APPLICATION OF ENZYME-DIGESTED SOY PROTEIN HYDROLYSATE ON HYDROPONIC-PLANTED LETTUCE: EFFECTS ON LETTUCE PHYTOCHEMICAL CONTENTS, BIOCHEMICAL PROFILES AND PHYSICAL PROPERTIES

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MASTER OF SCIENCE

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By

SHAILA A/P MOHANA DASS

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ABSTRACT

APPLICATION OF ENZYME-DIGESTED SOY PROTEIN HYDROLYSATE ON HYDROPONIC-PLANTED LETTUCE: EFFECTS ON LETTUCE PHYTOCHEMICAL CONTENTS, BIOCHEMICAL PROFILES AND PHYSICAL PROPERTIES

SHAILA A/P MOHANA DASS

The global population is growing exponentially over time and it is vital to produce much more food to meet the increase in demand for food. Similarly, more traditional agricultural arable lands are diminishing due to excessive infrastructure development, extreme climate change, and natural disasters. Advanced cultivation technologies have been developed and practiced worldwide to overcome challenges such as climate change as well as to boost sufficient food production to meet the rising demands as well as to improve food security. Hydroponic farming has been reported as one of the agricultural methods of growing crops indoors, especially in urban areas and it has become an alternative approach for food production to cope with food supply challenges as well as helps to decrease the need for more land farming. The hydroponic system is a modern agriculture method of growing crops using minerals-rich nutrient solutions instead of soil where the plant roots are suspended in the nutrient solution. Generally, parameters such as nutrient solution pH and electrical conductivity (EC) are set to the desired value according to the crop type in the hydroponic system to achieve optimal crop growth. Protein hydrolysate is a mixture of various sizes of peptide fragments and free amino

acids produced through hydrolysis of a protein-rich source. Protein hydrolysate can be produced from animal or plant protein that can further be used as bio-stimulants in agriculture to stimulate crop cultivation. On the other hand, soybean waste is also known as Okara, a by-product of soy milk and soy tofu production. Currently, soybean waste is utilized as food additives, animal feed as well as a source of fertilizer. A considerable amount of this soybean waste is also discarded in landfills which causes environmental problems. At present, the application of soybean waste in hydroponic planting remains unexplored.

In this study, soybean waste-derived protein hydrolysate was produced via an enzymatic hydrolysis approach by using protease (alcalase). After the enzymatic hydrolysis process, the prepared protein hydrolysate was tested on hydroponic-planted green coral lettuce. The effects of soy protein hydrolysate (SPH) as a hydroponic nutrient supplement were assessed by determining the physical properties, phytochemical contents, and biochemical profiles of the hydroponic-planted lettuce. Based on the results obtained, the length and fresh weight of the hydroponic-planted lettuce were at a peak when treated with 0.01 mg/mL of SPH whereas, for other physical properties such as lettuce leaf surface area, root length, and root weight, no significant difference was detected. There were increasing concentrations of total phenolic content (TPC), total flavonoid content (TFC), and total hydroxycinnamic acid content (THC) observed in lettuces that were treated with 0 to 0.01 mg/mL of SPH compared to control. Whereas, lettuce treated with 0.01 mg/mL of SPH has the highest Vitamin C content. In addition, the concentrations of chlorophyll *a*, chlorophyll *b*, and carotenoid in hydroponic-planted lettuce were the highest when treated with 0.01

mg/mL of SPH compared to control. The presence of five mineral elements (iron, copper, zinc, magnesium, and calcium) was detected in all lettuce. However, there was no significant difference detected in mineral contents of SPH-treated hydroponic-planted lettuces, compared to the control group except for a higher magnesium content detected in lettuce samples treated at the highest SPH concentration (0.1 mg/mL). Lastly, higher catalase and superoxide dismutase contents were detected in lettuces treated with SPH concentrations ranging from 0.001 to 0.1 and 0.01-0.1 mg/mL, respectively, compared to the control group.

KEYWORDS: Food security; phytochemical; nutritional contents; biochemical profile; hydroponic; protein hydrolysate; soybean waste

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APPROVAL SHEET

This dissertation entitled **"APPLICATION OF ENZYME-DIGESTED SOY PROTEIN HYDROLYSATE ON HYDROPONIC-PLANTED LETTUCE: EFFECTS ON LETTUCE PHYTOCHEMICAL CONTENTS, BIOCHEMICAL PROFILES AND PHYSICAL PROPERTIES"** was prepared by **SHAILA A/P MOHANA DASS** and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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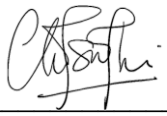

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Date: 10/2/2022

SUBMISSION OF DISSERTATION

It is hereby certified that **SHAILA A/P MOHANA DASS, (ID No: 20ADM01091)** has completed this dissertation entitled "**APPLICATION OF ENZYME-DIGESTED SOY PROTEIN HYDROLYSATE ON HYDROPONIC-PLANTED LETTUCE: EFFECTS ON LETTUCE PHYTOCHEMICAL CONTENTS, BIOCHEMICAL PROFILES AND PHYSICAL PROPERTIES**" under the supervision of Dr Wong Fai Chu from the Department of Chemical Science, Faculty of Science, and Dr Chai Tsun Thai from the Department of Chemical Science, Faculty of Science.

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Yours truly,

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DECLARATION

I **SHAILA A/P MOHANA DASS** hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

(SHAILA A/P MOHANA DASS)

Date: 10/2/2022

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BSA	Bovine Serum Albumin
PBS	Phosphate Buffer Saline
EC	Electrical Conductivity
DCPIP	Dichlorophenol indophenol
OPA	o-phthalaldehyde
EDTA	Ethylenediaminetetraacetic acid
DNA	Deoxyribonucleic acid
ROS	Reactive Oxygen Species
NBT	Nitroblue Tetrazolium
NFT	Nutrient Film Technique
SPH	Soy Protein Hydrolysate
TPC	Total Phenolic Content
TFC	Total Flavonoid Content
THC	Total Hydroxycinnamic acid Content
FAAS	Flame Atomic Absorption Spectrometer
CAT	Catalase
SOD	Superoxide Dismutase
LECA	Lightweight Expanded Clay Aggregate
H_2O_2	Hydrogen Peroxide
HNO_3	Nitric Acid
Chl $_a$	Chlorophyll a
Chl $_b$	Chlorophyll b
C_{x+c}	Carotenoids
Ca	Calcium
Cd	Cadmium
Cu	Copper
Fe	Iron
Zn	Zinc
Mg	Magnesium
Pb	Lead

Al	Aluminium
P	Phosphorus
K	Potassium

CHAPTER 1

INTRODUCTION

1.1 Background information and Introduction

The global population is growing exponentially over time and it is vital to produce much more food to meet the increasing demands for food. In the year 2019, According to the United Nations, by 2050, the global population will have surpassed 9 billion people, demanding a 70% increase in overall food production above current levels to ensure sheltered food security. (Za, 2020). Food security challenges develop when a country cannot afford to feed its rising population, and it can only be achieved when everyone has access to inexpensive, sufficient, high-quality, and nutritious food on a daily basis (Naharul, 2021). According to Firdaus et al. (2020), more traditional agricultural arable lands are diminishing due to excessive infrastructure developments whereas remaining food production activities and crop yields are reducing due to extreme climate change and natural disasters such as long droughts and floods that unfortunately contribute to a massive uncertainty to the food production activities.

The majority of environmental experts agree that traditional farming activities will drop drastically in the next 50 years as excessive deforestation sadly leads to the acceleration of extreme climate change. This would disrupt the balance of the carbon cycle and will get even worse if nothing is being done on

a global scale (Despommier, 2011). Recently, well-engineered greenhouse technology has evolved to provide a safer and more reliable agriculture alternative to produce nutritious crops all year round regardless of the weather or location (Besthorn, 2013). A controlled environment agriculture culture should be emphasized and practiced in both rural and urban areas in a country to overcome the food production challenges. Hence, the hydroponic system is a soilless crop cultivation technology where the crops are grown by suspending the plant roots directly to the mineral-rich nutrient solution under a highly controlled environment in which the temperature, light, humidity, air, and water levels, as well as the nutrient solution pH, are constantly monitored (Za, 2020).

Compared to traditional soil agriculture, a hydroponic system reduces water consumption, reduces the usage of pesticides, prevents pest infections, and protects the crop from unfavorable weather. In addition, since there is no dependence on climate, seasonal crops can be produced all year round which leads to a continuous supply of food to the consumers throughout the year (Chow, et al., 2017). The hydroponic system ensures food safety because hydroponic farming has the potential to produce contamination-free crops as the crops are fully grown in a controlled environment which is less likely for the crops to get infested by pests or pathogens. After all, soil-grown crops easily tend to be infected by pathogens that cause foodborne illness because the soil itself contains pathogens including animal droppings and toxins run-off (Beecher, 2020). However, the hydroponic planting system is also known as vertical farming and this evolution of agriculture activity has attracted

supporting attention from a few global regions including Japan, China, the United Kingdom, Singapore, South Korea, Canada, and Italy (Besthorn, 2013).

On the other hand, organic waste production is increasing worldwide from various sources but mostly from the food industries, and these organic wastes can be utilized wisely to improve fertility and compensate for the nutrient levels in the soil (Chiew, et al., 2015). For instance, in Asian countries manufacturing of soy milk and tofu generates a large quantity of soybean by-product which is often regarded as waste (Khare, et al., 1995). Currently, this soybean waste is mainly being used either as fertilizer or animal feed, and in worst cases, it is simply dumped in the landfills. Meanwhile, discarding large quantities of soybean waste causes environmental odor problems because soybean waste is high in moisture content that causes them susceptible to decomposition (Almaraz, et al., 2009). According to Anbu and Saranraj (2016), soybean meal, also called soybean waste is used as fertilizer to cultivate crops because soybean waste releases nitrogen into the soil which improves soil fertility and increases crop yields. In addition, soybean waste is rich in fiber and protein that help in the growth and development of crops.

According to Kielland et al. (2006), soil predominantly contains low molecular mass protein as nitrogen, and plants easily uptake amino acids as the nitrogen sources for growth and development. However, proteins and peptides are insufficiently known as potential nitrogen sources for plant growth. Besides,

since proteins are interwoven with cellular structures and execute specialized tasks such as hormones, antibodies, and enzymes, they are a vital component in all organisms. Intact proteins are breakdown into protein hydrolysate by digestive enzymes such as pepsin, trypsin, chymotrypsin, bacterial and fungal peptidases like alcalase and flavourzyme via enzymatic hydrolysis to produce peptide fragments and amino acids (Bhat, et al., 2015). Enzymatic digestion of protein hydrolysate can be used to make animal supplements or as a bio-stimulant in crop cultivation (Silva, 2017). Maize plants treated with protein hydrolysate grew faster than maize plants cultivated with inorganic nitrogen, according to a prior study. Protein hydrolysate also serves as a source of nutrients, boosting soil fertility and enhancing biological activity and nutrient cycling (Santi, et al., 2017).

In this study, soybean processing wastes are selected to test, as it is commonly available in desired quantity in the common food industry and daily cooking processes, and soybean is a potential source of high protein content. Hence, soybean waste-derived protein hydrolysates were produced via an enzymatic hydrolysis approach by using protease such as alcalase. The prepared soy protein hydrolysate (SPH) was tested on hydroponic-planted Green Coral Lettuce. Lettuce was chosen because of the relatively shorter growing period. The effect of SPH as an effective hydroponic nutrient supplement was assessed firstly by the physical properties of the hydroponic-planted lettuce because it is a directly linked factor to hydroponic yield and profitability. In addition, to assess the effect of SPH-treated lettuce on the phytochemical contents and biochemical profiles were determined using several biochemical assays such as

total phenolic, flavonoid, hydroxycinnamic, vitamin C contents, mineral contents using flame atomic absorption spectrometer (FAAS) as well as catalase, and superoxide dismutase assays to test for the levels of catalase and superoxide dismutase, respectively.

1.2 Research Rationale

Protein hydrolysates have been derived from many different plant and animal sources through various protein hydrolysis methods. Many studies showed that these plant-derived and animal-derived protein hydrolysate are being used as bio-stimulant and supplements to enhance the growth and yearly yield of soil cultivated crops. The use of protein hydrolysate as a nutrient supplement for hydroponic plants has not been much reported in the literature and there is still a wide range of unclear statements to discover and study the benefits of using enzymatically hydrolyzed protein hydrolysate in growing hydroponic crops. In addition, there is limited information available on the effect of protein hydrolysate on the phytochemical content, biochemical profile as well as physical properties of hydroponic-planted crops. Besides, organic waste from food industries such as soybean processing waste is discarded in landfills in large amounts apart from using it as fertilizer or animal feed. The discarded soybean waste causes environmental odor problems due to rapid decomposition. Therefore, soybean waste has been chosen as the source to derive protein hydrolysate as well as to utilize the protein-rich potential bio-stimulant.

1.3 Objectives

1. To investigate the effects of soy protein hydrolysate on the physical parameters of lettuces cultivated in hydroponic system.

2. To investigate the effects of soy protein hydrolysate on the phytochemical contents of lettuces cultivated in hydroponic system.

3. To investigate the effects of soy protein hydrolysate on the biochemical profiles of lettuces cultivated in hydroponic system.

CHAPTER 2

LITERATURE REVIEW

2.1 Hydroponic System

Agriculture is the major source of economic backbone in developing countries worldwide. As the world population is increasing year after year, the concern about the ability of agricultural activities to keep up with the increase in food demand arises. The existing traditional soil agriculture system might not be able to fulfill the demand for food supplies in the future as the system is facing challenges such as soil infertility, deforestation, soil erosion, and limited arable agricultural lands (Khan, et al., 2018). In addition, the crop production yield decreases due to several factors including decreasing irrigation, declining investments in agricultural research, and infrastructure, as well as increasing water scarcity. In warmer and tropical environments, climate change results in drastic rainfall events between dry periods and reduces water resources for irrigation promoting more pests and diseases on crops and soil. Hence, advanced crop cultivation technologies have been developed and practiced worldwide to overcome challenges such as climate change as well as to boost sufficient production to meet the rising food demands to ensure and improve food security (Rosegrant & Cline, 2003).

Hydroponic farming has been reported as one of the agricultural methods of growing crops indoors, especially in urban areas, and has become an alternative approach for food production to cope with food supply challenges and helps to decrease the need for more land farming (Gumisiriza, et al., 2020). The hydroponic system is a modern agriculture method of growing crops using minerals-rich nutrient solutions instead of soil (Silva, et al., 2018). According to Lakkireddy et al. (2012), a hydroponic system is also known as soilless agriculture or water culture and the term ‘hydroponic’ is derived from a Greek word in which ‘hydro’ means water and ‘ponics’ means labor. Hydroponic systems are more reliable compared to traditional soil agriculture as the hydroponic system can be utilized fully in urban areas which particularly provides food assessability to the consumers living in the cities which indirectly leads to the reduction of environmental interferences, the effects of climate change, food exportation loads and carbon emissions from the transportations (Verma & Sanjay, 2020).

In a hydroponic system, the roots of plants are suspended in a minerals-rich nutrient solution and it is reported that crops grown in a hydroponic system have higher yield percentages than soil-planted crops because all the minerals required for the crop growth are directly provided to the plant roots. Therefore, a hydroponic system can increase productivity to overcome food demands (Gashgari, et al., 2018). Inert mediums such as rock-wool, peat moss, coconut fiber, gravel, perlite, and lightweight expanded clay aggregate (LECA) pebbles

are generally used to provide mechanical support to the growing plants and various types of crops including leafy vegetables, peppers, tomatoes, cucumbers, strawberries and many more can be grown hydroponically (Sharma, et al., 2018). To promote optimal crop growth in a hydroponic system, characteristics such as nutrient solution pH and electrical conductivity (EC) are often tuned to the desired value based on the crop type (Palande, et al., 2018).

Many crops have been successfully grown in a hydroponic system with a positive outcome. Based on a study performed by Abdelmawgoud et al. (2021), the report showed that the tomato grown hydroponically has a higher production yield compared to the conventional soil agriculture method. They also reported that the production of tomatoes in hydroponics depends mainly on the nutrient solution and growing conditions such as solution pH. In addition, tomatoes that are grown in three different systems such as soil, drip irrigation, and deep-water culture showed that there were less water transpiration and more water efficiency in the two hydroponic systems whereas the levels of β-carotene and lycopene, as well as the quality of deep-water cultured tomatoes, was higher than the other two systems (Verdoliva, et al., 2021). Leal et al. (2020) reported a linear increase in hydroponically grown spinach leaves fresh weight and sodium concentration due to the increase in nutrient solution salinity. In addition, two ornamental plant species namely *Globba schomburgkii* Hook. F. and *Globba marantina* L. were grown using hydroponic nutrient film technique (NFT) and soil, showed higher leave area, shoot length, and stem diameter, have more flowers as well as higher

stomatal conductivity in hydroponic conditions (Phantong, et al., 2018). Photosynthetic pigments content including chlorophyll *a*, chlorophyll *b*, and carotenoid has shown a remarkable increase in Red Flash caladium plantlets grown in the hydroponic system. Also, the increase in several morphological characteristics such as the number of leaves and diameter of leaves are highly related to the increase in chlorophyll content in the hydroponic plantlet leaves (Yuan-Shan, et al., 2019).

2.2 Types of Hydroponic Systems

The hydroponic system is used in various techniques according to the use of the nutrient solution and supporting media where they are categorized into two groups, open and closed systems. The nutrient solution in an open system is not reused or recycled and vice versa. However, closed systems are more sensitive to water salinity and cost-effective than open systems (Jensen, 1999; Lippert, 1993). In addition, each technique is slightly diverse from one another to optimize the growing conditions for plants. Commonly use hydroponic systems are the ebb and flow, drip, wick, deep-water culture or Kratky method, nutrient film technique (NFT), and aeroponic (Sharma, et al., 2018; Lee & Lee, 2015; Kratky, 1993).

2.2.1 The Ebb and Flow System

The ebb and flow system was one of the earliest commercially available closed-hydroponic systems. It works on the flood and drains principle in which the plants on the grow bed are momentarily flooded with the nutrient solution through a water pump and stay on the grow bed at a certain level for some time to provide the plants with nutrients and moisture before draining out into the reservoir for recirculation. This system required continuous observation to control the level of water provided to the system. Besides, various kinds of plants can be grown using the ebb and flow system, however, a system with a filtration unit is required to prevent root rot, mold, and algae-related problems (Buwalda, et al., 1994; Nielsen, et al., 2006). One of the major drawbacks of the ebb and flow system is the host infections caused by bacteria, viruses, and fungal pathogens that can migrate through the recirculating system. However, previous research has shown that when compared to spray systems, the danger and severity of disease spread are lower (Ferrarezi, et al., 2015). Other than the filtration method, heat, pressure, and ultraviolet (UV) radiation can be used to eliminate infectious pathogens in the recirculated nutrient solution (Martínez, et al., 2010).

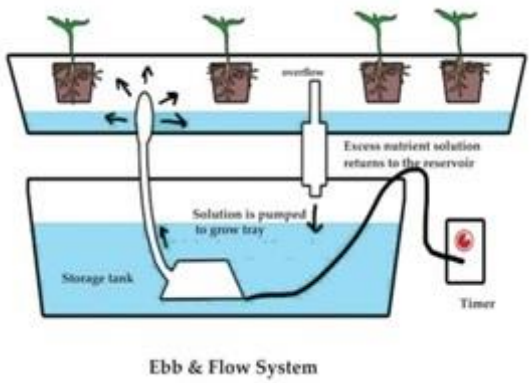

Figure 2.1: Diagram illustration of the ebb and flow system (Sharma, et al., 2018).

2.2.2 The Drip System

The drip system is also known as the drip irrigation system is another type of hydroponic system that is widely used in both home and commercial cultivations to grow long-term crops where an appropriate amount of nutrient solution from the reservoir is delivered to each plant through drip emitters on a timed basis (Rouphael & Colla, 2005). For instance, the growing medium will be flushed with nutrient solution approximately 10 minutes every hour providing the plants with fresh nutrients, oxygen, and water. A reservoir drain line is set below the pot of perlite or rock-wool where the plants are planted to collect the excess nutrient solution dripping out (Sheikh, 2006).

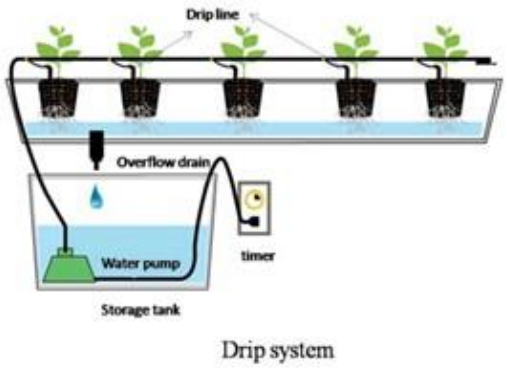

Figure 2.2: Diagram illustration of the drip system (Sharma, et al., 2018).

2.2.3 The Wick System

The wick system is the most basic hydroponic system since it does not rely on power to provide the nutrient solution to the plant growing medium (Sharma, et al., 2018). However, the nutrients are supplied to the plants through capillary action via nylon wick between the nutrient solution reservoir and plant roots that are placed in an absorbent medium such as perlite or coco coir. In addition, the wick system works well for plants that require less water such as small plants, herbs, and spices (Son, et al., 2006). As the wick system operates in a closed method, it avoids nutrient runoff and permits high water and nutrient use efficiency. Besides, optimum wick system conditions including wick length and width, water depth for wick contact, growing containers that prevent water evaporation as well as the composition of growing medium for root wetting and moisture maintenance contributes to the quality and uniformity of plants productions (Son, et al., 2006; Kang, et al., 2009; Semananda, et al., 2018).

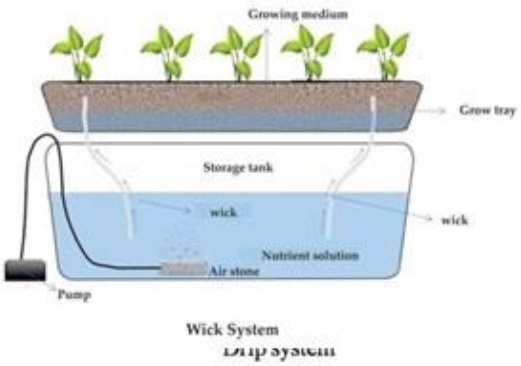

Figure 2.3: Diagram illustration of the wick system (Sharma, et al., 2018).

2.2.4 Deep-water culture and Kratky method

The nutrient solution is continuously supplied to plant roots in a deep-water culture and Kratky system, ensuring that the plant roots are always submerged in water and oxygen. Therefore, the system is highly oxygenated and requires less maintenance and monitoring time (Saaid, et al., 2013; Kratky, 1993). The nutrient solution must be brought to the pH required for the particular plant before transplanting the plant into the system to produce good quality plants (Spinu, et al., 1998). The difference between deep-water culture and the Kratky method is that the nutrient solution in deep-water culture is aerated using an air pump whereas in the Kratky method the solution is non-aerated (Ali, et al., 2021).

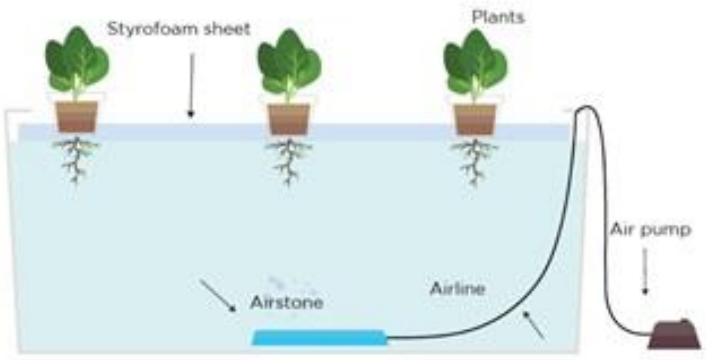

Figure 2.4: Diagram illustration of the deep-water culture (Max, 2021).

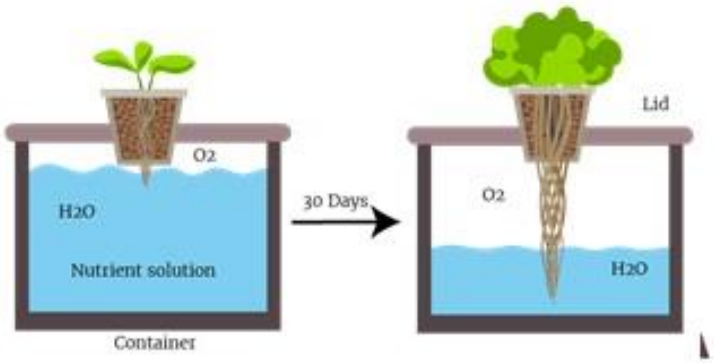

Figure 2.5: Diagram illustration of the Kratky method (Max, 2021).

2.2.5 Nutrient Film Technique (NFT)

In the Nutrient Film Technique, plants are placed in tubes or mesh pots and their roots are exposed hanging to the recirculating nutrient solution. Furthermore, the growing tray is slanted to allow a thin film of nutrient solution to run through the plant roots, keeping them moist and draining back into the reservoir, and the nutrient solution circulates the growth tray constantly via a water pump with no timer. (Domingues, et al., 2012). The system can be widely

adjusted for both short- and long-term crop production such as lettuce, herbs, leafy vegetables, cucumber, and tomatoes (Mohammed & Sookoo, 2016).

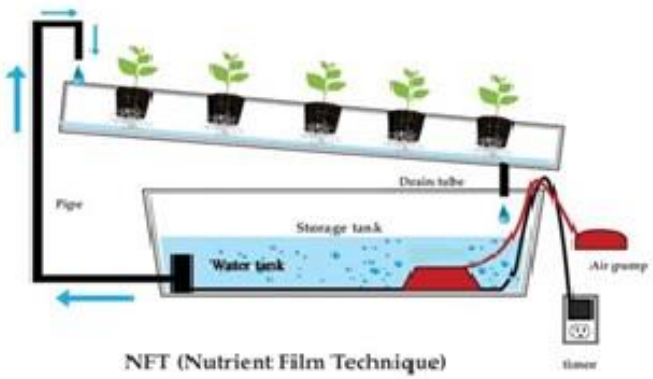

Figure 2.6: Diagram illustration of the nutrient film technique (Sharma, et al., 2018).

2.2.6 The Aeroponics System

Aeroponics is an air-water culture cultivation technique in which plants grow in the air with the help of artificial support, without the use of soil or medium, and plant roots are exposed to a misted nutritional solution via atomization nozzles (Sheikh, 2006; Osvald, et al., 2001). Hence, the system uses a limited amount of nutrient solution in recirculation, it can conserve water and energy input. The oxygen in the air of the aeroponics system is a few times more than in other hydroponics systems that optimize root aeration which helps the plant and the root system to develop faster (Ritter, et al., 2001). The size of the nutrient solution droplets impacted the rate of plant growth, with finer droplets

allowing more oxygen to reach the root system and promoting long-term lateral root growth. This system requires a constant power supply to run the system and interruption in the power supply may lead to irreversible damages to the plants (Lakhiar, et al., 2018).

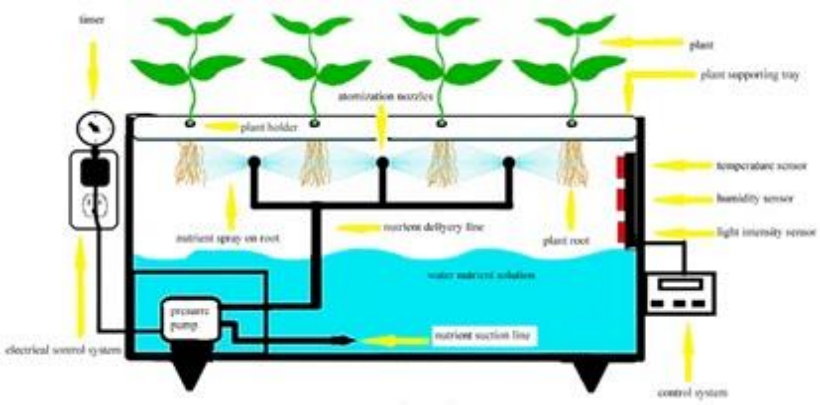

Figure 2.7: Diagram illustration of the aeroponics system (Lakhiar, et al., 2018).

2.3 Nutrient Solution Compositions and Nutrient Availability in The Solution

The nutrient solution's composition has a big impact on the quality and yield of hydroponically grown crops (Valentinuzzi, et al., 2015). The water-based nutrient solution is composed of various minerals that are necessary to support the crop growth while the crops are cultivated with organic media such as rock wool and inorganic media such as vermiculite, LECA pebbles, or perlite to provide support to the plant (Sharma, et al., 2018). It is reported that the

nutrient equilibrium in the solution could be affected by several factors including the temperature of the solution. Besides, the physiological levels of the crop are impacted in locations where the temperature is somewhat higher due to an increase in the temperature of the fertilizer solution. (Ilahi, et al., 2017). The nutrient solution can be provided in a closed system where the solution is recirculated in the system or drained after a single use in the open system (Song, et al., 2004). Recycling the nutrient solution helps in water and biostimulant savings. However, reusing exhausted nutrient solutions in the system will lead to a shortage of some key macro-and micronutrients, and increase solution salinity which might cause problems in crop development and growth (Carvalho, et al., 2018; Carmassi, et al., 2005).

In a hydroponic system, proper support of pH and electrical conductivity (EC) of the nutrient supplement arrangement is required because they affect mineral accessibility for plants to achieve optimal growth and execution. In this way, the pH and EC of the nutrient supplement arrangement are powerful tools that aid harvested crops. (Gruda, 2009). The optimum range of pH and EC values vary according to the plants and in general, leafy vegetables grow well in nutrient solutions with pH 5.5 to 6.5 and EC 1.5 to 2.5 dS m^{-1} (Kim, et al., 2005; Sharma, et al., 2018). However, the nutrient solution with lower pH inhibits plants growth due to nutrient deficiency in the solution whereas higher EC prevents nutrient absorption by the plant roots due to changes in the nutrient solution osmotic pressure in which both situations severely affect plant performance and yield

(Savvas & Gruda, 2018; Sambo, et al., 2019). Besides, the pH of the nutrient solution is frequently changing due to the uptake of water and nutrients by the plants and is adjusted using acidic or alkaline solutions (Kim, et al., 2005). Saturation conditions can occur when specific cations and anions in a solution approach their maximum concentration limit, as the cations and anions in an aqueous solution experience the precipitation reaction, generating precipitates. This condition can be influenced by factors including surrounding temperature that causes water evaporation, the nutrient solution pH, and EC where cations form insoluble hydroxides at lower pH whereas at higher pH macronutrients such as calcium and magnesium can precipitate as carbonates. Therefore, the pH and EC of the nutrient solution must be constantly monitored to prevent nutrient saturation which can affect the physiological state of crops (Sambo, et., 2019; Tomasi, et al., 2015).

Studies that have been conducted on the nutrient solution reported that there are approximately 17 elements categorized as macro and micro-nutrients required for proper plant growth. Plants obtain components like hydrogen, carbon, and oxygen from the air and water, while the remainder of the nutrients is normally found in the soil or, in the case of hydroponics, the nutrient solution. (Khan, et al., 2018). These nutritional solution ingredients and their roles are summarised in Table 2.1 below.

Table 2.1: The elements of nutrient solution and their function in plant growth (Khan, et al., 2018).

Elements	Function
Nitrogen	The major component of chlorophyll, amino acids, and protein that involve in photosynthesis
Phosphorus	Photosynthesis and plant growth
Potassium	Enzyme activation and regulate ATP production
Hydrogen	Photosynthesis and plant respiration
Oxygen	Cellular respiration
Carbon	Photosynthesis, respiration, and formation of organic compounds for plant growth
Calcium	Plant tissue formation and cellular activities
Magnesium	Enzyme activation
Sulfur	Involve in the defense of plants against oxidative stress
Iron	Component of enzymes and pigments, involved in photosynthesis
Boron	Regulate plant hormones and growth
Chlorine	Used in photosynthesis
Copper	Involve in photosynthesis and respiration
Manganese	Plant growth and development
Zinc	Used in photosynthesis and enzyme activation
Molybdenum	Involve in nitrogen fixation
Cobalt	Involve in plant growth and development

2.4 Advantages and Disadvantages of Soil and Hydroponic Planting Systems

Soil agriculture has been practiced for many centuries and it is claimed as the traditional method for cultivating crops. The occurrence of the agriculture revolution has shifted the attention to better alternative cultivation methods to reach the demand for food as the global population increase year by year. However, soil agriculture has its advantages and disadvantages for growing crops where soil provides a supportive medium for crop roots to retain water and supply required nutrients for crop production (Atkinson, et al., 2005). Soil plating system can be set up at a low cost where there are no high maintenance technology systems required and it is stated that crop grown in soil, in this study, strawberry has higher fresh weight and moisture content (Treftz & Omaye, 2015). According to Verdoliva et al. (2021), soil agriculture required more water used due to the frequent watering of the crops, and most of the water runoff or disperse through the soil result in lower efficiency of water intake by crops. Hence, water conservation is difficult to achieve. In addition, excessive water supply to the soil can wash away the fertilizers or pesticides on the soil into water resources and may cause pollution. More labor force is required in soil agriculture due to activities such as watering, weeding, or spraying fertilizer or pesticides. Moreover, crops grown in soil have a lower survival rate due to soil-borne diseases or pest outbreaks (Treftz & Omaye, 2015).

The hydroponic system has known as an effective modern agriculture technique for crop cultivation. However, many key factors need to be taken into consideration when setting up a hydroponic system as the system exhibits several advantages and disadvantages in terms of growing crops, resource management as well as system maintenance. Hydroponic planting has more benefits than drawbacks in which a hydroponic planting system enhances crop productivity by increasing crop yield. High crop productivity can be achieved when the growing systems are uniform and controlled providing optimum growing conditions. In addition, the hydroponic system can control plant nutrition accurately in which the important nutrients required for the plant growth and development are directly provided to the plant root causing the plant to grow faster and thus, increasing the yield. Hence, it is shown that hydroponic crops have a faster growth rate compared to soil cultivation (Bello, et al., 2019; Silberbush & Ben-Asher, 2001; Savvas, 2003). Besides, water is one of the most vital resources in agriculture and the hydroponic system uses less water for irrigation as the nutrient solution is recirculated in the system and there is no frequent crop watering activity involved. Hence, water is conserved in a hydroponic system compared to soil agriculture (Maucieri, et al., 2018).

According to Barbosa et al. (2015), the system is very versatile and has a wide range to easily set up in a backyard as well as as a highly maintained commercial enterprise. Various crops such as tomatoes, peppers, cucumbers, strawberries, leafy vegetables, and many more can be grown using hydroponics.

Crops grown hydroponically are not influenced by climate and therefore, they can be produced year-round without depending on the weather or season. This factor leads to an increase in crop production (Manzocco, et al., 2011). Furthermore, several traditional agricultural practices such as watering, weeding, and spraying pesticides can be eliminated in a hydroponic system and that indirectly leads to the reduction of labor forces. In conjunction with no weeding activity existing in a hydroponic system, it helps to reduce the problem of pest and soilborne diseases spreading to the hydroponic crops while increasing the lifespan as well as the quality of the crop. (Jovicich, et al., 2003). Despite many benefits, a hydroponic system has some limitations including higher initial system setup cost as well as the need for fundamental technical knowledge to properly operate a hydroponic system at a commercial level. On the other hand, water-borne diseases can be easily spread among the plants as the hydroponic plants share the same nutrient solution (Sharma, et., 2018; Ikeda, et al., 2002). The frequent monitoring of power supply, nutrient solution pH, and EC, as well as the supply of appropriate nutrients to the crops, are of prime importance in a hydroponic and neglecting these components might limit the crop production and can result in the loss of crops (Sardare & Admane, 2013).

2.5 Green Coral Lettuce

Lettuce, *Lactuca sativa* L., a member of the Asteraceae family, is a commonly consumed vegetable produced mostly for its fleshy leaves. Although lettuce is widely consumed, it was formerly rejected as a nutritious diet because

of its high water content. (about 95%). Lettuce can be classified into different groups based on diversity including the color, shape, and size of the leaves. These diversities in lettuce are due to morphologically similar traits and genetic similarities. (Kim, et al., 2016). According to Křístková, et al. (2008), lettuce can be grouped into six types depending on the leaf size, shape, texture, head formation, and stem. They are called butterhead lettuce (*Lactuca sativa var. capitata*), romaine lettuce (*Lactuca sativa var. longifolia*), and crisphead lettuce (*Lactuca sativa* L.), leaf or cutting lettuce (*Lactuca sativa var. acephala*), stem or stalk lettuce (*Lactuca sativa var. angustana* Irish) and Latin lettuce. Particularly, green coral lettuce has loose curly bright green leaves (Figure 2.7) and is generally added to salads and eaten raw which helps the nutrients to retain compared to other vegetables such as potatoes that are preferably cooked before consumption (Xiao, et al., 2012). It is reported that lettuce is low in total calories such as sugar alcohols and saturated fatty acids (SFAs) but contains polyunsaturated fatty acids (PUFAs) as well as rich in dietary fiber, which is beneficial for health. As a result, lettuce is frequently included in diets to help with weight loss, cardiovascular disease prevention (by lowering LDL cholesterol and blood pressure), diabetes prevention (by increasing glucose metabolism), and colon cancer prevention (by increasing glucose metabolism). However, lettuce, like most other green vegetables, is low in protein. (Kaur, et al., 2014; Hansen, et al., 2012; Yao, et al., 2014).

Figure 2.8: Image Green Coral Lettuce, *Lactuca sativa* L. (Tadimalla, 2021).

Minerals from the diet are necessary for optimal health and metabolism. For example, sodium (Na) and potassium (K) are essential for maintaining body water and electrolyte balance, calcium (Ca), magnesium (Mg), and phosphorus (P) are essential for bone health, and iron (Fe) and zinc (Zn) are essential for hemoglobin formation, oxygen transport, cellular function, and immune and antioxidant function (Soetan, et al., 2010; Gupta & Gupta, 2014; Kloubert & Rink, 2015). Apart from the minerals needed for good health, lettuce has a lower contribution of Na, K, Ca, P, Mg, Fe, and Zn than other vegetables, according to studies. On the other hand, it is a good source of K, Mg, and Fe. The difference between minerals contributions among different types of lettuce can be due to the general genetic and morphological state, cultivation conditions, soil properties, or the growing seasons (Baslam, et al., 2013).

Vitamins are essential micronutrients that play important roles in body metabolism. Vitamins such as folate (vitamin B9), vitamin C, and E are commonly found in lettuce. Folates play important roles in single-carbon transfer reaction, DNA synthesis, and reducing the risk of birth defects whereas folate deficiency affects cellular functions, growth and development as well as increases the risk of cancer (Scott, et al., 2000; Shohag, et al., 2012). According to Wang et al. (2013), When compared to other vegetables, lettuce is a good source of folate. Vitamin C, commonly known as ascorbic acid, is a water-soluble vitamin that is necessary for the immune system, metabolism, and antioxidant properties. Vitamin C can be easily excreted from the body due to its higher soluble in water. Therefore, a regular intake of dietary is important to sustain the vitamin C in the body (Carr & Frei, 1999; Gallie, 2013). In a study by Llorach et al. (2008), Despite having a lower vitamin C level than other green vegetables like kale and spinach, lettuce is a particularly strong source of vitamin C, according to the study. Furthermore, α-tocopherol is the most abundant and biologically active form of vitamin E. Vitamin E is a lipid-soluble antioxidant that aids in the prevention of heart disease, degenerative illnesses, and cancer (Knecht, et al., 2015). Besides, vitamin E is present in the forms of α- tocopherol and γ- tocopherol in the lettuce (Nicolle, et al., 2004). On the other hand, Vitamin A comes in two forms: retinol, which comes from animal sources, and carotenoids, which come from plant sources. Generally, vitamin A is important for the immune system, reproduction, vision, and embryonic development while the antioxidant properties of carotenoids help in reducing the risk of chronic diseases (Chapman, 2012). Carotenoid is a fat-soluble pigment found in yellow-orange fruits and vegetables, as well as dark-green leafy vegetables (Maiani, et

al., 2009). Mou (2005), stated that the primary carotenoids in lettuce are β-carotene and lutein. In his study, the level of these chemical compounds was higher in the outer leaves than in the inner leaves, probably due to higher exposure to light intensity, which influences carotenoid production. As a result, lettuce is touted as a good source of lutein and β-carotene.

Secondary metabolites in plants aid in plant defense and influence the sensory and nutritional properties of plant-based foods (Dai & Mumper, 2010). Phenolic compounds are classified into two groups; phenolic acids and flavonoids depending on their chemical structures (Karakaya, 2004). These phenolic compounds have greater antioxidant properties compared to vitamin C and are effective against inflammation, oxidative stress, diabetes, cancer, cardiovascular diseases, and age-related neurodegeneration (Seeram, et al., 2006; Shukitt-Hale, et al., 2009). The phenolic content in lettuce is majorly represented as phenolic acids and flavonoids are the minority. Chlorogenic acid and caffeic acid are common phenolic acids found in lettuce, as are flavonoids such anthocyanins, quercetin, flavone luteolin, and kaempferol derivatives. The amount of total phenolic acid and flavonoids in lettuce varies depending on whether the leaves are green or red (Llorach, et al., 2008) and the cultivation conditions, for instance, lettuce grown in open areas has a higher total phenolic content due to more exposure to light intensity and UV radiation to enhance photosynthesis rates (Zhao, et al., 2007). It is reported by Luna et al. (2013), that

red leave lettuce has a higher total phenolic content compared to green leave lettuce.

2.6 Protein Hydrolysate as a Potential Bioactive Peptide

Protein hydrolysate is a mixture of various sizes of peptide fragments and free amino acids, the building blocks of protein produced through partial hydrolysis of a protein-rich source. These oligopeptides are inactive inside the sequence of a protein molecule until they are activated by metabolic activities. Based on their specific sequence composition, bioactive peptides have greater activity and health benefits than native protein molecules, including antioxidant, antihypertensive, antimicrobial, anti-obesity, antioxidative, antithrombotic, and immunomodulatory properties when compared to an equivalent mixture of free amino acids (Dziuba & Dziuba, 2014; McCarthy, et al., 2013; Umayaparvathi, et al., 2014). Protein hydrolysate characteristics are mostly determined by the molecular size, amino acid content, and amino acid sequence that make up the protein's structure (Putra, et al., 2018). During the hydrolysis process, the peptide bonds holding the protein structure are broken down leading to the release of peptide fragments and free amino acids that have higher permeability and absorbability due to the smaller molecular size and possess biological activities as stated above (Budseekoad, et al., 2018; Hou, et al., 2017; Jain & Anil Kumar, 2016). Generally, animals, plants, microbes, and marine species serve as the major protein sources in protein hydrolysate such as rice, corn, chickpeas,

soybean, milk, peanut, eggs fish, and other marine species (Malaguti, et al., 2014).

Protein hydrolysate can be produced from animal or plant protein that can further be used as biostimulants in agriculture to stimulate crop cultivation (Nardi, et al., 2016). Protein hydrolysate can be produced through chemical, enzymatic, and microbial hydrolysis. However, the protein hydrolysate produced via enzymatic and microbial hydrolysis can improve several physiological properties including the solubility, emulsification, viscosity, and gelation of the peptides and amino acids (Dieterich, et al., 2014). On the other hand, the protein hydrolysis method should be chosen based on the protein source. For instance, acidic, alkaline, or bacterial keratinases treatment will be used to hydrolyze protein from harder protein sources such as horns, beaks, wool, feathers, and keratin structure. Besides, enzymatic and microbial hydrolysis is often used to hydrolyze animal or plant-derived protein where intact microorganisms and proteases isolated from either bacteria, plants, or yeast will be used in microbial and enzymatic hydrolysis respectively (Hou, et al., 2017).

According to Dai et al. (2014), acid hydrolysis is performed at high temperatures at which the protein is treated with hydrochloric acid for a shorter period to produce peptides. The acid protein hydrolysates are mainly used as flavor enhancers in food industries. There are pros and cons of using the acid hydrolysis method in which, this process is low cost but it causes a partial loss

of certain amino acids, conversion of asparagine into aspartame, and glutamine into glutamate as well as the destruction of tryptophan. Whereas, in the alkaline hydrolysis method, a shorter period of hydrolysis time is performed at lower temperatures to generate peptides in the food industry. However, with the help of alkaline agents like sodium, potassium hydroxide, and calcium, high temperatures can be utilized to hydrolyze proteins. Alkaline hydrolysis is a low-cost method and has the highest rate of recovery of tryptophan whereas this method destroys most amino acids. Examples of products produced via alkaline hydrolysis are foaming agents as a substitute for egg proteins and fire extinguisher foams. This method is not widely used in the food industry (Pasupuleti, 2010). However, the usage of hydrochloric acid and sodium hydroxide to hydrolyze protein in both acid and alkaline hydrolysis respectively result in poor functionality and lower nutritive value of the protein hydrolysates (Kristinsson & Rasco, 2000).

Microbial and animal-derived proteases have different degrees of protein hydrolysis efficiency and specificity and therefore, choosing a protease to hydrolyze a protein should be based on the region of a peptide bond in the protein or the terminal or internal region of the protein structure. Enzymatic hydrolysis is a complex process as the accessibility of peptide bonds to the enzymes is very specific and several factors such as specificity of enzymes, temperature, and pH can affect the reaction. Besides, extreme temperature and pH could denature and deactivate the enzyme resulting in an unsuccessful enzymatic reaction

(Clemente, 2000). Several advantages of enzymatic hydrolysis are that required only mild temperature and pH, minimal side reactions, do not cause any loss of amino acids, and the enzymes used are more precise to control the degree of protein hydrolysis. In contrast, the drawback of enzymatic hydrolysis includes the presence of natural enzyme inhibitors in the raw protein sample and its high cost (Andriamihaja, et al., 2013). Alcalase is an example of protease which is produced from the bacteria *Bacillus lincheniformis* and is one of the well-performed enzymes used in fish-derived protein hydrolysate production (Muzaifa, et al., 2012).

In enzymatic hydrolysis, proteases such as alcalase are used to produce protein hydrolysate. Alcalase used in this research is purchased from Sigma-Aldrich and it is a commercial proteinase extracted from *Bacillus licheniformis*, *Subtilisin* A. The specific activity range of this commercial Alcalase is 2.4 U/g, with an ideal pH range of 6.5-8.5 and a temperature range of 60^{o}C. Previous studies from our laboratory have also shown an optimal enzyme: substrate ratio of 1: 10 for protein hydrolysate production (Chai, et al., 2015; Chai, et al., 2021). Other Alcalase characteristics and mode of action include the following: it is a serine alkaline protease that has broad specificity and hydrolyzes most peptide bonds, particularly those consisting of aromatic amino acid residues. Alcalase hydrolyzes peptide bonds on the carboxyl side of phenylalanine, tyrosine, tryptophan, glutamic acid, methionine, leucine, lysine, and glutamine (Doucet, et al., 2003).

Microorganisms hydrolyze the extracellular protein into peptides and free amino acids via protease hydrolysis. Microorganisms release protease to hydrolyze extracellular proteins present around them. Protein fermentation is divided into two types, solid and liquid-state. In solid-state, the fermentation is performed under low-moisture conditions which can help to shorten the time for the protein hydrolysate to dry whereas, in liquid-state fermentation, the process is carried out under high moisture fermentation conditions (Smid & Lacroix, 2013). According to Hou et al. (2017), the advantages of microbial hydrolysis to generate protein hydrolysate are the removal of anti-nutritional and hyper-allergic factors from the protein during the hydrolysis reaction. However, relatively high cost, maintenance as well as inconsistency in the peptides and free amino acids production due to the changes of microbial activity under various fermentation conditions are the disadvantages of this microbial hydrolysis method.

Three alternative methods were used to determine the protein content. Bradford assay was used to determine the total protein in a sample by binding the protein molecule to Coomassie blue dye under an acidic condition. The protein-dye complex will be measured at 595 nm. However, this assay is not suitable to determine the total peptide concentration as the dye does not bind to peptides with low molecular weight (Noble & Bailey, 2009). OPA assay was used to determine the total peptide in a sample. OPA reacts to primary amines and this assay is useful in the determination of primary amines and peptides

(Colombini, et al., 2011). The reduction of the color from purple to yellow is assessed at 340 nm. Ninhydrin assay was used to determine the total amino acid in a sample. The alpha-amino group is contained in all proteins, peptides, and amino acids. It is due to the reaction between ninhydrin and the amino group of free amino acids. Hence, ninhydrin binds to free amino acid and predominantly leads to purple or blue color development absorbs at 570 nm (Friedman, 2004).

2.7 Sources and Applications of Protein Hydrolysate in Agriculture

Countless agricultural activities produce tons of organic wastes that have the potential to produce protein hydrolysates when undergoing further processing that can be used as an environmentally friendly organic bio-stimulant to cultivate crops (Xu & Geelen, 2018). As the alternative to chemical bio-stimulants, these protein hydrolysates have been used as a bio-stimulant to enhance plant growth, development, and quality. Protein hydrolysates have been used as a plant bio-stimulant as an alternative to chemical bio-stimulants to enhance plant growth and quality. Food waste stream is the main important precursor in protein hydrolysate production. Besides, protein hydrolysates are developed from food waste, manures, composts, vermicompost, aquaculture, fish processing waste, and sewage-treated products (Xu & Geelen, 2018; Yakhin, et al., 2017). A large number of fish, poultry, and livestock processing industries produce the by-product waste in either solid or liquid form. These wastes are either discarded or reused to make organic compost or convert into protein hydrolysate to be used in animal feed or as plant bio-stimulant

(Chalamaiah, et al., 2012). To date, milk-based products including casein are the greatest source of isolated bioactive peptides. Other plant sources such as wheat and soy both protein and fiber-rich, have been used to produce protein hydrolysates to play a role as natural herbicides (Pasupuleti, et al., 2010).

For over a decade now, enzyme hydrolyzed plant-derived protein hydrolysates have been contributing a huge role in agriculture bio-stimulant applications due to their agronomic value and multiple benefits in growing crops organically (Colla, et al., 2015). Protein hydrolysates have been used in seed treatments for field crops like maize, wheat, and soybeans, for example (Rouphael, et al., 2017). Plant bio-stimulants are composed of a vast variety of organic components including peptides, amino acids, polysaccharides, and phytohormones. In agricultural research, protein hydrolysates are utilized as bio-stimulants to enhance the growth and quality of crops as well as to reduce the use of inorganic bio-stimulants. These bio-stimulant compounds are produced through the hydrolysis of animal or plant-derived proteins (Colla, et al., 2013).

Protein hydrolysate application in crop cultivation has positively affected several parameters of the crop. For instance, according to Nurdiawati et al. (2019), the application of liquid chicken feather-derived protein hydrolysate enhanced the growth and annual production of mung bean plants, as well as the leaf surface area and chlorophyll content of patchouli plants. Similarly, chicken

feather-derived protein hydrolysate application has increased the nutrient contents including chlorophyll, reducing sugar, protein, and amino acid levels in bananas (Gurav & Jadhav, 2013). In maize seedlings, the application of alfalfa-derived protein hydrolysate improved root system strength, nitrogen absorption, and antioxidant activity (Ertani, et al., 2013). According to a study by Sestili et al. (2018), the application of legume-derived protein hydrolysate to tomato stimulated root growth by regulating the expression of genes encoding for ammonium and amino acids transporters, upregulating genes for nitrogen assimilation, increasing nitrogen content in leaves, and increasing plant growth by upregulating genes for nitrogen assimilation, increasing nitrogen content in leaves, and increasing plant growth. Moreover, protein hydrolysate Trainer has also raised the root system, chlorophyll synthesis, and proline accumulation in lettuce, as well as plant growth, photosynthetic rate, antioxidant activity, and calcium, phosphorus, phenols, and ascorbic acid content in perennial wall rocket leaves (Rouphael, et al., 2017; Caruso, et al., 2019).

2.8 Protein Hydrolysates Effect on Crops

The animal and plant-derived protein hydrolysates enhance plant growth and development by modulating the root growth by improving the efficiency of resources and nutrients uptake, triggering the essential enzymes to stimulate the metabolism of nitrogen and carbon, stimulating the secondary metabolites by increasing the antioxidant capacity, and induce the hormone-like activities similar to gibberellins and auxin (Rouphael, et al., 2017). According to the report

by (Ertani, et al., 2016), The use of protein hydrolysate in plant cultivation disrupts carbon and nitrogen metabolism, which increases macro and micronutrient uptake and aids in biomass accumulation and rapid plant growth. Plants can be treated with protein hydrolysate via foliar application or soil drenching. Protein hydrolysate is absorbed by the cuticle, epidermal cells, and stomata before reaching mesophyll cells in foliar applications, whereas they are absorbed through root epidermal cells and redistributed to different regions of the plant through the xylem in soil drenching applications. (Subbarao, et al., 2015).

However, the rate of protein hydrolysates absorption in leaves is higher compared to absorption through roots due to the lesser microbial competition on the plant foliar and the presence of soil microbial activity as the soil microorganisms use most of the applied protein hydrolysates for respiration and biomass production. Protein hydrolysates stimulate lettuce growth by enhancing naturally occurring microorganisms like P-solubilizing, N2-fixing, and indoleacetic acid-producing bacteria via microorganism-mediated bio-stimulants, according to a recent study (Moe, 2013; Colla, et al., 2015). Protein hydrolysate, according to Calvo et al. (2014), enhance nitrogen absorption, which is essential for plant growth and development. A study conducted by Wilson, et al. (2018) reported that protein hydrolysates-based bio-stimulant on maize plants enhanced the growth and development of the plant by enhancing the activity of several essential enzymes such as nitrate reductase, NAD-

dependent glutamate dehydrogenase, and malate dehydrogenase. Apone et al. (2010) reported that protein hydrolysates enhance plant defense and tolerance to abiotic and biotic stress such as drought, extreme temperatures, and oxidative conditions by inducing plant secondary metabolism.

In addition, protein hydrolysates application to maize plants influences glycolysis and the Kreb cycle as well as stimulates enzymes that are involved in carbon metabolisms such as citrate synthase, malate dehydrogenase, and isocitrate dehydrogenase. Although protein hydrolysates contain peptides, amino acids, and hormones that could operate as signaling molecules in a variety of cellular metabolic processes in plants, the exact mechanism is yet unknown. Alfalfa-derived protein hydrolysates have been shown to boost enzyme activity and up-regulate gene expression in the secondary metabolism, which involves the synthesis of phenolic chemicals (Ertani, et al., 2013). When the auxin-like action of protein hydrolysates was tested on corn coleoptile elongation rate, it was discovered that corn treated with plant-derived protein hydrolysates had a higher rate of coleoptile elongation than the control. Furthermore, plant-based protein hydrolysate treated tomato plants had greater shoots, root dry weight, root length, and root area than untreated plants. (Colla, et al., 2015). When gibberellin-deficient dwarf pea plants are treated with plant-based protein hydrolysate Trainer, the shoot length increases compared to the control plant. While, compared to gibberellic acid-treated lettuce, the application of a protein

hydrolysate-based bio-stimulant boosted lettuce shoot length, demonstrating that protein hydrolysates have high gibberellin-like activity. (Ertani, et al., 2009).

2.9 Soybean waste Compositions and Nutritional Values

Substances that are either liquid or solids produced from the cultivation processes of fertilizers, crop residues, pesticides, and animal waste are unusable and often categorized as agricultural wastes (Shehrawat, et al., 2015). Food processing industries are one of the main industries in the world that produce billions of tons of agricultural wastes which often creates environmental concerns. The majority of these agricultural wastes are disposed of in landfills or have to undergo further treatments to convert them into compost or use as animal feed (Choi, et al., 2012). These agricultural wastes generally contain high levels of nutrients and moisture which leads to environmental problems by causing bad odors due to rapid decay in the open air and landfill leachates. In addition, disposing of waste in landfills can be expensive due to limited landfill areas around the globe. Therefore, it is suggested that the utilization of agricultural waste can help to achieve a zero-waste society as well as may leave a positive impact on environmental and economic issues (Choi, et al., 2015; Pfaltzgraff, et al., 2013).

Soybean waste is also known as Okara, soybean residue or soy pulp is a by-product of mainly soy milk and soy tofu production. Soybean waste is considered a major agricultural waste because approximately 250 kg of soybean residues are produced for every 1 Litre of soy milk all over the world which causes environmental problems (Nguyen, et al., 2013). Malaysia is one of the largest soy-based food production industries in Asia. About 15% of the imported soybean quantity is used to produce soy milk and tofu leads to a larger quantity of soybean waste produced. However, only a limited amount of this soybean waste is utilized in animal feedstock and the remaining will be discarded in the landfill leading to environmental odor problems (Hui, 2019). Soybean waste spoils easily when is not refrigerated due to the high water and protein contents. The waste contains 70%-80% of moisture, 50% carbohydrates including fermentable sugars such as glucose, mannose, and galactose as well as 50% crude fiber consisting of hemicellulose, cellulose, lignin, and pectin, a complex polymer consisting of galacturonic acid bound to monosaccharides such as rhamnose and arabinose (Choi, et al., 2015; Nguyen, et al., 2013).

On the other hand, soybean waste is rich in protein content and also widely known for its high nutritional and functional properties. However, this composition of soybean waste is varied according to the country and processing (Cheng, et al., 2015). The soybean waste is mainly composed of ruptured cell wall polysaccharides-rich components such as cotyledon cells and the soybean seed coat. It is considered a good dietary fiber in which these cellulose

components can be fermented in the large intestine by microbes and cannot be digested in the small intestine. Thus, soybean waste has several health benefits to the body including reducing blood fat, cholesterol, and blood pressure, regulating blood sugar levels, and preventing coronary heart disease, constipation as well as colon cancer (Rinaldi, et al., 2000). The soluble fiber present in soybean waste has anti-inflammatory and anti-carcinogenic when treated with high hydrostatic pressure. Furthermore, insoluble fiber helps in the reduction of gastrointestinal transit time and increases fecal bulk (Mateos-Aparicio, et al., 2010; Bosaeus, 2004).

According to Wang & Cavins (1989), soybean wastes are rich in protein sources especially essential amino acids compared to soy products where about 27% of the protein sources in dried soybean waste are present in the form of amino acids. Studies showed that functional properties of proteins including solubility, foaming and emulsifying are improved remarkably in acid-treated soybean waste (Chan & Ma, 1999). In addition, protein isolate from soybean waste has lower solubility levels can be due to protein aggregation and approximately 53% of protein isolate can be obtained from soybean waste when the protein is extracted at pH 9 and 80°C for 30 minutes. The fermented soy protein isolates produce soy peptides and free amino acids which shows that soybean waste has great potential for the development of protein resources (Vishwanathan, et al., 2011). Studies have reported that soybean is rich in phenolic compounds and isoflavones. Soybean isoflavones is a plant chemical

that has a similar structure to a hormone namely estrogen and possesses several biochemical activities. The primary isoflavones including glycosides such as daidzein, genistein, and phenolic compounds such as syringic, chlorogenic, ferulic, and gallic acids that are present in soybean contribute to several health-promoting activities (Kim, et al., 2006). Isoflavones play a vital role in reducing the risks of cancer, osteoporosis, and cardiovascular diseases as well as contribute to antibacterial inflammation. However, during the process of soy tofu making, most of the isoflavones are left in the soybean waste residue due to the existence of these isoflavones mainly in the plumular axis of the soybean (Jackson, et al., 2002; Lee, et al., 2005).

Moreover, soybeans contain a functional compound known as soyasaponins which is a group of nonvolatile, amphiphilic molecules. The compound is commonly found in the cells of the cotyledons of legumes such as soybeans, lentils, peas, and so on and thus left in soybean residue waste after soybean product processing. It is reported that soyasaponins have several health-promoting properties including antiviral, hepatoprotective, immunostimulatory, and antitumorigenic (Bae, et al., 2002; Gurfinkel & Rao, 2003). Other functional compounds include minerals, lignans, phytates, phytosterols, and coumestans that have various therapeutical properties such as antioxidant capacity, chemo-preventive agents for certain cancer, and prevent cardiovascular diseases are present in soybean waste (Quitain, et al., 2006). Soluble nutritional factors are utilized in the bean curd-making process and thus nutrients such as vitamin B

and fat-soluble nutrients including linoleic acid, linolenic acid, lecithin, tocopheryl, phytosterol, and vitamin D are left in the soybean waste (Li, et al., 2013).

The utilization of underutilized resources or edible by-products from food industries is one of the alternatives to overcome food waste problems in developing countries. Currently, soybean waste is utilized as food additives, animal feed as well as a source of fertilizer (Wickramarathna & Arampath, 2003). However, the soybean waste utilization ratio is considered lower compared to the production worldwide. Soybean waste is a good nutrient-rich raw material that can be further processed and transformed into reusable materials in various ways. In addition, some useful components such as protein, pectic polysaccharides, and dietary fiber, can be produced from wet or dry soybean waste under different treatment conditions (Redondo-Cuenca, et al., 2008). For instance, poultry industries reduce animal feeding costs by utilizing the by-products from food industries as animal feed. Thus, due to the high nutritional values and lower cost of soybean waste, it is used as feed for animals such as pigs, goats, chickens, and cattle. Soybean waste serves as a potential alternative for an organic protein source that helps in fulfilling the requirement of protein content in poultry production (Li, et al., 2012). Using bacteria strains derived from water and soil in the Amazon region, bioconversion of fibrous wastes generated from soybean protein manufacturing into value-added industrial goods such as enzymes (Heck, et al, 2002). Identifying the

enzymatically digestible and indigestible components of soybean waste using enzymes such as cellulase and pectinase (Kasai, et al., 2004). Development of dietary fiber-rich functional food with prebiotic effect due to the presence of indigestible (non-starch polysaccharides, lignin) and digestible (protein, oil) fractions as well as the bifidogenic capacity of soybean waste (Martos & Ruperez, 2009).

On the other hand, the microbial protein feed can be produced through solid-state fermentation of soybean waste where the fibers in soybean waste will be degraded into low molecular weight (LMW)-carbohydrates by mold during the fermentation process. These LMW-carbohydrates will further be treated by yeast to synthesize protein as well as reduce the anti-nutritional factors such as saponin, trypsin inhibitor, and lectin present in soybean waste during the fermentation process thus, providing a highly nutrient-rich product that can be used as a replacement for animal protein (Yuan, et al., 2017). Besides, the effluent of hazardous synthetic dyes that are used in textiles, paper, plastic, tannery, and many other materials in wastewater is highly visible and results in environmental odor problems as well as the removal of these dyes from the wastewater is not easy due to the structure, high thermal, photo-stability and toxicity of the dye structure (Gao, et al., 2011; Aksu, 2005). A study conducted by Gao et al. (2015) showed that soybean waste has effectively served as an adsorbent to remove Reactive Brilliant Blue KN-R (RBB) dye from wastewater at pH value 2.0 and therefore, it can be used as an alternative, low-cost adsorbent

in wastewater treatments. Ma et al. (2018) reported in the soybean enzyme-assisted aqueous extraction processing (EAEP) residue, the mechanism of covalent intermolecular cross-linking between the two carboxyl groups of citric acid and the hydroxyl groups of polysaccharides improves water uptake reduction, resulting in smooth, transparent, and hydrophobic surfaces suitable for food packaging.

2.10 Oxidative Stress

Free radicals are molecules containing one or more than one unpaired electron that cause this free radical molecule to act as an electron acceptor. Generally, free radicals are very reactive and tend to receive an electron from another molecule through an oxidation reaction When reacting with other molecules at high energy input, free radicals can form new radicals during the process such as reactive oxygen species (ROS) which is derived from oxygen (Sen, 2001). An oxidative stress state occurs when the level of ROS is higher than antioxidants which can result in cell damage. Even though free radicals exert positive effects on the immune system and essential metabolisms, they are often involved in many diseases and cause aging (Cooper, et al., 2002). ROS act against antigens during phagocytosis in inflammation, serve as cell messenger in cellular signals, involve in enzyme activation as well as essential in muscular contraction (Fehrenbach & Northoff, 2001; Reid, 2001; Coombes, et al., 2001). Despite some positive effects, ROS can induce apoptosis and cause inflammation in healthy cells as well as alter normal cellular functions which

can result in cell aging, cancer, Parkinson's, or Alzheimer's disease (Golden, et al., 2002).

An antioxidant is a chemical that combines with ROS to generate a less reactive radical or disrupts the free radical reaction chain to help reduce oxidative stress on substrates like carbohydrates, proteins, DNA, and lipids (Powers & Lennon, 1999). Antioxidants can be naturally synthesized in the body enzymatically or consumed through diet. Enzymatic antioxidants such as catalase (CAT), superoxides dismutase (SOD), glutathione peroxidase (GPX), and non-enzymatic antioxidants such as vitamin A, ascorbic acid, vitamin E, phenolic compounds, and flavonoids are efficient in reacting against ROS (Finaud, et al., 2006). These antioxidant enzyme production in cells is regulated by deoxyribonucleic acid (DNA) is vital in inhibiting lipid peroxidation and hence, protects healthy cells from oxidative stress (Gill & Tuteja, 2010). SOD is a metalloenzyme and plays an important role in the dismutation of oxygen to hydrogen peroxide which will then be catalyzed by CAT or GPX. Whereas CAT is found in the peroxisomes and the main function is to detoxify the process initiated by SOD by decomposing hydrogen peroxide into water and molecular oxygen during the oxidation of fatty acids (Chelikani, et al., 2004). Besides, GPX activity depends on selenium and it takes place mainly in the mitochondria where the enzyme breaks down hydrogen peroxide in water and lipid peroxides (Góth & Páy, 2004). The non-enzymatic antioxidants serve as free radical scavengers and neutralize the free radicals by donating electrons to produce stable or less reactive complexes. In this process, the antioxidants become less reactive to free

radicals. Phenolic compounds donated a hydrogen atom from their hydroxyl group to inhibit the oxidation of LDL. As a result, it lowers the risk of gastrointestinal cancers, cardiovascular disorders, colon, breast, and ovarian cancers, as well as neurological diseases (Paran, et al., 2009; Riemersma, et al., 2001). According to Corti et al. (2010), vitamin C is a water-soluble antioxidant capable of reacting with ROS via hydrogen atom transfer (HAT), inactivating singlet oxygen, and removing molecular oxygen.

2.11 Photosynthetic Pigments

Chlorophylls and carotenoids are photosynthetic pigments that play a vital role in the photosynthesis process in which the changes in the levels of these pigments indicate the rate of photosynthetic activity and abiotic stress in plants (Minocha, et al., 2009). Chlorophyll is the green pigment that absorbs and converts sunlight into energy during photosynthesis chlorophyll utilizes solar energy to fix carbon dioxide into carbohydrates and provides energy for plant growth and development (Rinawati, et al., 2020). In higher plants, it consists of chlorophyll a as the major and chlorophyll b as accessory pigments, respectively, in addition, the ratio of chlorophyll a and b can be influenced by internal and environmental factors as well as is often used to evaluate the physiological status in the plants (Vicas, et al., 2010). On the other hand, carotenoids also known as carotene provides colored pigments in a variety of plants. This pigment contributes positive benefits to human health by serving as an antioxidant, boosting the immune system, and protecting against cancer (Chew & Park,

2004). Chlorophyll can efficiently deliver magnesium and oxygen in the blood to all cells and tissues, improve oxygen supply by stimulating the red blood cells, involve in assimilating and chelating calcium as well as other heavy minerals, and prevent cell damage by neutralizing free radicals with the help of other vitamins such as A, C, and E. In addition, (Kizhedath & Suneetha, 2011). Lutein, zeaxanthin, and β-carotene are carotenoids present in leafy vegetables that have positive effects on human health. For instance, lutein and zeaxanthin have improved human vision (Landrum & Bone, 2001; Wisniewska & Subczynski, 2006). The presence of lutein and zeaxanthin in the diet can benefit in reducing the incidence of age-related eye diseases (Wisniewska & Subczynski, 2006).

The overproduction of chlorophyll may lead to plant cell death and growth retardation by generating reactive oxygen species (ROS). Therefore, regulating the levels of chlorophyll is important to maintain a healthy and enhance plant growth (Tanaka & Tanaka, 2006). There are three main phases of chlorophyll metabolism where the first phase involves the synthesis of chlorophyll *a* from glutamate, the second phase is known as the chlorophyll cycle which involves the interconversion of chlorophyll *a* and chlorophyll *b*, and the third phase is also the final phase that involves the chlorophyll a degradation (Rüdiger, 2002; Eckhardt, et al., 2004All photosynthetic and some non-photosynthetic organisms produce carotenoids in the presence of photosynthetic organisms. Carotenoids in photosynthetic organisms contribute to the presence of pigments in the yellow to red range in a variety of plants, fruits, and

vegetables, whereas carotenoids in non-photosynthetic organisms contribute to the presence of pigments in the yellow to red range in a variety of plants, fruits, and vegetables (Cazzonelli & Pogson, 2010; Ruiz-Sola & Rodríguez-Concepción, 2012). Carotenoids can be only provided through dietary uptake as humans and nearly most animals could not be able to synthesize carotenoids (McGraw, et al., 2006). The condensation of carotenoid precursors such as isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) initiates the synthesis of carotenoids (Rodríguez-Concepción, 2010).

CHAPTER 3

MATERIAL AND METHODS

3.1 Overview of the Study

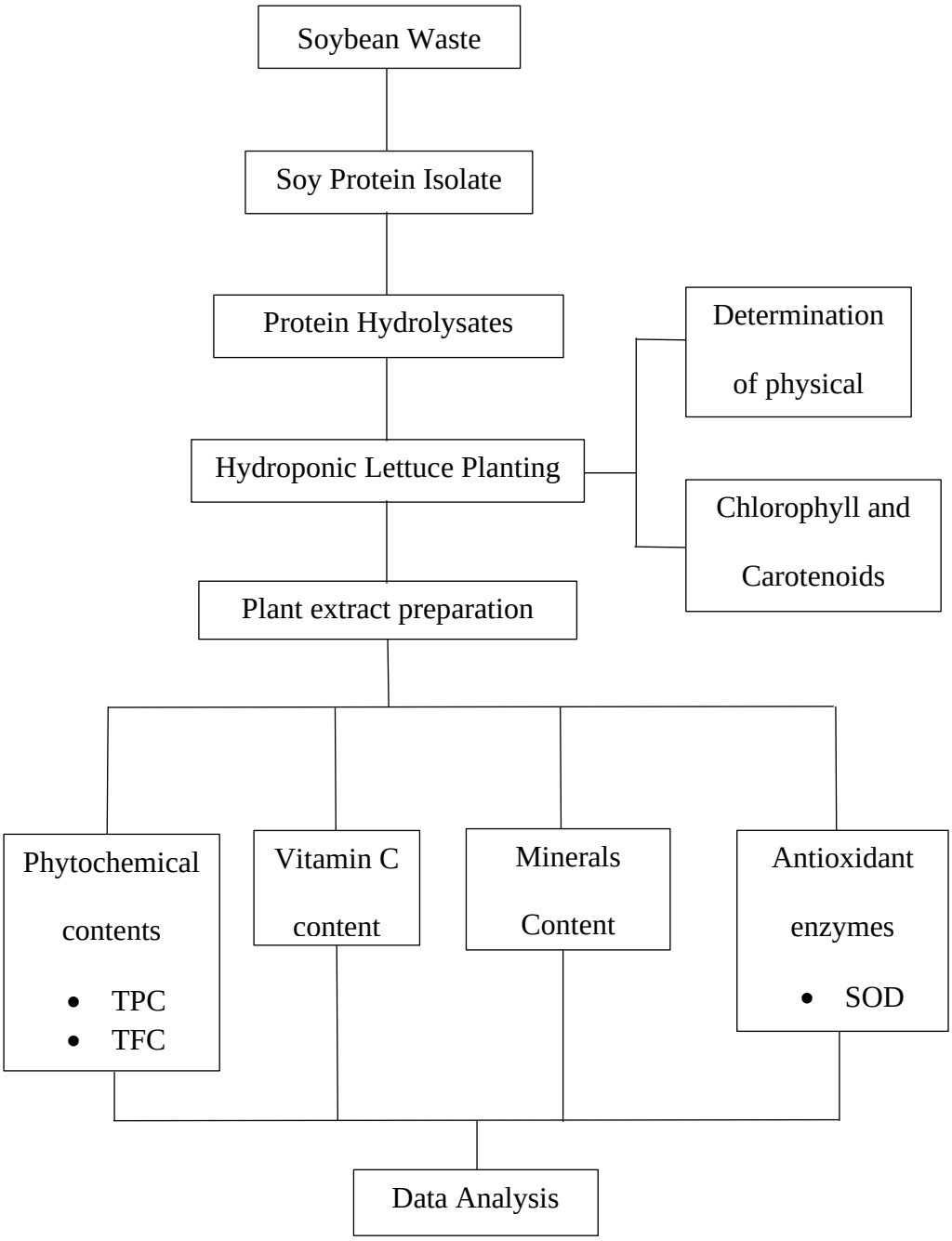

Figure 3.1: Overview of Study

3.2 Preparation of Protein Hydrolysates

Soy waste was collected from a local shop at Taman Bandar Baru, Kampar, Perak. Firstly, to make soy protein isolate, 100 g of soy waste was suspended in 500 mL deionized water at a 1:5 ratio, stirred for 1 hour at room temperature, then heated for 20 minutes at 90^{o}C. After that, the mixture was centrifuged for 25 minutes at 4000 rpm to separate the water-insoluble components. To separate the precipitated soy protein from the mixture, the collected supernatant was adjusted to an ammonium sulphate saturation of 80% and stirred for 1 hour at 4^{o}C before centrifugation at 10 000 rpm for 1 hour. The separated soy proteins were subsequently dialyzed for 24 hours at 4^{o}C using dialysis tubing (molecular weight cut-off: 6000-8000 Da) and then stored at -20^{o}C for future usage (Quah, et al., 2017). Bradford assay was used to determine the protein content of soy protein isolate using a bovine serum albumin (BSA) reference curve (Bradford, 1976). Protein hydrolysate was prepared by incubating 0.5 g of soy protein isolate, 100 mL of 50 mM Phosphate Buffer Saline (PBS), and 0.05 g of protease (alcalase) for 6 hours at 50^{o}C in a 1:10 ratio (protein isolate: protease). After incubation, the protease was heat-inactivated for 20 minutes at 100°C, cooled on ice for a few minutes, and the resulting protein hydrolysate was kept at -20°C for further use. (Quah, et al., 2017). To prepare the 0.1 mg/mL protein hydrolysate, 38 mL of the prepared protein hydrolysate (5 mg/mL) will be diluted in 1862 mL of hydroponic nutrient solution.

3.3 Nutrient Solution Preparation

The commercial hydroponic solution A (Ca, NO_3, NH_4, Fe, K) and B (H_2PO_4, SO_4, K, Mg, B, Cu, Mo) from Well Grow Seeds of 5 mL were diluted with 2 L of water to make the hydroponic nutrient and the solution's pH was adjusted to 6.0.

3.4 Protein Hydrolysates treated Hydroponic Green Coral Lettuce

Green Coral Lettuce (*Lactuca sativa* L.) seedlings were germinated in mesh pots and transplanted into covered hydroponic containers using the Kratky method, where they were subjected to natural photoperiod and daily temperatures (Kratky, 2005; Silva, et al., 2018). Seedlings that germinated successfully were separated into two groups, one with SPH treatment and the other without. SPH was added to the hydroponic nutrient solution at doses of 0.001, 0.01, and 0.1 mg/mL to treat the hydroponic-planted lettuces. The hydroponic lettuces were grown at pH 6.0 for 9 weeks, with the pH and EC of the solution being checked three times a week. The lettuces were then harvested to determine the effect of SPH in the treated and untreated lettuce groups.

3.5 Protein Hydrolysates treated Hydroponic Crop Extract Preparation

In a chilled mortar and pestle, 1 g of the freshly harvested fresh hydroponic crop was homogenized with 10 mL of extraction solution containing 50 mM phosphate buffer (pH 7.4), 0.5 mM ascorbate, and 1 mM EDTA. After centrifuging the mixture for 15 minutes at 10,000 rpm, the supernatant was used for further analysis (Malar, et al., 2014).

3.6 Determination of Total Chlorophyll and Carotenoids

The total chlorophyll and carotenoid content were determined according to Lichtenthaler & Buschmann (2001). From the harvested crop, a fully grown leaf was selected and sliced into strips. The leaf was then incubated in 10 mL of 95% acetone overnight at 4ºC. For chlorophyll *a*, chlorophyll *b*, and carotenoids, the absorbance was measured at 661.6, 644.8, and 470 nm, respectively. The following equations were used to calculate the concentrations of chlorophyll *a* (c*a*), chlorophyll *b* (c*b*), and total carotenoids (c*x+c)*:

c_a (µg/ml) = $11.24A_{661.6} - 2.04A_{644.8}$

c_b (µg/ml) = $20.13A_{644.8} - 4.19A_{661.6}$

$c_{(x+c)}$ (µg/ml) = $(1000A_{470}-1.90c_a-63.14c_b)/214$

3.7 Determination of Phytochemical Contents

The total phenolic content in lettuce extracts was determined using the Folin-Ciocalteu colorimetric technique, which was slightly modified. To begin, the reaction mixture of 0.1 mL extract and 0.2 mL 10% (v/v) Folin-Ciocalteu reagent was incubated at room temperature for 3 minutes. After that, 0.8 mL of 700 mM sodium carbonate was added to the reaction mixture, which was then incubated at room temperature for 2 hours. The absorbance was measured at 765 nm, and the standard curve was created using gallic acid concentrations ranging from 0 to 0.05 mg/mL. The total phenolic content was measured in mg of gallic acid equivalents per g of dry matter (mg GAE /g DM) (Ainsworth & Gillespie, 2007). The total flavonoid content in the harvested crop extracts was determined according to Zou, et al. (2004) with some slight modifications. At room temperature, a reaction mixture containing 0.2 mL of harvested crop extract and 0.15 mL of 5% (w/v) sodium nitrite was incubated for 6 minutes. The reaction mixture was then added 0.15 mL of 10% (w/v) aluminium chloride hexahydrate and incubated for another 6 minutes. The reaction mixture was then added 0.8 mL of 10% (w/v) sodium hydroxide and incubated for 15 minutes before the absorbance was measured at 510 nm. The standard curve was made with quercetin dissolved in 80% ethanol at concentrations ranging from 0 to 0.5 mg/mL. The amount of total flavonoid was measured in mg of quercetin equivalents per g of dry matter (mg QE /g DM). To determine the Total Hydroxycinnamic acid Content, the reaction mixture containing 1 mL of lettuce extracts, 6 mL of deionized water, 1 mL of 0.1 M hydrochloric acid, 1 mL of 1 M sodium hydroxide, and 1 mL of Arnow's reagent (10 g sodium nitrite and 10

g sodium molybdate added to 100 mL distilled water) were mixed in a tube, and the absorbance was measured at 490 nm immediately. The standard curve was created using caffeic acid concentrations ranging from 0 to 0.2 mg/mL. The amount of total hydroxycinnamic acid was measured in mg of caffeic acid equivalents per g of dry matter (mg CAE /g DM) (Matkowski, et al., 2008).

3.8 Determination of Vitamin C content

A burette containing 0.02% 2, 6-dichlorophenol indophenol (DCPIP) solution was placed under a conical flask holding 1 mL of lettuce extract and 9 mL of 0.5% oxalic acid. Drop by drop, the DCPIP solution was added to the reaction mixture in the flask until it turned a faint pink tint. To calculate the average volume of DCPIP solution required, the process was repeated three times. Ascorbic acid was used as a positive control and a vitamin C tablet was used as a reference. The amount of vitamin C in plant extract was measured in mg/g. (Dinesh, et al., 2015).

3.9 Determination of Antioxidant Enzymes Contents

With slight changes, the superoxide dismutase (SOD) activity of collected crop extracts was measured according to Malar (2014). The 3 mL reaction mixture, which included 100 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 2.25 mM nitroblue tetrazolium (NBT),

extract, and 60 M riboflavin, was incubated for 15 minutes under fluorescent illumination. The reaction mixture including extract was maintained in the dark as a blank, while the reaction mixture without extract was kept in the light as a control. The absorbance was then measured at 560 nm. The inhibition of NBT reduction by SOD at 50% was computed as follows:

Inhibition of NBT reduction by SOD (%) = $(A_{control} - A_{sample})/A_{control} \times 100\%$

The absorbance of the control reaction (without extract) is $A_{control}$, while the absorbance of the sample reaction is A_{sample} (with extract). The result was expressed in Units per mg of protein. The 3 mL reaction mixture for determining catalase concentration contained 50 mM phosphate buffer, 20 mM hydrogen peroxide, H_2O_2, and 100 mL extract. The mixture's absorbance was measured at 240 nm for 3 minutes with 15 second intervals at 240 nm. The extinction coefficient, 43.6 M-1 cm-1, was used to calculate the H_2O_2 decomposition. Unit/mg protein/min was used to express the result (Malar, 2014).

3.10 Determination of Mineral Contents - Atomic Absorption Spectroscopy (AAS)

With slight adjustments, sample preparation for metal content measurement using atomic absorption spectroscopy (AAS) was determined according to Uddin, et al. (2016). The fresh lettuce was oven-dried for three

days at 60ºC before being processed into a fine powder with an electric grinder. About 10 mL of 65% nitric acid, HNO_3, was added to 0.1 g of powdered plant and heated for 15 minutes. The mixture was then allowed to cool to ambient temperature before being filtered with filter paper to eliminate any remaining contaminants. Using distilled water, the filtrate was then brought up to a final amount of 50 mL. A flame atomic absorption spectrometer (FAAS) was used to examine the minerals aluminium (Al), cadmium (Cd), calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg), lead (Pb), and zinc (Zn) (Agilent Flame Atomic Absorption Spectrometer 280FSAA). The mineral determinations were made based on the conditions listed in Table 3.1.

Table 3.1: Experimental conditions for Al, Ca, Cd, Cu, Fe, Mg, Pb, and Zn determination using FAAS.

Element	Standard solution concentration (mg/L)	Wavelength (nm)	Slit (nm)	Lamp intensity	Type of gas	Gas Flow (L/min)	
						Acetylene	Oxidant
Al	20-80	309.3	0.5	100	N_2O/C_2H_2	6.35	11.00
Ca	0.4-1.6	422.7	0.5	100	N_2O/C_2H_2	6.35	11.00
Cd	0.3-1.2	228.8	0.5	40	Air/C_2H_2	2.00	13.50
Cu	0.75-3.0	324.8	0.5	100	Air/C_2H_2	2.00	13.50
Fe	1.25-5.0	248.3	0.2	15	Air/C_2H_2	2.00	13.50
Mg	5-20	285.2	0.5	100	Air/C_2H_2	2.00	13.50
Pb	2.5-10	217.0	1	20	Air/C_2H_2	2.00	13.50
Zn	0.3-1.2	213.9	1	100	Air/C_2H_2	2.00	13.50

3.11 Data Analysis

Statistical analysis was performed using the SAS System (Version 9.4) and data are presented as mean ± standard error. The ANOVA test was used to analyze the data, and Fisher's Least Significant Difference (LSD) test was used to separate the means of significant differences at the 0.05 level of probability.

3.12 Materials and Reagents

Table 3.2: List of Materials and Reagents Used and Their Manufacturer

Material and Reagent	Manufacturer
Ammonium sulfate	Bendosen
Dialysis Tube	Fisher Scientific
Braford Reagent	Sigma Aldrich
Bovine Serum Albumin (BSA)	Sigma Aldrich
Phosphate Buffer Saline (PBS)	OXOID
Alcalase	CALBIOCHEM
Hydroponic Solution A and B	Well Grow Seeds Co.
Green Coral Lettuce Seeds	Well Grow Seeds Co.
Potassium dihydrogen phosphate	SYSTERM
Dipotassium hydrogen phosphate	SYSTERM
Ascorbic acid	HmbG Chemicals
Ethylenediaminetetraacetic acid, EDTA	HmbG Chemicals
Disodium tetraborate decahydrate	QReC
Ethanol	QReC
Acetone	Emsure
Folin-Ciocalteu Reagent	Chemiz
Sodium carbonate	QRec
Gallic acid	R&M Chemical
Sodium nitrite	QReC
Aluminium chloride hexahydrate	QReC
Sodium hydroxide	Merck

Material and Reagent	Manufacturer
Quercetin	ACROS ORAGNICS
Hydrochloric acid	QReC
Sodium molybdate	R&M Chemical
Caffeic acid	Sigma Aldrich
2,6-dichlorophenolindophenol, DCPIP	Chem Soln
Oxalic acid	Bendosen
Vitamin C table	Appeton
Methionine	Chem Soln
Nitroblue tetrazolium, NBT	Sigma Aldrich
Riboflavin	Sigma Aldrich
Hydrogen peroxide	ChemAR
Nitric acid	Emsure
Aluminium	Merck
Cadmium	Merck
Calcium	Merck
Copper	Merck
Iron	Merck
Magnesium	Merck
Lead	Merck
Zinc	Merck

3.13 Equipment

Table 3.3: List of Equipment Used and Their Model/Brand

Equipment	Model/Brand
Centrifuge Machine	Velocity 14R
Water bath	SASTEC
Hot plate	STUART
Microplate Reader	FLUOstar OMEGA, BMG Labtech
Drying Oven	Memmert
Electronic Balance	Sartorius
Refrigerator	LG
pH Meter	Mettler Toledo
Vortex	Genie Scientific Industries
UV-Visible Spectrophotometer	THERMO Scientific (GENESYS 10)
Flame Atomic Absorption Spectrometer (FAAS)	Agilent FAAS 280FSAA

CHAPTER 4

RESULT

4.1 Physical Characteristics of Soy Protein Hydrolysate Treated Hydroponic-planted Lettuce

For 9 weeks, hydroponic-planted lettuces were treated with four different concentrations of soy protein hydrolysates (0, 0.001, 0.01, 0.1 mg/mL). To distinguish the effect of SPH between treated and non-treated hydroponic-planted lettuces, a set of hydroponic-planted lettuces was not treated with SPH (0 mg/mL). The effects of SPH on the hydroponic-planted lettuces were determined using the physical parameters of harvested lettuces. In comparison to lettuces treated with various SPH concentrations and untreated lettuce, lettuce treated with 0.01 mg/mL of SPH has the highest fresh weight (3.28 ± 0.53^{a}) (Figure 4.1).

When comparing lettuce from the control group to lettuce from the experimental group, the length of the lettuce reached a peak of 25.73 ± 1.76^{a} when treated with 0.01 mg/mL of SPH (Table 4.1). At the highest SPH concentration (0.1 mg/mL) there was a decrease in lettuce length of $22.08 \pm 1.83^{a, b}$, however, there was no SPH concentration-dependent trend. Other physical parameters such as lettuce leaf surface area (Table 4.1), root length, and root weight (Table 4.2) did not show a significant difference when compared to the control group.

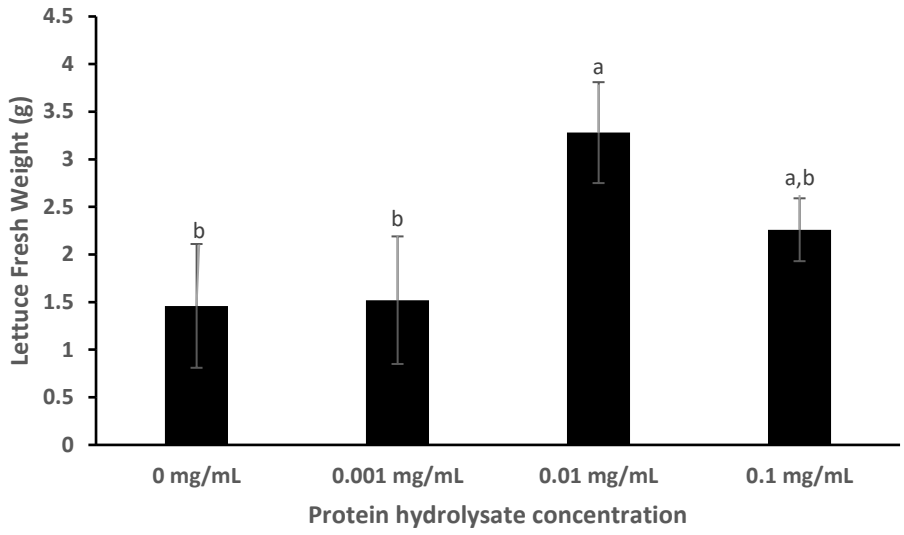

Figure 4.1: The fresh weight of soy protein hydrolysates treated hydroponic-planted lettuce. Data are reported as mean ± SE values (n=4). Different superscripts (a-b) indicate statistically significant differences ($p < 0.05$).

Table 4.1: The length and leave surface area of soy protein hydrolysates treated hydroponic-planted lettuce.

SPH Conc. (mg/mL)	Lettuce length (cm)	Leave surface area (cm^2)
0	$17.60 \pm 2.51^{b, c}$	493.76 ± 77.18^{a}
0.001	14.88 ± 2.26^{c}	248.44 ± 40.06^{b}
0.01	25.73 ± 1.76^{a}	531.65 ± 47.65^{a}
0.1	$22.08 \pm 1.83^{a, b}$	563.49 ± 65.55^{a}

Data are reported as mean ± SE values (n=4). Different superscripts (a-c) indicate statistically significant differences ($p < 0.05$).

Table 4.2: The length and weight of soy protein hydrolysates treated hydroponic-planted lettuce root.

SPH Conc. (mg/mL)	Root length (cm)	Root weight (g)
0	12.60 ± 2.37^{a}	0.05 ± 0.03^{a}
0.001	13.58 ± 2.93^{a}	0.08 ± 0.04^{a}
0.01	18.28 ± 1.84^{a}	0.13 ± 0.02^{a}
0.1	15.78 ± 0.96^{a}	0.10 ± 0.01^{a}

Data are reported as mean ± SE values (n=4). Different superscripts (a) indicate statistically significant differences ($p < 0.05$).

4.2 Phytochemical contents in Soy Protein Hydrolysate Treated Hydroponic-planted Lettuce

The phytochemical contents of harvested hydroponic-planted lettuce, including total phenolic content (TPC), total flavonoid content (TFC), and total hydroxycinnamic acid content (THC), as well as their ascorbic acid content, were examined to determine the effect of SPH. In lettuces treated with SPH doses ranging from 0 to 0.01 mg/mL, increased amounts of TPC, TFC, and THC were observed. However, at the greatest SPH concentration (0.1 mg/mL), the concentrations of these phytochemical contents declined. The ascorbic acid content followed a similar pattern. As a result, when compared to untreated lettuces (0 mg/mL of SPH), the overall rise in phytochemical and ascorbic acid varied from 1.10 to 1.13 times (Table 4.3).

Table 4.3: Soy protein hydrolysate treated hydroponic-planted lettuce extracts tested for their total phenolic content (TPC), total flavonoid content (TFC), total hydroxycinnamic acid content (THC), and ascorbic acid.

SPH Conc. (mg/mL)	TPC (mg GAE/ g DW)	TFC (mg QE/ g DW)	THC (mg CAE/ g DW)	Ascorbic acid (mg/g)
0	0.27 ± 0.00^{c}	3.18 ± 0.03^{c}	1.53 ± 0.02^{c}	0.39 ± 0.02^{b}
0.001	0.29 ± 0.00^{b}	3.44 ± 0.03^{b}	1.59 ± 0.02^{b}	0.37 ± 0.01^{b}
0.01	0.30 ± 0.00^{a}	3.58 ± 0.04^{a}	1.69 ± 0.00^{a}	0.59 ± 0.01^{a}
0.1	0.26 ± 0.00^{d}	$3.53 \pm 0.04^{a, b}$	1.67 ± 0.02^{a}	0.29 ± 0.00^{c}

Data are reported as mean ± SE values (n=4). Different superscripts (a-c) indicate statistically significant differences ($p < 0.05$) for total phenolic content (TPC), total flavonoid content (TFC), total hydroxycinnamic acid content (THC), and ascorbic acid (Vit C).

4.3 Chlorophyll and carotenoid contents in Soy Protein Hydrolysate Treated Hydroponic-planted Lettuce

The chlorophyll and carotenoid levels of freshly harvested hydroponic-planted lettuces were spectrophotometrically determined after being immersed in acetone overnight. When treated with 0.01 mg/mL of SPH, the amounts of chlorophyll *a* (9.89 ± 0.08^{a} g/mL), chlorophyll b (3.69 ± 0.08^{a} g/mL), and carotenoid (2.73 ± 0.02^{a} g/mL) in hydroponic-planted lettuce were the highest. When the lettuces were treated with a greater dosage of SPH (0.1 mg/mL), both

chlorophyll and carotenoid levels decreased. According to Table 4.4, the rise in chlorophyll and carotenoid contents in SPH treated lettuces was 1.71 to 1.88 times that of non-treated lettuces (Table 4.4).

Table 4.4: The chlorophyll a (Chl $_a$), chlorophyll b (Chl $_b$), and carotenoid (C_{x+c}) contents in PH treated hydroponic-lettuces.

SPH Conc. (mg/mL)	Chl $_a$ (µg/ml)	Chl $_b$ (µg/ml)	C_{x+c} (µg/ml)
0	5.44 ± 0.03^{d}	1.96 ± 0.07^{d}	1.60 ± 0.02^{d}
0.001	6.49 ± 0.03^{c}	2.60 ± 0.02^{c}	1.97 ± 0.01^{c}
0.01	9.89 ± 0.08^{a}	3.69 ± 0.08^{a}	2.73 ± 0.02^{a}
0.1	9.18 ± 0.02^{b}	3.13 ± 0.04^{b}	2.61 ± 0.01^{b}

Data (n=4) are reported as mean ± SE values. The superscripts (a-d) indicate significant differences ($p < 0.05$), for chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoids (Cx+c).

4.4 Mineral contents in Soy Protein Hydrolysate Treated Hydroponic-planted Lettuce

The effect of mineral absorption and bio-accumulation in SPH treated hydroponic-grown lettuce was investigated using a flame atomic absorption spectrometer. Eight minerals were evaluated, including aluminium, cadmium, lead, iron, copper, zinc, magnesium, and calcium, and none of the lettuce extracts

contained aluminium, cadmium, or lead (data not shown). Other five mineral elements (iron, copper, zinc, magnesium, and calcium) were detected in all lettuce samples (Figure 4.2). It was observed that no significant difference in mineral content in the SPH treated hydroponic lettuces compared to those in the control group in our lettuce study. However, the greater magnesium content detected in lettuce samples grown at the highest SPH concentration (0.1 mg/mL) was an interesting exception (Figure 4.2). When compared to the control group, the magnesium content is 1.2-fold higher.

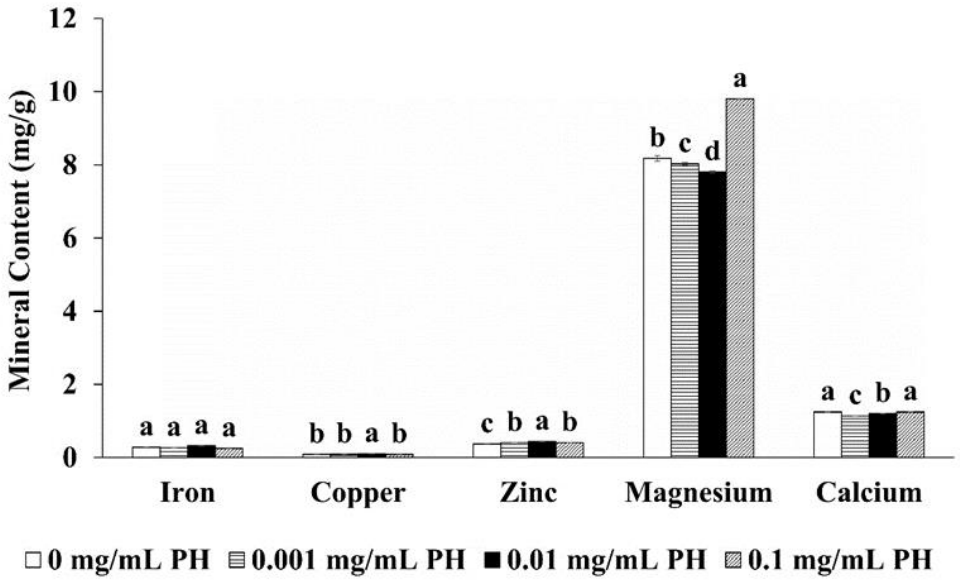

Figure 4.2: The mineral contents of PH treated hydroponic-lettuce. Data are reported as mean ± SE values (n=4). Different superscripts (a-d) indicate statistically significant differences ($p < 0.05$).

4.5 Antioxidant enzyme contents in Soy Protein Hydrolysate Treated Hydroponic-planted Lettuce

After SPH treatment, antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) were measured in the harvested hydroponic lettuces (SOD). According to the result (Figure 4.3), hydroponic-lettuces treated with SPH 0.001 and 0.1 mg/mL had greater CAT and SOD levels, respectively. In comparison to the control group (0 mg/ml PH), increased CAT (2.17-3.04 folds) and SOD (1.23-1.31 folds) were observed in these SPH concentration ranges. However, we could not rule out the possibility that the elevated CAT and SOD levels in SPH-treated lettuces were due to greater stress levels.

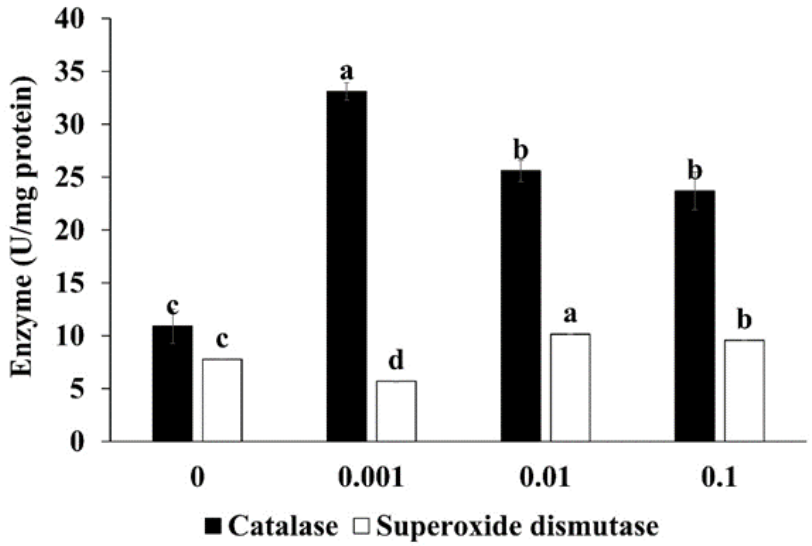

Figure 4.3: Antioxidant enzymes contents in PH treated harvested hydroponic-lettuces, reported as Unit (U) per milligram of protein. Data are reported as mean ± SE values (n=4). Different superscripts (a-b) indicate statistically significant differences ($p < 0.05$).

CHAPTER 5

DISCUSSION

5.1 The Effect of Soy Protein Hydrolysate on the Physical Characteristics of Hydroponic-planted Lettuce

Soon, the food production sectors may undergo a challenge to produce sufficient as well as quality food to compensate for the increasing world population. According to Colla, et al. (2015), the application of protein hydrolysate in crop cultivation can be an advanced technology to overcome food crisis challenges also, enhance crop production worldwide regardless of the seasons. It is also reported in several studies that the application of protein hydrolysate improves crop productivity by enhancing crop growth, development, phytochemicals, tolerance against abiotic stresses, increases shoot and root length as well as the crop biomass (Calvo, et al., 2014; Colla et al., 2015). In this study, green coral lettuce was grown hydroponically and treated with soybean waste-derived protein hydrolysates at various concentrations such as 0, 0.001, 0.01, and 0.1 mg/mL for 9 weeks. The physical properties and biochemical contents of the hydroponic-planted lettuces were harvested and tested to determine the effects of SPH. Physical properties such as lettuce length and leaf surface areas, which may influence consumer purchase decisions, as well as aspects such as root length and weight, which may be influenced by the plant's nutrition uptake, were evaluated.

As a result of the findings, hydroponic-planted lettuce treated with 0.001 mg/mL of SPH had the greatest length and fresh weight compared to other lettuces treated with SPH at doses of 0.001 and 0.1 mg/mL and the non-treated lettuce group. When lettuces were treated with 0.1 mg/mL of SPH, however, their length and fresh weight decreased. Other variables, such as leave surface area, root length, and root weight, on the other hand, show no significant changes among the four groups of hydroponic-planted lettuces, necessitating future research to determine the impacts of SPH on these specific elements.

A previous study by Colla, et al. (2014) reported that plant-derived PH namely PH Trainer and treated corn (*Zea mays* L.) have accelerated coleoptile elongation compared to the non-treated control group of corn. The authors stated that due to the presence of bioactive peptides and tryptophan, a major precursor of indole-3-acetic acid biosynthesis, the PH Trainer exhibited the auxin-like activity which enhances plant growth and development. Similarly, the application of protein hydrolysate Trainer to gibberellin-dwarf pea (*Pisum sativum* L.) has enhanced the shoot length compared to the control group. Thus, it showed a gibberellin-like activity has been exhibited by the protein hydrolysate. Moreover, foliar application of legume-derived protein hydrolysate improved the yield of fresh tomatoes (Colla, et al., 2017). In contrast, Colla et al. (2013) reported that foliar application of plant-derived protein hydrolysate exhibited no obvious effect on the fresh and dry weight of lettuce. Compared to the control, spinach treated with fish waste-derived protein hydrolysate (FPH)

has a higher fresh weight except for the highest FPH dose (Dewang & Devi, 2021). On the other hand, it is reported in previous studies that protein hydrolysate enhanced the activities of several enzymes that involve in plant growth and development such as malate and glutamate dehydrogenase, nitrate reductase, glutamine synthetase, and glutamate synthase in crops including corn, corn leaves, and bean (*Phaseolus vulgaris*) (Bulgari, et al., 2015; Ertani, et al., 2009; Baglieri, et al., 2014).

Despite the positive effects of protein hydrolysate on crop growth, several factors might lead to the inhibition of crop growth. Also, it is stated that protein hydrolysate can be affected differently depending on the growing medium and plant species (Xu & Mou, 2017). The excess use of fertilizer can cause the inhibition of growth and eventually lead to soft rot damage on the crops. The precipitation of fertilizer compounds in the hydroponic nutrient solution, accumulation of phytotoxic compounds such as ammonium ions, or the application of organic fertilizer in nutrient solution may cause a reduction of dissolved oxygen and can inhibit plant growth (Kano, et al., 2021). Botta (2013) reported that, although protein hydrolysate has improved tolerance against abiotic stress such as heat, cold, salinity, and nutrient deficiency, it did not directly affect the lettuce growth. In addition, it is also concluded that a specific range of feather-derived protein hydrolysate has a positive effect on spinach fresh weight and yield, however, when the dose upsurge from the optimum dose

of this feather-derived protein hydrolysate, it did not show a dose-dependent effect on the spinach growth (Dewang & Devi, 2021).

5.2 Determination of Phytochemical and Vitamin C contents

Phytochemicals are naturally occurring bioactive compounds in most dietary plants and these natural compounds play vital roles in the discoveries of new drugs to treat multiple diseases as well as to prevent the causes of these diseases. Relatively, natural bioactive compounds can be safer and more effective alternatives with no or fewer side effects compared to synthetic drugs (Cos, et al., 2006). Additionally, Sasidharan et al. (2011) summarized that plant phytochemicals possess several biological activities including anticancer, antioxidant, antidiarrheal, antimicrobial activities, and wound healing properties. The phytochemical composition of plants is connected to their bioactivities and pharmacological potentials in several studies. (Cao, et al., 2017; Teng, et al., 2019; Wong, et al., 2016). Polyphenolics are the most popular phytochemicals in plants and these polyphenolics consist of phenolic acids and flavonoids (Puupponen-Pimiä, et al., 2005). Because they are a large collection of secondary metabolites that are universally distributed in green plants and are vital in signaling molecules, detoxifying, spore germination stimulations, acting as UV-filters, drought resistance, and pollinator attractants, phenolic acids and flavonoids are an important part of human and animal diets. (Michalak, 2006; Samanta, et al., 2011).

In this study, the phytochemical contents including total phenolic content (TPC), total flavonoid content (TFC), and total hydroxycinnamic acid content (THC) were tested to determine the effect of SPH on hydroponic-planted lettuces. The result obtained in this study shows the concentrations of TPC, TFC, and THC contents have gradually increased in hydroponic-planted lettuces when treated with 0 to 0.01 mg/mL of SPH (Table 4.3). However, compared to the non-treated control group, the phytochemical contents decreased in hydroponic-planted lettuce treated with the highest SPH concentration which is at 0.1 mg/mL. Some previous studies showed the positive effects of protein hydrolysate application in enhancing the phytochemical contents in plants. Protein hydrolysates derived from the alfalfa plant (*Medicago sativa* L.) enhance the flavonoid contents in corn (*Zea mays* L.) plants by possibly simulating the flavonoid biosynthesis pathway. The flavonoid contents can be detected by the consistent increase of key enzymes involved in phenylpropanoid biosynthesis. In addition, the stimulation of nitrogen assimilation in the plant due to protein hydrolysate application can further stimulate phenylpropanoid biosynthesis (Ertani, et al., 2013; Shetty & McCue, 2003). Fish-derived protein hydrolysate treated fava bean germination has shown an increased phenolic content (Randhir & Shetty, 2003). Moreover, soybean plants treated with fish-derived protein hydrolysate showed a significant increase in phenolic content due to a potential stimulation of pentose-phosphate pathway (PPP) activity as a sugar-phosphate precursor source to stimulate phenolic synthesis to enhance plant development (Horii, et al., 2007; Shetty, 2004). The hydroxycinnamic amides have been induced in tomatoes by plant-derived protein hydrolysate application where this

compound contributes to mediating root growth as well as ROS signaling (Paul, et al., 2019; Mukherjee, 2018).

Furthermore, ascorbic acid, or vitamin C, is a powerful water-soluble antioxidant. Vitamin C deficiency, on the other hand, can be detected by some symptoms, including weak joints, skin discoloration caused by ruptured blood vessels, and bleeding gums (Gallie, 2013). Ascorbic acid is vital in the synthesis of collagen, an important component in skin, ligaments, tendons, and blood vessels, repair, and maintenance of bones, and teeth, wound healing, and improve hon-heme iron absorption from plant-based foods (Hancock & Viola, 2005). In addition, ascorbic acid improv plant nutritional value by serving in redox buffer, a cofactor in enzyme reactions, an antioxidant compound, and regulating cell division and signal transduction (Pinto & Gara, 2004; Smirnoff & Wheeler, 2000). Also, Athar et al. (2009) reported that the application of ascorbic acid to plants improves the activities of several key antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD).

In addition, the ascorbic acid content was at its highest when the hydroponic-planted lettuce was treated with 0.01 mg/mL of SPH (Table 4.3). However, the level of ascorbic acid decreased drastically and was lower than the non-treated lettuces when the lettuces were treated with 0.1 mg/mL of SPH. According to Colla et al. (2017), the ascorbic acid content in tomatoes has

increased when treated with legume-derived protein hydrolysate compare to the untreated tomatoes. Similarly, legume-derived protein hydrolysate treated greenhouse perennial wall rocket has improved the ascorbic acid content (Caruso, et al., 2019). According to El-Nakhel et al. (2021), the application of protein hydrolysate Trainer on dill microgreens enhances the ascorbic acid content by possibly promoting ascorbic acid biosynthesis. In addition, the increased ascorbic acid concentration is possibly linked to the enhanced minerals uptake by protein hydrolysate-treated plants (Rouphael, et al., 2017).

5.3 Determination of Chlorophyll and Carotenoid Contents

The determination of chlorophyll and carotenoid contents in PH-treated hydroponic-planted lettuce were tested for chlorophyll *a*, chlorophyll *b*, and carotenoids where freshly harvested plant leaves were cut into strips and incubated in acetone overnight at 4ºC and the extract was tested spectrophotometrically at respective wavelengths. The amounts of chlorophyll *a*, chlorophyll *b*, and carotenoids in hydroponic-planted lettuce treated with 0.01 mg/mL of SPH were the greatest compared to non-treated and lettuces treated with 0.001, and 0.1 mg/mL of SPH, according to the results obtained.

Chlorophyll and carotenoids are important pigments in plants that play a critical role in the photosynthesis process. Chlorophyll is the green and

carotenoids provide colored pigments in the vegetables, and fruits of the plant. Carotenoids are a good source of antioxidants, able to boost the immune system and provide protection against cancers. The main functions of these pigments in process of photosynthesis are the utilization of solar energy, triggering the fixation of carbon dioxide into carbohydrates, and lastly provision of energy for plant development (Rinawati, et al., 2020). Chlorophyll consists of chlorophyll *a* and chlorophyll *b* where the major and accessory pigments respectively. Besides, the changes in the ratio of chlorophyll a and b have been used to indicate the incidence of abiotic stress in the plants (Vicas, et al., 2010). The extraction of pigments from plant tissues is highly dependent on the extraction techniques, type of solvent used, and the duration of extraction. The commonly used solvents are methanol, acetone, ethanol, N, N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), and chloroform (Minocha, et al., 2009). Acetone is stated as one of the best solvents as it gives sharp chlorophyll peaks, and can extract the least polar carotenoids (Vimala & Poonghuzhali, 2015; Vicas, et al., 2010). In addition, **S**u et al. (2010) mentioned that acetone is the best to use for a quick and reliable chlorophyll extraction protocol and it provides a stable environment over time. Other than the type of solvent, the leaf anatomy and tissue structure equally affect the plant pigment extraction.

A previous study reported that fish-derived protein hydrolysate has significantly increased total chlorophyll content in soil-grown lettuce (Xu & Mou, 2017). Similarly, plant-derived protein hydrolysate application has

enhanced chlorophyll content in tomato and corn seedlings, perennial ryegrass, and tomato leaves (Colla, et al., 2013; Ertani, et al., 2013, Botta, 2013; Cerdán, et al., 2013). The addition of amino acids to hydroponic nutrient solution enhanced the chlorophyll concentration in tomato leaves (Garcia, et al., 2011). The insoluble component in tomato plant-derived protein hydrolysate increased chlorophyll content whereas the soluble components did not affect the chlorophyll content in bean plants (Baglieri, et al., 2014). Total chlorophyll *a*, *b* and carotenoids contents increased in tomato leaves, eggplant, and pepper when treated with increasing concentrations of micro-granule plant-derived protein hydrolysate. However, the ratio of chlorophyll *a* and *b* reduced progressively with the increasing concentrations of a micro-granule plant-derived protein hydrolysate, in which it is also indicated that the presence of higher N concentration will result in chlorophyll *a* and *b* ratio reduction (Rouphael, et al., 2021). Foliar application of whey-protein protein hydrolysate has increased these photosynthetic pigments in pea plants in a concentration-dependent manner (Osman, et al., 2021). The increase in chlorophyll concentration and photosynthesis rate in plant-derived protein hydrolysate can be due to the presence of a high level of amino acids (Luziatelli, et al., 2016).

5.4 Determination of Mineral Contents

Flame atomic absorption spectroscopy was used to detect the presence of minerals in the samples. Selected minerals including aluminium (Al), cadmium (Cd), lead (Pb), iron (Fe), copper (Cu), zinc (Zn), magnesium (Mg), and calcium

(Ca) were studied in SPH treated hydroponic-planted to study the minerals absorption and bio-accumulation. Based on the result obtained in this study, Al, Cd, and Pb were not detected in all of the sample extracts. Except for magnesium, the other five minerals, Fe, Cu, Zn, Mg, and Ca, were detected in the sample extracts, but no significant changes were found between the protein hydrolysate treated and non-treated hydroponic-lettuces. In hydroponic lettuce treated with 0.1 mg/mL of PH, a greater quantity of magnesium was observed.

In a study, the application of a high dose of microgranular-based protein hydrolysate has significantly increased the concentrations of Ca and Mg in tomato plant leaves compared to control. Signaling peptides in protein hydrolysate elicit auxins hormone-like activities that promote nutrient uptake by roots, enhance nutrient utilization and mineral profile as well as improve the plant and root growth of pepper, tomato, and eggplant. Even though protein hydrolysate is generally not considered a fertilizer, it enhances nutrient uptake by plants when applied at higher doses (Rouphael, et al., 2021). According to Anwar et al. (2020), Mg is involved in chlorophyll biosynthesis in leaves where Mg is covalently linked with N atoms. In addition, the increase of Mg in leaves can correlate with the increase of chlorophyll in tomato leaves. Consentino et al. (2020), reported that protein hydrolysate-treated celery plants have increased Mg concentration but no significant effects on Ca. In contrast, plant-derived protein hydrolysate has effectively increased the Ca but not Mg contents in Perennial Wall rocket (Caruso, et al., 2019). A study by Celletti, et al. (2020) reported that

tomato plants treated with protein hydrolysate affected the macro and micronutrients such as Ca, Mg, Zn, Cu, and Fe in the plant. In that study, protein hydrolysate application significantly increase Mg, Cu, and Fe concentrations in shoots while reducing and did not affect Zn and Ca respectively.

However, based on the result obtained in this study, the magnesium content in 0.1 mg/mL of SPH treated hydroponic-planted lettuce was at the highest compared to other minerals. The plant-derived protein hydrolysate can modify and enhance the root system of a crop that facilitates nutrient uptake by increasing the root absorbing area (Ertani, et al., 2013; Rouphael, et al., 2017). In addition, a recent study by Ertani et al. (2018), showed an induced expression of nutrient transporters and accumulation of minerals in plant foliar were observed in alfalfa-based protein hydrolysate treated tomato plant leaves. Hence, it explains the enhanced root system improved the uptake of minerals including phosphorus, potassium, and magnesium. Legume-derived protein hydrolysate at the highest concentration (5.0 ml L^{-1}) influenced the absorption, translocation, and accumulation of macronutrients in tomato leaf tissue better than lower protein hydrolysate concentrations (0.0 and 2.5 ml L^{-1}) (Rouphael, et al., 2017).

5.5 Determination of Antioxidant Enzyme Contents

Plants required sodium for photosynthesis in which sodium affects plant growth positively at lower concentrations, whereas at higher concentrations it can be toxic to plants by reducing water potential, affecting plant growth as well as causing ion imbalance in the plant system. Salinity can result in the generation of ROS as the response to abiotic and biotic stress which major alter the metabolic process, protein synthesis, and N assimilation in plants (Parida, et al., 2004). To prevent alterations in plant systems, plants use non-enzymatic and enzymatic antioxidants to manage the level of ROS. Two major antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), were investigated in this study, and an increase in both enzymes indicates that the plant is under stress (Hoque, et al., 2007).

The application of pumpkin seed protein hydrolysates treatment has increased the antioxidant enzymes such as SOD, CAT activities on beans, *Phaseolus vulgaris* to enable the plant to tolerate the negative effects caused by salt stress and maintain plant life under normal conditions (Sitohy, et al., 2020). The plant activated the defense system to protect against abiotic stress as well as quench ROS (Kaur & Asthir, 2015). A study by Rouphael et al. (2017) reported that antioxidant enzyme, especially CAT activity was significantly higher in plant-derived protein hydrolysate treated lettuces compared to untreated lettuce plants. Chicken feather-derived protein hydrolysate application on wheat cultivars decrease the ROS level by increasing the antioxidant enzymes such as

SOD but significantly did not affect the activity of CAT (Genc & Atici, 2018). Vasconcelos et al. (2009), stated that the application of amino acid-derived biostimulants contributes to reducing the abiotic stress for the plant by increasing SOD and CAT activities. The application of amino acid-based bio-stimulant in soybean crops increased CAT enzyme activity (Teixeira, et al., 2017). The quality, as well as the antioxidant activity of tomato, were enhanced in the foliar application of legume-derived protein hydrolysate (Colla, et al., 2017). Besides, Horii et al. (2007) reported that fish protein hydrolysate increased the free radical scavenging antioxidant activity in soybean but did affect the antioxidant activity in tomatoes. The antioxidant activity in greenhouse tomatoes is enhanced when treated with legume-derived protein hydrolysate in a dose dependent-manner (Rouphael, et al., 2017).

CHAPTER 6

CONCLUSION

In summary, we were able to prepare protein hydrolysates from soybean processing waste by using the enzymatic digestion method. The produced protein hydrolysates were successfully added to the hydroponic nutrient solution at various concentrations such as 0, 0.001, 0.01, and 0.1 mg/mL. G green coral lettuces were then cultivated in these nutrient-rich solutions for 9 weeks with a change of nutrient solution every 3 weeks. After harvesting, the effect of protein hydrolysate application on the physical properties of hydroponic-planted lettuce was determined. When compared to non-treated lettuce, hydroponic-planted lettuces treated with 0.1 mg/mL soy protein hydrolysate (SPH) had the greatest length and fresh weight. Other physical properties such as leave surface area, root length, and weight did not show and significant difference between SPH treated and non-treated hydroponic lettuces. Besides, SPH application has positively affected the phytochemical contents, chlorophyll and carotenoids, superoxide dismutase, and catalase contents in hydroponic-lettuce cultivation compared to non-treated lettuces. However, there was no significant difference in mineral contents were observed in SPH treated hydroponic lettuce compared to the control except for magnesium, where the lettuce treated with 0.1 mg/ml of PH has the highest magnesium content compared to lettuces treated with lower SPH concentration and control.

As a result, it can be concluded that using enzyme digested protein hydrolysate in hydroponic lettuce production could increase the above-mentioned parameters. This study highlighted the possible application of SPH to improve hydroponic crop production, as well as helping to ensure food supply for increasing demand. Additionally, the application of protein hydrolysates in hydroponic crop cultivation will further help in producing quality and nutrient-rich food for the consumers.

LIMITATIONS OF STUDY

There were several limitations to accomplishing this study. The effect of protein hydrolysates on the other biochemical profile including radical scavenging activities of hydroponic-planted lettuce was not able studied due to time and resource constraints. We were not able to test the other minerals such as potassium and phosphorus was not able to test due to limited sample extracts.

FURTHER STUDIES

Protein hydrolysates can be further developed as crop bio-stimulant in addition to chemical fertilizers. In addition to lettuce, other varieties of crops and herbs can be grown hydroponically and tested with the addition of enzyme-digested protein hydrolysates to study the effects in future studies. Furthermore, a study on the comparison of the effect of protein hydrolysates in soil and hydroponic crop cultivation is necessary. Lastly, more studies on the protein hydrolysates treated crops are necessary before providing the crops to consumers.

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Appendix A

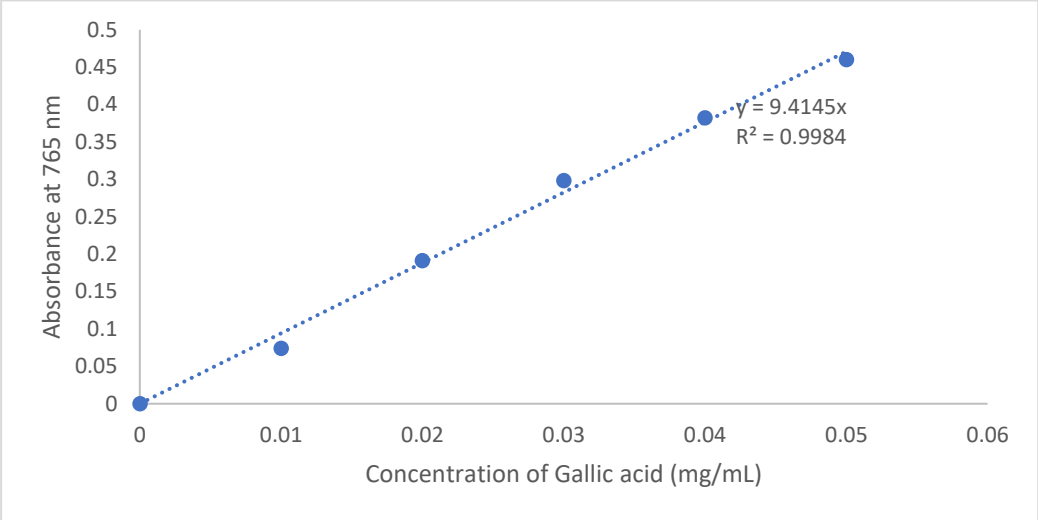

Figure 1: Standard curve of Total Phenolic Content (TPC) using gallic acid as standard.

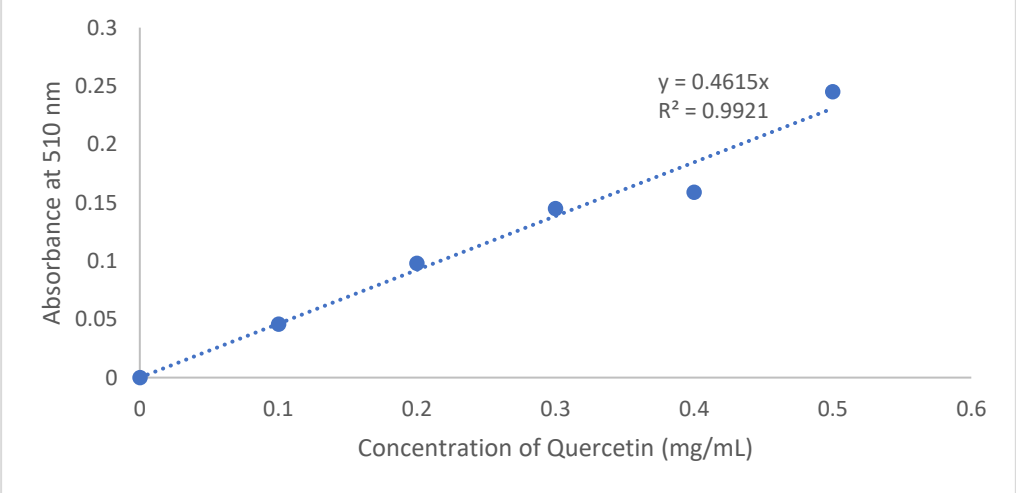

Figure 2: Standard curve of Total Flavonoid Content (TFC) using quercetin as standard.

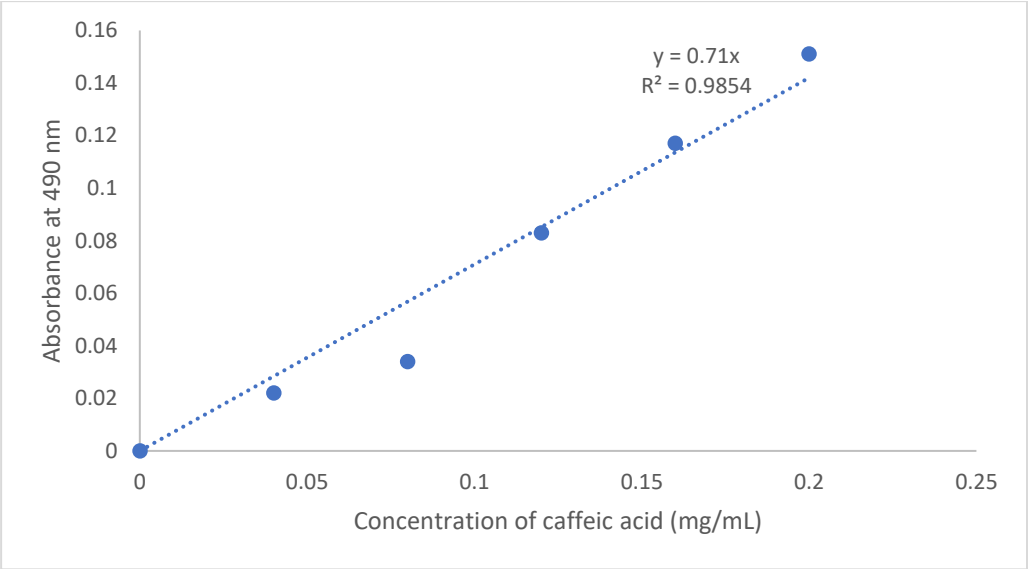

Figure 3: Standard curve of Total Hydroxycinnamic acid Content (THC) using caffeic acid as standard.

Table 1: Fresh weight of soy protein hydrolysate (SPH) treated hydroponic-planted lettuces.

SPH Concentration	Lettuce weight (g)
0 mg/mL	1.46 ± 0.65^{b}
0.001 mg/mL	1.52 ± 0.67^{b}
0.01 mg/mL	3.28 ± 0.53^{a}
0.1 mg/mL	$2.26 \pm 0.33^{a, b}$

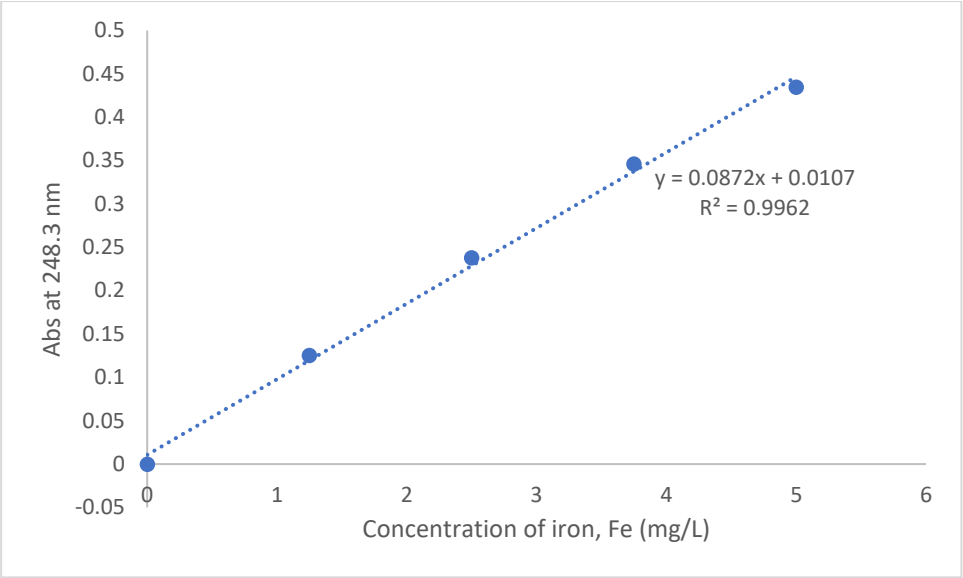

Figure 4: Standard curve of iron, Fe obtained through Flame Atomic Absorption Spectrometer (FAAS).

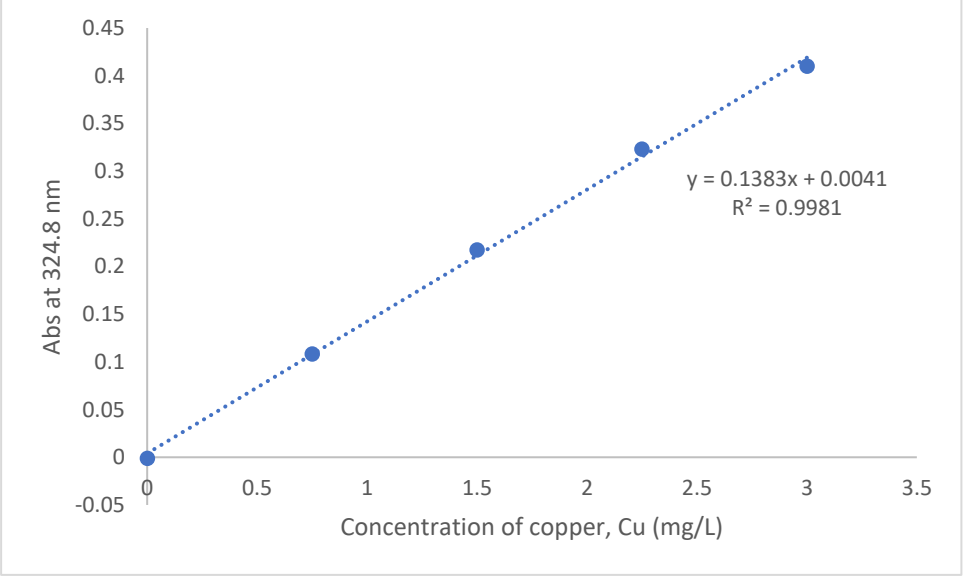

Figure 5: Standard curve of copper, Cu obtained through Flame Atomic Absorption Spectrometer (FAAS).

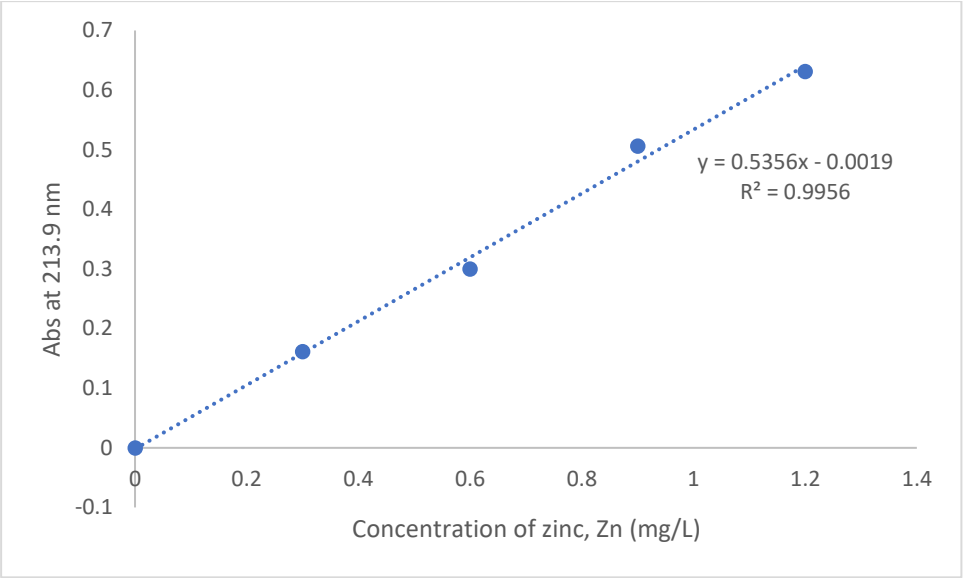

Figure 6: Standard curve of zinc, Zn obtained through Flame Atomic Absorption Spectrometer (FAAS).

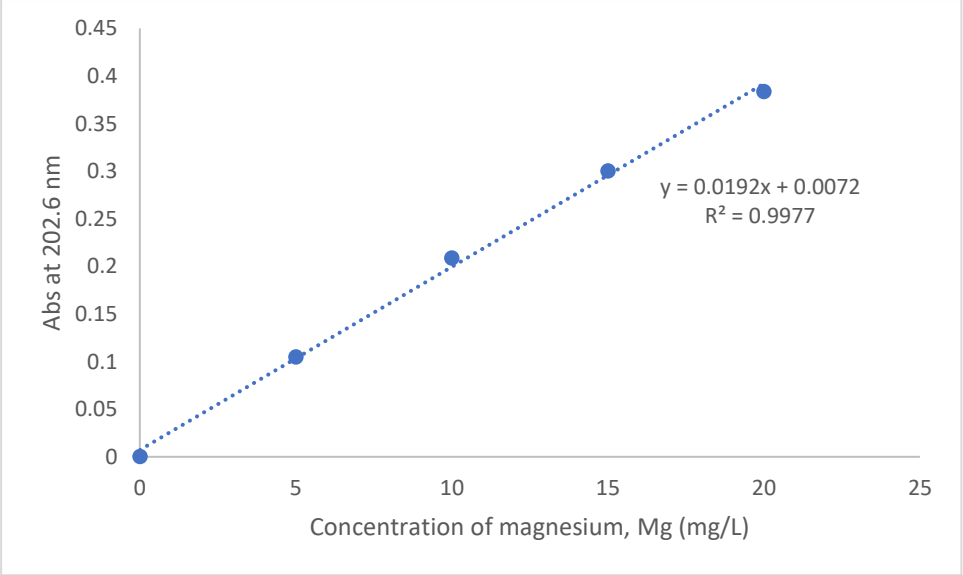

Figure 7: Standard curve of magnesium, Mg obtained through Flame Atomic Absorption Spectrometer (FAAS).

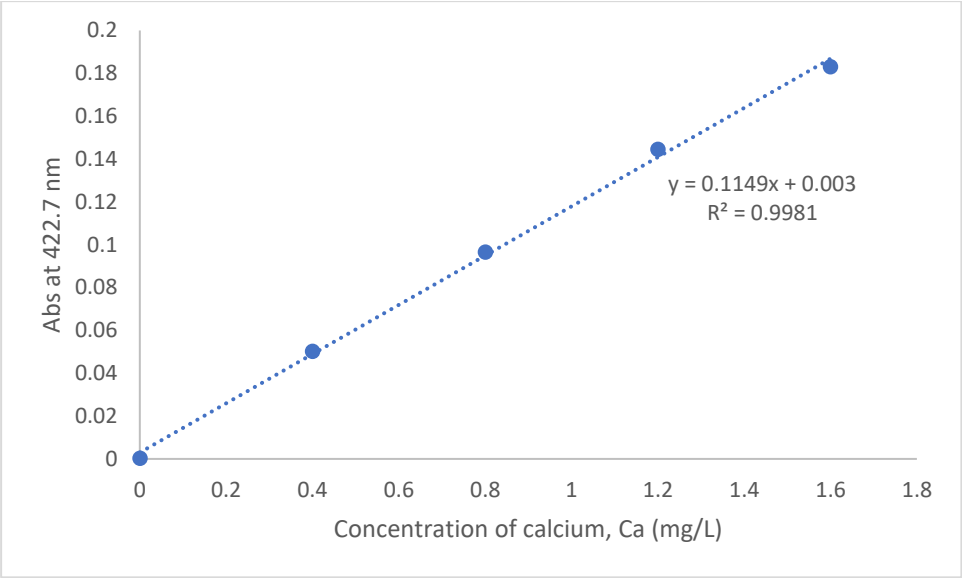

Figure 8: Standard curve of calcium, Ca obtained through Flame Atomic Absorption Spectrometer (FAAS).

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