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SCHOOL OF CHEMICAL AND BIO ENGINEERING

Utilization of coffee husk as a substrate for vinegar production using *Acetobacter aceti bacteria*

A THESIS PROPOSAL SUBMITTED TO THE SCHOOL OF CHEMICAL AND BIO ENGINEERING IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN CHEMICAL ENGINEERING (BIOCHEMICAL STREAM)

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

This is to certify that the thesis prepared by Piniel Belachew entitled "The Utilization of Coffee Husk as a Substrate for Vinegar Production using *Acetobaceter aceti bacteria*" and submitted in partial fulfillment of the requirements for the Degree of Master of Science in Chemical and Bio Engineering (Under Biochemical Engineering Stream) complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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By my signature below, I declare that this thesis entitled "The Utilization of Coffee Husk as a Substrate for Vinegar Production using *Acetobaceter aceti bacteria*" 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 Information taken from published and unpublished work of others has been acknowledged in the text and a list of references is given. I have followed all ethical principles of research in the preparation, data collection, data analysis and completion of this thesis. The work was under the guidance of Dr.Eng.S.Anuradha Jabasingh, instructor in Addis Ababa University, School of Chemical and Bio Engineering.

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Abstract

Advancement in industrial biotechnology offer potential opportunities for the economic utilization of agro-industrial residues. Coffee husk is a fibrous by-product obtained during the processing of coffee cherries by dry process. These wastes cause pollution to the environment, unless treated properly. This study employs coffee husk as a substrate to produce acetic acid using *acetobacter aceti* bacteria in a submerged fermentation process. Vinegar was formulated after the production of acetic acid from coffee husk. Three factors; temperature, ethanol concentration and time were optimized by conducting 17 experiments according to the Design Expert Software 11, using the response surface methodology. The optimum acetic acid concentration (16.59%) was found at 33.76 ℃, 7.84% (ethanol concentration), and 68h respectively and this was analyzed using High Performance Liquid Chromatography (HPLC) followed by the formulation of vinegar to 5% acetic acid.

Keywords: Agro-industrial residues, Coffee husk, Acetic acid, Vinegar, Fermentation, A*cetobacter aceti*, Optimization.

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1. INTRODUCTION

1.1. Background

In recent years, globalization and population growth globally has raised questions to how to utilize agricultural waste and waste management from different agricultural raw materials. These agricultural wastes are produced as a result of various agricultural operations. Nowadays, the world is putting a great political and social pressure to reduce the pollution arising from various human activities. (Achankeng, 2003)

Ethiopia, one of the fast growing countries in Africa has based its economy on agriculture and not yet has adopted the system of utilizing the resulting agricultural wastes. In recent years, there has been an increasing interest towards efficient utilization and value addition of agro-industrial residues such as coffee wastes. Coffee production is important to the Ethiopian economy with about 15 million people directly or indirectly deriving their livelihoods from coffee. Coffee is also a major Ethiopian export commodity generating about 25% of Ethiopia's total export earnings. Ethiopia is the largest producer of coffee in Sub-Saharan Africa and is the fifth largest coffee producer in the world. This huge industry causes environmental pollution due to the large amounts of processing by-products such as coffee pulp and husk that is not being efficiently processed and utilized (Tefera, 2013).

Biotechnological applications in the field of agricultural residues management promote sustainable development of country's economy. With the introduction of biotechnological innovations, mainly in the area of enzyme and fermentation technology, many new opportunities have opened for the utilization of coffee by-products. Coffee by-products are rich in organic content, and thus are a

suitable substrate for fermentation process. The objectives pertaining to food processing by-products, waste and effluents include the recovery of fine chemicals and production of precious metabolites via chemical and biotechnological processes. Pre-treatments, followed by recovery procedures provide value-added products (natural antioxidants, vitamins, enzymes, cellulose, starch, lipids, proteins, aroma, and pigments) of high significance to the pharmaceutical, cosmetic and food industries. (Pushpa and Murthy, 2010).

With the background of high coffee crop production in Ethiopia, there is an imperative need to counterpart this production with some utilization and industrial application of coffee by-products since coffee industry emerges enormous amounts of coffee by-products which are good source of nutrients (Pushpa and.Murthy, 2010). The difficulty concerning food processing by-products, waste and effluents is the recovery of fine chemicals and production of valuable metabolites via chemical and biotechnological processes. Several processes have been developed that utilize these as raw material for the production of bulk chemicals and value-added fine products. Organic acids are among the value added products that can be produced from processing coffee waste. (Pandey, 2000).

An organic acid is an organic compound with acidic properties and has a carbon skeleton. Organic acids are produced by living things, such as plants and animals. The organic acids are utilized in plastic making, tanning, textiles, paper, metal, pharmaceuticals, food industries, beverage making as well as and in manufacturing of cosmetics. Citric acid, acetic acid, lactic acid, tartaric acid, malic acid, gluconic acid, propionic acid and fumaric acid are some of the organic acids used widely in various industries. Acetic acid is a very well-known organic acid used to make vinegar. They are also used in pharmaceutical production, leather tanning, and the manufacture of other organic chemicals (Stellman, 1998).

Organic acids are obtained as the end-products or sometimes as the intermediate components of a particular biochemical cycle. Generally, organic acids are produced commercially either by chemical synthesis or fermentation. Nonetheless, fermentation processes are the most commonly used method. Depending on the substrate type, moisture level and other factors, fermentation process can either be in solid state or submerged state. Submerged fermentation is commonly used in the industrial production of Vinegar. Vinegar is an aqueous solution of acetic acid and trace chemicals that may include flavorings. Vinegar typically contains 5–20% acetic acid by volume. Usually the acetic acid is produced by the fermentation of ethanol or sugars by acetic acid bacteria. (Nakayama, 1959)

This research aims to utilize the byproducts of coffee waste specifically the coffee husk as a substrate for optimizing the synthesis of acetic acid using *acetobacter bacteria* by applying biochemical engineering principles.

1.2. Statement of the problem

The agriculture sector holds the core place in Ethiopia's economy. The generation and accumulation of wastes through this sector is undeniably in large quantities, thus bringing with it a great deal of opportunities to promote sustainable development of the country's economy through the management and efficient utilization of these wastes in various application. Coffee is a major agricultural produce in Ethiopia, which consequently comes up with a challenge by producing large amount of wastes, such as coffee husk and pulp. About 600,000 hectares of the country's agricultural land is planted to coffee, and of each ton harvest of coffee fruit, and in the year 2018/19 Arabica coffee production in Ethiopia is estimated at 7.1 million 60-kilo bags (~426,000 metric tons), leading to an annual release of more than 240,000 tons of coffee husk into the environment.

Currently, wastes from coffee processing industries are accumulated, inefficiently being utilized or generally dumped in the soil and later discharged into the nearby water sources (rivers and lakes). These wastes cause major environmental pollution, due to the presence of compounds like caffeine, tannins and polyphenols. their direct release into the environment could inhibit plant root growth and lead to an increase in greenhouse gas emissions through anaerobic decomposition.

Despite its disadvantage with the lack of proper handling, coffee wastes have several benefits as it is composed of useful organic compounds. By the application of biochemical principles, these wastes can be bio transformed into useful value added products, such as acetic acid. Acetic acid is most commonly known to be used in vinegar production. Vinegar is used as a nutrition, beverage, pesticide, and cleaning agent.

1.3. Objectives

1.3.1. General objective

- The general objective of the study was the utilization of coffee husk as a substrate for vinegar production using *Acetobacter aceti bacteria*.

1.3.2. Specific objectives

- To carry out the anaerobic conversion of reducing sugar to ethanol by *Saccharomycese cereviseae,* using submerged fermentation.
- To optimize the aerobic fermentation time, ethanol concentration, and temperature, for the production of acetic acid using *acetobacter aceti* bacetria.
- To determine and characterize the produced acetic acid using High Performance Liquid Chromatography.
- To formulate vinegar after the production of acetic acid.

1.4. Scope of the study

The aim of this research was to use coffee husk as a substrate for the production of acetic acid using *acetobacter aceti* bacteria in a submerged fermentation. Prior to the fermentation/optimization process, Size reduction, hydrolysis, characterization (moisture content, pH, organic matter, total nitrogen and carbon content) was carried out. The selected operational parameters for the optimization process were, temperature, fermentation time, and ethanol concentration. The produced acetic acid was separated from the fermentation broth by liquid-liquid separation using ethyl acetate as a solvent. After the concentration of acetic acid was estimated, the experiment run number with the optimum acetic acid concentration was taken for further analysis using high performance liquid chromatography (HPLC).

2. Literature Review

2.1. Coffee waste

Coffee (*Coffea* sp.) is one of the most important agricultural commodities in the world. *Coffea arabica* and *Coffea robusta* are the two principal varieties of the genus cultivated all over the world for commercial production. Coffee is cultivated in over 80 countries worldwide. Ethiopia is the origin of the coffee plants, and one of the top coffee producers in the world. The scrubs or coffee trees start blooming after three to four years and provide a full harvest for the subsequent six years(Murthy and Naidu, 2010). During the different processes to obtain the beans, large amounts of by-products are generated as approx. 50% of the coffee fruit is not used for the production of green coffee beans. From the dry coffee processing method, the main by product produced is the coffee husk, whereas the coffee pulp and coffee silver skin are produced from the wet processing method. Another by-product which is obtained after brewing is spent coffee grounds, also called coffee extract residue (Tehrani et al., 2015).

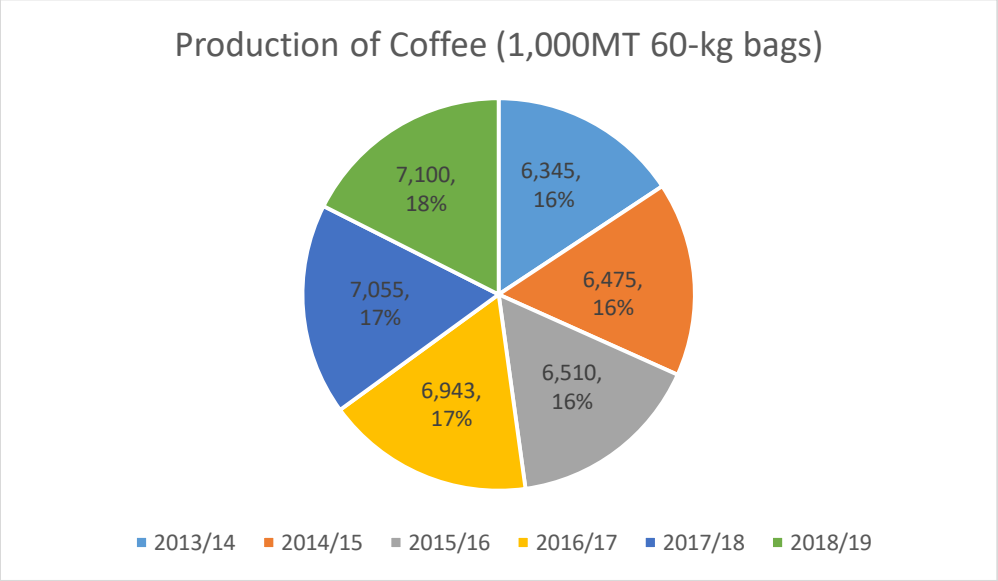

Figure 1: Ethiopia's annual coffee production. (Source: Official USDA PSD for MY 13/14-MY 15/16 and Post Estimates for MY 16/17-18/19)

2.1.1. Chemical composition of coffee waste

The three main characteristic features of coffee are acidity, aroma and taste. It is derived from over 1500 chemical & substances, 850 volatile and 700 soluble substances. When coffee is extracted in water, most of the hydrophobic compounds, including oils, lipids, triglycerides, and fatty acids remain in the grounds, as do insoluble carbohydrates like cellulose and various indigestible sugars. Structural lignin, protective phenolics and the wonderful aroma-producing essential oils are also present in coffee. (Padmapriya, 2013) The composition of coffee pulp differs from that of coffee husk, although the nature of the compounds present in both are largely similar. There may be difference in the percent composition of the constituents, depending upon the processing mode and efficiency, crop variety, cultivation conditions such as soil type, etc. (Elias and Braham, 2009). The main covetted chemical compounds of coffee husk, as in coffee and the other by-products, are secondary metabolites such as caffeine and other phenolic compounds, e.g. hydroxycinnamate acids and flavonoids, desired for their beneficial antioxidant properties (Farah and Donangelo, 2006).

Caffeine is an active compound, one of the nature's most powerful and addictive stimulants. It is the principal substance causing the mild stimulation effect of coffee. It is also present in coffee pulp and husk at about 1.3% concentration on dry weight basis. Tannins are generally thought to be an anti-nutritional factor and prevent coffee pulp from being used at greater than 10% of animal feed. (Trevilo et al., 1992.) has discussed about the anti-nutritional effect of tannins in animal feed. Depending upon the type of cultivar, the tannin contents may differ also. For example, coffee pulp from a yellow-fruited cultivar was significantly richer in condensed tannins (proanthocyanidins) than pulp from the associated red-fruited cultivar (Colmenares, 1993).

The composition of phenolic compounds in coffee pulp was studied by (Ramirez-Martinez, 1988). Chlorogenic acid (5-caffeoylquinic acid) was the main constituent (42.2%). Epicatechin (21.6%,

isochlorogenic acid I, II and III, 5.7, 19.3, 4.4%, respectively), catechin (2.2%), rutin (2.1%), protocatechuic acid (1.6%), and ferulic acid (1.0%) were the other compounds. Chlorogenic acids are per definition hydroxycinnamates ester of quinic acid with, most commonly, caffeic and ferulic acid as substituent (Jaiswal and Matei, 2012). The major chlorogenic acid classes, which all have at least three isomers, are caffeoylquinic acid (CGA), feruloylquinic acid (FGA), and dicaffeoylquinic acid (diCGA) (Clifford, 2000). The chlorogenic acids contribute to the pigmentation and astringency in coffee after roasting due to its incorporation in the melanoidins.

No qualitative or quantitative differences were detected between cultivars of coffee plants resistant and susceptible to coffee leaf rust. The author noted that these values should not be considered as the absolute values, as the contents of the compounds in coffee pulp could vary from time to time (Pandey, 2000).

Table 1 Components of Coffee Waste

Component	Coffee husk	Coffee pulp	Coffee spent ground
Proteins	9.2 – 11.3	8.5 – 12.1	10.3 – 12.2
Lipids	2.0 – 2.3	1.5 – 2.0	15.2 – 17.9
Cellulose	13.2 – 27.6	15.1 – 20.3	13.2 – 18.4
Ash	3.3 – 4.1	5.5 – 6.8	4.5 – 6.3
Tannins	4.5 – 5.4	1.8 – 2.4	1.2 – 1.5
Caffeine	0.8 – 1.1	0.5 – 0.7	0.02 – 0.08

Few have specified the species and origin of the analyzed coffee husk. Also, the coffee husk studied in literature originated from different parts of the world, e.g. Brazil (Ferraz , 2009), Ethiopia (Bekalo and Reinhardt, 2009) and India (Murthy and Naidu, 2014). (Navya and Pushpa,

2013) coffee husk from both Arabica and Robusta, but made no difference between them in their study, extracting antioxidants and functional compounds from different coffee by-products. As the dry method is mainly used for Robusta (Mussatto and Carnerio, 2011), a possible conclusion could be that it is the conditions on which the coffee scrub grows which makes the difference.

2.1.2. Use of coffee husk

According to different studies on coffee husk, the by-product consists of compounds usable in food production. (Murthy and Naidu, 2010) Concluded that the coffee husk is a useful substrate for mold, yeast and enzyme production due to its high amount of fermentable sugars. In 2012, they found coffee husk as a possible useful dietary supplement due to its high concentrations of dietary fibers and natural antioxidant components (Murthy and Naidu, 2014). The coffee husk is also rich in nutrients and could be used for extraction of caffeine and polyphenols. (Esquivel, 2012) concluded the coffee husk is a potential functional ingredient in food production as a source for phytochemicals.

Traditionally, coffee pulp and husk had found only a limited application as fertilizers, livestock feed, compost, etc. These applications utilized only a fraction of available quantity and were not technically very efficient. Recent attempts have focused on its application as substrate in bioprocesses and vermi-composting. Attempts have also been made to detoxify it for improved application as feed, and to it use as an efficient substrate for producing several value-added products such as enzymes, organic acids, flavor and aroma compounds, mushrooms, etc. Since these sub-products contain a good amount of fermentable sugars, these constitute an appropriate substrate for the cultivation of molds and yeasts (Pandey, 2000).

In recent years, there has been an increasing trend towards efficient utilization and value-addition of agro-industrial residues such as coffee pulp and husk, cassava bagasse, sugar cane bagasse,

sugar beet pulp, apple pomace, etc. (Pandey and Soccol, 2000). Several processes have been developed that utilize these as raw material for the production of bulk chemicals and value-added fine products (Pandey, 2000). Many application approaches have been studied for coffee cherry husks re-utilization, as substrate for biogas and alcohol production, biosorbents for cyanide, biosorbents for the removal of heavy metals from aqueous solutions, biosorbents for the removal of dyes from aqueous solutions, biosorbent for defluoridation of water, biosorbent for lead (II), for preparing ion exchange material, converted into fuel pellets or extracted for bioactive substances recovery. Besides, coffee husks demonstrated to be suitable candidates for a more direct use as substrate for edible mushrooms production or composting (Lenka Blinová, 2017). Coffee husks are also utilized as a potential functional ingredient in food production (using the ground coffee husk as a food supplementary for usage in smoothies, granolas and juices. The high concentration of caffeine and tannins in coffee husks, which are negative in environmental perspective, could be extracted for use in "energy drinks". The high content of dietary fibers in coffee husk constitutes a problem for the development of a beverage, but the fibers can be included in a food product of "energy bars", by grinding the whole coffee husk, and thereby including all antioxidants and fiber into the product. The coffee husk could also be launched as allergic-friendly, since it is naturally gluten free. Using the coffee husk for brewed tea is called coffee cherry tea. Coffee husk is a useful substrate for the mold, yeast and enzyme production, owing to its high amount of fermentable sugars (Bondesson, 2015).

Figure 2: Coffee husk

2.2. Acetic acid

Organic acids are categorized as the third largest products among biologically produced compounds and their application has been continuously increasing. Organic acids are known to hold extensive and versatile applications in many industries such as those for food and beverage preservation, animal feed production, soap manufacturing, medicines and pharmaceutical products, industrial solvents, perfumes preparation, oil and gas stimulation treatments, among others. Organic acids such as acetic acid, citric acid, lactic acid, succinic acid, fumaric acid, humic acid, oxalic acid, gluconic acid, and gallic acid have been produced commercially both by chemical synthesis and biological fermentation. Several authors have evaluated the potential use of organic solid waste such as sugarcane bagasse, cassava bagasse, coffee husk, kiwi fruit peel, wheat bran, rice bran, pineapple waste or apple pomace in organic acid production (Pandey and Ashok, 2000). Acetic acid which is categorized under organic acids is the major characterizing component of vinegar and its concentration determines the strength of the vinegar. The word is derived from the French term ‘vinaigre’ vin (wine) and aigre (sour) meaning ‘sour wine’. The available definition from the Codex Alimentarius (1987) states that vinegar is ‘‘a liquid, fit for human consumption,

produced from a suitable raw material of agricultural origin, containing starch, sugars, or starch and sugars, by the process of double fermentation, first alcoholic and then acetous".

Figure 3: Structure of Acetic acid (Ashok, 2000)

2.2.1. Production Technology of acetic acid

Vinegar production dates back at least to 200 BC, and it is an illustrative example of microbial biotransformation. However, its production was always considered a chemical process. (Solieri and Laura, 2009) Although vinegar has always been considered among the lowest quality products of fermented foods, it has also been used as a food condiment, as a preservative agent and, in some countries as a healthy drink (Pandey and Ashok, 2000). The use of vinegar to flavor food is centuries old. It has also been used as a medicine, a corrosive agent, and as a preservative. In the middle Ages, alchemists' poured vinegar onto lead in order to create lead acetate. (Solieri and Laura, 2009) In 1732, the Dutchman Boer have noted that the "mother of vinegar" was a living organism (Mas and Albert, 2014). Vinegar contains about 5% acetic acid in water, varying amounts of fixed fruit acids, coloring matter, salts and a few other fermentation products which impart a characteristic flavor and aroma to the product (Bhat, Akhtar, & Amin, 2015). Acetic acid is the predominant flavoring and antimicrobial component in vinegar. It is formed naturally due to spoilage of wine. Therefore, literally vinegar means "sour wine". Industrially for the first time it was produced in open vats, which however, was a very slow process. But the process was

increased many folds in the 19th century by surface fermentation technique in which trickling generators were used (Maria Gullo and Elena Verzelloni, 2014).

In traditional vinegars, the transformation of ethanol into acetic acid is performed by a static culture of acetic acid bacteria at the interface between the liquid and air. The barrels are filled to 2/3 capacity to leave an air chamber, which is kept in contact with the outside air using one of various types of openings. This production system is called "surface culture," and this process is considered the traditional method (Mas, and Albert, 2014). The vinegars produced by this traditional system are generally considered of high quality because of their organoleptic complexity. In fact, the product quality results from (i) the raw material (wine or other substrate), (ii) the metabolism of the acetic acid bacteria, which produce some additional transformations (mostly oxidation reactions, but also ester formations, e.g.) on top of the basic transformation (ethanol to acetic acid), (iii) the interaction between the vinegar and the wood from the barrels, and (iv) the aging process, which integrates all of the previously mentioned characteristics (Mas, and Albert, 2014). Commercially acetic acid is produced by two methods, surface fermentation process and submerged fermentation process. If materials with low alcohol content are used such as wine, whey, malt or cider there is no need of addition of any component to constitute a complete nutrient solution. However, if potato or grain spirits or technical alcohol is used, nutrients must be added to obtain optimal growth and acetic acid production. Nutrient concentration that is used in submerged fermentation is generally five times greater than surface fermentation (Maria Gullo and Elena Verzelloni, 2014).

Orleans Method

The earliest vinegar manufacturing method, which was originated in France, was known as the 'Orleans process' or also known as the 'slow process'. wooden barrels are used and are filled with

alcohol fermenting liquid to almost three quarters full. Holes are drilled into the ends of the barrel a few inches above the liquid level in order to allow air circulation. The holes are left open and netting or screens are placed over the holes in order to prevent insects from getting into the barrels. Fresh vinegar is added to the barrel to acidify the liquid to the point of optimum growth necessary for the vinegar bacteria. The vinegar bacteria settle down into the liquid and a slimy layer develops on the top of the liquid. This is a slow process because the bacteria come in contact with the air and the substrate only at the surface. The air is supplied through the holes in the barrel. The fermented liquid is allowed to sit for several months with free access to air. Once the alcohol fermenting liquid becomes acetified or is converted to vinegar through activity of organisms growing on or close to the surface of the liquid, a proportion of the vinegar may then be drawn off and replaced with alcohol fermenting liquid and the process is restarted. The acetic acid may become oxidised if the alcohol sources are not constantly added to the vinegar. Orleans method is reported to be the best method to produce the finest quality vinegar. (M.Cheryan, 2009)

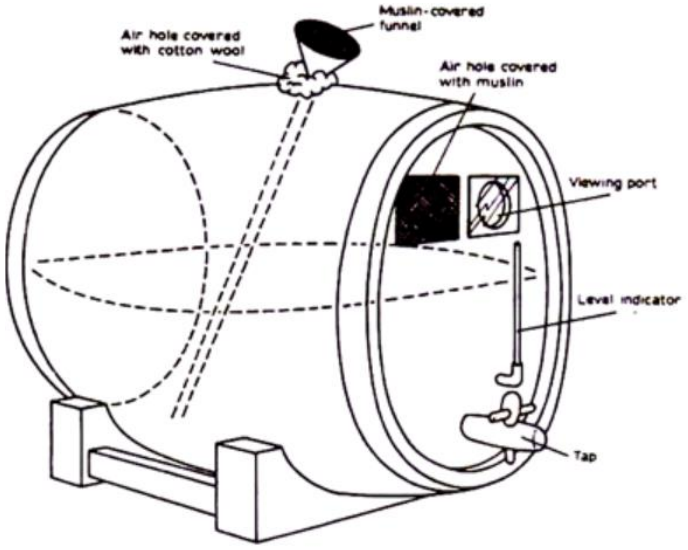

Figure 4: orleans method of vinegar production

Surface Fermentation

For surface fermentation, generally trickling generator is used for the acetic acid production. First, the ethanol is transferred into the generator from the inlet pump at the top. The ethanol is trickled through the column of wood shavings. The bacteria is inoculated at the bottom, which converts the ethanol into acetic acid through their microbial activity. A solution is cooled and pumped back to the top and again passed through the birch of wood shavings. The process is repeated again and again till the conversion of 80-90% of the ethanol to acetic acid. The concentration of ethanol should not be less than 0.2% as it can increase the death rate of the microorganisms producing acetic acid. Therefore, for the optimum growth of microorganisms and production of vinegar, the concentration of ethanol must be in between 0.2-5.0%. The significant advantage of surface fermentation is that it leaves little residue with a high yield of acetic acid. (Acetic Acid Production, Supriya N)

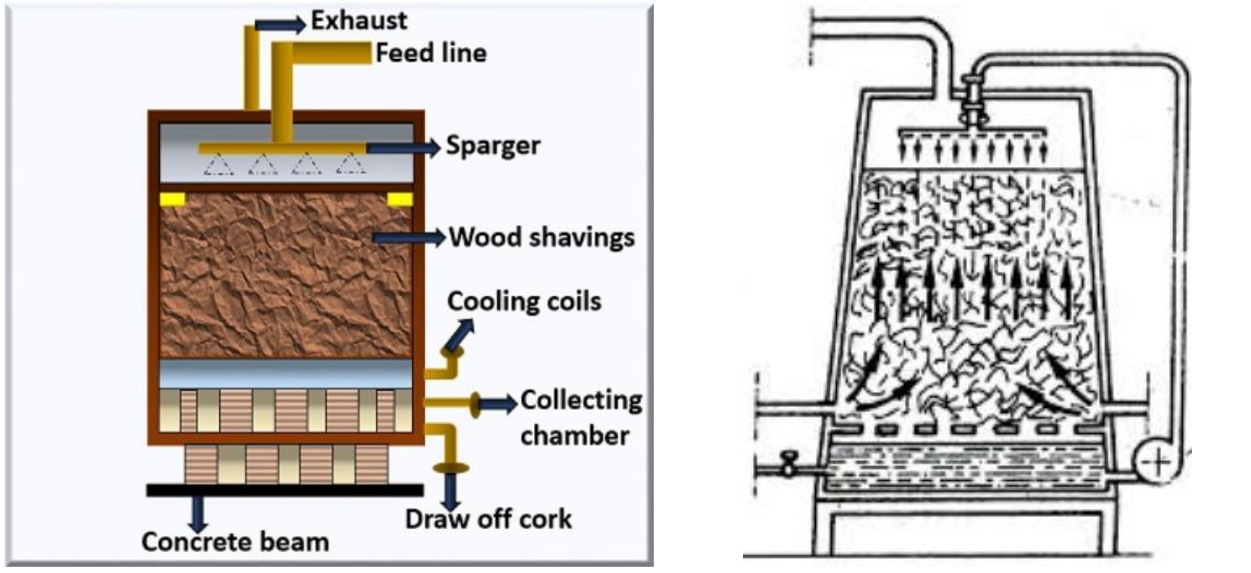

Figure 5: Trickling generator for vinegar production

Submerged Fermentation

At the industrial scale, vinegar is mainly produced by submerged fermentation, which refers to an aerobic process in which the ethanol in beverages such as spirits, wine or cider is oxidized to acetic acid by AAB. Submerged fermentation requires robust AAB strains that are able to oxidize ethanol under selective conditions to produce high-titer acetic acid. Currently submerged fermentation is conducted by unselected AAB cultures, which are derived from previous acetification stocks and maintained by repeated cultivation cycles (Maria Gullo and Elena Verzelloni, 2014). A submerged system has several advantages over other techniques (e.g. SSF and surface fermentation), including high yield and process speed. Over the last few decades, many studies have examined process variables (oxygen availability,temperature, acetic acid and ethanol content), and a number of strategies for process control have been developed. As a result, acetic acid fermentation systems and the modern vinegar industry benefitfrom robust processes and optimization tools (De Ory I, Romero LE, 1999). The basic requirements for submerged processes are the availability of suitable alcoholic stocks, uninterrupted aeration and AAB strains that tolerate high concentrations of acetic acid and ethanol, which are not sensitive to phage infections and that require small quantities of nutrients, to produce high amounts of acetic acid (Ebner anf Sellmer, 1996). One of the most important features of the bioreactors used in these processes is the aeration system. This system consists of a hollow body turbine supported by a non-rotating stator. The turbine sucks air from the outside and releases it into radial holes that open in the opposite direction of rotation; the action of turbines results in very fine air bubbles and homogenous air–liquid dispersion. The air–liquid emulsion is pushed upwards and diverted by deflectors. All of the mass is maintained in a constant state of agitation to prevent the formation of low oxygen tension areas, which are unfavorable for

the metabolic activity of AAB. The heat generation during SF is unavoidable because acetic acid fermentation is an exothermic reaction, producing approximately 8.4 MJ for every liter of oxidized ethanol. Moreover, during charge of substrate and discharge of product, the temperature can vary greatly. Fermentation breakdown due to temperature variation is generally avoided by heating and cooling systems. (Schlepütz and Gerhards, 2013)

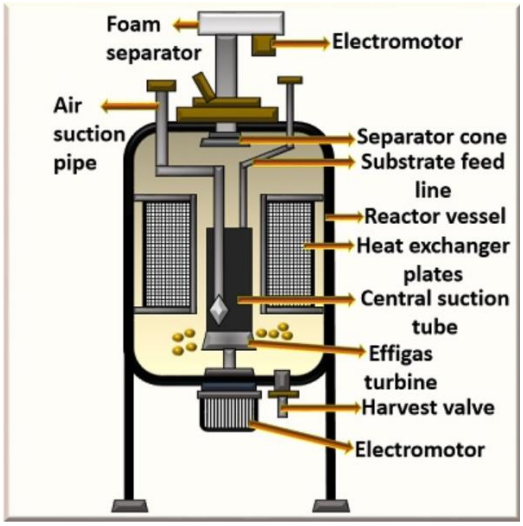

Figure 6: Submerged fermentation of vinegar

Biosynthesis of acetic acid

Acetic acid can be produced either by the synthetic or biological method. The synthetic process involves some chemical reactions to produce acetic acid, whereas a biological method involves microorganisms where they degrade the substrate into a product known as Acetic acid.

Vinegar is produced by a two-step bioprocess. In the first step, fermentable sugars are transformed into ethanol by the action of yeast. In the second step, acetic acid bacteria oxidize the ethanol into acetic acid in an aerobic process. Acetic acid bacteria are well known for their ability to spoil wines because they can produce large amounts of acetic acid from ethanol and other compounds present in wines (A. Joyeux, and S. Lafon-Lafourcade, 1984). The raw materials commonly used for

vinegar production include rice, grapes, malt, apple, honey, potatoes, whey or any other sugary food. In human history, vinegar appears at the beginning of agriculture with the discovery of alcoholic fermentation from fruits, cereals and vegetables. The genesis of vinegar can hardly be distinguished from the origin of wine. (Pandey and Ashok, 2000)

There are several kinds of vinegar: cider vinegar, apple vinegar, white distilled vinegar, rice vinegar, etc. The differences between them are primarily associated with the kind of material used in the alcoholic fermentation, e.g. fruit juices, sugar and hydrolyzed starchy materials. The products are made by the alcoholic and subsequent acetous fermentations of the apple juice. Yeast fermentation is used for the production of alcohol. The alcohol is adjusted to 10–13%, then it is exposed to acetic acid bacteria (*Acetobacter* species), whereby oxygen is required for the oxidation of alcohol to acetic acid. The desired temperature for *Acetobacter* is 15–34 °C. (Mas, and Albert, 2014).

2.2.2. Acetic acid bacteria (AAB)

Microbial species involved in fermentations may range from yeast and lactic acid bacteria (LAB) to molds and acetic acid bacteria. Depending on the environmental factors (temperature, pH, water activity) or the nutrients (carbon sources) and the microbial diversity present in the raw material, different biotransformation could take place. The microorganisms involved in the elaboration of vinegars are mainly yeasts and acetic acid bacteria. The former being responsible for the alcoholic fermentation, and the latter needed for the acetification. (Bhat et al., 2015)

Figure 7: Acetic acid bacteria (AAB)

Vinegar is the product made from the conversion of ethyl alcohol to acetic acid by a genus of bacteria, Acetobacter. Therefore, vinegar can be produced from any alcoholic material from alcohol-water mixtures to various fruit wines. Vinegar bacteria, also called acetic acid bacteria (AAB), are members of the genus acetobacter and characterized by their ability to convert ethyl alcohol (C_2H_5OH) into acetic acid (CH_3COOH) by oxidation.

Acetic acid bacteria are Gram negative, rod-shaped, peritrichously or polarly flagellated when motile, mesophilic and obligate aerobes. Their size varies between 0.4-1 μm wide and 0.8-4.5 μm long. They are observed as individual cells, in pairs or in chains. They show a strict aerobic metabolism with oxygen as the terminal electron acceptor, and are catalase positive and oxidase negative (Joyeux, and Lafon-Lafourcade, 1984). These bacteria are capable of oxidizing sugars, sugar alcohols and alcohols to corresponding acids (Komagata and Iino , 2014). They also exhibit resistance to high acetic acid concentrations at low pH. Acetic acid bacteria not only play a positive role in the production of a variety of foods and beverages, such as vinegars, kombucha, cocoa and nata de coco, but they can also occur as spoilers of other foods and beverages, such as wine, soft drinks and fruits (Raspor, 2008).

Five genera retain most species of acetic acid bacteria: *Acetobacter* (25 species), *Gluconobacter* (14), *Gluconacetobacter* (11), *Asaia* (8) and *Komagataeibacter* (13). The remaining 13 genera are monotypic, with the exception of the genus *Neokomagataea*, which has 2 species. Two genera, *Acetobacter* and *Komagataeibacter*, are generally responsible for the wine vinegar production process. In recent years, acetic acid bacteria have been the object of extensive research, resulting in a significant restructuration of their taxonomy and advances in understanding their physiology, metabolism and molecular biology and in methods for their isolation and identification (Raspor, 2008). The famous ability of this group of bacteria to oxidize ethanol to acetic acid is due to two key membrane-bound enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). Both these enzymes are bound to the cytoplasmatic membrane and face the periplasmic space (Sievers, 2005). This special type of metabolism differentiates them from all other bacteria. The species of AAB are well known to have a high capability to oxidise alcohols, aldehydes, sugars or sugar alcohols in the presence of oxygen (Adachi et al, 2007).

Ethanol oxidation

AAB partially oxidize EtOH by two successive catalytic reactions of the ADH and a membrane-bound aldehyde dehydrogenase (ALDH) that are bound to the periplasmic side of the cytoplasmic membrane. The complete oxidation of EtOH occurs at cytoplasmic level by a NAD-ADH and NAD-ALDH. The AcOH produced can be further utilized by acetyl CoA synthase and via TCA cycle.

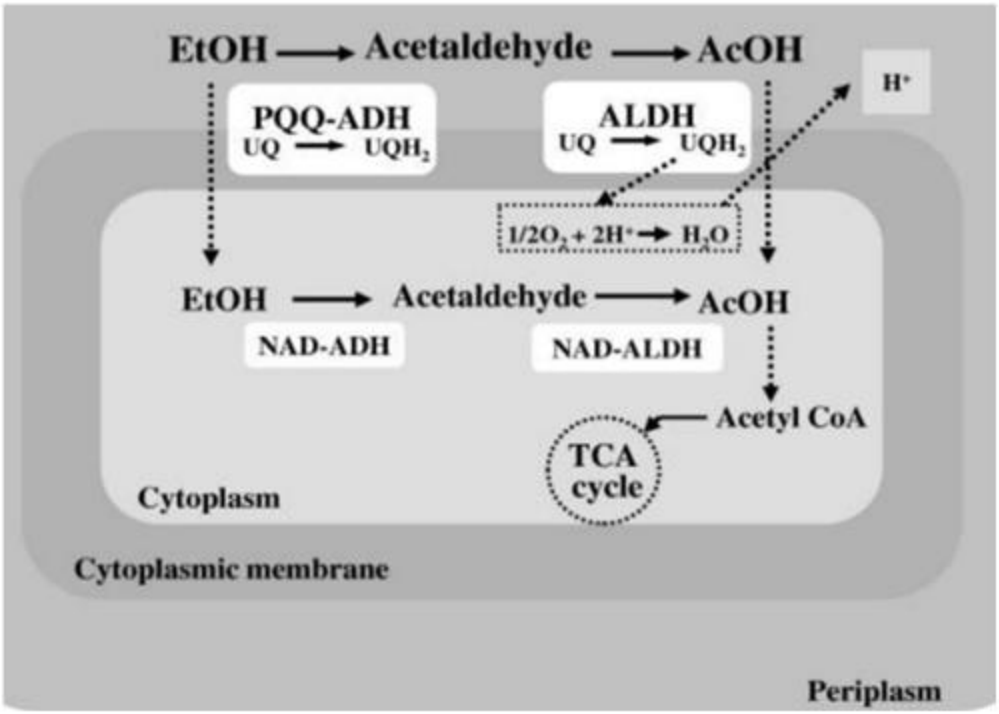

Figure 8: Ethanol oxidation by PQQ-ADH and ALDH at the outer surface of cytoplasmic membrane and by NAD-ADH and NAD-ALDH in the cytoplasm

AAB strains oxidise ethanol to acetic acid by two sequential catalytic reactions. First, ethanol is oxidised to acetaldehyde, which is catalysed by membrane-bound pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase (ADH). Then, the generated acetaldehyde is immediately oxidised to acetate by membrane-bound aldehyde dehydrogenase (ALDH), located near ADH (Yakushi, 2010)During alcohol oxidation, no aldehyde liberation is observed, indicating that ADH and ALDH form a multienzyme complex in the bacterial membrane and function sequentially to produce acetic acid from ethanol (Adachi et al., 2007). The produced acetic acid is released into the growth medium, where it accumulates to a maximum 5–10% in *Acetobacter* species and 10–20% in *Komagataeibacter* species (Andrés-Barro et al, 2012). Some genera can further oxidise the produced acetic acid to CO_2 and H_2O, resulting in so-called

acetate oxidation (overoxidation). This ability is useful for distinction from the genus *Gluconobacter*, which does not have the same capability. This condition depends on the composition of the medium, especially when ethanol is used by the bacteria (Gullo and Verzelloni, 2014).

Sugars oxidation

AAB are known to have a high oxidative ability against sugars, mainly glucose but also arabinose, fructose, galactose, mannose, ribose, sorbose and xylose. (De Ley and Gillis, 1984). They can catabolize sugars through the cytoplasmic hexose monophosphate pathway (Warburg-Dickens pathway) (Drysdale , 1988). The Entner-Doudoroff pathway occurs only in cellulose synthesizing Acetobacter and Gluconacetobacter strains, where it appears to be more active than the hexose monophosphate cycle (White , 1964). *Acetobacter* species can use sugars through the hexose monophosphate pathway and also through the Embden-Meyerhof-Parnas and Entner-Doudoroff pathways (Attwood and van Dijken, 1991). Sugars are further metabolised to $CO_2$and H_2O via the TCA pathway, which is not functional in *Gluconobacter*. Sugar is more preferred as carbon source by *Gluconobacter* than by *Acetobacter* because the species of this genus can obtain energy more efficiently by the metabolisation of the sugars via pentose phosphate pathway. (De Ley and Gillis 1984)

Organic acid oxidation

The ability of *Acetobacter and Gluconacetobacter* strains to oxidize AcOH (Fig. 1) generating the so-called acetate overoxidation occurs via TCA. Other acids such as lactic, pyruvic, malic, succinic, citric, and fumaric are similarly metabolized. Although the optimum pH for the oxidation of organic acids by AAB is near 6.0, there is evidence that it occurs at lower values (3.5–4.0). In

vinegar, for instance, *Acetobacter* species exhibits a biphasic growth curve, where the first corresponds to an EtOH oxidation with AcOH production, and the second to an overoxidation cycle. (Matsushita, 2004)

2.2.3. Factors affecting the production of acetic acid

Pure culture

The use of pure cultures of acetic acid bacteria for the production of vinegar has not been popular. (Shimwell, 1948) claimed that the use of a pure culture of species possessing the most desirable characteristics is advantageous. The organism should be able to tolerate a higher concentration of alcohol, convert ethyl alcohol to acetic acid rapidly, oxidize ethyl alcohol incompletely and produce good-flavoured vinegar. (Shimwell, 1948) claimed that the type of bacterium best sui ted for the "quick" vinegar process is one with a minimum tendency to over-oxidation, rapid utilization of high concentrations of alcohol and a minimum of film production. In using pure cultures in any industriel fermentation, the culture should be able to stand prolonged sub-cultivation in the laboratory without changing its characteristics. Possibly uniformity of product is the greatest advantage gained by the use of pure cultures.

Medium

Acetic acid may be manufactured from almost any product capable of yielding alcohol by fermentation. (Shimwell, 1948) said that in the manufacture of spirit- vinegar industrial alcohol diluted to a suitable concentration may be used and nutrients added to supply the nitzogen requirements of the bacteria. (Cosbie et al, 1943) observed that the oxidation of alcohol to acetic acid by *Acetobacter turbidans* varies directly as the concentration of the yeast water in the media;

therefore to a certain limit the concentration of nutrients in the medium affects the amount of acid produced.

(Wa1ker and Kulka, 1949) observed that a medium consisting of ethyl alcohol and yeast water encourages the growth of Acetobacter species but not that of ethers. (Rao, Raghavendra and Stokes, 1953) stated that acetic acid bacteria are dependent upon carbohydrates for growth initiation, and that once this has occurred, the bacteria use ethyl alcohol as a source of carbon and energy, and oxidize ethy1 a1coho1 to acetic acid. The ethy1 alcohol content of the medium is important in determining whether Acetobacter species produce acetic acid from ethyl alcohol. Although some au thors claim that alcohol in a concentration of 10 to 13 % is readily fermented, the general observation was that Acetobacter species vary in their ability to utilize different concentrations of ethyl alcohol.

(Vaughn, 1942) claimed that concentrations of alcohol between 14 and 15% by volume are very inhibitory to acetic acid bacteria, but concentrations of 5 to 12% are not inhibitory. (Walker, and Tosic, 1946)asserted that an ethyl alcohol content in media greater than 4% prolongs the stationary and lag phases of growth of the bacteria, but an alcohol content of more than 6% inhibits growth of some species. when a1coho1 concentrations of less than 1 or 2 percent are used acetic acid may be lost.

Oxygen supply

Since the conversion of ethyl alcohol to acetic acid is an oxidative reaction, large quantities of air must be present during fermentation. Sorne species of Acetobacter oxidize acetic acid to carbon dioxide and water; these are undesirable for fermentation. Some species in a limited amount of air produce acetic acid, but in a large amount of air produce carbon dioxide and water. Therefore, the

amount of oxygen must be controlled in relation to the activities of the bacterium causing the oxidation of ethyl alcohol. Pasteur showed that the fermentation must be arrested when fermentation is completed, otherwise the "mycoderms" transform the acetic acid into water and carbon dioxide (Ames, 1946).

Temperature

At temperatures below 12°C to 15°C. acetic acid bacteria grow slowly, and at temperatures of 42°C to 45°C, cells are produced that lose the power to function normally. From 15°C to 34°C, acetobacter appear to develop normally. Too low a temperature favours a slow fermentation, but too high a temperature favors the loss through evaporation of alcohol, acetic acid and the volatile substances important in the production of flavor and aroma (Prescott and Dunn, 1949).

pH

(Wa1ker and Kulka, 1949) claimed that many acetic acid bacterie have a pH range extending well below 3.5 for growth. It appears that in a natural environment oxygen is the hydrogen accepter in the conversion of ethyl alcohol to acetic acid; and that under anaerobic conditions, especially at high pH values (7.6 to 8.4), and to some extent in an aerobic environment, acetaldehyde may act as the hydrogen accepter for acetaldehyde hydrate (Porter, 1948). The result is a dismutative change or Cannizzaro reaction. The amount of alcohol, acetaldehyde and acetic acid present at any given stage of the process depends upon the pH of the medium and other factors as well.

2.2.4. Downstream process of acetic acid

In the biosynthesis process, important quantities of aqueous solutions are produced, from which acetic acid should be economically recovered (Tesfaye et al, 2002). The separation of carboxylic acids (acetic acid in particular) from aqueous solutions by simple distillation or azeotropic

distillation is difficult, requiring a column with many stages, a high reflux ratio or a great amount of azeotropic agent, which leads to a very expensive process (Sebastiani and Lacquaniti, 1967). Recent studies concerned to the efficient and economical separation of acetic acid refer to the use of the esterification agents, based on difference of acetic acid and acetic ester solubility in water (Bianchi et al, 2003). Tertiary amines are considered highly efficient as extractants, especially used in capsulated systems in order to avoid emulsion formation and toxicity effect towards microorganisms. The problem of toxicity of solvent can be partly solved by using membrane processes: ultrafiltration, electrodialysis, pervaporation, reverse osmosis, or pertraction (Matsumoto and Otono, 2001).

Other processes can be used for separation, depending on the acetic acid concentration in the solution. For acid concentrations between 50 and 70% w/w, extractive distillation was studied (Further and Cook, 1967). In the extractive distillation, a convenient selection of the solvent is fundamental to ensure an effective and economical process. For example, for acid concentrations lower than 40% w/w, liquid-liquid extraction can be an appropriate process Typical solvents used for acetic acid recovery via liquid-liquid extraction are ethyl acetate, isopropyl acetate, and Methyl tert-butyl ether (Lei et al, 2004). Acetic acid is one of the most widely used organic acids, which has many industrial applications. The pure acetic acid is extensively used as an additive in the food industry. Therefore, the efficient separation of acetic acid from aqueous solutions, by solvent extraction technique, is of considerable economic importance in the chemical industry (McCullough, 1976). The type of solvent is one of the most important factors, which influence the equilibrium characteristics of extraction of the acid from aqueous solutions.

Solvents that are used in the liquid-liquid extraction process must have the following properties; High capacity that decreases the amount of solvent required. Capacity determines the conditions

and size of the separator; High selectivity that leads to products produced with desirable purity; Capability to form two phases at reasonable temperatures and capability of rapid phase separation; Boiling point temperature of solvent should be as varied as possible from the boiling point temperature of components of mixture; Low toxicity, non- corrosiveness and finally, the use of solvent is desired economic justification. Many organic solvents have been tested as extractants for the recovery and purification of acetic acid from water. Heavy alcohols, ketones and ethers have been used for extraction of acetic acid from aqueous solutions (Afolabi, 2014).

Seperation factor which is a quantitative index of effectiveness of separation is calculated from solubility and tie line data. It is a measure of the solubility of solvents to separate acetic acid from aqueous solution. Separation factor is the ratio of distribution coefficient of solute (acetic acid) to the distribution coefficient of diluent (water). It is denoted by S. Distribution coefficient of water is the ratio of weight fraction of diluents in solvent phase to that in diluents phase. (Sumona Haque et al, 2008) tested proper solvent for the separation of acetic acid from aqueous solution, and the mutual solubility data and tie line data were obtained using organic solvents such as, ethyl acetate, iso-butanol, n-butanol, amyl alcohol. According to (Sumona Haque et al, 2008) distribution coefficient of acetic acid were obtained by the ratio of weight percent of acetic acid in solvent phase to the weight percent of acetic acid in water phase, which was larger than 1 for all the system but acetic acid-water-amyl alcohol system showing the highest value (3.0), indicates that acetic acid has preferential solubility between water and alcohol which is the key factor in the extraction process.

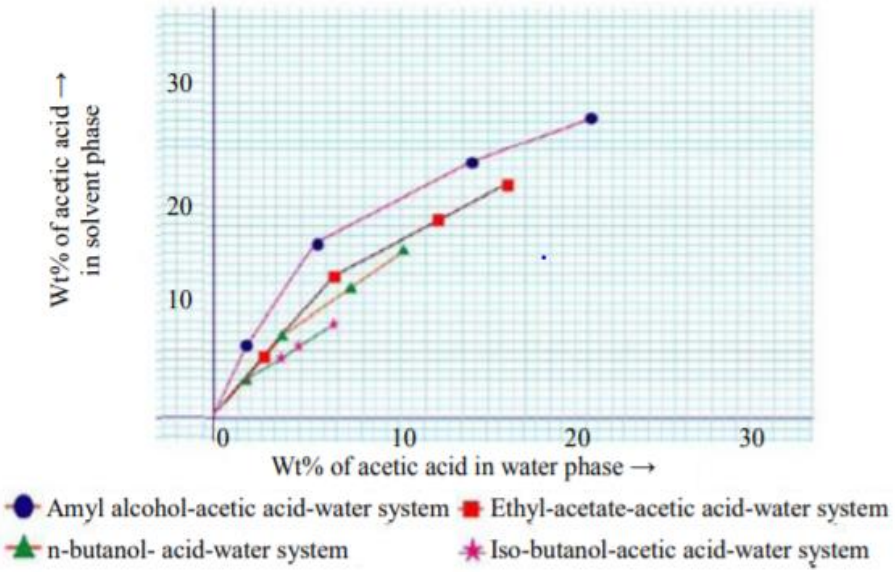

Figure 9: Equilibrium diagram for acetic acid –water-solvents system.

(Sumona Haque et al, 2008) obtained the selectivity diagram shown above directly from the data determined for the distribution or (tie-line data) and binodal curves. The above Figure reveals that amyl alcohol and ethyl acetate shows the highest selectivity than other solvents. Normally, more low boiling extraction agents are used. Characteristics like solubility in water, absorption capacity, distribution coefficient, price, availability and composition of the azeotrope, and requirements in terms of environmental and health protection must be taken into account for the purpose of this selection.

The economic viability of the overall process greatly depends on the energy requirements of the solvent rectification. The difference in boiling temperatures between the pure acetic acid (118°C) and the azeotropic point provides clue on the size of the reflux ratio. (Sumona Haque et al, 2008) The energy consumption, however, also depends on the vaporisation enthalpy of the azeotropic mixture, which in turn is determined by the proportion of water in the azeotrope. It can be shown

that these observations only apply up to certain feed concentrations of acetic acid, which, for example, amount to approx. 15wt% in the case of ethyl acetate. If the feed concentrations are higher, then the quantity of extraction agent must be increased in accordance with the balance and vapour-liquid equilibrium, which consequently causes an increase in the operating costs.

3. MATERIALS AND METHODS

3.1. Materials

The materials and equipments used for the laboratory work are: grinder, sieve, volumetric flask, beaker, petri dish, autoclave, laminar flow chamber, UV-spectrophotometer, balance, pipette, shaker incubator, and microscope. The chemicals and reagents used for media and standard preparation were: Dextrose, Yeast Extract, sulphuric acid, potassium dichromate, and others. The materials and chemicals used were collected from Addis Ababa Institute of Technology, Biochemical engineering laboratory. The yeast strain, cultured *S.cerevisiae* was collected from Ethiopian Biodiversity Institute, Addis Ababa. Freeze dried form of *Acetobacter aceti* was purchased from NCIMB Ltd, Ireland. Coffee husk was collected from processed coffee residuals dumping site located in Dilla, found in the southern part of Ethiopia.

3.2. Experimental Overview

The production of vinegar involves: (1) Inoculum preparation, (2) Alcoholic fermentation by *Saccharomyces cerevisiae*, and (3) Oxidation of the alcohol to acetic acid by *Acetobacter aceti* bacteria.

The type of raw material/substrate used is one essential part of vinegar production. In this study, coffee husk was used as a substrate/nutrient source for the production of vinegar. Coffee husk is subjected to hydrolysis by concentrated acid, prior to the fermentation process, in order to bereak down and avail the nutrients to the microorganisms. After the hydrolysis and sterilization of the coffee husk, the temperature and pH were adjusted to 30℃ and 5.5 respectively for ethanol fermentation. Subsequently, acetic acid production was optimized using Design Expert 11.0, Box Behnken response surface. 17 experiments were conducted in order to determine the optimum

temperature, ethanol concentration and production period. Finally, the produced acetic acid was analyzed by using high performance chromatography (HPLC).

3.3. Experimental Methods

The experiment was carried out in three phases.

3.3.1. Phase One: Substrate preparation

I. Physiochemical Characterization

The coffee husk was ground and sieved to obtain smaller sizes (100µM). The characterization was conducted both at AAIT Laboratory and PASTOR Institute.

Figure 10: Coffee husk grinding and sieving

Determination of Moisture Content

For the determination of moisture content of a coffee husk sample, S**AS 1289 B1.1-1977:** Oven Drying Method was used. The weights of empty crucibles were measured. 10g of raw coffee husk was added on to each of the two crucibles and placed into an oven drier 60°C. After 24hours, the weight of the coffee husk was measured in order to determine the moisture content using the formula;

$$\text{Mositure } \% = \frac{W_2 - W_1}{W_2} \times 100 \qquad Eq(1)$$

Where, W_1 is the weight of the coffee husk before drying (g)

W_2 is the weight of the coffee husk after drying (g)

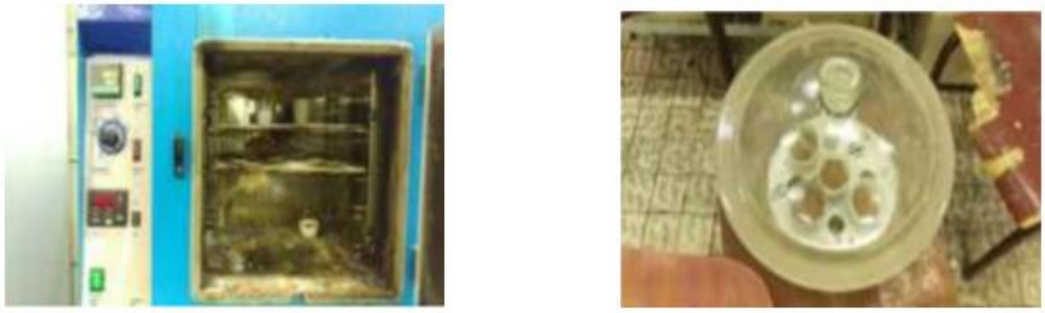

Figure 11: a) Coffee husk in an Oven drier, b) Coffee husk in a Desiccator

Determination of organic matter

The organic matter content was determined by the loss on ignition of the dry mass at 550°C. (Dean, 1974). The coffee husk was oven dried using the procedure for moisture determination. Then samples were kept in a furnace at 550 °C for 6h. The difference in weight before and after ignition represents the amount of the OM that was present in the sample. The percentage was determined by using the formula:

$$\text{Organic matter } (\%) = \frac{W_2 - W_1}{W_1} \times 100 \qquad Eq(2)$$

Where, W_1 is the weight coffee husk before ignition (g)

W_2 is the weight of the coffee husk after ignition (g)

Determination of nitrogen content

The method used for the determination of total nitrogen is Kjeldahl method. There are three main steps in the Kjeldahl method: (I) Digestion, (II) Neutralization/distillation, and (III) Titration. The analysis starts with an acid digestion of the sample organics, converting organic nitrogen to ammonia. This requires boiling the sample in concentrated sulfuric acid, potassium sulfate, and a copper catalyst to convert the organic nitrogen to ammonia. The speed of this reaction is increased by increasing the digestion temperature to 395° C. The second part of the method is a repeat of the distillation describe above, however, the acidic digestion sample pH, must be raised to 9.5 with the addition of concentrated sodium hydroxide. At this pH ammonia gas forms and the gas is transferred by distillation into the acidic trapping/absorbing solution where it is converted back to ammonium. Finally, the nitrogen concentrations within the receiving solution is determined by using titrimetic methods.

II. Concentrated acid hydrolysis

Hydrolysis of coffee husk is an essential step for the proceeding process of vinegar fermentation. The pretreatment is carried out in order to improve the formation of sugar, destroy lignin shell protecting cellulose and hemicelluloses, decrease crystallinity of cellulose, increase porosity, and to break this shell for enzyme to access the substrate. Concentrated acid hydrolysis was employed for this experiment.

This stage consisted of three steps: de-crystallization, hydrolysis reaction, and product separation. The first two steps can be considered as two-step concentrated acid hydrolysis. The de-crystallization step was performed in order to breakdown the crystalline structure of the lignocellulose material separating the cellulose away from the lignin in the cell walls for the next step of hydrolysis. The coffee husk was soaked in a diluted solution of 20% sulfuric acid for 30

minutes at room temperature, then the soluble portion was separated from the insoluble portion by filtration method. The separated insoluble portion was then proceeded with the next step of concentrated hydrolysis process. For this stage, the coffee husk was soaked in 80% of sulfuric acid at a temperature of 121℃ for 2hours. Consequently, the soluble portion was separated from the insoluble portion by using filtration method. Finally, the pH was adjusted at 5.5 for the next step of ethanol fermentation. (Schlepütz and Gerhards et. al, 2013)

III. Estimation of reducing sugar

The total sugar content of the hydrolyzed coffee husk was estimated using the phenol-sulfuric acid method by taking anhydrous D– glucose as a standard solution (Dubois et.al, 1956). The stock solution was prepared by dissolving 0.5g of glucose into 100ml of distilled water. Six tubes were prepared by cleaning thoroughly to avoid error. The standard solution was prepared by adding 20%, 40%, 60%, 80%, and 100% of the stock solution and distilled water. 1mL of the sample and 1 mL of phenol solution (5 g/100mL) were added into the test tubes, and the contents were mixed. Then 5 mL of concentrated sulphuric acid was added and the test tubes were shaken to facilitate good mixing. Following 10 minutes of shaking, the test tubes were placed placed in a water bath (25–30 ℃) to cool and display blue color. Blank solution was prepared using only distilled water. Finally, the absorbance of the sample was measured at 490 nm by UV-visible spectrophotometer using glucose as standard. The graph was plotted with concentration of D—glucose standard in the X-axis and Absorbance in the Y-axis.

Figure 12: Standard dilution for reducing sugar estimation

3.3.2. Phase Two: Ethanol fermentation

I. Inoculum preparation

S.cerevisiae was used for the production of ethanol. 40g Potato dextrose, 4g hydrated magnesium sulphate, 4g urea, 1g yeast extract, and 400ml distilled water was prepared as growth media for the yeast (Mebrahtom, 2014). After autoclaving the media @ 121℃ and 15psi for 15 minutes, *S.cerevisiae* was then inoculated in to the media under sterile condition and it was placed in a shaker incubator at 30℃ for 24h.

II. Ethanol fermentation

The prepared hydrolysate was sterilized in an autoclave at 121℃ for 15minutes. Then after cooling the hydrolysate, the yeast (1:10) was inoculated under sterile condition and placed in a shaker incubator at 30℃ for 72hours.

III. Ethanol determination

Total ethanol concentration in the coffee husk was estimated by titration using chromic acid method (Caputi A, 1968). The experiment was performed by dissolving 1g of potassium dichromate in a 100mL solution containing 5 mL sulfuric acid. In a separate flask, 40g of Rochelle salt was dissolved in 100ml of distilled water. Six clean and dry test tubes were prepared, then

pure ethanol with known concentration was added in to each tubes as; 0%, 25%, 50%, 75%, 100% respectively. The test tubes were made up to 10mL by adding distilled water. 10mL of the sample was added to a test tube. Then 3ml of prepared chromic solution reagent and 1mL of Rochelle salt was added to the tubes, and then they were placed in a water bath for 20minutes at 60^0C, and a purple color was noticed. Finally, the absorbance was measured at a wavelength of 600nm, using UV- visible spectrophotometer.

Figure 13: Standard Dilution for Ethanol Estimation

3.3.3. Phase Three: Acetic acid production

I. Inoculum preparation

Acetobacter aceti bacteria was used for the production of acetic acid. Yeast glucose media, containing; 2g of yeast extract, 1g of glucose, and 100mL distilled water was prepared and autoclaved. Then, the bacteria was transferred to the growth media under sterile conditions and placed in a shaker incubator at 30℃ for 24h under aerobic condition.

II. Optimization of the acetic acid production

There are various factors that can affect the production of acetic acid, and among these factors; ethanol concentration, time, and temperature were selected as the main variables affecting the optimization of acetic acid production process from coffee husk.

The ethanol attained after the fermentation of the coffee husk was adjusted to 2%, 5%, and 8%, Concentration for the proceeding steps, was sterilized in an autoclave for better result. Then, the bacteria was inoculated (1:10) in to the media under sterile condition. Consequently, the flask was kept in a fermenter shaker at the selected levels of ethanol concentration, temperature and incubation period. These procedures were repeated for each of the 17 experiments according to the output parameters of Desin Expert 11.0.

III. Separation of Acetic acid

The final produced acetic acid needs to be separated from the fermentation broth in order to measure the concentration and formulate vinegar. Liquid-liquid extraction method using ethyl acetate as a solvent was selected for this process. 1:1 solvent to feed ratio was used. (Usman et al, 2015) After adding the solvent to the broth, it was kept overnight at 30℃, then it was placed in a water bath for an hour at 60℃. Consequently, by using a separating funnel, the phase separation was carried out. This process was repeated for all the 17 experiments. The solvent was then separated from acetic acid by using their difference in boiling point.

IV. Estimation of Acetic acid

Acid-base titration was used to determine the concentration of acetic acid. Sodium hydroxide was used as the standard reactant solution for this titration. Phenolphthalein was used as a coloring acid/base indicator of the titration end point. An acid/base indictor is a colored substance with two or more different colors depending on the value of the pH of the solution.

By using a double burret-clamp on a ring stand, 50 mL burret loaded with standard NaOH solution was attached. 5 ml of acetic acid was added into 250mL Erlenmeyer flask. It was made to 20mL by adding distilled water. Three drops of phenolphthalein indicator was added to the solution in the flask and mixed by swirling gently. By carefully opening the burret stop-cock, the NaOH

solution was allowed to drain into the flask slowly while swirling it to insure continuous mixing. NaOH solution was continuously added till one drop turned the color in the solution to faint pink. The solution was kept swirling until the faint pink color persisted for 30 sec. These process was repeated for each of the 17 experiments to estimate the concentration of acetic acid. The reaction between acetic acid and sodium hydroxide is as follows;

$$CH_3COOH_{(aq)} + NaOH_{(aq)} \longrightarrow NaC_2H_3O_{2(aq)} + H_2O_{(l)} \qquad Eq(3)$$

As the stoichiometric relationship between acetic acid and sodium hydroxide is 1:1, nNaOH = n CH_3COOH. Therefore;

Moles of CH_3COOH = Moles of NaOH = $V_{NaOH}M_{NaOH}$, Where; $V_{NaOH} = V_2 - V_1$

Consquently, the concentration of acetic acid (CH_3COOH) can be determined;

$$C_{CH_3COOH}\left(\frac{mol}{L}\right) = \frac{n_{CH_3COOH}}{V_{CH_3COOH}} \qquad Eq(4)$$

The percentage concentration is determined using;

$$\text{Acetic acid \%} = C_{CH_3COOH}\left(\frac{mol}{L}\right) / \text{density} \times Mwt_{CH_3COOH} \times 100 \qquad Eq(5)$$

V. Determination of acetic acid content using HPLC

The method is applicable for the determination of organic acids in different samples. After filtration of the solution, the acetic acid content is determined by HPLC (High Pressure Liquid Chromatography) with RI-detection. Peaks are identified on the basis of their retention times. Quantification is performed according to the external standard method on peak areas or peak heights. The apparatus used for this method were; Agilent HPLC with RID; Hi-plex H,300 x

7.7mm, 8um column; 0.45µm Syringe filter; Syringe; Volumetric flask; Beaker; Pipette; Measuring cylinder; and Ultrasonic bath. The reagents used in this process were; H_2SO_4, HPLC grade water, Acetic acid standard Deionized water, Methanol, Acetic acid Stock solution (10%). 1.0 ml of 99.5 % acetic acid standard was measured and transferred into a 10ml volumetric flask, and made to final volume with HPLC grade water. Then, 0.2, 0.4, 0.8, and 1 ml aliquots of stock solution was transferred to respective 10ml volumetric flasks and diluted to the final volume with HPLC grade water. These represent: 0.2, 0.4, 0.8 and 1% acetic acid. The sample solution was filtered through 0.45µm syringe filter to provide a sample solution ready for chromatographic analysis. Then the solution was transferred into a 2ml vial and analyzed using HPLC.

Instrumental condition

- Mobile phase: 14 mM H2SO4
- Column type: Hi-plex H,300 x 7.7mm, 8um
- Flow rate: 0.6 mL/min
- Column temperature: 75°C
- Detector temperature: 35 °C
- Injection volume: 10µL
- Detector: refractive index detector
- Run time: 25 min.

Calculation and expression of result

Concentration of Acetic acid in the sample (%) = C x DF

Where C = reading of the sample (%); DF = dilution factor (if applicable)

4. RESULT AND DISCUSSION

4.1. Coffee husk characterization

Coffee husk which is one of the wastes from coffee processing industries, has the characteristics of being fibrous, high in cell wall components, whereas protein digestibility and nitrogen retention are the major factors limiting the use of coffee husk (Kassu et al, 2014)which can make it toxic and slow degradation in nature. However, coffee husk is rich in lignocelluloses materials, which makes it an ideal substrate for microbial processes (Dzung et al, 2013). The types of nutrients that are required include those that supply energy, carbon and additional necessary materials. In this study the coffee husk obtained from Dilla, Ethiopia was characterized at PASTOR Institue of Health.

Table 2: Characterization of coffee husk

Components	Coffee Husk	(Gouvea et al., 2009)	(Soccol, 2011)
Moisture Content (%)	50	15	-
pH	5	-	-
Organic Matter (%)	94.7	-	-
Total Nitrogen (%)	4	7	5.2
Total Carbon (%)	52.611	72.3	35.0

The moisture content 50% have a big difference with coffee husk used by (Gouvea et al., 2009). This might be due to the environment variation of the storage place. High moisture content affects the product quality. The analysis of total moisture is used to determine other analytical results such as volatile matter, ash content and fixed carbon The nitrogen content seems to be in fair comparison with (Gouvea et al., 2009) and (Brand et al, 2011). Higher values of nitrogen is desirable because it makes the coffee husk a good nutrient for the microorganism. It is assumed that a mixture of pure proteins will contain 16% nitrogen. Thus, the protein content of a sample is calculated from the determined nitrogen content by multiplying by a nitrogen to protein conversion factor 6.25. The total carbon content for the coffee husk was found to be 52%, which is much lesser than 72.3% but higher thant the result found by (Brand et al, 2011), which was 35%. Carbon is necessary for microbial growth and it plays vital role in the proper cultivation of microorganisms.

4.2. Hydrolysis of coffee husk

Coffee husk is lignocelluloses rich agricultural residue that can liberate free sugar when acid hydrolyzed. Lignocellulosic materials contain several high value substances such as sugars, minerals and protein. They are mainly composed of three groups of polymers, namely cellulose, hemicellulose, and lignin. Unless these components are degraded or broken down, microorganisms won't be able to utilize the waste effectively, therefore pretreatment should be done prior to the fermentation process. In this study, the total reduced sugar content through concentrated hydrolysis process was investigated to be 51.76g/ml. Efficiency of ethanol fermentation decreases with increase in sugar concentration resulting in increased osmotic pressure of the media or the number of cells that over capacity due to the high concentration of substrate. The high concentration of substrate inhibits the growth of yeast cells that lead to high osmotic pressure and low water concentration. This causes the yeast cells become dehydrated (Soni, N. Bansali et.al,

2009). Yeasts are fairly tolerant of high concentrations of sugar and grow well in solutions containing 40% sugar. There are only a few yeasts that can tolerate sugar concentrations of 65-70% and these grow very slowly in these conditions (Board, 1983)

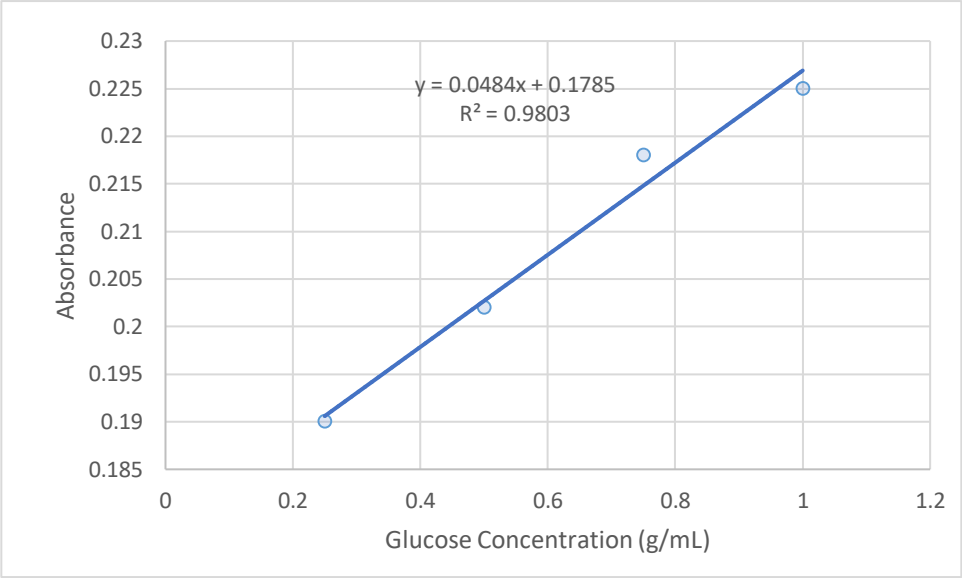

Figure 14: Glucose concentration Vs Absorbance Graph

4.3. Ethanol Fermentation

After the hydrolysis of coffee husk, ethanol fermentation was carried out, using the yeast *S. cerevisiae* for 72 hours under the optimum condition of temperature 30℃ and pH 5.5. The total ethanol concentration in the fermented coffee husk was estimated by using the chromic acid method. Potassium dichromate and sulfuric acid was prepared as the standard solution. After following the standard procedure, UV-spectrophotometry at 600nm was used to read the absorbance of both the sample and the diluted standard solution. The graph plotted for the standard curve is shown in the graph below.

As shown in Figure 11, the coefficient of determination (R^2) value is 0.98, which is closer to 1. R-squared is a statistical measure of how close the data are to the fitted regression line. 100% or values closer to 1 indicates that the model explains all the variability of the response data around

its mean and how strong the linear relationship is. By inserting the absorbance value of the fermented hydrolysate in the equation above, 42.76% of ethanol was obtained.

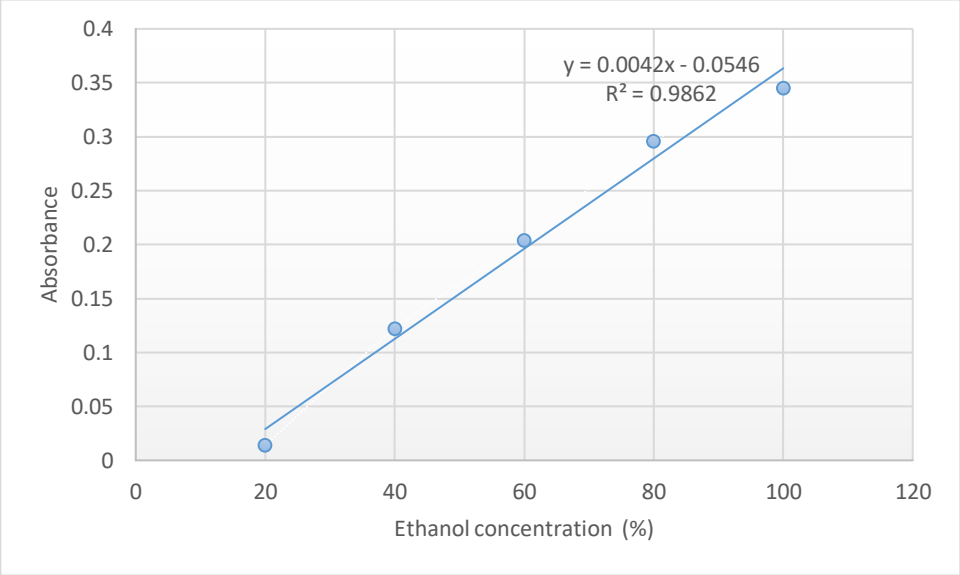

Figure 15: Ethanol Concentration Vs Absorbance Graph

4.4. Optimization of Acetic acid production

In order to determine the effect of temperature, ethanol concentration, and time on the production of acetic acid, Box Behnken response surface method of analysis was performed using design expert analysis software. All the three factors were done on three selected levels: temperature at 30℃, 35℃, and 37℃; ethanol concentration at 2%, 5%, and 8%; time at 24h, 48h, and 72h. After performing each of the 17 experiments, the concentration of acetic acid was estimated by using titration method, consequently, the results were measured and fed in to Design expert 11.0.

Table 3: Experimental Results of Acetic acid Concentration (%)

Block	Factor 1 A:Temperature Degree Celsius	Factor 2 B:Ethanol Concentration %	Factor 3 C:Time Hour	Response 1 Acetic acid Concentration %
Block 1	30.00	8.00	48.00	15.499
Block 1	30.00	5.00	72.00	14.893
Block 1	30.00	2.00	48.00	4.976
Block 1	30.00	5.00	24.00	5.205
Block 1	35.00	5.00	48.00	5.8965
Block 1	35.00	5.00	48.00	5.685
Block 1	35.00	5.00	48.00	5.013
Block 1	35.00	5.00	48.00	5.851
Block 1	35.00	2.00	24.00	4.473
Block 1	35.00	5.00	48.00	4.9
Block 1	35.00	8.00	72.00	16.598
Block 1	35.00	8.00	24.00	6.293
Block 1	35.00	2.00	72.00	3.529
Block 1	37.00	5.00	72.00	7.741
Block 1	37.00	2.00	48.00	2.551
Block 1	37.00	8.00	48.00	6.512
Block 1	37.00	5.00	24.00	1.602

4.4.1. Development of Experimental Model

The model equation developed by the analysis software is quadratic equation as shown in the equation below:

Final Equation in Terms of Coded Factors:

Acetic acid Concentration = +6.75 -2.77A +3.99B +3.39C -0.50A^2 +1.14B^2 +1.11C^2 -1.48AB

-1.13AC +2.81B C Eq(6)

Parameters such as R-squared and Adequacy precision are useful in order to check whether the model equation is accurate or not. The R–square value of 0.9843, is closer to 1 which indicates that the model equation is acceptable. The "Pred R-Squared" of 0.7993 is in reasonable agreement

with the "Adj R-Squared" of 0.964. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. A ratio of 22.785 indicates an adequate signal. Therefore, This model can be used to navigate the design space.

4.4.2. Adequacy check

ANOVA and diagnosis

The ANOVA analysis indicates whether the experiments are statistically significant or not. The ANOVA of all the 17 experiments is indicated in APPENDIX I. From the output results there are three significant terms. The Model F-value of 48.77 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 (Temprature), B (Ethanol Concentration), C (Time), B^2, C^2, AB, AC, BC are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit F-value" of 5.94 implies there is a 5.91% chance that a "Lack of Fit F-value" this large could occur due to noise. Lack of fit is bad -- we want the model to fit. The diagnosis analysis serves as a key for the comparison of the results of the actual experiments and the results predicted by the analysis software. There are mainly three graphs that are used for the diagnosis analysis.

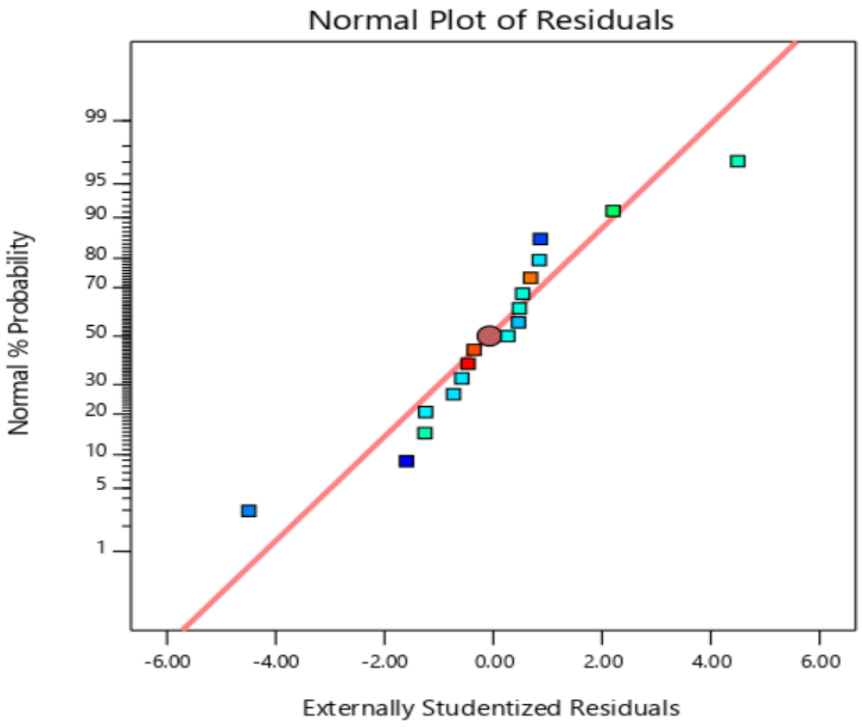

Figure 15: Normal Probability Graph

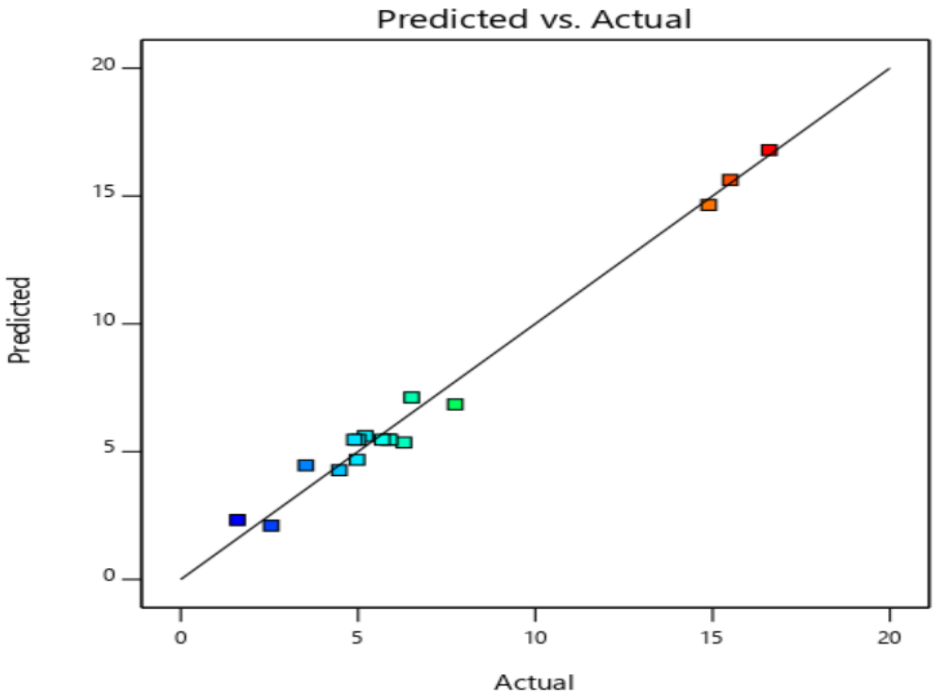

Figure 16: Predicted vs Actual Graph

The distribution of experimental results as shown in the normal probability graph are relatively closer to the straight line. Since the results does not deviate from the straight line, the data are closer to normal. Therefore, it can be acceptable. The predicted Vs Actual graph shows how much the actual results deviate from the predicted result. By calculating the error, it can be concluded how much the actual value deviates from the predicted.

$$error(\%) = \frac{Actual - Predicted}{Actual} \times 100 \qquad Eq(7)$$

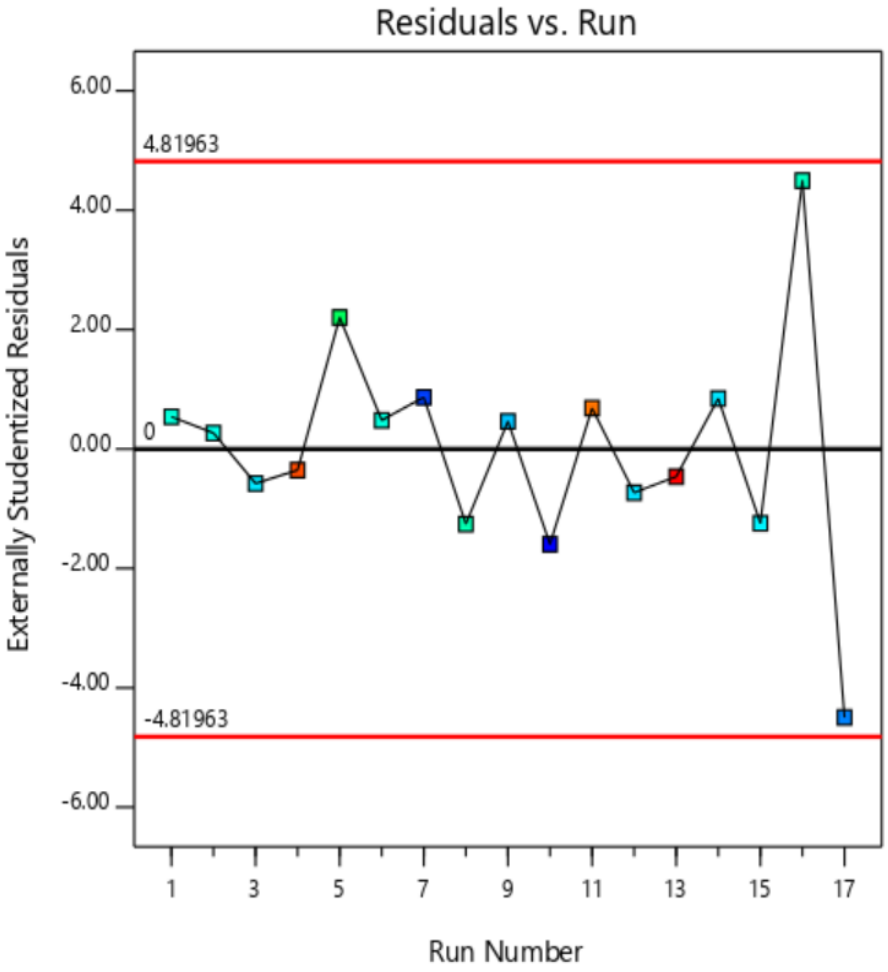

Figure 17: Residuals Vs Run Graph

From the residual vs run graph, it is can be observed that the run's are dispersed randomly above and below the residual = 0 line. these shows that the residuals exhibiting normal random noise around the residual = 0 line which suggests that there is no serial correlation.

4.4.3 Interaction Effect

4.4.3.1. Direct Effect

The direct effect of each of the parameters does not show the actual effect of the factors on the whole experiment but it can be used to indicate the point where all the factors have equal effect. The perturbation graph which shows the direct effect of the factors is indicated below. The perturbation plot helps to compare the effects of all the factors at a particular point in the design space. The response is plotted by changing only one factor over its range while holding all the other factors constant.

A steep slope or curvature in a factor shows that the response is sensitive to that factor. A relatively flat line shows insensitivity to change in that particular factor. If there are more than two factors, the perturbation plot could be used to find those factors that most affect the response. These influential factors are good choices for the axes on the contour plots. The perturbation plot shown above shows the effect of each of the independent variables on the response, which is acetic acid concentration. For the case of factor A (temperature), the concentration of acetic acid is at its highest when it is at 30℃ and declines as it goes to 37℃. As in the case of factor B (ethanol concentration) and factor C (time) it can be observed that the acetic acid concentration increases as both the ethanol concentration and time increases.

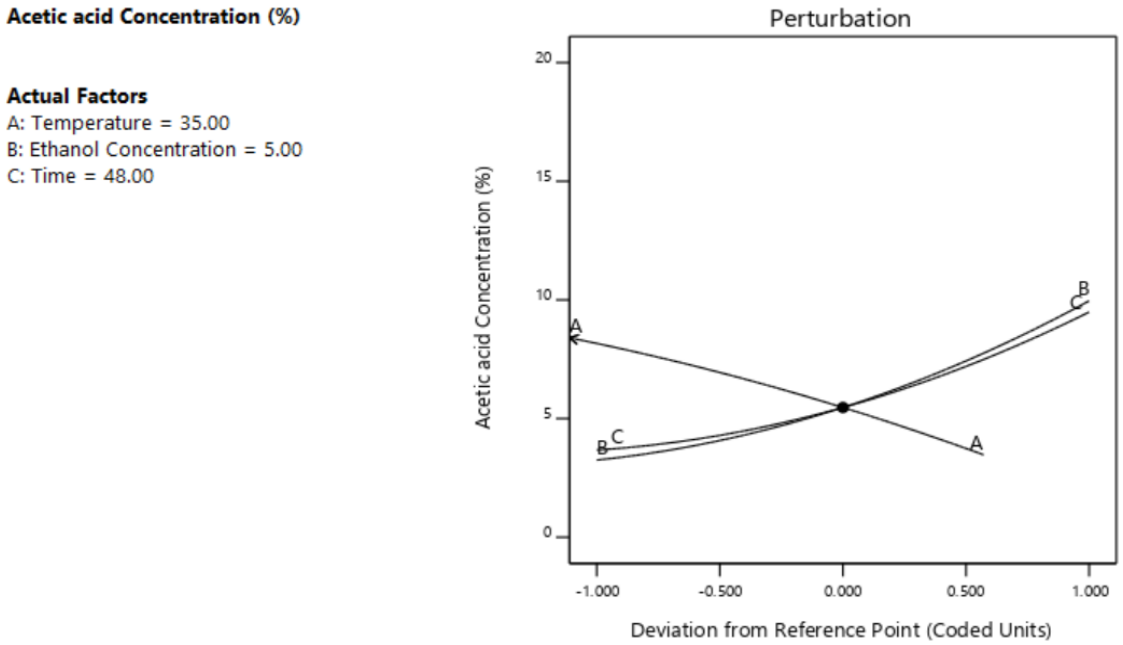

Figure 18: Direct Effect of each Factor on Acetic acid Concentration

4.4.3.2 Combined effect

In the first run of the experiments, four experiments with a temperature of 30℃ were conducted. The maximum acetic acid concentration is recorded to be 15.49% at 8% ethanol concentration and 48h, whereas the minimum acetic acid concentration of 4.98% was recorded at 2% ethanol concentration and 48h. From this, it can be observed that the temperature (30℃) and time (48h) are similar for both the maximum and minimum results, so for this case it can be concluded that the variation is caused due to ethanol concentration.

In the second set of experiments, nine experiments at a temperature of 35℃ were conducted. The maximum acetic acid concentration of 16.59% was recorded at 8% and 72hours of time, while the minimum acetic acid concentration of 3.53% was recorded at 2% ethanol concentration and 72 hours of time. The temperature and the time are similar for both the maximum and minimum values, thus the variation is caused by the difference in ethanol concentration. Five experiments were

repeated at 35℃, 5% ethanol concentration, and 48h. The recorded result fluctuated in the range between 4.9 and 5.89, these might be due to external factors.

In the final set of experiments, four experiments at a temperature of 37℃ were conducted. The maximum and minimum values of acetic acid concentration are recorded to be 7.74% and 1.6% respectively. At 5% ethanol concentration and at a time from 24 to 72 hours, the acetic acid concentration increased from 1.6% to 7.74%. Similarly, for a time of 48 hours and ethanol concentration of 2% and 8%, the acetic acid concentration was recorded to be 2.55% and 6.51% respectively. The result indicates that variation in ethanol concentration shows to have a more significant effect on the result. The combined effect of ethanol concentration and temperature at a fixed time, ethanol concentration and time at a fixed temperature, and temperature and time at a fixed ethanol concentration is extracted from the analysis software.

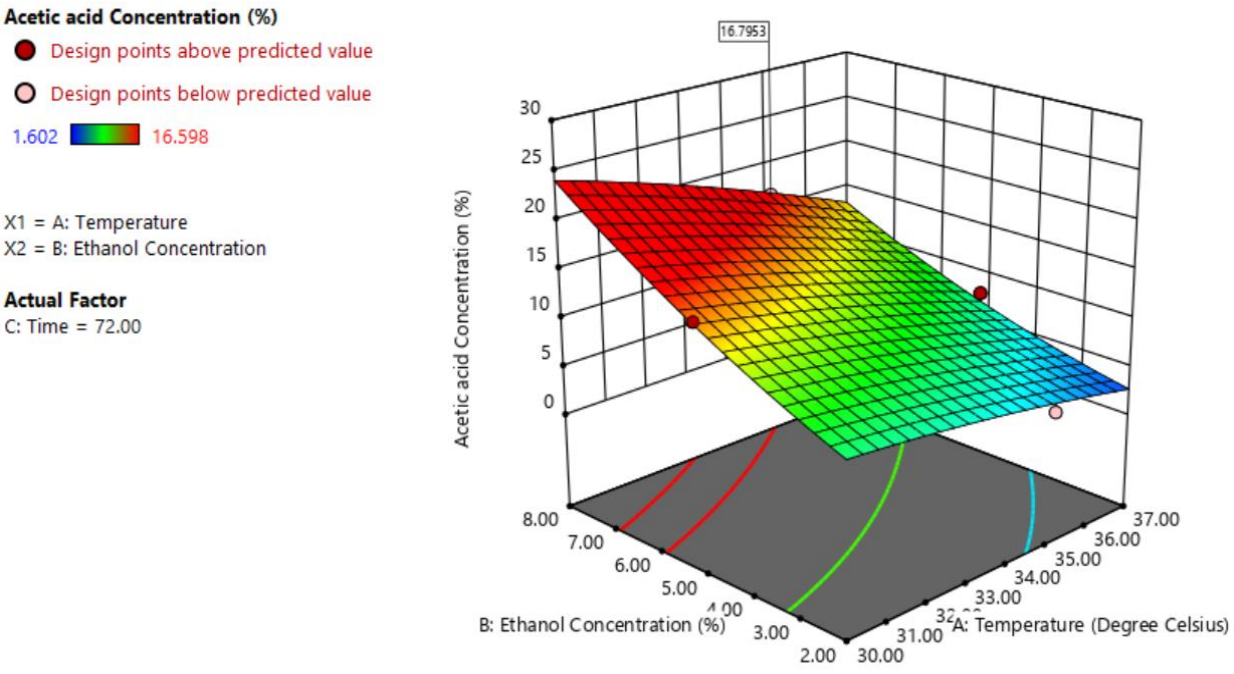

Figure 19: Interaction between ethanol concentration and temperature

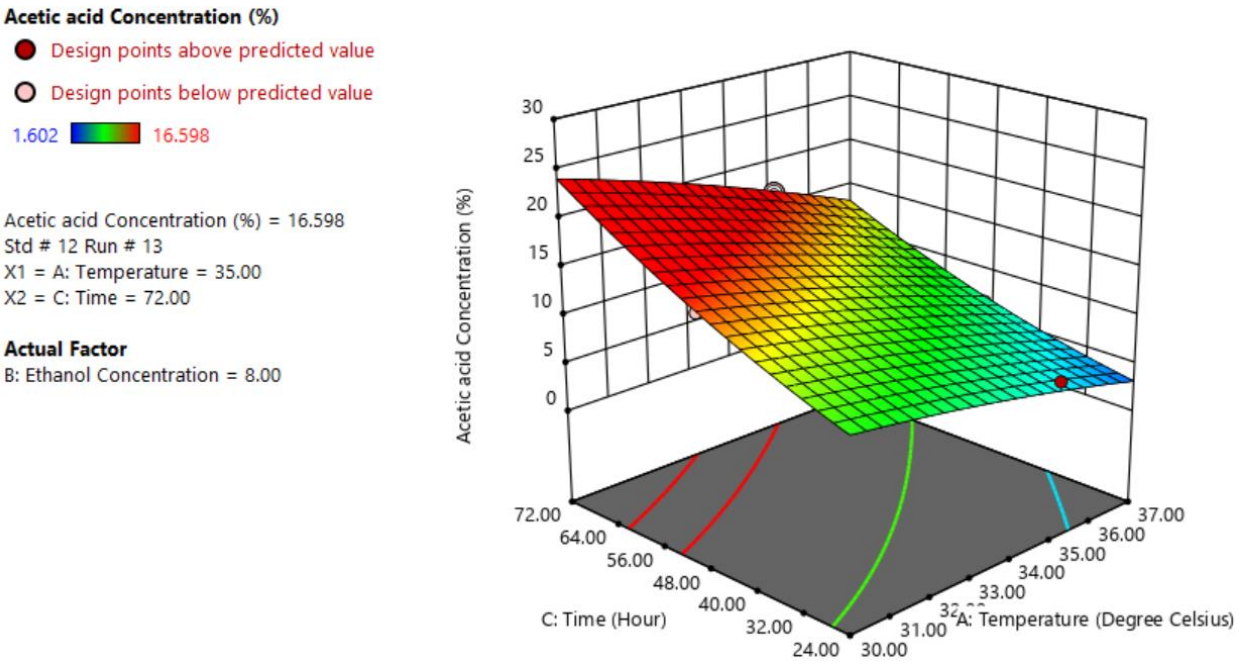

Figure 20: Interaction between temperature and time

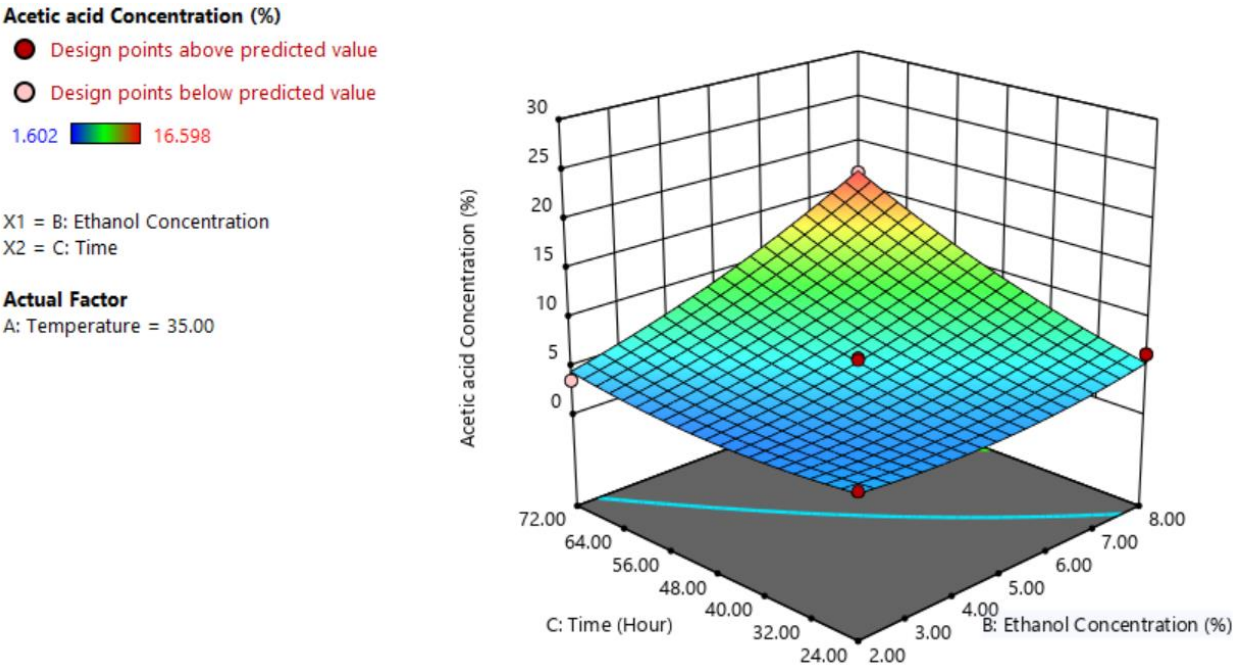

Figure 21: Interaction between ethanol concentration and time

4.4.4. Optimization

According to the values of acetic acid concentration fed to the analysis software, 100 solutions are suggested for the value of temperature, pH and incubation time by numerical optimization. The optimum values for each factors is shown in a ramp plot as shown below. Ramps are a graphical view of each optimal solution. The desirability is equal to one as shown in Figure 19. This shows that the optimized parameters have the highest response. The value is 1 or 100% because the experiments were conducted without replication and the probability of occurrence of error is low.

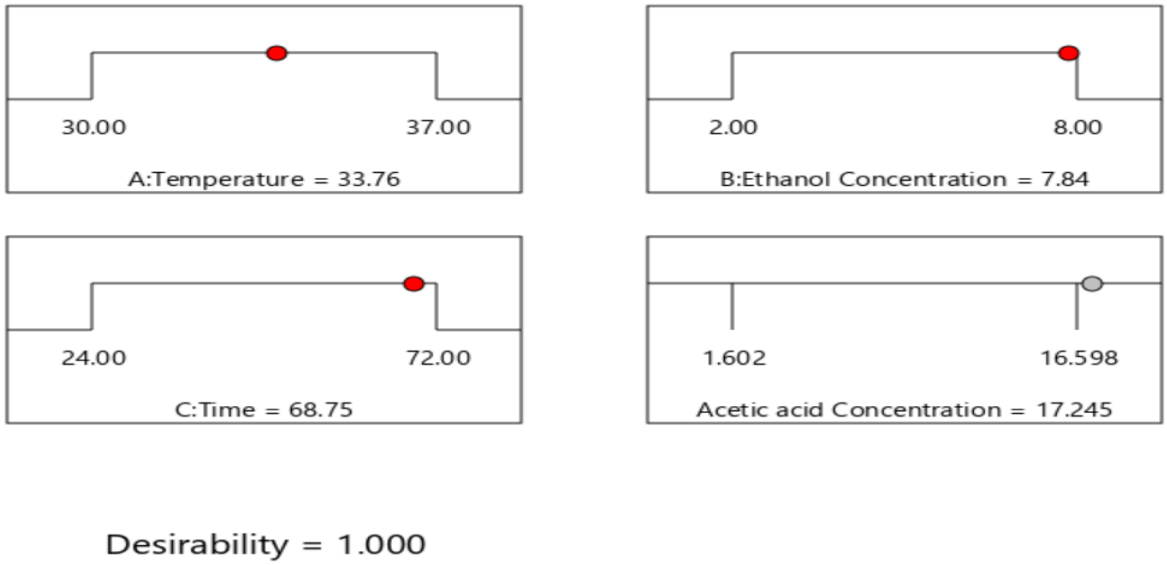

Figure 22: Ramp Plot

4.5. Liquid-Liquid Separation of Acetic acid

Separation factor is the ratio of distribution coefficient of solute (acetic acid) to the distribution coefficient of diluent (water). It is a measure of the solubility of solvents to separate acetic acid from aqueous solution. According to (Sumona Haquel et al, 2008) distribution coefficient of acetic acid were obtained by the ratio of weight percent of acetic acid in solvent phase to the weight percent of acetic acid in water phase, which was larger than 1 for all the system but acetic acid-

water-amyl alcohol system showing the highest value (3.0), indicates that acetic acid has preferential solubility between water and alcohol which is the key factor in the extraction process.

In this study, the laboratory scale extraction of acetic acid from an aqueous solution was carried out using ethyl acetate as a solvent. According to(Usman et al, 2015), as the solvent to feed ratio is increased, so will the separation process. S=1L was selected for this study; where S and L are the volumes of the solvent and the feed respectively. As the acetic acid concentration is expected to be less than 40%w/w, simple evaporation method was used to separate the two liquids. The temperature of the evaporator was set to 77.1℃, as ethyl acetate will evaporate at that temperature. Since acetic acid have a higher boiling point of 110℃, it will remain in the flask. After evaporation the volume of the solution decreased from 130ml to 50ml, which is one of the indications of ethyl acetate evaporating from the solution.

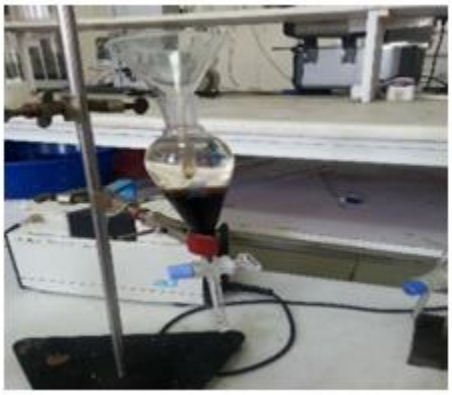

Figure 23: Liquid-liquid Separation in a Funnel

The minimum acetic acid concentration is recoded in experiment 17, where the temperature is 37℃, ethanol concentration 5%, and the time 24h, while the Maximum acetic acid concentration was found in experiment 11, at a temperature of 35℃, ethanol concentration of 8%, and time of 72h. From this result, it can be observed that the two results have a big gap between them, this can

be due to the ability of the microorganism to tolerate high temperature, and the short amount of time during its adoption. Whereas, the maximum concentration was achieved on an intermediate temperature of 35℃, which is more or less close to the optimum temperature for growth of *acetobacter aceti* bacteria, 30℃. Moreover, the ethanol concentration and the time for the maximum concentration of acetic acid (1.902mol/L) was found to be 8% and 72hours respectively, thus it can be concluded that the bacteria have good tolerance to high ethanol concentration (8%) and produces more acetic acid at a longer time (72h).

According to (Vaughn, 1942) concentrations of alcohol between 14 and 15% by volume are very inhibitory to acetic acid bacteria, but concentrations of 5 to 12 % are not inhibitory. However, (Walker and Tosic, 1946) asserted that an ethyl alcohol content in media greater than 4 per cent prolongs the stationary and lag phases of growth of the bacteria, but an alcohol content of more than 6% inhibits growth of some species. When alcohol concentrations of less than 1 or 2% are used acetic acid may be lost, because *Acetobacter* species starts to oxidize acetic acid to carbon dioxide and water. Hence, in this study, an ethyl alcohol concentration of 8% did not inhibit the growth of *acetobacter aceti*, thus the production of acetic acid. The result seems to be in accordance to the studies of (Vaughn, 1942).

Table 4: Result for Acetic acid Concentration (mol/L)

Experiment	Temperature (℃)	Ethanol Concentration (%)	Time (h)	Concentration of Acetic acid (M)
1	30	8	48	1.71
2	30	5	72	0.604
3	30	2	48	0.869
4	30	5	24	0.909
5	35	5	48	1.031
6	35	5	48	0.994
7	35	5	48	0.876
8	35	5	48	1.023

9	35	5	48	0.782
10	35	2	24	0.857
11	**35**	**8**	**72**	**1.902**
12	35	8	24	1.101
13	35	2	72	0.617
14	37	5	72	0.353
15	37	2	48	0.446
16	37	8	48	1.138
17	**37**	**5**	**24**	**0.28**

Acid/base Titration

From the formula; $n_b V_b M_b = n_a V_a M_a$, M_a can be calculated as;

$$Ma = \frac{M_b V_b}{V_a} \qquad Eq(8)$$

$$\% = \frac{M_a}{p} \times M_{wt} \times 100\% \qquad Eq(9)$$

Where, M_a (mol/l) is the concentration of acetic acid from Table 6

p (g/cm3) is the density of acetic acid

M_{wt} (g/mol) is the molar mass of acetic acid

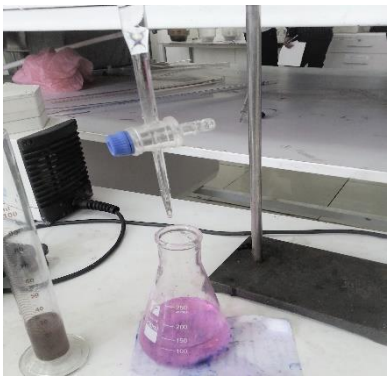

Figure 24: Titration of Acetic acid with 0.5M NaOH

4.6. HPLC Analysis for Acetic acid concentration determination

A modern refinement in chromatographic methods is HPLC, or high-performance liquid chromatography. HPLC makes use of high-pressure pumps that speed the movement of the protein molecules down the column, as well as higher-quality chromatographic materials that can withstand the crushing force of the pressurized flow. Further acetic acid identification was achieved by using HPLC technique. The sample with the optimum acetic acid concentration (16.9%) was selected for this analysis. As shown in Table 7, a known concentration of acetic acid was diluted into different concentration, in order to construct the standard curve.

Table 5:Calibration Sample Statistics

Compound: Acetic acid **Signal:** RID1 A, Refractive Index Signal

Line#	Location	Inj#	RT [min]	Unit	Area	Height	Sample Name
1	1	1	15.71	%	170411.42	7040.60	0.2%EtOH and AA2
2	2	1	15.68	%	328932.94	13435.33	0.4%EtOH and AA3
4	4	1	15.79	%	659859.00	26591.43	0.8%EtOH and AA5
5	5	1	15.67	%	789493.94	32351.17	1.0%EtOH and AA6

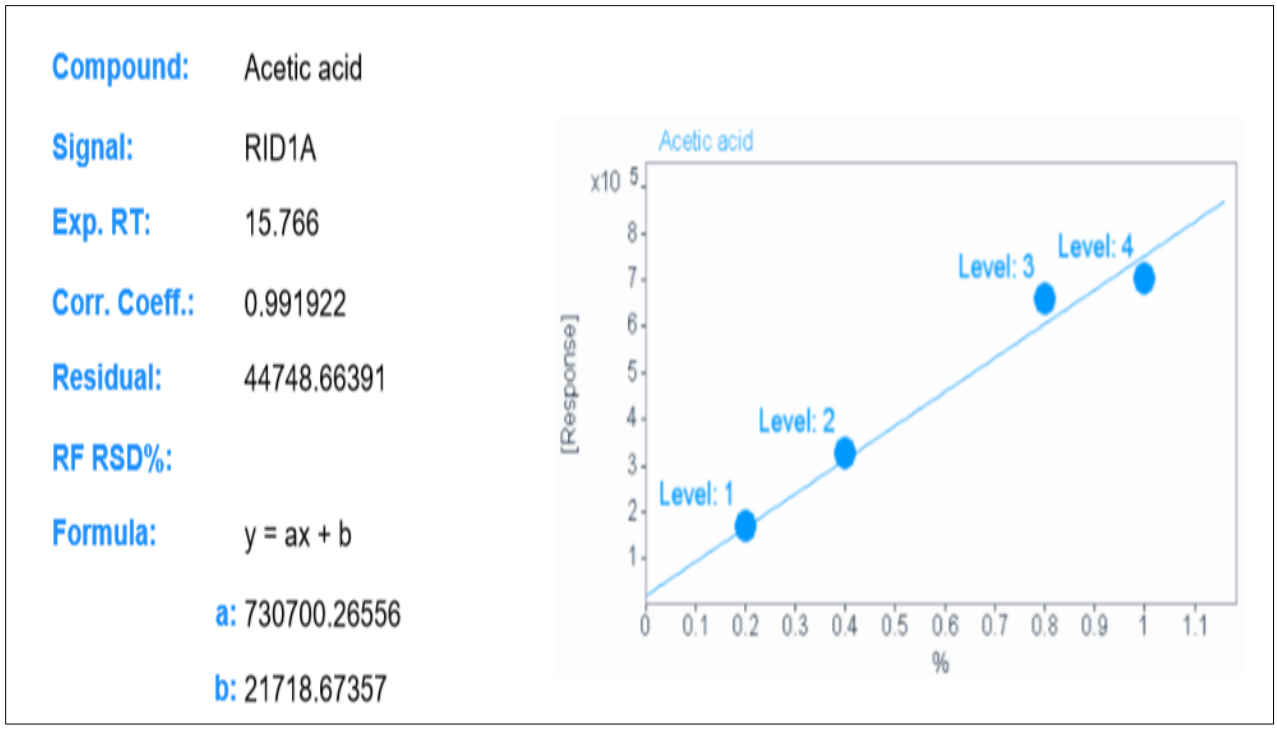

Figure 25: Standard Acetic acid Curve

Table 6: Sample Statistics

Sample statistics:

Compound: Acetic acid **Signal:** RID1 A, Refractive Index Signal

RT [min]	Amount Unit	Area	Height	Sample Name
15.670	15.5701 %	476919.3719	7438.28809	Acetic acid

Quantitative analysis was used, so that the percentage of acetic acid present in the sample could be determined. From table 6, the acetic acid concentration of the selected sample is shown to be 15.57%, which is slightly different from the concentration estimated by Acid/Base titration (16.6%). This variation is most likely to be due to other organic acids present in the sample.

After the analysis of acetic acid concentration, it was diluted to 5% to formulate the vinegar. The acetic acid typically ranges from 4% to 8% by volume for table vinegar and up to 18% for pickling. In this study, the final acetic acid concentration was diluted to 5%, which the standard levels of concentration for vinegar. By using equation (10) and equation (11), the amount of distilled water required to dilute the acetic acid was determined

$$V_1 C_1 = V_2 C_2 \qquad Eq(10)$$

$$V_2 = \frac{V_1 C_1}{C_2} \qquad Eq(11)$$

Where V_1 is the volume of the acetic acid (50 ml), V_2 is the volume of the distilled water, C_1 is the concentration of the fermented acetic acid (15.57 %), C_2 is the final concentration of acetic acid (5%).

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

Coffee husk which is one of the wastes from coffee processing industries, has the characteristics of being fibrous, and high in cell wall components. The coffee husk used in this study was obtained from Dilla, Ethiopia. Concentrated acid hydrolysis was used to as a chemical treatment for the coffee husk to make it suitable as a substrate. Phenol-sulfuric acid method was used to determine the total sugar concentration of the coffee husk hydrosylate, and it was estimated to be 51.76 g/mL. After the hydrolysis of coffee husk, ethanol fermentation was carried out, using the yeast *S. cerevisiae* for 72 hours under the optimum condition of temperature 30 ℃ and pH 5.5. Chromic acid method and plotting a standard curve, the total ethanol concentration in the fermented coffee husk was estimated to be 42.76%. In order to determine the effect of temperature, ethanol concentration, and time on the production of acetic acid, Box Behnken design using the response surface method of analysis was performed using design expert analysis software. After performing each of the 17 experiments, the concentration of acetic acid was estimated by using acid/base titration and HPLC analysis. The temperature, ethanol concentration, and time for the optimum acetic acid concentration (16.6%) were 33.76℃, 7.84%, and 68 h respectively. The optimum acetic acid concentration was analyzed using HPLC. The acetic acid produced was formulated to be vinegar with 5% acetic acid concentration.

5.2. Recommendations

Ethiopia is one of the major coffee producers in the world, thus, the solid wastes generated from this industry are in large amount. As coffee waste is a major pollutant to the environment, the government, investors, and experts should give higher attention in handling and utilizing these wastes. Coffee waste has good aroma and it is composed of essential nutrients that can be used in the production of different biological or other products.

The culture *acetobacter aceti* bacteria, used in this study was purchased from abroad, due to its unavailability in the country, thus I recommend for microbiologist or other concerned individuals/organizations should culture this important microorganism.

The liquid-liquid separation of acetic acid from the fermentation broth was performed using ethyl acetate as a solvent. The effect of the solvent (ethyl acetate) on the final concentration of acetic acid, needs further research.

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Appndex I

HPLC Result

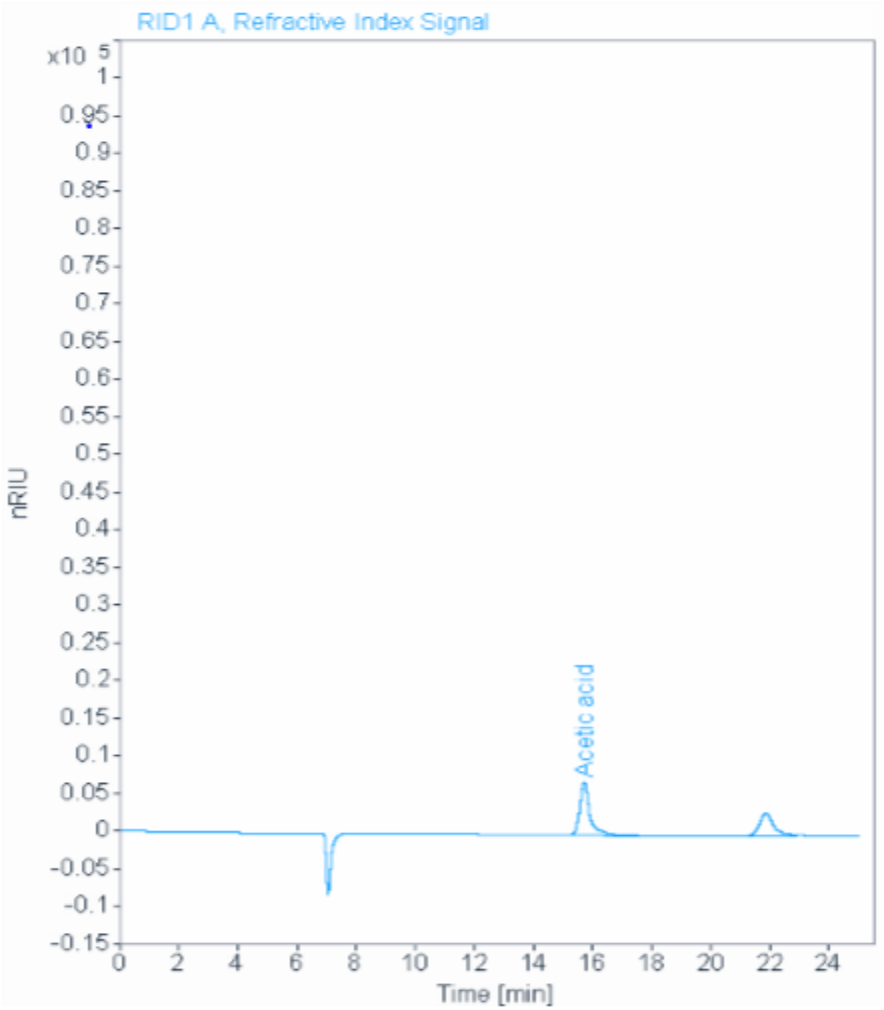

Figure 26: 0.2% concentration of acetic acid

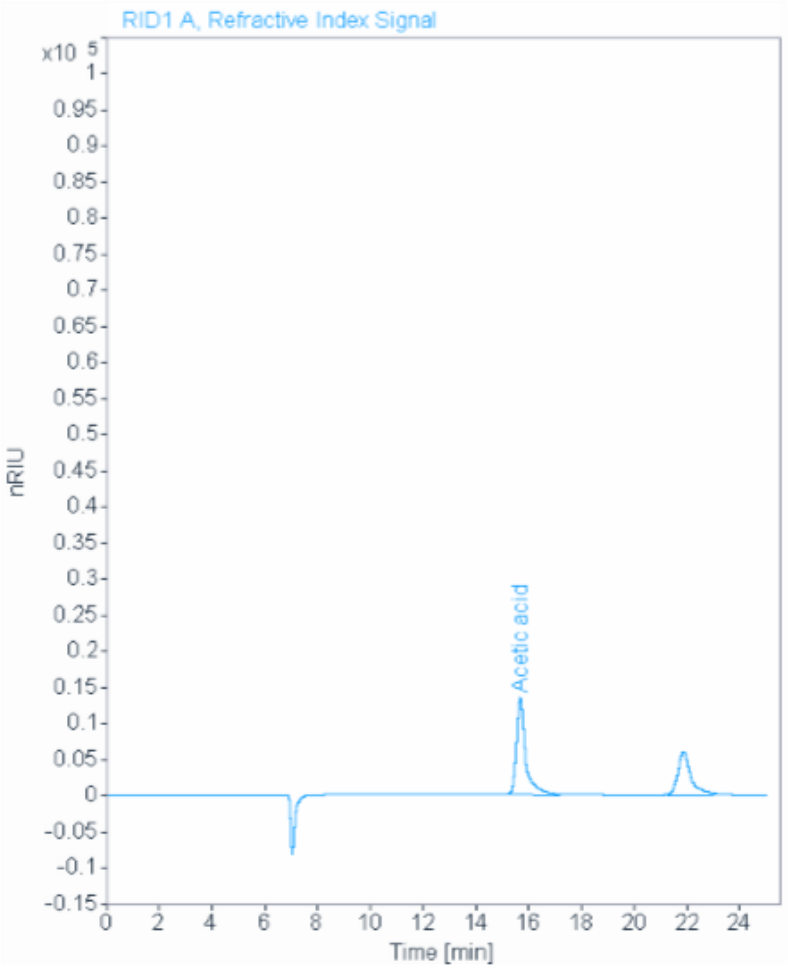

Figure 27: 0.4% concentration of acetic acid

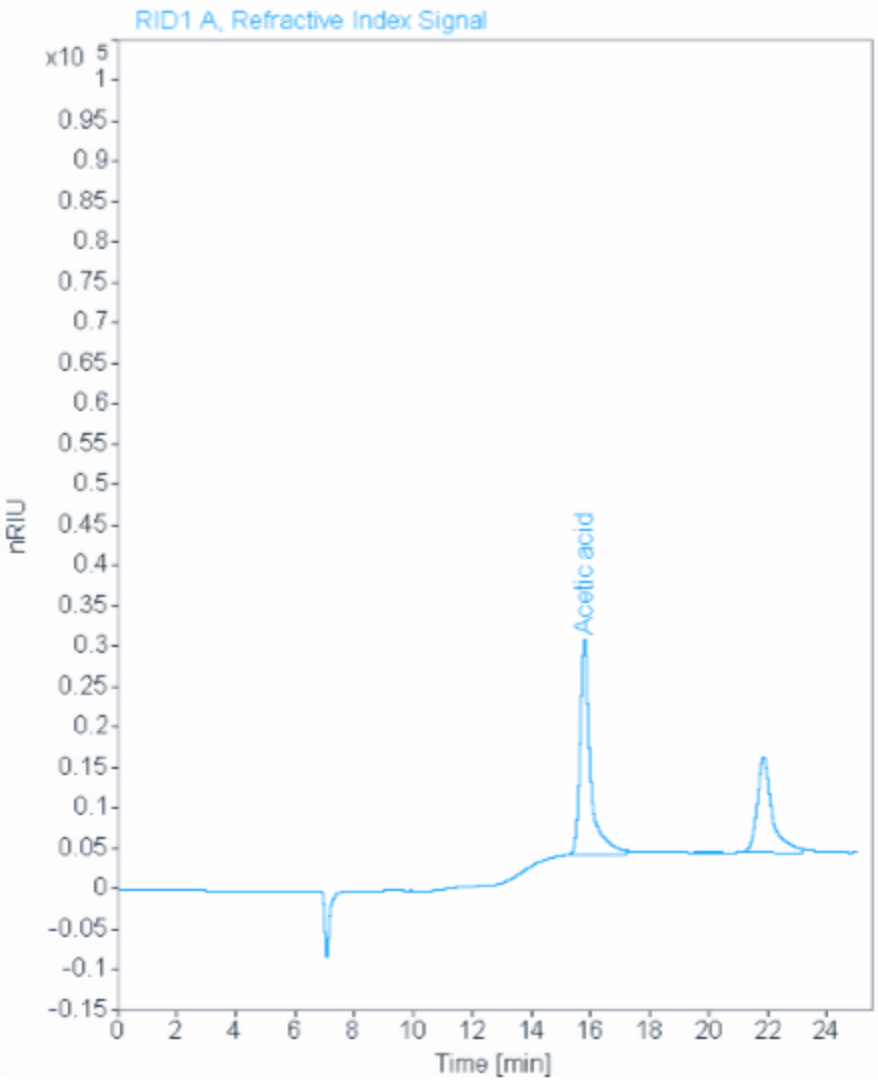

Figure 28: 0.8% concentration of acetic acid

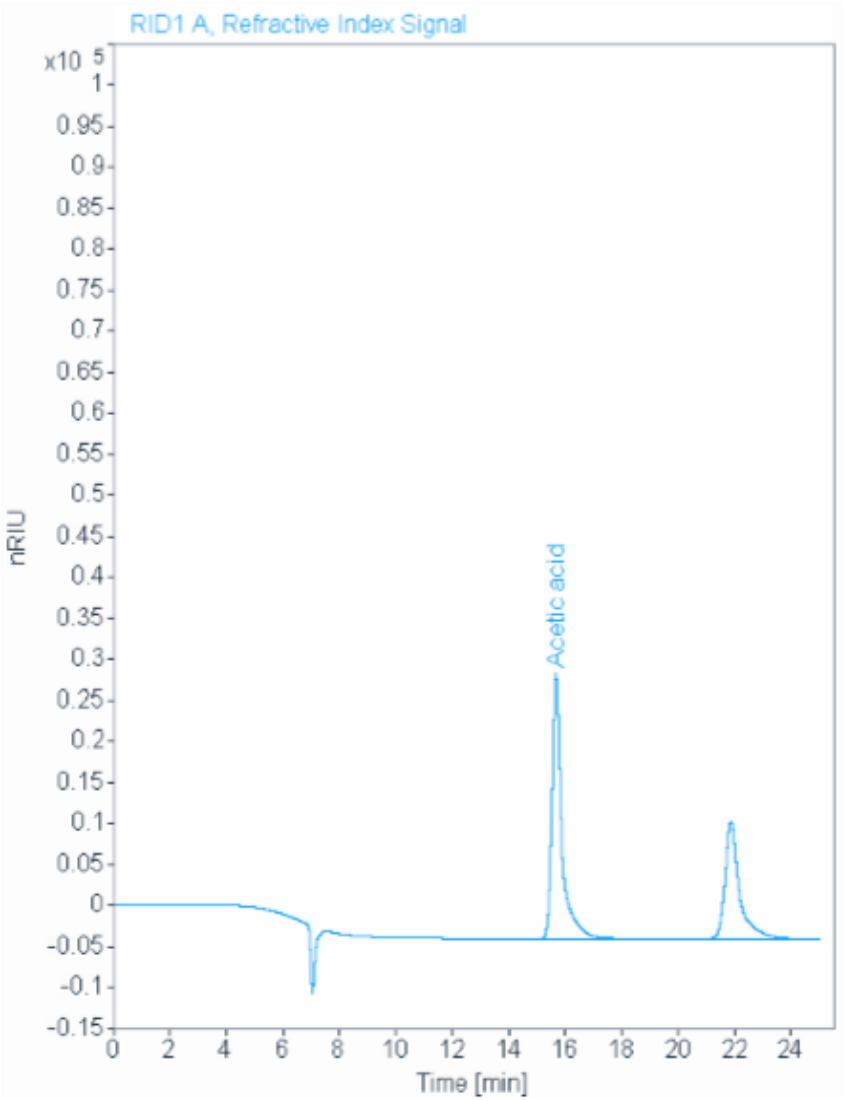

Figure 29: 1% concentration of acetic acid