EXTRACTION OF DEFATTED RICE BRAN PROTEIN BY SOLID-STATE FERMENTATION AND CHARACTERIZATION OF THE PROTEIN

UGYEN

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in Food Science and Technology - (Type A 1)
2022

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has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Food Science and Technology - (Type A 1) of Naresuan University

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ABSTRACT

Defatted rice bran (DRB) is an abundant by-product of rice bran oil industries and a potential source of nutritious and hypoallergenic protein content. The most common alkaline extraction of defatted rice bran protein (DRBP) possed undesirable characteristics, making nutritional and functional compromises. However, enzymatic extraction could extract protein with improved protein properties despite being expensive. Therefore, this thesis exploited solid-state fermentation (SSF), which is an economically sustainable and natural approach to facilitate the extraction of DRBP using two fermentation starters, Loog-pang (Thai wine starter) and Koji.

The feasibility of two fermentation starters, Loog-pang and Koji, in producing enzymes and their efficacy in extracting protein from DRB were investigated. SSF of DRB was carried out at different times and Loog-pang and Koji fermented DRB obtained was further hydrolyzed for 24 h to enhance the protein extraction. The results obtained indicated that both the fermentation starters could secrete cellulase and protease (acid and neutral) enzymes with almost similar effectiveness in DRBP extraction. The fermented DRB (72 h) followed by hydrolysis (24 h) process revealed maximum protein extraction with both the fermentation starters. Loog-pang and Koji fermentation could extract protein of 65.66 and 65.67 g/100 g

DRB, respectively. However, the defatted rice bran protein hydrolysate (DRBPH) presented a significant quantity of ash content which impaired the protein purity (27.83 and 29.36 g/100 g DRBPH with Loog-pang and Koji, respectively). Therefore, the DRB was dephytinized at pH 2 to obtain dephytinized defatted rice bran (DDRB) and SSF was carried out with a Loog-pang fermentation starter to evaluate the protein extraction. The result revealed that 48 h of SSF followed by 24 h hydrolysis could extract maximum protein content of 59.44 g/100 g DDRB. The DDRBPH prepared contained very low ash content (2.73 g/100 g DDRBPH) with better protein purity (37.23 g/100 g DDRBPH).

The SDS-PAGE protein profile showed that 72 h fermented DRB for Loog-pang and Koji obtained protein of diverse molecular weights ranging from 10-100 KDa. These polypeptides were later hydrolyzed and fragmented into smaller molecular weight peptides during 24 h hydrolysis and enhanced protein solubility. Glutamic acid followed by aspartic acid, leucine, arginine, alanine, and glycine were the most abundant amino acid present in both non-fermented and fermented DRBP. SSF assisted to extract protein without altering the amino acid profiles to that of non-fermented DRBP. This indicated the effectiveness of SSF on DRBP extraction which helped to improve protein yield and maintained the amino acid profile.

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ABBREVIATIONS

^{o}C	Degree Celsius
CFU	Colony Forming Unit
CMC	Carboxymethyl Cellulose
DNS	Dinitro Salicylic Acid
DRB	Defatted Rice Bran
DRBP	Defatted Rice Bran Protein
DRBPH	Defatted Rice Bran Protein Hydrolysate
DDRB	Dephytinized Defatted Rice Bran
DDRBP	Dephytinized Defatted Rice Bran Protein
DDRBPH	Dephytinized Defatted Rice Bran Protein Hydrolysate
DSM	Defatted Soybean Meal
FF-RB	Full Fat Rice Bran
FAO	Food and Agriculture Organization
g	Gram
GRAS	Generally Recognized as Safe
h	Hour
HCl	Hydrochloric Acid
HPLC	High-Performance Liquid Chromatography
HSDRB	Heat-stabilized Defatted Rice Bran
KDa	Kilo Dalton
Kg	kilogram
M	Molar
µL	Microlitre
mL	Millilitre
min	Minute
nm	Nanometer
NaCl	Sodium Chloride
NPR	Net Protein Ratio
NPU	Net Protein Utilization
PER	Protein Efficiency Ratio

pH	Potential of Hydrogen
RBO	Rice Bran Oil
rpm	Revolution Per Minute
SSF	Solid-state Fermentation
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TCA	Trichloroacetic Acid
USDA	United States Department of Agriculture
WHO	World Health Organization

CHAPTER I

INTRODUCTION

1.1 Introduction

Rice bran is an abundant by-product of the rice milling industry and is identified as one of the most nutritious agricultural wastes with 11-17% protein content (Fabian and Ju, 2011; Fathi et al., 2021). Defatting or extraction of oil from the full-fat rice bran (FF-RB) for food application owing to its unique health benefits due to the presence of high nutraceutical content in the rice bran oil (RBO) (Danielski et al., 2005) further raises the protein content to about 15-18% (Alexandri et al., 2020). Protein extracted after the removal of oil content from the FF-RB is called defatted rice bran protein (DRBP). DRBP has gained attention in the world for more than a decade and proved to possess a superior quality of protein mainly in terms of its nutritional and functional properties. DRBP possesses a hypoallergenic (Helm and Burks, 1996) and anti-cancer activity (Kawamura and Muramoto, 1993), thereby, making it the ideal ingredient for infant food formulation (Zhuang et al., 2019). Moreover, the nutritional content of the DRBP is comparable to the protein of animal and other plant-origin as protein efficiency ratio (PER), net protein ratio (NPR), and net protein utilization (NPU) were 2.39, 3.77, and 70.70, respectively (Han et al., 2015). Rich bran protein contains all the essential amino acids and the amino acid composition is significantly closer to the model recommended by the Food and Agriculture Organization and World Health Organization (Wang et al., 2016; Zaky et al., 2020). The lysine amino acid, which is lacking in most cereals, is 3-4% in rice bran protein which is greater than rice endosperm protein or protein from any other cereals (Juliano, 1985; Shih et al., 1999; Yeom et al., 2010) and the digestibility and biological value of the protein are reported as 0.90 and 72.6, respectively (Han et al., 2015). Despite these various health benefits and the superior nature of the DRBP, the search for a feasible extraction process is a never-ending process due to the lack of efficient extraction methods.

DRBP consists of a mix of 37% albumin, 36% globulin, 22% glutelin, and 5% prolamin making the protein complex in nature. During the oil extraction process, rice

bran goes through a heat stabilization step to inactivate the lipase enzyme to preserve the quality of the oil. Moreover, DRB contains phytic acid (5.0-8.7%) (Kortekangas et al., 2020) which binds with the protein extensively making it unavailable for separation (Tang et al., 2002). Therefore, DRB has been utilized for fuel production (Isha, 2020) and mostly as animal feed (Forster Jr et al., 1994; Palo and Sell, 1996; Gadberry et al., 2006; Ranjan et al., 2018; Chen et al., 2019; Kumar et al., 2019; Fan et al., 2020; Huang et al., 2021a; Huang et al., 2021b) which otherwise should have been directly utilized as a protein source for the growing population.

Alkaline and enzymatic extraction are the two most commonly explored methods. Although the protein solubility increases concurrently with the pH, it has several drawbacks such as protein denaturation and discoloration resulting in decreased nutritional properties. The sulfur amino acid and histidine are also greatly reduced due to extreme alkaline treatment. Furthermore, the extreme alkali treatment results in the formation of a toxic compound, lysinoalanine (Hamada, 1999) which make the protein obtained unfit and unsafe for food application. While the enzyme treatment can extract protein at neutral pH without any limitation or with hardly any nutritional loss. However, the expensive nature of the enzyme limits its application urging a cheaper source of enzyme exploration.

Solid-state fermentation (SSF), a type of fermentation that takes place in a solid substrate with minimum moisture content (Pandey, 2003; Singhania et al., 2009; Sharma et al., 2020), is an effective technique for utilizing agricultural waste products and converting them into value-added products. This biological process aids in reducing environmental pollution caused otherwise by wastes from agro-food industries (Ranjan et al., 2019; Chilakamarry et al., 2022). And above all, SSF holds a record for its potential in enzyme production in enzyme production such as amylase, cellulase, and protease with the abundant by-product, rice bran, and other agricultural wastes (Chancharoonpong et al., 2012; Ali and Vidhale, 2013; Hoa and Hung, 2013; Utharalakshmi et al., 2014; Pandey et al., 2016; Basak and Rangan, 2018) mostly with fungus due to their GRAS (Generally Recognized as Safe) status.

Loog-pang and Koji are the types of fermentation starters popularly used for alcoholic beverages in Thailand and Japan, respectively. Both the fermentation starters are used as enzyme sources to produce alcohol by fermentation. Loog-pang (Thai wine

starter) is dominantly used by the population of Thailand and its production process is maintained as a secret and passed down to their generation. However, the works of the literature suggest that it is a consortium of bacteria, yeast, and fungi mixed with herbs such as *Allium sativa*, *Zingiber officinale*, and *Alpinia siamensis*. It is known as Chinese yeast cake by Westerners as it is shaped like a ball. Whereas, Koji, known by the name Koji-kin in Japan, is prepared by inoculating Koji starter or Koji mold, onto steaming cereal such as rice and barley in temperature and humidity-regulated conditions (Yamashita, 2021). Molds in koji production are the main source of enzymes and these are often *Aspergillus oryzae* or *Rhizopus oryzae* which have GRAS status. Koji molds contribute to the production of the characteristic color, flavor, and aroma of its fermented products such as soy sauce, miso, and douche (Chou, 1995; Zhu and Tramper, 2013; He et al., 2019).

Therefore, this research work aims to extract protein from the abundant agro-industrial waste, DRB by SSF with the fungus Loog-pang and Koji, and study the characteristics of the extracted protein.

1.2 Research Aim

To extract protein from DRB by SSF with Loog-pang and Koji and to study some characteristics of the extracted protein.

1.3 Research Objectives

1. To evaluate and investigate the feasibility of the fermentation starter, Loog-pang and Koji in enzyme production and its efficacy in protein extraction from DRB.
2. To study some characteristics of the extracted protein.

1.4 Research Scope

1. The variability of enzymes produced during fermentation by each culture, Loog-pang, and Koji, with DRB substrates will be investigated.
2. After SSF with Loog-pang and Koji, protein from the DRB will be extracted.
3. Proximate compositions, molecular size, and amino acid profiles of the extracted protein powder will be studied.

1.5 Hypothesis

SSF of DRB with Loog-pang and Koji would produce enzymes that will assist in the extraction of available protein from the DRB.

CHAPTER II

LITERATURE REVIEW

2.1 Rice and Rice grain structure

Rice (*Oryzae sativa* L.) is one of the world's most important cereal crops serving as a staple food for a large fraction (50%) of the global population and its demand is expected to increase by almost 28% in 2050. The consumption of rice when compared to any other staple cereals is very high. As a result, rice is strongly linked to both the political and food security of the world, especially in developing countries (Nadathur et al., 2016; Hussain et al., 2020). FAO reported a rice production of 759.6 million tons in 2017 (Pan et al., 2019). Due to its vast flexibility and tolerance to many climatic circumstances, rice can grow in a variety of climates, but it is primarily grown in warmer temperate areas and wet tropics (Uraipong, 2016). Because of this, rice is farmed in more than 100 nations across all continents except Antarctica. Rice is sometimes referred to as the "gold of the orient". According to the United States department of agriculture (USDA), approximately 500 million tons of milled rice have been produced globally during the 2019/2020 marketing year with China being the top producer followed by India and Thailand being one of the top producers.

Paddy rice needs to undergo numerous processing steps before it is ingested by consumers. The essential components of paddy rice include 70% endosperm, 20% rice husk, 8% bran, and 2 % rice germ (Van Hoed et al., 2006). The milling process of rice grain involves the removal of bran and germ portion from the paddy rice to obtain white rice (endosperm). As a result, the main by-product generated from the rice milling process includes hull, germ, and bran which hold tremendous potential to be used as an important source for value-added food products and other non-food applications. However, they are either disposed of as unwanted material or underutilized as low-cost feed for animals. Such valuable material disposal in the environment could also compromise public health.

The rice grain, as illustrated in Figure 1, shows that it constitutes hull and bran as outer layers and endosperm and germ on the inner layer. The outermost layer, the hull/husk plays a protective role and is composed of mostly fibrous tissue such as

cellulose (38-48%); hemicellulose (23-28%); lignin (12-16%); protein (1.9-3.7%), and fats (0.3-0.8%) (Nadathur et al., 2016). Minerals including calcium, copper, iron, potassium, magnesium, manganese, zinc, sodium, phosphorus, and silica are also present in it. The amorphous silica which is about 18-20% by weight (Luh, 1991) remains bound to cellulose and lignin which makes the hull resistant to water and fungal decomposition. The rice husk or hull is quite difficult for humans to digest but it is mostly used for energy production such as fuel. The removal of the hull from the rice grain leaves bran, germ, and endosperm and is collectively called "brown rice". Endosperm forms the edible white rice after further milling and polishing the brown rice. The level of protein, minerals, and vitamins is very less in white rice. But it is very starchy and therefore, serves as a staple food for a large portion of the world.

The bran fraction constitutes a thin fibrous pericarp, seed coat, and aleurone layer (Figure 1). The outermost fibrous pericarp mostly contains pigments that provide color to the colored rice (Juliano and Tuaño, 2019) whereas the seed coat is less fibrous and contains lipids and protein. The most underneath layer of rice bran is the aleurone layer and it has the most protein in the bran (Nadathur et al., 2016).

On average, the rice kernels, when milled, produce almost 8-11% rice bran (Dhankhar and Hissar, 2014) which is roughly 10% of the grain's yearly production in the world. This was estimated to exceed 500 thousand metric tons in the 2020/2021 harvest (da Rocha Lemos Mendes et al., 2021). The oil content in rice bran provides unique and superior health benefits to consumers due to the presence of high nutraceutical content. Hence, there is a high demand for rice bran oil in the market, and is therefore, dominantly utilized in the oil extraction industries. In India, roughly 10 million metric tons of rice bran are produced annually, and 50-60% of the total rice bran by-product produced uses solvents to extract oil content from them, producing about 8 million metric tons of DRB as a by-product (Rajam et al., 2005). In general, every 1 kg of milled paddy rice generates 0.05-0.1 Kg of rice bran of which 78-85% accounts for DRB production after undergoing the oil extraction (15-22%) process (Alexandri et al., 2020).

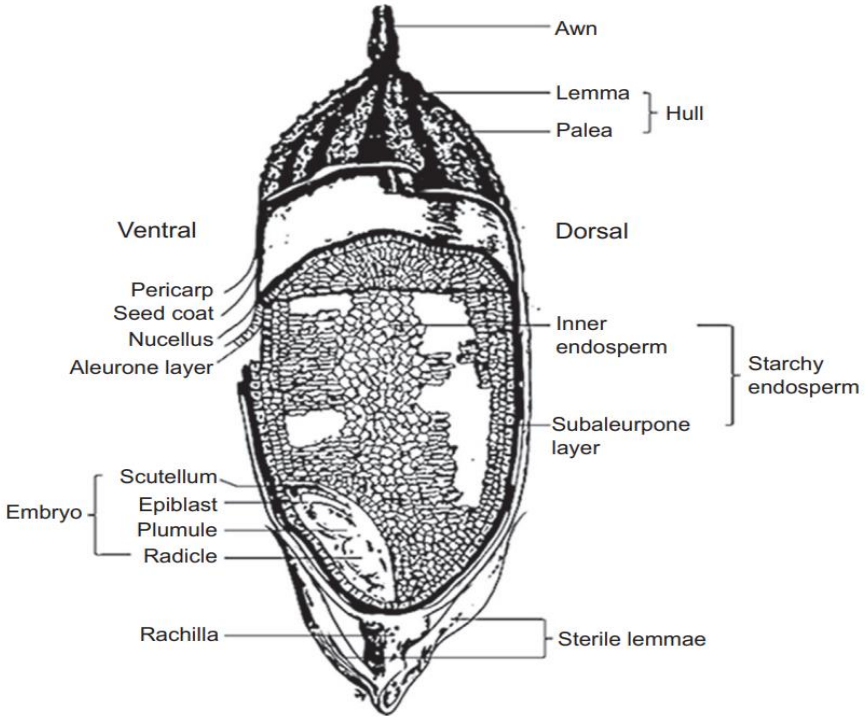

Figure 1 Longitudinal Structure of Rice Grain

Source: Juliano and Tuaño (2019)

2.2 Rice bran and its compositions

Rice bran is the brown-colored outer layer of rice kernel which is mainly comprised of pericarp, aleurone, and germ. It is a by-product of the polishing or whitening step of the milling process of brown rice to produce edible white rice. Because of the large amount of rice cultivation and production all over the world, rice bran generation is also abundant. However, it has been most of the time under-utilized when otherwise could be directly utilized as per its potential such as a valuable source of oil, protein, and dietary fiber for the growing population.

Rice bran, amongst all other byproducts generated in rice mills, is identified as one of the most nutritious parts with a proximate composition of 10–15%, 12–22%, 11–17%, 34.1–52.3%, 6–14%, and 8–17% moisture, lipids, protein, carbohydrates, fibers, and minerals content respectively (Fabian and Ju, 2011; Fathi et al., 2021). The nutrient amount of rice bran is far better than in rice/endosperm. It also contains a significant quantity of vitamin E, thiamin, and niacin and minerals like aluminum, calcium, chlorine, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc

(Sharif et al., 2014). However, the rice bran composition values vary with the rice variety and the efficiency of the milling system (Rosniyana et al., 2007). Moreover, the climatic conditions of the rice growing environment and the processing conditions also alter the rice bran compositions. However, rice bran after defatting is concentrated with protein and dietary fibers along with the anti-nutritional agent, phytic acid, and other components (Kortekangas et al., 2020).

2.2.1 Dietary fiber

DRB contains about 32.9 % of total dietary fiber, of which almost 90% are insoluble and mostly includes cellulose (34%), hemicellulose (55%), and lignin (6%) (Daou and Zhang, 2014). Arabinoxylans are the most common hemicellulose. However, the amount and composition vary on the rice cultivar, degree of milling, and analytical method (Sapwarobol et al., 2021). Rice bran dietary fiber is about 25.30 g per 100 g which can meet the dietary fiber intake recommended for an adult (approx. 27.00 g/ day) (Devi et al., 2021). Cellulose is a long-chain homopolymer of D-glucose linked together by a β-(1,4) bond while arabinoxylan consists of a β-(1,4)-linked xylose backbone, with substitution of arabinose residue at the second and third carbon positions. Neither of those components are digested by humans.

2.2.2 Phytic acid

Figure 2 is the molecular structure of Myo-inositol-1,2,3,4,5,6 hexakisphosphoric acid (IP6), commonly known as phytic acid. DRB comprises a considerable quantity of phytic acid and its salt form phytate. It is the main storage of phosphorus in plants and rice bran consists of 5.0–8.7% phytic acid (Kortekangas et al., 2020). It is primarily found in the outer layer of bran, especially pericarp and aleurone. Phytic acid has 12 ionizable protons which are responsible for its unique structure and characteristic properties, especially the chelate-forming ability with metal ions such as calcium, zinc, and iron, resulting in insoluble salts called phytates (Bloot et al., 2021).

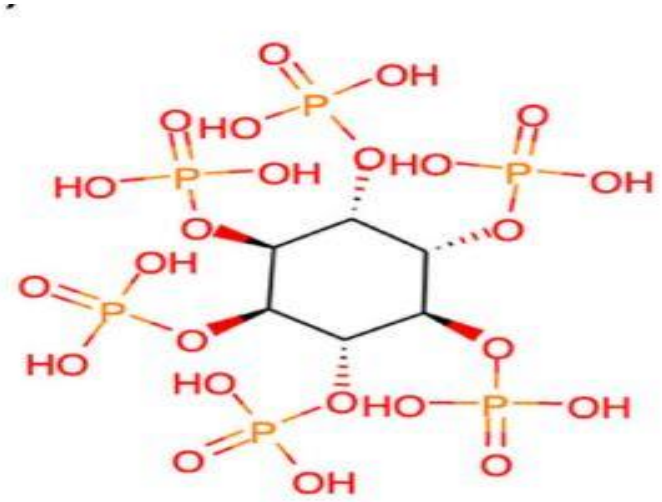

Figure 2 Molecular structure of phytic acid

Source: Wang and Guo (2021)

A large number of scientific reports have suggested numerous health benefits and applications of phytic acid. Due to its chelating ability of phytic acid, it has been demonstrated to possess antioxidant activity by binding the iron, and inhibiting the hydroxyl radical formation (•OH). Moreover, it is considered to possess the potential to prevent diseases such as cancer (Vucenik and Shamsuddin, 2006), diabetes (Lee et al., 2006), and coronary heart diseases (Obata and Nakashima, 2016).

On the other hand, because of its strong negatively charged inositol phosphate, phytic acid is also considered an anti-nutritional agent as shown in Figure 3. It chelates minerals, forming various complexes with proteins that are mostly insoluble, thereby decreasing the bioavailability, digestibility, and nutritional value of feed (Servi et al., 2008; Sharif et al., 2014; Kortekangas et al., 2020). Therefore, some studies have also focused on the removal of phytic acid from rice bran.

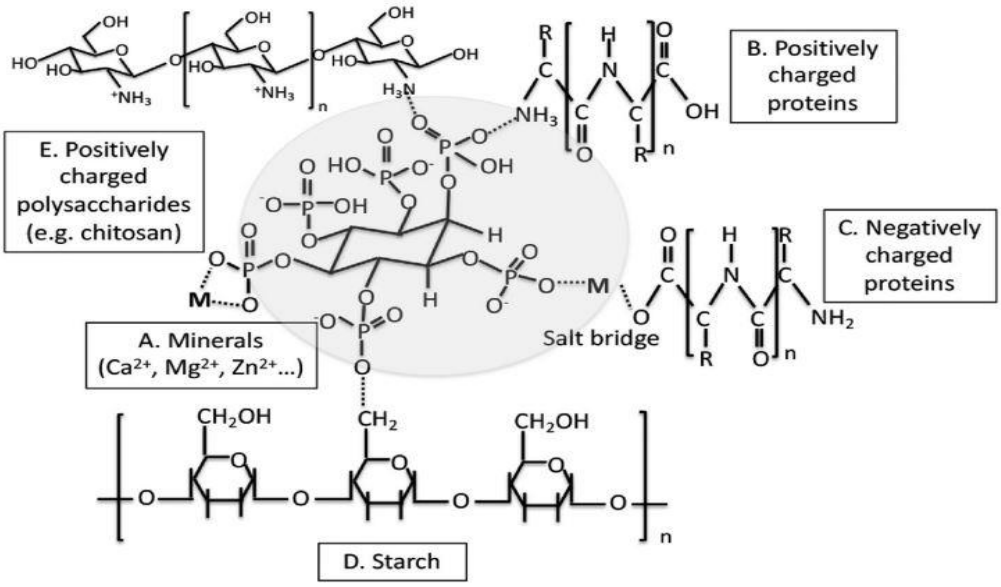

Figure 3 Interaction of phytic acid with minerals (A), proteins (B) and (C), starch (D), and polysaccharides (E).

Source: Wang and Guo (2021)

2.3 Dephytinization of DRB and other agro-industrial wastes

Extraction or removal of phytic acid or phytate from rice bran or legumes and cereals can be achieved by hydrolysis with either endogenous phytase or with the application of microbial enzymes. Various food processing techniques such as soaking, malting, and biological process such as fermentation activates and produces phytase enzyme which initiates the catalytic hydrolysis of phytic acid to Myo-inositol and orthophosphate via intermediate myo-inositol phosphates. But other processing methods such as blanching, baking, autoclaving, and frying which involve heat cause autolysis of phytic acid (Servi et al., 2008; Liu et al., 2019). The author also reported the improvement of mineral bioavailability in cereals food products as fermentation (8 h) of wheat significantly reduced almost phytic acids by 3 times.

Furthermore, dephytinization cane also be accomplished by hydrolysis with acidic solvents such as hydrochloric or sulfuric acids (Han, 1988; Fuh and Chiang, 2001; Saad et al., 2011). At normal pH, phytic acid is negatively charged and forms a strong bond with positively charge substances very easily. However, the solubility of

the phytic acid increases proportionally with the acidic pH and high purity of phytic acid could be extracted at pH 2 (Fuh and Chiang, 2001). Dephytinization at higher pH compromised the purity as the separation of phytic acid from the extract was found difficult as the macromolecules such as starch were suspected to undergo degradation under high pH.

2.4 Rice Bran Protein

Many studies have recently focused on the use of rice bran because large amounts of the grain's outer layers are removed during the processing of whole rice. This concentrates the nutrients in the bran and makes it an important source of nutrients for the food industry and human consumption (Faria et al., 2012). DRB, also called a defatted rice bran meal, is the main residue obtained after the extraction of oil from the full-fat rice bran. DRB residue from the agro-industrial is extensively explored to be utilized as the feed for cattle (Forster Jr et al., 1994; Gadberry et al., 2006), poultry (Palo and Sell,1996; Chen et al., 2019), fish diet (Kumar et al., 2019; Ranjan et al., 2019), and pigs (Fan et al., 2020; Huang et al., 2021a; Huang et al., 2021b), as well as for the fuel production (Isha, 2020) while the increasing global population still suffers from the shortage of dietary protein. Protein extracted from rice bran mostly after extraction of oil from them is called rice bran protein. The defatting process from the rice bran further concentrates the protein content (Prakash and Ramanatham, 1994; Ali et al., 2010; Alexandri et al., 2020) in them but most of the time it is used as animal feed.

Rice bran has been a point of attraction for decades owing to its high nutritional and functional properties such as its well-balanced amino acid composition. Moreover, protein from rice bran has been accepted as nutritionally superior to any other cereal proteins mainly due to its hypoallergenic (Helm and Burks, 1996) and anticancer activity (Kawamura and Muramoto, 1993). These properties make it the best and most suitable for the formulation of infant foods (Zhuang et al., 2019). The rice bran contains approximately 10-15% protein, and it is raised to 15-18% with the defatting process (Alexandri et al., 2020). The protein includes albumin, globulin, glutelin, and prolamin, comprehending all the essential amino acids and its amino acid composition is much closer to that of the FAO/WHO-approved model than rice protein (Yu et al., 2019). And

the amino acid composition of rice bran protein shows the available lysine content of 3-4% which is considered to possess greater than that of rice endosperm protein or any other cereal bran protein (Juliano, 1985; Shih et al., 1999; Yeom et al., 2010). Table 1 shows the amino acid composition of essential amino acids from the DRBP extracted by alkaline solvent and its comparison with the FAO/WHO recommended model (Wang et al., 2016; Zaky et al., 2020). The essential amino acid compositions in rice bran meet the standards of FAO/WHO and are, therefore, recommended for infant consumption.

Han et al. (2015) Claimed that the nutritional quality of protein from defatted rice bran can be comparable to any other protein of animal and plant origin. The nutritional quality, as indicated by the PER of rice bran (2.0 to 2.5) could be compared to that of casein (2.5) with a digestibility greater than 90% (Wang et al., 1999). Furthermore, with superior digestibility and biological value of 0.90 and 72.6, it can be a good source of protein for human consumption. They also reported that the PER, NPR, and NPU were 2.39, 3.77, and 70.7 which is just comparable to animal protein. Huang et al. (2021a) also claimed that defatted rice bran might be utilized as a maize and soybean alternative, as well as a valuable addition to protein feed for pigs.

Despite all these various health benefits and high nutritional content with huge production of rice bran as a result of paddy processing all over the world, there has not yet been found any feasible protein extraction method with the highest protein recovery while retaining its functional benefits.

Table 1 Essential amino acid composition of DRB (g/100 g protein)

Amino acid	DRB	FAO/WHO Recommendation	
		Child	Adult
Histidine (His)	2.15 ± 0.12	1.9	1.6
Isoleucine (Ile)	4.27 ± 0.18	2.8	1.3
Leucine (Leu)	5.76 ± 0.23	6.6	1.9
Lysine (Lys)	6.47 ± 0.41	5.8	1.6
Phenylalanine (Phe)	5.16 ± 0.15	6.3	1.9
Methionine (Met)	1.65 ± 0.09	2.5	1.7
Valine (Val)	4.54 ± 0.17	3.5	1.3
Threonine (Thr)	3.32 ± 0.42	3.4	0.9
Tryptophan (Trp)	1.07 ± 0.05	-	-

The values are the mean ± standard deviation of three replicates. DRB: defatted rice bran, FAO: Food and Agricultural Organization, WHO: World Health Organization

Source: Wang et al. (2016) and Zaky et al. (2020)

2.5 Rice bran protein application

Due to various superior functional and nutritional qualities, rice bran protein has found its way to improve and enriched the human diet system.

1. Rice bran protein in food products

A systematic review of literature by Zheng et al. (2019) discusses some of the major and important applications such as protein enrichment in some bakery products, protein sources for infants, and protein-sensitive populations. The application of rice bran protein concentrate in the bread has shown better nutritive as it enriches the protein

content in the bread (Jiamyangyuen et al., 2005). Furthermore, the addition of rice bran to biscuits (10-15%) and cookies (10-12%) enhance the nutraceutical content in the product without compromising customer acceptance (Ali et al., 2010).

2. Protein supplement

The hypoallergenic property of rice bran protein has offered a great deal of promise for us as an appropriate protein source for baby formula, weaning meals, and limited formula for kids and adults with dysfunctions like lactose intolerance or celiac disease (Fabian and Ju, 2011).

3. Flavor enhancer

Since rice bran protein consists of about 61% of total amino acid content as glutamic, aspartic acid, arginine, leucine, and glycine, it can be applied to improve and enhance the flavor of certain food such as soup, sauce, and poultry along with amino acid enrichment in the food products (Hamada, 2000). The addition of rice bran protein could also intensify the salty aroma of foods (Kaewka et al., 2009).

2.6 Extraction of Rice Bran Protein

The extraction method used immensely determines the functional and physicochemical, and nutritional properties of the extracted protein (Ghanghas et al., 2020). Numerous methods have been explored in an attempt to maximize the extraction with little or no loss of its native functional and nutritional properties from rice bran. The rice bran protein consists of a mix of 37% albumin, 36% globulin, 22% glutelin, and 5% prolamin which makes the protein complex in nature and also, contains about 1.7% of phytic acid which binds with the protein extensively making it unavailable for separation (Tang et al., 2002; Adebiyi et al., 2009). Various extraction methods such as alkaline, physical/mechanical, and enzymatic methods have been studied and explored by many researchers to extract the protein from the rice bran to either substitute animal protein with plant-based protein or produce protein industrially utilizing milling waste rice bran from the rice processing industries (Fabian and Ju, 2011; Zheng et al., 2019; Ghanghas et al., 2022). However, the commonly used method for rice bran protein

extraction is solvent extraction which employs alkaline conditions followed by either acid at pH 4.5 or heat precipitation and enzyme extraction.

2.6.1 Alkali Extraction

The alkaline extraction method usually involves a pH shift from high (pH 9-12) to the isoelectric pH (4-4.5) of rice bran protein. The alkaline solution increases the solubilization of the protein by cleaving the hydrogen, amide, and disulfide bonds in the protein and precipitation with the use of either heat or acid at pH 4.5 (Sun et al., 2017). Most literature has concluded that the protein extraction yield increases with the increase in pH and temperature of the solution (Chen and Houston, 1970; Bera and Mukherjee, 1989; Shih et al., 1999). This method is the most widely utilized approach for protein extraction because of its simplicity and the easy availability of the reagents used (Jiamyangyuen et al., 2005).

Table 2 highlights some of the key findings from the literature on the alkali extraction of DRBP. The alkaline condition was effective in solubilizing the rice bran protein as hydrogen, amide, and disulfide bonds could be easily broken (Fabian and Ju, 2011; Zheng et al., 2019). The alkaline extraction method could extract protein from the DRB in the range of 9.6-48%. However, it can be improved and enhanced by the application of enzymes.

Table 2 Alkaline extraction of rice bran protein from various studies

Raw material	Conditions	Protein Yield (%)	Purity (%)	References
DRB	pH 1, 25°C for 1 h	37.0	85.0	Chen and Houston (1970)
DRB	pH 9.5, room temperature for 58 min	9.6	72.63	Jiamyangyuen et al. (2005)
DRB	pH 9.5 and agitation (500 rpm) for 45 min	44.4	NR	Theerakulkait et al. (2006)
DRB	pH 9.5, 30-75°C for 1 h	21-48.1	71-79.9	Gupta et al. (2008)
DRB	pH 11, 60°C for 60 min	13.2	37.6	Yadav et al. (2011)
DRB	pH 9.5, stirring (300 rpm) at 50°C for 2 h, precipitation at pH 3.8	32.9	NR	Zhang et al. (2012)
DRB	pH 11, precipitation at pH 4.5	30.7	57.1	Piotrowicz and Salas-Mellado (2017)

NR: not reported

But several studies have also agreed with Wang et al. (1999) in the statement of protein denaturation and hydrolysis, and protein discoloration which results in the decreased nutritional quality of rice bran protein extracted in a high alkaline solution. Although the solubilization of protein is found to be maximum when high temperature and concentration treatment of alkaline solution is applied, the protein extracted is of undesirable quality making it unfit for food use. The above conditions reduce the nutritional properties due to extensive hydrolysis resulting in undesirable flavor and toxic formation of lysinoalanine (Hamada, 1999). Kelly and Ballew (1982) also reported that the protein efficiency in soy protein was affected as the bioavailability of total sulfur amino acids and histidine was reduced by 71% and 80%, respectively due

to severe alkaline treatment. Bera and Mukherjee (1989) also reported that at low pH below its isoelectric point (4.5), phytate, a component in rice bran, can interact with protein leading to decreased solubility of the protein. Therefore, alkaline extraction is not a feasible method to be employed for protein extraction from rice bran even though it can improve the solubility of the protein.

2.6.2 Enzymatic Extraction

The major components in the rice bran are cellulose and hemicellulose unless commercial rice bran contains a considerable amount of starch depending on the degree of milling and its conditions and also the portion of endosperm if included. The application of enzymes to digest and extract protein from rice bran has been widely explored. Protease and carbohydrase are the two main enzyme groups that have been mostly used to fulfill this purpose. The starch and non-starch polysaccharides are digested by the carbohydrase making the proteins bonded to them available for extraction while protease improves the solubility of proteins as it breaks them into smaller peptides. Many studies have suggested that the use of enzymes can also give a high yield of protein without any limitations or change in the nutritional properties under neutral pH, unlike the solvent extraction process. The application of enzymes such as amylase, cellulase, and hemicellulase hydrolyzes starch, cellulose, and hemicellulose and makes the protein bonded to them available for extraction (Shih et al., 1999). Viscozyme L, which is a mixture of carbohydrases such as arabinase, cellulase, hemicellulase, and xylanase, was found to efficiently break the links within the polysaccharide matrix, allowing the more intercellular protein to be liberated according to Guan and Yao (2008). Ansharullah et al. (1997) showed a higher protein extraction yield of 57% with Viscozyme L which was much higher than the alkali extraction of 47% from the same bran used in the work of Ansharullah (1992).

Table 3 summarizes the reports of heat-stabilized defatted rice bran (HSDRB) and DRBP extraction using different enzymes from various works of literature. The enzymatic approach is much better in terms of protein extraction form DRB and other agro-industrial wastes. The table shows that protein extraction can be in the range of 12.1-87.6%, which is much better than alkaline extraction. Although the use of enzymes can greatly improve protein recovery rates when compared to alkaline extraction, the

only drawback in protein extraction is that enzymes are costly (Fabian and Ju, 2011). Therefore, there is a need to explore cheap and safe enzyme sources for protein extraction from agro-industrial byproducts such as DRB.

Table 3 Enzymatic extraction of DRBP

Raw Material	Enzyme	Conditions	Protein Yield (%)	Purity (%)	References
DRB	Alcalase 24 L	0.025 g at pH 8, 50°C, DH 7.5	81.4	27.6	Hamada (2000)
DRB	Flavourzyme	0.025 g at pH 8, 50°C, DH 8.8	87.6	29.9	Hamada (2000)
HSDRB	Amylase	1.1×104 Unit at pH 6.5, 200 rpm, 45°C for 3.5 h	45.4	**NR**	Tang et al. (2003)
HSDRB	Viscozyme	pH 4.5 at 45°C, 200 rpm for 3.5 h	28.5	**NR**	Tang et al. (2003)
HSDRB	Celluclast	pH 5.5 at 55°C, 200 rpm for 3.5 h	12.1	**NR**	Tang et al. (2003)
HSDRB	Alcalase 24 L	250 u/g, pH 10 at 50°C, 300 rpm for 2 h	44.8	NR	Zhang et al. (2012)
DRbB	Flavourzyme	1 wt. %, pH 8 at 50°C for 4 h	74.9	23.7	Thamnarathip et al. (2016)
DRbB	Alcalase	1 wt. %, pH 8 at 50°C for 4 h	54.7	13.5	Thamnarathip et al. (2016)
DRbB	Neutrase	1 wt. %, pH 7 at 50°C for 6 h	36.6	12.3	Thamnarathip et al. (2016)

HSDRB: heat-stabilized defatted rice bran; DRB: defatted rice bran; DRbB: defatted rice-berry bran, NR: not reported

2.7 Solid-State Fermentation of Defatted Rice Bran

Fermentation is a process that uses microbes to break down big organic molecules into smaller ones (Sharma et al., 2020) and this SSF process is also described as fermentation that occurs in a solid substrate with simply enough moisture content to support the growth and metabolisms of microorganisms (Pandey, 2003; Singhania et al., 2009). SSF is considered a green process utilizing and converting various agricultural wastes into value-added products and thereby, solving the environmental pollution issues caused due to disposal of these solid wastes (Chilakamarry et al., 2022). It has also been proven to be particularly beneficial in increasing nutritional content and bioavailability, therefore adding value to the product and opening up new possibilities for its application.

SSF holds a promising account in the production of enzymes depending on the strain and substrates, and selection process parameters (Pandey et al., 2000). Many microorganisms, particularly filamentous fungi, may thrive on solid surfaces to a substantial extent in the absence of free water (Ranjan et al., 2019) and they release intracellular or extracellular enzymes that break down nutrients in the substrate to grow and survive. SSF supports the growth of microorganisms even in the presence of a low amount of water as it imitates the natural habitat of most microbes, especially fungi, and molds. Moreover, it is less vulnerable to bacterial contamination allowing the enzymes to work efficiently. However, the production of enzymes is influenced by the organic content present in the substrate, microorganism, and processing conditions applied (Šelo et al., 2021).

Table 4 shows the production of various enzymes by the SSF technique. SSF has proven to be an effective technique in producing various enzymes such as protease, cellulase, amylase, and xylanase with different starter cultures and with rice bran as the growing medium.

Table 4 Enzyme production by SSF of rice bran and other agricultural waste

Substrate	**Microorganisms**	**Enzyme**	**References**
RB	*Rhizopus* sp.	Neutral protease	Sumantha et al. (2006)
RB	*Fusarium oxysporum*	Protease	Ali and Vidhale (2013)
RB	*Aspergillus flavus* SB4	Cellulase	(Utharalakshmi et al., 2014)
RB	*Neurospora crassa* and *Fusarium oxysporum*	Cellulase	Basak and Rangan (2018)
RB + wheat bran	*Aspergillus oryzae* S.	Amylase, Neutral and alkaline protease	Chancharoonpong et al. (2012)
RB, rice husk, soybean residue, grapefruit peel, sugarcane bagasse	*Aspergillus oryzae*	Cellulase and pectinase	Hoa and Hung (2013)
RB, wheat bran, barley bran, olive spinet, oats bran, chopped date stones, chopped dried fish	*Streptomyces* sp. CN902	Alkaline protease	Lazim et al. (2009)
Wheat bran	*Rhizopus Oryzae* SN5	Cellulase and Xylanase	Pandey et al. (2016)

RB: rice bran

DRB contains mainly carbohydrates including cellulose and hemicellulose, protein, and phytic acid (Zhuang et al., 2019), and fermentation of DRB as substrate with different microorganisms leads to the consumption of these components releasing proteins bonded to them during the various stages of microbe's metabolism and helps in enrichment and increases the available protein for extraction in the bran.

Numerous studies have explored the effectiveness of SSF with various microorganisms, mostly GRAS-natured fungus has been sprayed on the substrates to increase the available protein in the rice bran for extraction and to study some of its properties. Silveira and Badiale-Furlong (2009) reported that the solid-state fermentation of DRB increased the protein content in the bran fraction by approximately 69% in the fermented rice bran biomass with *Rhizopus* Sp. after 72 h of fermentation time. Moreover, the solubility of the protein obtained was found to have increased with fermentation time up to a certain time when compared to the unfermented raw material. The phytic acid content in rice bran which is reported by many researchers to have anti-nutritional properties (Wang et al., 1999; Fabian and Ju, 2011) was found to reduce by 53% during fermentation at 30^{o}C for 48 h using *Lactobacillus plantarum* EM as a starter culture, and hot air-dried at 55^{o}C for 16 h (Moon and Chang, 2021). The protein extracted from yeast and natural fermented defatted rice bran showed high denaturation temperature with other essential functional properties such as higher essential amino acid content and antioxidant activity, water and oil absorption capacities, and lighter color suggesting their potential as functional ingredients in baked products (Chinma et al., 2014).

Table 5 recapitulates the protein recovery from the rice bran including both FF-RB and other agricultural residues as substrates by solid-state fermentation. The protein content from various substrates by SSF technique can be recovered as high as 91% as reported by Li et al. (2019) from the substrate mixture of soybean meal and anchovy residue proving the potential of SSF in protein recovery from various agro-food industrial wastes.

Table 5 Protein recovery by SSF

Substrate	Microorganism	Fermentation Time (h)	Protein recovery (%)	References
FF-RB	*Rhizopus oryzae*	120 h	26.60	Kupski et al. (2012)
DSM	*Aspergillus oryzae*	24 h	57.35	Zhao et al. (2018)
Soybean meal + Anchovy hydrolysis residue	*Bacillus subtilis* N-2 strain	144 h	91.00	Li et al. (2019)
HSDRB	*Bacillus subtilis* (natto) Takahashi	61.01 h	64.60	Bisly et al. (2022)

FF-RB: full-fat rice bran; DSM: defatted soybean meal; HSDRB: heat-stabilized defatted rice bran

2.8 Loog-pang

Loog-pang is a Thai traditional fermentation culture shaped like a ball that plays a significant part in Sato manufacturing quality and flavor. It is also referred to as a Chinese yeast cake by the Western populations. Numerous Asian nations have employed this kind of fermentation starter under different regional names, including banh men in Vietnam, bubod in the Philippines, chu (vinegar) in China, koji in Japan, murcha in India, nuruk in Korea, ragi in Indonesia, and ragi tapai in Malaysia (Limtong et al., 2005). It contains a variety of herbs such as *Allium sativa*, *Zingiber officinale*, and *Alpinia siamensis*, mixed with bacteria, yeast, and fungi and it is the source of beneficial microorganisms for ethanol production (Paewlueng et al., 2019).

Loog-pang has been used as a fermentation starter culture by the people of Thailand for alcoholic production and much literature has been published on its effectiveness in ethanol production (Chaijamrus and Mouthung, 2011; Manysoat et al., 2013; Duangwang and Sangwichien, 2015) due to various species of yeast, molds, and

acid-producing bacterial dominance. *Amylomyces rouxii*, *Aspergillus oryzae*, *Aspergillus niger* group, *Aspergillus* spp., *Mucor* spp., *Penicillium* spp., and *Rhizopus* spp. are the mold species that have been often recorded to exist in Loog-pang but only a few species have reported to play a significant role in fermented product production (Limtong et al., 2005). Since the Loog-pang production process is limited only to certain households, the diversity of microbial content also varies to households and also, with different geographical sources. *Sacchromycopsis fibuligera*, which is well known for the production of amylase and acid protease (Chi et al., 2009), has been identified as a dominant yeast present in the Loog-pang. It also generates enzymes that break down cellulose, which is crucial to the process of saccharifying lignocellulosic substances (Ma et al., 2015; Van Zyl et al., 2016). *Saccharomyces* spp. and *Pichia anomala* were also identified in the Loog-pang (Limtong et al., 2002). These microorganisms, when cultured in the DRB substrates through SSF, will produce various enzymes that will consume all the carbohydrates present in the raw material, thereby, producing ethanol and other byproducts. Thus, it can be explored for its efficacy in extracting protein that is left, after the carbohydrate and starch content has been consumed. Moreover, there is no single literature work published for its applicability in this respect.

2.9 Koji

Koji production is achieved by inoculating Koji starter or Koji mold, onto steaming cereal and cultivating it while regulating the temperature and humidity. If it is cultivated with rice or barley as the substrate, then it is called rice Koji or barley Koji (Yamashita, 2021). The most widely used Koji starter *Aspergillus oryzae*, a fungus of the *Aspergillus* strain, which was recognized as the Japanese national microorganism, has been extensively used in the Koji manufacture in Japanese fermentation industries. Molds in Koji are the major source of hydrolytic enzymes including amylases, proteases, lipases, and a variety of others that hydrolyze substrate components (Chou, 1995). Koji can not only produce amylase, carbohydrase, and protease that attack starch, carbohydrate, and protein substrates but also contributes to the production of the characteristic color, flavor, and aroma of fermented products (Zhu and Tramper, 2013).

CHAPTER III

RESEARCH METHODOLOGY

3.1 Materials

Rice bran from Khao Dawk Mali 105 rice cultivar, defatted by hexane was supported by Surin Bran Oil Co., Ltd. Loog-pang was purchased from local producers in Nan province, Thailand, whereas, Koji-kin (Vision Brewing, Western Australia) was purchased from a supplier in Thailand.

(a) (b)

Figure 4 Fermentation starters (a) Loog-pang; (b) Koji used for fermentation of DRB

3.1.1 Chemicals used

1. 99% Sodium chloride (RCI Labscan, Thailand)
2. Ethanol (RCI Labscan, Thailand)
3. Sulfuric acid (RCI Labscan, Thailand)
4. Boric acid (QReC, New Zealand)
5. Kjeldahl catalyst tablets (QReC, New Zealand)
6. Dinitro salicylic acid (DNS) (Fluka, Switzerland)
7. Sodium acetate trihydrate (RCI Labscan Limited, Thailand)
8. Potassium sodium (+) tartrate tetrahydrate (RCI Labscan, Thailand)

9. Hydrochloric acid (RCI Labscan, Thailand)
10. Carboxyl methyl cellulose (CMC)
11. Casein
12. Glucose
13. L-tyrosine (Fluka, Germany)
14. Bovine hemoglobin (Sigma, USA)
15. Bovine serum albumin (Fluka, Switzerland)
16. Trichloroacetic Acid (TCA) (Fisher Scientific., UK)
17. 99% Sodium carbonate (LOBA CHEMIE PVT. LTD., India)
18. Folin-Ciocalteu phenol reagent (LOBA CHEMIE PVT. LTD., India)
19. Polyacrylamide gel (Bio-rad Laboratories, Inc., USA)

3.1.2 Equipment used

1. Protein digestion Unit (Digestion Unit Model: BÜCHI, B-435)
2. Distillation Unit (Distillation Unit Model: BÜCHI, B-323)
3. Autoclave (HARAYAMA brand, model HV-501)
4. Mixer/blender
5. Water boiler
6. pH meter (Starter 3100, Ohaus, USA)
7. Magnetic stirrer
8. Hot plate (IKA C-MAG: HS7)
9. Water bath (LIO lab LTD. Part, WB-710M)
10. Spectrophotometer (Thermo fisher, Model 4001/4)
11. Over-head stirrer (KITTISIT ENTERPRISE Co. LTD., RW 20 digital)
12. Centrifuge (Panta metrology Co. Ltd, model K240 R S 10036)
13. Tray drier (Model DH410)
14. Automatic moisture analyzer
15. Spray drier (Ohkawara kakohki Co. Ltd., Model L-8)
16. Desiccator

3.2 Method

The overall methodology employed in this study is shown in Figure 5.

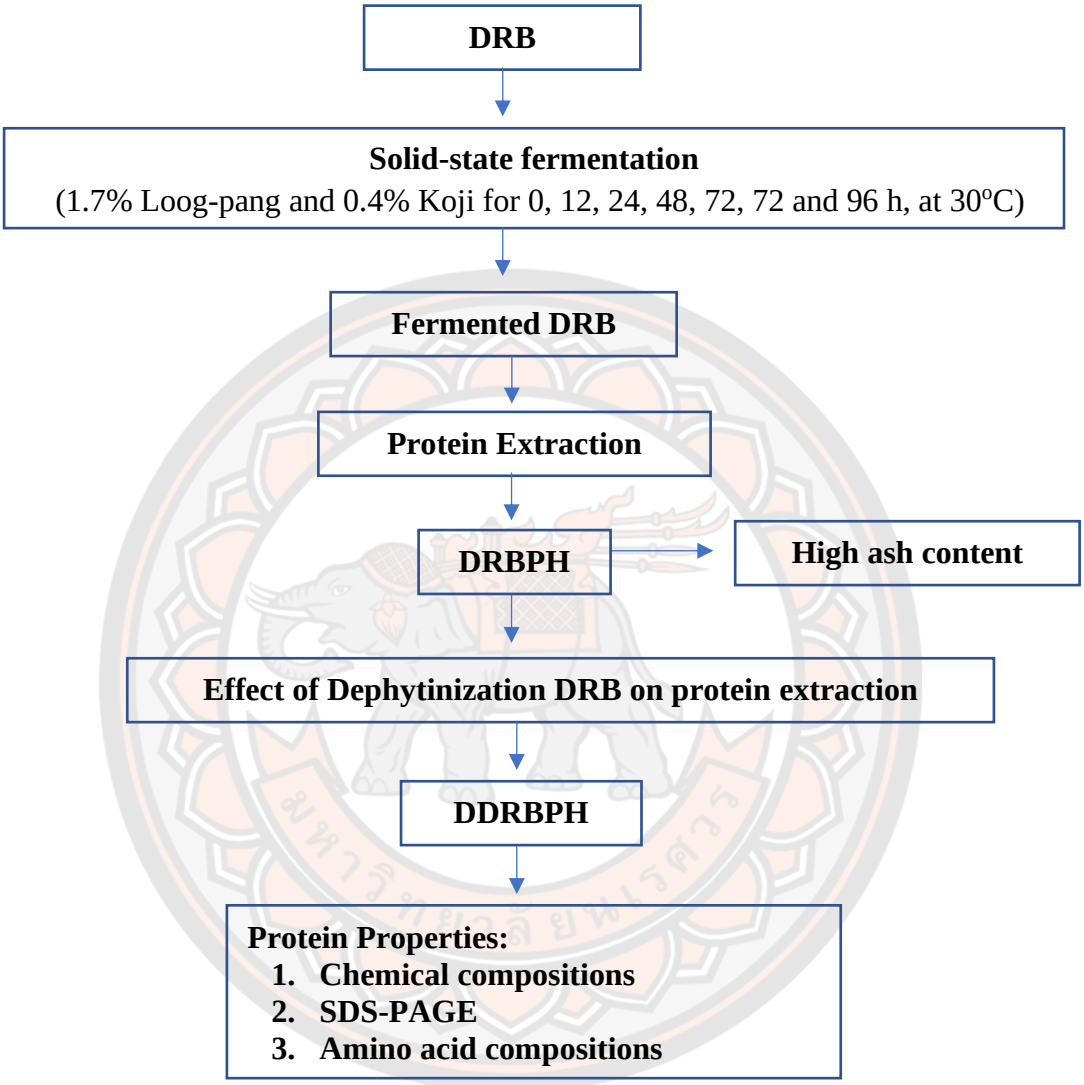

Figure 5 Flowchart of overall methodology

3.2.1 Determination of the proximate composition of DRB

The DRB was sieved using a 20 mesh (841-micron opening) sieve to obtain uniform particle size and the proximate composition was determined as follows:

- Moisture (AOAC, 2005)
- Protein content (Kjeldhal method) (AOAC, 2005)
- Crude fiber content (AOAC, 2005)
- Fat content (Soxhlet) (AOAC, 2005)
- Carbohydrate content (AOAC, 2005)

3.2.2 Loog-pang and Koji preparation

Loog-pang starter culture was stored at ambient room temperature and was used within 6 months, whereas Koji was stored at 4^{o}C until its use in the experiment. When needed, at least three Loog-pang balls were randomly selected and activated by heating at 30^{o}C for 3 h after which they were made into powder using a mortar and pestle and mixed thoroughly.

3.2.3 Solid-state fermentation of DRB

DRB substrate, adjusted to about 50% moisture content with deionized water at a ratio of 1:1 (w/w), was taken in a glass jar (300 mL for 10 g DRB) and sterilized using an autoclave at 121^{o}C for 20 mins. The sterilized DRB was then cooled to room temperature and inoculated with 1.7% Loog-pang and 0.4% Koji (w/w, dry basis) containing yeast and mold count of 2.805×10^{8} and 5.4×10^{7} CFU/g Loog-pang and Koji, respectively which was analyzed by spread-plate method (Tournas et al., 2001) But for natural fermentation, no inoculum was added. The amount of Loog-pang and Koji inoculum utilized was according to the recommendation of the producers. The substrate and inoculum mixture was then properly mixed using a heat and alcohol-sterilized glass rod. The Loog-pang starter, before inoculation, was ground to a fine powder using a motor and pestle. The mixture was then cultured at 30^{o}C and maintained by an incubator at different times (0, 12, 24, 48, 72, and 96 h) as shown in Figure 6. All the fermentation was carried out in duplicate. The fermented DRB obtained after indicated fermentation time was analyzed for pH change, reducing sugar concentration, and enzymatic activities.

Figure 6 Solid-state fermentation of DRB at 30^{o}C

3.2.3.1 Determination of pH change and reducing sugar (mg/g dry DRB)

To measure pH, 10 g of fermented DRB was mixed with 60 mL of deionized water and the suspension mixture was then homogenized by using a mixer. The change in the pH of the fermentation culture was then monitored using a digital pH meter (Starter 3100, Ohaus, USA). Reducing sugars at different fermentation points was also analyzed using the dinitro salicylic acid (DNS) method according to Miller (1959).

3.2.3.2 Evaluation of enzymatic activities (U/g DRB, dry basis)

a. Preparation of the enzyme extract

NaCl (0.9 %) solution (Denardi de Souza et al., 2019) was used the extract the enzymes from the fermented DRB biomass at different times. Briefly, 50 mL of NaCl solution was added to 10 g of fermented DRB, and orbital shaking at ambient room temperature for 60 mins was carried out. Then, the suspension mixture was centrifuged at 3560 × g for 30 min. The supernatant obtained was used as the enzyme extract and was analyzed for the cellulase (Denardi de Souza et al., 2019) and protease activity including both acid and neutral protease (Su et al., 2011) with certain modifications. Detailed methods are described below.

b. Total cellulase activities

Total cellulase activities were analyzed using Carboxymethyl Cellulose (CMC) as substrate according to Denardi de Souza et al. (2019) with slight modifications. Briefly, 0.5 mL of 0.5% CMC substrate was taken in a test tube followed by 0.5 mL of samples, and the reaction mixture was incubated at 50^{o}C for 30 min. During the incubation time, working glucose standards were prepared by taking 0.5 mL of standard solutions with 0.5 mL of DNS reagent. Exactly after 30 min, the reaction in the mixture was terminated by adding 0.5 mL of DNS reagent but for the control (0 min), DNS stopping reagent was added to the test tube before the addition of the enzyme sample (0.5 mL). The reaction mixture along with the standards prepared was heated until the orange-red color was seen (approximately 5 min) in the boiling water.

Then, 2.5 mL of deionized water was added to the mixture after cooling and a spectrophotometer at 540 nm will be used to measure its absorbance. Preparation of working glucose standard: 1 mg/mL glucose stock solution was prepared with 0.1 g glucose in 100 mL of deionized water.

One Unit of cellulase activity was defined as the amount of cellulase enzyme required to produce color equivalent to 1 µmol of reducing sugar in 1 min under assay conditions.

c. Neutral and acid protease activities

The neutral and acid protease activities were analyzed according to Su et al. (2011) using casein and bovine hemoglobin as the substrate for neutral and acid protease, respectively, with some necessary changes. The only difference between neutral and acid protease was the pH of the buffer solution, pH 7 for neutral and 4.8 for acid protease activity. 0.25 mL of substrate solutions were taken in a 2 mL micro-tube followed by a 0.25 mL enzyme sample with proper dilution with phosphate buffer. The reaction mixture was incubated at 37^{o}C for 30 min.

During this 30 mins incubation time, the L-tyrosine standard curve was prepared with the following dilutions as shown in Table 6. Exactly after 30 mins, 0.5 mL of trichloroacetic acid (TCA) was added to stop the reaction except for control samples, where TCA was added before the addition of the enzyme sample. The reaction mixtures were then centrifuged at 10,000 × g for 2 min. The supernatant/standard dilutions (0.25

mL) was added to 1.25 mL of 0.4 M sodium carbonate followed by 0.25 mL of 2.0 N Folin-Ciocalteu phenol reagent. The reaction was then allowed to stand for 30 mins at 37^{o}C after which absorbance was read against the blank (deionized water) at 660 nm.

One unit of protease activity was defined as the amount of neutral and acid protease required to produce color equivalent to 1 µmol of tyrosine in 1 min under the assay conditions.

Table 6 L-tyrosine standard curve for determination of neutral and acid protease activity

Concentration (mg/mL)	Volume of tyrosine (µL)	Volume of distilled water (µL)	Final volume (µL)
0.04	50	200	250
0.08	100	150	
0.12	150	100	
0.16	200	50	
0.20	250	0	

3.2.4 Protein extraction and determination of extracted protein (g/100 g DRB, dry basis)

Fermented DRB for different times were homogenized with deionized water in the ratio of 1:6 (w/v) using a mixer and hydrolysis for 24 h at 150 rpm and 55^{o}C was achieved by using an orbital shaker (Figure 6) after which filtration was carried out using a nylon filter bag (200 microns). The residue obtained was washed with boiled deionized water in the ratio of 1:6 (w/v) and combined with the filtrate. The filtrate mixture was then boiled using a hotplate magnetic stirrer for enzyme and microbial inactivation before drying at 60^{o}C for 24 h using a hot air oven. The dried defatted rice bran protein hydrolysates (DRBPH) were ground and sieved into homogeneous powder using a 0.6 mm mesh.

The protein content was then analyzed using the Kjeldahl method (AOAC, 2005) with 5.95 (Bisly et al., 2022) as a protein conversion factor followed by the calculation of extracted protein content by the equation given below. Then the DRBPH with the highest extracted protein was selected and spray-dried.

$$\text{Extracted protein (\%)} = \frac{\text{Protein content (g) in DRBPH}}{\text{Protein content (g) in the raw material}} \times 100$$

The proximate composition of DRBPH obtained from different fermentation times was also analyzed according to AOAC (2005).

3.2.4.1 DRBPH powder preparation by Spray drying and analysis of proximate compositions

Fermented DRB with the highest extracted protein was selected for preparation of DRBPH by spray drying technique. The hydrolysis process of fermented DRB was carried out using an overhead stirrer maintained at 55^{o}C using a water bath for 24 h (Figure 7) after which it was filtered using a nylon mesh (200 microns). The residues obtained after filtration were washed with boiled deionized water in the ratio of 1:6 (w/v) and combined with the filtrate obtained during filtration to obtain DRBPH.

Figure 7 Hydrolysis of fermented DRB in a water bath maintained at 55^{o}C

The spray drying conditions were according to the conditions optimized by Mansor et al. (2020) with some modifications. The heating temperature adjusted at 160^{o}C with a 5 mL/min sample inlet was applied to the spray dryer (Figure 8). The DRBPH obtained was immediately packed airtight in an aluminum bag and stored in the desiccator for further analysis.

Proximate compositions of spray-dried DRBPH were analyzed according to AOAC (2005). Kjeldahl technique (AOAC, 2005) and a protein conversion factor of 5.95 (Bisly et al., 2022) were used.

Figure 8 Spray dryer used for preparing DRBPH from 72 h fermented DRB

3.2.5 Effect of dephytinization on protein extraction from DRB

3.2.5.1 Preparation of DDRB and determination of protein and ash content

Since the DRBPH contained a significant amount of ash, the raw material DRB was dephytinized at pH 2 according to the conditions optimized by Fuh and Chiang (2001) with some adjustments to remove the anti-nutritional agent, phytic acid (Figure 9). For control, dephytinization was carried out in deionized water without pH adjustment. The DRB raw material was dephytinized with the procedure as shown in Figure 8 and the retentate (bran) obtained was named dephytinized defatted rice bran

(DDRB). The protein and ash content of DDRB obtained were analyzed according to AOAC (2005). A protein conversion factor of 5.95 was used.

3.2.5.2 Solid-state fermentation and protein extraction of DDRB

SSF of DDRB and protein extraction was carried out as indicated in the above sections 3.2.3 and 3.2.4. However, fermentation was carried out with Loog-pang at different times (0, 12, 24, 48, 72, and 96 h). During SSF, change in pH, reducing sugar concentration, and enzyme activities were analyzed. Then, fermented DDRB obtained was first determined for extracted protein. The highest extracted protein was selected for the preparation of protein powder and study some of its characteristics.

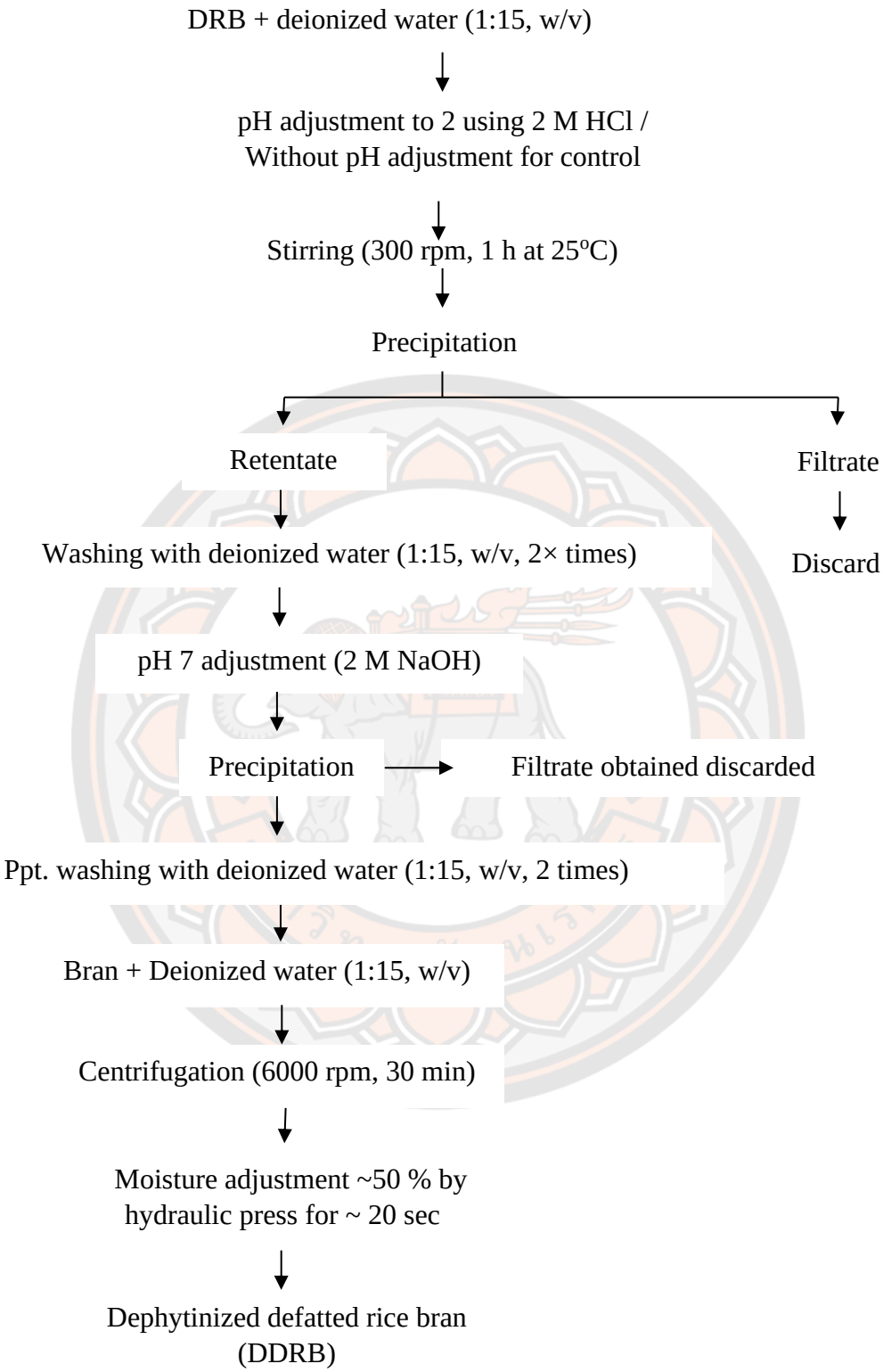

Figure 9 Dephytinization of DRB

3.2.5.3 Preparation of DDRBPH powder and determination of protein properties

The highest extracted protein content obtained after fermentation followed by 24 h hydrolysis was used to carry out the preparation of DDRBPH powder according to the method indicated above in method 3.2.4. The protein powder from just the fermented DDRB without the hydrolysis process was also prepared and named dephytinized defatted rice bran protein (DDRBP).

After obtaining the protein samples (DDRBP and DDRBPH), the following properties were analyzed;

a. Proximate composition

The proximate composition including protein, ash, and moisture content of DDRBP and DDRBPH prepared was analyzed according to AOAC (2005).

b. Protein profile by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Analysis of molecular weight distribution of fermented DRBP and DRBPH was conducted using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method performed previously by Laemmli (1970) with some required adjustments. Concisely, 0.05 g of Loog-pang and Koji fermented DRBP and DRBPH was dissolved in 200 µL deionized water. After a proper mixing using a vortex, the suspension was centrifuged at 3000 × g for 30 min to obtain supernatant. A 4× loading buffer (3:1 v/v) containing 0.1 M Tris-HCl, (pH 6.8), 2% SDS, 5% β-mercaptoethanol, and 15% glycerol was mixed with 15 µL supernatant and the mix was heated for 5 min followed by cooling in ice for 10 min. 15 µL sample was loaded and electrophoresis was carried out with 15% acrylamide separating gel in a Mini-Protean Tetra Cell (BIO-RAD) at a constant current of 100 V for approximately 180 min. Gel staining procedures for overnight involved 0.25% Coomassie brilliant blue R-250 in a methanol/acetic acid/water (50:10:40, v/v/v) solution and de-staining with 10% ethanol and 10% acetic acid. A protein marker with a molecular weight range of 10-250 KDa was used to determine the sample's molecular weight (BIO-RAD).

c. Amino acid compositions

The sample was analyzed for total amino acid (with acid hydrolysis) in Central Instrument Facility, Mahidol University, Bangkok by High-Performance Liquid Chromatography (HPLC) (Waters Alliance 2965, USA). All the samples were prepared in three replicates before dried powders were combined at an equal proportion before sampling for analysis.

3.3 Experimental design and data analysis

All experiments were carried out in duplicates and the results were expressed as mean ± SD. The statistical examination of the data was performed using the SPSS program. A difference in mean values was analyzed, using an analysis of variance (ANOVA) test. These means were compared, using Duncan's Multiple Range Test (DMRT), and a significant value of $P \leq 0.05$ was applied to establish the significant difference between the mean values.

CHAPTER IV

RESULTS AND DISCUSSION

This chapter presents and discusses all the results obtained in the experiment according to the following order:

4.1. Proximate composition of DRB

4.2. Solid-state fermentation of DRB

4.3. Extracted protein content in Loog-pang and Koji fermented DRBPH

4.4. Proximate compositions of DRBPH obtained as a function of SSF time

4.5. Effect of dephytinization on protein extraction from DRB

4.1 Proximate composition of DRB

The proximate composition of DRB used in this study is shown in Table 7. DRB contained 7.89, 16.61, 11.51, 1.32, 10.32, and 52.35 g/100 g DRB (dry basis) for moisture, protein, ash, fats, fiber, and carbohydrates, respectively. Kumari et al. (2018) reported 4.8 g moisture, 13.80 g protein, 11.60 g ash, 0.04 g fats, 13.10 g fiber, and 61.46 g carbohydrate in 100 g DRB (dry basis), while Tajasuwan et al. (2022) reported moisture, protein, ash, fats, and carbohydrate as 5.82, 16.60, 9.76, 2.23, and 66.12 g/100 g DRB (dry basis). The values of the proximate composition of DRB (Table 7), were in the range reported by other authors. However, the value fluctuations in the results confirm that several factors, including the genotype of the rice, climatic influences, the milling process, and the analytical method, have an impact on the chemical composition (da Rocha Lemos Mendes et al., 2021).

Table 7 Proximate composition of DRB

Compositions	Content (g/100 g DRB, dry basis)
Moisture	7.89 ± 0.27
Protein	16.61 ± 0.19
Ash	11.51 ± 0.09
Fats	1.32*
Crude fiber	10.32 ± 0.21
Carbohydrate	52.35

±: standard deviation of duplicates except *

DRB was composed of 16.61 g protein in 100 g DRB which aligns with Alexandri et al. (2020) who reported that the protein content in DRB was in the range of 15-18 g/100 g DRB (dry basis) but the value varies with the varieties of rice cultivar and mainly on DRB production process. The ash content was 11.51 g/100 g DRB indicating DRB as an excellent source of mineral content. The crude fiber content in this study (10.32 g/100 g DRB) was also comparable to 9.50 and 11.7 g/100 g DRB (dry basis) reported by Gupta et al. (2008) and Yadav et al. (2011), respectively. The variation in the values was reported due to the difference in the rice cultivar and degree of rice milling (Rosniyana et al., 2007).

4.2 Solid-state fermentation of DRB

Change in pH, production of reducing sugar, and enzyme activities analyzed after fermentation of DRB for different time are given below.

4.2.1 Determination of pH change

Figure 10 represents the metabolic activities of the microbial strains present in Loog-pang and Koji causing the change in the culture pH at different SSF times. It can be noted that the initial pH of the DRB was 6.60. As the fermentation time proceed, the culture pH significantly deviated from the initial pH. These changes were caused due by various metabolites produced as the microorganism started to grow and utilize the nutrients present in the DRB.

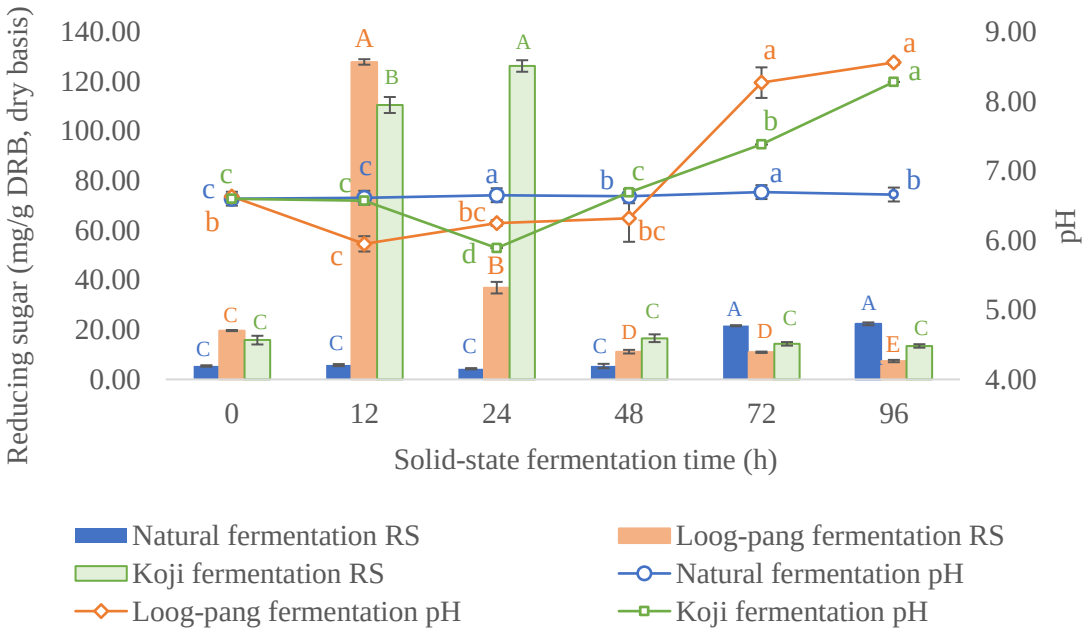

Figure 10 pH changes and reducing sugar (RS) concentrations as a function of SSF time

Different letters (a-d) down the column indicates significant difference ($p \leq 0.05$) between the samples using Duncan's test analysis of data.

In general, it was discovered that the pH drastically dropped at the start of the fermentation before gradually rising after 12 h for Loog-pang and 24 h for Koji. However, the natural fermentation could significantly alter the pH from its initial pH but the pH remained around 6.60-6.69 probably due to inadequate active microbes to degrade the nutrients present in the DRB substrate.

At the initial lag phase of the microbial growth during the SSF of DRB, the microbes started to digest the nutrient content especially fibrous polysaccharides such as cellulose and hemicellulose in the substrates for their use. This leads to the production of various metabolites such as alcohol and organic acid. Due to the substantial production of organic acid, a reduction in the pH of the fermented biomass was observed (0-12 h for Loog-pang and 0-24 h for Koji fermentation). But over time (24-96 h for Loog-pang and 48-96 Koji fermentation), as a result of nutritional loss, microorganisms might have exploited organic acid as a source of nutrients, raising the pH.

4.2.2 Determination of reducing sugar (mg/g DRB, dry basis)

Reducing sugars are present in the DRB in the form of polysaccharides such as starch, cellulose, and hemicellulose. During the SSF, these polysaccharides should be converted to reducing glucose or disaccharides through enzymatic hydrolysis secreted by microbes. According to Daou and Zhang (2014), DRB contains about 32.9 % total dietary fiber, from which 93.80% is insoluble dietary fiber while 6.10% is soluble dietary fiber. The insoluble dietary fiber contained 33.4% cellulose, 54.5% hemicellulose, and 5.8% lignin. Those dietary fibers are the main source of carbon required for the growth and reproduction of fungi and bacteria during the fermentation process. Therefore, for their easy assimilation, the microbes need to degrade the large polysaccharides such as starch and cellulose into their smaller and absorbable monomers such as glucose or reducing sugar.

The reducing sugar quantity obtained after a distinct fermentation time in this study is shown in Figure 10. The reducing sugar drastically increased at the early stage of the fermentation at 12 h with Loog-pang and 24 h with Koji, with maximum reducing sugar/glucose concentrations of 127.90 and 126.27 mg/g DRB, respectively. However, in the case of natural fermentation, due to the presence of limited natural microbes in DRB, the release of reducing sugar took a longer duration and lesser concentration after 72 h. After the indicated period, the reducing sugar levels gradually decreased and depleted at 72 h, except for natural fermentation, indicating the consumption for microbial growth and enzyme secretion.

Due to the active production of an extracellular enzyme such as cellulase at the initial stage of microbial growth, at 12 h for Loog-pang and 24 h for Koji, there involved a maximum breakdown of the DRB cell matrix. This resulted in the production of glucose which was later consumed by the microorganisms for their proliferation.

The changing pattern obtained in this study was also observed by several authors in their respective studies (Oliveira et al., 2010; Mansor et al., 2019; Liu et al., 2021). But Oliveira et al. (2010) reported a maximum reducing sugar production of only 47.6 mg/g DRB after 48 h which is much lower than the value obtained in this study. This discrepancy might be due to differences in the microorganisms and SSF conditions applied. Moreover, the difference in the fermentation conditions might have played a major role in the production of reducing sugar.

4.2.3 Enzyme activity (U/g DRB, dry basis)

Rice bran contains a substantial quantity of nutrients that can support bacterial and fungal growth by providing the necessity such as carbon, nitrogen, sugar, and protein (Sumantha et al., 2006). SSF of DRB with three starters, namely, Loog-pang, Koji, and natural fermentation, could detect various enzymes such as cellulase, neutral, and acid protease depending on the substrate nutrients and culture conditions. The enzyme activity trends are shown in Figures 11-13.

a. Cellulase activity

The extracellular cellulase enzyme was produced to hydrolyze the cellulose content of the DRB cell wall matrix. As a result, absorbable reducing sugar or glucose monomers were generated (Figure 10). Figure 11 depicts the activity of cellulase enzymes produced by Loog-pang, Koji, and during natural DRB fermentations.

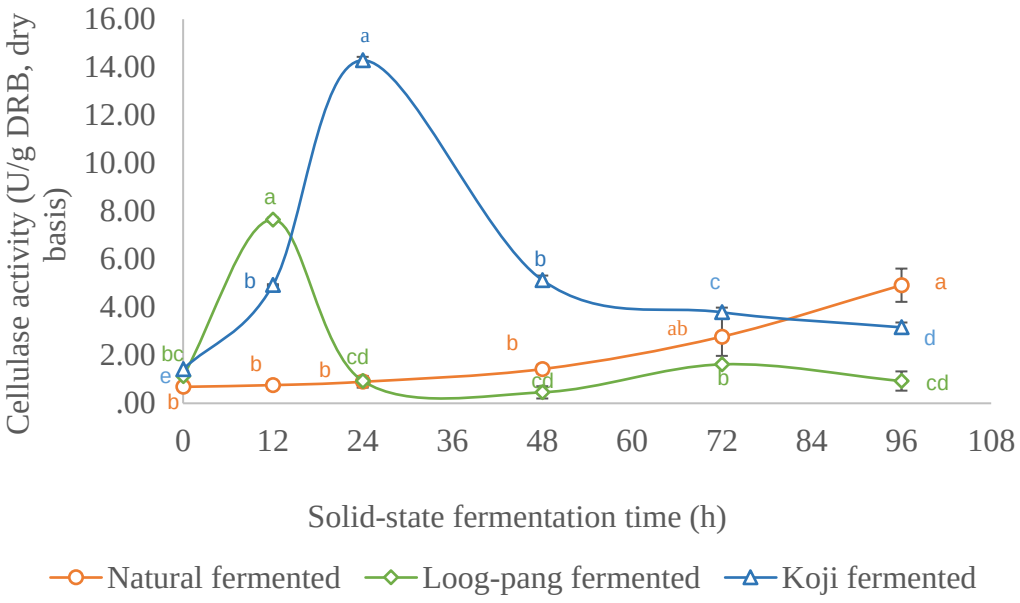

Figure 11 Cellulase activity as a function of SSF time

Different letters (a-e) within the same pattern indicate significantly different ($P \leq 0.05$)

A drastic increase in cellulase activity was observed in the early stage of the fermentation with Loog-pang and Koji, attaining the highest level at 7.65 and 14.29 U/g DRB at 12 h and 24 h, respectively. However, in the case of natural fermentation, it could detect a maximum cellulase enzyme activity of 4.92 U/g DRB, only after 96 h indicating a longer time requirement. After the indicated time, the activity gradually declined. Enzyme activity rises as fermentation times lengthen and falls as nutrients become scarce. It mimics the microbial growth curve (Maftukhah and Abdullah, 2018). At the beginning of the fermentation, due to the high content of cellulose in the DRB substrate, the microbes in Loog-pang and Koji actively produced cellulase enzymes. These extracellular cellulase enzymes actively hydrolyzed cellulose into simple sugar monomers which can be absorbed by the microbes. When the sugar concentration was increased, it indicated the complete hydrolysis of the cellulose matrix in the DRB and the cellulase activity gradually decreased with time. Alam et al. (2009) and Basak and Rangan (2018) have also reported similar activity profile, with cellulase activity increased at first followed by a gradual decrease by *Trichoderma harzianum* and *N. crassa* of rice bran and oil palm empty fruit bunches fermentation.

The maximum cellulase activity obtained at 12 h and 24 h for Loog-pang and Koji fermentation and sugar concentration (Figure 10) were also found consistent.

b. Neutral cellulase activity

Protease enzyme was produced to hydrolyze the large peptide bonds of protein into smaller peptides and amino acids, which are crucial for cell differentiation and development. The optimum pH for the neutral protease activity ranges between 6-8 (Souza et al., 2015). In this study, neutral protease was produced to hydrolyze DRBP when the fermentation culture attained its optimum pH, around 7-8 as indicated in Figure 10. Neutral protease production is usually reported after the exponential growth phase when the microorganisms enter the stationary phase and start the sporulation process. They are believed to contribute to the production of spores (Zhang et al., 2021).

Therefore, neutral protease activity (0-700 U/g DRB) during the SSF of DRB was also traced in this study as shown in Figure 12. Generally, all the fermentation (Natural, Loog-pang, and Koji fermentation) followed a similar activity profile, with a gradual increase, from 12-72 h, followed by a decrease at the final fermentation time.

The neutral protease activity profile revealed its consistent relationship with different stages of microbial growth: 0-12 h was for Loog-pang and Koji spore germination, 12-72 h was for cell growth and differentiation, and 72-96 for sporulation (Zhao et al., 2018; Zhang et al., 2021). The microorganisms at the beginning of the fermentation process, being new to the environment took time to adapt, prepared to germinate, and started to produce enzymes. As the cell started to grow at 12 h, various enzymes were produced to digest large DRBP and polysaccharides into micro-molecules that can be absorbed. Maximum activity was observed at the end of the exponential phase (72 h) with Koji (655.52 U/g DRB) hitting the highest when compared to Loog-pang (427.57 U/g DRB). However, after some time, the activity dwindled, perhaps as a result of decreased humidity, a lack of substrates, inhibition of catabolism, pH changes, and the formation of amino acids and low-molecular-weight molecules (Klapper et al., 1973; Belmessikh et al., 2013).

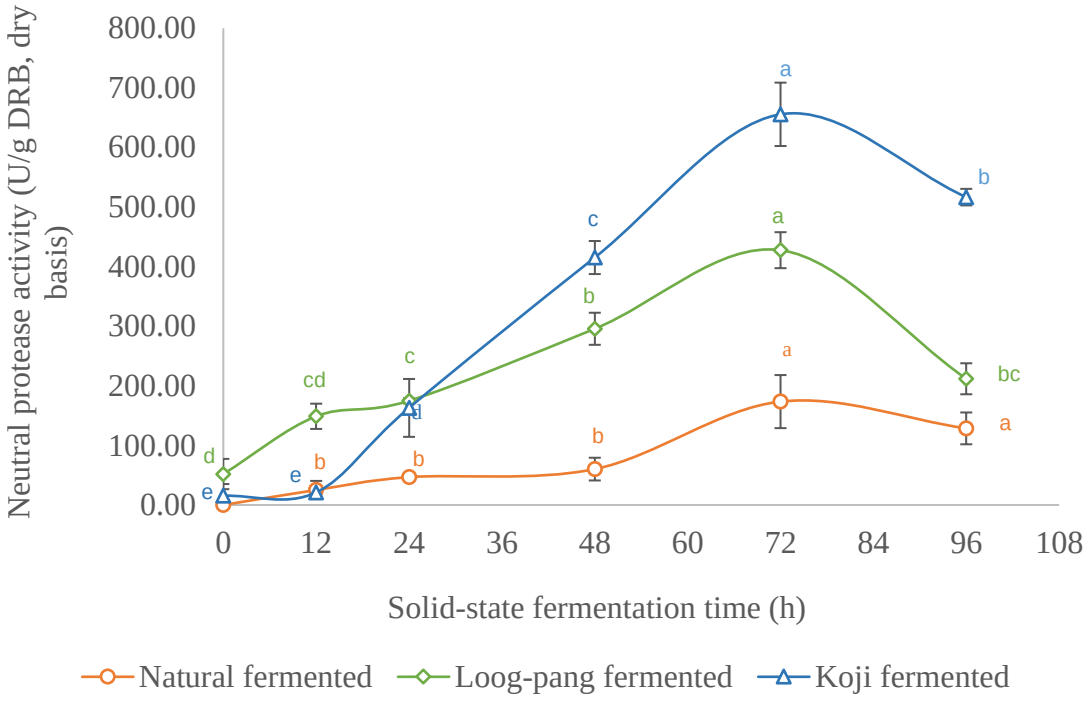

Figure 12 Neutral protease activity as a function of SSF time

Different letters (a-e) within the same pattern indicate significantly different ($P \leq 0.05$)

c. Acid protease activity

Besides neutral protease, acid proteases (Figure 13) were also detected during the SSF of DRB. The peak enzyme production was seen at 24 h with Loogpang fermentation, 72 h with Koji, and natural fermentation. Nevertheless, the acid protease generation in any of the fermentations did not coincide with either the pH changes or the amount of extracted protein as shown in Table 8. Thus, it is not likely that acid proteases were involved in protein hydrolysis as the pH during fermentations as reported in Figure 10 was not in the optimal range for acid protease to be active.

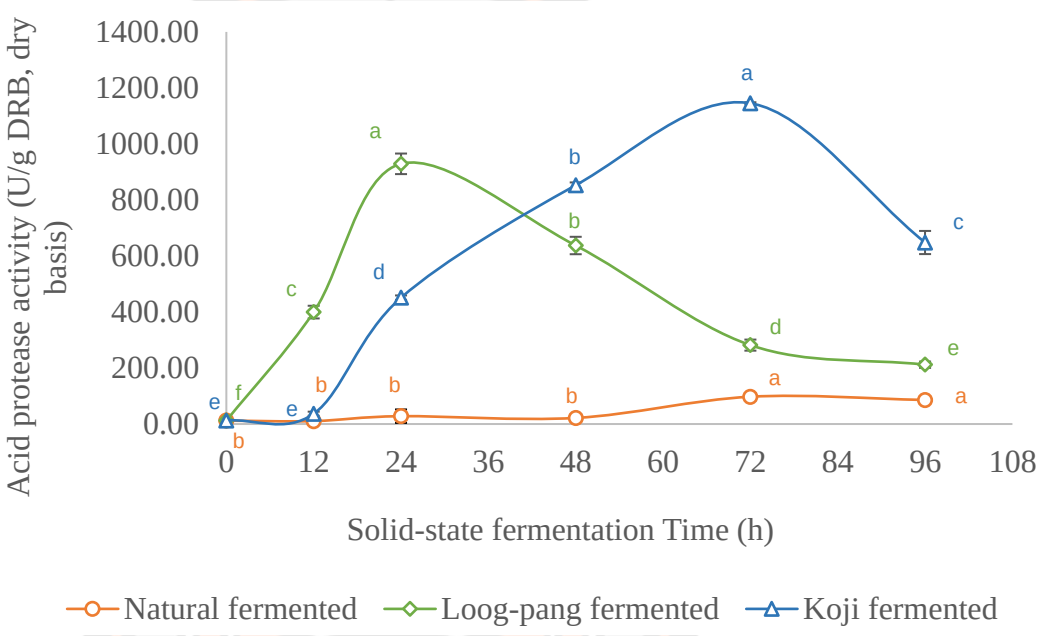

Figure 13 Acid protease activity as a function of SSF time

Different letters (a-e) within the same pattern indicate significantly different ($P \leq 0.05$)

4.3 Extracted protein in Loog-pang and Koji fermented DRBPH

The extracted protein in the DRBPH shown in Table 8 was obtained by determining the total nitrogen content and it indicated the efficiency of hydrolysis by enzymes produced during the SSF.

The result exhibited a significant increment of protein extraction from Loog-pang and Koji fermented DRB with a maximum extracted protein content of 65.66 and 65.67 g/100 g DRB respectively, after 72 h of SSF.

Several authors (Wang et al., 1999; Hamada, 2000; Tang et al., 2003) prepared HSDRB or DRBPH using commercial enzymes such as protease, cellulase, Alcalase, and phytase. They reported that the enzymes were effective in loosening protein from the carbohydrate-protein complex and the large protein was solubilized into medium-sized and small peptides. Likewise, enzymes produced in this study were effective to enhance the protein extraction from DRB biomass. Most importantly, owing to the neutral protease being very efficient (Opazo-Navarrete et al., 2022), its activity (Figure 12) was also found to be consistent with the highest protein extracted after 72 h of SSF time.

Table 8 Extracted protein content in Loog-pang and Koji fermented DRBPH

Fermentation time (h)	Extracted protein content (g/100 g DRB, dry basis)	
	Loog-pang fermentation	Koji fermentation
0	35.80 ± 2.99^{Ad}	29.93 ± 2.48^{Ac}
12	47.59 ± 0.21^{Ac}	31.92 ± 0.33^{Bc}
24	43.25 ± 0.46^{Ac}	61.32 ± 0.59^{Ab}
48	57.60 ± 2.64^{Ab}	61.14 ± 0.65^{Ab}
72	65.66 ± 1.16^{Aa}	65.67 ± 2.22^{Aab}
96	66.74 ± 1.71^{Aa}	68.87 ± 4.30^{Aa}

±: Standard deviation of fermentation duplicates; Different letters (a-c) down the column (A-B) within the row indicates significant difference ($p \leq 0.05$) between the samples using Duncan's test analysis of data.

Despite Loog-pang and Koji producing cellulase and protease enzymes of different activities, they could extract almost similar protein content (~66 g/100 g DRB). This is because the carbohydrase responsible for exposing protein from the DRB matrix produced no significant difference in protein extraction as reported by Tang et al. (2003). The author varied the concentration of enzyme additions and studied the protein extraction from HSDRB. It was found that the addition of food-grade enzyme,

Celluclast in different concentrations (0-700 EGU) did not cause any significant effect on the protein extraction from HSDRB (10.3-12.1 g/100 g HSDRB, dry basis). And also, Jodayree et al. (2012) extracted protein from defatted oat bran (DOB) by altering the concentration of Celluclast (5, 30, and 60 U/g DOB) and extracted protein of almost similar values (53, 53, and 52 g/100 g DOB, respectively). However, amylase and protease when together were able to extract protein of approximately 61 g/100 g HSDRB (dry basis) (Tang et al., 2002). Thus, it can be noted that carbohydrase such as Celluclast is only responsible for exposing and releasing the protein from the DRB matrix by digesting the carbohydrate and plays no role in hydrolyzing the exposed protein. Accordingly, in our investigation, cellulase and protease also collaborated to generate the same results.

The extracted protein content in this study, both the hydrolysates produced from both Loog-pang and Koji fermented DRB after 72 h fermentation was much better and higher than that of Tang et al. (2003), who extracted protein of 45.4 g/100 g HSDRB using amylase with proteolytic activity. And also Bisly et al. (2022) could extract protein of 64.6 g/100 g from HSDRB fermented with *B. subtilis* (natto) Takahashi culture after 61 h. However, the result values were slightly better in this study and this might be because of different enzymes as well as different fermentation starters utilized along with different fermentation conditions.

Due to the effectiveness of this study's preparation of DRBH with a high percentage of extracted protein, SSF with Loog-pang and Koji is demonstrated as a feasible method for extracting and preparing hydrolysates from any other agro-food wastes. Furthermore, since the extracted protein content in the hydrolysate was significantly maximum at 72 h for both the Loog-pang and Koji fermentation, 72 h was considered for further preparation of DRBPH preparation by spray drying technique.

4.4 Proximate compositions of DRBPH obtained as a function of SSF time

DRBPH prepared from Loog-pang and Koji fermented DRB at different times was analyzed for its proximate compositions. The results obtained from the experiment are shown in Tables 9 and 10. Longer fermentation periods weren't investigated since it was thought that they were economically undesirable. For the production of commercial enzymes manufacturing process, the duration of the incubation time is of

utmost importance (Abdullah et al., 2016). The release of reducing sugar and the maximum enzyme production in natural fermentation took a longer duration (72 h) with significantly low values when compared to the Loog-pang and Koji cultures. Thus, natural fermentation was not carried out to omit time consumption.

Table 9 Proximate compositions of Loog-pang fermented DRBPH

Fermentation time (h)	Content (% dry basis)			
	Moisture	Protein	Crude fiber	Ash
0	4.86 ± 0.79^{bc}	12.57 ± 1.25^{e}	1.04 ± 0.00^{b}	19.07 ± 0.71^{c}
12	7.92 ± 1.80^{a}	14.79 ± 0.34^{d}	2.93 ± 0.32^{a}	18.88 ± 0.21^{c}
24	4.54 ± 0.15^{c}	21.45 ± 0.01^{c}	1.42 ± 0.06^{b}	25.18 ± 0.15^{a}
48	4.81 ± 0.17^{bc}	25.98 ± 1.40^{b}	1.04 ± 0.00^{b}	25.02 ± 0.77^{a}
72	6.44 ± 0.99^{ab}	30.09 ± 0.53^{a}	2.65 ± 0.15^{a}	23.29 ± 1.16^{b}
96	5.18 ± 0.15^{bc}	30.82 ± 0.99^{a}	2.59 ± 0.24^{a}	23.64 ± 0.20^{b}

±: Standard deviation of fermentation duplicates; Different letters (a-e) within the column indicate a significant difference ($p \leq 0.05$) between the samples using Duncan's test analysis of data.

Table 10 Proximate composition of Koji fermented DRBPH

Fermentation time (h)	Content (% dry basis)			
	Moisture	Protein	Crude fiber	Ash
0	4.89 ± 0.17^{c}	10.09 ± 0.04^{e}	1.46 ± 0.00^{b}	18.56 ± 0.48^{b}
12	5.49 ± 0.13^{bc}	11.01 ± 0.01^{d}	1.68 ± 0.36^{b}	18.91 ± 0.02^{b}
24	6.87 ± 0.14^{ab}	19.41 ± 0.05^{c}	3.19 ± 0.02^{a}	18.91 ± 0.59^{b}
48	5.92 ± 0.07^{abc}	28.80 ± 0.68^{b}	1.69 ± 0.01^{b}	25.27 ± 0.44^{a}
72	7.13 ± 1.78^{a}	33.76 ± 0.11^{a}	0.96 ± 0.14^{c}	26.19 ± 1.15^{a}
96	5.84 ± 0.30^{abc}	34.05 ± 0.12^{a}	-	25.41 ± 0.49^{a}

-: not determined; ±: Standard deviation of fermentation duplicates; Different letters (a-e) within the column indicate a significant difference ($p \leq 0.05$) between the samples using Duncan's test analysis of data.

The fermentation process entails growing microorganisms to obtain metabolic activity changes that have the desired effects and to increase the availability of nutrients in raw materials. The biochemical modifications related to microbial metabolism and enzyme activities during this fermentation process are the main factor that influences the nutritional quality of the fermented products (Nisa et al., 2020). Likewise, SSF of DRB with Loog-pang and Koji followed by 24 h hydrolysis in this study, also helped to improve the nutritional quality of the substrates, enhancing protein content in DRBPH of both Loog-pang fermented (29% to 82%) and Koji fermented (16% to 103%) after 24 h of fermentation. The ash content was also enhanced from 66% to 119% in Loog-pang fermentation and 61% to 127% in the case of Koji fermentation, whereas the fiber content was reduced up to 90% in both Loog-pang and Koji fermentation.

However, the significant increment of the ash content in the fermented DRBPH could be due to the contribution by the microbial ash especially fungus as the fungal ash content varied between 1% to 29% (Griffin, 1996). Several authors (Oduguwa et al., 2008; Oliveira et al., 2010) have reported the same increment of ash content in fermented rice bran. The moisture content was in the range of 4% to 8% which is low enough for the DRBPH to maintain stability and shelf-life which otherwise could cause undesirable changes such as color and falling pH (Tsoraeva and Zhurbenko, 2000).

Omarini et al. (2019) emphasized that the inoculum uses a repertoire of extracellular enzymes to carry out SSF, which enables it to absorb nutrients and alter the chemical composition of the substrate in addition to creating additional metabolites. The results obtained in this study also suggested that DRBPH is a potential source of protein and minerals which can be used as a nutrient supplementation in functional food products as well as functional ingredients in food and pharmaceutical applications.

When it comes to nutrition, fermented DRB offered a tempting way to supply protein and other nutrients just by utilizing an economically sustainable SSF method. A short fermentation period was sufficient, as evidenced by the fact that the protein

enhancement in DRB substrates required only about 72 h (3 days) and that prolonged fermentation periods did not significantly alter the nutritional content of DRB. Thus, DRB fermented for 72 h was selected for further preparation of protein powder by spray drying technique.

4.4.1 Spray-dried DRBPH powder and proximate compositions

Since the 72 h Loog-pang and Koji fermented DRBPH displayed the highest extracted protein content (Table 8), it was selected and spray-dried to obtain DRBPH powder. The moisture, protein, fiber, and ash content of spray-dried 72 h Loog-pang and Koji fermented and hydrolyzed DRBPH powder are given in Table 11. The moisture content was about 8 g/100 g DRBPH which is safe enough to prevent spoilage during storage. The statistics of protein content in both spray-dried DRBPH prepared from Koji fermentation (29.36 g/100 g DRBPH) and Loog-pang fermentation (27.83 g/100 g DRBPH) were significantly the same. This might be due to the similar effectiveness and hydrolysis rate of cellulase and protease produced which exposed and cleaved DRBP enhancing protein content in the fermented DRB.

Table 11 Proximate compositions of spray-dried DRBPH prepared from 72 h fermented and 24 h hydrolysis

DRBPH	Compositions (g/100 g DRBPH, dry basis)			
	Moisture	Protein	Fiber	Ash
Loog-pang fermented	8.11 ± 0.09^{a}	27.83 ± 0.21^{a}	0.51 ± 0.57^{a}	24.73 ± 0.05^{a}
Koji fermented	8.22 ± 0.17^{a}	29.36 ± 0.67^{a}	0.47 ± 0.21^{a}	23.36 ± 0.12^{b}

±: standard deviation of fermentation duplicates; Different letters (a-b) within the column indicate the significant difference ($p \leq 0.05$) between the DRBPH using one-way ANOVA.

However, the ash content was still significantly high in both the Loog-pang (24.73 g/100 g DRBPH) and Koji (23.36 g/100 g DRBPH) fermented DRBPH. Ash content refers to the minerals compositions and DRB contains mainly calcium, phosphorus, iron, potassium, and magnesium (Kumari et al., 2018) which are strongly bonded to phytic acid as well as dietary fibers (Ekholm et al., 2003) making the mineral content of DRB highly insoluble. The phosphate groups of phytic acid are negatively charged in the normal pH range, allowing them to interact with positively charged substances like proteins and minerals (Oatway et al., 2001). Therefore, two possible reasons were assumed for the high minerals content in the spray-dried DRBPH powder; 1) the production of phytase enzyme along with consumption of dietary fiber by fungus released the minerals linked to them; and 2) The fungus development further contributed to the DRB ash content (Griffin, 1996; Oliveira et al., 2010). This high ash content in the DRBPH powder hampered the protein purity and phytic acid was assumed to be responsible as it is reported to possess an anti-nutritional property chelating protein, minerals (ash), and other components (Servi et al., 2008; Sharif et al., 2014; Kortekangas et al., 2020; Wang and Guo, 2021).

It was necessary to remove the phytic acid from the DRB which indirectly also removes minerals attached to it. Thus, the effect of dephytinization on DRBP extraction was studied.

4.5 Effect of dephytinization on protein extraction from DRB

4.5.1 Dephytinization effect on protein and ash content of DDRB

The dephytinization of DRB was carried out by hydrolysis at pH 2 using HCl since phytic acid solubility was greater at pH $\leq$ 2 (Champagne et al., 1985; Bloot et al., 2021) and dephytinization using deionized water, without setting pH was also carried out and used as control. The results of chemical compositions in DDRB were shown in Table 12 and were compared to DRB.

Table 12 Chemical compositions of DDRB obtained after dephytinization of DRB

Bran	Compositions (g/100 g DDRB, dry basis)		
	Moisture	Protein	Ash
DRB	7.89 ± 0.274^{b}	16.61 ± 0.19^{b}	11.51 ± 0.09^{a}
Control	54.91 ± 1.15^{a}	21.27 ± 0.74^{a}	6.56 ± 0.34^{b}
DDRB	54.13 ± 1.44^{a}	20.04 ± 0.10^{a}	3.09 ± 1.18^{c}

DRB: defatted rice bran; Control: dephytinization in deionized water, DDRB: dephytinized at pH 2; ±: Standard deviation of triplicates; Different letters (a-c) within the column indicates significant difference ($p \leq 0.05$) between the samples using Duncan's test analysis of data.

The dephytinization process was focused on the indirect removal/reduction of the ash content in DRB by removing the anti-nutritional agent, phytic acid. However, the process also posed some changes in protein content. The protein content in the DRB was 16.61 g/100 g DRB. After dephytinization, both the control and DDRB contained significantly high and similar protein content of 21.27 and 20.04 g/100 g DDRB, respectively. Dephytinization process significantly enhanced protein content. This increase in the protein content may be due to the release of protein attached to phytic acid and the reduction of mineral content from the DRB.

The change in the ash content in DDRB was analyzed and compared to the DRB. The DRB contained an ash content of 11.51 g/100 g DRB which was significantly higher than the control and DDRB. Dephytinization using HCl (pH 2) and distilled water (normal pH) proved their effectiveness in decreasing ash/mineral from the DRB. Dephytinization with deionized water in the case of control significantly reduced ash content and reached 6.56 g/100 g DDRB which was about a 43% reduction from the DRB. However, dephytinization at pH 2 using HCl, in the case of DDRB changed the ash content to 3.09 g/100 g DDRB and this was about a 73% reduction when compared with the control. This value indicates and aligns with Champagne et al. (1985) who reported that the solubility of phytic acid is pH-dependent, and at pH ≤ 2, the solubility

is greater. This method of the dephytinization process indirectly favored the reduction of the ash content in the DRB. After dephytinization, Fuh and Chiang (2001) reported that the majority of the important minerals, including calcium, phosphorus, potassium, magnesium, and sodium, were lost, probably because the minerals chelated away with phytic acid.

Therefore, the dephytinized bran at pH 2 (DDRB) was selected to proceed with fermentation owing to the significantly low ash content and evaluate the protein extraction from the DDRB.

4.5.2 Solid-state fermentation of DDRB

Unlike Loog-pang, SSF with Koji did not show any visible fungal growth even after 48 h. Thus, the SSF of DDRB and extraction of protein from DDRB were not further investigated.

4.5.2.1 Determination of pH change and reducing sugar change, and evaluation of enzymatic activity

Figure 14 indicates the observations of pH change and concentration of reducing sugar produced in fermented DDRB after different SSF times with Loog-pang. The change in pH shows that it followed a trend of initial fall followed by a gradual rise in the pH. This observation was similar to the results obtained from SSF of DRB as shown in Figure 10, with a gradual decrease for some time followed by a gradual increase.

Nonetheless, the outcome shows that the DDRB fermentation took place in acidic circumstances, perhaps as a result of the acid residue left during the dephytinization process along with the further acidic makeup of the DDRB substrate during fermentation.

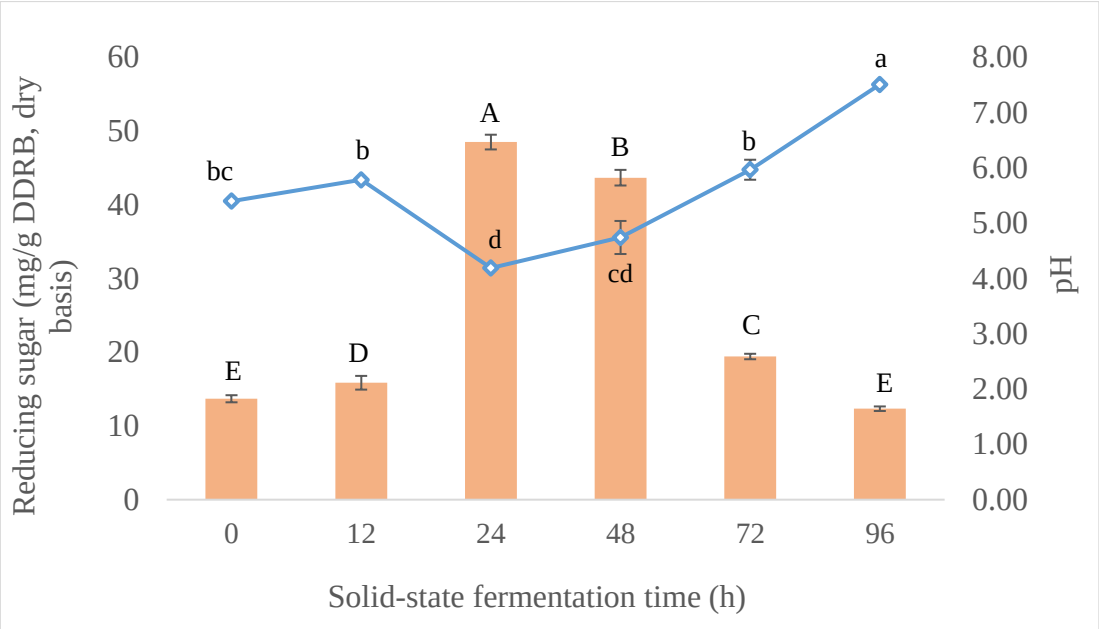

Figure 14 pH change and reducing sugar content in Loog-pang fermented DDRB as a function of SSF time

Different letters (a-e) within the column indicates significant difference ($p \leq 0.05$) between the samples using Duncan's test analysis of data.

The concentration of reducing sugar in the fermented DDRB for a distinct time indicates that the concentration of reducing sugar concurrently increased with the fermentation time until 24 h obtaining a reducing sugar of 48.51 mg/g DDRB. This reducing sugar, sugar/glucose was produced as a result of cellulose breakdown by the enzyme produced during the fermentation process which was later consumed by the microbes. The concentration reached 12.34 mg/g DDRB after 96 h of fermentation. However, the maximum reducing sugar concentration obtained at 24 h was much lower than the concentration obtained during DRB fermentation (127.90 mg/g DDRB) at 12 h of SSF. This might be due to the reduction of cellulose and hemicellulose content in the DDRB substrates caused by inorganic acid, HCl (Qi et al., 2015). Since HCl, unlike enzymes, will hydrolyze any glycosidic bonds present in polysaccharides (Lee et al., 2009) producing glucose and fructose monomers. These might have been discarded along with water during the washing step of DDRB.

4.5.2.2 Enzyme activities (U/g DDRB, dry basis)

As indicated above, enzymes that involve cellulase and proteases were produced by the microorganisms in Loog-pang and Koji to cleave cellulose and DDRB protein components, respectively, and utilize them for their development during the fermentation process. Therefore, enzyme activities including cellulase, neutral, and acid protease from Loog-pang fermentation of DDRB were also traced and shown in Table 13.

a. Cellulase activity

As mentioned earlier the cellulase was secreted for cleaving cellulose to produce reducing sugar/glucose. The cellulase activity significantly increased up to the first 24 h hitting the maximum value of 4.96 U/g DDRB. The peak reducing sugar concentration obtained in this study after 24 h (Figure 14) coincides with the peak cellulase activity value. However, due to the reduction of cellulose substrates by HCl (Qi et al., 2015) in DDRB, the peak activity did not match with the maximum value (7.65 U/g DRB) obtained during SSF of DRB with Loog-pang (Figure 10). DRB dephytinization with HCl in the dephytinization process leads to the breakage of dietary fiber such as cellulose and hemicellulose into smaller monomers (Lee et al., 2009; Qi et al., 2015) which might be washed off with the water during the washing step. Thus, the available dietary fiber decreased, affecting the reduction of cellulase production by the microorganisms.

Table *13* Enzyme activities of Loog-pang fermented DDRB as a function of SSF time

Fermentation time (h)	Activity (U/g DDRB, dry basis)		
	Cellulase	Neutral protease	Acid protease
0	0.44 ± 0.6^{c}	6.25 ± 2.95^{d}	55.56 ± 5.24^{e}
12	0.59 ± 0.08^{c}	8.33 ± 0.00^{d}	76.54 ± 3.49^{e}
24	4.96 ± 1.00^{a}	62.50 ± 1.96^{c}	707.41 ± 2.62^{d}
48	3.07 ± 1.0^{b}	112.50 ± 1.96^{b}	1583.33 ± 2.62^{c}
72	2.75 ± 0.47^{b}	265.28 ± 5.89^{a}	2183.33 ± 2.62^{a}
96	2.50 ± 0.14^{b}	283.33 ± 6.29^{a}	2098.15 ± 23.57^{b}

±: standard deviation of three replicates of fermentation duplicates; Different letters (a-e) within the column indicates significant difference ($p \leq 0.05$) between the samples using Duncan's test analysis of data.

b. Neutral protease activity

Generally, the neutral protease activity was found to increase with the increasing SSF time and an almost similar trend was observed when compared with the SSF of DRB. The maximum activity in both the SSF with Loog-pang was obtained at 72 h with 427.57 and 265.28 U/g of DRB and DDRB, respectively.

However, the activity values in SSF of DDRB (Table 13) were much lower than the activity obtained in DRB fermentation (Figure 12). This makes sense because most of the time SSF of DDRB took place in an acidic condition (pH 4-6) which did not allow the neutral protease to function properly even if it was present in the fermentation culture. When the pH climbed toward neutral after 72 h (Figure 14), the activity significantly increased and reach the maximum.

c. Acid protease activity

Unlike DRB fermentation, the acidic protease activity during this time showed a gradual increase up to 72 h of fermentation followed by a decrease in activity. The acid protease enzyme followed a different activity pattern when compared to activity obtained during the SSF of DRB (Figure 13) but was quite similar to the neutral protease enzyme activity obtained during the SSF of DRB (Figure 12). Neutral and acid protease activity patterns obtained in the SSF of DRB and DDRB, respectively show a similar fact that when the pH change is around their optimum range, pH 7-8 in the case of neutral and 4-5 in the case of acid protease, the enzymes became more active. Thus, the highest acid protease activity (2183.33 U/g DDRB) was obtained after 72 h of fermentation of DDRB. However, with the shift of pH to neutral (pH 7.51), a significant decrease (96 h) in the activity was observed.

The highest acid protease activity (2183.33 U/g DDRB) obtained at 72 h of SSF time was much higher than the highest activity (929.06 U/g DRB) obtained at 24 h of

SSF of DRB with Loog-pang (Figure 13) as most the SSF of DDRB took place in acidic medium.

4.5.3 Extracted protein content as a function of SSF time

The amount of extracted protein from the DDRB fermented at different times with Loog-pang is shown in Table 14. The results reveal that the extracted protein content increased with increasing the length of fermentation. The highest protein (65.66 g/100 g DRB) was extracted after 72 h of fermentation with Loog-pang from DRB (Table 8). However, Loog-pang fermentation of DDRB obtained the highest extracted protein content (59.44 g/100 g DDRB) after 48 h of fermentation. According to Zhao et al. (2018), the extracted protein from defatted soybean meal as a function of SSF time did not coincide with the increasing trend of SSF time due to the interaction of various enzymes in fermented defatted soybean meal which the *Aspergillus oryzae* produced and due to the shifts in equilibrium between substrates, intermediates, and end products as a result of development and metabolism of *Aspergillus oryzae*. Similarly, the extracted protein content obtained in this study from DDRB did not match with the highest neutral (265.28 U/g DDRB) and acid protease activity (2183.33 U/g DDRB) obtained at 72 h of SSF.

Moreover, the protein extracted was much lower than the protein extracted from DRB (65.66 g/100 g DRB) after 72 h of SSF. Two possible reasons are, 1) due to the loss of bran and protein during the dephytinization process and 2) the enzyme especially neutral protease (6-283.33 U/g DDRB), produced during the fermentation of DDRB (Table 13) was much lower than DRB fermentation (51.95-427.57 U/g DRB).

Table 14 Extracted protein content in Loog-pang fermented DDRBPH

Fermentation Time (h)	Extracted protein content (g/100 g DDRB, dry basis)
0	15.45 ± 1.53^{d}
12	18.05 ± 0.56^{d}
24	48.89 ± 1.52^{b}
48	59.44 ± 4.40^{a}
72	51.29 ± 1.98^{b}
96	42.39 ± 1.57^{c}

±: standard deviation of two fermentation duplicates; Different letters (a-d) within the column indicates significant difference ($p \leq 0.05$) between the samples using Duncan's test analysis of data.

4.5.4 Chemical Composition of DDRBPH

Table 15 captures the chemical composition, especially protein and ash content of the DDRBPH obtained after fermentation for different times followed by 24 h hydrolysis. Since this part was carried out to investigate the reduction of ash content which hampered the protein content or purity. It was shown that both protein and ash content increased with the SSF time. After 96 h of SSF, the highest protein content (49.00 g/100 g DDRBPH) was obtained which was a 145 % increment when compared to DDRB (20.04 g /100 g DDRB). The ash content was also significantly the highest (5.60 g/100 g DDRBPH) in the DDRBPH obtained after 96 h SSF time. This indicated that SSF has the potential to enrich protein and mineral content and improve the nutrition of any substrate.

However, the ash content in the DDRBPH was much lower (2-6 g/100 g DDRBPH) than DRBPH (18-25 g/100 g DRBPH). This indicated that the dephytinization process was much more efficient in removing most of the ash content and probably, the ash content in the DDRBPH was contributed by the fungal cells as it is found to increase with the SSF time when compared to DDRB (3.09 g/100 g DDRB).

Table 15 Chemical compositions of Loog-pang fermented DDRBPH

Fermentation Time (h)	Compositions (g/100 g DDRBPH, dry basis)		
	Moisture	Protein	Ash
0	6.79 ± 0.73^{c}	16.79 ± 1.66^{e}	3.39 ± 0.06^{c}
12	7.44 ± 0.02^{c}	17.27 ± 0.53^{e}	2.94 ± 0.01^{d}
24	13.48 ± 0.43^{b}	28.84 ± 0.90^{d}	2.52 ± 0.09^{e}
48	16.27 ± 0.79^{a}	37.04 ± 2.74^{c}	3.26 ± 0.14^{c}
72	15.46 ± 0.55^{a}	45.39 ± 1.75^{b}	4.50 ± 0.00^{b}
96	13.12 ± 3.57^{b}	49.00 ± 1.82^{a}	5.60 ± 0.00^{a}

±: standard deviation of fermentation duplicates; Different letters (a-e) within the column indicates significant difference ($p \leq 0.05$) between the samples using Duncan's test analysis of data.

4.5.5 DDRBPH properties

a. Chemical compositions analysis

The moisture, protein, and ash content of DDRBP and DDRBPH prepared from the 48 h Loog-pang fermented and hydrolyzed DDRB is given in Table 16. The result shows that after the dephytinization process, the ash content in the protein has significantly decreased when compared to the protein obtained from DRB fermentation. However, the protein content hydrolysis of fermented DDRB seemed to play a major role in the enhancement of protein content, as the 24 h hydrolyzed, DDRBPH contained significantly high protein content when compared to DDRBP (non-hydrolyzed). This protein content was found to be better when compared to protein content in 72 h Loog-pang fermented DRBPH. This was due to the massive reduction of ash content from the DRB during the dephytinization process.

Table 16 Chemical compositions of DDRBP

Sample	Compositions (g/100 g sample, dry basis)		
	Moisture	Protein	Ash
DDRBPH	22.63 ± 0.36[a]	37.23 ± 0.21[a]	2.73 ± 0.25[a]
DDRBP	14.57 ± 0.24[b]	24.51 ± 0.04[b]	2.80 ± 0.04[a]

DDRBPH: dephytinized defatted rice bran protein hydrolysate; DDRBP: fermented dephytinized defatted rice bran protein; ±: standard deviation of fermentation duplicates. Different letters (a-b) within the column indicate the significant difference ($p \leq 0.05$) between the DRBPH using one-way ANOVA.

The protein content obtained in this study is much better than Thamnarathip et al. (2016) who obtained protein content of 12.3%, 13.5%, and 23.7% with Neutrase, Alcalase, and Flavourzyme, respectively from defatted riceberry bran and comparable to Hamada (2000) who reported of 29.9% and 27.6% with Flavourzyme and Alcalase 24 L from DRB. The differences in these values are due to different extraction techniques and different bran with different cultivars but indicate that the SSF with Loog-pang is also an economical and comparable approach to extract protein from DRB.

b. SDS-PAGE profile of DRBP

Rice bran protein hydrolyzed enzymatically comprises of protein with bands of different molecular weights that correspond to prolamin (13 and 16 KDa), globulin (26 KDa), acidic and basic subunits of glutelin (22-23 KDa and 37-39 KDa, respectively), and pro-glutelin (57 KDa) (Zang et al., 2019). Protease enzyme produced during the fermentation process has influenced and altered the distribution of high- and low-molecular-weight protein components of hydrolysates prepared in this study as shown in Figure 15. The molecular weight of peptides in the range of 10-100 KDa was observed in both the Loog-pang and Koji-fermented DRBP. However, all those medium

and large-sized peptides were further broken down into smaller molecular weights by hydrolysis process.

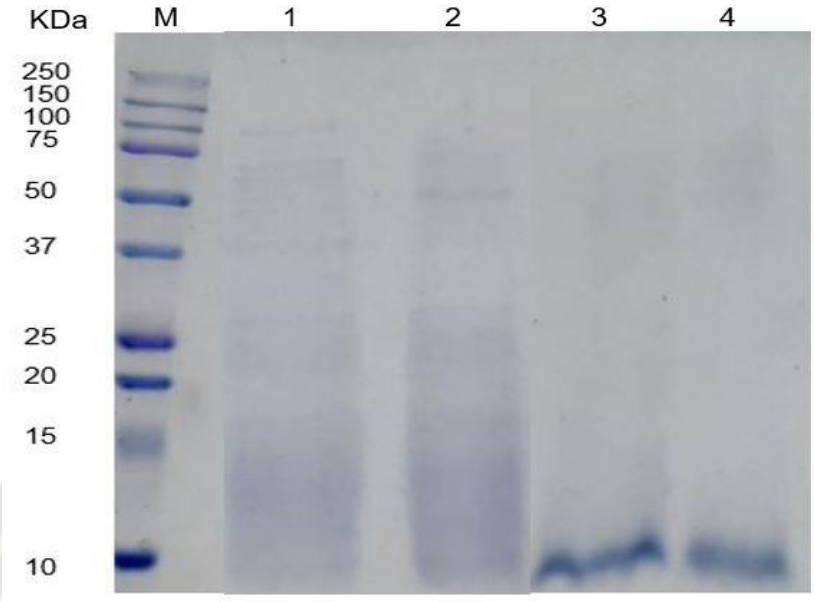

Figure 15 SDS-PAGE profile of 72 h fermented DRBP

M= marker; 1 & 2= Koji & Loog-pang fermented DRBP; 3 & 4 = 24 h hydrolyzed Koji and Loog-pang fermented DRBP, respectively.

c. Amino acid composition

The amino acids composition represents the both nutritional and physicochemical properties of protein content in any food materials. The amino acid compositions of the samples, non-fermented DRBP, fermented DRBP, and DRBPH in this study are shown in Figure 16. The figure shows that all the samples exhibited variations in the amino acid concentration due to diverse microbial growth and enzyme activities which influenced the protein hydrolysis rate and fragmentation of the DRB polypeptides (Chinma et al., 2014).

The total amino acid content in non-fermented DRBP was 18.71 mg/100 mg DRBP and after 48 h of fermentation, the total amino acid in fermented DRBP reached 19.85 mg/100 mg DRBP. This was because the enzyme, cellulase produced during the fermentation helped to break the protein-carbohydrate interaction and release the protein from the DRB cell matrix. Then the protease produced fragmented the DRBP

into various polypeptides just enough for microbial utilization. Thus, this led to an increase in the protein content and the total amino acid content increment was about 6.12%.

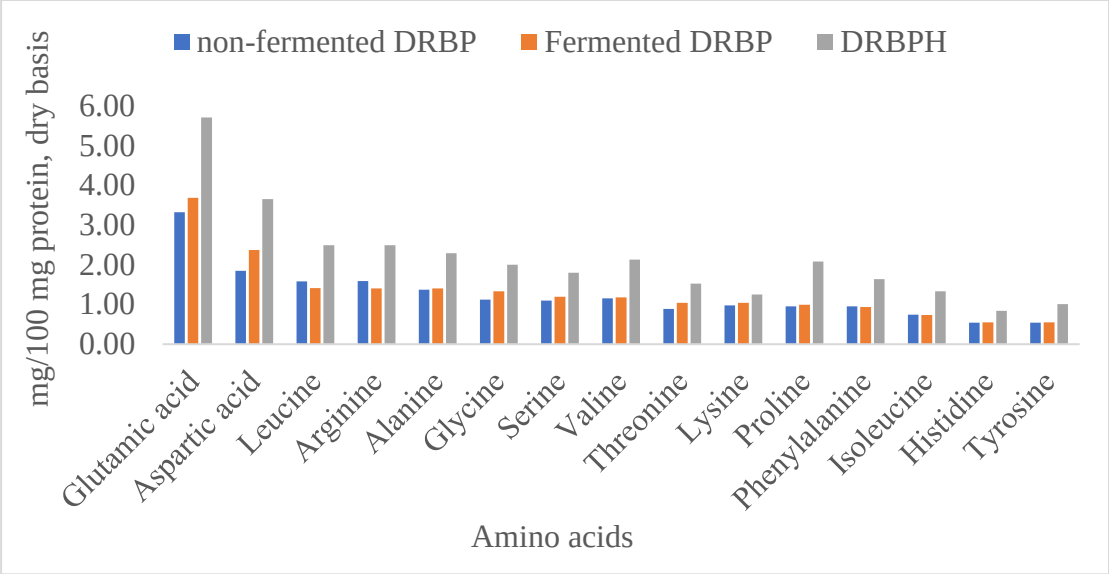

Figure 16 Amino acid compositions of DRBP

Non-fermented DRBP: Solubilized in deionized water followed by pH 4 precipitation; Fermented DRBP: DRBP obtained after 48 h of fermentation; DRBPH: 48 h fermented followed by 24 h hydrolyzed DRBP

However, on further hydrolysis of fermented DRBP, the total amino acid changed to 32.26 mg/100 mg DRBPH, which was about 62.50% and 72.44% increase when compared to fermented and non-fermented DRBP, respectively. This was because hydrolysis of fermented DRBP accelerated and fragmented the exposed DRBP into mostly free amino acids, thereby improving and enhancing the amino acid content in DRBPH (Hamada, 2000).

The amino acid profile of DRBP in this study comprehends with the findings by Wang et al. (2016) who reported that rice bran protein and protein fractions (albumin, globulin) contains glutamic and aspartic acid as the most abundant amino acids. Likewise, Jarunrattanasri et al. (2005) also reported an amino acid profile in DRBP similar to this study with glutamic acid, arginine, aspartic acid, alanine, leucine,

and glycine exhibiting the highest abundance and accounting for 61% of the total amino acid compositions.

All the samples in this study also comprised glutamic acid as the most abundant amino acid followed by aspartic acid, leucine, arginine, alanine, and glycine accounting for 57.96% of the total amino acids in protein extracted from DRB. Fermentation increased amino acid content as mentioned previously, however, the amino acid profile in fermented DRBP and DRBPH remained the same as the non-fermented DRB. Glutamic acid, aspartic acid, Leucine, arginine, alanine, and glycine were still dominant even after the fermentation process accounting for 58.55% and 57.81% of the total amino acids in fermented DRBP and DRBPH, respectively.

The fact that the fermented DRBP had a higher amino acid content while maintaining the same amino acid profile as the non-fermented DRBP demonstrated the efficacy of SSF on DRBP extraction, which helped increase protein yield without altering the amino acid profile.

CHAPTER V

CONCLUSION

This chapter consists of the conclusions of the results obtained and recommendations to further improve and study more on the physicochemical and functional properties as well as applications of the extracted protein.

5.1 Conclusions

This study demonstrated the protein extraction from DRB by SSF with Loog-pang and Koji. Cellulase, neutral, and acid proteases were detected during SSF with Loog-pang and Koji. Despite the different amounts of enzymes produced, both fermentation starters successfully enhanced protein content extraction with no difference in the extracted yield. The results obtained showed that DRBPH obtained after 72 h of SSF followed by 24 h hydrolysis obtained the highest extracted protein but DRBPH presented significantly high ash content in both Loog-pang and Koji fermentation. However, SSF of DDRB with Loog-pang for 48 h, and 24 h hydrolysis extracted the highest protein and the DDRBPH with improved protein content and significantly low ash content was obtained.

The DRBP contained polypeptides of molecular weights in the range of 10-100 KDa and DRBPH with smaller molecular weight peptides. Moreover, the amino acid compositions in non-fermented and fermented DRBP and DRBPH were mostly glutamic acids, aspartic acid, leucine, arginine, and glycine.

5.2 Recommendations

1. The functional properties such as protein solubility, emulsion activity and emulsion stability, foaming capacity and foaming stability, thermal properties, and physical properties such as color analysis of the protein extracted could be further studied.

2. Since the DRBP contains glutamic acid as the dominant amino acid composition, therefore DRBPH can be used as an excellent flavor-enhancing ingredient in various food applications.

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APPENDIX

APPENDIX A DETERMINATION OF pH CHANGE AND REDUCING SUGAR (mg/g DRB, DRY BASIS) FOR FIGURE 9

Fermentation	Natural fermentation		Loog-pang fermentation		Koji fermentation	
Time (h)	pH	Reducing Sugar	pH	Reducing Sugar	pH	Reducing Sugar
0	6.60 ± 0.14^{c}	5.39 ± 0.29^{c}	6.63 ± 0.03^{b}	19.67 ± 0.22^{c}	6.60 ± 0.01^{c}	15.82 ± 1.75^{c}
12	6.61 ± 0.01^{c}	5.76 ± 0.41^{c}	5.95 ± 0.11^{c}	127.90 ± 1.10^{a}	6.57 ± 0.01^{c}	110.55 ± 3.22^{b}
24	6.69 ± 0.01^{a}	4.29 ± 0.25^{c}	6.25 ± 0.04^{bc}	36.95 ± 2.33^{b}	5.89 ± 0.07^{d}	126.27 ± 2.32^{a}
48	6.67 ± 0.00^{b}	5.39 ± 0.87^{c}	6.32 ± 0.34^{bc}	11.17 ± 0.73^{d}	6.69 ± 0.11^{c}	16.59 ± 1.56^{c}
72	6.69 ± 0.01^{a}	21.63 ± 0.21^{a}	8.27 ± 0.22^{a}	10.93 ± 0.28^{d}	7.38 ± 0.01^{b}	14.32 ± 0.76^{c}
96	6.66 ± 0.00^{b}	22.38 ± 0.57^{a}	8.56 ± 0.03^{a}	7.39 ± 0.47^{e}	8.28 ± 0.35^{a}	13.48 ± 0.70^{c}

±: standard deviation of fermentation duplicates; Different letters (a-d) down the column indicates significant difference ($p \leq 0.05$) between the samples using Duncan's test analysis of data.

APPENDIX B CELLULASE ACTIVITY OF FIGURE 10

Fermentation Time (h)	Cellulase activity (U/g DRB, dry basis)		
	Natural fermented	Loogpang fermented	Koji fermented
0	0.69 ± 0.11^{Bb}	1.13 ± 0.23^{Abc}	1.42 ± 0.11^{Ae}
12	0.76 ± 0.16^{Cb}	7.65 ± 0.08^{Aa}	4.92 ± 0.04^{Bb}
24	0.90 ± 0.24^{Bb}	0.93 ± 0.05^{Bcd}	14.29 ± 0.14^{Aa}
48	1.43 ± 0.18^{Bb}	0.46 ± 0.25^{Cd}	5.13 ± 0.19^{Ab}
72	2.78 ± 3.02^{Aab}	1.62 ± 0.06^{Ab}	3.79 ± 0.19^{Ac}
96	4.92 ± 0.69^{Aa}	0.93 ± 0.40^{Ccd}	3.16 ± 0.20^{Bd}

±: standard deviation of two replicates of fermentation duplicates

Different letters (A-C) within the row and (a-f) down the column indicates significant difference ($p \leq 0.05$) between the samples using Duncan's test analysis of data.

APPENDIX C NEUTRAL PROTEASE ACTIVITY FOR FIGURE 11

Fermentation Time (h)	Neutral protease activity (U/g DRB, dry basis)		
	Natural fermented	Loog-pang fermented	Koji fermented
0	nd	51.95 ± 2.43^{Ad}	15.53 ± 1.65^{Ae}
12	25.17 ± 15.56^{Bb}	148.85 ± 2.19^{Acd}	21.25 ± 4.62^{Be}
24	46.75 ± 2.54^{Bb}	174.82 ± 4.24^{Ac}	162.93 ± 4.55^{Ad}
48	60.23 ± 19.07^{Bb}	295.70 ± 6.16^{Ab}	415.22 ± 2.74^{Ac}
72	173.51 ± 44.50^{Ca}	427.57 ± 5.51^{Ba}	655.52 ± 3.17^{Aa}
96	128.56 ± 26.70^{Ba}	211.79 ± 4.38^{Bbc}	516.57 ± 3.87^{Ab}

nd: not detected; ±: standard deviation of fermentation duplicates

Different letters (A-C) within the row and (a-f) down the column indicates significant difference ($p \leq 0.05$) between the samples using Duncan's test analysis of data.

APPENDIX D ACID PROTEASE ACTIVITY FOR FIGURE 12

Fermentation Time (h)	Acid protease activity (U/g DRB, dry basis)		
	Natural fermented	Loogpang fermented	Koji fermented
0	11.99 ± 8.48^{Ab}	11.99 ± 2.83^{Af}	12.74 ± 5.30^{Ae}
12	10.49 ± 9.18^{Bb}	399.60 ± 2.60^{Ac}	36.71 ± 7.42^{Be}
24	27.97 ± 12.02^{Cb}	929.06 ± 6.73^{Aa}	451.04 ± 8.53^{Bd}
48	21.73 ± 1.06^{Cb}	637.36 ± 3.08^{Bb}	852.64 ± 9.60^{Ab}
72	97.40 ± 12.72^{Ca}	281.72 ± 4.78^{Bd}	1145.22 ± 6.56^{Aa}
96	85.91 ± 2.46^{Ca}	211.79 ± 1.30^{Be}	648.10 ± 4.32^{Ac}

±: standard deviation of fermentation duplicates

Different letters (A-C) within the row and (a-f) down the column indicates significant difference ($p \leq 0.05$) between the samples using Duncan's test analysis of data.

APPENDIX E pH CHANGE AND REDUCING SUGAR CONTENT IN LOOG-PANG FERMENTED DDRB FOR FIGURE 13

Fermentation time (h)	Fermentation pH	Reducing sugar (mg/g DDRB, dry basis)
0	5.40 ± 0.01^{bc}	13.69 ± 0.48^{e}
12	5.79 ± 0.01^{b}	15.86 ± 0.93^{d}
24	4.19 ± 0.03^{d}	48.51 ± 1.00^{a}
48	4.74 ± 0.30^{cd}	43.68 ± 1.06^{b}
72	5.97 ± 0.81^{b}	19.42 ± 0.37^{c}
96	7.51 ± 0.03^{a}	12.34 ± 0.31^{e}

±: standard deviation of fermentation duplicates. Different letters (a-e) within the column indicates significant difference ($p \leq 0.05$) between the samples using Duncan's test analysis of data.

APPENDIX F AMINO ACID COMPOSITIONS OF DRBP FOR FIGURE 15

Amino acid Compositions	Quantity (mg/100mg DRBP, dry basis)		
	non-fermented DRBP	Fermented DRBP	DRBPH
Glutamic acid	3.32	3.69	5.71
Aspartic acid	1.85	2.38	3.66
Leucine	1.58	1.42	2.49
Arginine	1.59	1.40	2.49
Alanine	1.37	1.40	2.29
Glycine	1.12	1.33	2.00
Serine	1.10	1.19	1.80
Valine	1.15	1.18	2.13
Threonine	0.89	1.04	1.53
Lysine	0.98	1.04	1.25
Proline	0.95	0.99	2.08
Phenylalanine	0.95	0.94	1.64
Isoleucine	0.74	0.74	1.33
Histidine	0.55	0.55	0.84
Tyrosine	0.55	0.55	1.01