ENZYMATIC HYDROLYSIS OF SOY OKARA AND FERMENTATION USING *Lactobacillus sp.* TO IMPROVE ITS NUTRITIONAL AND FUNCTIONAL BENEFITS AS FUNCTIONAL FOOD INGREDIENT

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by

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LIST OF SYMBOLS

®	Registered trademark
℃	Degree Celsius
µ	Micro
%	Percentage
±	Plus-minus Sign

LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ATCC	American Type Culture Collection
ANOVA	Analysis Of Variance
ATP	Adenosine Triphosphate
CO2	Carbon Dioxide
CE	Capillary Electrophoresis
CCD	Central Composite Design
C/O	Carbon Per Oxygen
CoA	Coenzyme A
DF	Dietary Fiber
DH	Degree of Hydrolysis
DPPH	2,2-diphenyl-1-picrylhydrazyl
DNA	Deoxyribonucleic Acid
Da	Dalton
DNS	3, 5-dinitrosalicylic acid
EDS	Energy-Dispersive X-ray spectroscopy
ETO	Enzymatic Treated Okara
Calcium EDTA	Ethylenediaminetetraacetic Acid Calcium Disodium Salt Hydrate
FAO	The Food and Agriculture Organization
FO	Fermented Okara
FRAP	Ferric Reducing Ability of Plasma

FT	Fourier Transform NMR
FTIR	Fourier Transform Infrared Spectroscopy
FBG	Fungal β-glucanase
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HPH	High-Pressure Homogenization
HPLC	High Performance Liquid Chromatography
ISF	Insoluble Fiber
kV	kilovolt
keV	Kilo electron Volts
kPa	Kilopascal
LAB	Lactic Acid Bacteria
LC-MS	Liquid Chromatography-Mass Spectrometry
MS	Mass spectrometry
MPa	Mega Pascal Pressure Unit
MRS	De Man, Rogosa and Sharpe
MSTFA	N-methyl-N-(trimethylsilyl)-trifluoroacetamide
m/z	Mass Divided by Charge Number
NMR	Nuclear Magnetic Resonance Spectroscopy
NIR	Near-infrared Spectroscopy
NaCl	Sodium Chloride
OP	Okara Protein
Ok	Okara

pH	Potential of Hydrogen
PUFA	Polyunsaturated Fatty Acids
PCA	Principal Component Analysis
PLS-DA	Partial Least Square Discriminant Analysis
PCR	Principal Component Regression
Psi	Pounds Per Square Inch
PEP	Phosphoenolpyruvate
RNA	Ribonucleic Acid
RO	Raw Okara
rpm	Revolution Per Minute
SDF	Soluble Dietary Fiber
SDGs	Sustainable Development Goals
SEM	Scanning Electron Microscopy
SF	Soluble Fiber
SY	Soya
SPSS	Statistical Program for the Social Sciences
TCA	Trichloroacetic Acid
TMCS	Trimethylchlorosilane
UPLC	Ultra Performance Liquid Chromatography

LIST OF APPENDICES

HIDROLISIS ENZIMATIK OKARA SOYA DAN PENAPAIAN MENGGUNAKAN *Lactobacillus* sp. UNTUK MENINGKATKAN FAEDAH PEMAKANAN DAN FUNGSINYA SEBAGAI BAHAN MAKANAN BERFUNGSI

ABSTRAK

Okara adalah sisa industri utama, dihasilkan sebagai produk sampingan daripada pengilang susu soya. Oleh kerana okara mempunyai nilai pemakanan yang tinggi, menggunakannya sebagai substrat untuk penapaian adalah pilihan ekonomi dan mesra alam. Fermentasi bakteria asid laktik digunakan untuk meningkatkan pecahan aromatik, dengan mengurangi heksanal, yang bertanggung jawab untuk rasa tidak enak dan meningkatkan keton dengan buah dan nota mentega dalam makanan yang ditapai. Tahap pertama kajian ini bertujuan untuk mengoptimumkan pra-rawatan soya okara pada pengekstrakan gula penurun dengan menggunakan kompleks multi-enzim, Viscozyme. Keadaan optimum mengikut reka bentuk komposit pusat untuk pengekstrakan gula penurun maksimum (4.78 mg/mL) ialah 8.0% (b/v) enzim, 50°C dan pH 3.0 untuk masa hidrolisis 4 jam, mewakili kenaikan sebanyak 158.33 kali, berbanding sampel okara mentah. Kromatogram kromatografi cecair berprestasi tinggi (HPLC) menunjukkan karbohidrat seperti glukosa, fruktosa, arabinosa, raffinosa, dan stakiosa meningkat setelah hidrolisis. Mikroskopi elektron imbasan (SEM) mengesahkan matriks okara yang sangat berstruktur terganggu oleh tindakan pra-rawatan enzim sementara spektroskopi sinar-X penyebaran tenaga (EDS) menyampaikan perubahan komponen unsur dalam okara terawat enzim (ETO). ETO menunjukkan kapasiti antioksidan yang lebih tinggi

berbanding sample kawalan oleh ujian DPPH, ABTS, dan FRAP. Seterusnya, kajian ini memfokuskan pada tiga permulaan yang disaring berdasarkan daya hidup sel dan kandungan protein total setelah penapaian ETO selama 48 jam. Keupayaan sel dan kandungan protein *L. plantarum* SY11 mencapai 16.40 log CFU/mL dan 14.76% (b/b), yang merupakan yang tertinggi berbanding *L. casei* ATCC 393 dan *L. acidophilus* ATCC 4356. Untuk mendapatkan pandangan mengenai mekanisme tersebut, analisis metabolomik yang tidak disasarkan dilakukan dengan kromatografi gas-spektrometri jisim (GC-MS). Secara keseluruhan, 55 metabolit dikesan dan dikelaskan kepada asid organik, asid lemak, asid amino, karbohidrat, alkohol, aldehid, fitosterol, dan pelbagai kumpulan. Data tersebut menunjukkan aliran tenaga dan metabolik ke arah asid amino dan asid lemak, yang sejajar dengan peningkatan pengeluaran asid amino dan asid lemak okara difermentasi (FO) oleh *L. plantarum* SY11. Analisis jarak menunjukkan bahawa protein kasar meningkat setelah fermentasi. Lebih-lebih lagi, FO mempunyai aktiviti antioksidan yang lebih tinggi (DPPH, ABTS, dan FRAP) berbanding okara mentah. Penemuan ini membuka kemungkinan menggunakan okara yang difermentasi sebagai makanan berfungsi yang berpotensi, menyediakan bahan simbiotik dalam aplikasi makanan dan makanan haiwan yang menjanjikan.

ENZYMATIC HYDROLYSIS OF SOY OKARA AND FERMENTATION USING *Lactobacillus* sp. TO IMPROVE ITS NUTRITIONAL AND FUNCTIONAL BENEFITS AS FUNCTIONAL FOOD INGREDIENT

ABSTRACT

Okara is a major industrial-waste, generated as a by-product from the soymilk manufacturer. Since okara has a high nutritional value, reusing it as a substrate for fermentation is an economical and environmentally friendly option. Lactic acid bacteria fermentation is being used to improve the aromatic fraction, by reducing hexanal, which is responsible for off-flavour and increase ketones with fruity and buttery notes in fermented food. The first stage of this study was aimed to optimize the pre-treatment of soy okara on the extraction of reducing sugars by applying a multi-enzyme complex, Viscozyme. The chemical composition and the morphological structure of enzymatic-treated okara (ETO) were scrutinized. The optimal conditions according to the central composite design for maximum reducing sugars extraction (4.78 mg/mL) were 8.0% (w/v) of the enzyme, 50°C and pH 3.0 for 4h hydrolysis time, representing a 158.33 times increment, compared to the sample raw okara (RO). The chromatogram from high-performance liquid chromatography (HPLC) revealed the carbohydrates such as glucose, fructose, arabinose, raffinose, and stachyose increased after hydrolysis. Scanning electron microscopy (SEM) confirmed the highly structured matrix of okara was disrupted by the action of enzyme pre-treatment while energy-dispersive X-ray spectroscopy (EDS) imparted the changes of elemental components in ETO. The ETO showed higher

antioxidant capacity than the control by DPPH, ABTS, and FRAP tests. Next, the study focused on three starters that were screened based on cell viability and total protein content after 48 h fermentation on enzymatic treated okara (ETO). The cell viability and protein content of *L. plantarum* SY11 reached 16.40 log CFU/mL and 14.76% (w/w), which was the highest among *L. casei* ATCC 393 and *L. acidophilus* ATCC 4356. To gain insight into the mechanism, untargeted metabolomics analysis was performed by gas chromatography-mass spectrometry (GC-MS). In total, 55 metabolites were detected and classified into organic acids, fatty acids, amino acids, carbohydrates, alcohols, aldehyde, phytosterol, and miscellaneous groups. The data revealed the energy and metabolic flux towards the amino acid and fatty acid pathways, which is in line with the increased amino acids and fatty acid production in fermented okara (FO) by *L. plantarum* SY11. The proximate analysis showed that crude protein was increased after fermentation. Moreover, FO had higher antioxidant activity (DPPH, ABTS, and FRAP) than raw okara. The findings open up the possibility of employing fermented okara as potential functional food, providing a symbiotic ingredient with promising food and feed applications.

CHAPTER 1

INTRODUCTION

1.1 Research background

Food processing by-products are a frequent industrial practice that causes socio-environmental difficulties and economic loss, thus finding new uses for them has gotten a lot of attention in recent years. Okara (in Japanese) is a cellulose by-product formed from soybeans, also known as *douzha* (in Chinese). It is the insoluble carbohydrate residue left over after the soymilk or bean curd has been filtered (William Shurtleff & Akiko Aoyagi, 2014).

There are a few restricting factors in the food chain that impede the economic adoption of okara. It is a low-nutritive by-product with the downside of having large levels of flatulence-causing carbohydrates (Liener, 1981). Fresh okara has an incredibly high (~70-80%) moisture content, render it vulnerable to rapid putrefaction (Khare et al., 1995). Drying is a costly waste residue solution. Industries thus favor either incinerating this by-product or burying waste in landfills. Secondly, high temperatures used during the soymilk/tofu output cooking process extensively denature soybean proteins and rendering the residue (okara) insoluble (Tao et al., 2019). This hampers the direct reuse of okara in the food supply chain. Other than that, high fiber content would also be found in Okara. However, high fiber content caused an unsavory feel for feeding, and so the use of Okara has been challenging. Therefore, before the re-use of this food waste, preparation methods must be utilized.

Although okara possess several problems but it still holds many nutrients (on a dry weight basis, approximately 50% carbohydrates, 20% proteins, as well as minerals and phytochemicals), making it a suitable substrate for biotransformation (Vong and Liu, 2016). Its high fiber content means that it could also be used as a dietary supplement to prevent diabetes, obesity, and hyperlipidemia (Li et al, 2012). Okara cell wall contains polysaccharides that can be hydrolyzed into soluble sugar and used as fermentable sugar by lactic acid bacteria. Its proper use would lead to economic advantages and a reduction in the potential for polluting the environment. Enzymatic treatment and lactic acid bacteria fermentation can improve its nutritional quality.

The employment of the biotechnological method of enzyme hydrolysis to the okara has less been recognized in the food industry, and the priority is to enhance the usage of nutrients profile from raw okara. Treatment for protein hydrolysis with proteases is common, but less studied is the hydrolysis of carbohydrates in okara by a mixture of carbohydrases (Rosset & Beléia, 2014). The addition of the multi-complex enzyme (carbohydrases) in okara may result in enzyme-treated okara with a composition different from the untreated okara. Due to the presence of these carbohydrases, the insoluble polysaccharides could be partially hydrolyzed by producing sugars or oligosaccharides and proteins, possibly changing the composition and texture of enzyme-treated okara. The hydrolysis of okara-insoluble polysaccharides can also facilitate the antioxidant release and sugars like glucose, galactose, and arabinose that would increase the sweetness of okara (Cai et al., 2021).

It should be considered that the bulk of the published study on okara uses agri-waste in its raw form. The enzymatic pre-treatment and subsequent bio-fermentation for

the enhancement of the nutrient profile is a comparatively recent domain that has yet to be thoroughly investigated. Different types of bacteria have been used in the biotransformation of raw okara recently, *Yarrowia lipolytica* (Vong et al., 2016), *Saccharomyces cerevisiae* (Queiroz Santos et al., 2018), *Rhizopus oligosporus,* and *Lactobacillus plantarum* (Gupta et al., 2018), *Eurotium cristatum* (Chan et al., 2019), and *Bacillus subtilis* WX17 (Mok et al., 2019). The positive outcomes of these studies show the need for further research in this field.

While much work has been undertaken to consider the potential of okara as a source of nutrition, there is still space for improvement in the nutritional profile of okara. In addition, there is a growing need for natural, green bioactive compounds rather than chemically synthesized compounds. This thesis aims to address these gaps, by enzymatic treatment and subsequent fermentation with food-grade microbes for enhancing the nutritional quality of okara.

1.2 Research scope

The scope of the study is limited to recruiting okara as the only substrate. Enzyme hydrolysis of okara was first performed with three different parameters used included enzyme dosage (0.5, 1.0, 3.0, 5.0, 7.5, and 10.0 % w/w of fresh okara), pH (3.5, 4.5, 5.0, and 5.5), and temperature (25, 30, 45, 50, and 55 ℃). The optimum parameters condition obtained was used for further analysis such as proximate, HPLC, SEM and EDS analysis, and antioxidant capacity of okara. These studies incorporated the usage of three Lactobacilli, *Lactobacillus casei* ATCC 393, *L. plantarum* SY 11, and *L. acidophilus* ATCC 4356 fermented with enzyme-treated okara for 48 h, only *L. plantarum* SY 11 was

used in the later stages of the study since it yielded better results in the preliminary stage. The latter study was a metabolomics study using GC-MS analysis and a nutritional study on proximate analysis and antioxidant capacity of fermented okara.

1.3 Objectives outlines and overall flow chart of this thesis

Okara, a by-product that remains after the production of soymilk or tofu. Putrefaction and bioactive nutrient inaccessibility are the key concerns with this food waste. Valorisation treatments with the Viscozyme® L were then selected for subsequent using microbial fermentation will render okara suitable for use as a functional food or nutraceutical while still being environmentally friendly. A flow chart of the overall research approach is shown in Figure 1.1.

Objectives

i. To investigate the enzymatic hydrolysis of soy okara using different parameters such as enzyme dosage, pH, and temperature to produce fermentable sugars.
ii. To screen the capability of three different *Lactobacillus* species to grow on enzyme-treated okara.
iii. To determine the nutritional values (such as proximate analysis and antioxidant capacity) and metabolomics analysis on fermented okara by selected *Lactobacillus* species.

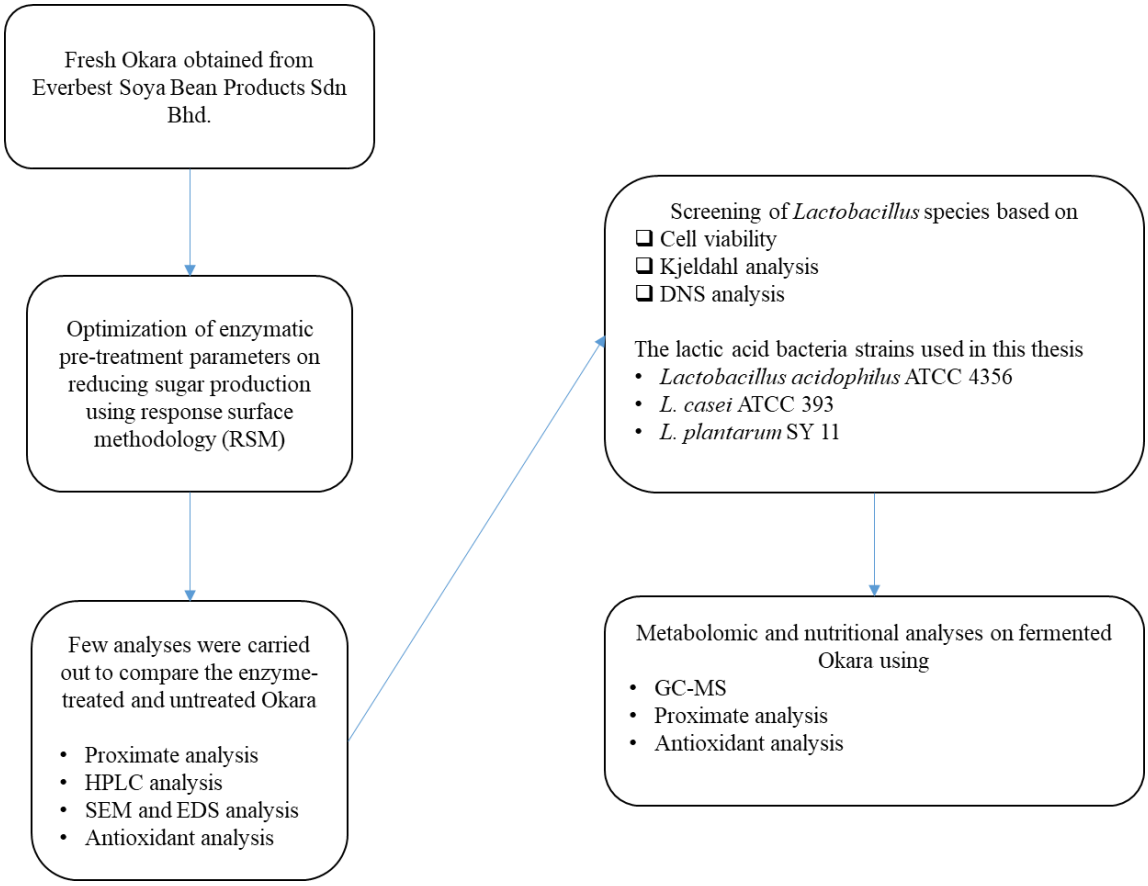

Figure 1.1 Flow chart of overall research approach.

CHAPTER 2

LITERATURE REVIEW

2.1 Sustainable development goals (SDGs)

All United Nations Member States adopted the Sustainable Development Goals (SDGs) in 2015, which were described as an action to end poverty, protect the environment, and ensure that all citizens live in peace and prosperity by 2030, as stated by the United Nations (United Nations Development Programme, 2021). There were 17 goals set by SDGs with one of the goals related in this thesis, the name Zero Hunger (SDG 2). This mission aims to end hunger and ensure year-round access to safe, nutritious, and sufficient food for all people, especially the poor and those in vulnerable situations (Alexandre Jeanblanc, 2018), but there are many other goals related to food system challenges. Food security is a priority of Zero Hunger, which aims to eliminate hunger, enhance food security and nutrition, and encourage sustainable agriculture. As a result, meeting the population needed, in terms of foods, resources, and nutrition, as well as advancing sustainable agriculture, creates favourable conditions for improving health, mental, and labour capacity, as well as economic growth and urbanization. As the world population continues to grow, this target becomes much more important and more effort has to be carried out, as preventing food waste is a recipe for SDGs. Furthermore, the Food and Agriculture Organization (FAO) emphasizes that global food insecurity is induced by unequal and undemocratic resource allocation and access, not an issue with global food production. Fermentation technologies are critical in ensuring the food

security of millions of people around the world, particularly those who are marginalized and vulnerable.

2.2 Waste

In general terms, waste refers to any material or object discarded or intended or needed to be discarded by the holder (Amanda Forni, 2019). Waste generation is directly proportional to a given urbanization and industrialization rate of geographic location. The effect of waste on the atmosphere may be expressed in air, water, and soil contamination, which has a direct impact on human health. Food waste or food loss is one of the wastes in this category.

2.2.1 Food waste

Food waste that brings the effect to the environment and the cause of this is manifold such as food spoilage due to improper harvesting, storage, transport, edible food from leftovers, discarding expired foods, etc. It includes peels and skins from meats, vegetables, and fruits (stalks and husks from grains and legumes or soy) (Gupta et al., 2018). Soybean by-product waste is one of the largest wastes among other plant-based waste. It spoils easily, has an unpleasant smell, and is unpalatable, so soy food producers dispose of it - which contributes to food waste (Wan, 2017). The sources of food waste such as homes, food manufactures, food distributors, food courts, food retailers, farms, etc. Most food waste exists in growing countries because of foods left unconsumed at home, in restaurants, or in supermarkets (Whitmee et al., 2015). This suggests that consumers can dramatically reduce food waste in high-income nations by changing their

eating habits and by voluntarily forcing supermarkets and eating venues to report and take steps to reduce food pollution by their public power. In low-income nations, by comparison, the majority of food losses exist between farms and markets due to bad farming methods, as well as poor food storage and distribution networks, including transportation (MacReady, 2015; Whitmee et al., 2015).

2.3 What is Okara

2.3.1 Okara

Soybean (Glycine max) is a fit to be eaten legume which has recently gained popularity due to the increase in vegetarianism, veganism, and lactose intolerance in the world (Gupta et al., 2018; Mok et al., 2019). It is primarily used for the processing of soymilk and tofu. Another major commercially viable commodity that is harnessed from soybeans is soy vegetable oil. Soy hulls, soybean feed, okara, and soybean straw are the waste that comes from soybean harvesting and processing.

Okara is one of the major agri-industrial wastes currently generated in the world. It is the tofu and primary by-product of the soybean industry. Botanically, it consists of soybean seed coat and ruptured cotyledon cells. It is a fiber-rich residue that is white or yellowish, insoluble in water and has a bland flavour. It is one of the agri-wastes that can be harnessed as a practical food with tremendous potential. Tofu is usually made from soy milk that has been heated, washed, and then processed with a coagulant at a high temperature (similar to rennet used in cheese making) that precipitates the proteins while simultaneously releasing curds and whey; the curds are then filtered off and formed under pressure into form. A large portion of the initial soybean mass is lost during the first

filtration process; the fibrous, protein- and oil-rich by-product formed at this point is known as okara, and it is used in both human and animal feeding (Rackis, 1981).

The current interest in okara is its potential value has ignited a whirlwind of related studies, the results of which are summarized in Table 2.1.

Table 2.1 Okara is used as a raw material.

Research	**Reference**
Characteristic and use	O'Toole, (1999)
Protein profile	Stanojevic et al., (2012)
Composition, nutrition, and utilization	Li et al., (2012)
Hypoglycemic effects	Lu et al., (2013)
Composition, utilization, and limiting factors	Li et al., (2013)
Isoflavone extraction	Jankowiak et al., (2014)
Bioethanol production	Choi et al., (2015)
As gluten-free flour	Ostermann Porcel et al., (2016)
Okara (soybean residue) valorization for food and nutrition	Vong & Liu, (2016)
As carbon source for developing photothermal nanomaterials	Weng et al., (2019)
Acid precipitation modifies the structural and functional properties of okara protein	Cai et al., (2020)

2.3.2 Chemical composition of okara

The composition of the okara in the proximate study will be different and was relies on soybean cultivation (growing environment) and the methods of processing (Kumar et al., 2006). As Li et al., (2013) reported that the proximate composition of the okara will depend on the amount of water phase extracted from the ground soybeans and whether further water is added to extract residual extractable components. If moisture is reduced to very low levels, then residual soy milk and its soluble and colloidal components will also be reduced to extremely low levels. The Chinese method involves soaking, rinsing, and grinding soybeans before filtering out the okara; the Japanese method involves cooking rehydrated soybeans before grinding and filtering (O'Toole, 1999). Table 2.2, Table 2.3, Table 2.4, and Table 2.5 provide an overview of the proximate composition of okara on a dry weight basis based on various studies.

Table 2.2 Proximate compositions of okara on a dry weight basis. (g/100g dry matter).

Crude Fiber	Proteins	Crude Fat/Oil	Ash	Carbohydrates	References
58.1-61.4	22.5-22.7	-	3.5-3.7	-	(Yu & Yang, 2019)
57.3-60.5	30.9-32.5	-	5.0-5.6	-	de Figueiredo et al., (2018a)
42.4-58.1	15.2-33.4	8.3-10.9	3.0-4.5	3.8-5.3	Vong & Liu et al., (2016)
55.5	28.5	9.8	4.5	5.1	Redondo et al., (2008)
54.3	33.4	8.5	3.7	3.9	Ma et al., (1996)

Okara is high in dietary fiber (DF). It also contains a minor amount of fucose, rhamnose, mannose, uronic acid, xylose, and arabinose had been uses as supplementation to enhance DF content in food. For this reason, enzymatic pre-treatment and microorganism fermentation have been used to improve the soluble dietary fiber (SDF)

content on it. Research proved that hydrolysis to be an effective approach in converting okara into a potentially value-added functional foods ingredient. The effective processing method to produce fermentable sugars and modify the microstructure of black soybean okara using bio-ionic liquid (bio-IL) pre-treatment and ultrasound-promoted enzymatic hydrolysis was investigated by (Yu & Yang, 2019). They reported bio-IL choline acetate ([Ch][OAc]) pre-treatment of black soybean okara, the TRS production of enzymatic hydrolysis was further increased to 5.2 times that without ultrasound in 4 h of reaction. de Figueiredo et al., (2018a) used the enzyme-assisted extraction was carried out under different temperatures (37–53 ℃), enzyme concentrations (1.5–4%), and pH values (5.5–6.5), resulting in an improved release of soluble fiber content. Hydrolysis under ideal conditions (0.8 % enzyme, 46 °C, 3 h) resulted in an increased soluble fiber content according to Yoshida & Prudencio, (2020). Li-zhen et al., (2009) reported the SDF content increased with the use of 4% cellulase, 50 ℃, and pH 5. The valuable oligosaccharides (including raffinose and stachyose) in okara, according to O'Toole, (1999), can serve as a prebiotic functional food ingredient. Next, fermentation effectively lowers the crude fiber contents of the okara and this will improve their digestibility by animals. *Lactobacilli* fermentation resulted in the breaking of polysaccharide glycoside bonds, resulting in the conversion of insoluble fiber (ISF) to soluble fiber (SF) (Tu et al., 2007).

Nonetheless, okara protein (OP) has a high nutritive value and a superior protein quality ratio, indicating that it may be a low-cost plant protein source for human foods (Muliterno et al., 2017). Also, there were several minor amounts of essential and non-essential amino acids that include lysine, valine, isoleucine, serine, glycine, alanine, threonine, proline, histidine, and cysteine + methionine was found in okara. These amino

acids can act as a food additive that gives a flavor to food and also some act as a supplement. Previous work had been carried out to extract or applied hydrolysis methods to further the production of okara protein found from the okara with a growing emphasis on extending its applications in the food industry. Ma et al., (1996) succeeded in extracting protein under these conditions (pH 9.0, 80 °C for 30 mins), resulting in a protein recovery of 53 %. They also found that the okara protein isolates have essential amino acid profiles that closely match the FAO scoring pattern when compared to the sample without enzymatic pre-treatment. de Figueiredo et al., (2018a) found that the optimal conditions (53 °C, pH 6.2, and enzyme concentration of 4%) resulted in a protein content of 56 % (dry weight basis). Fayaz et al., (2019) observed that high-pressure homogenization (HPH) contributes to a 90% protein extraction yield at 150 MPa for 5 passes. The structural disruption of okara particles was related to an increase in HPH strength, resulting in mechanically stable homogenates with increasing viscosity. This was largely due to increased okara solubility as a result of fiber and protein release.

Redondo-Cuenca et al., (2007) reported that okara contained around 18-22% of fat that comes from soybean seed. Polyunsaturated fatty acids (PUFA) such as linolenic acid (C18:3) and linoleic acid (C18:2) were the most abundant. It also contains a significant quantity of oleic acid (C18:1) and a low quantity of saturated fatty acids like palmitic acid (C16:0) and stearic acid (C18:0). The health benefits associated with the intake of these fatty acids are the reduction of cardiovascular diseases, the nervous system, anti-inflammatory, anti-allergic effects, and the potential protection against certain types of cancer (Álvarez & Lamas, 2021). Hence, PUFA of okara had been representing an excellent raw material that can be utilized for dietary protein fortification.

Table 2.3 Vitamins composition found in okara. (mg/100g dry matter).

Vitamins	Riboflavin	Thiamine	Nicotinic Acid	References
	0.03-0.04	0.48-0.59	0.82-1.04	Stanojevic et al., (2014); van der Riet et al., 1989)
	0.59	0.04	-	Khare et al., (1995)

Table 2.4 Carbohydrates composition found in okara. (g/100g dry matter). Extracted from (Gupta et al., 2018).

Carbohydrates	g/100g Dry Matter
Fucose	0.45
Rhamnose	0.85
Mannose	1.26
Uronic Acid	5.03
Xylose	5.14
Arabinose	6.35
Galactose	10.83
Glucose	15.0
Raffinose	30.0
Starch	59.0
Stachyose	140.0
Sucrose	230.0

Table 2.5 Minerals composition found in okara. (mg/100g dry matter).

Minerals	References		
	van der Riet et al., (1989)	Khare et al., (1995)	Li et al., (2012); Mateos-Aparicio et al., (2010); Vong & Liu, (2016)
Copper (Cu)	1.1-1.2	-	0.1–1.2
Manganese (Mn)	2.3-3.1	-	0.2-3.1
Zinc (Zn)	3.5-6.4	-	0.3-3.5
Iron (Fe)	6.2-8.2	6.0	0.6-11
Sodium (Na)	16.2-19.1	-	16.0-96.0
Magnesium (Mg)	158.0-165.0	163.0	130.0-165.0
Calcium (Ca)	260.0-428.0	260.0	260.0-428.0
Phosphorus (P)	396.0-444.0	-	-
Potassium (K)	1046.0-1233.0	1046.0	936.0-1350.0

The mineral content of okara has been analyzed extensively. It was rich in macro- and micronutrients, which enhances its nutritional value (Stanojevic et al., 2014). Mateos-Aparicio et al., (2010) mentioned that potassium is the most abundant mineral in the macro-element group, while iron is the most abundant micro-element. There are no published details on the contents of Cd, Co, Cr, and Pb in okara, even though the quantities of these elements are known.

Approximately 12-30 % of soybean Isoflavone remaining on it after soy milk processing. (Kamble & Rani, 2020). These compounds have a range of physiological and therapeutic properties, including antioxidant involvement, cardiovascular disease mitigation, and effective cancer chemo preventive agents (Quintana et al., 2017). According to Jackson et al., (2002), the major Isoflavone in okara is glucosides and aglycones, with acetyl genistein accounting for a smaller quantity. Izumi et al., (2000) discovered that glucosidase could hydrolyze the isoflavone glucosides to their aglycone stage enzymatically, resulting in increased human bioavailability. Furthermore, since the chosen fermentative microbes secrete glucosidase, the conversion of Isoflavone glucosides to aglycones by fermentation adds additional value (Bhatia et al., 2002).

2.4 Characteristic of okara structure

The morphological surface characteristic of okara has been studied using scanning electron microscopy techniques (Voss et al., 2018). SEM is a cutting-edge method for verifying the structure of foods by assessing the interaction of starch within the protein matrix (Kamble et al., 2019). Figure 2.1 shows the results of a study conducted by Santos et al., (2019) to analyze the structure of okara flour particles using SEM. The results

further implied that the surface structure of okara flour was either loose or agglomerated (a heterogeneous composition was observed in the micrograph). They further discovered the surface structure of okara flour had irregular and ambiguous forms, making them difficult to discern. Furthermore, okara surface particles are geometric forms with a few holes, resulted to have a high prevalence of permeable pores. Because the pores will cause high water absorption, flour component are described as hygroscopic structure. Voss et al., (2018) investigated the surface structure of fresh okara (stored at 4°C and -18°C) and thermally dried okara samples (stored at 80°C/5 h and 200°C/1 h). The key difference between the samples was the moisture level, which was lower in dried okara samples at 80°C and 200°C than in fresh okara samples. Because the okara structure had a higher porosity for those dried at 200 °C/1 h as due to the moisture induced alteration in it.

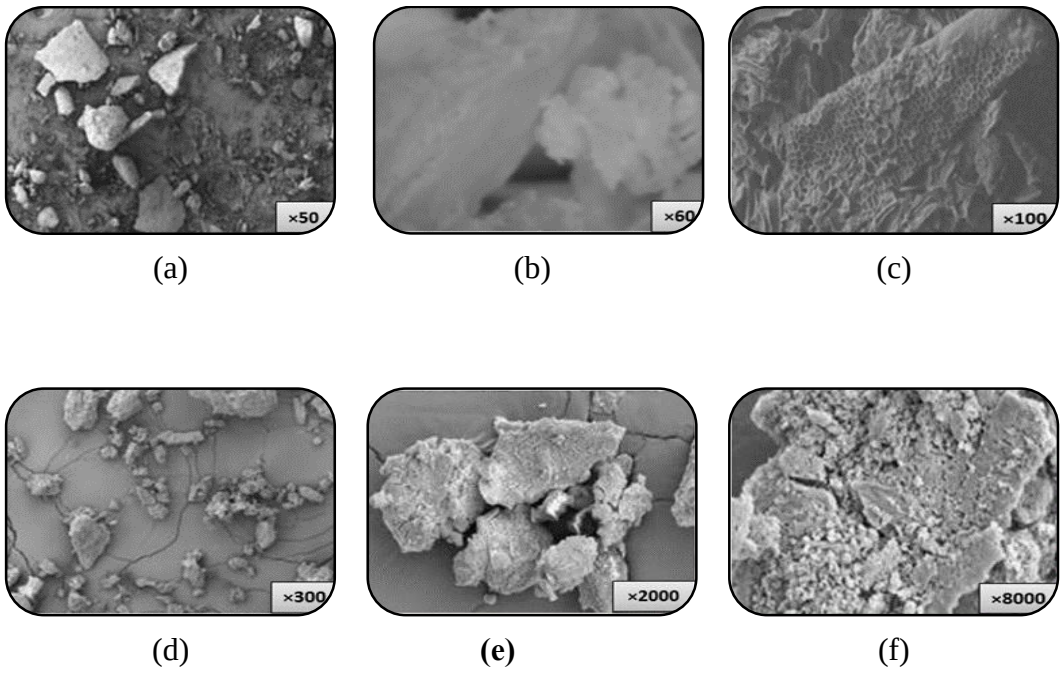

Figure 2.1 Microstructure of dried raw okara. Extracted from (Santos et al., 2019).

2.5 Enzyme hydrolysis treatment on okara

The high content of okara fibers and the unsavoury texture of the food has made them difficult to use (Ma et al., 1996). Nevertheless, the okara plant cell wall is composed of advanced complex structures (Kasai et al., 2003). Kasai et al., (2004) stated that both enzymes, Pectinex Ultra SP and pectolyase, were able to digest the secondary cell wall (consisting of galacturonic acid, protein, and neutral sugars) and resulted in yields of 85 % from raw okara. As a result, enzyme hydrolysis allows for the reduction of cell wall rigidity, resulting in a surface structure with more porous, better hydration characteristics, and thus improved chemical composition (including fermentable sugar and proteins during specific optimization conditions) of okara (Huang et al., 2015).

Recently, there was a few research have performed under specific optimization conditions on enzyme hydrolysis. Pereira et al., (2019) achieved the highest degree of hydrolysis (DH) of 22 % using a combination of alcalase (A) and flavourzyme (F) under optimal conditions of 40°C, 5.0 %E/S, pH 7.1, and 90/10 A/F. The aglycone Isoflavone content of all protein hydro lysates was three times greater than that of the okara protein concentrate. Wu et al., (2012) achieved optimized enzymatic oligosaccharide preparation conditions using Viscozyme® L with 3% (w/w of fresh okara), 45 ℃, and at pH 3.5 yielding a maximum of 10% (w/w). They discovered that these oligosaccharides contain a high amount of neutral sugar and are composed of galactose, xylose, rhamnose, arabinose, mannose, and glucose. Later, Yoshida & Prudencio, (2020) used the same enzyme under optimized parameters (0.8 % enzyme, 46 °C, 3 h), resulting in a huge increase in soluble fiber content. They discovered that enzyme-treated okara particles were more brittle and scattered than control particles. The procedure also changed the

colour and chemical composition of the fibers, resulting in less insoluble fibers and more soluble fibers and sugars. Enzymatic hydrolysis with a multi-enzyme complex improved the physicochemical properties of okara, which are important for its use in the food industry.

2.5.1 A multi-enzyme complex

Viscozyme® L is a multi-enzyme complex that comprises several distinct carbohydrases (composed of cellulase, arabanase, hemicellulases, beta-glucanase, and xylanase) which are produced by a selected strain of the fungus *Aspergillus aculeatus*. It also breaks down plant cell walls (branched pectin-like compounds) and has an optimum activity (a pH range of 3.3–5.5 and a temperature range of 25–55 °C). The processing of plant cell protoplasts from lettuce leaves is one of its more interesting applications in the school laboratory. Degrading the non-starch polysaccharides that are often attached to starch in plant products, may increase the supply of starch in fermentation. It decreases the viscosity of plant-derived products in general and can increase extraction yields (Viscozyme Product sheet, 2018).

2.5.2 Application of multi-enzyme complex

Table 2.6 List of applications of the multi-enzyme complex on a different substrate.

Substrate	Respond on extracted compound	Reference
Edible seaweed, Sargassum horneri	Superior and scavenged radical activities.	Park et al., (2004)
Oat bran	56.2% of the protein was extracted.	Guan & Yao, (2008)
Aloe vera gel	Extracted active polysaccharides have the highest yield and the best scavenging activities against DPPH, hydroxyl, and alkyl radicals.	Kang et al., (2014)
Rapeseed Meal	A rise in protein content from 41 to 68 % and an 80 % carbohydrate extraction yield.	Rodrigues et al., (2014)
Defatted soy flour	Reducing sugar (168.8% of glucose increased) increased and crude fiber decreased.	Chen et al., (2015)
Jujube Fruit	Quercetin, complete phenolic, and flavonoids were found to have higher levels of radical-scavenging capacity.	Kim et al., (2020)

2.6 Fermentation

Fermentation is the process of microorganisms producing enzymes that cause chemical changes in an organic substrate (such as food waste, agri-industrial crop waste, and kitchen and garden waste). Carbohydrates and associated compounds are partly oxidized with the release of energy in the absence of any external electron acceptors. Final electron acceptors are organic compounds formed directly from the breakdown of carbohydrates, and only a small amount of energy is released (Erkmen & Bozoglu, 2016). Bacterial fermentation will result in a safer fermented food with many benefits, including a longer shelf life than traditional foods, the elimination of unnecessary ingredients from raw materials (flatulence factors in soybeans), improved nutritional profiles, and higher in vitro antioxidant ability. Lactic acid bacteria, for example, is the most common micro

biota found in all fermented foods and beverages, it contributing to health benefits to fermented foods and beverages. (Şanlier et al., 2019; Sharma et al., 2020).

2.6.1 Lactic acid bacteria

The most important bacteria in desirable food fermentation are LAB bacteria, which can produce lactic acid from carbohydrates. They convert carbohydrates to lactic acid, carbon dioxide, and other organic acids in the absence of oxygen. The most popular LAB genera are *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus,* and *Streptococcus.* They are Gram-positive, non-motile, non-respiring, non-spore-forming rods or cocci that require carbohydrates for energy and produce lactic acid as a major end product of carbohydrate fermentation (Wedajo, 2015; Erkmen & Bozoglu, 2016). The majority of lactic acid sources are anaerobes, which can only expand in small amounts of oxygen. They are members of the *Lactobacillus* genus. Amino acids, B vitamins, and nucleic acid bases (purine and pyrimidine) are needed for LAB growth; some strains can grow at high temperatures (up to 45 °C), and some strains can grow at a variety of pH levels, but do best at 4.0–4.5. (and some at 3.2-9.6) (Erkmen & Bozoglu, 2016; Rakhmanova et al., 2018).

2.6.2 Characteristics of LAB used in this study

The *Lactobacillus* spp. have been classified into two categories based on how they manufacture carbohydrates as an end-product. To begin with, homofermentative *Lactobacillus* spp. produce lactic acid as their primary product, and they are unable to ferment pentoses or gluconates. The optimum temperature for their growth activity is

between 37 and 40 °C, with lactic acid production reaching up to 3%. The heterofermentative *Lactobacillus* spp. will then use hexoses to produce lactic acid, acetic acid, ethanol, formic acid, and CO_2, as well as pentoses. Furthermore, at the optimum growth temperature of 30 °C, they can contain up to 1.5 % lactic acid. The growth characteristics of this species can be classified into three classes, which are summarised in Table 2.7. Three types of Lactobacilli that would be used in this study included *L. acidophilus*, *L. casei*, and *L. plantarum*. A healthy intestinal bacterium that produces -galactosidase and lives in the small intestine, *L. acidophilus*. Lactose is metabolized, and large amounts of D (-) lactic acid, ranging from 1.2 to 2.0 %, are produced. *L. casei subsp. casei* then ferments lactose to produce L (+)-lactic acid, which is used in fermented dairy products. In addition, (DL)-lactic acid is produced when lactose is fermented with *L. plantarum.*

Table 2.7 Summary of growth characteristics. Extracted from (Erkmen & Bozoglu, 2016).

Thermobacterium	**Streptobacterium,**	**Letabacterium.**
Ferment hexoses (and disaccharides such as lactose and sucrose) produce mainly lactic acids and do not ferment pentoses (such as ribose, xylose, and arabinose). Produce lactic acid (up to 3%) with an optimum growth temperature of 40°C. Ferment hexose through the EMB pathway (homofermentative), not pentoses.	Species either produce lactic acid or a mixture of lactic, acetic, and formic acids, ethanol, and CO_2 (facultative heterofermentative) which depending on the number of carbohydrates. With an optimum growth temperature of 30°C, produce lactic acid (up to 1.5%), acetate, ethanol, CO_2, and formate. Hexoses and pentoses are fermented through the EMB and phosphoketolase pathways, respectively.	Ferment carbohydrates to a mixture of lactate, acetate, ethanol, and CO_2 (heterofermentative). Produce mixtures of lactate, acetate, ethanol, and CO_2. Use phosphoketolase pathway for fermentation of pentoses.

2.6.3 Food fermentation is made up of several functional lactic acid bacteria

Table 2.8 Examples of commercial starters for food fermentation are made up of several functional microorganisms. Extracted from (Tamang et al., 2016).

Lactic acid bacteria	Product
Enterococcus durans	Cheese
E. faecium	Soybean, dairy, meat, vegetables
Lactobacillus acetototolerans	Ricotta cheese
L. acidophilus	Milk, probiotics, vegetables
L. alimentarius	Sausages; ricotta; meat, fish
L. buchneri	Malolactic
L. casei subsp. casei	Dairy starter; cheese ripening; green table olives
L. delbruecki subsp. bulgaricus	Yogurt
L. helveticus	Starter for cheese; cheese ripening, vegetables
L. oeni	Wine
L. paracasei subsp. paracasei	Probiotics, meat, wine
L. plantarum subsp. plantarum	Fermentation of vegetables, malolactic fermentation, green table olives; dairy, meat
L. salivarious subsp. salivarius	Cheese fermentation
Lactococcus lactis subsp. lactis	Dairy starter, Nisin (as protective culture)

2.7 Primary and secondary natural product

In general, a natural product is referred to something produced by life, which includes biotic substances, bio-based substances, bodily fluids, and other different natural substances that were once found in living organisms. The natural product is defined as any organic substance synthesized by a living organism (Soderberg, 2016). They serve as excellent models for the creation of synthetic molecules that imitate their bioactivity. They are also currently being used in the healthcare and food industries as safe, natural, and

effective alternatives to synthetic compounds. Furthermore, it can be classified into primary and secondary metabolites (Abernethy, 1957).

Primary metabolites are organic molecules that play a role in normal growth, development, and reproduction. There are the basic building blocks (nucleic acids, amino acids, sugars, and fatty acids) that go into making the large macromolecules (DNA, RNA, proteins, carbohydrates, and lipids) that keep existence going. Secondary metabolites, on the other hand, are organic molecules that serve an ecological purpose but are not directly involved in the growth, development, or reproduction of an organism. The integration of primary and secondary metabolism is depicted in Figure 2.2.

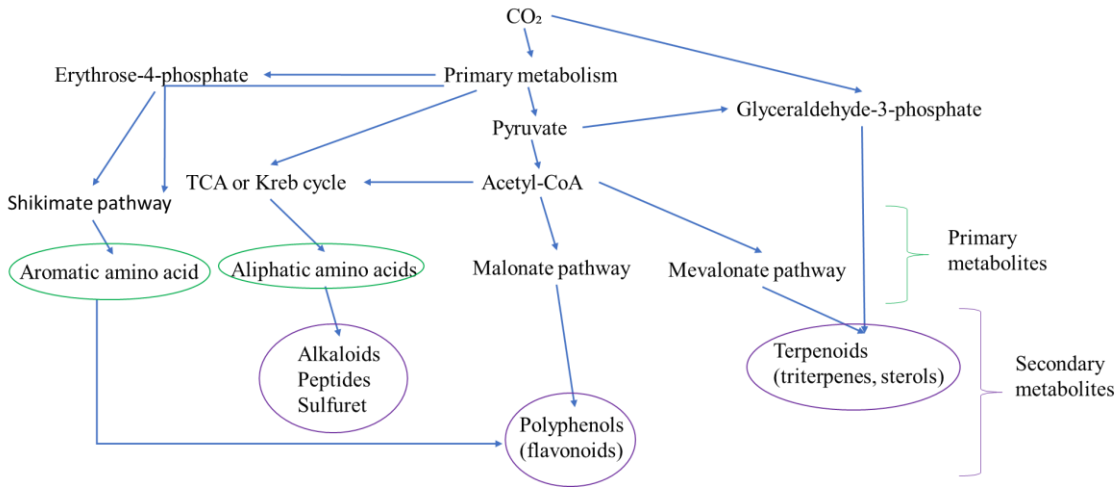

Figure 2.2 Diagram depicts the integration of primary and secondary metabolism adapted from (Ruchika et al., 2019).

2.8 Metabolomics, last member of the ‘Omics’ family

Genomic, transcriptomic, proteomic, and metabolomics methods are considered useful tools for understanding the biology of an organism and its response to environmental stimuli. Figure 2.3 depicts the Omics flow, which starts at the genomic level (‘genomics'), which encompasses the analysis of the entire genome. It was primarily used in the field of genomics, to detect genetic variants linked to illness, medical research, and patient prognosis in the future. Next, followed by ‘Transcriptomics’ which explores RNA levels throughout the genome, both qualitatively and quantitatively. The central dogma of biology regarded RNA as a molecular intermediate between proteins and DNA, which are primary functional readouts of DNA (Dunham et al., 2012). The term ‘proteomics' refers to a technique for determining peptide abundance, alteration, and interaction. MS-based approaches, for example, have been used in this omic for protein analysis and quantification.

The term "metabolomics" refers to a promising approach for biomarker discovery that includes both targeted and non-targeted studies of endogenous and exogenous small-molecule metabolites (<1500 Da). For example, small-molecule biomarkers such as carbohydrates, amino acids, nucleic acids, organic acids, inorganic species, polyphenols, and alkaloids act as a functional phenotype in a cell or organism. Technological advances in metabolomics have allowed the separation and identification of these small molecules. High-resolution MS, NMR, CE, HPLC, and UPLC technology are among the cutting-edge technologies that can detect metabolites in as little as a few minutes. These analyses are usually classified into either targeted or untargeted. The targeted research focuses on a specific cluster of intended metabolites, necessitating quantification and identification as a result. They are more complicated and necessitate higher levels of extraction and purification before the

examination. Untargeted metabolomics research is more general and focused on detecting a wide variety of metabolites to obtain fingerprints or trends (Seyler et al., 2020).

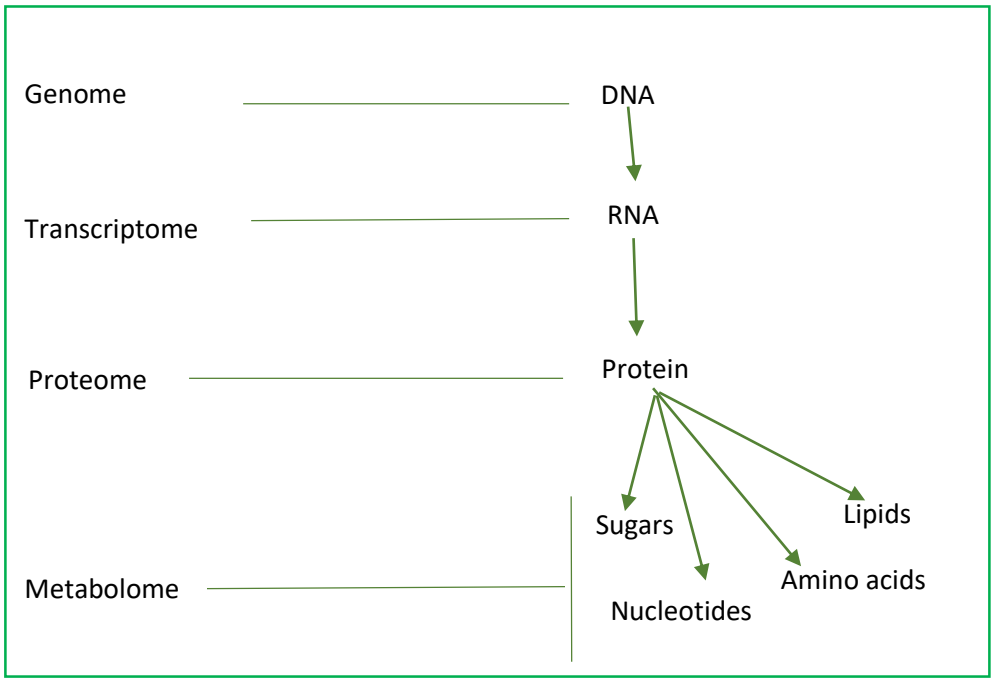

Figure 2.3 Schematic diagram of the central dogma of molecular biology and its corresponding omics disciplines. Extracted from (Seyler et al., 2020).