



ADDIS ABABA UNIVERSITY
ADDIS ABABA INSTITUTE OF TECHNOLOGY
SCHOOL OF CHEMICAL AND BIO ENGINEERING

**Utilization of Banana Peel for Citric Acid Production on
Solid-State Fermentation Using *Aspergillus Niger***

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This is to certify that the thesis prepared by Solomon Kahsay, entitled: *Utilization of Banana Peel for Citric Acid Production on Solid-State Fermentation Using Aspergillus Niger* and submitted in partial fulfillment of the requirement for the degree of Master of Science (Chemical and Bio Engineering) complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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I declare that this thesis entitled “*Utilization of Banana Peel for Citric Acid Production on Solid-State Fermentation Using Aspergillus Niger*” has not been submitted in any form for another degree, diploma or an award at any university or other institution of the tertiary education. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature and discussions. Information taken from published and unpublished work of others has been acknowledged in the text and a list of references is given. The work was under the guidance of Professor Eduardo Ojito instructor in Addis Ababa University, School of Chemical and Bio Engineering.

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ABSTRACT

*Citric acid is a tricarboxylic acid which is soluble in water with a pleasant taste. It is an important acid used in food Industries. It exists in nature when carbohydrates are oxidized to carbon dioxide. Because of its high solubility, palatability and low toxicity it can be used in food, biochemical, and pharmaceutical industries. The aim of this study is to produce citric acid by *Aspergillus niger* using banana peel as a substrate and to evaluate its quality and concentration. In this study, the utilization of banana peel to use as a raw material for citric acid production under different fermentation conditions with solid state fermentation method in the laboratory using 250 mL Erlenmeyer flask as the small-scale laboratory fermenter was investigated. Design of experiment (DOE) for response surface methodology (RSM) was used to analyze and optimize the simultaneous effect of solid substrate loading (18g, 36g, and 54 g), incubation time (3days, 6days and 9days) and fermentation temperature (25 °C, 30 °C and 35 °C) on citric acid production. The minimum citric acid was found to be 28.39 g/L at 36 g, 3 days, and 25 °C. A maximum citric acid yield of 45.55 g/l was achieved under optimal experimental conditions (36 g, 6 days and 30 °C). A three-variable, three-level Box-Behnken design was used to develop a statistical model to describe the relationship between citric acid concentration and the chosen independent variables and to optimize the production of citric acid using RSM. The model was statistically significant ($p < 0.0001$) with a low standard deviation (0.66). Besides, it did not show lack of fit ($R^2 = 0.9948$) and the Pred R-Squared of 0.9353 is in reasonable agreement with the Adj R-Squared of 0.9880. Citric acid production was significant at moderate levels of solid loading, incubation time and fermentation temperature. From the model regression equation developed, the linear terms of temperature and time had a positive effect and a substrate loading had a negative effect on response yield. A maximum citric acid yield of 45.59 g/L was also achieved under selected optimum values (31.92 g, 5.82 days and 31.55 °C) with high value of combined desirability. Validation of the statistical model showed an insignificant difference between experimental and model-predicted results.*

Keywords: Citric acid, *Aspergillus niger*, Banana peel, Substrate, Solid State Fermentation

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ACRONYMS

AAS	Atomic Absorption Spectroscopy
<i>A. niger</i>	<i>Aspergillus niger</i>
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
ATCC	American Type Culture Collection
BBD	Box -Bhenken Design
df	Degree of Freedom
DOE	Design of Experiment
EPHL	Ethiopian public Health Institute
FAAS	Falme Atomic Absorption Spectroscopy
FTIR	Fourier Transform Infrared Spectroscopy
HPLC	High Performance Liquid Chromatography
KHP	Potassium Hydrogen Phyhalate
PDA	Potato Dextrose Agar
Ppm	Parts Per Million
rpm	Revolution per Minute
RSM	Response Surface Methodology
<i>S.lipolytica</i>	<i>Saccharomycopsis Lipolytica</i>
SmF	Submerged Fermentation
SNNPRS	Southern Nations Nationalities and Peoples' National Regional State
SSF	Solid State Fermentation
TCA	Tricarboxylic Acid

1. INTRODUCTION

1.1 Background

Citric acid (2-hydroxypropane-1, 2, 3-tricarboxylic acid, $C_6H_8O_7$) is a tricarboxylic acid (TCA) with a molecular weight for anhydrous and monohydrate is 192 and 210g/mol respectively. It occurs as a natural constituent in citron, lime, pineapple, pear, peach and similar fruits. It is also found in animal tissues (Abbas et al., 2016). Citric acid is an intermediate in the TCA cycle and therefore is ubiquitous in living organisms (B. Journal et al., 2005). The popular forms of citric acid are the anhydrous forms, the monohydrate and sodium salts of the acid. At the end of the nineteenth century, it has been known as a natural plant substance (Kumar et al., 2003). It is soluble in water with a pleasant taste and the most important acid used in the food industries (Dhandayuthapani et al., 2008). It is solid at room temperature, melts at 153 °C, and decomposes at higher temperatures (Maharani et al., 2014). It also is responsible for the tart taste of various fruits in which it occurs, e.g., lemons, limes, oranges, pineapples, and gooseberries (Ciriminna et al., 2017).

Citric acid is an important commercial product, its global production has reached to 1.7 million tons per year and its annual increasing growth rate is 5% (Faisalabad & Faisalabad, 2013). Considerable amounts of citric acid are required in several industrial processes. Approximately 70% of citric acid produced is used in the food and beverage industry as pH adjuster, flavor enhancer, a preservative in processed food and as an acidulant in drinks (Dhandayuthapani et al., 2008). Pharmaceutical industry consumes 12% of citric acid to enhance flavors of medicines, as an acidulant and anti-coagulator, Cosmetic industry uses citric acid in the composition of different cosmetic products (Dhillon et al., 2010). Citric acid has also found other application such as metal cleaning, electroplating, fabric dyeing; detergent etc. (Faisalabad & Faisalabad, 2013).

Citric acid production can be carried out by different groups of microorganisms. Although yeast, bacteria and other *Aspergillus* species can produce citric acid, *Aspergillus niger* (*A. niger*) remained the organism of choice for commercial production, due to ease of handling, its ability

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to ferment a variety of cheap raw materials and produces more citric acid per time unit (Kishore et al., 2008).

A cost reduction in citric acid production can be achieved by using cheap agricultural wastes (Rahman, 2013). It is possible to produce citric acid by bacteria, however, not able to produce commercially acceptable yields due to the fact that citric acid is a metabolite of energy metabolism and its accumulation rises in appreciable amounts only under conditions of drastic imbalances (Yalcin et al., 2010).

The problem in the production of citric acid by yeast is the simultaneous formation of isocitrate (Show et al., 2015a). Recently, a wide range of citric acid production has been reported in response to different levels of nutrient supplementation under different articles. *A. niger* is capable of producing very high levels of citric acid, about 90% of the theoretical yield from a carbohydrate source.

A high rate of acidogenesis in *A. niger* is observed only under conditions of high glycolytic metabolism and can be induced by the addition of an excess amount of sucrose or other carbohydrates (Ali, 2002). Citric acid accumulation is known to be stimulated by high sugar concentration suggesting it may be influenced by osmotic stress (Ali & Haq, 2014).

The production of citric acid using cheap carbon source from agro-industrial byproducts provides considerable advantage combined in the benefit of waste management as well as a decrease in the cost of citric acid production. The use of alcohols as a stimulant in citric acid production may enhance the yield of citric acid. The stimulatory effect of ethanol and methanol on citric acid production can be explained in terms of mycelia morphology as well as pellet shape and size. Citric acid production could be increased by exploiting available resources and adding the stimulatory agents to the fermentation medium (Amenaghawon et al., 2015).

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Citrate accumulation in substantial amounts depends on certain environmental parameters. It is accumulated when several nutrient factors are present. Two of these factors are the concentration and type of sugar. The production of citric acid also depends strongly on an appropriate strain and on operating conditions such as aeration, type, and concentration of the carbon source, nitrogen and phosphate limitation, pH, the concentration of trace elements and the morphology of the producer organism (Amenaghawon et al., 2015).

To date, most industrial processes are carried out with submerged fermentation of *A. niger* (Barrena et al., 2017). Although citric acid can be obtained by chemical synthesis, the cost is much higher than using fermentation. It is mainly produced by submerged fermentation by the filamentous fungus *A. niger*. *A. niger* has been used commercially for the first time in 1923 for citric acid production. In order to decrease the cost of citric acid production using *A. niger*, solid-state fermentation has been studied as a potential alternative to submerged fermentation (Gupta & Sharma, 2002). There are a lot of studies reporting the use of filamentous fungi, mainly chosen in solid-state fermentation (SSF) because of their ability to produce thermostable enzymes of high commercial value (Barrena et al., 2017).

SSF, also known by « Koji » process, was first developed in Japan where abundant raw materials such as fruit wastes and mainly rice bran are available. It has been an alternative method for using agro-industrial residues. Solid state culture is characterized by the development of microorganisms in a low-water activity environment on an insoluble material that acts both as physical support and source of nutrients. The major advantages of SSF over submerged fermentation include higher yields, low water requirement and lower operating costs (Vandenberghe et al., 1983).

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Generally, the substrate in SSF is solid and it is moistened to about 70% moisture, depending on the substrate absorption capacity. The initial pH of the material is normally adjusted to 3.0 – 6.0 and the temperature of incubation is about 28 – 30 °C, depending on the microorganism used. The solid culture process is completed within 96 – 120 hours under optimal conditions (Atere, 2013).

The decline in concentration of citric acid higher than the optimum period may have been due to decay in the enzyme systems responsible for the production of citric acid upon exhaustion of fermentable sugars, or could be attributed to citric acid break down due to the toxic effect of accumulated waste product (Vandenberghe et al., 1983) .

Since it uses agro-industrial residues that are cheaper sources of substrates, solid state fermentation process is especially ideal for developing countries like Ethiopia. A cost reduction in citric acid production can be achieved by using cheap agricultural wastes such as banana peel and grape pomace, orange peel, kiwi fruit peel, cotton waste, okara soy-residue and cane molasses.

Dessert banana and plantain (*Musa* sp.) are the fourth most important staple food crops in the world after rice, wheat, and maize. Dessert banana, in particular, is a commercially important crop in the global trade, both by volume and value, as a leading fruit. In 2010, world commerce in banana was valued at US 8.05 billion and the total world production of banana is about 106,541,709.00 tons. For many African, Asian and Latin American countries, banana is as well one of the most important crops for foreign exchange earnings (Woldu et al., 2015).

Dessert banana is also the major fruit crop that is most widely grown and consumed in Ethiopia. It is cultivated in several parts where the growing conditions are favorable. Especially in the south and southwestern parts of the country, it is of great socioeconomic importance contributing significantly to the overall well-being of the rural communities including food security, income generation, and job creation. Banana in Ethiopia covers about 59.64% (53,956.16 hectares) of the total fruit area, about 68.00% (478,251.04 tones) of the total fruits produced, and about 38.30% (2,574,035) of the total fruit producing farmers. On the other hand, about 68.72% (37,076.85 hectares) hectares of land covered by banana, about 77.53% (370,784.17 tones) of the banana

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produced and 22.38% (1,504,207) of the banana producers in Ethiopia are found in the Southern Nations Nationalities and Peoples' National Regional State (SNNPRS). Gamo-Gofa, Bench-Maji and Sheka zones are among the major banana producing zones of the SNNPRS, of which Gamo-Gofa zone alone covers over 70% of the total banana marketed across the major market outlets in Ethiopia (Woldu et al., 2015).

The major commercial cultivars grown by small-scale growers across the survey areas are Dwarf Cavendish, Giant Cavendish, and Poyo, with Williams and Grand Naine recently coming into the picture in Gamo-Gofa zone and across the large-scale commercial farms of Ethiopia. Others like Robusta and Butuzua are also among the Cavendish banana cultivars recently released through Melkasa Agricultural Research Center. The rest are less popular landraces grown to a very limited extent in certain localities across the country (Woldu et al., 2015).



Figure 1-1: Local Banana Cultivars Grown in Different Parts of Ethiopia (Woldu et al., 2015)

Despite the above-stated facts and the concerted effort being made by the government of Ethiopia to promote and diversify its agricultural outputs as well as exports at large, the attention given to banana peels in terms of research, disposal method, and overall the banana wastes value-chain management has been very limited. In most parts of the country, banana peels are disposed of simply in the street which is easily rubbishing the environment.



Figure 1-2: Local Banana Peels Disposed in the Street

To minimize the environmental effect of banana peel, our country could utilize banana peel for the production of citric acid. According to Ethiopian central statistics agency (ECSA), Ethiopia is the country that imports citric acid from different countries. The amount of citric acid import from India, Iran, Saudi Arabia and USA increases from time to time following the growth of beverage, food, detergent and other factories. Although Ethiopia produces a large number of agricultural products and residues which are substrates for citric acid, it expends a lot of money in hard currency. Thus, there is an urgent need to find stable applications of these waste products.

1.2 Statement of the Problem

As explained in the background, banana in Ethiopia covers about 59.64% (53,956.16 hectares) of the total fruit area, about 68.00% (478,251.04 tones) of the total fruits produced, and about 38.30% (2,574,035) of the total fruit producing farmers, which means the people do cultivate and harness banana in different ways. Harnessing and daily consumption of banana in high amount do lead to banana peels. Daily disposal of these wastes usually represents a problem that is further aggravated by legal restrictions. The management of these wastes which produce odor and soil pollution, air pollution and other environmental problems, represents a major problem for the food industry and the nearby community.

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Generally, the generation of banana peel is dramatically increasing each year. The problems related to banana peel disposal have become more pronounced in recent years due to the rapid pace of development in our country. Most of the banana peel is generated from household, agricultural areas and industrial areas, etc. which are normally used as animal feed, incinerated or go to landfill sites. However, incineration is an expensive disposal method and causes air pollution, while in landfills the banana peel is broken down by microorganisms and forms leachate that can contaminate the groundwater. In addition, the degradation of banana peel in these conditions produces methane, which is 25 times more harmful greenhouse gas compared to carbon dioxide. Incorrect waste management practices can result in public health and environmental problems, regarding issues such as odor and diseases. Nevertheless, banana peels are comprised of materials rich in sugars, minerals, and proteins that could be used for other processes as substrates or raw materials. Since the cultivation of microorganism requires carbon, nutrient, and moisture, banana peel could be a good candidate to provide the appropriate conditions for the development of microorganisms. Thus, new aspects concerning the use of these wastes as by-products for further exploitation on the production of food additives or supplements with high nutritional value have gained increasing interest because these are high-value products and their recovery is economically attractive.

Finally, the rationale of this study is to investigate the potential of the most important by-products of banana peel processing as a source of valuable compounds particularly citric acid, and awareness of disposal of banana wastes and its packaging. In this view, SSF is presented as a promising technology for waste valorization through the bioconversion of banana peel used as a substrate, whilst *A. niger* has played a role in the degradation of banana peel into its constituents, and then convert them into high value-added byproducts. SSF is showed sustainable characteristics in the bioconversion of solid wastes that have been proved to be able to give high efficiency in terms of product yields and productivities, low energy consumption, and solving disposal problems.

1.3 Objective

1.3.1 General Objective

The general objective of the study is to utilize banana peel for the production of citric acid using *A. niger* on solid-state fermentation under different fermentation time, temperature and substrate levels.

1.3.2 Specific Objectives

The specific objectives of this research are:

- To characterize the physical and chemical composition of the banana peel
- To study the effect of substrate loading, fermentation time and temperature on citric acid production
- To determine the optimum operating conditions which maximize citric acid production
- To characterize the quality of the product

1.4 The significance of the Study

- ❖ The research would able to play an important role by providing a remedy for the environmental pollution due to the disposal of banana peel.
- ❖ If it is scaled up to large industry, it could create job opportunity and save foreign currency.
- ❖ The import of citric acid could be minimized.

1.5 The scope of the Study

The study was performed at laboratory scale. The thesis work generally covers fermenting organism (*A. niger*) and inoculum preparation, banana peel characterization, production of citric acid using banana peel by *A. niger* and characterization of produced citric acid . It was done using standard procedures and test methods.

2. LITERATURE REVIEW

2.1 History of Citric Acid

Citric acid was first isolated by Karls Scheels in 1874, in England, from the lemon juice imported from Italy (El-holi & Al-delaimy, 2003). Italian manufacturers had a monopoly for its production for many years, and it was sold at high cost. This led to extensive attempts all over the world to investigate an option for its production, which included chemical and microbial techniques. In 1893, Wehmer observed the presence of citric acid as a byproduct of calcium oxalate produced by a culture of *Penicillium glaucum* (Show et al., 2015b). Other investigations showed the isolation of two varieties of fungi belonging to genus *Citromyces* (namely *Penicillium*). However, industrial trials did not succeed due to contamination problems and long duration of fermentation.

It was the work of Currie which opened up the way for the successful industrial production of citric acid (Shetty, 2015). The industrial process was first opened by Currie, in 1917, who found that *A. niger* had the capacity to accumulate significant amounts of citric acid in the sugar-based medium. He also showed that high concentrations of sugar favored its production, which occurred under the limitation of growth. In the thirties, some units were implanted in England, in the Soviet Union, and in Germany for the commercial production. However, the biochemical basis was only cleared in the fifties with the discovery of the glycolytic pathway and the TCA cycle. Consequently, an improved process employing submerged fermentation was developed in United States (Vandenberge et al., 1983).

The submerged fermentation (SmF) process is the commonly employed technique for citric acid production. It is estimated that about 80% of world production is obtained by SmF (Yalcin et al., 2010). Several advantages such as higher yields and productivity and lower labor costs are the main reasons for this (Abbas et al., 2016). Two types of fermenters, conventional stirred fermenters, and tower fermenters are employed, although the latter is preferred due to the advantages it offers on price, size, and operation. Preferentially, fermenters are made of high-grade steel and require the provision of aeration system, which can maintain a high dissolved oxygen level (Yalcin et al., 2010). Fermenters for citric acid production do not have to be built as

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pressure vessels since sterilization is performed by simply steaming without applying pressure, Cooling can be done by an external water film over the entire outside wall of the fermenter (Parvez et al., 1998).

In SmF, different kinds of media are employed such as sugar and starch based media. Molasses and other raw materials demand pretreatment, addition of nutrients and sterilization. Inoculation is performed either by adding a suspension of spores, or of pre-cultivated mycelia. When spores are used, a surfactant is added in order to disperse them in the medium. For pre-cultivated mycelia, an inoculum size of 10% of fresh medium is generally required (Maghsoodi & Yaghmaei, 2010). Normally, SmF is concluded in 5 to 10 days depending on the process conditions. It can be carried out in batch, continuous or fed-batch systems, although the batch mode more frequently used. In recent years, the use of solid substrate fermentation has shown some potential as an alternative for the production of this organic acid (Bezalwar et al., 2013). Solid substrate fermentation involves the growth of microorganisms on moist solid substrates in the absence or near absence of free-flowing water.

2.2 Biochemistry of Citric Acid Production by *Aspergillus niger* (*A. niger*)

2.2.1 Formation of Citric Acid

The biochemistry of citric acid formation is complex. The production of citric acid is caused by the collective effect of different nutritional conditions in the medium (Show et al., 2015a). Nutritional conditions synergistically affect the production yield of citric acid, including carbon source concentration, dissolved oxygen, hydrogen ions, and suboptimal concentrations of phosphate and trace metals (Dhandayuthapani et al., 2008). Some studies report that limitation of the nitrogen source and deficiency of manganese or phosphate in the fermentation medium will inhibit the anabolism of *A. niger*, resulting in protein degradation which, in turn, leads to a higher concentration of ammonium ions (Parvez et al., 1998).

Excessive citrate can be produced by *A. niger* through an active glycolytic pathway (Figure 2-1). Citrate is a well-known inhibitor of glycolysis, thus making it an area of research interest. Under

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Protein breakdown occurs as a result of manganese deficiency and this leads to an increase in intracellular NH_4^+ concentration. The phenomenon is known as the ‘ammonium pool’ and it inhibits the enzyme phosphofructokinase, which is vital for the conversion of fructose and glucose to pyruvate. As a result, there is a flux through glycolysis and citric acid formation. High glucose and NH_4^+ concentrations will strongly limit 2-oxoglutarate dehydrogenase formation and eventually inhibit the catabolism of the citric acid present in the TCA cycle (Gupta & Sharma, 2002).

Show et al. (2015b) reported that the presence of an intracellular ammonium pool does not account for the inhibition of phosphofructokinase. Ammonium ions enter cells to form glucosamine by combining with glucose, rather than just being simply deposited inside the cell.

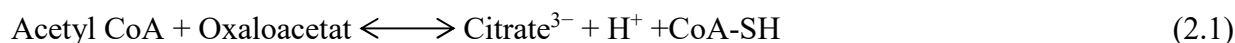
Thus, inhibition of the phosphofructokinase enzyme was found not to be a result of the ammonium pool. This is caused by several factors such as the release of synthesized glucosamine into the fermentation broth, the very low concentration of intracellular ammonium ions (about one-hundredth of the external pH) and the intracellular pH (Papagianni, 2007). Dhillon et al. (2010) recommended further investigation into the relationship of the enzymes phosphofructokinase, 2-oxoglutarate dehydrogenase and glucosamine synthase with the high levels of glucose and ammonium ions within the TCA cycle. The enzymes of *A. niger* act as the driving force in citric acid formation. These enzymes include invertase (a membrane-bound enzyme), which hydrolyzes sucrose into glucose and fructose, which are transported into the cell as the reaction pathway from sucrose to citric acid begins outside the cell. Hexokinase is more abundant than other enzymes in *A. niger*, and it has an affinity for glucose over fructose in a ratio of 1000:1. Citrate inhibits glucokinase non-competitively. Kumar et al. (2003) showed that mutation can be used to produce an *A. niger* strain with higher affinity for sucrose growth).

Glucose oxidase stimulates *A. niger* to oxidize glucose to gluconic acid. The enzyme is most potent under a high concentration of glucose, with strong aeration and low concentrations of other nutrients (typical conditions for citric acid fermentation). The effect of glucose oxidase is limited; however, as it is deactivated at pH lower than 3.5. The pH is reduced as a result of the accumulation of protons in the fermentation broth. It is not known whether the gluconic acid will be used later in the citric acid formation during fermentation. Phosphofructokinases (PFK1 and

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PFK2) are responsible for the phosphorylation of fructose-6-phosphate. PFK1 phosphorylates at the C₁ position to produce fructose-6-phosphate. PFK1 phosphorylates at the C₁ position to produce fructose-1,6-bisphosphate. High concentrations of ATP, citrate, and manganese inhibit PFK1, but it can be activated by the products of the PFK2 reaction, such as Zn²⁺, Mg²⁺, NH₄⁺ and fructose-2,6-bisphosphate (Majumder et al., 2010).

Citric acid is formed via the glycolytic reactions pathway. The glycolytic and pentose phosphate reaction pathways act as a channel for *Aspergillus* species to utilize glucose and other carbohydrates for biosynthesis and maintenance. Pyruvate kinase was previously found to be an essential regulatory stage in citric acid synthesis, but a study showed that the enzyme in its pure form was only marginally affected by the metabolic levels of inhibitors (Karaffa et al., 2001). Citrate synthase is a catalyst for the reversible condensation reaction between acetyl coenzyme A (acetyl CoA) and oxaloacetate. This reaction favors citrate production and, as a result, thioester hydrolysis occurs during the reaction (Majumder et al., 2010). The equation is shown below:



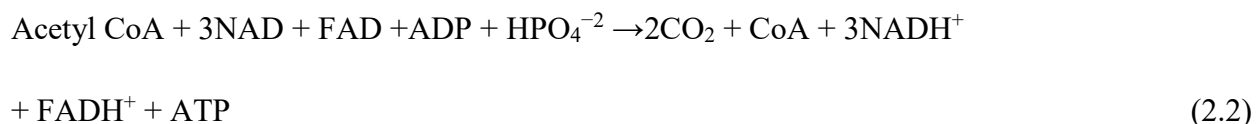
Three different isocitrate dehydrogenase isoenzymes exist in *A. niger*, which can be differentiated from each other by the recipient that they utilize for the removal of protons from isocitrate. These enzymes are known as NADP⁺-dependent forms (one exists in mitochondria and another in the cytoplasm) and NAD⁺-dependent isocitrate dehydrogenases (which exists only in mitochondria). The NAD⁺-dependent enzymes are found in lower quantities than the NADP⁺-dependent enzymes (Karaffa et al., 2001).

2.2.2 Accumulation of Citric Acid

In general, the build-up of citric acid involves deactivation of aconitase and/or isocitrate dehydrogenase. Activity in the Krebs cycle produces intermediates necessary for biomass formation during the formation of citric acid. The Krebs cycle is a series of eight reactions that take place in the mitochondrion. These reactions take a two-carbon molecule (acetate) and completely oxidize it to carbon dioxide (Show et al., 2015a).

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The cycle is summarized in the following chemical equation:



Therefore, the accumulation of citric acid probably results from enhanced (deregulated) biosynthesis instead of inhibited degradation. Parvez et al. (1998) explained the accumulation of citric acid by associating it with the tricarboxylate transporter competing for citric acid with aconitase. Under conditions where the tricarboxylate transporter has a greater affinity for citric acid than for aconitase, the enzyme removes citric acid from the mitochondria of the cell without inhibiting the enzymes present in the cycle.

Glutamine synthase is linked with the effects of pH, ammonia and manganese ions on the accumulation of citric acid. The low pH value during the Frontiers in Production phase ($\text{pH} < 2$) reduces the risk of contamination by other microorganisms and inhibits the production of unwanted organic acids (gluconic and oxalic acids), which facilitates recovery of the product. The presence of Mn^{2+} or Mg^{2+} ions in the enzyme structure determines its form. The isozymes of isocitrate dehydrogenase in *A. niger*, which are present in the mitochondria as well as the cytoplasm, require Mg^{2+} or Mn^{2+} for reaction, and their inhibition by α -ketoglutaric and citric acid ensures citric acid accumulation (Parvez et al., 1998).

Isocitrate undergoes a two-phase conversion process to become α -ketoglutarate. First, protons are detached to form oxalosuccinate. Secondly, carbon dioxide is formed and released, completing the conversion. The metal ions Mg^{2+} and Mn^{2+} are essential for the reaction, as they are attached to the active sites by these enzymes. They are responsible for the stabilization of oxalosuccinate (intermediate reaction product) and the substrate-enzyme binding. Either Mg^{2+} or Mn^{2+} can be utilized by the enzymes without adverse effects on their activity. Citric acid accumulation is made possible as citrate and α -ketoglutarate inhibit the NADP^+ -dependent form of the enzyme (Show et al., 2015a).

Generally, citric acid or citrate, the ionic form of citric acid, is a major intermediate compound produced by the TCA cycle. Pyruvate ($\text{HO}(\text{CO})_2\text{CH}_3$), a product resulting from the oxidation

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of glucose, namely glycolysis is the compound required to initiate the TCA cycle. Pyruvate must first be converted to acetaldehyde (CH_3COH) and then into Acetyl CoA to activate the TCA. The first intermediate compound produced by the TCA cycle is citrate ($\text{C}_3\text{H}_5\text{O}(\text{COO})_3^{3-}$) which is the ionic form of citric acid ($\text{CH}_2\text{HOC}(\text{COOH})_3\text{CH}_2$). In *A. niger*, the TCA cycle occurs in the matrix of the mitochondrion.

Once formed, Citrate can be used as a major intermediate in the TCA cycle, to generate oxaloacetate and several compounds used to store energy such as ATP and NADPH or can be excreted outside the cell wall if conditions prohibit the second stage of the TCA cycle. If nutrients become limited, the citrate accumulated outside the cell wall can be reabsorbed and degraded within the TCA cycle by the enzyme aconitase (Kulkarni, 2015).

2.3 Microbial production of Citric Acid

Large number of microorganisms including fungi (*Aspergillus* species) and bacteria such as *Arthrobacter paraffinens*, *Bacillus licheniformis* and *Corynebacterium ssp.*, *A. niger*, *A. aculeatus*, *A. carbonarius*, *A. awamori*, *A. foetidus*, *A. fonsecaeus*, *A. phoenicis* and *Penicillium janthinellum*; and yeasts such as *Candida tropicalis*, *C. oleophila*, *C. guilliermondii*, *C. citroformans*, *Hansenula anamola* and *Yarrowia lipolytica* have been employed for citric acid production (Shetty, 2015).

Most of them, however, are not able to produce commercially acceptable yields due to the fact that citric acid is a metabolite of energy metabolism and its accumulation rises in appreciable amounts only under conditions of drastic imbalances. Among the mentioned strains, the fungus *A. niger* has remained the organism of choice for commercial production because it produces more citric acid per time unit (Adeoye et al., 2015). The problem in the production of citric acid for yeasts is the simultaneous formation of isocitric acid (Yalcin et al., 2010). The main advantages of using *A. niger* are its ease of handling, its ability to ferment a variety of cheap raw materials, and high yields. Industrial strains which produce commercial citric acid are not freely available and only a few can be obtained from international culture collections (Profile & Profile, 2010).

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The improvement of citric acid producing strains has been carried out by mutagenesis and selection. The most employed technique has been by inducing mutations in parental strains using mutagens. Mutants of *A. niger* are used for commercial production. Among mutagens, γ -radiation, UV radiation, and chemical mutagens are often used. To obtain hyper-producer strains, UV treatment can frequently be combined with some chemical mutagens. The »single-spore technique« and the »passage method« are the principal methods of selecting strains. The first one has the disadvantage that mineral acid and organic acids (gluconic and oxalic acids) simulate the presence of citric acid (Yigitoglu, 1992).

Different methods of fermentation can lead to different yields of citric acid production by the same strain. Thus, a strain which produces good yields in the solid fermentation or liquid surface is not necessarily good producer in the submerged fermentation. In that way, each method and raw material of industrial interest should be tested with known producer strains (Soccol et al., 2006).

2.4 Raw Materials

Although citric acid is mostly produced from starch or sucrose-based media using liquid fermentation, a variety of raw materials such as molasses, several starchy materials and hydrocarbons have also been employed. Rohr et al. classified raw materials used for citric acid production in to two groups: (i) with a low ash content from which the cations could be removed by standard procedures (e.g. cane or beet sugar, dextrose syrups and crystallized dextrose); (ii) raw materials with a high ash content and high amounts of other non-sugar substances (e.g. cane and beet molasses, crude unfiltered starch hydro-lysates) (Papagianni, 2007).

Several attempts have been made to produce citric acid using molasses, which is preferred due to its low cost and high sugar content (40 – 55%). The composition of molasses depends on various factors, e.g. the kind of beet and cane, methods of cultivation of crops and fertilizers and pesticides applied during cultivation, conditions of storage and handling (e.g. transport, temperature variations), production procedures, etc. Both cane and beet molasses are stable for citric acid production. However, beet molasses is preferred due to its lower content of trace metals. Generally, cane molasses contains calcium, magnesium, manganese, iron, and zinc,

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which have a retarding effect on the synthesis of citric acid. Consequently, some pre-treatment is required for the removal/reduction of trace metals. Despite that, cane molasses possess difficulties in achieving good fermentation yields (Maharani et al., 2014).

Various other agro-industrial residues such as banana peel, apple pomace, cassava bagasse, coffee husk, wheat straw, pineapple waste, sugar beet cosset, kiwi fruit peel, etc. have been investigated with solid state fermentation techniques for their potential to be used as substrates for citric acid production (Vandenberghe et al., 1983). In fact, these residues are very well adapted to solid-state cultures due to their cellulosic and starchy nature. However, despite the fact that these solid residues provide rich nutrients to the microorganisms, and are good substrates for growth and activity of micro-organisms, much remains to be done for developing commercially feasible process utilizing these residues (Soccol et al. , 2006).

2.5 Citric Acid Production Techniques

Citric acid production synthesis 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, technical skills required are lower, energy consumption is lower and frequent power failures do not critically affect the functioning of the plant (Bagasse et al., 2014).

Citric acid production by fermentation can be divided into 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 out in three different ways: by submerged fermentation, surface fermentation and solid-state fermentation or »Koji« process. All of these methods require raw material 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 remainder mineral of the raw material and add other nutrients such as phosphorous, magnesium and nitrogen for development of the mycelium and a good production of the citric acid (Majumder et al., 2010).

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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.. Classically, the solid-state process has been carried out in trays, which facilitates aeration. Higher yields (347 g/kg dry cassava bagasse) were obtained in flasks without any aeration, and very little sporulation was observed. Equivalent yields (309 g/kg of dry cassava bagasse) were obtained in column reactors only with variable aeration. This fact showed great perspective in using solid culture process for citric acid production in simple tray type fermenters. In fact, the scale-up study of the production of citric acid showed that in-tray bioreactors with 4 cm bed thickness 263 g/kg of dry cassava bagasse were attained (Soccol et al., 2006).

2.5.1 Submerged Fermentation

Submerged fermentation is a process involving the development of microorganisms in a liquid broth. This liquid broth contains nutrients and it results in the production of industrial enzymes, antibiotics or other products. The substrates are utilized quite rapidly; hence need to be constantly replaced/supplemented with nutrients. This fermentation technique is best suited for microorganisms such as bacteria that require high moisture (Maghsoodi & Yaghmaei, 2010). The process involves taking a specific microorganism such, as fungi, and placing it in a small closed flask containing the rich nutrient broth. A high volume of oxygen is also required for the process. The production of enzymes then occurs when the fungi interact with the nutrients in the broth resulting in them being broken down. At industrial level, this production of yeasts has become a major output of microbiological industries as a result of improved fermentation technologies. Fermentation in industries is carried out using fermenters which are large vessels which can store huge volumes. In an effort to reduce nitrogen and carbon levels, microorganisms secrete enzymes in the selected medium (Ravichandran & R, 2012).

There are two common methods by which submerged fermentation takes place; they are batch-fed fermentation and continuous fermentation. In batch-fed fermentation, sterilized growth nutrients are added to a culture. It is most common in bio-industries as it occurs during the growth of biomass in the fermenter. It helps raise the cell density in the bioreactor and it is typically highly concentrated to stop dilution. The rate of growth in the culture is maintained by

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adding nutrients, this also reduces the risk of overflow metabolism. An open system is constructed for continuous fermentation. Then sterilized liquid nutrients are slowly and continuously added to the bioreactor at the same rate at which the converted nutrient solution is being recovered from the system. This results in a steady-rate production of the fermentation broth. In order to maintain a successful fermentation certain variables must be monitored, for example, temperature, pH, as well as oxygen and carbon dioxide levels (Of, 2006).

2.5.2 Surface Fermentation

The first individual process for citric acid production was the liquid surface culture, which was introduced in 1919 (Belgium) and in 1923 by Chas Pfizer & Co. in the US. After that, other methods of fermentation, such as SmF were developed. Although this technique is more sophisticated, the surface method required less effort in operation and installation and energy cost (Vandenberghe et al., 1983).

In the classical process for citric acid manufacture, the culture solution is held in shallow trays (capacity of 50 –100 L) and the fungus develops as a mycelial mat on the surface of the medium. The trays are made of high purity aluminum or special grade steel and are mounted one over another in stable racks. The fermentation chambers are provided with an effective air circulation in order to control temperature and humidity. Fermentation chambers are always in aseptic conditions, which might be conserved principally during the first two days when spores germinate. Frequent contamination is mainly caused by *Penicilia*, other *Aspergilli*, yeast and lactic bacteria (Soccol et al., 2006). Refined or crude sucrose, cane syrup or beet molasses are generally used as sources of carbon. When applied, molasses is diluted to 15 –20% and is treated with hexacyanoferrate (HFC).

2.5.3 Solid State Fermentation (SSF)

SSF utilizes solid substrates, like bran, bagasse, and paper pulp. The main advantage of using these substrates is that nutrient-rich waste materials can be easily recycled as substrates. In this fermentation technique, the substrates are utilized very slowly and steadily, so the same substrate can be used for long fermentation periods. Hence, this technique supports controlled release of nutrients. SSF is best suited for fermentation techniques involving fungi and microorganisms that require less moisture content (Bakhiet & Al-mokhtar, 2015). Solid-state culture is characterized by the development of microorganisms in a low-water activity environment on an insoluble material that acts both as physical support and source of nutrients. Some similarities are observed with the surface process since the fungus also develops on the material surface (Shetty, 2015).

The most common organism used in SSF is *A. niger*. However, there have also been reports with yeasts. The strains with large requirements of nitrogen and phosphorus are not ideal microorganisms for solid culture due to lower diffusion rate of nutrients and metabolites occurring at lower water activity in the solid-state process. The presence of trace elements may not affect citric acid production so harmfully as it does in submerged fermentation, thus, substrate pretreatment is not required. This is one of the important advantages of the solid culture (Iralapati & Kummari, 2015).

Table 2-1: Raw Materials for Citric Acid Fermentation(Show et al., 2015b)

Type of fermentation	Raw materials
Surface	Brewery waste, carob sugar, cotton waste, tubers of <i>Asphodelus aestivus</i> , turnip whey (enriched with molasses)
Submerged	Bagasse hydrolysates, beet molasses, black strap molasses, brewery wastes, cane molasses, carob pod extract, coconut oil, corn starch, date syrup, glycerol, hydrolysate starch, n-paraffin, palm oil, olive oil, soya bean oil, rapeseed oil, pent grain liquor, wood hemicellulose, whey

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	permeate, xylan hydrolysate, yam bean starch
Solid state	Apple pomace, carob pod, carrot waste, cassava bagasse, cellulose hydrolysate and sugar cane, coffee husk, corncob, deoiled rice bran, grape pomace, banana peel, kiwi fruit peel, kumara, molasses, mussel processing wastes, okara, orange waste, pineapple waste, rice bran, sucrose, sugarcane-pressmud, wheat bran

2.6 Factors Affecting Citric Acid Production

2.6.1 Medium and Its Components

Carbon source: Citric acid accumulation is strongly affected by the nature of the carbon source. The presence of easily metabolized carbohydrates has been found essential for the good production of citric acid. Bagasse et al. (2014) showed that sucrose was the most favorable carbon source followed by glucose, fructose, and galactose. Galactose contributed to a very low growth of fungi and did not favor citric acid accumulation (Kulkarni, 2015). Other sources of carbon such as sorbose, ethanol, cellulose, manitol, lactic, malic and α -acetoglutaric acid, allow a limited growth and low production. Starch, pentoses (xyloses and arabinoses), sorbitol and pyruvic acid slow down growth, though the production is minimal (Atere, 2013).

According to Yigitoglu (1992), initial sugar concentration was critical for citric acid production and other organic acids produced by *A. niger*. El-holi & Al-delaimy (2003) reported that *A. niger* strains needed an initial sugar concentration of 10-14% as optimal; no citric acid was produced at sugar concentration of less than 2.5%. Papagianni (2007) showed that immobilized cells of *A. niger* needed lower concentrations of sucrose than free cells culture, in order to obtain high yields (200 g of citric acid/L for free cells culture, and 120 g/L for immobilized cells). Bakhiet & Al-mokhtar, (2015) reported the influence of different sources of carbon on citric acid production by *A. niger* and *Saccharomycopsis lipolytica* (*S. Lipolytica*). Glucose, maltose, galactose, xylose,

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and arabinose were tested. Fermentation was carried out in 8 and 4 days, respectively, at 30 °C and 180 revolutions per minute (rpm). Better results were found for *A. niger* with 0.45 g of citric acid/ g of glucose corresponding to 27 g/L. *S. Lipolytica* produced 0.41 g/g of glucose or 9 g/L which was not so bad (Kulkarni, 2015).

As presented previously, several raw materials can be employed successfully for citric acid production. There are some critical factors (costs, need for pretreatment), which should be considered for substrate determination. One another aspect is the presence of trace elements, which can act as inhibitors or stimulants. Consequently, sometimes it is necessary to conduct a pre-treatment, e.g.; precipitation of trace metals of molasses by potassium ferrocyanide (Majumder et al., 2010).

Nitrogen source: Citric acid production is directly influenced by the nitrogen source. Physiologically, ammonium salts are preferred, e.g. urea, ammonium sulfate, ammonium chloride, peptone, malt extract, etc. Nitrogen(N) consumption leads to pH decrease, which is a very important point in citric acid fermentation (Yalcin et al., 2010). However, it is necessary to maintain pH values in the first day of fermentation prior to a certain quantity biomass production. Urea has a tampon effect, which assures pH control. The concentration of nitrogen source required for citric acid fermentation is 0.1 to 0.4 N /liter. A high nitrogen concentration increases fungal growth and the consumption of sugars but decreases the amount of citric acid produced (Parvez et al., 1998).

Phosphorous source: In the first works it was verified that the presence of phosphate in the medium had a great effect on the yield of citric acid (Kudzai et al., 2016). Low levels of phosphate have positive effects on citric acid production. This effect acts at the level of enzyme activity and not at the level of gene expression. On the other hand, the presence of an excess of phosphate leads to a decrease in the fixation of carbon dioxide, which in turn increases the formation of certain sugar acids, and the stimulation of growth (Majumder et al., 2010).

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Trace elements: Trace element nutrition is probably the main factor influencing the yield of citric acid (Yalcin et al., 2010). A number of divalent metals such as zinc, manganese, iron, copper, and magnesium have been found to affect citric acid production by *A. niger*. However, it is crucial to take into account the interdependence of medium constituents in SmF and, probably, in SSF. Zinc favored the production of citric acid if added with KH_2PO_4 . On the other hand, the presence of manganese ions and iron and zinc (in high concentrations) could cause the reduction of citric acid yields only in phosphate-free medium (Karaffa et al., 2001). Parvez et al. (1998) noticed that there were few differences in the response of *A. niger* to metal ions and minerals in SSF and in SmF systems. SSF systems were able to overcome the adverse effects of the high concentrations of these components in the medium. As a consequence of this, the addition of chelating agents such as potassium ferrocyanide to the medium proved to be of no use (Amenaghawon et al., 2015).

Copper was found to complement the ability of iron at an optimum level, to enhance the biosynthesis of citric acid. Manganese deficiency resulted in the repression of the anaerobic and TCA cycle enzymes with the exception of citrate synthetase. This led to an overflow of citric acid as an end product of glycolysis. A low level of manganese (ppm) was capable to reduce the yield of citric acid by 10% (Sanchez-Marroquin et al., 1970). Citric acid accumulation decreased by the addition of iron, which also had some effect on mycelia growth (Karthikeyan & Sivakumar, 2010). Bakhiet and Al-mokhtar (2015) reported that the presence of different copper concentrations in the pellet formation medium was very important in order to enhance a stable structure, related to cellular physiology, for citric acid production. The optimal initial $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ concentration was 78 mg/L. Magnesium is required both for growth as well as for citric acid production. The optimal concentration of magnesium sulfate was found in the range of 0.02 – 0.025% (Yalcin et al., 2010).

Lower alcohols: Lower alcohols added in pure material inhibit citric acid production but if added to crude carbohydrates these alcohols enhance the production. Methanol, ethanol, n-propanol, isopropanol or methylacetate neutralize the negative effect of the metals in citric acid production generally in amounts about 1 to 5%. Even so, the optimal amount of methanol and ethanol depends upon the strain and the composition of the medium (M & G, 2014). Alcohols

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have been shown to act principally on membrane permeability in microorganisms by affecting phospholipid composition. Other studies showed that alcohols stimulate citric acid production by affecting growth and sporulation on space organization of the membrane or changes in lipid composition of the cell wall (Iralapati & Kummari, 2015).

Other compounds: Oils and fats are used in citric acid production to control the foam formation. In addition, some oils stimulate productivity and maintenance of fermentation processes. These lipids act as carbon sources and they are consumed during the fermentation, which is necessary to maintain its level above 0.05 – 0.3% (Soccol et al., 2006).

2.6.2 Process parameters

pH: The pH of a culture may change in response to microbial metabolic activities. The most obvious reason is the secretion of organic acids such as citric, acetic or lactic acids, which will cause the pH to decrease. Changes in pH kinetics depend highly also on the micro-organism. With *Aspergillus sp.*, *Penicillium sp.* and *Rhizopus sp.*, pH can drop very quickly until less than 3.0. For other groups of fungi such as *Trichoderma*, *Sporotrichum*, *Pleurotus sp.*, pH is more stable (between 4 and 5). Besides, the nature of the substrate also influences pH kinetics (Lingappa et al., 2007).

Generally, a pH below 4.0 is required for the optimum production of citric acid. A low initial pH has the advantage of checking contamination and inhibiting oxalic acid formation. A pH of 2.2 was reported to be optimum for the growth of the mold as well as for the production of citric acid whereas; a higher pH i.e. 5.4 and 6.0 - 6.5 has been found optimum for citric acid production in molasses medium (Soccol et al., 2006).

Aeration: Since citric acid production is an aerobic process, oxygen supply has a determinant effect on its production. Increased aeration rates lead to enhanced yields and reduced process time (B. Journal et al., 2005). An interruption of aeration during batch fermentation is quite harmful. Dissolved oxygen concentration influences the citric acid formation directly. It is important to maintain the oxygen concentration above 25% saturation. Critical dissolved oxygen tension is 9 – 12% of air saturation for growth phase and 12 – 13 % of air saturation for the production phase. The high demand for oxygen is reached by constructing appropriate aeration

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devices, which is also dependent on the viscosity of the fermentation broth. This is an additional reason why small compact pellets are the preferred mycelial forms of *A. niger* during the production. When the organism turns into development of filaments, the dissolved oxygen tension rapidly falls to less than 50% of its previous value, even if the dry mass has not increased by more than 5% (Bakhiet & Al-mokhtar, 2015).

Aeration should be performed through the medium during the whole process with the same intensity, even though, due to economic reasons, it is usually preferred to start with low aeration rates. The incorporation of the oxygen together with air in submerged process results in an increment of citric acid production, but it is economically unviable. However, it is possible to circulate the oxygen in the fermenter since the carbonic gas is removed from the process. High aeration rates lead to high amounts of foam, especially during the growth phase, so the addition of antifoaming agents and the construction of mechanical »defoamers« are required to tackle this problem.

It has been reported that forced aeration at the beginning of the process in solid-state fermentation packed-bed reactor affects metabolic rates, and thus, citric acid productivity. The study of citric acid production by *A. niger* in SSF revealed the importance of a CO₂ rich atmosphere. Vandenberghe et al. (1983) showed that an environment with high concentrations of CO₂ has a positive effect on citric acid synthesis. The high partial pressure of CO₂ probably retards spore liberation of the filamentous fungi and favours citric acid synthesis with cassava bagasse as support/substrate. In fact, low oxygen environment is directly involved in the growth limitation, which is crucial for citric acid production. Low aeration rates are supposed to limit the respiration activity of *A. niger* and, consequently, to turn the metabolism to citric acid synthesis and not to biomass production. It was also observed that strongly aerated cultures increased sporulation. As the sporulation increased, the accumulation of citric acid decreased.

Temperature: Microbial growth in citric acid production by fermentation generates significant amount of metabolic heat. The temperature of the fermentation media heavily affects yields as well as rates of citric acid fermentations. Establishment of temperature gradients and localized overheating of the substrate occurs because of inefficient removal of heat from the substrate. Heat transfer problem in SSF includes temperature gradients that may cause belated microbial

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activity, dehydration of the medium and undesirable metabolic deviations. Heterogeneous materials make heat removal difficult; this is due to low transfer coefficient and low thermal conductivity (Selahzadeh & Roehr, 2003).

Temperature can rise rapidly, because there is little water to absorb the heat or in other words mean specific heat capacity of the fermenting mass is much lower than that of water. Therefore, heat generated must be dissipated immediately as most of the microorganisms used in SSF are mesophilic, having optimal temperature for growth between 25 and 35 °C. Different methods are used for dissipation of heat such as cooling of metal trays using circulating coolant and intermittent agitation. Other methods include forced aeration, water circulation through a jacket surrounding the fermenter, agitation of the solids and covering the external surface of fermenter with water soaked burlaps. However, forced aeration is generally the technique of choice for heat removal (Çevrimli et al., 2009).

2.7 Product Recovery

The recovery of citric acid from liquid fermentation broth is generally accomplished by three basic procedures, precipitation, extraction, and adsorption and absorption (mainly using ion exchange resins) (Wu, 2009). Citric acid extraction has been described by the Food and Drug Administration of the United States. Citric acid extracted by this method has been recommended stable for use in food and drugs. Precipitation is the classical method and it is performed by the addition of calcium oxide hydrate (milk of lime) to form the slightly soluble tricalcium citrate tetrahydrate (Pazouki & Panda, 1998). The precipitated tri-calcium citrate is removed by filtration and washed several times with water. It is then treated with sulphuric acid forming calcium sulphate, which is filtered off. Mother liquor containing citric acid is treated with active carbon and passed through cation and anion exchangers. 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 (Wu, 2009).

Some methods were developed to decrease the cost of the recovery, trying to overcome the drawback of precipitation scheme, which is responsible for the formation and disposal of enormous amounts of calcium sulfate, leading to pollution problems. Electrodialysis is an electrochemical separation process in which electrically charged membranes and electrical potential difference are used to separate ionic species from aqueous solutions. This technique was tested in citric acid recovery and shows that the separation of ionic species from clarified fermentation broths is more economical. The problem is that electrodialysis technique costs were found to be about 50% greater than current industrial-scale citric acid recovery process. The great specific electromembrane and electric energy costs appear to limit the electrodialysis applications only to high value-added products. The use of electrodialysis would need the development of new integrated fermentation processes to minimize waste formation and enhance productivity (Pazouki & Panda, 1998).

2.8 Applications of Citric Acid

Citric acid is a versatile and innocuous alimentary additive. It is accepted worldwide as GRAS (generally recognized as safe), approved by the Joint FAO/WHO Expert Committee on Food Additives (Ciriminna et al., 2017). The food and pharmaceutical industries utilize citric acid extensively because of its general recognition of safety, pleasant acid taste, high water solubility and chelating and buffering properties. Citric acid is used in cosmetics and toiletries as buffer, and in a wide variety of industrial applications as a buffering and chelating agent. Citric acid is also a reactive intermediate in chemical synthesis. In addition, its carboxyl and hydroxyl groups permit the formation of a variety of complex molecules and reactive products of commercial interest (Abbas et al., 2016).

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Table 2-2: Citric Acid Application (Ciriminna et al., 2017)

Application	Industry	Function
Beverage	Wines and ciders	Prevents browning in some white wines Prevents turbidity of wines and ciders. Used in pH adjustment.
	Soft drinks and syrups	Provides tartness. Stimulates natural fruit flavor. As acidulant in carbonated and sucrose-based beverages.
Food	Jellies, jams, and preservatives	Used in pH adjustment. Acts as acidulant. Provides the desired degree of tartness, tang, and flavor. Increases the effectiveness of antimicrobial preservatives.
	Dairy products	As emulsifier in ice creams and processed cheese. Acidifying agent and antioxidant in many cheese products.
	Candies	Acts as acidulant. Provides tartness. Minimizes sucrose inversion. Produces dark color in hard candies. Prevents crystallization of sucrose.
	Frozen fruits	Protects ascorbic acid by inactivating trace metals. Lowers pH to inactivate oxidative enzymes.
	Fats and oils	Synergist for other antioxidants, as sequestrant. Stabilizing action.
Agriculture		Micronutrient evaluation in fertilizers. Enhances P availability in plants.
Pharmaceutics		As effervescent in powders and Tablets in combination with bicarbonates. Anticoagulant.

3. MATERIALS AND METHODS

The experimental work was conducted in the laboratory of the School of Chemical and Bio Engineering of the Faculty of Engineering, Addis Ababa Institute of Technology, Addis Ababa University, Addis Ababa, during 7 months from November 2017 to May 2018.

3.1 Chemicals and Analytic Reagents

The chemicals and analytic reagents' grades used were 0.1 M of sodium hydroxide, 0.1 N sulfuric acid, phenolphthalein as indicator, distilled water, ethanol absolute, activated carbon, deionized water, potato dextrose agar (OXOID, England), 1 M hydrochloric acid, sucrose(D(+) – saccharide sugar), 0.1 N chlorhydric acid, 4% sodium sulphate solution, 40% solution of sodium hydroxide, petroleum ether, boiling point 40 – 60 °C mercuric oxide, potassium sulphate, boric acid indicator solution, sulphuric acid solution 0.255 N, sodium hydroxide solution 0.313 N free of sodium carbonate, antifoam (e.g. octyl alcohol or silicone), ethyl alcohol at 95% (v/v), chlorhydric acid solution at 1% (v/v), tween 80 solution, magnesium sulfate ,calcium chloride , potassium dihydrogen phosphate, ammonium sulfate.

3.2 Equipment

The equipment used during this study includes laboratory autoclave, magnetic stirrer, Erlenmeyer flasks 250 mL, volumetric flask 200 mL, rotary incubator (SHKA4450-1CE), whatmann №.42 and № .1 , membrane filter paper 45 µm, test tubes 50 mL, burette 100 mL, balance (precision, ±0.0001), oven (MB45, OHAUS, Switzerland), different size of conical flasks and beakers 50 mL, 100 mL, 250 mL, measuring cylinders 50 mL, 100 mL, 250 mL, centrifuge (UNIVERSAL 320R), PH meter, thermostatic incubator, flow fast V cabinet, water bath (HWS-26), aluminum foil, furnace, porcelain crucibles, crucible furnace, dryer, 600 mL flat-bottomed balloon flask with roughened neck, melting point apparatus, condensation unit for flask, 11 Kitazato flask, buchner funnel, filtration crucible, rubber cones, 500 mL retort,gloves, Petridis, thermometer, vacuum filter (KIF, LABOPORT), Soxhlet extraction apparatus, Laboratory kiln set at 105°C, Extraction thimbles, Kjeldahl digestion and distillation apparatus, 500 mL Kjeldahl flasks, glass beads, haemocytometer, freeze drier, atomic absorption spectroscopy(Buck of scientific 210 VGP AAS,USA), Atomic Absorption Spectroscopy (AAS),

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AA-6300, SHIMADZU, and Spectrophotometer(T60U model,PG Instruments Ltd) spectrophotometer CM 600d (KONICA MINOLTA INC.).

3.3 Substrate Preparation and Characterization

Fresh banana peels were collected from fruit vendors, substrates were obtained in a quantity sufficient for all the experiments. Hundred g of banana peels were removed and air dried for 72 hours and then oven dried at 60 °C to constant weight. The sample was ground and stored in polythene container for characterization. The nutritional, mineral and anti-nutritional content of banana peel were characterized.

3.3.1 Determination of Nutritional Content

The sample was analyzed for proximate composition (moisture, ash, volatile content, organic matter, crude protein, lipids, carbohydrate and crude fiber). The moisture content of the peel was determined by oven drying to a constant weight at 105 °C (Nagarajaiah & Prakash, 2011). The Lipid was extracted with petroleum ether using a Soxhlet apparatus for six hours. The Micro-Kjeldahl procedure was adopted for the determination of protein. Carbohydrate was determined by difference (Aina et al., 2012).

3.3.1.1 Determination of Moisture Content

In balancing the ration it is essential to know the water content of each component; also, moisture in prepared feed must be monitored because levels over 8% favor the presence of insects, and over 14% there is the risk of contamination by fungi and bacteria. The method is based on drying a sample in an oven and determining moisture content by the weight difference between dry and wet material. Approximately 10 g of air dried (for 72 hours) and cleaned fresh banana peel was taken in a crucible and dried in an oven at a temperature of 105 °C for 24 hours and the weight was taken after every 12 hours. The procedure was repeated until a constant weight was obtained. After every 12 hours, the sample was removed from the oven and cooled in desiccator for 30 minutes. It was then removed and re-weighed.

The percentage loss of weight gave the percentage of moisture in the sample. The percentage of moisture content is computed as follows:

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$$M_c(\%w/w) = \frac{W_1 - W_2}{W_1 - W_3} \times 100 \quad (3.1)$$

Where M_c = moisture content (%)

W_1 = weight of the sample and container, g

W_2 = weight of dry sample and container, g

W_3 = weight of the container, g

3.3.1.2 Ash Content

A dry, tarred porcelain dish containing 10 g of dry sample (free of moisture) of banana peel was placed in a muffle furnace set at 550 °C for 2 hours, and the sample was cooked in a desiccator and weighed. The ash content was calculated using the equation as described in an association of official analytical chemists (AOAC) (Aina et al., 2012). This method is used to determine ash content in feedstuffs by calcination. Ash is considered as the total mineral or inorganic content of the sample.

$$\text{Ash } (\%w/w) = \frac{M_1 - M_2}{M_3} \times 100 \quad (3.2)$$

Where M_1 = mass of empty dish and ash, g

M_2 = mass of the empty dish, g

M_3 = mass of the dry sample, g

3.3.1.3 Crude Fiber Determination

Three g of the sample was weighed into a dry 100 mL conical flask. Two hundred mL of 0.25 N H_2SO_4 acid was added to the sample and then brought to boiling point. It was boiled gently for 30 minutes. The flask was rotated every few minutes in order to mix the contents and to remove particles from the sides. A funnel with a filter paper was prepared. At the end of the 30 minutes boiling period, the acid mixture was allowed to stand for one minute and poured immediately into a shallow layer of hot water under gentle suction in the prepared funnel. The suction was adjusted so that at the end, 200 mL of the filtrate was completed within 10 minutes. The

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insoluble matter was washed with warm water gently until the washing was free of acid and then washed back into the conical flask by means of a wash bottle containing 200 mL of 0.25 N hydroxide solution and then boiled it for 30 minutes as in previous boiling.

After boiling it was allowed to stand for one minute and then filtered immediately through a stable filter paper. The insoluble matters were then transferred into an already weighed crucible, then placed in a muffle furnace and heated at 600 °C. The crude fiber was then determined by subtracting the weight of ash sample from the weight of the dried matter. The percentages crude fiber was calculated as follow (Egbebi & Bademosi, 2011):

$$\% \text{Crude fiber} = \frac{C_1 - C_2}{\text{Weight of original sample}} \quad (3.3)$$

Where C_1 = weight of the crucible with dry residue

C_2 = weight of the crucible with ash

3.3.1.4 Determination of Crude Lipid Contents

A clean, dried 500 cm³ round bottom flask containing few anti-bumping granules was Weighed (W_1) with 300 cm³ petroleum ether (40-60 °C) for extraction poured into the flask filled with Soxhlet extraction unit. The extractor thimble weighing twenty grams was fixed into the Soxhlet unit. The round bottom flask and a condenser were connected to the Soxhlet extractor and cold water circulation was connected/put on. The heating mantle was switched on and the heating rate adjusted until the solvent was refluxing at a steady rate. Extraction was carried out for 6 hours. The solvent was recovered and the oil dried in an oven set at 70 °C for 1 hour. The round bottom flask and oil were then Weighed (W_2). The lipid content was calculated as a percentage.

$$\% \text{ Crude lipid} = \frac{W_2 - W_1}{\text{Weight of the sample}} \times 100 \quad (3.4)$$

Where W_1 = weight of a clean dry flask

W_2 = weight of the flask with fat

3.3.1.5 Protein Content Determination

Digestion

Because of its cost, this is the most important dietary nutrient in a commercial operation; proper evaluation of it means that the quality of protein intake or of the feed being provided can be controlled. The analysis is by Kjeldahl's method, which evaluates the total nitrogen content of the sample after it has been digested in sulphuric acid. In this study, to milligram precision, 1 g of dry sample was weighed out into 300 cm³ Kjeldahl flask; then 10 g potassium sulfate, 0.7 g mercuric oxide and 20 mL concentrated sulphuric acid were added. The sample was digested until a clear green color was obtained. The digest was cooled and diluted with 100 cm³ with distilled water.

Distillation

Into 500 cm³ Kjeldahl flask containing antibumping chips and 40 cm³ of 40% NaOH was slowly added to the flask containing mixture of 50 cm³ 2% boric acid and 3 drops of mixed indicator was used to trap the ammonia being liberated. The conical flask and the Kjeldahl flask were then placed on Kjeldahl distillation apparatus with the tubes inserted into the conical flask, heat was applied to distill out the NH₃ evolved with the distillate collected into the boric acid solution. The distillate was then titrated with 0.1 M HCl.

$$\%N_2 = \frac{14 \times M \times V_t \times V}{\text{Weight of sample (mg)} \times V_a} \quad (3.5)$$

$$\% \text{ crude protein} = \%N_2 \times 6.25$$

Where M = Actual Molarity of Acid

V = Titre Value (Volume) of HCl used

V_t = Total volume of diluted digest

V_a = Aliquot volume distilled

3.3.1.6 Determination of Carbohydrate

The total carbohydrate was determined by difference. The sum of the percentage moisture, ash, crude lipid, crude protein and crude fiber was subtracted from 100.

$$\% \text{ Total carbohydrate} = 100 - (\% \text{ moisture} + \% \text{ Ash} + \% \text{ lipid} + \% \text{ Protein} + \% \text{ Fibre}) \quad (3.6)$$

3.3.1.7 Determination of Volatile Matter

The dried sample after moisture removal was taken in a crucible and placed in an electrically heated oven at a temperature of 920 °C for 10 minutes and then it was cooled in desiccator for 30 minutes. It was then removed and reweighed. The percentage of weight loss gave the volatile matter content (Hamid et al. , 2016). The percentage of volatile matter is computed as:

$$\text{Volatile Solids (\%)} = \frac{W_1 - W_2}{W_1} \times 100 \quad (3.7)$$

Where W_1 = Initial mass (dried) of the sample as delivered and

W_2 = Mass of the sample after drying

Determinations of fixed and volatile solids do not distinguish precisely between inorganic and organic matter because the loss on ignition is not confined to organic matter. It includes losses due to decomposition or volatilization of some mineral salts (Kabenge et al., 2018).

3.3.2 Determination of Mineral Content

An amount of 2 g of banana peels was dried in an air oven at 105 °C for 3 hours. The dried sample was next charred until it ceased to smoke. The charred sample was then ashed in a muffle furnace at 550 °C until a whitish or greyish ash was obtained. The ash was treated with concentrated hydrochloric acid transferred to a volumetric flask and made up to 100 mL before submission to atomic absorption spectrophotometry (AAS) (Aina et al., 2012).

For AAS, a SHIMADZU, atomic absorption flame emission spectrophotometer model AA-6300 with an air-acetylene flame, and wavelength respectively set to 422.7 nm for calcium, 285.213 nm for magnesium, 248.3 nm for iron and 589.0 nm for sodium determination was used. Stock solutions (1000 ppm) of calcium, magnesium, iron, and sodium were used to prepare working standard solutions with at least four concentrations within the analytical range. To eliminate

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phosphorus interference, lanthanum chloride was added to working standard solutions of calcium and to the test ash solution destined to calcium determination so that the final solutions contained 1% La. Concentration of each mineral contained in test solutions was calculated from the standard curve prepared (Fisher et al., 2008).

3.3.2.1 Basic Principles of Analyzing Metals Using Atomic Absorption Spectrometry

Flame atomic absorption Spectroscopy (FAAS) is a very common technique for detecting metals and metalloids in environmental samples. The technique of FAAS requires a liquid sample to be aspirated, aerosolized and mixed with combustible gases, such as acetylene and air or acetylene and nitrous oxide. The mixture is ignited in a flame whose temperature ranges from 2100 to 2800 °C. During combustion, atoms of the element of interest in the sample are reduced to free, unexcited ground state atoms, which absorb light at characteristic wavelengths. The characteristic wavelengths are element specific and accurate to 0.01 – 0.1 nm. To provide element specific wavelengths, a light beam from a lamp whose cathode is made of the element being determined is passed through the flame. Metal ions in a solution are converted to the atomic state by means of a flame. Light of the appropriate wavelength is supplied and the amount of light absorbed can be measured against a standard curve (Fisher et al., 2008).

3.3.3 Determination of Hydrogen Cyanide (HCN)

Ten g of sample was soaked in a mixture of 200 cm³ of water and 10 cm³ of orthophosphoric acid. The mixture was left for 12 hours to release all bounded hydrocyanic acid. A drop of anti-foaming (paraffin) and antibumping agents were added and the solution distilled until 150 cm³ of the distillate was collected. Twenty cm³ of distillate was taken into a conical flask and diluted with 40 cm³ of water, 8 cm³ of 6.0 moldm⁻³ ammonium hydroxide (NH₄OH) and 0.002 L 5% (w/v) potassium iodide (KI) solutions were added. The mixture was titrated with 0.02 moldm⁻³ silver nitrate (AgNO₃) using a micro burette until a faint but permanent turbidity was obtained (1 cm³ 0.02 moldm⁻³ AgNO₃ $\equiv$ 1.08 mg HCN) (AOAC, 1990).

3.3.4 Determination of Phytate

Two g of the sample was weighed into a 250 mL conical flask. One hundred mL 2% concentrated HCl was used to soak sample for 3 hours and then filtered with a Whatman No.1

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filter paper. Fifty cm³ of the filtrate and 10 cm³ of distilled water were added in each case to give proper acidity. Ten mL 0.3% ammonium thiocyanate solution was added into the solution as indicator and titrated with standard Iron II Chloride solution containing 0.00195 g Iron/mL, endpoint observed to be yellow which persisted for 5 minutes (Aina et al., 2012). The percentage phytate was calculated thus:

$$\% \text{ Phytate} = y \times 1.19 \times 100 \text{ where, } y = \text{titre value} \times 0.00195 \text{ g} \quad (3.8)$$

3.3.5 Determination of Oxalate Content

Ground sample (1.0 g) was placed in a 250 cm³ volumetric flask, 190 mL of distilled water and 10 mL of 6.00 M HCl was added. The mixture was warmed on a water bath at 90 °C for four hours; the digested sample was centrifuged at a speed of 2000 rpm for five minutes. The filtrate was diluted to 250 mL, three 50 mL aliquots of the filtrates were evaporated to 25 mL, and the brown precipitate was filtered off and washed. The combined solution and washing were titrated with concentrated ammonia solution until a faint yellow coloration was obtained using methyl orange as indicator. The solution was then heated at 90 °C and the oxalate was precipitated with 10 mL of 5% calcium chloride solution. The solution was left overnight and then diluted to 125 mL with water after warming at 90 °C, and titrated against 0.05 M KMnO₄ (1 mL 0.05 M KMnO₄ = 2.2 mg Oxalate) (Aina et al., 2012).

3.3.6 Determination of Saponins

A measured weight (5 g) of the powdered sample of banana peel was mixed with 50 mL of 20% aqueous ethanol solution in a flask. The mixture was heated with periodic agitation in a water bath for 90 minutes at 55 °C; it was then filtered through Whatman filter paper (№ 42). The residue was extracted with 50 mL of 20% ethanol and both extracts were poured together and the combined extract was reduced to about 40 mL at 90 °C and transferred to a separating funnel where 40 mL of diethyl ether was added and shaken vigorously. Re-extraction by partitioning was done repeatedly until the aqueous layer becomes clear in color. The saponins were extracted, with 60 mL of normal butanol. The combined extracts were washed with 5% aqueous sodium chloride (NaCl) solution and evaporated to dryness in a pre-weighed evaporation dish. It was dried at 60 °C in the oven and reweighed after cooling in a desiccator. The process was repeated

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two more times to get an average (Ezeabara et al., 2014). Saponin content was determined by difference and calculated as a percentage of the original sample thus:

$$\text{Percent saponins (\%)} = \frac{W_2 - W_1}{\text{weight of the sample before drying}} \times 100 \quad (3.9)$$

Where w_1 = Weight of evaporating dish

w_2 = Weight of evaporating dish + sample after drying

3.4 Pre-treatment of Banana Peel

The banana peels collected from the fruit vendor were thoroughly washed with deionized water to remove loosely sticking flour or dirt, filtered, then air dried for 72 hours and finally, oven dried at 60 °C for 2 hours. The dried substrates were pulverized in a dry grinder, passed through standard sieves with a pore size of 2 mm and stored in polythene container for citric acid production. This pretreatment of banana peel was done to make their chemical constituents more accessible and their physical structure more susceptible to mycelial penetration.

3.5 Microorganism and Inoculum Preparation

A. niger ATCC 10535(American Type Culture Collection), obtained from the Microbiology (Traditional and modern medicine) division of the Ethiopian public health Institute (EPHL), Addis Ababa, Ethiopia was used throughout the study as the fermenting organism. It was kept on potato dextrose agar (pH 5.6) slants. The potato dextrose agar medium was prepared by dissolving 4 g of PDA in approximately 80 mL of distilled water and rising up to 100 mL, and then cooked for 15 minutes while constantly stirring until a clear solution formed.

The pH of the medium was maintained at 5.6 by 0.1 N of HCl and NaOH. Approximately 5.0 mL of this medium was decanted into individual test tubes. The test tubes were cotton plugged and sterilized in an autoclave at 121 °C for 15 minutes. After sterilization, the test tubes were maintained in a slanting position at an angle of about 30° to increase the surface area. The potato dextrose agar slants were then inoculated by transferring a small amount of *A. niger* conidia from the culture provided and incubated at 28 °C for 5 days for maximum sporulation. After 5 days the cultures were stored at 4 °C in a lab cool place for analysis.

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3.6 Methods

The overall structure of the experimental work is shown in Figure 3-1

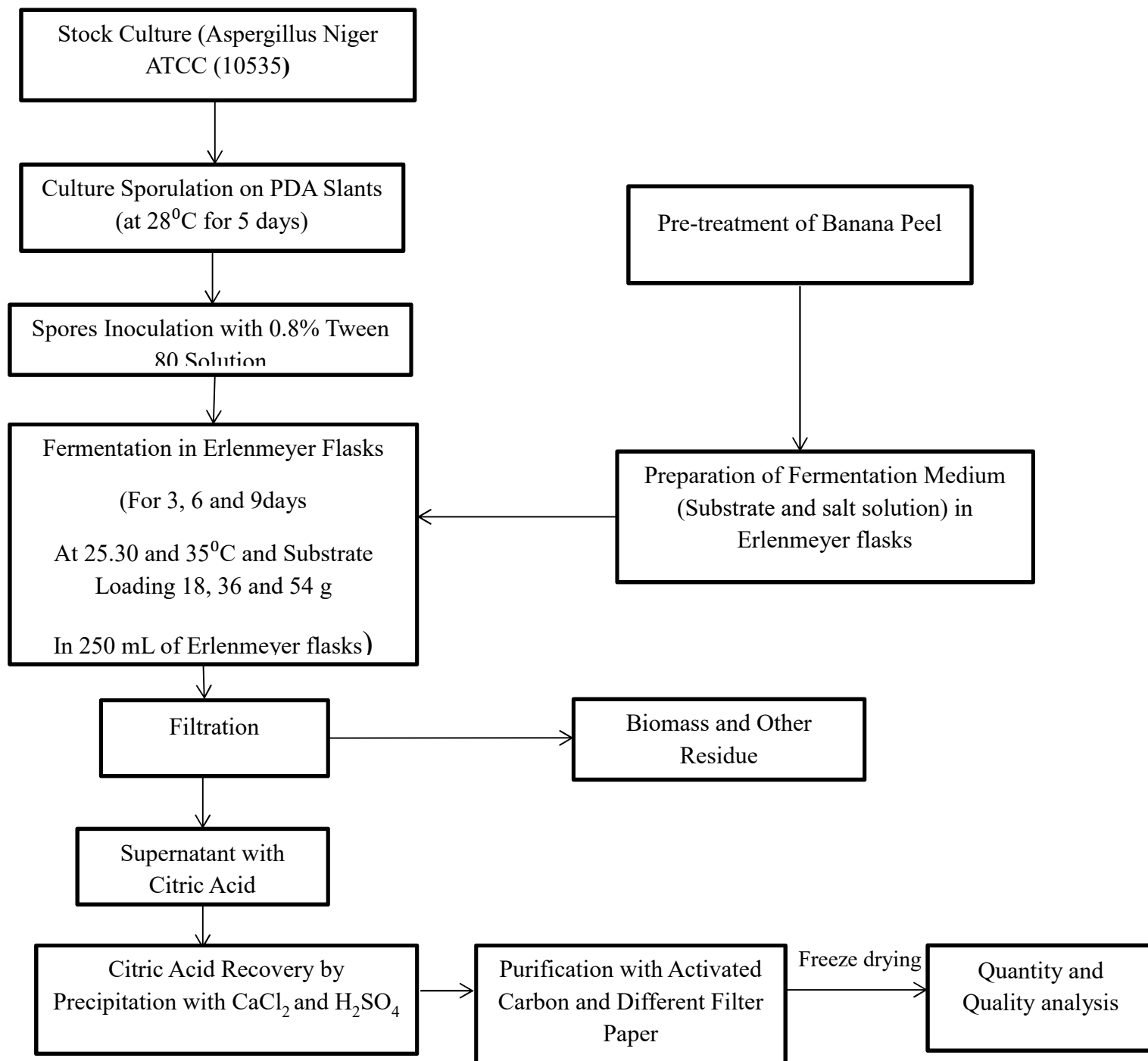


Figure 3-1: Frame Work of the Exneriment

3.7 Experimental Design

In this investigation, citric acid was produced using SSF. A three-variable Box-Behnken design (BBD) for response surface methodology (RSM) was used to develop a statistical model to describe the production of citric acid from banana peel. There were three-factors with three levels for each variable i.e. substrate loading (18 g, 36 g, 54 g), temperature (25 °C, 30 °C, 35 °C) and incubation time (3 days, 6 days, 9 days). The range of the variables that were optimized is shown in Table 3-1. The BBD is stable for the exploration of quadratic response surfaces and this design can be used to develop a second-degree polynomial model which can be utilized for optimization purposes. The number of an experimental run for this design was obtained from Equation (3.10).

$$N = K^2 + K + C_p \quad (3.10)$$

Where K is the number of factors and C_p is the number of replications at the center point. The experimental design was developed using Design Expert 7.0.0. The coded and actual values of the independent variables were calculated using Equation (3.11).

$$x_i = (X_i - X_0) / \Delta X_i \quad (3.11)$$

Where x_i and X_i are the coded and actual values of the independent variable respectively. X_0 is the actual value of the independent variable at the center point and ΔX_i is the step change of X_i .

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

Variables	Symbols	Coded levels		
		-1	0	+1
Solid loading(g)	X1	18	36	54
Incubation time(days)	X2	3	6	9
Temperature (°C)	X3	25	30	35

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The following generalized second order polynomial equation was used to estimate the response of the dependent variable (Montgomery & Runger, 2002).

$$Y_1 = b_0 + \sum b_i X_i + \sum b_{ij} X_i X_j + \sum b_{ii} X_i^2 + e_i \quad (3.12)$$

Where Y_1 is the dependent variable or predicted response, X_i and X_j are independent variables, b_0 off set terms, b_i and b_{ij} are the single and interaction effect coefficient e_i is the error term. The Design Expert software was used for regression and graphical analysis of the experimental data. The goodness of fit of the model for citric acid production was evaluated by the coefficient of determination (R^2) and analysis of variance (ANOVA). The optimum values of the variables tested was obtained by numerical optimization based on the criterion of desirability.

3.8 Experimental Setup

3.8.1 Fermentation Conditions Preparation and Settings

The experiment was conducted as shown in the above Table 3-1. A total of 17 experiments were carried out based on the response surface methodology a three variable BBD.

3.8.1.1 Fermentation Temperature

Temperature plays a very important role in microbial growth and metabolism. The effect of the temperature on citric acid production was studied by carrying out the fermentation at different temperature ranging from 25 °C – 35 °C for different incubation time and substrate loading. The three levels of temperature were selected because of the fact that most filamentous fungi are mesophilic and require for growth optimal temperature between 25 °C and 35 °C.

3.8.1.2 Fermentation Time

The optimum time of incubation for maximum citric acid production varies both with the organism and fermentation conditions. The other factor studied in citric acid production in this study was the incubation time at different temperature and substrate loading. To observe the influence of fermentation the incubation time was studied at different day ranging from 3 – 10 days. The three levels of the time were selected because of the fact that fermentation process is carried out between 3 and 10days.

3.8.1.3 Substrate Loading

Citric acid accumulation is strongly affected by the nature of the carbon source. The presence of easily metabolized carbohydrates has been found essential for the good production of citric acid. In this study, the banana peel was used as carbon source. To observe the influence of carbon source Substrate loading was carried out at different substrate level ranging 18 g – 54 g. The three levels of substrate loading were selected because the fact that carbon source should range between 50 – 240 g/l. Considering at amounts below 50 g/L carbon source, citric acid biosynthesis is not realized and therefore, experiments always started from the threshold of 50 g/L and upwards (Çevrimli et al., 2009).

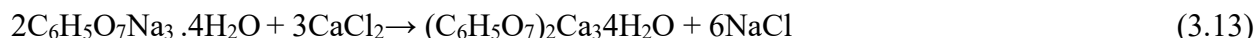
3.9 Preparation of Fermentation Media

Solid-substrate fermentation was carried out to produce citric acid in this study. The basal medium was prepared by introducing banana peels under different levels in a separate 250 mL Erlenmeyer flasks with a working volume of 150 mL. The composition of fermentation media for citric acid production was NH_4SO_4 0.3 g, KH_2PO_4 0.23 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.077 g, 3%(v/w) ethanol and banana peel 18 g, 36 g and 54 g (quantity of ingredients for 150 mL⁻¹). Sucrose was added to the medium (100 g/1000 g) to boost carbon substrate. The initial pH of the substrates was adjusted to about 3.5 with NaOH or HCl. The contents were thoroughly mixed, cotton plugged and autoclaved at 121 °C for 15 minutes.

Spores suspension of *A. niger* ATCC 10535 was prepared by washing 5 days old culture potato dextrose agar slant with sterilized 0.8% Tween 80 solution by shaking vigorously for 1 minute. Spores were counted by a haemocytometer to adjust the count to approximately 1×10^7 spores/ml. After cooling, the sterilized substrate including media was inoculated with 1 mL (1×10^7 spores/ml) of inoculum and then incubated at varying conditions in a rotary shaking incubator and the shaking speed was kept at 200 rpm. The solid substrate loading, time and the temperature were varied according to the experimental design. At the end of fermentation, the medium was diluted with sterilized distilled water (1:4 w/v) and shaken for 60 minutes at 121 rpm to mix all the content. The content then filtered through filter paper and the filtrate was used for analysis.

3.10 Product Recovery

Different separation mechanisms can be used to separate citric acid from the fermented broth. However, in this research, the precipitation recovery method was implemented for separation citric acid from the fermented media after 3, 6 and 9 days of fermentation period. The filtrate that was obtained after the filtration process from the production medium of the different substrate level of banana peels with ethanol was recovered by using calcium chloride. Sodium hydroxide was added to the sample prior to the addition of calcium chloride, as direct addition of the latter to the sample would have resulted in the formation of hydrochloric acid which was considered undesirable (Tweel, 1995). Trisodium citrate was formed when the PH of the sample with sodium hydroxide reached between 8 and 9.



For partial recovery citric acid, calcium chloride solution (28.5 g/70 mL) was added gradually to continuously agitated filtrate at 70 °C (placed on hot plate magnetic stirrer). After the pH of medium reached 7.0, white tricalcium precipitate was formed as result of an exothermic reaction. The white precipitate was recovered by centrifuging the suspension at 3000 revs/min for 10minutes, then filtered using a Whattman №.1 filter paper and washed several times with sterilized distilled water. Finally, these precipitate was converted to soluble citric acid and insoluble calcium sulfate after heavily diluted sulphuric acid (98 %) was added to the calcium citrate based on the stoichiometric relationship in equation 3.14. The amount sulphuric used was depends on the amount of calcium citrate recovered, hence less sulphuric acid was used than needed in order to make sure that it was all consumed. Any leftover sulphuric acid is very difficult to separate from the citric acid. Whereas any leftover calcium citrate can be filtered off easily. Following the addition of sulphuric acid, the mixture of insoluble calcium citrate and sulphuric acid were stirred with a magnetic stirrer for 90 minutes at 90°C to allow the calcium sulfate to precipitate.



The resultant suspension obtained from the above reaction was centrifuged at 3000 rev/min for 10 minutes and filtered the calcium sulfate with whatmann №.42 filter paper. Then the filtrate

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was subjected to activated carbon and vacuum filtration for the removal of colorants. Finally, the supernatant (presumed to be citric acid in solution) were filtered by micro-membrane filter paper to remove the residues of fungi.

3.11 Citric Acid Determination

The concentration of citric acid in the supernatant was estimated titrimetrically by using 0.1M NaOH and phenolphthalein as indicator. Hence, a known volume of the analyte (presumed to be a citric acid solution) was placed in a titration flask and the burette filled by standard solution (0.1 M NaOH) until it reached the zero mark using a funnel. Before the titration was started, three drops of phenolphthalein were poured into the titration flask with citric acid. A 0.1 M NaOH (standard solution) was slowly added from the burette, drop by drop with continual in magnetic stirrer to keep it thoroughly mixed. The titration continues to drop by drop until the phenolphthalein achieves the intermediate color (weak pink) between that of acid and the color of the base (El-holi & Al-delaimy, 2003).

The titration ceases when the system was neither acidic nor basic referred to as the endpoint. The endpoint was corresponding to a perfect stoichiometric relationship between the acid and the base. Once the endpoint was reached, the volume of NaOH used(titer) on the burette was read and the titration is complete and the final calculation was done using citric acid, 1 mL 0.1 M NaOH is equivalent to 0.064 g citric acid (Kudzai et al., 2016). The amount of citric acid was expressed in terms of g/L citric acid factor (0.064) formula.

$$\text{g/L citric acid} = \frac{\text{Titre (volume of NaOH)} \times 0.064 \times 100 \times 10}{X(\text{ml analyte}) \times 1000 \text{ml}} \quad (3.14)$$

3.11.1 Preparing a Standard Base

Prior to performing the titration to determine the amount of acid in the analyte (presumed to be a citric acid solution), the exact concentration of the base was prepared for the purpose of titration. Standardizing the base was involved in performing a two titration with a known amount of a stable acid, which in this case is the monoprotic acid potassium hydrogen phthalate (abbreviated KHP). The reaction of KHP and NaOH is:



3.11.1.1 PROCEDURE (Standardization of NaOH)

The buret was cleaned and rinsed with deionized water. Next, the buret was rinsed twice with 10 mL of the NaOH solution that was standardized at the end. Finally, the buret was filled with the NaOH solution. Approximately 0.05 g KHP, to the nearest thousandth of a gram, was accurately weighed and it was transferred to one of the 250 mL Erlenmeyer flasks. Approximately 25 mL of deionized water and 2 to 3 drops of the phenolphthalein indicator was added. The flask was swirled until all the KHP was dissolved. The initial volume of NaOH solution in buret was read to the nearest hundredth of a mL. Then, slowly added the NaOH solution to the dissolved KHP until the first faint lasting pink color appears. A white piece of paper was placed under the Erlenmeyer flask which helped to better saw pink color changed. The final volume was read in the buret when the final pink color was reached. The molarity of the NaOH solution was calculated. This procedure was repeated two more times and calculated the average molarity of the NaOH solution.

3.12 Crystallization of Citric Acid

Freeze drying is often regarded as the best method of water removal to obtain a final product of the highest quality. Because of the absence of liquid water and the low temperature required for the process, most of the deterioration reaction and microbiological activities are prevented, which give a final product of excellent quality. The mother liquor containing citric acid was finally concentrated in a freeze dryer, and finally forming citric acid.

3.13 Characterization of the product

3.13.1 Assessment of Purity of the Product

A melting-point apparatus was used to assess the purity of the product obtained. The temperature at which the product crystals melted was compared with the melting temperature of pure, anhydrous citric acid crystals.

In addition to the melting point analysis, Citric acid was estimated gravimetrically following the recommended pyridine-acetic anhydride method by Merrier and Boulet in 1958. In brief, four gram of the sample was accurately weighed and dissolved in 40 mL of distilled water, then for

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each 1 mL of sample, 1.3 mL of pyridine was added and mixed well. The solution was added with 5.7 mL of acetic anhydride and placed incubated at 32 °C for 30 minutes in water bath. The optical density values of the sample were recorded at 405 nm using UV visible spectrophotometer (Kishore et al., 2008). The citric acid concentration of the sample was estimated from the reference run in parallel after citric acid standard was prepared from citric acid monohydrate by dehydrating it at 90 °C for 72 hours to constant weight.

3.13.2 Determination of the Sulfated Ash

Silica crucible was dried over a burner for 10 minutes, cooled in a desiccator containing silica up to room temperature. Then five gram of the sample was weighed accurately in a tared crucible. It was ignited in a muffle furnace at 650 °C for 2 hours, gently at first, until the material was thoroughly charred. The residue was cooled, moistened with 1 mL of sulphuric acid and ignited till the carbon source was completely oxidized. The crucible was cooled in a desiccator and weighted.

$$\text{Sulfated ash, percent by mass} = \frac{M_1}{M_2} \times 100 \quad (3.16)$$

Where M_1 = Mass, in g, of the residue

M_2 = Mass, in g, of the material taken for the test

3.13.3 Preparation of Samples for Determination of Heavy Metals

3.13.3.1 Digestion of Samples

The sample was prepared for each metal according to a wet-ashing technique developed by Maria, 2002. One gram of the sample was weighed out into acid washed glass round bottom flask. Samples were digested by addition of 20 mL of aquaregia (a mixture of HCl and HNO₃, ratio 3:1). Condenser was fitted to the round bottom flask that reflux for 2 hours at 90 °C. The round bottom flask wall was washed with distilled water and the sample was filtered out through whatman filter № 1 to separate the insoluble solids from the supernatant liquid. The volume was adjusted to 100 mL with distilled water. All samples and blanks were stored in plastic container. Standard solution of each sample As, Pb and Fe was prepared according to Sc 2000 standard

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manufacturer producer for Atomic absorption spectroscopy (Buck scientific model 210 VG AAS, USA and ZEEit 700, German).

3.13.3.2 Determination of Metal Concentration of Each Sample

After digested, the sample was connected to AAS to read concentration of each metal ions present in the sample. Then As, Fe and Pb were analyzed with AAS (Buck Scientific model 210 VGP and ZEEit 700, German) equipped with deuterium arc background corrector and standard air-acetylene flame system using external calibration curve after the parameters (burner and lamp alignment, slit width and wavelength adjustment) were optimized for minimum signal intensity of the instrument. Four replicate determinations were carried out on each sample. Hollow cathode lamps operated at the manufacturer recommended conditions were used at their respective primary source line. The concentration of the metal ions presented in the sample was determined by reading their absorbance using AAS and comparing it to the respective standard curve.

3.13.4 Titrimetric Method for Better Quality Assay

Known Molarity of Sodium Hydroxide (0.01 N NaOH Standardized solutions) was used for the titration. Phenolphthalein was used as an indicator (Hotha et al., 2014). The burette was rinsed twice with a few milliliters of Milli Q water followed by the standardized 0.01 N NaOH solution. The burette was filled above the zero mL mark with the standardized 0.01 N NaOH. The NaOH solution was drained until the tip is filled and all air bubbles are removed. The sample solution was kept on a magnetic stir plate with a magnetic stir bar into the conical flask. A white paper was placed under your conical flask to make the color change more noticeable. The tip of the burette was placed inside the neck of the conical flask. The magnetic stir plate was turned on and the speed was adjusted to prevent splashing of the mixture up the sides of the flask. 5 drops of the phenolphthalein indicator was added into the sample and slowly 0.01 N NaOH was added from the burette to the conical flask until the pink color of the indicator lasts for a few seconds when the base first enters the solution. The flow of the base was adjusted from flow to a drop at a time. If Pinkish color persists for 30 seconds; the titration is at the endpoint. The same procedure was repeated for the other experiments. The formation of the light pink color is the significant in outcome of the accurate result. Dark pink color was observed when excess base was added gives

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the inaccuracies in the result. The precision of test method was evaluated by analyzing six test samples of free citric acid by titrimetric analysis (N, 2007).

3.13.5 Color Analysis

Color, flavor, and texture are the three principal quality attributes that determine food acceptance, and color has a far greater influence on our judgment than most of us appreciate. Hence, in this study, the color of the produced citric acid was determined for quality analysis by spectrophotometer CM 600d (KONICA MINOLTA INC.). CIELAB color space was used with the parameters $L^*a^*b^*$. L^* indicates lightness (0 to 100) with 0 being black and 100 being white. The coordinate a^* is for red (+) and green (−), and b^* is for yellow (+) and blue (−). The limits for a^* and b^* are approximate + or −80. It is very effective for measuring color differences.

The quality of the produced citric acid was compared with the product of Citrique Belge which is one the greatest citric acid producer in the world scorecard for citric acid quality because they know that, for their customers, the color must be “right.” Food scientists who establish quality control specifications for their product are very aware of the importance of color and appearance. While subjective visual assessment and use of visual color standards are still used in the food industry, instrumental color measurements are extensively employed. Objective measurement of color is desirable for research and industrial applications, and the ruggedness, stability, and ease of use of today’s color measurement instruments have resulted in their widespread adoption.

3.13.6 Fourier Transformation Infrared (FTIR) Spectroscopy

The structural and vibrational studies of citric acid are of chemical, biochemical, and technological interest. Many previous studies in relation to the chemical and spectroscopic properties of this acid as monomer have been reported while an ab-initio/HF force field study for the vibrations of citric acid and citrate trianion was published by Tarakeshwar and Manogaran. In the latter study, the calculated geometries and vibrational frequencies only for the monomer citric acid were used to assign the vibrational spectra. A complete vibrational characterization of this acid is very important to obtain information about chemical and physical components in food, biological materials, and pharmaceutical products at the molecular level. So far, there are few publications about experimental or high-level theoretical studies on the geometries and force

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field for the dimer citric acid. Hence, obtaining reliable parameters by experimental study is an appealing alternative to have a precise knowledge of the normal vibration modes (Kosa et al., 2017). In this study, the experimental study of dimer citric acid by infrared spectroscopy in order to effect a complete assignment of every observed band in the vibrational spectra by taking into account the presence of the dimeric species in the solid phase was carried out.

FTIR Spectroscopy is a technique based on the determination of the interaction between an IR radiation and a sample that can be solid, liquid or gaseous. It measures the frequencies at which the sample absorbs, and also the intensities of these absorptions. The frequencies are helpful for the identification of the sample's chemical make-up due to the fact that chemical functional groups are responsible for the absorption of radiation at different frequencies. The concentration of component can be determined based on the intensity of the absorption. The spectrum is a two-dimensional plot in which the axes are represented by intensity and frequency of sample absorption (Simonescu, 2012).

Fourier transform infrared spectroscopy was used to examine the framework of vibration. The functional group of the produced citric acid was tested by FTIR on spectrum 65 FTIR with the resolution of 4 cm^{-1} and scanning range from 4000 cm^{-1} to 400 cm^{-1} using the conventional KBr technique. The functional group was determined based on the interpretation of the infrared spectrum obtained by comparing it with the standard spectrum group frequencies. The following procedure was followed to perform the analysis: First, the sample for the analysis was milled in a ceramic pestle and mortal to powdery conditions. The powder was then mixed with KBr particles to make it stable to infrared analysis. The mixture was then pressed to a small thickness, slightly below 1mm, required for FTIR analysis. Then the power of the instrument was turned on and the initialization started. Then the sample holder was cleaned by acetone with kimwipes and the spectrum software was launched. From the window that appeared, the instrument setup button was selected and scans and instrument set up dialog popped up. The sample name, scan range, and scan number was inputted. The sample was placed on the sample holder, apply and start button were pressed to collect the spectrum (Kosa et al., 2017). Finally, the data was saved and the instrument was switched off. The FTIR was be graphed by using origin lab.

4. RESULTS AND DISCUSSION

4.1 Biochemical Analysis of Banana Peel

The biochemical analysis of the banana peel such as nutritional, mineral and anti-nutritional content was characterized. The result of mineral content (Table 4-1) shows the concentration of potassium to be the highest (70.67mg/g). The concentration (mg/g) of calcium, sodium, iron, and manganese were 15.20, 18.18, 0.19 and 67.59 respectively. All the concentrations indicated that banana peel can be used as a source of mineral for *A. niger* for the production of citric acid by SSF without much pretreatment requirement. The appreciable high content of potassium also signifies that if the peel is taken, it will help in the regulation of body fluids and maintain normal blood pressure for the future. It will also help in controlling kidney failure, heart oddities, and respiratory flaw. Iron concentration was the lowest. Iron carries oxygen to the cells and is necessary for the production of energy, synthesis of collagen and the proper functioning of the immune system. Its low concentration implies that banana peel will be an idyllic source of iron since its excess is implicated in abnormal functioning of the immune system, cell growth, and the heart. Manganese known to aid formation of skeletal and cartilage was also found to be high (67.59 mg/g). Manganese dearth is scarce but could affect glucose tolerance, normal reproductive, skeletal and cartilage formation. Generally, the banana peel can have different purposes in addition to the production of citric acid (Hamid et al., 2016).

The moisture content of the banana peel was found to be 87% and 9.25% which varied due to the time the banana peels were put on the oven. If fresh peel dries directly, it will have 85% – 88% moisture content. Air-dried banana peel for 3 – 4 days has less than 50% moisture which is recommended for biochemical analysis (Hamid et al., 2016). The 9.25% found in this study was relatively low, and this may be due to the time at which it was harvested. The low value also designate that the peel can be amassed for a long time without growing moldy. The ash content was found to be 13.51%. This value is analogous to other staples measured as good sources of minerals. The organic matter content was found to be 86.49%. Organic matter measures the nutritional value (lipids, proteins, and carbohydrate) of a plant material. The high value indicates that banana peels are good sources of nutrients for microorganisms and other living things.

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The contents of protein, lipid, carbohydrates and crude fiber (Table 4-2) were found to be 5.46 %, 3.62%, 41.52% and 26.64% respectively. The values indicate that the peel could be a good source of carbohydrates and fiber. The high fiber content also indicates that the peels could help treat constipation and improve general health and wellbeing. High values of volatile matter content of 74.54% for banana peel was recorded. This signified the possibility of a high organic load, a high susceptibility to thermal degradation status and a high bio-oil potential of the banana peel. Generally, banana peel could be a good source of minerals and carbohydrate for *Aspergillus niger* and other species which can be used as a fermenting organisms during SSF.

The results of anti-nutrients indicated the concentrations of hydrogen cyanide were found to be 1.03 mg/g. The result obtained falls within the threshold value (0.5-3.5 mg/g) reported as safety limit (Anhwange, B.A. et al., 2009). Hydrogen cyanide is an extremely poisonous substance formed by the action of acids on metal cyanides. A large dose of hydrogen cyanide can cause death within few minutes. While smaller dosages may result to stiffness of the throat, chest, palpitation and muscle weakness.

The oxalate content of the peel was found to be 0.31 mg/g. Oxalate consumption had been associated with kidney diseases which may result in death. It decreases the availability of essential minerals like calcium. Phytate content was found to be 0.18 mg/g which can be used as a source of phosphors for *A. niger*. Saponin content was 19 mg/g. saponin consumption can result in paralysis of the sensory system. It is found to inhibit growth in swine and poultry; though it increases the excretion of cholesterol in the body.

The study of the anti-nutrient content of the peel indicates generally low values except for saponins, hence, banana peel could be used as substrate for citric acid production without much pretreatment requirement. The chief concern about study the anti-nutrient is that they can bind to certain minerals which mean high amount of anti-nutrient leads to low amount of free minerals. In addition to this, if the peels are properly processed could be a good source of feed for livestock, hence, in addition to citric acid production, we can use the banana peel for different purposes.

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Table 4-1: Minerals Composition of Banana peels

Elements	Concentration (mg/g) (present finding)	Anhwange, B.A. et al. (2009)
Potassium	70.67	78.10
Calcium	15.20	19.20
Iron	0.19	0.61
Magnesium	67.59	76.20
Sodium	18.18	24.30

Table 4-2: Proximate Composition and Anti - nutritional Content of Banana Peel

Parameters	Concentration	Anwange, B.A. et al. (2009)
Moisture (%)	9.25	6.70
Ash (%)	13.51	8.50
Organic Matter (%)	86.49	91.50
Protein (%)	5.46	0.9
Crude Lipid (%)	3.62	1.70
Carbohydrate (%)	41.52	59
Crude fibre (%)	26.64	31.7
Volatile content (%)	74.54	-----
Hydrogen cyanide(mg/g)	1.03	1.33
Oxalate(mg/g)	0.31	0.51
Phayte(mg/g)	0.18	0.28
Saponins(mg/g)	19	24

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4.2 Statistical Analysis

Table 4-3 shows the result of the 17 experimental runs, whereas, Table 4-4 also illustrates the observed value and predicted value carried out according to the BBD. BBD is the commonly used experimental design model for three-level three-factor experiments. BBD always has three levels for each factor and is purpose-built to fit a quadratic model. The BBD does not have runs at the extreme combinations of all the factors but compensates by having better prediction precision in the center of the factor space. While a run or two can be botched in these designs the accuracy of the observations in the remaining runs is critical to the dependability of the model. In this study, Design-Expert Software 7.0.0 was used in the least squares regression ANOVA. The statistical software program was used to generate the model equation, interaction effects of the independent variables and surface plots using the fitted equation obtained from the regression analysis holding one of the independent variables constant.

Table 4-3: Box-Behnken Design Matrix for Citric Acid Production

Factors							Response
Run №	Coded value			actual value			Citric acid(g/L)
	X ₁	X ₂	X ₃	X ₁	X ₂	X ₃	Observed
1	-1	-1	0	18	3	30	33.59
2	+1	-1	0	54	3	30	30.78
3	-1	+1	0	18	9	30	34.51
4	+1	+1	0	54	9	30	31.81
5	-1	0	-1	18	6	25	30.78
6	+1	0	-1	54	6	25	34.51
7	-1	0	+1	18	6	35	40.12
8	+1	0	+1	54	6	35	34.89
9	0	-1	-1	36	3	25	28.39
10	0	+1	-1	36	9	25	34.89
11	0	-1	+1	36	3	35	39.14

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12	0	+1	+1	36	9	35	33.29
13	0	0	0	36	6	30	44.75
14	0	0	0	36	6	30	44.75
15	0	0	0	36	6	30	45.55
16	0	0	0	36	6	30	45.55
17	0	0	0	36	6	30	45.55

Table 4-4: Diagnostics Case Statistics

Standard order	Actual value	Predicted value	Residual
1	33.59	33.25	0.34
2	30.78	31.44	-0.66
3	34.51	33.85	0.66
4	31.81	32.15	-0.34
5	30.78	31.35	-0.57
6	34.51	34.08	0.43
7	40.12	40.55	-0.43
8	34.89	34.32	0.57
9	28.39	28.16	0.23
10	34.89	34.98	-0.091
11	39.14	39.05	0.091
12	33.29	33.52	-0.23
13	44.75	45.23	-0.48
14	44.75	45.23	-0.48
15	45.55	45.23	0.32
16	45.55	45.23	0.32
17	45.55	45.23	0.32

4.3 Effect of the Operating Conditions

The effect of individual operating conditions such as substrate loading, fermentation time and temperature on the yield was investigated.

4.3.1 Effect of Temperature on the Citric Acid Yield

The temperature of fermentation medium is one of the critical factors that have a profound effect on the production of citric acid. The *A. niger* grows well at 30 °C and produces maximum amount of citric acid (Maharani et al., 2014). In this study maximum citric acid was found at 30 °C (45.55g/L) as illustrates in the Table 4-3. This result was in agreement with finding of M. Journal (2010) and which has reported maximum amount of citric acid production at 30 °C. When the temperature of medium was low, the enzyme activity was also low, giving low impact on the citric acid production. Similarly when the temperature of medium was increased above 30 °C, the biosynthesis of citric acid was decreased relative to the amount of citric acid at 30 °C. It might be due to enzyme denaturation and inhibition, excess moisture loss and growth of arrest (Adinarayana et al., 2004). It might be also due to the accumulation of by-products such as oxalic acid. Different workers have also used 30 °C as the cultivation temperature and obtained higher values of actual product (Ali, 2002).

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Design-Expert® Software

citric acid

● Design Points

X1 = C: temperature

Actual Factors

A: Substrate Loading = 54.00

B: fermentation time = 6.00

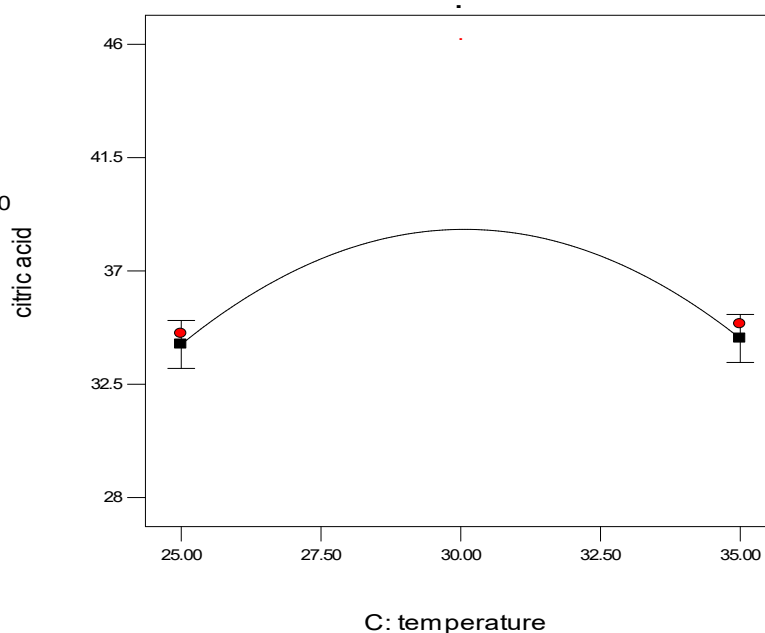


Figure 4-1: Effect of Temperature on Citric Acid Production

4.3.2 Effect of Incubation Time on Citric Acid Yield

Citric acid production was recorded at different incubation time for a banana peel. Citric acid recorded at three, six, and nine incubation days showed citric acid production increment after four days and decline after six days. In the present study, maximum amount of citric acid was found at six days and thereafter it showed a slight decrease at nine days of incubation time. Similarly, six days of fermentation period was found to be optimum in other studies as well (Abbas et al., 2016). The decrease in citric acid production after six days can be accounted to decrease in sugar content; the growth phase of fungi, decrease in the amount of available nitrogen and the presence of inhibitor produced by the fungi itself in the fermentation medium. A decrease in pH values from 3.5 to 2.5 was noted at the end fermentation. The pH value maintained at the beginning of fermentation was important for a specific biomass formation. The progressive decrease in pH was noted as incubation time increased which was due to the formation and accumulation of citric acid (Rahman, 2013).

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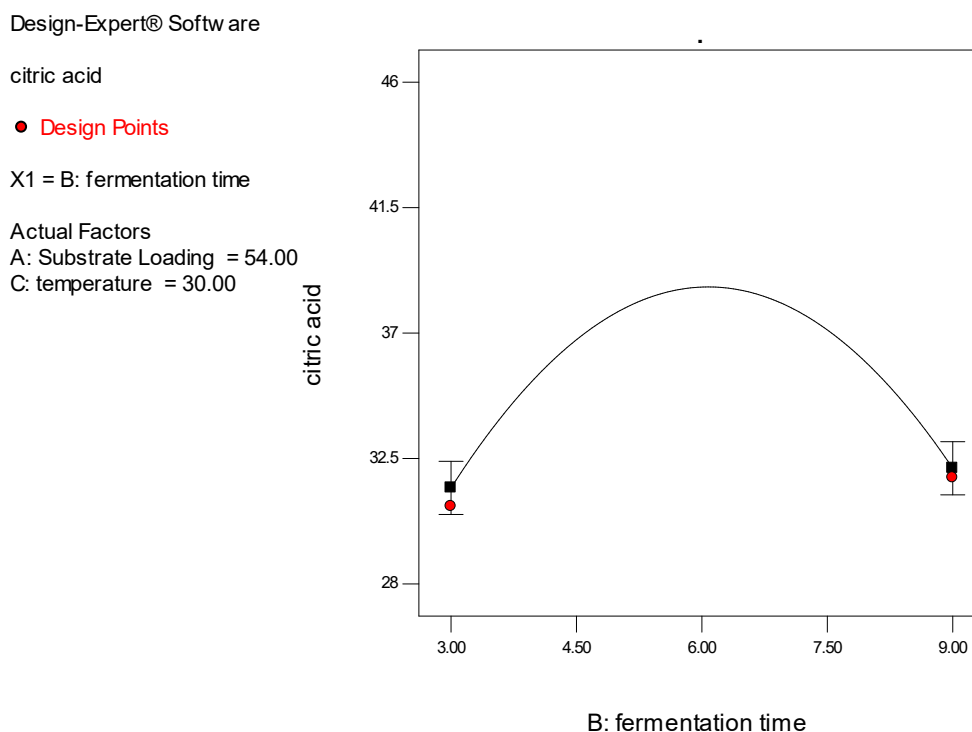


Figure 4-2: Effect of Incubation Time on Citric Acid Production

4.3.3 Effect of Substrate Loading on Citric Acid Production

The final yield of citric acid in fermentation by *A. niger* is strongly dependent on the type and concentration of carbon source. The effect of substrate loading on citric acid yield is shown in Figure 4-3. From the plot, as substrate loading increases from 18 g to 36 g, the citric acid yield was increased. Above and below 36 g citric acid production was found to decline in the fermentation broth. This is because of a low substrate loading leads to low carbon source, which consequently results in increase competition for nutrients, and also further increase carbon source causes reduction in the solubility of nutrients of the substrate and low degree of swelling. The amount of substrate loading has been described in different literature and the general requirements (substrate loading) for production of citric acid with *A. niger* is 50 – 240g/L (Çevrimli et al., 2009). This result is similar with the finding of Abbas et al. (2016).

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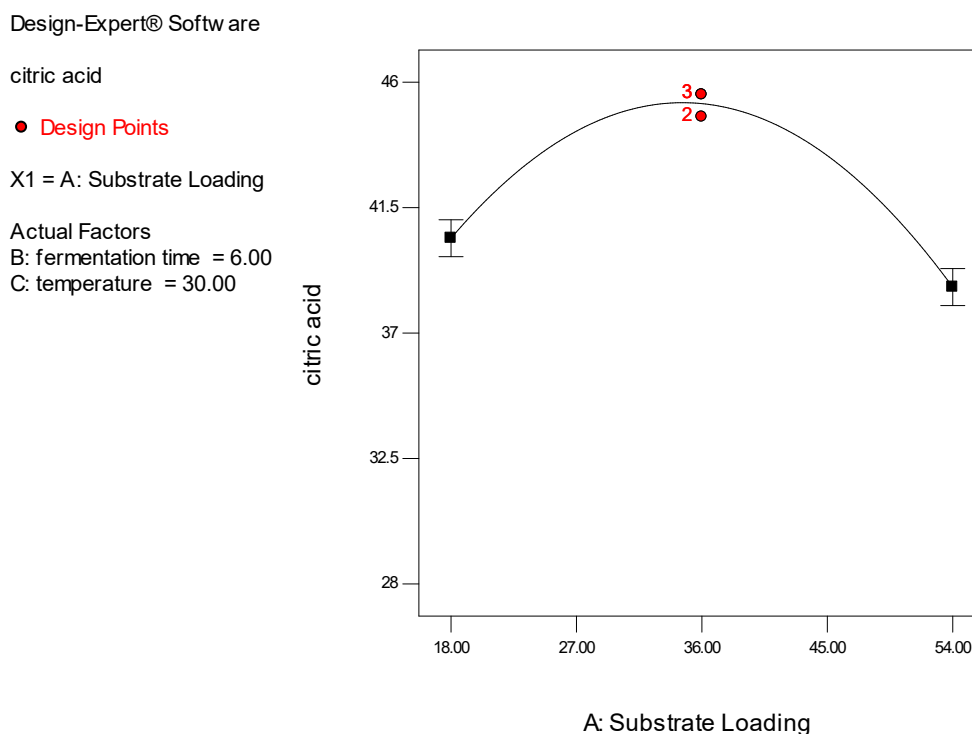


Figure 4-3: Effect of Substrate Loading on Citric Acid Production

4.4 Experimental Design Analysis

The experimental design selected for ANOVA was RSM. Under response surface methodology, BBD was selected. The response of the analysis was extraction yield. The response yield was arranged from 28.39 – 45.55 g/L. The ratio of maximum to minimum yield was 1.574. A ratio greater than 10 usually indicates a transformation is required. For a ratio less than 3, the power transformation has a little effect. The aim of the model fit summary was maximizing the adjusted R-Squared and predicted R-Squared. Model significances was checked for both model and model factor, linear model factors substrate loading (A,) fermentation time (B) and temperature (C) and, quadratic model factors; pure quadratic terms (A^2 , B^2 , C^2) and interaction quadratic terms (AB, AC, BC) depending on the F and P values.

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Table 4-5: Analysis of variance (ANOVA) for the Response of Citric Acid yield of Banana Peel

Source	Sum of square	Df	Mean square	F value	p-value prob > F	
Model	574.99	9	63.89	147.60	< 0.0001	Significant
A-Substrate Loading	6.14	1	6.14	14.19	0.0070	
B-fermentation time	0.85	1	0.85	1.95	0.2050	
C-temperature	44.51	1	44.51	102.83	< 0.0001	
AB	3.025E-003	1	3.025E-003	6.989E-003	0.9357	
AC	20.07	1	20.07	46.37	0.0003	
BC	38.13	1	38.13	88.09	< 0.0001	
A ²	137.04	1	137.04	316.61	<0.0001	
B ²	197.71	1	197.71	456.78	<0.0001	
C ²	83.38	1	83.38	192.63	<0.0001	
Residual	3.03	7	0.43			
Lack of fit	2.26	3	0.75	3.93	0.1097	not significant
Pure error	0.77	4	0.19			
Core total	578.02	16				

The model was statistically significant as seen from the very low p-value (<0.0001) as shown in Table 4-5. The model F-value of 147.60 implies the model is significant. There is only a 0.01% chance that a “Model F- Value” this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case, A, C, AC, BC, A², B², C² are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. This shows that the substrate loading, fermentation temperature, interaction between substrate loading and fermentation temperature, interaction between fermentation temperature and incubation time, square of the substrate loading, square of incubation time and the square of fermentation time affect the yield of citric acid production significantly.

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The "Lack of Fit F-value" of 3.93 implies the Lack of Fit is not significant relative to the pure error. There is a 10.97% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good because we want the model to fit. The model fit summary statistics were listed in Table 4-6.

Table 4-6: Analysis of variance (ANOVA) for the Response of Citric Acid yield of Banana Peel

Std.Dev	0.66	R-Squared	0.9948
Mean	37.23	Adj R-Squared	0.9980
C.V. %	1.77	Pred R-Squared	0.9353
Press	37.39	Adeq R-Squared	33.837

The "Pred R-Squared" of 0.9353 is in reasonable agreement with the "Adj R-Squared" of 0.9880. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. In this case, the ratio of 33.837 indicates an adequate signal. This model can be used to navigate the design space.

Table 4-7: Citric Acid Model Coefficient Estimate and Confidence Interval

Factors	Coefficient estimate	Df	Standard error	95% CI Low	95% CI High	VIF
Intercept	45.23	1	0.29	44.53	45.93	
A-Substrate Loading	-0.88	1	0.23	-1.44	-0.33	1.00
B- fermentation time	0.32	1	0.23	-0.23	0.88	1.00
C- temperature	2.36	1	0.23	1.81	2.91	1.00
AB	0.028	1	0.33	-3.02	-1.46	1.00
AC	-2.24	1	0.33	-3.02	-1.46	1.00
BC	-3.09	1	0.33	-3.87	-2.31	1.00
A2	-5.71	1	0.32	-6.46	-4.95	1.01
B2	-6.85	1	0.32	-7.61	-6.09	1.01
C2	-4.45	1	0.32	-5.21	-3.69	1.01

The application of RSM offers an empirical relationship between the response and the independent variables. The mathematical relationship between the response and the independent

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variables such as substrate loading(A), incubation time(B) and fermentation temperature(C) in terms of coded and actual factors can be determined by Design Expert Software. The model equation that correlates the response (Y) to the extraction process variables were given in equation below.

Final Equation in Terms of Coded Factors:

$$\begin{aligned} \text{citric acid} = & +45.23 - 0.88 * A + 0.32 * B + 2.36 * C + 0.028 * A * B - 2.24 * A * C \\ & - 3.09 * B * C - 5.71 * A^2 - 6.85 * B^2 - 4.45 * C^2 \end{aligned} \quad (4.1)$$

Where A= Substrate loading

B=Incubation time

C=Fermentation temperature

From the regression model equation developed in terms of coded factors, the response yield was affected by linear terms substrate loading(A) , incubation time(B) and fermentation time (C) and, quadratic terms, pure quadratic terms (A^2 , B^2 , C^2) and interaction quadratic terms (AB, BC, AC). On the basis of the coefficient in the equation , it was evident that the response yield increases with an increase in the fermentation temperature (C), and incubation time which have positive linear effect on extraction yield but fermentation temperature has a more weighty linear effect on yield compare to incubation time. pure quadratic terms (A^2 , B^2 , C^2) have negative effects on the response yield but the pure quadratic term (B^2) has a substantial effect than the other quadratic terms. Interaction of incubation period and substrate loading (AB) has a positive effect on response yield. Interaction of substrate loading and fermentation temperature (AC), and interaction of incubation time and fermentation temperature (BC) has a negative quadratic effect on yield.

Final Equation in Terms of Actual Factors:

$$\begin{aligned} \text{citric acid} = & - 242.07000 + 1.96271 * \text{Substrate Loading} + 15.4016 * \text{fermentation time} \\ & + 13.28275 * \text{temperature} - 0.017608 * \text{Substrate Loading}^2 - 0.76139 \\ & * \text{fermentation time}^2 - 0.17800 * \text{temperature}^2 + 5.09259\text{E-}004 \\ & * \text{Substrate Loading} * \text{fermentation time} - 0.024889 * \text{Substrate Loading} \\ & * \text{temperature} - 0.20583 * \text{fermentation time} * \text{temperature} \end{aligned} \quad (4.2)$$

Model equation of both coded and actual factors can be used to make prediction about the response for given levels of each factors. The coded model equation is useful for identifying the relative impact of the comparing the factor coefficients.

4.5 Model Adequacy Check

The model was tested for adequacy by analysis of variance. The regression model was found to be highly significant with the correlation coefficients of determination of R^2 -Squared, adjusted R^2 -Squared and predicted R^2 -Squared having a value of 0.9948, 0.9880 and 0.9353 respectively. The quality of the model developed could be evaluated from their coefficients of correlation. The value of R^2 -squared for the developed correlation is 0.9948. It implies that 99.48% of the total variation in the percentage of conversion is attributed to the experimental variables studied. The graph of the predicted values obtained using the developed correlation versus actual values is shown in Figure 4-4. The results in Figure 4-4 demonstrate that the regression model equation provided a very accurate description of the experimental data, in which all the points are very close to the line of perfect fit. This result indicates that it was successful in capturing the correlation between the three variables to the production of citric acid. The adequacy of the model was further checked with ANOVA, based on a 95% confidence level, F-value is a test for comparing model variance with residual (error) variance. If the variances are close to be the same, the ratio will be close to one and it is likely that any of the factors have a significant effect on the response with the P-value less than 0.05. It was calculated by model mean square divided by residual mean square. The effectiveness of the model could also be measured so as to assure its approximation to the true value. Thus, regression coefficient, R^2 , could be used for checking its adequacy. The regression value is between 0 and 1, and as it approaches to 1 it fits well to the experimental data otherwise it indicates failure of approximation. In this case, R^2 , 0.9948 was obtained, which was close to one and the value Adj- R^2 was 0.9880. and it is in a reasonable agreement with R^2 .

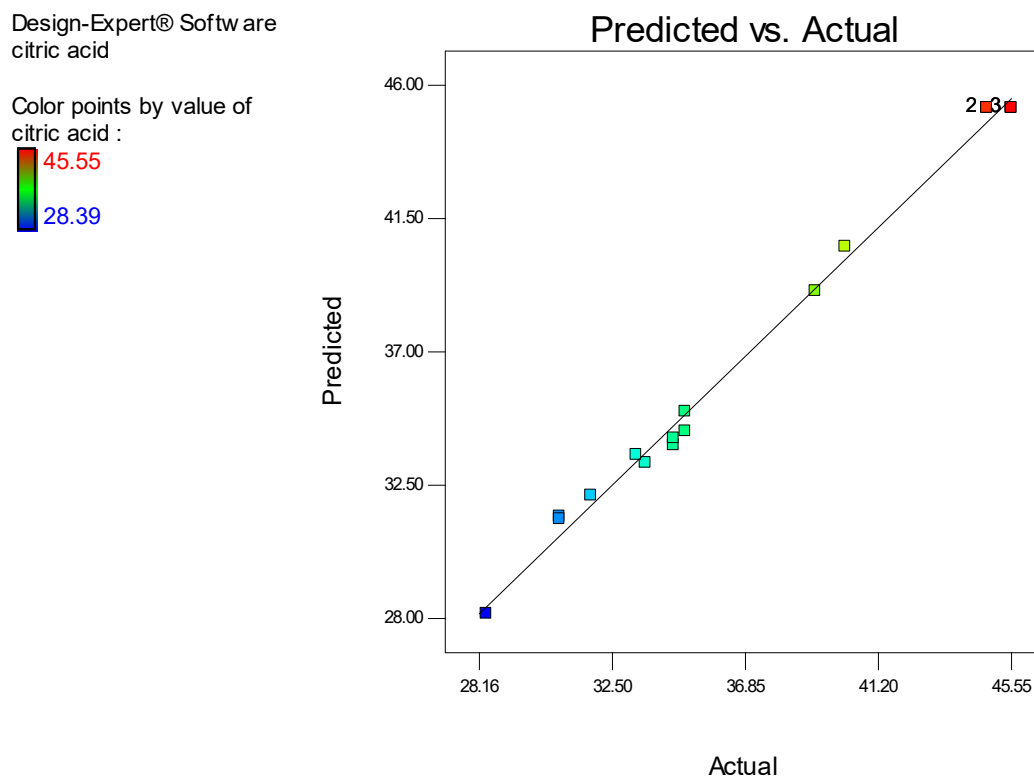


Figure 4-4: Predicted Vs Actual Experimental Value for Citric Acid Yield

4.6 Effect of Interactive Parameters between Process Variables

The interaction response was also significantly affected by two interactive variables at fixed third variable. Three dimensional (3D) response surface plots (plotted in order to understand the interaction between the variables and the optimum level of each variable) and contours plots (presented the effect of two variables) were generated to determine the optimum levels of the variables that were investigated in this study. The plots were generated by keeping one variable constant at the center point and varying the others within the experimental range. The resulting response surfaces showed the effect of solids loading, incubation time and fermentation temperature on citric acid production. The significance of the interaction between the corresponding variables was indicated by saddle nature of the contour plots.

An interaction occurs when the response was different depending on the setting of two factors. Plots make it easy to interpret two factors interact. They would appear with two non-parallel

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lines, indicating that the effect of one factor depends on the level of other. If the plotted points fall outside of the range, the differences are unlikely to be caused by error alone and can be attributed to the factor. If the “I beams” overlap there is no significant difference (95% confidence is default) between the two points. We can then choose the most economical or continent level for that factor. As it can be seen from equation 4.1 the interaction factor effects on the citric acid yield can be understood easily by the coefficient of interaction factors. There were three interaction factors analyzed by the model equation. These are:

- AB- Substrate loading and Incubation time
- AC- Substrate loading and fermentation temperature
- BC- Incubation time and fermentation temperature

Among these three interaction factors interaction of incubation time and fermentation temperature (BC) was the most significant factor for citric acid production as a response, because it has the highest coefficient (3.09) of the rest. The sign of the coefficient of the interaction factor indicates the effect of interaction factor on citric acid yield. Therefore, the interaction factors with positive sign have positive effect on citric acid yield (as interaction factors increase citric acid yield increase). Whereas, interaction factors with negative signs have a negative effect on citric acid production (as the interaction factors increase citric acid yield decrease). The 3D response surface and contour plots of the effect of the interaction of incubation period, temperature and substrate loading with the response of citric acid production are discussed below.

4.6.1 Effect of Substrate Loading and Fermentation Temperature on Citric Acid Yield

Figure 4-5 shows the effect of solid loading and fermentation temperature on citric acid production. The trend observed indicates that citric acid production was not favored at high solids loading and low solids loading relative to the amount at the center point. This is evident from the increase in citric acid concentration when the solid loading was increased but when the number of solids loading at the very high amount the production citric acid was decreased because of the polyalcohol forming (Çevrimli et al., 2009).

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The amount of solids used for SSF determines the number of fermentable sugars that can be liberated for the purpose of producing citric acid. Since citric acid production is enhanced at increased sugar concentration, it will be expected that increasing the solid loading will also have a similar effect but the amount of sugar concentration should not be more than the optimum amount, which is 240 g/L. The increase in citric acid concentration in the course of fermentation could be attributed to the consumption of sugar substrate by the *Aspergillus niger* cells to produce citric acid (Barrena et al., 2017). These results are in agreement with those reported by Abbas et al., (2016), a similar increase in citric acid concentration with an increase in sugar for the production of citric acid from banana peel but decrease when the amount goes beyond the optimum one.

As it can be observed in the Figure 4-5, with rising substrate loading and fermentation temperature at fixed incubation time, the citric acid production was increased but declined when the fermentation temperature and substrate loading increased further. The maximum yield was found at the moderate substrate loading (36 g) and fermentation temperature (30 °C). The optimum fermentation temperature of the fermentation medium displayed a significant overall positive effect on citric acid concentration as shown in Figure 4-5. This is evident from the relationship between the optimum temperature and the production of citric acid. This result is also corroborated by the negative value of the coefficient estimate of the model term representing substrate loading (i.e. A) as shown in Table 4-7. At moderate solid loading and fermentation temperature citric acid production was observed maximum. This observation could be attributed to the fact that an optimum temperature and substrate loading are required to produce high amount citric acid. This is because when the temperature of the medium was low, the enzyme activity was also low, giving low impact on the citric acid production. Similarly, when the temperature of the medium was increased above 30 °C, the biosynthesis of citric acid was decreased relative to the amount of citric acid at 30 °C. It might be also due to the accumulation of by-products such as oxalic acid. And also further increase in carbon source causes a reduction in the solubility of nutrients in the substrate and low degree of swelling (Adinarayana et al., 2004).

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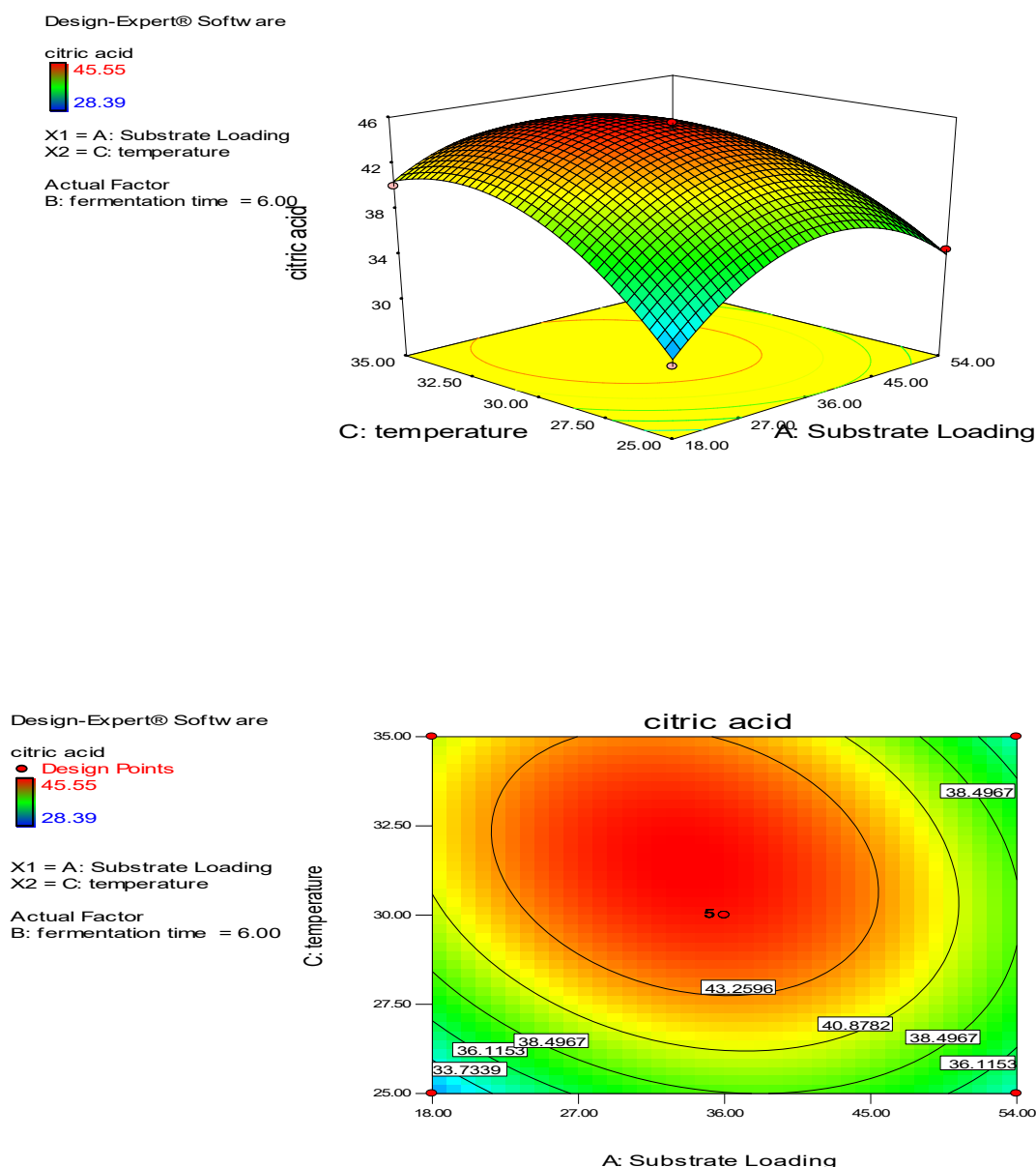


Figure 4-5: 3D and Corresponding Contour Plot Showing the Effect of Solid Loading and Fermentation Temperature on Citric Acid Concentration at Six Days

4.6.2 Effect of Incubation Time and Fermentation Temperature on Citric Acid Yield

Incubation time is an important fermentation variable that has been reported to affect citric acid production (Company et.al., 2008). Citric acid production displayed a quadratic relationship with

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the incubation time showing that the citric acid concentration increased within incubation time to a certain level beyond which any further increase in incubation time did not positively affect citric acid production as shown in Figures 4-6 and 4-7. This observation has also been reported by previous researchers. According to Papagianni (2007), metabolite formation during fermentation generally increases with an increase in incubation time up to a particular limit.

At low values of incubation time, there is a good production of citric acid as a result of high amount of carbon source and these results in an increment in the production of metabolic products. The decrease in citric acid production at the incubation time is beyond the optimum amount be accounted to decrease in sugar content, the growth phase of fungi, decrease in the amount of available nitrogen and the presence of inhibitor produced by the fungi itself in the fermentation medium. The effect of fermentation time and incubation temperature on the response yield of extract by fixing the substrate loading at its center point was shown in the form of 3D and surface contours as illustrated in the Figure 4-6. The maximum citric acid yield was found at moderate incubation temperature and fermentation time. As the fermentation time and incubation temperature rise to the optimum amount the yield of citric acid was increased but above and below the center point the yield was declined.

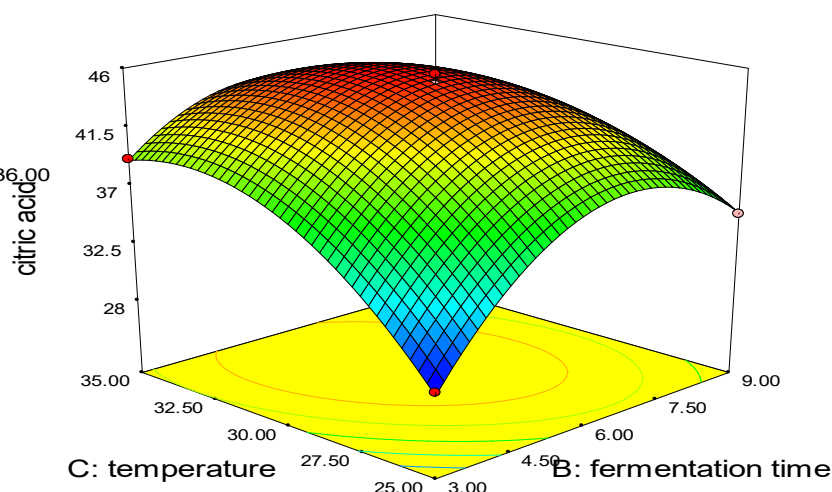
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citric acid
45.55
28.39

X1 = B: fermentation time
X2 = C: temperature

Actual Factor

A: Substrate Loading = 36.00



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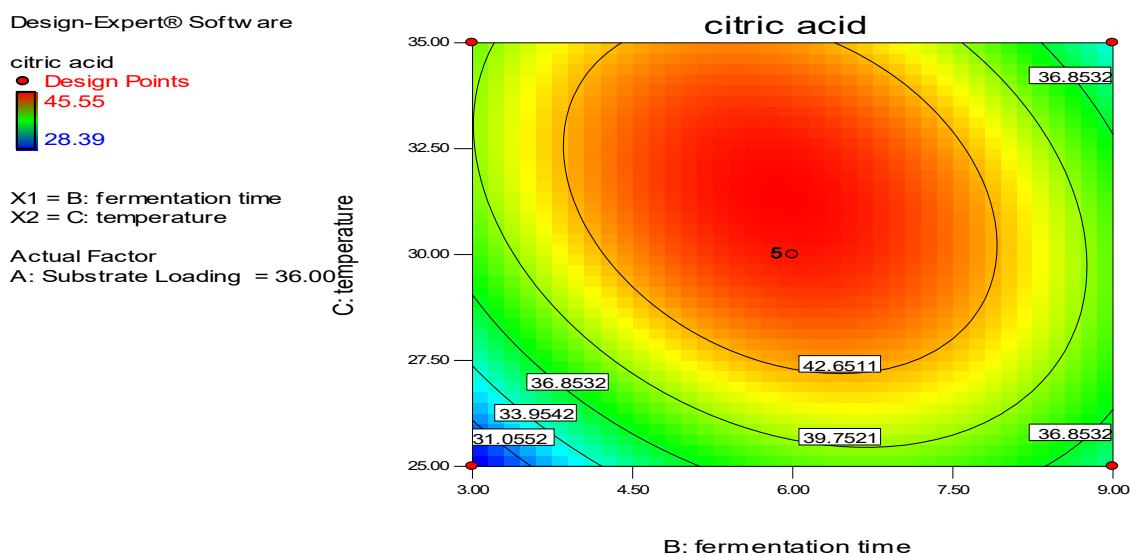
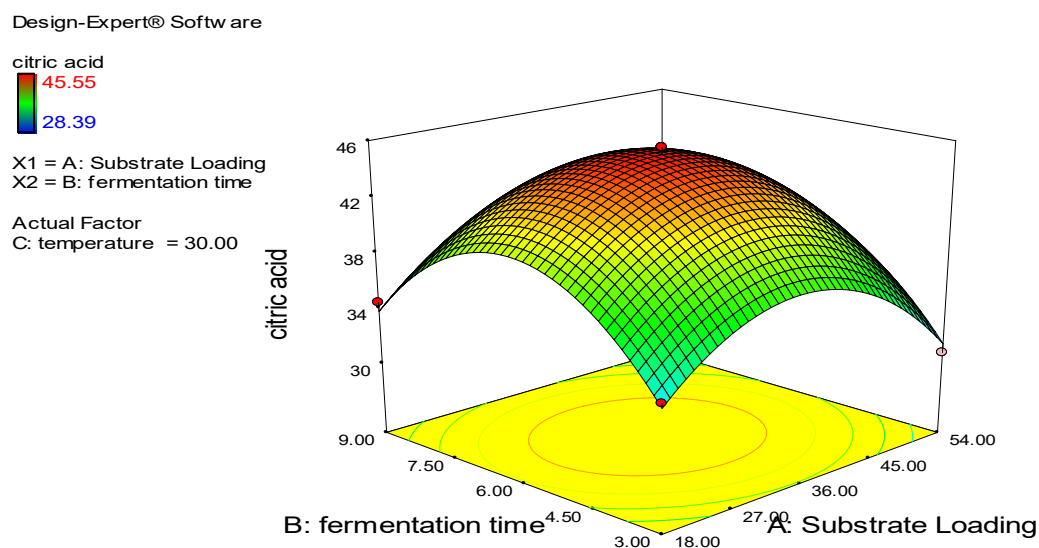


Figure 4-6: 3D and Corresponding Contour Plot showing the Effect of Incubation Time and Fermentation Temperature on Citric Acid Concentration at 36g



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medium. The low amount of citric acid in lower incubation time is due to; fungi need enough amount time to ferment the entire carbon source, to adapt the environment as well.

4.7 Experimental Optimization

One of the objectives of the present stud was to find the optimal process variables for a better citric acid yield at minimum process economy. The process variables such as incubation time, substrate loading and fermentation temperature were optimized. In optimizing the fermentation process, fermentation temperature, incubation time and substrate loading are a set of process variables held to be in range while the citric acid yield was the response that needs to be maximized. Table 4-8 showed the summary of factors, goals and the corresponding set of specific objectives that could optimize the process condition to have the highest responses. The expert design has three different optimization choices, namely, numerical optimization (set goal for each response), graphical optimization (set minimum and maximum limits for each response and then create an overlay highlighting an area of operability) and point predication optimization (enter desired operating conditions and discover predicted response values with confidence interval). In numerical optimization choice, depending on constraints selected different alternatives solution of optimization was given by expert design. For this study, numerical optimization was selected to obtain the better highest response of extract.

Table 4-8: Constraints Applied for Optimization

Name	Goal	Lower limit	Upper limit	Lower weight	Upper weight	Importance
Substrate loading(g)	Is in range	18	54	1	1	3
Incubation time(d)	Is in range	3	9	1	1	3
Temperature (C ⁰)	Is in range	25	35	1	1	3
Yield(g/L)	Maximize	28.39	45.55	1	1	3

The design expert under numerical optimization gave 30 different optimization solutions. Depending on the parameters by compromising yield, economy and energy carrying, the best solution was selected among the alternatives. Undertaking the consideration of these constraints,

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31.92 g, 5.82 days and 31.55 °C were selected which gave 45.609 g/L yield among optimizing alternatives given by the design expert software. Desirability function was used to identify the optimum level of factors and get a maximum desirable response. The optimized batch was selected with maximum combined yield desirability value i.e. 1.00 and the graph of the optimized solution for the fermentation condition is shown in Figure 4-9.

As shown in Table 4-8, the numerical optimization can be taken as an optimal value because the predicted value is close enough with the actual value. The histogram shows how well each variable satisfied the criteria and values near one are good.

Table 4-9: Different Alternative Optimization

Number	Substrate loading	Incubation time	Fermentation temperature	Citric acid	Desirability
1	33.34	6.00	31.03	45.6002	1.000
2	33.26	5.71	31.71	45.6409	1.000
3	34.04	6.18	31.04	45.5631	1.000
4	34.24	5.88	31.27	45.6361	1.000
5	35.17	5.66	31.53	45.5761	1.000
6	34.74	5.93	31.64	45.6227	1.000
7	32.46	5.91	31.72	45.6342	1.000
8	34.03	5.63	31.88	45.6063	1.000
9	32.96	5.73	31.76	45.6413	1.000
10	35.45	5.72	31.39	45.5719	1.000
11	34.59	5.66	31.83	45.6177	1.000
12	31.66	5.99	31.50	45.5808	1.000
13	33.74	5.61	31.41	45.5939	1.000
14	32.71	5.65	31.61	45.6146	1.000
15	34.26	5.71	31.68	45.6285	1.000
16	31.74	5.61	31.80	45.5686	1.000
17	34.72	5.84	31.82	45.6043	1.000

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18	34.09	5.62	31.49	45.6044	1.000	
19	33.30	5.65	31.74	45.627	1.000	
20	33.64	5.62	31.28	45.5853	1.000	
21	31.92	5.82	31.55	45.609	1.000	Selected
22	35.71	5.75	31.23	45.5506	1.000	
23	31.96	5.78	31.58	45.6106	1.000	
24	33.18	5.95	31.30	45.6376	1.000	
25	34.37	5.74	31.56	45.6323	1.000	
26	33.93	5.81	31.37	45.6441	1.000	
27	32.64	5.83	31.12	45.5936	1.000	
28	31.53	5.71	31.66	45.5767	1.000	
29	34.79	5.90	31.53	45.6271	1.000	
30	33.14	5.83	32.22	45.5921	1.000	

The ramp plot of the optimization solution for the fermentation conditions was shown in Figure 4.8. The ramp display combines the individual graphs for easier interpretation and the dot on each ramp reflects the factor setting or response prediction for that solution.

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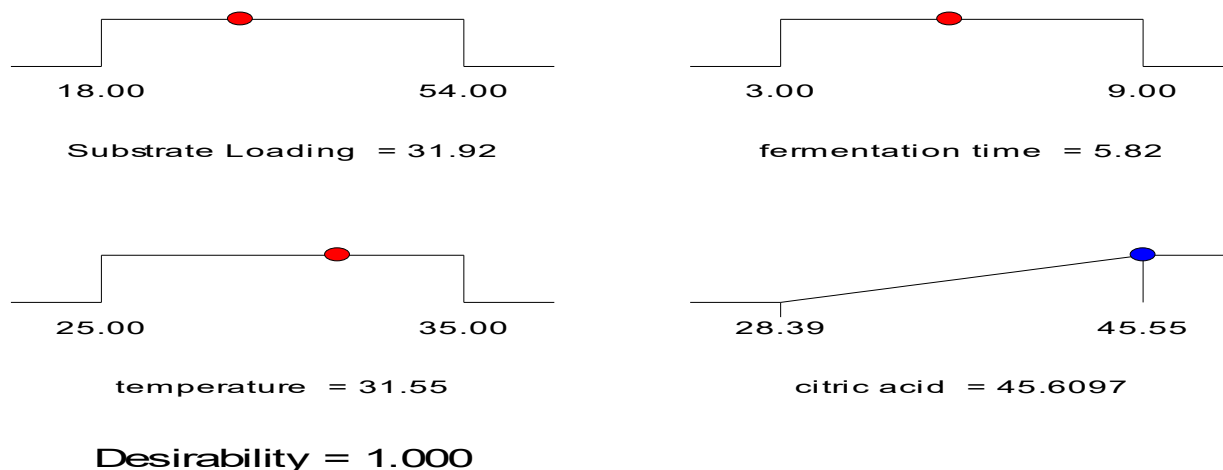


Figure 4-8: Ramp Plot of Optimization Solution for the Response

The predicted highest screened yield was produced from a solution of optimization at substrate loading (31.91 g), incubation time (5.82 days) and temperature (31.55 °C) where the screened yield was at 45.609 g/L as summarized in Table 4-10. In order to verify this prediction, the experiment was conducted (repeated five times) and the results were computable with prediction. This is good in terms of cost efficiency for commercial scale, as the only small amount of energy power and moderate chemicals are a need for the quality citric acid production.

Table 4-10: Summary of Optimization Conditions

Substrate	Time	Temperature	Citric acid yield(predicted)	Experimental
31.92g	5.82days	31.55°C	45.609 g/L	45.59 g/L

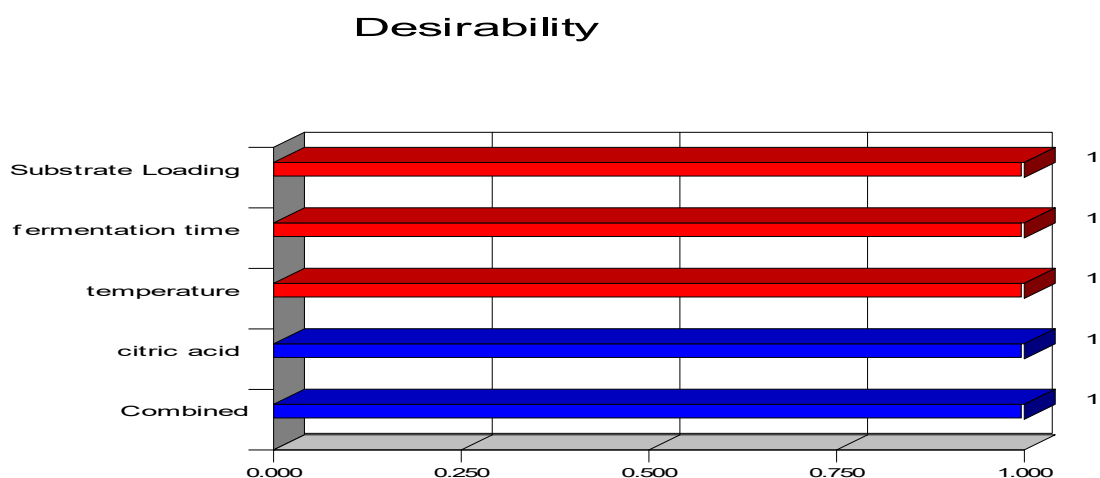


Figure 4-9: Desirability Plot of Optimization Solution for the Response

4.8 Characterization and comparing of the product quality with Standard

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

4.8.1 Sulphated Ash

The sulfated ash test follows a procedure to measure the amount of residue substance not volatilized from a sample when the sample is ignited in the presence of sulphuric acid. The test is usually used for determining the content of inorganic impurities in an organic substance.

Total sulfated ash content of the product was calculated by direct substitution in the relationship expressed in equation 3.16 . The result of a current study in terms of sulfated ash content was satisfactory as compared to the standard IS 13186 and Citrique Belge as mentioned in the Table 4-11. The sulfated ash content of the extracted citric acid in this study was lower than the standard which was 0.02 ppm <0.05 ppm. The amount of residue obtained did not exceed the

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limit specified in the standard. This implies that the product has good quality when compared to the standard value.

4.8.2 Arsenic

Arsenic is a poisonous trivalent and pentavalent solid element; it is odorless and tasteless heavy metal. Inorganic arsenic is a known carcinogen and can cause cancer of the skin, lungs, liver, and bladder. Lower level exposure can cause nausea and vomiting, decrease production of red and white blood cells, abnormal heart rhythm, damage to blood vessels, and a sensation of pins and needles in hands and feet. Therefore, in order to overcome the problem caused by intake of this heavy metal the quality of the product was assessed by Atomic Absorption Spectroscopy (Buck of Scientific VGP AAS, USA). As illustrated in Table 4-11, the result of the elemental analysis in this study was found to be BDL (<0.06) which showed that the concentration of arsenic in the sample was below the value specified in the standard (1 ppm max).

4.8.3 Iron

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

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

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Table 4-11: Physical and Chemical Properties of Citric Acid Produced at Optimized Fermentation

Parameter	Present result	IS 13186, 2010 standard	Citrique Belge
Appearance	White powder	Colorless crystals or white powder	Colorless crystals or white powder
Odor	Odorless	Odorless	Odorless
Colour (%)	94.42	-----	Minimum 98
Assay (%)	99.4	99.5	99.8
Melting point(⁰ C)	151	153	153
Lead(ppm)	BDL(<0.05)	3ppm max	0.5ppm max
Iron (ppm)	3	5ppm max	1ppm max
Arsenic (ppm)	BDL(<0.06)	1ppm max	1ppm max
Ash (%)	0.024	-----	0.02 max
Sulphated ash (%)	0.03	0.05ppm	0.05max

4.8.4 Lead

Lead is one of the limited classes of elements that can be described as purely toxic. Lead is a highly toxic metal and a very strong poison. Lead poisoning is a serious and sometimes fatal condition. It occurs when lead builds up in the body. Most other elements thought toxic and high concentration is actually required nutrient at a lower level. There is no exposure level below which lead appears to be safe. High level of lead is particularly of great concern especially due to the fact that citric acid was consumed by a human who is uniquely susceptible to the effect of lead. As it can be illustrated in Table 4-11, the result showed the maximum amount of lead (BDL<0.05) in the sample was significantly lower than that of the standard (1 ppm max).

4.8.5 Fourier Transformation Infrared (FTIR) Analysis of Citric Acid Yield

FTIR spectrum of the produced citric acid after purification is demonstrated in Figure 4-10. The strong peak at 3498 cm^{-1} is attributed to $\text{O}_{19}-\text{H}_{21}$ bond stretching vibration for dimer. The other strong band at 3291 cm^{-1} is assigned to $\text{O}_{22}-\text{O}_{24}$ bond stretching vibration for dimer citric acid. The very strong Band at 1725 cm^{-1} is assigned to $\text{C}=\text{O}_3$ anti-symmetric stretching vibration for

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the dimer. Medium peak at 1430 cm^{-1} is attributed to $\text{C}_{29}\text{-C}_{30}$ stretching vibration dimer citric acid. The other medium band at 1242 cm^{-1} assigned to symmetric stretching vibration for the dimer. The strong band at 1140 is attributed to $\text{C}_{38}\text{-O}_{40}$, $\text{C}_1\text{-O}_3$, and $\text{C}_8\text{-O}_{13}$ stretching vibration for dimer citric acid. The medium band at 1053 cm^{-1} assigned to $\text{C}_8\text{-C}_5$ and $\text{C}_8\text{-C}_{15}$ stretching vibration for dimer citric acid. The weak band at 904 cm^{-1} attributed to $\text{C}_{29}\text{-O}_{31}$ stretching vibration for dimer citric acid. The strong peak at 782 cm^{-1} is assigned to COO_2 scissoring vibration for dimer citric acid. The strong band at 599 cm^{-1} attributed to $\text{O}_{40}\text{-H}_{42}$ torsion vibration, to COO_5 , Scissoring vibration, to $\text{C}_{31}\text{-C}_{29}\text{-C}_{30}$ scissoring vibration and to COO_5 rocking vibration for dimer citric acid. Moreover, FTIR spectrum of the purified citric acid product is self-same to the analytic grade of 99.9% pure citric acid, which is further validated for the quality of produced citric acid.

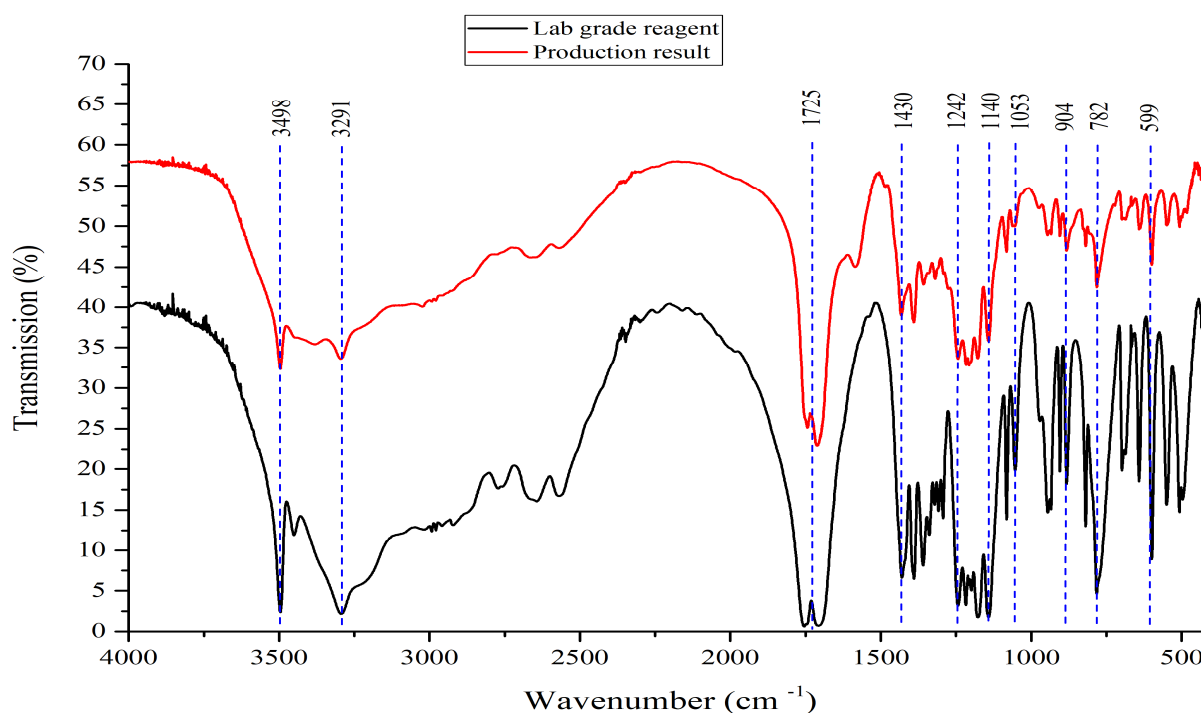


Figure 4-10: FTIR Spectrums of Production Result and Lab Grade Reagent

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Citric acid production from banana peel by solid state fermentation using *A. niger* was investigated in this study. A three-variable, three-level BBD was used to study the simultaneous effect of solid loading, incubation time and fermentation temperature. A statistically significant model ($p < 0.0001$) was developed to describe the relationship between citric acid concentration and the chosen independent variables. The fermentation conditions were optimized using RSM. The statistical model showed a good fit with the experimental data ($R^2 = 0.9948$) with a low standard deviation. Based on the experimental result obtained, it was found that the process variables revealed a significant interaction effect on citric acid yield result except for the interaction between substrate loading and incubation time.

According to the model regression equation developed, extraction yield was positively affected by linear effects except substrate loading and negatively affected by all quadratic effects. In this study based on the analysis of experimental results, it is realized that the individual factors and their interaction effects are significant model terms on citric acid yield. This shows that the capability of the design of the experimental analysis is successfully capturing these effects. Moderate level of solid loading, incubation time and fermentation temperature was favorable for citric acid production. Optimal values of extraction conditions which gave maximum citric acid yield were selected using numerical optimization of design expert. The maximum citric acid yield obtained after conducting the experiment at selected optimum condition was 45.59 g/L at 31.92 g, 5.82 days and 31.55 °C with a high value of combined desirability. These values were selected from 30 alternative optimal solutions set by design expert, based on considerations and compromise of yield, energy, and economy during extraction.

The observed quantitative difference in the quantity of the citric acid produce was due to substrate loading, incubation time and fermentation temperature variability. Thus, determination of the appropriate amount of the substrate loading, optimum incubation time and fermentation temperature needs to have the consideration to get the maximum amount of citric acid. Based on the study conducted, it was found that the properties of citric acid produced at the optimum

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condition (31.92 g of substrate loading, 5.82 days incubation time and 31.55 °C fermentation temperature) were assessed by using different methods and the findings are fulfill the standard requirement as listed in the Table 4-11. It can be concluded from these findings that comparable good quality citric acid can be produced from banana peel by using solid state fermentation which is the most environmentally friendly type of fermentation process.

Finally, it can be concluded that utilization of banana peels for citric acid production does not only solve the problem of waste disposal but also save valuable foreign exchange by reducing the citric acid imported from abroad.

5.2 Recommendations

It has been given the demonstration possibility of utilizing banana peels for citric acid production but; further works are still important. Future research that would be complementary to the current study aiming at the investigation of the better citric acid yield and concerning the environment is necessary. Hence, the following points are recommended strongly.

- Experiments performed on fermentation of banana peels showed that good quality grade citric acid can be produced from banana peels. But ahead of implementing, further study, better process design, and feasibility studies need to be carried out.
- One can also investigate the effective species of *A. niger* and effective type of low-grade alcohols such as ethanol, methanol on citric acid yield.
- Storage techniques of banana peels could also be studied for the production of citric acid and further investigation on the elemental analysis of the banana peels must be studied.
- Further studies are needed on various topics related to utilization and best incorporation techniques of other waste material in citric acid production.
- It is recommended to conduct researches to measure the optimum amount of PH, inoculum density, particle size and trace elements on citric acid yield.
- It is recommended to conduct research on purification using ion exchange.
- Further studies are needed to determine the concentration of citric acid by using HPLC.

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APPENDICES

Appendix A: Proximate Analysis of Banana Peel

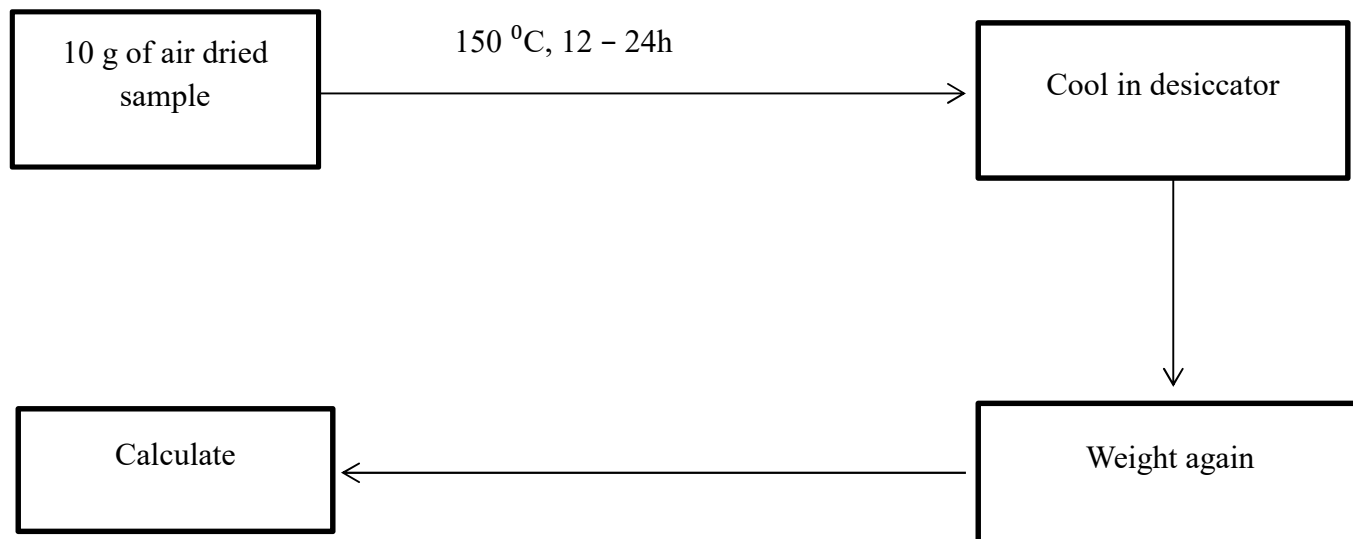


Figure A - 1: Determination of Humidity Content in Feed Ingredients

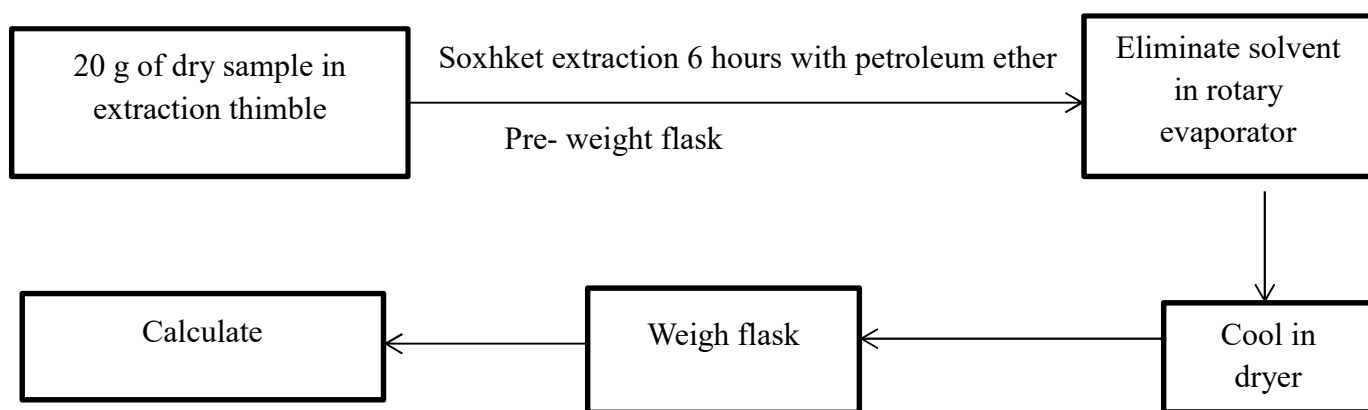


Figure A - 2: Determination of lipids by Soxhlet's method

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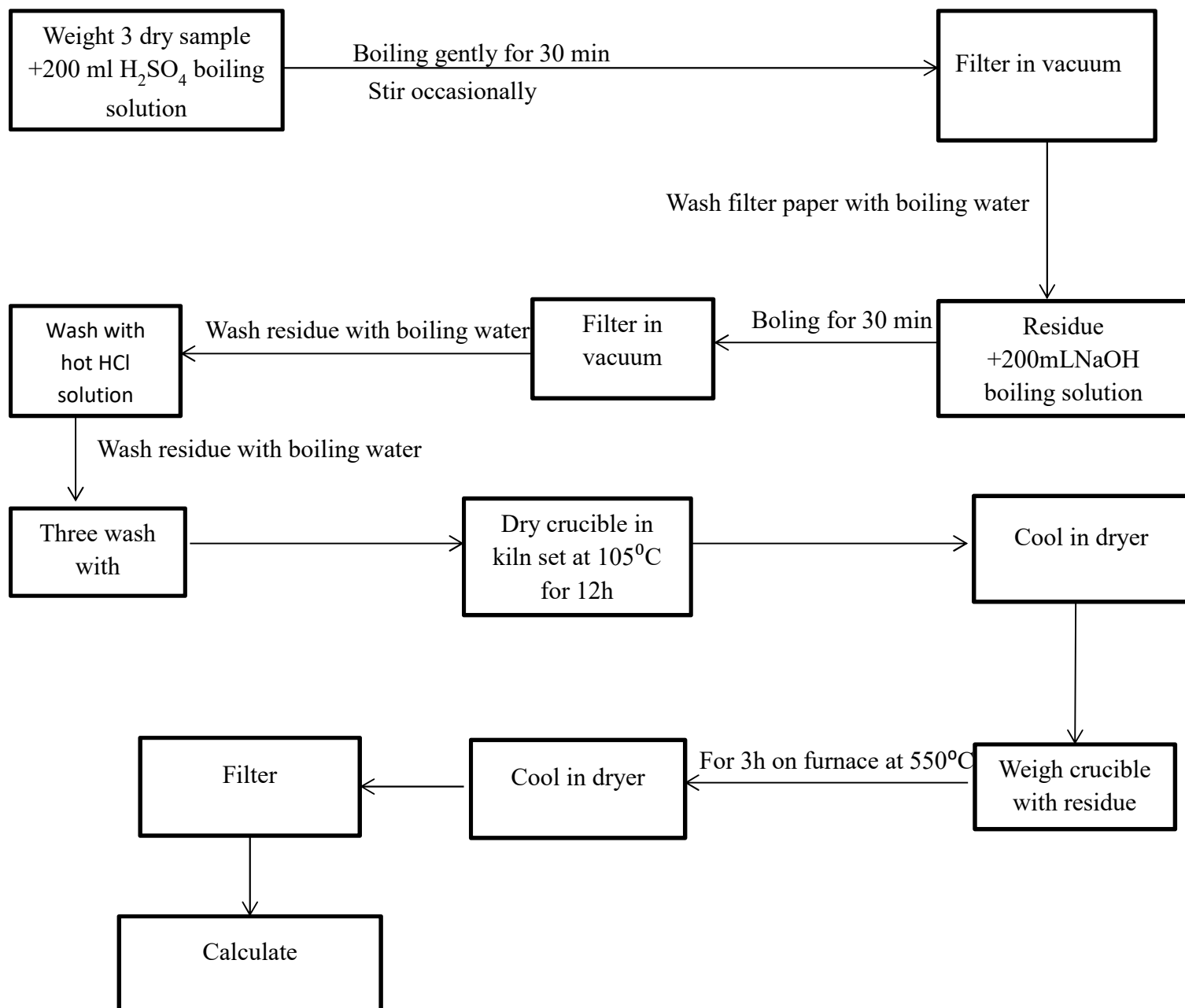


Figure A - 3: Determination of Crude Fiber

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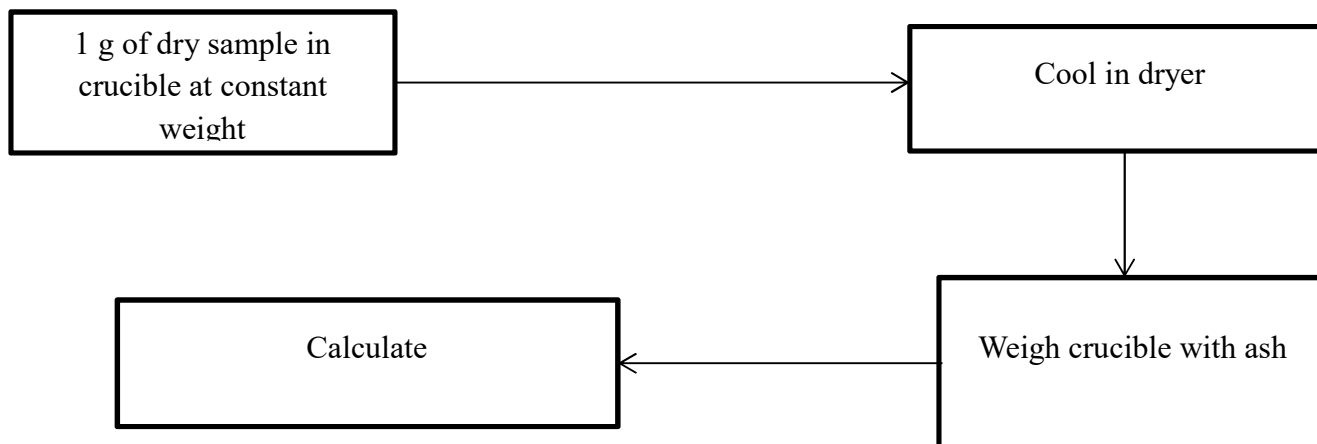


Figure A - 4: Determination of Ash Content in Feed Ingredients

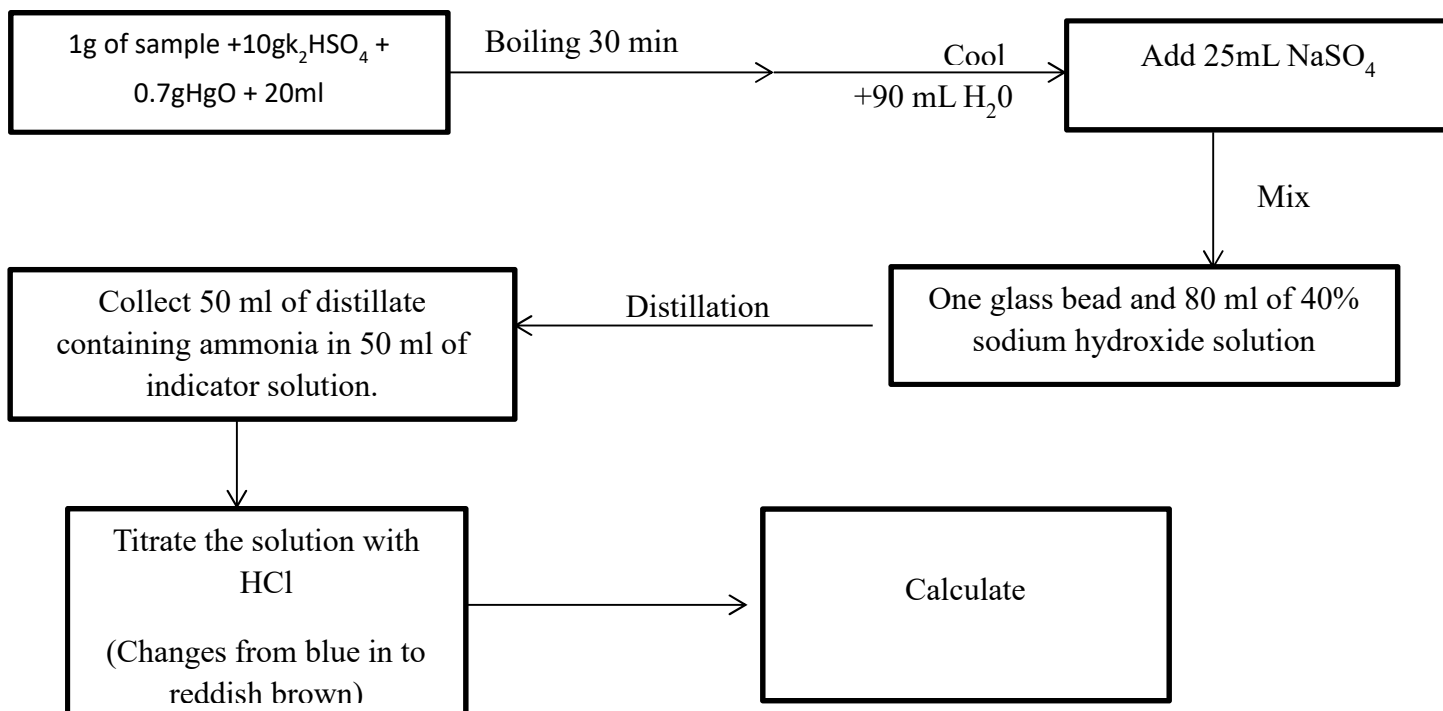


Figure A - 5: Determination of crude protein by Kjeldahl's method

Appendix B: Qualitative Analysis of the Recovered Citric Acid

Table B - 1: Color Analysis

L* (lightness)	a* [-a* (red) and +a* (green)]	b* [+ b* (yellow) and (-b* blue)]
96.68	-0.29	4.48

Intensity(C) and whiteness (w) of the color of the produced citric acid were calculated as follow:

$$W = 100 - \sqrt{(100 - 96.68)^2 + (-0.29)^2 + (4.48)^2}$$

$$= 94.42\% \text{ whiteness}$$

$$C = \sqrt{(0.29)^2 + (4.48)^2}$$

$$= 4.48 \text{ color of intensity}$$

Table B - 2: Gravimetric Analysis

	Optical density	Citric acid (g/L)
Standard	0.09	35.73
Citric acid produced	0.07	29.97

The difference was: $35.73 - 29.97 = 5.76 \text{ g/L}$

Deviation was observed from the pure standard citric acid by 5.76 g/L.

Appendix C: Calculation Part

C1: Amount of H₂SO₄ Used During Citric Acid Recovering

90 g calcium citrate tetrahydrate(selected 1 out 17 calcium citrate for this calculation) was recovered (it exists in its tetrahydrate form, meaning it has four water molecule associated with it).

$$M/W = 570.49 \text{ g/mol}$$

$$\neq \text{ of mol} = 90/570.49 = 0.178 \text{ mol}$$

3 H₂SO₄ for each mole of calcium citrate

$$\neq \text{mol H}_2\text{SO}_4: 3 \times 0.178 \text{ mol} = 0.473 \text{ mol}$$

Used slightly less than needed~95%

$$\neq \text{mol} = 0.473 \times 0.95 = 0.4496 \text{ mol}$$

$$\text{Mass of H}_2\text{SO}_4: 0.4496 \text{ mol} \times 98.08 \text{ g/mol} = 44.098 \text{ g}$$

44.098 g pure H₂SO₄, but commercial H₂SO₄ is 98%

$$\text{Mass of 98\%} = 44.098 / 0.98 = 44.998 \text{ g}$$

$$\text{Volume} = \text{mass/density} = 44.998 \text{ g} / 1.84 \text{ g/mL} = 24.456 \text{ mL}$$

C2: Titrimetric Method for Better Quality Assay

Citric acid was determined by titration using 0.01N NaOH and Phenolphthalein as an indicator and calculated as a percentage according to the following formula:

$$\text{Normality of Citric acid} = \text{normality of NaOH} \times \text{NaOH volume} \div \text{volume of Citric acid}$$

$$\text{The concentration of Citric acid} = \text{Citric acid normality} \times \text{equivalent} \times 100 \div \text{volume of distillation (Equivalent} = 96)$$

Appendix D: Experimental design and analysis

Design-Expert® Software
citric acid

Color points by value of
citric acid :

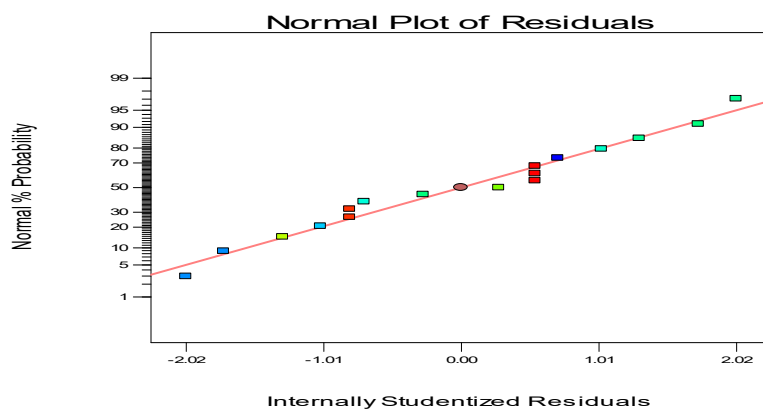


Figure D - 1: Normal Plot Residuals

Design-Expert® Software
citric acid

Color points by value of
citric acid :

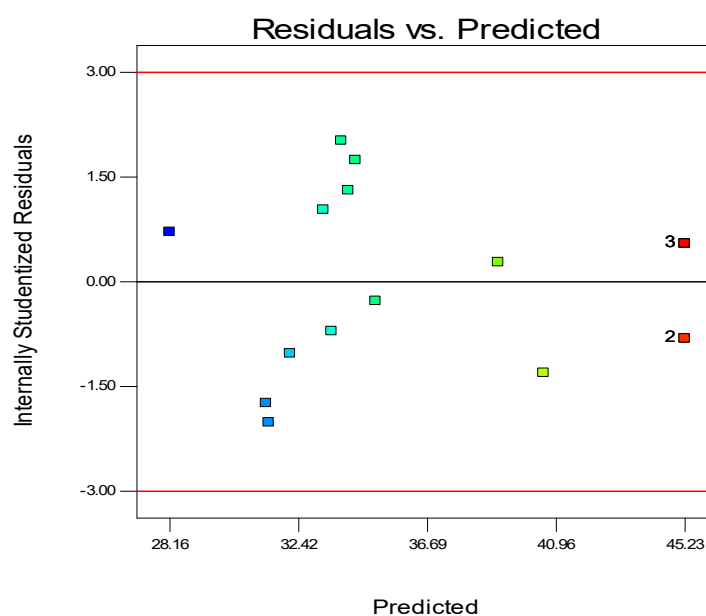


Figure D - 2: Residuals Vs Predict

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Design-Expert® Software are
citric acid

Color points by value of
citric acid :

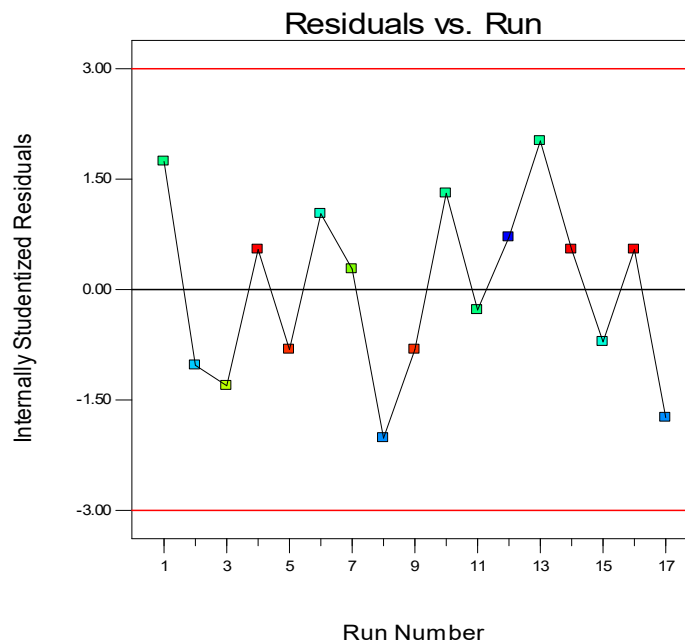


Figure D - 3: Residuals Vs Run

Design-Expert® Software are
citric acid

Lambda

Current = 1

Best = 1.93

Low C.I. = -0.44

High C.I. = 4.36

Recommend transform:

None

(Lambda = 1)

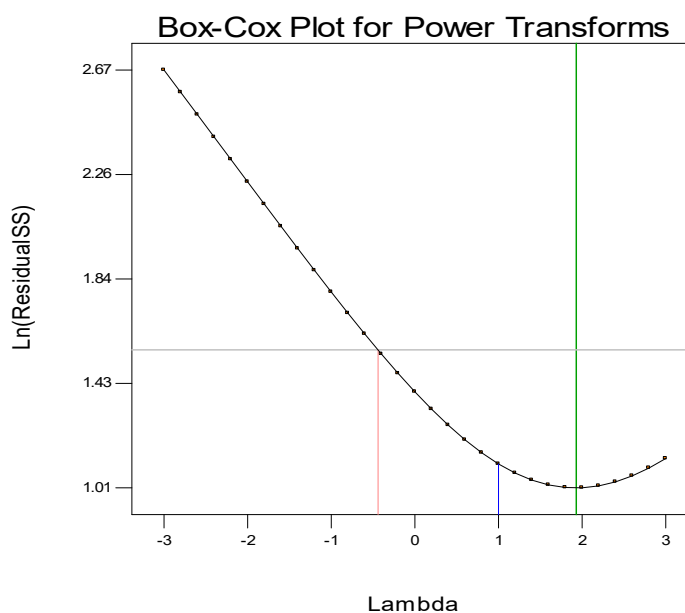


Figure D - 4: Box-Cox

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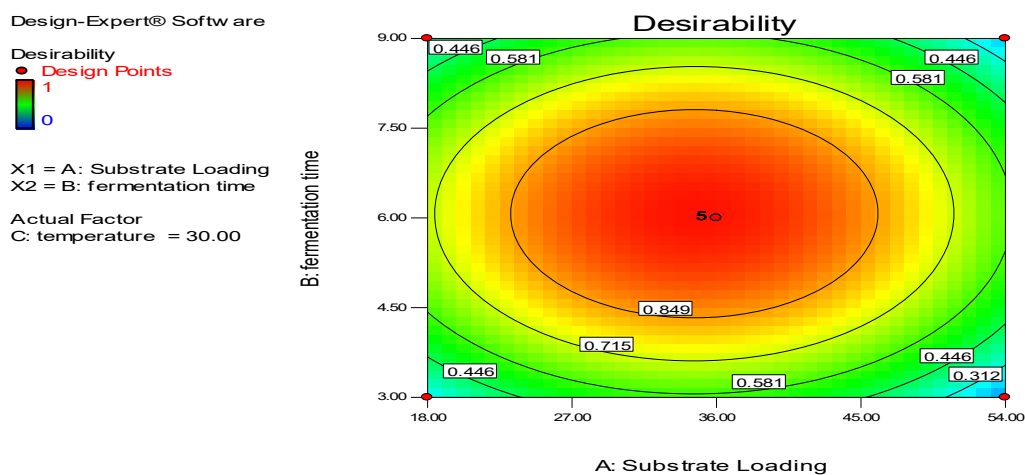


Figure D - 5: Experimental optimization

Appendix E: Laboratory Equipment's and Results

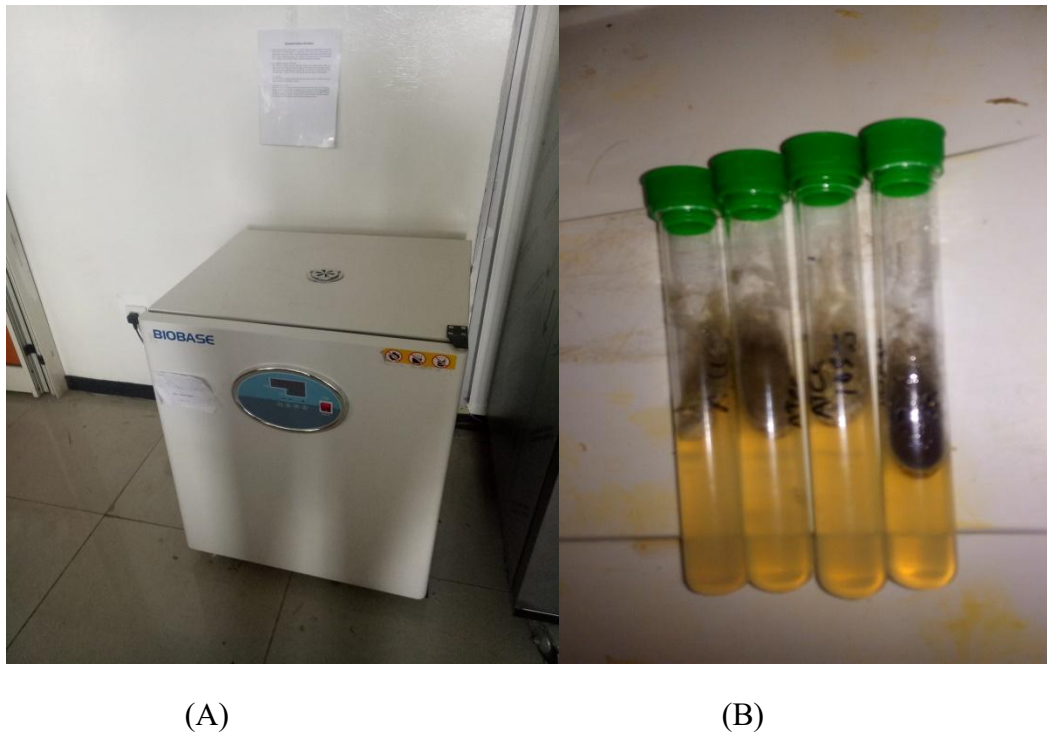


Figure E - 1: (A) Laboratory Incubator and Cultured and (B) *Aspergillus niger* After 5days

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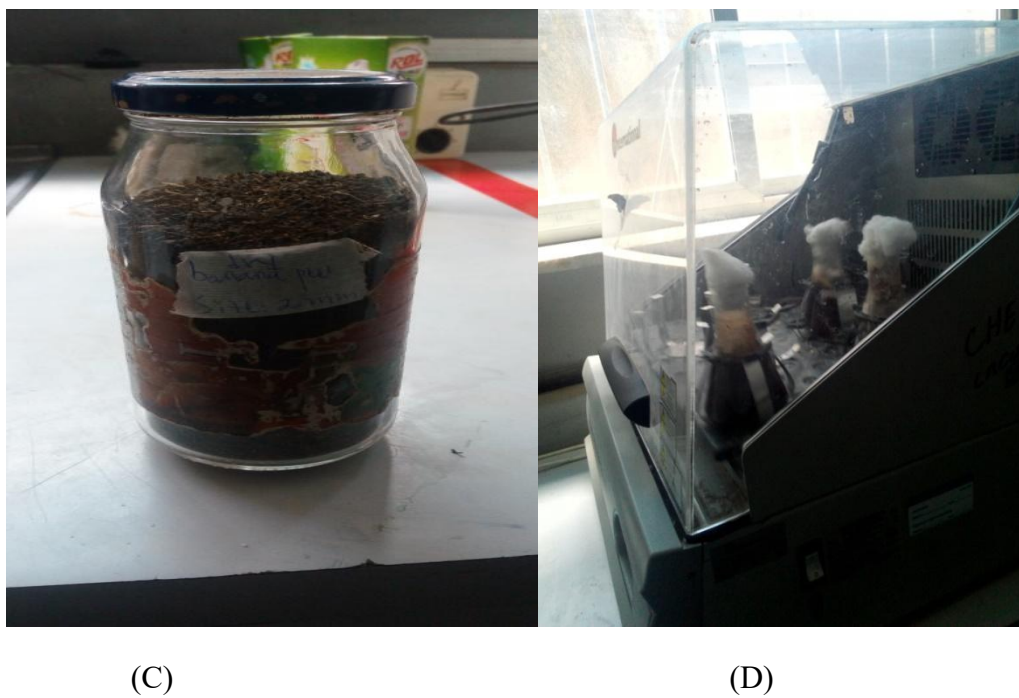


Figure E - 2: (C) Crushed Banana Peel (2mm size) and (D) Incubator Shaker (During fermentation)

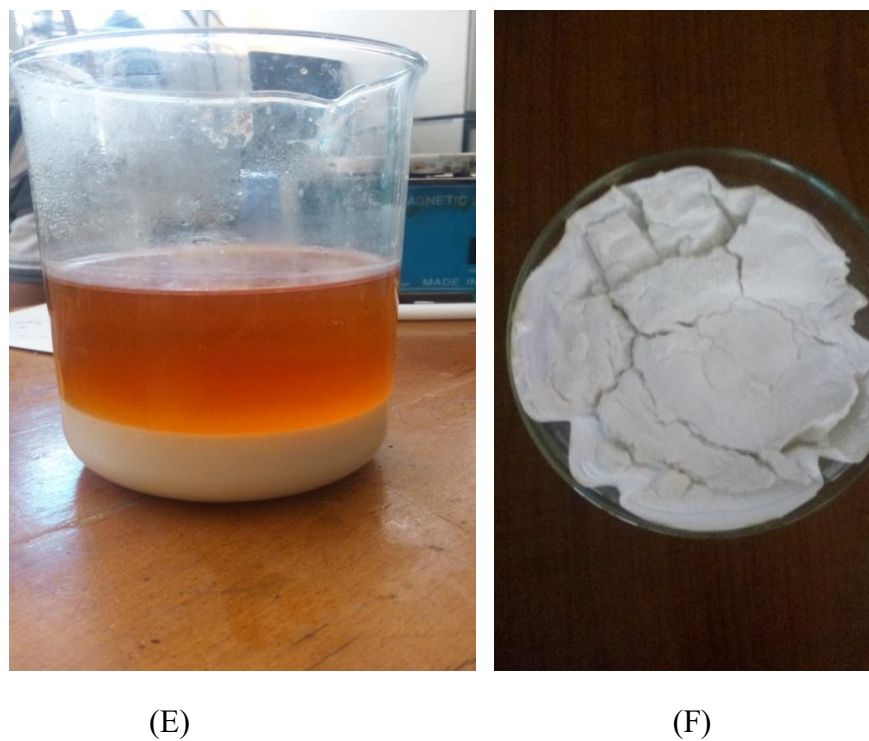


Figure E - 3: (E) Precipitated Calcium Citrate and (F) Dried Calcium Citrate

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(G)



(H)

Figure E - 4: (G) Calcium Sulphate (Gypsum) and (E) Analyte(presumed to be citric acid solution)



(I)



(K)

Figure E - 5: (I) Apparatus Setup (During titration) and (K) weak pink after titration

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(L)



(M)

Figure E - 6: (L) Frieze Dryer and (M) produced Citric Acid (for Characterization)