CERTIFICATE

This is to certify the dissertation entitled "**WATERMELON VINEGAR**" submitted by **Ms. SUMAN MAYURI** in partial fulfilment of the requirement for the degree of **Master of Science in Applied Microbiology**, KIIT School of Biotechnology, KIIT University, Bhubaneswar, bearing Roll No. '***1662018***' & Registration No. **16530750272** is a *bona fide* research work carried out at CSIR-CFTRI by her under my guidance and supervision from **20-12-2018** to **03-05-2018.**

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CERTIFICATE

This is to certify that the dissertation entitled "**WATERMELON VINEGAR**" submitted by '**Suman Mayuri, *Roll No.* 1662018, Registration No 16530750272**.to the School of Biotechnology, KIIT University, Bhubaneswar-751024, for the degree of **Master of Science** in **Applied Microbiology** is her original work, based on the results of the experiments and investigations carried out independently by her during the period from **20-12-2017 to 03-05-2018** of study under my guidance.

Further, it is also to certify that the above said work has not been previously submitted for the award of any degree, diploma, or fellowship in any Indian or foreign University.

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Watermelon Vinegar

Dissertation submitted in partial fulfillment for the degree of

Master of Science in Applied Microbiology

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MAY - 2018

DECLARATION

I hereby declare that the dissertation entitled "**Watermelon Vinegar**" submitted by me, for the degree of Master of Science to KIIT University is a record of *bona fide* work carried by me under the supervision and guidance of '***Dr. S.V.N Vijayendra,*** **Principal Scientist',Microbiology and Fermentation Department, CSIR-Central Food Technological Research Institute, Karnataka, India.**I further declare that, the results of the work have not formed the basis for the award of any other degree to any candidate of any university.

Date:

Place: ***(Suman Mayuri)***

ABSTRACT

Watermelon (*Citrullus Lanatus*) is a warm season fruit and is grown worldwide. Natural vinegar can be produced using watermelon juice and can be used as a food additive. Watermelon (*Citrullus Lanatus*) juice was initially fermented with yeast culture *Saccharomyces cerevisiae* KTP to produce alcohol and the alcohol thus produced was subjected to fermentation by *Acetobacter aceti* to get vinegar. It was a slow and long process. The acetic fermentation was carried out with ethanol concentration 6-10% (v/v). and analyses of acetic acid, ethanol, total polyphenol content, total sugar, reducing sugar were conducted. The experiment showed that the optimum condition for acetic fermentation is with 6% (v/v) of ethanol. The vinegar obtained had a concentration of 2.664% acetic acid. Major polyphenols noticed were Myristic acid- 2.3722µg/ml and Syringic acid- 2.1917µg/ml. Reducing sugar was was found to be 0.7734 g/L.

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Date:

Place: ***(Suman Mayuri)***

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS/ACRONYMS

Kg Kilogram (s)

L Litre

mg Milligram

Nm Nanometer

Ppm Parts per million

HPLC High Performance Liquid Chromatography

YEPDA Yeast Extract Peptone Dextrose Agar

DNS Dinitro Salicylic Acid

KMS Potassium Meta bisulphite

DW Distilled Water

TSS Total Soluble Solids

RT Retention Time

CHAPTER 1

INTRODUCTION

1.1 Background and Context

Vinegar, from the French *vin aigre*, meaning "sour wine," can be made from almost any carbohydrate source, including wine, molasses, dates, sorghum, apples, pears, grapes, berries, melons, coconut, honey, beer, potatoes, beets, malt, grains, and whey.

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. The vinegar whether naturally produced during fermentation or commercially prepared acetic acid (4%) retards the microbial growth and contributes sensory properties to a number of foods. The wide diversity of products containing vinegar (sauces, ketchup, etc.) and the current fall in wine consumption have favoured an increase in vinegar production (De Ory et al 2002).

Acetic acid is the predominant flavouring and antimicrobial component in vinegar. Earlier processes used for making vinegar were the Orleans process (slow process), generator process (quick process) and the submerged culture process. The quick process and submerged culture process were developed and are used for commercial vinegar production.

Acetic acid is formed in a three-step reaction involving anaerobic conversion of sugars to ethanol by yeast fermentation, conversion of ethanol to hydrated acetaldehyde, and dehydrogenetion to acetic acid by aldehyde dehydrogenase (Nichol 1979; Canning 1985). The last two steps are performed aerobically with the aid of acetic acid prodcuing bacteria. 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.

Most bacteria strains derived from vinegar factories are able to oxidize acetic acid to Carbon dioxide and water (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.

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 reason for choosing watermelon as a precedence was, watermelon is nominally 60% flesh, and about 90% of the flesh is juice that contains 7 to 10% (w/v) sugars. Thus, over 50% of a watermelon is readily fermentable liquid.

1.2 Scope And Objectives

- Optimization of juice production from the watermelon
- Standardization of the fermentation parameters for vinegar production.

CHAPTER -2

REVIEW OF LITERATURE

2.1 Watermelon

Citrullus lanatus commonly called as watermelon is a popular fruit in many parts of the world and it is notable for its high water content and attractive look. The fruit comes in various shapes, sizes and rind pattern (Wehner, 2008).

The plants have weak stems and climb by tendrils, which hang from tree, the watermelon fruit matures on the ground. The drying of the tendril at the point of attachment the fruit stem to the vine is also considered a sign of maturity (Soteriadou, 1969).

Fruit shape is often spherical but can be globular, oval or oblong. The watermelon rind consists of two layers. The thin, glossy outer layer. The thick inner layer of the rind, or mesocarp, is wet, white and hard. Underneath the rind is the watery fruit flesh or endocarp, the portion of the fruit that is usually eaten. The colour of the rind varies in shades of green from a pale yellowish green to a deep blackish-green.

Early in development, the fruit flesh is hard, white or otherwise pale-coloured, and insipid.

In citron watermelons, the fruit flesh remains hard, nearly colourless and tasteless to fruit maturity (Xu *et al*., 2012). In sweet dessert watermelons, the flesh of the maturing fruit becomes tender and accumulates carotenoid pigments and sucrose (Elmstrom and Davis, 1981; Brown and Summers, 1985; Soteriou *et al*., 2014). Colour begins to accumulate between 2 and 3 weeks after anthesis, first around the developing seeds and thereafter gradually spreading throughout the endocarp (Perkins-Veazie *et al*., 2012). Depending on the genotype, the flesh of ripe watermelon fruits can range in colour from red to pink, orange, yellow, a mixture of these colours, green and white (Gusmini and Wehner, 2006). The range in texture of the ripe fruit flesh has been variously described as crisp, soft or liquefied, and coarsely or finely grained. Each fruit can contain 200 or more seeds that, to the casual

observer, are seemingly scattered throughout the flesh and, to the consumer, are of great annoyance. The seeds of sweet watermelons are hard, flat and oval and, depending on cultivar, generally range in length from 8 to 16 mm and can be black, brown, tan, white, yellow or red, and can be patterned with a second colour.

Because of consumer demand for sweet and flavorful watermelons, total sugar content is an important quality factor. One way to determine field maturity before harvest is to cut a few melons taken from random parts of the field and test their sugar level using a hand refractometer. High quality watermelon should have a sugar content (measured as total soluble solid) of 10 percent or more in the flesh near the centre of the melon (William, 1999).

Watermelon is rich in vitamin C, vitamin A, vitamin B, amino acid and also carotenoid lycopene. The red flesh of watermelon contains some vitamin A (Anon, 2008). Watermelon is rich in vitamin B that is primarily responsible for the production of energy in your body. Hence, consuming watermelon can boost the energy levels. Watermelon can be viewed as a more nutritious alternative to having energy drinks or supplements prior to exercise. Watermelons are also a good source of vitamin C, which is an essential nutrient for humans, because it aids in the synthesis of collagen in addition to protecting against oxidative damage. Vitamin C consumption has been shown to protect against stomach, oral, and lung cancers, improve cholesterol, and prevent scurvy (Fontham *et al*., 1988; Block, 1991; Ness *et al*., 1996).Vitamin C is very sensitive to heat and degrades very quickly during pasteurization.

Watermelon is rich in amino acid called citrulline, which helps us to produce another amino acid called arginine. According to the USDA Nutrient Database (2009), watermelons contain 40% more lycopene (per 100 g) than raw red ripe tomatoes.

Lycopene is a carotenoid that provides the red color to tomatoes, watermelons, and red bell pepper, among other fruits and vegetables. Lycopene is a powerful antioxidant and has been shown to prevent various cancers and help against heart disease (Rao and Agarwal, 1999). Lycopene quenches free radicals which prevents oxidative damage which subsequently prevents many cancers. Fish et al. (2002) stated that watermelon is a gastronomically pleasing food and is rich in lycopene which makes it a highly

desirable source of this phytochemical. The seeds are also reported to possess medicinal properties and are used to treat chronic or acute eczema.

Due to its low acidity and growing conditions, watermelon is regarded as a potentially hazardous food (FDA, 2001). In the fruit juice industry, juice is typically pasteurized by high temperature short time (HTST) pasteurization. This process uses plate heat exchangers to heat the sample quickly at least 78°C. Although this method is effective at inactivating microorganisms and enzymes, it can cause detrimental effects on the quality of the juice. Heat treatment may cause color change, separation of particles, and a change in flavor and/or smell (Qin *et al.,* 1995). If heat treatment is not performed rapidly or at a reasonably low temperature, the juice will begin to separate due to the destruction of pectin (Goodman *et al*., 2002). Compared with thermal pasteurization, non-thermal processing offers the advantages of low process temperatures, which results in a better retention of flavors and nutrients (Vega-Mercado *et al*., 1997). Thermal pasteurization can cause pigments to degrade which may reduce the color and appearance of fruit juice.

Traditionally, the seed of *C. lanatus* is said to be medicinal because it can relieve inflammation/irritation; causes increased passing of urine and gives tonic effects (Okunrobo et al., 2012; Varghese et al., 2013).

2.2 Vinegar

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).

2.2.1 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).

2.2.2 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.

2.2.3 Medicinal Uses of Vinegar

- **Anti-infective Properties**

The use of vinegar to fight infections and other acute conditions dates back to Hippocrates (460-377 BC; the father of modern medicine), who recommended a vinegar preparation for cleaning ulcerations and for the treatment of sores. Oxymel, a ancient medicine composed of honey and vinegar, was prescribed for persistent coughs by Hippocrates and by physicians up to modern day. (Diggs L. Vinegar 200)The formulation of oxymel was detailed in the *British Pharmacopoeia* (1898) and the *German Pharmacopoeia* (1872), and, according to the *French Codex* (1898), the

medicine was prepared by mixing 4 parts of virgin honey, with 1 part of white wine vinegar, concentrating and clarifying with paper pulp.(Felter HW 2006)

Similarly, experts caution against using vinegar as a household disinfectant against human pathogens because chemical disinfectants are more effective.(Rutala et al 2000) However, undiluted vinegar can be used for cleaning dentures, and, unlike bleach solutions, vinegar residues left on dentures were not associated with mucosal damage(Shay K 2000)

Although investigations have demonstrated the effectiveness of diluted vinegar (2% acetic acid solution at pH 2) for the treatment of ear infections (otitis externa, otitis media, and granular myringitis), (Jung HH et al) the low pH of these solutions may irritate inflamed skin and damage cochlear outer hair cells.(Dohar JE) Immediate vinegar application at the site of jellyfish stings is practiced at various coastal locations around the world (Macrokanis et al 2004) (Vilan A) because vinegar deactivates the nematocysts. However, hot-water immersion is considered the most efficacious initial treatment for jellyfish envenomation because the venom is deactivated by heat. (Perkins RA et all),(Nomura JT et al).

- **Anti-tumor Activity**

In vitro condition, sugarcane vinegar induced apoptosis in human leukemia cells, (Mimura A et al 2004) and a traditional Japanese rice vinegar inhibited the proliferation of human cancer cells .(Nanda K et al 2004)Vinegars are also a dietary source of polyphenols,(US. Food and Drug Administration 2006)compounds synthesized by plants to defend against oxidative stress. Ingestion of polyphenols in humans enhances in vivo antioxidant protection and reduces cancer risk.(Nishino H et al 2005)

2.2.4 Vinegar Formation

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).

Initially, yeasts ferment the sugars to alcohol. Next, acetic acid bacteria (*Acetobacter*) convert the alcohol to acetic acid.

The alcoholic fermentation and acetification processes play a key role in vinegar production. Different biotransformations can take place depending on the environmental factors (temperature, pH,) or the nutrients (sugar sources) and the microbial diversity present in the raw material. Microbial species involved in fermentations may range from yeast and lactic acid bacteria to molds and AAB (Nanda et al., 2001; Haruta et al., 2006; Wu et al., 2010).

Alcoholic fermentation (AF) is a fermentation step common to all vinegars. This is a biological process in which sugars, such as glucose, fructose, and sucrose, are converted into cellular energy, ethanol and carbon dioxide (CO_2). This process is mainly carried out by yeast. Among yeast, *Saccharomyces cerevisiae* is the most widespread species (Ribéreau-Gayon et al., 2006; Aruta et al., 2006; Wu et al., 2010)

Acetification is commonly known as the oxidation of the ethanol. Once the sugar has been converted into ethanol, the second bioprocess is carried out by AAB and consists of an oxidation that is highly dependent on the availability of oxygen. In vinegar production, the use of back slopping is a very common practice to start the acetification. It consists of using part of a previously acetified batch, which is called "mother vinegar", to inoculate a new batch. This practice makes the process more reliable and faster than the spontaneous one.

The vinegar mother is an undefined starter culture that increases the initial number of AAB cells. It is composed of mainly cellulose.

Commercial vinegar is produced by either fast or slow fermentation processes. For the quick methods, the liquid is oxygenated by agitation and the bacteria culture is submerged permitting rapid fermentation. The slow methods are generally used for the production of the traditional wine vinegars, and the culture of acetic acid bacteria grows on the surface of the liquid and fermentation proceeds slowly over the course of weeks or months. The longer fermentation period allows for the accumulation of a nontoxic slime composed of yeast and acetic acid bacteria, known as the mother of vinegar. Most manufacturers filter and pasteurize their product before bottling to prevent these organisms from forming. After opening, mother may develop in stored vinegar; it is considered harmless and can be removed by filtering. Many people advocate retaining the mother for numerous, but unsubstantiated, health effects.

The chemical and organoleptic properties of vinegars are a function of the starting material and the fermentation method. Acetic acid, the volatile organic acid that identifies the product as vinegar, is responsible for the tart flavor and pungent, biting odor of vinegars. However, acetic acid should not be considered synonymous with vinegar. The US Food and Drug Administration (FDA) states that diluted acetic acid is not vinegar and should not be added to food products customarily expected to contain vinegar.(US Food and Drug Administration. Acetic Acid - Use in Foods.) Other constituents of vinegar include vitamins, mineral salts, amino acids, polyphenolic compounds (e.g, gallic acid, catechin, caffeic acid, ferulic acid), and nonvolatile organic acids (eg, tartaric, citric, malic, lactic).[4,5]

White distilled vinegars generally have 4% to 7% acetic acid, whereas, cider and wine vinegars contain 5% to 6% acetic acid. Specialty vinegars are grouped as herbal or fruit vinegars. Herbal vinegars consist of wine vinegars or white distilled vinegars, which may be seasoned with garlic, basil, cinnamon, clove, or nutmeg. Fruit vinegars are wine and white vinegars sweetened with fruit or fruit juice to produce a characteristic sweet-sour taste. Traditional vinegars are produced from regional foods according to well-established customs. The balsamic vinegar is made from the white Trebbiano grapes, which are harvested as late as possible, fermented slowly, and concentrated by aging in casks of various woods. Traditional rice wine vinegars are produced in Asia, coconut and cane vinegars are common in India and the Philippines, and date vinegars are popular in the Middle East.

2.2.5 Yeasts for Alcohol Fermentation

Yeasts are the most important group of microorganisms during AF because they influence fermentation speed, wine flavor and other wine qualities (Pretorius, 2000; Fleet, 2003; Loureiro and Malfeito-Ferreira, 2003; Jolly et al., 2006). The *Saccharomyces* genus is the most commonly used in beverage industry. The *Saccharomyces* genus has several unique characteristics that are not found in other genera, such as their higher capacity to ferment sugars (Fleet and Heard, 1993). This ability allows them to colonize sugar-rich media.

2.2.6 Vinegar Bacteria

The ninth edition of Bergey's Manual of Systematic Bacteriology classifies the acetic acid bacteria in the family Acetobacteriaceae and Gluconobacter (Buchanan and Gibbons 1974). Acetic acid bacteria are Gram-negative or Gram-variable bacteria, ellipsoidal to rod-shaped cells that have a required aerobic metabolism with oxygen as the terminal electron acceptor (Gonzalez et al 2004).And they are catalase positive and oxidase negative.

These bacteria are usually found in substrates containing sugar and/or ethanol. These substrates include fruits, flowers, food and fermented beverages, such as fruit juices, wine, cider, beer, cocoa and vinegar (Thompson et al., 2001; Nielsen et al., 2007; Yamada and Yukphan, 2008)

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.

2.2.7 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 1**) from Kehrer 1921:

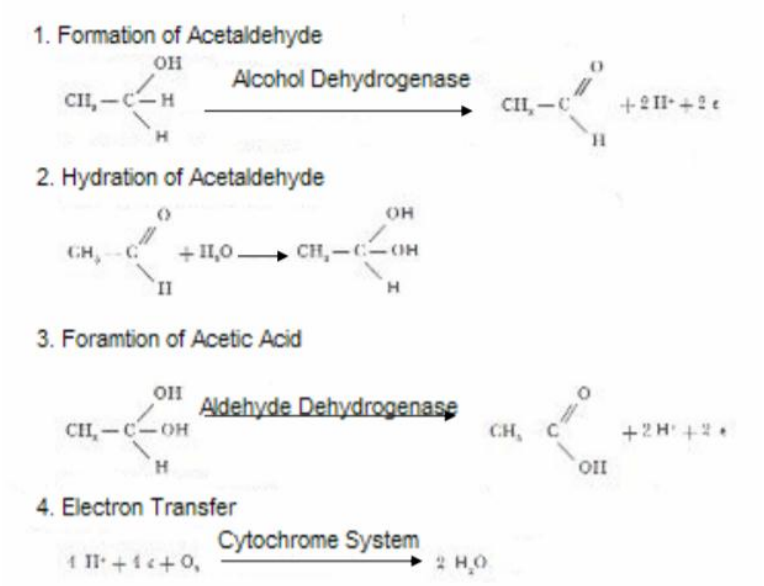


Figure-1. Conversion of alcohol to acetic acid

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.

2.2.8 Production Methods

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)

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 cannot 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) It is a static method that is traditionally employed for the manufacture of high-quality vinegars.

In this case, the presence of AAB is limited to the surface of the acidifying liquid. In other words, they are placed on the air–liquid interface in direct contact with air and hence with the available atmospheric oxygen to allow the conversion of alcohol into acetic acid (Laguno and Polo, 1991).

In this process, wood barrels from (Cruess 1958) are used and filled with alcohol fermenting liquid to approximately ¾ full (**Figure 2**). 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.

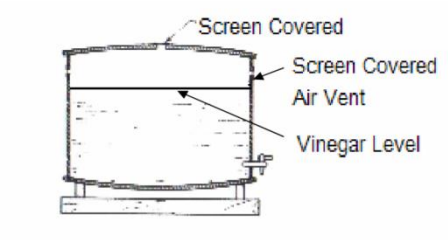


Figure-2 Orleans Process Barrel

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).

The main drawback of this method is the long period of time required to obtain a high acetic acid concentration, resulting in increased production time and costs.

2. Generator Fermentation

Early in the nineteenth century, a vinegar-making system called the trickle method [now called generator fermentation or quick process] 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 3**) from (Cruess 1958) of 2000 cubic feet capacity above a perforated wood grating floor.

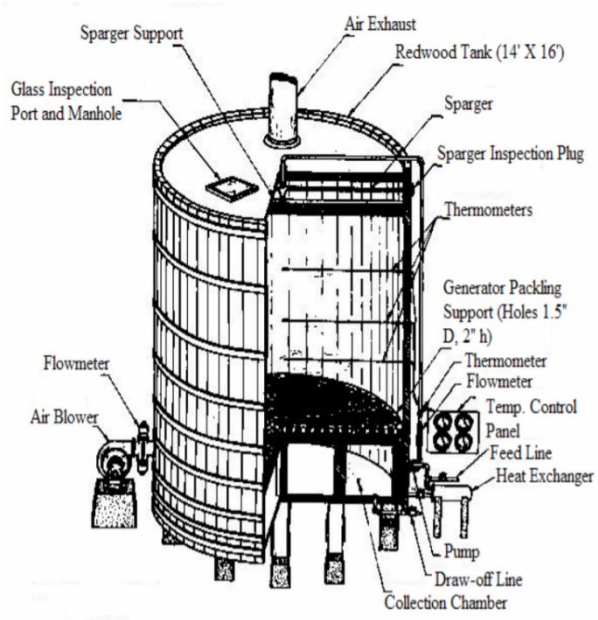


Figure-3 Vinegar Generator

The wood shavings (**Figure 4**) from (Peppler and Beaman 1967) are generally made of air-dried beech wood sliced to form a coil about 2 inches long and 1 inch in diameter.

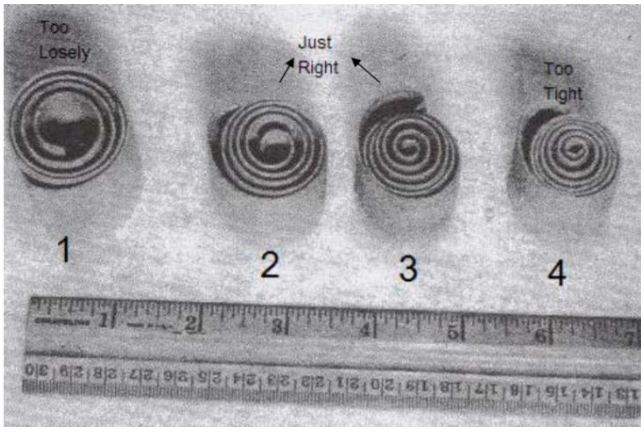


Figure- 4 Beech wood shavings

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* sp. 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).

3. Submerged Fermentation

Currently, the most common production method is submerged culture (Figure 5) 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 6) from (De Ory et al 2002). This operation consists of the development of successive discontinuous cycles of acetification. At the 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).

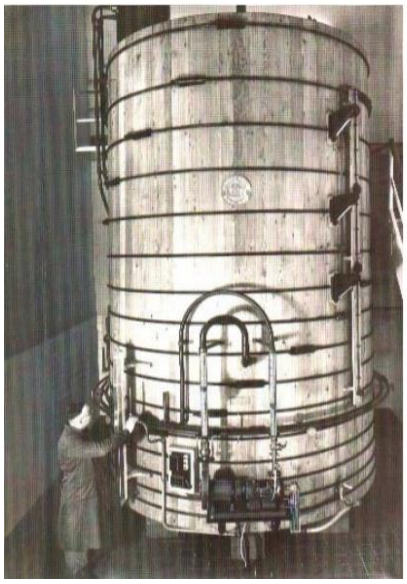


Figure 5- Submerged Process

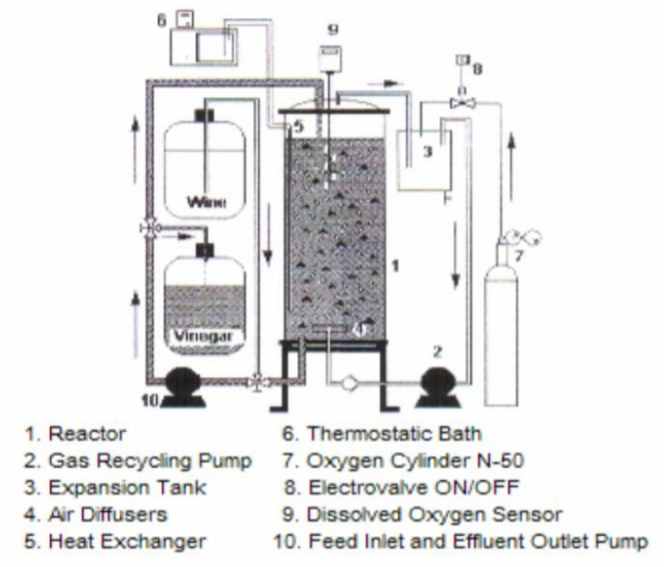


Figure-6 Semi-Continuous Process

The main advantages of the submerged method in comparison to the traditional one include a high acetification speed, which is capable of producing a high acetic acid concentration in a short time (1-2 days), the production of large volumes of vinegar, and control of the environment to create the optimal conditions for AAB acetification. However, one of the main problems with this method is loss of volatile compounds, such as ethanol, acetic acid or ethyl acetate, due to the recirculation system. This system reduces the production yield and the quality of the product, and it increases the operational costs (Romero and Cantero, 1998).

2.3 Vinegar Quality 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).

2.3.1 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, 20 acetoin, praline, and total acidity-oxidation index quotient. Figure 7 (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.

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

	Vinegar categories									
	Submerged 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

Figure 7 Acid and Volatile Compounds in Vinegars

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

AIMS AND OBJECTIVES

Vinegar is the product made from the conversion of ethyl alcohol to acetic acid by a genus of bacteria, *Acetobacter* sp. Vinegar can be prepared from almost any carbohydrate source, like wine, apples, pears, grapes, berries, melons, coconut, honey, beer, potatoes, beets, malt, etc. Vinegar can be made from any vegetables or fruits which have high sugar content, if the sugar content is less, then sucrose can be added for the production of good quality of alcohol. The alcohol can then be converted to acetic acid in 2^{nd} step of fermentation.

Watermelon has high water content as well as sugar. Watermelon is rich in vitamin B that is primarily responsible for the production of energy in your body. Hence, consuming watermelon can boost the energy levels. Watermelon can be viewed as a more nutritious alternative to having energy drinks or supplements prior to exercise. Watermelons are also a good source of vitamin C, which is an essential nutrient for humans, because it aids in the synthesis of collagen in addition to protecting against oxidative damage.

Hence, for its good sugar content and high nutritious value, watermelon was used as a substrate for the production of vinegar

The main aim of the study was to optimize the parameters for the production of vinegar from watermelon juice.

The objectives are:

- Standardization of watermelon juice for vinegar production.
- Optimization of vinegar production using watermelon juice.

CHAPTER-3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Raw materials:

Dark green watermelons procured from HOPCOM vegetable vending shop located in CFTRI campus, Mysuru.

3.1.2 Culture

Yeast culture *Saccharomyces cerevisiae* KTP strain and *Acetobacter aceti* available at from the Department of Microbiology and Fermentation Technology, CFTRI, Mysuru, were used for the production of watermelon vinegar.

3.1.3 Chemicals

The 3,5 – dinitrosalicyclic acid , chromic acid, sulphuric acid , phenol, sodium hydroxide, gallic acid (Sigma-Aldrich), Folin Ciocalteu reagent, sodium carbonate, sucrose (Hi Media), yeast extract (Hi Media), peptone (Hi Media), dextrose, phenolphthalein indicator, potassium bisulphite were used in this study.

3.1.4 Apparatus

Borosil flasks, Borosil test tubes, beakers, Petri plates, scissors, forceps, Eppendorf's, falcon tubes and other laboratory glassware.

3.2 EQUIPMENTS:

Equipments involve those required for specific operation in the whole course of study. These includes the operation of Weighing, Sterilization, Incubation, Aseptic Environment for microbiological analysis, Refrigeration, Centrifugation, Distillation unit, Spectrophotometer, Autoclave, Water Bath, High Performance Liquid Chromatography, Laminar Air Flow, Hot air oven, Refractometer and other essential lab equipment.

3.3 METHODS USED FOR ANALYSING SAMPLES

3.3.1 Physical Parameters

Total soluble solids were determined using hand refractometer (range from 0-50%).

3.3.2 pH

The pH of the watermelon juice and natural fruit vinegar was checked by using a microprocessor controlled digital pH meter.

3.3.3 Method for Estimation of Reducing Sugar (Miller,1959)

Materials Required:

Reagent Preparation- DNS Reagent- Dinitrosalicylic acid-1 gram was dissolved in 20 ml 2 N NaOH, 30 grams of Sodium Potassium Tartarate was added and boiled to get a clear solution.

Standard Stock- One gram of glucose was dissolved in 100 ml of distilled water in a volumetric flask, giving a concentration of 10 mg/ml.

Working Standard- One milliliter of stock solution was made up to 10 ml using distilled water to get a concentration of 1 mg/ml.

Procedure:

Preparation of standard curve

- Different aliquots of standard solution (0.1-1 ml) were taken in test tubes.
- The volumes were made up to 1 ml with distilled water.
- To all these test tubes 1 ml of DNS reagent was added.
- The test tubes were covered and heated in a boiling water bath for 5 mins.
- To all these test tubes 10 ml of distilled water was added.
- Colour developed was observed using UV spectrophotometer at 540 nm against a reagent blank.
- A standard graph was obtained by plotting concentration of glucose on X-axis and optical density on Y-axis.

Sample

- Watermelon juice was diluted appropriately and used.
- One milliliter of watermelon juice was taken and volume was made up to 10 ml using distilled water.

3.3.4 Method for Estimation of Total Acidity

Materials Required:

Reagent- 0.1N NaOH Solution, 0.4 grams of NaOH was dissolved in 100 ml distilled water in a volumetric flask.

0.1N Oxalic acid solution, 0.63 grams of oxalic acid was dissolved in 100 ml of distilled water.

Phenolphthalein Indicator

Procedure:

- Standardization of 0.1N NaOH using 0.1N oxalic acid
- Five milliliters of 0.1 N oxalic acid solution was transferred to a conical flask.
- Two drops of phenolphthalein indicator was added to the solution.
- From the burette, 0.1N NaOH was added gradually with continuous swirling of the solution.
- The addition of NaOH was continued until the colour of the solution passes from colourless to faint pink.
- The above four steps were repeated three times.
- The volume of NaOH consumed was recorded.

Sample

- One milliliter of watermelon juice was taken and diluted with 9ml of distilled water.

- Five milliliters of diluted watermelon juice was taken in a Borosil flask and 2 drops of phenolphthalein was added to it.
- The mixture was titrated against 0.1N NaOH.
- The volume of NaOH consumed was recorded.

3.3.5 Method for Estimation of Total Alcohol by Chromic Acid Method (Caputi et al., 1968):

Materials Required:

Reagent: Chromic acid solution, 8.5 grams of potassium dichromate was dissolved in 125 ml of distilled water and was cooled ice water bath. A 81.25 ml concentrated sulphuric acid was added slowly. The final volume was made up to 250 ml with distilled water.

Standard Stock: Different concentration (2-10%) of ethanol was prepared and made up to 50 ml using distilled water.

Distillation: Two milliliters from each of the concentration was taken and volume was made up to 50 ml using distilled water in a measuring cylinder.

- The solution was transferred to a distillation flask.
- Distillation was carried out at 55 ºC and first 15 ml of distillate was collected.

Procedure

Preparation of Standard Curve

- Aliquots of different concentrations of ethanol (2-10%) were prepared using distilled water.
- A 2.5 ml of distillate was taken in test tubes and to it 4.166 ml chromic acid and 1.666 ml distilled water were added.
- The test tubes were incubated at 50 ºC for 30 minutes.
- The test tubes were cooled to room temperature and the colour developed was observed in a UV Spectrophotometer at 600 nm against a reagent blank.

- A standard graph was obtained by plotting concentration of ethanol on X-axis and optical density on Y-axis.
- Alcohol concentration in wine was estimated by following the same procedure and using standard graph for ethanol.

3.3.6 Method for Estimation of Total Sugar by Phenol Sulphuric Acid Method (Duboise Method)

Materials Required

5% Phenol - 5 grams of phenol was added to 100 ml of distilled water.

Standard Stock - 100 mg of Glucose was dissolved in 100 ml of distilled water in a volumetric flask.

Working solution - 1 ml of standard stock solution, made up to 10ml using distilled water. Then again it was diluted with distilled water in the ratio 1:10.

Procedure:

Preparation of Standard Curve:

- Different aliquots of standard solution (0.2-1 ml) were taken in test tubes.
- The volumes were made up to 1 ml with distilled water.
- One milliliter of 5% phenol was added to all the test tubes.
- Five milliliters of sulphuric acid was added to all the test tubes.
- Test tubes were cooled at room temperature.
- Colour developed was observed using UV Spectrophotometer at 490 nm against a reagent blank.
- A standard graph was obtained by plotting concentration of glucose on X- axis and optical density on Y-axis.

3.3.7 Method for Estimation of Total Polyphenol using Folin & Ciocalteu's Phenol Reagent:

Materials Required:

20% Sodium Carbonate- 20 grams of sodium carbonate in 100 ml of distilled water

50% Folin Ciocalteu's Phenol Reagent - 5 ml of Folin & Ciocalteu's Phenol Reagent was added to 10 ml of distilled water

Standard stock- 10 mg of gallic acid was dissolved in 10 ml of distilled water.

Working solution- 400 µl of standard stock solution was added to 4 ml of distilled water.

Procedure:

Preparation of Standard Curve:

- Different aliquots of standard solution (200 -1000 µl) were taken in test tubes.
- Six milliliters of distilled water, 0.5 ml of FCR, and 1.5 ml of sodium carbonate were added to each test tube.
- A 1.8 ml, 1.6 ml, 1.4 ml, 1.2 ml, 1 ml distilled water was added to 200µl to 1000µl solutions sequentially.
- The test tubes were incubated for 15 minutes at room temperature.
- Colour developed was observed using UV Spectrophotometer at 760 nm against a reagent blank.
- A standard graph was obtained by plotting concentration of gallic acid on X- axis and optical density on Y-axis.

3.3.8 High Performance Liquid Chromatography for Polyphenols (Mradu et al., 2012)

Sample Preparation-

- Solvent Extraction was performed for estimation of phenolics compounds of watermelon juice samples.
- Two milliliters samples were taken in Eppendorf tubes and were centrifuged at 8000 rpm for 10 minutes.
- The supernatant was collected and again centrifuged at 8000 rpm for 10 minutes.
- The supernatant was collected and it was filtered through membrane filter of 0.45µM.
- After filtration the samples were ready to carry out high performance liquid chromatography.

Procedure:

- Liquid Chromatography was carried out in Shimadzu LC-10 system coupled with Photo Diode Array (PDA) detector (wavelengths 280 nm and 320 nm) using a C-18 column.
- The run time for each sample was 60 minutes.
- Injection volume was 20 µl.
- Flow rate was 1 ml/min.
- Two mobile phases were used for the chromatography- Solvent A- Acetonitrile and Solvent B- Milli-Q water and ortho-phosphoric acid (500 µl of ortho-phosphoric acid was dissolved in 500 ml of Milli-Q water.)
- The gradient program was as follows:-

0-15 min- A- 8%; B-92%
15-30 min- A- 8%; B-92%
30-45 min- A-22%; B-78%
45-55 min- A-78%; B-22%
55-60 min- A-8%; B-92%

3.3.9 High Performance Liquid Chromatography for Organic Acids (Kelebek et al., 2009):

Sample Preparation-

- Solvent Extraction was performed for estimation of phenolics compounds of watermelon juice samples.
- Two milliliters samples were taken in Eppendorf tubes and were centrifuged at 8000 rpm for 10 minutes.
- The supernatant was collected and again centrifuged at 8000 rpm for 10 minutes.
- The supernatant was collected and it was filtered through membrane filter of 0.45µM.
- After filtration the samples were ready to carry out high performance liquid chromatography.

Procedure:

- Liquid Chromatography was carried out in Shimadzu LC-10 system coupled with Photo Diode Array (PDA) detector (ƛ- 210 nm and 254 nm) using a C-18 column.
- The run time for each sample was 30 minutes.
- Injection volume was 20 µl.
- Flow rate was 0.7 ml/min.
- One mobile phase was used for the chromatography- Milli-Q water and sulphuric acid (220 µl of sulphuric acid dissolved in 500 ml of Milli-Q water).
- The gradient program used was - Isocratic.

3.4 Selection of Yeasts

3.4.1 Culture- *Saccharomyces cerevisiae KTP* was used for the fermentation of watermelon juice.

3.4.2 Inoculum Preparation:

YEPD (Yeast Extract Peptone Dextrose) broth medium was used to prepare the yeast inoculum. It consists of yeast extract, peptone and dextrose at 1, 2, 2%, respectively. Pure slant culture of *Saccharomyces cerevisiae KTP* strain preserved in the Department of Microbiology and Fermentation Technology, CFTRI, Mysuru, Karnataka, India, was collected and subcultured using YEPD broth.

A 100 ml of YEPD broth was prepared in the Erlenmeyer flask of 250 ml capacity and autoclaved. A loopful of yeast culture was added into the YEPD broth after sterilization and incubated at 28 ±1 ºC in a shaking incubator at 150 rpm for 24 hours.

The incubated YEPD broth was centrifuged at 8000 rpm for 15 minutes. After centrifugation the yeast cell suspension was collected into a separate sterilized container. The entire process was carried out under aseptic condition.

3.5 Production of alcoholic beverage exclusively with watermelon juice:

Fresh mature watermelons were purchased from a HOPCOM shop located in CFTRI campus. Total weight of the fruit was checked using a balance. The fruits were thoroughly washed with distilled water to remove dirt, dust, pesticide residues. All glassware and knives were cleaned and sanitized prior to usage. The watermelons were cut into quarters and the flesh was scooped out and cut into small cubes. Watermelon seeds were removed. The cubes were placed in a juice processor. Then the extracted juice was then filtered through cheese cloth.

Sugar content of the juice was determined with hand refractometer as brix (total soluble solids). And final sugar concentration was adjusted to 20° brix by adding sucrose.

The entire volume was divided into two sterilized 500 ml Erlenmeyer flasks. Two flasks were subjected to two different conditions. To one flask 100 ppm of sulphur

dioxide was added in the form of potassium metabisulphite. And the other flask is subjected to boiling for 10 minutes. The boiled watermelon juice was allowed to cool.

A 10% yeast inoculum (culture suspension) was added to both the flasks.

The flasks were closed with cotton plug and allowed to ferment and incubated at 30 ºC incubator for 24 hours.

The selection of yeast was based on the growth of the yeast to produce high alcohol and suitable fermentation time.

3.6 Vinegar Production from watermelon alcohol

The fermented watermelon juice (beverage) produced by yeast fermentation was used as the substrate for the production of vinegar.

3.6.1 Subculturing *Acetobacter aceti* (smooth mat producing strain)

Medium composition- Yeast Extract- 2 grams, Glucose - 0.6 grams, Agar agar -1.9%, Distilled water-100 ml (pH adjusted to 7.4).

Medium was autoclaved and *Acetobacter aceti* smooth strain was subcultured from pure culture collected from Department of Microbiology and Fermentation Technology, CFTRI, Mysuru.

After inoculation, the medium was incubated at 30 ºC.

3.6.2 Determination of *Acetobacter aceti*'s (smooth strain and rough strain) morphology

A loopful culture of *Acetobacter aceti* smooth and rough strain were taken and smear were made on glass slides and heat fixed and allowed to dry.

Gram staining was done.

- The smear were flooded with crystal violet and left for 1 minute. Then gently rinsed with tap water.
- Then the smear were flooded with Gram's Iodine and left for 1 minute. And then rinsed with tap water.

- Decolourised with 95% ethanol. Immediately rinsed with tap water.
- Lastly, the smear were flooded with safranin to counterstain for 45 seconds and then rinsed with tap water.
- The smears were viewed using a light microscope under oil-immersion.

3.6.3 Subculturing the Mother of Vinegar or Scoby

Grape wine prepared previously and kept in the cold storage of CFTRI was collected. A 100 ml of wine is poured in to a sterile flask and a lump of Mother of Vinegar (smooth strain) was added to it and cotton plugged, incubated at 30º C.

3.6.4 Production of Vinegar from fermented watermelon beverage

After 24 hours of yeast fermentation, the sub cultured *Acetobacter aceti* (smooth strain) scoby was added to both flasks i.e, KMS and boiled. And the flasks were incubated at 30 ºC.

3.7 Addition of 5% sucrose solution

5% Sucrose solution was added to both the flask to decrease the alcohol content and make it suitable for Acetobacter aceti to grow.

3.8 Inoculation of Mother of Vinegar (rough strain)

After subculturing the rough strain scoby, a lump of rough strain scoby was inoculated in both the flask.

3.9 Determination of quality parameters

3.9.1 Physical Parameters:

The sample was checked for pH by using a digital pH meter and Total Soluble Solids (ºBrix) was observed by using a hand refractometer.

3.9.2 Method for Estimation of Reducing Sugar (Miller, 1959):

Appropriately diluted samples were taken and measured as procedure described in watermelon juice analysis.

3.9.3 Method for Estimation of Total Acidity:

Vinegar samples after 1:10 dilution were taken and measured as procedure described in watermelon juice analysis.

3.9.4 Method for Estimation of Total Alcohol content (Caputi et al,1968):

2.5 ml of distillate were taken and measured as procedure described in watermelon juice analysis.

3.9.5 Method for Estimation of Total Sugar (Duboise Method):

Appropriately diluted sample were taken and measured as procedure described in watermelon analysis.

3.9.6 Method for Estimation of Total Polyphenol:

Diluted samples were taken and measured as procedure described in watermelon juice analysis.

3.9.7 High Performance Liquid Chromatography for Polyphenols (Mradu et al., 2012):

Samples were extracted and analyzed as per the procedure described in watermelon juice analysis.

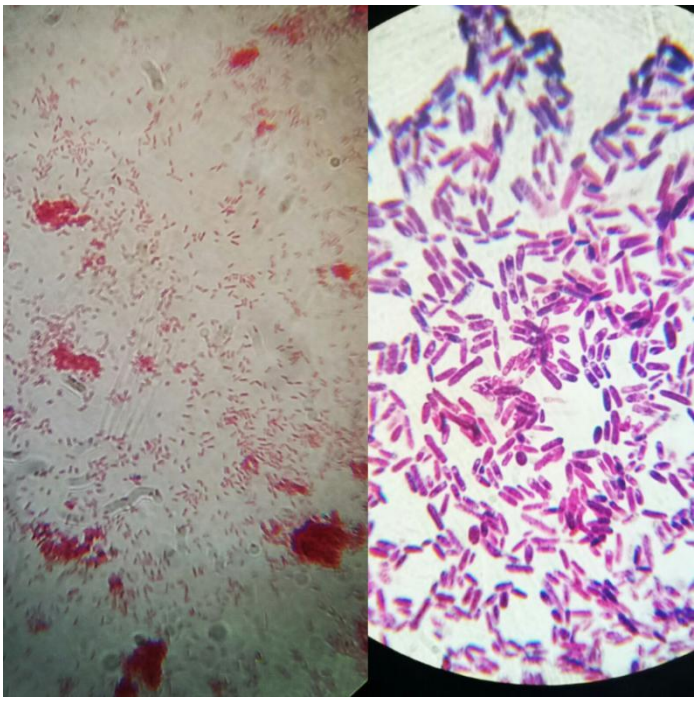
3.9.8 High Performance Liquid Chromatography for Organic Acids (Kelebek et al 2009):

Samples were extracted and analyzed as per the procedure described in watermelon juice analysis.

CHAPTER 5

RESULTS

5.1 Gram Staining of *Acetobacter aceti* strains



A B

Figure 8 Morphology and Gram reaction of *Acetobacter aceti* strains

A- *Acetobacter aceti* smooth mat producing strain

B- *Acetobacter aceti* rough mat producing strain

From the Gram's staining it was observed that the smooth mat producing strain is Gram negative and are very small in size, whereas, the rough mat producing strain is rod shaped and bigger in size as compared to smooth mat producing strain and has shown Gram positive reaction.

5.2 Growth of *Acetobacter aceti* strain

The *Acetobacter aceti* smooth mat producing strain was unable to grow in the watermelon alcohol samples. No growth was observed till 30 days.

The *Acetobacter aceti* rough mat producing strain was also unable to grow in the watermelon samples even after triggering the initial acidity.

But after lowering the alcohol content, the *Acetobacter aceti* rough mat producing strain could grow after 3 days in alcohol produced using water melon juice as a substrate. The rough mat producing strain gave a powdery pellicle on the top of the fermented watermelon solution.

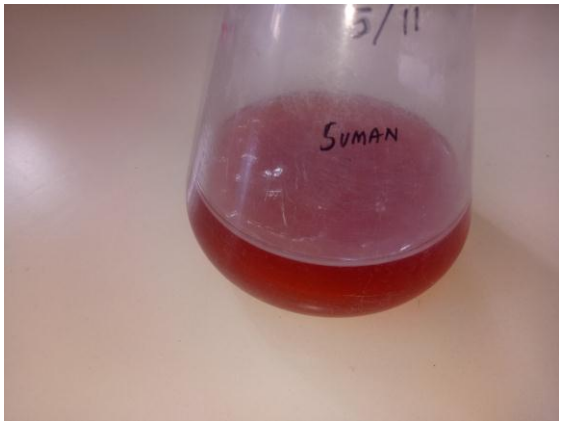


Figure 9- Smooth mat producing strain of *Acetobacter aceti*

Figure 10 -Rough mat producing strain of *Acetobacter aceti*

5.3 Sample Analysis

The following experiment was carried out by taking watermelon juice as a substrate. The following quantitative and qualitative analysis were performed and the fermentation was carried out.

Note- Sample

- 1-KMS 2nd Batch
- 2-Boiled 2nd Batch
- 3-KMS 3rd Batch
- 4-Boiled 3rd Batch
- 5A-Boiled 1st Batch
- 5B-Boiled 1st Batch (more aeration)
- 6A-KMS 1st Batch
- 6B-KMS 1st Batch (more aeration)

5.3.1 ºBrix

The total sugar content of the watermelon juice was determined using a handheld refractometer and the initial Brix was adjusted to 20° before fermentation and it was fermented with a yeast culture to produce alcohol and the results are provided in Table -1. The total sugar content dropped to 6-7 deg Brix within 24 h of fermentation and the drop was very slow thereafter.

Table 1: ºBrix of the water melon juice samples

Days	**Watermelon juice samples**							
	1	**2**	**3**	**4**	**5A**	**5B**	**6A**	**6B**
0	20	20	20	20	20	20	20	20
1	6	7	7	7	6	6	6	6
3	5	4	5	4	4	4	5	5
6	5	4	5	4	4	4	5	5
9	5	4	5	4	4	4	5	5
38	-	-	2	2	-	-	-	-
44	2	3		-	-	-	-	-
46	-	-	1	1	-	-	-	-
52	0	3	1	1	-	-	-	-
58	0	3	-	-	2	2	3	1
66	-	-	-	-	1	1	2	1
72	-	-	-	-	0	0	2	0

5.3.2 pH

The pH of the watermelon juice samples and the fermented watermelon juice was determined and the results are provided in Table-2.

Table 2: pH of Watermelon sample before and after fermentation

Days	Watermelon juice samples							
	1	**2**	**3**	**4**	**5A**	**5B**	**6A**	**6B**
0	6.2	6.0	5.98	5.95	6.1	6.1	5.97	5.97
1	4.50	4.46	4.60	4.34	4.33	4.33	4.43	4.43
3	4.49	4.45	4.61	4.35	4.34	4.34	4.40	4.40
6	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-
12	4.47	4.46	4.59	4.37	4.32	4.32	4.44	4.44
38	-	-	4.16	4.18	-	-	-	-
44	4.11	3.9	-	-	-	-	-	-
46	-	-	4.5	4.55	-	-	-	-
52	4.5	3.93	4.52	4.54	-	-	-	-
58	4.5	3.6	-	-	4.45	4.07	4.02	3.8
66	-	-	-	-	4.52	4.35	3.7	4.29
72	-	-	-	-	4.89	4.3	3.68	4.92

(-) : Not determined

5.3.3 Reducing sugar

The reducing sugar content of the watermelon juice samples before and after fermentation was determined and the results are provided in Table-3.

Table 3: Reducing sugar content in watermelon sample

Days	Watermelon juice samples							
	1	2	3	4	5A	5B	6A	6B
0	1.525	1.538	1.592	1.571	1.531	1.534	1.491	1.491
1	0.719	0.753	0.842	0.886	0.7141	0.7141	0.828	0.828
6	0.7183	0.7479	-	-	-	-	-	-
38	-	-	0.0703	0.8602	-	-	-	-
44	0.6929	0.710	-	-	-	-	-	-
58	-	-	-	-	0.6950	0.6823	0.6632	0.7734

5.3.4 Total Acidity

The Total acidity content of the watermelon juice samples before and after fermentation was determined and the results are provided in Table-4.

Table 4: Total acidity of Watermelon sample

Days	**Watermelon juice samples**							
	1	2	3	4	5A	5B	6A	6B
0	0.15	0.125	-	-	0.19	0.19	0.145	0.145
1	0.24	0.2407	-	-	0.38	0.38	0.31	0.31
3	-	-	-	-	-	-	-	-
18	-	-	0.312	0.32	-	-	-	-
23	0.39	0.39	0.51	0.48	-	-	-	-
38	-	-	0.632	0.485	-	-	-	-
44	0.97	1.3	-	-	-	-	-	-
46	-	-	-	-	-	-	-	-
52	-	-	-	-	-	-	-	-
58	--	-	-	-	0.29	1.16	1.275	2.664
66	-	-	-	-	-	-	-	-

5.3.5 Total Alcohol Content

The alcohol content of the fermented watermelon juice samples was determined and the results are presented in Table-5.

Table 5: Alcohol content of watermelon samples

Days	**Fermented watermelon juice samples**							
	1	2	3	4	5A	5B	6A	6B
0	>1	>1	>1	>1	>1	>1	>1	>1
1	<10	<10	<10	<10	<10	<10	<10	<10
3	<10	<10	<10	<10	<10	<10	<10	<10
46	-	-	>1	>2	-	-	-	-
52	>2	>6	-	-	-	-	-	-
66	-	-	-	-	>2	>2	>1	0

5.3.6 Total Sugar

Table 6: Total Sugar of watermelon samples

Days	1	2	3	4	5A	5B	6A	6B
0	1.872	1.783	1.93	1.853	1.895	1.895	1.901	1.901
1	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-
38	-	-	-	-	-	-	-	-
44	0.453	0.587	-	-	-	-	-	-
58	-	-	-	-	0.542	0.511	0.882	--

5.1.7 Total Polyphenol:

The total polyphenol content of the watermelon juice, in the alcohol produced from this juice and the acetic fermeted alcohol of watermelon juice was determined by HPLC method and the results are presented in Table-7.

Table 7: Total Polyphenol Content in Watermelon samples

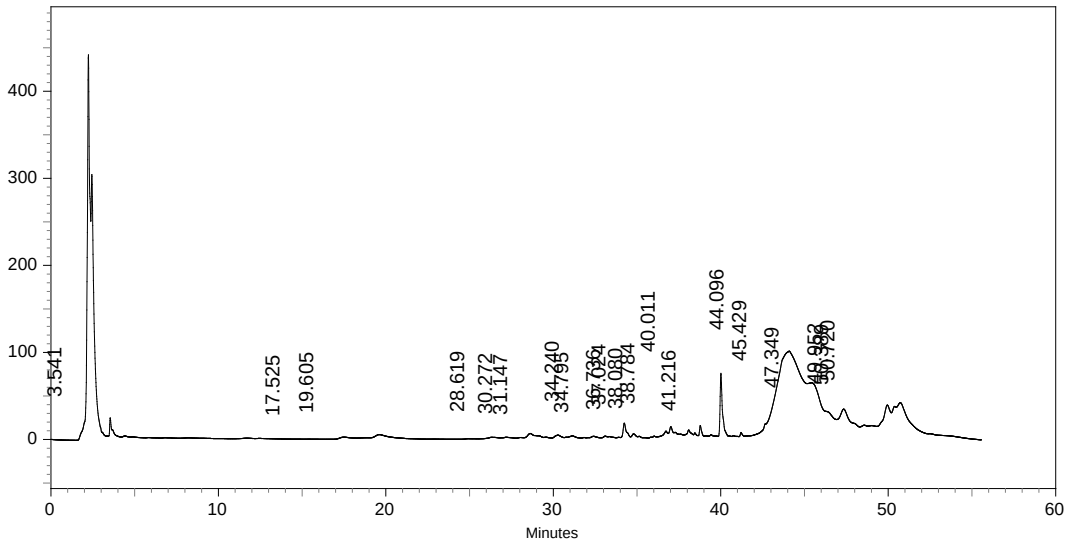
Days	Watermelon sample							
	1	**2**	**3**	**4**	**5A**	**5B**	**6A**	**6B**
0	0.0806	0.0807	0.0803	0.0804	0.0809	0.0809	0.0802	0.0802
1	0.0804	0.0808	-	-	-	-	-	-
6	0.0805	0.0804	-	-	-	-	-	-
38	-	-	0.0804	0.0804	-	-	-	-
44	0.0807	0.0806	-	-	-	-	-	-
58	-	-	-	-	0.0805	0.0806	0.0806	0.0807

5.1.8 High Performance Liquid Chromatography for Phenolics:

The chromatograms recorded at different wavelengths using HPLC showed that most compounds were detected at 280 nm and 320 nm. The results obtained allowed identification of a series of phenolic compounds.

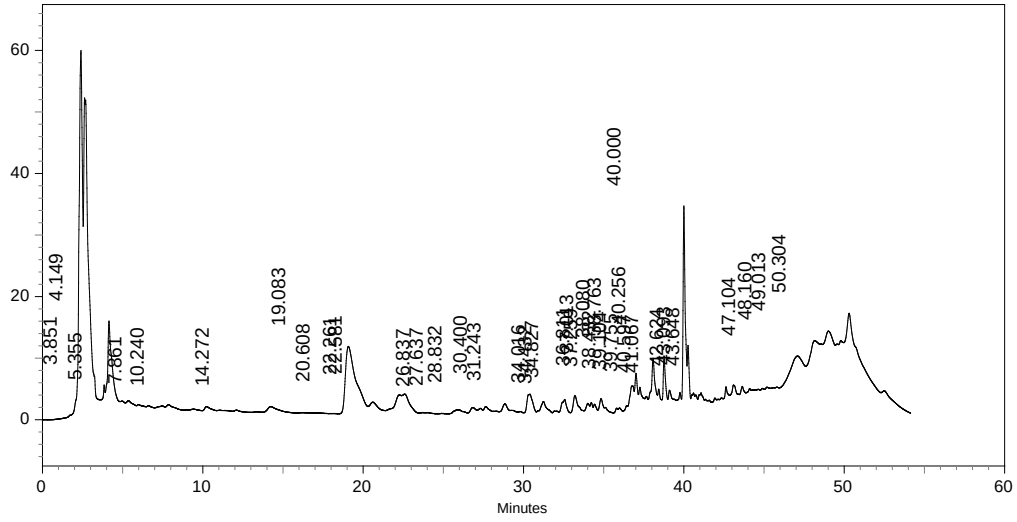
Sample- 1 (Day-0)

Chromatogram was obtained at 280 nm with PDA detector on Day 0



Sample- 1 (Day-6)

Chromatogram was obtained at 280 nm with PDA detector on Day 6



Sample- 6B (Day-58)

Chromatogram was obtained at 280 nm with PDA detector on Day 58

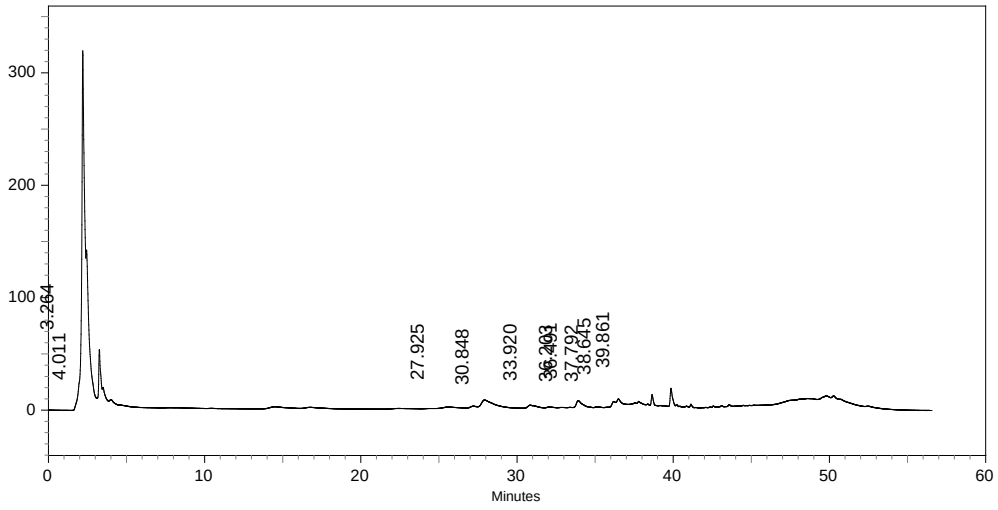


Table-8. Change in phenolics concentration during fermentation of watermelon juice

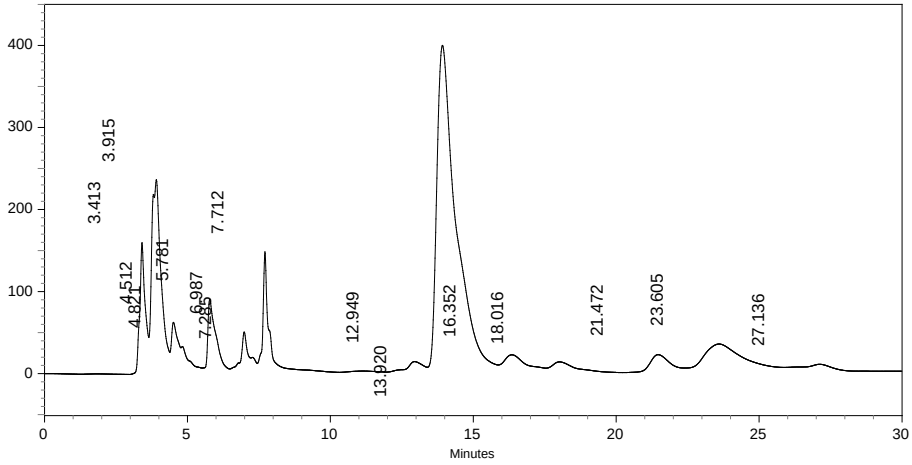
Acids (µg/ml)	Retention Time	Sample-1 Day 0	Sample 1 Day 6	Sample 6B Day 58
Gallic acid	5.796	0.187	-	-
Catechin	23.816	0.66135	-	-
Epicatechin	30.194	3.638	3.568	-
Methyl catechol	31.295	1.486	1.4201	-
Ferulic acid	35.790	-	-	-
Trans cinnamic acid	42.007	-	-	-
Vanillin	24.612	-	-	-
Chlorogenic acid	33.387	-	-	0.7040
Ethyl catechol	42.951	0.2166	-	-
Kaempferol	40.325	0.27002	6.3271	-
Caffeic acid	27.245	0.0948	-	-
Isovanillin	30.721	0.4411	-	0.5924
Myristic acid	39.565	0.2256	-	2.3722
Syringic acid	27.917	0.0696	-	2.1917
p-coumaric acid	34.307	0.10120	4.0424	-

Note- Sample 1- 2nd Batch KMS , Sample 6B- 1st Batch KMS (more aeration)

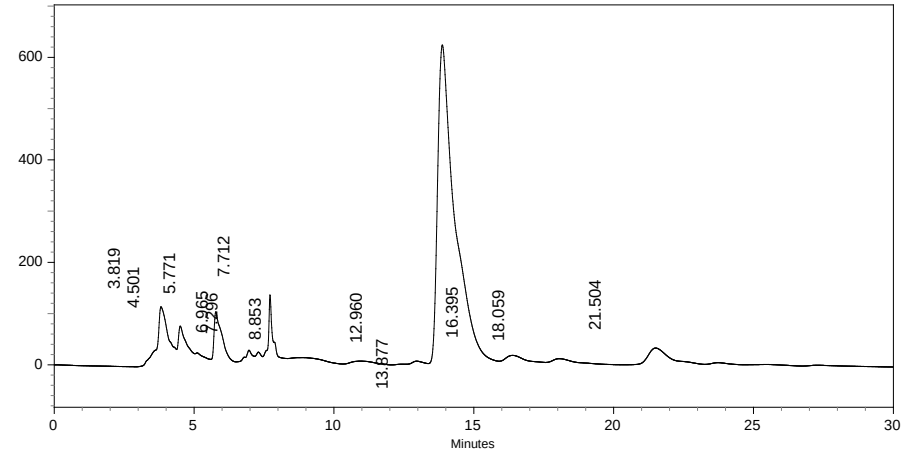
5.1.9 High Performance Liquid Chromatography for Organic Acids

The coelution of other components of a complex biological sample matrix with the targeted compound can be checked by means of PDA spectral data acquisition.

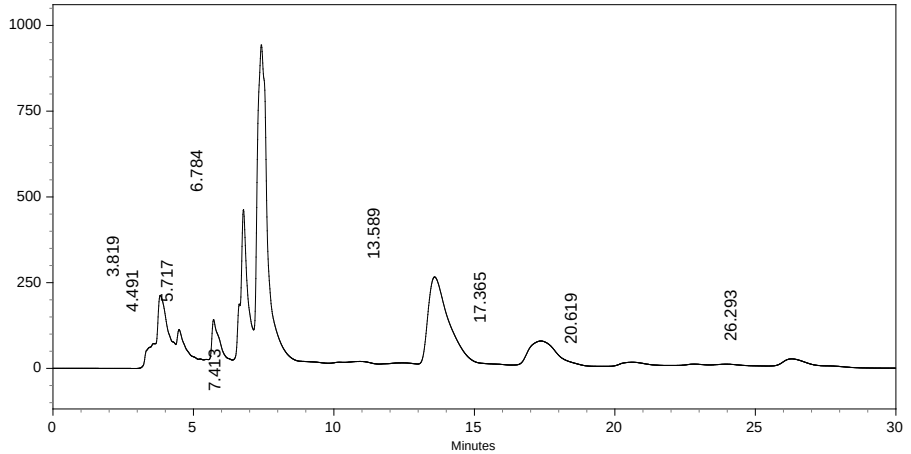
Sample 1- (Day 0)
Chromatogram was obtained at 210 nm with PDA detector on Day 0



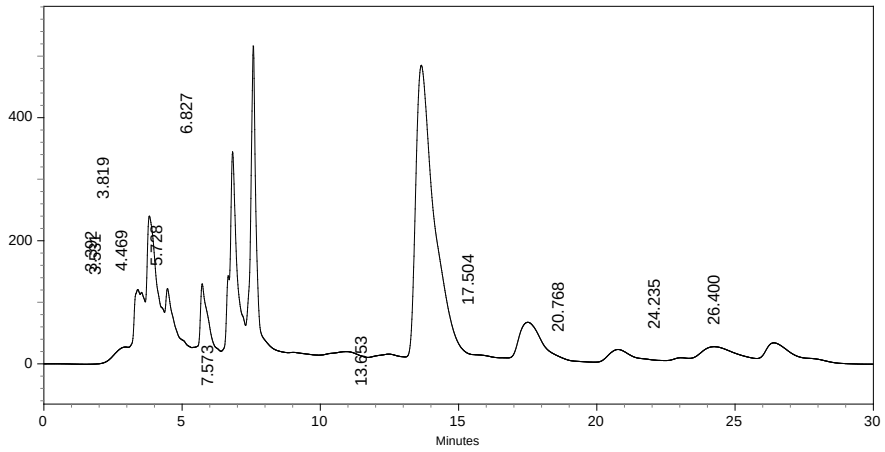
Sample 1 (Day 1)
Chromatogram was obtained at 210 nm with PDA detector on Day 1



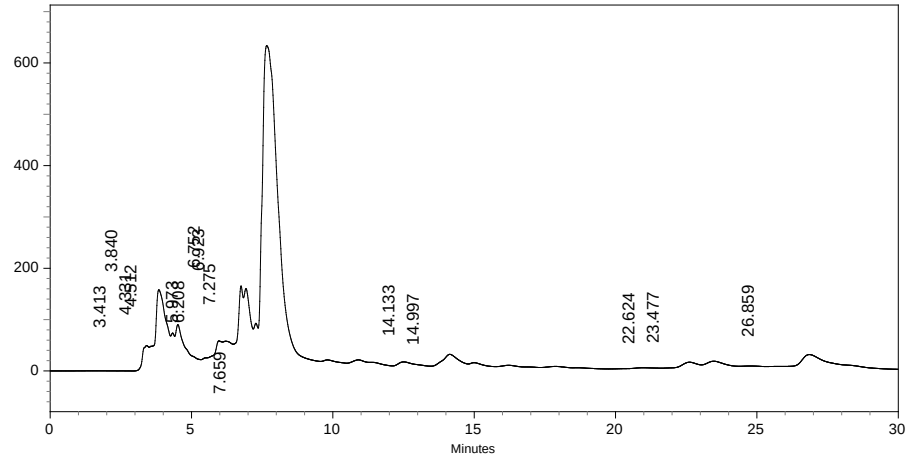
Sample 1- (Day 6)
Chromatogram was obtained at 210 nm with PDA detector on Day 6



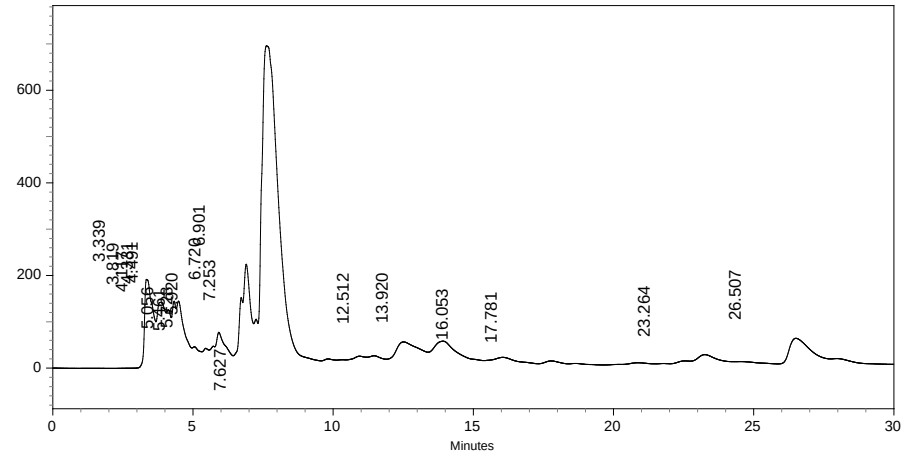
Sample 2 (Day 6)
Chromatogram was obtained at 210 nm with PDA detector on Day 6



Sample- 2 (Day- 58)
Chromatogram was obtained at 210 nm with PDA detector on Day 58



Sample- 6B (Day- 58)
Chromatogram was obtained at 210 nm with PDA detector on Day 58



Sample- 6A (Day- 72)
Chromatogram was obtained at 210 nm with PDA detector on Day 72

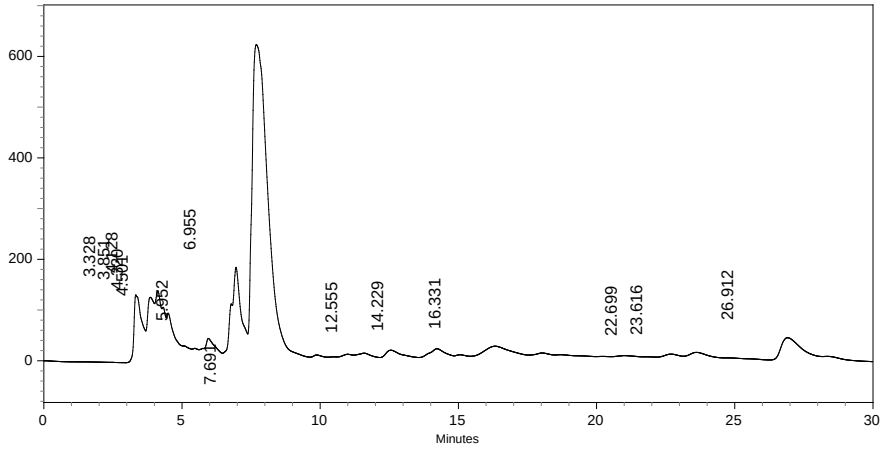


Table-9. Organic acids concentration during 1st step fermentation

Acids(µg/ml)	Retention time	Sample 1 (Day 0)	Sample- 1 (Day 1)	Sample-1 (Day6)	Sample-2 (Day 6)
Galacturonic acid	13.72	5985.23	131502.917	4456.378	7625.22
Succinic acid	10.56	-	-	-	-
Lactic acid	9.524	-	-	-	-
Isocitric acid	9.287	-	-	-	-
Citric acid	9.392	-	-	-	-
Fumaric acid	5.68	0.54280	0.62237	0.71161	0.661491
Tartaric acid	4.63	0.125226	2.4199	2.25939	2.03048
Malic acid	4.79	-	-	-	-
Pyruvic acid	13.07	0.08404	0.04780	5.2662	-
Glutaric acid	12.74	-	-	-	-
Acetic acid	6.539	0.096518	0.04888	1.5617	1.31901

Table-10. Organic acid concentration after 2nd step fermentation

Acids(µg/ml)	Retention time	Sample- 2 (Day-58)	Sample-6B (Day-58)	Sample-6A (Day-72)
Galacturonic acid	13.72	239.849	409.615	-
Succinic acid	10.56	-	-	-
Lactic acid	9.524	-	-	-
Isocitric acid	9.287	-	-	-
Citric acid	9.392	-	-	-
Fumaric acid	5.68	0.04609	0.00593	0.17283
Tartaric acid	4.63	1.20129	1.055113	0.5457
Malic acid	4.79	-	-	-
Pyruvic acid	13.07	-	-	-
Glutaric acid	12.74	-	6.35548	
Acetic acid	6.539	0.105531	0.068434	

Note- Sample 1-KMS 2nd Batch, Sample 2-Boiled 2nd Batch, 6A-KMS 1st Batch, 6B-KMS 1st Batch (more aeration)

CHAPTER-6

DISCUSSION

6.1 Growth of *Acetobacter aceti* strain

It was observed that the smooth mat producing strain of *Acetobacter aceti* was unable to grow in watermelon alcohol even after lowering the alcohol content.

But the rough mat producing strain could grow and the growth (a layer of the mat) was observed after 3 days of lowering the alcohol content and by adding sugar solution to increase the sugar content.

After lowering the alcohol content and addition of scoby (culture in the form of mat) to later batches, growth was observed in 3 days, but acid yield was low even after 2 weeks of acetic fermentation.

When the 50 days old batch sample was treated with alcohol lowering process by way of adding additional sugar solution along with the scoby of *Acetobacter*, and by providing more aeration by transferring the entire content into two Erlenmeyer flasks, growth was observed and the acid content was much high than the earlier trails just in 7 days.

Hence, the growth of *Acetobacter aceti* rough mat producing strain and acid yield was faster if the alcohol sample is old and provided with more aeration.

6.2 Morphology of *Acetobacter aceti* smooth and rough strains

It was observed that the smooth mat producing strain is Gram negative and are small in size, whereas, the rough mat producing strains are rod shaped and bigger in size and are Gram positive. Reports indictaed that *Acetobacter aceti* is a Gram variable bacterium.

In case of smooth culture the pellicle is smoother and the other rough strain pellicle is at first powdery on the top then after fermentation it settles down and the pellicle becomes less smooth.

6.3 Sample Analysis

The experiment was carried out and the quantitative and qualitative analyses were performed to observe the changes in the parameters throughout the fermentation process.

6.3.1 ºBrix

The Total Soluble Solids (TSS) of the sample were measured by using hand held refractometer and the results were expressed as unit ºBrix (Table 1).

Watermelon juice ºBrix depends on TSS, if it is higher then ºBrix also increases. The brix values decreases during the fermentation period, indicating the utilization of solids by the growing organism. The values were in the range of (7- 4) after 1st step of alcoholic fermentation and then again decreases after the inoculation of *Acetobacter aceti* pellicle and the values range between (4-1).

6.3.2 pH

The pH of the product has an important impact in the taste of the natural vinegar. Sequential observations were recorded with help of digital microprocessor pH meter (Table 2). Fresh watermelon juice sample pH range between 5.9 to 6.10 .After alcoholic fermentation the watermelon alcohol sample pH range between 4.2 and 4.7. And after scoby inoculation the pH range between 3.6 and 4.10.

The pH value was 3.41 at the beginning of fermentation and decreased slightly to 3.15 at day 11.

The pH value was 4.2-4.3 after the alcoholic fermentation. After the acetic fermentation the pH value decreased to 3.6-3.8.

6.3.3 Reducing Sugar

Reducing sugar carries a free aldehyde group and ketonic group. Reducing sugar was found to decrease during the fermentation process, may be due to the utilization of sugars by yeasts to produce alcohol. It was also observed that the samples treated with KMS have more reducing sugar content in comparison to Boiled treatment samples.

6.3.4 Total Acidity

It measures the amount of acid present in the sample. The titratable acidity of fruits is used, along with the sugar content, as an indicator of maturity. Generally, the higher the maturity, the lower the acid content during the ripening process. It is one of the important aspects in analyzing the quality of a product. Acids are an important component of the flavour and aroma of watermelon wine as well as watermelon vinegar.

Different range of acidity values was evaluated. From the analysis it was observed that the acidity increased exponentially (Table 4).

The acidity increases as the acetic fermentation proceeds. But after a due course of time the acetic fermentation ceases and acid is consumed and hence acidity decreases, if the samples were not filtered properly after fermentation.

From the two step fermentation system in case of onion vinegar, the total yield of acetic acid was 37.9 g/L reported (Tohru Kanno, 2000).

Whereas, the highest acetic acid yield from watermelon vinegar was 0.80577 µg/ml.

6.3.5 Total Alcohol

Alcohol content was determined by using chromic acid method. On 0^{th} day the alcohol content was less than 1%. After the alcoholic fermentation the alcohol content was more than 10%. But the *Acetobacter aceti* grows with an alcohol content of 6-8%. So the alcohol content was decreased up to 7-8% by adding 50% boiled sucrose solution. And after the *Acetobacter aceti* growth the alcohol content was utilized by the bacteria for acetic fermentation. At the end of fermentation the alcohol content was found to be 1 or less than 2% (Table 5).

During Pine vinegar production, the ethanol content decreased steadily from 7.0% at day 0 of acetic fermentation to 4.7% at day 6 acetic fermentation and then decreased fast to 0.4% at day 11 as reported by (Ya-Hui Chen 2011)

In case of Watermelon vinegar, during acetic fermentation, the alcohol content of the sample decreased from 8% to less than 2 %. Because the alcohol present were consumed in the acetic fermentation.

6.3.6 Total Sugar

The total sugar was found to decrease as the fermentation proceeds, due to the utilization of sugars by yeast to produce alcohol (Table 6)

6.3.7 Total Polyphenol

As per the Table 7, the total polyphenol content of the watermelon sample before alcohol fermentation and after acetic fermentation were almost the same.

6.3.8 High Performance Liquid Chromatography for Phenolics:

The polyphenols found in watermelon wine are Epicatechin, Methyl Catechol, Kaempferol and p-coumaric acid. The watermelon vinegar contains Chlorogenic acid-0.7040µg/ml, Isovanillin- 0.5924µg/ml, Myristic acid- 2.3722µg/ml and Syringic acid- 2.1917µg/ml.

In case of watermelon juice, many polyphenol were found such as Gallic acid, Catechin, Epicatechin, Methyl Catechol, Ethyl catechol, Kaempferol, Caffeic acid, Isovanillin, Myristic acid, Syringic acid, p-coumaric acid. Amon all these , the major polyphenol found was Epicatechin and Methyl Catechol having concentration 3.638µg/ml and 1.486µg/ml, respectively.

The polyphenols found in watermelon wine are Epicatechin, Methyl Catechol, Kaempferol and p-coumaric acid. Major polypheno;s are Epicatechin and Kaempferol having concentration 3.568µg/ml and 6.3271µg/ml, respectively.

The watermelon vinegar contains Chlorogenic acid-0.7040µg/ml, Isovanillin-0.5924µg/ml, Myristic acid- 2.3722µg/ml and Syringic acid- 2.1917µg/ml.

6.3.9 High Performance Liquid Chromatography for Organic acids:

The organic acid found before acetic fermentation having highest concentration was Tartaric acid -2.4199µg/ml.

The organic acids found after acetic fermentation are Fumaric acid, Tartaric acid, Glutaric acid and Acetic acid.

In Cider vinegar the organic acids found were Acetic acid, lactic acid and Succinic acid having concentration 40.585g/L, 3.64g/L, 0.275g/L, respectively, as reported by Maria TOFANA (2012).

In watermelon juice sample the organic acids found after acetic fermentation are Fumaric acid, Tartaric acid, Glutaric acid and Acetic acid having concentration 0.17283µg/ml, 0.5457µg/ml, 2.4120µg/ml , 0.8057µg/ml, respectively.

Gallic acid, Catechin, Epicatechin, Chlorogenic acid, Caffeic acid, Syringic acid, p-coumaric acid and ferulic acid were detected in grape juice, wine and vinegar samples The content of catechin in industrial vinegar (27.50 mg/L). Traditional vinegar had higher amounts of chlorogenic and syringic acids than the industrial vinegar ($P > 0.05$), as stated by Budak in NCBI.

CHAPTER 7

CONCLUSION

Production of alcohol from juice was carried out by making use of yeast *Saccharomyces cerevisiae KTP*. Temperature needs to be maintained at 30ºC for acetic acid production. KTP strain is used because it has an ability to ferment higher amount of glucose to yield appreciable amount of alcohol. Before alcoholic fermentation the fruit juice pH range between 5.9- 6.10. After 1st step of fermentation the watermelon sample pH range between 4.2- 4.7. The alcohol content of the fermented juice should be 6-8% for the growth of the vinegar bacteria and vinegar production,

The *Acetobacter aceti* grows well and gives higher acid yield when supplied with more aeration.

The polyphenol found after alcoholic fermentation are Gallic acid, Catechin, Epicatechin, Ethyl catechol, Kaempferol, Myristic acid, Isovanillin, Syringic acid, Caffeic acid.

The polyphenols found after the 2nd step fermention are: Chlorogenic acid, Isovanillin, Myristic acid and Syringic acid

The organic acids found after acetic fermentation are Fumaric acid, Tartaric acid, Glutaric acid, Acetic acid and Galacturonic acid.

7.1 SUMMARY

Watermelon juice collected and brix was adjusted to 20. Two treatments were done one is Boiled another one is KMS. At first alcoholic fermentation was carried out by *Saccharomyces cerevisiae KTP*. Then to the fermented juice sample, smooth strain *Acetobacter aceti* was added. Till 30 days no growth was observed. Then the rough strain *Acetobacter aceti* pellicle was added to the watermelon sample after lowering its alcohol content. And acidity was also triggered by adding old concentrated vinegar. Growth was observed within 2-3 days. It was found that when older watermelon wine is used for vinegar production, the *Acetobacter aceti* strains grows faster and yield more acid. The polyphenol found after vinegar production are Myristic acid and Syringic acid.

7.2 FUTURE WORK

Further more studies required to increase the acetic acid yield of watermelon vinegar samples.

Batch to batch variation needs to be studied further by controlling aeration, size of inoculum and temperature management.

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