Some observations on the performance of *Aspergillus oryzae* and *Saccharomyces cerevisiae* in the manufacture of Rice vinegar

By

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DECLARATION

I hereby declare that the work reported in the project report was exclusively carried out by me, under the supervision of Mr. T.D.W. Siriwardana and Mrs. Indira Wickramasinghe. Any part of this project report has not been submitted earlier or concurrently for same or any other degree.

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DEDICATED TO MY PARENTS, BROTHERS

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ABSTRACT

Vinegar is the liquid produced from a suitable raw material containing starch or sugar or starch and sugar by the process of double fermentation, alcoholic and acetous, and which contains at least 4% w/v acetic acid. Rich vinegar is grain vinegar, which has over 4.2% acidity as acetic acid.

Vinegar is used as a flavoring and preservative to make some ketchups, sauces and chopped pickles. To make rice vinegar rice, yeast, culture of *Aspergillus oryzea* and water are used. Variety of Bg 358 is used as the rice. Aspergillus oryzea, Saccharomyces cerevisiae and Acetobacter aceti are involved in the manufacture of rice vinegar.

First of all by giving anaerobic condition two samples were prepared by adding steamed rich, culture of Aspergillus oryzea, water and yeast. One of the sample was kept in room temperature and other was kept in cold room at 150C.

Another experiment was carried out by giving same environment. But yeast wasn't added to the mash during the activity of Aspergillus oryzea. Yeast was added at the initiation stage of the alcoholic fermentation

Another experiment was carried out by giving aerobic condition. In this stage too yeast was added at the initiation stage of the alcoholic fermentation.

Here increase in brix value was observed due to the performance of Aspergillus oryzea. Alcoholic fermentation was carried out by using Saccharomyces cerevisiae. Acetobacter bacteria was used in acitic acid fermentation, to convert ethanol in to acetic acid. Aerobic condition was used at this stage. Quality of the water is very important in whole fermentation process.

The produced rich vinegar total acidity as acetic acid 4.5%. Oxidation value was 952 . Alkaline oxidation value was 40. loding value was 360 and Formol value was 0.5 According to the vinegar analytical values and statiscally analyzed data the produced rice vinegar is with good quality attributes. So can introduce this produce rice vinegar successfully in to the competent vinegar market.

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CHAPTER 1

Introduction

The consumption of vinegar as a flavouring and preservation in Sri Lanka exceeds 400,000 Litres per annum. Vinegar in Sri Lanka broadly can be divided in to two groups viz artificial & natural vinegar. Artificial vinegar is diluted acetic acid where as natural vinegar constitutes those obtained from natural sources. Traditionally the vinegar made from the fermented sap of coconut or coconut toddy is preffered in Sri Lanka. Other sources of natural vinegar consist of fermenting coconut water with added sugar or use of molasses from sugar cane industry for subsequent fermentation. There has been an increasing trend in vinegar impart to Sri Lanka paticullary fruit based vinegar, spiced wine vinegar. Possibilities exist to evaluate effect microbiological hydrolysis and subsequent alcoholic and acid formation to manufacture vinegar, using rice as the substrate.

Rice (*Oryzae sativa* L.)Is the most important cereal crops in the developing countries and is the staple foods of over half the world's population. More than 250 million tons of rice is produced every year and over 95% of the world production is used for human consumption. In Sri Lanka is the most important food and more yielding product in Yala and Maha seasons.

The name vinegar is in fact derived from the French 'vin aigre' for 'sour wine' and even today the most popular types of vinegar in a region usually reflect the local alcoholic beverages for example malt vinegar in the U.K., wine vinegar in France.

Vinegar is the liquid produced from a suitable raw material containing starch or sugar or starch and sugar by the process of double fermentation, alcoholic and acetous, and with contains at least 4% w/v acetic acid. (Pearson,1979).It is also a dilution solution of acetic acid In the first stage yeast convert sugar in to ethanol anaerobically while in the second ethanol is oxidized to acetic acid aerobically by bacteria.

To make rice vinegar rice, yeast, culture of spore of mold and water are used, High quality rice is used for vinegar manufacture. It is necessary to remove the bran and 25%-30% of the weight oh the original kernels during polishing for high quality vinegar. (Wood,1985).

A speculated mold culture is produced by culturing *Aspergillus oryzae* on soaked, steamed polished rice at 25^0C -30^0C for 5 or 6 days or until there is abundant spoulation. The sproulated

Mold is than used to inoculate larger quantities of steamed rice to produce heavy mycelial growth and maximum enzyme production or and through the rice. The most important enzymes in sporulated mold are the amylases. But proteases and lipases are also present.

Saccharomyces cerevisiae is used to convert sugar ($C_6H_{12}O_6$) in to alcohol, which is called ethanol (C_2H_5OH). The oxidation of ethanol to acetic acid is performed by member of the genera *Acetobacter* and *Gluconobacter*. These are gram negative, catalase, positive, oxidase negative, strictly aerobic bacteria. *Acetobacter spp*. And the better acid producers and more common in commercial vinegar production.(Gostineau et al.1979).

Objectives of this study are;

1. To evaluate the possibility of the manufacture of vinegar using rice.
2. To study the performance of *Aspergillus oryzae* hydrolysis of rice starch.
3. Performance of *Saccharomyces cerevisiae* and *Acetobaceter* in the conversion of sugar to alcohol and acetic acid respectively.
4. To evaluate the quality of the product.

CHAPTER 2
LITERAURE REVIEW

2.1 Origin of vinegar

The production of vinegar is one of the oldest fermentation processes employed by man. In the past it has served a wide variety of different function, but today its use is largely confined to food flavoring, production and preservation of pickles, souces, chutneys and in other manufactured food products.

Vinegar is believed to be the oldest fermented product knows to man. It's origin is associated with the first time an alcoholic drink went sour. The ancients were quick to find the remarkable versatility of vinegar. The Babylonians used it as a preservatives and as a condiment and it was they who began flavoring it with herbs. Roman legionnaires used it as a beverage. Cleapatra demonstrated its solvent property by dissolving precious pearls in it to win wager that she could consume a fortune in a single meal. Hippocrates extolled its medicinal qualities and indeed, it was probably one of our earliest remedies. Biblical references show how it was much used for its soothing and healing properties and when Hennibal crossed the Alps, it was vinegar, which helped pave the way. Obstructive boulders were heated and doused with vinegar, which cracked and crumbled them.

The first large scale production of vinegar occurred in France during the 16th century for use by the French as well as for export to the British isles and to various European countries. The first major quantities of vinegar were reportedly produced in England by processing soured beer and ale. So also in 1782 canning of vinegar was introduced by a Swedish chemist. Knowledge of vinegar and its production appear
To have spread to other countries from the Middle East and perhaps china. The Japanese techniques of vinegar making, believed to have originated from china were introduced between AD 369 and 404.

In the late 1800s chemists learned to make acetic acid, manufactures added water to reduced its strength to 5%, coloured it and sold it as vinegar. Imitation vinegar is still manufactured and by law the label must state that it is diluted acetic acid. Diluted acetic acid is inexpensive and lacks the vitamins, minerals and esters found in fermented vinegar, its flavor and aroma are also inferior. (Anerine, et. Al. 1980)

2.1.1 Background of vinegar production

Vinegar, which in general terms may be defined as a condiment is not only a food commodity indispensable to almost all households and hotels. But is also of extensive use in the preservation of fruit and vegetables and the preparation of pickles, souses, chutneys and other manufactured food production. Although a wide variety of sugar or starch bearing material may be used as the raw material for the manufacture of vinegar.

In Sri Lanka vinegar production is a middle scale industry, which is distribution in coconut triangular areas. According to the Sri Lanka custom report / External trade statistics can be expressed current vinegar import market.

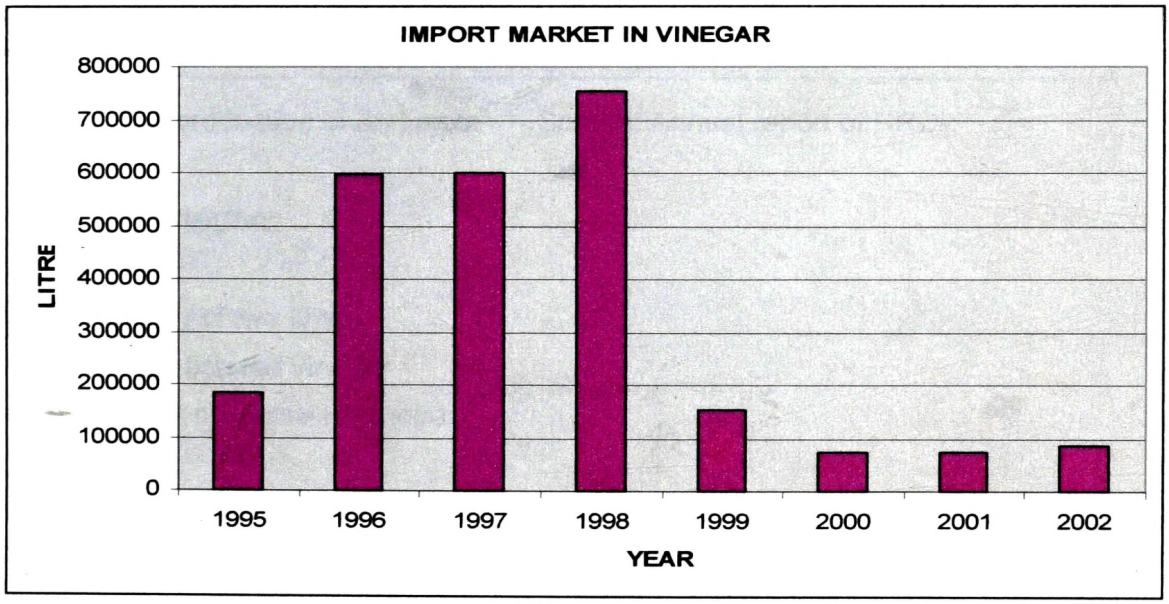

Figure 2.1 Import market in Vinegar Source: Custom report 1995-2002

2.1.2 Background of Rice production in Sri Lanka

Rice is one of the leading food crops of the world, the staple food of over half the world's population. It is generally considered a semi aquatic, annual, grass plant. In Sri Lanka, last few years rice production of paddy was increased, but farmers have been facing the serious problem during the cultivation seasons. Such as low price in during cultivation seasons, not enough store rooms and also stocks were destroyed due to natural disasters.
The following Figure 2.2 illustrates the Total Rice production in Sri Lanka.

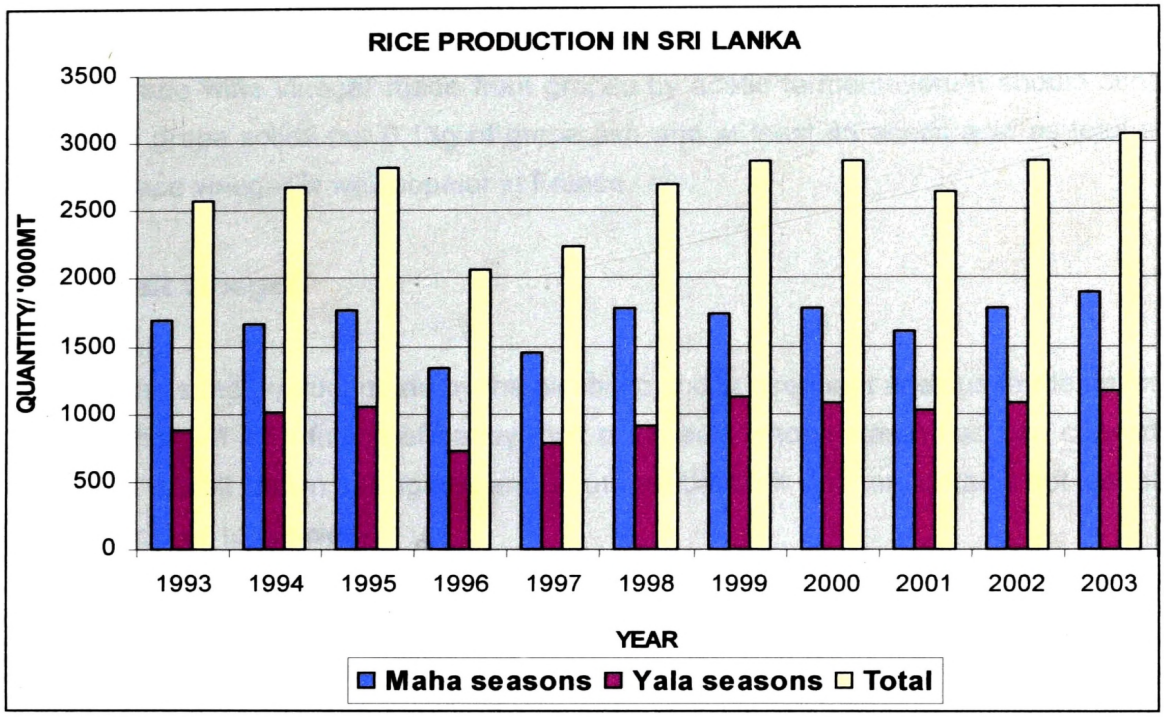

Figure 2.2 Rice production in Sri Lanka Source: Annual report of RRDI

2.2 Types of vinegar

Mainly vinegar are of two types

1. Natural or brewed vinegar
2. Artificial or non brewed vinegar

2.2.1 Natural or Brewed vinegar

By definition the United States Food and Drug Administration recognizes six types of vinegar distinguished by their sourceses.

2.2.1.1 Cider vinegar

Cider vinegar made from apple juice by fermentation is called "apple cider vinegar " or simply "cider vinegar ". It should contain at least 1.6g of apple solid per 100ml of which more than 50% are reducing sugars and at least 4% of acetic acid as total acidity. Cider vinegar is particularly well known in the United States, Switzerland and Austria because of its of its desirable aroma.

2.2.1.2 Wine vinegar

Wine or grape wine vinegar made from grapes by acetic fermentation. It should contain at least 1g of grape solids per 0.13g of grape ash and at least 45 acetic acid as total acidity. Wine or grape vinegar is well popular in France.

2.2.1.3 Malt vinegar

malt vinegar is the product made by the alcoholic and subsequent acetous fermentation, with out distillation, of an infusion of barley malt or cereals whose starch has ben converted by the malt. It is well known in England and South Africa. Malt vinegar contains not les than 4% of acetic acid as total acidity.

2.2.1.4 Sugar vinegar

Sugar vinegar is made by the alcoholic and subsequent acetic acid fermentation of sugar syrup, molasses of refiner's syrup.

2.2.1.5 Glucose vinegar

Glucose vinegar is made by the alcoholic and subsequent acetous fermentation of glucose solutions

2.2.1.6 Spirit vinegar

This type of vinegar is made by the acetic fermentation of dilute ethyl alcohol. It should contain at least 4% of acetic acid as total acid as total acidity. It may be colored with caramel. This vinegar is also called "Distilled vinegar".

2.2.2 Specialty vinegar

Specialty vinegars make up a category of vinegar products that are formulated or flavoured to provide a special or unusual taste when added to food. Specialty vinegars are favorities in the gourmet market.

2.2.2.1 Herbal vinegar

Wine or white distilled vinegars are sometimes flavoured with the addition of herbs, spices or other seasonings. Popular flavorings are garlic, basil and farragen but cinnamon, clove and nutmeg flavored vinegars can be tasty and aromatic addition to dressings.

2.2.2.2 Fruit vinegar

fruit or fruit juice can also be infused with wine or white vinegar. Raspberry flavored vinegars for example create sweetened vinegar with a sweet-sour taste.
So also vinegar can be categories as follows.

Table 2.1 Kind of vinegar

Classification	Acidity	Materials(per liter)	Kinds
Grain vinegar	Over 4.2%	Over 40 gram of grain	Rice vinegar Sake less vinegar Malt vinegar
Fruit vinegar	Over 4.5%	Over 300 gram of fruit vinegar	Apple vinegar Wine vinegar Balsamic vinegar
Synthetic vinegar	Over 4.0%	Made by blending diluted solution of acetic acid with saccharide.	

Source: Anerine, M.A.(1980)

2.3 Uses of vinegar

2.3.1 Medicinal uses

Today the quantities used for this purpose are probably insignificant; vinegar remains a popular remedy in some circles. Cider vinegar is widely sold in health food shops and books have been published describing the alleged beneficial effects or rice vinegar on a remarkable range of ailments. Undoubtedly, vinegar is often simply a nostrum but is some cases there does appear to be some basis for its use in its antibacterial and solvent properties. Nowadays vinegar is found to be almost a universal preservative and a cure to all. It also can kill infection, soothe caughs, ease the pain of throat, calm nausea, relieve varicose veins, ease arthritis, Sade headache away, treat burns, sooth aching feet, cool sun

burn, reduse itoh of welts and hives, stops hiccups, tread bee sting, remover corn and calluses, protects the skin from ravages of the sun, fade age spots and minimize memory loss.

2.3.2 Food uses

Vinegar is a good solvent for the essential oils of herbs and spices and has been a ubiquitous sauce ingredient throughout history. The Babylonians are known to have added a wide variety of herbs to the vinegars they used in food preparation and preservation. Normally, vinegars flavoured with tarragen, chilli, garlic, rosemary and the like, are produced commercially for used in home produced marinades and in salad dressings.

The range of other food products that can be prepared using vinegar was enormous, including mayonnaise, ketchups, sauces, chopped pickles and brined vegetables.
The preservative action of vinegar is due to its acetic acid content. As little as 0.1% of the undissociated acid will inhibit the growth of most food poisoning and spore
Forming bacteria and 0.3% will prevent the growth of mycotoxigenic molds.

2.3.3 Non food uses

Vinegar is well recognized as a cleaning sanitizing agent. It is especially effective in removing inorganic soils and mineral deposits such as hard water films. As a sanitizer, it is effective against a broad range of bacteria, yeasts and molds, destroying or reducing these organisms to acceptable levels.

Additionally, vinegar has been found to be effective as a rinse agent in reducing levels of E.coli on various countertop surfaces (e.g. Laminate. wood, tile, concrete, stainless steel and granite). The effectiveness of natural products and commercial disinfectants against human pathogens, researchers found that vinegar had "substantial activity "against Pseudomonas aeruginosa and Salmonella choleraesuis, but was not effective against E.coli 0157:H7 and staphylococcus aureus.

Vinegar's chemical properties make it a cleaner and sanitizer with several important advantages.

- Biodegradable-a mold organic acid
- Easy to dispense and control
- Safe for stainless steel used by the food industry
- Relatively nontoxic and stable-safer for handlers
- Less likely to leave harmful residues
- Pleasant "clean" odor

Where environmental compatibility and toxicity are especially important, vinegar has been used.

- To reduce microorganisms in slaughterhouses and poultry plants
- To reduce mineral and lime deposits in lavatory pipes
- To prevent milkstone buildup in tanks used by the milk industry
- To clean vehicles and equipment used in the construction industry
- To wash and rinses walls and ceilings in restaurants and food establishments.

2.4.Production of rice vinegar

Steamed rice,Culture of aspergillus oryzae, Yeast and Water are used.

2.5.Variety of Bg 358

Bg 358 is a samba type varity released in 1999. it is mother varieties were Bg 12-11 and Bg 1492. it can be harvested after 105 days. It get the highest yield 9.5 MT per ha. It was the 3rd most popular variety in 2003. Among the Cultivated extant in sri lanka 164094 ha as (15047%) all Cultivation land comes under this variety. It is resistant to Brown plant hopper, blast Bacterial leaf bright and moderately tolerant to iron.

Following characteristics can be seen in variety of Bg 358

Table 2.2 Main characteristics of Bg 358

Parameter	% or extent
Brown rice	78.9%
Total mill rice	74.5%
Head grain	66.7%
Broken grain	7.8%
Size/shape	Short/Round
Chalkiness	White center
Colour	White
Amylose	High
Gelatinization Temperature	High

Source: Annual report of rice production, RRDI

Following chemical composition can be seen in variety of Bg 358 (Brown rice)

Table 2.3 Chemical composition of Brown rice(dry matter basis)

Chemical composition	Percentage(%)
Protein	8.5%
Fat	2.6%
Available carbohydrate	74.8%
Thiamin	0.34%
Riboflavin	0.05%
Niacin	4.07%
Lysine	3.8%
Threonine	3.6%
Tryptophan	1.1%

Source: Chemical aspect of rice grain quality,(1979)

2.6. Rice polishing

The outer layer or germ of unpolished rice contains an excess of husks and vitamins for the yeast and culture of *Aspergillus oryzae* to propagate, and for the acceleration of

fermentation. Unpolished rice also contains proteins and fats which if excessively contained, would affect the smell or taste of the resultant liquid. These excesses make it difficult to control the fermentation process, and thus have to be removed through the process called rice vinegar.

2.6.1 Purpose of rice vinegar

The outer layer of germ and grain contains a lot of protein, fat, ashes and vitamins. They accelerate the growth of *Aspergillus oryzea* and yeast, breaking the harmonized alcohol quality. These components also cause coloration or sloppy taste in the resultant alcohol. Deteriorating the alcohol quality.

2.7 Rice washing

In a process called rice washing. The polished rice is washed with water to eliminate the sugar remaining on the surface. Rice washing wears away the surface of rice by 1%-2% which effectively leads to secondary rice polishing.

Potassium, protein and starch flow out and lost during the process of rice washing. Instead the rice absorbs water that accounts for 10%-20% of the weight of rice.

2.8 Rice soaking

The washed rice is then immediately put into a soaking basin to absorb water until it penetrates each grain by 25% to 30%. The soaking is aimed at completely steaming rice, normally turning it in to α state(water enters the solid crystal structure of raw starch in rice, which then swells or turns in to paste as a result of heating) with water sufficiently soaked to the center of each grain.

The following equation shows the relation between the moisture content and the amount of water absorbed in rice during washing and soaking.

$$Y = -2.8 x + 66$$

Where, Y is the percentage of water absorbed in soaked rice and
X is the percent moisture content of the rice.

2.9 Draining

After the scheduled soaking time is over, discharge the water from the soaking tank.

2.10 Rice steaming

Approximately moisture rice is heated up with a vapour that turns the raw starch in to α state, produced by culture of *Aspergillus oryzae*. The steaming also has a sterilization effect on rice, facilitating the safe condition of the fermentation process.

Good steamed rice is easy to handle and has hard outer surface as well as soft inner components. That means rice with a complete α state, appropriate hardness and free from a sticky surface. Establishing the hardness and softness of steamed rice is a very important process, because it greatly affects the culture of *Aspergillus oryzae* production control and thereafter the dissolution of rice in fermentation solution.

2.10.1 Time

Steam permeates in to the center of the rice, easing the saccharification of starch and the decomposition of protein. Normally rice is steamed for 60 min after vapour has been generated inside the steamer.

2.11 Culture of *Aspergillus oryzae*

Culture of *Aspergillus oryzae* that is grown on and within steamed rice grains and that accumulates various enzymes useful for degradation.

About 50 kinds of enzymes found in culture of *Aspergillus oryzae* . The most important are amylases and proteases. Alfa-amylase liquefies starch and the amount of glucose formed in the mash, by glucoamylase regulates yeast growth and fermentation. Acid protease decomposes proteins to from amino acids and peptides and helps amylase action, as discussed below cultural conditions influence the production of enzymes by the molds. In general, the higher the cultivation temperature (up to 42^{0}C) the greater the amylase activity. Lower temperature favors the development of protease activity. Aflatoxin producing strains have not been found among the Japanese industrial strains of *Aspergillus oryzae* molds.

2.11.1 Preparation of culture

The general procedure of culture making is as follows. Steamed rice cooked to about 35^{0}C-40^{0}C is transferred in to the incubation device or room where the temperature and humidity are controlled at a level suitable for growth of the molds. After inoculation with seed molds, the mixture is incubated for about 24 hrs. After one day mixture is mixed well. Next it is kept in the incubator to incubate for about 48hrs. After two days mycelia develop to cover and penetrate the grains. At this time *Aspergillus oryzae* has spores. Next sproulated rice is packed and kept in refrigerator.

2.12 Yeast

Yeast is a unicellular organism as large approximately 5µm(5-8 µm in length * 4-6 µm in width). Yeast is widespread all over the world of nature, with various types of strains each with its own characteristics.

Nitrate potassium is used to control the multiplication of yeast and shall not exceed 0.1g per liter. The amount of three minerals (Potassium, Magnesium and phosphorus) required for the growth and fermentation of yeast has been found as follows.

Potassium: 150 to 160 mg/liter for growth and 80mg/liter for fermentation

Magnesium: 10mg/liter for growth an 5mg/liter for fermentation

Phosphorus: 30mg/liter for growth and 15mg/liter for fermentation

2.13 Water

Generally it is desirable that the water contains a very low level of toxic ingredients like iron and manganese, while appropriately containing active principles can easily be supplemented by the addition of an ingredient, and through processing. How ever, it is difficult to remove the active principles, so it is necessary to choose water with fewer active principles.

Following requirements are essential for water as a fermentation medium.

Table 2.4 Requirements of water for fermentation

CHARACTERISTICS	REQUIREMENTS
Color	Transparent
Smell	No abnormality
pH	Neutral or slightly alkaline
Iron	0.02ppm or less
Manganese	0.02ppm or less
Organic matter	5ppm or less
Nitritoid nitrogen	None
Ammonia nitrogen	None
Bacterial acidity	2ml or less
Bacterial generating acid	None
Coliform group	None

Source: Food processing and preservation technology(2002)

2.13. 1 Treatment of water

If there is no requirements in water following processing chemicals can be added to achieve the treatment. Processing and purification can be applied for the water to obtain good quality water.

2.13.1.1 Processing

Chemicals should be added if the active is scare. The preferred types and amount of chemicals are showing Table 2.5.

Table: 2.5 Processing of water

Components	Inorganic salt	Required amount
Potassium (K)	Acid phosphate potassium(KH_2PO_4)	3.5
Phosphorus(PO_4)	Acid phosphate calcium($Ca(H_2PO_4)2H_2O$) Acid phosphate potassium(KH_2PO_4)	1.3 1.4
Magnesium(Mg)	Sulfate magnesium($MgSO_4.7H_2O$)	10.1
Calcium(Ca)	Acid phosphate calcium($Ca(H_2PO_4)_22H_2O$) Crystal sulfate calcium($CaSO_4.2H_2O$)	6.3 4.3
Chlorine(Cl)	Salt (NaCl 99% or more) Heien(Another type of salt, NaCl 96% or more)	1.7 1.8
Nitric acid (NO_3)	Sulfate potassium(KNO_3)	1.6

Source: Food processing and preservation technology(2002)

2.13.1.2 Purifcation

(a) Well change

The well has to be changed before the fermentation process, than 10g to 20g of bleaching powder or hypochlorite sodium agent is to the well for sterilization.

(b) Iron elimination method

Water contains various types of iron, which has to be taken in to consideration in the adoption of a purification method. Filtration is used for isolating minute particles like hydroxide iron. Oxidation is used for ionic iron with a low molecular weight. The oxidation/ adsorption or cohesion method is used for high molecular iron that is combined with organic mater, as in the case of corrosion.

Table: 2.6 The purification of fermentation water

Method		Object
Oxidation/Sterization	Treatment of chlorine	Microorganism, Iron, Manganese
Oxidation	Expose	Iron, Manganese, Gas
	Contact oxidation	Iron
	Sand filtration	Iron, Impurities, Microorganism
	Limestone filtration	Iron
	Filtration of active carbon / Granulation charcoal	Odour, Iron, Organic matter, Ammonia, Cl odour
	Manganese zeolite filtration	Manganese
	Waterlite filtration	Iron, Ammonium Organic matter
	Zeolite filtration	Hardness component
	Unglazed metal filter and other	Microorganism, Impurities
	Diaphragm, Filter filtration	Microorganism, Impurities
	Celite filtration	Microorganism, Impurities
	Filter press, Cottton filter	Impurities
	Adsorption to fixed, Tannin	Iron
	Adsorption to chealate resin	Iron, Manganese
Ion exchange	Rasin treatment for ion exchange	Inorganic component
Cohesion	PAC,AS	Iron, Manganese, Silicic acid, Organic matter

Source: Food processing and technology(2002)

To secure a new source of water for fermentation, the water chose should be fre from any toxic components, as much as possible. Various purification methods have been established to remove toxic components and to make clan water. However, purification requires specialized equipment and considerable effort. There is no single method to purify all the toxic components, So a range of purification method is used, depending on the water quality.

2.14 Acetic acid bacteria

Several distinct forms of bacteria have been applied in vinegar manufacture, and they may be found distributed throughout the liquid in such numbers that the 'gyle' has a silky streamlined appearance. In liquids of low alcohol concentration, the bacteria usually cohere in gelatinous masses to from the slimy, tough and almost transparent zoogloeal mat known as 'vinegar flower' or 'mother of vinegar'. This pellicle is actually extra cellular bacterial cellulose encapsulating the bacterial cells. If the liquid is undisturbed, the film remains on the surface until all the alcohol is changed to acetic acid and water. A very slight disturbance causes the film to sink, and a new film takes its place and with occasional disturbance a succession of films can from can from a submerged jelly like mass in the vinegar.

2.15 Main steps of vinegar production

Three distinct types of biochemical processes make vinegar, all the result of the action of microorganisms. The first process is brought about by the action of Aspergillus oryzea, which change starch in to glucose under controlled conditions. The second process is brought about by the action of yeast (*Sachomyces cerevisiae*), which change sugar (glucose) to alcohol under controlled conditions. This is called the alcoholic fermentation. The third process results from the action of a group of bacteria (*Acetobacter aceti*) upon the alcohol portion, converting it to acetic acid . This is the acetic or acid fermentation that forms vinegar. Proper bacterial cultures are important, timing is important, and fermentation should be carefully controlled.

The chemical reactions involved in these three processes can be represented as follows.

- $(C_6H_{12}O_6)nH_2O \longrightarrow C_6H_{12}O_6$
- $C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + 2CO_2$
- $2C_2H_5OH \longrightarrow 2CH_3COOH + H_2O$

2.15.1 Activity of *Aspergillus oryzea*

Aspergillus oryzae produce two most important enzymes such as amylases and protease. A-amylase liquefies starch and acid protease decomposes protein to form amino acids and peptides.

2.15.2 Alcoholic fermentation

Normally the yeast used in alcoholic fermentation is a strain of the species *Saccharomyces cerevisiae*. The first fermentation is anaerobic. If any wild acetobacter is present and produce more than 0.5% acetic acid, the growth of *Saccharomyces cerevisiae* will be inhibited. For the reason, 125ppm sulphur dioxide or an equivalent amount of bisulphate can be added to the fermentation solution to prevent the growth of mold, wild yeast and lactic acid bacteria.

Acidity conditions favor the production of ethanol by yeast, pH range for growth of *Saccharomyces cerevisiae* is between 2.4 to 5 and the optimum temperature is 20^0C and 30^0C.

The transformation of a sugar by the *Saccharomyces cerevisiae* can be represented chemically by the Gay-Lussac.

$$\underset{180g}{C_6H_{12}O_6} \longrightarrow \underset{92g}{2C_2H_5OH} + \underset{88g}{2CO_2}$$

During alcoholic fermentation, anaerobic condition is created, realizing carbon dioxide, which dissolve in the solution and generate carbon acid. Because of this pH drops further and the ethanol concentration rise.

Since the catabolism of sugar provides the yeast with a source of biosynthetic intermediates as well as energy, not all the sugar is converted to ethanol. Some is diverted to the production of yeast cell biomass, glycerol and succinic acid.

2.15.2.1 Biochemistry of alcoholic fermentation

The reaction of alcoholic fermentation are involved in several steps. The hexose (6-carbon) sugars being fermented are isomerized if necessary and phosphorylated to fructose -1,6-diphosphate which is split in to two triose units. The triose units are converted to pyruvic acid and this is decarboxylated to acetaldehyde. The decarboxylation step is irreversible, as is hexokinase and, although the other steps theoretically are reversible, in practice the energy input necessary is too high for significant reversal of the phosphofructokinases or phosphopyruvic transphosphorylase steps.

The acetaldehyde is reduced to ethanol by accepting hydrogen from reduced nicotinamide adenine dinucleotide (NADH), the heat stable coenzyme with alcohol dehydrogenase. During the initial induction phase of alcoholic fermentation no acetaldehyde is present and 3-phosphoglycerate is converted to glycerol. This is one example of the diversion of sugar to products other than ethanol that explains why the Gay-Lussac equation represents only the theoretical maximum yield. As acetaldehyde accumulates, it becomes the hydrogen acceptor(in place of dihydroxyacetone phosphate) and react with NADH to produce ethyl alcohol. During the rest of a normal fermentation this process predominates and little glycerol is formed. If acetaldehyde is not available glycerol is produced instead of ethanol.

In the presence of a high concentration of sulfur dioxide in acid solution, acetaldehyde, carbon dioxide and glycerol is he primary products and alcohol a by –product. If the sulfite solution is alkaline, acetaldehyde, glycerol, alcohol and carbon dioxide are all produced. Certain lactic acid bacteria, for example, by the same Embden-Meyerhof pathway, can divert some of the sugar to lactic acid production. The Krebs tricarboxylic acid cycle enzymes also exist in yeasts and are responsible for the conversion of pyruvate completely to carbon dioxide and water in aerobic conditions. This system can explain other by-products of fermentation occurring in alcohol such as succinic acids. Other enzymatic systems also exist which divert sugar in to amino acids, nucleosides, and other building blocks for yeast cells and by-products which appear in alcohol, thus also lowering the conversion to ethanol from the theoretical.

For conversion of glucose or fructose to ethanol, note that no fewer than 12 enzymes are required, at least 3 sets of co-factors (ADP-ATP, NAD^+ -NADH, TPP) and several inorganic Ions. The overall reaction becomes. (Anerine et al.1980)

$$C_6H_{12}O_6 + 2ADP + 4H^+ + 2HPO_4^{-2} \longrightarrow 2C_2H_5OH + 2CO_2 + 2ATP + 2H_2O + Heat$$

2.15.3 Acetic acid fermentation

Acetification is the oxidation of ethanol by bacteria to produce acetic and water. The process can be represented chemically by:

$$C_2H_5OH + O_2 \longrightarrow CH_3COOH + H_2O$$
$$46g \quad 32g \qquad\qquad 60g \quad 18g$$

Acetic acid fermentation is brought about by acetic acid bacteria *Acetobacter aceti.* These are strongly aerobic. Their activity is greatly reduced or inhibited by direct sunrays.

Acetic acid bacteria require for their growth; nutrients, which are generally present in the alcoholic liquor made from fruit juices or sugary substances. If, however, distilled alcohol is used, addition of food for the bacteria becomes essential . Usually malt sprouts, Phosphoric acid, Potassium carbonate, Trisodium phosphate and Ammonium hydroxide are used. For acetic acid fermentation, the alcoholic content of the fermented liquid is adjusted to 7% to 8% alcohol. Because acetic acid bacteria do not function properly at higher strengths. Mother vinegar containing acetic acid bacteria is then added to it in order to check the growth of undesirable microorganisms and to hasten the process. It is generally added at the rate of one part to three parts of the fermented solution.

The range of pH for acetic acid bacteria is 4.5 to 7.5 and temperature is 5^0C to 42^0C. Buchanon and Gibbons (1974) listed only three species of acetic acid bacteria, namely *Acetobacter aceti, Acetobacter pasteuriensis* and *Acetobacter peroxydens.* These three organisms consume alcohol and produce acetic acid, and as results the pH goes down. From the stoichiometry of the equation it is apparent that 1 liter of ethanol should produce 1.036 kg of acetic acid and 0.313 kg of water. The slight increase in volume that occurs during fermentation.(Adams et.al.1999).

Also apparent from the equation is the need for oxygen, supplied as air. Since it is only sparingly soluble in aqueous media (8.1 mg/ liter in water at 250C, and less as the temperature and solute content increase), oxygen availability is often the rate limiting factor

in acetification. The different approaches to this problem of oxygen mass transfer are one of the distinguishing features of the various techniques used.

2.15.3.1 Biochemistry of Acetic acid fermentation

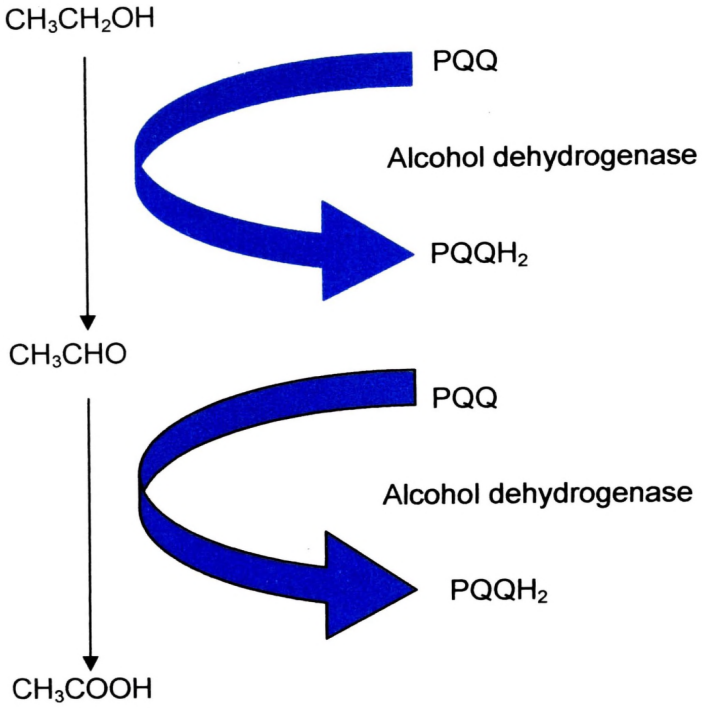

Figure 2.3 Oxidation of ethanol by acetic acid bacteria

Oxidation of ethanol to acetic acid is the relatively simple pathway by which acetic acid bacteria derive their energy. It occurs in two steps mediated by an alcohol dehydrogenase and on aldehyde dehydrogenase. Both enzymes are associated with the cytoplasmic membrane and have pyrroloquinoline quinine (PQQ) as a co-enzyme. PQQ act as a hydrogen acceptor which then reduces a cytochrome. The consequent electron transport establishes a proton motive force across the membrane, which can be used to synthesize ATP. (Adams, et.al.1999).

STARCH

$(C_6H_{12}O_6)_n + H_2O \xrightarrow{\text{enzymes}} x\,C_6H_{12}O_6 + y\,C_6H_{12}O_5$

	Starch	glucose	sucrose
MW	162n + 18	180	342

FERMENTABLE MONOSACCHARIDES

ALCOHOLIC FERMENTATION

$C_6H_{12}O_6 \xrightarrow[\text{Anaerobic condition}]{\text{yeast}} 2C_2H_5OH + 2CO_2$

	Glucose	ethyl alcohol	carbon dioxide
	MW 180	46	44

ETHYT ALCOHOL

ACETIFICATION

$C_2H_5OH + O_2 \xrightarrow[\text{Air}]{\text{Acetic acid bacteria}} CH_3COOH + H_2O$

	Ethyl alcohol	Acetic acid
	60	MW46

ACETIC ACID

Theoretical conversion

1g glucose ⟶ 0.51g ethyl alcohol ⟶ 0.67g acetic acid

Figure: 2.4 Schematic outline of Rich vinegar production

2.15.4. Influence factors of fermentation

There are most important factors, which affect the process of alcoholic fermentation. Most yeast (strains of S. cerevisiae) can grow on a medium, which provides utilizable sources of energy and carbon, nitrogen and certain inorganic salts.

2.15.4.1. Carbon and Energy sources

Certain sugars, particularly glucose, fructose, sucrose and maltose are the normal substrate for yeasts, but they do not ferment lactose, pentose, dextrins or starch. They also can grow on a variety of other carbon sources especially aerobically. Acetic acid, for example, can be utilized by yeasts and during the early stages of fermentation appreciable amounts of acetic acid can disappear. Most yeasts ferment glucose more rapidly than fructose even though fructofuranose has an affinity for hexokinase twice that of any from of glucose.

2.15.4.2. Alcohol

Alcohol itself has an inhibiting effect on fermentation, which increases with temperature. This effect is of course, related to the maximum yield of alcohol, which can be expected from various sugar concentrations.

2.15.4.3.Carbon dioxide and pressure

The effect of carbon dioxide in alcoholic fermentation is too often neglected. The amount of carbon dioxide content of 15g per liter(about7.2 atm) essentially stopped yeast growth. The CO2 effect on yeast growth did not prevent alcoholic fermentation A mush higher carbon dioxide pressure up to 30 atm is necessary to halt alcoholic fermentation. Carbon dioxide pressure is specially inhibitory to yeasts at low pH or high alcohol level.

2.15.4.4 Acid

little attention has been paid to the effects of fixed organic acidson the alcoholic fermentation of musts. If the pH is very low; 3.0 or lower fermentation is somewhat reduced. Yeasts are, however, not very sensitive to the amounts of fixed organic acids present in normal musts. There may be some effect of organic acids on the by products of alcoholic fermentation. The acids, are however, important in maintaining the pH low enough so a to inhibit the growth of many undesirable bacteria, thus giving a growth advantage to yeast. Fatty acids such as acetic, butyric and propionic, do have a decided inhibitory effect on yeasts.

2.15.4.5 Nitrogen

Normal yeasts can synthesize their own needed amino acids from ammonium ions or certain other simple nitrogen sources and sugar carbon. Although they have no absolute requirements for amino acids, the amino acids of musts are important as nitrogen sources and do stimulate the rate of yeast growth.

2.15.4.6 Growth factor

Yeast may respond to accessory growth factors. Among those found desirable or necessary with certain yeasts are biotin, inositol, nicotic acid, pantothenic acid, p-aminobenzoic acid, pyridoxine and thiamin. Biotin is an absolute requirements of most strains of Saccharamyces.

2.15.4.7 Mineral

The normal course of alcoholic fermentation requires Magnesium, Potassium, Zinc, Cobolt, Iodine, Iron, Calcium, Copper and anions of Phosphorus and Sulfur. For growth alone, yeasts require Copper, Iron, Magnesium, Potassium, Phosphorus and Sulfur.
(Anerine, et.al.1980)

2.16.1 Causes for spoilage of vinegar

2.16.1 Vinegar flies

Known as *Drosophila cellaris*, vinegar flies are small flies, which propagate in piles of fermenting pomace or rotten,fruits. Although they do not propagate by themselves in any way affect the quality of the vinegar, they hinder work. They can , however be kept away by screening the premises and by ensuring sanitary conditions.

2.16.2 Vinegar eels

One of the most troublesome natural contaminants of vinegar, during manufacture is the vinegar eel or eelworm. This is a free living nematode, *Anguillula aceti,* from 1-2 mm in length and about 0.04 mm in breadth, which lives its life cycle in vinegar. It requires very little oxygen and tolerates temperatures of 0-37^{0}C. Its pH 1.6 and 11.It is harmless to man, but they destroy the acid in vinegar.

They can, however, be destroyed by heating the vinegar to 60^{0}C or by filtration. They do not grow if the container is filled to the brim.

2.16.3 Lactic acid bacteria

Lactic acid bacteria are generally found in fermented juices. They cause cloudiness and produce disagreeable mousy flavors in the fermented juices, besides producing lactic acid and other acids. The bacteria interfere with acetic acid fermentation and lower the quality of the vinegar. In alcoholic fermentation; they may be avoided by using a starter of pure yeast, and by adding, during fermentation, they may be avoided by using a starter of pure yeast, and by adding, during fermentation 20% to 25% of unpasteurized vinegar to the fermented alcoholic juice.

2.16.4 Wine flowers

This is a kind of film yeast. If the fermented juice is unnecessary exposed to air, wine flowers grow on the surface of the liquid. They destroy the alcohol and also cause cloudiness. Their growth can, however, be checked by, spreading a neutral oil like liquid paraffin over the surface of the fermented liquid or adding 20% to 25% of unpasteurized vinegar or filling the barrels to the brim.

2.16.5 Vinegar louse

These rarely become a serious pest. The louse is a small from of aphid and develops only around generators under certain conditions.

2.16.6 Vinegar mites

These mites are the enemies of acetic acid bacteria. They multiply rapidly and interfere with the oxidation of alcohol. When they die, their bodies settles to the liquid and begin putrefy. The putrefactive bacteria so produced sooner or later overpower the acetic acid bacteria.

CHAPTER 3
MATERIAL AND METHODOLOGY

3.1 Materials

3.1.1 Location

The experiments were carried out at the food science laboratory and microbiology laboratory of the Food Research Unit of Department of Agriculture. Other analysis of the product was conducted at the Food science laboratory of the Faculty of Applied sciences of Sabaragamuwa University.

3.1.2 Raw materials and equipment used for the production of the rice vinegar

1 Rice
2 Culture of *Aspergillus oryzae*
3 Water
4 Yeast
5 5L white and amber glass bottles
6 Plastic bottles
7 Autoclave

3.1.3 Vinegar Analysis

3.1.3.1 Equipment used for the determination of Brix value

3.1.3.1.1 Apparatus

1 Refractometer (Erma Hand refractometer, range 0-32^0)

3.1.3.2 Equipment and reagent used for the determination of reducing sugar

3.1.3.2.1 Apparatus

1 Conical flask (250ml)
2 Burette
3 Pipettes (5ml, 10ml)
4 Electric balance
5 Volumetric flasks (100ml, 250ml, 500ml, 1000ml)

6 Beakers

7 Magnetic stirrer

8 Dropper

9 Muslin cloths

10 Stands

3.1.3.2.2 Reagent

1 Clear crystals of copper sulphate

2 Sodiumpotassium tartarate (Rochelle salt)

3 Sodium hydroxide

4 1% Methylene blue

5 Distilled water

3.1.3.3 Equipment and reagent used for the determination of Alcohol strength

3.1.3.3.1 Apparatus

1 Distillation unit

2 Specific gravity bottles

3 Thermometer

4 beakers (100ml, 50ml)

5 Measuring cylinders

3.1.3.3.2 Reagent

1 M Sodium hydroxide

2 Distilled water

3.1.3.4 Equipment used for the determination of pH

3.1.3.4.1 Apparatus

1 pH meter (Corning 215)

2 Beakers (10ml)

3.1.3.5 Equipment and reagent used for the determination of Total acidity

3.1.3.5.1 Apparatus

1 Burette

2 Pipette (10ml)

3 Conical flasks

4 Dropers

3.1.3.5.2 Reagent

1 0.1M NaOH solution

2 Phenolphthalein indicator solutions

3.1.3.6 Equipment and reagent used for the determination of Specific gravity

3.1.3.6.1 Apparatus

1 Specific gravity bottles

2 Electric balance

3 Piece of cloth

3.1.3.6.2 Reagent

1 Water

3.1.3.7 Equipment and reagent used for the determination of oxidation value

3.1.3.7.1 Apparatus

1Distillation apparatus

2 250ml glass stoppered bottles

3 Burette

4 Pipette

5 beakers

6 Droppers

7 Thermometer

8 Conical flasks

3.1.3.7.2 Reagent

1 Dilute sulpuric acid

2 0.02M potassium permanganate

3 10% potassium iodide

4 0.02M sodium thiosulphate

5 Starch solutions

3.1.3.8 Equipment and reagent used for the determination of Alkaline Oxidation value

3.1.3.8.1 Apparatus

1 250ml glass stoppered bottle

2 Pipette

3 Beakers

4 Conical flasks

5 Distillation apparatus

6 Droppers

3.1.3.8.2 Reagent

1 Distilled water

2 10% sodium hydroxide

3 0.02M potassium permanganate

4 Dilute sulphuric acid

5 Potassium iodide

6 0.02M sodium thiosulphate

7 Starch solution

3.1.3.9 Equipment and reagent used for the determination of oxidation value

3.1.3.9.1 Apparatus

1 250ml glass stoppered bottle

2 Pipette

3 Beakers

4 Distillation apparatus

5 Droppers

3.1.3.9.2 Reagent

1 Distilled water

2 10M potassium hydroxide

3 0.02M sodium thiosulphate

4 Dilute sulphuric acid

5 1M potassium hydroxide

6 0.1M iodine

3.1.3.10 Equipment and reagent used for the determination of Mineral acid

3.1.3.10.1 Apparatus

1 Pipette

2 Beakers

3 Droppers

3.1.3.10.2 Reagent

1 Alcohol

2 Methyl orange

3.1.3.11 Equipment and reagent used for the determination of Formol titration

3.1.3.11.1 Apparatus

1Pipette

2 Burette

3 Beakers

4 Droppers

3.1.3.11.2 Reagent

1 0.5M sodium hydroxide

2 0.05M sodium hydroxide

3 Phenolphthalein

4 Formalin

3.1.3.12. Sensory evaluation for rice vinegar

1 Three sample

2 Water glass

3 Questionnaire papers

4 Pens

3.2 Methodology

3.2.1 Preparation of culture of *Aspergillus oyzae*

It was necessary to get more purify rice to prepare culture. Before steaming, to remove the unwanted materials, rice was soaked in water basin for 32(s) and by using tap water. It was washed well. After washing rice was dipped in spring water basin. Time was taken in this step was 7 min. Then rice was kept to drain the excess absorption water. After draining rice was steamed at least 1 hr to get sterilization effect. After steaming rice was kept to cool, before inoculating the "mother culture". 1g of mother culture was inoculated in to 1kg of steamed rice in the lamina flow and to get the well growth condition. It was kept in incubator at 32^{0}C. After one day it was taken from the incubator and kept in Lamina flow. Crystal of rice can be seen due to the growth of *Aspergilus oryzae*. Rice was mixed well to mix fungi in all rice grains. After mixing, it was kept in incubator to grow the fungi well. To obtain the sporulated mold culture to manufacture rice vinegar. After two days culture was taken from

the incubator and crystals were break in to small parts. Then sporulated mold culture was packed and kept in refrigerator.

3.2.2 Preparation of steamed rice

First of all steamed rice was prepared by using steamer. Before steaming to remove the Unwanted materials rice was soaked in water basin for 32(s) and by using tap water, it was washed well. After washing rice was dipped in spring water basin which is described in 2.13. time was taken in this step at 7 min. then rice was kept drain the excess absorption water. After draining, rice was steamed at least 1 hr to get sterilization effect and facilitate the safe condition of the fermentation process.

3.2.3 Formula development

To make the rice vinegar formula was used.

Steamed rice	1kg
Culture of *A.oryzea*	250 g
Water	2 L
Yeast	½ tea spoon

Steamed rice, culture and water were used according to the following ratio.

Culture: Steamed rice	Streamed rice: water
1 : 2	1 : 4

All of the samples were included in above ratio between three raw materials.

3.2.4 Manufacture of Rice vinegar

First of all by giving anaerobic condition two samples were prepared by adding steamed rice, culture of *Aspergillus oryzae* water and yeast.5L large white glass bottles were used to make rice vinegar. One of the sample was kept in room temperature and other was kept in cold room at 15°C. Every day brix value was recorded the two samples.Maxmimum brix values were recorded after few days, fermentation solution was taken out from glass bottles. The sample which was in room temperature immediately showed the maximum brix value. The sample, which was in cold temperature, showed maximum brix value, but it took a longer time than the room temperature.

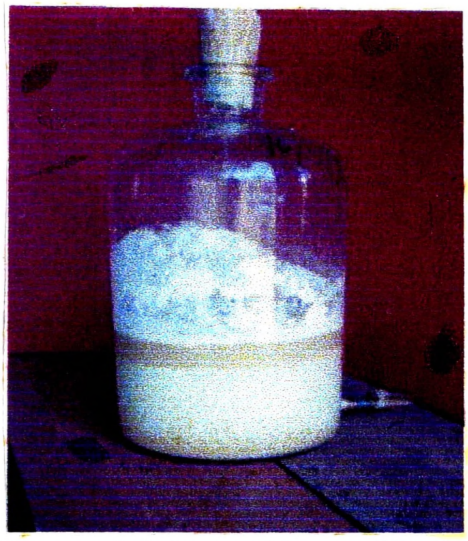

Figure: 3.1 Anaerobic condition

Then another experiment was carried out to obtain maximum brix value from the samples. So to give the optimum condition for *Aspergillus oryzea*, *Saccharomyces cerevisiae* wasn't add together as in the above explained experiment, but it was added at the initiation stage of the alcoholic fermentation. Because some unfavorable reactions can be happen due to the activity of *Saccharomyces cerevisiae*. These two samples were prepared in anaerobic condition by using steamed rice, culture and water. One sample was kept in room temperature and the other one was kept in cold temperature at 15^{0}C. Every day brix value was recorded. In this experiment recorded higher brix value was recorded than in the previous one.

Then another experiment was carried out by giving aerobic condition instead of anaerobic condition. First, steamed rice, culture and water were mixed together. One of the sample was kept in room temperature and the other one was kept in low temperature at 15^{0}C. This experiment showed the highest brix values than in the above two experiments. Reducing sugar was determined by the Lane and Eynon's method.

After few days the highest brix value came in to a constant level. Next steps of rice vinegar production was carried out with the highest brix value samples. These samples were autoclaved and cooled. After cooling, the samples, *Saccharomyces cerevisiae* was introduced in to the solution to carry out the alcoholic fermentation of vinegar production. Alcoholic fermentation was carried out in anaerobic condition. Alcoholic strength was measured in the samples which gave a high amount of brix value.

Next step was the Acetic acid fermentation. After destroying yeast cells by autoclaving the "mother culture" was added in to the fermentation liquid to make vinegar. By using a fish tank aerator O_2 was inserted to the samples. pH and total acidity were calculated on every other day. When pH and total acidity reached the standard values, Acetic acid bacteria were destroyed by using autoclave. When the residues were settled down in to the bottom of the container, the vinegar bottling was done.

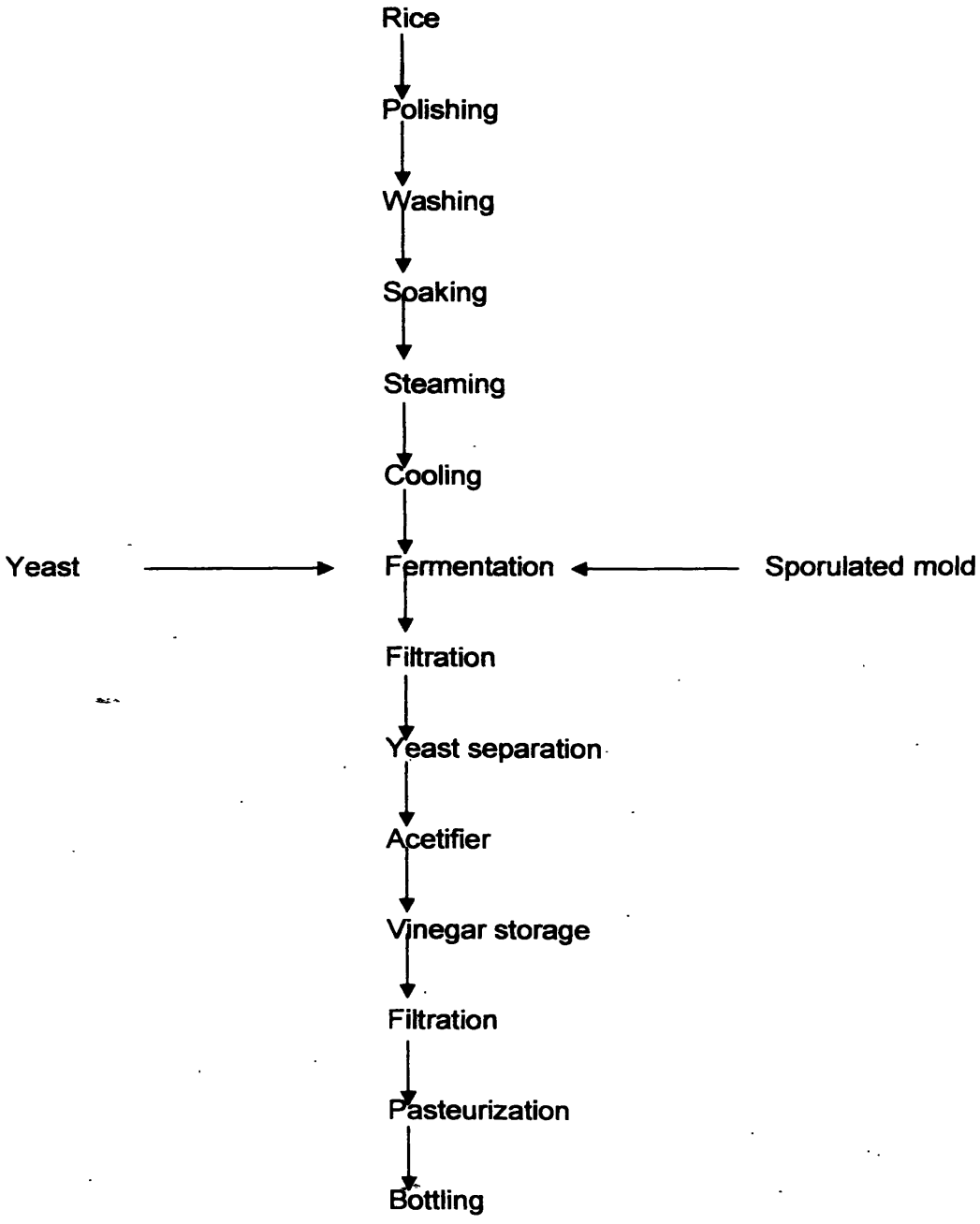

Figure: 3.2 Process flow chart of Rice vinegar production

3.3 Vinegar Analysis

3.3.1 Determination of Brix value

Few drops of samples were kept on the glass of Refractometer and readings were recorded.

3.3.2 Determination of Reducing sugar

Equal quantities (5ml each) of Fehling A solution and Fehling B solutions were peppet out separately in to a cleaned 250ml conical flask. 10ml of the sample solution was added to the conical flask from the burette. The content in the flask was brought to boiling by keeping on the electric heater and then the sample solution was added to the flask till blue color change to faint blue, while doing vigorous boiling. At this point, two drops from 1% methylene blue indicator was added to the flask and titration was continued till brick red color obtained and the burette reading was recorded and the corresponding invert sugar equivalent was taken from the table given in appendix 1. (Pearson. 1979)(Solutions preparation were attached I Appendix v).

The reducing sugar content present in fermentation solution was calculated as follows.

Reducing sugar = $\frac{F \times V_1 \times V_3 \times 100}{T \times V2 \times W}$ (mg invert sugar/ 100ml solution)

F – Factor corrected after standardization

T – Burette reading

V_1 – Volume to which original solution is diluted

V_2 – Aliquot of solution taken

V_3 – Volume to which solution is further diluted

W – Weight of the sample

3.3.3 Determination of Alcohol strength

Measured out 100ml sample in a volumetric flask at 20°C and washed in to the distillation flask with 50ml water, neutralized any acidity with M sodium hydroxide and distilled slowly in to the same 100ml volumetric flask. Collected 90-95ml made up to 100ml with water at 20°C and determined the specific gravity at 20°C/20°C preferably using a specific gravity bottle. At 20°C empty specific gravity bottle was weighted (W_1). Then specific gravity bottle was filled

with water and weight recorded (W_2). Then dry bottle was taken and filled with alcohol sample and weight recorded (W_3). The alcohol content was estimated as percentage by volumes and percentage by weight from the appropriate customs and exercise table was given in Appendix 11. (Pearson. 1976)

$$\text{Specific gravity} = \frac{W_3 - W_1}{W_2 - W_1}$$

3.3.4 Determination of Acetic acid

10ml of vinegar samples was taken in to a conical flask and diluted up to 10ml was taken from the diluted samples and drops of phenolphthalein indicator were added. Then it was titrated carefully to a faint pink color end-point with 0.1M NaOH solution. Repeated three times for each samples. Total acidity was tested every day expert holidays during the conversion of Alcoholic solution in to acetic acid.

The Total acidity content present in fermentation solution was calculated as follows.

% acetic acid (m/v) = T x 0.6
Where T = mean titre (in ml) of 0.1M NaOH solution required to neutralize the acidity in 10ml of the vinegar sample.

3.3.5 Determination of pH

pH value was tested every day during the acetic acid fermentation of Rice vinegar samples by using Corning 215 pH meter.

3.3.6 Determination of Specific gravity

Weight of empty bottle was weighted by using electric balance (W_1g). Then weight was taken with water (W_2g). Then dry bottle was taken and fill with vinegar sample and weighted (W_3g). All the readings were recorded.

$$\text{Specific gravity} = \frac{W_3 - W_1}{W_2 - W_1}$$

3.3.7 Determination of Oxidation value

Distillation: - 60ml of vinegar sample was distilled from a distillation unit fitted with a small tap funnel. When 45ml of distillate had come, 15ml of distilled water was added to the flask down the tap funnel and distillate a further 15ml to give a total volume of distillate of 60ml.
5ml of the distillate was added to a 250ml glass stoppered bottle by using a 5ml pipette. 10ml of dilute sulphuric acid (1+3) and exactly 15ml of 0.02M potassium permanganate were added by using 25ml pipette. It was allowed to stand at about 180C for 30 min and then 5ml of 10% potassium iodide solution was added in to the solution. Then liberated iodine was titrated with 0.02M sodium thiosulphate solution (a ml) using starch near the end point. A blank titration was carried out at the same time using 5ml of distilled water in place of vinegar (titration b ml). Procedure was repeated three times. (Pearson. 1976)(Solutions preparation were attached in Appendix v).

The Oxidation value of Rice vinegar was calculated as follows.

For 5ml of distillate,
Oxidation value = 40(b – a)

3.3.8 Determination of Alkaline Oxidation value

Distillation was done as above explained in 3.3.7 and 2ml of the distillate was added in to a 250ml glass stoppered bottle by using a 5ml pipette. 100ml of water, 10ml o 10% sodium hydroxide solution and exactly 10ml of 0.02M potassium permanganate were added in to the solution. It was allowed to stand for 30 min and then 10ml dilute sulphuric acid (1+3) and 0.5g of potassium iodide was added. Then liberated iodine was titrated with 0.02M sodium thiosulphate solution (a ml), using starch near the end point. A blank titration was carried out at the same time using 2ml of distilled water in place of vinegar.(titration b ml) (Pearson.1976)(Solutions preparation were attached in Appendix v).
The Alkaline Oxidation value of Rice vinegar was calculated as follows.

Alkaline Oxidation value= 8 (b - a)

3.3.9 Determination of Iodine value

Distillation was done as above explained in 3.3.7 and 5ml of the distillation was added to a 250ml glass stoppered bottle by using a 5ml pipette. Then distillate was made neutral to litmus with 10M potassium hydroxide. Then 10ml of M potassium hydroxide and exactly 10ml of 0.1M iodine were added. It was allowed to strand in the dark for 15 min and 10ml dilute sulphuric (1+3) was added in to the solution. Then liberated iodine was titrated with 0.02m sodium thiosulphate solution (a ml), using starch near the end point. A blank titration was carried out at the same time using 5ml of distilled water in place of vinegar.(titration b ml)(Pearson. 1976)(Solutions preparation were attached in Appendix v).

The Iodine value of Rice vinegar was calculated as follows.

For 5ml of distillate,
Iodine value = 40(b – a)

3.3.10 Determination of Formol value

10ml of vinegar sample was taken in to a stoppered conical flask by using a 10ml pipette. Few drops of phenolphthalein were added and then it was titrated with 0.1M NaOH to rose pink. 5ml of formaldehyde was also neutralized to the same tint with 0.1M NaOH solution and phenolphthalein. Then the two solutions were mixed in the stoppered conical flask and allowed to stand for 5-10 minutes. When pink color had disappeared, titrated back with 0.1M NaOH and phenolphthalein, to the same tint. The amount of 0.1M NaOH solution required for the final titration was only recorded. Same procedure was repeated three times for each vinegar sample. (Pearson.1976)(Solutions preparation were attached in Appendix v).

The Formol value of Rice vinegar was calculated as follows.

Formol value = amount of 0.1M NaOH solution (in ml), required for the final titration only.

3.3.11 Determination of Mineral value

2ml of vinegar sample was mixed with 2ml of alcohol and drops of orange indicator solution. (Pearson.1976).

3.3.12 Determination of Vinegar eels

25ml of vinegar sample was taken in to a sparkling clear glass beaker and checked for vinegar eels under direct sunlight by naked eyes.
So also a drop of vinegar sample was placed on a slide and viewed through the light microscope. Checked for vinegar eels.

CHAPTER 4
RESULT AND DISSCUSSION

4.1 Manufacture of rice vinegar

The rice vinegar was processed using steamed rice, culture, yeast and water. Unpolished rice, which contains high amounts of protein, lipid and minerals. Is considered undesirable for fermentation. Purpose of the rice polishing is to remove the undesirable substances as much as possible. The purpose of rice washing is to eliminate the sugar remaining on the surface. Also Potassium, Protein and Starch flow out and are lost during the process of rice washing. The soaking is aimed at completely steaming rice, namely turning it in to α state with water sufficiently soaked to the center of each grain. So also a large amount of enzymatic activity is to be expected. Indeed, appreciable enzymatic conversion of sucrose in to reducing sugars. Other reports of increase in reducing sugars and phenolic compounds have appeared. Steaming gave a sterilization effect on rice, facilitating the safe continuation of the fermentation process. So also the heat treatment has several other effects. Enzymes present in the grains were largely inactivated. Grain discoloration occurs due to the Maillard reaction. Antioxidants in rice were inactivated. Bg 358 variety has high gelatinization temperature and has an influence on the steaming quality of rice. It means steaming take a long time to this purpose. Normally time is given at least 1 hr for steaming.

4.2 Water for fermentation

Water has different types of minerals. Some of them should be less than 5ppm due to their high activity. Normally well water is used for fermentation, although recently a some factors have started to use tap water or industrial water. Particular care should be taken with tap water, because it often contains iron. Isolation chlorine is used as a disinfectant and responds to organic matter as soon as the fermentation process commences. The chlorine eliminates odours, so it does not require as much care as tap water. To add water to vinegar production, water is filtered with activated charcoal to remove the isolated chlorine. Iron is the active principle to be most actively avoided, because it thickens the color of fermentation solution, and affect the taste and smell. Like ammonia and nitrous acid, the presence of many organic matters in water means there may be rotten animals or plants present

4.3 Role of microorganism

At the beginning of the Rice vinegar production activity of *Aspergillus oryzae* and fermentation process were carefully controlled. *Aspergillus oryzae* have important enzymes involve in saccharification process, which are named the amylases, proteases and lipase. Mycelium of *Aspergillus oryzae* growing not desirable for alcoholic fermentation, so by using the autoclave fungi were the very important stage. *Saccharomyces cerevisiae* showed their high activity during 130 hrs. So time was more important to control the alcohol strength. Yeast cells dead due to the undesirable environment made in the medium. Before going to the next step autoclaving was done to destroyed the yeast cell and their enzymes. Acetic acid fermentation was carefully controlled by giving optimum condition. Dark bottles were used through out the acetic acid fermentation process. Because acetic acid bacteria activity is greatly reduced or inhibited by direct sunrays. Even diffused day light checks their growth. This process took normally long time than alcoholic fermentation. Usually the time taken to complete one acetification cycle is in the order of 14 days. Extending of acetic acid fermentation may help to get low quality due to over oxidation of alcohol. So to stop over oxidation, pasteurization was used by giving in optimum condition.

4.4 Change in Brix value during the activity of *Aspergilus oryzae* at room temperature

Table 4.1 Changes in Brix value during activity of *Aspergillus oryzae*

Day	Brix value (Aerobic condition)	Brix value (Anaerobic condition)
0	0.0	0.0
1	1.3	0.6
2	2.2	1.1
3	3.4	1.7
4	4.3	2.3
5	5.6	3.2
6	7.8	5.3
7	8.9	6.1
8	10.0	7.0
9	11.2	7.0
10	11.2	7.0

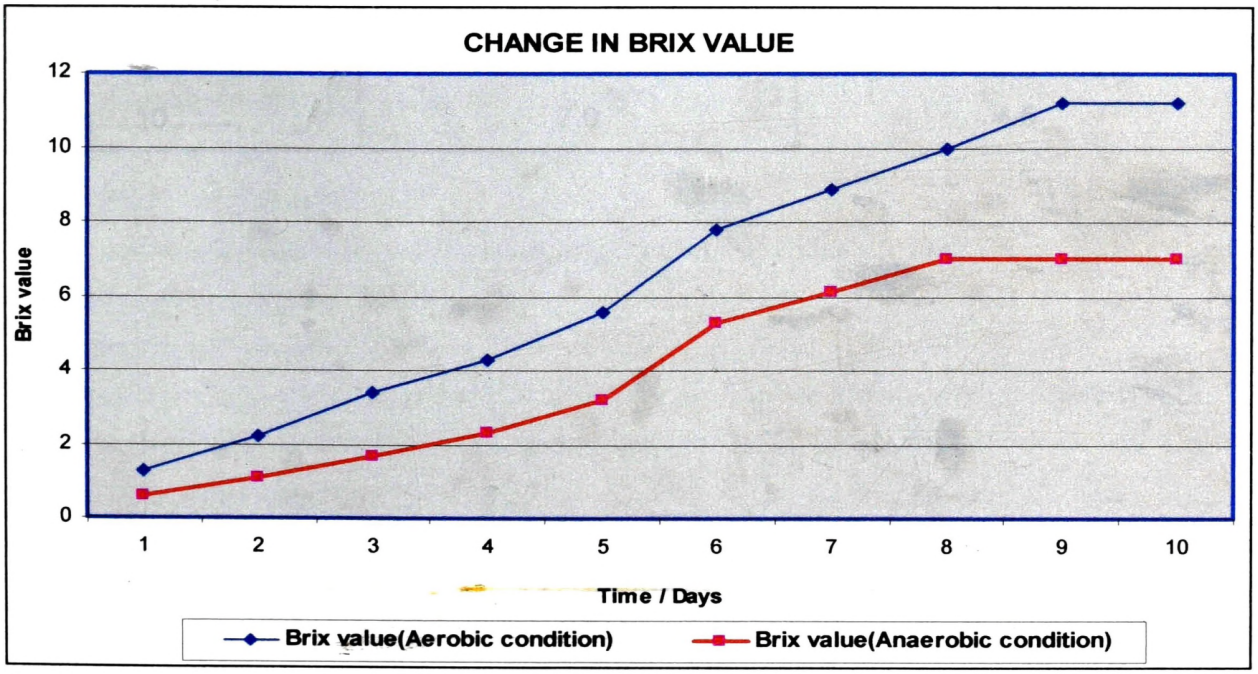

Figure 4.1 Change in brix value at room temperature

A result of the Table 4.1 is graphically expressed in Figure 4.1.From the above explained results (Table 4.1 & Figure 4.1) following expressions can be made.

- ❖ The Brix value increased gradually with time
- ❖ After two weeks later, maximum Brix value was shown in aerobic condition than in anaerobic condition.
- ❖ These readings were recorded in fermentation solution with out yeast cells.

4.5 Change in Brix value during activity of *Aspergillus oryzae* at cold temperature (15^0C).

Table 4.2 Changes in Brix value during activity of *Aspergillus oryzae*

Day	Brix value (Aerobic condition)	Brix value (Anaerobic condition)
0	0.0	0.0
1	1.1	0.3
2	1.8	0.7
3	2.5	1.2
4	3.1	1.7
5	3.9	2.3
6	5.3	3.4
7	6.2	4.2
8	7.0	4.4
9	7.0	4.5
10	7.0	4.5

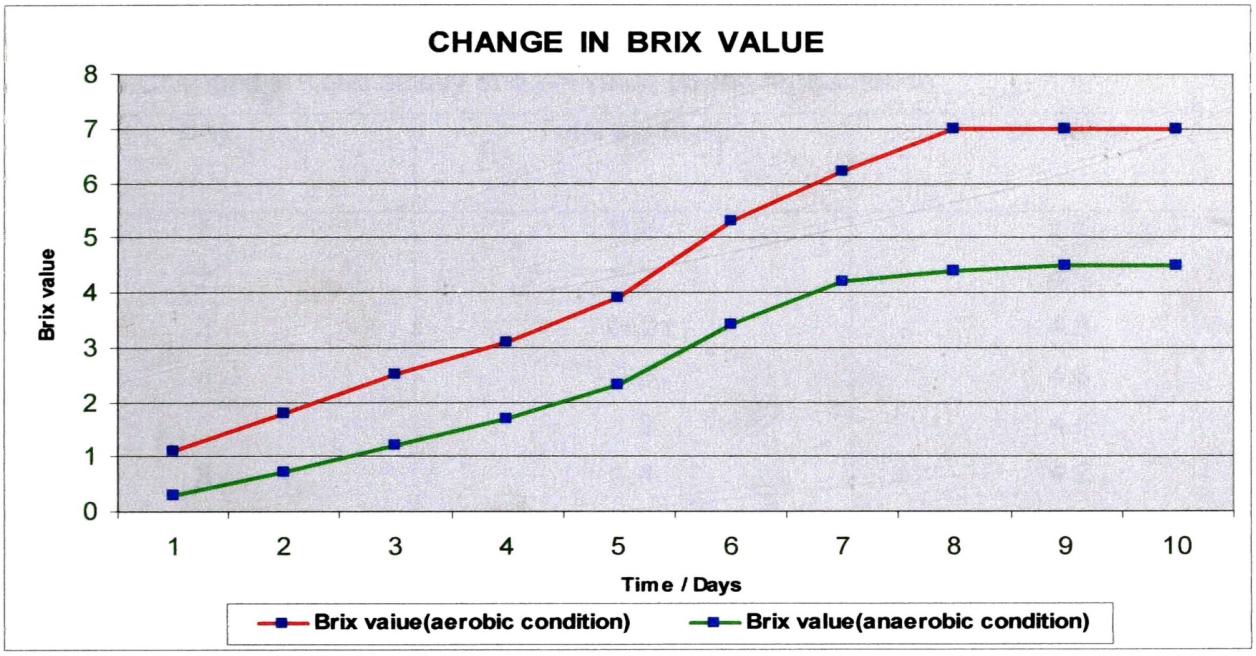

Figure 4.2 Change in brix value at cold temperature

Result of the Table 4.2 is graphically expressed in Figure 4.2. According to the above results expressed in Table 4.2 and Figure 4.2 within the given time period at cold temperature (15^0C) the brix values are not increased as in normal temperature conditions.

4.6 Reducing sugar

End of the activity period of Aspergillus oryzae, reducing sugar was grained as 19.75%.
(Data were attached in Appendix I)

4.7 Alcoholic strength

The present alcoholic strength of rice vinegar samples were as follows.
% of alcohol by weight = 5.71%
% of alcohol by volume = 7.16%
Above alcoholic percentage desirable for acetic acid fermentation. High amount of alcohol is used to inhibit the acetic acid bacteria. Alcohol strength was calculated by using the table shown in Appendix II.

4.8 Change in Total acidity and pH value during Acetic acid fermentation

Table 4.3 Changes in Total acidity and pH value during fermentation

Day	Total acidity%	pH value
1	0.2	5.1
2	0.3	4.9
3	042	4.8
4	0.8	4.6
5	1.2	4.5
6	1.4	4.2
7	1.7	4.0
8	1.86	3.9
9	2.0	3.8
10	2.24	3.6
11	2.6	3.4
12	2.9	3.3
13	3.2	3.1
14	3.45	3.0
15	3.7	2.8
16	3.94	2.75
17	4.2	2.6
18	4.5	2.52
19	4.7	2.34
20	4.92	2.2
21	5.1	2.0
22	5.2	1.84

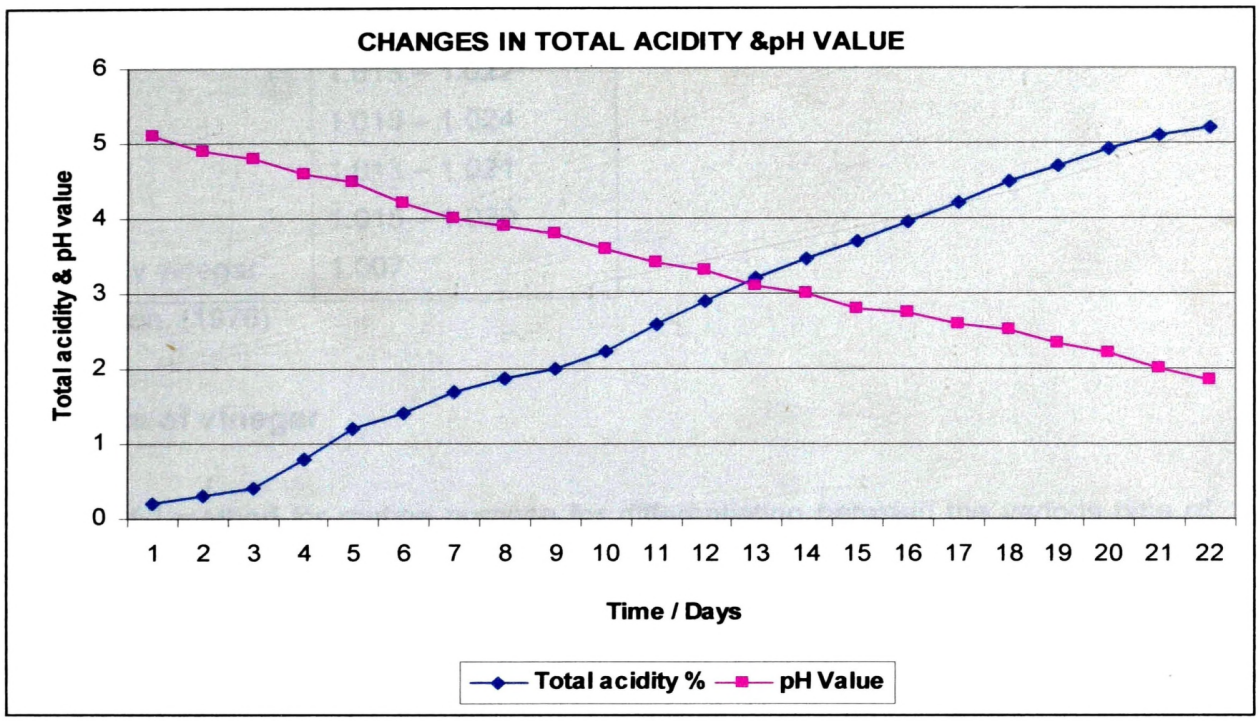

Figure 4.3 Changes in Total acidity% & pH value during Acetic acid fermentation

Results of the Table 4.3 is graphically expressed in Figure 4.3. Following expressions can be made according to the Table 4.3 & Figure 4.3.

- The pH value decreased gradually with time.
- The acetic acid content increased gradually with time.
- After 17 days, acetic acid content reached approximately 4.0(w/v) the legal acetic acid content of vinegar.
- Afterwards, the rate of acetic acid formation declined
- Due to the low pH value, vinegar used as a preservative especially in the pickle manufacturing.

4.9 Specific gravity of vinegar

Specific gravity of Rice vinegar was 1.0048. It can be predicted as a good reading when comparing with the standard reading of other given vinegars in Table 4.4.

Table 4.4 Value of specific gravity

Vinegar type	Specific gravity
Malt vinegar	1.013 – 1.022
Cider vinegar	1.013 – 1.024
Wine vinegar	1.013 – 1.021
Sprit vinegar	1.015 – 1.020
Coconut toddy vinegar	1.007

Source: Pearson. (1976)

4.10 Analysis of vinegar

The most useful method for routine purpose for differentiating between the various type of vinegars are based on the values obtained on the volatile substances present. I.e. the Oxidation, Alkaline oxidation and Iodine value. Brewed vinegar gave comparatively high values, but those for artificial products are low as the latter are almost devoid of volatile reducing substances. The formol titration represents a useful rapid method for deciding whether vinegar is brewed or not. When comparing with other vinegars, values in tables 4.5, 4.6 and 4.7. The produced rice vinegar comes within this range and with good quality attributes.

Table 4.5 Analysis reading of Rice vinegar

Characteristics	Requirements
Total acidity as acetic acid	4.5%
Oxidation value	952
Alkaline oxidation value	40
Iodine value	360
Formol value	0.5

4.10.1 SLS standard for vinegar

Sri Lanka SLS standards are more important to make Rice vinegar to obtain good quality parameters. Table 4.6 is shown the above characteristics that were published in the minimum level of the SLSI.

Table 4.6 SLS standards of vinegar

Characteristics	Requirements
Total acidity as acetic acid g/100ml, (minimum)	4%
Oxidation value,(minimum)	750
Alkaline oxidation value,(minimum)	80
Iodine value,(minimum)	160

Source:SLS 1194 hand book

Table 4.7 Comparison reading of other vinegar

Characteristics	Malt vinegar	Cider vinegar	Wine vinegar	Sprit vinegar
Total acidity%	4.3 – 5.9	3.9 – 9.0	4.4 – 7.4	11.5 – 12.2
Oxidation value	500 – 1800	up to 3500	600 – 2000	90 – 650
Alkaline oxidation value	70 – 180	_	60 – 180	3 – 20
Iodine value	380 - 1500	_	380 - 1000	0 - 20

Source: Pearson (1976)

4.10.2 Mineral acid in vinegar

Rice vinegar was indicated orange color due to the low mineral acid. Traces of sulphate are usually present in vinegar, but if the amount exceeds 0.03% (as H2SO4).It would be indicated by red color. The red color indicates a low pH due to added mineral acid.

4.11 Sensory evaluation to determination the Taste, Color and Odour

The samples given for the panelists as follows.

Sample 1 – Coconut toddy vinegar

Sample 2 – Red rice vinegar

Sample 3 – Rice vinegar

Scoring sheet was attached in Appendix III

4.11.1 Taste

Sensory evaluation for taste was determined by using Kruskal-wallis test

Table 4.8 Analysis results of sensory evaluation for taste

Factors	N	Medium	Ave.Rank	Z
Sample 1	15	3.000	17.3	-2.07
Sample 2	15	7.000	29.7	2.41
Sample 3	15	4.000	22.1	-0.34
Overall	45		23.0	

H = 6.80 DF = 2 P = 0.033

H = 6.96 DF = 2 P = 0.031 (adjusted for ties)

H_o: There is no significant between vinegar samples at 5% level

H_1: There is a significant between vinegar samples at 5% level

According to above results P value is less than 0.05 significant level. So there is a significant difference between vinegar samples at 5% level. Sample which got the lowest average rank samples 1 is the best sample and the sample which got the next average rank sample 3 is also good.

4.11.2 Color

Sensory evaluation for color was determined by using Kruskal-wallis test

Table 4.9 Analysis results of sensory evaluation for color

Factors	N	Medium	Ave.Rank	Z
Sample 1	15	3.000	18.9	-1.47
Sample 2	15	7.000	31.6	3..11
Sample 3	15	4.000	18.	-164
Overall	45		23.0	

H = 9.66 DF = 2 P = 0.008

H = 9.84 DF = 2 P = 0.007 (adjusted for ties)

H_o: There is no significant between vinegar samples at 5% level

H_1: There is a significant between vinegar samples at 5% level

According to above results P value is less than 0.05 significant level. So there is a significant difference between vinegar samples at 5% level. Sample which got the lowest average rank samples 3 is the best sample and the sample which got the next average rank sample 1 is also good.

4.11.3 Odour

Sensory evaluation for odour was determined by using Kruskal-wallis test.

Table 4.10 Analysis results of sensory evaluation for odour

Factors	N	Medium	Ave.Rank	Z
Sample 1	15	4.000	20.0	-1.07
Sample 2	15	6.000	27.7	1.70
Sample 3	15	4.000	21.3	-0.63
Overall	45		23.0	

H = 2.95 DF = 2 P = 0.229

H = 2.99 DF = 2 P = 0.224 (adjusted for ties)

H_o: There is no significant between vinegar samples at 5% level

H_1: There is a significant between vinegar samples at 5% level

According to above results P value is less than 0.05 significant level. So there is a significant difference between vinegar samples at 5% level. Sample which got the lowest average rank samples 1 is the best sample and the sample which got the next average rank sample 3 is also good.

Collection data of sensory evaluation was attached in Appendix IV.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The main aim of production of this type of product as a flavouring and preservative is to fulfill the market requirements. By introducing new Rice vinegar can diversify the vinegar market. During the rice harvesting seasons Rice vinegar can be produced by using the excess rice production, and can utilize surplus rice effectively.

Rice vinegar contains 5.2% of acetic acid, 1.84 of pH value, 952 of oxidation value, 40 of Alkaline oxidation value, 360 of Iodine value and 0.5 of Formol value. It has a low amount of minerals and the specific gravity of the produced Rice vinegar is 1.00048. The characteristics of the produced vinegar are within the standard range. Also the organoleptic attributes of the produced Rice vinegar are statistically accepted. So can introduce the new Rice vinegar in to the market without hesitation.

5.2 Recommendations for further studies

1. Odour of the product can be improved by developing the raw material by adding some fruit vinegar.
2. The percentage of the utilization of rice starch due to the activity of *Aspergillus oryzae.*
3. The amount of protein and minerals should be analyzed.

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APPENDX I

Invert sugar table for 10ml Fehling's solution

ml of sugar solution required	Invert sugar factors*	mg invert sugar per 100ml
15	50.5	336
16	50.6	316
17	50.7	298
18	50.8	282
19	50.8	267
20	50.9	254.5
21	51.0	242.9
22	51.0	231.8
23	51.1	222.2
24	51.2	213.3
25	51.2	204.8
26	51.3	197.4
27	51.4	190.4
28	51.4	183.7
29	51.5	177.6
30	51.5	171.7
31	51.6	166.3
32	51.6	161.2
33	51.7	156.6
34	51.7	152.2
35	51.8	147.9
36	51.8	143.9
37	51.9	140.2
38	51.9	136.6
39	52.0	133.3
40	52.1	130.1
41	52.1	127.1
42	52.2	124.2
43	52.2	121.4
44	52.3	118.7
45	52.3	116.1
46	52.4	113.7
47	52.4	111.4
48	52.5	109.2
49	52.5	107.1
50	52.5	105.1

* mg of invert sugar corresponding to 10ml of Fehling's solution

APPENDIX II

Relationship between Specific gravity of Alcoholic solution at 20°C

Specific gravity in air at 20°C/20°C	Reading at 20°C% Alcohol	
	By weight	By volume
0.7904	100.0	100.00
0.7936	98.98	99.37
0.8000	96.85	98.02
0.8100	93.38	95.69
0.8200	89.71	93.06
0.8300	85.89	90.18
0.8400	81.94	87.07
0.8500	77.89	83.75
0.8600	73.77	80.25
0.8700	69.60	76.59
0.8800	65.38	72.78
0.8900	61.12	68.80
0.9000	56.80	64.66
0.9100	52.41	60.33
0.9200	47.93	55.77
0.9300	43.31	50.94
0.9400	38.42	45.68
0.9500	33.16	39.85
0.9600	27.25	33.09
0.9700	20.28	24.88
0.9800	12.65	15.68
0.9900	5.71	7.16
1.0000	0.00	0.00

APPENDIX III

Hedonic Rating Test

Name :- **Date:-**

Please consume the samples in order to evaluate all attributes starting from left side.

Rinse your mouth with water after tasting each samples.

Please answer the following questions by completely filling in the square that best reflects your feelings about this sample.

1. How would you the COLOR of this sample

Extremely Dislike Extremely Like

---

2. How would you rate the Taste of this sample

Extremely Dislike Extremely Like

---

3. How would you rate the ODOUR of this sample

Extremely bad Acceptable odour

POINT SCALE	POINT
Like extremely/ Acceptable odour	1
Like very much	2
Like moderately	3
Like slightly	4
Neither likes or dislike	5
Dislike slightly	6
Dislike moderately	7
Dislike very much	8
Dislike extremely / Extremely bad	9

Thank you

APPENDIX IV

Collection data of sensory evaluation

1. Color

Sensory aspect	Sample 1	Sample 2	Sample 3
1	8	7	6
2	4	2	9
3	9	2	4
4	9	2	5
5	6	4	3
6	2	6	3
7	6	4	3
8	5	6	4
9	1	8	2
10	4	8	3
11	1	8	2
12	3	7	2
13	4	8	3
14	3	9	2
15	2	5	1

2. Taste

Sensory aspect	Sample 1	Sample 2	Sample 3
1	8	7	6
2	5	2	9
3	1	4	7
4	6	4	5
5	2	9	4
6	4	4	4
7	3	5	4
8	2	7	3
9	2	7	8
10	4	8	6
11	1	7	2
12	4	5	3
13	5	3	2
14	2	2	3
15	3	7	2

3. Odour

Sensory aspect	Sample 1	Sample 2	Sample 3
1	9	6	7
2	5	2	9
3	7	2	4
4	6	5	4
5	2	9	3
6	4	3	3
7	4	5	6
8	1	8	1
9	2	6	7
10	2	8	5
11	8	4	3
12	6	4	2
13	3	8	5
14	1	7	3
15	2	5	4

APPENDIX V

1. PREPARATION METHOD OF 0.1M NaOH SOLUTION

- 4g of NaOH were measured by using the electronic balance.
- It was placed in a standardized stoppered 1000ml flask and little amount of distilled water was added.
- Then it was shaked thoughraly until all NaOH was dissolved.
- Distilled water was added up to the point carefully.

2. PREPARATION METHOD OF 0.02M $Na_2S_2O_3$ SOLUTION

- 4.963g of Na2S2O3 were measured by the electronic balance.
- It was placed in a standardized stoppered 1000ml flask and amount of distilled water was added.
- Then it was shaked thoughraly until dissolving completed.
- Distilled water was added up to the point carefully.

3. PREPARATION METHOD OF 0.02M $KMnO_4$ SOLUTION

- 3.16g of KMnO4 were measured by using the electronic balance
- It was placed in a standardized stoppered 1000ml flask and little amount of distilled water was added.
- Then it was shaked thoughraly until dissolving completed.

4. PREPARATION METHOD OF FEHLING A SOLUTION

- 34.639g of clear crystals of copper sulphate ($CuSO_4\,5H_2O$) were measured by using the electronic balance.
- It was placed in a cleaned 500ml volumetric flask and little amount of distilled water was added.
- Then it was shaked thoroughly until all the crystals were dissolved.

- 2 drops of conc.H2SO4 was added and then distilled water was added up to the point carefully.
- Distilled water was added up to the point carefully.

5. PREPARATION METHOD OF FEHLING B SOLUTION

- 173g of Sodium potassium tartarate (Rochelle salt) and 5g of NaOH was measured by using the electric balance.
- It was placed in a cleaned 500ml volumetric flask and about 4000ml distilled water was added.
- Then it was shaked thoughraly until dissolving completed.
- It was allowed to stand over night, and then it was filtered through a gooch crucible.

6. PREPARATION METHOD OF 1% METHYLENE BLUE SOLUTION

- 1.000g of methylene blue was measured by using an electrical balance.
- It was dissolved in 100ml of distilled water.
- The solution was preparation freshly when need.

7. PREPARATION METHOD OF 0.1M IODINE

- 12.69g of iodine and 18g of potassium iodide were measured by using the electronic balance.
- It was placed in a standardized stoppered 1000ml flask and amount of distilled water was added.
- Then it was shaked thoughraly until dissolving completed.
- Distilled water was added up to the point carefully.

8. PREPARATION METHOD OF 10M POTASSIUM HYDROXIDE

- 561.1g was measured by using the electronic balance
- It was placed in a standardized stoppered 1000ml flask and amount of distilled water was added.
- Then it was shaked thoughraly until dissolving completed.
- Distilled water was added up to the point carefully.

9. PREPARATION METHOD OF 0.5M NaOH SOLUTION

- 20g NaOH were measured by the using the electronic balance.
- It was placed in a standardized stoppered 1000ml flask and little amount of distilled was added.
- Then it was shake thoughraly until all NaOH was dissolved.
- Distilled water was added up to the point carefully.

10. PREPATION METHOD OF 0.05M NaOH SOLUTION

- 0.20g of NaOH were measured by the using the electronic balance.
- It was placed in a standardized stoppered 1000ml flask and little amount of distilled water was added.
- Then it was shaked thoughraly until all NaOH was dissolved.
- Distilled water was added up to the point carefully.

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