TIME COURSE EVALUATION OF BIOCHEMICAL CONTENTS AND BIOCATALYTIC ACTIVITIES OF JIAOSU FROM FRUIT WASTES DURING ONE-YEAR NATURAL FERMENTATION

By

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A dissertation submitted to the
Department of Allied Health Sciences,
Faculty of Science,
Universiti Tunku Abdul Rahman,
in partial fulfillment of the requirements for the degree of
Master of Science
March 2024

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ABSTRACT

TIME COURSE EVALUATION OF BIOCHEMICAL CONTENTS AND BIOCATALYTIC ACTIVITIES OF JIAOSU FROM FRUIT WASTES DURING ONE-YEAR NATURAL FERMENTATION

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Jiaosu is a multifunctional organic solution derived from fermentation of a mixture of fruit or vegetable wastes, sugar and water for a typical period of three months. The production of jiaosu is an inexpensive approach to reduce food waste. However, the significance of a fermentation period of three months or longer remains to be ascertained. Therefore, the present study evaluated the changes in pH, concentrations of proteins, phenolics, carbohydrates, alcohols, and organic acids (oxalic, tartaric, malic, lactic, acetic, citric, and succinic) as well as amylase, protease, and lipase activities of different groups of fruit peel jiaosu throughout one year of natural fermentation. Three jiaosu groups, each with different types of fruit peels were prepared: orange-papaya-watermelon (OPW), grapefruit-mango-pineapple (GMP), and durian-jackfruit-passion fruit (DJP). A total of 19 jiaosu samples (day 0, 7, 14, 21, 28, 42, 56, 70, 84, 90, 120, 150, 180, 210, 240, 270, 300, 330, and 360) were analysed for each group. Using day 90 as a reference point for all three jiaosu groups, fermentation caused significant increases ($p < 0.05$) in phenolics (from 0.17-0.84 to 2.25-5.66 mg GAE/mL), alcohols (from 0% to 4.47-10.47%), malic acid (from 67.36-159.63 to 206.32-403.49 μg/mL), lactic acid (from 67.30-133.41 to 493.19-8353.75

μg/mL), acetic acid (from 61.97-171.84 to 3591.78-6265.55 μg/mL), citric acid (from 32.49-52.67 to 443.73-981.95 μg/mL), and succinic acid (from 433.61-584.63 to 1258.85-2969.13 μg/mL) whereas significant decreases ($p < 0.05$) were observed in pH (from 4.29-5.19 to 3.11-3.24), carbohydrates (from 52.58-105.31 to 1.48-9.49 mg/mL), and amylase activity (from 237.31-2507.97 to 60.35-118.38 μmol/min/μg protein) compared to before fermentation (day 0). Throughout the one-year fermentation period, the pH, the concentrations of proteins, phenolics, carbohydrates, alcohols, and lactic acid, the amylase, protease, and lipase activities were significantly different ($p < 0.05$) between all three groups of jiaosu. Notably, GMP showed the highest total protein and phenolic concentrations and the lowest protease activity ($p < 0.05$) while DJP exhibited the highest lipase activity and lactic acid concentration, and the lowest total alcohol concentration ($p < 0.05$). The results indicated that the biochemical content and enzyme activities of jiaosu could be influenced by fermentation duration and the types of fruit peels used for fermentation.

Keywords: Total phenolic concentration; total alcohol concentration; organic acid; amylase; protease; lipase

Subject Area: QD415-436 Biochemistry

ACKNOWLEDGEMENTS

I would like to express my utmost appreciation and gratitude towards my supervisor, Dr. Sit Nam Weng and my co-supervisor, Dr. Wong Kam Huei for their guidance, trust and assistance throughout the duration of this project. I truly cherish their unwavering support, patience and motivation in helping me to overcome the challenges I faced while conducting this research and when writing the dissertation and journal papers. Moreover, I would like to thank Universiti Tunku Abdul Rahman (UTAR) for providing a conducive working environment with proper facilities and equipment to conduct my research and for funding this project via UTAR Research Fund (UTARRF).

Furthermore, I would like to extend my gratitude and appreciation to the panel of judges during my presentations, Mr. Looi Sing Yan, Ms. Kokila Thiagarajah and laboratory officers especially Mr. Saravanan Sivasangaran and Mr. Nicholas Ooh for their guidance and helpful advice in carrying out this research. I would also like to thank my lab mates in D213A and D213B, who were always willing to lend a helping hand and offer words of encouragement.

Last but not least, I would like to express my sincere gratitude and respect to my parents for their unconditional support and for providing me the opportunity to further my studies.

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LIST OF ABBREVIATIONS

4-NPP	4-nitrophenyl palmitate
BSA	Bovine serum albumin
CTAB	Hexadecyltrimethylammonium bromide
DJP	Durian, jackfruit and passion fruit
DNSA	3,5-dinitrosalicylic acid
FCR	Folin-Ciocalteu's reagent
GAE	Gallic acid equivalent
GMP	Grapefruit, mango and pineapple
HDPE	High-density polyethylene plastic
HPLC	High-performance liquid chromatography
OPW	Orange, papaya and watermelon
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
Tris-HCl	Trisaminomethane -hydrochloric acid
UV	Ultraviolet
UNEP	United Nations Environment Programme

CHAPTER 1

INTRODUCTION

In 2019, the worldwide pandemic of the novel coronavirus disease (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has not only resulted in a severe healthcare crisis but a social and economic crisis globally. Countries have implemented drastic actions to contain the spread of the virus, including several preventive measures, test screening of high-risk individuals, quarantine of patients and close-contact individuals and the enforcement of lockdown and movement restrictions (World Health Organization, 2021).

In Malaysia, the government implemented the nationwide Movement Control Order (MCO) as an initiative to control the spread of the virus. Besides its effect on the business and economic sectors, the stay-at-home order has resulted in an increase of activity at home which led to a significant rise in the percentage of household wastes, predominantly food waste (Ismail, et al., 2020). The 20-30% increase in household wastes have been contributed by food consumption at home and bulk purchasing of food (Das, et al., 2021).

In addition, fruit and vegetable markets, supermarkets, restaurants and food industries produce high amounts of decomposable pre-consumer wastes such as fruits, vegetables and their peels which contain high percentage of organic matter (Arun and Sivashanmugam, 2015a). Organic waste management, treatment and safe disposal have become a major worldwide problem especially in developing countries (Arun and Sivashanmugam, 2015b).

Food waste is extensively known as a global predicament not only in the social and economic aspect but in an environmental one as well. According to the United Nations Environment Programme's (UNEP) 2021 Food Waste Index Report, each year, approximately 931 million tonnes of food are wasted, of which 569 million tonnes are household wastes, followed by 244 and 118 million tonnes of wastes from food service and retail sectors, respectively (United Nations Environment Programme, 2021). When food is wasted, all of the energy and water it took to grow, harvest, transport and package it is wasted and when food ended up in landfills start to rot, methane, which is a greenhouse gas, will be emitted (World Wildlife Fund, 2021). According to the National Solid Waste Management Department Malaysia, landfills are the primary source of methane gas emission, which is more potent than carbon dioxide and contributes to the depletion of the ozone layer. The decomposition of food waste in landfills was highly associated to about 12% of the global methane emissions (Shakil, Azhar, Othman, 2023). Furthermore, food waste management via combustion is an inefficient method due to the high moisture content in food that can cause air pollution (Lytras, et al., 2021).

Generally, food waste can be divided into avoidable and unavoidable waste. Avoidable food wastes are edible food that are wasted instead such as leftovers and expired food whereas unavoidable food wastes are non-edible food such as fruit and vegetable dregs, bones, tea leaves, etc (Schott, et al., 2013). In Malaysia, based on the reported statistic sourced from Solid Waste Management and Public Cleansing Corporation, out of the 17,000 tonnes of daily food waste, 24% are avoidable and 76% are unavoidable food waste (Zainal, 2021). Initiatives to reduce food wastage are primarily focused on the avoidable food waste, however reduction of unavoidable food waste should be given as much attention as the amount is comparatively higher than avoidable food waste and thus, comparatively more detrimental to the environment.

One of the solutions to possibly reduce unavoidable food waste is the recovery of its beneficial compounds and its transformation into other functional bio-products. Previous research has shown that by-products of processed plant materials contain valuable nutrients that have the potential to be developed into new useful ingredients (Oreopoulou and Tzia, 2007). One of the ideas to convert the beneficial components that are found in unavoidable food wastes into valuable bio-products was introduced by Dr. Rosukon Poompanvong who utilised fruit and vegetable wastes to create a multipurpose solution known as garbage enzyme (Ho, Ling and Manaf, 2014).

Garbage enzyme, which is also known as eco-enzyme, bio-enzyme or jiaosu, is a three-month fermented solution containing fruit and/or vegetable wastes, sugar and water. It has been reported to be a multifunctional liquid as it contains components such as proteins, carbohydrates, phenolic compounds, enzymes, organic acids and alcohols (Arun and Sivashanmugam, 2015a). The term jiaosu was used in this research as it best suited its definition.

Previous research done on jiaosu mostly focused on the use of different types fruit and/or vegetable waste to study on the characteristics and effectiveness of the jiaosu that is typically subjected to a fermentation period of three months as per Dr. Rosukon's instructions. However, there were no studies that explained the significance of a fermentation period shorter or longer than three months. Therefore, this research aims to study the significance of various fermentation periods on the jiaosu quality based on the changes of its biochemical contents throughout the fermentation process. The key objective of this research is to quantify the total protein concentration, total carbohydrate concentration, organic acid concentration, total alcohol concentration, total phenolic concentration and amylase, protease and lipase activities during a one-year fermentation period of three different jiaosu groups prepared from different fruit peels.

CHAPTER 2

LITERATURE REVIEW

2.1 Garbage Enzyme and Fermentation

Dr. Rosukon Poompanvong, a researcher who established the Organic Agriculture Association of Thailand, introduced garbage enzyme as a means to transform kitchen waste into a useful product through fermentation process. It is a complex organic solution containing protein chains (enzyme), mineral salts and organic acids that functions correspondingly to enzymes in attaining a high level of degradation within a brief amount of time. Garbage enzyme is produced from fruit and/or vegetable wastes and sugar (jaggery, brown sugar or molasses) mixed with water and fermented for three months in a dark room temperature environment (Arun and Sivashanmugam, 2015a).

As the main ingredient for garbage enzyme production, vegetable and fruit wastes are mostly regarded as the inedible parts of vegetables and fruits that are not useful and often thrown away. However, scientific research has revealed that all parts of plants including flowers, stems, stalks, barks, peels, fruits, leaves, roots and seeds contain bioactive phytochemicals (Khattak and Rahman, 2017). A study conducted by Singh and Immanuel (2014) showed that the fruit peels of

pomegranate contained a high phenolic concentration of 249.41 mg/g. Carota, et al. (2020) used orange peel waste as a liquid medium to produce biodiesel from oleaginous yeasts and the biodiesel yields of 31.9% and 36.9% were obtained from the strains *Papiliotrema laurentii* (formerly *Cryptococcus laurentii*) and *Rhodotorula toruloides* (formerly *Rhodosporidium toruloides*), respectively. Another study reported that fibres from apple, passion fruit and banana peels helped to preserve the viability and promote the growth of *Lacticaseibacillus casei* (formerly *Lactobacillus casei*), *Lactobacillus acidophilus*, *Lacticaseibacillus paracasei* (formerly *Lactobacillus paracasei*) and *Bifidobacterium animalis* subsp. *lactis* in the production of fibre-rich skim yoghurts (Do Espírito Santo, et al., 2012).

Fermentation is a method that involves the utilisation of the growth of microorganisms and its metabolic activities for the conversion and preservation of food materials. Metabolites produced by microorganisms prevent spoilage of food and therefore help to prolong the shelf life of perishable produce. Fermentation allows the formation of characteristic flavour, aroma, texture, nutritional enhancement and helps to remove toxins and anti-nutritional factors in food materials (Terefe and Augustin, 2020). According to the study conducted by Song, et al. (2008), the protein level in unfermented soybean meal increased from 47% to 50.16% when subjected to natural fermentation and increased to 52.08%, 52.14% and 58.08% when fermented with *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*), *Bifidobacterium animalis* (formerly *Bifidobacterium lactis*) and *Saccharomyces cerevisae*, respectively. Soybean meal also has anti-nutritional factors such as phytic acid that has a

strong binding affinity to calcium, magnesium, iron, and zinc which makes these minerals insoluble and unabsorbable in the intestines. Two strains of *Rhizopus oligosporus* used for tempeh fermentation, two strains of *Aspergillus oryzae* used for soy sauce fermentation and six strains of *Aspergillus oryzae* used for miso fermentation, have been reported to secret phytases that hydrolyse phytic acid and therefore, inactivates its anti-nutritional property (Chen, et al., 2013). The advantageous properties of fermentation have appeared to be a heightened interest in different research areas not only in the food and beverage industry but in the agricultural and environmental fields as well and a good example of this is the development of garbage enzyme.

Garbage enzyme is produced based on a 3:1:10 (w/w/w) ratio of fruit and/or vegetable wastes, sugar and water respectively in an air-tight container placed in a dark, room temperature environment and allowed to ferment for three months. The resulting solution is dark brown and has a strong fermented scent (Novianti and Muliarta, 2021). According to Dr. Rosukon, the container should not be filled up to the brim and to allow some space for the gas being produced during the fermentation process. The use of plastic containers is advisable instead of glass for safety reason due to the pressure build-up from the gas produced. During the first month of fermentation the container's cover has to be opened daily to allow trapped gas to be released. After at least three months of fermentation, the garbage enzyme can be filtered and the liquid is ready for use. The remaining solid residues can be dried and used as fertiliser or be added to a new batch of garbage enzyme (Enzymesos, 2015).

2.2 Garbage Enzyme Characteristics

2.2.1 Proteins

Samriti, Sarabhai and Arya (2019) produced a three-month fermented garbage enzyme made from fruit peels (papaya, banana, sapodilla and pomegranate) that showed a higher protein concentration of 4.225 mg/mL as compared to that of control vinegar of 2.575 mg/mL. Different types of jiaosu that fermented for six months, which were watermelon jiaosu, cantaloupe jiaosu, orange jiaosu, watermelon-cantaloupe jiaosu and orange-cantaloupe jiaosu showed total protein concentrations of 8.90, 5.98, 9.46, 6.54 and 7.91 mg/mL, respectively. This study highlighted that different types of fruit peels can have an effect on the total protein content of the jiaosu (Jiang, et al., 2021).

2.2.2 Enzymes

Arun and Sivashanmugam (2015a) reported that garbage enzyme made from tomato, cauliflower, pineapple, orange and mango dregs fermented for three months with molasses and water showed a lipase activity range of 2500 to 3000 U/mL, amylase activity range of 2.5 to 3.0 U/mL and protease activity range of 0.08 to 0.09 U/mL.

The garbage enzyme made from papaya, banana, sapodilla and pomegranate peels was analysed for its enzyme activity using agar plate diffusion method and the zones of clearance were 1.6, 2.35, 1.13 and 1.49 cm for protease, lipase, amylase and papain activities, respectively (Samriti, Sarabhai and Arya, 2019). A study reported a protease activity of 0.129 U/mL and amylase activity of 7.261

U/mL in fermented orange peels, which were comparatively higher than in fermented mixed fruit that showed protease and amylase activities of 0.041 U/mL and 0.615 U/mL, respectively (Chin, et al., 2018).

2.2.3 Phenolic Compounds

Jiang, et al. (2021) reported that watermelon jiaosu, cantaloupe jiaosu, orange jiaosu, watermelon-cantaloupe jiaosu and orange-cantaloupe jiaosu showed total phenolic concentrations of 0.40, 0.32, 0.54, 0.41 and 0.40 mg/mL after fermentation for six months, respectively. The total polyphenol content of mulberry jiaosu increased from 508.19 mg/L to 1734.73 mg/L after one month of fermentation (Zhang, et al., 2023). Rusdianasari, et al. (2021a) reported an increase in total phenolic concentration of orange, pineapple and papaya peels garbage enzyme from 184552 mg/L before fermentation to 762200 mg/L after three months of fermentation.

2.2.4 Carbohydrates

According to a study by Chin, et al. (2018), the carbohydrate content of orange peels, pineapple peels, banana peels and mixed fruit peels (pomelo, watermelon and melon) after three months of fermentation were 37.87, 11.98, 10.60 and 13.10 mg/mL, respectively. Another study reported that the carbohydrate content of garbage enzyme made from papaya, banana, sapodilla and pomegranate peels was higher with a concentration of 14.295 mg/mL compared to that of vinegar which was 11.51 mg/mL (Samriti, Sarabhai and Arya, 2019).

2.2.5 Organic Acids

The study by Zhang, et al. (2023) reported a total organic acid concentration of 14.00 g/L in mulberry jiaosu before fermentation that drastically increased to 39.91 g/L at day 30. Similarly, Arun and Sivashanmugam (2015a) reported that the acetic acid concentration increased drastically from 11.12 g/L at day 15 to 78.14 g/L at day 90 in the garbage enzyme made from cauliflower, tomato, mango, pineapple and orange peels. The study also showed lactic, oxalic, malic and citric acid concentrations of 26.02, 44.81, 11.05 and 39.05 g/L, respectively at day 15 and all decreased to less than 10 g/L at day 90.

2.2.6 Alcohols

Jiang, et al. (2021) reported different alcohol content of six-month fermented jiaosu produced from a single type of fruit and mixed fruits. The results showed that the orange jiaosu had the highest alcohol content of 56.51 μL/mL followed by orange-cantaloupe jiaosu, cantaloupe jiaosu, watermelon-cantaloupe jiaosu and watermelon jiaosu which was 38.92, 29.15, 22.44 and 15.37 μL/mL, respectively. The alcohol content in the garbage enzyme made from banana, papaya, sapodilla and pomegranate peels was 0.18 mL/mL, which was higher than the alcohol content of commercial vinegar, 0.09 mL/mL (Samriti, Sarabhai and Arya, 2019).

2.3 Application of Garbage Enzyme

2.3.1 Natural Fertiliser

Inorganic fertilisers that mostly contain nitrogen, phosphorus and potassium, which are the basic nutrients for plants, will eventually cause negative effects on agricultural soil by draining the nutrients from the soil and also making it infertile (United States Environmental Protection Agency, 2020). Garbage enzyme has been reported to function as an effective organic fertiliser that improve soil quality by increasing the soil organic matter and total nitrogen and thus, serve as a solution to eradicate the negative effects of inorganic fertilisers. Three types of garbage enzyme made from dragon fruit peel, apple peel and eggplant peel were fermented for six months. The filtered liquid was diluted to a ratio of 1:800 and irrigated to soil with organic matter and total nitrogen background of 24.32 g/kg and 1.61 g/kg, respectively. The soil sample's total nitrogen after four weeks of irrigation with dragon fruit peel, apple peel and eggplant peel garbage enzyme gradually increased to 3.17, 4.13 and 4.27 g/kg, respectively. The total organic matter of the soil sample increased to 49.33 g/kg after four weeks of irrigation with eggplant garbage enzyme. This study concluded that fruit and vegetable peels when subjected to fermentation, produce organic acids and various enzymes that potentially increased soil nutrient (Tong and Liu, 2020).

Fadlilla, Budiastuti and Rosariastuti (2023) reported that undiluted eco-enzyme fermented from different types of fruit and vegetable waste for three months contained nitrogen (N), phosphorus (P), potassium (K), carbon (C) and enzymes

which were lipase, trypsin, and amylase. The results concluded that even though the NPK and organic C levels were less than 2% which is below the quality standards levels for liquid organic fertiliser, the eco-enzyme can still be used as an eco-friendly addition to other organic fertilisers to boost nutrient levels in the soil.

In another study, the profitability of organic tomato farming was evaluated by replacing chemical fertiliser with eco-enzyme fertiliser. The results showed that the profits were increased by U$ 114.18/ha (5.3%) and U$ 288.10/ha (13.4%) after using eco-enzyme dosages of 150 and 300 L/ha, respectively on the plants. These eco-enzymes dosages or even higher dosages can increase the profits in tomato farming because it showed a 2:1 ratio of total revenue to total cost (Ardiyanta, et al., 2022).

2.3.2 Natural Pesticide and Insecticide

Based on the report by Zhang, et al. (2020), agricultural jiaosu made from jujube wastes and fermented for three months showed antifungal activity against *Botrytis cinerea* that causes the formation of grey mould in plants, with a half-maximal inhibitory concentration (IC_{50}) of 9.24%. In a qualitative study, garbage enzyme made from orange peels and tap water as the control were applied separately on pieces of bread that were packed in plastic bags for seven days. The results on the eighth day showed a visually moderate fungal growth on the bread that was applied with the garbage enzyme as compared to the control bread that showed more fungal growth (Lakra, Saini and Saini, 2022).

Three jiaosu groups made from orange-papaya-watermelon (OPW), grapefruit-mango-pineapple (GMP), and durian-jackfruit-passion fruit (DJP) exhibited larvicidal activity against *Aedes albopictus* and *Aedes aegypti* larvae after 24h post-treatment with a median lethal concentration (LC_{50}) of 6.52–14.55% v/v and 2.14–5.16% v/v, respectively (Punniamoorthy, et al., 2024).

2.3.3 Plant Growth and Soil Sustainability

Garbage enzyme can enhance photosynthesis in plants and this results in the increased intake of nutrients and water, thus, improving the quality of plant growth (Sethi, et al., 2021). Kajal, et al. (2020) evaluated the effectiveness of a three-month fermented garbage enzyme produced from vegetable and fruit wastes on chick pea seed germination and plant height for a period of four weeks. The soil added with garbage enzyme resulted in a seed germination rate of 100% on the 4^{th} day and plant height from zero to 7.8 cm on the 7^{th} day. The negative control of cow dung fertiliser showed a seed germination rate of 10% on the 4^{th} day and plant height from zero to 3 cm on the 7^{th} day (Kajal, et al., 2020).

Microorganisms in soil assist in the formation of soil nutrients, organic carbon metabolism and pollutant decomposition, which in turn is crucial in improving soil fertility and promoting nutrient absorption in plants. Thus, garbage enzyme, which is rich in various nutrients, microorganisms, bioactive enzymes and secondary metabolites, can enhance soil fertility and environment, promote crop growth and increase crop yield (Zhu, et al., 2020). Kiwifruit garbage enzyme at 1:800 dilution could enhance the growth of castor and had the optimum

inhibition on copper, zinc, cadmium and lead absorption by castor, with a maximum decrease of 21% to 42% (Zhu, et al., 2020).

Another research was done using garbage enzyme produced from watermelon and orange peels fermented for three months to study the potential biocatalytic property and influence on the remediation of soils contaminated by used motor-oil (Bulai, et al., 2021). Results showed a maximum reduction of 54% oil content at 5% (w/w) of oil pollution levels and reduction of 57% grease content at 10% (w/w) of oil pollution levels after six weeks of treatment by orange and watermelon garbage enzymes, respectively. The orange garbage enzyme resulted in 74% and 62% removal of total organic carbon in oil contamination loads of 10% and 5%, respectively. The watermelon garbage enzyme showed an overall total organic carbon removal efficiency of 39% and 45% for the oil contamination levels of 10% and 5%, respectively. This research suggested that watermelon and orange garbage enzyme possess the ability to remove oil from soil and could be applied for biocatalytic rectification of soils contaminated by oil (Bulai, et al., 2021).

2.3.4 Waste Management

Garbage enzyme's degradation function has allowed its utilisation as a low-cost option to improve wastewater treatment processes through the removal of contaminants, harmful sludge and bacteria, which consecutively promotes recycling of waste back into the earth. A study was conducted to assess the ability of 5% to 75% (v/v) of garbage enzyme produced from vegetable and fruit

biomass in decreasing the level of pollutants in domestic wastewater for five days. The results showed that 9% garbage enzyme solution in wastewater was the most economical as it removed ammonia nitrogen concentration from 3 mg/L to zero at the fourth day and removed phosphorus concentration from 4.33 mg/L to zero on the second day, and in neutralising the wastewater from pH 7.6 to pH 7 (Tang and Tong, 2011).

Greywater is one of the major sources of water pollution. A study was conducted to investigate the effectiveness of garbage enzyme made from fruit and vegetable peels as a 30-day treatment method in eradicating ammonia nitrogen and phosphates in synthetic greywater. A 10% solution of the garbage enzyme showed 100% removal of ammonia nitrogen and phosphates which were initially 8 to 10 mg/L and 100 to 120 mg/L, respectively (Fazna and Meera, 2013).

In the treatment and disposal of sludge activated by dairy waste obtained from milk processing, the stability of the sludge is important to aid in the recycling process. Due to the biocatalytic and pathogen-inhibiting property of garbage enzyme, it has the ability to enhance the stability of sludge by removing the solids and suppressing the activity of microbes in the sludge. The garbage enzyme made from tomato, cauliflower, pineapple, orange and mango dregs possessed lipase, protease and amylase activities. It also reduced 38.6% and 37.2% of suspended and total solids, respectively and reduced 99% of pathogens in the sludge activated by dairy waste (Arun and Sivashanmugam, 2015a).

Another study reported the potential use of garbage enzyme as a pre-treatment for aquaculture sludge to minimise the negative impact of the sludge disposal to the environment. A 10% dilution of garbage enzyme made from tomato and orange wastes was able to remove the total suspended solids, volatile suspended solids, total phosphorus, total ammonia nitrogen and chemical oxygen demand by 87%, 67%, 99%, 91% and 77%, respectively. Furthermore, the garbage enzyme made from orange waste showed higher removal percentages compared to tomato garbage enzyme due to the high total organic acid content (Rasit, Lim and Ghani, 2019).

Rasit and Ooi (2018) reported on the use of garbage enzyme as a low-cost pre-treatment for palm oil mill effluent (POME) which is the waste produced from palm oil milling activities. When POME was pre-treated with 10% garbage enzyme made from orange, pineapple, tomato, and mango dregs, there were removals of 70 to 80% of oil and grease, 40 to 50% total suspended solids and 20 to 30% chemical oxygen demand compared to 5% garbage enzyme that reduced oil and grease content, total suspended solids and chemical oxygen demand of POME by 50 to 60%, 30 to 40% and 10 to 20%, respectively.

2.3.5 Detergent and Disinfectant

Gu et al. (2021) studied the potential of garbage enzyme made from apple waste and Chinese honeylocust fruit powder to be used as a detergent. The garbage enzyme showed an amylase activity of 0.33 to 0.36 mg/min·g, cellulose activity of 0.6 to 0.8 μg/h·g and lipase activity of 2 to 3 μ/g. The same garbage enzyme

at a 100-fold dilution showed a removal efficiency of 100% and 15 to 20% on soil and oil stains, respectively and a whitening power of 3.86%. This study suggested that the garbage enzyme can be an effective and eco-friendly alternative to commercial synthetic detergents. The study also reported on the ability of garbage enzyme to detoxify pak-choi treated with dichlorvos and chlorpyrifos pesticides. The results showed that the garbage enzyme diluted to 1:100 had a pesticide residue removal rate of 90 to 100% which was higher than that of a commercial detergent that had a removal rate of 40 to 70%.

Compared to commercial chemical cleaning products, garbage enzyme is a cost-effective and environmentally safer disinfectant (Vama and Cherekar, 2020). A study tested the antibacterial activity of six types of garbage enzyme made from lime, pineapple, pomegranate, papaya, mixed fruits and vegetable peels fermented for 12 weeks. The garbage enzyme produced from vegetable peels only showed antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*, while the garbage enzyme produced from the lime, pineapple, pomegranate, papaya and mixed fruits showed antimicrobial activity against *S. aureus, Bacillus* spp., *Salmonella enterica* serotype Typhi, *E. coli*, *Shigella* spp., and *Pseudomonas aeruginosa* except for the mixed fruit and papaya garbage enzyme which did not show any antimicrobial activity against *S.* Typhi and *S. aureus*, respectively. The study concluded a positive antimicrobial activity with Gram-positive and Gram-negative bacteria and therefore, the garbage enzyme can possibly be used to inhibit bacteria growth in different settings (Neupane and Khadka, 2019).

Another study showed that eco-enzyme prepared from domestic organic waste which are rambutan fruit skin, corn cobs and chayote skin with the addition of 10% frangipani flower (*Plumeria alba*) extract fermented for ten days inhibited *S. aureus* growth with zones of inhibition that ranged from 31.85 to 34.41 mm compared to the positive control amoxicillin that had an average of 23.72 mm of zone of inhibition, therefore the eco-enzyme has a high potential to be used as a natural disinfectant (Rahayu and Situmeang, 2021).

The eco-enzyme made from orange, papaya and pineapple peels fermented for three months possessed a pH value ranging from 4.5 to 5.5 that meets the standard quality requirements of hand sanitiser pH value. Antibacterial activity test showed that the eco-enzyme hand sanitiser with a dilution ratio of 5:40 showed comparatively lesser bacterial growth on nutrient agar medium than commercial hand sanitiser (Rusdianasari et al., 2021b).

Three jiaosu groups made from orange-papaya-watermelon (OPW), grapefruit-mango-pineapple (GMP), and durian-jackfruit-passion fruit (DJP) were tested for its antimicrobial activities. The results of the study showed that the jiaosu samples exhibited antibacterial activity against *Bacillus cereus*, *E. coli, Klebsiella pneumoniae*, *P. aeruginosa* and *S. aureus* at a minimum inhibitory concentration (MIC) values of 25% to 50% v/v. All three jiaosu groups showed antifungal activity against *Cryptococcus neoformans* at a minimum fungicidal

concentration (MFC) of 12.5 to 50% v/v, however only OPW was effective against *Aspergillus fumigatus* (Punniamoorthy, et al., 2024).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

All the chemicals/reagents and instruments/equipment were provided by the Faculty of Science of Universiti Tunku Abdul Rahman (UTAR), Perak. The chemicals and reagents used in this research are shown in Table 3.1 and the list of instruments used are given in Table 3.2. The preparation methods of the reagents are listed in Appendix A.

Table 3.1: List of chemicals and reagents.

Chemicals/reagents	**Brand/Manufacturer**	**Country**
α-amylase from porcine pancreas	Sigma-Aldrich	USA
3,5-dinitrosalicylic acid (DNSA)	Sigma-Aldrich	USA
4-nitrophenyl palmitate (4-NPP)	Sigma-Aldrich	USA
4-nitrophenol	Sigma-Aldrich	USA
Acetic acid	Sigma-Aldrich	USA
Black vinegar	Mother brand	Malaysia

Table 3.1: (continued)

Bradford reagent (Protein Assay CBB solution)	Nacalai Tesque	Japan
Brown sugar	MSM Gula Prai	Malaysia
Bovine serum albumin (BSA)	HiMedia	USA
Calcium acetate hydrate	Merck	USA
Casein from bovine milk	Merck	USA
Citric acid	Sigma-Aldrich	USA
D-glucose	Sigma-Aldrich	USA
Disodium tetraborate	R&M Chemicals	India
Ethanol	Sigma-Aldrich	USA
Folin-Ciocalteu's phenol reagent	Merck	USA
Gallic acid	Biobasic	Canada
Glacial acetic acid	R&M Chemicals	India
Hexadecyltrimethylammonium bromide (CTAB)	Nacalai Tesque	Japan
Lactic acid	Sigma-Aldrich	USA
Lipase from porcine pancreas	Sigma-Aldrich	USA
Malic acid	Sigma-Aldrich	USA

Table 3.1: (continued)

Maltose	R&M Chemicals	India
Oxalic acid	Sigma-Aldrich	USA
Phenol crystals	Bendosen	Malaysia
Protease from bovine pancreas	Sigma-Aldrich	USA
Potassium permanganate	Bendosen	Malaysia
Potassium sodium tartrate tetrahydrate	Merck	USA
Sodium acetate trihydrate	Bendosen	Malaysia
Sodium carbonate	Bendosen	Malaysia
Sodium dihydrogen phosphate monohydrate (NaH_2PO_4)	Merck	USA
Sodium dodecyl sulfate (SDS)	Fisher Scientific	USA
Sodium hydroxide	Merck	USA
Sodium hydrogen phosphate (Na_2HPO_4)	Merck	USA
Sodium sulfite	Merck	USA
Starch	Systerm	Malaysia
Succinic acid	Sigma-Aldrich	USA
Sulphuric acid	R&M Chemicals	India
Tartaric acid	Sigma-Aldrich	USA
Trichloroacetic acid	Fisher Scientific	USA
Tris-base	Fisher Scientific	USA
Tris-hydrochloric (HCl) acid	Biobasic	Canada
Triton X-100	Fisher Scientific	USA
Tyrosine	Biobasic	Canada
White vinegar	Lee Kum Kee	China

Table 3.2: List of apparatus and equipment.

Apparatus/Equipment	Manufacturer	Country
C18 column	Merck, Purospher STAR RP-18 endcapped 5 µm (column length and internal diameter: 25 cm × 4.6 mm; particle size: 5 µm)	Darmstadt, Germany
High-performance liquid chromatography (HPLC)	Agilent 1100 LC	California, USA
LogTag® UTRIX-16 data logger.	LogTag North America Inc.	New Jersey, USA
Microcentrifuge	Beckman Coulter, microfuge 16 centrifuge	California, USA
Microplate (non-treated flat-bottom 96-well plates)	NEST	Jiangsu, China
pH meter	Ohaus Aquasearcher™ AB23PH bench meter	Nänikon, Switzerland
Spectrophotometric microplate reader	BMG Labtech FLUOstar Omega	Ortenberg, Germany
Water bath	Memmert	Schwabach, Germany
Weighing balance	Ohaus V11P30 Valor 1000 scale	Nänikon, Switzerland

3.2 Collection of Fruit Peels

The types of fruit peels used in this research were randomly selected based on their availability during this study. The fruit peels were sourced from households, fresh fruit sellers and fruit juice shops in Ipoh, Batu Gajah and Tanjung Tualang, Perak. Nine different types of fruit peels were used, which were durian, grapefruit, jackfruit, mango, orange, papaya, passion fruit, pineapple and watermelon as shown in Figure 3.1.

3.3 Experimental Design

The jiaosu samples were prepared in three groups and each group was prepared in triplicate, therefore a total of nine containers of jiaosu were prepared. Each group contained three different types of fruit peels. The groups were named based on the first initial of the fruit peels it contained in alphabetical order and its triplicates were labelled as A, B and C. The names of the jiaosu sample groups were as follows: group 1: orange, papaya and watermelon (OPW), group 2: grapefruit, mango and pineapple (GMP) and group 3: durian, jackfruit and passion fruit (DJP).

3.4 Jiaosu Sample Preparation

The raw materials were weighed using a weighing balance (Figure 3.1) and mixed based on a 3:1:10 (w/w/w) ratio (Enzymesos, 2015), 6 kg of three different fruit peels (2 kg each fruit peel) were combined with 2 kg of brown sugar and 20 L of deionised water in an airtight 30 L high-density polyethylene

plastic barrel. The barrels were placed in a dark, dry, room temperature environment and allowed to ferment naturally for one year.

Figure 3.1: Collected fruit peels. (a): orange, papaya and watermelon (OPW), (b): grapefruit, mango and pineapple, (c): durian, jackfruit and passion fruit (DJP).

3.5 Jiaosu Sampling

Samplings were taken before fermentation (day 0), weekly during the first month of fermentation (day 7, 14, 21 and 28), bi-weekly during the second and third months of fermentation (day 42, 56, 70 and 84), day 90 (three months of fermentation), and monthly until one year of fermentation period. Figure 3.2

shows an example of OPW jiaosu sample. During each sampling, approximately 120 mL of sample were collected in four separate 50 mL centrifuge tubes using a sterile serological pipette and stored in a -20 ℃ freezer. The samples collected from each time point were centrifuged at 10,000 rpm (6932 x *g*) at room temperature for 15 minutes and the supernatant was used for analysis in triplicates.

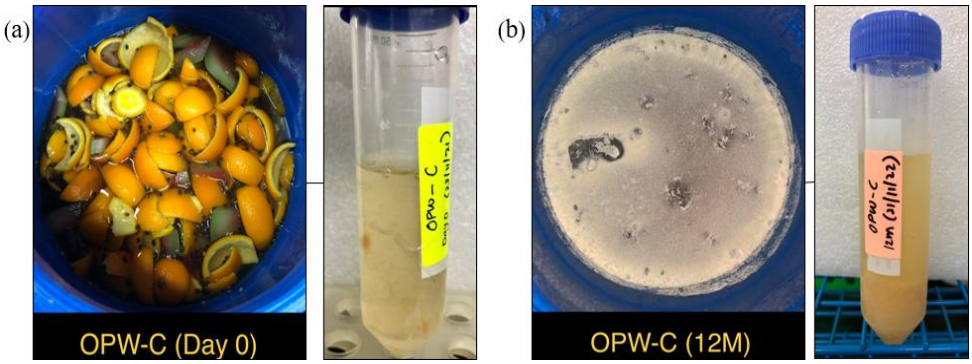

Figure 3.2: Orange, papaya and watermelon (OPW) jiaosu in a plastic barrel and approximately 30 mL sample collected in a centrifuge tube at (a) day 0 and (b) 12th month.

3.6 pH Measurement

The pH of each sample collected was measured using a calibrated pH meter.

3.7 Total Protein Concentration

The total protein concentration was measured using a linearised Bradford protein assay (Ernst and Zor, 2010) with bovine serum albumin (BSA) (1.25, 2.50, 5.00, 10.00, 15.00 and 25.00 μg/mL) as the standard to construct a calibration curve. The standard solutions, samples, 100 % white vinegar and 100 % black vinegar as the positive controls of 100 μL each were added with an equal volume of Bradford reagent in a microplate which was then covered with aluminium foil and incubated for 5 minutes at room temperature. The negative control was 200 μL of deionised water. After incubation, the absorbance was read at 450 nm and 590 nm using a spectrophotometric microplate reader. The calibration curve was plotted using the absorbance ratio (590 nm/450 nm) vs BSA concentration (μg/mL).

3.8 Biocatalytic Characterisation of Jiaosu

3.8.1 Amylase Activity

The amylase activity was measured using the 3,5-dinitrosalicylic acid (DNSA) method (Bezerra et al., 2006). To construct a calibration curve, 1 mL of standard solutions of maltose ranging from 100 to 1000 μg/mL prepared from a stock solution of 1 mg/mL was added with 1 mL of DNSA reagent, incubated for 10 minutes in a 95°C-water bath and left to cool at room temperature for 5 minutes. The solutions were transferred to a microplate and the absorbance was read at 570 nm using the spectrophotometric microplate reader.

To estimate the total amylase activity of the samples, 1% starch solution in 0.1 M phosphate buffer (pH 6.6), was prepared. In separate glass test tubes, 250 µL of the centrifuged sample, deionised water as the negative control or α-amylase (1 mg/mL) as the positive control were added with 2.5 mL of the 1% starch solution, the mixtures were incubated for 10 minutes in a 37℃-water bath. Next, 1 mL of DNSA reagent was added to the mixtures and incubated for 10 minutes in a 95℃-water bath. After the solutions cooled down, it was transferred to a microplate and the absorbance was read at 570 nm using the spectrophotometric microplate reader. One unit of enzymatic activity is defined as the amount of enzyme that produces 1 µmol/min of maltose. The enzyme activity was calculated and expressed as units per micrograms of protein.

$$\text{Enzyme activity (µmol/min/mL)} = \left(\frac{\text{µg of maltose released} \times 1000}{\text{Molecular weight of maltose (342.3 g/mol) x Incubation time}}\right) \times \left(\frac{1\ \text{mL}}{\text{sample volume (mL)}}\right)$$

$$\text{Specific enzyme activity (µmol/min/µg of protein)} = \frac{\text{µmol/min enzyme activity per mL}}{\text{µg/mL of protein}}$$

3.8.2 Protease Activity

The protease activity was estimated by using a non-specific protease assay that employed casein as the substrate and tyrosine as the standard (Cupp-Enyard, 2008). Firstly, 0.65% casein solution and enzyme diluent of pH 7.5 was prepared as described in Appendix A. To construct the tyrosine calibration curve, a stock solution of the standard at 0.2 mg/mL was diluted to concentrations ranging from 20 to 100 μg/mL. In glass test tubes, each of the standard solutions was added with 5 mL of 0.5 M sodium carbonate and 1 mL of 0.5 M Folin-Ciocalteu's phenol reagent. The solutions were mixed by swirling and filtered using a 0.45 μm nylon syringe filter to remove insoluble particles. The solutions were then transferred to a microplate and the absorbance was measured at 660 nm using a spectrophotometric microplate reader.

For the enzymatic assay, an enzyme solution was first prepared by adding 0.5 mL of the enzyme diluent to separate glass test tube containing 0.5 mL centrifuged sample, deionised water as the negative control and protease (1 mg/mL) as the positive control. Next, 5 mL of 0.65% casein was added into each of the glass test tubes and incubated in a 37°C-water bath for 5 minutes. Next, 0.5 mL of enzyme diluent was added to each of the test tubes and incubated in the 37°C-water bath once again for 10 minutes. After the incubation, another 0.5 mL enzyme diluent was added, followed by 5 mL of 0.11 M trichloroacetic acid solution and incubated in the 37°C-water bath for 30 minutes. The solutions were filtered using a 0.45 μm nylon syringe filter after incubation, added with 5 mL of 0.5 M sodium carbonate and 1 mL of 0.5 M Folin-Ciocalteu's phenol reagent.

The solutions were then transferred to a microplate and the absorbance was measured at 660 nm using a spectrophotometric microplate reader. One unit of enzymatic activity is defined as the amount of enzyme that produces 1 µmol/min of tyrosine. The amount of tyrosine released from the reaction was determined using the calibration curve and the enzyme activity was calculated using the formula below and expressed as units per micrograms of protein.

$$\text{Enzyme activity (µmol/min/mL)} =$$

$$\left(\frac{\text{µg of tyrosine released} \times 1000}{\text{Molecular weight of tyrosine (181.19 g/mol) x Incubation time}}\right) \times \left(\frac{1\text{ mL}}{\text{sample volume mL}}\right)$$

$$\text{Specific enzyme activity (µmol/min/µg of protein)} = \frac{\text{µmol/min enzyme activity per mL}}{\text{µg/mL of protein}}$$

3.8.3 Lipase Activity

The lipase activity was determined based on a spectrophotometric assay in which 4-nitrophenol was used as the standard and 4-nitrophenyl palmitate (4-NPP) as the substrate (Ha et al., 2021). Firstly, 5 mM substrate solution (A) and (B), 3 mM substrate solution (A) and (B) and Tris-hydrochloric (HCl) buffer (pH 7) were prepared as described in Appendix A.

The standard curve was established by using 4-nitrophenol solution with concentrations ranging from 5.00 to 50.00 μg/mL. A volume of 100 μL of 3 mM substrate solution (A) was added into each well of a microplate and pre-incubated in the microplate reader at 30°C for 5 minutes. After that, 100 μL of the prepared standards were added and the absorbance was measured. The blank was 100 μL of deionised water.

For the enzymatic assay, 100 μL of 3 mM substrate solution (B) was added into each well of a microplate and pre-incubated in the microplate reader at 30°C for 5 minutes. After that, 100 μL of the centrifuged samples were added into the wells and the absorbance was measured at 410 nm every 5 minutes for 1 hour. The negative control was 100 μL of 3 mM substrate (B) solution mixed with 100 μL of deionised water and the positive control was 100 μL of lipase (1 mg/mL) mixed with 100 μL of deionised water. The highest sample absorbance was used to determine the 4-nitrophenol concentration from the calibration curve and enzyme activity expressed in μmol/min/μg of protein was calculated based on the formula below. The amount of lipase liberating 1 μmol of 4-nitrophenol per minute represents one unit of lipase activity.

$$\text{Enzyme activity } (\mu\text{mol/min/mL}) =$$

$$\left(\frac{\mu\text{g of 4-nitrophenol released} \times 1000}{\text{Molecular weight of 4-nitrophenol (139.11 g/mol) x Incubation time}}\right) \times \left(\frac{1\text{ mL}}{\text{sample volume (mL)}}\right)$$

$$\text{Specific enzyme activity } (\mu\text{mol/min/}\mu\text{g of protein})$$

$$= \frac{\mu\text{mol/min enzyme activity per mL}}{\mu\text{g/mL of protein}}$$

3.9 Total Phenolic Concentration

The total phenolic concentration was quantified using the Folin-Ciocalteu method (Herald et al., 2012). A standard stock solution of 2 mg/mL gallic acid was prepared and serially diluted to 15.63, 31.25. 62.50, 125, 250, 500 and 750 µg/mL to construct a calibration curve. In a microplate, each well was added with 75 µL of deionised water, followed by 25 µL of either centrifuged sample, standard, deionised water (negative control) or undiluted white vinegar and black vinegar as the positive controls, and 25 µL of 50% Folin-Ciocalteu's phenol reagent. The solutions were mixed and incubated for 6 minutes at room temperature. Then, 100 µL of 75 g/L of sodium carbonate solution was added to each well, mixed and the microplate was covered and incubated in the dark for 90 minutes at room temperature. After incubation, the absorbance was read at 765 nm using the spectrophotometric microplate reader. The total phenolic concentration of each sample was expressed as mg gallic acid equivalent (GAE)/mL sample using the formula below, where A is the concentration of

gallic acid based on the calibration curve in μg/mL, V is the total volume of the reaction medium used in the assay and Vs is the sample volume.

$$\text{Total phenolic concentration (mg GAE/mL)} = \left(\frac{\text{A (μg/mL)}}{1000}\right) \times \left(\frac{\text{V}}{\text{Vs}}\right)$$

3.10 Total Carbohydrate Concentration

The total carbohydrate concentration was analysed by using a microplate phenol–sulfuric acid method as modified and optimised by Masuko et al. (2005). Glucose stock solution at a concentration of 0.2 mg/mL was used as the standard to construct a calibration curve with concentrations ranging from 200 to 1000 μg/mL.

For the standard and sample analysis, 100 μL of standards or centrifuged samples were pipetted into microcentrifuge tubes and mixed with 200 μL of 96% sulphuric acid, followed by immediate addition of 80 μL of 5% phenol solution. The standards or samples were incubated for 5 minutes in a 90°C-water bath and cooled at room temperature for 5 minutes. Lastly, 100 μL of the standards or samples were then transferred to a microplate and the absorbance was measured at 490 nm using a microplate reader.

3.11 Organic Acid Analysis

The organic acid concentration was analysed by high-performance liquid chromatography (HPLC) coupled with an ultraviolet detector (Model 1100, Agilent Technologies Inc., CA, USA) set to 210 nm (Lee et al., 2012). A C18 column (Purospher STAR RP-18, Merck KGaA, Darmstadt, Germany) with an internal diameter of 4.6 mm, length of 150 mm and particle size of 5 μm was used for the organic acid separation at 30°C. The mobile phase of 20 mM sodium dihydrogen phosphate (NaH_2PO_4) at pH 2.7 was used at a flow rate of 1.0 mL/min (Lee et al., 2012). The prepared mobile phase was filtered using 0.45 μm nylon filter membrane before use.

The peak areas of standard solutions of tartaric, malic, lactic, acetic, citric, and succinic acids from 100 to 1000 μg/mL and oxalic acid from 20 to 100 μg/mL were used to construct the respective calibration curves. The standards were dissolved using the mobile phase and filtered using 0.22 μm nylon syringe filters before use. The samples were centrifuged at 10,000 rpm for 15 minutes, the supernatant was filtered using a 0.22 μm nylon syringe filter and diluted 1:1 with the mobile phase. A mixture of deionised water and mobile phase in the ratio of 1:1 was used as the blank. The organic acid concentrations in the jiaosu samples were expressed in μg/mL.

3.12 Total Alcohol Concentration

The total alcohol concentration was determined using a spectrophotometric method using ethanol and glucose as the standards (Zhang et al., 2019). The samples were pre-treated with an equal volume of 20% trichloroacetic acid solution and centrifuged at 10,000 rpm for 5 minutes. The supernatant was filtered with nylon syringe filters (0.22 μm) and then added with 20% hexadecyltrimethylammonium bromide (CTAB). The mixture was incubated at 65℃ for 10 minutes and centrifuged again at 10,000 rpm for 10 minutes. The resulting supernatant was used for the 3,5-dinitrosalicylic acid (DNSA) assay and potassium permanganate assay.

For the DNSA assay, 20 μL of the pretreated samples were diluted with 180 μL of deionised water. To construct a calibration curve, glucose with concentrations ranging from 0.156 to 2.500 mg/mL was used as the standard. In a microcentrifuge tube, 200 μL of sample or standard solutions were added with 600 μL DNSA reagent and incubated in a 95℃-water bath for 5 minutes. After cooling at room temperature for 5 minutes, the mixtures were transferred into a microplate and the absorbance was measured at 550 nm using the microplate reader.

For the potassium permanganate assay, 2 μL of the pretreated sample was diluted with 198 μL of deionised water. Ethanol and glucose were used as the standards to construct two calibration curves ranging from 0.039 to 0.625 μL/mL and 0.016 to 0.250 mg/mL, respectively. In a microcentrifuge tube, 200 μL of potassium permanganate solution was added to 200 μL of the sample or the standards

(glucose and ethanol) and incubated for 90 minutes in a 40℃-water bath. After 5 minutes of cooling at room temperature for, the mixture was then transferred into a microplate for absorbance measurement at 526 nm. After subtracting the portion of absorbance increase contributed by reducing sugars from the DNSA assay, the remaining absorbance decrease was used to calculate the total alcohol concentration in the jiaosu samples. The total alcohol concentration of the jiaosu samples was expressed in percentage (%).

3.13 Data Analysis

All data were presented in mean ± standard deviation of three replicates. Due to software limitations, two sets of data representing the first three months of fermentation (day 0, 28, 56, and 84) and from the 3rd month to one year (day 84, 6th month, 9th month, and 12th month), respectively were examined for statistical significance ($p < 0.05$) by multivariate analysis of variance (MANOVA) with repeated measures using JMP statistical software version 16.2 (JMP Statistical Discovery LLC, Cary, North Carolina, USA). Wilk's Lambda test was used as the multivariate test and the Greenhouse-Geisser Epsilon (G-G) estimates of sphericity were applied when Mauchly's test indicated that the assumption of sphericity had been violated ($p < 0.05$).

CHAPTER 4

RESULTS

4.1 pH of Jiaosu Samples

Figure 4.1 shows the pH values of OPW, GMP and DJP that drastically decreased from a range of 4.29 ± 0.06 to 5.19 ± 0.11 at day 0 to 3.05 ± 0.01 to 3.43 ± 0.03 after a week of fermentation. However, the pH decreased to the lowest of 2.60 ± 0.02 and 2.82 ± 0.04 at the 8^{th} month for OPW and GMP, respectively. For DJP, the lowest pH value of 2.61 ± 0.01 was noted at the 7^{th} month. Slight increase was noted for all three jiaosu groups from the 10^{th} to 12^{th} month with a pH range of 3.24 ± 0.02 to 3.39 ± 0.06 at the end of the one-year fermentation period. Among the three jiaosu groups, GMP had significantly lower pH ($p < 0.05$) values throughout the one-year fermentation process.

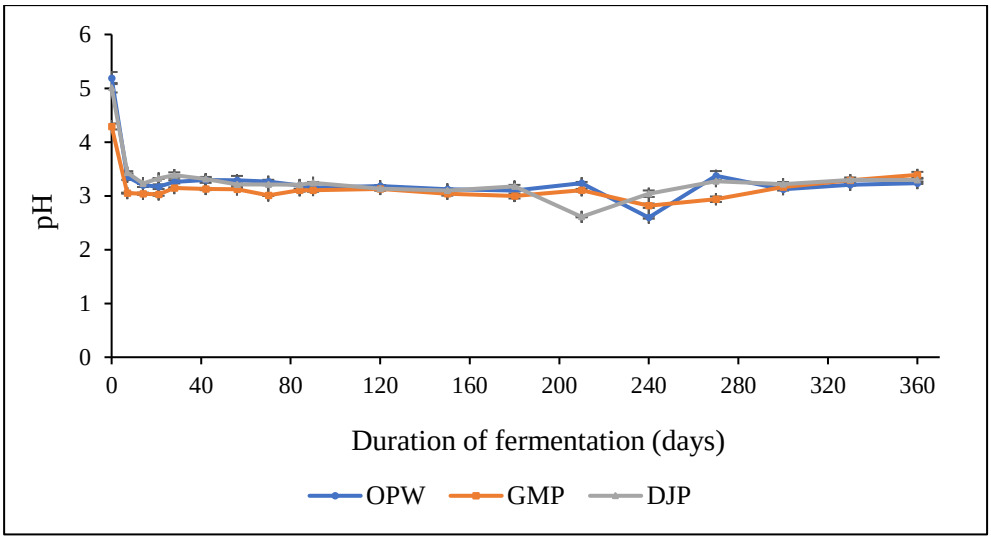

Figure 4.1: pH of orange, papaya and watermelon (OPW), grapefruit, mango and pineapple (GMP) and durian, jackfruit and passion fruit (DJP) jiaosu from day 0 to 12th month.

4.2 Total Protein Concentration

The total protein concentration of OPW decreased from 5.00 ± 0.47 µg/mL at day 0 to 2.97 ± 0.35 µg/mL at day 90. For GMP and DJP, the lowest total protein concentration was noted at day 0 with a value of 3.13 ± 0.38 µg/mL and 1.20 ± 0.12 µg/mL, respectively. The total protein concentration gradually increased to 5.76 ± 0.29 µg/mL for OPW, 19.88 ± 0.75 µg/mL for GMP and 3.17 ± 0.21 µg/mL for DJP at the 6th month. The total protein concentrations for OPW, GMP and DJP decreased to 2.98 ± 0.36, 9.57 ± 0.48 and 1.72 ± 0.20 µg/mL, respectively at the 12th month. Statistically, there was a notable difference in the total protein concentration ($p < 0.05$) between all three jiaosu groups at the different time points of fermentation (Figure 4.2).

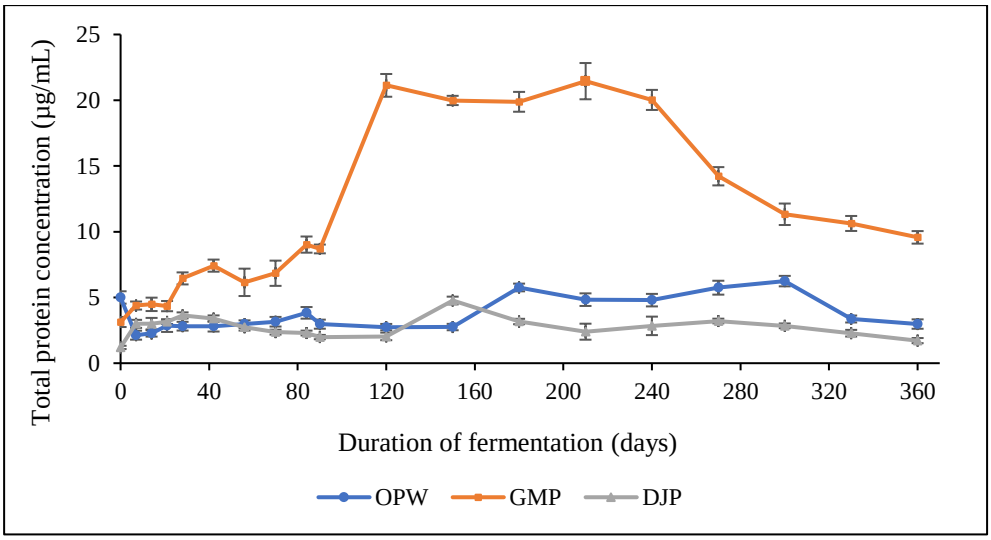

Figure 4.2: Total protein concentration (µg/mL) of orange, papaya and watermelon (OPW), grapefruit, mango and pineapple (GMP) and durian, jackfruit and passion fruit (DJP) jiaosu from day 0 to 12th month.

4.3 Biocatalytic Activity

4.3.1 Amylase Activity

For OPW, there was a sharp increase of amylase activity from 2507.97 ± 15.32 µmol/min/µg of protein at day 0 to the highest activity of 5725.36 ± 73.84 µmol/min/µg of protein at day 7. At day 0, the amylase activity was the highest at 1209.24 ± 25.45 µmol/min/µg of protein for GMP and 237.31 ± 10.47 µmol/min/µg of protein for DJP. As shown in Figure 4.3, the amylase activities of GMP and DJP declined whereas in OPW it increased after one week of fermentation. At day 90, the amylase activities of OPW, GMP and DJP were 60.35 ± 0.91, 118.38 ± 1.69 and 65.10 ± 5.37 µmol/min/µg of protein, respectively. At the 12th month, the amylase activities of OPW and GMP decreased to 43.25 ± 3.08 µmol/min/µg of protein and 18.34 ± 2.79 µmol/min/µg of protein, respectively. However, the amylase activity of DJP increased to

124.05 ± 25.84 µmol/min/µg of protein at the 12th month. Statistically, there was a notable difference in the amylase activity ($p < 0.05$) among all three jiaosu groups throughout the one-year fermentation process.

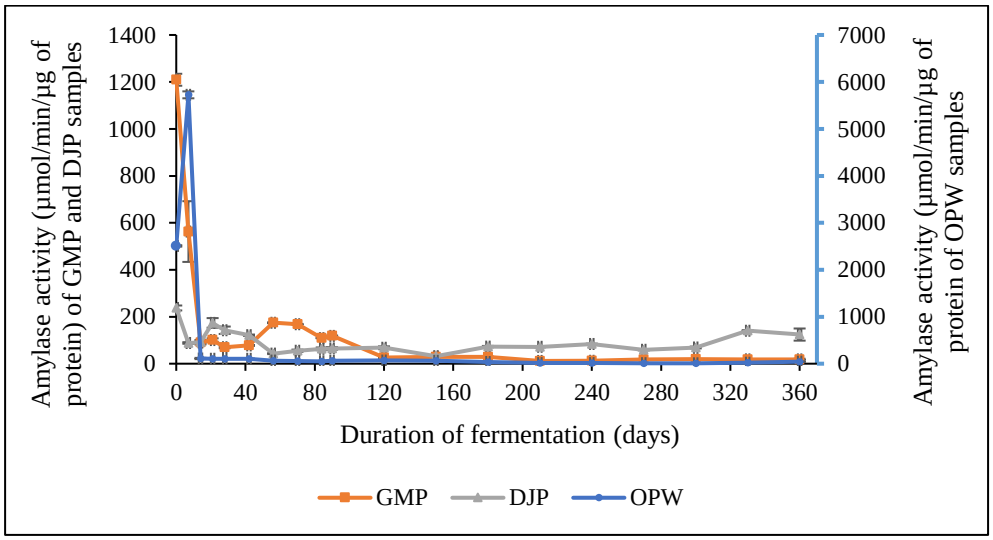

Figure 4.3: Amylase activity (µmol/min/µg of protein) of orange, papaya and watermelon (OPW), grapefruit, mango and pineapple (GMP) and durian, jackfruit and passion fruit (DJP) jiaosu from day 0 to 12th month.

4.3.2 Protease Activity

During fermentation, the protease activity of OPW increased, whereas the protease activities of GMP and DJP decreased. As shown in Figure 4.4, the protease activity of OPW increased from 3.70 ± 0.20 µmol/min/µg of protein at day 0 to the highest of 9.42 ± 0.29 µmol/min/µg of protein after one week of fermentation. The protease activities of GMP and DJP were the highest before fermentation (day 0) with 4.26 ± 0.23 µmol/min/µg of protein and 10.29 ± 0.44 µmol/min/µg of protein, respectively. At the 12th month of fermentation, the protease activities of the three jiaosu groups decreased to 5.55 ± 0.22, 1.51 ±

0.07 and 6.73 ± 0.12 µmol/min/µg of protein for OPW, GMP and DJP, respectively. There was a statistically notable difference in the protease activity ($p < 0.05$) between all three jiaosu groups throughout the one-year fermentation process as shown in Figure 4.4.

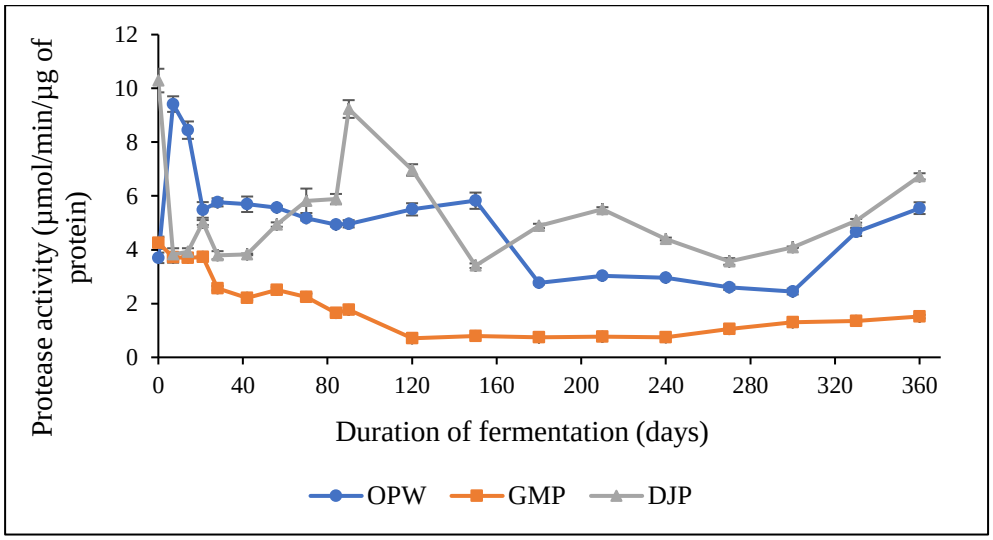

Figure 4.4: Protease activity (µmol/min/µg of protein) of orange, papaya and watermelon (OPW), grapefruit, mango and pineapple (GMP) and durian, jackfruit and passion fruit (DJP) jiaosu from day 0 to 12th month.

4.3.3 Lipase Activity

The lipase activity increased for OPW and DJP, but decreased in GMP. For OPW, the lipase activity was at its lowest of 1.89 ± 0.96 µmol/min/µg of protein at day 0 and gradually increased to the highest activity of 7.47 ± 3.02 µmol/min/µg of protein the 12th month. The lipase activity for GMP was the highest before fermentation (day 0) at 5.49 ± 1.16 µmol/min/µg of protein that decreased to 1.83 ± 0.50 µmol/min/µg of protein at the 3rd month. At the end of fermentation (12th month), the lipase activity of GMP slightly increased to 2.31 ± 1.73

μmol/min/μg of protein. For DJP, the lipase activity increased from 17.86 ± 3.48 μmol/min/μg of protein at day 0 to the highest activity of 27.33 ± 6.39 μmol/min/μg of protein at the 6th month. At the 12th month, the lipase activity of DJP decreased to 10.81 ± 0.80 μmol/min/μg of protein. As shown in Figure 4.5, there was a statistically notable difference in the lipase activity ($p < 0.05$) of DJP as compared to OPW and GMP throughout the one-year fermentation process.

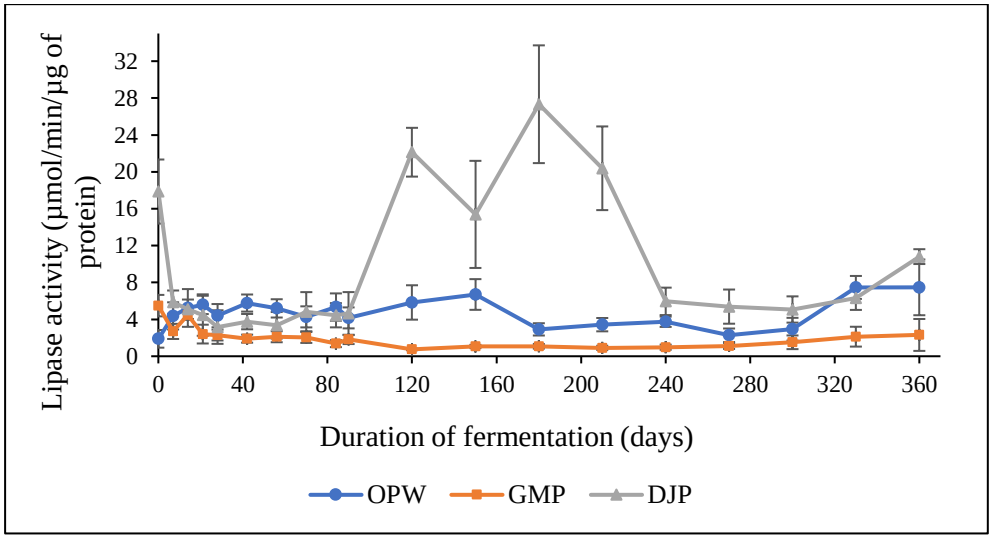

Figure 4.5: Lipase activity (μmol/min/μg of protein) of orange, papaya and watermelon (OPW), grapefruit, mango and pineapple (GMP) and durian, jackfruit and passion fruit (DJP) jiaosu from day 0 to 12th month.

4.4 Total Phenolic Concentration

The total phenolic concentrations of OPW, GMP and DJP were the lowest at day 0 at 0.60 ± 0.04, 0.84 ± 0.02 and 0.17 ± 0.00 mg GAE/mL, respectively. The total phenolic concentration then drastically increased to 3.20 ± 0.12 mg GAE/mL for OPW, 5.66 ± 0.09 mg GAE/mL for GMP and 2.25 ± 0.05 mg GAE/mL for DJP at day 90 (Figure 4.6). After that, the total phenolic concentrations of OPW, GMP and DJP declined to 1.85 ± 0.05, 4.36 ± 0.05 and 1.82 ± 0.01 mg GAE/mL, respectively at the 12th month. There was a statistically notable difference in the total phenolic concentration ($p < 0.05$) between all three jiaosu groups at the different time points of fermentation.

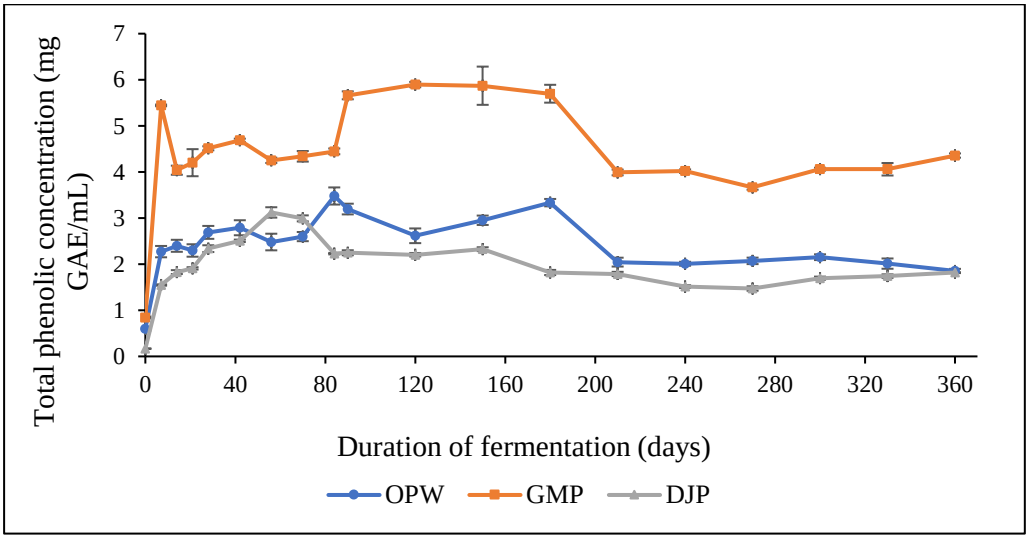

Figure 4.6: Total phenolic concentration (mg GAE/mL) of orange, papaya and watermelon (OPW), grapefruit, mango and pineapple (GMP) and durian, jackfruit and passion fruit (DJP) jiaosu from day 0 to 12th month.

4.5 Total Carbohydrate Concentration

The total carbohydrate concentrations for OPW, GMP and DJP were the highest before fermentation (day 0) with 104.98 ± 0.71, 105.31 ± 2.46 and 52.58 ± 1.43 mg/mL, respectively. At day 90, the total carbohydrate concentration declined drastically to 1.48 ± 0.04 mg/mL for OPW, 9.49 ± 0.62 mg/mL for GMP and 2.18 ± 0.04 mg/mL for DJP (Figure 4.7). At the 12th month, OPW, GMP and DJP had a total carbohydrate concentration of 2.42 ± 0.04, 3.09 ± 0.16 and 3.04 ± 0.12 mg/mL, respectively. Statistically, there was a notable difference in the total carbohydrate concentration ($p < 0.05$) between all three jiaosu groups throughout the one-year fermentation period.

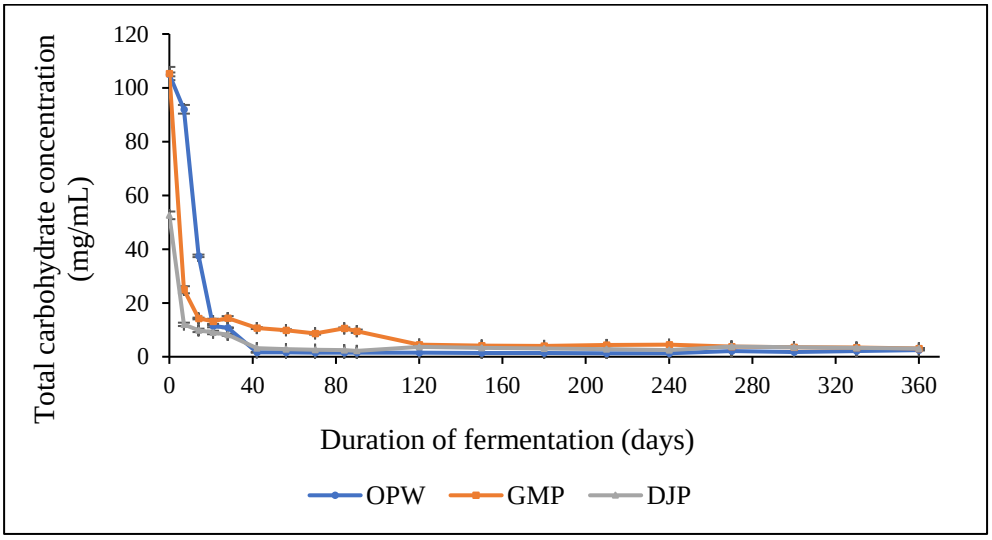

Figure 4.7: Total carbohydrate concentration (mg/mL) of orange, papaya and watermelon (OPW), grapefruit, mango and pineapple (GMP) and durian, jackfruit and passion fruit (DJP) jiaosu from day 0 to 12th month.

4.6 Organic Acid Analysis

The concentrations of seven types of organic acid in OPW, GMP and DJP were analysed using high-performance liquid chromatography (HPLC). The ascending order of retention times of the organic acids were 1.94, 2.25, 3.02, 3.82, 4.06, 5.52 and 6.94 minutes for oxalic acid, tartaric acid, malic acid, lactic acid, acetic acid, citric acid and succinic acid, respectively. Acetic acid was the most abundant in OPW and GMP, whereas lactic acid was the most abundant in DJP. Oxalic acid was the least abundant in all three jiaosu groups. Examples of the chromatograms from the HPLC analysis for OPW, GMP and DJP at day 0, day 90 and the 12th month are shown in Appendix C.

4.6.1 Oxalic Acid

Based on Figure 4.8, the oxalic acid concentration for OPW increased from 44.52 ± 0.33 μg/mL at day 0 to its highest concentration of 73.75 ± 0.46 μg/mL at day 7. Thereafter, the oxalic acid concentration in OPW declined to its lowest of 15.55 ± 0.33 μg/mL at the 12th month. In GMP, the initial oxalic acid concentration of 12.88 ± 0.38 μg/mL at day 0 increased to a concentration of 36.21 ± 0.22 μg/mL at day 90. At the 12th month, the oxalic acid concentration of GMP decreased sharply to 7.09 ± 0.24 μg/mL. For DJP, the oxalic acid concentration was the lowest before fermentation (day 0) at 5.85 ± 0.31 μg/mL and gradually increased to the highest concentration of 38.11 ± 0.46 μg/mL at day 90. At the 12th month, the oxalic acid concentration of DJP decreased to 18.46 ± 0.24 μg/mL. It was also noted that the oxalic acid concentration of OPW and GMP at the 12th month was lower than it was at day 0, whereas the oxalic

acid concentration of DJP at the 12th month was higher than it was at day 0. Statistically, there was no notable difference in the oxalic acid concentration ($p > 0.05$) among the three jiaosu groups throughout the one-year fermentation process.

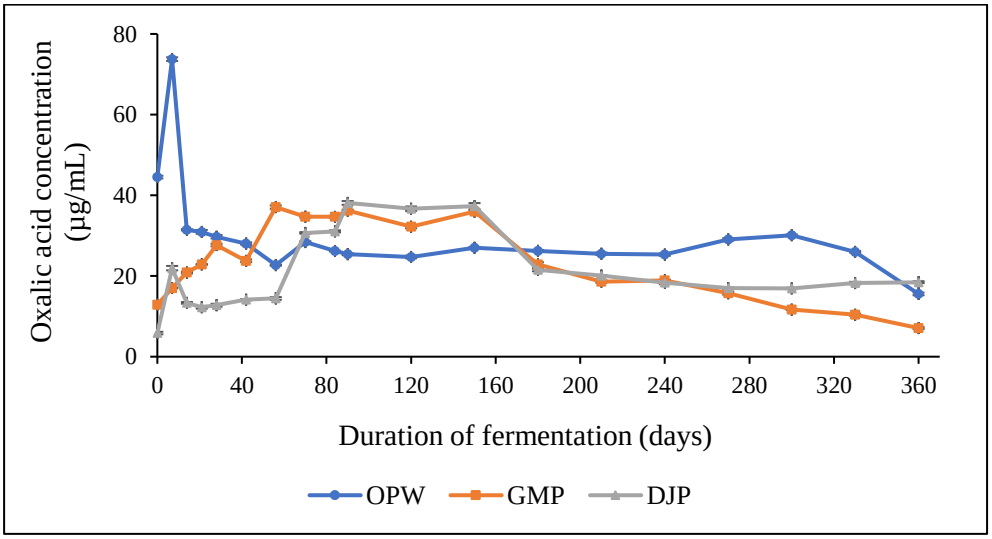

Figure 4.8: Oxalic acid concentration (µg/mL) of orange, papaya and watermelon (OPW), grapefruit, mango and pineapple (GMP) and durian, jackfruit and passion fruit (DJP) jiaosu from day 0 to 12th month.

4.6.2 Tartaric Acid

The tartaric acid concentration of OPW was at its highest of 2100.63 ± 11.02 µg/mL before fermentation (day 0). It then drastically decreased to a concentration of 174.17 ± 2.70 µg/mL at day 90. At the 12th month, the tartaric acid concentration of OPW increased to 220.60 ± 1.39 µg/mL. For GMP, the tartaric acid concentration of 189.83 ± 3.08 µg/mL at day 0 gradually increased to 499.08 ± 11.10 µg/mL at day 90, followed by a decrease to its lowest concentration of 97.77 ± 2.27 µg/mL at the 12th month. For DJP, the lowest

tartaric acid concentration of 38.11 ± 2.20 µg/mL at day 0, increased to the highest concentration of 692.83 ± 5.86 µg/mL at day 90, which then decreased to 334.18 ± 1.87 µg/mL at the 12th month of fermentation. Overall, fermentation decreased the tartaric acid concentration in OPW but increased in GMP and DJP as shown in Figure 4.9. Statistically, there was no notable difference in the tartaric acid concentration among the three jiaosu groups ($p > 0.05$).

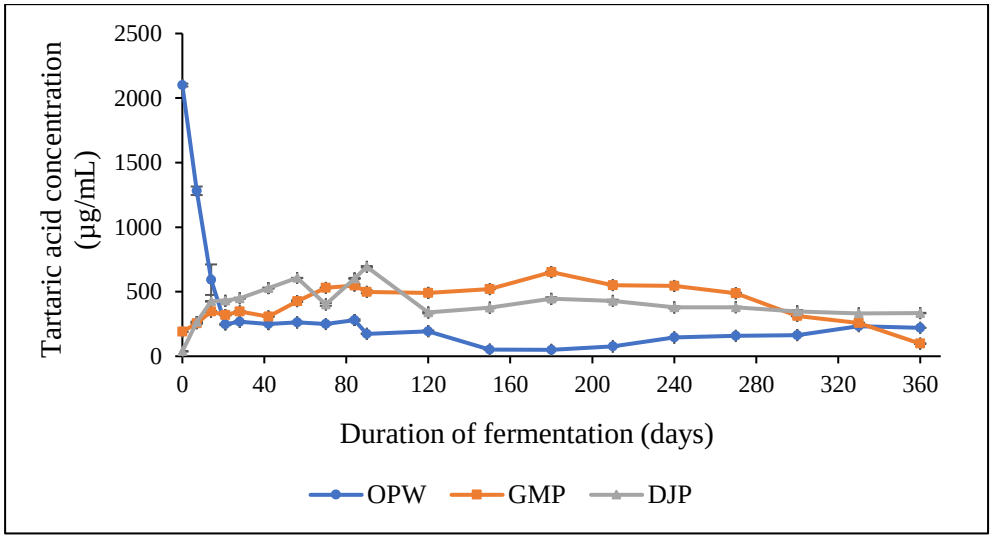

Figure 4.9: Tartaric acid concentration (µg/mL) of orange, papaya and watermelon (OPW), grapefruit, mango and pineapple (GMP) and durian, jackfruit and passion fruit (DJP) jiaosu from day 0 to 12th month.

4.6.3 Malic Acid

Figure 4.10 shows the malic acid concentrations of all three jiaosu groups which were the lowest before fermentation (day 0) at 125.83 ± 4.75, 159.63 ± 2.51 and 67.36 ± 1.93 µg/mL for OPW, GMP and DJP, respectively. At day 90, the malic acid concentration gradually increased to a concentration of 206.32 ± 2.74

μg/mL for OPW, 403.49 ± 0.92 μg/mL for GMP and 373.63 ± 5.61 μg/mL for DJP. At the end of the one-year fermentation period (12th month), the malic acid concentrations of OPW, GMP and DJP decreased to 150.56 ± 2.19, 202.81 ± 0.60 and 167.23 ± 2.50 μg/mL, respectively. Statistically, there was a no notable difference in the malic acid concentration ($p > 0.05$) among the three jiaosu groups throughout the one-year fermentation process.

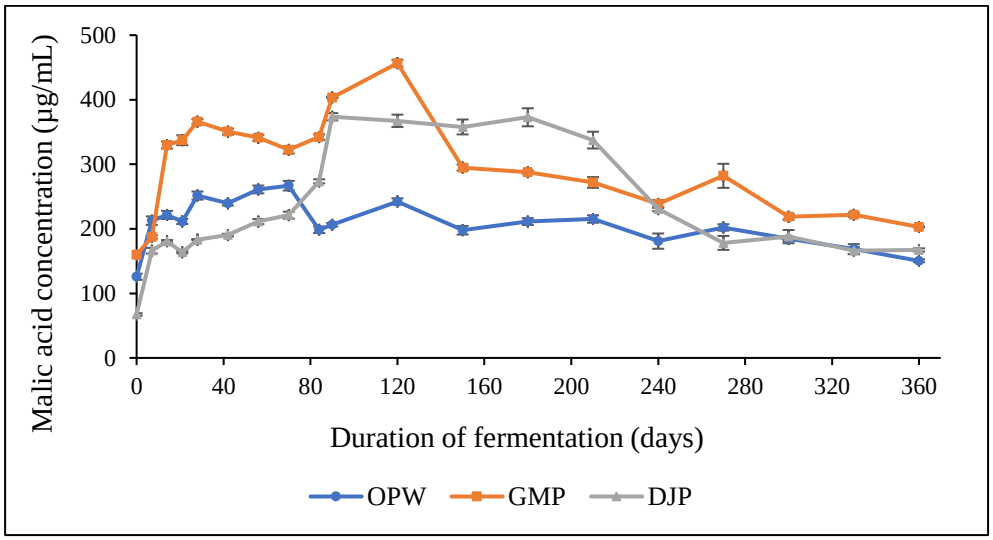

Figure 4.10: Malic acid concentration (μg/mL) of orange, papaya and watermelon (OPW), grapefruit, mango and pineapple (GMP) and durian, jackfruit and passion fruit (DJP) jiaosu from day 0 to 12th month.

4.6.4 Lactic Acid

Initially at day 0, the lactic acid concentrations of OPW, GMP and DJP were the lowest at 133.41 ± 2.26, 129.05 ± 3.60 and 67.30 ± 1.10 μg/mL, respectively. At day 90, the lactic acid concentration drastically increased to a concentration of 5075.77 ± 15.81 μg/mL for OPW, 493.19 ± 19.98 μg/mL for GMP and 8353.75

± 50.86 µg/mL for DJP. At the 12th month, the lactic acid concentrations of OPW, GMP and DJP declined to 953.10 ± 26.93, 269.54 ± 2.52 and 8216.75 ± 30.99 µg/mL, respectively. Statistically, DJP ($p < 0.05$) had a significantly higher concentration of lactic acid compared to OPW and GMP as shown in Figure 4.11.

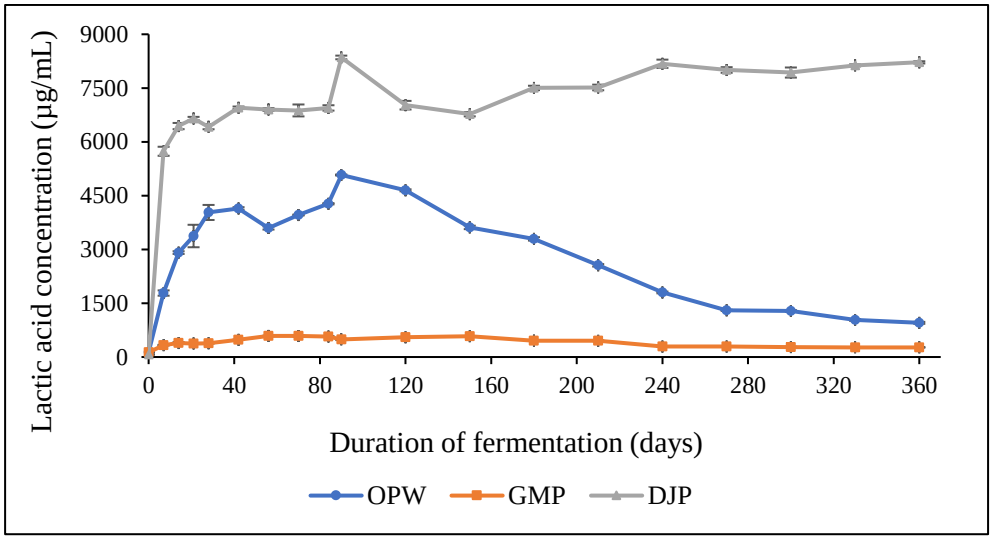

Figure 4.11: Lactic acid concentration (µg/mL) of orange, papaya and watermelon (OPW), grapefruit, mango and pineapple (GMP) and durian, jackfruit and passion fruit (DJP) jiaosu from day 0 to 12th month.

4.6.5 Acetic Acid

Based on Figure 4.12, the acetic acid concentrations were the lowest at day 0 for all three jiaosu groups at 171.84 ± 8.62 µg/mL for OPW, 124.26 ± 19.63 µg/mL for GMP and 61.97 ± 6.74 µg/mL for DJP. At day 90, the acetic acid concentrations sharply increased to 5856.09 ± 59.07, 6265.55 ± 57.91 and 3591.78 ± 39.95 µg/mL for OPW, GMP and DJP, respectively. At the 12th month, the acetic acid concentration gradually decreased to 785.69 ± 6.78 µg/mL for

OPW, 3514.53 ± 28.25 µg/mL for GMP and 2395.83 ± 18.28 µg/mL for DJP. Overall, the acetic acid concentration increased in all three jiaosu groups. However, there is no statistically notable difference in the acetic acid concentration ($p > 0.05$) among the three jiaosu groups throughout the one-year fermentation process.

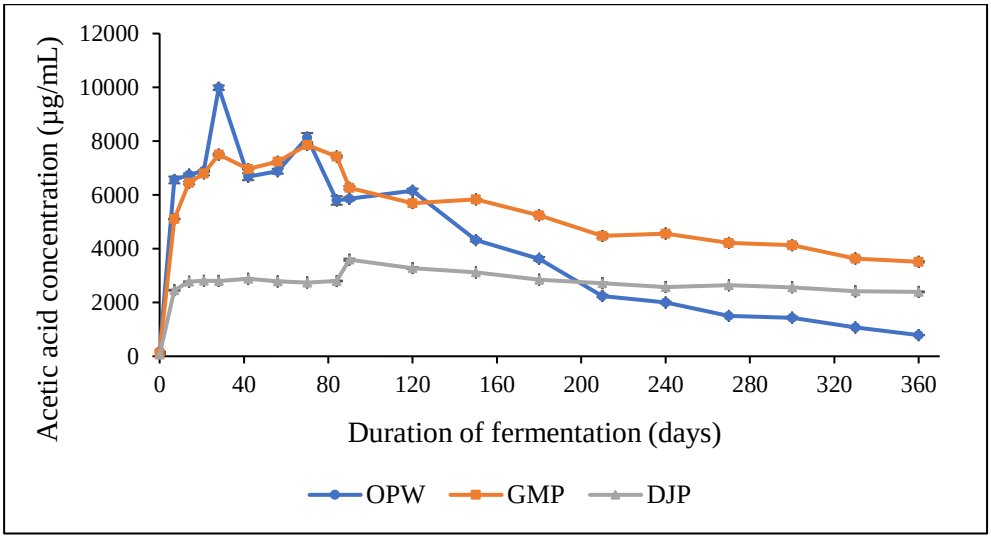

Figure 4.12: Acetic acid concentration (µg/mL) of orange, papaya and watermelon (OPW), grapefruit, mango and pineapple (GMP) and durian, jackfruit and passion fruit (DJP) jiaosu from day 0 to 12th month.

4.6.6 Citric Acid

Based on Figure 4.13, the citric acid concentration of all three jiaosu groups were the lowest before fermentation (day 0) at 52.67 ± 1.13 µg/mL for OPW, 32.49 ± 2.24 µg/mL for GMP and 43.93 ± 2.22 µg/mL for DJP. At day 90, the citric acid concentrations increased to 443.73 ± 4.69, 713.01 ± 11.41 and 981.95 ± 23.43 µg/mL for OPW, GMP and DJP, respectively. At the 12th month, the citric acid concentrations declined to 184.90 ± 6.31, 253.52 ± 10.43 µg/mL and 465.18 ±

17.78 µg/mL for OPW, GMP and DJP, respectively. Overall, the citric acid concentration increased in all three jiaosu groups. However, there was no statistically notable difference in the citric acid concentration ($p > 0.05$) among the three jiaosu groups throughout the one-year fermentation process.

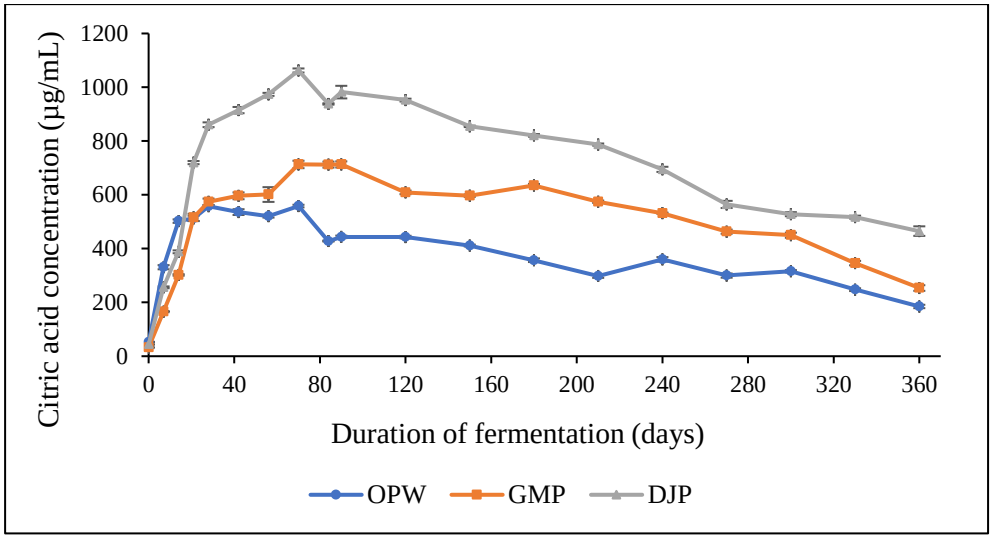

Figure 4.13: Citric acid concentration (µg/mL) of orange, papaya and watermelon (OPW), grapefruit, mango and pineapple (GMP) and durian, jackfruit and passion fruit (DJP) jiaosu from day 0 to 12th month.

4.6.7 Succinic Acid

The succinic acid concentrations of OPW, GMP and DJP at day 0 were 566.54 ± 7.27, 584.63 ± 12.03 and 433.61 ± 12.60 µg/mL, respectively. At day 90, the succinic acid concentrations increased to 1258.85 ± 9.51, 2969.13 ± 24.18 and 2312.63 ± 10.72 µg/mL for OPW, GMP and DJP, respectively. At the 12th month, the succinic acid concentrations of OPW, GMP and DJP decreased to 1678.35 ± 66.87, 1946.63 ± 22.85 and 912.64 ± 19.75 µg/mL, respectively. Based on Figure

4.14, there was no statistically notable difference in the succinic acid concentration ($p > 0.05$) among the three jiaosu groups throughout the one-year fermentation process.

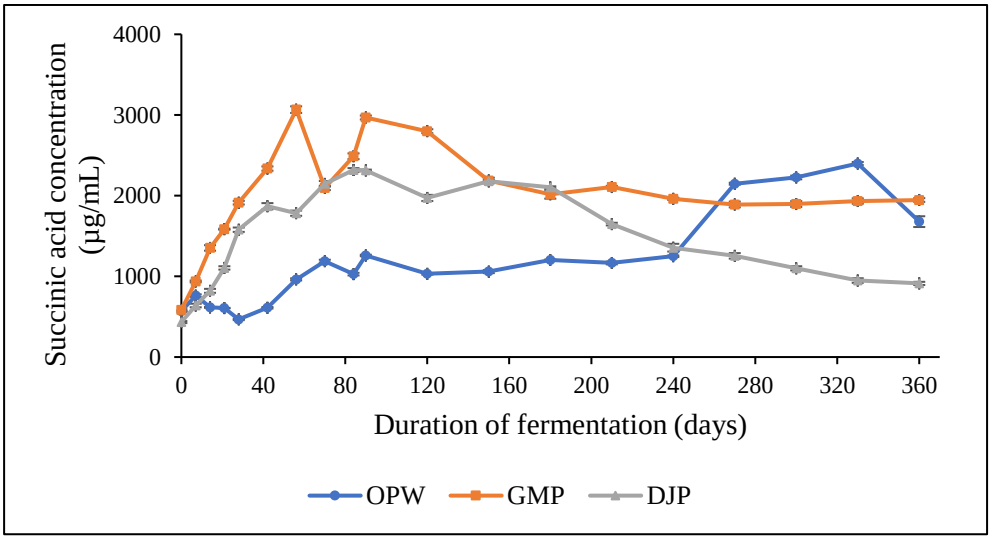

Figure 4.14: Succinic acid concentration (µg/mL) of orange, papaya and watermelon (OPW), grapefruit, mango and pineapple (GMP) and durian, jackfruit and passion fruit (DJP) jiaosu from day 0 to 12th month.

4.7 Total Alcohol Concentration

Figure 4.18 shows an absence of alcohol before fermentation (day 0) for all three jiaosu groups. The presence of alcohol was only detected at day 7 for GMP at 0.93 ± 0.37% and at day 14 for both OPW and DJP at 0.90 ± 0.24% and 0.52 ± 0.24%, respectively. At day 90, the total alcohol concentration increased to a concentration of 7.49 ± 0.15% for OPW, 10.47 ± 0.16% for GMP and 4.47 ± 0.23% for DJP. The total alcohol concentration then gradually declined to a concentration of 0.49 ± 0.10, 0.63 ± 0.31 and 0.22 ± 0.19% for OPW, GMP and

DJP, respectively at the 12th month. Among the three jiaosu groups, DJP had a significantly lower total alcohol concentration, whereas GMP had the highest total alcohol concentration ($p < 0.05$).

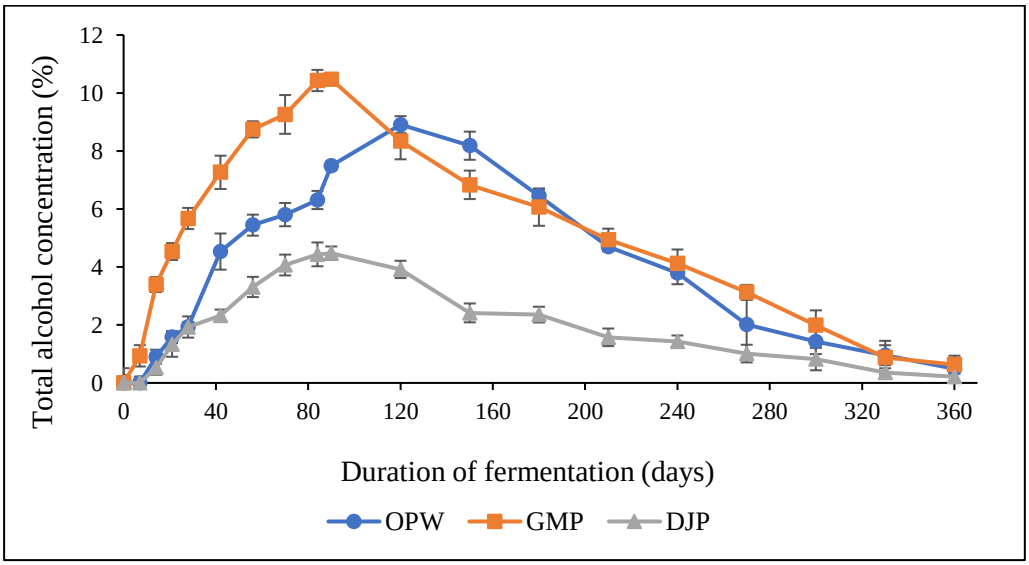

Figure 4.15: Total alcohol concentration (%) of orange, papaya and watermelon (OPW), grapefruit, mango and pineapple (GMP) and durian, jackfruit and passion fruit (DJP) jiaosu from day 0 to 12th month.

CHAPTER 5

DISCUSSION

5.1 pH of Jiaosu Samples

Experimental results in Figure 4.1 showed that OPW, GMP and DJP became more acidic after a week of fermentation and the pH values remained fairly stable throughout the fermentation process except from the 7^{th} to 8^{th} month where the pH dropped to its lowest for all three jiaosu groups. Based on previous research, the acidic pH of different types of garbage enzyme fermented for 3 months ranged from 2.9 to 3.7 (Pasalari, et al., 2024). The reduction in pH gives an indication of the fermentation process that causes elevated conversion of the bulk of sugars present in the jiaosu into organic acids and also due to the organic acids naturally present in the fruit waste that may have been drawn out into the fermented fruit waste solutions by microbial activity (Arun and Sivashanmugam, 2015a). Based on the statistical analysis, the different types of fruit peels used could have an influence on the pH of the jiaosu as the pH value ($p < 0.05$) of GMP was significantly different from OPW and DJP.

5.2 Total Protein Concentration

Based on Figure 4.2, fermentation increased the total protein concentration of GMP and DJP but decreased in OPW. These results coincide with the protease activity as shown in Figure 4.4. Protein is broken down by protease into amino acids via the addition of water to peptide bonds (Nalladiyil, Prakash and Babu, 2023). In OPW, as the protease activity increased, the total protein concentration decreased. In GMP and DJP, the total protein concentration increased due to the decrease in protease activity.

The total protein concentration noted before fermentation (day 0) could be from the release of proteins that were already present in the various fruit peels into the jiaosu solution. Moreover, the commercial brown sugar used in the preparation of the jiaosu fermentation did not contain protein (MSM Prai Berhad, 2017). Pranoto, Anggrahini and Efendi (2013) reported that the increase of protein in naturally fermented sorghum flour is associated with the microbial hydrolysis of bound proteins which in turn increases the soluble protein concentration. Another study suggested that a significant increase in protein content in pearl millet after fermentation for 24 hours was related to the loss of carbohydrates (Osman, 2011).

The total protein concentrations of OPW, GMP and DJP declined towards the end of fermentation (12^{th} month). Carbohydrates are one of the main energy sources for microbial growth during fermentation, however its depletion may lead to the utilisation of protein as a secondary source of energy. The use of

protein as an essential nutrient for microbial growth and production of enzymes would most likely cause the decrease in protein concentration (Nkhata, et al., 2018). Additionally, the decrease in protein content in fermented foods can occur if the amino acids are hydrolysed into ammonia and volatile compounds that act as flavour compounds (Pranoto, Anggrahini and Efendi, 2013).

The increase and decrease in protein concentration during fermentation is highly regulated by microbial activity. Chelule, et al. (2010) showed that the addition of bread flour, sucrose and yeast to maize meal fermentation resulted in a 77% increase in protein concentration compared to less than 3% increase in protein concentration for the maize meal fermented with only sucrose and bread flour. The study suggested that the addition of yeast into the fermenting solution favourably increased the protein concentration. Thus, the number of microorganisms present in the fermenting solution can have an additive effect on the fermentation process and lead to higher protein concentration (Chelule, et al., 2010). In the present study, the significantly higher total protein concentration in GMP compared to OPW and DJP could be due to higher number of microorganisms during the fermentation.

5.3 Biocatalytic Activity

During fermentation, microorganisms excrete enzymes to digest potential food source such as organic and inorganic materials in the fermenting solution for microbial growth (Chadha, 2023). In the present study, fermentation resulted in an increased amylase and protease activities in OPW, but reduced amylase and

protease activities in GMP and DJP. The fruit peels and brown sugar serve as a carbon source for microbial growth and enzyme production (Chin, et al., 2018). This could explain the highest specific amylase and protease activities at the first week of fermentation as the total carbohydrate concentration was the highest at the beginning of fermentation.

Garg, et al. (2021) reported that *Lactiplantibacillus plantarum* fermented mango stone waste had an amylase activity of 0.0573 U/mL/min at day 7 that increased to 0.4424 U/mL/min at day 13 and reduced to 0.3626 U/mL/min at day 28. However, *L. plantarum* fermented banana pseudo stem had a decreasing trend of amylase activity of 0.0768 U/mL/min, 0.0146 U/mL/min and zero amylase activity at day 7, 13 and 28, respectively. The higher amylase activity of fermented mango stone waste compared to fermented banana pseudo stem was due to higher *L. plantarum* count from day 7 to 28 in the fermented mango stone waste that ranged from 6.80×10^7 CFU/g to 7.46×10^8 CFU/g compared to that of the fermented banana pseudo stem that ranged from 7.10×10^6 CFU/g to 8.20×10^6 CFU/g (Garg, et al., 2021). A study done by Chin et al. (2018) showed that orange peels fermented for three months had a higher protease activity of 0.129 U/mL compared to that of pineapple peels with a protease activity of 0.046 U/mL. The study suggested that higher protease activity could be attributed to lower reducing sugar content for orange peels as compared to pineapple peels which correlates to the amount of sugar as energy source that is used up for microbial enzyme production (Chin et al., 2018). Therefore, the increase and decrease in amylase and protease activities could be affected by the amount of

microorganisms in the jiaosu solutions and the depletion in carbohydrate content during fermentation.

The lipase activity was found to be increased in OPW and DJP but decreased in GMP. The presence of enzymes in a fermenting solution is commonly due to microbial production of extracellular enzymes (Shu, Xu and Lin, 2006). Therefore, the contrasting trend of the lipase activities in OPW, GMP and DJP could mainly be due to the type and number of lipase-producing microorganisms present in the jiaosu samples. Shu, Xu and Lin (2006) reported that the lipase production by the fungus *Antrodia cinnamomea* not only increased steadily with the duration of fermentation but it was also enhanced from 15.03 U/mL to 26 U/mL by adding 0.01% olive oil into culture medium. Nutrient composition analysis showed a crude fat content of 5.64 to 8.70 g/100 g in orange peels, 1.8 to 12.61 g/100 g in watermelon peels, 0.42 to 4.72 g/100 g in mango peels, 1.10 to 5.31 g/100 g in pineapple peels, 1.71 to 7.47 g/100 g in jackfruit peels and 0.9% crude fat in durian peels (Alighiri et al., 2020; Prusty et al., 2024). Thus, the varying crude fat content in the fruit peels and presence of lipase-producing microorganisms may have an effect on the lipase activity of jiaosu.

The specific enzyme activity can also be affected by the composition of fruit peels used. The amylase, protease and lipase activities in garbage enzyme were the highest and lowest for pineapple and citrus fruit waste at a ratio of 6:4 and 1:9, respectively. The study suggested that the waste composition ratio can have a significant effect on hydrolytic enzyme activity of garbage enzyme solution

(Arun and Sivashanmugam, 2017). Selvakumar and Sivashanmugam (2017) reported that pomegranate, orange and pineapple wastes at a ratio of 35:20:35 resulted in a lipase activity of 9.7 ± 0.20 U/mL, which was 49% higher than the composition at 10:40:40 with a lipase activity of 6.8 ± 0.12 U/mL. These results indicated that fruit peel compositions have a considerable effect on lipase activity of garbage enzyme.

5.4 Total Phenolic Concentration

The total phenolic concentrations of OPW, GMP and DJP drastically increased during the one-year natural fermentation. Phenolic compounds are secondary plant metabolites that typically exist in different forms, such as free or as conjugated forms via hydroxyl groups with sugar and glycosides (Lattanzio, 2013). Fermentation can cause microorganism-induced breakdown of the structural integrity of cell walls of the fruit peels and lead to the release of the phenolic compounds. During fermentation, microbial enzymes hydrolyse glycosides which leads to the liberation of bound phenolics (Wang, Wu and Shyu, 2014). Therefore, the sharp increase in total phenolic concentrations in all three jiaosu groups after a week of fermentation can be due to the release of free-form phenolics from the different types of fruit peels into the jiaosu solution and the enzymatic breakdown of bound phenolics in the fruit peels by microorganisms. The total phenolic content could be affected by the type of microorganism involved in fermentation. A study conducted by Wang, Wu and Shyu (2014) showed that the total phenolic content of walnut extracts increased from 22.8 mg GAE/g extract in the native unfermented sample to 33.8 mg

GAE/g extract in the extract fermented with *Bacillus subtilis* and 28.6 mg GAE/g extract in the extract fermented with *Lactiplantibacillus plantarum*.

Another study reported that the total phenolic content of unfermented barley of 16.4 mg GAE/g extract increased to 20.1 mg GAE/g extract and 18.5 mg GAE/g extract when fermented with *Lactobacillus rhamnosus* and *Saccharomyces cerevisiae*, respectively (Đorđević, Šiler-Marinković and Dimitrijević-Branković, 2010). Similarly, the methanol extract of non-fermented black soybeans showed a total phenolic content of 15.94 mg GAE/g extract that increased to 23.43 mg GAE/g after fermentation with *Bacillus subtilis* (Juan and Chou, 2010). The study also reported that *B. subtilis* produced β-glucosidase that catalysed the release of phenolics and flavonoids from the black soybean substrate during fermentation that led to an increase in those compounds (Juan and Chou, 2010). In contrast, the phenolic acid content of orange peels fermented for 24 hours with *L. plantarum* was 16 to 17 mg/g dry weight. After 48 hours of fermentation, the phenolic acid content reduced to 12 to 13 mg/g dry weight which was lower than that of unfermented orange peels of 14 to 15 mg/g dry weight (Razola-Díaz, et al., 2024). These studies suggest that the type of microorganisms present during fermentation have an influence on the phenolic concentration and declined microbial activity can lead to a reduction in the total phenolic concentration.

Moreover, the total phenolic concentration can also be affected by the types of fruit peels used due to the different natural phenolic content. Citrus fruits highly

contain flavonoids which is one of the main groups of phenolic compounds (Ignat, Volf and Popa, 2011). A study showed that the total phenolic content in peels of grapefruits and oranges were 15% higher than those in the peeled fruits. Peeled grapefruit and orange contained total polyphenols of 135 mg/100 g and 154 mg/100 g, respectively, whereas the peels of grapefruit and orange contained total polyphenols of 155 mg/100 g and 179 mg/100 g, respectively (Gorinstein, et al., 2006). Li, et al. (2006) reported that pomegranate peels contain a total phenolic content of 249.4 mg GAE/g compared to the pulp which contain 24.4 mg GAE/g.

5.5 Total Carbohydrate Concentration

Carbohydrates in the fruit peels consist of a mixture of soluble carbohydrates, for example glucose, sucrose and fructose and insoluble carbohydrates such as cellulose and starch, which can be broken down by various types of microorganisms present in the jiaosu solutions (Chin, et al., 2018). In the present study, fermentation drastically reduced the total carbohydrate concentrations of OPW, GMP and DJP. This can be attributed to the activity of microbial enzymes such as α-amylase, β-amylase and maltase that hydrolyses starch into maltodextrins and simple sugars (Osman, 2011). Based on Figure 4.7, the sharp decline in total carbohydrate concentration in all three jiaosu groups coincides with the high amylase activity during the first week of fermentation as shown in Figure 4.3.

A study by Huang, et al. (2024) showed that the carbohydrate content of unfermented lychee juice at 12.7 g/100 g reduced to 6 g/100 g after fermentation. Another study reported that the total carbohydrate content of fermented mixed fruit (pomelo, watermelon and melon) sample alone after three months was 13.10 mg/mL. However, the mixed fruit sample added with *Saccharomyces cerevisiae* and yoghurt (lactic acid bacteria) had a lower carbohydrate content of 4.30 mg/mL and 8.80 mg/mL, respectively at the third month of fermentation (Chin, et al., 2018). Thus, the carbohydrate concentration can be influenced by the type of microorganisms present during fermentation.

5.6 Organic Acid Analysis

Organic acids such as oxalic acid, tartaric acid, malic acid and citric acid generally originate from fruits, whereas organic acids such as acetic acid, lactic acid and succinic acid are normally produced by microorganisms during fermentation. The variation in the type and content of organic acids during fermentation may also depend on the type of fruit peels used (Fu, et al., 2015). In the present study, the concentrations of seven types of organic acids increased in all three jiaosu groups, except for the tartaric acid concentration in OPW that reduced during fermentation.

Based on the results obtained in this study, the organic acid with the highest concentration in OPW and GMP was acetic acid (Figure 4.15) and in DJP, lactic acid was the most abundant (Figure 4.14). Acetic acid production is due to the fermentation process in an anaerobic environment where complex organic compounds are hydrolysed into simple molecules by microbial activity, for

example the breakdown of carbohydrates into simple sugars. The alcohols produced through the glycolysis of sugar during fermentation are broken down by bacteria into acetaldehyde and water, in which acetaldehyde will be converted into acetic acid (Gomes, et al., 2018). Therefore, if the carbohydrate concentration is high, the acetic acid concentration significantly increases during fermentation (Sroka and Tuszyński, 2007). During fermentation, the acetic acid concentrations for all three jiaosu groups increased. A similar trend was reported in a study where the acetic acid level of a mixture of tomato, cauliflower, pineapple, orange and mango peels fermented for three months, increased from 11.12 g/L at day 15 to 78.14 g/L at day 90 (Arun and Sivashanmugam, 2015b).

In DJP, lactic acid was the most abundant as compared to the other six types of organic acids tested. The increase in lactic acid concentration in DJP can be due to the presence and type of lactic acid-producing microorganisms in the fermenting solution. Studies have shown that increased lactic acid production can be attributed to certain strains of bacteria or fungi that can thrive in acidic environment and the presence of a mixture of different types of these microorganism in the fermenting solution. Cui, Li and Wan (2011) reported that during the fermentation of pretreated corn stover by combined cultures of *Lactobacillus brevis* and *Lactobacillus rhamnosus*, a lactic acid concentration of 0.70 g/g was obtained, which was 29.6% and 18.6% higher than that by single cultures of *L. brevis* and *L. rhamnosus*, respectively. This study highlighted that the conversion efficiency of substrates into lactic acid is increased by the presence of mixed cultures of lactic acid-producing microorganisms.

Oxalic acid was the organic acid with the lowest concentration in OPW, GMP and DJP. The initial oxalic acid concentration in all three jiaosu groups at day 0 could have originated from the fruit peels (Hanifah, et al., 2022). A study reported that the concentration of oxalic acid in blueberry wine declined sharply from 110.08 mg/L before fermentation to 26.17 mg/L after 4 weeks of yeast fermentation and aging (Fu, et al., 2015). Another study reported that the oxalic acid content of naturally fermented Huyou (*Citrus aurantium*) peel was 0.15 mg/g, whereas Huyou peel fermented with 5.18% lactic acid bacteria had an oxalic acid content of 0.18 mg/g (He, et al., 2023). Thus, the increase or decrease in the oxalic acid concentration during fermentation can be attributed to the metabolic activity of microorganisms.

In this study. the tartaric acid concentration of OPW decreased from its highest concentration at day 0 as the duration of fermentation increased, whereas the tartaric acid concentration of GMP and DJP increased. A study reported that fig pulps have a tartaric acid concentration of 122.00 μg/mL before fermentation which increased to 340.10 μg/mL after natural fermentation and 1921.79 μg/mL after fermentation with *Lactobacillus plantarum* for 70 days (Yao, et al., 2024). Similarly, fermented *Rosa roxburghii* Tratt fruit juice was found to have 21 times higher tartaric acid concentration compared to unfermented *Rosa roxburghii* fruit juice (Luo, et al., 2024). The tartaric acid concentration in avocado juice reduced from 0.10 mg/mL at the first week of fermentation to 0.05 mg/mL after 8 months (Liawruangrath, et al., 2015). The reduction in tartaric acid concentration could

be due to the conversion of tartaric acid into oxaloacetic acid and then into acetic acid, lactic acid, and carbon dioxide by tartaric acid dehydratase produced by bacteria during fermentation (Zheng et al., 2023).

The citric, malic and succinic acid concentration increased in all three jiaosu groups. Citric acid can be produced from the metabolic activity of microorganisms via the tricarboxylic acid cycle (TCA) that involves the conversion of carbohydrates, proteins and fats into carbon dioxide and water for microbial growth (Angumeenal and Venkappayya, 2013). Similarly, reduction of citric acid concentration could be related to its role in the cyclic metabolism of tricarboxylic acid in which it is converted to lactic acid by bacterial enzymes (Yao et al., 2024).The report by Zhang, et al. (2008) on fermented apple juice inoculated with *S. cerevisiae* showed an increase of citric acid concentration from 116.52 mg/L at day 0 to 512.89 mg/L at day 18, whereas the study by Arun and Sivashanmugam (2015b) showed a decrease of citric acid content to less than 20.00 g/L at day 90 from a concentration of 39.05 g/L at day 15 in the naturally fermented fruit and vegetable wastes. Similarly, the citric acid concentration of unfermented fig pulps was 765.71 µg/mL and reduced after natural fermentation and fermentation with *L. plantarum* to 169.47 µg/mL and 24.03 µg/mL, respectively (Yao, et al., 2024).

The production of malic acid during fermentation is due to the tricarboxylic acid (TCA) cycle in which malic acid is either produced from oxaloacetic acid via malate dehydrogenase or from fumaric acid (Suto and Kawashima, 2022).

Unfermented fig pulps had a malic acid concentration of 96.40 μg/mL that increased after natural fermentation and fermentation with *L. plantarum* to 426.97 μg/mL and 898.30 μg/mL, respectively (Yao, et al., 2024) Malic acid can be broken down by microorganisms to form lactic acid during malolactic fermentation which can account for the decrease in malic acid concentration during fermentation (Suto and Kawashima, 2022). Zhang, et al. (2008) reported that the malic acid concentration in the apple juice inoculated with *Saccharomyces cerevisiae* reduced from of 7171.20 mg/L at day 0 to 5783.10 mg/L at day 18.

Increase of succinic acid during fermentation could be due to microbial TCA cycle in which succinic acid is produced from fumaric acid via fumarate reductase (Suto and Kawashima, 2022). Zhang, et al. (2008) showed an increase of succinic acid concentration from 19.21 mg/L at day 0 to 290.53 mg/L at day 18 in *S. cerevisiae*-fermented apple juice. He, et al. (2023) reported that the succinic acid concentration of naturally fermented Huyou (*Citrus aurantium*) peel was 1.56 mg/g, whereas Huyou peel fermented with 5.18% lactic acid bacteria had a higher succinic acid concentration of 14.82 mg/g.

Overall, natural fermentation increased the concentrations of oxalic, tartaric, malic, lactic, acetic, citric and succinic acids in all three jiaosu groups except for the tartaric acid in OPW. Organic acid concentrations in the jiaosu solution were highly regulated by the type of microorganisms, its microbial activities and the type of fruit peels utilised.

5.7 Total Alcohol Concentration

In anaerobic fermentation, glucose is broken-down into pyruvic acid by glycolysis. Pyruvic acid will be converted by pyruvate decarboxylase into acetaldehyde, which is then is converted to ethanol and carbon dioxide by the action of alcohol dehydrogenase (Rusdianasari, et al., 2021b). In this study, the total alcohol concentrations of OPW, GMP and DJP increased after a week of fermentation. The increase in total alcohol concentration was likely due to the microbial enzyme activities that converted sugar to alcohol. Chitranshi and Kapoor (2021) measured sugar content using specific gravity in the alcoholic fermentation process. The study showed that at the peak of ethanol concentration, the specific gravity of fermented Indian blueberries was reduced to 0.875 and remained constant which can indicate the end of fermentation. Similarly, in this study the total alcohol concentrations increased (Figure 4.18) as the total carbohydrate concentrations declined drastically (Figure 4.7). The total alcohol concentration could also be affected by the amount of fermenting microorganisms in the jiaosu solutions. A study showed that concentrations of *Saccharomyces cerevisiae* of 12%, 9%, 6% and 3% were able to produce the highest ethanol concentration in 2, 3, 5 and 7 days, respectively in fermented banana peels (Singh, et al., 2014). The study showed that the increase in fermenting microorganisms can reduce the time required to produce maximum level of alcohol.

The total alcohol concentrations of OPW, GMP and DJP declined from the 5th month to 12th month. The reduction of alcohol concentration can be attributed to the hydrogenation of alcohol to form acetaldehyde which is then converted by aldehyde dehydrogenase to form acetic acid during fermentation (Bhat, Akhtar and Amin, 2014). Trinh, Masniyom and Maneesri (2016) showed that coconut water fermented with baker's yeast, produced approximately 6% (v/v) ethanol concentration within 1 day. Subsequent fermentation by *Acetobacter aceti* starter powder produced 6.27% acetic acid and the ethanol concentration reduced to less than 0.1% (v/v) within 18 days, thus attaining 89% fermentation efficiency.

CHAPTER 6

CONCLUSIONS

6.1 Overview

The novelty of this research is that this is the first study documenting the changes of biochemical composition and biocatalytic activities of jiaosu derived from fruit waste over a one-year fermentation period. In the present study, three different groups of fruit peel jiaosu prepared using orange, papaya and watermelon (OPW), grapefruit, mango and pineapple (GMP) and durian, jackfruit and passion fruit (DJP) were analysed for their concentration of selected biochemical compounds and biocatalytic activities during one-year natural fermentation. All three jiaosu groups showed decreased pH and total carbohydrate concentration and increases in total protein concentration, total phenolic concentration and total alcohol concentration. After fermentation, the amylase and protease activities of OPW and lipase activities of OPW and DJP increased. The amylase and protease activities of GMP and DJP and lipase activity of GMP decreased. Oxalic, tartaric, malic, lactic, acetic, citric and succinic acid concentrations increased in all three jiaosu groups except for the tartaric acid concentration in OPW that decreased. Acetic acid was the most abundant in OPW and GMP samples and lactic acid was the most abundant in DJP sample. Oxalic acid was the least abundant among the seven organic acids tested in all three jiaosu groups.

The results of this study were able to provide a useful evaluation on how the duration of jiaosu fermentation and type of fruit peels can have an effect on the end-product quality of jiaosu. The results of this study showed that the increase in biochemical contents and biocatalytic activity occurred after 2 months of fermentation onwards and therefore this supports Dr. Rosukon's recipe of a three-month natural fermentation period for preparation of jiaosu. However, up to one year of fermentation is not suitable as the decrease in biochemical content and biocatalytic activity can be attributed to decreased substrate availability as the fermentation period increases. Hence, a more efficient preparation of jiaosu based on the effective level of bioactive compounds is required for specific applications at a commercial or industrial level.

The possible uses of the three jiaosu groups prepared can be suggested based on the results of this study. GMP is best suited to be used as a natural fertiliser as it contained higher phenolic and protein concentrations as compared to OPW and DJP. Due the highest lipase activity in DJP, it can be used as a natural detergent. However, as DJP contained the lowest total alcohol concentration, it is the least suitable to be used as a natural disinfectant as compared to OPW and GMP.

The simplicity of jiaosu production and the utilisation of fruit waste makes it cost-effective and environmentally friendly. Natural fermentation may cause uncertain variations in the biochemical content of jiaosu. For industrial or commercial use of jiaosu, a more controlled fermentation process can be studied

to maintain product consistency and to effectively reduce the fermentation duration. Production of jiaosu as a solution to reduce food waste at a household level constitutes as one of the solutions recommended in the Food Waste Index Report 2024 that emphasizes on a collective effort to combat food waste that can eventually lead to environmental problems and food insecurity (United Nations Environment Programme, 2024).

6.2 Limitations and Future Studies

The present study has a few limitations, one of which is the unknown microbial population in the fermenting solution. The study on the various types of microorganisms responsible for the natural fermentation of the different fruit peels can provide a more comprehensive understanding on the changes of the biochemical contents throughout the fermentation process. Advanced analytical techniques such as gas chromatography for alcohol analysis, HPLC for carbohydrate analysis and the study on the total flavonoids content can be included in future research to provide a more comprehensive understanding on the biochemical contents in the jiaosu.

The fruit peels used in this study were randomly selected and therefore, future studies on how the different fruit peels and their nutritional content can affect the biochemical contents in jiaosu can be studied. The fruit peel combinations used in this study can be further investigated for its applications as a natural disinfectant and/or detergent, organic fertiliser, natural pesticide, waste management, animal feed and etc. Future studies on the long-term stability and

shelf life of jiaosu subjected to various storage conditions can be useful for commercial purposes. The potential nutritional benefits for health purposes and the impact of large-scale production of jiaosu on food waste reduction can be researched in the future.

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APPENDIX A

PREPARATION OF REAGENTS AND SOLUTIONS

Reagents/Solutions	Preparation
0.65% casein solution	0.65 mg casein from bovine milk in 100 mL of 0.05 M potassium phosphate buffer of pH 7.5. The solution was mixed by gentle stirring for 10 minutes with a gradual increase in temperature to 85℃.
DNSA reagent (amylase assay)	On 95℃ hot-plate with continuous stirring, 80 mL of deionised water was added with 1 g of 3,5-Dinitrosalicyclic acid and 30 g potassium sodium tartrate tetrahydrate, followed by 20 mL of 2N sodium hydroxide that was made by dissolving 8 g of it in 100 mL deionised water. The reagent was filtered into an amber bottle using filter paper and stored for no longer than 2 weeks.
DNSA reagent (alcohol assay)	3.15 g DNSA, 131 mL of 2 M NaOH solution, 92.5 g potassium sodium tartrate, 2.5 g crystallization of phenol and 2.5 g sodium sulfite in 500 mL deionised water.
Enzyme diluent pH 7.5	0.680 g of 0.01 M sodium acetate trihydrate with 2.9 mL of 0.005 M glacial acetic acid and 0.395 g of 0.005 M calcium acetate hydrate made up to a volume of 500 mL with deionised water. The pH was adjusted to 7.5 by adding 1 M sodium hydroxide solution.
0.5 M Folin-Ciocalteu's reagent (FCR)	125 mL FCR dissolved in 375 mL deionised water.
20% hexadecyltrimethylammonium bromide (CTAB)	20 g of the powder little at a time to 100 mL of deionised water heated to 65℃ with gentle stirring
20 mM sodium dihydrogen phosphate (NaH_2PO_4) pH 2.7	2.76 g of NaH_2PO_4 in 1000 mL deionised water, the pH adjusted to 2.7 with concentrated sulphuric acid
5% phenol solution	5 g of phenol crystals in 100 mL deionised water
0.1 M phosphate buffer pH 6.6	2.76 g/200 mL Sodium dihydrogen phosphate monohydrate (NaH_2PO_4) added to

	1.42 g/100 mL of di-Sodium hydrogen phosphate (Na_2HPO_4).
0.05 M potassium phosphate buffer of pH 7.5	0.8709 g dipotassium hydrogen phosphate (K_2HPO_4) in 100 mL deionised water was slowly added with 1.3609 g potassium dihydrogen phosphate (KH_2PO_4) in 200 mL deionised water.
Potassium permanganate reagent	0.395 g potassium permanganate and 10 g disodium tetraborate in 250 mL of 98% sulfuric acid.
1% Starch solution	1 g of starch in 100 mL of 0.1 M phosphate buffer pH 6.6. The solution was boiled for 5 minutes and let cool at room temperature. Prior to assay, it was incubated at 37°C for 5 minutes.
75 g/L of sodium carbonate	4.054 g Sodium carbonate in 50 mL deionised water.
5 mM substrate solution (A)	0.1442 g of sodium dodecyl sulfate (SDS) with 0.3234 g of Triton X-100 in 100 mL of deionised water. The mixture was incubated in a 65 ℃-water bath for 20 minutes with continuous stirring and cooled to room temperature.
5 mM substrate solution (B)	0.1888 g of 4-nitrophenyl palmitate (4-NPP) with 0.1442 g SDS and 0.3234 g Triton X-100 in 100 mL of deionised water. The substrate solution was incubated at 65℃ for 20 minutes with continuous stirring and cooled to room temperature.
0.11 M trichloroacetic acid (TCA) solution	1.8 g TCA in 100 mL deionised water.
20% trichloroacetic acid (TCA)	10 mL of 100% TCA solution in 40 mL deionised water.
Tris- hydrochloric (HCl) buffer	0.7882 g Tris-HCl in 100 mL deionised water and the pH was adjusted to 7 by a basic solution which was made by mixing 0.6057 g Tris-base in 100 mL of deionised water

APPENDIX B

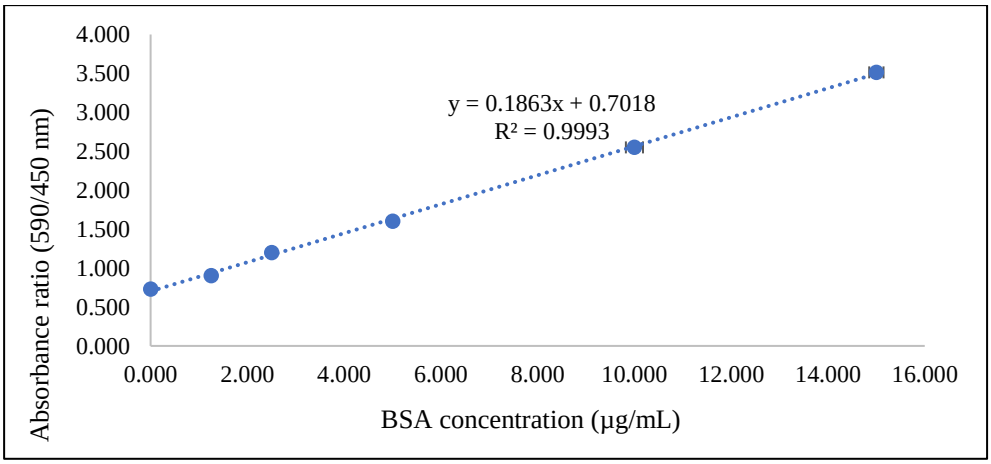
LIST OF LINEAR EQUATIONS OF THE CALIBRATION CURVES

Parameter	Linear Equation	R^2 value
Total protein concentration	y = 0.1863x + 0.7018	0.9993
Amylase activity	y = 0.0005x - 0.0372	0.9997
Protease activity	y = 0.0065x + 0.0034	0.9995
Lipase activity	y = 0.0129x + 0.0008	0.9996
Total phenolic concentration	y = 0.0067x + 0.0608	0.9996
Total carbohydrate concentration	y = 0.0029x + 0.0331	0.9996
Total alcohol concentration	y = 0.1743x + 0.0168	0.9994
Oxalic acid concentration	y = 19.546x + 51.987	0.9986
Tartaric acid concentration	y = 2.3831x + 38.114	0.9989
Malic acid concentration	y = 1.3099x - 17.144	0.9992
Lactic acid concentration	y = 1.0651x - 13.809	0.9995
Acetic acid concentration	y = 0.7326x + 5.5664	0.9985
Citric acid concentration	y = 1.4076x + 17.552	0.9988
Succinic acid concentration	y = 0.2249x + 7.5623	0.9981

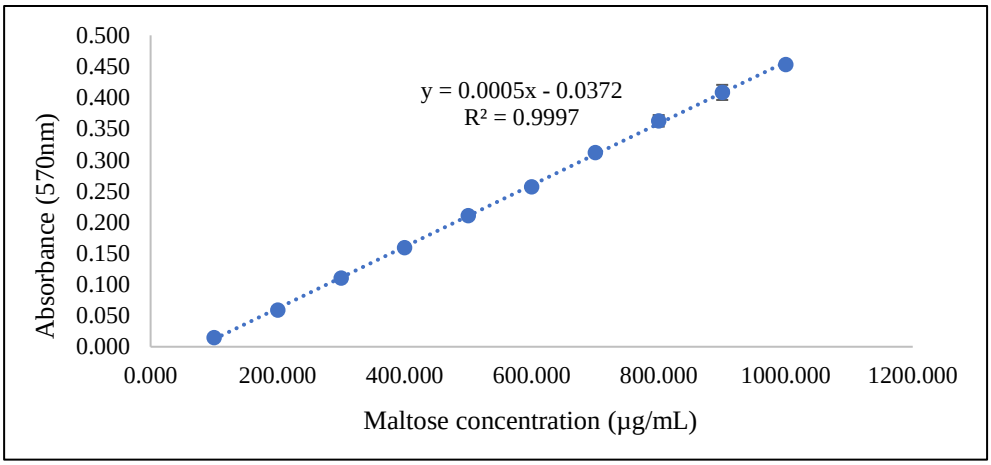
APPENDIX C

EXAMPLES OF THE CALIBRATION CURVES

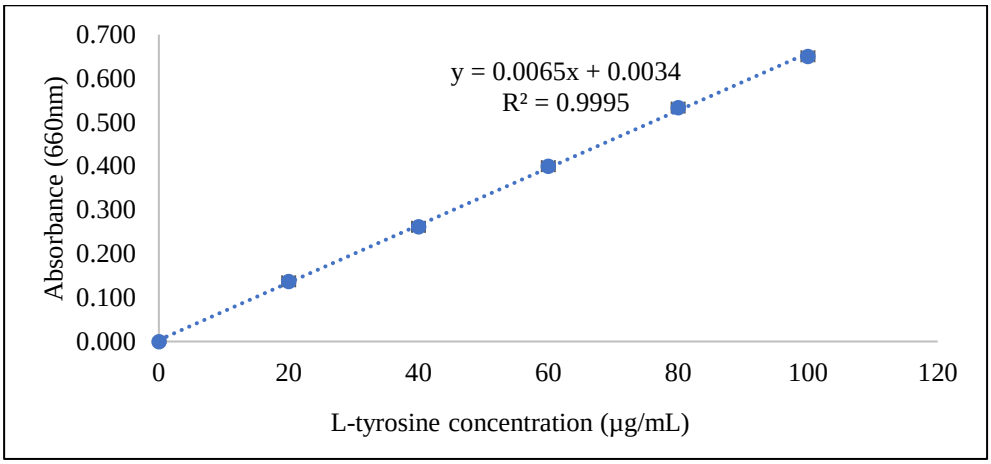
1. Total protein concentration



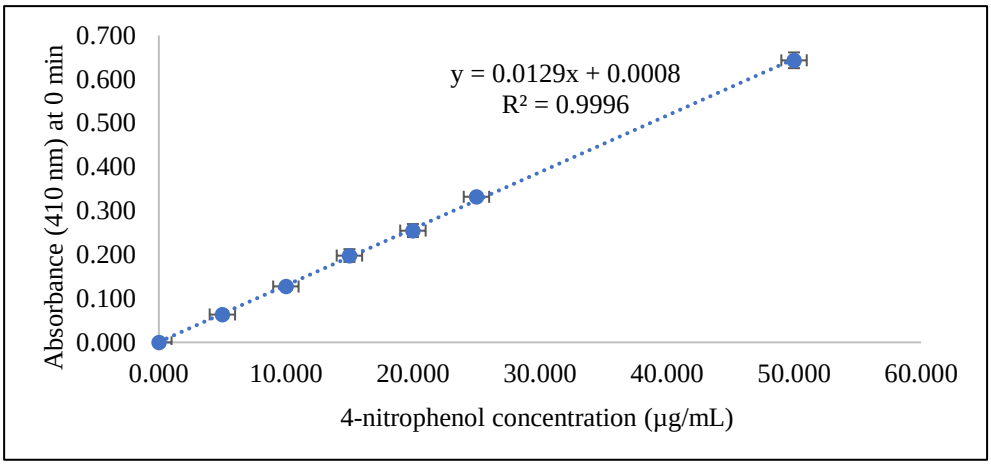
2. Amylase activity



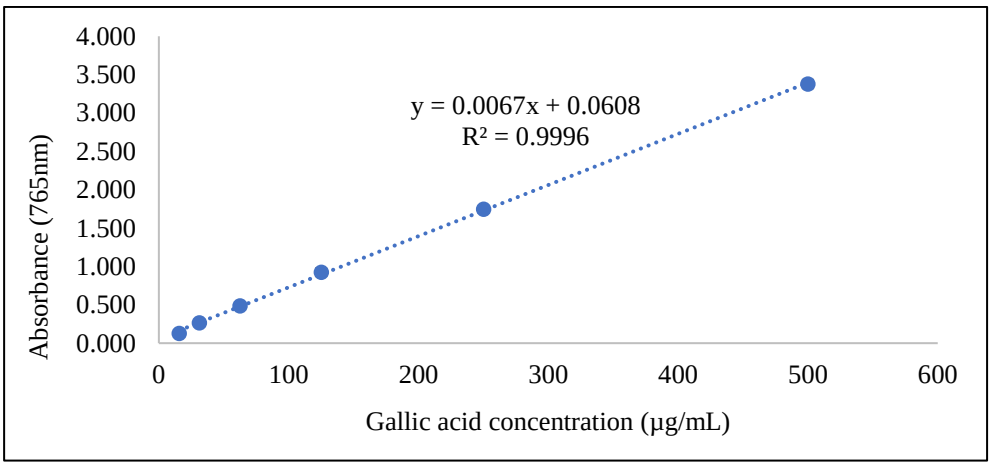
3. Protease activity



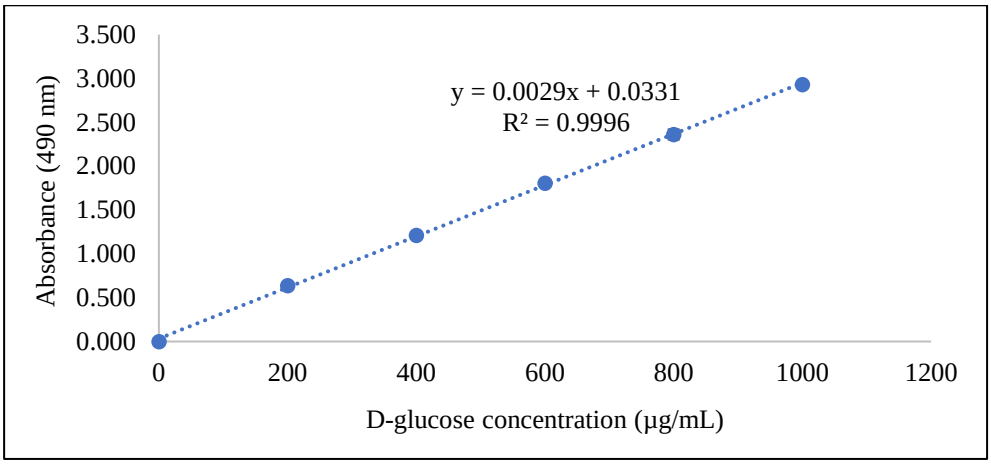
4. Lipase activity



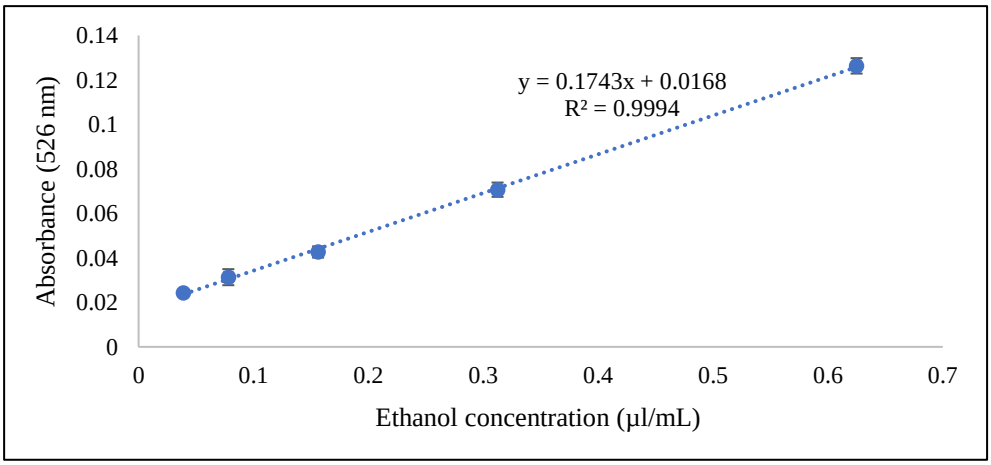
5. Total phenolic concentration



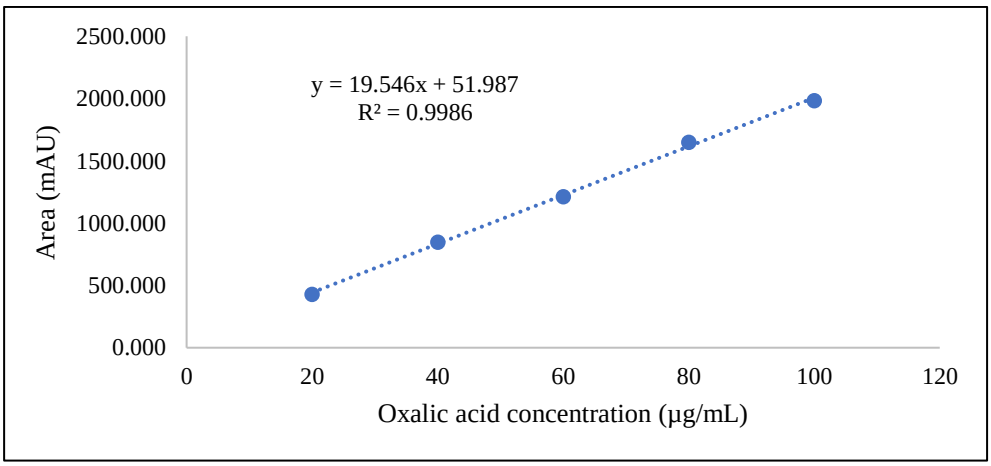
6. Total carbohydrate concentration



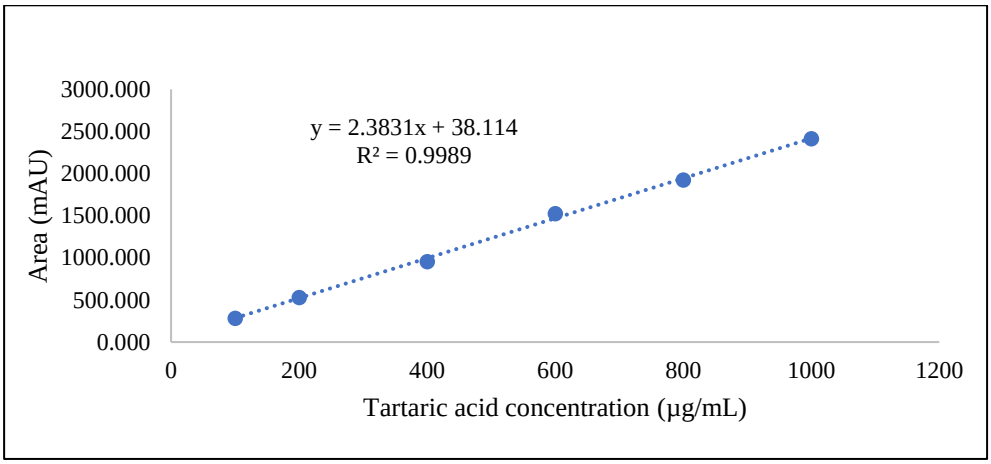
7. Total alcohol concentration



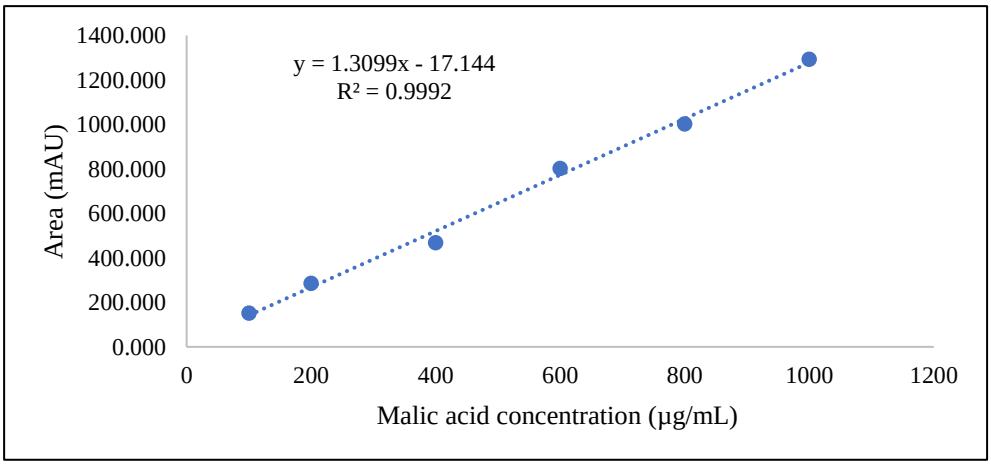
8. Oxalic acid concentration



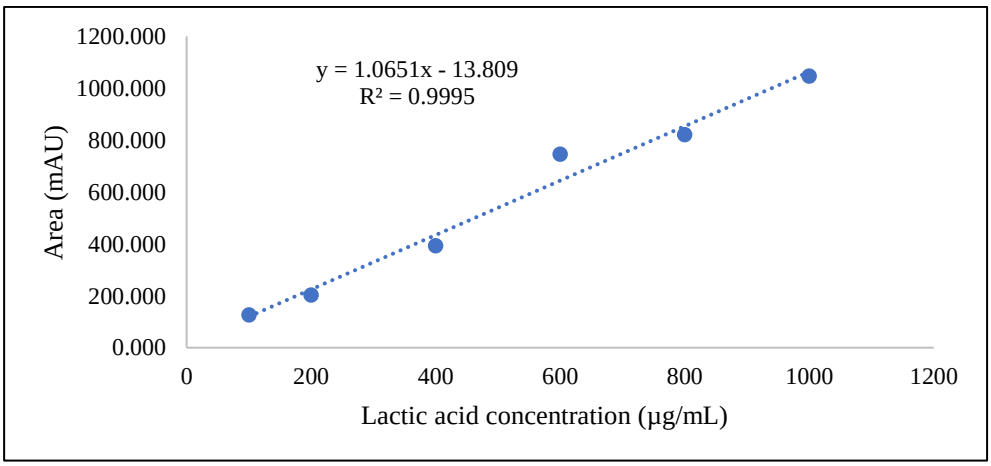
9. Tartaric acid concentration



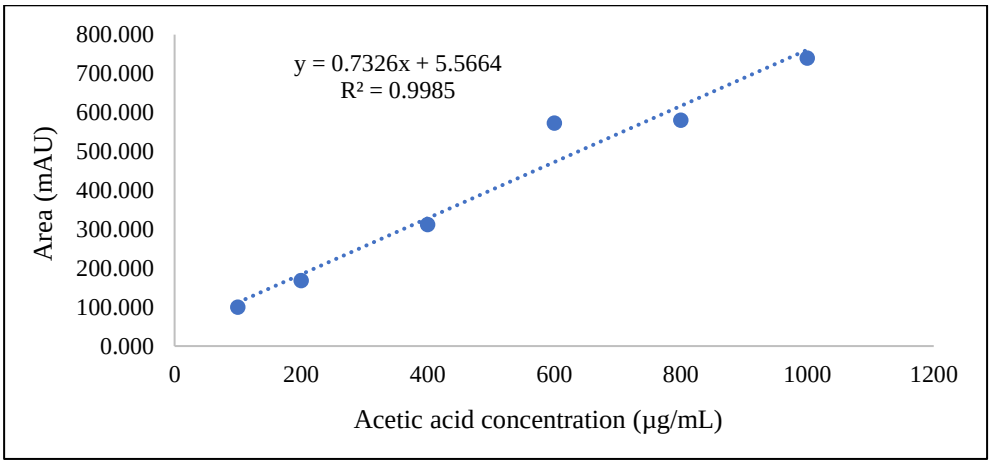
10. Malic acid concentration



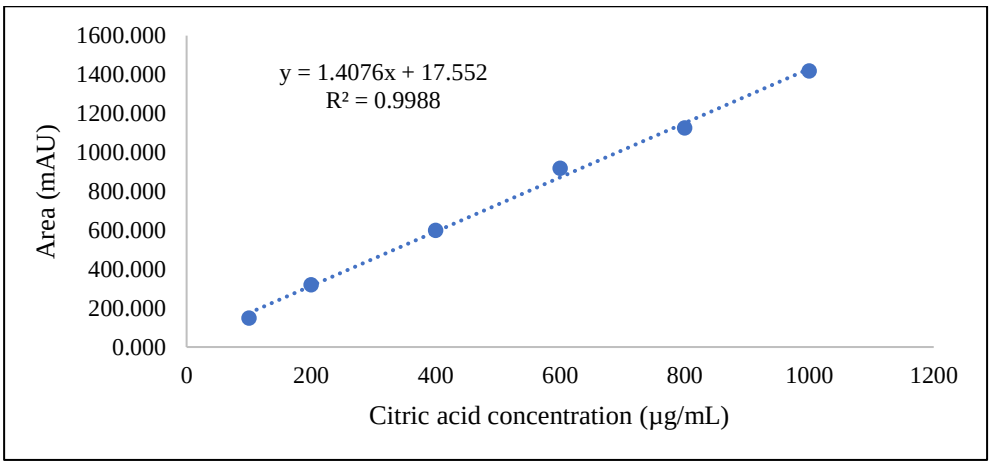
11. Lactic acid concentration



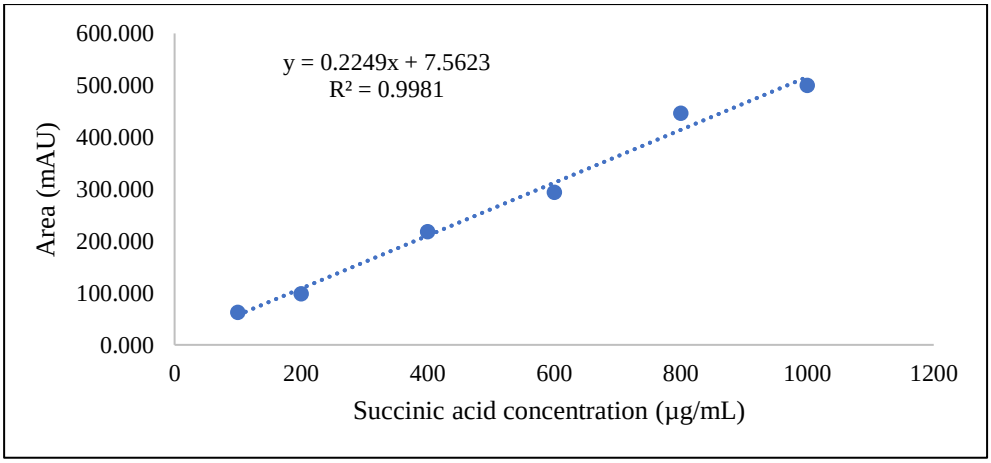
12. Acetic acid concentration



13. Citric acid concentration



14. Succinic acid concentration



APPENDIX D

STATISTICAL ANALYSIS DATA

Day 0, Day 28, Day 56 and Day 90

Parameters	F-Test	Mauchly's Test of Sphericity	Statistical interpretation
pH	<0.05	$\chi2(2) = 7.87$, $p = 0.16$	F (3,4) = 767.63, $p < 0.05$); reject null hypothesis
Total protein concentration	<0.05	$\chi2(2) = 8.87$, $p = 0.11$	F (3,4) = 28.71, $p < 0.05$); reject null hypothesis
Amylase activity	<0.05	$\chi2(2) = 48.39$, $p < 0.05$	Wilk's $\Lambda < 0.05$, G-G Epsilon correction: F (1.01, 6.04) = 433.86, $p < 0.05$; reject null hypothesis
Protease activity	<0.05	$\chi2(2) = 5.14$, $p = 0.40$	F (3,4) = 31.63, $p < 0.05$); reject null hypothesis
Lipase activity	<0.05	$\chi2(2) = 11.32$, $p = 0.05$	F (3,4) = 15.05, $p < 0.05$); reject null hypothesis
Total phenolic concentration	<0.05	$\chi2(2) = 5.37$, $p = 0.38$	F (3,4) = 670.46, $p < 0.05$); reject null hypothesis
Total carbohydrate concentration	<0.05	$\chi2(2) = 42.39$, $p < 0.05$	Wilk's $\Lambda < 0.05$, G-G Epsilon correction: F (1.01, 6.06) = 2235.60, $p < 0.05$; reject null hypothesis
Total alcohol concentration	<0.05	$\chi2(2) = 7.73$, $p = 0.17$	F (3,4) = 35.97, $p < 0.05$); reject null hypothesis
Oxalic acid concentration	0.56	$\chi2(2) = 7.75$, $p = 0.17$	F (3,4) = 1.23, $p = 0.41$); accept null hypothesis
Tartaric acid concentration	0.39	$\chi2(2) = 17.34$, $p < 0.05$	Wilk's $\Lambda < 0.05$, G-G Epsilon correction: F (1.30, 7.81) = 6.10, $p < 0.05$; reject null hypothesis

Malic acid concentration	0.30	$\chi2(2) = 7.70$, $p = 0.17$	F (3,4) = 6.13, $p = 0.06$); accept null hypothesis
Lactic acid concentration	<0.05	$\chi2(2) = 17.22$, $p < 0.05$	Wilk's $\Lambda < 0.05$, G-G Epsilon correction: F (1.56, 9.35) = 12.94, $p < 0.05$; reject null hypothesis
Acetic acid concentration	0.15	$\chi2(2) = 15.38$, $p < 0.05$	Wilk's $\Lambda < 0.05$, G-G Epsilon correction: F (1.72, 10.33) = 16.76, $p < 0.05$; reject null hypothesis
Citric acid concentration	0.33	$\chi2(2) = 6.66$, $p = 0.25$	F (3,4) = 16.90, $p < 0.05$); reject null hypothesis
Succinic acid concentration	<0.05	$\chi2(2) = 10.26$, $p = 0.07$	F (3,4) = 22.62, $p < 0.05$); reject null hypothesis

Day 90, 6 months, 9 months and 12 months

Parameters	**F-Test**	**Mauchly's Test of Sphericity**	**Statistical interpretation**
pH	<0.05	$\chi2(2) = 2.67$, $p = 0.75$	F (3,4) = 83.20, $p < 0.05$); reject null hypothesis
Total protein concentration	<0.05	$\chi2(2) = 2.64$, $p = 0.76$	F (3,4) = 1114.13, $p < 0.05$); reject null hypothesis
Amylase activity	<0.05	$\chi2(2) = 19.11$, $p < 0.05$	Wilk's $\Lambda < 0.05$, G-G Epsilon correction: F (1.14, 6.85) = 152.19, $p < 0.05$; reject null hypothesis
Protease activity	<0.05	$\chi2(2) = 13.90$, $p < 0.05$	Wilk's $\Lambda < 0.05$, G-G Epsilon correction: F (2.43, 7.29) = 56.79, $p < 0.05$; reject null hypothesis
Lipase activity	<0.05	$\chi2(2) = 28.45$, $p < 0.05$	Wilk's $\Lambda < 0.05$, G-G Epsilon correction: F (1.04, 6.27) = 18.78, $p < 0.05$; reject null hypothesis
Total phenolic concentration	<0.05	$\chi2(2) = 23.38$, $p < 0.05$	Wilk's $\Lambda < 0.05$, G-G Epsilon correction: F (1.16, 6.93) = 191.40, $p < 0.05$; reject null hypothesis

Total carbohydrate concentration	<0.05	$\chi 2(2) = 1.64$, $p = 0.90$	F (3,4) = 42.20, $p < 0.05$); reject null hypothesis
Total alcohol concentration	<0.05	$\chi 2(2) = 5.28$, $p = 0.38$	F (3,4) = 45.56, $p < 0.05$); reject null hypothesis
Oxalic acid concentration	0.93	$\chi 2(2) = 10.25$, $p = 0.07$	F (3,4) = 10.15, $p < 0.05$); reject null hypothesis
Tartaric acid concentration	0.36	$\chi 2(2) = 13.53$, $p < 0.05$	Wilk's $\Lambda = 0.06$, G-G Epsilon correction: F (1.56, 9.35) = 2.15, $p = 0.17$; accept null hypothesis
Malic acid concentration	0.29	$\chi 2(2) = 19.74$, $p < 0.05$	Wilk's $\Lambda = 0.05$, G-G Epsilon correction: F (1.11, 6.67) = 5.05, $p = 0.06$; accept null hypothesis
Lactic acid concentration	<0.05	$\chi 2(2) = 23.42$, $p < 0.05$	Wilk's $\Lambda = 0.09$, G-G Epsilon correction: F (1.38, 8.25) = 8.16, $p < 0.05$; reject null hypothesis
Acetic acid concentration	0.34	$\chi 2(2) = 9.77$, $p = 0.08$	F (3,4) = 7.45, $p < 0.05$); reject null hypothesis
Citric acid concentration	0.14	$\chi 2(2) = 10.70$, $p = 0.06$	F (3,4) = 22.49, $p < 0.05$); reject null hypothesis
Succinic acid concentration	0.56	$\chi 2(2) = 3.30$, $p = 0.65$	F (3,4) = 1.18, $p = 0.22$); accept null hypothesis

APPENDIX E

CHROMATOGRAMS OF ORGANIC ACIDS

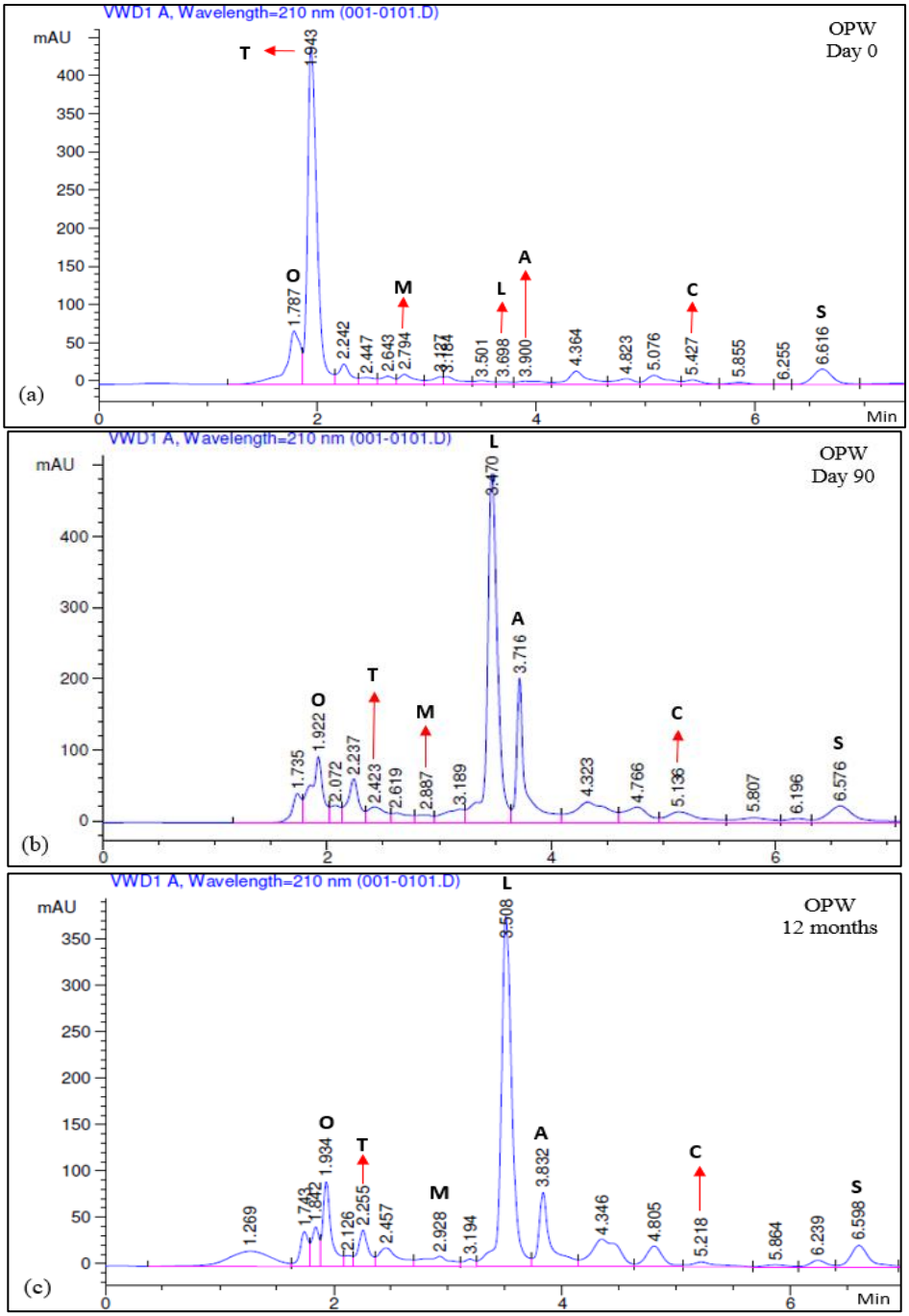


Figure 1: Chromatograms of organic acids for orange, papaya and watermelon (OPW) jiaosu at (a): day 0, (b): day 90 and (c): 12th month (Peaks were assigned as follows: O: oxalic acid, T: tartaric acid, M: malic acid, L: lactic acid, A: acetic acid, C: citric acid and S: succinic acid).

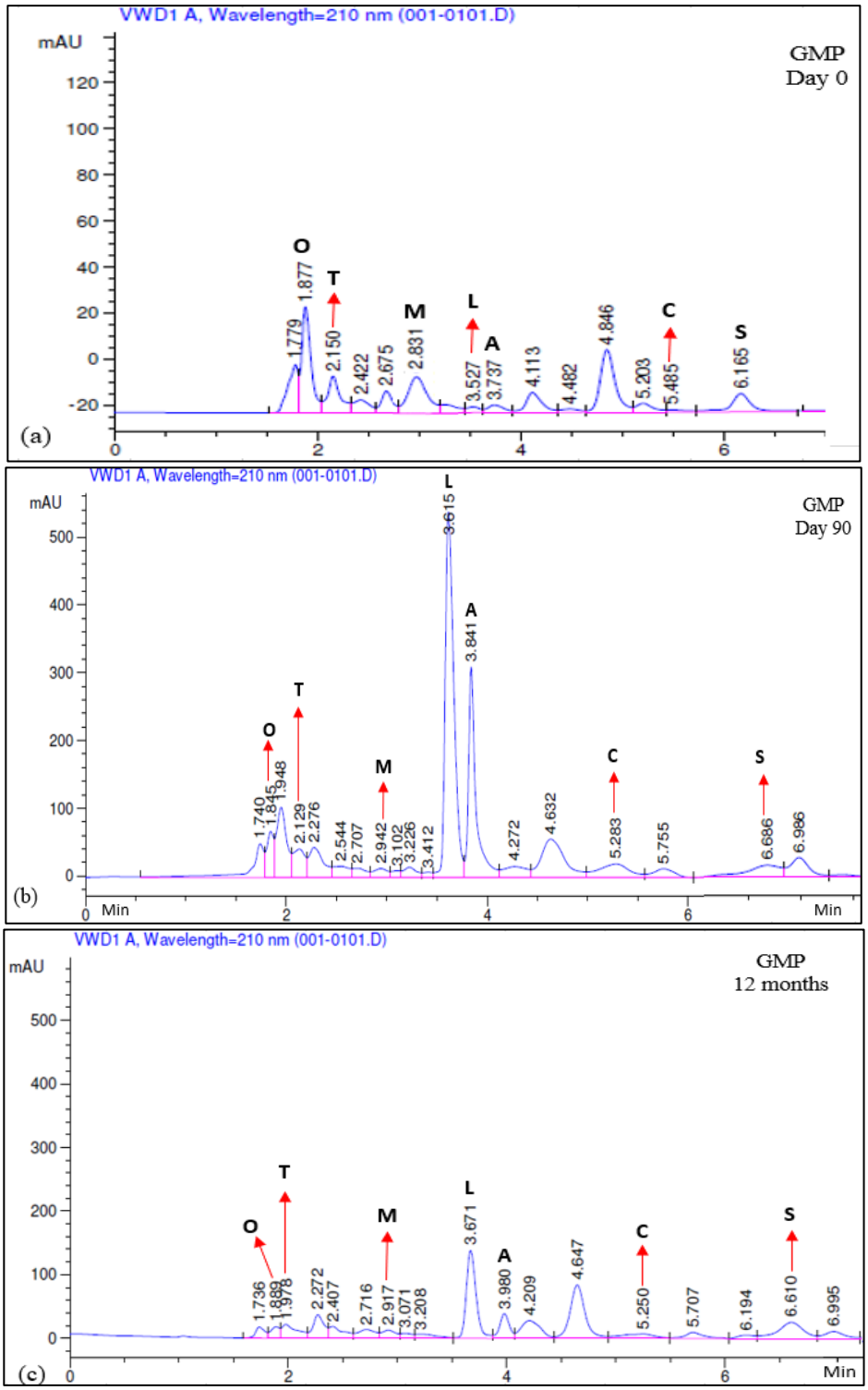


Figure 2: Chromatograms of organic acids for grapefruit, mango and pineapple (GMP) jiaosu at day 0, day 90 and 12th month (Peaks were assigned as follows: O: oxalic acid, T: tartaric acid, M: malic acid, L: lactic acid, A: acetic acid, C: citric acid and S: succinic acid).

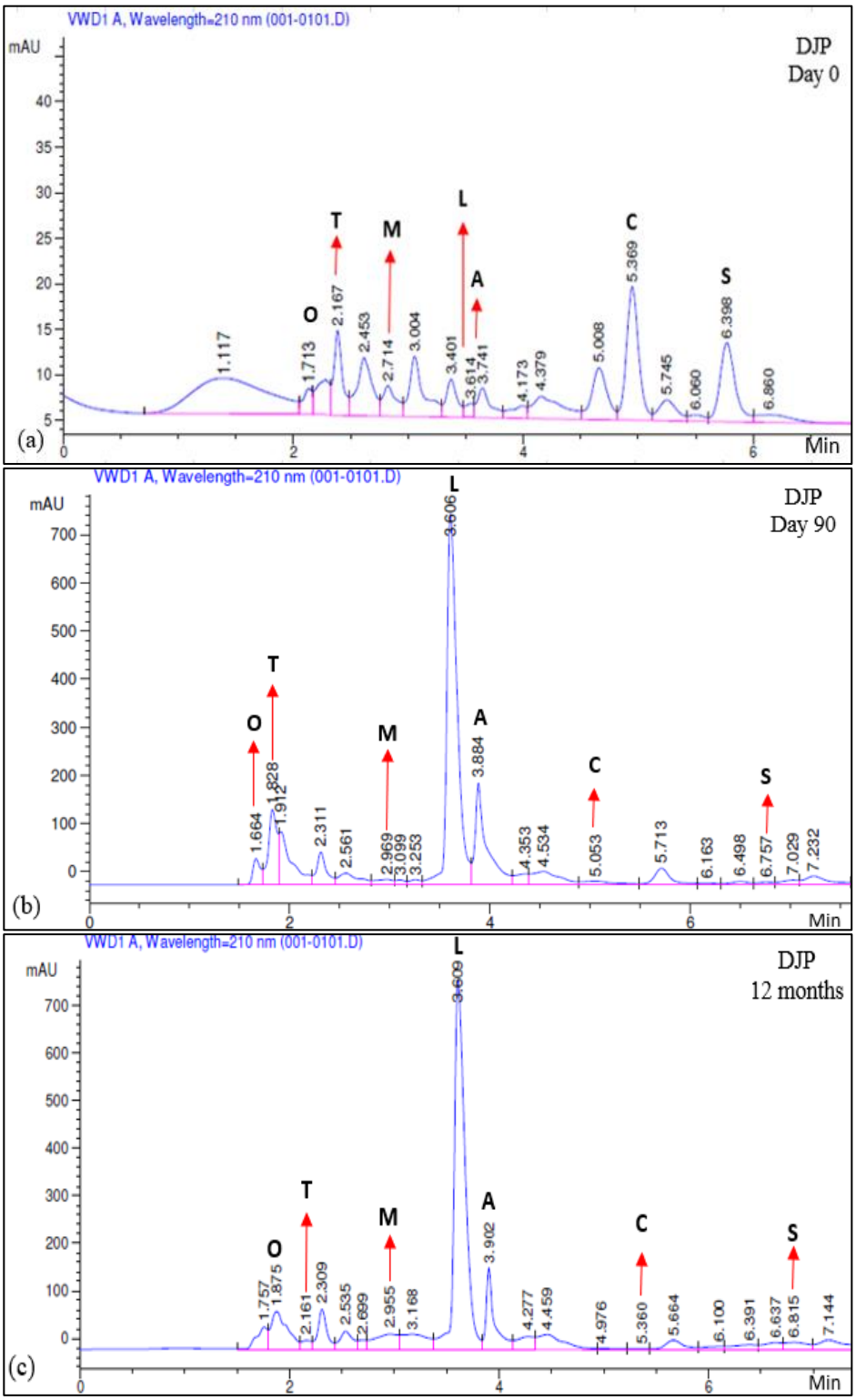


Figure 3: Chromatograms of organic acids for durian, jackfruit and passion fruit (DJP) jiaosu at day 0, day 90 and 12th month (Peaks were assigned as follows: O: oxalic acid, T: tartaric acid, M: malic acid, L: lactic acid, A: acetic acid, C: citric acid and S: succinic acid).