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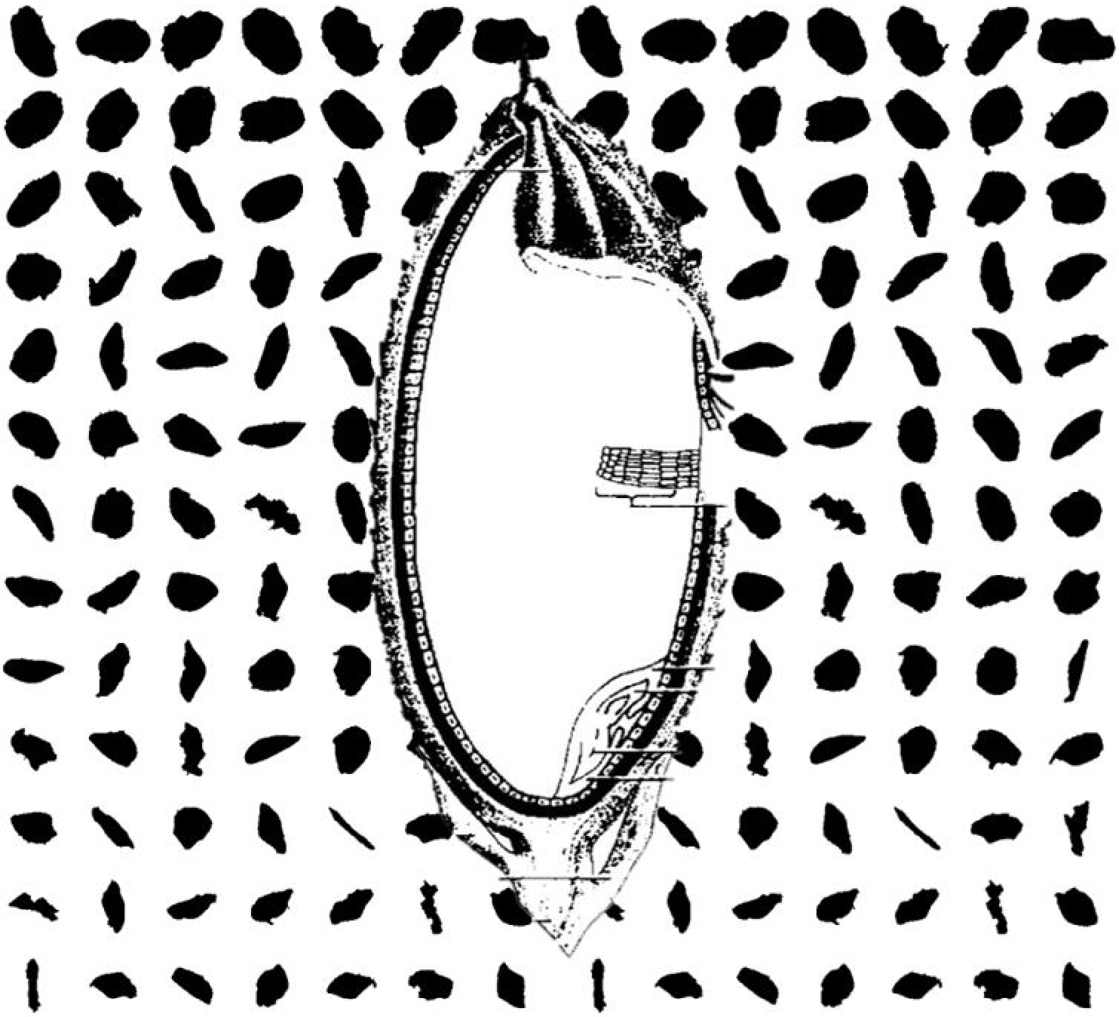

Extraction of Rice Bran Protein

Screening of enzymatic digestion, solubilization using pH shift and mechanical disruption through bead milling

Master's thesis in Biotechnology

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Department of Biology and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2020

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Cover: Background: Unprocessed rice bran particles captured by Image analysis. Foreground: Longitudinal section of rice grain. [Bienvenido O. J., 1993]. Reproduced with permission.

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Abstract

Due to the amino acid composition, protein digestibility and protein functionalities, the abundant side stream rice bran has potential as a plant-based protein source for human nutrition. Valorizing this side stream would reduce food losses in an already existing food processing chain and thereby reduce climate impact. This project aimed to find a protein extraction method suitable for rice bran. Three methods were investigated, separately and in combination: Enzymatic digestion, solubilization using pH shift, and mechanical disruption using bead milling in water phase. The protein yield from different extraction conditions were compared and analyzed. In addition, the protein yields were compared between rice bran with different pretreatments. Defatted, heat stabilized, and unprocessed rice bran were evaluated. It was concluded unprocessed rice bran consistently resulted in the highest protein yields. The best extraction method was mechanical disruption through bead milling in combination with solubilization using pH shift. Using several extraction steps also increased the protein yield. The study confirms previous studies where pH shift and mechanical disruption increased protein yields. Further studies are needed to understand how these treatments affect unprocessed rice bran protein functionalities and taste.

Keywords: Rice bran, protein extraction, bead mill, alkaline, xylanse, acid cellulase, protein function

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Elise Larsson, Zürich, March 2020

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1

Introduction

This Master thesis was performed at Bühler Management AG, in the department Corporate Technology, division Future of Food. The topic and aim of this project were chosen not only for purposes of potential future commercial opportunity, but also to be able to work towards the strategic sustainability goals of a 50% waste, 50% energy, 50% water reduction within Bühler customers' value chains. This Master thesis contributed to this aim through the work of screening for a method of protein extraction from the side stream rice bran. Finding a method for rice bran protein extraction may contribute to valorizing this side stream of the rice milling process, which is otherwise used for animal feed or fuel. This could lead to a more sustainable use of natural resources and protein, and help reduce process induced losses in the production process of white rice by Bühler's customers.

Three types of rice bran were investigated. Defatted rice bran (DRB) was purchased from Green Rich Agro products in India. Heat stabilized rice bran (HSRB) was kindly provided from Herba rice, Spain. Finally, unprocessed rice bran (UPRB) was donated from Reismühle Brunnen, Switzerland.

1.1 Background

Food production and consumption have one of the greatest climate impacts. Alone it stands for nearly 20% of Global Carbon footprint from a lifecycle perspective (Smith et al., 2017). One way to decrease the climate impact from the food sector, is to move from animal to plant based foods for protein. Another method to reduce climate impact from food production is to reduce food losses in already existing food processing chains, through valorization of so-called side streams. This master thesis aims to investigate protein extraction from rice bran- a side product from the whitening process of white rice.

The outer layer of the rice, underneath the hull, is called rice bran. Approximately 337 million metric tons of white rice is processed each year globally for consumption by humans. The side-product rice bran is thereby also produced with a volume of approximately 60 to 68 million metric tons each year (Ryan, 2011). Despite the large volumes produced each year, rice bran is often overlooked as a source of nutrition for humans. Instead it is used as animal feed or fuel. (Fabian and Ju, 2011). Bioactive food components in rice bran may include ferulic acid (Liu et al., 2017), -oryzanol, tocopherols, tocotrienols, polyphenols, carotenoids and micronutrients (eg, magnesium, calcium, phosphorous, manganese, and vitamin B1), all beneficial to the human body (Ryan, 2011). Many studies have also shown the potential of rice bran as a source of protein, (Han, Chee and Cho, 2015). The protein content in rice bran is around 15% and it has a desirable amino acid profile for human consumption (Adebiyi et al., 2009). Rice bran includes the essential amino acids (Ryan, 2011) and have an efficiency ratio, net protein ratio, net protein utilization, and biological value which are comparable to the qualities of animal proteins (Han, Chee and Cho, 2015). The main challenge however, when using rice bran as a protein source for human nutrition is the hydrolytic rancidity and oxidation. Just hours after the bran layers are removed from the endosperm, the fat fraction comes in contact with lipase and lipoxygenase (S Vallabha et al., 2015) which eventually causes off-flavors (Ramezanzadeh et al., 1999). There are strong reasons to believe products from the oxidation reactions affects the proteins and this may hinder protein separation and remove nutritional value of the protein (Zhou et al., 2017), (Lund et al., 2011). Many rice mills use defatting or heat stabilization of their rice bran to avoid off-flavor.

Several protein extraction methods of rice bran have been investigated. Among them alkaline processes, or processes with a combination of solvents (Fabian and Ju, 2011), (Adebiyi et al., 2009), extractions using enzymes (Ansharullah, Hourigan and Chesterman, 1997), (Sozer et al., 2017a) and physical processes (Fabian and Ju, 2011), (Anderson and Guraya, 2001). Research concerning rice bran protein recuperation is not new. Studies of rice bran nutritional qualities, stabilization, and extraction methods have been conducted since the sixties. This project aims at assessing whether novel processing conditions could enable the extraction of rice bran protein.

1.2 Relevant Studies

Numerous studies have been published about rice bran protein extraction. This section will summarize and describe the studies which have served as the main inspiration for this Master thesis. However these studies and also additional ones will be described more in depth in the theory section of this thesis. Valuable ideas and knowledge on how to build up the method section of this master thesis came from the work by Anderson and Guraya (Anderson and Guraya, 2001). This publication describes how the physical processes of colloid milling and homogenization affect rice bran protein extraction. It also compares the protein yields from DRB, HSRB and UPRB. In addition, an SDS-page study describes the differences of the proteins obtained from the different kinds of rice bran. This section served as inspiration for the theory part of this thesis.

Other important information on how to build up the enzymatic digestion experimental protocol came from (Wang et al., 1999) who used xylanase and phytase with DRB for protein extraction. It also describes protein properties and potential applications useful for the outlook section of this thesis. Another study (Adebiyi et al., 2009) optimized protein extraction yield by a combination of solvents. The

study helped set expectations in terms of what is possible in protein extraction and provided extensive information about the different groups of rice bran protein. The study by (Sozer et al., 2017) describes the benefits, potential, and applications of rice bran protein. In addition, an overview of existing extraction processes is described together with advantages and disadvantages of each method. This information was valuable both for developing the method and for the discussion.
Finally, the patent filed by (Hammond, 1994), describes in detail a method of protein extraction and separation developed to be used in industry. Detailed explanations of methods, flow charts, and examples of potential end products made this patent a very valuable inspiration.

2

Aim and Objectives

2.1 Project Aim

The aim of this project is to examine and screen different methods of protein extraction from three kinds of rice bran: DRB (defatted rice bran), HSRB (heat stabilized rice bran) and UPRB (unprocessed rice bran).

2.2 Objectives

As a by-product from white rice production, rice bran is produced in immense amount each year globally. In addition, rice bran is a nutritious fraction of rice suitable for human consumption not the least due to its high protein content. Owing to the fact of a complex cell matrix and the quick evolvement of off-flavor and rancidity, extracting protein from rice bran without damaging the proteins and with acceptable sensory performance is challenging. This project aims at screening and investigating three protein extraction methods on three types of rice bran. After a high protein extraction yield is achieved, the advantages and disadvantages with the best methods are discussed. This project contributes to increase the knowledge about the three chosen methods of rice bran protein extraction: mechanical disruption through bead milling, solubilization using pH shift, and enzymatic digestion. In addition, these three methods are applied to three types of rice bran: DRB, HSRB and UPRB. To examine the impact of mechanical disruption, protein extraction rates from milled rice bran were studied. Then enzymatic digestion or a pH shift was added and their respective protein extraction rates were compared. Finally, the best results from the screening of mechanical disruption, pH shifted solubilization and enzymatic digestion were optimized to achieve the highest protein extraction rates possible in the scope of this project.

3

Theory

The Theory section of this Master thesis gives the necessary theoretical background to understand rice bran protein extraction and the methods used for sample analysis. First, the structure of the rice kernel, rice bran and rice bran protein are described. Then, the methods of enzymatic digestion, solubilization using pH shift, and mechanical bead milling are presented. Finally, the functionalities of rice bran protein are discussed.

3.1 Rice and rice bran structure

Paddy rice (the whole rice grain), illustrated Figure 3.1 is constituted out of the outer layers hull and bran, and the inner parts endosperm and germ.

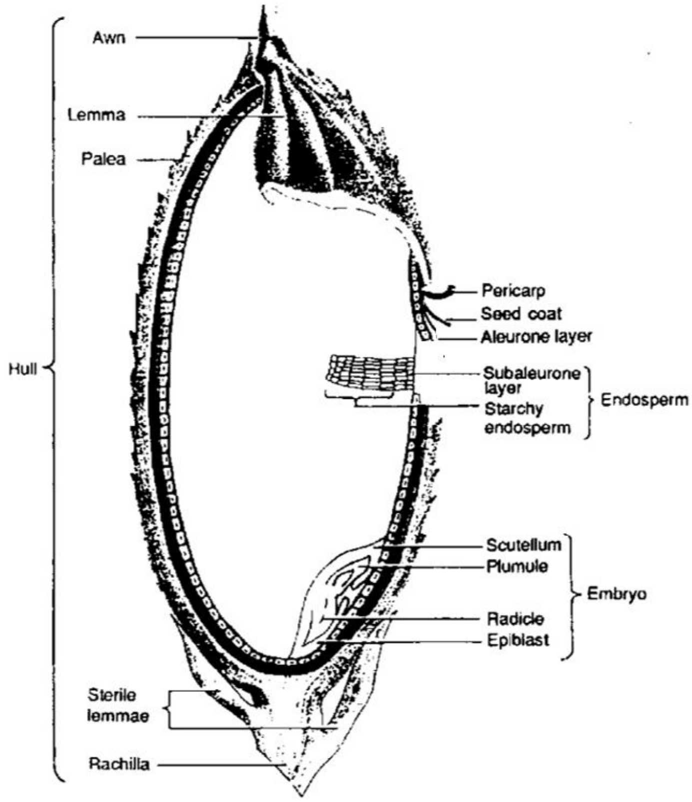


Figure 3.1: Longitudinal section of rice grain. Reproduced with permission by Food and Agriculture Organization of the United Nations (Bienvenido O. J., 1993).

The hull is made up of palea and lemma. Hull, or husk, is a fibrous protective layer, difficult to digest for humans. Nevertheless, it is useful as an energy source for fuel. White rice is constituted out of the endosperm part of the rice kernel. The outer layer is the subaleurone layer which contains a high amount of protein bodies. (Nadathur, Wanasundara, & Scanlin, 2017). The major carbohydrase in the starchy endosperm is, as the name suggests, starch (Shih et al., 1999). The amount of protein, minerals and vitamins are low. However, with the high level of carbohydrase it is an important staple food for a large part of the world. Germ is the second part of the endosperm fraction of the rice paddy. It is situated at the bottom center of the rice kernel and is made up out of plumule, scutellum and radicle.

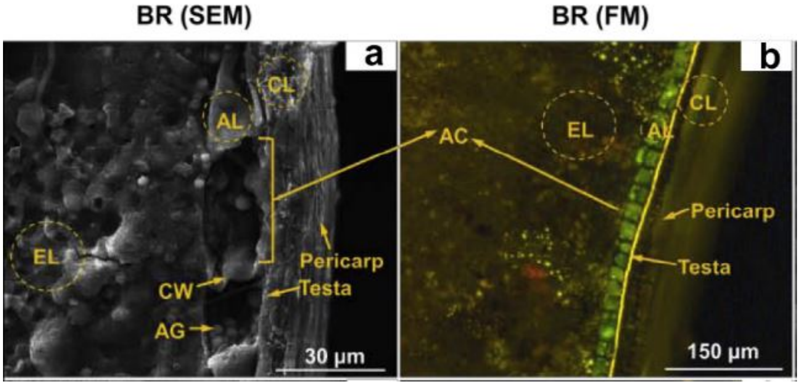


Figure 3.2: Used with permission by (Wu et al., 2016). SEM (Scanning electron microscopy) and FM (Fluorescence microscopy) micrographs of BR (Brown Rice). Cuticular layer (CL), aleurone layer (AL) and endosperm layer (EL), all circled in the image. Aleurone grain (AG), aleurone cell (AC) and cell wall (CW) are shown with arrows. The different layers of brown rice (BR) are stained to differentiate the cuticular layer (CL), aleurone layer (AL) and endosperm layer (EL). In the Fluorescence microscopy image, May Grunwald's reagent was used as a dye. The dye consists of equal amount eosin Y and methylene blue dissolved in methyl alcohol (Wu et al., 2016).

The bran fraction is made of pericarp, seed coat/testa, and aleurone layers. SEM (scanning electron microscopy) and FM (fluorescence microscopy) micrographs of rice bran are shown in Figure 3.2. The two outer, closely appressed, layers pericarp and seed coat together constitute the cuticular layer (Wu et al., 2016). Pericarp is the fibrous outermost layer and contains pigmets in coloried rice (Nadathur, Wanasundara, & Scanlin, 2017). It is dense and free from pinhole porosity. The seed coat underneath is rich in lipids (Wu et al., 2016). The seed coat is also less fibrous and rich in protein. The bottom layer is the aleurone layer which contains the highest amount of protein in the bran (Nadathur, Wanasundara, & Scanlin, 2017). The aleurone cells are loosely packed with scattered aleurone grains, lipid bodies and intracellular voids (Wu et al., 2016). The main carbohydrates in the bran fraction are cellulose and hemicellulose (Shih et al., 1999).

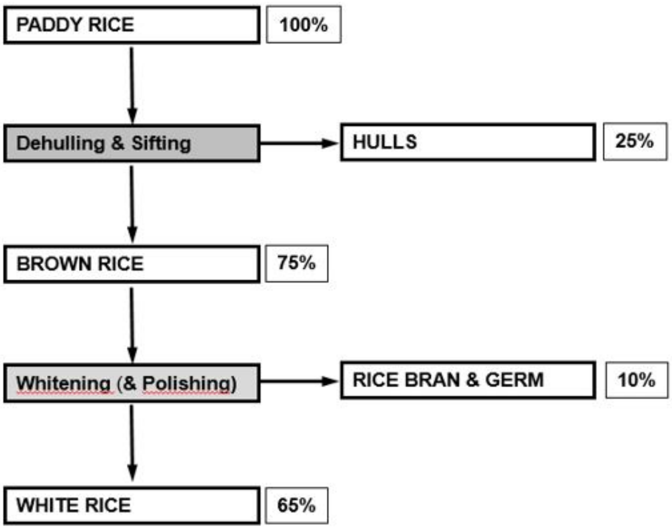


Figure 3.3: Flow chart of white rice production. The gray boxes represent the processing steps and the white boxes represent the parts of the paddy rice that is being processed. All percentages are ratios of the original paddy rice. Statistics from (Nadathur, Wanasundara, & Scanlin, 2017).

The paddy rice kernel goes through several processing steps before becoming the final product white rice. As visualized in a flow chart in Figure 3.3, brown rice is created from paddy rice after dehulling and sifting. This produces the by-product husk. White rice and the by-product rice bran, are produced after one or several whitening steps. Sometimes also a polishing step is added to have an even surface on the white rice kernels. The by-product produced by the whitening machine is mainly the bran fraction of rice. However, according to the suppliers of rice bran for this project, the rice germ is in most cases also extracted in the first or second pass in whitening machine of rice, and therefore is a part of what we call the rice bran by-product.

Important to note is that the whitening process of the brown rice greatly affects the quality of the rice bran produced and the protein fraction in the product. The degree of milling, or the number of passes through the mill the brown rice are put through, determines how much of the subaleurone layer and the starchy endosperm that will be included in the rice bran by-product. (Nadathur, Wanasundara, & Scanlin, 2017). Schramm et al. showed how the outer layer of the rice bran contains a higher amount of protein than the inner layer of rice bran (Schramm et al., 2007). This could be due to the higher amount of starchy endosperm in the latter fraction, which contains less protein. If a polishing step is added to the process, this too will affect the by-product rice bran as more starchy endosperm might end up in the rice bran fraction. UPRB was put through three passes in the whitening machine, according to information given by Reismühle Brunnen. Herba rice reported that the HSRB received was put through two passes in the whitening machine and one pass through a polishing machine. The supplier for DRB, Green Rich Agro Product, did not give out information regarding number of passes through the whitening machine.

3.2 Rice bran protein

In order to obtain a protein isolate from rice bran there are two main steps involved: extraction of protein and separation of protein. For the extraction step it is important to have extensive knowledge about protein solubility and for the separation step it is vital to have knowledge about protein sizes. This Master thesis focuses on the extraction step and therefore this section will focus on protein properties influencing extraction yields. Rice bran mainly contains storage proteins. A summary of properties of storage proteins are presented in Table 3.1. The proteins are divided into different groups based on their solubility in various solutions, according to the Osbourne solubility classification.

Table 3.1: The fractions of the four kinds of proteins in rice bran, their size, their solubility, and nutritional qualities. The mixture of different solubility makes for a challenging protein extraction process. (Fabian and Ju, 2011), (Nadathur, Wanasundara, & Scanlin, 2017).

Protein	**Albumin**	**Globulin**	**Glutelin**	**Prolamin**
Fraction in rice bran	34%	15-26%	11-27%	4%
Solubility	Water-soluble. Possess a lower amount of disulfide crosslinkings	Salt-soluble. Often co-extracted with albumin due to the presence of salts in the plant material	Alkali-soluble. Extensive aggregation, disulfide bonding and glycosylation contributes to a difficult extraction and solubilization	Alcohol-soluble. 60-70% ethanol needed for solubilization
Size	10 to 200 kDa. 60-kDa glycoprotein as one of the major constituents	25 kDa and 16 kDa (polypeptides stabilized by interchain disulfide bonds)	45 to 150 kDa	12–17 kDa
Nutrition	Readily digestible and absorbable. Exhibits antioxidant activity	Moderately high amounts of cysteine and methionine	Some glutelin types are rich in lysine	High levels of glutamic acid or glutamine, alanine, glycine, and arginine

3.3 Extraction by enzymatic digestion: xylanase and acid cellulase

Use of enzymatic digestion for extraction of cell wall plant proteins have been widely investigated. Mainly, the two enzyme groups proteases and carbohydrases have been tested for extraction of rice bran protein with varying results. Proteases produce smaller proteins and peptides that are more soluble in water than the original larger molecules (Hamada, 1999). The use of proteases have shown good results in previous studies (Fabian and Ju, 2011). However, by hydrolyzing proteins the functionality of the proteins change. According to the patent filed by Janse A., there is a risk the high degree of hydrolysis produced by proteases lead to a bitter tasting end product. A degree of hydrolysis below 16% is preferable (Janse A., 2014). Because of these reasons, proteases were not used in this study.

The enzyme group carbohydrases disintegrate the cell wall tissue and is therefore useful in extracting protein. Wang et al. studied rice bran protein extraction with help of xylanse and phytase (55ºC, pH 5, incubation 2h). With phytase the extraction rate was 57% and with xylanse 56%. With both enzymes combined an extraction rate of 74.6% was achieved (Wang et al., 1999). In a study by Anscharullah et al., a 57.9% protein yield was achieved using the enzyme Viscozyme L (50ºC, pH 3.8, incubation 5h). Viscozyme L is a mixture of different carbohydrases including arabanase, cellulase, hemicellulase and xylanase. The ability to cleave a wide range of linkages proved to be successful when extracting protein. (Ansharullah, Hourigan and Chesterman, 1997). Given that the major carbohydrates in rice bran are cellulose and hemicellulose, the enzymes acid cellulase and xylanase were chosen for this study. By hydrolyzing cellulose and hemi-cellulose they should be effective in releasing bound protein (Shih et al., 1999). Another reason for the choice are the good results achieved by cellulase and xylanase in literature, and that they each affect different compounds in the cell matrix. The amount of enzyme should be in accordance to the recommendations of the enzyme supplier. If not provided an estimation guide is 0.1-5% of the rice bran by weight (Lane, H. R., 2001).

3.4 pH shift facilitated protein solubilization

Alkaline conditions are the most common method used for rice bran protein extraction and the reason is the high protein yields achieved (30-80% at pH 7-12) (Fabian and Ju, 2011). When using NaOH to reach a higher pH the solubility of the proteins are increased by the breakage of hydrogen, amide and disulfide bonds in the proteins (Sun, Lv and He, 2017). However, research has shown alkaline conditions also lead to unwanted effects. High pH could lead to increased Maillard reaction which causes dark colored products (Wang et al., 1999). It could also lead to denaturation of protein (Sun, Lv and He, 2017). In addition, an increase in pH is favorable for lysinoalanine formation through intermolecular cross-coupling and rearrangement of proteins. Proteins containing lysinoalanine have been proved to be toxic for rats (Karayiannis wt al., 1980). The effect on humans is still unknown. However, the potential risk calls for caution. Lysinoalanine also leads to a decrease in protein digestibility. (Ayim et al., 2018). Finally, alkaline conditions also increase solubility of other components than protein, components which might coprecipitate with protein in the final product and thereby reduce protein functionality and nutrition. (Wang et al., 1999) Using alkaline conditions for improved solubility does indeed come with issues, however by combining a smaller increase in pH and a physical process of the rice bran, a high extraction yield can still be achieved. A study conducted by Sun Lv and He, compared protein extraction yields between only using alkaline conditions (48.96%), using ultrasonic-assisted alkali method (57.89%) and using homogenization-assisted alkali method (52.83%). These results show how physical processes in combination with a lower alkaline treatment can have a positive impact on protein extraction yields (Sun, Lv and He, 2017). In this study, pH shifts were used to increase solubilization of rice bran protein both with and without physical processes.

3.5 Extraction by mechanical disruption: bead milling in water phase

Physical or mechanical methods for cell wall disruption have been proved to increase protein yields (Fabian and Ju, 2011). It is an economic and easily operated method of extracting rice bran protein and therefore these methods are of high interest to conduct research on and improve (Sun, Lv and He, 2017). Anderson and Guraya used colloid milling with 10% dry matter and 30 minutes of milling time for rice bran, as an aid to increase protein extraction yields. The protein content of the supernatant fraction of UPRB increased from 21.8 to 33.0% after colloid milling. After using homogenization as an additional treatment the yield increased to 38.2%. This study proves that mechanical disruption does have an impact on rice bran protein extraction yields (Anderson and Guraya, 2001). In the study by Rosa-Sibakov et al., wet milling was also used to increase solubility of proteins. Wheat bran was put through a colloid mill in seven passes at 5% dry matter content. Nonetheless, the treatment did not succeed in increasing the solubility of wheat bran protein (Rosa-Sibakov et al., 2015). One reason could be the elevated temperatures used to inactivate the enzymes in the protocol. Another reason is the difference in structure between wheat bran and rice bran.

Another method of wet grinding is bead milling. Bead mills are traditionally used for the manufacturing of paints/lacquers and grinding of minerals. The application of bead milling has in recent years expanded to also include grinding of biologic materials such as yeast, cyanobacterium and microalgae (Postma et al., 2017). By reducing the particle sizes of these materials, proteins can be released. In the patent by Lane, their enzymatic digestion protocol is performed on food grade milled rice bran with the particle sizes of 0.1-50 microns. It is stated that the most preferable particle sizes are between 5-10 microns. It is also suggested in this patent to mix one part rice bran with ten parts water (Lane H. Robert, 2001). The advantages of using bead milling for particle size reduction are the easy operation and mild manner of treating the biologic material. Several parameters can be changed to reach the highest efficiency of cell matrix disintegration and protein release. Firstly the water/dry matter ratio can be adjusted to an optimum based on both scientific factors and economic profit. The process parameters agitator speed, material throughput, bead diameter, bead filling ratio and bead type are important to choose in accordance to the particle size distribution needed. Lastly, the time spend in the mill and the choice between recirculation or single/multiple pass settings are important to decide upon (Postma et al., 2017). In this thesis we have defined the ratio of parameters Specific Energy (kWh/t) as a function of pressure (p), material throughput (m), and time (t). Specific energy is used to differentiate between bead milling settings and is calculated with Equation 3.1.

$$\Sigma E = \frac{p - p_0}{\mathrm{m}} \cdot t \tag{3.1}$$

Another ratio of parameters defined in this Master thesis is the theoretical number of passes through the bead mill. Theoretical number of passes in recirculation mode is calculated by Equation 3.2 using material throughput (m), time (t), and mass of the material (M). Longer milling time in recirculation mode leads to a higher amount of theoretical passes in the bead mill. This leads to the fact that a higher percentage of particles in the samples are milled, which in turn leads to a more narrow PSD (particle size distribution).

$$\text{Theoretical passes through bead mill} = \frac{m \cdot t}{\mathrm{M}} \tag{3.2}$$

3.6 Functionality of rice bran protein

Functionalities of rice bran protein have been studied in several publications and give an indication of potential future food applications of rice bran protein concentrates and isolates. The value of protein can be divided into the categories shown in Table 3.2: Nutrition, taste, color and functional properties. All are of great importance to determine if protein can be used in food. Furthermore, functional properties are essential to estimate economic value. Therefore, a more in depth review of six functionalities can be found in Table 3.3.

Table 3.2: Values of protein divided into categories of nutrition, taste, color and functional properties. The results of rice bran protein in the Taste category are derived from studies where wheat flour have been exchanged for rice bran protein concentrates in baked goods. These results does not represent the taste of all potential rice bran food products

Protein value	**Rice bran protein result**	**Source**
Nutrition	Amino acid profile good for 2- to 5 year old children, similar to casein and soy isolate protein. Protein digestibility greater than 90%. Hypoallergenic. Anti-cancer activity detected.	(Wang et al., 2014) (Nadathur, Wanasundara, & Scanlin, 2017)
Taste	Over 10% substitution rate in biscuits affected sensory quality adversely. Over 10% substitution rate in bread affected sensory quality adversely.	(Chinma et al., 2015) (Yadav, Yadav and Chaudhary, 2011)
Color	Brown. Maillard reaction leads to darker color (increased by heat-treatment or alkaline conditions).	(Wang et al., 1999)
Functional properties	See Table 3.3	See Table 3.3

Rice bran has good results in all of the categories In Table 3.2 and is therefore interesting as a potential future food ingredient. In particular, the results for Nutrition are promising. For Taste however, it is difficult to predict how it will affect every food product. Other ingredients in the product as well as the protein extraction method used, have a big impact. When hydrolyzing proteins with proteases for extraction, more bitter flavors are created than for less hydrolyzed rice bran protein (Janse, A, 2014). On the other hand it has been shown that enzymatic hydrolysis enhances the antioxidative activity of rice bran proteins. Albumin and glutelin peptides, hydrolyzed by pepsin, has higher antioxidative activity compared with the hydrolysates of globulin and prolamin (Adebiyi et al., 2009).

Table 3.3: Functional properties of rice bran protein. All results are shown as a comparison to standard benchmarks. The benchmarks are chosen because they are commonly used as benchmarks due to their high values in their respective functional property.

Functional property	**Rice bran protein result**	**Food system examples**	**Sources**
Emulsifying	High, but lower than bovine serum albumin. Stable results with varying pH, salt and sugar concentrations.	Comminuted meats, cakes, soups, salad dressings, non-dairy milk, desserts.	(Wang et al., 1999a). (Chandi and Sogi, 2007) (Park, Hyun-Jun et al, 2009). (Nadathur, Wanasundara, & Scanlin, 2017)
Foaming	High, similar to egg white albumin.	Ice cream, cakes, whipped toppings, mousses, desserts.	(Wang et al., 1999a). (Chandi and Sogi, 2007) (Park, Hyun-Jun et al, 2009). (Nadathur, Wanasundara, & Scanlin, 2017)
Water binding capacity	High in comparison to casein.	Comminuted meats, low fat meat products, bakery products	(Chandi and Sogi, 2007) (Nadathur, Wanasundara, & Scanlin, 2017)
Oil binding capacity	High in comparison to casein.	Sausages, cake batters, mayonnaise and salad dressings	(Chandi and Sogi, 2007)
Bulk density	Low bulk density compared to casein and tomato seed protein.	Packaging of food product. Low bulk density is required for the formulation of weaning foods.	(Chandi and Sogi, 2007)
Nitrogen solubility	High at higher pH. Comparable to soy bean protein isolate. Around 80% at pH 10 obtained.	Influence other properties such as emulsifying and foaming.	(Fabian and Ju, 2011) (Zhang et al., 2012) (Wang et al., 1999)

All results shown in Table 3.2 and Table 3.3 are promising for the chosen functionalities of rice bran protein concentrates and isolates. Nevertheless, the varying properties of the rice bran concentrates, as well as the different pre-treatments of the rice bran, makes for a difficult definite conclusion as to what functional properties rice bran protein extracted in this Master thesis would have. Several researchers have studied differences in functional properties of rice bran protein concentrates with different extraction methods. An interesting conclusion is discussed by Silventoinen et al. They showed how finer fractions of rice bran produced by dry fractionation influenced functionalities of rice bran protein. Fine fractions showed improved protein solubility and colloidal stability (Silventoinen et al., 2019). Another interesting study about functionality differences, is the study by Zhang et al. They compared the functional properties of alkaline (pH 10) and enzyme (alcalase) extracted heat stabilized and defatted rice bran protein. The nitrogen solubility, emulsifying properties, foaming capacity, foaming stability, and water and oil absorption capacity were all higher for enzyme extracted rice bran protein than for alkaline extracted rice bran protein. Zhang et al, stated that their results show that rice bran protein has potential as a nutraceutical ingredient in food applications (Zhang et al., 2012).

4

Method and Material

This section will describe all protocols used to achieve the Aim and Objectives of this Master thesis. First, some information about the raw material and enzymes will be provided. Then all protocols used for the laboratory work will be described. Table 4.1 summarizes the protocols used for all three types of rice bran.

Table 4.1: All protocols used and for which type of rice bran: DRB (defatted rice bran), HSRB (heat stabilized rice bran), UPRB (unprocessed rice bran), UM (unmilled), CM (coarsely milled) and FM (finely milled). “Untreated” indicates that the protein extraction yield was tested on a rice bran ratio of 10:90 (bran/H2O mixture, w/v) without additional treatment. ”Enzyme: xylanase” and “Enzyme: acid cellulase” mean that an enzyme was added to increase protein yields. “pH screening” means several pH values were tested and “pH 10” that pH 10 was tested. “Higher solids content” indicates that a rice bran ratio of 20:80 (bran/H2O mixture, w/v) was used for the testing.

Type of rice bran	DRB		HSRB			UPRB		
Milling setting	UM	CM	UM	CM	FM	UM	CM	FM
Untreated	✓	✓	✓	✓	✓	✓	✓	✓
Enzyme: xylanse	✓	✓	✓	✓		✓	✓	
Enzyme: acid cellulase	✓	✓	✓	✓		✓	✓	
pH screening			✓	✓				
pH 10	✓	✓	✓	✓		✓	✓	✓
Second centrifugation		✓		✓			✓	
Second centrifugation, pH 10							✓	✓
Higher solids content								✓

4.1 Raw material and enzymes

Rice bran was provided from three different sources. DRB was purchased from Green Rich Agro products in India. HSRB was provided from Herba rice, Spain. Finally, UPRB was donated from Reismühle Brunnen, Switzerland. The protein contents of each raw material were measured with the Dumas method with Truespec N nitrogen analyser (Leco corporation, St: Joseph, USA) at the Bühler lab. Thereafter, the moisture content was measured with the TGA701 oven (LECO corporation, St: Joseph, USA) The enzymes xylanase and acid cellulase were purchased from CapriEnzymes, India. Also the protein content of the enzymes were measured with the

Dumas method. The moisture content was determined with the help of a Halogen dryer HG63 (Mettler Toledo, Greifensee, Switzerland). The supplier Caprienzymes provided information about temperature and pH range of operation for each enzyme. Acid cellulase operational pH range was pH 3.5-5.5, while the optimal range was pH 4-5. The operational temperature ranged from 40 – 70° C, and the optimum range was 50 – 65° C. For xylanse the activity temperature was 30-75° C, and operational pH range was 3.0-7.0. No optimum ranges were provided for xylanse. Based on this information the protocols for enzymatic digestion of the rice bran were prepared.

4.2 Particle size determination

All three types of rice bran and all milled rice bran/water products were measured with a laser scattering particle size distribution analyzer LA-950V2 (Horiba, Japan), to determine the PSD (particle size distribution). The PSD of the unmilled rice bran was measured by mixing approximately 3 g rice bran with distilled water in the measurement chamber. The PSD of the milled material was determined by pipetting approximately 30 drops of the rice bran/water mix in the measurement chamber with distilled water. The principle behind laser scattering is the correlation between particle size and the angle and intensity of scattered light. As shown in Figure 4.1, the light aimed at a sphere can either be reflected, refracted, absorbed and re-radiated, or diffracted. To measure the particle size of a sphere the focus is to measure the diffracted light since light is diffracted more strongly and at a smaller angle from a large particle than from a small one. An algorithm uses the data of scattered light and transforms it to information about the particle size.

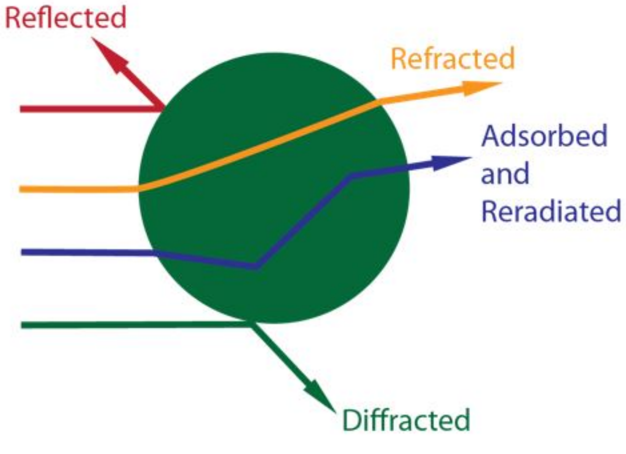


Figure 4.1: Light aimed at a spherical particle. Used with permission from HORIBA Instruments Incorporated.

Important to consider is that all rice bran particles are approximated as spheres, which in reality is not the case as rice bran particles have different compositions and shapes ("Laser Diffraction for Particle Size Analysis - HORIBA", 2020).
In addition, the PSD of the three untreated types of rice bran were measured with Image analysis for comparison. QICPIC RODOS/LVIBRI/L (Sympatec GmbH,

Clausthal-Zellerfeld, Germany) was used to determine the PSD as well as to generate images of the rice bran particles. To determine the PSD, QICPIC uses a pulsed light source with nanosecond range times. The particles are then captured with a high speed camera with a frequency of 450 frames per second. The set-up is shown in Figure 4.2. In contrast to laser diffraction, QICPIC's dynamic image analysis does not only deliver the PSD, various other particle parameters can be obtained as well, such as circumference, sphericity, aspect ratio, convexity and various diameters. ("QICPIC", 2020).

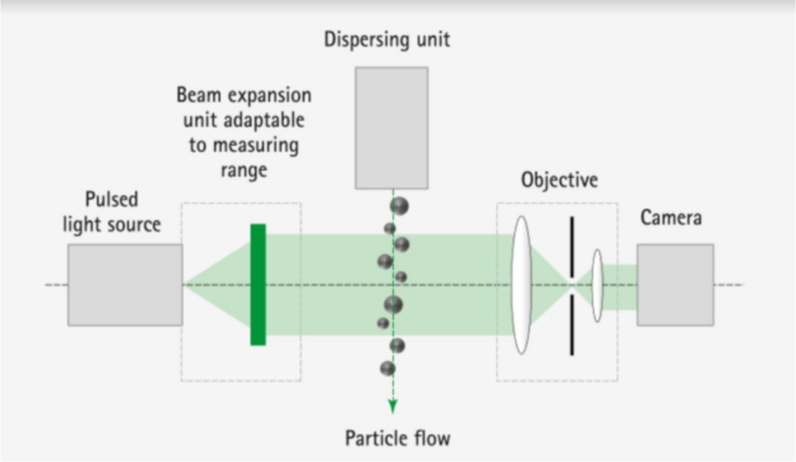


Figure 4.2: Set-up of QicPic machinery. The dispersing unit is composed of RODOS and VIBRI units to properly disperse the particles. The camera, objective and pulsed light source captures the images. The beam expansion unit makes QICPC more adaptable to different measurement ranges. Used with permission from Sympatec GmbH.

When visualizing PSD the D-value parameters D10, D50 and D90 were used. The D10 value corresponds to the diameter value above which 90% of the particle diameters are higher and 10% are lower. The D50 is the median diameter, meaning that 50% of the particles are smaller than the given value and 50% of the particles are larger. Finally the D90 value is the diameter of which 90% of the diameters are lower and 10% are higher.

4.3 Mechanical disruption through bead milling

Rice bran with a ratio of 10:90 (bran/H2O mixture, w/v) was put in a vessel heated to 45°C before recirculating in the laboratory bead mill PLM2-Centex S2 (Bühler, Uzwil, Switzerland) for a set time. All three types of rice bran were sieved at below d=2mm before milling. The water was first heated up in the vessel before the rice bran was slowly poured in the vessel during 4 minutes. The rice bran was suspended and continuously agitated by stirring before being pumped in the treatment chamber. A screening of different times of milling (10, 20, 30, 40 and 50 min) was performed and two times were chosen for further experiments based on their particle size distributions.

The coarsely milled material of DRB, HSRB and UPRB was milled for 20 minutes with a bead size of 2.5 mm. The finely milled material of DRB, HSRB and UPRB was milled for 40 min with a bead size of 2.0 mm. For all trials the bead milling chamber was filled to 80% with beads, had a rotor tip speed of 10m/s. The throughput started at 50kg/h and was then slowly increased to 70kg/h with time. The milling was done in re-circulation.

One protocol was performed with a rice/bran water mix with pH 10. For this protocol the rice bran and water was mixed in a 5 l beaker and NaOH (5M) was added during stirring until pH 10 was reached. The pH was measured with a pH-meter. Before measuring the pH meter was calibrated with buffer solution of pH 7.0 and pH 4.0. When the required pH was reached the water fraction of the mix was transferred to the heated vessel. Once the water reached 45 °C, the soaked fraction of rice bran from the NaOH-treated mix was added to the water in the vessel and milled. One protocol used rice bran with a ratio of 20:90 (bran/H2O mixture, w/v) to have a more viscous material with higher protein content/liter. Otherwise, everything was performed exactly as with the finely milled rice bran with a ratio of 10% rice bran.

4.4 Enzymatic digestion

Enzymatic digestion of unmilled and coarsely milled rice bran with a ratio of 10:90 (bran/H2O mixture, w/v) and an enzyme dosage (ED) of 0.04g/g bran was performed. For the samples with acid cellulase, HCL (1 M) was added to the rice bran/water mix and adjusted with the help of a pH meter to a pH of 5.0. For the samples containing both xylanase and acid cellulase the pH was changed to 5.0 as well. However, the pH was unchanged for the samples with only xylanase. Before measuring the pH meter was calibrated with buffer solution of pH 7.0 and pH 4.0. All samples were incubated at 50°C for 4h with stirring at 640 rpm. For each trial, a control without the addition of the enzymes was performed. Controls included only the rice bran/water mix at a ratio of 10:90 (bran/H2O mixture, w/v).

4.5 Solubilization using pH shift

Incubation with different pH (pH 5, 7, 8, 9 and 10) was performed with unmilled, coarsely milled, and finely milled rice bran with a ratio of 10:90 (bran/H2O mixture, w/v). For which types of rice bran different pH treatments was performed, see Table 4.1. HCL (1 M) or NaOH (1M) was added to the rice bran/water mix and adjusted with the help of a pH meter to the required pH. Before measuring the pH meter was calibrated with buffer solution of pH 7.0 and pH 4.0. The samples were incubated at room temperature for 1h with stirring at 640 rpm. For each trial, a control without change of pH was performed. Controls included only the rice bran/water mix at a ratio of 10:90 (bran/H2O mixture, w/v).

4.6 Protein quantity and dry matter ratio determination

Rice bran/water samples of 50 g each were centrifuged at 3500 x G for 20 min in the Allegra X15R swing bucket rotor centrifuge (Beckman Coulter Inc, Brea, USA). The supernatant was carefully poured in a beaker and weighed. Then 3.5 ml was pipetted into an aluminum foil container to measure the dry matter ratio and later the protein quantity with the Dumas method in the Truespec N nitrogen analyzer (Leco corporation, St: Joseph, USA).

There are two main methods used for crude protein analysis: The Kjeldahl and Dumas methods. In this Master thesis the Dumas method was chosen for all protein analyses. The Dumas method measures both organic nitrogen and inorganic fractions like nitrite and nitrate while the Kjeldahl method detects only organic nitrogen and ammonia. Therefore, when comparing crude protein analysis results from the two methods, the results could be slightly different. Dumas is an automated, faster and safer method than Kjeldahl, and it is becoming common as a reference method as well as Kjeldahl. The Dumas method uses combustion of the material of interest and determines the nitrogen content by converting it to N2 in high temperatures in an oxygen atmosphere. Other gas products are trapped or separated from the N2. By using a thermal conductivity detector and a protein conversion factor, the fraction of crude protein in the sample can be calculated. The Dumas (and Kjeldahl) method do not give a measure of true protein, as it registers non-protein nitrogen, and different correction factors are needed for different proteins because they have different amino acid sequences (Müller, 2017). For rice bran, the conversion factor used is 5.95 (Tang et al., 2003). For enzymes, the standard protein conversion factor 6.25 was used (Müller, 2017).

All samples were measured in two or three replicates and dried for 4h in a convection oven at 105ºC. After the drying the aluminum foil containers with sample were weighed again and the dry matter ratio was calculated. Finally, the aluminum was folded and shaped like a ball before being put in the Dumas nitrogen analyzer to determine the protein ratio of the dried sample. Two protocols included a second centrifugation step with the same settings. First, the first supernatant was poured into a beaker. Then water or a solution with pH 10 was added to the pellet in the tube and mixed thoroughly with a stirrer. After centrifugation, the second supernatant was poured into a beaker and analyzed for dry matter ratio and protein quantity.

Total protein extraction yields are calculated with the Equation 4.1. Protein extraction yields from enzyme trials are calculated using Equation 4.2.

$$\text{Protein yield (\%)} = \frac{\text{Total protein of supernatant}}{\text{Total protein of rice bran}} \text{x } 100\% \tag{4.1}$$

$$\text{Protein yield (\%)} = \frac{\text{Total protein of supernatant}}{\text{Total protein of rice bran + Total protein of enzyme}} \text{x } 100\% \tag{4.2}$$

For all experiments, paired and two-tailed t-tests with a p-value of 0.05 were performed using Microsoft Excel. This simple statistical analysis was done in order to determine the significance of the differences in protein yield between two experimental conditions.

5

Results and Discussion

In the Results and Discussion section of this Master thesis all results from previously described protocols are presented and discussed. The first part contains results from initial trials performed to attain background knowledge needed before the protein extraction trials. Here, the particle size and protein ratio of the raw materials (DRB, HSRB and UPRB) are discussed. In addition, the results from the screening of bead milling settings with HSRB are presented further.

The second part of the Results and Discussion section discusses how different extraction and solubilization treatments influence protein extraction yields for the three types of rice bran. First, the impact of mechanical disruption through bead milling and enzymatic digestion are analyzed. The results are compared between DRB, HSRB and UPRB. Secondly, the results from the solubilization method using pH shift are discussed for HSRB, and then for all three types of rice bran. The best protocols for protein extraction are then reproduced and investigated closer to compare the impact of PSD and pre-treatment of rice bran on protein yield. Finally, the impact of an extra step of centrifugation and higher ratio of rice bran in the bead milling step are discussed.

5.1 Raw material: Protein content and particle size

Protein and moisture quantity analyses were performed for all three types of untreated rice bran to be able to calculate and understand protein yield values of the final rice bran products after extraction. The results are shown in Table 5.1. All measurements were done in triplicates.

Table 5.1: Protein and moisture content with standard deviations of DRB (defatted rice bran), HSRB (heat stabilized rice bran) and UPRB (unprocessed rice bran).

Rice bran type	**Protein ratio (wet based)**	**Moisture ratio**
DRB (%)	17.0 +/- 0.68	12.0 +/- 0.14
HSRB (%)	13.3 +/- 0.04	8.4 +/- 0.02
UPRB (%)	13.1 +/- 0.06	9.4 +/- 0.01

The rice bran was sieved below d=2mm before any extraction and solubilization treatment. The sieving was done in order to not clog the bead mill and for all trials to be comparable. Results are shown in Table 5.2.

Table 5.2: Ratios of rice bran particles above and below d=2mm for DRB (defatted rice bran), HSRB (heat stabilized rice bran) and UPRB (unprocessed rice bran).

Rice bran type	Ratio of d<2mm	Ratio of d>2mm
DRB (%)	78	22
HSRB (%)	95	5
UPRB (%)	98.6	1.4

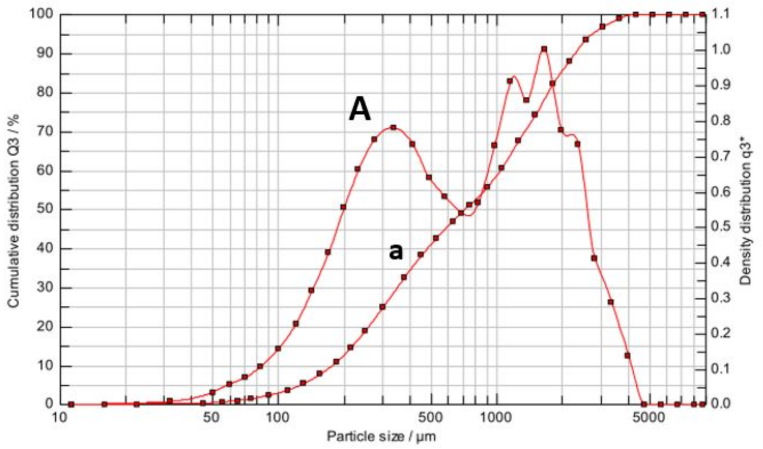
Figure 5.1: a) DRB (defatted rice bran), (b) HSRB (heat stabilized rice bran) and (c) UPRB (unprocessed rice bran). All are divided into fractions above d=2mm (left) and below d=2mm (right).

Figure 5.1 (a, b, c) depicts images of the fractions of rice bran above and below d=2mm. The fraction DRB above d=2mm is with high likelihood pellets processed for animal feed (a). The same fraction of HSRB is probably starch particles from broken rice kernels and rice bran particles clogged together due to heat treatment (b). The larger rice bran fraction of UPRB is broken rice kernels, as can be observed in (c).

Table 5.3: Difference in PSD (particle size distribution) between sieved, untreated DRB (defatted rice bran), HSRB (heat stabilized rice bran) and UPRB (unprocessed rice bran). The differences in D-values between image analysis and laser scattering are also presented.

Rice bran type	DRB		HSRB		UPRB	
Method	Image analysis	Laser scattering	Image analysis	Laser scattering	Image analysis	Laser scattering
D10 (μm)	176.5	92.6+/-6.8	221.5	13.3+/-3.3	113.3	6.3+/-2.6
D50 (μm)	717.2	397.5+/-27.4	633.8	318.6+/-73.0	340.6	145.8+/-25.4
D90 (μm)	2291.3	1077.5+/-62.5	1708.6	1143.7+/-256.0	1264.6	844.5+/-180.3

Particle sizes of all sieved, untreated rice bran were measured in order to understand the impact different bead milling settings imposed on the particle sizes. The PSD of unmilled DRB, HSRB and UPRB measured with laser scattering and image analysis are shown in Table 5.3. The particles measured with laser scattering are in water suspension. Laser scattering and image analysis provide very different results and there are several explanations for this. One reason is that the laser scattering measured the particles in water suspension, which makes for different particle characteristics. The other reason is how laser scattering approximates all particles as spheres while image analysis uses a more complex algorithm with the help of image technology. The image analysis values does not have a standard deviation as an automatic average is generated from the whole measurement range.

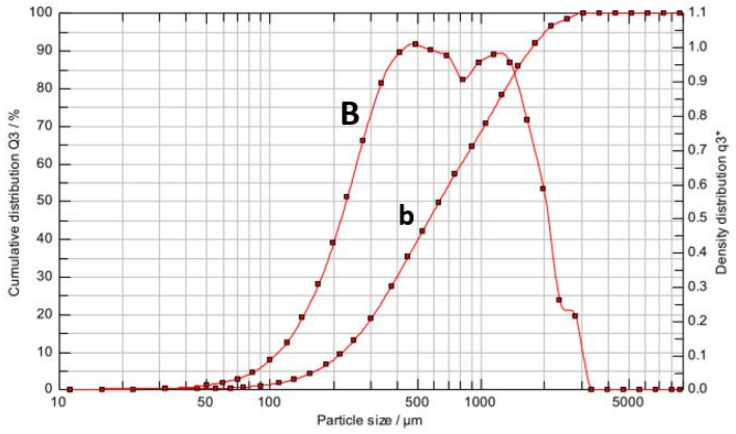


Cumulative distribution

x_0/µm	Q_3/%	x_0/µm	Q_3/%	x_0/µm	Q_3/%	x_0/µm	Q_3/%
22.57	0.00	155.00	7.64	625.00	46.85	2550.00	93.51
45.00	0.21	185.00	10.93	750.00	51.12	3050.00	96.72
55.00	0.50	215.00	14.56	900.00	55.63	3650.00	98.96
65.00	0.91	250.00	18.90	1050.00	60.52	4350.00	100.00
75.00	1.37	300.00	24.80	1250.00	67.41	5150.00	100.00
90.00	2.20	375.00	32.34	1500.00	74.20	6150.00	100.00
110.00	3.56	450.00	38.14	1800.00	82.12	7350.00	100.00
130.00	5.20	525.00	42.42	2150.00	88.08	8750.00	100.00

x_0/µm = Particle size (upper limit of class)
Q_3/% = Cumulative distribution $<x_0$ in %

Figure 5.2: Image analysis result: The cumulative distribution (a) and density distribution (A) of DRB (defatted rice bran) particles.

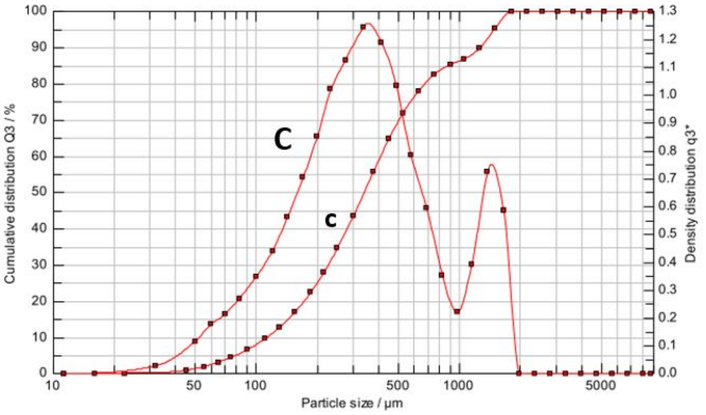


Cumulative distribution

x_0/µm	Q_3/%	x_0/µm	Q_3/%	x_0/µm	Q_3/%	x_0/µm	Q_3/%
22.57	0.00	155.00	4.17	625.00	49.46	2550.00	98.35
45.00	0.06	185.00	6.53	750.00	57.18	3050.00	100.00
55.00	0.16	215.00	9.32	900.00	64.33	3650.00	100.00
65.00	0.30	250.00	13.00	1050.00	70.73	4350.00	100.00
75.00	0.49	300.00	18.75	1250.00	78.13	5150.00	100.00
90.00	0.86	375.00	27.41	1500.00	85.67	6150.00	100.00
110.00	1.60	450.00	35.21	1800.00	91.90	7350.00	100.00
130.00	2.58	525.00	41.96	2150.00	96.42	8750.00	100.00

x_0/µm = Particle size (upper limit of class)
Q_3/% = Cumulative distribution $<x_0$ in %

Figure 5.3: Image analysis result: The cumulative distribution (b) and density distribution (B) of HSRB (heat stabilized rice bran) particles.



Cumulative distribution

x_0/µm	Q_3/%	x_0/µm	Q_3/%	x_0/µm	Q_3/%	x_0/µm	Q_3/%
22.57	0.00	155.00	16.94	625.00	77.78	2550.00	100.00
45.00	0.76	185.00	22.34	750.00	82.48	3050.00	100.00
55.00	1.75	215.00	27.89	900.00	85.24	3650.00	100.00
65.00	3.02	250.00	34.57	1050.00	86.70	4350.00	100.00
75.00	4.33	300.00	43.48	1250.00	89.66	5150.00	100.00
90.00	6.46	375.00	55.53	1500.00	95.38	6150.00	100.00
110.00	9.48	450.00	64.92	1800.00	100.00	7350.00	100.00
130.00	12.66	525.00	71.84	2150.00	100.00	8750.00	100.00

x_0/µm = Particle size (upper limit of class)
Q_3/% = Cumulative distribution $<x_0$ in %

Figure 5.4: Image analysis result: The cumulative distribution (c) and density distribution (C) of UPRB (unprocessed rice bran) particles.

Results from image analysis of untreated rice bran types are presented in Figure 5.2, 5.3 and 5.4. Image analysis provides two curves describing the PSD. The cumulative distribution curve (a,b and c) measures the range of particles divided into classes shown in the table below the graphs. Each class ends at an upper diameter limit, and represents a percentage of the total particles in the measurement range. The density distribution (A, B and C) shows which particle size fractions are covered by the most particles. The higher the peaks the larger the fraction of particles at a certain particle size range. The width of the peaks shows the character of the particle size distribution, i.e. if the sample material is distributed over a wide size range or if it is distributed over a narrow size range.

Both the D-values and the image analysis PSD curves show how the three types of rice bran have different PSD. DBR has the largest particles and UPRB the smallest. Given that the rice bran originates from three different rice mills it is no surprise there is a clear difference in particle size. In addition, DRB and HSRB have been through hexane and heat pre-treatment respectively which affects the material. One explanation to the small particle sizes of UPRB is the number of passes the rice paddy was put through in the whitening step. The particle size changes and gets finer after the first pass. UPRB was put through three passes in the whitening machine. In comparison, HSRB was put through two passes in the whitening machine and one pass through a polishing machine. The information about passes in the whitening machine was not available for DRB.

5.2 Bead milling screening

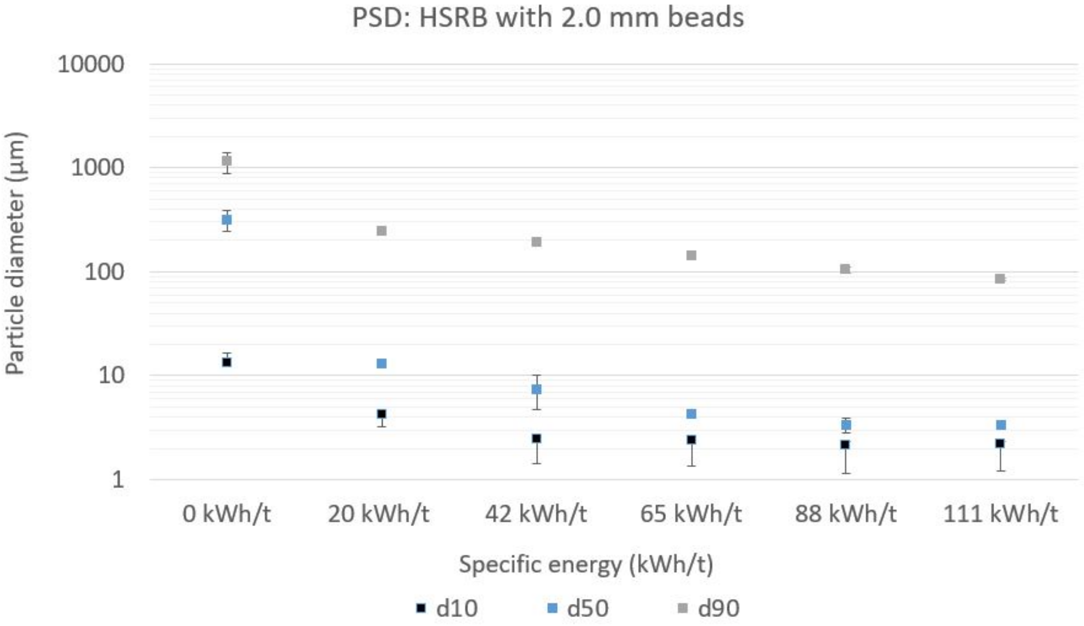


Figure 5.5: Difference in PSD (particle size distribution) of HSRB (heat stabilized rice bran) after bead milling performed with 2mm beads and with different specific energy. The values of specific energy on the x-axis correspond to the milling times 0, 10, 20, 30, 40 and 50 minutes respectively from left to right.

Table 5.4: D-values from Figure 5.5.

Specific energy (kWh/t)	**0**	**20**	**42**	**65**	**88**	**111**
D10 (μm)	13.3+/-3.3	4.2	2.4+/-0.1	2.4	2.1+/-0.2	2.2+/-0.1
D50 (μm)	318.6+/-73.0	13.2+/-0.8	7.4+/-2.7	4.3	3.3+/-0.5	3.4+/-0.3
D90 (μm)	1143.7+/-256.0	244.4+/-16.2	190.5+/-2.0	140.3+/-8.2	103.9+/-6.1	84.5+/-1.8

The screening of milling times trial was performed to choose one time for processing of coarsely milled bran and one time for processing of finely milled bran. This was important in order to have comparable results between trials. HSRB was used for screening of bead milling parameters (throughput, time and bead size). As the time increased, the throughput was also increased from 50kg/h to 70kg/h in order to raise the number of theoretical passes through the bead milling chamber. Other parameters remained constant: 2.0 mm beads and 80% filling degree of chamber. Results of the screening trial are presented in Figure 5.5 and Table 5.4. After evaluating the PSD from the screening trial, all bead milling parameters were chosen. After 40 minutes milling time, resulting in the specific energy 88kWh/t and 11.6 theoretical passes through the bead mill, the D-values did not decrease as significantly as before. Rice bran which was milled for 50 minutes, led to 111kWh in specific energy and 18.5 theoretical passes through the bead mill. However, this did not provide a large change towards a smaller PSD. Therefore the 40 minute setting was used for future trials to produce finely milled rice bran. The time of 20 minutes was chosen for coarsely milled bran, with the additional change to a 2.5 mm bead size. A larger bead size produces more coarsely milled material. The reason for these choices was to have a PSD in between unmilled and finely milled rice bran.

5.3 Impact of bead milling and enzymatic digestion on DRB, HSRB & UPRB

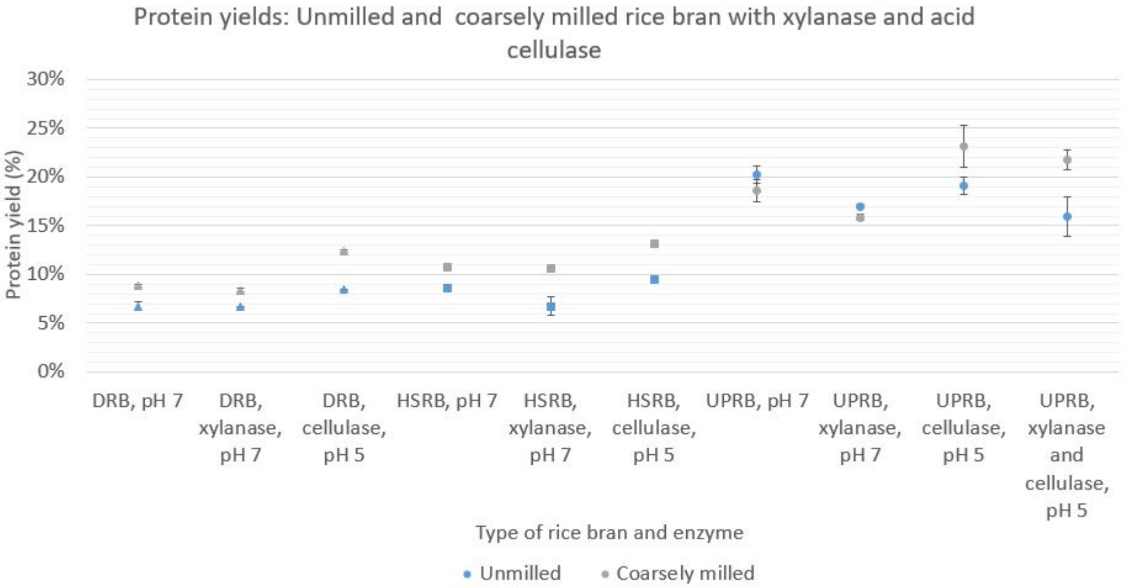


Figure 5.6: Protein yields after extraction using enzymatic digestion and/or bead milling from DRB (defatted rice bran), HSRB (heat stabilized rice bran) and UPRB (unprocessed rice bran). Coarsely milled bead milling settings= 2.5 mm beads, 80% filling degree, 20 min milling time.

The enzymes acid cellulase and xylanase were used to increase the rice bran protein extraction yield by enzymatic digestion. All measurements were done in duplicates and all protein measurements in two replicates. Protein yields are shown in Figure 5.6. Acid cellulase significantly ($p < 0.05$) increased the protein yield, while xylanase significantly decreased the protein yield with the same significance level. Addition of both enzymes showed no significant difference in either direction. The highest increase in yield by an enzyme was 4% with acid cellulase for UPRB. In conclusion, acid cellulase was the best enzyme although no enzymatic digestion treatment produced a high increase in protein yield.

Enzymes are a type of protein and this protein also have to be included in the protein yield calculations. The initial protein is calculated as the product of both rice bran protein and the added protein from the enzymes. However, the protein in the final product is calculated as if only rice bran protein and no enzyme protein is extracted in the extraction process. This means the calculation assumes no protein from the enzyme ends up in the final product. In reality however, protein from the added enzymes might have also been extracted and ended up in the final product. This calculation method for enzymatic extraction yields is likely one reason why the results presented in Figure 5.6 are inconsistent. Another explanation of the inconsistency and the low protein yields derived by the enzymes, is the storage time of the enzymes. As the supplier did not share any information about maximum storage time it is unclear whether the enzymes were still active at the time of use. Total storage time in the lab fridge at Bühler was two months. The storage time before this is unknown. The activity of the enzymes is due to this fact an unknown factor. To be certain of using enough enzyme for digestion of the rice bran cell wall, an enzyme weight corresponding to 4% of the rice bran weight was added. However, since the activity parameters were unknown, there was no way of predicting the protein yields that theoretically could be extracted with enzymes.

In this trial the impact of coarse bead milling, with and without enzymes, was also tested and is presented in Figure 5.6. Comparison between unmilled and coarsely milled samples indicates that bead milling significantly ($p < 0.05$) increases the rice bran protein extraction yield.

5.4 Impact of bead milling and alkaline conditions on HSRB protein yields

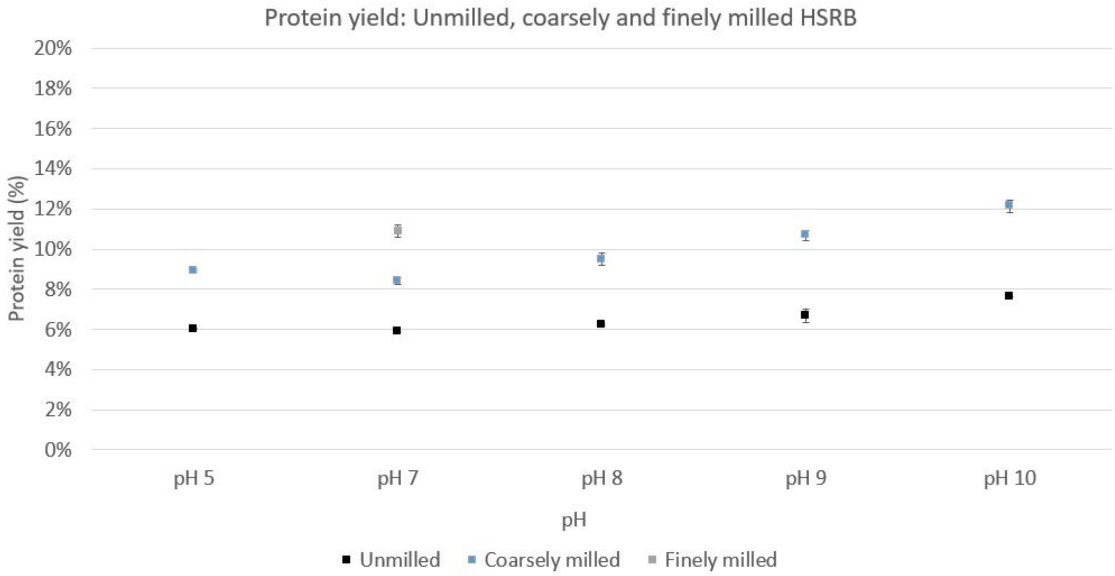


Figure 5.7: pH screening with and without bead milling of HSRB (heat stabilized rice bran). Coarsely milled bead milling settings= 2.5 mm beads, 80% filling degree, 20 min milling time. Finely milled bead milling settings= 2.0 mm beads, 80% filling degree, 40 min milling time.

A trial testing different alkaline conditions, course and fine milling of HSRB was performed in order to decide the optimal pH and bead milling settings for future trials. Results of protein extraction yields of HSRB at different pH and milling settings are presented in Figure 5.7. All measurements were done in duplicates and all protein measurements in two replicates.

The graph depicts a clear pattern towards higher protein yields at more alkaline conditions. A change to pH 10 from pH 7 leads to a significantly ($p<0.05$) higher protein yield. The increased protein yields of alkaline samples are expected as NaOH breaks hydrogen, amide and disulfide bonds in the proteins and thereby makes them more soluble. The glutelin fraction of total rice bran protein is alkaline soluble. As the glutelin fraction accounts for 11-27% of total rice bran protein it is very likely more glutelin is solubilized in the samples with higher pH. However, as pointed out by Wang et al (1999), alkaline conditions also cause negative effects in protein functionality and nutritional value. In addition, it might generate production of potentially toxic substances such as lysinoalanine.

When considering an extraction method for a potential future food ingredient, a tradeoff between high protein yield and quality of proteins should be considered when deciding what pH to use. The second clear pattern in Figure 5.7 is how coarsely milled HSRB have 3-5% higher protein yields than unmilled HSRB. The finely milled HSRB adds an additional 3% increase in protein yield at neutral pH. Comparison between unmilled and coarsely milled samples with and without pH changes shows how course bead milling significantly ($p < 0.05$) increases the rice bran protein extraction yield.

Table 5.5: PSD (particle size distribution) of HSRB (heat stabilized rice bran) at different specific energy in the bead mill. Coarsely milled bead milling settings (47kWh/t)= 2.5 mm beads, 80% filling degree, 20 min milling time. Finely milled bead milling settings (111kWh/t)= 2.0 mm beads, 80% filling degree, 50 min milling time.

Specific energy (kWh/t)	0	47	111
D10 (μm)	13.3+/-3.3	2.4+/-0.1	2.2+/-0.1
D50 (μm)	318.6+/-73.0	7.4+/-2.7	3.4+/-0.3
D90 (μm)	1143.7+/-256.0	190.5+/-2.0	84.5+/-1.8

The PSD of unmilled, coarsely milled and finely milled HSRB are presented in Table 5.5. The low D10 value of d=2.2 µm for finely milled HSRB at 111 kWh/t means there are a high amount of very small particles in this sample. Here, it is important to consider the ability to remain in suspension for these small particles. The high protein yield of the finely milled rice bran, might be generated by small insoluble protein particles which are not centrifuged down to the pellet. Since the centrifugation setting only go up to 3500 x G there might very well be insoluble particles in the supernatant fraction. These particles might be constituted out of protein.

5.5 Impact of bead milling and alkaline conditions on DRB, HSRB & UPRB protein yields

A trial testing extraction rates at pH 10 for all types of unmilled and coarsely milled rice bran was performed. The trial was conducted in order to investigate the increase in protein yield for coarsely milled rice bran in comparison to unmilled rice bran, and to evaluate difference in yield between DRB, HSRB and UPRB at pH 10. All samples were incubated at pH 10 due to the previously calculated highest protein extraction yield at pH 10 for HSRB (as shown in Figure 5.7).

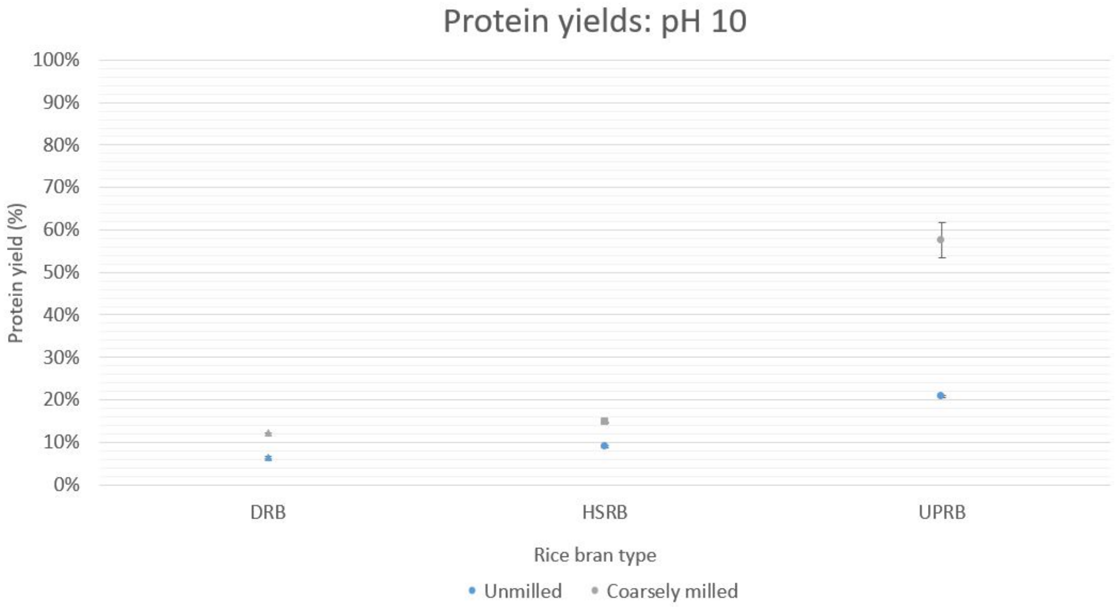


Figure 5.8: Protein yields of DRB (defatted rice bran), HSRB (heat stabilized rice bran) and UPRB (unprocessed rice bran) at pH 10. Coarsely milled bead milling settings= 2.5 mm beads, 80% filling degree, 20 min milling time. Finely milled bead milling settings= 2.0 mm beads, 80% filling degree, 40 min milling time.

Protein extraction yields of unmilled and coarsely milled samples are presented for all types of rice bran in Figure 5.8. As expected based on literature and previous trials, all milled samples had higher protein yields than the unmilled samples. For DRB and HSRB the increase is in the range of 4-8%. However, for UPRB the increase in protein yield between unmilled and coarsely milled rice bran is approaching 40%. Coarsely milled UPRB at pH 10 had the highest protein yield of nearly 58%.

Table 5.6: Difference in PSD (particle size distribution) for all materials used in the extraction trial at pH 10. DRB (defatted rice bran), HSRB (heat stabilized rice bran) and UPRB (unprocessed rice bran). Coarsely milled bead milling settings= 2.5 mm beads, 80% filling degree, 20 min milling time.

Rice bran type	DRB	HSRB	UPRB
D10 (μm)	5.3+/-0.0	3.4+/- 0.9	2.2+/-0.1
D50 (μm)	59.2+/-3.5	11.7+/-1.1	24.6+/-2.1
D90 (μm)	318.0+/-28.8	185.7+/-5.7	240.5+/-18.8

The PSD of each type of rice bran after course milling are presented in Table 5.6. In order to evaluate the effect of bead milling in rice bran protein extraction, all three types of rice bran were milled with the same settings for coarsely milled rice bran, described in Section 5.2. However, due to clogging of the bead mill when milling DRB, the material could only be milled for 16 minutes. This fact is clearly visible in Table 5.6, as the D-values of DRB are higher. This might be one reason for the low protein yields of DRB in Figure 5.8. The PSDs of HSRB and UPRB are more similar. Conceivably, the enhancement of the protein extraction is due to more protein release rather than protein particles in suspension. The difference in protein extraction between rice bran types could instead be explained by the difference in pre-treatment. The low protein solubility due to hexane pre-treatment of DRB might be the major reason for the low protein yield. The high yields of UPRB could be explained by the lack of pre-treatment of both hexane and heat stabilization.

5.6 Impact of PSD on highest protein yields

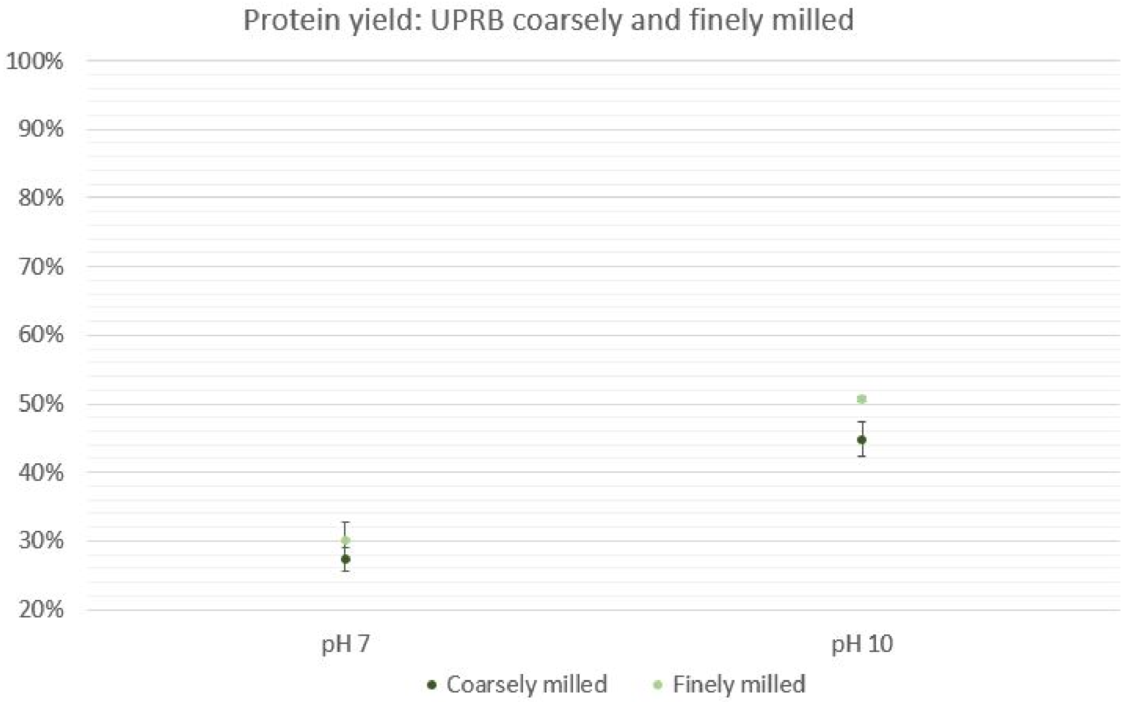


Figure 5.9: Protein yields of UPRB (unprocessed rice bran) with different pH and milling settings. Coarsely milled bead milling settings= 2.5 mm beads, 80% filling degree, 20 min milling time. Finely milled bead milling settings= 2.0 mm beads, 80% filling degree, 40 min milling time.

After evaluating rice bran protein yield results from the previous extraction trials; the most promising settings were reproduced and refined. Coarsely milled UPRB as well as finely milled UPRB with and without increase in pH were tested. The results are presented in Figure 5.9. Comparison between coarsely and finely milled samples indicates that finely milled settings significantly ($p < 0.05$) increased the rice bran protein extraction yield. Also the comparison between samples with pH 7 and pH 10 shows that pH 10 significantly ($p < 0.05$) increased the rice bran protein extraction yield for UPRB.

Table 5.7: Difference in PSD (particle size distribution) and protein yields of milled UPRB (unprocessed rice bran) extracted at pH 10. The UPRB milled with 43 kWh/t and 49 kWh/t were milled with settings= 2.5 mm beads, 80% filling degree, 20 min milling time. The UPRB with specific energy 78 kWh/t was milled with settings= 2.0 mm beads, 80% filling degree, 40 min milling time.

Specific energy (kWh/t)	43	49	78
Protein yield (%)	44.9+/-2.5	57.6+/-4.1	50.6+/-1.8
D10 (μm)	1.3+/-0.5	2.2+/-0.1	1.3+/-0.0
D50 (μm)	17.0+/- 8.4	24.6+/-2.1	1.9+/-0.1
D90 (μm)	340.3+/-244.5	240.5+/-18.8	86.4+/-5.4

Considering the previously achieved protein yield of 58% for coarsely milled UPRB at pH 10, the yield presented in Figure 5.9 are unexpectedly low. The protein yield did not get higher than 45% for coarsely milled UPRB at pH 10 in this trial. One explanation for the difference in yields is the specific energy and particle size differences between the trials. Particle size difference between the three milled batches of UPRB are presented in Table 5.7, together with their respective protein extraction yields. In this trial the specific energy is 78 kWh/t for finely milled UPRB, and for coarsely milled 43 kWh/t. However, for coarsely milled UPRB in the previous trial, the specific energy was 49 kWh/t. This was due to a slightly lower volume of UPRB used because of lack of raw material supply at the time of the trial. Nevertheless, this small difference in specific energy between the coarsely milled UPRB does not fully explain the large difference in protein yield. The experiments were done at different days and explanation could be raw material variability or inhomogeneity.

Table 5.8: Difference in PSD (particle size distribution) and protein yield of finely milled HSRB (heat stabilized rice bran) and UPRB (unprocessed rice bran). Finely milled bead milling settings= 2.0 mm beads, 80% filling degree, 40 min milling time.

Rice bran type	HSRB	UPRB
Protein yield (%)	10.9+/-0.3	30.2+/-2.7
D10 (μm)	2.2+/-0.1	1.3+/-0.0
D50 (μm)	3.4+/-0.3	1.9+/-0.1
D90 (μm)	84.5+/-1.8	86.4+/-5.4

To further evaluate the role of PSD in rice bran protein yields, the PSD of finely milled HSRB and UPRB are compared together with their respective protein extraction yields at pH 7. Table 5.8 presents the differences of these values. Although there is a difference in PSD between the two finely milled materials, this relatively small difference most likely does not explain the protein yield difference of approximately 20%. A more probable explanation is the difference in pre-treatment of the material. Literature and previous trials both suggest that heat treatment leads to insoluble and damaged protein. Therefore, it is expected that UPRB have the highest protein extraction yield.

5.7 Impact of a second centrifugation step

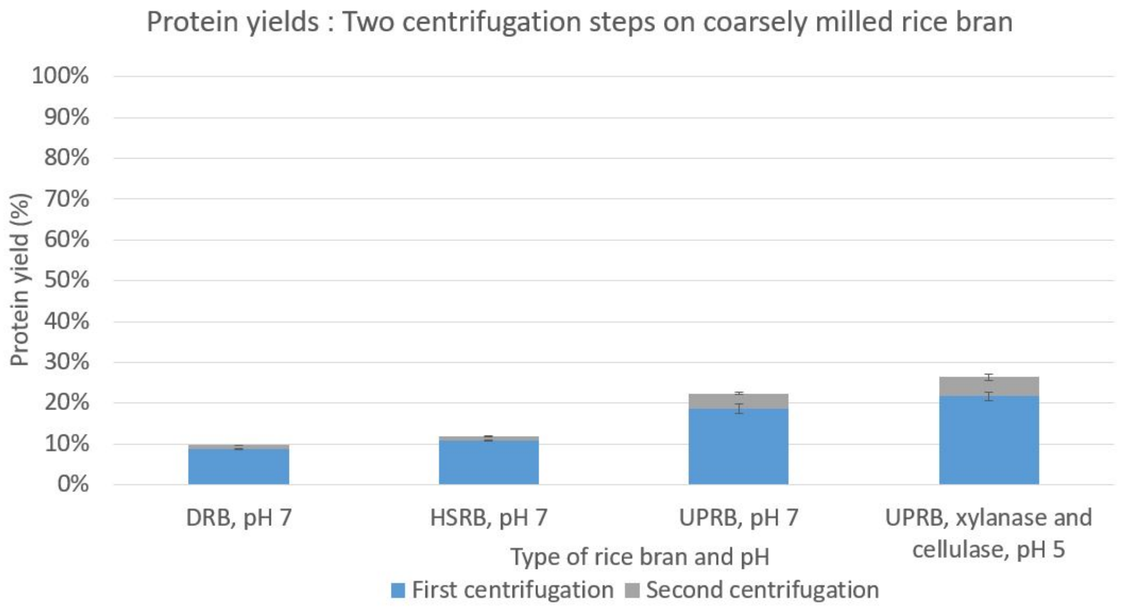


Figure 5.10: Protein yields from two centrifugation steps of DRB (defatted rice bran), HSRB (heat stabilized rice bran) and UPRB (unprocessed rice bran). The ratio of protein extracted after the first centrifugation step is represented as the blue part of the bars. The gray part of the bars represents the protein extracted after the second centrifugation step. Coarsely milled bead milling settings= 2.5 mm beads, 80% filling degree, 20 min milling time.

In order to evaluate how much soluble protein was left in the pellet fraction of the three types of rice bran after centrifugation, a second centrifugation protocol was applied. The protein yield results from both the first and second supernatants are presented in Figure 5.10. The results prove that there is indeed still soluble protein in the pellet fraction after the first centrifugation step, since the second supernatant also contained protein. This is expected as the water-soluble albumin fraction of rice bran protein is 34% of total protein and the first supernatant contained only around 7-22% of total protein for the different samples. With a second centrifugation the highest combined total yield of 26% was attained by UPRB incubated with xylanase and acid cellulase, where the second supernatant accounted for 4% of the combined yield.

5.8 Impact of a second centrifugation step with changed pH and higher solids content

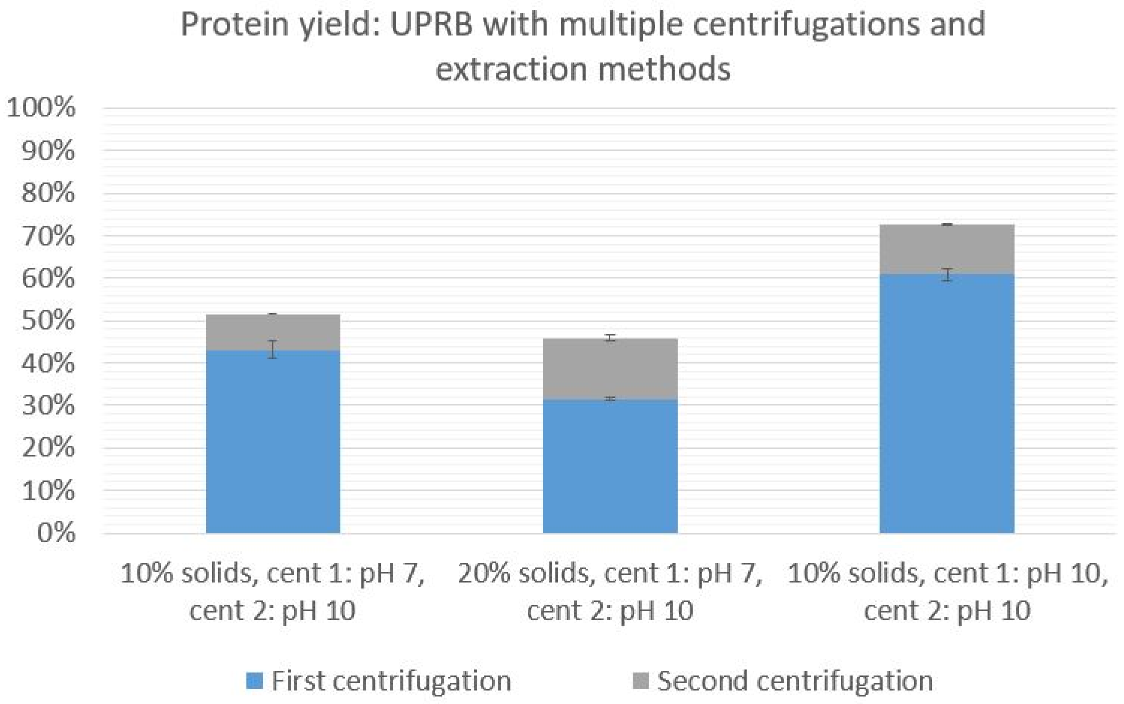


Figure 5.11: Protein yields from two centrifugation steps with pH change of finely milled UPRB (unprocessed rice bran). The labels on the x-axis indicate the ratio of rice bran in the rice bran/water mix used for bead milling. The first pH-value on the x-axis label indicates the pH applied during milling and the first centrifugation. The second pH on the x-axis label indicates the pH applied for the second centrifugation step. The ratio of protein extracted after the first centrifugation step is represented as the blue part of the bars. The gray part of the bars represents the protein extracted after the second centrifugation step. Finely milled bead milling settings= 2.0 mm beads, 80% filling degree, 40 min milling time.

In Figure 5.11 the results from the final trial are presented. All these parameters were tested in order to optimize the rice bran protein yields in this Master thesis. The best combination of parameters (rice bran ratio of 10:90 (bran/H2O mixture, w/v), and two centrifugation rounds with pH 10) reached a protein yield of nearly 72%. This is plausible given both the solubility of rice bran protein and protein extraction results reported by literature. Nadathur, Wanasundara, & Scanlin (2017), report that the water and alkaline soluble protein in rice bran together only add up to 45-61% of the rice bran protein. However, the 34% salt soluble protein group is often co-extracted with the water soluble albumin protein fraction. Therefore a 72% yield is reasonable to expect. In addition, the 72% yield achieved in this Master Thesis is in line with earlier reported alkaline protein extraction yields of 30-80% reviewed by Fabien and Ju (2011).

The first conclusion is the expected observation that pH 10 significantly ($p < 0.05$) increases the rice bran protein extraction yield in comparison to pH 7. This has been shown before when changing pH after bead milling. However, this trial shows that the extraction yield is significantly improved also when changing the pH of rice bran before bead milling. The second conclusion is that a second centrifugation round of finely milled UPRB with pH 10 greatly increases the protein yield of all samples compared to having only one round of centrifugation. In addition, when comparing the yields of a second centrifugation step at pH 10 with finely milled UPRB, they are significantly ($p<0.05$) higher than yields from a second centrifugation step at pH 7 performed with coarsely milled UPRB. This indicates that finer milling and pH 10 has a positive impact of protein yields from the second centrifugation step.

The third conclusion is that a rice bran ratio of 20:80 (bran/H2O mixture, w/v) instead of 10:90 (bran/H2O mixture, w/v), does not increase the protein yield. Comparison between 10% rice bran content and 20% rice bran content indicates that using only 10% solids content significantly ($p < 0.05$) increases the rice bran protein extraction yield. However, the protein yields are calculated as a ratio between total protein in the final product and total protein in the original product. Since the total protein in the original product is the double amount due to the double amount of rice bran used, the amount of protein will be higher for the 20% rice bran water mix when comparing weight of protein in the final products. Despite this fact, a rice bran ratio of 10% has been used consistently throughout this thesis. The reason for this is the separation process that will be used to obtain a protein concentrate after the extraction step. However, this separation process is not in the scope of this thesis.

6

Conclusion and Outlook

6.1 Conclusion

This Master thesis compares protein extraction yields of three types of rice bran treated either with enzymatic digestion, alkaline conditions, mechanical disruption, or a combination of these treatments. By comparing protein yields of DRB, HSRB and UPRB under all these treatments, it was confirmed that UPRB consistently got the highest protein yields. Studies performed in the scope of this thesis also confirmed that alkaline conditions in combination with mechanical disruption was the most efficient method to solubilize and extract rice bran protein. Further, it was confirmed that more alkaline conditions and more energy used for mechanical disruption, consistently increased the protein yields. On an industrial scale the energy used for bead milling could be lowered by increasing the throughput, and thereby a more sustainable processing solution could be acquired. The adverse effects of alkaline protein extraction would have to be balanced with higher protein yields.

On the other hand, enzymatic digestion did not achieve a large increase in protein yield, probably due to lack of activity in the enzymes used for the trial. Finally it could be observed that several centrifugation steps helped extract all the soluble protein from the samples. Adding alkaline conditions to the second centrifugation step helped to further increase the yield.

6.2 Outlook

To optimize the best protein extraction methods described in this thesis, more tests are required. Extraction using enzymatic digestion has been proved successful in literature and could be investigated closer. If more information about the activity of the enzymes is available, more accurate trials could be performed. Only if activity of the enzymes used for the trials could be guaranteed, could the method of enzymatic digestion be completely ruled out as unsuccessful. A protocol using finely milled unprocessed rice bran could be optimized further using two or more centrifugation steps. For each centrifugation step different extraction methods can be used. Active enzymes in combination with a solubilization and separation step using pH 10, could lead to a large increase in protein yield.

To obtain a protein concentrate or isolate, further separation of the protein after the extraction and centrifugation steps is required. To complement the work performed in this thesis, a review of different separation methods would be needed. In order to have a rice bran protein ingredient on the market, a spray drying step might also be necessary.

The functional properties and taste of a rice bran protein concentrate or isolate should also be investigated in order to continue the work performed in this thesis. These two factors will both be important in order to bring a food product to the market. Literature suggest nutritional qualities, emulsifying capacity and foaming properties all are functionalities worth investigating closer for rice bran. Finer fractions of rice bran have been shown to improve protein solubility and colloidal stability which is promising for the finely milled rice bran used in this project.

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