DYNAMIC CHANGES IN BIOCHEMICAL AND PHYSICOCHEMICAL PROPERTIES OF CASSAVA VINEGAR WITH AND WITHOUT AGARWOOD LEAF INFUSION

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DYNAMIC CHANGES IN BIOCHEMICAL AND PHYSICOCHEMICAL PROPERTIES OF CASSAVA VINEGAR WITH AND WITHOUT AGARWOOD LEAF INFUSION

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ABSTRACT

DYNAMIC CHANGES IN BIOCHEMICAL AND PHYSICOCHEMICAL PROPERTIES OF CASSAVA VINEGAR WITH AND WITHOUT AGARWOOD LEAF INFUSION

LAM WEI SHAN

Vinegar is a condiment made from raw agricultural ingredients containing starch and sugar through a double fermentation process: alcoholic and acetic. Although vinegar's functional properties are well known, its physicochemical and biochemical changes during fermentation with the incorporation of plant-derived materials such as agarwood leaves remain underexplored. The objectives of this study are to assess the saccharification rate through reducing sugar and free amino acid analysis, and to determine ethanol production during the alcoholic fermentation stage of cassava., to study the dynamic changes in biochemical and physicochemical properties throughout the acetic acid fermentation process, and to determine the effect of agarwood leaf infusion on the biochemical and physicochemical properties of cassava-based vinegar. Cassava was first saccharified using ragi for two days, followed by 14-day alcoholic fermentation with wine yeast. Two sets were prepared: one with agarwood leaf infusion and one with water. Mother vinegar was added to initiate acetic acid fermentation. During fermentation, sugar (DNS test), free amino acids (ninhydrin test), alcohol (potassium dichromate test), pH, total titratable acidity, acetic acid (HPLC), and total phenolic content (TPC) (Folin-Ciocalteu

method) were monitored and compared between both sets. The sugar content of cassava increased from 3.58% to 16.28% (db) after saccharification. The agarwood leaf-based sample had a lower pH (4.89), higher TPC (0.1402 mg GAE/mL), and lower alcohol content (5.56%) compared to the water-based sample (pH 5.15, TPC 0.1215 mg GAE/mL, alcohol 6.48%). The acetic acid concentration for both sets varied between 0.68 and 4.39 mg/mL. Phytochemical tests showed that alkaloids, flavonoids, and coumarins were present in both samples. Tannins were only detected in the agarwood leaf-based vinegar, and quinones only in the water-based vinegar. The overall physicochemical and biochemical profile of the vinegar was significantly improved by the addition of agarwood leaves infusion, as indicated by higher TPC and increased fermentation efficiency.

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DECLARATION

I hereby declare that this final year project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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LIST OF ABBREVIATIONS

% (v/v)	Percentage volume per volume
% (w/v) AAE	Percentage weight per volume acetic acid equivalent
% db	Percentage on dry basis
× g	Relative centrifugal force (RCF)
®	Registered
°C	Degree Celsius
µL	Microlitre
AAB	Acetic acid bacteria
ABV	Alcohol by volume
DNS	3,5-dinitrosalicylic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
g	Gram
GAE	Gallic acid equivalent
h	Hour
HPLC	High-Performance Liquid Chromatography
L	Litre
LAB	Lactic acid bacteria
M	Molar
mg/mL	Milligram per millilitre
min	Minute
mL	Millilitre
N	Normality
PDA	Potato dextrose agar

rpm	Revolutions per minute
sp.	Species
TBP	Tri-n-butyl phosphate
™	Trade mark
TPC	Total phenolic content

CHAPTER 1

INTRODUCTION

Vinegar is a widely consumed acidic condiment and preservative that has been recognized due to its distinctive taste as well as its medicinal and functional properties. Vinegar can be divided into two categories depending on the raw materials used, which are grain and fruit vinegars. In East Asia, rice vinegar is deeply rooted in traditional cuisines, especially in countries such as China, Japan, and Korea. It is commonly used as a seasoning and flavor enhancer in dishes (Murooka and Yamashita, 2008). In Asian countries, most vinegar such as Kurozu vinegar (traditional Japanese black vinegar), and Shanxi aged vinegar (traditional Chinese vinegars) are typically produced using solid-state fermentation. Meanwhile, European vinegars such as balsamic and apple cider vinegar are mainly produced by liquid-state fermentation (Xia et al., 2020).

Rice and malt vinegar are the examples of grain vinegar. They are produced from cereals rich in starch, hence require an additional saccharification step to break down starch into fermentable sugars. This process is commonly done by enzymatic or microbial action before alcoholic fermentation begins (Wang et al., 2023). Fruit vinegars are made from fruits like apples or grapes that are rich in simple sugar. As a result, they can be blended and proceed directly to alcoholic fermentation without saccharification step (Luzon-Quintana et al., 2021). Since vinegar has been produced and sold for about 5000 years, its production method has changed (Budak et al., 2024). Traditionally, it is produced through surface

methods, at which the bacteria grow at the liquid-air interface while aeration is often applied in modern submerged fermentation to accelerate vinegar production and hence increase yield (Luzon-Quintana et al., 2021).

Vinegar is produced through a two-step sequential fermentation process, at which yeast first converts the sugars into ethanol, followed by ethanol oxidation into acetic acid by acetic acid bacteria. This process enriches vinegar with diverse bioactive compounds such as organic acids, amino acids, polyphenols, vitamins and melanoidins, which are responsible for both functional and organoleptic properties of vinegar (Budak et al., 2024; Chen et al., 2016).

Although vinegar is widely known for its functional and beneficial properties, most studies focus on fruit and grain vinegars. There are limited studies on cassava as a vinegar source. Given that cassava is one of the most abundantly cultivated staple crops in tropical and subtropical regions, its application in vinegar production could provide both nutritional and economic value. In addition, the time-based biochemical and physicochemical changes in cassava vinegar also remain poorly documented. Studies have shown that fermentation of herbs can enhance the functional properties by increasing phenolic content and transforming complex compounds into more bioactive forms. For instance, Leksono and Murtini (2021) showed that fermentation of agarwood leaves for 24 hours doubled the total phenolic content and antioxidant activity. While the resinous heartwood is the main commercial product of *Aquilaria* trees, the leaves are often treated as by-products in the agarwood industry. In recent years,

agarwood leaves have gained increasing attention for their use in teas and aromatic oil due to their bioactive compounds and associated pharmacological benefits (Wu et al., 2023). However, their potential application in vinegar production remains underexplored.

Hence, this study aims

1. To assess the saccharification rate through reducing sugar and free amino acid analysis, and to determine ethanol production during the alcoholic fermentation stage of cassava.
2. To unravel the dynamic changes in biochemical and physicochemical properties throughout the acetic acid fermentation process.
3. To determine the effect of agarwood leaf infusion on the biochemical and physicochemical properties of cassava-based vinegar.

CHAPTER 2

LITERATURE REVIEW

2.1 Vinegar as a Product of Fermentation

Vinegar is a condiment containing high amounts of acetic acid. It is produced from sugar- and starch-rich agricultural raw materials such as grapes, apples and rice through a two-stage fermentation process involving alcoholic fermentation followed by acetic acid fermentation. Vinegar can be produced through solid-state and liquid-state fermentation, depending on geographical areas. Besides acetic acid, vinegar also contains a variety of other constituents such as organic acids, polyphenols, vitamins and minerals, depending on the raw materials and production technique used. These constituents are vital in shaping the vinegar's sensory attributes, including taste, aroma, antioxidant and blood glucose-regulating effects. Nowadays, vinegar is used as a natural additive to improve both sensory qualities and microbial safety in the food industry (Garcia-Parrilla et al., 2017; Xia et al., 2020).

2.1.1 Benefits of Vinegar

One of the most widely studied benefits of vinegar is its impact on metabolic health. According to Santos et al. (2019), consuming vinegar with carbohydrate-rich meals can effectively improve postprandial glycemic response. This is done by inhibiting α-amylase activity to slow down starch digestion and rate of glucose absorption. In addition, studies have shown that vinegar consumption can enhance satiety, resulting in a reduction of daily energy intake by roughly

200 to 275 kcal. Over time, this reduction may support gradual weight loss (Budak et al., 2014).

According to Shahinfar et al. (2022), daily vinegar consumption can support cardiovascular health by regulating the blood pressure. For instance, a daily consumption of around 30 mL of vinegar has caused a reduction in both systolic and diastolic blood pressure by roughly 3 mmHg. Besides, fermented vinegar also exhibits antioxidant and antimicrobial activities. Phenolic compounds and other phytochemicals present in different vinegars have been shown to neutralize free radicals, thereby reducing oxidative stress (Liu et al., 2019). Overall, vinegar offers a wide range of health benefits that extend beyond its traditional culinary use.

2.1 Steps involved in Vinegar Production

2.2.1 Saccharification Process

Saccharification refers to the process of conversion of starch into fermentable sugars. There are two types of saccharification process: acidic and enzymatic. Acidic saccharification employs acids such as sulfuric or hydrochloric acid under elevated temperature and pressure to convert cellulose into sugars (Chang et al., 2018). In contrast, enzymatic saccharification is widely used in the food industry. Cellulases and hemicellulases are the key extracellular enzymes produced by fungi such as *Penicillium roqueforti*. These enzymes hydrolyze complex polysaccharides into free sugars, which serve as substrates for various

fermentations like alcohol fermentation. They generally work best at pH range of 4 to 6 and temperature between 38 and 60 °C, depending on the enzyme used. Compared to chemical hydrolysis, enzymatic saccharification offers advantages such as higher sugar yields, lower energy requirements, milder operating conditions and fewer undesirable by-products (Ferraz et al., 2017; Musdalifa, Laga and Rahman, 2024).

2.2.2 Alcoholic Fermentation

Alcoholic fermentation is a key metabolic pathway in which yeasts, particularly *Saccharomyces cerevisiae*, convert sugars such as glucose and fructose into ethanol and carbon dioxide under anaerobic conditions. Alcoholic fermentation usually takes place at temperatures ranging from 18 to 30 °C, while the total fermentation time depends on factors such as fermentation temperature, the yeast strain employed and the concentration of available substrates (Walker and Stewart, 2016). During alcoholic fermentation, *S. cerevisiae* metabolized sugars via glycolysis and produced pyruvate as a key intermediate compound. Under anaerobic environments, pyruvate is decarboxylated to acetaldehyde by pyruvate decarboxylase, which is then reduced to ethanol by alcohol dehydrogenase. At the same time, NAD+ is regenerated to maintain glycolytic activity (Topaloglu et al., 2023; Walker and Stewart, 2016).

In beverage industries like winemaking and brewing, *S. cerevisiae* is the dominant yeast due to its high fermentation efficiency and strong ethanol tolerance (Querol et al., 2003). It is a unicellular fungus that requires a minimum

water activity above 0.65 for survival. Being a facultative anaerobe, *S. cerevisiae* is capable of growth within a temperature range of 20 to 30 °C and thrives best in slightly acidic conditions, usually between pH 4.5 and 6.5 (Walker and Stewart, 2016).

Besides alcohol and carbon dioxide, *S. cerevisiae* also produces various secondary metabolites such as esters, higher alcohols, and organic acids. These metabolites enhance the distinctive sensory attributes of the final product, especially its aroma and flavor. Alcoholic fermentation not only supports microbial energy metabolism, but also plays a vital role in food biotechnology, driving both product yield and sensory quality (Maicas, 2020; Mussatto et al., 2010).

2.2.3 Acetic Acid Fermentation

In acetic acid fermentation, acetic acid bacteria oxidize alcohols and sugars into acetic acid in the presence of oxygen. There are two types of acetic acid fermentation: surface culture and submerged fermentation. Surface culture fermentation is a traditional method used to produce vinegar. The acetic acid bacteria grow as a film on the liquid surface to utilize oxygen for ethanol oxidation. *Acetobacter* is the most abundant acetic acid in surface culture fermentation, yielding low acetic acid content in longer period due to limited oxygen (Gomes et al., 2018; Ge et al., 2025). On the other hand, submerged fermentation required a shorter fermentation duration than surface culture method. This is done by the utilization of pumps to incorporate air bubbles into

the fermenting solution, thereby increasing the air-liquid interfaces for oxidation process to take place. Hence, faster ethanol oxidation, higher acid concentrations and improved productivity can be achieved (Mas et al., 2014).

The efficiency of acetic acid fermentation is influenced by environmental and microbial factors. Temperature is one of the most important parameters, with most acetic acid bacteria growing optimally at 30 °C, only some thermotolerant strains can withstand up to 42°C (Kourouma et al., 2022). Besides, oxygen availability is also crucial for acetic acid fermentation as hypoxia will lead to reduced activity and lower yield (Qiu, Zhang and Hong, 2021).

2.3 Cassava as a Fermentation Substrate

Cassava, scientifically known as *Manihot esculenta*, is one of the starchy root crops commonly consumed by humans. Sweet and bitter cassavas are the two commonly known varieties of cassavas (Bovell-Benjamin and Roberts, 2015). Raw and uncooked cassavas contain significant amounts of cyanogenic glycosides, primarily in the form of linamarin and small amount of methyl linamarin. When cassava tissues are damaged during peeling, cutting, or storage, linamarin will undergo enzymatic hydrolysis by linamarase to form toxic hydrogen cyanide. The concentrations of linamarin are high in the leaves and epidermis layers of cassavas. However, sweet cassava contains up to 50 times lower amounts of cyanogenic glucosides compared to bitter cassava (Cardoso et al., 2004). Appropriate processing methods such as boiling, drying,

fermenting and peeling cassava can significantly diminish these toxins, rendering them safe for human consumption (Borku, Tora and Masha, 2025).

Despite its high natural toxicant content when improperly processed, cassava is often consumed as an energy source in tropical and subtropical regions owing to its high starch content. Cassava tubers are rich in carbohydrates, including starch levels ranging from 34.2 to 35.1% (Charles et al., 2004). However, cassava tubers possess a significantly lower protein content than cassava leaves, varying from 0.8% to 1.1% (Charles et al., 2004).

2.3.1 Cassava-Based Fermented Foods and Beverages

In order to remove toxins, cassavas are further processed into cassava starch, cassava flour and fermented cassava products. For example, cassavas are fermented by lactic acid bacteria into a traditional food in Africa called *gari* over a period of 3 to 5 days, dependent upon the preferred flavor (Oguntoyinbo and Dodd, 2010). Besides, a study indicates that the solid-state fermentation of cassava using *Daqu*, a traditional starter in Chinese liquor brewing, resulted in a 57.39% rise in reducing sugar content and elevated amounts of leucine, valine, and phenylalanie after 6 days of fermentation. The high reducing sugar content makes cassava a desired fermentation substrate capable of providing energy to sustain microbial development in later stages (Yang et al., 2025). According to Galapia, Wasin and Labog (2018), the kamoteng kahoy (cassava) vinegar with 4% total acidity was successfully produced using dry yeast. In a sensory evaluation, 15 untrained panelists rated the vinegar from ‘like very much’ to

'like moderately'. This indicates that the product is generally acceptable in terms of sensory quality.

2.4 Ragi as a Saccharification Starter Culture

Ragi tapai is a widely used starter culture in fermenting starchy foods. It usually appeared in dried cake form of round and flattened shape (Gunam et al., 2021). Ragi tapai is produced by blending rice flour with spices such as pepper and garlic together with water. The mixture is then shaped into small cakes and dried (Delva, Arisuryanti and Ilmi, 2022). It contains diverse microbial communities that typically include yeasts from the *Saccharomyces* sp. and moulds such as *Rhizopus* sp., *Mucor* sp. and *Amylomyces* sp. The microbes are important in breaking down complex starch into simpler sugar form for subsequent fermentation steps (Anasa et al., 2019).

2.4.1 Application of Ragi in Fermenting Food

Ragi has been used in fermenting various foods such as *tapai pulut*, *tempe* and *oncom*. In *tapai pulut*, ragi is mixed with the cooked glutinous rice and fermented anaerobically. A successfully fermented tapai pulut will exhibit a sweet and sour taste with mild alcoholic note. This is due to the increase in reducing sugar content and decrease in pH resulting from lactic acid bacteria (Anasa et al., 2019). In tempeh production, ragi is mixed with cooked beans and incubated for 1 to 2 days to promote mycelial growth, which binds the beans into the characteristic white tempeh (Nout and Kiers, 2005).

2.5 Mother Vinegar as Acetic Acid Fermentation Starter Culture

Mother vinegar, which is generally raw, unfiltered, and unpasteurized vinegar. It is often used as a natural starter culture because it contains a variety of living populations of acetic acid bacteria that maintain and promote ongoing fermentation (Yetiman and Kesmen, 2015). Mother vinegar contains a mixture of phenolic compounds, carbohydrates and essential minerals. The addition of mother vinegar delivers the microbial inoculum needed for converting ethanol into acetic acid. This ensures the acetic acid fermentation progress. Besides, it also acts as a carrier or bioactive molecules which provide antioxidant capacity and other health-enhancing properties (Gomes et al., 2018; Han et al., 2024).

2.6 Agarwood Leaf

Agarwood leaf is derived from *Aquilaria malaccensis* Lamk, which is widely produced into tea leaf. It offers a wide range of pharmacological benefits. This is mainly due to the presence of various phytochemicals compounds such as flavonoids, tannins, terpenoids, alkaloids and phenolic acids in the agarwood leaf which are released upon extraction or infusion (Xie et al., 2024). For example, flavonoids in agarwood leaves contribute to strong antioxidants against low-density lipoprotein (LDL) (Adam, Lee and Mohamed., 2017). Studies have shown that agarwood leaves demonstrate wound-healing properties. The spray-dried leaf extract formulated into ointments containing alkaloids, flavonoids, and triterpenoids, displayed accelerated healing process of second-degree burns in experimental animals (Desmiaty et al., 2024). Besides, agarwood leaf extracts also possess anti-inflammatory activity, as

evidenced by their ability to reduce swelling and pain in experimental models by suppressing the excessive production of nitric oxide in immune cells (Xie et al., 2024).

2.6.1 Effects of Agarwood Leaf Addition during Fermentation

Studies have shown that fermentation of agarwood leaf tea with *Lactobacillus plantarum* resulted in a decrease in total phenolic and flavonoids content due to the enzymatic oxidation of polyphenols. However, the antioxidant activity increased as lactic acid bacteria produced aglycone with strong antioxidant properties (Apridamayanti and Sari, 2024). Furthermore, Suwanposri et al. (2025) found that the incorporation of agarwood leaves into herbal kombucha resulted in increased acetic acid concentration, higher total phenolic content, greater DPPH antioxidant activity, as well as inhibition of *Staphylococcus aureus* and *Escherichia coli*.

2.6.2 Effects of Other Plant Materials on Food and Beverage Fermentation

According to Lee et al. (2021), the incorporation of peppermint during the fermentation of doenjang, a traditional Korean fermented soybean paste, resulted in the highest antioxidant activity compared to coriander and Korean mint. Metabolites such as lactic, malonic, and succinic acids in fermented doenjang showed a strong correlation with the observed antioxidant effects.

Besides, brown rice vinegar fermented with 5% lemongrass showed higher total phenolic content, lower pH, and higher total acidity compared to plain brown rice vinegar. The sensory properties of the vinegar were also enriched, as incorporation of lemongrass imparted a fresh herbal note and more complex flavor then plain brown rice vinegar (Yi, Kang and Bu., 2017).

The wine fermentation with roselle (*Hibiscus sabdariffa*) calyces' incorporation significantly enhances the nutritional, functional, and sensorial qualities of the wine. The calyces are rich in phenolics and anthocyanin pigments, which contributed to the increased total phenolic content and a deep red coloration of the wines. The 100% roselle-based wine also exhibited the lowest pH, highest total soluble solid and alcohol content compared to the roselle-date blended wine (Sobowale, Omosebi and Animashuan, 2021).

2.7 Physicochemical Profiles of Vinegar

The physicochemical profiles of vinegar are typically assessed through the measurement of parameters such as pH, total titratable acidity, and residual alcohol content. These parameters indicate the fermentation efficiency and overall quality of the vinegar produced.

2.7.1 pH

pH is expressed as the negative logarithm (base 10) of hydrogen ion concentration ($pH=-\log[H^+]$) and serves as a scale to indicate the acidity or alkalinity of an aqueous solution. pH provides a quantitative measure of how

acidic (pH<7), neutral (pH=7), or alkaline (pH>7) a substance is. The measurement of pH is done by a pH meter equipped with a glass electrode, which detects the electrochemical potential related to hydrogen ion activity. pH meter provides a more accurate reading than simple indicator papers (Vivaldi et al., 2021).

The pH of most vinegar ranges from 2.4 to 3.9, depending on the types of raw material used and production method. Homemade vinegar usually has higher pH compared to commercial vinegar due to different processing stages. The pH value of vinegar serves as a key indicator of its acidity, the progress of fermentation, and its microbiological safety. The decrease in pH during fermentation reflects the accumulation of acids, which contributes to the distinctive sour taste of vinegar (Cosmulescu et al., 2022; Sengun, Kilic and Ozturk, 2019).

2.7.2 Total Titratable Acidity (TTA)

Total titratable acidity is a method used to quantify all the titratable acids in vinegar. It is commonly expressed as the percentage of acetic acid equivalent in a sample. Titration is a conventional method for determining total titratable acidity, which involves titrating a sample with defined volume with a standardized sodium hydroxide solution until phenolphthalein changes color, indicating the endpoint (Cosmulescu et al., 2022). Titratable acidity reflects only the amount of hydrogen ions neutralized by the base at the endpoint. Hence,

it is usually lower than the actual total acidity of the product, as some acids may remain in undissociated form during titration (Darias-Martin et al., 2003).

According to Cosmulescu et al. (2020), the total titratable acidity usually ranges from 0.32% to 5.09% for homemade vinegar, while it ranges from 4.14% to 9.63% for commercial vinegar. The observed differences between homemade and commercial vinegars are due to the variations in production methods. Homemade vinegars rely on spontaneous fermentation with limited process control, whereas commercial vinegars are produced through controlled and standardized protocols, resulting in higher and more consistent acidity values.

2.7.3 Alcohol Content

Alcohol content is known as the amount of ethanol present in a solution. It is usually reported as alcohol by volume (ABV), which expresses the volume of ethanol contained in 100 mL of solution (Peter, Speers and Dawn., 2017)

The concentration of alcohol is a critical parameter in acetic acid fermentation, as ethanol acts as the essential substrate for acetic acid bacteria (AAB) to convert into acetic acid. Maintaining an optimum alcohol content is essential, since insufficient concentrations reduce acid formation while excessively high alcohol levels inhibit bacteria growth and activity. Previous studies have indicated that the most effective fermentation is achieved when the initial ethanol content is maintained within 5 to 10% (v/v), depending on the alcohol

tolerance of the acetic acid bacteria used (Kong et al., 2023). According to Jamaludin et al. (2016), the maximum allowable alcohol content in vinegar in Malaysia is at 1% (v/v). This limit is intended to balance product safety with religious and regulatory compliance.

2.8 Biochemical Profiles of Vinegar

The biochemical profiles of vinegar are commonly evaluated through the determination of parameters such as total phenolic content (TPC), organic acids composition, and phytochemical screening. These measurements provide insights into the functional properties, nutritional value, and bioactive potential of the vinegar produced.

2.8.1 Total Phenolic Content (TPC)

Phenolic compounds are plant-derived secondary metabolites that include flavonoids, tannins and coumarins. They are widely recognized for their pharmacological benefits and antioxidant properties (Luna-Guevara et al., 2018). In vinegar, phenolic compounds act as major antioxidants and contribute significantly to its health-related benefits and sensory attributes such as color, flavor, and aroma. The total phenolic content (TPC) is commonly used to measure the antioxidant properties of a sample and is expressed as gallic acid equivalents (GAE). Higher levels of phenolics will enhance the antioxidant capacity and improve the functional characteristics of vinegar (Budak and Guzel-Seydim, 2010).

Homemade vinegars made by traditional methods contain a greater variety of phenolic compounds than commercial vinegar. For instance, gallic acid, catechin and chlorogenic acid were identified in traditional vinegars, whereas commercial vinegars contain significantly fewer of these compounds. The differences are mainly attributed to the production approach and raw materials used. For example, industrial processing that involves microfiltration to reduce vinegar turbidity has been shown to decrease its polyphenol content by up to approximately 15% (Cosmulescu et al., 2022; Lopez et al., 2005).

2.8.2 Organic Acids

Organic acids in vinegar are weak carboxylic acids and can be classified into volatile and non-volatile acids. They are produced from the microbial fermentation of sugars, alcohols, and other raw substrates. These acids directly affect the acidity, flavor and overall quality of vinegar (Hosseini et al., 2025).

In fruit-based vinegars, the predominant organic acids detected are acetic, tartaric, malic, citric, lactic and succinic acids, which largely reflect the natural composition of the raw fruits. Meanwhile, starch or cereal-based vinegars are mainly characterized by acetic and lactic acids as their primary acid components. The acetic acid concentration of vinegar typically ranges between 4% to 8% (Hosseini et al., 2025; Liu et al., 2019). According to Malaysia Food Regulations 1985 (Regulation 334), vinegar must contain at least 4% w/v of acetic acid and free from mineral acids (Ministry of Health Malaysia, 2023).

2.8.3 Phytochemical Screening

Phytochemicals are a broad category of bioactive compounds produced as secondary metabolites in plants. Examples include phenolics, flavonoids, alkaloids, terpenoids, saponins, and carotenoids. These compounds are recognized for their significant contributions to human health although they are not classified as essential nutrients. They possess diverse pharmacological functions such as antioxidants, antimicrobial, anti-inflammatory and anticancer activities (Kumar et al., 2023).

To identify these compounds in plant materials, preliminary phytochemical screening is commonly performed. This involves qualitative assays in which specific reagents are added to plant extracts, producing visible changes. For instance, the development of color or precipitation indicates the presence of particular phytochemical groups (Basumatary, 2016; Maheshwaran et al., 2024).

Phytochemicals provide several health benefits through their biological activities. They act as antioxidants to protect the body from oxidative damage by neutralizing reactive oxygen species. Some phytochemicals can reduce infection risk due to their antimicrobial and antiviral effects. In addition, phytochemicals also contribute to cardiovascular health, immune support, and overall well-being. This underpins their growing use in functional foods and nutraceuticals (Kumar et al., 2023; Budak and Guzel-Seydim, 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Sample Materials

Fresh cassavas were purchased from Econsave, Kampar, Perak. The cassavas were processed on the second day of purchasing to ensure its freshness. Ragi powder (Bee RizQie), wine yeast (Angel), and 100% agarwood leaf teabags (GHR) were purchased from an online shopping platform. The mother vinegar (Bragg apple cider vinegar) was obtained from Aeon, Ipoh, Perak.

3.1.2 Chemicals

The sources of chemicals used to prepare assay reagents are listed in Table 3.1.

Table 3.1: Sources of chemicals used for reagents preparation and analysis.

Chemicals	Sources	Used in Method
3,5-dinitrosalicylic acid	QReC™	3.6 Total reducing sugar content
Potassium sodium tartrate (Rochelle salt)	Systerm®	3.6 Total reducing sugar content
Sodium Hydroxide	Chemiz	3.6 Total reducing sugar content 3.9 Total titratable acidity

		3.13 Phytochemical screening
D-glucose anhydrous	ChemSoln	3.6 Total reducing sugar content
Tin Chloride	R&M Chemical	3.7 Free amino acid content
Ethylene Glycol	Merck	3.7 Free amino acid content
Ninhydrin	Merck	3.7 Free amino acid content
Sodium acetate	Bendosen	3.7 Free amino acid content
Acetic acid glacial	Chemiz	3.7 Free amino acid content
Glycine	Chemiz	3.7 Free amino acid content
Tributyl phosphate	ChemSoln	3.8 Alcohol content
Potassium dichromate	Bendosen	3.8 Alcohol content
Sulfuric Acid 95-98%	Sigma Aldrich	3.8 Alcohol content
		3.13 Phytochemical screening
		3.12.1 High-Performance Liquid Chromatography (HPLC)
99% ethanol	Systerm®	3.8 Alcohol content
		3.13 Phytochemical screening
Phenolphthalein	Bendosen	3.9 Total titratable acid
Gallic acid	R&M Chemical	3.11 Total phenolic content
Folin-Ciocalteu's phenol reagent	Merck	3.11 Total phenolic content
Sodium Carbonate anhydrous	GENE Chemicals	3.11 Total phenolic content
Iodine	R&M Chemical	3.13 Phytochemical screening
Potassium Iodate	Systerm®	3.13 Phytochemical screening

Hydrochloric acid	QReC^{TM}	3.13 Phytochemical screening
Iron (III) chloride hexahydrate	Nacalai tesque	3.13 Phytochemical screening
Chloroform	Merck	3.13 Phytochemical screening
Potassium Hydroxide	Qrec	3.13 Phytochemical screening
Ammonia	Merck	3.13 Phytochemical screening
Lactophenol cotton blue stain	Fluka Analytical	3.4 Morphology identification of ragi
Organic acid kit	Sigma aldrich	3.12.1 High-Performance Liquid Chromatography (HPLC)
Sodium chloride	Merck	3.4 Morphology identification of ragi
Potato dextrose agar	Merck	3.4 Morphology identification of ragi

3.1.3 Equipment

All the sources of equipment used for analysis are listed in Table 3.2.

Table 3.2: Sources of equipment used for analysis.

Equipment	Brand, Model, Country	Used in Method
Vortex Mixer	Scientific Industries, SI-0236, United States	3.7 Free amino acid content 3.8 Alcohol content 3.13 Phytochemical screening
Drying oven with fan convection	Binder, FD, Germany	3.5.1 Sample preparation
UV-Vis spectrophotometer	Thermo Scientific, Genesys 10S UV-VIS, United States	3.6 Total reducing sugar content 3.8 Alcohol content 3.11 Total phenolic content
Shaking incubator with cooling	INFORS HT, INF, Switzerland	3.3.2 Alcoholic fermentation 3.3.3 Acetic acid fermentation 3.7 Free amino acid content
Centrifuge machine	Newton Scientific, United Kingdom	3.6 Total reducing sugar content 3.7 Free amino acid content 3.8 Alcohol content
Inverted microscope	Evident Scientific, Olympus-CKX31SF, Japan	3.4 Morphology identification of ragi

Incubator	Memmert, ICO105, Germany	3.4 Morphology identification of ragi
Water bath	Memmert, WNB, Germany	3.6 Total reducing sugar content
Hot plate	IKA, RCT basic, Malaysia	3.6 Total reducing sugar content 3.7 Free amino acid content 3.13 Phytochemical screening
pH meter	Mettler Toledo, FiveEasyTM FE 30, Switzerland	3.10 pH
HPLC	Shimadzu Pump: LC-20AD Autosampler: SIL-20AC Diode Array Detector: SPD-M20A Column Oven: CTO-10AS VP, Japan	3.12 Organic acids concentrations
Weighing balance	Mettler Toledo, ME1002, Switzerland	Used to weigh chemicals for all tests except 3.10 pH
Moisture analyzer	A&D, MX50, Japan	3.5.2 Moisture content determination
TLC visualizer	CAMAG, TLC Visualizer, Switzerland	3.13 Phytochemical screening

Microplate reader	BMG Labtech, Fluostar Omega, Germany	3.7 Free amino acid content
Laminar flow	ESCO, AHC 4D1, Singapore	3.4 Morphology identification of ragi

3.2 Experimental Design and Data Collection

Figure 3.1 shows the process flow of cassava-based vinegar production with and without agarwood leaf infusion. The procedure involves cassava preparation, saccharification, alcoholic fermentation, and acetic acid fermentation. The key parameters such as sugar, amino acids, alcohol, pH, total titratable acidity, total phenolic content, HPLC analysis, and phytochemical properties are analyzed.

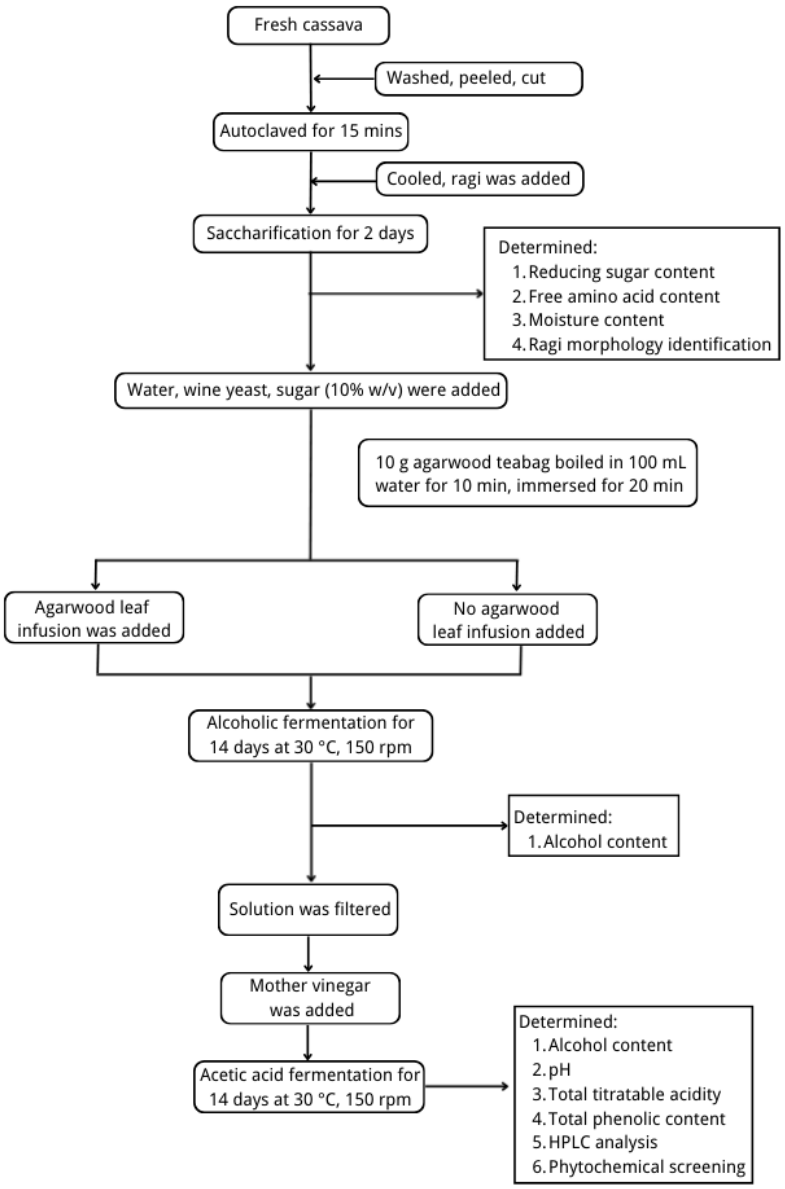

Figure 3.1: Flow chart of experimental design.

One batch of cassava samples was purchased from Econsave. For each treatment, duplicate experimental sets were prepared. The analysis done throughout this research took duplicate results from each experimental set, resulting in quadruplicate results for each treatment type.

3.3 Saccharification, Alcoholic Fermentation, and Acetic Acid Fermentation

Part of the methodology described below were adapted from several studies (Jimoh and Amire, 2024; Komlaga et al., 2021; Ghosh et al., 2012) with slight modifications.

3.3.1 Saccharification of cassava

Three hundred grams of cassavas were peeled and cut into cube shape before placing into a 1 L glass bottle with lid. The cassavas together with the glass bottle were subjected to autoclave for 15 min at 121 °C. Upon cooling, 7.5 g of ragi was added to the cassava to initiate saccharification process. The cassava was stirred once a day using a sterile spatula under sterile condition. The saccharification process proceeded for 2 days.

3.3.2 Alcoholic Fermentation

The saccharified cassavas were divided equally into 4 different 1 L glass bottles, with 65 g cassavas each. A volume of 325 mL of distilled water and 32.5 g of sugar were added to each bottle, together with 1.3 g of wine yeast for water-based sample. Two agarwood teabags (10 g) were boiled in 100 mL distilled water for 10 min, followed by immersion for 20 min. The cooled agarwood leaf infusion was then filtered before being added to the glass bottle. Meanwhile, 292.5 mL of distilled water, 32.5 mL of agarwood leave infusion, 32.5 g of sugar and 1.3 g of wine yeast were added to each of the other two bottles. All the processes were carried out under sterile conditions in a laminar flow. The glass

bottles were allowed to ferment in a shaking incubator at 150 rpm and 30°C for 14 days.

3.3.3 Acetic Acid Fermentation

After the completion of alcoholic fermentation (≥ 5% alcohol in each sample), the solution was sterilely filtered using a mesh cloth to remove the cassavas. One millilitre of mother vinegar was added per 100 mL of alcoholic solution to initiate the acetic acid fermentation. The lid of the glass bottle was replaced with a mesh cloth to ensure good aeration throughout the fermentation. The glass bottles were incubated at 30°C and 150 rpm for 10 days before the collection of products for further analysis.

3.4 Morphology Identification of Ragi

3.4.1 Growing of Ragi

The procedure was conducted according to the methods described by Rosana et al. (2014) and Saxena and Gupta (2019) with slight modifications. A sterile inoculating needle was dipped into the ragi powder and stabbed into the centre of a potato dextrose agar (PDA) plate. The plate was incubated in an upright position at 30°C for 2 days. A 1 x 1 cm PDA block containing fresh mycelia was cut using a sterile scalpel and transferred to a new PDA plate. Then, a sterile coverslip was gently placed onto the block with slight pressure to ensure adherence. The plate was incubated at 30°C for 4 days.

3.4.2 Staining and Microscopic Observation

The coverslip was placed on a microscope slide and stained with Lactophenol cotton blue. The slide was air-dried and observed under the microscope at 10X magnification.

3.5 Analysis of Sample at Different Stage of Fermentation

3.5.1 Sample Preparation

Saccharified cassavas were dried in a petri dish using drying oven at 70°C for 16 h to remove excessive moisture content. The dried samples inside petri dish were cooled and sealed using Parafilm for further analysis.

3.5.2 Moisture Content Determination

The moisture content of dried cassava sample was measured using a moisture analyser and calculated with the following equation:

$$Moisture\ content\ (\%) = \frac{W_i - [W_f \times \left(1 - \frac{M_{wb}}{100}\right)]}{W_i} \times 100$$

W_i= Weight of sample before drying (g)

W_f= Weight of sample after drying (g)

M_{wb}= residual moisture content (%) obtained from moisture analyser

3.6 Total Reducing Sugar Content

3.6.1 Solid-liquid Extraction of Reducing Sugar

The dried cassavas were ground into smaller pieces using mortar and pestle before subjected to extraction process. A solid-liquid extraction of reducing sugar from cassava was done using distilled water in a ratio of 1:10 (w/v) in a Falcon tube. The tube was incubated in a water bath at 35°C for 40 min. Upon incubation, the tube was centrifuged at 2795 × g for 10 min and the supernatant was used for analysis. The extracted solution was diluted 10 times before analysis (Martínez-Avila, Llenas, and Ponsá, 2021).

3.6.2 Chemicals and Stock Solution Preparation

DNS (3,5-dinitrosalicylic acid) reagent was prepared by dissolving 30 g potassium sodium tartrate (Rochelle salt) in 50 mL distilled water while heated until completely dissolved. One gram of DNS was added to the solution and was stirred until dissolved. Forty millilitres of NaOH (1M) were added slowly into the mixture before transferred to a volumetric flask and distilled water was used to make up the volume to 100 mL (Saqib and Whitney, 2011). Glucose stock solution (1 mg/mL) was prepared by dissolving 50 mg of glucose in distilled water and making up the volume to 50 mL in a volumetric flask. Glucose standard solutions of 0.1, 0.2, 0.4, 0.6, and 0.8 mg/mL were prepared by diluting the glucose stock solution with distilled water.

3.6.3 DNS Assay

Four millilitres of DNS reagents were added to 1 mL of diluted sample in a Falcon tube. The tube was incubated in a boiling water bath for 5 min, then cooled to room temperature. The absorbance was subsequently measured at 540 nm using a UV-vis spectrophotometer (Saqib and Whitney, 2011). The reducing sugar content (% db) was calculated by using the following equation:

$$Glucose\ (\%\ db) = \frac{Glucose\ (\%\ wb)}{100 - Moisture\ (\%\ wb)} \times 100$$

3.7 Free Amino Acid Content

3.7.1 Extraction of Free Amino Acid

Four grams of dried sample was added to 20 mL of water in a 50 mL conical flask. The mixture was shaken using a shaker at 200 rpm for 15 min for the extraction of free amino acid. Upon extraction, the mixture was centrifuged, and the supernatant was collected for further analysis (Jones, Owen, and Farrar, 2002).

3.7.2 Chemicals and Stock Solution Preparation

The tin chloride solution was prepared by dissolving 1 g of $SnCl_2$ in 10 mL ethylene glycol. One hundred millilitres of ninhydrin stock solution was prepared by adding 2 g ninhydrin to 75 mL ethylene glycol, followed by top up the solution until 100 mL using 5.5M acetate buffer. The ninhydrin working solution was prepared by adding 25 µL of tin chloride solution to 1 mL of

ninhydrin stock solution. Glycine stock solution (1 mg/mL) was prepared by dissolving 50 mg of glycine in 50 mL of distilled. Serial dilution was performed to produce glycine standard solutions of 0.1 to 0.5 mg/mL.

3.7.3 Ninhydrin Assay

In a 15 mL Eppendorf tube, 30 µL of the sample was added to 200 µL of the ninhydrin working solution. The tube was boiled in a boiling water bath for 10 min. After boiling, 2.8 mL of cold water was added to the solution and was mixed by vortex. Two hundred microlitres of solution was transferred to a microplate and the absorbance was measured at 575 nm using a microplate reader (Abernathy, Spedding, and Starcher, 2012). The free amino acids content (% db) was calculated by using the following equation:

$$Free\ Amino\ Acid\ (\%\ db) = \frac{Free\ Amino\ Acid\ (\%\ wb)}{100 - Moisture\ (\%\ wb)} \times 100$$

3.8 Alcohol Content

3.8.1 Alcohol Extraction

One millilitre of Tri-n-butyl phosphate (TBP) was added to 1 mL of liquid sample in a Falcon tube. The tube was vortex vigorously for 1 min and centrifuged at 3,420 ×g for 5 min. Upon centrifugation, the upper TBP phase was collected for further analysis.

3.8.2 Chemicals and Stock Solution Preparation

Potassium dichromate reagent (10% w/v $K_2Cr_2O_7$ in 5 M H_2SO_4) was prepared by dissolving ten grams of $K_2Cr_2O_7$ in 60 mL of distilled water and 27mL of concentrated H_2SO_4 was added slowly into the solution while stirring. The solution was cooled and transferred to a 100 mL volumetric flask. A 10% (v/v) alcohol stock solution was prepared by diluting 1 mL of absolute ethanol to 9 mL of distilled water. Serial dilution was performed to produce alcohol standard solutions ranging from 0.5% to 7% (v/v).

3.8.3 Potassium Dichromate Assay

In an Eppendorf tube, 0.5 mL of TBP solution was mixed with 0.5 mL of potassium dichromate reagent and was vortexed vigorously for 1 min to ensure thorough mixing. The sample was incubated at room temperature for 10 min. A volume of 0.1 mL of the bottom layer of the extracted sample was diluted with 0.9 mL of distilled water prior to absorbance measurement at 595 nm using an UV-vis spectrophotometer (Sriariyanun, et al., 2019).

3.9 Total Titratable Acid

3.9.1 Standardization of NaOH

The freshly prepared 0.2 N sodium hydroxide (NaOH) solution was standardized by titration with 5 ml of 0.25 N sulfuric acid using phenolphthalein as the indicator. The volume of NaOH used was recorded and the actual molarity of NaOH was calculated using the following equation:

$$M_{NaOH} = \frac{V_{H2SO4} \times M_{H2SO4} \times 2}{V_{NaOH}}$$

V= volume of sulfuric acid or sodium hydroxide solution used (mL)

M= molarity of sulfuric acid or sodium hydroxide solution (mol/L)

3.9.2 Titration

Fifty microlitres of phenolphthalein indicator was added to the 10 mL sample. The sample was titrated with 0.2 M NaOH until the endpoint. The volume of NaOH used was recorded. The total titratable acidity (% w/v acetic acid equivalent) was calculated using the following equation:

$$\% \text{ w/v acetic acid equivalent} = \frac{M_{NaOH} \times V_{NaOH} \times 60.05}{V_{sample}}$$

V_{NaOH}= volume of sodium hydroxide solution used (mL)

M_{NaOH}= molarity of sodium hydroxide solution (mol/L)

V_{sample}= volume of sample used (mL)

3.10 pH

Ten microlitres of sample was put in a beaker. The pH of the sample was recorded using a calibrated pH meter.

3.11 Total Phenolic Content

3.11.1 Stock Solution Preparation

Gallic acid stock solution (1 mg/mL) was prepared by dissolving 10 mg of gallic acid in 10 mL distilled water. Gallic acid standard solutions from 0.02 to 0.06 mg/mL were prepared by diluting the gallic acid stock solution with distilled water.

3.11.2 Folin-Ciocalteu Assay

The Folin-Ciocalteu colorimetric assay was used to quantify the total phenolic contents in the sample. The procedure was conducted according to the method of Kupina et al. (2019), with slight modifications. An aliquot of 0.2 mL sample, 0.8 mL of distilled water, and 0.1 mL of Folin-Ciocalteu reagent were mixed in an Eppendorf tube and incubated at room temperature for 3 min in the dark. Then, 0.3 mL of Na_2CO_3 (20% w/v) was added to the sample. The sample was incubated at room temperature for 120 min at dark. The absorbance was measured at 765 nm against the blank (distilled water) using a UV-Vis spectrophotometer.

3.12 Organic Acid Concentrations

3.12.1 High-Performance Liquid Chromatography (HPLC)

The organic acids in both agarwood leaf-based and water-based samples were analysed by HPLC based on the method of Mignard et al. (2022), with slight

modifications. The organic acids were analysed using a Rezex ROA-Organic Acid (H+) column (300 x 7.8 mm, Phenomenex). The HPLC system was equipped with a diode array detector (DAD), with the wavelength set to 210 nm. A 0.005 N sulphuric acid solution was used as the mobile phase for the separation of acids under isocratic mode. The flow rate of the mobile phase was 0.5 mL/min at a column temperature of 35°C. An injection volume of 20 µL was used for each run. Phytic acid (0.05-0.75 µmol/20 µL), malonic acid (0.15-1.20 µmol/20 µL), lactic acid (0.50-2.50 µmol/20 µL), and acetic acid (0.50-4.00 µmol/20 µL) were used as standard solutions to construct organic acid calibration curves.

3.13 Phytochemical Screening

Table 3.3 summarizes the qualitative assays applied to identify major classes of secondary metabolites. The listed methods include step-by-step test procedures and the corresponding positive indicators, such as specific colour changes or the formation of precipitates. These procedures were adapted from established protocols with minor modifications and serve to confirm the presence of compounds such as alkaloids, flavonoids, triterpenoids, phlobatannins, coumarins, quinones and anthraquinones.

Table 3.3: Phytochemical tests procedures for alkaloids, flavonoids, triterpenoids, phlobatannins, coumarins, quinones and anthraquinones with slight modifications (Ishtiaq et al., 2024; Jabeen, et al., 2023; Sabri, et al., 2019; Tamene and Endale, 2019; Aziz, 2015).

Secondary metabolites	**Procedure**	**Positive results observation**
Alkaloids	Wagner's test: A few drops of 0.44 M Wagner's reagent were added to 2 mL of sample from the side of test tube.	Formation of reddish-brown precipitate
Flavonoids	A few drops of 5% NaOH was added to 1 mL of sample to form a deep-yellow solution. After the reaction, a few drops of 1M HCl was added into the same test tube.	Yellow coloration upon addition of sodium hydroxide. Discoloration after addition of hydrochloric acid.
Tannins	A few drops of 5% $FeCl_3$ was added to 5 mL of sample.	Bluish black colouration of the sample.
Triterpenoids	Two microlitres of sample was added to 1 mL of chloroform solution and vortex before further analysis. The chloroform layer was collected and concentrated H_2SO_4 was added to the side of the test tube.	Formation of red brown colour at the interface

Phlobatannins	Two microlitres of sample was added to 2 mL of 1% HCl. The mixture was boiled in boiling water bath for 5 min.	Formation of red colour precipitates
Coumarins	A filter paper moistened with 1M NaOH was used to cover the test tube containing 2 mL of sample. The test tube was heated in a boiling water bath for 5 min. Then, the filter paper was removed from the test tube and was placed under UV light.	Yellow fluorescence under UV light
Quinones	Two microlitres of sample was added to 1 mL of 5% alcoholic KOH.	Blue-green, red or purple colouration of the sample
Anthraquinones	Five millilitres of chloroform was added to 1 mL of sample in a test tube. The tube was shaken for 5 min. In another test tube, 1 mL of 100% ammonia solution was added to 1 mL extract.	Ammonia lower layer turns pink, violet or red colour.

3.14 Statistical Analysis

All statistical analyses were performed using SPSS software. Descriptive statistics such as mean, standard deviation, standard error, and coefficients of

variances were used to summarize and organize the raw data. Normality testing was carried out to evaluate the distribution of the data prior to performing the t-test and ANOVA test. An independent sample t-test was performed at a significance level of $\alpha = 0.05$ to compare the initial and final contents after saccharification and fermentation in the same samples such as reducing sugar, free amino acids, moisture content, and alcohol content. A two-way ANOVA test was performed to evaluate the significant differences across fermentation days and between agarwood leaf-based and water-based sample at a 95% confidence interval. When significant differences were detected in the ANOVA test, post-hoc test such as Tukey's HSD was performed to conduct pairwise comparisons.

CHAPTER 4

RESULTS

4.1 Morphology Identification of Ragi

Referring to Figure 4.1, the morphology of ragi observed consisted of broad, non-septate hyphae with unbranched sporangiophore bearing round sporangium at their tips, containing numerous sporangiospores. Based on these morphological characteristics, the observed sample corresponds to *Mucor* sp.

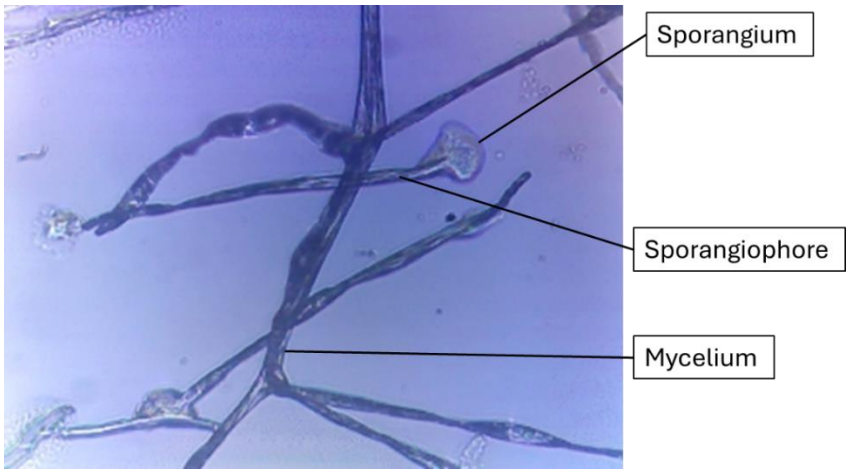

Figure 4.1: Microscopic observation of ragi under 10× magnification.

4.2 Changes of Composition During Saccharification on Cassava

At Day 0, the cassava cubes were firm and coated with ragi. After 2 days of saccharification, the cassava cubes showed reduced firmness with partial disintegration, and their surfaces appeared moist as shown in Figure 4.2.

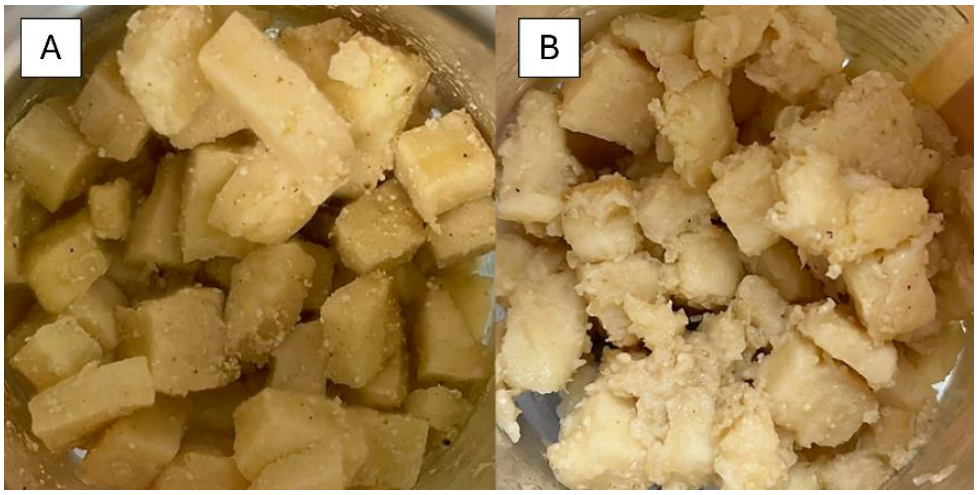

Figure 4.2: Cassava on Day 0 (A) and Day 2 (B) of saccharification.

4.2.1 Reducing Sugar and Free Amino Acid Content

Figure 4.3 shows the standard curve obtained, with r^2=0.9902. The equation y=1.235x derived from the standard curve was used to calculate the sugar content in cassava samples. Figure 4.4 shows the standard curve (r^2= 0.9997), and the derived equation y=1.7511x was used to calculate the free amino acid content in the cassava samples.

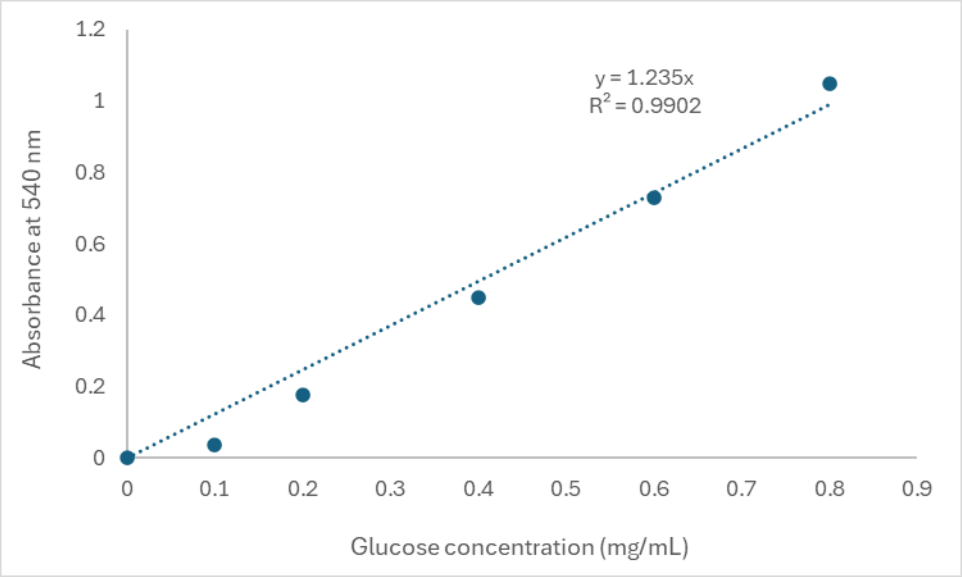

Figure 4.3: Absorbance at 540 nm against different glucose concentrations (mg/mL).

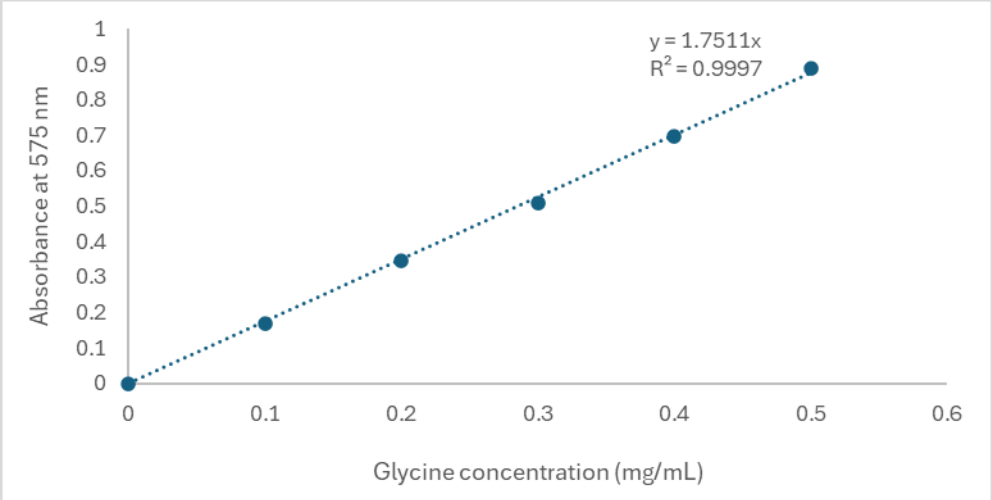

Figure 4.4: Absorbance at 575 nm against different glycine concentrations (mg/mL).

Table 4.1.1 shows the sugar and free amino acid content in cassava before (Day 0) and after saccharification (Day 2). The sugar content in cassava increased significantly ($p < 0.05$) from 3.58% db to 16.28% db after two days of saccharification. However, no significant difference ($p > 0.05$) was detected

between the initial and final free amino acid content of cassava after saccharification. The initial free amino acid content is 0.52% db while final free amino acid content is 0.46% db.

Table 4.1.1: Sugar and free amino acid content in cassava at Day 0 and Day 2 of saccharification.

Day	Sugar content (% db)	Free amino acid content (% db)
0	3.58 ± 0.08 [a]	0.52 ± 0.04 [a]
2	16.28 ± 0.14 [b]	0.46 ± 0.02 [a]

Values are expressed as mean ± standard deviation (n=3).

Different superscript letters within the same column indicate significant differences ($p < 0.05$) according to the independent samples t-test.

4.2.2 Moisture Content

Table 4.1.2 shows the moisture content of cassava samples at Day 0 and Day 2 of saccharification. The moisture content of cassava on Day 0 (57.65%) was significantly lower ($p < 0.05$) than the moisture content on Day 2 (61.80%).

Table 4.1.2: Moisture Content of cassava samples at Day 0 and Day 2 of saccharification.

Day	Moisture content (%)
0	57.65 ± 1.00 [a]
2	61.80 ± 1.15 [b]

Values are expressed as mean ± standard deviation (n=3).

Different superscript letters within the same column indicate significant differences ($p < 0.05$) according to the independent samples t-test.

4.3 Alcohol Fermentation Stage

After the saccharification process, cassava cubes underwent alcohol fermentation under a submerged condition. The solution of the water-based sample appeared whitish and clear, with some cassava cubes floating on the surface on Day 0. After 14 days of fermentation, the solution became more turbid with a slightly beige colour. All cassavas cubes were observed at the bottom of the solution. Bubbles were also present on the surface of the solution (**Figure 4.5**). The agarwood leaf-based sample exhibited the same observation as shown in **Figure 4.5**.

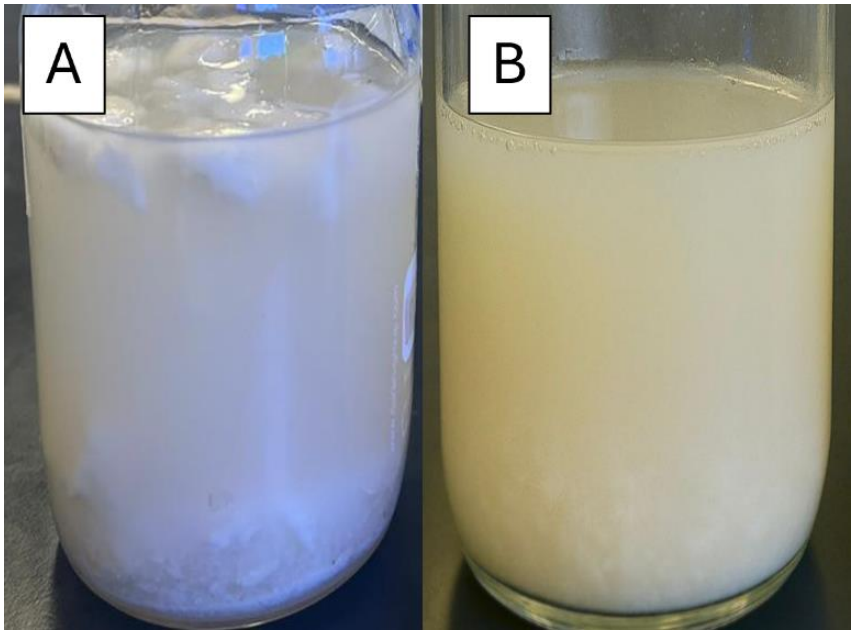

Figure 4.5: Water-based sample at Day 0 (A) and Day 14 (B) of alcoholic fermentation.

4.3.1 Comparison of Alcohol Content between Agarwood leaf-based and Water-based Samples

Figure 4.6 shows the standard curve (r^2=0.9983) and the derived equation y=0.1227x was used to determine the alcohol content of agarwood leaf-based and water-based samples in both alcoholic and acetic acid fermentation.

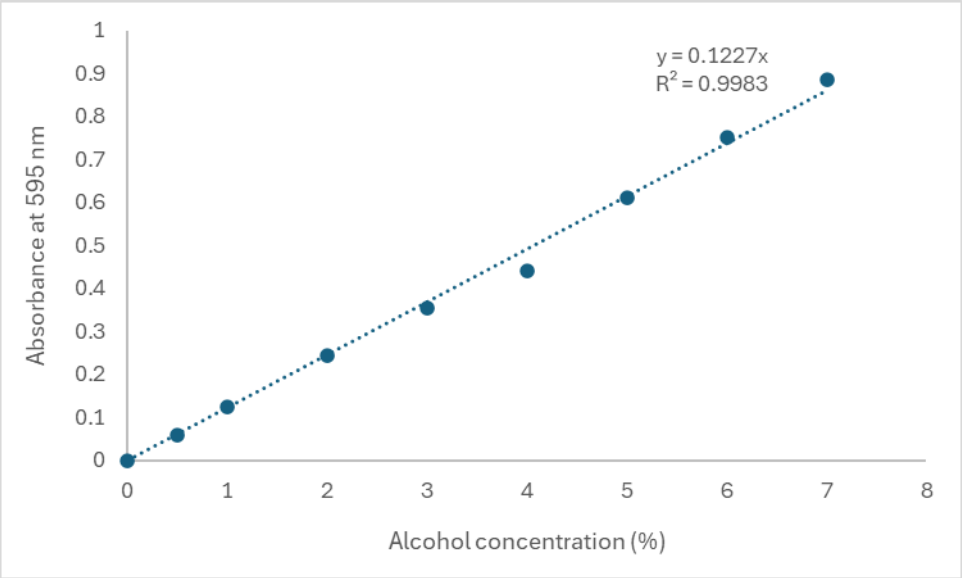

Figure 4.6: Absorbance at 595 nm against alcohol concentration (%).

Table 4.2 shows the alcohol content in agarwood leaf-based and water-based samples at day 0 and day 14 of alcoholic fermentation. The alcohol content for both samples increased significantly ($p < 0.05$) from day 0 to day 14 of alcoholic fermentation. Water-based sample showed a higher increase in alcohol content, which is from 0.18% to 6.48%, whereas the alcohol content of agarwood leaf-based sample increased from 0.14% to 5.56% after 14 days of alcoholic fermentation.

Table 4.2: Alcohol content in agarwood leaf-based and water-based samples at different stages of alcoholic fermentation.

Day	Alcohol content (%)	
	Agarwood leaf-based	**Water-based**
0	0.14 ± 0.00^{a}	0.18 ± 0.00^{b}
14	5.56 ± 0.04^{a}	6.48 ± 0.05^{b}

Values are expressed as mean ± standard deviation (n=4).

Different superscript letters within the same row indicate significant differences ($p < 0.05$) between samples according to the independent samples t-test.

4.4 Physicochemical and Biochemical Properties of Vinegar Samples

Table 4.3 shows the physicochemical and biochemical properties, including alcohol content, total phenolic content (TPC), pH, and total titratable acid (TTA) of agarwood leaf-based and water-based vinegar at different stages of acetic acid fermentation.

Table 4.3: Alcohol content, Total phenolic content (TPC), pH and total titratable acid (TTA) in agarwood leaf-based and water-based samples at different stages of acetic acid fermentation.

Day	Alcohol content (%)		TPC (mg GAE/mL)		pH		TTA (% w/v)	
	Agarwood leaf-based	Water-based	Agarwood leaf-based	Water-based	Agarwood leaf-based	Water -based	Agarwood leaf-based	Water-based
1	5.83 ± 0.13 ^{e*}	6.68 ± 0.18 ^{e}	0.1253 ± 0.0007 ^{d*}	0.1077 ± 0.0009 ^{c}	3.30 ± 0.01 ^{a*}	3.35 ± 0.03 ^{a}	6.33 ± 0.11 ^{e*}	5.52 ± 0.09 ^{e}
2	3.32 ± 0.05 ^{d*}	4.22 ± 0.04 ^{d}	0.1166 ± 0.0011 ^{b*}	0.0946 ± 0.0012 ^{a}	3.37 ± 0.00 ^{b*}	3.41 ± 0.01 ^{a}	5.04 ± 0.14 ^{d}	5.29 ± 0.09 ^{d}
4	2.91 ± 0.01 ^{c*}	3.09 ± 0.01 ^{c}	0.1122 ± 0.0007 ^{a*}	0.0983 ± 0.0009 ^{b}	3.66 ± 0.01 ^{c}	3.64 ± 0.03 ^{b}	2.28 ± 0.06 ^{c}	2.14 ± 0.09 ^{c}
5	1.84 ± 0.04 ^{b}	1.81 ± 0.03 ^{b}	0.1201 ± 0.0011 ^{c*}	0.1000 ± 0.0007 ^{b}	4.76 ± 0.01 ^{d*}	4.86 ± 0.06 ^{c}	0.39 ± 0.07 ^{b}	0.48 ± 0.06 ^{b}
7	1.59 ± 0.01 ^{a}	1.66 ± 0.05 ^{ab}	0.1325 ± 0.0023 ^{e*}	0.1136 ± 0.0009 ^{d}	5.03 ± 0.03 ^{f*}	5.09 ± 0.03 ^{d}	0.34 ± 0.00 ^{a*}	0.28 ± 0.01 ^{a}
10	1.51 ± 0.02 ^{a*}	1.55 ± 0.01 ^{a}	0.1402 ± 0.0014 ^{f*}	0.1215 ± 0.0006 ^{e}	4.89 ± 0.01 ^{e*}	5.15 ± 0.01 ^{d}	0.23 ± 0.00 ^{a*}	0.28 ± 0.01 ^{a}

Values are expressed as mean ± standard deviation (n=4).

Different lowercase superscript letters within the same column indicate significant differences ($p < 0.05$) among days for that sample (Tukey's test).

Asterisks (*) indicate significant differences ($p < 0.05$) between agarwood leaf-based and water-based samples on the same day.

4.4.1 Alcohol Content

Table 4.3 shows that the alcohol content of water-based sample was significantly higher than the agarwood leaf-based sample on most fermentation days ($p < 0.05$). The alcohol content of agarwood leaf-based sample decreased significantly ($p < 0.05$) from day 1 (5.83%) to day 7 (1.59%), with no significant difference observed between day 7 and day 10. While the alcohol content of water-based sample decreased significantly ($p < 0.05$) from day 1 (6.68%) to day 5 (1.81%), with no significant differences ($p > 0.05$) observed between day 5 and day 7, and between day 7 and day 10.

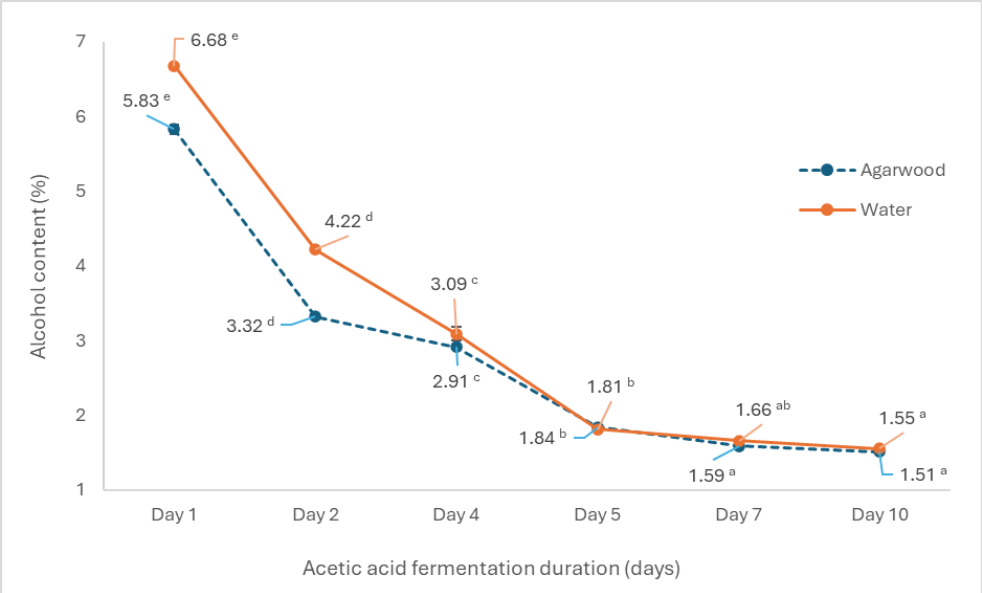

Figure 4.7: Alcohol content in agarwood leaf-based and water-based samples from day 1 to day 10 of acetic acid fermentation.

4.4.2 Total Phenolic Content

Figure 4.8 shows the standard curve obtained from gallic acid as a source of phenolic compounds, with r^2=0.9991. The equation y=14.323x + 0.0111 derived from the standard curve was used to calculate the total phenolic content of both samples.

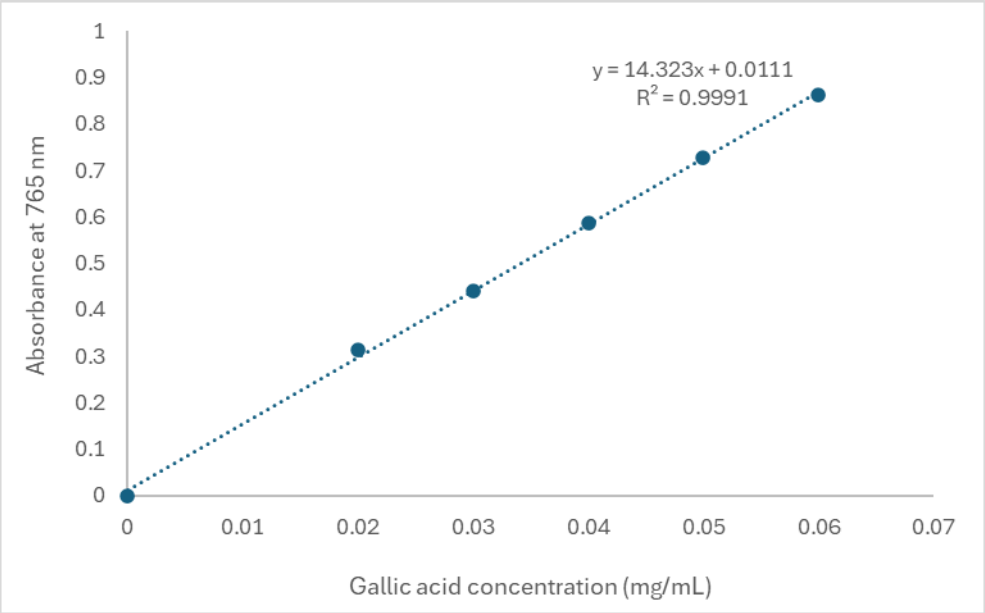

Figure 4.8: Absorbance at 765nm against gallic acid concentrations (mg/mL).

Table 4.3 shows that the agarwood leaf-based sample had significantly higher ($p < 0.05$) total phenolic content than the water-based sample. The total phenolic content of agarwood leaf-based sample decreased significantly ($p < 0.05$) from Day 1 (0.1253 mg GAE/mL) to Day 4 (0.1122 mg GAE/mL), before increasing significantly to 0.1402 mg GAE/mL by Day 10. While for water-based sample, the total phenolic content of 0.1077 mg GAE/mL on Day 1 decreased significantly ($p < 0.05$) to 0.0946 mg GAE/mL on Day 2. The TPC was then

increased significantly ($p < 0.05$) to 0.1215 mg GAE/mL by Day 10, with no significant difference ($p > 0.05$) observed between Day 4 and Day 5.

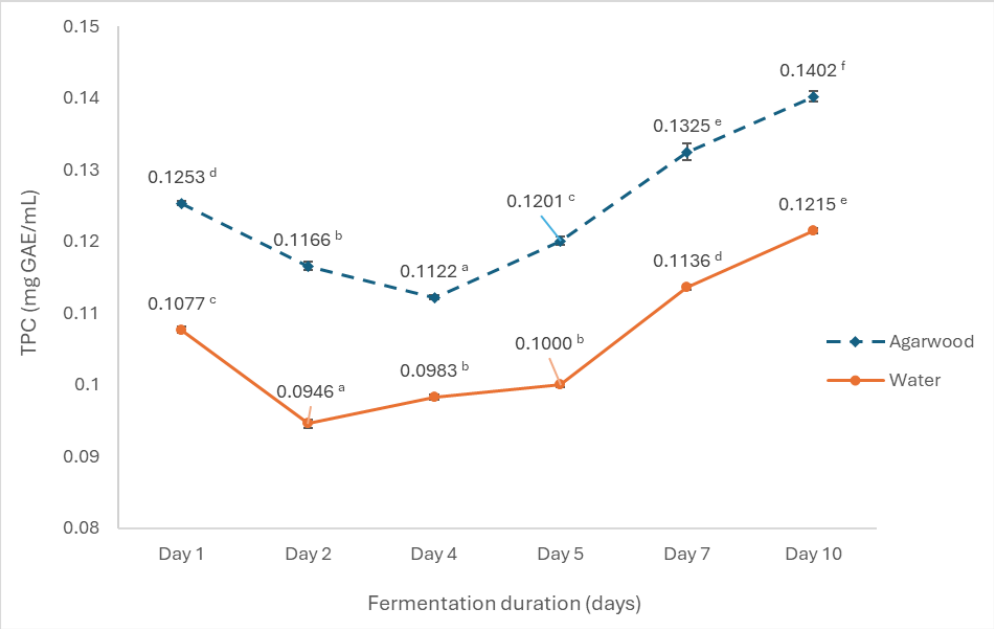

Figure 4.9: Total phenolic content in agarwood leaf-based and water-based samples from Day 1 to Day 10 of acetic acid fermentation.

4.4.3 pH

Table 4.3 shows that the water-based sample had a significantly higher ($p < 0.05$) pH than the agarwood leaf-based sample on most fermentation days. The pH of agarwood leaf-based sample pH increased significantly ($p < 0.05$) from 3.30 on Day 1 to 5.03 on Day 7 and followed by a significant decreased to 4.89 ± 0.01 on Day 10. In the water-based sample, the pH increased significantly ($p < 0.05$) from Day 2 (3.41) to Day 7 (5.09) and remained statistically unchanged ($p > 0.05$) by Day 10 (5.15).

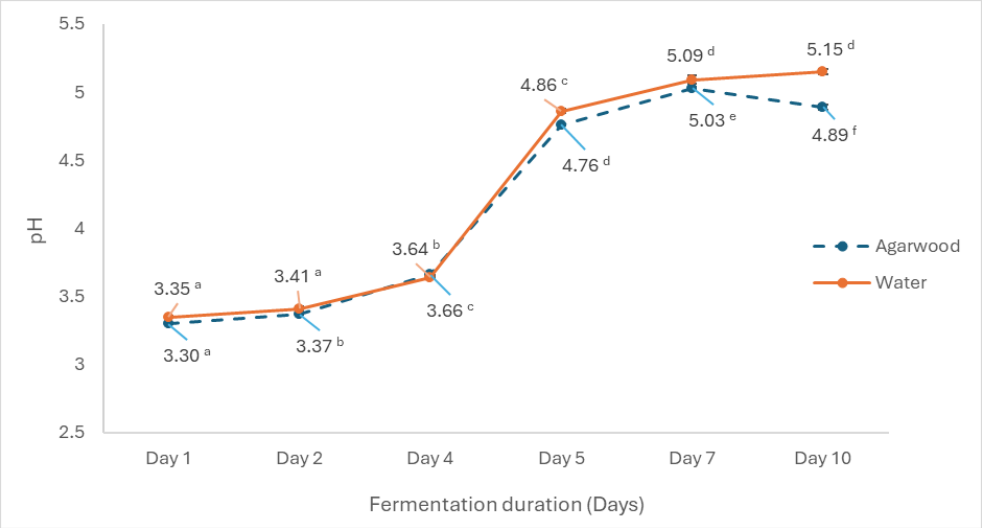

Figure 4.10: pH in agarwood leaf-based and water-based samples from Day 1 to Day 10 of acetic acid fermentation.

4.4.4 Total titratable acidity

According to Table 4.3, the total titratable acidity of agarwood leaf-based samples was generally higher than water-based samples, with significant differences observed on certain fermentation days ($p < 0.05$). The total titratable acidity for both agarwood leaf-based and water-based samples decreased significantly ($p < 0.05$) from Day 1 to Day 5, from 6.33% w/v acetic acid equivalent (AAE) to 0.39% w/v AAE and from 5.52% w/v AAE to 0.48% w/v AAE, respectively. No significant differences ($p > 0.05$) were observed in both samples between Day 7 and Day 10.

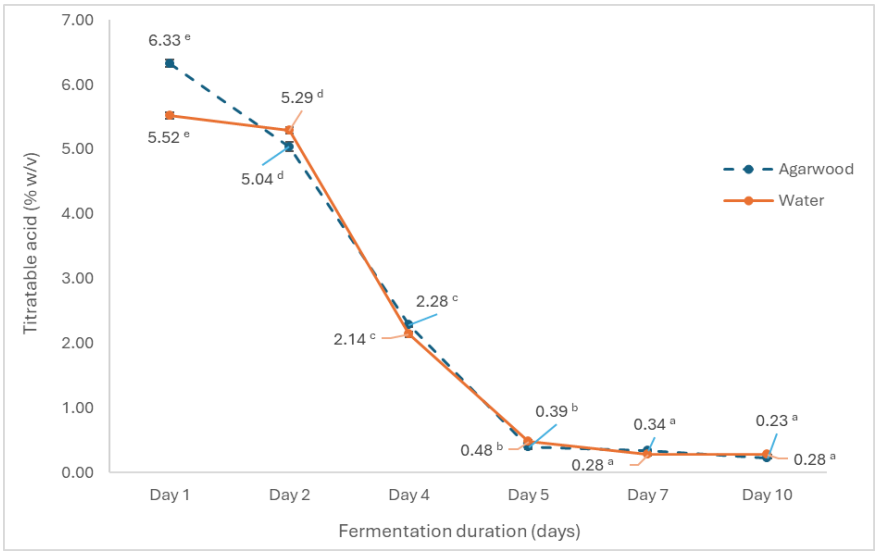

Figure 4.11: Total titratable acid content in agarwood leaf-based and water-based samples from Day 1 to Day 10 of acetic acid fermentation.

4.5 Organic Acid Concentrations

Figure 4.12 to 4.15 show the standard curve of various organic acid as determined by High Performance Liquid Chromatography (HPLC).

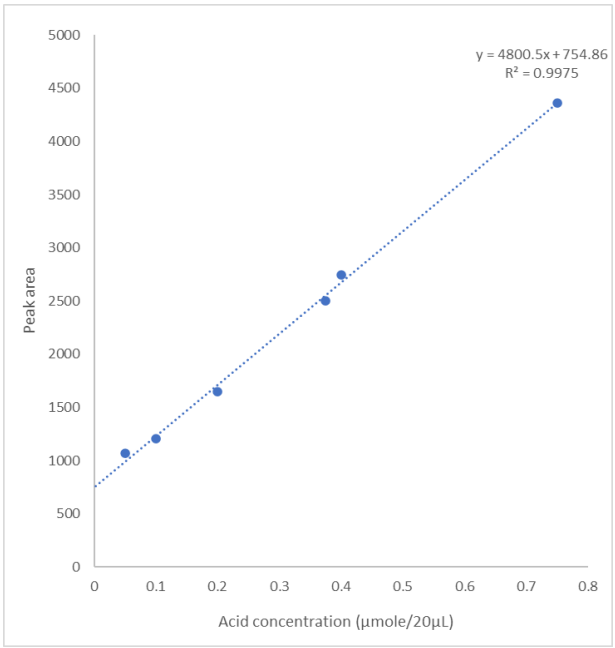

Figure 4.12: Peak area against phytic acid concentration (µmole/20µL).

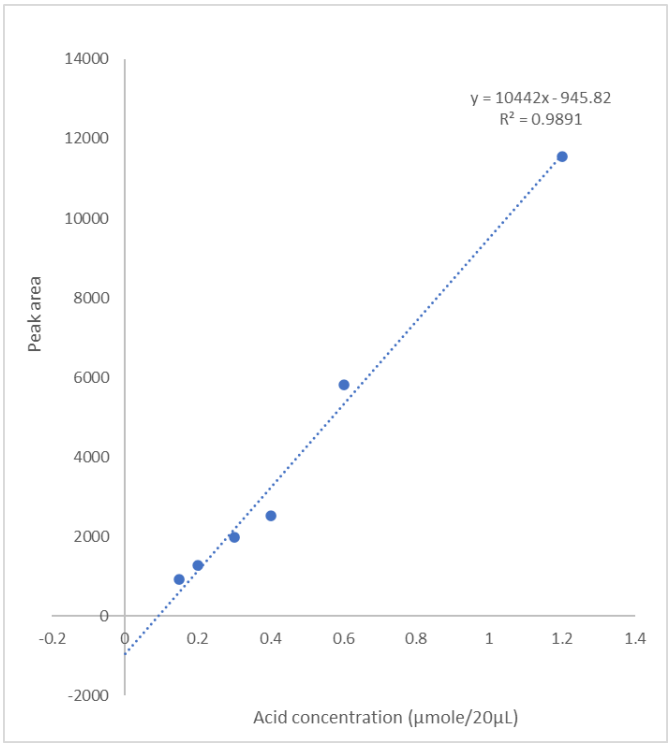

Figure 4.13: Peak area against malonic acid concentration (µmole/20µL).

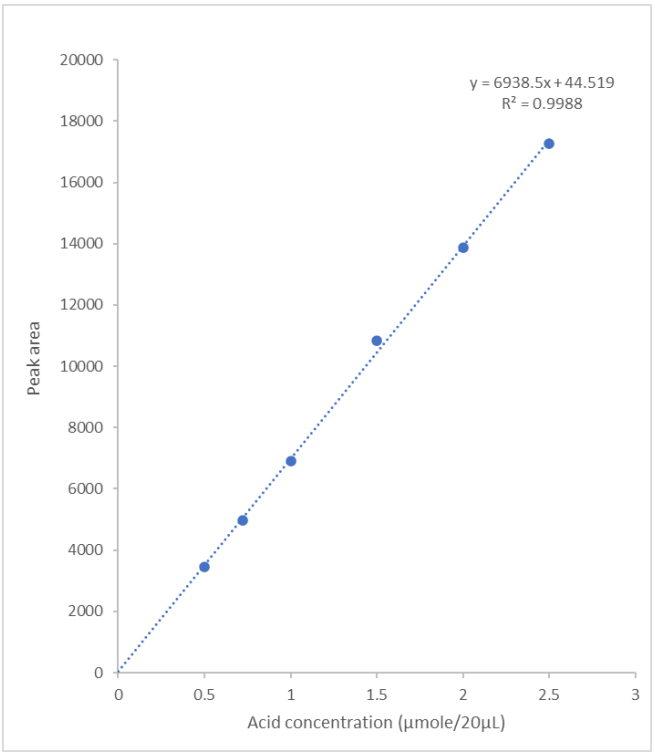

Figure 4.14: Peak area against lactic acid concentration (µmole/20µL).

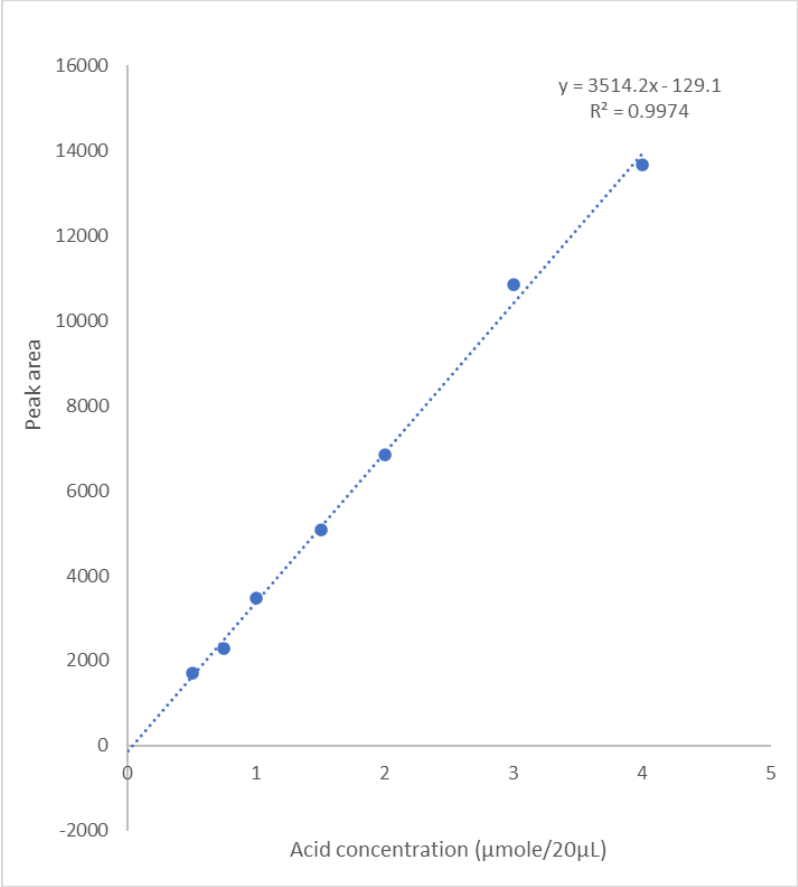

Figure 4.15: Peak area against acetic acid concentration (µmole/20µL).

Table 4.4.1 summarizes the concentration range, retention time and standard curve equation for phytic, malonic, lactic and acetic acid. Table 4.4.2 shows the phytic, malonic, lactic, and acetic acid concentrations of agarwood leaf-based and water-based vinegar at different stages of acetic acid fermentation.

Table 4.4: Standard curve equation for different types of acids.

Type of Acids	**Acid Concentrations Ranges (µmole/20 µL)**	**Retention Time**	**Standard Curve Equation**	**r^2**
Phytic acid	0.05-0.75	8.49	y=4800.5x + 754.86	0.9975
Malonic acid	0.15-1.2	12.50	y=10442x – 945.82	0.9891
Lactic acid	0.5-2.5	15.76	y=6938.5x + 44.519	0.9988
Acetic acid	0.5-4	18.80	y=3514.2x -129.1	0.9974

Table 4.5: Phytic, malonic, lactic, and acetic acid concentration in agarwood leaf-based and water-based samples during acetic acid fermentation.

Day	Phytic acid (mg/mL)		Malonic acid (mg/mL)		Lactic acid (mg/mL)		Acetic acid (mg/mL)	
	Agarwood leaf-based	Water-based	Agarwood leaf-based	Water-based	Agarwood leaf-based	Water-based	Agarwood leaf-based	Water-based
0	10.04 ± 0.02 ^{a*}	9.65 ± 0.03 ^{ab}	1.16 ± 0.00 ^{e*}	1.30 ± 0.01 ^{e}	6.52 ± 0.06 ^{e}	6.48 ± 0.18 ^{g}	4.22 ± 0.12 ^{e}	4.39 ± 0.30 ^{d}
1	9.77 ± 0.21 ^{a}	9.52 ± 0.02 ^{a}	1.13 ± 0.06 ^{de}	1.03 ± 0.01 ^{d}	6.47 ± 0.25 ^{de}	6.21 ± 0.01 ^{f}	4.17 ± 0.20 ^{de}	3.93 ± 0.01 ^{c}
2	10.78 ± 0.01 ^{a*}	9.87 ± 0.03 ^{c}	1.04 ± 0.00 ^{d*}	0.84 ± 0.00 ^{c}	6.05 ± 0.02 ^{d*}	5.49 ± 0.00 ^{e}	3.84 ± 0.03 ^{d*}	2.89 ± 0.00 ^{b}
4	10.73 ± 0.11 ^{a*}	9.82 ± 0.03 ^{bc}	0.61 ± 0.07 ^{a}	0.75 ± 0.00 ^{b}	4.18 ± 0.13 ^{c}	4.21 ± 0.01 ^{d}	1.27 ± 0.01 ^{b*}	1.04 ± 0.04 ^{a}
5	11.10 ± 0.03 ^{a*}	11.66 ± 0.07 ^{d}	0.65 ± 0.01 ^{ab*}	0.59 ± 0.01 ^{a}	2.07 ± 0.27 ^{b}	1.76 ± 0.07 ^{c}	1.19 ± 0.03 ^{b*}	1.03 ± 0.06 ^{a}
7	13.30 ± 0.41 ^{b}	13.25 ± 0.11 ^{f}	0.75 ± 0.01 ^{bc}	0.76 ± 0.01 ^{b}	1.27 ± 0.06 ^{a}	1.39 ± 0.14 ^{b}	0.68 ± 0.01 ^{a*}	0.99 ± 0.03 ^{a}
10	13.85 ± 1.17 ^{b}	12.74 ± 0.10 ^{e}	0.81 ± 0.01 ^{c}	0.78 ± 0.02 ^{b}	1.18 ± 0.08 ^{a*}	0.87 ± 0.08 ^{a}	2.09 ± 0.23 ^{c*}	1.24 ± 0.22 ^{a}

Values are expressed as mean ± standard deviation (n=3).

Different lowercase superscript letters within the same column indicate significant differences ($p < 0.05$) among Days for that sample (Tukey's test).

Asterisks (*) indicate significant differences ($p < 0.05$) between agarwood leaf-based and water-based samples on the same Day.

4.5.1 Phytic Acid Concentration

Table 4.5 shows that the phytic acid concentration in agarwood leaf-based sample was significantly higher than water-based sample on most fermentation day ($p < 0.05$). The phytic acid concentration in agarwood leaf-based samples remained statistically constant from Day 0 to Day 5 and increased significantly ($p < 0.05$) by Day 10, reaching 13.85 mg/mL. In the water-based samples, phytic acid concentration increased significantly from Day 1 to Day 7 (9.52 mg/mL to 13.25 mg/mL), before significantly decreasing to 12.74 mg/mL on Day 10 ($p < 0.05$).

4.5.2 Malonic Acid Concentration

According to Table 4.5, the malonic acid concentration in agarwood leaf-based samples was significantly higher ($p < 0.05$) than in water-based samples on most fermentation day. In agarwood leaf-based samples, malonic acid concentration decreased significantly from Day 0 (1.16 mg/mL) to Day 4 (0.61 mg/mL), followed by a significant increase from 0.65 mg/mL on Day 5 to 0.81 mg/mL on Day 10 ($p < 0.05$). A similar trend was observed in the water-based sample, where the concentration decreased significantly ($p < 0.05$) from 1.30 mg/mL on Day 0 to 0.59 mg/mL on Day 5. It increased significantly ($p < 0.05$) at Day 7 (0.76 mg/mL), after which no significant difference was observed between Day 7 and Day 10 ($p > 0.05$).

4.5.3 Lactic Acid Concentration

Table 4.5 shows the lactic acid concentration in agarwood leaf-based sample was significantly higher than in water-based sample ($p < 0.05$). Lactic acid concentration showed a similar decreasing trend in both agarwood leaf-based and water-based samples over the 10-day fermentation. In agarwood leaf-based samples, lactic acid decreased significantly ($p < 0.05$) from Day 0 to Day 7 (6.52 mg/mL to 1.27 mg/mL), with no significant difference observed between Day 7 and Day 10 ($p > 0.05$). In water-based samples, lactic acid decreased significantly ($p < 0.05$) from 6.48 mg/mL on Day 0 to 0.87 mg/mL on Day 10.

4.5.4 Acetic Acid Concentration

Referring to Table 4.5, the acetic acid concentration in agarwood leaf-based sample was significantly higher than water-based sample on most fermentation days ($p < 0.05$). The agarwood leaf-based sample's acetic acid concentration decreased significantly ($p < 0.05$) from 4.22 mg/mL on Day 0 to 3.84 mg/mL on Day 2 and further decreased to 0.68 mg/mL on Day 7. A significant increase ($p < 0.05$) was observed on Day 10, reaching 2.09 mg/mL in agarwood leaf-based sample. For water-based sample, the acetic acid concentration decreased significantly ($p < 0.05$) from 4.39 mg/mL on Day 0 to 1.04 mg/mL on Day 4 and showed no significant difference from Day 4 to Day 10.

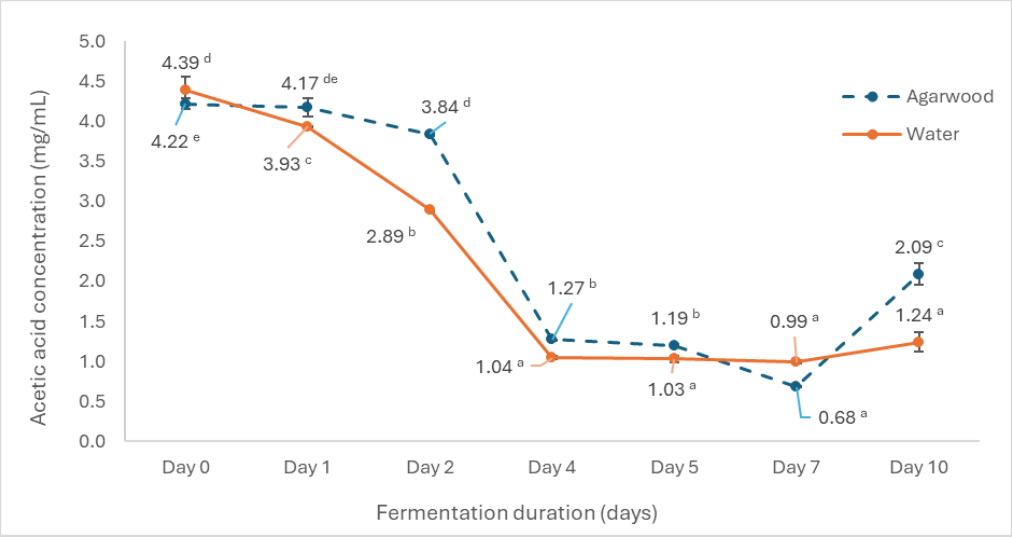

Figure 4.16: Acetic acid concentration in agarwood leaf-based and water-based samples at different stages of acetic acid fermentation.

4.6 Phytochemical tests

Table 4.5 shows the results of phytochemical test for alkaloids, flavonoids, tannins, triterpenoids, phlobatannins, coumarin, quinone, and anthraquinones in the final products of agarwood leaf-based and water-based samples. A positive result indicates the presence of the respective phytochemical compound.

Table 4.6: Phytochemical tests results of agarwood leaf-based and water-based samples after acetic acid fermentation.

Phytochemicals	Agarwood leaf-based	Water-based
Alkaloids	Positive	Positive
Flavonoids	Positive	Positive
Tannins	Positive	Negative
Triterpenoids	Negative	Negative
Phlobatannins	Negative	Negative
Coumarin	Positive	Positive
Quinone	Negative	Positive
Anthraquinones	Negative	Negative

According to Table 4.6, both agarwood leaf-based and water-based samples contained alkaloids, flavonoids, and coumarin (**Figure 4.17, 4.18,** and **4.22**), whereas triterpenoids, phlobatannins, and anthraquinones were absent in both samples (**Figure 4.20, 4.21,** and **4.24**). Tannins were detected in the agarwood leaf-based left-based sample, indicated by the solution turning bluish-black colour, but were absent in the water-based sample (**Figure 4.19**). In contrast, quinones were detected only in the water-based sample, forming a pale red colour in the solution, and were absent in the agarwood leaf-based sample (**Figure 4.23**).

In alkaloids test, black precipitates were observed in both samples after the addition of Wagner's reagent (**Figure 4.17**). Figure 4.18 shows that both agarwood leaf-based and water-based samples turned yellow colour upon the

addition of NaOH solution, followed by a colourless solution after adding HCl during flavonoids testing. For coumarin test, fluorescence was observed in both samples under UV light (**Figure 4.22**).

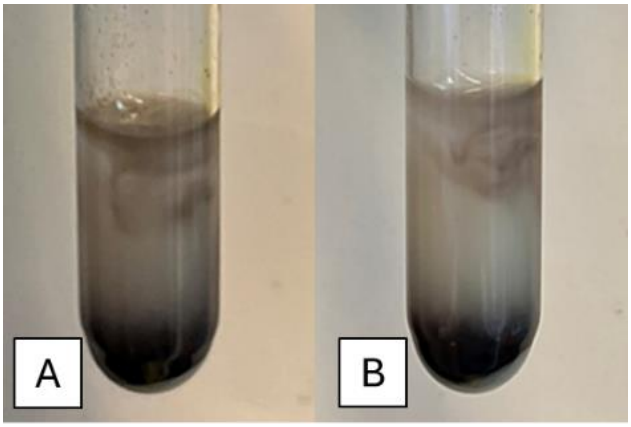

Figure 4.17: Positive test results for alkaloids in agarwood leaf-based (A) and water-based (B) samples.

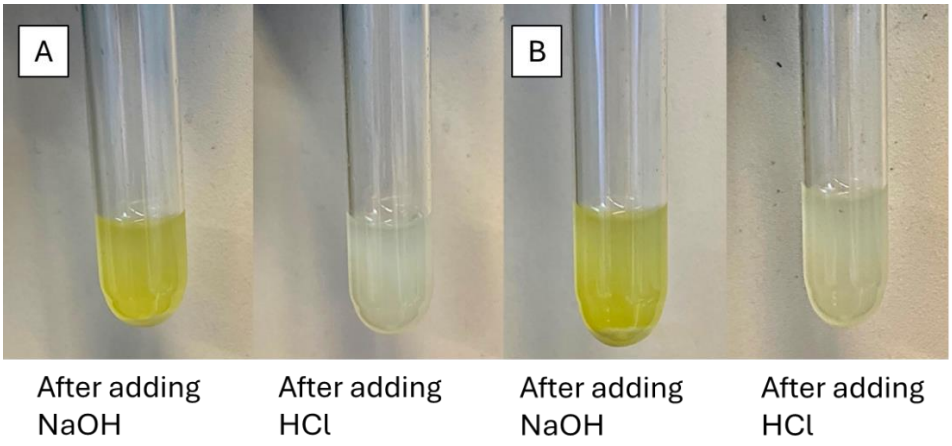

Figure 4.18: Positive test results for flavonoids in agarwood leaf-based (A) and water-based (B) samples.

Figure 4.19: Positive test result for tannins in agarwood leaf-based (A) and negative test result in water-based (B) sample.

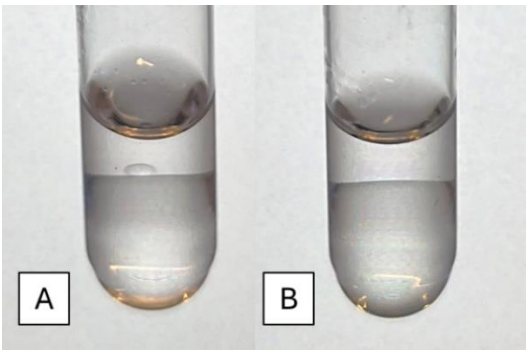

Figure 4.20: Negative test results for triterpenoids in agarwood leaf-based (A) and water-based (B) samples.

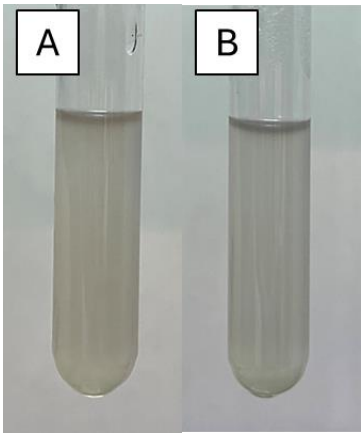

Figure 4.21: Negative test results for phlobatannins in agarwood leaf-based (A) and water-based (B) samples.

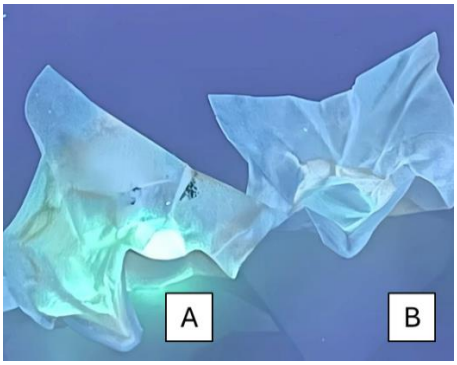

Figure 4.22: Filter papers containing agarwood leaf-based (A) and water-based (B) samples fluoresce under UV light.

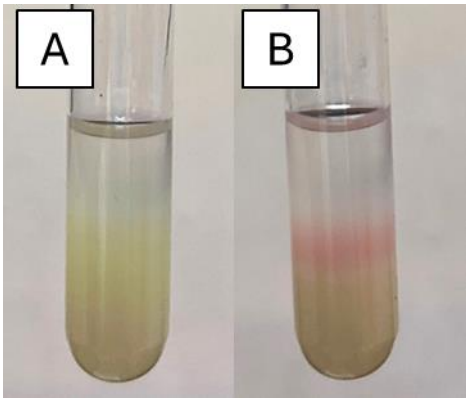

Figure 4.23: Negative test result for quinone in agarwood leaf-based (A) and positive test result in water-based (B) sample.

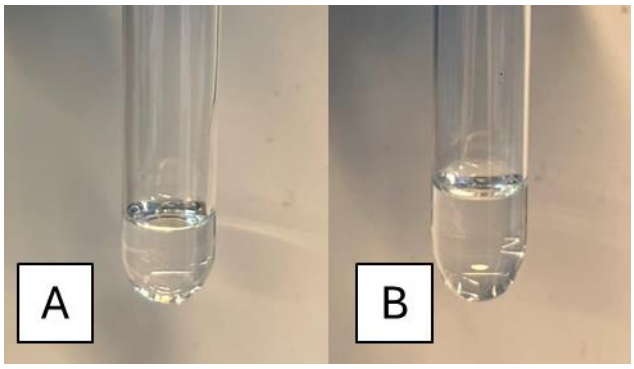

Figure 4.24: Negative test results for anthraquinones in agarwood leaf-based (A) and water-based (B) sample.

CHAPTER 5

DISCUSSION

5.1 Morphology Identification of Ragi

The fungi commonly found in Ragi are *Amylomyces* sp., *Mucor* sp. and *Aspergillus* spp. (Delva, Arisuryanti and Ilmi, 2022). The microscopic observation of Ragi (**Figure 4.1**) was examined and compared with the characteristic features of these fungi. Based on the morphological features shown in **Figure 5.1** and **Figure 5.2**, the fungus was identified as *Mucor* sp. This is supported by the absence of rhizoids, which are usually present in *Rhizopus* spp. (**Figure 5.3**), and the absence of chlamydospores, which are characteristic of *Amylomyces* sp. (**Figure 5.4**). These morphological traits are consistent with descriptions of *Mucor* spp. provided in the University of Adelaide (2024).

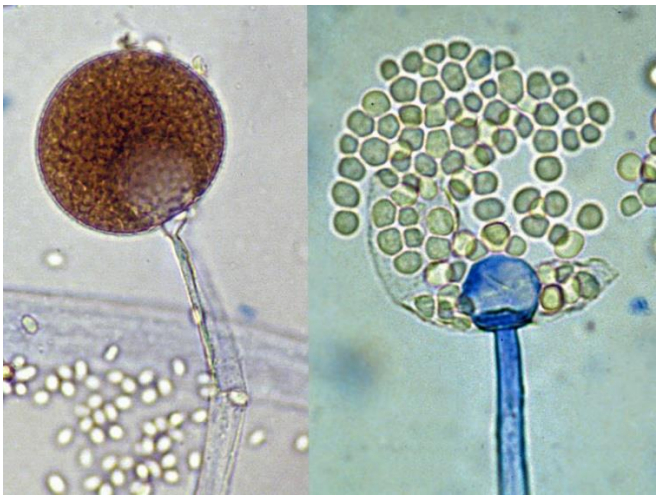

Figure 5.1: Microscopic features of *Mucor* spp. (University of Adelaide, 2024).

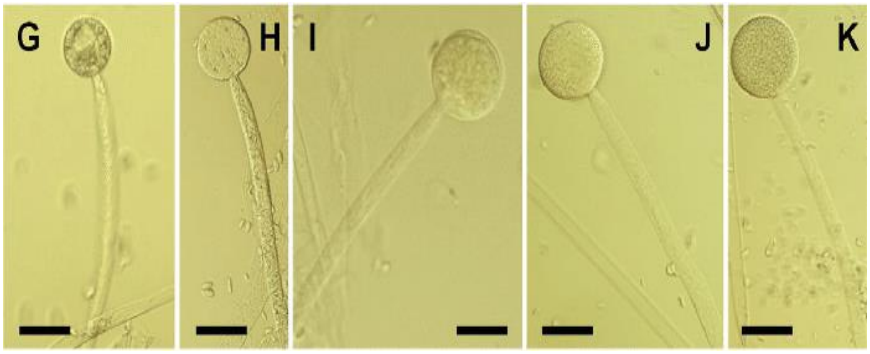

Figure 5.2: Morphology of *Mucor* sp. showing young and mature sporangia (G-K) (Nguyen, Duong and Lee, 2016).

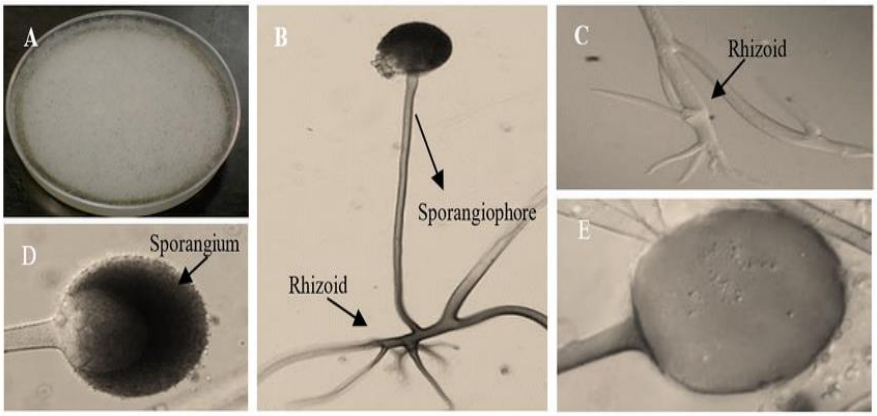

Figure 5.3: Microscopic photos of *Rhizopus oryzae* (Shaima, Yousef and Hosny, 2024).

Figure 5.4: Microscopic photos of *Amylomyces rouxii* with chlamydospores (Roongrojmongkhon et al., 2020).

Mucor is important in fermented food as it contributes to fermentable sugars and improves product quality. *Mucor* contains glucoamylases that convert starch into simpler sugars, which act as precursor for subsequent fermentation (Yang et al., 2022). The growth of *Mucor* mycelia on the surface of tofu stabilizes its structure while enzymatic proteolysis softens the interior part. This will contribute to the characteristic smooth and creamy consistency valued in sufu (Chen et al., 2024). Studies have shown that *Mucor racemosus* releases proteases and peptidases that hydrolyze proteins into smaller peptides and amino acids in sufu fermentation. This enhances the nutritional value as well as the flavor of the food as free amino acids are major contributors to umami, sweet, and bitter tastes (Xie et al., 2023).

5.2 Changes of Composition During Saccharification on Cassava

The cassava cubes exhibited softer texture and moist appearance after 2-day saccharification (**Figure 4.2**). This is mainly due to microbial enzymatic degradation of cassava cell wall and starch matrix. Wakem et al. (2023) reported that cassava softening is strongly associated with pectin degradation in the cell wall, which causing a measurable reduction in tissue firmness. According to Amande, Ado and Adebayo-Tayo (2022), *Mucor* sp. secretes pectinolytic enzymes, including polygalacturonase and pectin lyase, which depolymerise cassava cell wall polysaccharides. This leads to cell separation and tissue disintegration (Ngea et al., 2016). In addition, studies have shown that enzymatic hydrolysis of cassava starch during saccharification will increase its water-holding and water-binding capacity by exposing the hydrophilic groups and disrupting starch structure, hence increase the water absorption ability of

cassava (Cornejo et al., 2022). The microbial catabolism of carbohydrates also generates metabolic water as a by-product of oxidative reactions, which may further contribute to the increase in moisture content as observed during saccharification (Weiner et al., 2023).

5.2.1 Reducing Sugar Content

The reducing sugar content in saccharified cassava increased (**Table 4.1.1**), which is due to the enzymatic hydrolysis of starch by fungi that are present in ragi. According to Delva, Arisuryanti and Ilmi (2022), ragi usually contains amylolytic fungi such as *Mucor* sp., *Amylomyces rouxxi* and *Rhizopus* spp. These fungi will secrete enzymes such as amyloglucosidase that can degrade cassava starch into simpler sugars such as glucose. This enzymatic activity dominates during the first 48 to 72 h of cassava tapai fermentation, resulting in the accumulation of dextrin and glucose (Cempaka, 2021). In addition, enzymatic studies of cassava starch hydrolysis have shown that the addition of amyloglucosidase results in a 30-50% increase in total reducing sugars, confirming that saccharification directly increases the reducing sugar levels (Collares et al., 2012).

5.2.2 Free Amino Acid Content

The free amino acid content of cassava cubes remained statistically constant after saccharification (**Table 4.1.1**). Amylolytic activity is the predominant activity in ragi tapai starters. This is because microbial investigations have shown that none of the 41 yeasts isolated from ragi starters possessed proteolytic

capacity, while most of them have amylolytic capacity (Nout and Aidoo, 2010). This indicates that starch degradation is the dominant enzymatic process initiated by ragi molds, generating glucose to sustain yeast and bacterial growth during the early stage of fermentation. Since cassava roots contain only about 1-2% protein, the limited substrate availability further restricts protease action and amino acid release (Bayata, 2019).

5.3 Alcohol Fermentation Stage

After 14 days of fermentation, the solution became more turbid and developed a light beige colour (**Figure 4.5**). The increase in turbidity is due to the build-up of microbial cells and fermentation-derived colloidal substances such as proteins, polyphenols, and polysaccharides. These compounds scatter light and make the solution appear hazy and turbid (Mastanjevic et al., 2018).

The alcohol content in agarwood leaf-based sample was significantly lower than in water-based sample (**Table 4.2**). This is because of the presence of bioactive compounds in agarwood leaves. According to Batubara et al. (2021a), phytochemical screening of ethanolic extracts of agarwood leave has confirmed the presence of flavonoids, tannins, and triterpenoids, which are compounds associated with antimicrobial activity. In antifungal assays, a 20% ethanolic extract of agarwood leaves produced clear zones of inhibition against fungi such as *Candida albicans* and *Trichophyton* species on nutrient agar. This indicates that these phytochemicals can interfere with fungal growth.

Besides, studies on fermented Miang (tea leaf) have shown that tannins at concentration of 12.5 mg/mL can inhibit *Saccharomyces cerevisiae* by chelating essential ions, altering membrane integrity, and disrupting metabolic pathways during wine fermentation (Phovisay et al., 2024). Similarly, lignin-derived derivatives at concentrations around 20 g/L have been shown to prolong the lag phase and reduce sugar utilization as well as ethanol yield (Xue et al., 2018). As these phytochemicals compounds are also present in agarwood leaves, it is reasonable to suggest that they could exert comparable inhibitory effects on the yeast metabolism, which contribute to the reduced alcohol yield in agarwood leaf-based sample.

5.4 Dynamic Changes in Physicochemical and Biochemical Properties of Vinegar Samples

Alcohol, pH and total titratable acidity are the key indicators of fermentation progress, since they reflect the substrate utilization, acid production, and acid-base balance thorough fermentation process. Biochemical properties of vinegar samples are used to determine the nutritional value and functional properties during acetic acid fermentation. These properties were assessed through measurement of total phenolic content, organic acids concentration, and phytochemical screening.

5.4.1 Alcohol Content, Lactic Acid and Acetic Acid Concentration

The alcohol content in water-based sample was significantly higher than in agarwood leaf-based sample, which may be due to the higher ethanol yield

produced by wine yeast during water-based alcoholic fermentation. The alcohol content in both samples continued to decline during acetic acid fermentation (**Figure 4.7**). However, the acetic acid concentration in both samples also decreased from Day 0 to Day 7, before increase again on Day 10 (**Figure 4.16**).

This can be due to the microbial activity and fermentation constraints. In submerged vinegar fermentation, ethanol is sequentially oxidized to acetic acid. The ethanol is first oxidized to acetaldehyde by alcohol dehydrogenase (ADH), followed by oxidation to acetic acid by aldehyde dehydrogenase (ALDH). Both ADH and ALDH enzymes are located on the periplasmic side of the bacterial membrane. They work under strictly aerobic conditions using oxygen as the terminal electron acceptor (Hata et al., 2022). When oxygen transfer is limited, the activity of aldehyde dehydrogenase (ALDH) in *Acetobacter* strains is restricted, resulting in the accumulation of acetaldehyde rather than complete oxidation to acetic acid (Mamlouk and Gullo, 2013). However, acetaldehyde is highly volatile and can evaporate from the broth easily. This leads to ethanol depletion before complete conversion to acetic acid. Besides evaporating, acetaldehyde is also actively metabolized by yeast. For example, acetaldehyde can be reduced back to ethanol via alcohol dehydrogenase, converted into acetate, or used for cytoplasmic acetyl-CoA formation which is required for lipid synthesis (Guittin et al., 2023). These reasons support the reduction in both alcohol content and acetic acid concentrations in agarwood leaf-based and water-based vinegar samples.

In addition, the lactic acid concentrations in both samples decreased across acetic acid fermentation (**Table 4.5**). Freire et al. (2015) reported that *Saccharomyces cerevisiae* ferments sugars into ethanol while lactic acid bacteria (LAB) simultaneously convert sugars into lactic acid. The LAB coexist with *Saccharomyces cerevisiae* during alcoholic fermentation as they are both anaerobes. During the early stage of the acetic acid fermentation, lactic acid bacteria that present in the fermentation matrix may compete with acetic acid bacteria for both nutrients and oxygen. Besides, LAB will also synthesize bacteriocins that suppress acetic acid bacteria growth. This will alter the microbial population in the fermentation matrix and hence affecting the fermentation progress (Mota and Vilela, 2024). When fermentation continues, the prolong exposure of LAB to aerobic environments will cause oxidative stress and hence reduces their ability to compete with acetic acid bacteria. Reactive oxygen species are toxic to lactic acid bacteria as they lack catalase and superoxide dismutase, which are key enzymes for neutralizing them. As a result, ROS can damage proteins, DNA, and lipids, ultimately leading to cell death. Hence, the reduction of lactic acid bacteria allows obligate aerobes acetic acid bacteria become gradually dominate the fermentation system and continue to produce acetic acid (Feng and Wang, 2020; Siciliano et al., 2019). This is consistent with the decreased in alcohol content (**Figure 4.7**) and the increased in acetic acid concentration observed on Day 10 (**Figure 4.16**), indicating that the alcohol was oxidized to acetic acid.

5.4.2 pH, Total Titratable Acidity and Organic Acid Concentrations

The pH was lower but total titratable acidity (TTA) was higher in agarwood leaf-based sample than in water-based sample. This is related to the higher organic acid concentrations observed in agarwood leaf-based sample (**Table 4.5**) across fermentation even lower alcohol content at initial of acetic acid fermentation (**Figure 4.7**). Suwanposri et al. (2025) showed that the peppermint kombucha added with agarwood leaves produced significantly higher acetic acid concentrations and lower pH values than the controls without agarwood leave incorporation after 14 days of fermentation. However, this phenomenon was not observed in roselle-agarwood leaf kombucha, as roselle contributed to lower initial pH during fermentation, so incorporating agarwood leaves did not change the pH. Since cassava does not contribute to a low pH like roselle, it is suggested that incorporating agarwood leaves will exhibit the same effect as observed by Suwanposri et al. (2025). This explains the higher acetic acid concentration in agarwood leaf-based sample across fermentation (**Figure 4.16**).

Across the fermentation of both agarwood leaf-based and water-based samples, the pH increased while the total titratable acidity (TTA) declined in the early stages and then stabilized. pH and TTA showed an inverse relationship throughout the fermentation.

The observed pH changes across both treatments are related to the buffering capacity of different organic acids present in the medium. This is because buffering capacity is the strongest when the solution pH is close to the acid's

pKa, as both dissociated and undissociated forms coexist to resist pH shifts as described by the Henderson-Hasselbalch equation (Shaw and Gregory, 2022). In the samples, the pH values were closer to the pKa of lactic acid (3.8) and the first pKa of manolic acid (~2.83), making them act as the main buffers during the initial stages of acetic acid fermentation. Strong buffering components like lactic acid neutralize most of the released hydrogen ions, so more acid is required to cause a measurable change in pH. This is supported by the study of Li et al. (2015), which showed that the wort buffering capacity is positive correlated with the final pH of the beer, as stronger buffering resists the pH change during fermentation. However, lactic acid concentrations decreased during acetic acid fermentation, as observed in both samples (**Table 4.5**). This is due to lactic acid can be oxidized into pyruvate under nutrient limitation and aerobic conditions. The pyruvate will be further converted to acetate and 1,2-propanediol to provide energy for lactic acid bacteria (Ganzle, 2015). As lactic and malonic acids decreased over time, the buffer capacity weakened. This allowed the sample pH to rise as the concentration of acetic acid (pKa=4.76) was still low, providing insufficient buffering (Susjenka et al., 2024).

Phytate, the salt form of phytic acid, is the primary phosphorus storage compound in cassava tissues. It forms an insoluble phytate-mineral complexes when its phosphate groups interact with divalent cations such as calcium and iron (Gupta et al., 2015; Lopez et al., 2002). During acetic acid fermentation, the slightly acidic solution with pH around 4 to 5 favours phytase activity that mainly originates from fermenting microorganisms rather than the cassava itself. Cassava contains only limited endogenous phytase, but lactic acid bacteria

present in fermentation are known to secrete phytase, which becomes active at this pH (Afolabi and Popoola, 2004; Terefe, Omwamba and Nduko, 2022). This causes the phytate-mineral complexes in cassava tissues become destabilized, leading to the release of free phytic acid into the fermentation broth (Lopez et al., 2002). Even though phytic acid increased over time, it did not lower the pH values or raise the TTA. This is because phytic acid acts as a weak polyacid at the sample pH, remaining largely protonated due to its high pKa values (Humer, Schwarz and Schedle, 2014).

The decreasing trend in TTA followed by stabilization (**Figure 4.11**) is due to the organic acid dynamics driven by microbial metabolism. In the early phase, lactic acid bacteria metabolize sugar to form lactic acid, leading to higher acidity. However, as fermentation progresses, lactic acid may be consumed by other microorganisms such as acetic acid bacteria as a carbon source, reducing the overall titratable acid pool (Xia et al., 2022). Because the wine yeast was not inactivated prior to acetic acid fermentation, it is plausible that a multispecies fermentation occurred in the initial stage. During this period, viable wine yeast and other bacteria could compete with acetic acid bacteria, depending on nutrient availability, oxygen levels, and microbial interactions. Under these conditions, microbial succession may lead to the accumulation and subsequent depletion of organic acids. This shows that organic acids function not only as fermentation products, but also as microbial substrates (Han et al., 2024).

5.4.3 Total Phenolic Content

The total phenolic content (TPC) in agarwood leaf-based sample is significantly higher than in water-based sample as flavonoids, tannins and phenolic compounds are extracted through boiling of agarwood leaves. Though their hydroxyl groups, these compounds react with the Folin-Ciocalteu reagent, resulting in higher TPC values (Batubara et al., 2021b).

The total phenolic content (TPC) in both agarwood leaf-based and water-based samples exhibited an initial decrease followed by increased trend (**Figure 4.9**). The initially high total phenolic content at Day 1 is due to the high ethanol concentration in the solution, which acted as an effective solvent, as measured by the alcohol content in **Table 4.3.** According to Xu et al. (2019), phenolic extraction from fermented glutinous rice infused with Fu brick tea at 80% ethanol concentration have been shown to be more efficient than water extraction. Besides, the enzymatic activity of *Saccharomyces cerevisiae* produces β-glucosidases that is capable of hydrolysing β-glucosidic linkages. Hence, bound phenolics are freed and liberated into the medium as free phenolics, results in high TPC at the initial of acetic acid fermentation (Schmidt et al., 2011; Zhao et al., 2021).

The decline in TPC in both samples is due to the oxidative degradation of flavonol glycosides and the conversion of monomeric phenolics into polymeric tannins, which cannot be detected by Folin-Ciocalteu assay (Kim et al., 2011). From Day 4 onwards, the TPC in both samples increased again and reached the

highest values by Day 10. This is due to the enzymes produced by yeasts and acetic acid bacteria such as glucosidase, cellulase and pectinase can depolymerize larger condensed tannin into smaller phenolics, including catechin and epicatechin (Kim et al., 2023). Although condensed tannins contribute to TPC, their depolymerization into smaller and more soluble phenolics during fermentation increases the pool of Folin-reactive compounds. Hence, this increase in free hydroxyl groups and antioxidative phenolics contributes to the increase in TPC (Zhao et al., 2021).

5.5 Phytochemicals

Phytochemicals screening is important to detect bioactive compounds that contribute to the nutritional and functional properties of food products. Alkaloids, flavonoids, and coumarin were present in both agarwood leaf-based and water-based samples (**Table 4.6**). This is consistent with the study of Lehmane et al. (2023) which showed that alkaloids, flavonoids, and coumarin are the secondary metabolites that is naturally present in cassava tubers.

While triterpenoids, phlobatannins, and anthraquinones were absent in both samples (**Table 4.6**), as these compounds are not found in cassava tissues but only in cassava leaves (Sarma et al., 2023). Similarly, phlobatannins and anthraquinones were not detected in agarwood leaves in previous studies. Although triterpenoids are present in agarwood leaves, they are mainly non-polar and extractable primarily with organic solvents such as ethanol (Batubara et al., 2021a). Since the agarwood leaves in this study were incorporated by hot

water extraction, triterpenoids were not solubilized and extracted; therefore, they remained absent in the agarwood leaf-based sample.

Tannins were observed in agarwood leaf-based sample but absent in water-based sample (**Table 4.6**). This is because tannins are highly prone to hydrolysis and oxidation under acidic and aerobic environments. They are hydrolysed by microbial tannase into ellagic acid, hence reducing their concentration in the fermentation broth (Yang et al., 2023). Agarwood leaves are rich in tannins, the polyphenolic compounds that are highly soluble in water. This allows tannins to be extracted during hot water infusion of agarwood leaves (Batubara et al., 2021b). However, although cassava tubers contain tannins, they exist in lower amounts compared to agarwood leave (Lehmane et al., 2023). Most of them are degraded by enzymes during fermentation, hence showing a negative result to tannins.

However, quinones were detected only in the water-based sample (**Figure 4.23**). This may be due to quinones are strong electrophiles and highly reactive, making them prone to react with nucleophiles. Tannins and flavonoids present in the agarwood leaf-based sample acted as nucleophiles that reacted with quinones, thereby reducing their concentration to an undetectable level. While in water-based samples, the free cassava phenolics were oxidized during fermentation, and quinones were accumulated to detectable levels. Since tannins were absent in water based-sample, fewer quinones were neutralized (Schieber, 2018; Seczyk, Gawlik-Dziki and Swieca, 2021).

5.6 Study Limitations and Future Study Recommendations

There are some limitations in this study. First, the dynamic changes in physicochemical and biochemical properties of cassava vinegar were only monitored up to Day 10 due to time constraints. However, studies indicate that natural fermentation may take up to 3 weeks to reach an acidity level comparable to the 12- days fermentation using isolated acetic acid bacteria (Mathew et al., 2019). Third, mother vinegar was not revived prior to inoculation, possibly reducing AAB viability and fermentation success rate. This is because the acetic acid bacteria in mother vinegar are exposed to high acetic acid concentrations, which may introduced acid stress and consequently reduce their cell numbers or viability (Qiu, Zhang and Hong, 2021). The sensory impact of the high TPC on cassava vinegar taste was not evaluated. According to Pedan et al (2019), the perception of bitterness is highly correlated with total phenolic content.

For further studies, the acetic acid fermentation should be extended beyond Day 10 to capture full biochemical and physicochemical changes in the vinegar for more complex study The mother vinegar culture should be revived and tested for viability before inoculation to ensure an active AAB population, hence further enhance the success rate of fermentation. Lastly, sensory testing can be done to evaluate the impact of high TPC on vinegar taste and consumer acceptability.

CHAPTER 6

CONCLUSION

In conclusion, the biochemical and physicochemical profile of cassava-based vinegar was successfully characterized across different stages of fermentation, and the dynamic changes during acetic acid fermentation were clearly established. The incorporation of agarwood leaf infusion further influenced the biochemical and physicochemical properties of cassava-based vinegar.

The morphological identification comfirmed that the ragi used for saccharification was *Mucor* sp., which significantly increased the reducing sugar content of cassava after 2 days as starch was broken down to simple sugars. However, free amino acids remained constant as cassava roots are a poor source of protein, thereby restricting protease action. The incorporation of agarwood leaf infusion during alcoholic fermentation caused significantly lower alcohol content compared to the water-based sample due to the antifungal properties of agarwood leaves.

During acetic acid fermentation, alcohol and acetic acid concentrations in both agarwood leaf-based and water based samples showed a declining trend. This was due to oxygen limitation, which restricted aldehyde dehydrogenase activity in *Acetobacter*, causing acetaldehyde to accumulate and be lost through volatilization or yeast metabolism. As fermentation progressed, acetic acid

bacteria dominated, leading to increased acetic acid. Whereas lactic acid bacteria declined under oxidative stress after initial competition for nutrients and oxygen.

The agarwood leaf-based sample showed lower pH and higher titratable acidity due to higher organic acid levels. As lactic and malonic acids declined during fermentation, the buffering effect weakened, and pH began to rise. Phytic acid was the most abundant acid in both samples, as phytase activity was favoured at pH 4-5, releasing it from cassava phytate-mineral complexes. Although phytic acid increased, it did not affect pH or TTA as it remained largely protonated at the sample pH.

The agarwood leaf-based sample showed higher total phenolic content (TPC) as flavonoids, tannins and phenolic compounds were extracted through boiling to obtain the infusion. The high initial TPC was due to ethanol acting as an effective solvent, followed by a decline from oxidative degradation and polymerization. At later stages, microbial enzymes depolymerized condensed tannins into smaller catechin and epicatechin, expanding the pool of Folin-reactive compounds and elevating TPC.

Phytochemical screening showed that alkaloids, flavonoids, and coumarin were present in both samples due to their natural occurrence in cassava tubers and agarwood leaves. Tannins were observed only from agarwood leaf-based sample due to their high abundance and solubility. Phlobatannins, and

anthraquinones were absent in both samples as they are not present in either cassava tubers or agarwood leaves. Triterpenoids were absent in cassava but present in agarwood leaves, where their non-polar nature requires extraction with organic solvents instead of hot water. Quinones appeared only in the water-based sample. as they were neutralized by tannins and flavonoids in the agarwood-leaf based sample.

This study was limited by monitoring the acetic acid fermentation process only until Day 10, while natural fermentation typically required up to three weeks to reach full maturity. The mother vinegar was not revived prior to inoculation, which may have reduced acetic acid bacteria viability. The sensory impact of high total phenolic content was not assessed. Future studies should extend the fermentation period, ensure mother vinegar viability, and include sensory testing for consumer acceptability.

Overall, this study demonstrates the potential of incorporating agarwood leaves into cassava vinegar fermentation to enhance its nutritional, functional, and phytochemical properties, providing a basis for developing value-added herbal vinegars.

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APPENDICES

APPENDIX A

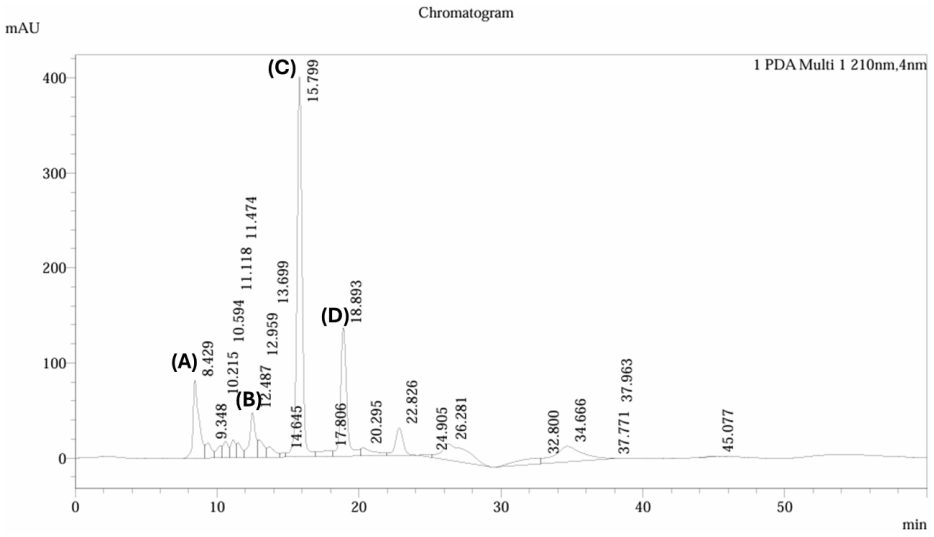

Figure A1: Chromatogram of Organic Acid Content in Agarwood Leaf-Based Sample on Day 0. Peak Identification and Retention Time: (A) Phytic Acid, 8.429 min; (B) Malonic Acid, 12.487 min; (C) Lactic Acid, 15.799 min; (D) Acetic Acid, 18.893 min

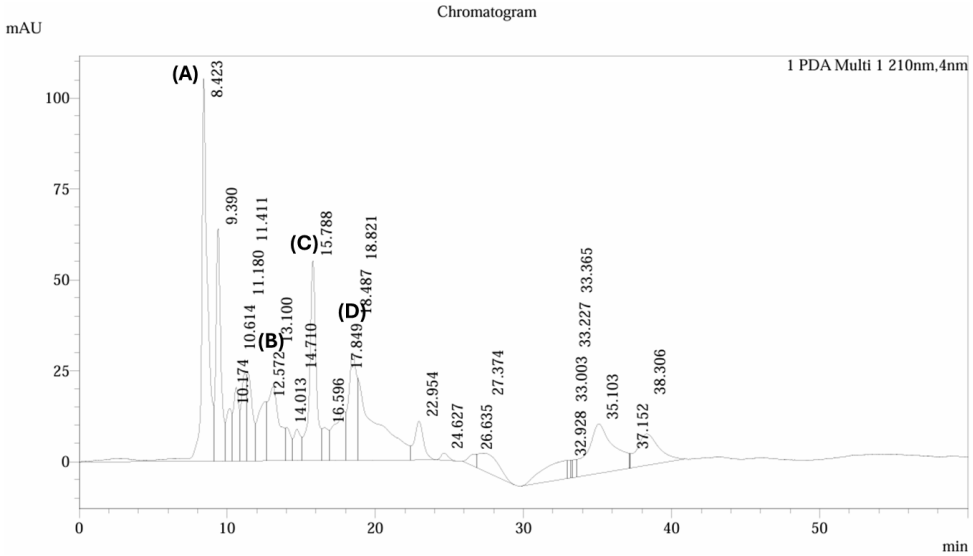

Figure A2: Chromatogram of Organic Acid Content in Agarwood Leaf-Based Sample on Day 10. Peak Identification and Retention Time: (A) Phytic Acid, 8.423 min; (B) Malonic Acid, 12.572 min; (C) Lactic Acid, 15.788 min; (D) Acetic Acid, 18.487 min

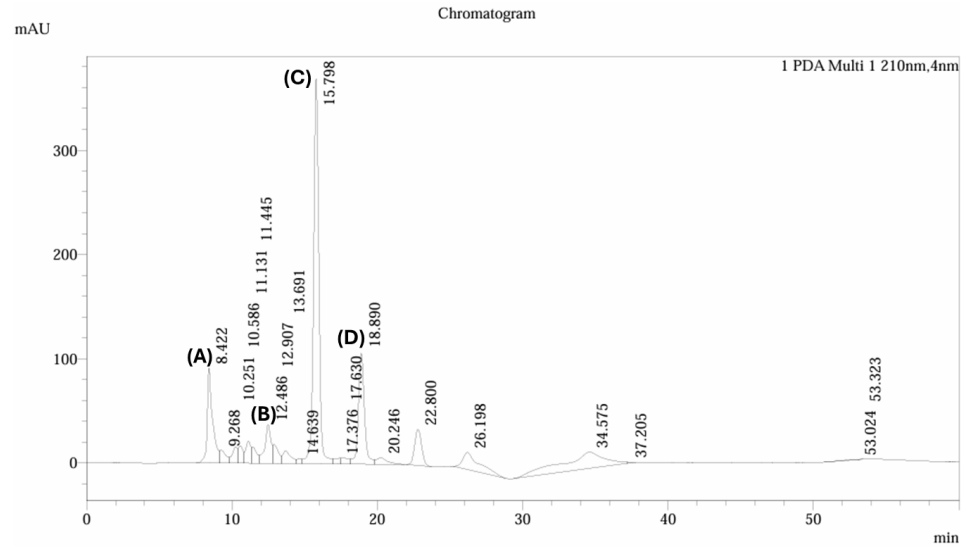

Figure A3: Chromatogram of Organic Acid Content in Water-Based Sample on Day 0. Peak Identification and Retention Time: (A) Phytic Acid, 8.422 min; (B) Malonic Acid, 12.486 min; (C) Lactic Acid, 15.798 min; (D) Acetic Acid, 18.890 min

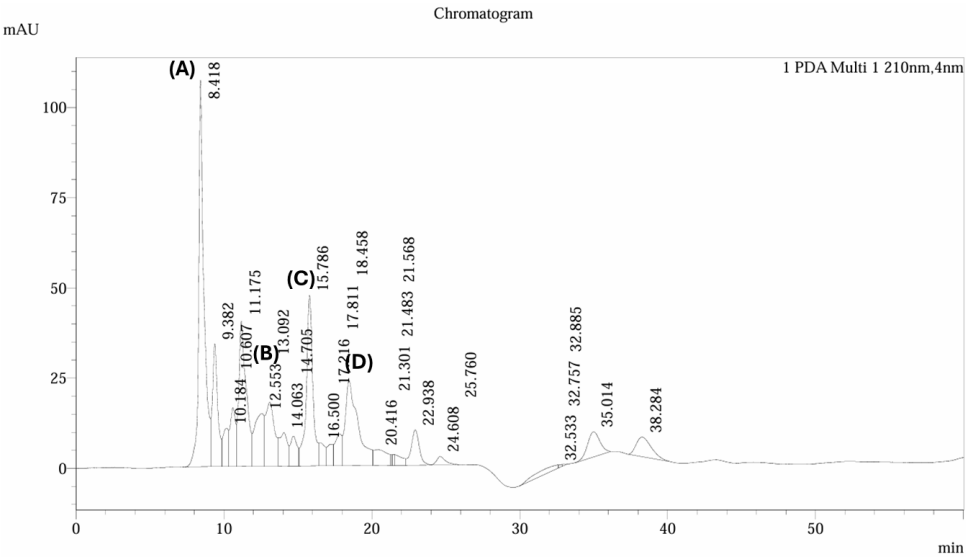

Figure A4: Chromatogram of Organic Acid Content in Water-Based Sample on Day 10. Peak Identification and Retention Time: (A) Phytic Acid, 8.418 min; (B) Malonic Acid, 12.553 min; (C) Lactic Acid, 15.786 min; (D) Acetic Acid, 18.458 min

APPENDIX B

Table B1: Normality test results for reducing sugar content.

Tests of Normality

Saccharification day		Cassava	Kolmogorov-Smirnov[a] Statistic	df	Sig.	Shapiro-Wilk Statistic	df	Sig.
Day 0	Sugar content	Cassava	0.219	3		0.987	3	0.780
Day 2	Sugar content	Cassava	0.335	3		0.858	3	0.263

a. Lilliefors Significance Correction

Table B2: Independent sample t-test results for reducing sugar content.

Independent Samples Test

		t-test for Equality of Means							
				Significance		Mean	Std. Error	95% Confidence Interval of the Difference	
		t	df	One-Sided p	Two-Sided p	Difference	Difference	Lower	Upper
Sugar content	Equal variances	-134.071	4	0.000	0.000	-12.70333	0.09475	-12.96640	-12.44026
	Equal variances not assumed	-134.071	3.002	0.000	0.000	-12.70333	0.09475	-13.00478	-12.40188

Table B3: Independent sample t-test results for free amino acid content.

Independent Samples Test

		t-test for Equality of Means							
				Significance			Std. Error	95% Confidence Interval of the Difference	
		t	df	One-Sided p	Two-Sided p	Mean Difference	Difference	Lower	Upper
FAA content	Equal variances	2.233	4	0.045	0.089	0.06000	0.02687	-0.01461	0.13461
	Equal variances not assumed	2.233	3.180	0.053	0.107	0.06000	0.02687	-0.02285	0.14285

Table B4: Normality test results for alcohol content.

Tests of Normality

Fermentation day		Sample	Kolmogorov-Smirnov[a] Statistic	df	Sig.	Shapiro-Wilk Statistic	df	Sig.
Day 1	Alcohol content	Agarwood	0.287	4		0.820	4	0.142
		Water	0.306	4		0.761	4	0.049
Day 2	Alcohol content	Agarwood	0.271	4		0.897	4	0.416
		Water	0.287	4		0.931	4	0.598
Day 4	Alcohol content	Agarwood	0.260	4		0.827	4	0.161
		Water	0.441	4		0.630	4	0.001
Day 5	Alcohol content	Agarwood	0.231	4		0.968	4	0.828
		Water	0.262	4		0.895	4	0.408
Day 7	Alcohol content	Agarwood	0.364	4		0.840	4	0.195
		Water	0.305	4		0.798	4	0.099
Day 10	Alcohol content	Agarwood	0.210	4		0.982	4	0.911
		Water	0.250	4		0.945	4	0.683

Table B5: ANOVA results for alcohol content.

Tests of Between-Subjects Effects

Dependent Variable: Alcohol content

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	136.077[a]	11	12.371	2462.840	0.000
Intercept	431.996	1	431.996	86005.050	0.000
Sample	1.332	1	1.332	265.264	0.000
Day	132.953	5	26.591	5293.847	0.000
Sample * Day	1.792	5	0.358	71.348	0.000
Error	0.181	36	0.005		
Total	568.254	48			
Corrected Total	136.258	47			

a. R Squared = .999 (Adjusted R Squared = .998)

Table B6: Tukey's test results for alcohol content.

Multiple Comparisons

Dependent Variable: Alcohol content

						95% Confidence Interval	
(I) Fermentation day	(J) Fermentation day		Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Tukey HSD	Day 1	Day 2	2.4858*	0.03544	0.000	2.3791	2.5924
		Day 4	3.2539*	0.03544	0.000	3.1473	3.3605
		Day 5	4.4306*	0.03544	0.000	4.3239	4.5372
		Day 7	4.6275*	0.03544	0.000	4.5208	4.7341
		Day 10	4.7270*	0.03544	0.000	4.6204	4.8336
	Day 2	Day 1	-2.4858*	0.03544	0.000	-2.5924	-2.3791
		Day 4	.7681*	0.03544	0.000	0.6615	0.8748
		Day 5	1.9448*	0.03544	0.000	1.8382	2.0514
		Day 7	2.1417*	0.03544	0.000	2.0351	2.2483
		Day 10	2.2412*	0.03544	0.000	2.1346	2.3479
	Day 4	Day 1	-3.2539*	0.03544	0.000	-3.3605	-3.1473
		Day 2	-.7681*	0.03544	0.000	-0.8748	-0.6615
		Day 5	1.1766*	0.03544	0.000	1.0700	1.2833
		Day 7	1.3736*	0.03544	0.000	1.2669	1.4802
		Day 10	1.4731*	0.03544	0.000	1.3665	1.5797
	Day 5	Day 1	-4.4306*	0.03544	0.000	-4.5372	-4.3239
		Day 2	-1.9448*	0.03544	0.000	-2.0514	-1.8382
		Day 4	-1.1766*	0.03544	0.000	-1.2833	-1.0700
		Day 7	.1969*	0.03544	0.000	0.0903	0.3035
		Day 10	.2964*	0.03544	0.000	0.1898	0.4031
	Day 7	Day 1	-4.6275*	0.03544	0.000	-4.7341	-4.5208
		Day 2	-2.1417*	0.03544	0.000	-2.2483	-2.0351
		Day 4	-1.3736*	0.03544	0.000	-1.4802	-1.2669
		Day 5	-.1969*	0.03544	0.000	-0.3035	-0.0903
		Day 10	0.0995	0.03544	0.079	-0.0071	0.2062
	Day 10	Day 1	-4.7270*	0.03544	0.000	-4.8336	-4.6204
		Day 2	-2.2412*	0.03544	0.000	-2.3479	-2.1346
		Day 4	-1.4731*	0.03544	0.000	-1.5797	-1.3665
		Day 5	-.2964*	0.03544	0.000	-0.4031	-0.1898
		Day 7	-0.0995	0.03544	0.079	-0.2062	0.0071

Table B7: ANOVA results for pH.

Tests of Between-Subjects Effects

Dependent Variable: pH

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	33.574[a]	13	2.583	4021.106	0.000
Intercept	932.770	1	932.770	1452319.810	0.000
Sample	0.075	1	0.075	116.844	0.000
Day	33.410	6	5.568	8669.763	0.000
Sample * Day	0.089	6	0.015	23.158	0.000
Error	0.027	42	0.001		
Total	966.371	56			
Corrected Total	33.601	55			

a. R Squared = .999 (Adjusted R Squared = .999)

Table B8: Tukey's test results for pH.

Multiple Comparisons

Dependent Variable: pH

(I) Fermentation day	(J) Fermentation day		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Day 0	Day 1	0.0000	0.01267	1.000	-0.0392	0.0392
		Day 2	-.0625*	0.01267	0.000	-0.1017	-0.0233
		Day 4	-.3213*	0.01267	0.000	-0.3605	-0.2820
		Day 5	-1.4825*	0.01267	0.000	-1.5217	-1.4433
		Day 7	-1.7338*	0.01267	0.000	-1.7730	-1.6945
		Day 10	-1.6938*	0.01267	0.000	-1.7330	-1.6545
	Day 1	Day 0	0.0000	0.01267	1.000	-0.0392	0.0392
		Day 2	-.0625*	0.01267	0.000	-0.1017	-0.0233
		Day 4	-.3213*	0.01267	0.000	-0.3605	-0.2820
		Day 5	-1.4825*	0.01267	0.000	-1.5217	-1.4433
		Day 7	-1.7338*	0.01267	0.000	-1.7730	-1.6945
		Day 10	-1.6938*	0.01267	0.000	-1.7330	-1.6545
	Day 2	Day 0	.0625*	0.01267	0.000	0.0233	0.1017
		Day 1	.0625*	0.01267	0.000	0.0233	0.1017
		Day 4	-.2588*	0.01267	0.000	-0.2980	-0.2195
		Day 5	-1.4200*	0.01267	0.000	-1.4592	-1.3808
		Day 7	-1.6712*	0.01267	0.000	-1.7105	-1.6320
		Day 10	-1.6312*	0.01267	0.000	-1.6705	-1.5920
	Day 4	Day 0	.3213*	0.01267	0.000	0.2820	0.3605
		Day 1	.3213*	0.01267	0.000	0.2820	0.3605
		Day 2	.2588*	0.01267	0.000	0.2195	0.2980
		Day 5	-1.1613*	0.01267	0.000	-1.2005	-1.1220
		Day 7	-1.4125*	0.01267	0.000	-1.4517	-1.3733
		Day 10	-1.3725*	0.01267	0.000	-1.4117	-1.3333
	Day 5	Day 0	1.4825*	0.01267	0.000	1.4433	1.5217
		Day 1	1.4825*	0.01267	0.000	1.4433	1.5217
		Day 2	1.4200*	0.01267	0.000	1.3808	1.4592
		Day 4	1.1613*	0.01267	0.000	1.1220	1.2005
		Day 7	-.2512*	0.01267	0.000	-0.2905	-0.2120
		Day 10	-.2112*	0.01267	0.000	-0.2505	-0.1720
	Day 7	Day 0	1.7338*	0.01267	0.000	1.6945	1.7730
		Day 1	1.7338*	0.01267	0.000	1.6945	1.7730
		Day 2	1.6712*	0.01267	0.000	1.6320	1.7105
		Day 4	1.4125*	0.01267	0.000	1.3733	1.4517
		Day 5	.2512*	0.01267	0.000	0.2120	0.2905
		Day 10	.0400*	0.01267	0.043	0.0008	0.0792
	Day 10	Day 0	1.6938*	0.01267	0.000	1.6545	1.7330
		Day 1	1.6938*	0.01267	0.000	1.6545	1.7330
		Day 2	1.6312*	0.01267	0.000	1.5920	1.6705
		Day 4	1.3725*	0.01267	0.000	1.3333	1.4117
		Day 5	.2112*	0.01267	0.000	0.1720	0.2505
		Day 7	-.0400*	0.01267	0.043	-0.0792	-0.0008

Based on observed means.
The error term is Mean Square(Error) = .001.

*. The mean difference is significant at the .05 level.

Table B9: ANOVA test results for total titratable acidity.

Tests of Between-Subjects Effects

Dependent Variable: Titratable acid

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	265.194[a]	11	24.109	4282.078	<.001
Intercept	272.510	1	272.510	48402.424	<.001
Sample	.131	1	.131	23.201	<.001
Day	263.666	5	52.733	9366.311	<.001
Sample * Day	1.397	5	.279	49.619	<.001
Error	.203	36	.006		
Total	537.907	48			
Corrected Total	265.396	47			

a. R Squared = .999 (Adjusted R Squared = .999)

Table B10: Tukey's test results for total titratable acidity.

Multiple Comparisons

Dependent Variable: Titratable acid

	(I) Fermentation day	(J) Fermentation day	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Day 1	Day 2	.7600*	.03752	<.001	.6471	.8729
		Day 4	3.7158*	.03752	<.001	3.6029	3.8286
		Day 5	5.4893*	.03752	<.001	5.3764	5.6021
		Day 7	5.6173*	.03752	<.001	5.5044	5.7301
		Day 10	5.6738*	.03752	<.001	5.5609	5.7866
	Day 2	Day 1	-.7600*	.03752	<.001	-.8729	-.6471
		Day 4	2.9558*	.03752	<.001	2.8429	3.0686
		Day 5	4.7293*	.03752	<.001	4.6164	4.8421
		Day 7	4.8573*	.03752	<.001	4.7444	4.9701
		Day 10	4.9138*	.03752	<.001	4.8009	5.0266
	Day 4	Day 1	-3.7158*	.03752	<.001	-3.8286	-3.6029
		Day 2	-2.9558*	.03752	<.001	-3.0686	-2.8429
		Day 5	1.7735*	.03752	<.001	1.6606	1.8864
		Day 7	1.9015*	.03752	<.001	1.7886	2.0144
		Day 10	1.9580*	.03752	<.001	1.8451	2.0709
	Day 5	Day 1	-5.4893*	.03752	<.001	-5.6021	-5.3764
		Day 2	-4.7293*	.03752	<.001	-4.8421	-4.6164
		Day 4	-1.7735*	.03752	<.001	-1.8864	-1.6606
		Day 7	.1280*	.03752	.019	.0151	.2409
		Day 10	.1845*	.03752	<.001	.0716	.2974
	Day 7	Day 1	-5.6172*	.03752	<.001	-5.7301	-5.5044
		Day 2	-4.8573*	.03752	<.001	-4.9701	-4.7444
		Day 4	-1.9015*	.03752	<.001	-2.0144	-1.7886
		Day 5	-.1280*	.03752	.019	-.2409	-.0151
		Day 10	.0565	.03752	.663	-.0564	.1694
	Day 10	Day 1	-5.6738*	.03752	<.001	-5.7866	-5.5609
		Day 2	-4.9138*	.03752	<.001	-5.0266	-4.8009
		Day 4	-1.9580*	.03752	<.001	-2.0709	-1.8451
		Day 5	-.1845*	.03752	<.001	-.2974	-.0716
		Day 7	-.0565	.03752	.663	-.1694	.0564

Based on observed means.
The error term is Mean Square(Error) = .006.

*. The mean difference is significant at the 0.05 level.

Table B11: ANOVA test results for total phenolic content.

Tests of Between-Subjects Effects

Dependent Variable: TPC

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.009[a]	13	0.001	625.085	0.000
Intercept	0.745	1	0.745	671123.597	0.000
Sample	0.005	1	0.005	4268.698	0.000
Day	0.004	6	0.001	631.870	0.000
Sample * Day	7.346E-05	6	1.224E-05	11.030	0.000
Error	4.662E-05	42	1.110E-06		
Total	0.754	56			
Corrected Total	0.009	55			

a. R Squared = .995 (Adjusted R Squared = .993)

Table B12: Tukey's test results for total phenolic content.

Multiple Comparisons

Dependent Variable: TPC

(I) Fermentation day	(J) Fermentation day		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Day 0	Day 1	-0.000438	0.0005268	0.980	-0.002068	0.001193
		Day 2	.010463*	0.0005268	0.000	0.008832	0.012093
		Day 4	.010850*	0.0005268	0.000	0.009219	0.012481
		Day 5	.006038*	0.0005268	0.000	0.004407	0.007668
		Day 7	-.006988*	0.0005268	0.000	-0.008618	-0.005357
		Day 10	-.014738*	0.0005268	0.000	-0.016368	-0.013107
	Day 1	Day 0	0.000438	0.0005268	0.980	-0.001193	0.002068
		Day 2	.010900*	0.0005268	0.000	0.009269	0.012531
		Day 4	.011288*	0.0005268	0.000	0.009657	0.012918
		Day 5	.006475*	0.0005268	0.000	0.004844	0.008106
		Day 7	-.006550*	0.0005268	0.000	-0.008181	-0.004919
		Day 10	-.014300*	0.0005268	0.000	-0.015931	-0.012669
	Day 2	Day 0	-.010463*	0.0005268	0.000	-0.012093	-0.008832
		Day 1	-.010900*	0.0005268	0.000	-0.012531	-0.009269
		Day 4	0.000387	0.0005268	0.989	-0.001243	0.002018
		Day 5	-.004425*	0.0005268	0.000	-0.006056	-0.002794
		Day 7	-.017450*	0.0005268	0.000	-0.019081	-0.015819
		Day 10	-.025200*	0.0005268	0.000	-0.026831	-0.023569
	Day 4	Day 0	-.010850*	0.0005268	0.000	-0.012481	-0.009219
		Day 1	-.011288*	0.0005268	0.000	-0.012918	-0.009657
		Day 2	-0.000387	0.0005268	0.989	-0.002018	0.001243
		Day 5	-.004813*	0.0005268	0.000	-0.006443	-0.003182
		Day 7	-.017838*	0.0005268	0.000	-0.019468	-0.016207
		Day 10	-.025588*	0.0005268	0.000	-0.027218	-0.023957
	Day 5	Day 0	-.006038*	0.0005268	0.000	-0.007668	-0.004407
		Day 1	-.006475*	0.0005268	0.000	-0.008106	-0.004844
		Day 2	.004425*	0.0005268	0.000	0.002794	0.006056
		Day 4	.004813*	0.0005268	0.000	0.003182	0.006443
		Day 7	-.013025*	0.0005268	0.000	-0.014656	-0.011394
		Day 10	-.020775*	0.0005268	0.000	-0.022406	-0.019144
	Day 7	Day 0	.006988*	0.0005268	0.000	0.005357	0.008618
		Day 1	.006550*	0.0005268	0.000	0.004919	0.008181
		Day 2	.017450*	0.0005268	0.000	0.015819	0.019081
		Day 4	.017838*	0.0005268	0.000	0.016207	0.019468
		Day 5	.013025*	0.0005268	0.000	0.011394	0.014656
		Day 10	-.007750*	0.0005268	0.000	-0.009381	-0.006119
	Day 10	Day 0	.014738*	0.0005268	0.000	0.013107	0.016368
		Day 1	.014300*	0.0005268	0.000	0.012669	0.015931
		Day 2	.025200*	0.0005268	0.000	0.023569	0.026831
		Day 4	.025588*	0.0005268	0.000	0.023957	0.027218
		Day 5	.020775*	0.0005268	0.000	0.019144	0.022406
		Day 7	.007750*	0.0005268	0.000	0.006119	0.009381

Table B13: ANOVA (split fermentation day) test results for total phenolic content.

Tests of Between-Subjects Effects

Dependent Variable: TPC

Fermentation day	Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day 0	Corrected Model	.001[a]	1	0.001	5000.000	0.000
	Intercept	0.108	1	0.108	879895.878	0.000
	Sample	0.001	1	0.001	5000.000	0.000
	Error	7.350E-07	6	1.225E-07		
	Total	0.108	8			
	Corrected Total	0.001	7			
Day 1	Corrected Model	.001[b]	1	0.001	987.194	0.000
	Intercept	0.109	1	0.109	171588.626	0.000
	Sample	0.001	1	0.001	987.194	0.000
	Error	3.798E-06	6	6.329E-07		
	Total	0.109	8			
	Corrected Total	0.001	7			
Day 2	Corrected Model	.001[c]	1	0.001	725.485	0.000
	Intercept	0.089	1	0.089	67028.733	0.000
	Sample	0.001	1	0.001	725.485	0.000
	Error	7.987E-06	6	1.331E-06		
	Total	0.090	8			
	Corrected Total	0.001	7			
Day 4	Corrected Model	.000[d]	1	0.000	640.000	0.000
	Intercept	0.089	1	0.089	144617.804	0.000
	Sample	0.000	1	0.000	640.000	0.000
	Error	3.675E-06	6	6.125E-07		
	Total	0.089	8			
	Corrected Total	0.000	7			
Day 5	Corrected Model	.001[e]	1	0.001	922.674	0.000
	Intercept	0.097	1	0.097	110336.225	0.000
	Sample	0.001	1	0.001	922.674	0.000
	Error	5.268E-06	6	8.779E-07		
	Total	0.098	8			
	Corrected Total	0.001	7			
Day 7	Corrected Model	.001[f]	1	0.001	238.471	0.000
	Intercept	0.121	1	0.121	40548.330	0.000
	Sample	0.001	1	0.001	238.471	0.000
	Error	1.793E-05	6	2.988E-06		
	Total	0.122	8			
	Corrected Total	0.001	7			
Day 10	Corrected Model	.001[g]	1	0.001	575.951	0.000
	Intercept	0.137	1	0.137	113645.339	0.000
	Sample	0.001	1	0.001	575.951	0.000
	Error	7.227E-06	6	1.205E-06		
	Total	0.138	8			
	Corrected Total	0.001	7			

a. R Squared = .999 (Adjusted R Squared = .999)
b. R Squared = .994 (Adjusted R Squared = .993)
c. R Squared = .992 (Adjusted R Squared = .990)
d. R Squared = .991 (Adjusted R Squared = .989)
e. R Squared = .994 (Adjusted R Squared = .992)
f. R Squared = .975 (Adjusted R Squared = .971)
g. R Squared = .990 (Adjusted R Squared = .988)

Table B14: ANOVA (split sample) test results for total phenolic content.

Tests of Between-Subjects Effects

Dependent Variable: TPC

Sample	Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Agarwood	Corrected Model	.002[a]	6	0.000	230.579	0.000
	Intercept	0.434	1	0.434	277400.676	0.000
	Day	0.002	6	0.000	230.579	0.000
	Error	3.287E-05	21	1.565E-06		
	Total	0.436	28			
	Corrected Total	0.002	27			
Water	Corrected Model	.002[b]	6	0.000	538.778	0.000
	Intercept	0.315	1	0.315	481898.613	0.000
	Day	0.002	6	0.000	538.778	0.000
	Error	1.375E-05	21	6.545E-07		
	Total	0.318	28			
	Corrected Total	0.002	27			

a. R Squared = .985 (Adjusted R Squared = .981)

b. R Squared = .994 (Adjusted R Squared = .992)

Table B15: Tukey's HSD test results (split sample) for total phenolic content.

Multiple Comparisons

Dependent Variable: TPC

Sample		(I) Fermentation day	(J) Fermentation day	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
Agarwood	Tukey HSD	Day 0	Day 1	-0.000525	0.0008847	0.996	-0.003401	0.002351
			Day 2	.008225*	0.0008847	0.000	0.005349	0.011101
			Day 4	.012600*	0.0008847	0.000	0.009724	0.015476
			Day 5	.004725*	0.0008847	0.000	0.001849	0.007601
			Day 7	-.007675*	0.0008847	0.000	-0.010551	-0.004799
			Day 10	-.015300*	0.0008847	0.000	-0.018176	-0.012424
		Day 1	Day 0	0.000525	0.0008847	0.996	-0.002351	0.003401
			Day 2	.008750*	0.0008847	0.000	0.005874	0.011626
			Day 4	.013125*	0.0008847	0.000	0.010249	0.016001
			Day 5	.005250*	0.0008847	0.000	0.002374	0.008126
			Day 7	-.007150*	0.0008847	0.000	-0.010026	-0.004274
			Day 10	-.014775*	0.0008847	0.000	-0.017651	-0.011899
		Day 2	Day 0	-.008225*	0.0008847	0.000	-0.011101	-0.005349
			Day 1	-.008750*	0.0008847	0.000	-0.011626	-0.005874
			Day 4	.004375*	0.0008847	0.001	0.001499	0.007251
			Day 5	-.003500*	0.0008847	0.011	-0.006376	-0.000624
			Day 7	-.015900*	0.0008847	0.000	-0.018776	-0.013024
			Day 10	-.023525*	0.0008847	0.000	-0.026401	-0.020649
		Day 4	Day 0	-.012600*	0.0008847	0.000	-0.015476	-0.009724
			Day 1	-.013125*	0.0008847	0.000	-0.016001	-0.010249
			Day 2	-.004375*	0.0008847	0.001	-0.007251	-0.001499
			Day 5	-.007875*	0.0008847	0.000	-0.010751	-0.004999
			Day 7	-.020275*	0.0008847	0.000	-0.023151	-0.017399
			Day 10	-.027900*	0.0008847	0.000	-0.030776	-0.025024
		Day 5	Day 0	-.004725*	0.0008847	0.000	-0.007601	-0.001849
			Day 1	-.005250*	0.0008847	0.000	-0.008126	-0.002374
			Day 2	.003500*	0.0008847	0.011	0.000624	0.006376
			Day 4	.007875*	0.0008847	0.000	0.004999	0.010751
			Day 7	-.012400*	0.0008847	0.000	-0.015276	-0.009524
			Day 10	-.020025*	0.0008847	0.000	-0.022901	-0.017149
		Day 7	Day 0	.007675*	0.0008847	0.000	0.004799	0.010551
			Day 1	.007150*	0.0008847	0.000	0.004274	0.010026
			Day 2	.015900*	0.0008847	0.000	0.013024	0.018776
			Day 4	.020275*	0.0008847	0.000	0.017399	0.023151
			Day 5	.012400*	0.0008847	0.000	0.009524	0.015276
			Day 10	-.007625*	0.0008847	0.000	-0.010501	-0.004749
		Day 10	Day 0	.015300*	0.0008847	0.000	0.012424	0.018176
			Day 1	.014775*	0.0008847	0.000	0.011899	0.017651
			Day 2	.023525*	0.0008847	0.000	0.020649	0.026401
			Day 4	.027900*	0.0008847	0.000	0.025024	0.030776
			Day 5	.020025*	0.0008847	0.000	0.017149	0.022901
			Day 7	.007625*	0.0008847	0.000	0.004749	0.010501

Table B16: Normality test results for acetic acid concentration.

Tests of Normality

Fermentation day		Sample	Kolmogorov-Smirnov[a] Statistic	df	Sig.	Shapiro-Wilk Statistic	df	Sig.
Day 0	Acetic acid	Agarwood	0.303	3		0.909	3	0.414
		Water	0.270	3		0.949	3	0.564
Day 1	Acetic acid	Agarwood	0.283	3		0.934	3	0.504
		Water	0.243	3		0.972	3	0.680
Day 2	Acetic acid	Agarwood	0.337	3		0.854	3	0.253
		Water	0.231	3		0.980	3	0.732
Day 4	Acetic acid	Agarwood	0.243	3		0.972	3	0.682
		Water	0.373	3		0.780	3	0.067
Day 5	Acetic acid	Agarwood	0.243	3		0.972	3	0.680
		Water	0.327	3		0.872	3	0.301
Day 7	Acetic acid	Agarwood	0.288	3		0.928	3	0.481
		Water	0.251	3		0.966	3	0.645
Day 10	Acetic acid	Agarwood	0.337	3		0.854	3	0.252
		Water	0.323	3		0.878	3	0.320

a. Lilliefors Significance Correction

Table B17: ANOVA test results for acetic acid concentration.

Tests of Between-Subjects Effects

Dependent Variable: Acetic acid

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	83.603[a]	13	6.431	360.708	0.000
Intercept	233.283	1	233.283	13084.576	0.000
Sample	0.807	1	0.807	45.261	0.000
Day	80.788	6	13.465	755.221	0.000
Sample * Day	2.008	6	0.335	18.769	0.000
Error	0.499	28	0.018		
Total	317.385	42			
Corrected Total	84.102	41			

a. R Squared = .994 (Adjusted R Squared = .991)

Table B18: Tukey's test results for acetic acid concentration.

Multiple Comparisons

Dependent Variable: Acetic acid

(I) Fermentation day	(J) Fermentation day		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Day 0	Day 1	.253650*	0.0770906	0.038	0.009109	0.498191
		Day 2	.941300*	0.0770906	0.000	0.696759	1.185841
		Day 4	3.148267*	0.0770906	0.000	2.903725	3.392808
		Day 5	3.195550*	0.0770906	0.000	2.951009	3.440091
		Day 7	3.468417*	0.0770906	0.000	3.223875	3.712958
		Day 10	2.638717*	0.0770906	0.000	2.394175	2.883258
	Day 1	Day 0	-.253650*	0.0770906	0.038	-0.498191	-0.009109
		Day 2	.687650*	0.0770906	0.000	0.443109	0.932191
		Day 4	2.894617*	0.0770906	0.000	2.650075	3.139158
		Day 5	2.941900*	0.0770906	0.000	2.697359	3.186441
		Day 7	3.214767*	0.0770906	0.000	2.970225	3.459308
		Day 10	2.385067*	0.0770906	0.000	2.140525	2.629608
	Day 2	Day 0	-.941300*	0.0770906	0.000	-1.185841	-0.696759
		Day 1	-.687650*	0.0770906	0.000	-0.932191	-0.443109
		Day 4	2.206967*	0.0770906	0.000	1.962425	2.451508
		Day 5	2.254250*	0.0770906	0.000	2.009709	2.498791
		Day 7	2.527117*	0.0770906	0.000	2.282575	2.771658
		Day 10	1.697417*	0.0770906	0.000	1.452875	1.941958
	Day 4	Day 0	-3.148267*	0.0770906	0.000	-3.392808	-2.903725
		Day 1	-2.894617*	0.0770906	0.000	-3.139158	-2.650075
		Day 2	-2.206967*	0.0770906	0.000	-2.451508	-1.962425
		Day 5	0.047283	0.0770906	0.996	-0.197258	0.291825
		Day 7	.320150*	0.0770906	0.005	0.075609	0.564691
		Day 10	-.509550*	0.0770906	0.000	-0.754091	-0.265009
	Day 5	Day 0	-3.195550*	0.0770906	0.000	-3.440091	-2.951009
		Day 1	-2.941900*	0.0770906	0.000	-3.186441	-2.697359
		Day 2	-2.254250*	0.0770906	0.000	-2.498791	-2.009709
		Day 4	-0.047283	0.0770906	0.996	-0.291825	0.197258
		Day 7	.272867*	0.0770906	0.021	0.028325	0.517408
		Day 10	-.556833*	0.0770906	0.000	-0.801375	-0.312292
	Day 7	Day 0	-3.468417*	0.0770906	0.000	-3.712958	-3.223875
		Day 1	-3.214767*	0.0770906	0.000	-3.459308	-2.970225
		Day 2	-2.527117*	0.0770906	0.000	-2.771658	-2.282575
		Day 4	-.320150*	0.0770906	0.005	-0.564691	-0.075609
		Day 5	-.272867*	0.0770906	0.021	-0.517408	-0.028325
		Day 10	-.829700*	0.0770906	0.000	-1.074241	-0.585159
	Day 10	Day 0	-2.638717*	0.0770906	0.000	-2.883258	-2.394175
		Day 1	-2.385067*	0.0770906	0.000	-2.629608	-2.140525
		Day 2	-1.697417*	0.0770906	0.000	-1.941958	-1.452875
		Day 4	.509550*	0.0770906	0.000	0.265009	0.754091
		Day 5	.556833*	0.0770906	0.000	0.312292	0.801375
		Day 7	.829700*	0.0770906	0.000	0.585159	1.074241

Based on observed means.
The error term is Mean Square(Error) = .018.

*. The mean difference is significant at the .05 level.

Table B19: ANOVA test results (split sample) for acetic acid concentration.

Tests of Between-Subjects Effects

Dependent Variable: Acetic acid

Sample	Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Agarwood	Corrected Model	42.699[a]	6	7.117	455.765	0.000
	Intercept	130.765	1	130.765	8374.636	0.000
	Day	42.699	6	7.117	455.765	0.000
	Error	0.219	14	0.016		
	Total	173.683	21			
	Corrected Total	42.918	20			
Water	Corrected Model	40.097[b]	6	6.683	333.421	0.000
	Intercept	103.325	1	103.325	5155.080	0.000
	Day	40.097	6	6.683	333.421	0.000
	Error	0.281	14	0.020		
	Total	143.702	21			
	Corrected Total	40.378	20			

a. R Squared = .995 (Adjusted R Squared = .993)

b. R Squared = .993 (Adjusted R Squared = .990)

Table B20: Tukey's HSD test results (split sample) for acetic acid concentration.

Multiple Comparisons

Dependent Variable: Acetic acid

Sample		(I) Fermentation day	(J) Fermentation day	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
Agarwood	Tukey HSD	Day 0	Day 1	0.048200	0.1020277	0.999	-0.300182	0.396582
			Day 2	.381967*	0.1020277	0.028	0.033584	0.730349
			Day 4	2.947300*	0.1020277	0.000	2.598918	3.295682
			Day 5	3.028067*	0.1020277	0.000	2.679684	3.376449
			Day 7	3.538000*	0.1020277	0.000	3.189618	3.886382
			Day 10	2.128800*	0.1020277	0.000	1.780418	2.477182
		Day 1	Day 0	-0.048200	0.1020277	0.999	-0.396582	0.300182
			Day 2	0.333767	0.1020277	0.064	-0.014616	0.682149
			Day 4	2.899100*	0.1020277	0.000	2.550718	3.247482
			Day 5	2.979867*	0.1020277	0.000	2.631484	3.328249
			Day 7	3.489800*	0.1020277	0.000	3.141418	3.838182
			Day 10	2.080600*	0.1020277	0.000	1.732218	2.428982
		Day 2	Day 0	-.381967*	0.1020277	0.028	-0.730349	-0.033584
			Day 1	-0.333767	0.1020277	0.064	-0.682149	0.014616
			Day 4	2.565333*	0.1020277	0.000	2.216951	2.913716
			Day 5	2.646100*	0.1020277	0.000	2.297718	2.994482
			Day 7	3.156033*	0.1020277	0.000	2.807651	3.504416
			Day 10	1.746833*	0.1020277	0.000	1.398451	2.095216
		Day 4	Day 0	-2.947300*	0.1020277	0.000	-3.295682	-2.598918
			Day 1	-2.899100*	0.1020277	0.000	-3.247482	-2.550718
			Day 2	-2.565333*	0.1020277	0.000	-2.913716	-2.216951
			Day 5	0.080767	0.1020277	0.982	-0.267616	0.429149
			Day 7	.590700*	0.1020277	0.001	0.242318	0.939082
			Day 10	-.818500*	0.1020277	0.000	-1.166882	-0.470118
		Day 5	Day 0	-3.028067*	0.1020277	0.000	-3.376449	-2.679684
			Day 1	-2.979867*	0.1020277	0.000	-3.328249	-2.631484
			Day 2	-2.646100*	0.1020277	0.000	-2.994482	-2.297718
			Day 4	-0.080767	0.1020277	0.982	-0.429149	0.267616
			Day 7	.509933*	0.1020277	0.003	0.161551	0.858316
			Day 10	-.899267*	0.1020277	0.000	-1.247649	-0.550884
		Day 7	Day 0	-3.538000*	0.1020277	0.000	-3.886382	-3.189618
			Day 1	-3.489800*	0.1020277	0.000	-3.838182	-3.141418
			Day 2	-3.156033*	0.1020277	0.000	-3.504416	-2.807651
			Day 4	-.590700*	0.1020277	0.001	-0.939082	-0.242318
			Day 5	-.509933*	0.1020277	0.003	-0.858316	-0.161551
			Day 10	-1.409200*	0.1020277	0.000	-1.757582	-1.060818
		Day 10	Day 0	-2.128800*	0.1020277	0.000	-2.477182	-1.780418
			Day 1	-2.080600*	0.1020277	0.000	-2.428982	-1.732218
			Day 2	-1.746833*	0.1020277	0.000	-2.095216	-1.398451
			Day 4	.818500*	0.1020277	0.000	0.470118	1.166882
			Day 5	.899267*	0.1020277	0.000	0.550884	1.247649
			Day 7	1.409200*	0.1020277	0.000	1.060818	1.757582

Table B21: ANOVA test results (split fermentation day) for acetic acid concentration.

Tests of Between-Subjects Effects

Dependent Variable: Acetic acid

Fermentation day	Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Day 0	Corrected Model	.045[a]	1	0.045	0.881	0.401
	Intercept	111.259	1	111.259	2198.809	0.000
	Sample	0.045	1	0.045	0.881	0.401
	Error	0.202	4	0.051		
	Total	111.506	6			
	Corrected Total	0.247	5			
Day 1	Corrected Model	.085[b]	1	0.085	4.395	0.104
	Intercept	98.538	1	98.538	5074.672	0.000
	Sample	0.085	1	0.085	4.395	0.104
	Error	0.078	4	0.019		
	Total	98.701	6			
	Corrected Total	0.163	5			
Day 2	Corrected Model	1.343[c]	1	1.343	2633.345	0.000
	Intercept	67.935	1	67.935	133183.413	0.000
	Sample	1.343	1	1.343	2633.345	0.000
	Error	0.002	4	0.001		
	Total	69.280	6			
	Corrected Total	1.345	5			
Day 4	Corrected Model	.079[d]	1	0.079	118.076	0.000
	Intercept	8.045	1	8.045	12015.931	0.000
	Sample	0.079	1	0.079	118.076	0.000
	Error	0.003	4	0.001		
	Total	8.126	6			
	Corrected Total	0.082	5			
Day 5	Corrected Model	.040[e]	1	0.040	15.690	0.017
	Intercept	7.401	1	7.401	2928.143	0.000
	Sample	0.040	1	0.040	15.690	0.017
	Error	0.010	4	0.003		
	Total	7.451	6			
	Corrected Total	0.050	5			
Day 7	Corrected Model	.146[f]	1	0.146	321.764	0.000
	Intercept	4.211	1	4.211	9307.538	0.000
	Sample	0.146	1	0.146	321.764	0.000
	Error	0.002	4	0.000		
	Total	4.359	6			
	Corrected Total	0.147	5			
Day 10	Corrected Model	1.077[g]	1	1.077	21.280	0.010
	Intercept	16.683	1	16.683	329.534	0.000
	Sample	1.077	1	1.077	21.280	0.010
	Error	0.203	4	0.051		
	Total	17.962	6			
	Corrected Total	1.280	5			

a. R Squared = .180 (Adjusted R Squared = -.024)
b. R Squared = .524 (Adjusted R Squared = .404)
c. R Squared = .998 (Adjusted R Squared = .998)
d. R Squared = .967 (Adjusted R Squared = .959)
e. R Squared = .797 (Adjusted R Squared = .746)
f. R Squared = .988 (Adjusted R Squared = .985)
g. R Squared = .842 (Adjusted R Squared = .802)

Table B22: ANOVA results in phytic acid concentration.

Dependent Variable: Phytic acid

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	85.776[a]	13	6.598	200.597	0.000
Intercept	5197.253	1	5197.253	158007.377	0.000
Sample	1.480	1	1.480	44.982	0.000
Day	82.350	6	13.725	417.268	0.000
Sample * Day	1.946	6	0.324	9.861	0.000
Error	0.921	28	0.033		
Total	5283.950	42			
Corrected Total	86.697	41			

a. R Squared = .989 (Adjusted R Squared = .984)

Table B23: Tukey's HSD test results for phytic acid concentration.

(I) Fermentation day	(J) Fermentation day		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Day 0	Day 1	0.200650	0.1047099	0.487	-0.131503	0.532803
		Day 2	-.482383*	0.1047099	0.001	-0.814537	-0.150230
		Day 4	-.397800*	0.1047099	0.011	-0.729953	-0.065647
		Day 5	-1.630083*	0.1047099	0.000	-1.962237	-1.297930
		Day 7	-3.434900*	0.1047099	0.000	-3.767053	-3.102747
		Day 10	-3.207083*	0.1047099	0.000	-3.539237	-2.874930
	Day 1	Day 0	-0.200650	0.1047099	0.487	-0.532803	0.131503
		Day 2	-.683033*	0.1047099	0.000	-1.015187	-0.350880
		Day 4	-.598450*	0.1047099	0.000	-0.930603	-0.266297
		Day 5	-1.830733*	0.1047099	0.000	-2.162887	-1.498580
		Day 7	-3.635550*	0.1047099	0.000	-3.967703	-3.303397
		Day 10	-3.407733*	0.1047099	0.000	-3.739887	-3.075580
	Day 2	Day 0	.482383*	0.1047099	0.001	0.150230	0.814537
		Day 1	.683033*	0.1047099	0.000	0.350880	1.015187
		Day 4	0.084583	0.1047099	0.982	-0.247570	0.416737
		Day 5	-1.147700*	0.1047099	0.000	-1.479853	-0.815547
		Day 7	-2.952517*	0.1047099	0.000	-3.284670	-2.620363
		Day 10	-2.724700*	0.1047099	0.000	-3.056853	-2.392547
	Day 4	Day 0	.397800*	0.1047099	0.011	0.065647	0.729953
		Day 1	.598450*	0.1047099	0.000	0.266297	0.930603
		Day 2	-0.084583	0.1047099	0.982	-0.416737	0.247570
		Day 5	-1.232283*	0.1047099	0.000	-1.564437	-0.900130
		Day 7	-3.037100*	0.1047099	0.000	-3.369253	-2.704947
		Day 10	-2.809283*	0.1047099	0.000	-3.141437	-2.477130
	Day 5	Day 0	1.630083*	0.1047099	0.000	1.297930	1.962237
		Day 1	1.830733*	0.1047099	0.000	1.498580	2.162887
		Day 2	1.147700*	0.1047099	0.000	0.815547	1.479853
		Day 4	1.232283*	0.1047099	0.000	0.900130	1.564437
		Day 7	-1.804817*	0.1047099	0.000	-2.136970	-1.472663
		Day 10	-1.577000*	0.1047099	0.000	-1.909153	-1.244847
	Day 7	Day 0	3.434900*	0.1047099	0.000	3.102747	3.767053
		Day 1	3.635550*	0.1047099	0.000	3.303397	3.967703
		Day 2	2.952517*	0.1047099	0.000	2.620363	3.284670
		Day 4	3.037100*	0.1047099	0.000	2.704947	3.369253
		Day 5	1.804817*	0.1047099	0.000	1.472663	2.136970
		Day 10	0.227817	0.1047099	0.340	-0.104337	0.559970
	Day 10	Day 0	3.207083*	0.1047099	0.000	2.874930	3.539237
		Day 1	3.407733*	0.1047099	0.000	3.075580	3.739887
		Day 2	2.724700*	0.1047099	0.000	2.392547	3.056853
		Day 4	2.809283*	0.1047099	0.000	2.477130	3.141437
		Day 5	1.577000*	0.1047099	0.000	1.244847	1.909153
		Day 7	-0.227817	0.1047099	0.340	-0.559970	0.104337

Table B24: ANOVA test results for malonic acid concentration.

Tests of Between-Subjects Effects

Dependent Variable: Malonic acid

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1.548[a]	13	0.119	46.298	0.000
Intercept	31.178	1	31.178	12120.993	0.000
Sample	0.019	1	0.019	7.446	0.011
Day	1.423	6	0.237	92.179	0.000
Sample * Day	0.106	6	0.018	6.891	0.000
Error	0.072	28	0.003		
Total	32.798	42			
Corrected Total	1.620	41			

a. R Squared = .956 (Adjusted R Squared = .935)

Table B25: Tukey's HSD test results for malonic acid concentration.

Multiple Comparisons

Dependent Variable: Malonic acid

(I) Fermentation day	(J) Fermentation day		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Day 0	Day 1	0.065550	0.0292816	0.308	-0.027335	0.158435
		Day 2	.207350*	0.0292816	0.000	0.114465	0.300235
		Day 4	.468967*	0.0292816	0.000	0.376082	0.561852
		Day 5	.513733*	0.0292816	0.000	0.420848	0.606618
		Day 7	.395033*	0.0292816	0.000	0.302148	0.487918
		Day 10	.343517*	0.0292816	0.000	0.250632	0.436402
	Day 1	Day 0	-0.065550	0.0292816	0.308	-0.158435	0.027335
		Day 2	.141800*	0.0292816	0.001	0.048915	0.234685
		Day 4	.403417*	0.0292816	0.000	0.310532	0.496302
		Day 5	.448183*	0.0292816	0.000	0.355298	0.541068
		Day 7	.329483*	0.0292816	0.000	0.236598	0.422368
		Day 10	.277967*	0.0292816	0.000	0.185082	0.370852
	Day 2	Day 0	-.207350*	0.0292816	0.000	-0.300235	-0.114465
		Day 1	-.141800*	0.0292816	0.001	-0.234685	-0.048915
		Day 4	.261617*	0.0292816	0.000	0.168732	0.354502
		Day 5	.306383*	0.0292816	0.000	0.213498	0.399268
		Day 7	.187683*	0.0292816	0.000	0.094798	0.280568
		Day 10	.136167*	0.0292816	0.001	0.043282	0.229052
	Day 4	Day 0	-.468967*	0.0292816	0.000	-0.561852	-0.376082
		Day 1	-.403417*	0.0292816	0.000	-0.496302	-0.310532
		Day 2	-.261617*	0.0292816	0.000	-0.354502	-0.168732
		Day 5	0.044767	0.0292816	0.726	-0.048118	0.137652
		Day 7	-0.073933	0.0292816	0.189	-0.166818	0.018952
		Day 10	-.125450*	0.0292816	0.003	-0.218335	-0.032565
	Day 5	Day 0	-.513733*	0.0292816	0.000	-0.606618	-0.420848
		Day 1	-.448183*	0.0292816	0.000	-0.541068	-0.355298
		Day 2	-.306383*	0.0292816	0.000	-0.399268	-0.213498
		Day 4	-0.044767	0.0292816	0.726	-0.137652	0.048118
		Day 7	-.118700*	0.0292816	0.006	-0.211585	-0.025815
		Day 10	-.170217*	0.0292816	0.000	-0.263102	-0.077332
	Day 7	Day 0	-.395033*	0.0292816	0.000	-0.487918	-0.302148
		Day 1	-.329483*	0.0292816	0.000	-0.422368	-0.236598
		Day 2	-.187683*	0.0292816	0.000	-0.280568	-0.094798
		Day 4	0.073933	0.0292816	0.189	-0.018952	0.166818
		Day 5	.118700*	0.0292816	0.006	0.025815	0.211585
		Day 10	-0.051517	0.0292816	0.584	-0.144402	0.041368
	Day 10	Day 0	-.343517*	0.0292816	0.000	-0.436402	-0.250632
		Day 1	-.277967*	0.0292816	0.000	-0.370852	-0.185082
		Day 2	-.136167*	0.0292816	0.001	-0.229052	-0.043282
		Day 4	.125450*	0.0292816	0.003	0.032565	0.218335
		Day 5	.170217*	0.0292816	0.000	0.077332	0.263102
		Day 7	0.051517	0.0292816	0.584	-0.041368	0.144402

Table B26: ANOVA test results for lactic acid concentration.

Tests of Between-Subjects Effects

Dependent Variable: Lactic acid

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	208.185[a]	13	16.014	582.463	0.000
Intercept	630.270	1	630.270	22923.887	0.000
Sample	0.412	1	0.412	14.979	0.001
Day	206.885	6	34.481	1254.120	0.000
Sample * Day	0.888	6	0.148	5.386	0.001
Error	0.770	28	0.027		
Total	839.225	42			
Corrected Total	208.955	41			

a. R Squared = .996 (Adjusted R Squared = .995)

Table B27: Tukey's HSD test results for lactic acid concentration.

Multiple Comparisons

Dependent Variable: Lactic acid

(I) Fermentation day	(J) Fermentation day		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tukey HSD	Day 0	Day 1	0.158883	0.0957323	0.647	-0.144792	0.462559
		Day 2	.727333*	0.0957323	0.000	0.423658	1.031009
		Day 4	2.402467*	0.0957323	0.000	2.098791	2.706142
		Day 5	4.503533*	0.0957323	0.000	4.199858	4.807209
		Day 7	5.185000*	0.0957323	0.000	4.881325	5.488675
		Day 10	5.413433*	0.0957323	0.000	5.109758	5.717109
	Day 1	Day 0	-0.158883	0.0957323	0.647	-0.462559	0.144792
		Day 2	.568450*	0.0957323	0.000	0.264775	0.872125
		Day 4	2.243583*	0.0957323	0.000	1.939908	2.547259
		Day 5	4.344650*	0.0957323	0.000	4.040975	4.648325
		Day 7	5.026117*	0.0957323	0.000	4.722441	5.329792
		Day 10	5.254550*	0.0957323	0.000	4.950875	5.558225
	Day 2	Day 0	-.727333*	0.0957323	0.000	-1.031009	-0.423658
		Day 1	-.568450*	0.0957323	0.000	-0.872125	-0.264775
		Day 4	1.675133*	0.0957323	0.000	1.371458	1.978809
		Day 5	3.776200*	0.0957323	0.000	3.472525	4.079875
		Day 7	4.457667*	0.0957323	0.000	4.153991	4.761342
		Day 10	4.686100*	0.0957323	0.000	4.382425	4.989775
	Day 4	Day 0	-2.402467*	0.0957323	0.000	-2.706142	-2.098791
		Day 1	-2.243583*	0.0957323	0.000	-2.547259	-1.939908
		Day 2	-1.675133*	0.0957323	0.000	-1.978809	-1.371458
		Day 5	2.101067*	0.0957323	0.000	1.797391	2.404742
		Day 7	2.782533*	0.0957323	0.000	2.478858	3.086209
		Day 10	3.010967*	0.0957323	0.000	2.707291	3.314642
	Day 5	Day 0	-4.503533*	0.0957323	0.000	-4.807209	-4.199858
		Day 1	-4.344650*	0.0957323	0.000	-4.648325	-4.040975
		Day 2	-3.776200*	0.0957323	0.000	-4.079875	-3.472525
		Day 4	-2.101067*	0.0957323	0.000	-2.404742	-1.797391
		Day 7	.681467*	0.0957323	0.000	0.377791	0.985142
		Day 10	.909900*	0.0957323	0.000	0.606225	1.213575
	Day 7	Day 0	-5.185000*	0.0957323	0.000	-5.488675	-4.881325
		Day 1	-5.026117*	0.0957323	0.000	-5.329792	-4.722441
		Day 2	-4.457667*	0.0957323	0.000	-4.761342	-4.153991
		Day 4	-2.782533*	0.0957323	0.000	-3.086209	-2.478858
		Day 5	-.681467*	0.0957323	0.000	-0.985142	-0.377791
		Day 10	0.228433	0.0957323	0.241	-0.075242	0.532109
	Day 10	Day 0	-5.413433*	0.0957323	0.000	-5.717109	-5.109758
		Day 1	-5.254550*	0.0957323	0.000	-5.558225	-4.950875
		Day 2	-4.686100*	0.0957323	0.000	-4.989775	-4.382425
		Day 4	-3.010967*	0.0957323	0.000	-3.314642	-2.707291
		Day 5	-.909900*	0.0957323	0.000	-1.213575	-0.606225
		Day 7	-0.228433	0.0957323	0.241	-0.532109	0.075242

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