VINEGAR FERMENTATION

A Thesis

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ABSTRACT

Traditionally, the manufacture of vinegar provided a means of utilizing a large proportion of the cull fruit from apple-packing establishments and the waste from apple processing facilities. Most vinegar is now produced from distilled grain alcohol. Vinegar may be defined as a condiment made from various sugary and starchy materials by alcoholic and subsequent acetic fermentation. The vinegar bacteria, also called acetic acid bacteria, are members of the genus *Acetobacter* and characterized by their ability to convert ethyl alcohol (C_2H_5OH) into acetic acid (CH_3CO_2H) by oxidation. Vinegar can be produced from various raw materials like distilled alcohol, wine, rice wine and any kind alcoholic solution by several major production techniques for making vinegar such as the Orleans process, generator process and submerged acetification process.

The Orleans process consists of wood barrels filled with alcohol liquid fermented for about 1 to 3 months at 70ºF to 85ºF (21°C to 29°C). After fermentation, 1/4 to 1/3 of the vinegar is then drawn off for bottling and an equivalent amount of alcoholic liquid added. The generator process was introduced by Schutzenbach in 1823. Non compacting material is filled in the large upright wood tanks above a perforated wood grating floor. Re-circulated fermenting liquid trickles over packing material toward the bottom while air moves from the bottom inlets toward the top. The recirculation process takes about 3 to 7 days after which 2/3 of the final vinegar product is withdrawn from the tank and new alcohol solution is added. In 1955, Hromatka reported on a new method of making vinegar using submerged acetification. In this

process, supply air is forced into the alcohol liquid in the tank and the material is fermented at 86°F (30°C). At the end of every cycle, 1/3 of the liquid is discharged as final product, replaced with mash containing fresh alcohol solution and a new fermentation cycle begins.

The aim in the present study is to identify quality and microbial differences between the generator process and submerged acetification and to characterize the species of vinegar bacteria used in acetification.

CHAPTER 1 INTRODUCTION

Vinegar may be defined as a condiment made from various sugary and starchy materials by alcoholic and subsequent acetic fermentation (Cruess 1958).

Vinegar can be produced by different methods and from various raw materials. Wine (white, red, and sherry wine), cider, fruit musts, malted barley, or pure alcohol are used as substrates. Vinegar production ranges from traditional methods employing wood casks and surface culture to submerged fermentation in acetators (Morales *et al* 2001). Vinegar traditionally has been used as a food preservative. Whether naturally produced during fermentation or intentionally added, vinegar retards microbial growth and contributes sensory properties to a number of foods. The wide diversity of products containing vinegar (sauces, ketchup, mayonnaise, etc.) and the current fall in wine consumption have favored an increase in vinegar production (De Ory *et al* 2002).

Acetic acid is the predominant flavoring and antimicrobial component in vinegar. The following review will focus on the importance of acetic acid as a direct food additive or more recently as a food processing aid, to decontaminate food prior to distribution and consumption (Marshall *et al* 2000).

Earlier processes used for making vinegar were the Orleans process (which is also known as the slow process), the quick process (which is also called the generator process), and the submerged culture process. The quick process and submerged culture process were developed and are used for commercial vinegar production today.

Acetic acid is formed in a four-step reaction involving conversion of starch to sugar by amylases, anaerobic conversion of sugars to ethanol by yeast fermentation, conversion of ethanol to hydrated acetaldehyde, and dehydrogenation to acetic acid by aldehyde dehydrogenase (Nichol 1979; Canning 1985). The last two steps are performed aerobically with the aid of acetic acid forming bacteria. Acetic acid yield from fermented sugar is approximately 40%, with the remaining sugar metabolites either lost to volatilization or converted into other compounds. Acid yield improvements can be achieved using high rates aeration of during continuous production (Ghommidh *et al* 1986).

Vinegar bacteria, also called acetic acid bacteria, are members of the genus *Acetobacter* and characterized by their ability to convert ethyl alcohol, C_2H_5OH, into acetic acid, CH_3CO_2H, by oxidation as shown below;

Anaerobic Aerobic

$$\mathbf{2C_2H_5OH \rightarrow 2CH_3CHO \rightarrow 2CH_3CO_2H + 2H_2O}$$

Most bacteria strains derived from vinegar factories are able to oxidize acetic acid to CO_2 and H_2O (over-oxidation) and therefore are classified in the genus *Acetobacter* (De Ley *et al* 1984).

Common types of vinegar include white distilled vinegar, cider vinegar, wine vinegar, rice vinegar, and malt vinegar. Further processing of vinegar, following substrate conversion to acetic acid may include filtration, clarification distillation and pasteurization at 165.2°F (74°C) before it is bottled. Regulations in the United States require vinegar to contain at least 4% acetic acid resulting from acetic acid

fermentation of ethanol containing substrates. Labels identifying the diluents used to meet the listed concentration of acid are also required. Acetic acid concentration in vinegar may be expressed using the term "grain". For example, 100 grain distilled vinegar is a 10% acetic acid solution (Nickol 1979). If higher concentration of acetic acid is required, the dilute solution of acetic acid maybe heat distilled or frozen to slush. The slush is centrifuged to isolate the liquid portion (Nickol 1979; Ebner 1982). Concentration from 10-30% may be achieved using this technique (Chukwu and Cheryan 1996).

Vinegar plays an important role in salad dressings, ketchup, hot sauce and other sauces. This need demands industrial fermentation systems capable of producing a large amount of vinegar. These systems must maintain reliable controls and optimum conditions for acetic acid bacteria fermentation (De Ory *et al* 1999). Many techniques have been developed to improve industrial production of vinegar. Most try to increase the speed of the transformation of ethanol into acetic acid in the presence of the acetic acid bacteria (Tesfaye *et al* 2002). Today, the most common technology for the vinegar industry is based on the submerged culture (Hormatka and Ebner 1951) with diverse technical modifications which try to improve the general fermentation conditions (aeration, stirring, heating, etc.).

The overall aim in the present study is to identify the quality and microbial differences between the generator process and submerged acetification. Specific goals were to achieve 10-12% acidity using constructed lab scale production facilities and to characterize the species of vinegar bacteria used in acetification.

CHAPTER 2 LITERATURE REVIEW

2.1 Background

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 (Peppler and Beaman 1967). Its color and aroma are greatly dependent on the material from which it is made (Kehrer 1921).

2.1.1 Vinegar History

Vinegar is the world's oldest cooking ingredient and food preservation method. According to the Vinegar Institute (Vinegar Institute 2005), vinegar's use can be traced back over 10,000 years. In fact, flavored vinegars have been manufactured and sold for almost 5,000 years. The wide variety of vinegars available today is nothing new. Until the six century BC, the Babylonians were making and selling vinegars flavored with fruit, honey, malt, etc. to gourmets of the time. In addition, the Old Testament and Hippocrates recorded the use of vinegar for medicinal purposes (Kehrer 1921; Conner 1976).

There are other historical reports about vinegar. Albucases in 1100 made the statement that colorless vinegar must be distilled over a low fire. Basilius Venlentinus, a monk, in the fifteenth century found that by distilling weak vinegar, a stronger product could be obtained. The Geber in the seventeenth century discovered increasing the strength of wine vinegar by distillation. Chemist Stahl in the first half of eighteenth century discovered the sour principle of vinegar was acetic acid. In 1790,

Loewitz, reported that running weak acetic acid over charcoal would strengthen it. Durande in 1778 made a more concentrated product and called it glacial acetic acid. The first complete analysis of acetic acid was made by Berzelios in 1814. Dobereiner proved that alcohol was oxidized at the expense of oxygen and produced acetic acid and water. In 1823 Schutzenbach introduced the quick process of manufacturing vinegar based on Dobereiner's theory of formation of acetic acid from alcohol (Kehrer 1921). In 1955 Joslyn reported that Hromatka developed a new method of making vinegar called submerged acetification (Cruess 1958).

2.1.2 Production and Uses

According to AC Nielsen and the Vinegar Institute (Vinegar Institute 2005), vinegar sales grew at 15% from 2000 to 2002 and have been stronger than most other comparative categories including meat marinades, oriental sauces, Worcestershire sauce, cooking wine and sherry. According to the AC Nielsen data presented at the 2003 annual meeting, vinegar sales have increased 29% over the past 9 years (Figure 1) from Crisco Company 2005.

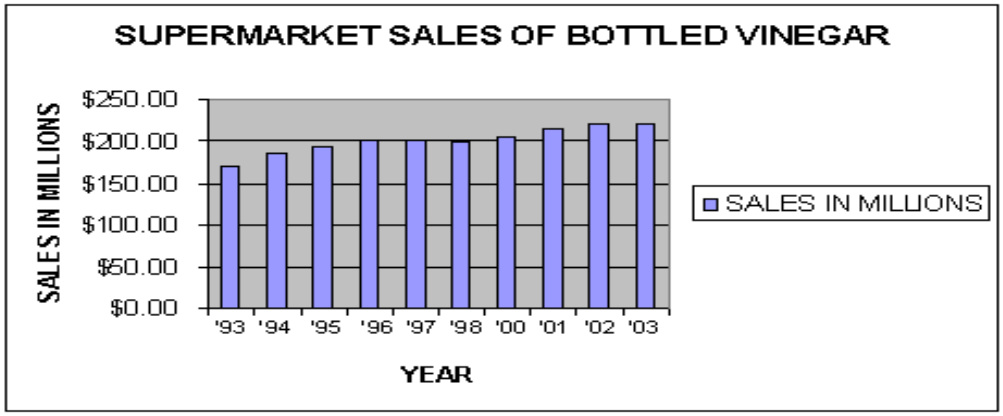


Figure 1: AC Nielsen Data Presented at 2003 Annual Meeting –Supermarket Sale

A summary of a survey provided by the Vinegar Institute in 1989, characterizing the production of vinegar by food category in the U.S.A is shown in Table 1 from Crisco Company 2005.

Table 1: Vinegar Institute Production Survey in 1989

Category of vinegar usage	**Percent of total production**
Bottled	33.7%
Dressings & Sauces	16.8%
Pickles	14.8%
Mustard	11.5%
Other Processed Foods	10.5%
Tomato Products	8.5%
Other	4.2%

According to the AC Nielsen Unit Share by Flavor (Figure 2) from Crisco Company 2005, there has been a slight decrease in the consumption of white distilled and cider vinegars. Red wine and other vinegar consumption was maintained during

the three year period 2000 to 2002. The use of balsamic and rice vinegar increased during this same time period. This increase may indicate that flavor is a key for the consumers.

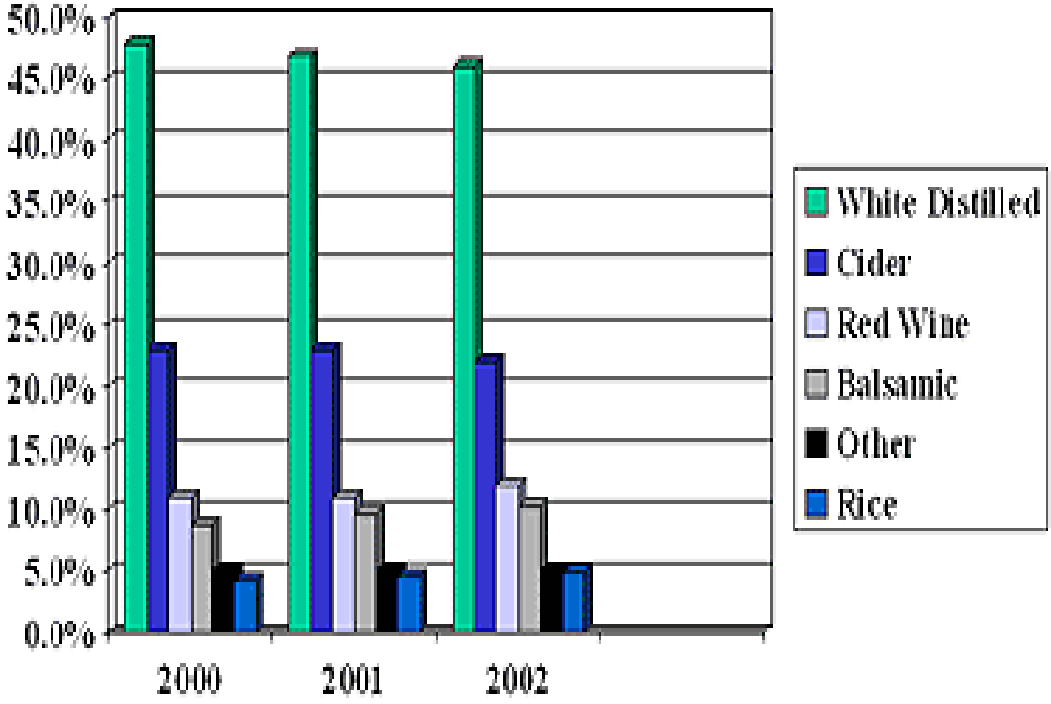


Figure 2: Vinegar Unit Shares by Flavor (2000 – 2002)

According to the Progressive Grocer in September 2001, 49.3% of U.S.A households purchased vinegar at least once (Crisco 2005). Each household spent about $3.79 per year on vinegar.

In addition, AC Nielsen reported that 53 million households buy vinegar and spend $4.07 each on the category (Crisco 2005). Vinegar sales are somewhat seasonal, with a peak in the summer months and a secondary peak in April. Vinegar buyers in the U.S.A like the 16/17 ounce size the best with the 32/34 ounce size as the second favorite.

There are some reports that suggest consumers are changing their vinegar purchasing habits. According to IRI (Information Resources, Inc) information from 1994 - 1998, of the 48% of households that purchased vinegar, 30% purchase white distilled vinegar, 14% purchase cider vinegar, 9% purchase red wine vinegar, 5% purchase balsamic vinegar and 3% purchase rice vinegar (Figure 3) from Crisco Company 2005.

According to the IRI (Information Resources, Inc.) data from 1994 – 1998, more vinegar is sold in the Northeast, Southeast and the Great Lakes area compared to the remainder of the U.S.A.

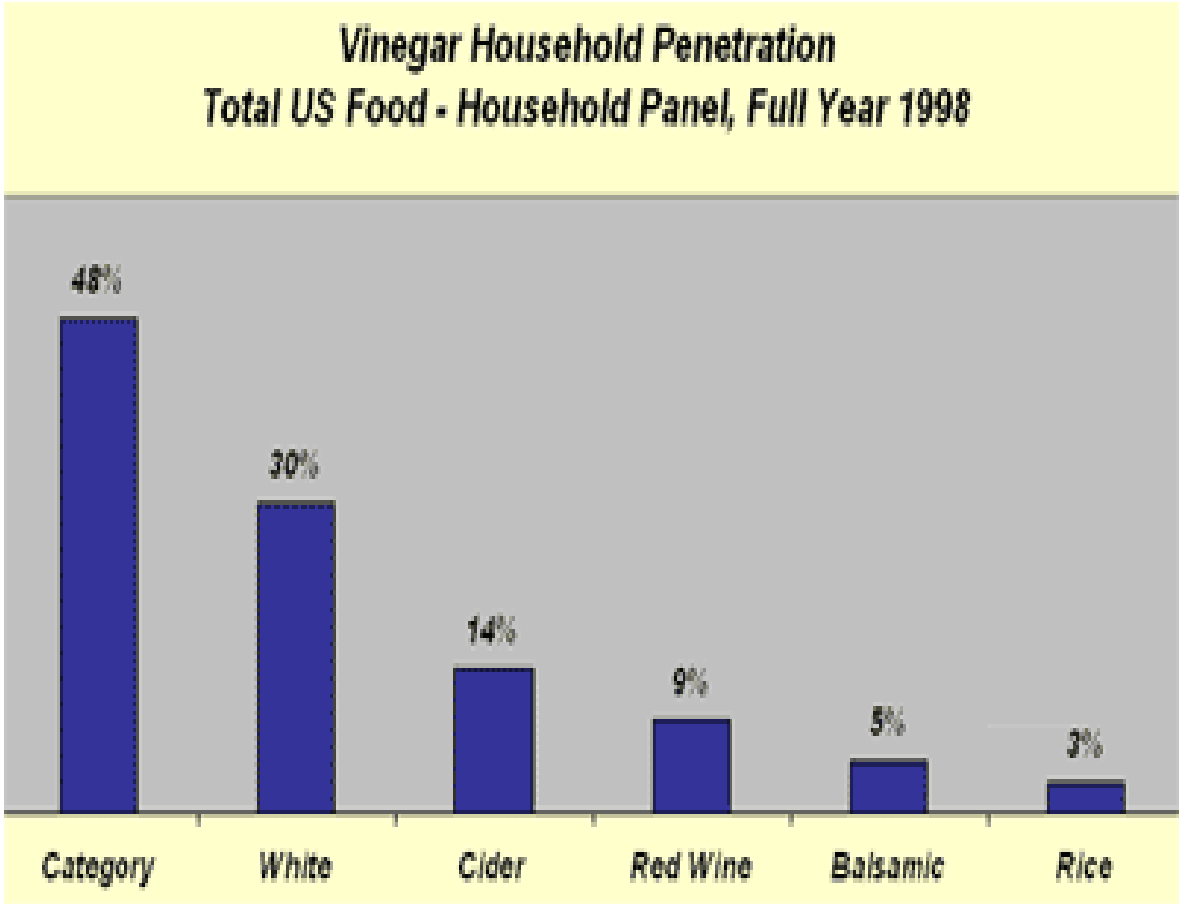


Figure 3: Vinegar Household Penetration in 1998

In 2003, AC Nielsen noted that white distilled remains the strongest in sales, although white and ciders are giving way slowly to increases in red wine, rice and balsamic vinegar (Crisco 2005).

An increased percentage of vinegar sales are moving through clubs and mass merchandisers. From 2000 to 2002, the percentage of sales in outlets other than supermarkets increased from 23% to 29% (Tables 2 and 3) from Crisco Company 2005.

Table 2: AC Nielsen Data Presented at 2003 Annual Meeting – Retail Outlets

Outlet	**% Buyers making at least one purchase in the retail outlet)**
Large Grocery Stores	71.0
Mass Merchandiser	10.0
Warehouse Clubs	9
Other Outlets	10.0

Table 3: Progressive Grocers, July 1999, "1999 Sales Manual/Multi Channel"

Outlet	**Dollar Sales (millions)**	**% Total Dollar Share**	**% Change from 1997**
Supermarkets	$215.61	95.4	-2.2
Mass Merchandisers	$9.27	4.1	19.1
Drug Stores	$1.13	0.5	18.7
Outlet Total	*$226.01*	*100*	*11.87*

2.1.3 Types of Vinegar

The predominant type of vinegar in the United States is white or distilled vinegar. Vinegar is usually described in terms of grain strength, the grain being ten times the acid percentage. For example 10% acid is referred to as 100 grain (Cruess 1958).

According to the Crisco Company, vinegar varieties vary greatly from country to country. Some of the most popular vinegars and their characteristics are shown below (Crisco Company 2005):

- **Balsamic vinegar** is brown in color with a sweet-sour flavor. It is made from the white Trebbiano grape and aged in barrels of various woods. Some gourmet Balsamic vinegars are over 100 years old.
- **Cane vinegar** is made from fermented sugarcane and has a very mild, rich-sweet flavor. It is most commonly used in Philippine cooking.
- **Champagne vinegar** has no bubbles. It's made from a still, dry white wine made from Chardonnay or Pinot Noir grapes (both of which are used to make Champagne).
- **Cider vinegar** is made from apples and is the most popular vinegar used for cooking in the United States.
- **Coconut vinegar** is low in acidity, with a musty flavor and a unique aftertaste. It is used in many Thai dishes.
- **Distilled vinegar** is harsh vinegar made from grains and is usually colorless. It is best used only for pickling.

- **Malt vinegar** is very popular in England. It's made from fermented barley and grain mash, and flavored with woods such as beech or birch. It has a hearty flavor and is often served with fish and chips.
- **Rice wine vinegar** has been made by the Chinese for over 5,000 years. There are three kinds of rice wine vinegar: red (used as a dip for foods and as a condiment in soups), white (used mostly in sweet and sour dishes), and black (common in stir-fries and dressings).
- **Sherry vinegar** is aged under the full heat of the sun in wooden barrels and has a nutty-sweet taste.
- **Wine vinegar** can be made from white, red, or rose wine. These vinegars make the best salad dressings.

2.2 The Formation of Vinegar

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 (Joyeux *et al* 1984; Drysdale *et al* 1984).

2.2.1 Vinegar Bacteria

The ninth edition of Bergey's Manual of Systematic Bacteriology classifies the acetic acid bacteria in the family *Acetobacteriaceae* and *Gluconobacter* (Figure 4) (Buchanan and Gibbons 1974). Acetic acid bacteria are Gram-negative, ellipsoidal to rod-shaped cells that have a required aerobic metabolism with oxygen as the terminal electron acceptor (Gonzalez *et al* 2004). The identification of the acetic acid bacterial species has traditionally been performed by studying physiological and

chemotaxonomic properties (De Ley *et al* 1984). Taxinomic studies based on partial sequence comparisons of 16S rRNA have shown that *Gluconoacetobacter* can be considered as a new genus which is present along with other species during wine fermentations (Yamada *et al.* 1997). Bacterial 16S rRNA sequences are attractive targets for developing identification methods because they represent conserved regions in all bacteria.

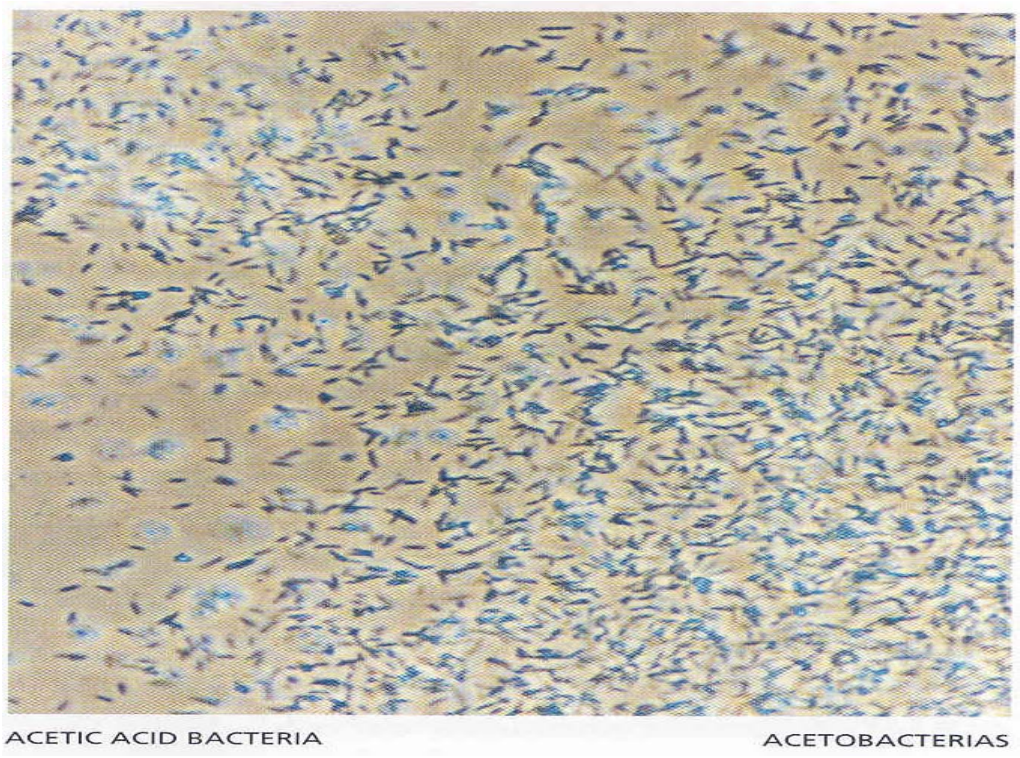


Figure 4: Acetic Acid Bacteria, Picture Provided by Frings Company

The restriction fragment length polymorphisms (RFLPs) of the genes coding for rRNAs show inter-species and intra-species differences in bacteria (Grimont 1986). The PCR-RFLP method is used for the rapid identification of acetic acid bacteria at the genus level and the identification of *Acetobacter*, *Gluconobacter* and *Gluconoacetobacter* species (Poblet *et al* 2000). PCR has been shown to be a suitably

accurate technique for identifying bacterial strains and for determining taxonomic relationships between bacterial species.

2.2.2 Chemical Reaction and Formulation

In 1822, Dobereiner established the theory of producing acetic acid from alcohol (Kehrer 1921) and the equation of the process is shown below (Figure 5) from Kehrer 1921:

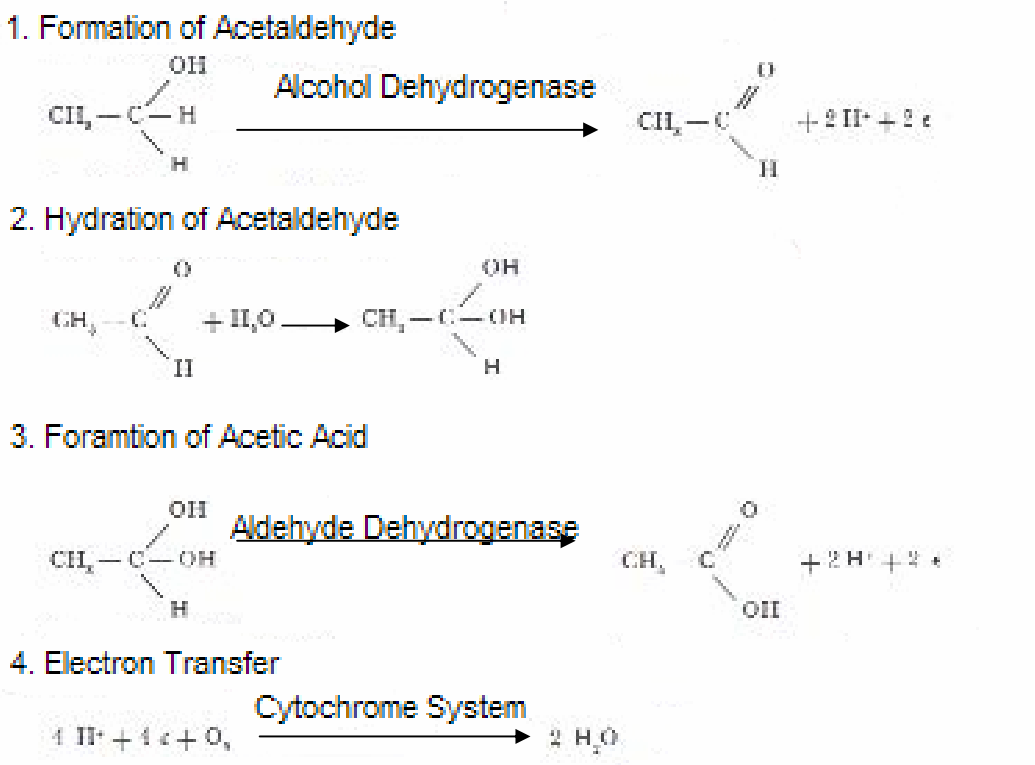


Figure 5: Conversion of Alcohol to Acetic Acid Reaction

Initially, alcohol is dehydrogenated to form acetaldehyde and two hydrogen ions and two electrons are released. In the second step, two hydrogen ions bind with oxygen to form water that hydrates acetaldehyde to form aldehyde. During step three, aldehyde dehydrogenase converts acetaldehyde to acetic acid and releases 2 hydrogen ions and 2 electrons.

2.3 Production Method

Vinegar production methods can range from traditional methods employing wood casks (Orleans Process) and surface culture (Generator Process) to submerged fermentation (Morales *et al* 2001). Vinegar is an important ingredient in many food products. The need for large amounts of the vinegar demands industrial fermentation systems that are capable of producing volumes that are reliably controlled (De Ory *et al* 1999). Many technical devices have been developed to improve the industrial production of vinegar. Generally, these improvements increase the speed of the transformation of ethanol into acetic acid in the presence of acetic acid bacteria (Tesfaye *et al* 2002).

2.3.1 Orleans Process

The slow method of acetifying wine which has been used in France since 1670 is called the French or Orleans process. In this process, alcohol solutions less than 5% in wine can not be acetified easily. Below this strength, phosphates and nitrogenous substances must be added to the mash and the products have to be sold under the name of "spirit vinegar". The Orleans process was the only method to make pure wine vinegar (Mitchell 1916), and was reported to be the best process to produce fine quality table vinegar (Hickey and Vaughn 1954). In this process, wood barrels (Figure 6) from (Cruess 1958) are used and filled with alcohol fermenting liquid to approximately ¾ full.

First, holes are drilled at the ends of the barrel a few inches above of the liquid surface. The holes are left open and covered with a fine screen.

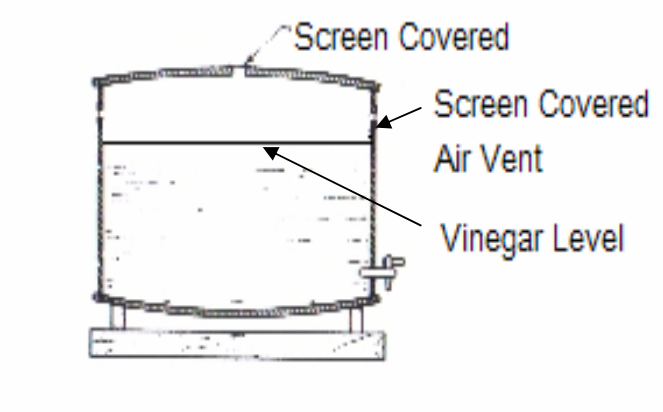


Figure 6: Orleans Process Barrel

Secondly approximately 20-25% of fresh vinegar is added into the barrel (Muspratt 1871). The function of adding the fresh vinegar is acidifying the liquid to the point of optimum growth for the vinegar bacteria (Cruess 1958). Vinegar bacteria settle into the liquid from the air and form a gelatinous slime layer on top of the liquid (Peppler and Beaman 1967). The liquid is fermented for about 1 to 3 months at 70ºF to 85ºF (Hickey and Vaughn 1954). After this time, 1/4 to 1/3 of the vinegar may then be drawn off for bottling purposes and an equivalent amount of alcoholic liquid added (Cruess 1958). Alcohol sources must constantly be added to the vinegar or the acetic acid might begin to oxidize (Cruess 1958).

2.3.2 Generator Fermentation

Early in the nineteenth century, a vinegar-making system called the trickle method [now called generator fermentation or quick process (Schnellessig)] was developed by German chemist Schutzenbach in 1832 (Hickey and Vaughn 1954). According to this process, the bacteria were grown and formed a thick slime coating around a non-compacting material like beech wood shavings, charcoal or coke (Peppler and Beaman 1967). The non-compacting material was packed into large

upright wood tanks (Figure 7) from (Cruess 1958) of 2000 cubic feet capacity above a perforated wood grating floor.

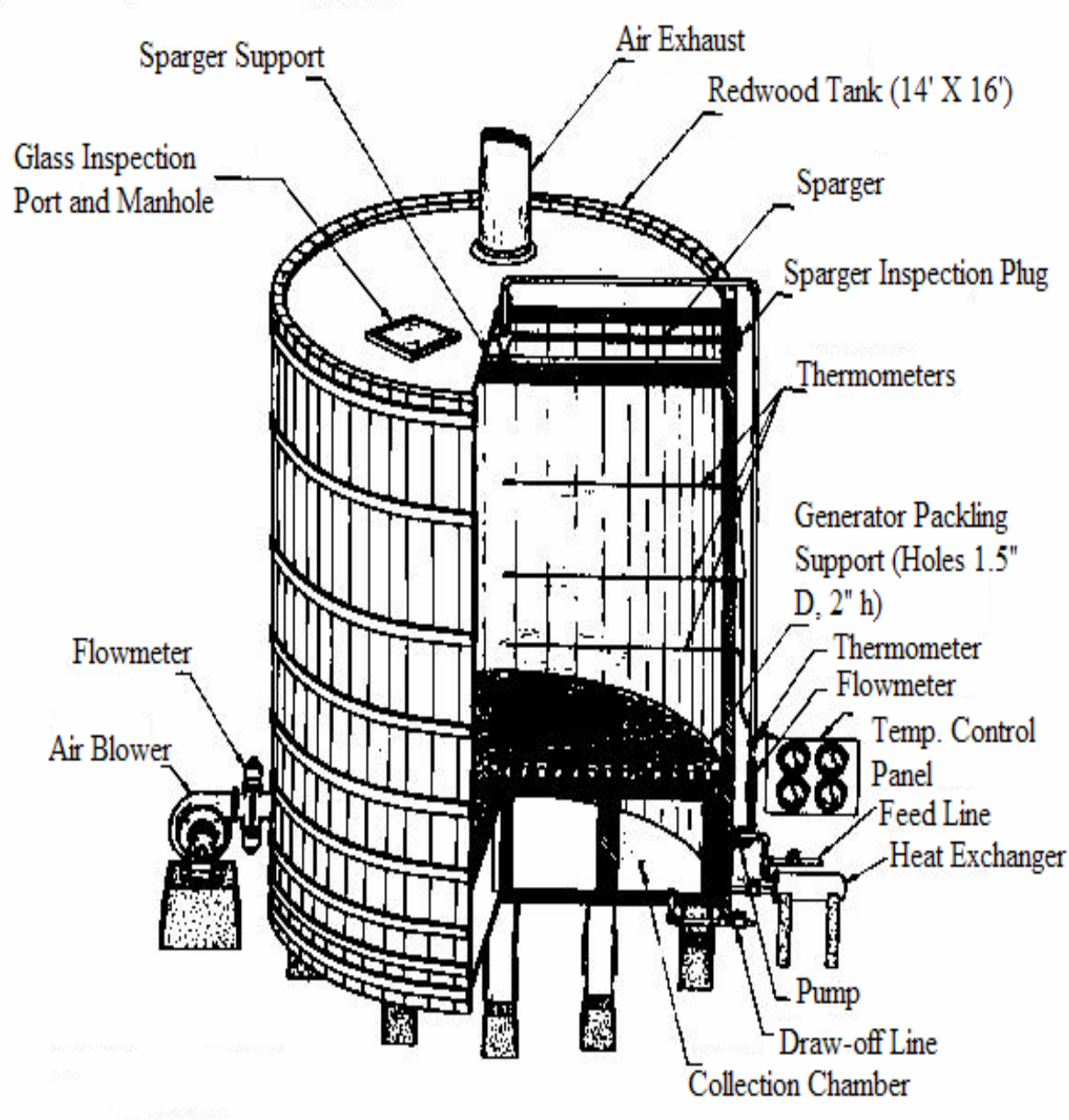


Figure 7: Vinegar Generator

The wood shavings (Figure 8) from (Peppler and Beaman 1967) are generally made of air-dried beech wood sliced to form a coil about 2 inches long and 1¼ inches in diameter.

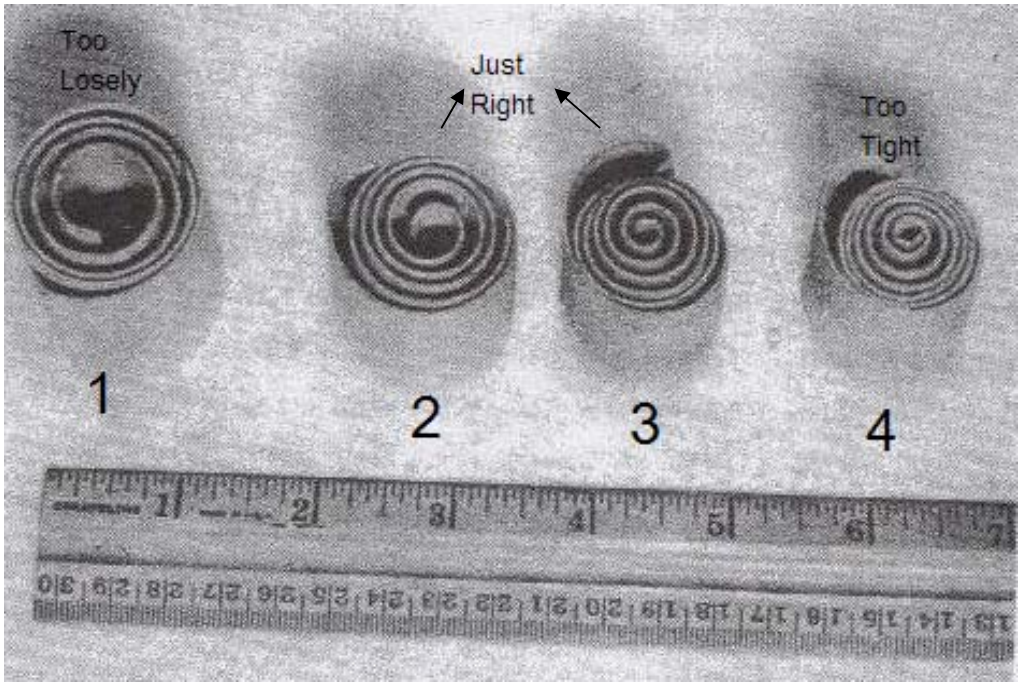


Figure 8: Beech Wood Shavings

Re-circulated fermenting liquid or mash trickles over the packing material toward the bottom while air moves from the bottom through inlets toward the top. The rate of acetification is dependent upon oxygen concentration (Cruess 1958). A limited air supply means limited acetic acid production and lower generator temperatures while an overabundant air supply creates over production and higher generator temperatures. The generators must be closely monitored to present over oxidation or unacceptable temperatures (Hassack 1922). The process takes about 3 to 7 days. Two thirds of the final vinegar product is withdrawn from the tank and fresh mash added (Cruess 1958). Replacement mash is slowly poured into the tank until the working level for acetification of the solution and a beginning temperature of 70°F (21.1°C) are reached. The optimum temperature for generator operation is 85 to 90°F (30 to 32.2°C) (Hickey and Vaughn 1954). Each gallon of 190 proof alcohol oxidized to acetic acid

releases about 30000 to 35000 Btu (32000000 to 37000000 Joules) (Hickey and Vaughn 1954). The optimum temperature for *Acetobacter* is about 86°F (30°C). A temperature control system is necessary to prevent overheating and consequent inactivation of the bacteria (Peppler and Beaman 1967).

2.3.3 Submerged Fermentation

Today, the most common production method is submerged culture (Figure 9) from (De Ory *et al* 1999) which improves the general fermentation conditions like aeration, stirring, heating, etc (Hromatka and Ebner 1951). As generator culture systems are slow and expensive, submerged culture fermentors have become widely used at industrial scales (Hromatka and Ebner 1951; Ormaechea 1991). In this process, the mash is stirred and aerated frequently (De Ley and Swings 1984). The fermenters are usually fitted with a heat exchanger for the maintenance of the optimum temperature during the fermentation process (De Ory *et al* 1999).

The typical operation mode in industrial submerged cultures (Adams 1985) is semi-continuous (Figure 10) from (De Ory *et al* 2002). This operation consists of the development of successive discontinuous cycles of acetification. At the

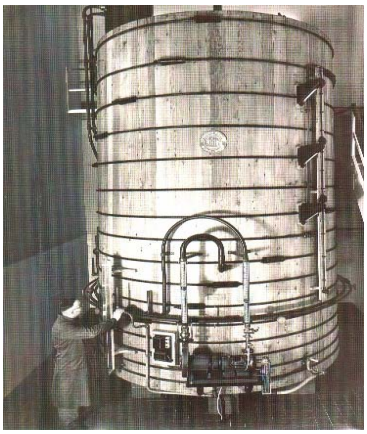


Figure 9: Submerged Process

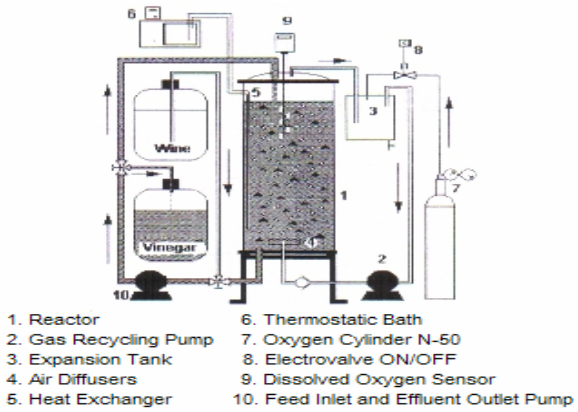


Figure 10: Semi-Continuous Process

end of every cycle, a given volume of acetic acid is discharged and refilled with mash (De Ory *et al* 2004). The best temperature for industrial production of 11 to 12% vinegar was 86°F (30°C) (Allgeier *et al* 1960; Adams 1985). Damage to the bacteria may occur above 86°F. In addition, the bacteria's condition also affects the concentration of acetic acid produced (Fregapane *et al* 2001).

2.4 Vinegar Qualities Characteristics

The vinegar qualities depend on process conditions including acetification speed. The rate of fermentation influences the sensory properties of the final vinegar, but some believe there are no differences between vinegars obtained at different fermentation speeds. Experts usually detect important sensory differences between vinegar manufactured by the submerged and generator processes (Nieto *et al* 1993).

2.4.1 Vinegar Aroma

The characterization of vinegar includes a wide range of values obtained from physicochemical and sensory parameters (Carnacini and Gerbi 1992). Various researches characterized the quality of vinegars using different analytical parameters as well as sensory analysis. Principal component analysis (PCA), cluster analysis (CA) and linear discriminant analysis (LPA) were applied to conventional wine vinegars obtained by submerged acetification process and wood cask aging wine vinegar (Guerrero *et al* 1994). Analysis using gas chromatography (GC) and high performance liquid chromatography (HPLC) of these two different processes of wine vinegars produced different linear functions involving the following variables: methanol, 1-propanol, ethyl propionate, 3-methyl-1-butanol, 2-methyl-1-butanol,

acetoin, praline, and total acidity-oxidation index quotient. Table 4 (Gerbi *et al* 1997) lists five organic acids found in wine vinegar: citric acid, tartaric acid, malic acid, lactic acid and acetic acid. There were fourteen volatile compounds found in white, wine vinegar with aging and without aging condition (Morales *et al* 2001). Acetic acid and ethyl acetate are the major compounds in wine vinegar and white distilled vinegar.

Table 4: Acid and Volatile Compounds in Vinegars

Mean values ($\bar{x}$) and standard error (σ_{n-1}) of studied parameters for the vinegar categories

	Vinegar categories									
	Subemerged Wine Vinegar		Submerged Wine Vinegar with Aging		Submerged White distil Vinegar		Wine Vinegar with Aging from Market (Wood Cask)		Wine Vinegar with Aging (Wood Cask)	
	$\bar{x}$	σ_{n-1}	$\bar{x}$	σ_{n-1}	$\bar{x}$	σ_{n-1}	$\bar{x}$	σ_{n-1}	$\bar{x}$	σ_{n-1}
Acetaldehyde[a]	61.3	45	34.1	20.2	37	19	23.5	21.6	19	24.5
Acetoin[a]	441	205	406	196	614	218	455	197	480	136
Methanol[a]	26.5	2	29.8	6.7	18.6	2.8	17	13.8	11.2	5.9
Ethanol[b]	10.5	7.9	19.7	20.1	5.1	8.9	4.5	3.3	1.2	0.9
2-Methyl-1-butanol[a]	5.4	2.3	7.3	2.6	7.2	3.2	11.6	9.6	1.9	2.1
3-Methyl-1-butanol[a]	30.9	14	37.7	21.9	47.3	12	11.6	9.6	4.2	3.6
meso-2,3-butanediol[a]	148	63	134	53.3	120	37	171	56	294	68.5
2-phenylethanol[a]	20.6	4.6	20.5	5	21.1	5.3	13.7	5.8	13.4	6.2
2-Methyl-1-propanol[a]	4.9	3.5	6.3	4	10.7	4.1	3.43	3.8	0	0
Ethyl acetate[a]	780	635	1634	1274	121	229	550	582	107	213
Methyl acetate[a]	3.7	1.4	7.9	4.3	0	0	7.7	7	6.5	6.2
Propyl acetate[a]	0	0	4.3	6.2	0	0	25.9	15.4	61.8	40.7
Ethyl formiat[a]	0	0	24.3	48.7	0	0	4083	5035	1138	1969
γ-Butyrolactone[a]	22.7	5.9	27.4	9.3	26	18	32.04	11.1	33.3	7
Dry extract[c]	1.5	0.5	1.8	0.6	1.35	0.1	1.71	0.45	1.9	0.5
Glycerol[b]	2.7	1.1	3.2	1.1	5.5	0.8	2.3	0.8	3.7	0.6
Proline[a]	296	155	324	152	381	144	371	80	477	111
Citric acid[b]	0.04	0	0.04	0.1	0.31	0.2	0.08	0	0.09	0.1
Tartaric acid[b]	2.3	0.6	2.4	0.8	2.6	0.9	1.9	0.7	2.5	0.9
Malic acid[b]	0.22	0.2	0.28	0.2	0.74	0.5	0.18	0.1	0.14	0.1
Lactic acid[b]	0.01	0	0.05	0	0.09	0.1	0.36	0.26	0.25	0.2
Acetic acid[b]	75.7	3.9	81.9	9.5	71.8	13	74.6	8.3	94.4	7.9

[a] mg/l [b] g/l [c] %p/p

So, vinegar not only contains acetic acid, it also contains at least four other organic acids. The flavor and aroma are dependent on the method of process, aging time and raw material used in the mash.

CHAPTER 3 MATERIAL AND METHODS

3.1 Vinegar Fermentation

Vinegar can be produced by different methods and from various raw materials like wine, rice wine and any kind alcoholic solution (Morales *et a*l 2001). There are several major production techniques for making vinegar such as the Orleans process, the generator process and the submerged process. The Orleans process consists of wood barrels filled with alcohol liquid fermented for about 1 to 3 months at 70ºF to 85ºF (21°C to 29°C) (Hickey and Vaughn 1954). After fermentation, 1/4 to 1/3 of the vinegar is drawn off for bottling purposes and an equivalent amount of alcoholic liquid or mash is added (Cruess 1958). The generator process was introduced by Schutzenbach in 1823 (Hickey and Vaughn 1954). Non compacting material is filled above a perforated wood grating floor in large upright wood tanks. Re-circulated fermenting liquid trickles over the packing material toward the bottom while air moves from the bottom inlets toward the top. The recirculation process takes about 3 to 7 days after which 2/3 of the final vinegar product is withdrawn from the tank and new alcohol solution is added (Cruess 1958). In 1955, Hromatka reported on a new method of making vinegar using submerged acetification (Cruess 1958). In this process, air is forcefully supplied to alcohol liquid in a tank and the material is fermented at 86°F (30°C). At the end of every cycle, 1/3 of the liquid is discharged as final product and the submerged fermentor is refilled with 1/3 mash or fresh alcohol solution. Then, a new fermentation cycle begins (De Ory *et al* 2004).

3.1.1 Generator Process

Vinegar fermentation was carried out by a lab scale generator pilot unit (Figure 11) which was constructed for this investigation.

Figure 11: Generator Pilot Unit

Basically, the generator fermented the vinegar from diluted alcohol using beech wood chips (Figure 12) packed loosely in a column. The column was arranged so that air could enter at the bottom and circulate up through the spaces between the beech wood chips.

The generator pilot unit used beech wood chips obtained from McIlhenny, Co. The chips (Figure 13) were approximately 1" X ½" X 0.125" (2.54cm X 1.27cm X 0.3175cm). Prior to use, the chips were heated with 5% vinegar solution in a kettle at 212°F (100°C) (Figure 14) to permeate the chips and prepare them to receive the

bacteria culture. After cooking, the wood chips were placed evenly on paper to dry (Figure 15). The heating process removed wild yeast and other bacteria from the surface of the chips.

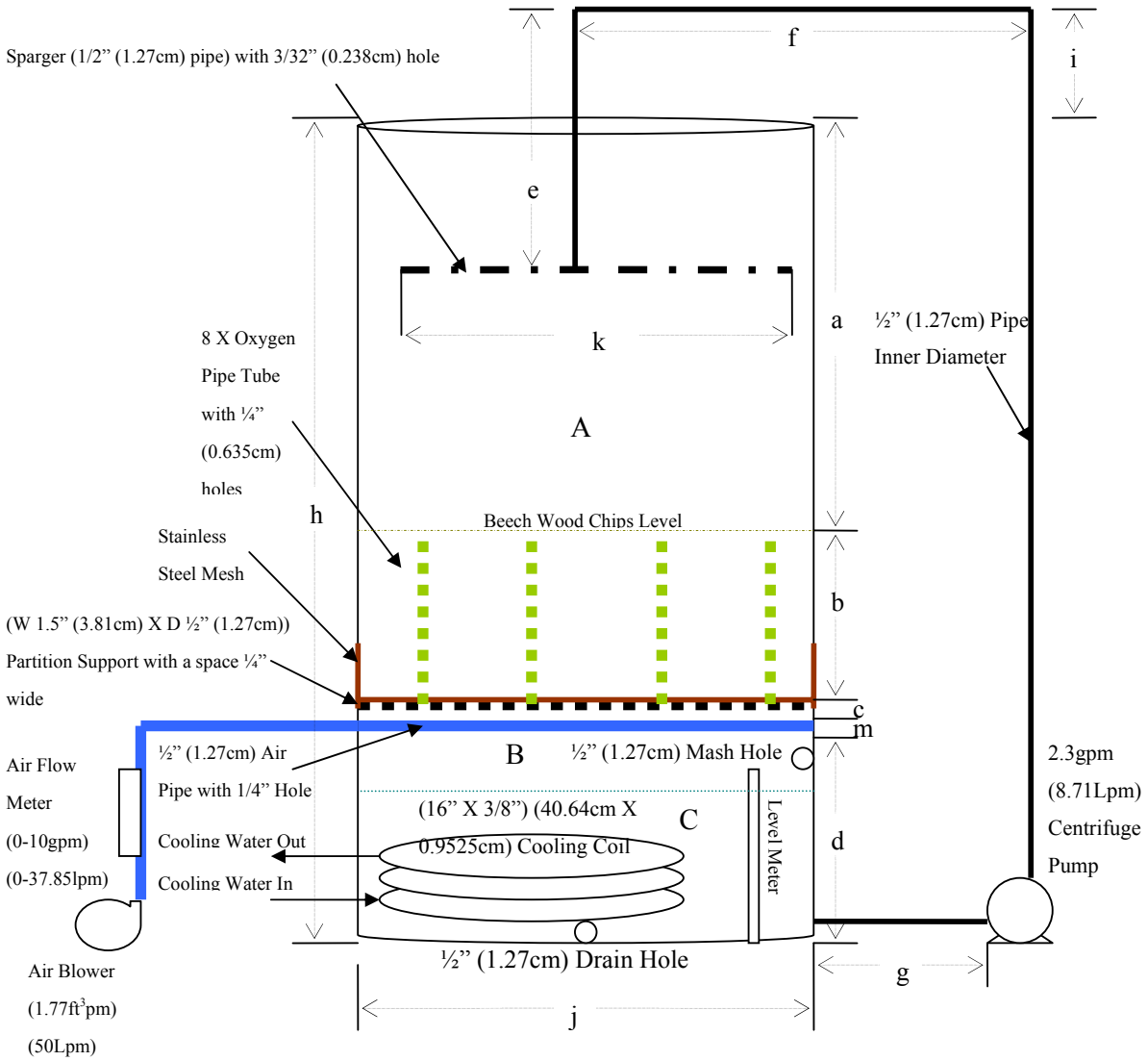
Figure 12: Beech Wood Chips

Figure 13: Beech Wood Chip Dimensions

Figure 14: Cooked Chips

Figure 15: Drying Chips

The generator pilot unit was made using a 50 gallon (227.31L) plastic barrel obtained from McIlhenny, Co. It was divided into three sections which were: beech wood area (A), clearance area (B) and ferment area (C). A schematic of the lab generator is shown in Figure 16.



Basic Areas of Generator:

A - Beech wood Chips area

B – Clearance & Partition area

C - Ferment area

Dimensions:

1. a – 15" (38.1cm)
2. b – 9" (22.86cm)
3. c – 0.5" (1.27cm)
4. d – 9.5" (24.13cm)
6. g – 10.5" (26.67cm)
7. h – 35" (88.69cm)
8. i – 8" (20.32cm)
9. j – 22" (55.88cm)
10. k – 15.5" (39.37cm)
11. m – 1" (2.54cm)

Figure 16: Generator Pilot Unit Drawing

The fermenting liquid was circulated using a centrifugal pump (Cole Palmer, IL) to the top of generator pilot unit. At the top of the unit was a 15.5" (39.37cm) long sparger tube constructed of 1/2" (1.27cm) PVC pipe with twenty 3/32" (0.238cm) holes (Figure 17) which evenly sprayed (Figure 18) fermenting mash down on to the top of beech wood chips.

Figure 17: Sparger

Figure 18: Liquid Sprays on Top of Chips

The pilot unit was filled to a 9" (22.86cm) depth (approximately, 5.78cu.ft (163671cu.cm)) of beech wood shavings in section A. The beech wood chips were held within a stainless steel mesh (Samuel Specialty Metals, LA) basket (Figure 21) supported with ½" (1.27cm) diameter CPVC pipes (Lowe's, LA) reinforced by insertion of 3/8" (0.9525cm) diameter stainless steel pipe (Southwest Stainless Inc, LA) (Figure 21). The wood partition (Figure 20) was placed above the CPVC pipe arrangement so that the stainless steel basket would be evenly supported. The partition was build of pine 1.5" (3.81cm) X ¾" (1.91cm) (Lowe's, LA). The partition (Figure 19) can handle up to 70lb (31.7513kg) of beech wood chips and the chips can be easily removed.

Figure 19: CPVC Pipes Support & Stainless Steel Mesh on Partition

Figure 20: Wood Partition

Figure 21: Stainless Steel Mesh

Section B represents about 1" (2.54cm) (approximately 1.56cu.ft (44174cu.cm)) clearance area below the chip basket to allow about 0.1059cu.ft (3L) per minute air flow through a half inch (1.27cm) pipe with twenty 3/32" (0.238cm) holes under the beech wood chips (Figure 22).

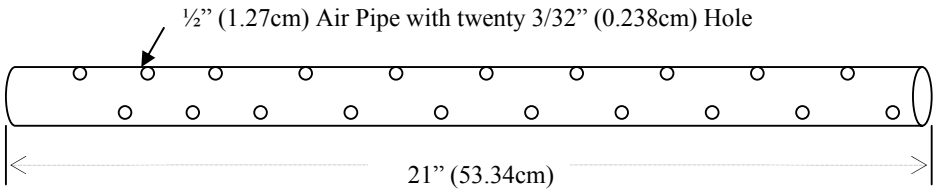


Figure 22: Air Pipe Drawing

At the initial run, section C was filled with 12.5 gallons (47.32L) of unfiltered vinegar supplied from National Vinegar Company (Houston, TX) containing a vinegar culture. This vinegar culture was used to inoculate the beech wood chips with bacteria and was re-circulated for 7 to 11 days.

Section C also contained a cooling coil (Figure 23 and 25) which used city water to remove the heat produced from fermentation. The city water (Figure 24)

temperature remained around 70°F (21.1°C) to 80°F (26.7°C) keeping the generator at 70°F (21.1°C) to 90°F (32.2°C). The most suitable temperature for industrial production of vinegar content of 11 to 12 percent acetic acid is 86°F (30°C). This is the temperature currently used in the industry (Allgeier *et al* 1960; Adams 1985). The cooling loops were made from 3/8" (0.9525cm) stainless pipe (Samuel Specialty Metals, LA). Each gallon of pure alcohol oxidized to acetic acid releases about 30,000 to 35,000 Btu (32000000 to 37000000 Joules) (Peppler and Beaman 1967). Based on this information, the length of cooling coil was calculated to be 11.22" (28.50cm). Assuming an overall heat transfer (U) value of 20 Btu/hr.ft$^{2.\circ}$R (W/hr.cm$^{2.\circ}$K), the calculations are shown below (Table 5):

Table 5: Cooling Coil Calculation

Tube Diameter	0.375 (0.9525)	In (cm)
Heat,q	35000 (8122777)	Btu/gal (J/L)
	26437.89 (6135688)	(0.755368 gallon (3.43L) of alcohol was used)
Coversoin time	5	days
Rate of heat, Q	220.3158 (232446)	Btu/hr (J/hr)
Overall heat transfer, U	20 (113.6)	Btu/hr.ft$^{2.\circ}$R (W/hr.cm$^{2.\circ}$K)
Area of surface	1.101579 (1023.4)	ft^2 (cm^2)
Length of tube, L	0.935049 (28.51)	ft (cm)
	11.22059 (28.5)	In (cm)
C_p	1 (4.1868)	Btu/lbm.°R (kJ/kg.$^\circ$K)
ΔT	10 (-12.22)	°F (°C)
Mass per hr	22.03158 (9.9933)	lbm/hr (kg/hr)
Water	8.33 (0.83)	lbm/gal (kg/L)
	2.644847 (0.58)	gal/hr (L/hr)

Figure 23: Cooling System Figure 24: Water Hose Figure 25: Cooling Coil

The air was supplied by a 1/125hp (5.97W) blower (Figure 20) (Grainger, LA) which had a free air capacity of 60cu.ft (1699 L) per minute flow rate. Because of back pressure in this pilot unit, the blower produced only 0.07062 to 0.1059cu.ft (2 to 3 L) per minute.

Figure 26: Air Blower

A sample of 150ml fermenting liquid was taken from the drain hose at the bottom of the pilot unit every 24 hours. The 150ml sample was kept in a 500ml sample cup and held in a cooler at 39.2°F (4°C) until analysis for pH, TA% and alcohol. Beech wood chips and ferment liquid temperatures were recorded every 24 hrs.

Figure 27: Drain Hose for Sampling

Using industry guidelines (National Vinegar Company, Houston, TX), two generator mashes were used for the generator pilot unit. Generator mash 1 (GM1) was taken from a 16000 gallon (72737.6L) mash tank at National Vinegar Company and GM2 was prepared in a 5 gallon (22.731L) volume in the lab (Table 6).

Table 6: Generator Mash Preparation

GM 1 mash [16000 gallon (72738L)]	**GM 2 mash [5 gallon (22.731L)]**
13500 gallon (61372L) process water	4.22 gallon (19.18L) distill water
150 gallon (682L) 10% filter vinegar	0.047 gallon (0.214L) 10% filter vinegar
2144 gallon (9747L) 190 proof alcohol	0.67 gallon (3.05L) 190 proof alcohol
80 lb (36.3kg) *nutrient	0.064 lb (0.029kg) *nutrient

* (Nutrient Incorporated, WI)

The generator pilot unit flow chart is shown in Figure 28:

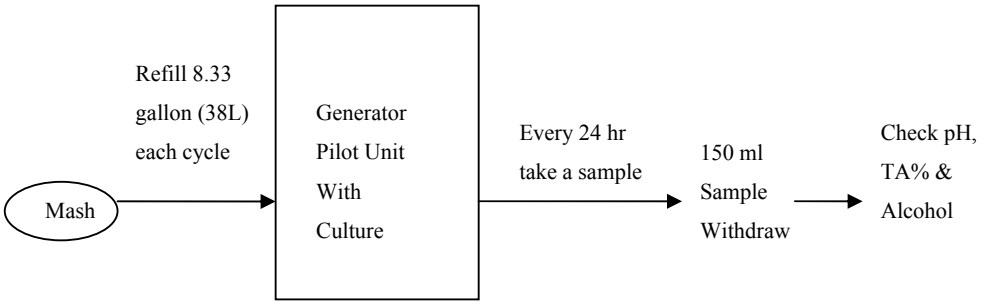


Figure 28: Generator Pilot Unit Flow Chart

At the start of the process, 12.5 gallon (56.8 L) of generator culture solution obtained from National Vinegar Company on June 20, 2005 was added to the generator pilot unit and re-circulated. After 7 days, the generator working level fell to 11 gallons (50 L) due to evaporation and absorption by the wood. Another 2.5 gallons (11.4 L) of the same generator culture solution was added into the generator pilot unit at this time. After an additional 5 days fermentation, 2/3 (8.33 gallon) of the generator solution was withdrawn, 8.33 gallons (38 L) of fresh GM 1 was added to the unit and fermented another 6 days. At that time, another 2/3 solution was removed and replaced with 8.33 gallons (38 L) of GM 2.The lab scale generator setup conditions are shown as below (Table 7):

Table 7: Generator Setup Condition

Air Flow	0.52 - 0.79 ft^3PM (2 - 3 LPM)
Cooling Temperature	70 - 80ºF (21.1 – 26.7°C)
Sparging Revolutions	27 rpm
Discharged Cycles	5-7 days
Working Volume	12.5 gallon (47.32L)
Discharge Volume	8.33 gallon (31.53L)
Removed and Replaced Time	30 min

3.1.2 Submerged Process 1

A small sample of mid range vinegar culture was taken from the National Vinegar Company (Houston, TX) which contained 8.4% of acid, 2.5% of alcohol and bacteria culture. This sample was brought back to LSU and about 100ml of this vinegar culture was placed into a 500ml flask (Figure 29) and covered with aluminum foil. Before the submerged process was begun, the culture was held in an incubator (Hotpack, IL) at 86°F (30°C) for 3 days (Figure 30 and 31). This was done to keep the high strain bacteria culture alive. If the culture sits at room temperature, the bacteria will die slowly.

Figure 29: Culture in Flask Figure 30: Culture in Incubator Figure 31: Incubator

After 3 days, the culture was taken from the incubator and used to initiate the submerged process. The process flow chart is shown below (Figure 32):

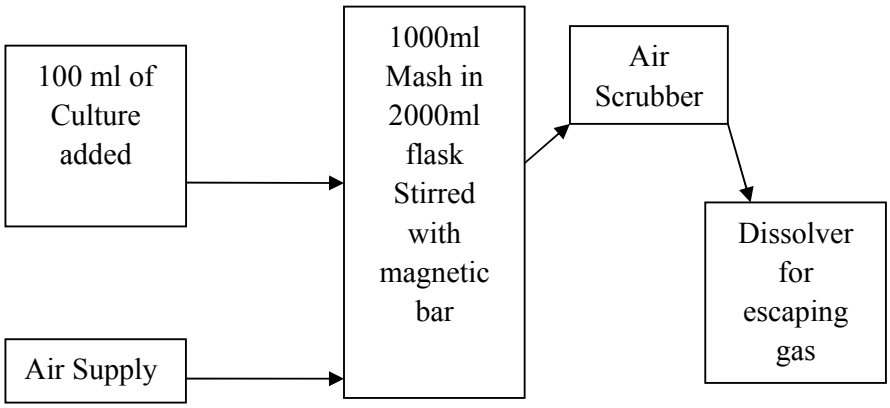


Figure 32: Submerged Process 1 Flow Chart

The starting solution was prepared as shown below in Table 8:

Table 8: Submerged Process 1 Starting Solution

Ingredients	Amount in Grams
Fring Nutrient*	0.72g
Distilled Water	1000g

*Dextrose, ammonium phosphate, citric acid, muriate of potash, soy protein, yeast and potassium phosphate (Nutrient Incorporated, WI)

Once the 1000ml mash mix was stirring well in a 2000ml fermenting flask, 100ml of vinegar culture was added into the flask. Figure 33 displays the setup. Compressed air was supplied through lab tubing. The dissolver was added to absorb the escaping alcohol and vinegar vapor. The dissolver was a 1000ml flask filled with 50ml of distilled water. Each day, the dissolver solution was poured back into the 2000 flask and additional 50ml distilled water was added to the dissolver. The scrubber was added between the fermenter and dissolver. This was done because the nutrient solution produced a lot of foam during aeration and the vacuum in the flask would draw the foam into the scrubber rather than contaminating the vacuum lines. Each day, 10ml of 190 proof alcohol was added to the fermenter. The bacteria would not tolerate a large amount of 190 proof alcohol placed into the flask at once. The additions would continue until the acid reached 12%.

Figure 33: Submerged Processes 1

Figure 34: Air Supply

Figure 35: Fermentor

Figure 36: Scrubber and Dissolver

The sample and mash was added through the pipe at the rubber stopper (Figure 37). A 10ml sample was taken out of the fermenting flask every 24 hrs and analyzed for TA% and pH. This was replaced with 10ml of liquid alcohol.

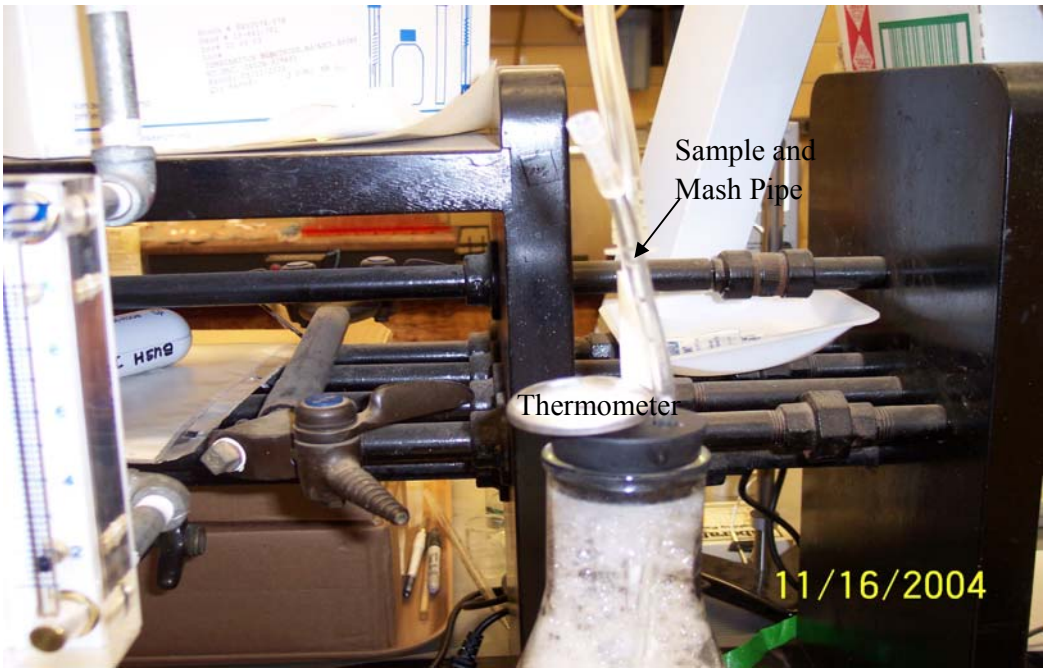


Figure 37: Thermometer and Sample Pipe

After adding alcohol for three weeks, the strength of acid did not increase as expected. The reason for failure could be contamination of the solution, dilution of ferment liquid by the 50ml dissolver solution per day or death of bacteria because of poor air supply.

After this experimental failure, a 9L lab scale fermenter was borrowed from Creole Fermentation Inc (Abbeville, LA) to run the Submerged Process 2 experiment.

3.1.3 Submerged Process 2

Vinegar fermentations were carried out by a semi-continuous process using a 9L lab scale fermenter shown in Figure 38. Basically, the semi-continuous process is the most common operation mode in the vinegar industry at the present time. This operation mode consists of successive discontinuous cycles of acetification, each one with conversion of ethanol into acetic acid. At the end of every cycle, a given volume of reactor is discharged (final product) and refilled with initial medium (fresh

alcoholic mash). Then, a new fermentation cycle begins (Ory et al 2004). Operating conditions can be found in Table 9.

Figure 38: 9L Creole Lab Scale Fermentor

Table 9: Lab Scale Fermentor Setup Condition

Air Flow	0.053 ft^3PM (1.5 LPM)
Cooling Temperature	86ºF (30°C)
Stirring Revolutions	3450 rpm (High)/ 2890 (Low)
Discharged Cycles	18-23 hr
Working Volume	1.87 gallon (8.5L)
Discharge Volume	0.593 gallon (2.7L)
Removed and Replaced Time	15 min

In this fermentation unit, the cooling system was built directly into the fermenter and consisted of a stainless steel coil. Each gallon of pure alcohol oxidized to acetic acid released about 30,000 to 35,000 Btu (32000000 to 37000000 Joules) (Peppler and Beaman 1967). Figures 39, 40 and 41 show the fermentor cooling coil and the temperature control.

Figure 39: Cooling Coil in the Fermentor

Figure 40: Cooling Coil Sit Above the Aerator

Figure 41: Cooling Temperature Control

The concentration of dissolved oxygen in the culture broth during fermentation has a significant effect on bacterial growth and on the production rate of acetic acid (Ghommidh *et al* 1982; Park *et al* 1989). The most important factors affecting dissolved oxygen are the oxygen transfer rate, the air flow rate and the oxygen partial pressure in the air supply to the bioreactor (Hipolito 2004). High aeration flow is undesirable for successful acetic acid production rate (Ghommidh 1982; De Ory *et al* 1999; Fregapane *et al* 1999). To reduce the loss of volatile components, a fermenter has been developed, equipped with a closed gas recirculation system (De Ory *et al* 1999). The air hole and aerator spinner are shown in Figures 42 and 43.

Figure 42: Air Hole

Figure 43: Aerator Sits on the Air Hole and Spins at 3450rpm

This fermenter can produce many tiny air bubbles in the liquid and provides plenty of dissolved oxygen to the culture broth. Figure 44 shows the air bubbles in the solution.

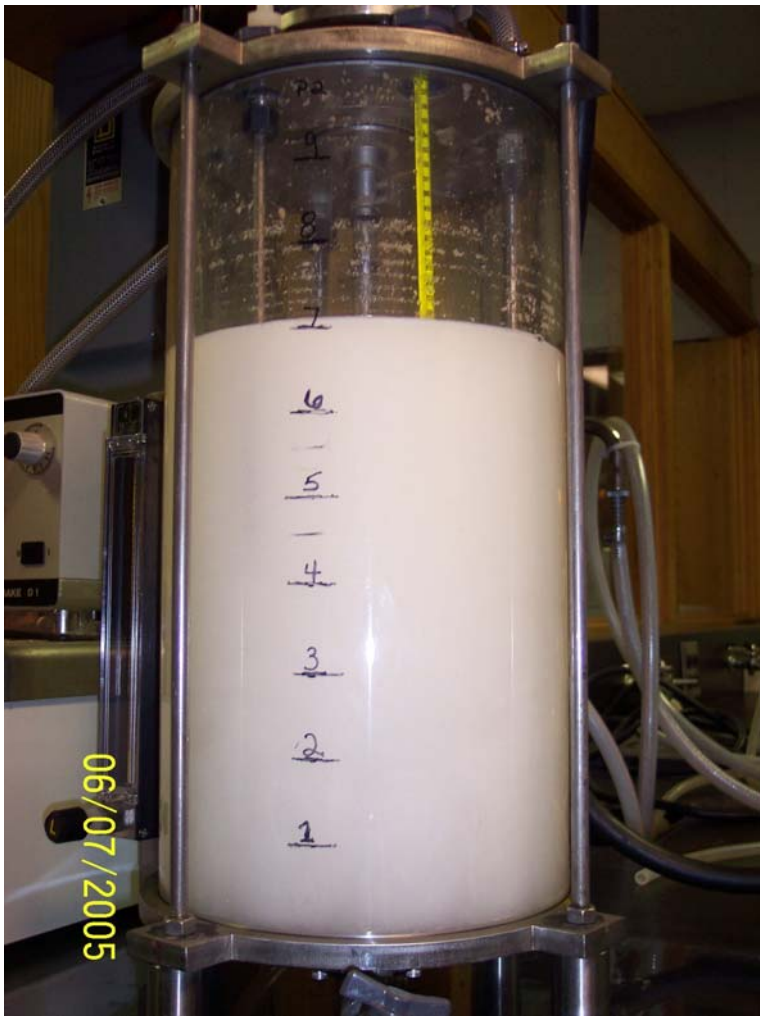


Figure 44: Tiny Air Bubbles Give the Solution a Milky Color

In this process, 1.87 gallons (8.5L) of mid range culture broth with 9.5% acidity and 3.35% alcohol was added into the 2.2 gallon (10L) fermenter. The mid range broth contained a large amount of vinegar culture. Fermentation temperature was controlled at 86°F (30°C). After 24, hours 1/3 of the 12.35% acidity liquid was discharged as final product and the fermentor was refilled with 1/3 of SM2 mash (Table 10) containing fresh alcohol solution. After addition of this mash the final concentration of alcohol in the fermenter overall was 4.6% and the acidity in the fermenter dropped to 8.25%. Then, a new fermentation cycle began.

The processes outline is shown below in Figure 45. Figure 46 contains a diagram of the fementor.

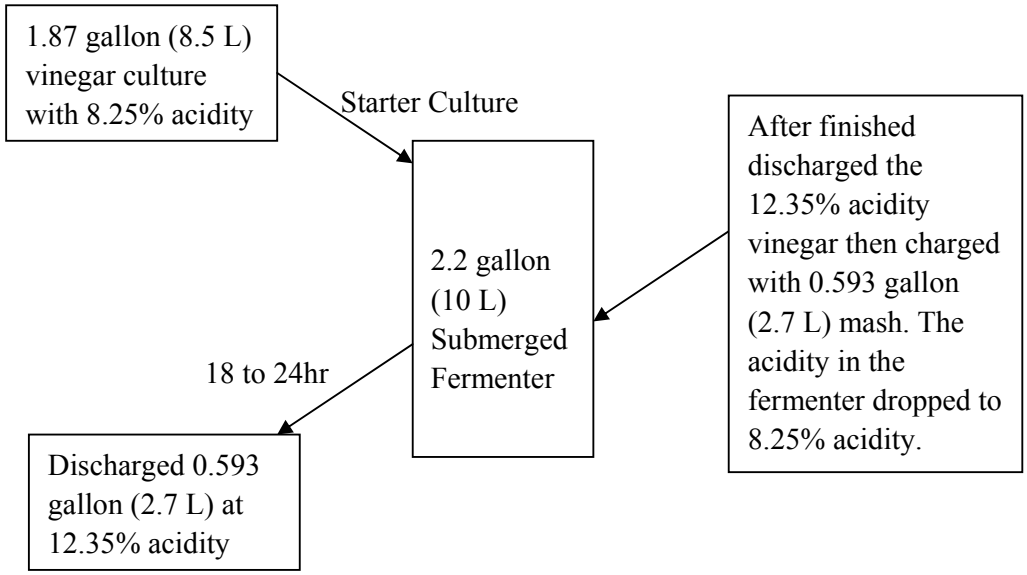


Figure 45: Submerged Acetification Process 2 Flow Chart

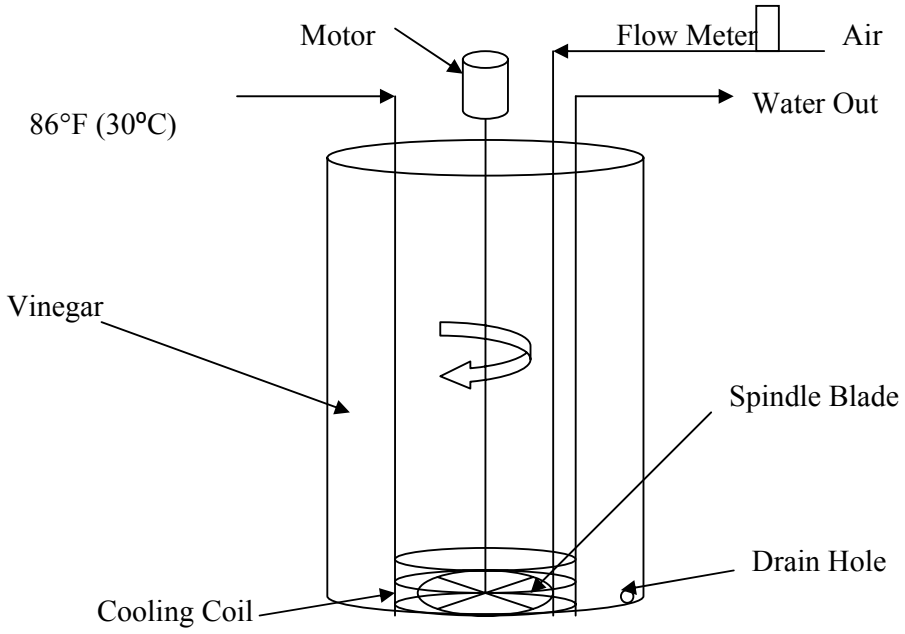


Figure 46: Diagram of Submerged Fermentor

The mash used in the fermentor was derived from standard industrial practice (Creole Fermentation Industries Incorporated, Abbeville, LA). Submerged mash 1 was taken from a 8400 gallon (38187 L) mash tank at Creole Fermentation Industries Incorporated and submerged mash 2 represents the amounts calculated for the 4.2 gallon (19.09 L) mash used in the pilot lab scale fermentor (Table 10).

Table 10: Submerged Mash Preparation

SM 1 mash [8400 gallon (38187L)]	**SM 2 mash [4.2 gallon (19.09 L)]**
7000 gallon (31822.7L) process water	3.5 gallon (15.9L) distill water
200 gallon (909.22L) 10% filter vinegar	0.1 gallon (0.45L) 10% filter vinegar
1200 gallon (5455.3L) 190 proof alcohol	0.6 gallon (2.73L) 190 proof alcohol
33.33 lb (15.12kg) [1]nutrient	0.064 lb (0.00771kg) [*]nutrient
66.67 lb (30.24kg) [2]dextrose	0.034 lb (0.0154kg) [^]dextrose

[1, 2] (Nutrient Incorporated, WI)

The discharged acid concentration from the commercial submerged acetification operation is typically 12.35% acid with an ending concentration of 0.5% alcohol in 24 hours. This 0.5% alcohol allows bacteria maintenance during the discharge or charge period. The vinegar culture continues to produce the vinegar and is not shocked when the new mash is added.

In this lab scale fermenter process, 150ml samples were taken from the drain hose at the bottom of the pilot unit. The 150ml sample was kept in a 500ml sample cup and put into a cooler at 39.2°F (4°C) until analysis for pH, TA% and alcohol. Ferment liquid temperature was recorded every 24 hrs.

3.1.4 Submerged Process 3

Additional experiments were performed using this fermentor running at the same conditions as submerged process 2. In submerged process 3 the vinegar was fermented in the presence of beech wood powder (Table 11). The beech wood powder was added at a level of 2% (0.0052g) in the first cycle of the lab scale fermentor. At the beginning of the second cycle, an additional 0.0052g was added for a total of 0.0104g. Similarly, at the beginning of the third and fourth cycles an additional 0.0052g was added yielding 0.0156g and 0.0208g total, respectively, to the fermentor.

Table 11: Submerged Setup Condition with Beech Wood Powder

Air Flow	0.053 ft^3PM (1.5 LPM)
Cooling Temperature	86ºF (30°C)
Stirring Revolutions	3450 rpm (High)/ 2890 (Low)
Discharged Cycles	18-23 hr
Working Volume	1.87 gallon (8.5L)
Discharge Volume	0.593 gallon (2.7L)
Removed and Replaced Time	15 min

At each sampling period, a 150ml sample was taken from the drain hose at the bottom of the pilot unit. The 150ml sample was kept in a 500ml sample cup and put into a cooler at 39.2°F (4°C) until analysis.

3.2 Physicochemical Analysis

The pH, titratable acidity and alcohol are very important parameters in the vinegar fermentation process. These parameters are used to predict the time of discharge and charge in the fermenter.

Gas chromatography (GC) can be used to detect flavor differences of vinegar samples. The GC was used to compare flavor profiles from the commercial generator process (National Vinegar Company) and submerged acitification (Creole Fermentation Inc.) as well as from the lab scale fermentors.

Polymerase chain reaction (PCR) and Gram stain are methods to identify the bacterial species in vinegar. The Gram stain can be used to narrow down identity of bacteria species to gram positive or negative. From the Gram stain, the bacteria then can be easily classified by using Polymerase Chain Reaction (PCR) to identify the bacterial strain.

3.2.1 pH and Titratable acidity

Titratable acidity and pH were measured using an Orion EA920 pH meter and Thermo Orion 915600 (Orion, MA) pH probe. Titratable acidity (TA) was determined as ml of 1N NaOH used to obtain a pink color endpoint with phenolphthalein (AOAC, 1990). Dry phenolphthalein (0.002 gram) was added into each 10ml sample vinegar solution. Acetic acid is the major organic acid in vinegar. The formula to calculate %TA as acetic is as below:

$$\%TA = \frac{(\text{ml of NaOH}) \times (\text{N of NaOH}) \times (60.05)}{10 \times \text{Sample Weight}}$$

3.2.2 Alcohol Measurement

According to the Frings Company (Germany) method for analysis of alcohol in vinegar fermentation, 100ml of vinegar and 50ml of distilled water are measured into volumetric flask before distillation (Figure 47) (Frings Company 2005).

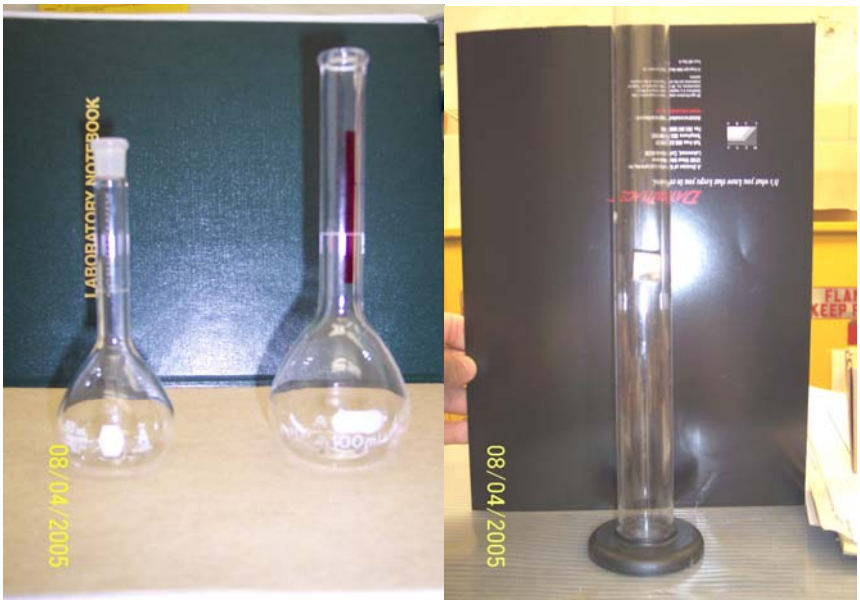


Figure 47: 50ml and 100ml Volumetric Flask and 200ml Cylinder

A little phenolphthalein powder (approximate 0.002 gram) is added into the vinegar and the solution is neutralized to pink color with 25% concentrated NaOH solution. Another 50ml of distilled water is then added and the entire mixture is placed into a round-bottom 1000 ml distillation flask. The sample is distilled at 212ºF (100ºC) for 25 minutes until 100 ml of liquid is condensed (Figure 48). An ice bath (Figure 50) is used to cool down the collected liquid to 68°F (4.5°C).

Figure 48: Distillation System

Figure 49: Alcohol Hydrometer

Figure 50: Ice Bath

The distillate is poured into a 200ml graduated cylinder and the Frings (Heinrich) 0% to 6% Alcohol Hydrometer (Figure 49) is used to measure the alcohol content on a volume/volume basis.

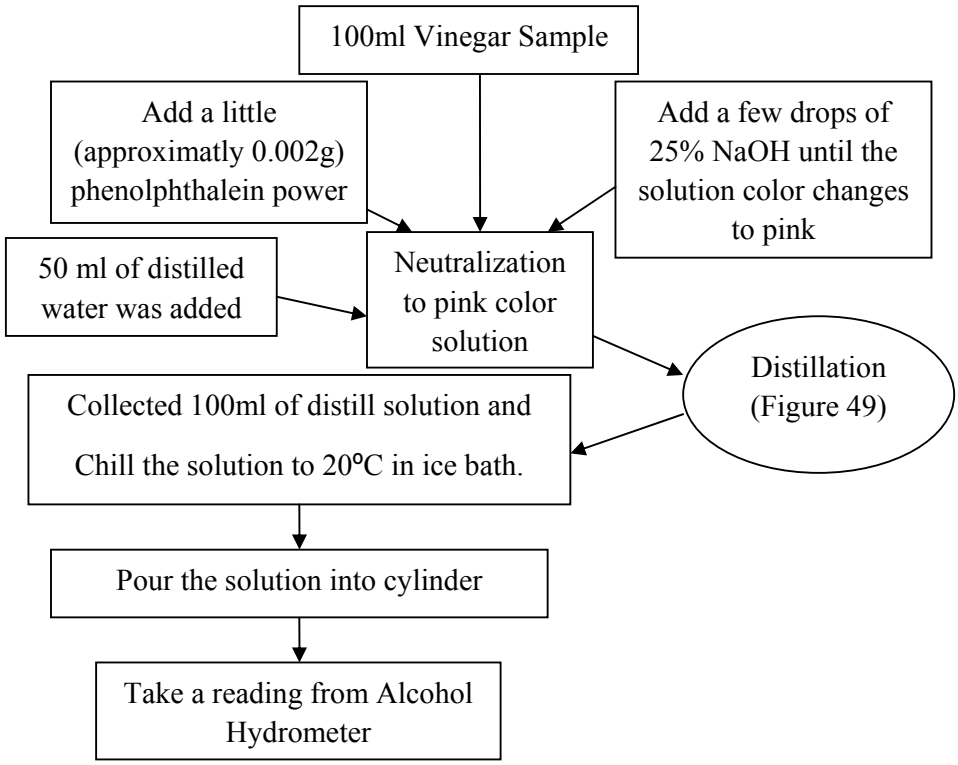


Figure 51: Alcohol Measurement Chart

3.2.3 Gas Chromatography

Eighteen vinegar samples were examined by GC analysis. Four samples were obtained from shipping tank trucks of National Vinegar Company (Houston, TX) and Creole Fermentation Inc (Abbeville, LA). Two samples were taken directly from the generator tank and submerged acetification tank at National Vinegar Company. Four samples were obtained from the lab fermenter used at Creole Fermentation Inc and four samples were produced with the submerged acetification lab fermenter using beech wood powder (2% (0.0052g), 4% (0.104g), 6% (0.156g) and 8% (0.208g)). All of the samples were kept in 500ml sample cup at 39.2°F (4°C) until analysis. Table 12 contains the sampling regime for the GC analysis.

Table 12: Gas Chromatography Samples Employed for the Study

Acetification Techniques	Sampling Sources	No. of Samples	Samples Codes
Submerged Process (Truck), Creole	Factory Delivery Truck	4	CSPT1-CSPT4
Generator Process (Truck), National	Factory Delivery Truck	4	NGPT1-NGPT4
Lab Fermenter (Creole)	Laboratory fermenter	4	LFC1-LFC4
Lab Fermenter (Creole), Beech Wood Poweder (2%, 4%, 6% & 8%)	Laboratory fermenter	4	LFCBWP1-LFCBWP4
Generator (Tank), National	Factory generator tank	1	GTN1
Submerged (Tank), National	Factory submerged tank	1	STN1

Volatile compounds were determined by a Varian CP-3800 gas chromatograph with FID (Varian, CA) (Figure 54) detector. A capillary column, SPB-1000 30m x 0.32mm x 0.25µm film thickness (Supelco Inc, PA), was used (Figure 52).

Figure 52: Capillary Column SPB-1000

Figure 53: Varian CP-3800 Oven

Chromatography conditions (Table 13) were taken from Morales *et al* (2001).

Table 13: Chromatography Condition Setup

Initial Temperatures	35°C
Initial Time	5 min
Program Rate	4°C/min
Final Temperature	150°C
Injector Temperature	220°C
Detector Temperature	250°C
Carries Gas Helium	1ml/min

Figure 54: GC Analysis Computer

Figure 55: Varian CP-3800 with FID Detector GC

Samples underwent direct injection into split mode (1:60) of 1µl; 1:10 dilutions of 4-methyl-2-pentanol were added as an internal standard. The sample was injected

using the sandwich method (Figure 56 and 57) in which the 1µl of sample is spaced between two 1µl samples of air. This assures the sample can be totally injected into the GC.

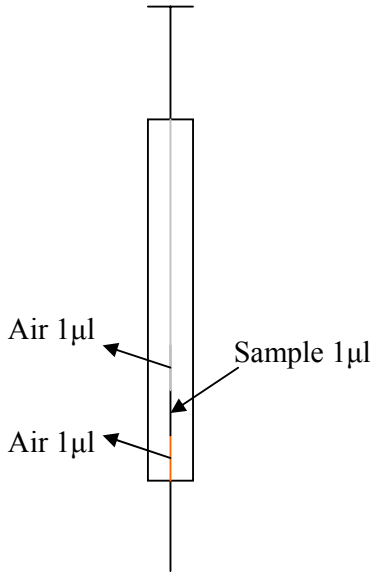


Figure 56: Injector Method

Figure 57: Injector

Another method of sample preparation for GC analysis was also performed. Headspace solid-phase microextraction (HS-SPME) has been applied to the analysis of aroma compounds in vinegar (Morales *et al* 2003). Four samples removed from National Vinegar Company generator process and Creole Fermentation Inc submerged process tank trucks were analyzed using GC-MS.

In the HS-SPME, a fiber is exposed in the head phase of a sample (Morales *et al*, 1999). Headspace solid-phase micro-extraction is used because the GC-MS detector can not tolerate the direct sample injection to the column. The extracted sample were injected onto a Varian GC-MS CP-3800 (Varian, CA). A capillary column, SPB-1000 30m x 0.32mm x 0.25µm film thickness (Supelco Inc, PA), was used (Figure 52).

Sample (5ml) was poured into a 50ml volumetric flask which had a wood cap with a small drilled hole. SPME silica fiber (Supelco Inc, PA) was inserted into the wood cap at the top of 50ml volumetric flack. The sampling assembly was (Figure 58) placed into 158°F (70°C) water bath for one hour. After an hour, the SPME fiber was removed and inserted into the GC-MS (Figure 59).

Figure 58: SPME Fiber and Holder

GC-MS conditions (Table 14) were taken from Morales *et al* (2001).

Table 14: GC-MS Condition Setup

Initial Temperatures	35°C
Initial Time	5 min
Program Rate	4°C/min
Final Temperature	150°C
Injector Temperature	220°C
Detector Temperature	250°C
Carries Gas Helium	1ml/min

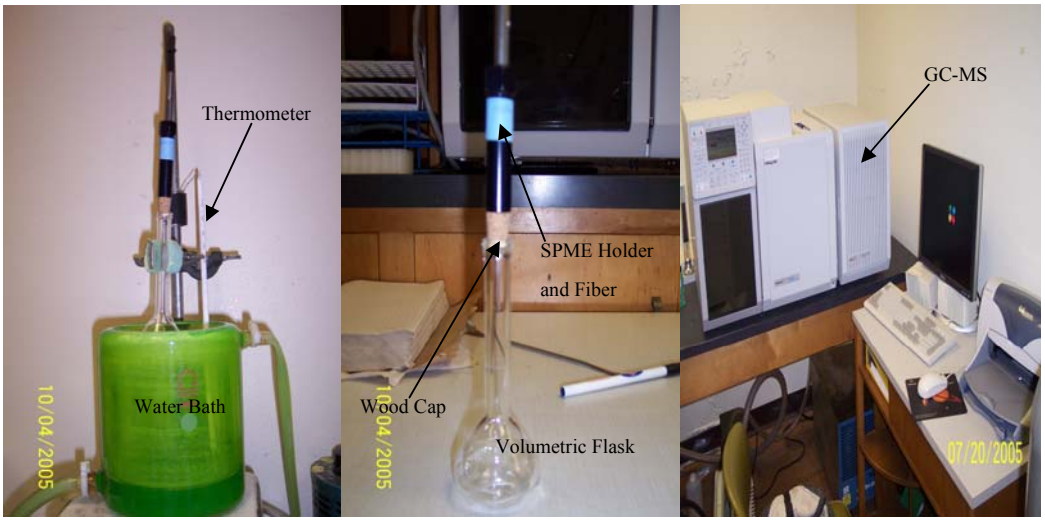


Figure 59: Water bath, SPME setup and GC-MS

3.3 Identification Bacteria

3.3.1 Gram Stain

The Gram stain method can be used to classify gram-positive or gram-negative bacteria. The gram stain kit used in the study was provided by Difco BBCTM Company (MI). Gram staining can narrow down the identity of vinegar cultures to gram-positive and negative classes, and then the cultures can be identified to a specific species by using the polymerase chain reaction (PCR).

The Gram stain flow chart is shown in Figure 60. For the Gram stain, 1ml of culture sample was placed into a 1.5ml Eppendoff^{TM} tube (Fisher Sci, PA) and centrifuged 5417C (Fisher Sci, PA) at 12000g for 8 min. A drop (approximate 0.18 gram) of the bacteria culture sample was removed from the tube, smeared on a slide, and allowed to dry. After drying, the bacteria were heat fixed to the slide. Crystal violet pigment was added to the smear for 1 minute. After 1 minute, the pigment was washed off with distilled water. Then iodine was applied for 1 minute. The iodine was

washed off with distilled water again and the smear was decolorized with 95% ethyl alcohol for 3 seconds. The alcohol was removed with distilled water and the smear was counterstained with safranin for 1 minute. The safranin was removed with distilled water and the slide dried with a paper towel.

After drying, the slide was mounted under a microscope (Optics, IL) with 10X100 magnification. A pink color demonstrates gram-negative character and a blue color indicates gram-positive. Vinegar cultures are predominantly gram-negative bacteria.

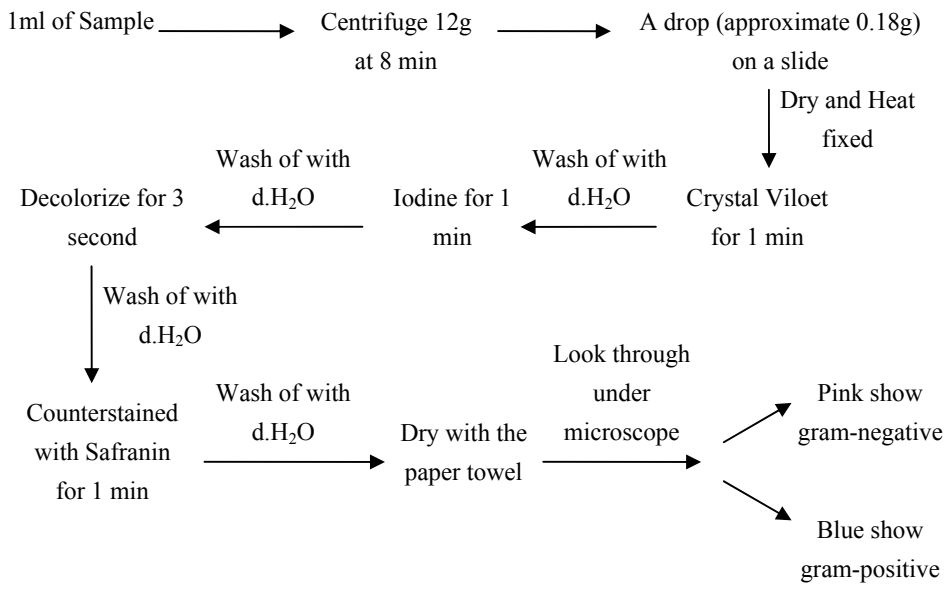


Figure 60: Gram Stain Process Flow Chart

3.3.2 PCR (Polymerase Chain Reaction)

Polymerase Chain Reaction (PCR) was used for identifying bacterial species in vinegar.

- Sampling

Two 500 ml samples of culture were collected from Creole Fermentation, Inc. (Abbeville,LA) and National Vinegar Company (Houston, TX) and kept in 500ml sample cup. The samples were put into a cooler box with ice for transport. Cultures were incubated at 86ºF (30°C) prior to analysis.

- Standard Preparation

An *Acetobacter pasteurianus* culture was obtained from ATCC (American Type Culture Collection, VA). This bacterium is slow growing and can be easily contaminated. A laminar flow hood (Class II A/B3 Biological Safety Cabinet, MN) was used to control the environment during inoculation to assure there was no contamination. The bacteria took up to four days to grow in agar and broth medium, prepared according to the ATCC, formulations as shown in Table 15. The medium was mixed in a 2000 ml flask on a hot plate. After boiling, the flask was placed into a plastic container tray and autoclaved at 250ºF for 30 minutes.

Table 15: Agar and Broth Medium Preparation

	Agar medium (200ml)	Borth medium (500ml)
Yeast Extract	1.0g	2.5g
Peptone	0.6g	1.5g
Mannitol	5.0g	12.5g
Agar	3.0g	N/A
Distilled Water	200ml	500ml

- DNA Extraction

One ml of sample from the inoculated culture was placed into a labeled Eppendoff™ tube. Samples were centrifuged for 8 minutes at 12000g. The liquid fraction was poured into bleach (to eliminate contamination in the lab). The pellet in the tube was re-dissolved with 500 µl of distilled water and the sample was vortexed well. The tube was put into a 203ºF (95°C) water bath for 5 minutes and then in ice bath 32ºF (0ºC) for 5 minutes.

- Primers Preparation

Oligonucleotide primers used to amplify part of the 16S rDNA gene were selected from conserved regions of rDNA bacterial sequences (http://www.ncbi.nlm.nih.gov). Alignments of 16S rDNA sequences were obtained from the GenBank database (Poblet *et al*. 2000). The accession numbers of 16S rDNA sequences were AJ012466 and NC004994 for *Acetobacter sp.* and *Acetobacter pasteurianus* respectfully. The forward Primer of the 16S rDNA sequence was 5' to 3' and the reverse primer was 5' to 3' (BioMMED, LA). The primer (Table 16) was diluted 1:20.

Table 16: PCR Primer Selection

Code of ATCC	Organism	Standard Size of Organism	Forward Primer of the 16S rDNA sequence (5' to 3')	Revere primer (5' to 3')
AJ012466	*Acetobacter sp.*	1481 bp	TTCCTCCACT AGGTCGGCGT	TCTCAAACTA GGACCGAGTC
NC004991	*Acetobacter pasteurianus*	1480 bp	CGAGAAGGGG CAAATTCTAA	GATTTAAGAA AAGCAGTCCA

- PCR Preparation

The Taq PCR Master Mix (QIANGEN, CA) was vortexed briefly, and 50 µl each was dispensed into PCR tubes. Five µl of each diluted primer mix was added into the PCR tubes containing the Master Mix (i.e. 5 µl x 4 = 20 µl) and then 25 µl of distilled water were added into the PCR tubes. Finally, 5 µl of template DNA (kept on ice) was added into the Perkin Elmer™ PCR tubes. The PCR tubes were then placed into the PCR (Perkin Elmer 2400, MA) and run using the general procedure of Poblet (Poblet *et al* 2000):

1. Initial denaturation: 5 min for 94°C (201.2°F).
2. 3-step cycling which was denaturation, amplification and annealing.
3. Denaturation: 1 min for 94°C (201.2°F).
4. Amplification: 2 min for 62°C (143.6°F).
5. Annealing: 2 min for 72°C (161.6°F).
6. Final extensions: 10 min 72°C (161.6°F).

Total cycle time is 35 minutes.

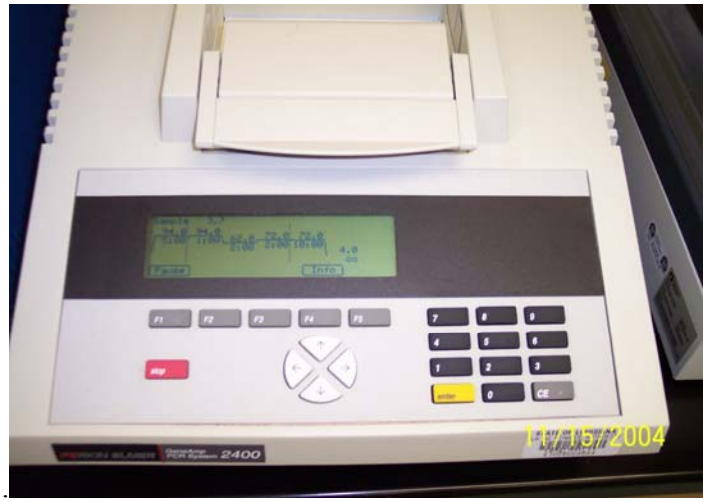


Figure 61: PCR Perkin Elmer 2400

- Gel Preparation

Thirty ml of 1X TBE (Tris Boric and EDTA) buffer with 0.24g of agarose added was poured into a 100 ml flask and placed on a hot plate to boil. After boiling, the solution became clear. After cooling 5 min, the gel solution was poured into the gel tray (Figure 62) to set.

Figure 62: Gel Tray

- Electrophoresis

Two µl of nucleic acid dye (QIAGEN, CA) and 8 µl of PCR sample from PCR tube were placed into the device tube. Five µl of mix solution were withdrawn from the tube into the gel. At that moment, 5 µl of ladder (100 base pair standard) were also added into the gel. The gel was placed into the electrophoresis unit (Figure 63) and 500 ml of 1X TBE solution was poured into the tray. The electrophoresis (Figure 64) conditions were 100 V for 1 hour and 45 minutes.

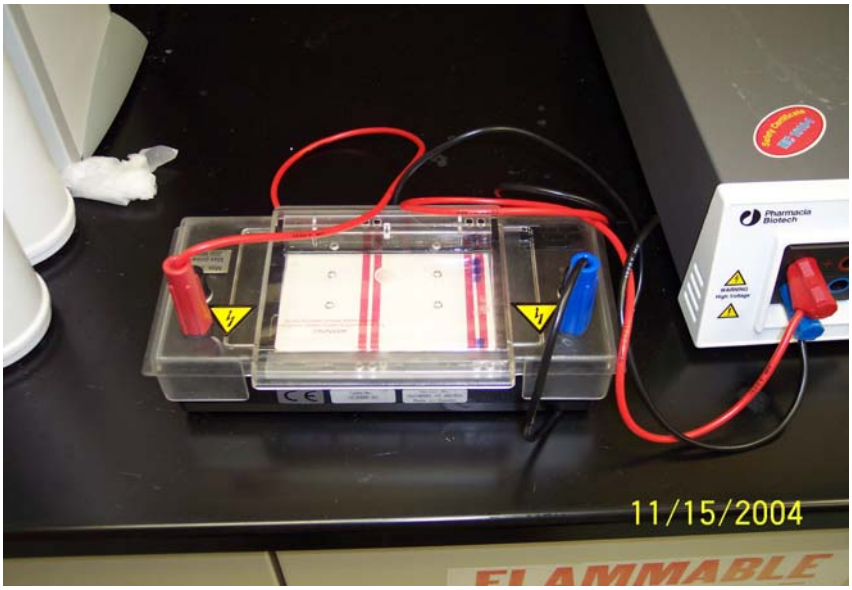


Figure 63: Electrophoresis Tray

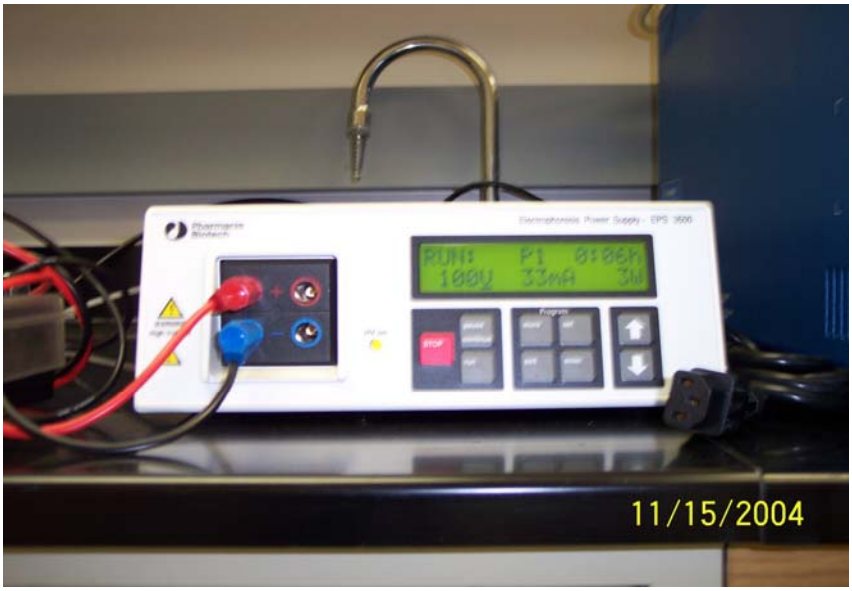


Figure 64: Electrophoresis

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Generator Pilot Unit Process

A complete diagram of all cycles in the lab scale generator process can be seen in Appendix 2. Mid range culture solution was obtained from National Vinegar Company (Houston, TX) commercial generator unit to start the pilot generator process. The 12.5 gallon (56.8 L) mid range culture solution with 8.47% acidity, 2.00% alcohol and pH 2.70 was used at the beginning of the starting cycle to inoculate the wood chips. The commercial generator unit starts each new cycle with 2.3% alcohol. According to National Vinegar Company, the mid range culture solution has a high concentration of vinegar culture. From the Figure 65 it can be seen that the acid increased slowly to 9.67% and the pH dropped to 2.48 at 142 hours. The alcohol content reached zero at the same time. The working level had dropped to 11 gal (50 L). Since the acidity strength had not reached 10% it was evident that the pilot generator unit was not ready to start so an additional 2.5 gallons of the same mid range culture solution was added into the unit. In commercial practice, 1% alcohol can be converted to 1% of acid (Hickey and Vaughn 1954).

From Table 17 theoretically the final acidity should have been 10.47% but actually was 9.67%. The 0.8% of acidity could have been lost during the starting cycle fermentation or become the culture failed to start quickly. So, 2.5 gallons of the same mid range culture solution was added at 142 hours to ensure viable vinegar cultures were living on the beech wood. After this charge and discharge was finished in 30 minutes, the first 150ml sample was taken from the pilot generator unit. The rest

of the samples were taken from the pilot generator unit every 24 hours. The alcohol content of this early sample did not appear to increase as it should have with the addition. This may have been due to poor circulation in the generator. The pump may not had enough time to mix the solution at the bottom of the fermentor. The alcohol reading did increase to 0.2 on the second day indicating circulation. After 238 hours, the final acidity of the starting cycle was 9.79%, the pH was 2.62 and alcohol content was zero.

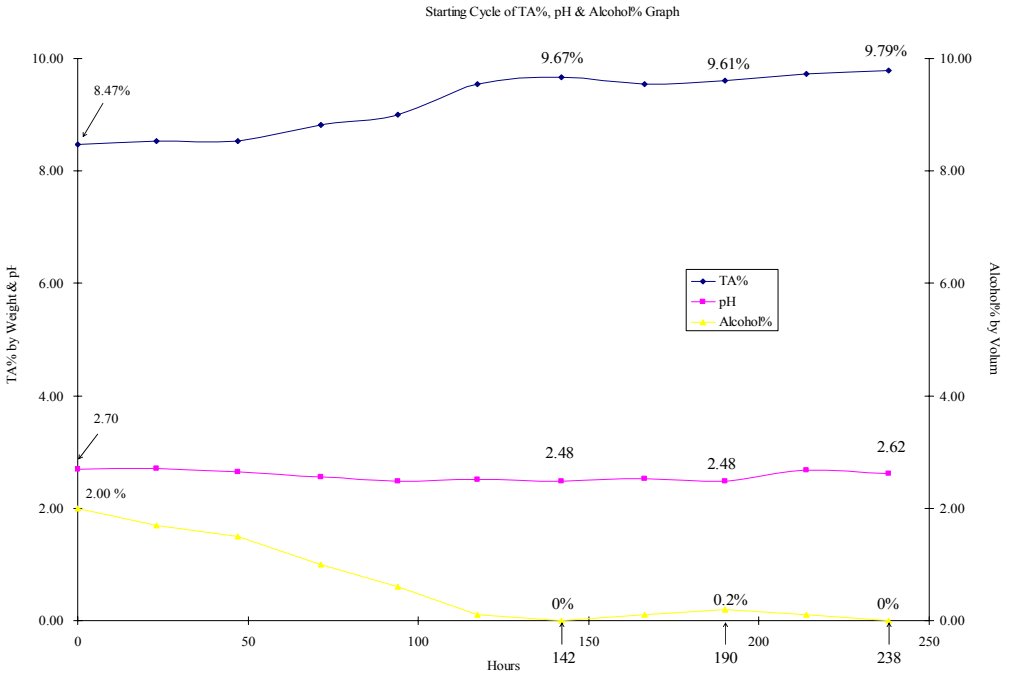


Figure 65: Generator Process Starting Cycle

Table 17: Result of Starting Cycle of Generator

Starting Acidity, %	Theoretical Final Acidity, %	Actual Final Acidity, %	Starting Alcohol, %	Theoretical Final Alcohol, %	Actual Final Alcohol, %
8.47	10.47	9.67 @ 142 hours	2.00	0	0 @ 142 hours

The initial cycle of the generator was started after 238 hours of the starting cycle. The first 2/3 volume of vinegar solution was discharged out of the 12.5 gallon (56.8 L) total capacity. Figure 66 shows that after discharge, 8.33 gallon (38 L) of fresh GM 1 mash was introduced into the generator and the pH increased from 2.62 to 2.84, the 9.79% acidity dropped to 7.09% and the alcohol content increased from 0% to 3.50%. After 118 hours the first cycle was considered complete.

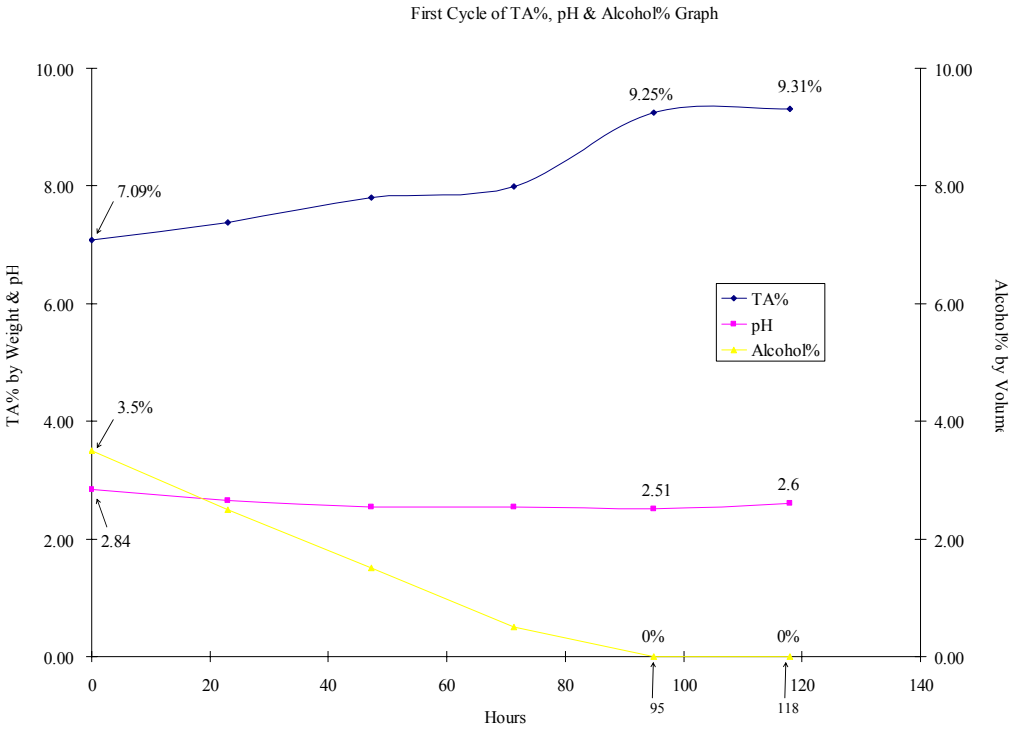


Figure 66: Generator Process First Cycles

Table 18 shows that the theoretically final acidity should have been 10.59% but the actually result was 9.31%. There was a discrepancy of 1.28% acidity lost during the first cycle fermentation. This final acidity at 118 hours represents 2.22% of alcohol converted rather than the 3.5%. This may be due to alcohol evaporation during the 118 hours or retention of alcohol in the beech wood chips. The normal

commercial results after addition of the GM1 mash in the generator process is 2.3% alcohol (National Vinegar Company, TX) and the acidity is 9.00% at the beginning of cycle fermentation. In the pilot scale generator, the pH dropped from 2.84 to 2.60 and the alcohol was content 0% at the end of cycle. The generator pilot unit was discharged with 2/3 of the solution being removed and recharged with 2/3 GM2 mash into the unit. The final acidity of the starting cycle was 9.31% at 118 hours, pH 2.6 and zero alcohol before the new mash was added.

Table 18: After First Cycle, Theoretically and Actually Result

Starting Acidity, %	Theoretical Final Acidity, %	Actual Final Acidity, %	Starting Alcohol, %	Theoretical Final Alcohol, %	Actual Final Alcohol, %
7.09	10.59	9.31 @ 118 hours	3.5	0	0 @ 118 hours

After recharge, the second cycle began with 6.79% acid, 3.5% alcohol and pH of 2.75 as shown in Figure 67.

After 144.3 hours the second cycle was concluded. Table 19 shows that the theoretical final acidity should have been 10.29% based on alcohol conversion but the actual result was 8.83%. There was an apparent 1.46% acidity lost during the second cycle fermentation. The final acidity represents 2.04% rather than 3.5% alcohol conversion. Figure 67 shows that the acid strength at 95.3 hours had reached 9.07%, the pH dropped to 2.40 and the alcohol dropped to 0%. After that the acid dropped from 9.07% to 8.59% in the vinegar solution at 120.3 hours. Apparently, the bacteria

had begun to convert acid because the alcohol had been depleted. By 144.3 hours, the acidity has returned to 8.83%. This may be due to the retention of alcohol in the beech chips during the re-circulation. At this time 2/3 of the volume was discharged and replaced with fresh GM2 mash.

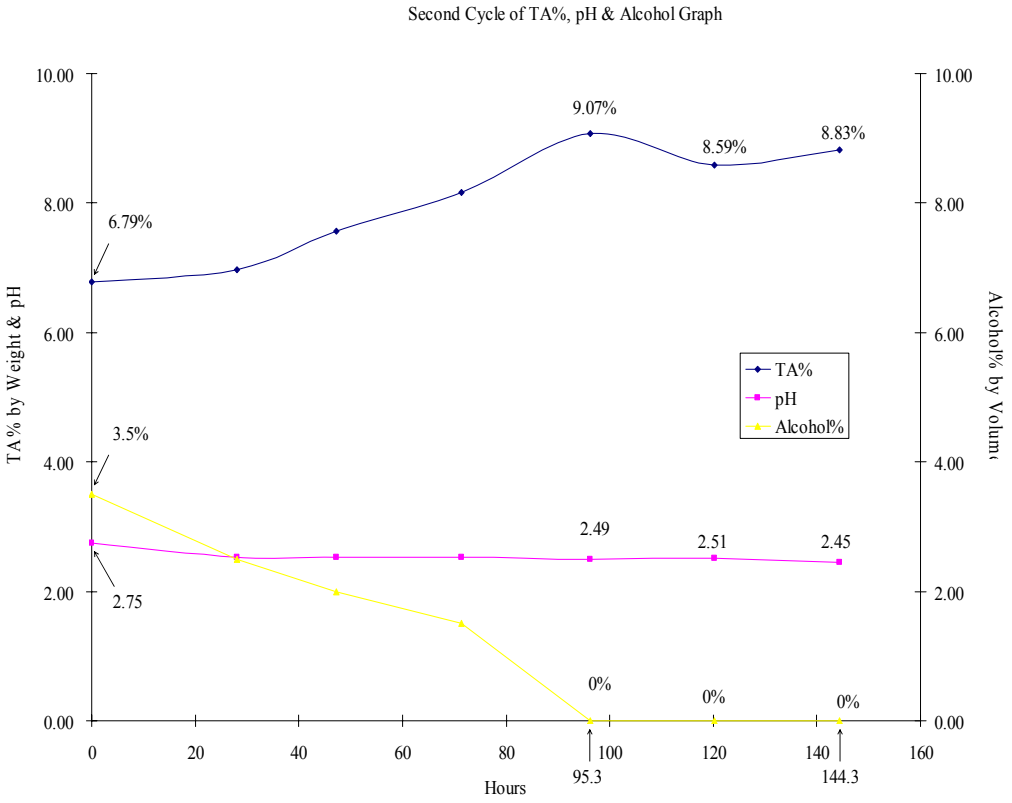


Figure 67: Generator Process Second Cycles

Table 19: After Second Cycle, Theoretically and Actually Result

Starting Acidity, %	Theoretical Final Acidity, %	Actual Final Acidity, %	Starting Alcohol, %	Theoretical Final Alcohol, %	Actual Final Alcohol, %
6.79	10.29	8.83 @ 95.3 hours	3.5	0	0 @ 95.3 hours

After fresh mash was added, the third cycle began with 8.11% acidity, pH at 2.49 and 1.5% alcohol. The third cycle of the process used the same GM2 mash as before. At the second cycle of the fermentation process 3.5% alcohol was present at the beginning of the cycle. In the third cycle of process, only 1.5% of alcohol was found at the beginning even though the same procedures were followed. This may be due to alcohol evaporation during the mash preparation, during storage or poor mixing before the sample was taken. After 95.3 hours, the acid strength increased from 8.11 % to 9.07%, pH dropped to 2.45 and alcohol dropped to 0%. After 93.5 hours acidity dropped probably because the bacteria attacking the acid since the alcohol were depleted without the food which is alcohol.

Table 20 shows that the theoretical final acidity should have been 9.61% but the actually result was 9.07%. There 0.54% was an apparent acidity lost during the third cycle fermentation.

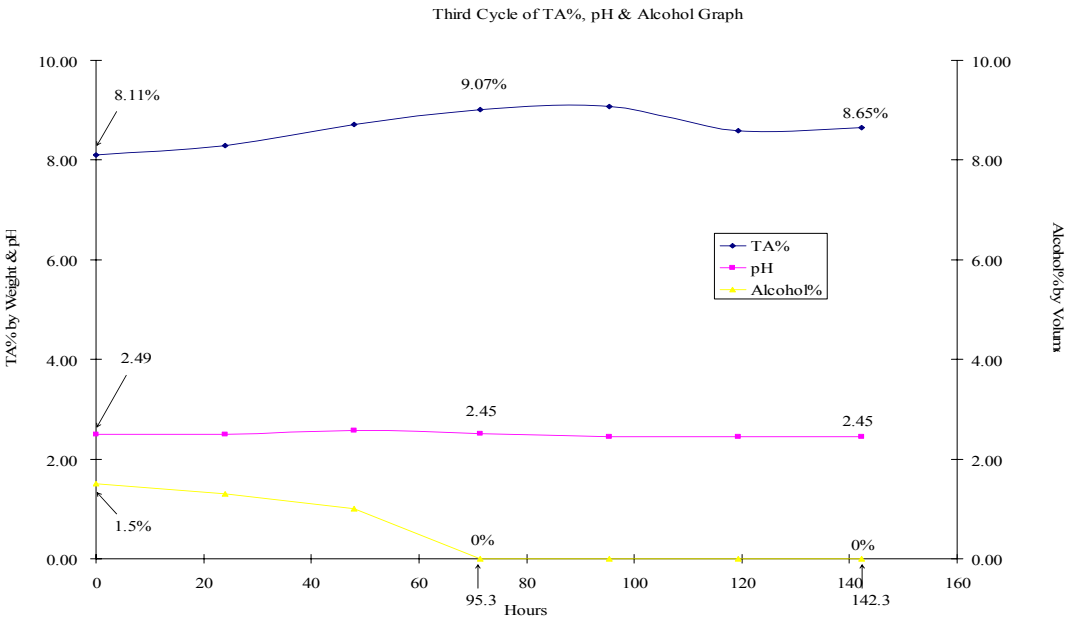


Figure 68: Generator Process Third Cycles

Table 20: After Third Cycle, Theoretically and Actually Result

Starting Acidity, %	Theoretical Final Acidity, %	Actual Final Acidity, %	Starting Alcohol, %	Theoretical Final Alcohol, %	Actual Final Alcohol, %
8.11	9.61	9.07 @ 95.3 hours	1.5	0	0. @ 95.3 hours

4.2 Submerged Process 1

In the submerged process 1, alcohol was added for three weeks but the percent of acidity did not increase. The reason for failure could be that the solution was contaminated; excessive dilution of the ferment liquid by 50ml addition of the dissolver solution per day or the bacteria may have died because of poor air supply. Following this, another fermentator was used to study the submerged acetification.

4.2 Submerged Process 2

A complete diagram of all cycles in the lab scale submerged process can be seen in Appendix 4. Mid range culture solution was obtained from a commercial submerged unit Creole Fermentation Inc (Abbeville, LA) to start the submerged process 2. The 1.87 gallons (8.5L) of mid range culture broth with 9.5% acidity and 3.35% of alcohol was added into the 2.2 gallon (10L) fermenter and used at the beginning of the starting cycle. Figure 69 shows the starting cycle of the submerged acetification. Acidity started at 9.5%, pH at 2.15 and alcohol at 3.35%. This is the mid range of culture solution taken from the commercial submerged process tanks during

the fermentation which explains why the initial alcohol content was 3.35%. The initial cycle begins with the mid-range unfiltered vinegar containing the culture source having an acidity of 8.5 to 9.5 percent. This assures the bacteria are in the exponential growth phase in a suitable environment. In fact, the fermentation process continued to 12.25 % acidity within 20.35 hours with a pH drop to 2.05. Table 21 contains the theoretical and actual results.

Theoretical final acidity should have been 12.85% but the actual result was 12.25%. Some alcohol appears to have been lost at the end of the starting cycle which may be due to alcohol evaporating from the cap of the thermometer holder. After 20.35 hours, the fermentor was discharged with 1/3 (2.6 L) of the volume being removed and replaced with fresh SM mash

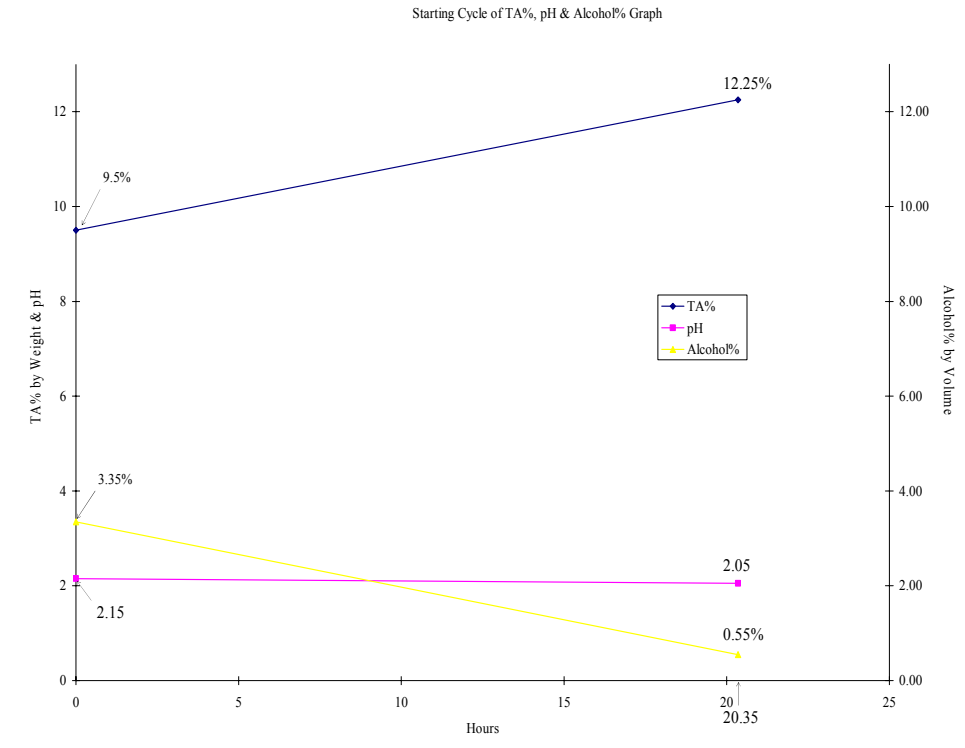


Figure 69: Starting Unit Vinegar Fermentation Submerged Process (Cycle Begin)

Table 21: After Starting Cycle, Theoretically and Actually Result in Submerged Process

Starting Acidity, %	Theoretical Final Acidity, %	Actual Final Acidity, %	Starting Alcohol, %	Theoretical Final Alcohol, %	Actual Final Alcohol, %
9.5	12.85	12.25	3.35	0.6	0.05

After new mash was added the acidity dropped to 8.25%, the pH rose to 2.29 and the alcohol content increased to 4.6%. The normal commercial standard of submerged process at the beginning of a cycle is 4.5 to 4.7% alcohols (Creole Fermentation, Inc. Abbeville, LA). Figure 70 shows the first cycle of the submerged fermentor with acetic acid beginning at 8.25% and ending at 12.35% after 19.4 hours. The pH dropped to 2.15 and the alcohol content dropped to 0.5%. After 19.4 hours 1/3 (2.6 L) vinegar was discharged and the fermentor was recharged with another 2.6 L SM mash.

Table 22 shows the theoretically final acidity should have been 12.85% but the actual result was 12.35%. The fixed leaking cap at the thermometer holder may have helped with the better recovery. The fermenter seems to be a very efficient process for vinegar production. This is because the environment is enclosed so that the fermentation is under control with little loss of volatiles. According to the results of the mass balance calculations shown in Table 22, the theoretical maximums were obtained.

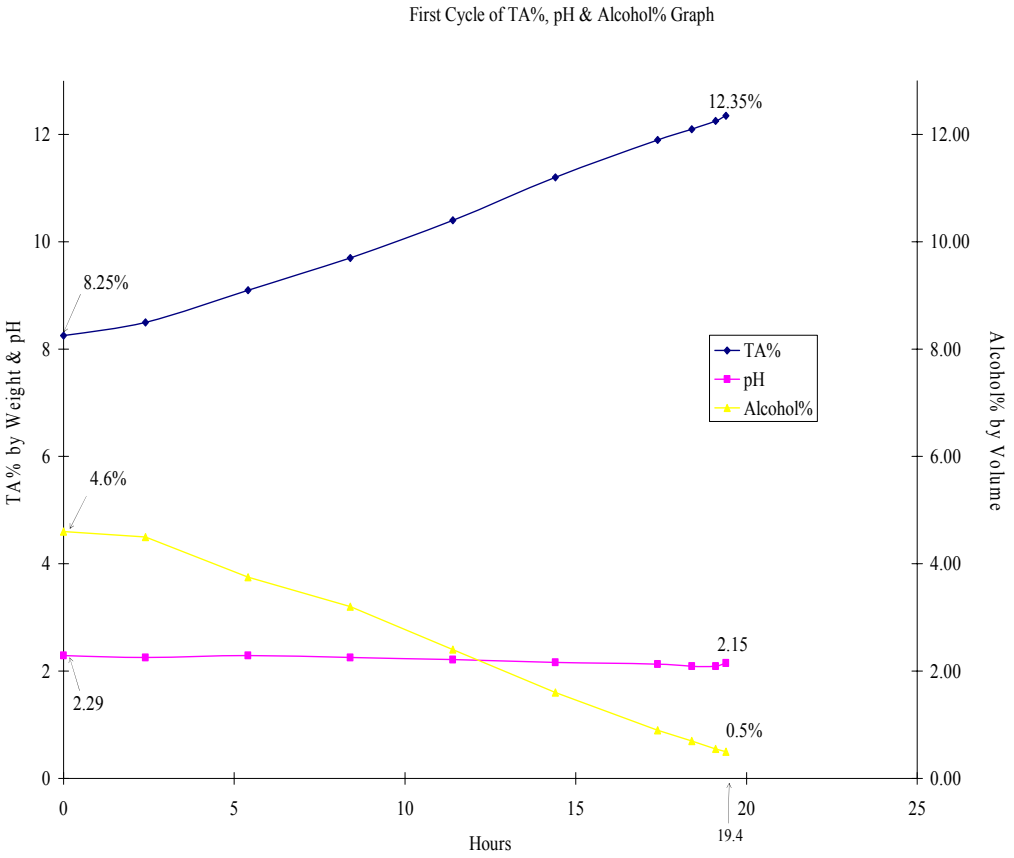


Figure 70: First Cycle after the First Discharged – Submerged Process

Table 22: First Cycle, Theoretically and Actually Result after Added SM Mash

Starting Acidity, %	Theoretical Final Acidity, %	Actual Final Acidity, %	Starting Alcohol, %	Theoretical Final Alcohol, %	Actual Final Alcohol, %
8.25	12.85	12.35. @ 19.4 hours	4.6	0.5	0.5 @ 19.4 hours

The second cycle is shown in the Figure 71 and the results are similar to the first cycle but the final acidity reached 12.35% at 20.45 hours. Table 23 shows the theoretical and actual results of the second cycle.

The theoretical final acidity was 12.8% at the end of second cycle but the actual result was 12.35%. The alcohol dropped to 0.45% in 20.45 hours. So, 4.05% alcohol had been converted to acid.

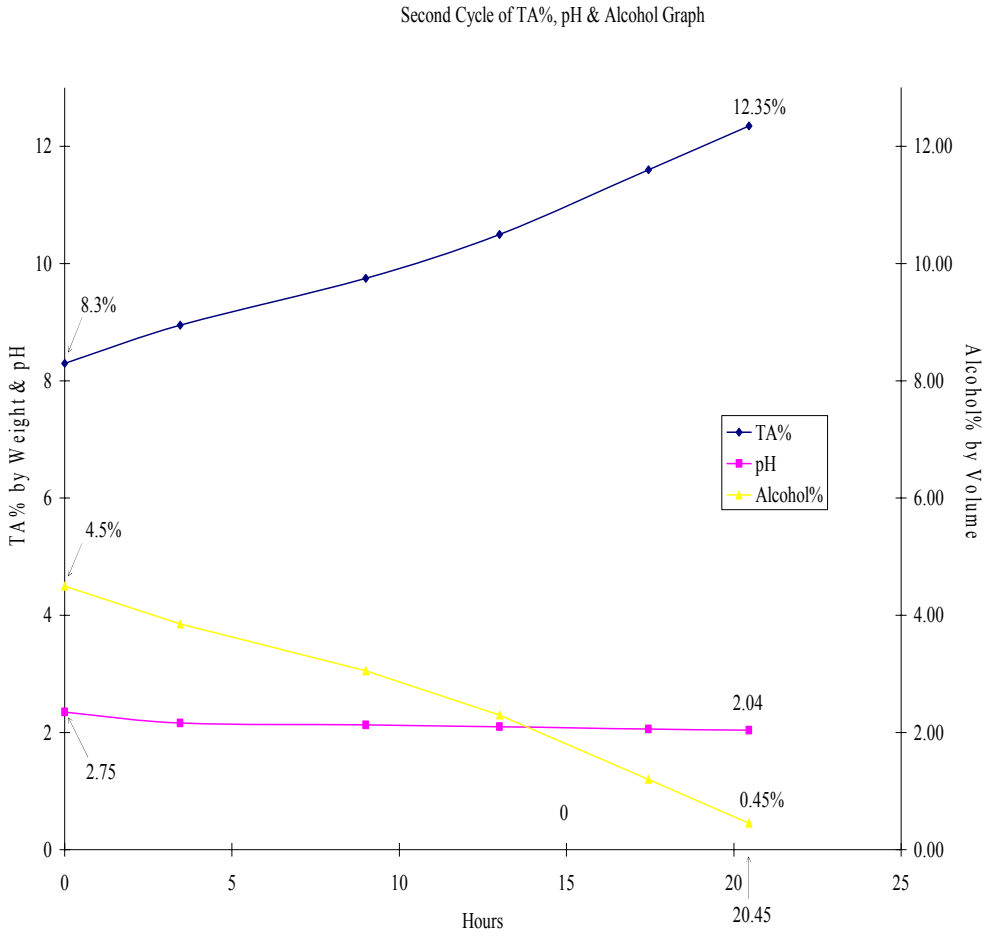


Figure 71: Second Cycle of Submerged Process

Table 23: Second Cycle, Theoretically and Actually Result after Added SM Mash

Starting Acidity, %	Theoretical Final Acidity, %	Actual Final Acidity, %	Starting Alcohol, %	Theoretical Final Alcohol, %	Actual Final Alcohol, %
8.30	12.8	12.35	4.5	0.45	0.45

Figures 72 shows the third cycle of the fermentation finishing in 39 hours with an acidity of 12.6%. The acid did not change from 24 hours on because the vinegar bacteria had exhausted the alcohol converting it to acid. The process was terminated at this point. The vinegar bacteria started to die because of lack of food supply and the liquid became clear after 39 hours with the bacteria setting to the bottom of the fermentor.

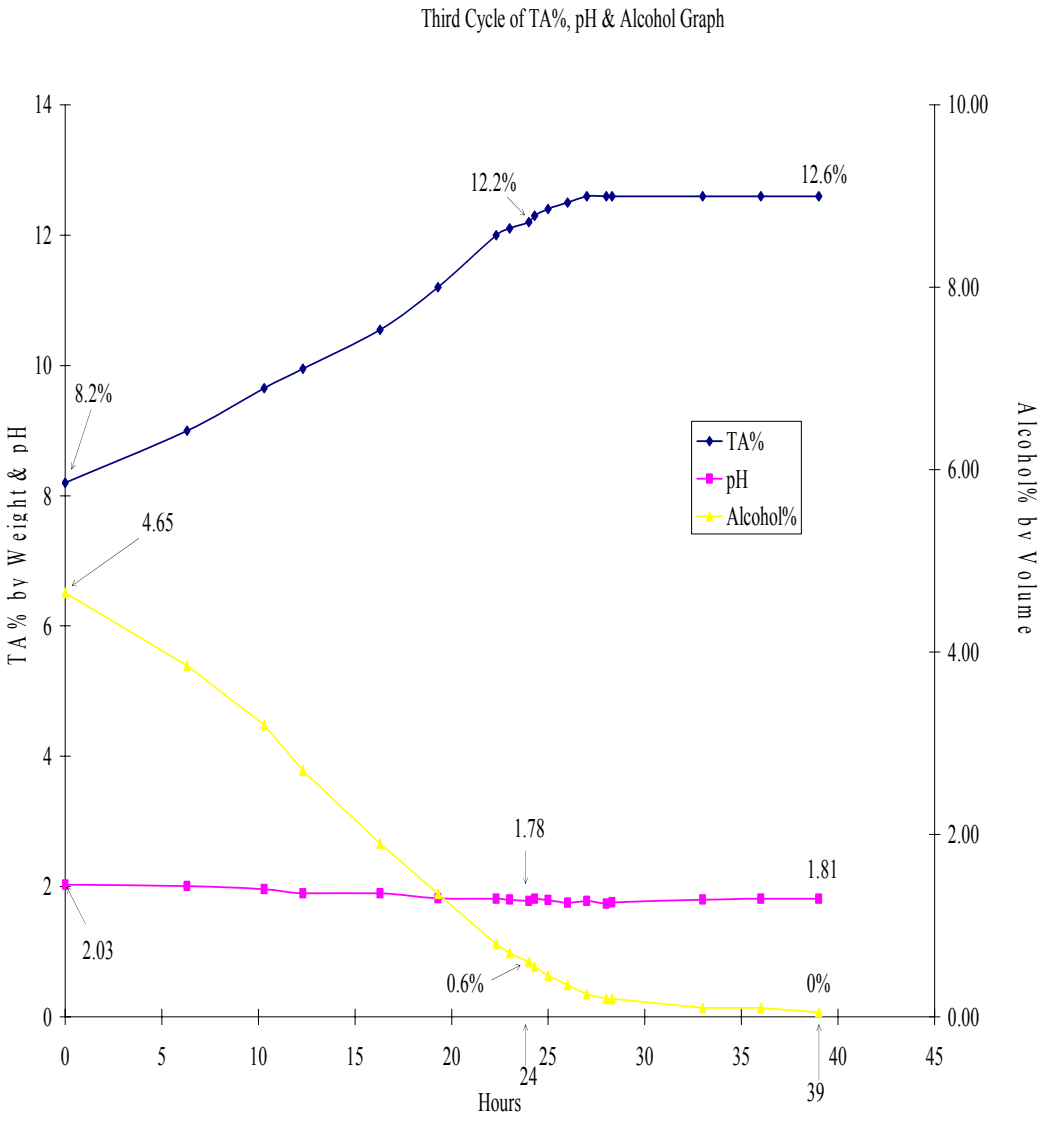


Figure 72: Third and Final Cycle – Submerged Process

4.4 Submerged Process 3

In the submerged process 3, the application of 2%, 4% and 6% beech wood powder showed poor color development and weak beech wood aroma. The final 8% beech wood powder application produced good color and aroma and was chosen for detailed analysis. The GC-MS profile from the 8% sample was used for comparison with the lab scale submerged samples without powder and with the lab scale generator process.

4.5 Gas Chromatography

Gas chromatography and mass spectroscopy were used to compare the profiles of the various vinegars produced in this study. Samples obtained from two commercial vinegar production facilities (National Vinegar Company and Creole Fermentation, Inc) along with samples from the laboratory generator and submerged unit were analyzed. This comparison was done to determine if there are distinct aroma or flavor profiles for vinegars produced by various means. The SPME method proved superior to the direct sample injection method for GC. The volatile compound acetic acid is present in the vinegar and damages columns because of its acidity (Charles *et al* 2000).

Figure 73 contains the GC-MS data comparison for the generator process vinegar produced by National Vinegar Company in Houston, Texas and the submerged process product from Creole Fermentation Inc in Abbeville, Louisiana. Using the SPME method, the generator process vinegar contains 13 identifiable compounds while the submerged process contained 15 compounds. Both vinegars contained high

concentrations of 2-propenoic acid at 1.3 minutes. In addition, both vinegar sources have similar amounts of acetic acid at 20.3 minutes and 1-methylethyl ester at 20.7 minutes. Both of them have low level of the 1-butene at 0.8 minute, 2-butyne-1 at 1 minute and pentyl ester at 6 minutes. The submerged process had a higher level of ethyl ester compared with the generator process.

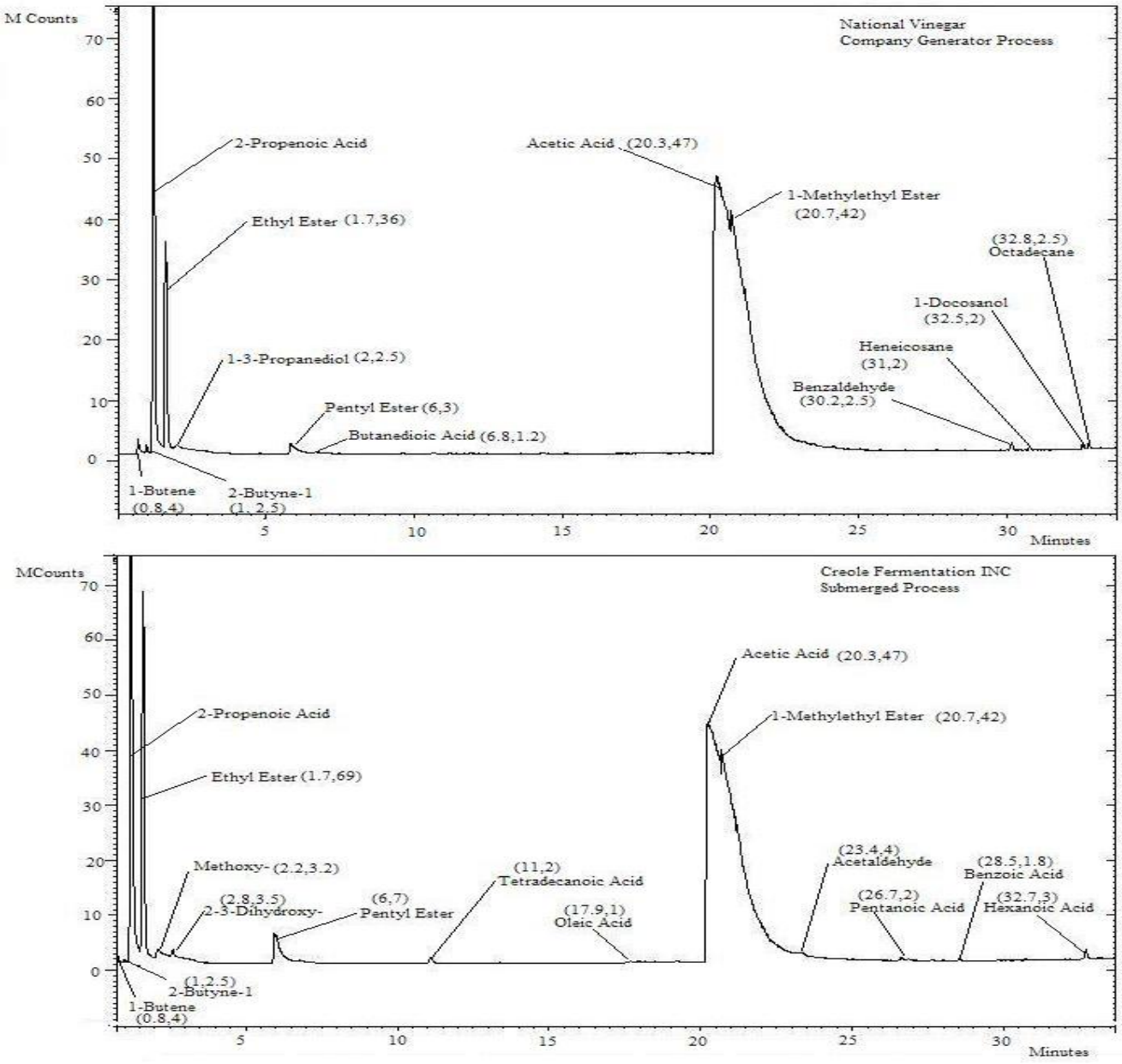


Figure 73: GC-MS Profiles of Vinegar from Commercial Generator and Submerged Processes

Six compounds in the generator process vinegar were present in minor amounts: 1-3-propanediol, butanedioic acid, benzaldehyde, heneicosane, 1-docosanol and octdecane. On the other hand in the submerged process, seven compounds were found in minor amounts: methoxy- group, 2-3-dihydroxy- group, tetradecanoic acid, oleic acid, acetaldehyde, pentanoic acid, benzoic acid and hexanoic acid.

From the results, it can be seen that the two processes have six or seven compounds in common. The aroma or flavor in vinegar fermentation is affected by the material used in the mash and the processing environment. There were also small amounts of residual ethanol in both fermentation processes. Typically, about 0.5% alcohol is left over during the discharge. This can not be shown clearly in the graph because of the scale.

Figure 74 demonstrate the differences in a lab scale submerged acetification process with and without beech wood powdered added. This was done to test whether compounds present in beech wood could affect the flavor and aroma of vinegar and simulate the results of vinegar produced by the generator process. The only detectable differences appear to be the presence of 4, 2 acetonitrile and octadecane in the beech wood powder fermentation.

Figure 75 contains an analysis of the pilot scale generator process vinegar in comparison to the commercial generator vinegar. Eight unique compounds were found in comparison to the vinegar produced by the National Vinegar Company generator process. These are methyl ester, benzene, octadecyl ester, tricosane, and 3-cyclohexene-1-methanol, pyrrolidine, butanoic acid and menthone. The pilot unit

produced less 2-propenoic aid and ethyl ester. Table 24 shows a comparison of compounds from all experiments.

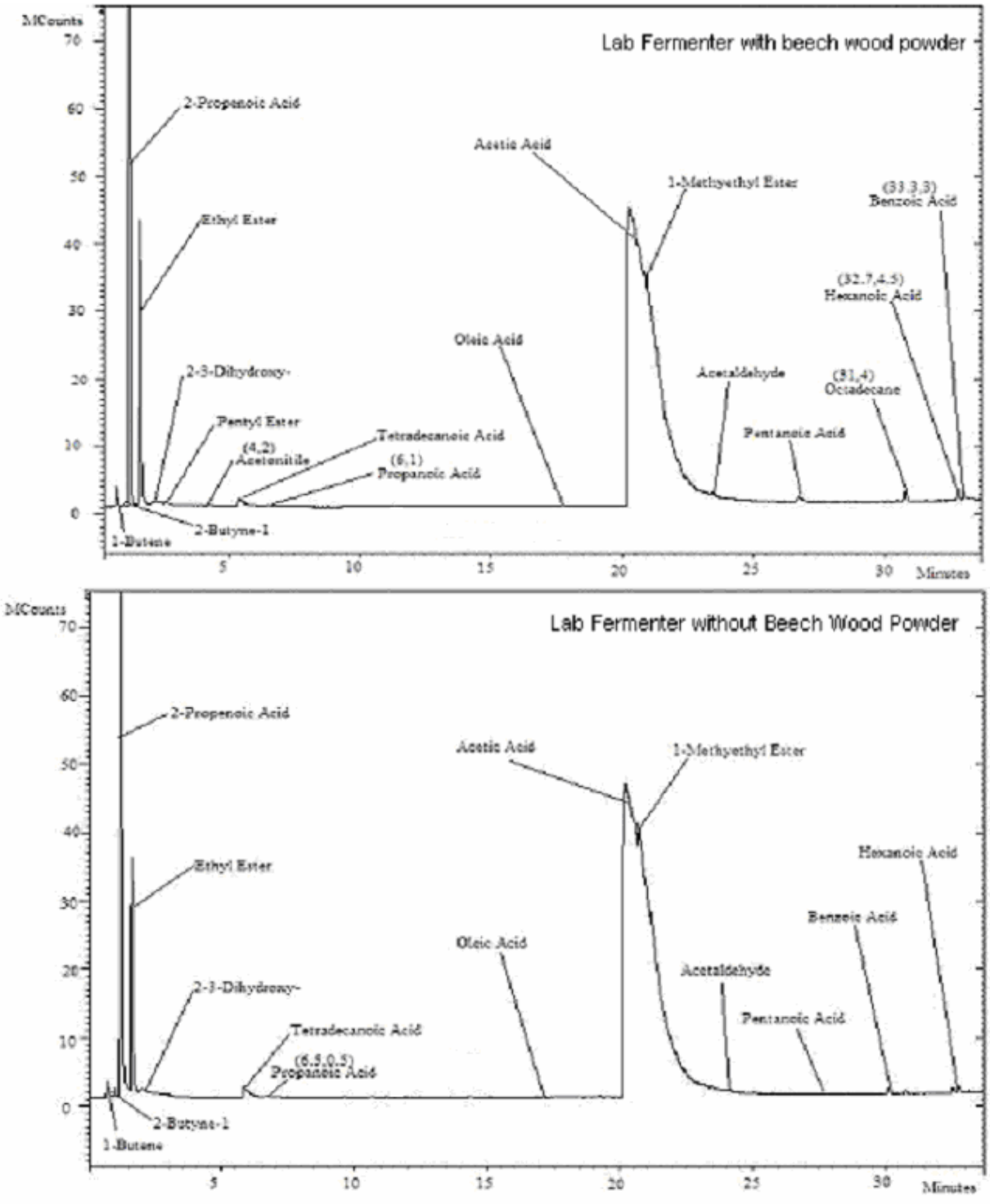


Figure 74: GC-MS Profiles of Lab Submerged Vinegars from Acetification with or without Beech wood

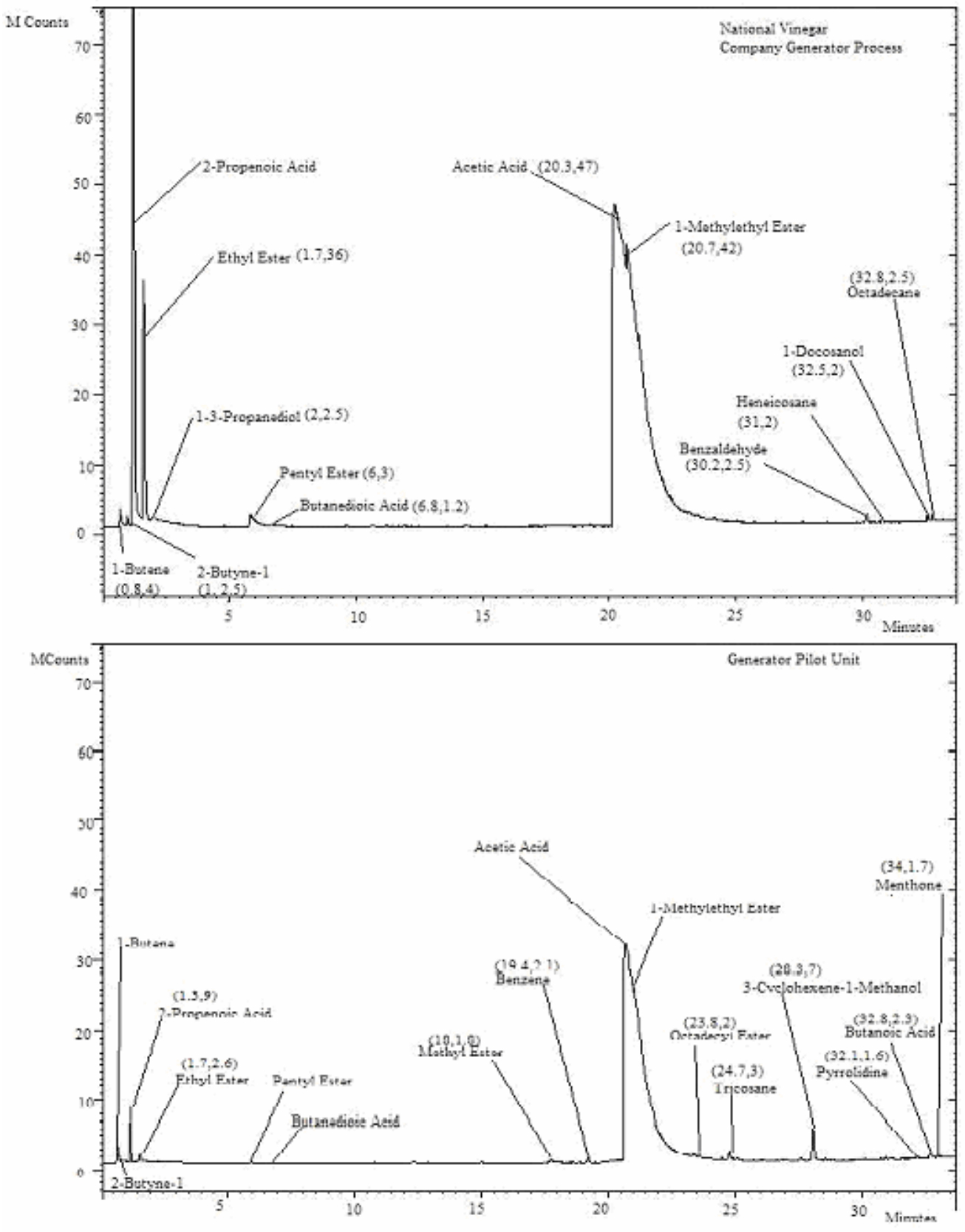


Figure 75: Comparison of generator pilot unit with National generator Unit GC Graph

Table 24: Summary of comparison Compounds for all experiments

Compounds	National Generator	Creole Submerged	L.Submerged w/ beech	L.Submerged w/o beech	Generator Pilot Unit
1-3-propanediol	x				
1-butene	x	x	x	x	x
1-doconanol	x				
1-methylethyl ester	x	x	x	x	x
2-3-dihydroxy		x	x	x	
2-butyne-1	x	x	x	x	x
2-propanoic acid	x	x	x	x	x
3-cyclohexane-1-methanol					x
acetaldehyde		x	x	x	
acetanitrile			x		
acetic acid	x	x	x	x	x
benzaldehyde	x				
benzene					x
benzoic acid		x	x	x	
butanedioic acid	x				x
butanoic acid					x
ethyl ester	x	x	x	x	x
heneicosane	x				
hexanoic acid		x	x	x	
methone					x
methoxy-		x			
methyl ester					x
octadecane	x		x		
octadecyl ester					x
oleic acid		x	x	x	
pentanoic acid		x	x	x	
pentyl ester	x	x	x		x
propanic acid			x	x	
pyrrolidine					x
tetradecanoic acid		x	x	x	
tricosane					X

4.6 Gram Stain

A Gram stain performed on a representative sample of vinegar from commercial submerged and generator processes indicated predominantly gram-negative bacteria. Figure 76 shows numerous gram-negative bacteria in the submerged process vinegar from Creole Fermentation, Inc. Figure 77 shows fewer gram-negative bacteria in the generator process vinegar from National Vinegar Company. This is not unexpected since most of the bacteria are retained on the non-packing substrate in the generator process.

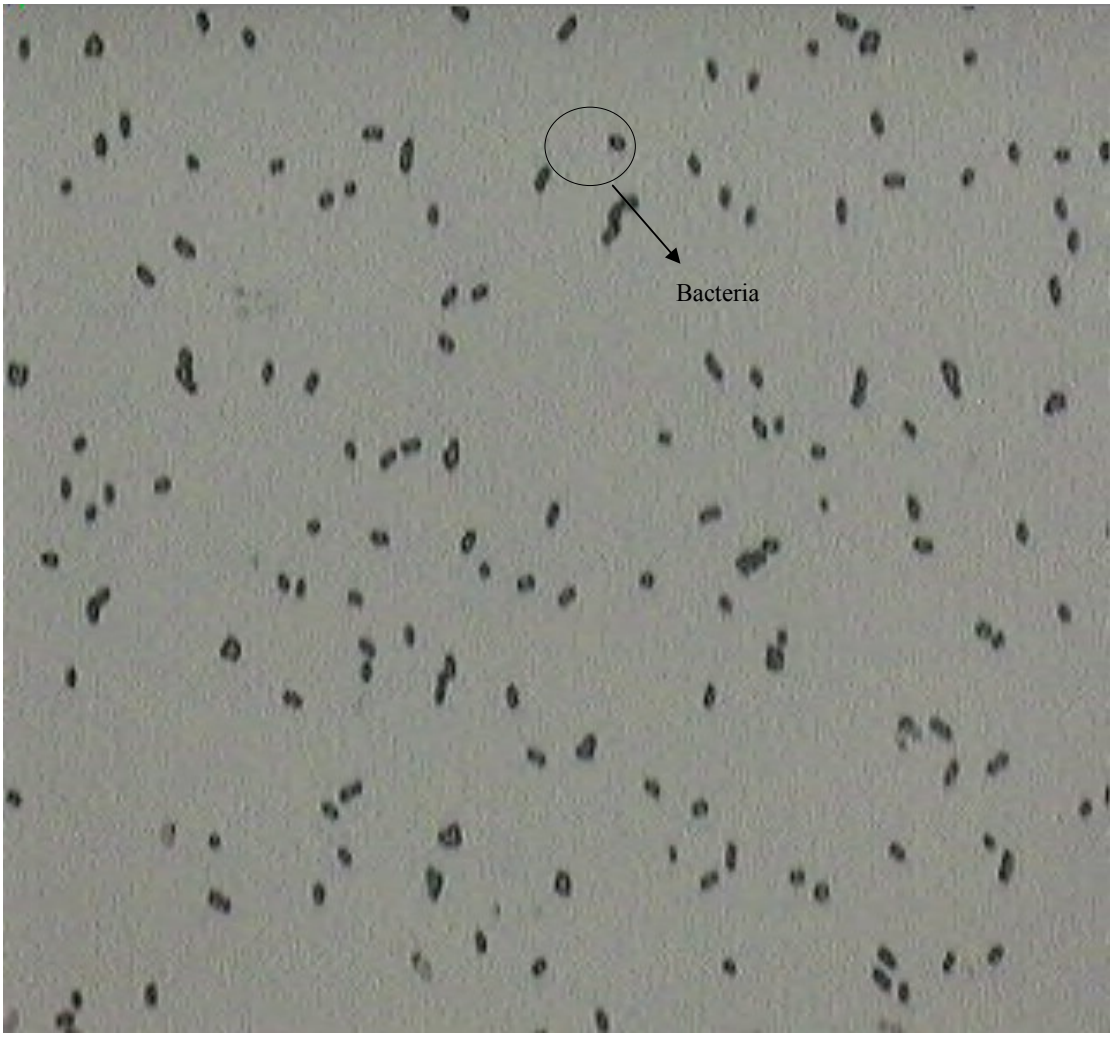


Figure 76: Gram-Negative Bacteria Found in the Submerged Process

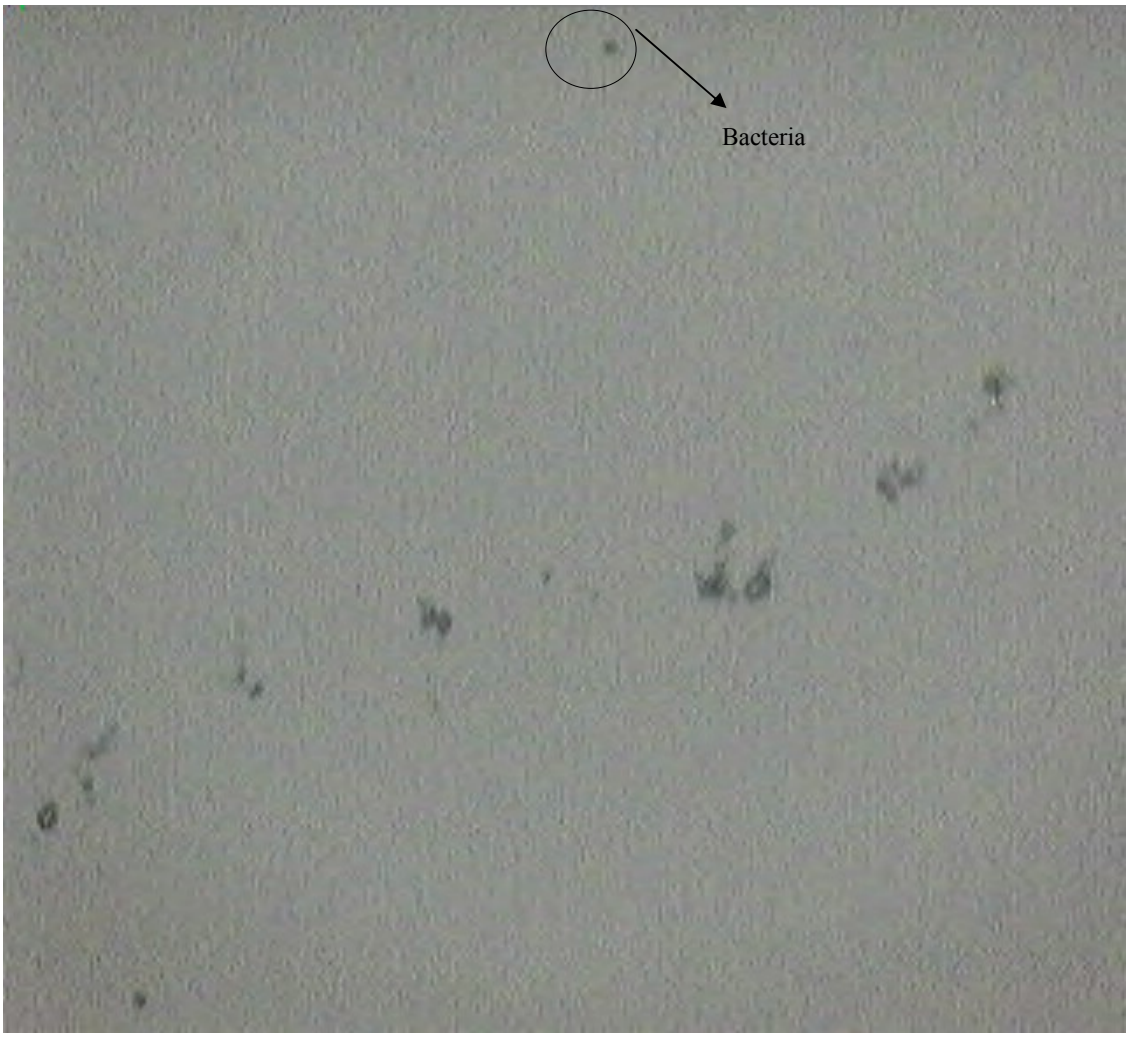


Figure 77: Gram-Negative Bacteria Found in the Generator Process

4.7 PCR (Polymerase Chain Reaction)

In an effort to identify the bacteria in the various vinegars, PCR was conducted. *Acetobacter pasteurianus* was used as the positive control (CON) and the base pair was 1440bp. The negative-control (N-CON) was *Listeria monocytogenes* used for comparison. N, N1, and N2 in increasing concentrations are the National Vinegar Company cultures from the generator process. C, C1, and C2 in increasing concentrations are the Creole Fermentation, Inc cultures from the submerged process (Table 25).

Table 25: Shows the Symbol Used for PCR

Symbol	Sample Treatment
N	1ml culture + centrifuge National – Generator Process
N1	2ml culture + centrifuge National – Generator Process
N2	3ml culture + centrifuge National – Generator Process
C	1ml culture + centrifuge Creole – Submerged Process
C1	2ml culture + centrifuge Creole – Submerged Process
C2	3ml culture + centrifuge Creole – Submerged Process

In Figure 78, it can be seen that the submerged fermentation bacteria from Creole Fermentation Inc. vinegar appear to be *Acetobacter sp.* The band appears only in the highest concentration sample (C2). In the test, the positive control should have given a similar band to the C2 at 1481bp rather than at 1250bp. Standard size of *Acetobacter sp.* is 1481 bp. The reason for the discrepancy is believed to be due to the age of the control culture. The culture was revived from the ATCC (American Type Culture Collection) dried culture one and half years previously and left in the freezer. It is possible that the base pair was lower because of deterioration or mutation.

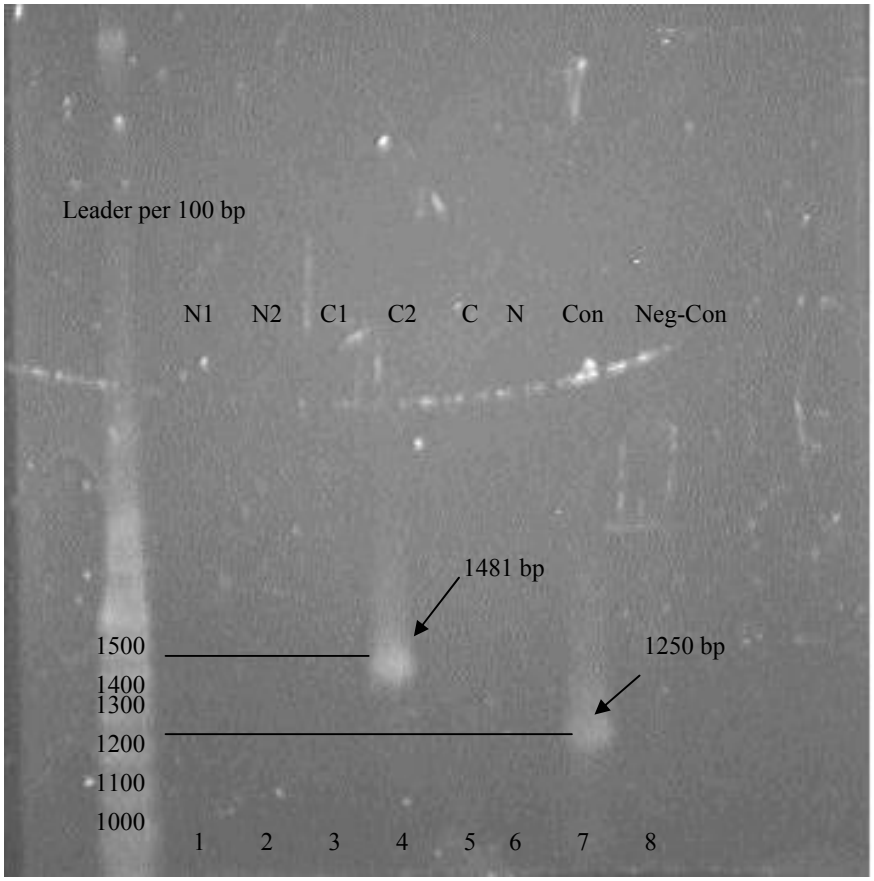


Figure 78: Agarose Image of *Acetobacter sp.* Family Primer

The bacteria in this vinegar appear to be *Acetobacter pasteurianus* (Figure 79). This band also appears in the highest concentration sample (C2). In the test, the control should have given a similar band to the C2 at 1440bp rather than at 1250bp. Standard size of *Acetobacter pasteurianus* is 1440 bp. A possible reason for the size to be lower may be due to bacteria mutation. Mutations found at this specific DNA target confirm previous reports on the mutagenic action of O_2 (Decuyper-Debergh 1987; Costa de Oliveira 1992; Agnez-Lima 1999).G→T transversion is the most frequent type of mutation induced by O_2 and has been associated with the presence of 8-oxodG, which is able to mispair with adenine (Wood 1990; Shibutani 1991). One of the bacteria used in the submerged fermentation in Creole Fermentation Inc is

Acetobacter pasteurianus. The band is not clear because the gel was exposed to light too long when the gel picture was taken.

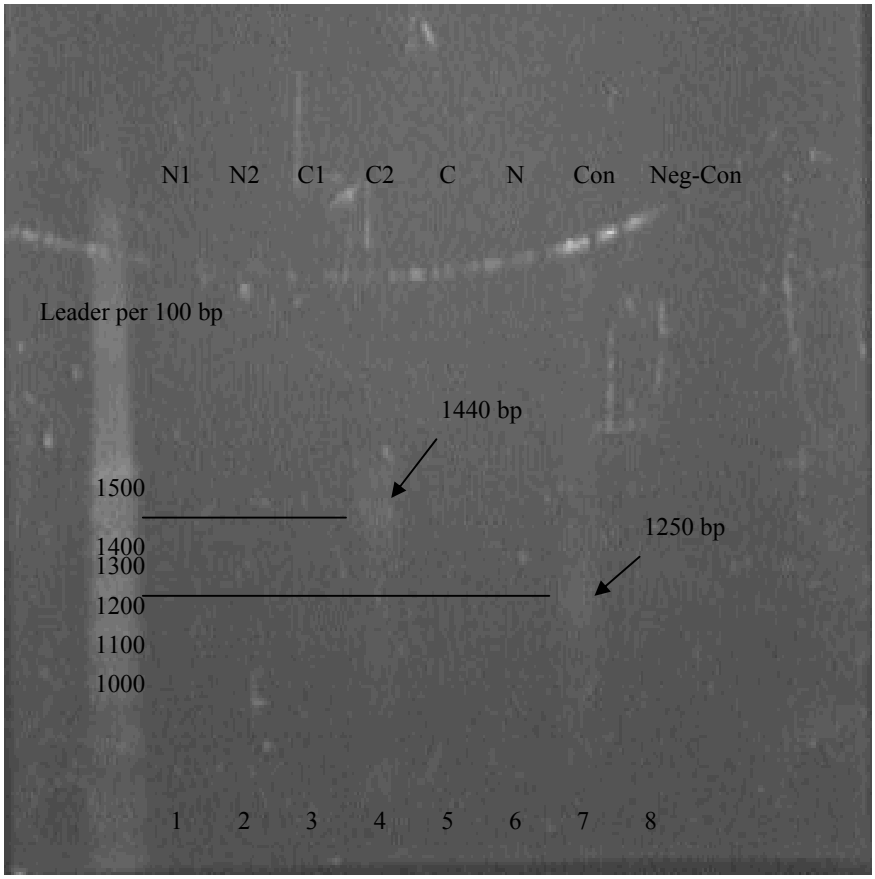


Figure 79: Agarose Image of *Acetobacter pasteurianus* Primer

CHAPTER 5 SUMMARY AND CONCLUSION

The generator pilot unit produced vinegar with an acid strength of 9.78% in 5 days. This was a slow process to produce vinegar and not very efficient. In addition, it appears that there was a loss of alcohol and acetic acid under this process possibly because the surface area was so large. The bacteria in the generator were slow growing even when the generator unit was operating under perfect conditions. It took 7 days to start this generator but sometimes as much as 1 or 3 months are needed to start a unit under perfect conditions.

The submerged process pilot unit was very efficient and produced vinegar with an acid strength of 12% or more. The highest acid strength produced by industry reported, so far, is 16%. In addition, this was closed process with controlled exposure of the fermenting liquid to air. This method minimizes the alcohol and acetic acid loss. The bacteria will grow easily in the aerated liquid under perfect conditions. The submerged process was easy to start compared to the generator process.

Many people believe the submerged and generator processes give different flavors to the vinegars. According to the GC-MS analysis, there were detectable differences between the processes. The differences might be due to the beech wood shavings as indicated by the submerged test with beech wood powder. The beech wood may impart flavors, just like aging of whiskey in the oak barrels.

Gram staining indicated that the predominant bacteria in all studied processes were gram-negative bacteria as it should be. The submerged acetification bacteria were identified through PCR as being *Acetobacter sp.* and *Acetobacter pasteurianus* the results of bacteria from the generator process were inconclusive.

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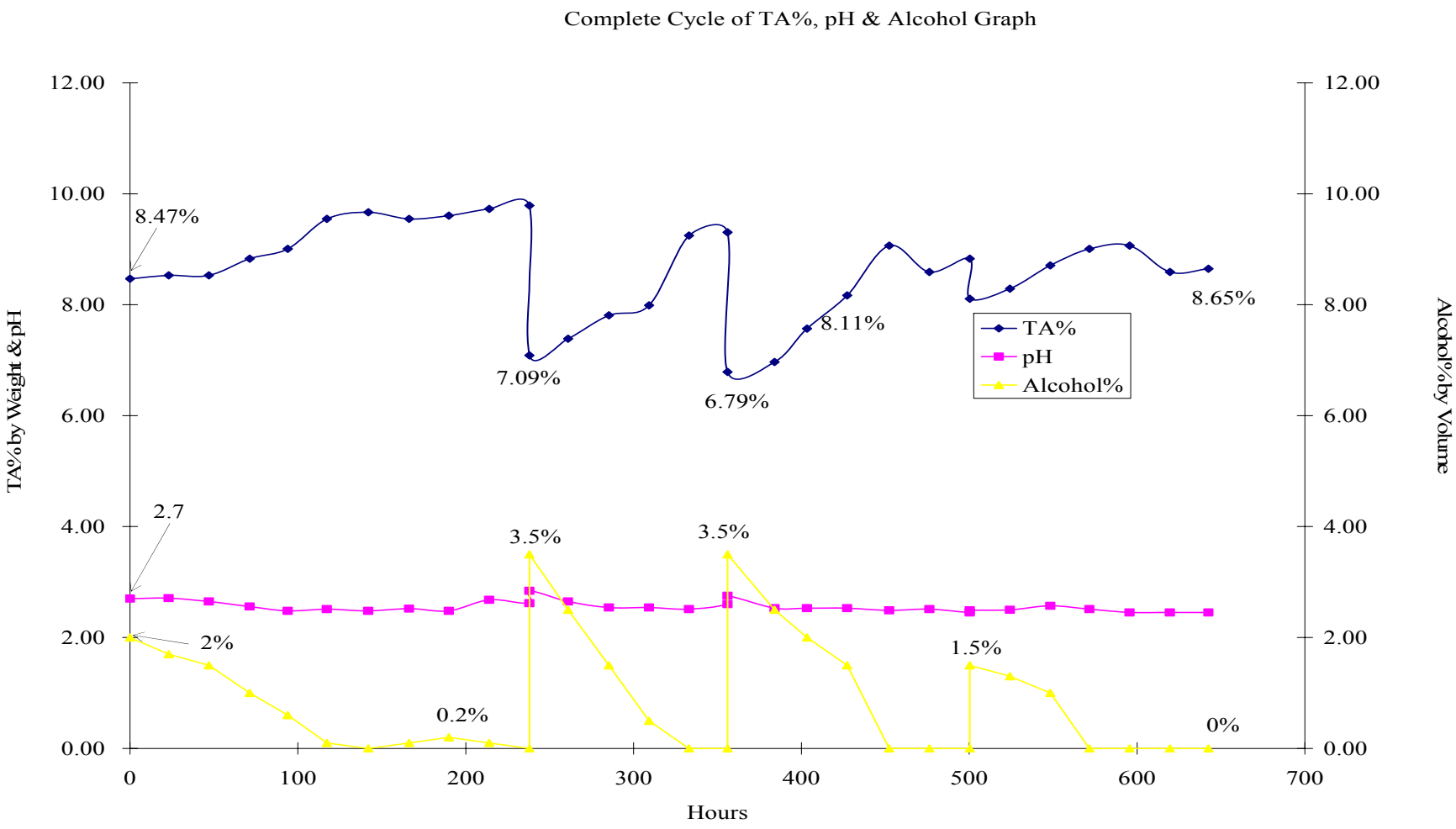
APPENDIX: ANALYTICAL DATA

A.1: Generator Pilot Unit Process Physicochemical Analysis

Date	Hours	Acid, %	pH	Alcohol, %	*Mix Temp, °F	*Gen Temp, °F	Level Gage	Air GMP	Remarks
27-Jul	0	8.47	2.7	2	74	80	normal	2	
28-Jul	23	8.53	2.71	1.7	76	80	normal	2	
29-Jul	47	8.53	2.65	1.5	76	80	normal	2	
30-Jul	71.3	8.83	2.56	1	79	80	normal	2	
31-Jul	94	9.01	2.48	0.6	82.94	86	normal	2	11gal
1-Aug	117.3	9.55	2.51	0.1	82.76	86	normal	2	<11gal
2-Aug	142	9.67	2.48	0	80.06	82.4	normal	2	11gal (Add)
2-Aug	166.3	9.55	2.52	0.1	80.42	82.4	normal	2	12.5gal
3-Aug	190	9.61	2.48	0.2	81.5	84.2	normal	2	
4-Aug	214	9.73	2.68	0.1	80.6	84.2	normal	2	
5-Aug	238	9.79	2.62	0	78.08	78.8	normal	2	w/4.17gal
5-Aug	0	7.09	2.84	3.5	78.26	80.6	normal	2	A/mash 4.17gal
6-Aug	23	7.39	2.65	2.5	80.06	80.6	normal	2	
7-Aug	47.3	7.81	2.54	1.5	84.5	87.8	normal	2	
8-Aug	71.3	7.99	2.54	0.5	85.64	89.6	normal	2	
9-Aug	95	9.25	2.51	0	84.56	87.8	normal	2	11gal
10-Aug	118	9.31	2.6	0	81.68	84.2	normal	2	w/4.17gal
10-Aug	0	6.79	2.75	3.5	81.14	84.2	normal	2	A/mash 4.17gal
11-Aug	28	6.97	2.53	2.5	83.3	86	normal	2	
12-Aug	47.3	7.57	2.53	2	86.18	89.6	normal	2	
13-Aug	71.3	8.17	2.53	1.5	87.62	89.6	normal	2	
15-Aug	96.3	9.07	2.49	0	83.12	86	normal	2	
16-Aug	120.3	8.59	2.51	0	81.86	84.2	normal	2	11gal
17-Aug	144.3	8.83	2.45	0	83.12	86	normal	2	w/4.17gal
17-Aug	0	8.11	2.49	1.5	83.12	86	normal	2	A/mash 4.17gal
18-Aug	24	8.29	2.5	1.3	83.84	87.8	normal	2	
19-Aug	48	8.71	2.57	1	86.54	89.6	normal	2	
20-Aug	71.3	9.01	2.51	0	88.52	91.4	normal	2	
21-Aug	95.3	9.07	2.45	0	86.36	89.6	normal	2	
22-Aug	119.3	8.59	2.45	0	81.68	84.2	normal	2	11gal
23-Aug	142.3	8.65	2.45	0	82.4	84.2	normal	2	w/4.17gal

*Mix Temp: Ferment Liquid Temperature, Gen Temp: Beech Wood Temperature

A. 2: Generator Pilot Unit Process Graph – Complete Cycle

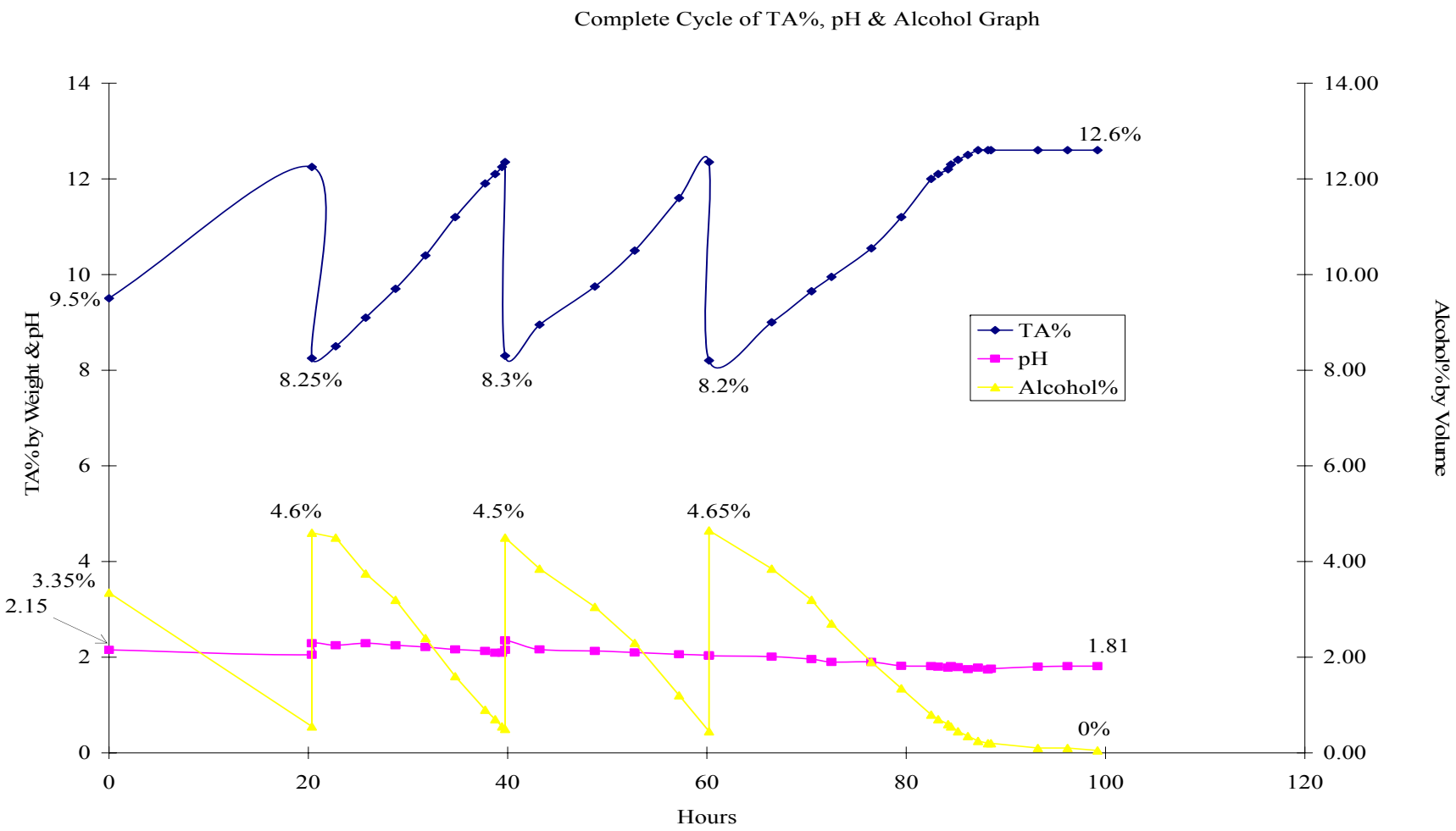


A.3: Lab Submerged Process Physicochemical Analysis

Date	Hours	*Volume, l	*TA,%	pH	Alcohol,%	Temperature	Remark
6-Jun	0	8	9.5	2.15	3.35	31.5	Starting from big tank
7-Jun	20.35	8	12.25	2.05	0.55	31.5	Discharge 2.6
7-Jun	0	8	8.25	2.29	4.6	31	
7-Jun	2.4	7.8	8.5	2.25	4.5	31.2	
7-Jun	5.4	7.6	9.1	2.29	3.75	31.3	
7-Jun	8.4	7.5	9.7	2.25	3.2	31.3	
7-Jun	11.4	7.4	10.4	2.21	2.4	31.5	
7-Jun	14.4	7.2	11.2	2.16	1.6	31.5	
8-Jun	17.4	7	11.9	2.13	0.9	31.5	
8-Jun	18.4	7	12.1	2.09	0.7	31.5	
8-Jun	19.1	7	12.25	2.09	0.55	31.5	
8-Jun	19.4	7	12.35	2.15	0.5	31.5	Discharge 2.6
8-Jun	0	8	8.3	2.35	4.5	30	
8-Jun	3.45	7.8	8.95	2.16	3.85	31.2	
8-Jun	9	7.7	9.75	2.13	3.05	31.2	
8-Jun	13	7.6	10.5	2.1	2.3	31.5	
8-Jun	17.45	7.5	11.6	2.06	1.2	31.2	
8-Jun	20.45	7.4	12.35	2.04	0.45	31.5	Discharge 2.6
8-Jun	0	8	8.2	2.03	4.65	30.5	
9-Jun	6.3	7.9	9	2.01	3.85	31.2	
9-Jun	10.3	7.8	9.65	1.96	3.2	31.2	
9-Jun	12.3	7.7	9.95	1.9	2.7	31.3	
9-Jun	16.3	7.5	10.55	1.9	1.9	31.2	
9-Jun	19.3	7.2	11.2	1.82	1.35	31.4	
9-Jun	22.3	7	12	1.81	0.8	31.4	
9-Jun	23	7	12.1	1.8	0.7	31.4	
9-Jun	24	6.9	12.2	1.78	0.6	31.4	Discharge 2.6
9-Jun	24.3	6.8	12.3	1.81	0.55	31.4	
9-Jun	25	6.7	12.4	1.79	0.45	31.4	
10-Jun	26	6.6	12.5	1.75	0.35	31.4	
10-Jun	27	6.5	12.6	1.78	0.25	31.4	
10-Jun	28	6.4	12.6	1.74	0.2	31.4	
10-Jun	28.3	6.3	12.6	1.76	0.2	31.4	
10-Jun	33	6.2	12.6	1.8	0.1	29.5	
10-Jun	36	6.1	12.6	1.81	0.1	29.5	
10-Jun	39	6	12.6	1.81	0.05	29.5	

* TA%: Titration Acidity in Percent, Volume l: Volume Represent in Litter

A.4: Lab Submerged Process – Complete Cycle



VITA

The author was born on February 19, 1979, in Johor Bahru, Malaysia. He spends his childhood life in Johor Bahru, Malaysia, until h graduated from Foon Yew High School. In January 2000, he started his first year of the college life in Nilai Inti College, Malaysia. Later he continued to pursue his studies in United States of America on August 2001. He graduated from University of Louisiana at Lafayette with a Bachelor of Science in Mechanical Engineering. After receiving his bachelor's degree, he entered the master's program in the Department of Food Science, Louisiana State University and Agricultural and Mechanical College, in the spring of 2004. He conducted research under Dr. Paul Wilson's supervision. He is a candidate for the degree of Master of Science in food science in December 2005.