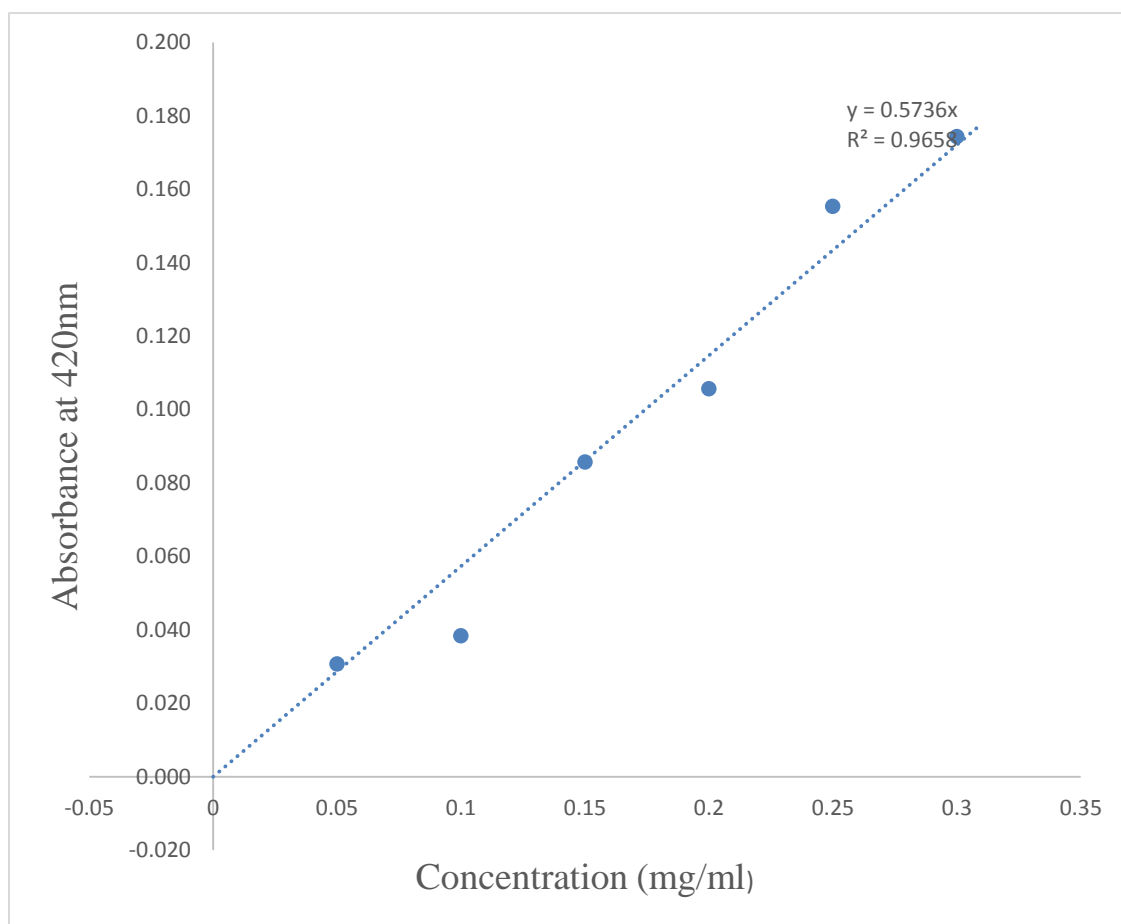
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APPENDICES

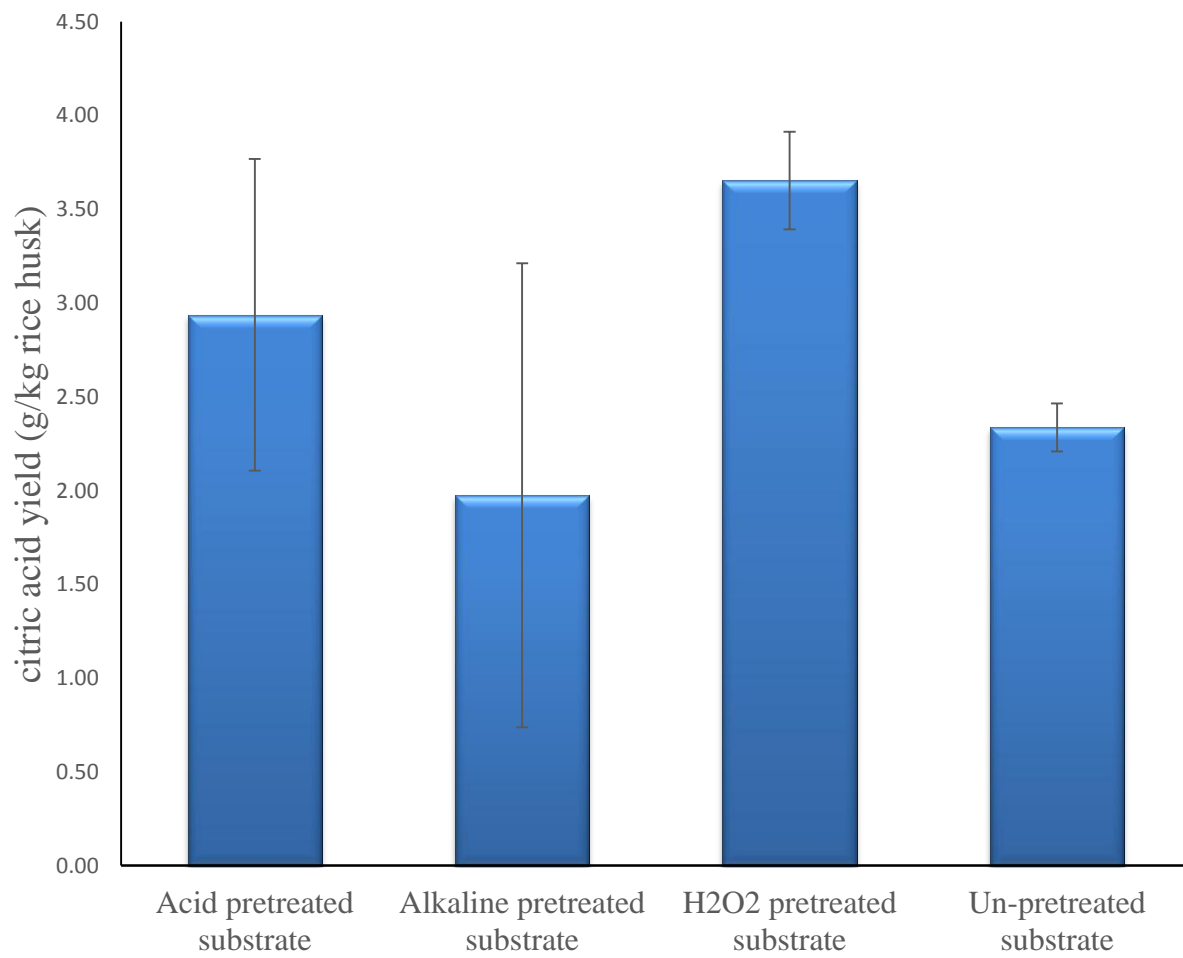
Appendix 1 Preparation of Citric Acid Standard

Citric acid standard was prepared from citric acid monohydrate by dehydrating it for three days at 90°C to get anhydrous citric acid. Anhydrous citric acid obtained by heating the monohydrate at 90°C to constant weight about 72 hours was used to prepare a stock solution (50 mg/ml) which is stable for at least one year at 0°C. Standards prepared from the stock solution are stable for one month at 0°C. Using dilution rule, $M_1V_1 = M_2V_2$. Concentration of 0.3 mg/ml, 0.25 mg/ml, 0.2 gm/ml, 0.15 gm/ml, 0.1 mg/ml and 0.05 gm/ml citrate solution were made. 60 µl, 50 µl, 40 µl, 30 µl, 20 µl and 10 µl per 10 ml distilled water were used respectively (Feleke, 2010).

Appendix 2 Citric Acid Standard Curve



Appendix 3 Duncan Multiple Range Test for Determining Best Pretreatment Method for Citric Acid Production



Appendix 4. Various Concentration of $(\text{NH}_4)_2\text{SO}_4$ at Fixed Concentration of Other Contributing Factors.

$(\text{NH}_4)_2\text{SO}_4$ Concentration g/L	Citric acid yield (g/kg) of Rice Husk \pmSD
1.5	1.541 \pm 0.006
2.0	1.832 \pm 0.007
2.5	1.543 \pm 0.004
3.5	1.183 \pm 0.005

Appendix 5. Various Concentration of NH_4NO_3 at Fixed Concentration of other Contributing Factors.

NH_4NO_3 Concentration g/L	Citric acid yield g/Kg Rice Husk \pm SD
1.0	0.939 \pm 0.006
1.5	1.223 \pm 0.001
2.0	2.841 \pm 0.003
3.0	1.801 \pm 0.006

Appendix 6 Various Concentration of KH_2PO_4 at Fixed Concentration of other Contributing Factors

KH_2PO_4 Concentration g/L	Citric acid yield g/Kg Rice Husk \pm SD
1.0	2.664 \pm 0.007
2.0	2.864 \pm 0.002
3.0	3.731 \pm 0.027
4.0	3.359 \pm 0.027

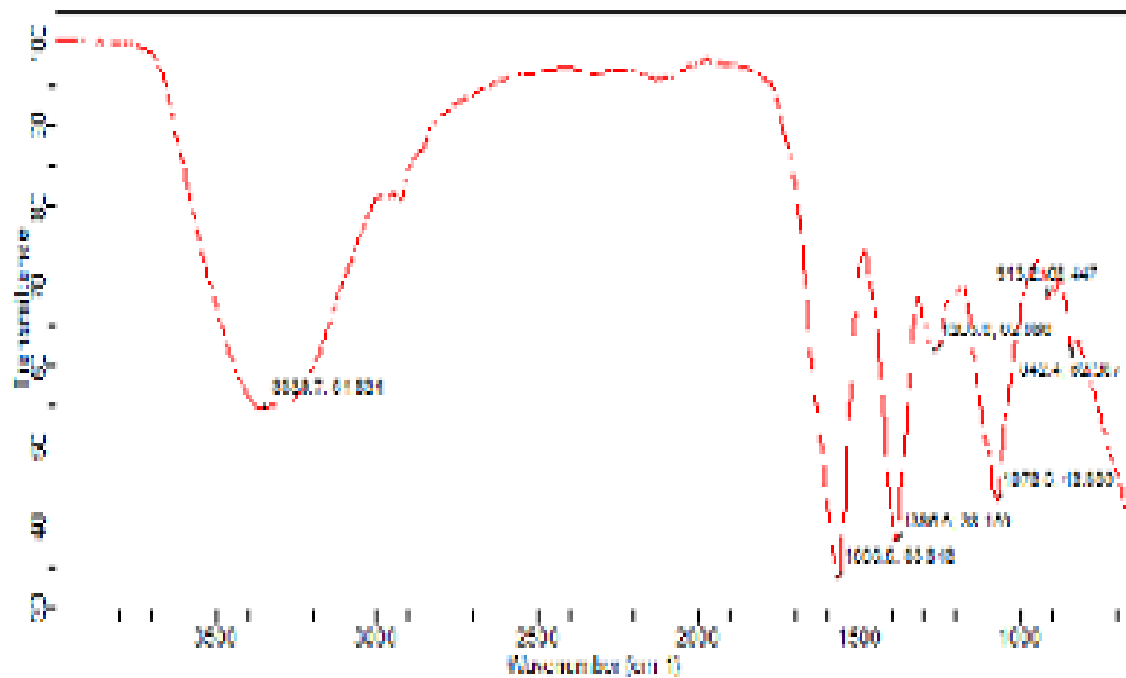
Appendix 7 Various Concentration of Molasses at Fixed Concentration of other Contributing Factors

Molasses Concentration×10⁻³ g/L	Citric acid yield g/Kg Rice Husk ±SD
120	1.771±0.002
160	3.138±0.046
200	5.595±0.002
220	6.051±0.010
240	4.796±0.057

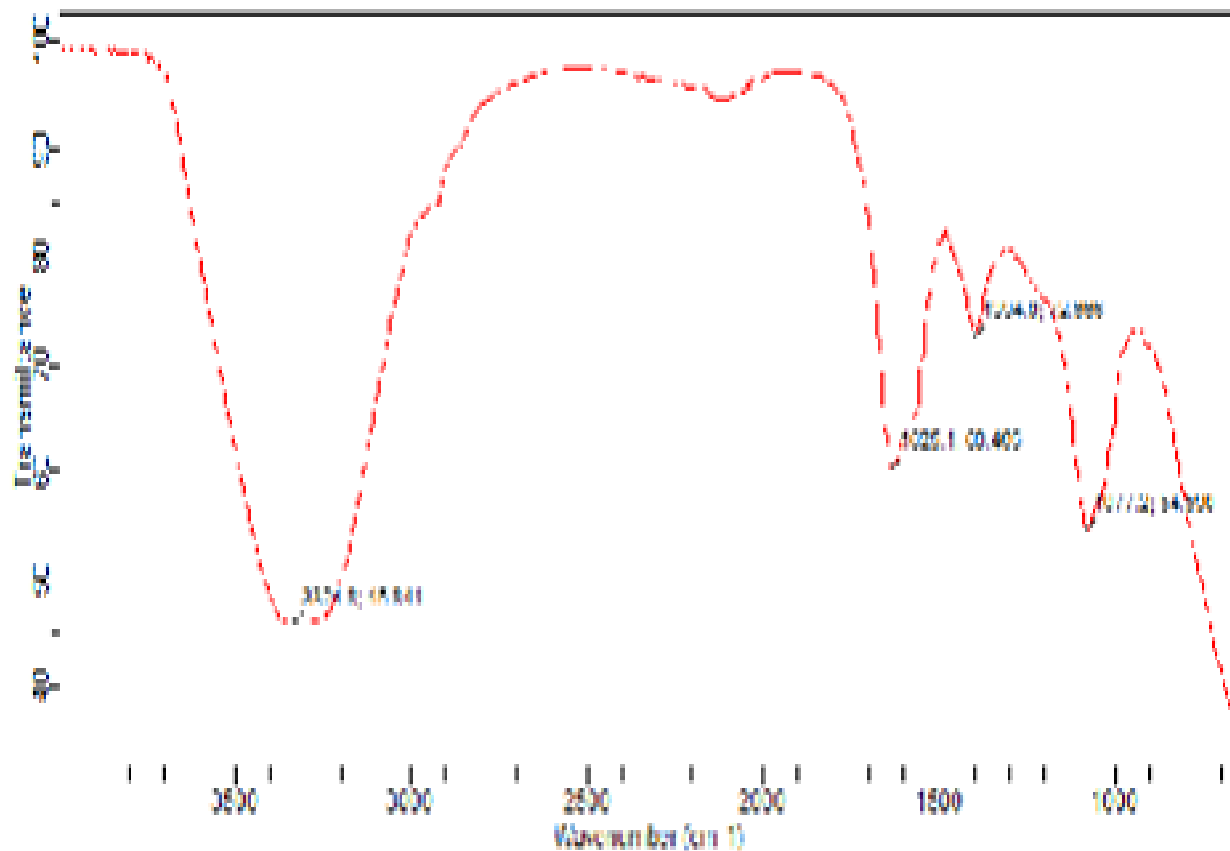
Appendix 8 Various Concentration of CuSO₄ at Fixed Concentration of other Contributing Factors

CuSO₄ Concentration×10⁻³ g/L	Citric acid yield g/Kg Rice Husk ±SD.
0.4	1.839±0.006
0.5	2.585±0.005
0.7	4.384±0.010
0.9	4.003±0.004

Appendix 10: Fourier Transform Infra-Red Spectrum of Standard Citric Acid



Appendix 11: Fourier Transform Infra-Red Spectrum of Microbial Produced Citric Acid Using Hydrogen Peroxide Pretreated Rice Husk.



Appendix 12: Contribution to Knowledge

1. These findings therefore validate the potential of using rice husk for citric acid production.
2. Best pretreatment for rice husk to produced citric acid was determined.
3. Model for the optimize conditions was developed.