Yessie Widya Sari

Biomass and its potential for protein and amino acids:

valorizing agricultural by-products

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Thesis committee

Promotor
Prof. Dr J.P.M. Sanders
Emeritus professor of Valorization of Plant Production Chains
Wageningen University

Co-promotor
Dr M.E. Bruins
Researcher, Wageningen UR Food and Biobased Research
Wageningen University and Research Centre

Other members
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Dr L.A.M. Pouvreau, Nizo Food Research, Ede

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Chapter 1

Introduction

1.1 Biorefinery: transition towards bio-based products

The use of biomass for industrial products is not new. Plants have long been used for clothes, shelter, paper, construction, adhesives, tools, and medicine [1]. With the use of fossil fuels in the early 20th century and development of petroleum based refinery, the use of biomass for industrial application declined. Since the late 1960s, the petroleum-based products have widely replaced biomass-based products [2]. However, depletion of fossil fuels, rising oil prices, and growing environmental awareness, push the attention and policy towards a transition from fossil into bio-based products.

Transition towards bio-based products will have consequences on the demand and processing of biomass to enable production of bio-based products, which are biofuels (biodiesel and bioethanol), bioenergy (heat and power), and bio-based chemicals and materials (such as succinic acid and polylactic acid) [3, 4]. It is important to develop and combine various feedstock, conversion techniques, and production routes. The integrated process of separating and converting biomass elements is known as biorefinery.

Biorefining biomass for higher value products is expected to improve the overall productivity and efficiency of biomass utilisation. Oilseed mills are an example of biorefinery that is already available nowadays. Here, a combination of food and feed products is produced. Aiming at 10% replacement of fossil fuel with biofuel in 2020 [5], more biorefinery facilities will be set up. Production of value-added products from residues can then serve as economic driver for low-cost biofuel production. To guide future developments on bio-based products, a road map on the biorefinery for bulk chemicals, known as *top twelve chemicals derived from biomass*, has been developed by PNN/NREL (Pacific Northwest National/National Renewable Energy Laboratory) [6]. The target of this roadmap is to produce value-added products from carbohydrates that can substitute petrochemical-based products. Examples of carbohydrate-based products are glycerol, succinic acid, hydroxypropionate, furfural, and sorbitol, which are building blocks for several products that are currently produced via the petrochemical route.

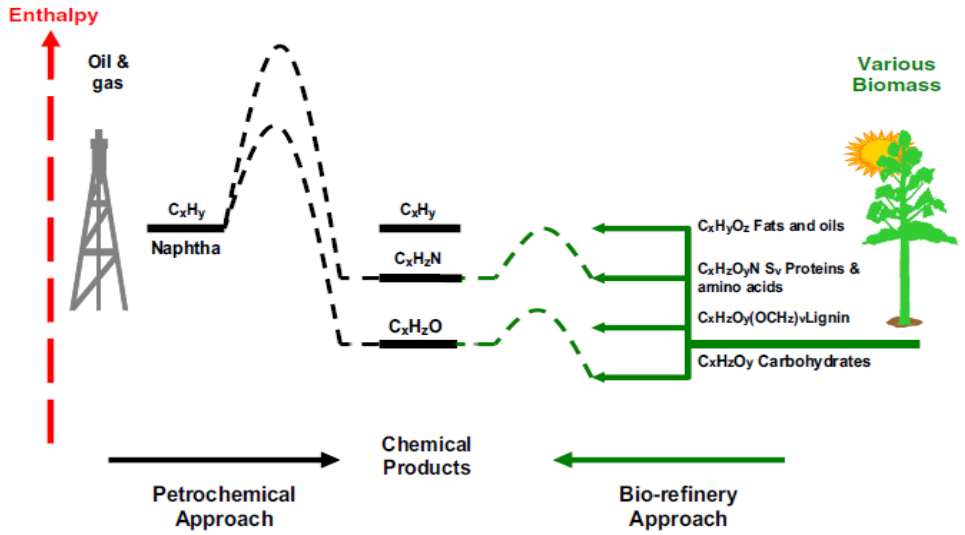

Figure 1. Biomass as feedstock of protein-derived bulk chemicals: more energy efficient [7].

Protein can be used to produce nitrogen-containing chemicals by taking advantage of the presence of the amine group (-NH_2) (Fig. 1). In petroleum-based conversion of crude oil into chemicals, co-reagents such as ammonia have to be used, and various process steps are involved. With the amine in protein, various co-reagent introducing process steps can be by-passed. With this biorefinery approach, less enthalpy is required compared to the petrochemical route to chemical products (Fig. 1). Section 1.4 discusses the details on how protein can be used as feedstock for bulk chemicals.

Biomass refinery for protein might not only be necessary for supplying feedstock for the chemical industry, before all, it is important to meet the world protein demand for food and feed. Section 1.2 illustrates the protein shortage in 2030 that we will encounter with the current uses of protein in the diet of both humans and animals. The worldwide protein production may provide this demand only if we consider the biomass refinery for protein and use the protein product in an effective and efficient way according the specific need of food, feed, and chemical industry.

1.2 World protein demand

The world population is currently growing with the rate of 1.14% per year increasing current population with 80 million per year [8]. Consequently, more food is needed. In 1992 world protein supply for food was 61 million tonnes and increased to 198 million tonnes in 2009 [9].Increase income earned by people in developing countries also contributes to an increased demand in protein. In 2009, people living in Europe and Asia, consumed 102 and 75 g protein/capita/day. For European people, representing those living in developed countries, that number is not so much different with their consumption in 1992, 98 g protein/capita/day. But for Asians, representing those living in developing countries, that number is considerably higher than their consumption in 1992, with only 63 g protein/capita/day. In addition to this, type of diet has also shifted. As income increases, people tend to eat more animal based protein than crop based one.

The increase in animal protein in diets leads to lower direct crop protein for food, yet a larger, indirect, increase in demand for feed.

In 2030, cereal and oilseed will be the major crop protein supplier, as in 2013 (Table 1). Cereal and oilseed productions in this period are predicted to be 2838 [10] and 686 million tonnes[1][10, 11], respectively. Protein from these crops can be directly consumed or indirectly in the form of animal protein products. For the latter, crop protein is required to be fed to animal. As much as 1200 million tonnes of cereal [10] and 173 million tonnes of oilseed are allocated for feed [10, 11]. These numbers correspond to 120 and 52 million tonnes of cereal and oilseed protein, respectively. In total, 172 million tonnes of protein from these crops are produced for feed. As people are projected to eat more animal protein than crop protein, we assume that animal protein supplies 2/3 of human protein demand. If total protein demand for human consumption in 2030 is 174 million tonnes[2], then as much as 116 million tonnes of animal based protein is required. Due to the inefficient conversion of crop protein into animal protein product[3] [13], 696 million tonnes crop protein is required to produce 116 million tonnes of animal protein. The worldwide cereal and oilseed protein production only supplies 25% of world demand for feed. Shifting the human diet to more crop and less animal protein is rather an optimist scenario. However, even with this scenario, more feed proteins are demanded than protein produced by cereal and oilseed crops. Grass is another important crop that supplies additional protein. However, the limited digestibility of grass, certainly from non-cultivated grassland, limits its protein intake by ruminants. With this, the available grass is predicted still not enough to meet the total worldwide feed protein demand. In addition to this, residues from agro-industry are used and needed to meet the total feed protein demand.

As much as 1406 million tonnes of cereal [10] and 68.6 million tonnes of oilseed [10, 11] are dedicated for food. For cereal, these numbers correspond to 140.6 million tonnes cereal protein. For oilseed, with 30% protein content, these numbers correspond to 20.6 million tonnes oilseed protein. In total, there will be 161.2 million tonnes of protein produced from these crops. However, only part of these cereal and oilseed proteins can meet the human food protein demand, since certain crops have lower protein quality than that is needed for humans. Protein Digestibility Corrected Amino Acid Score (PDCAAS), a parameter for assessing the quality of protein for human consumption can be used to measure the bioavailability of protein provided by crop [15]. The PDCAAS for cereals and oilseeds used in this calculation was 50% and 80%, respectively. Considering the PDCAAS, cereal and oilseed protein production will be 86.7 million tonnes. After considering both protein availability in quantity and quality, assuming an average daily uptake of 57 gram of protein per day, and assuming that people consume 1/3 their protein directly from crops, cereal and oilseed protein are enough to supply 58 million tonnes of direct crop-based protein demand. The remaining 28.7 million tonnes can be used to supply feed demand which is still not enough to fully supply feed demand.

[1] Extrapolated.

[2] Assumed that recommended daily intake protein is 57.5 g protein/capita/day and world population in 2030 is 8.3 billion people [12].

[3] As much as 6 kg crop protein is required to produce 1 kg animal protein [13, 14].

Table 1. Production of protein-rich crops in 2013 [16]

Crop	World	Europe	Asia
		(million tonnes)	
Corn	1016	117	304
Rice/Paddy	746	4	675
Wheat	713	225	319
Potato	368	113	180
Soybean	276	6	27
Barley	144	86	22
Rapeseed	72	26	23
Pea	64	3	2
Sorghum	61	12	9
Peanut, with cell	45	0.1	30
Sunflower	44	32	6
Oat	23	14	1
Chickpea	13	0.9	11

Calculation on total crop protein demand and total arable protein production can illustrate if there will be protein shortage or surplus in 2030. In previous paragraph we mention that 58 million tonnes protein is required directly from crop to be used in human food. If we consider the crop quality factor of 65%, as average PDCAAS, then the crop protein requirement for human food will be equal to 89 million tonnes. As feed demand is 696 million tonnes of protein, then a total of 785 million tonnes of crop protein is required to meet food and feed protein demand, assuming that animal protein supplies 2/3 world food protein demand. This crop protein demand is higher than the total protein production from total worldwide arable land, which can nowadays produce 600 million tonnes protein (from leaves and seeds) [17]. Grass can supply additional protein. Nowadays, grass supply only 24% of feed demand[4]. This equals with additional 174 million tonnes of protein. Total protein production from arable and grass land will be 774 million tonnes. Still, crop protein demand is higher than the total protein production, leading to biomass shortage. Certainly, without the need of protein for chemicals, the current protein resources cannot meet the demand for food and feed, as discussed above. This would mean that we have to lower the animal based protein consumption in order to reduce the worldwide protein demand, which is rather optimist. Another alternative to meet the protein demand is that we have to use protein efficiently and effectively isolate protein from crop, which can be approached through biomass refinery.

Biorefinery is expected to provide more protein as we consider collecting protein from the non-edible part of plants, such as stem, straw, bran, and hull. Even more protein is available if we also consider collecting protein from e.g. food waste, as one third of all food produced for human consumption is being wasted [19]. If development on biorefinery technology can increase worldwide protein yield from arable area and grassland area by 0.5% per year, by providing more protein resources and improving grass digestibility by cattle, then there will be 842 million tonnes of protein in 2030 which should be enough to meet world protein demand for food and feed. However, taking into

[4] Assuming that grazing system only supplies 24% of world beef production, then grass supplies 24% of world feed protein demand [18].

account that the protein resource are not well distributed in the world and that the mighty people or companies who work with protein may not divide the scarce protein resource, then we should be able make more proteins and give the tools to those people that need it. Development on biorefinery technology is also expected provide additional protein from novel sources such as microalgae. In addition to this, increasing the efficiency of crop protein usage, particularly in feed sector is expected to reduce the gross feed protein demand.

Current practice in animal feeding still provides room for improvement and for more efficient use of crop protein. It is common practice to feed animal with excess amount of protein [20, 21]. Livestock eat 6 times more protein than ends up in their product [13, 14]. The amount of protein intake can be reduced by addition of amino acids [22]. Pigs and poultry get lysine, methionine, threonine, and some tryptophan as supplements. Nowadays 0.6 million tonnes of amino acids are used in Europe to supplement 30 million tonnes of feed protein[5]. If we would have 38 million tonnes of the most limiting amino acids to supplement feed, then we can reduce 9 million tonnes of protein in feed sector. Assuming that the world feed production is 5 times than Europe's and after the population growth and the increased animal production in the world this will be 10 times of Europe's, with 38 million tonnes of amino acids are to be used for feed then 90 million tonnes of protein can be reduced from feed sector.

If we have enough protein for food and feed, protein can be used for chemical industry. If biorefinery is expected to supply 50% of feed demand on amino acids (38 million tonnes)[6], then 63 million tonnes of crop protein is required. Since only 6 amino acids (lysine, methionine, tyrosine, tryptophan, phenylalanine, and valine), are used for feed, the other amino acids, circa 44 million tonnes of amino acids, can be used for chemical industry.

Chemical industry is expected to replace fossil usage in chemicals sectors up to 20% in 2030 [23, 24]. Part of these chemicals contains nitrogen as a functional group. Estimation on the maximum demand of protein biomass needed for nitrogen-containing chemical industry in Europe has been made. To fully substitute the use of naphtha for N-containing for this type of chemicals, as much as 13 million tonnes of protein is needed per year, assuming a protein extraction yield of 50%. When protein extraction yield can be improved to 80%, then, only 8 million tonnes per year will be required [25]. The data on protein demand for chemical industry in Europe can be used to estimate the worldwide protein demand by taking into account that Europe supplies 20% of world chemical production. Extrapolating, 40 - 65 million tonnes of proteins will be required worldwide. If development on protein extraction can be made, it is possible that only 40 million tonnes is sufficient which can be sourced from the amino acids that are not used for feed as discussed in preceding paragraph. In this context, we can see the synergy of feed and chemical production provided by biorefinery.

Biorefinery of different protein resources can contribute to obtain products directed to specific needs for food, ruminant and non-ruminant feed, and chemical industry. With this scheme, biorefinery technologies and the use of protein in more

[5] Personal communication with Sanders J. (2015).
[6] Assuming that another 50% is supplied by amino acid from fermentation.

efficient way may avoid the protein shortage in 2030. In addition, the improved use of crops as protein and or amino acids resources leads to an increase in crop value.

1.3 Biomass pre-treatment

Exploiting and utilising crop protein, particularly crop by-products, is essential for world bio-based economy. Suitability of particular biomass as protein resources depends on availability and characteristics of this biomass and its protein. As example, in terms of availability, rice by-products and palm kernel meal may be suitable for Indonesia, while rapeseed meal may be suitable for European countries and Canada. Suitability of biomass as protein resource can be influenced by its composition, but also by treatments during production of its main product, e.g. oil. Production of the main product can improve protein extractability. For example, it is easier to extract protein from soybean meal than from soybean resulting higher protein yield in soybean meal [26]. However, too severe treatment may reduce protein functionality or can even make protein unsuitable for food. For these types of by-products, utilising their amino acids for food or feed additives or feedstock for bulk chemicals may be more suitable. These following subsections discuss protein-rich crops, focussing on processing methods for their main products, and its consequences for possible usage of their by-products as protein and/or amino acid resources. Processing methods for their main products is considered as pre-treatment for protein production. Information on pre-treatment of biomass is needed to get insight in what has been done that may affect protein extractability.

1.3.1 Oilseed-type biomass

Oilseeds are attractive biomasses in agricultural sector due to their oil and protein content. Oilseed crops are mostly used for the vegetable oil production. In smaller percentage, some other oilseeds are directly consumed as processed food. For example, soybean is processed into products such as soy flour, soy milk, and tofu.

Soybean, rapeseed, sunflower, and palm, are the four most important biomass for vegetable oil production [9]. Prior to oil extraction, oilseeds are cleaned, dried, dehulled, conditioned with steam and flaked. Conditioning temperatures range from 75 – 110 °C and its duration reaches up to 1.5 h [27]. High conditioning temperature is required for biomass having enzymes that can reduce the quality of extracted oil, such as rapeseed. Following conditioning, the oil in the flakes is extracted by solvent extraction, usually with hexane as the solvent. After extracting the oil, hexane is being removed by a desolventization. For soybean, this is the critical step determining if the meals are for feed or food. For feed purposes, toasting system at 105 °C is used. For food, where protein denaturation is to be minimized, flash system is used. In this system, protein denaturation is minimized by short desolventizing time [28].

In the end, oilseed processing produces by-products such as hull and meal. The latter is protein-rich oilseed residues following oil extraction. These are mainly used as animal feed. In lesser percentage, oilseed meals are also used in food. Oilseed meals are not optimally utilized, indicated by 4 million tonnes of oilseed meals being wasted during 2010/2011 [11].

1.3.2 Cereal-type biomass

Cereals occupy over half of the world's harvested area and are considered the most important food sources for human. Corn, rice, and wheat are the largest contributors to total world cereal production [9, 16]. In addition to these crops, barley, is also of considerable industrial importance [16]. Increased cereal production is expected in coming years due to the incentive to use cereals for non-food, such as ethanol production.

Industrially, cereals are being mostly used for the production of starch. Corn is dominating the starch market. In the last decades the use of wheat for starch production has increased. As residual from starch production, proteins are obtained, termed gluten.

For production of starch from corn, wet milling method is commonly used. Corn kernels are steeped for about 40 hours in warm water (about 50°C) to which sulphur dioxide is added to avoid undesirable fermentation. Steeping results in softening of the kernels matrix to facilitate the starch extraction process. Centrifugal separator is used to separate starch from other corn components. The main by-product is corn gluten, which is mainly used in feed.

For production of starch from wheat, dry milling method is commonly used. Wheat is ground to produce wheat flour. During milling process, the white wheat flour is separated from wheat germ and bran. These wheat components are then considered as by-products and known as wheat middling [29]. Following this, wheat flour is mixed with water to form stiff dough. During dough formation, gluten aggregates start to form larger particles. Having large particle, gluten is separated from starch by washed off the dough with water in mechanical screening machine.

Similar with wheat, dry milling is commonly used for starch production from rice. To obtain white rice endosperm, rice grain is exposed to 2 step dry milling. The first milling removes the hard protective hull (or husk). Once removed, the rice grain is known as brown rice. The second step is a gentle milling to remove the germ and bran from the grain thus exposing the white starchy rice endosperm. Rice hull, rice bran, broken rice and rice straw are by-products from rice processing and these are used as common ingredients in horticultural, livestock, industrial, household, building and food products [30]. Table 2 lists rice by-products and their protein content. Looking at the profile of rice bran and rice hull amino acid, particularly the essential amino acids, these two rice by-products have different pattern (Fig. 2). Using corn as reference for poultry feed, it is likely that rice bran can be used as alternative for feed. Even better, with higher essential amino acid content than corn and also having nutrition that comparable to animal protein, rice bran is expected to serve as novel food protein source [31]. On the other hand, rice hull contain less essential amino acids and may be not suitable for poultry feed but still possible for ruminants as these animals require lesser protein/amino acids compare to poultry.

Table 2. Protein content in rice by-products

	Protein content (%)	**Refs.**
Rice bran	15	[32]
Rice hull	2.3	[33]
Rice straw	4.7	[34]

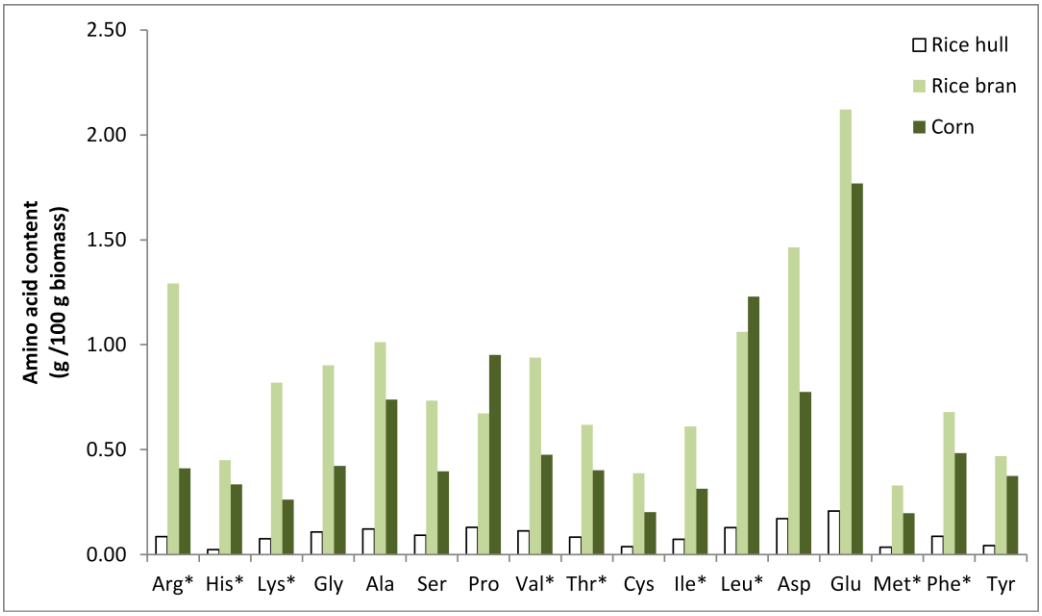

Figure 2. Amino acid content in rice hull [35], rice bran [35], and corn [36].
*: essential amino acids.

Barley is widely used as feedstock for the malting industry. For this purpose, barley starch has to be hydrolysed into sugar. Malting process includes step involving screening, cleaning, steeping (immersed in water for about 36-48 h) [37], germination, drying (up to 105 °C) [38], and de-culming (rootlets removal) [39]. Several barley by-products are available upon barley processing. Barley part that is not allowed to go to second step is called barley mill run. Barley rootlets are residue after malt deculming. Barley rootlets and barley mill run can also be combined and further known as malt by-products. With the protein content 10-26%, barley residues collected from malting currently are used as animal feed.

Cereal processing described above is a common practice for food application. In addition, cereals such as corn and wheat recently are also used as feedstock for bioethanol production. Processing steps for bioethanol production are milling, saccharification at which enzymes and water are added to hydrolysed starch into fermentable sugar, fermentation whereby the sugar is fermented into bioethanol and CO_2, distillation and rectification to concentrate the produced bioethanol, and finally dehydration or drying the bioethanol into 99% vol [40]. Residual thin liquid of distillation contains protein, fat, and other cereal constituents, is further processed for animal feed. This thin stillage may be directly evaporated to produce concentrated distillers solubles (CDS), or may be combined with coarse unfermented grain particle to produce wet distillers grain (WDG). WDG can then be directly dried to produce dried distillers grain (DDG) or dried in combination with CDS to produce dried distillers grains with solubles (DDGS) (Fig. 3) [41]. Protein and amino acid content in CDS, WDG, DDG, and DDGS varies due to different processing process (Table 3). Having starch removed, DDGS contains concentrated protein and essential amino acids than corn. DDGS colour is subjectively

used in feed industry to measure feed quality. Darker coloured DDGS has lower quality than lighter one. This may relate with the difference in lysine content, one of the limiting amino acids for animal feed. Dark coloured DDGS contents low lysine provided that this type of DDGS has undergone severe drying condition allowing glucose-lysine Maillard reaction and resulting dark colour. With less amount of lysine in dark coloured DDGS, less milk is produced by cow fed with dark coloured DDGS [42]. The quality of DDGS is also determined by the mixing ratio of WDG and CDS in DDGS (Table 4). DDGS having higher WDG content has higher quality provided by higher content of essential amino acids. Although inclusion CDS reduces the quality of DDGS, exclusion CDS in DDGS, in other words feeding with DDG instead DDGS, is less preferred as CDS contains fat that also required for animal growth. With only minor CDS is expected to be included in DDGS for feed, remaining protein or amino acids from CDS may be useful for technical application or bulk chemicals.

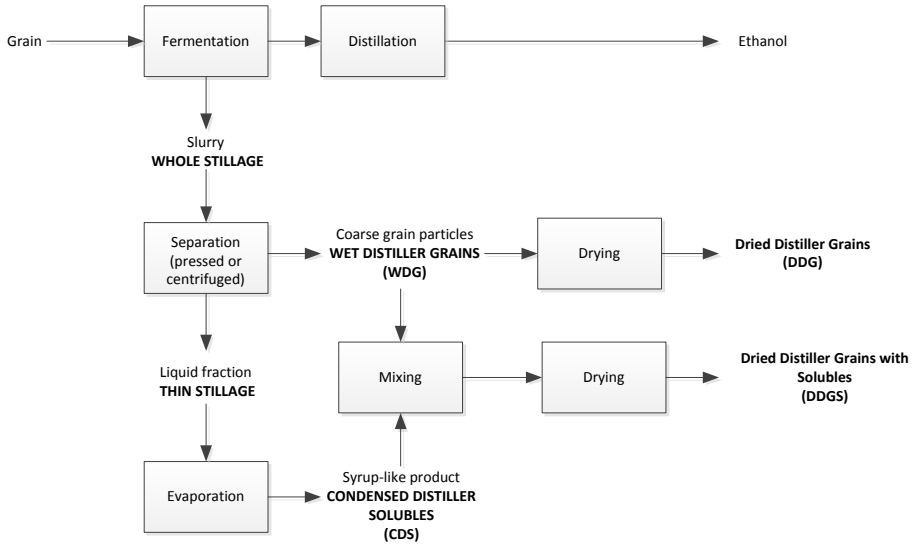

Figure 3. Ethanol production flowchart, adapted from [32].

Table 3. Protein content in corn by-products following ethanol production

	Protein content (%)	**Ref.**
Corn	10	[43]
CDS	17	[43]
WDG	25	[43]
DDG	27	[44]
DDGS	27-30	[44, 45]

Table 4. Significant limiting amino acids in corn and its DDGS (g/100 g dry matter)

Amino acid	Corn	WDG	CDS	DDGS	
				WDG:CDS = 9:1	WDG:CDS = 8:2
Lysine	0.24	0.33	0.95	0.95	0.88
Methionine	0.21	0.16	0.66	0.61	0.55
Threonine	0.39	0.32	1.2	1.15	1.06
Tryptophan	0.43	0.05	0.18	0.18	0.17

Reference for corn [45], WDG, CDS and DDGS [46].

1.3.3 Leaves, grass, and microalgae

Differ from oilseed and cereals which proteins are not their main products, leaves and grass are mostly known as protein resources for human and animal diet. Leaves and grass contain major protein namely as Rubisco, special protein involved in photosynthesis. Rubisco contains all essential amino acids that are required in human diet [47]. Leaves for food are not new and have been used as the main protein resources for developing countries, such as Zimbabwe, Nigeria, and Zambia, where meat proteins are relatively expensive [48]. In these countries, protein is consumed directly from fresh or dried leaves. However, the toughness of cell wall makes human bodies ineffectively absorb leaf proteins.

To improve human body absorption on protein, leaf protein concentrate is recommended instead of fresh or dried leaves. For this purpose, leaves are macerated, protein juice is produced, and further coagulated to form leaf protein concentrate. Despite the low protein extraction yield from this process [49], leaves provide potential sources of protein as leaves and oilseed have comparable protein yield per ha per year. As example, alfalfa leaf and soybean proteins production in US are counted as 0.90-1.5 and 0.96 tonnes per ha per year, respectively [50]. With development in leaf protein extraction [51], more proteins are expected in the future.

In addition to fresh leaves or grass, another source of leaf protein is tea leaf residue. World tea production in 2011 is counted as 4.5 million tonnes [52]. Fresh tea leaves are dried to a water content of 10-15%. For this purpose, fresh tea leaves follow steps of drying process up to 470 °C [53]. Later, hot water is used for immersing tea leaves to obtain tea extract. Industrial production of instant tea beverage ends up with tea residues which currently are considered as waste [54]. Considering that tea residue contains 20-30% [51], protein extraction from this will support the economy of instant tea production.

More leaves and grass proteins will be available with the incentive on bioethanol production. Possible protein resources include corn leaves, sugar beet leaves, sugar cane leaves, jatropha leaves, switch grass, dwarf elephant grass, and ryegrass. The protein production by-products not only will reduce the bioethanol price, but also it can mitigate the debate on food versus controversy [55, 56].

Microalgae are considered as novel protein resources. In term of capability to convert light energy into chemical energy during photosynthesis, with higher photon

conversion efficiency (3-8%) than terrestrial crop (0.5%), microalgae will grow at high rates and have higher biomass yield per hectare [57]. Currently microalgae are produced mainly as an ingredient for human nutrition [58]. Microalgae have high content of fatty acid, up to 40% varies by the strains, which also makes microalgae attractive for biofuel production [59, 60]. Nowadays, biofuel from microalgae can be produced technically. However, it is far more expensive than petroleum fuels [61]. Biofuel production may be economically feasible through integrated biorefinery of microalgae [60].

1.4 Amino acid production

The need for protein in food and feed is related to the inability of humans and animals to synthesize 9 amino acids; L-valine, L-leucine, L-isoleucine, L-lysine, L-threonine, L-methionine, L-histidine, L-phenylalanine, and L-tryptophan. These essential amino acids must be present in food or feed or can also be additionally supplied to humans or animals with specific demand, such as enhanced weight gain and livestock products or to supply deficiency of a particular amino acid.

The need of amino acids has increased the global amino acids market. The main amino acid market is in food, which absorbs about 50% of global market. (Fig. 4) [62]. Glutamic acid is the major portion of the used amino acids in food, and flavour enhancers that mainly contain this amino acid, accounted for 34% of total US amino acid market size in 2011[63]. Phenylalanine and aspartic acid are also highly demanded as these are the feedstock for producing aspartame, an artificial sweetener. In the feed sector, lysine, methionine, tryptophan and threonine are the leading products, accounting to over 31% of total market value in 2012 [64]. The amino acids market is forecasted to grow with 26% over the period 2012-2016 [65]. To meet the amino acids demand, three ways of production have been developed; extraction, chemical synthesis, and fermentation [66].

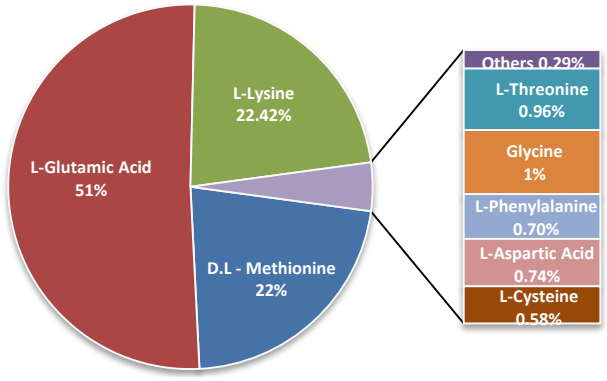

Figure 4. Global production of selected amino acids with the total amount of 1.5 million tonnes [62].

Historically, extraction was used for the industrial production of amino acids. The first original industrial application of an amino acid originated back to 1908 when Kikunae Ikeda isolated the unique flavour of seaweed that later became known as monosodium glutamate (MSG). Soon after this invention, MSG was also extracted from other biomass such as wheat gluten and soybean [66]. In addition to glutamic acid, some other amino acids are also extracted for food. Cysteine-is used as dough conditioner, leucine as flavour enhancer, isoleucine, and valine as dietary supplement. All can be extracted from feather and hair [67, 68]. The extraction method relies on the availability of biomass and the effectiveness of extraction process. To meet current industrial needs for amino acids, nowadays, extraction has been substituted and/or combined with microbial methods. However, within future biorefinery concepts, amino acids may once again be extracted from various agricultural residues.

Chemical synthesis has been conducted to produce a limited number of amino acids. Glutamic acid was chemically synthesised through the oxo reaction from acrylonitrile [69] and a reduction reaction from di-ester-alpha-nitroglutaric acid [70]. Glycine is manufactured by chemical synthesis from formaldehyde or monochloroacetic acid and ammonia [71]. Methionine is manufactured through a complex chemical synthesis involving mercaptan, propylene, and hydrogen cyanide [72]. Chemical synthesis of amino acids can only produce a mixture of D and L-forms of amino acids. The mixture limits the industrial use of chemical synthesis of amino acids for food which requires only L form. However, chemical synthesis is still used for manufacturing glycine and methionine for feed as glycine is an achiral molecule while for methionine, both L, and D-form have similar effect in animal [66]. To meet the demand on methionine, chemical synthesis has, nowadays, also been combined with fermentation methods [73, 74].

Currently, fermentation is the most commonly method to produce amino acids. The invention of microbial process for producing L-glutamic acid from soil bacterium, *Corynebacterium glutamicum*, using sugar and ammonia, paved the way for the success of fermentative production of amino acids [75, 76]. Since then, the extraction method on glutamic acid production has become less important by fermentation method. At the present time, fermentation method has also been used to produced L-lysine, L-phenylalanine, L-threonine, L-glutamine, L-arginine, L-tryptophan, L-valine, L-leucine, L-isoleucine, L-histidine, L-proline, and L-serine [66, 77].

1.5 Bio-based bulk chemicals: potential and challenge

Today's chemical industries still rely on crude oil as feedstock. Crude oil is refined into fractions such as naphtha, gasoline, kerosene, gas oil, and residues. Cracking, catalysing, and distillation are all involved in crude oil refinery. Naphtha is commonly used as platform for bulk chemicals. As naphtha only contains hydrocarbons, other elements such as oxygen, nitrogen, and chlorine have to be introduced to naphtha during bulk chemicals production [78]. Both the refining of crude oil and introduction of elements into naphtha require high amounts of energy. Biomass can be an alternative source for bulk chemicals

production that already contains elements like oxygen and nitrogen. With the presences of these elements, less energy is required to produce bulk chemicals from biomass.

A comparison between fossil and bio-based bulk chemicals production and its energy usage for ethanediamine production is given in Fig. 5. Ethanediamine is a bulk chemical used for rubber chemicals, pharmaceutical, and EDTA synthesis [7]. Ethylene, obtained from naphtha, is used as current platform to produce 1,2-ethanediamine. Using crude oil, or petrochemical route, ammonia is introduced twice; firstly during conversion of ethylene oxide into ethanolamine and secondly during conversion of ethanolamine into 1,2-ethanediamine. While using the bio-based route, ammonia is only introduced during conversion of ethanolamine into 1,2-ethanediamine. In final, total of 60 GJ and 44.5 GJ are required to produce 1 ton 1,2-ethanediamine using petrochemical and bio-based routes, respectively. This calculation indicates that less energy is required when the process involved is a bio-based production, using amino acid. Detailed energy calculations can be seen in Table 5.

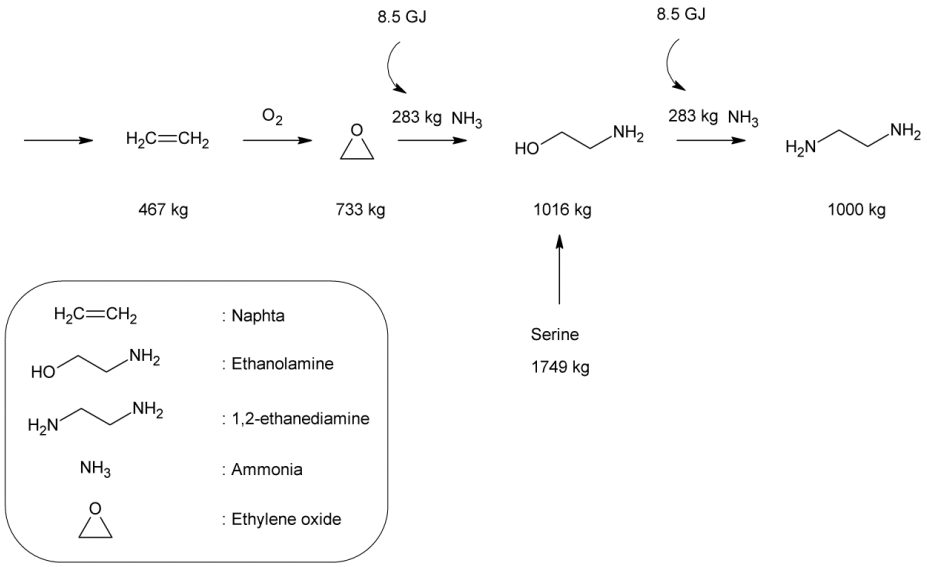

Figure 5. Bio-based vs petrochemical production of 1,2-ethanediamine [7].

Table 5. Comparison on energy required to produce 1 ton 1,2-ethanediamine using petrochemical and bio-based routes [7]

Petrochemical		**Bio-based**	
Step	Energy (GJ)	Step	Energy (GJ)
Calorific value feedstock	22	Calorific value feedstock	26
Conversion of ethylene to ethylene oxide	21	Conversion of serine to ethanolamine	5
Calorific value of ammonia	17	Conversion of ethanolamine to 1,2-ethanediamine	5
		Calorific value of ammonia	8.5
Total energy required	**60**	**Total energy required**	**44.5**

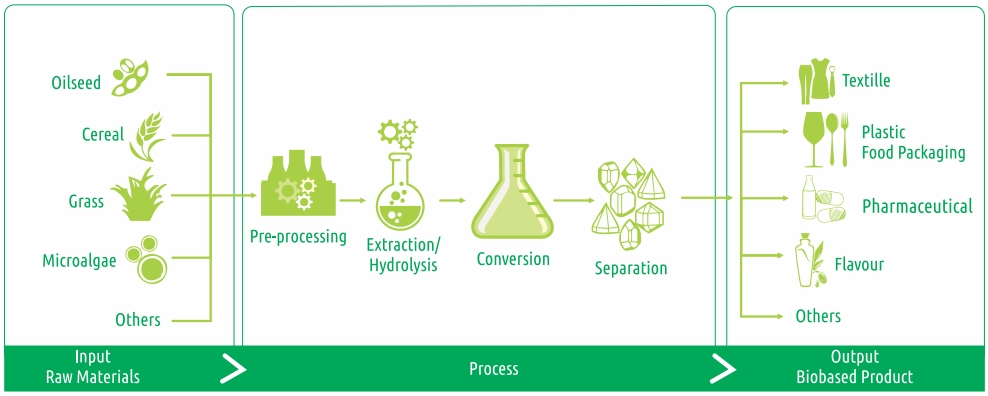

Figure 6. Schematic representation of the three different steps in protein biorefinery leading from raw materials to bio-based chemicals that can be used for various products.

Biorefinery for bulk chemicals contains three important aspects, which are raw materials as the input, biomass processing, and bio-based products as the output. In the case of protein refinery (Fig. 6), protein from several types of biomass is used as feedstock for producing several bio-based products such as textile, plastic food packaging, and pharmaceuticals. For this purpose, several biomass processing steps are required for isolating protein or amino acids from biomass. Parts of the biomass processing have already been conducted at laboratory scale at Wageningen University. Conversion of synthetic glutamic acid has been studied to produce bulk chemicals such as N-methyl pyrrolidone [79], succinonitrile [80, 81], and acrylonitrile [82]. Expecting that synthetic glutamic acid can later be replaced by the one isolated from biomass, the downstream process to separate amino acids from mixtures has also been conducted [83, 84]. In order to complete the overall biorefinery process from biomass to bio-based products, extraction or hydrolysis of protein and or amino acids from biomass should now get the highest priority [85].

1.6. Research questions and outline of this thesis

An initial approach of biomass refinery for protein is provided in this thesis. Alkaline protein extraction is first selected as the main method to be studied, based on previous studies at different pHs. Several research questions need to be answered. The first question is: How much protein can be extracted under alkaline conditions? Since different types of biomass may give different results, the next question to be answered is: Which types of biomass are suitable as protein feedstock and can we find criteria for selection? To further maximise the alkaline extraction, an additional question needs to be answered. Can enzymes, especially proteases, assist in protein extraction? To come to a process design, calculations on economic feasibility need to be conducted, in which the protein can be valorized as feed. The final research question is if it is technically feasible to further valorize the proteins to bulk-chemicals. For this a model process needed to be designed, based on an abundant agricultural residue (wheat gluten was chosen to

represent DDGS) and yielding an amino acid that has proven applicability for bulk chemicals production (in this case, glutamic acid)

To answer the above questions, **Chapter 2** provides a literature study on the currently available alkaline protein extraction methods, focussing on biomass types, processing conditions, and enzyme assistance with proteases and carbohydrolases. **Chapter 3** describes the possibility of extracting protein from 16 biomass resources that are considered as agricultural residues or related components. A three step alkaline extraction is performed and statistical analysis is conducted to determine how biomass chemical composition affects the protein extraction yield. **Chapter 4** describes the possibility of proteases in assisting protein extraction from oilseed meals. The effects of an enzyme type, dosage and time are discussed in detail. **Chapter 5** describes the possibility to mildly produce amino acids from wheat gluten. Considered as agricultural by-products, wheat gluten was selected as the source of glutamic acid. With the historical knowledge on glutamic acid production from wheat gluten using concentrated HCl, a relatively mild extraction and hydrolysis method is developed. **Chapter 6** discusses the overall results obtained in this study and evaluates the future application of this study, also putting it into an economical perspective.

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Chapter 2

Towards plant protein refinery: review on protein extraction using alkali and potential enzymatic assistance

Abstract

The globally increasing protein demands require additional resources to those currently available. Furthermore, the optimal usage of protein fractions from both traditional as new protein resources, such as algae and leaves, is essential. Here, we present an overview on alkaline plant protein extraction including the potentials of enzyme addition in the form of proteases and/or carbohydrolases. Strategic biomass selection, combined with the appropriate process conditions can increase protein yields after extraction. Enzyme addition, especially proteases, can be useful when alkaline protein extraction yields are low. These additions can also be used to enable processing at a pH closer to 7 to avoid the otherwise severe conditions that denature proteins. Finally, a protein biorefinery concept is presented that aims to upcycle residual biomass by seperating essential amino acids to be used for food and feed, and non-essential amino acids for production of bulk chemicals.

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Sari YW, Mulder WJ, Sanders JPM, Bruins ME. Towards plant protein refinery: review on protein extraction using alkali and potential enzymatic assistance.

1. Introduction

Protein has a broad range of applications for food and feed, indicating its efficacy. In addition, protein can also be used for producing nitrogen-containing chemicals by taking advantage of the presence of the amine group (-NH_2). In petroleum-based conversion of crude oil into chemicals, co-reagents such as ammonia have to be used, and various process steps are involved. With the amine in protein less enthalpy is required compared to the petrochemical route to chemical products and various co-reagent introducing process steps can be by-passed, [1]. As protein is highly demanded for food, feed, and chemicals, effective and efficient ways for protein usage are to be considered.

Cereal and oilseeds are two major vegetable protein resources [2] from which wheat gluten, soybean and corn proteins are the most widely used plant proteins. It is projected that the amount of protein produced from major crops such as wheat, soybean, and corn, will not meet the global protein demand for food and feed, let alone for chemicals. It is therefore suggested to seek alternative protein resources. Nowadays, only 35% of total protein (as equivalent to the nitrogen) produced from the agricultural sector is used for food. The remaining protein is allocated for feed and non-food applications, or even not used at all but regarded as waste [3]. With the growth in world population, more protein is required for food. As people tend to eat more meat, more protein is also needed for feed. Alternatives on protein resources and direction on protein usage for specific needs are therefore required. Protein from edible plant parts can be directed to food while the remainder can be used for feed or chemicals. The use of by-products as protein resources will provide more proteins, possibly after biorefinery. One of the challenges in protein refinery is the optimal extraction of protein from biomass.

This paper provides a review on plant protein extraction using alkali and is extended to extraction that also uses enzymes to assist this process. At first, a general perspective on protein refinery for food, feed, and chemicals is given. Subsequently, several types of biomass are discussed that serve as common or novel protein resources, followed by methods on protein extraction using alkali and enzymes. At last, concluding remark and outlook for research and innovation on protein extraction are provided.

2. Protein biorefinery for food, feed, and bulk chemicals

In the development of a biobased society, new resources or better usage of current resources is needed. Biorefinery is a means to aid in the valorization of product streams that are currently regarded as waste or less valuable side products. Protein fractions for instance will be an abundant residual product from the boost in production of transportation fuels. However, this protein is often severely treated during processing and as a consequence has lost its structural functionality, but has retained its nutritional value. Both food/feed and chemicals can be made out of it by using the essential amino acids as animal feed, and the other amino acids as building blocks for bulk functionalized chemicals. This solution can produce both “food and fuel”, thereby aiding society in effective land usage.

The success of new bio-based product manufacturing will heavily depend on engineering solutions, in which separation has a crucial role. In our group, we study the whole route from biomass, via protein and amino acids, to bulk chemicals. Alkaline extraction of protein from biomass is an important first step and is therefore the main focus for this article. Alkaline protein extraction is sometimes combined with the usage of proteases or carbohydrolases to increase extractability. Proteases can also be used subsequently to hydrolyse protein and peptides to single amino acids. This step is very important within the protein biorefinery, but still insufficiently developed. From the mixture, single amino acids can then be recovered. In the case of amino acids it is difficult to separate on size, and other methods have to be chosen. Electrodialysis can be used, if necessary combined with a conversion step [4, 5]. Another technique to be used is anti-solvent crystallisation (still under development). After separation, the essential amino acids can then be used for food or feed, while the non-essential ones can be converted to bulk chemicals. This concept we call the protein biorefinery to food, feed, and bulk chemicals.

3. Biomass type

Agricultural products can contain substantial amounts of proteins. A number of proteins, both derived from plants and animals, have been produced commercially for a long time. Traditionally, food and feed proteins can be found in different types of biomass; in cereals (such as gluten in wheat and zein in corn), legumes (such as pea), oil crops (such as soy seeds, sunflower seeds), in dairy products (such as casein in milk), meat, fish and tubers (such as potato).

The selection of biomass for protein extraction is the first step in the overall protein biorefinery. Because raw material cost are predominant in the overall balance, it can be important to look for cheap alternatives. In the production of amino acids from protein this means, looking at protein sources with sufficient protein present, but with relatively low biomass price. This e.g. excludes soybean meal as a source, since it can already be sold as high quality feed. However, rapeseed meal is much cheaper, and cannot be used for feed in large amounts, due to the presence of anti-nutritional components. Still, it contains sufficient amounts of protein for the production of amino acids for feed and chemicals production.

Many industrial crop protein resources come from crops that are mainly used as resources for other primary products. Wheat and corn, soybean and rapeseed are primarily used as starch and vegetable oil resources, respectively. In addition to these traditional protein resources, energy crop residues, with biofuel as their primary product, gain interest as they have high protein content [6]. With the vast production of both industrial and energy crops, enough protein resources are potentially available.

The different techniques used for the production the primary products like starch and oil, can be regarded as a pre-treatment for protein production. These are not always beneficial for protein quality and result in different levels of available protein. A beneficial pre-treatment is e.g. the mild technique used in starch production that actually contributes to the ease of cereal-based protein extraction [7]. The contrary is true for oilseed-based protein. To this protein a high temperature has already been applied during the hexane removal after vegetable oil extraction [8] leading to lower quality protein.

Next, different types of biomass will be discussed further as a protein source for alkaline extraction, with a focus on the pre-treatment that often occurs when extracting for the primary product. Oilseeds and cereals are chosen as important current sources of biomass protein. In addition, protein extraction from less conventional types of biomass like microalgae and leaves will also be discussed.

3.1 Oilseeds

After oil, protein is the second largest component that is present in oilseeds such as soybean, rapeseed, and sunflower. The oil in the oilseed is commonly collected by solvent extraction, usually with hexane. To facilitate oil release, cell wall hydrolysing enzymes, such as cellulase, hemicellulase, and pectinase, have been tested [9]. The oil removal often increases the relative protein extraction yield [9] after the mechanical [10, 11], hexane, or enzymatic [9] treatment during oil extraction. Following oil extraction, oilseed contains as much as 30-50% protein. With this high protein value, oilseed is one of the most important plant protein resources and is already used for that.

In terms of production, soybeans are by far the most important oilseeds. In current processing, soybeans are cleaned, dried, dehulled, conditioned with steam and flaked. The oil in the flakes is collected by counter-current solvent extraction, usually with hexane as the solvent. After oil extraction, hexane remains are removed by desolventization. This is conducted at high temperature, causing denaturation of protein. Soybean protein products range from full fat soybean flours (having a protein content of about 40%) till soybean protein isolates (protein contents above 90%). Defatted soybean flours, which are mainly used in feed but also in some food products, are the most important flours in terms of amounts being produced. They are prepared by milling defatted soybean flakes. Soybean protein isolates are obtained by alkaline extraction from defatted soybean flakes followed by precipitation of proteins at their iso-electric point. Most of oilseed protein extraction is conducted at pH 9-10 (Table 1). In this pH range as much as 71-84% [11, 12], 40-70% [10, 12], and 29-41% [13] of soybean, rapeseed, and sunflower protein was extracted, respectively. This indicates that protein from soybean is much easier obtainable than that of rapeseed, and both are easier than sunflower protein. For some oilseed meals, such as rapeseed meal, extraction yield is increased as pH is increased. However, very high pH does not increase protein yield considerably for soybean meal [12], as solubility of soybean protein is already high at pH 9-10 [14].

Oilseed proteins from meals that are applied in food and feed sometimes need special treatment before protein extraction. An example is phenol removal from sunflower oilseed meal as phenol is considered a toxic compound [13, 15]. Unfortunately, phenol removal can reduce the overall yield. In some cases only 20-35% sunflower was recovered compared to the case without phenol removal. However, it does increase the protein content in the concentrate, afterwards [15].

In the case of soybean, the precipitated proteins are, usually after neutralization, spray dried. Additional processing steps may be incorporated (e.g. jet cooking prior to spray drying) to improve functional properties of the proteins. Soybean proteins are mainly used as a functional protein ingredient in a large variety of food products.

3.2 Cereals

Cereals have a much lower protein content than oilseeds, typically about 10-12% [16]. This low value is the reason that cereals have been underutilized as a protein resource. However, with the abundant amount of cereal production for both food and biofuel production, vast cereal protein is and will be available. With 1500 million tonnes of cereal (rice, wheat, and oat) production in 2013, 180 million tonnes protein is produced. This value is comparable to the 200 million tonnes oilseed protein, coming from 400 million tonnes oilseed (soybean, rapeseed, and sunflower) production [17].

Industrially, cereals are being used for the production of starch. Aqueous treatment washes off cereal starch and leaves the insoluble protein; such as gluten in wheat. Cereal proteins are rich in sulphur containing amino acids [16]. The presence of these amino acids results in formation of disulfide cross-linking that lowers protein solubility [18]. The use of alkali can break these linkages [18]. In addition, all neutral and acidic amino acids will be ionized at alkali pH, thus increasing protein solubility. As example, an overnight treatment at pH 8.5 (55 °C) solubilized only 40% of wheat gluten protein [19]. When pH is increased into pH 10 (25 °C), more wheat gluten protein can be solubilized (85%) [20]. It is also possible to get to similar amounts of solubilized wheat gluten at lower pH, e.g. by adding salts such as KCl into a wheat gluten suspension at pH 8 [21].

Next to using the part of protein that is present alongside the starch in the kernel, it may be possible to extract protein from other parts of the grain, such as the hull or the bran. Although not commercially available [22], e.g. rice bran protein exhibits good emulsifying and foaming properties suitable for food protein [23]. Rice bran is a by-product after milling and de-husking rice grain [24] that is an example of an interesting protein source due to the vast availability of rice bran. Alkaline extraction at pH 11 and 60 °C resulted in only a 13% yield [23]. However, a multi-solvent sequential extraction left only 2% protein insoluble. Using water, NaCl, ethanol, acetic acid and NaOH at 20 °C, respectively 34, 15, 6, 11, and 32% protein was recovered [18].

Large amounts of protein-containing spent or distillers grain are produced in malt, brewery or bio-ethanol production. The ability to extract protein from distillers grain will not only give added value by taking advantage of producing high market value protein but also will improve the economic viability of ethanol facilities. However, the low protein extraction yield from distillers grain needs further optimization. Alkaline treatment of barley spent grain with 0.1 M NaOH at 60 °C extracted 41% of its protein [25]. Under similar condition, less than 15% of corn wet distillers grain with solubles was extracted. Surfactant addition (2% sodium dodecyl sulfate) increased this yield to 32% [26].

3.3 Novel protein sources

There is an increasing interest in new sources of protein rich biomass that is caused by the demand for alternative protein sources for food and feed and the increasing interest in protein usage for bio-based applications. The biorefinery of most of the new protein sources however is still in the research phase. The main protein-rich plant biomass that is currently studied are micro- and macro-algae, and green crops such as grass, alfalfa and leaves

3.3.1 Microalgae

Historically, microalgae have been considered as a source of proteins for foods, but were not considered interesting because of the co-extracted colour from non-protein components. Only after that microalgae gained interest again because of their oil for fuel production, their protein fraction was studied more intensively once more. Recently, a method has been developed to extract protein from *Tetraselmis* sp. with emphasis on discolouring the microalgae protein isolate to meet a broad range application of the final isolate. [27] This developed method still requires optimization as only 21% protein was extracted into the liquid phase, following microalgae mechanical extraction using bead milling.

Microalgae are easy to cultivate, fast growing, and having high energy per hectare leading to considerations for use as energy resource [28-30]. Protein concentration in algae can be as high as 40-50% (w/w) based on dry weight, depending on the strain used. Although analytical methods for determining protein content are available, no sufficient extraction methods have been published for the mild, preparative extraction of proteins from microalgae, in which the functional properties of the proteins are retained. Generally speaking, algae are hard to disrupt and protein extraction efficiencies are not high. Therefore more extreme, alkaline extraction might be an option. But also here, final protein recovery is not high. The effectiveness of the alkaline extraction is highly dependent on the microalgae strain. Alkaline treatment of *Nannochloropshis* spp at pH 11 and 60 °C extracted a maximum of 16% protein [31], while under similar conditions (pH 11, 60 °C) a maximum yield of 40% was obtained from *Chlorella fusca* [12].

3.3.2 Leaves

Green plants and leaves have a huge potential for production of proteins, due to their vast availability. Important crops that can be used for the production of proteins are grass, beet leaves, alfalfa, and spinach leaves. Protein production from leafy biomass coming from biofuel production can mitigate the debate on food versus fuel, particularly when leaf protein is co-produced in a biofuel plant [32].

Protein products from leaves are usually obtained by pressing or shearing the fresh crops, followed by heat coagulation of the proteins, centrifugation and drying. Because of the coagulation process that is performed at elevated temperature, the proteins loose most of their functional, but not their nutritional properties. Grass and lucerne juices have been fed to animals and it was shown that these leaf proteins could function as alternatives for e.g. fish protein or soybean meal.

Different types of alkali have been used to extract protein from several types of leaves. Not only NaOH or $Ca(OH)_2$, but also ammonia [33] were tested, sometimes in

combination. Although the amount of easy to solubilize protein from leaves may be low when only water extraction is used, addition of alkali can increase protein extraction yields. Even yields up to 95% protein can be obtained, but only when a combination of high temperature (95°C) and alkali was applied [34]. Only NaOH could be used here, because of the high pH that was needed in this case.

Combinations of alkali also enable an increase in protein yield. The effectiveness of ammonia as pre-treatment agent in extracting protein has been tested on dwarf elephant grass [35] and cassava leaves [36]. Following this, calcium hydroxide was used to extract protein. A combination of ammonia pre-treatment and calcium hydroxide seems to drastically increase protein extraction in dwarf elephant grass. A maximum of 12% and 53% of untreated and ammonia-treated dwarf elephant grass protein was extracted, respectively. However, this combination seems less effective for extracting protein from cassava leaves. Only 30% of ammonia-treated cassava leave protein was extracted. This value was only 10% higher than that of untreated cassava leaves. Another method to pre-treat biomass with ammonia is through ammonia fibre explosion (AFEX). As much as 20- 55% protein was extracted from pre-treated switch grass [32, 33]. The use of surfactant or reducing agent in combination with 3% ammonia did not significantly improve protein extraction yields [33].

4. Alkaline extraction and process conditions

After appropriate biomass selection, cell disruption and extraction of proteins are essential. Alkaline treatment simultaneously disrupts the cell wall and extracts protein. Protein extractability is influenced by extraction conditions such as biomass to solvent ratio, temperature, time, and pH. The data from different experiments (Table 1) were analysed and conclusions were drawn on the importance of each process parameter.

Table 1. Protein extraction under alkaline conditions

No.	Biomass	Pre-treatment	Biomass to solvent ratio	Solvent	pH	Temp. (°C)	Time (min)	Content in biomass (% w/w)	Yield (%)[a]	Content in supernatant (%)[b]	Refs.
1.	Soybean	Flaking	1:10	NaOH adjusted deionized water	9	50	60	39	71	n.a	[11]
		Extrusion	1:10	NaOH adjusted deionized water	9	50	60	40	57	n.a	
2.	Soybean	Deoiled and flaked	1:6	NaOH adjusted deionized water	7	60	180	61	55	n.a	[37]
3.	Soybean	Deoiled	1:20	NaOH adjusted deionized water	9	60	45	38	33	n.a	[38]
4.	Soybean	Deoiled	1:11	NaOH adjusted deionized water	9.5/10/11	60/60/60	180	36	84/80/80	n.a	[12]
5.	Rapeseed	Dehulled and deoiled	1:18	NaOH adjusted deionized water	9.5-12	room	30	46	40-70	n.a	[10]
6.	Rapeseed	Deoiled	1:15	NaOH adjusted deionized water	9.5/10/11	60/60/60	180	26	15/31/43	n.a	[12]
7.	Rapeseed	Deoiled	1:20	0.1 -0.4 % w/v NaOH		room	60	32	70-92		[39]
8.	Sunflower	Deoiled	1:10	NaOH adjusted deionized water	9	20	60	32	46	47	[15]
		Deoiled and dephenolisation with water	1:10	NaOH adjusted deionized water	9	20	60	32	35	76	
		Deoiled and dephenolsation with 0.1% w/v Na_2SO_3	1:10	NaOH adjusted deionized water	9	20	60	32	35	71	

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Table 1. *Continued from previous page*

No.	Biomass	Pre-treatment	Biomass to solvent ratio	Solvent	pH	Temp. (°C)	Time (min)	Content in biomass (% w/w)	Yield (%)[a)]	Content in supernatant (%)[b)]	Refs.
		Deoiled and dephenolisation with 70% v/v ethanol	1:10	NaOH adjusted deionized water	9	20	60	32	25	63	
		Dephenolisation with 80% v/v methanol	1:10	NaOH adjusted deionized water	9	20	60	32	22	60	
		Deoiled and dephenolisation with 92:8 v/v 1-buthanol in 0.005 N HCl	1:10	NaOH adjusted deionized water	9	20	60	32	19	59	
9.	Sunflower	Deoiled	1:15	NaOH adjusted deionised water	9	20	60	32	41	41	[13]
		Deoiled dephenolization with water	1:15	NaOH adjusted deionised water	9	20	60	32	26	66	
		Deoiled and dephenolization with 1 g/l Na_2SO_3	1:15	NaOH adjusted deionised water	9	20	60	32	29	62	
10.	Sunflower	Deoiled	1:10	Na_2SO_3+KOH	10.5	20/40/60	60	27	14/15/18	n.a	[40]
11.	Peanut	Deoiled	1:10	0.25 - 1 % NaOH		30	60	45	17-24	n.a	[41]
12.	Rubber seed kernel	Pre-dried 60 °C	1:10	0.1 N NaOH		60	60	17	78		[42]

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Table 1. *Continued from previous page*

No.	Biomass	Pre-treatment	Biomass to solvent ratio	Solvent	pH	Temp. (°C)	Time (min)	Content in biomass (% w/w)	Yield (%)[a)]	Content in supernatant (%)[b)]	Refs.
13.	*Jatropha curcas* kernel		1:20	Deionised water		20	30	23	15	14	[43]
			1:20	0.1/ 0.55/ 1M NaCl		20	30	23	17/23/34	12/7/6	
			1:20	0.01/ 0.055/ 0.1 M NaOH		20	30	23	43/70/66	34/36/30	
14.	*Jatropha curcas* seed	Deoiled	1:20	Deionised water		20	30		7	23	[43]
			1:20	0.1/ 0.55/ 1 M NaCl		20	30		10/15/19	14/7/5	
			1:20	0.01/ 0.055/ 0.1 M NaOH		20	30		32/65/61	48/53/52	
15.	Lupin	Flaking	1:10	NaOH adjusted deionized water	9	50	60	47	69	n.a	[11]
		Extrusion	1:10	NaOH adjusted deionized water	9	50	60	46	61	n.a	
16.	Mustard meal	Deoiled	1:30	NaCl adjusted thin stillage from wheat	10	25	120	32	60	n.a	[44]
17.	Guar		1:10 - 1:50	Deionized water		room	40	32	20-25	n.a	[45]
			1:10 - 1:50	5 % NaCl		room	40	32	32-40	n.a	
			1:10 - 1:50	0.1 N NaOH		room	40	32	71-85	n.a	
			1:10 - 1:50	0.1 N NaOH + 5% NaCl		room	40	32	75-87	n.a	
			1:50	Deionized water		room	10	32	18-25	n.a	
			1:50	5 % NaCl		room	10	32	28-40	n.a	
			1:50	0.1 N NaOH		room	10	32	69-84	n.a	
			1:50	0.1 N NaOH + 5% NaCl		room	10	32	70-87	n.a	

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Table 1. *Continued from previous page*

No.	Biomass	Pre-treatment	Biomass to solvent ratio	Solvent	pH	Temp. (°C)	Time (min)	Content in biomass (% w/w)	Yield (%)[a)]	Content in supernatant (%)[b)]	Refs.
18.	Pennycress seed		1:30	Deionized water		5/21/77		20	12/21/35	23/26/25	[46]
			1:30	0.5 M NaCl		5/21/77		20	17/12/14	50/36/31	
			1:20	70%EtOH/Water		5/21/77		20	<1	n.a	
			1:30	60% AcOH/Water		5/21/77		20	4/3/3	36/38/24	
			1:25	0.1 M NaOH		5/21/77		20	1/2/6	17/17/20	
19.	Pennycress seed	Deoiled	1:30	Deionized water		5/21/77		26	24/34/22	34/41/31	[46]
			1:30	0.5 M NaCl		5/21/77		26	25/13/9	83/73/59	
			1:20	70%EtOH/Water		5/21/77		26	<1	n.a	
			1:30	60% AcOH/Water		5/21/77		26	14/16/15	61/58/47	
			1:25	0.1 M NaOH		5/21/77		26	8/7/5	37/33/20	
20.	Rice bran	Deoiled	1:5	NaOH adjusted deionized water	11	30	45	18	48 mg/g	n.a	[47]
21.	Rice bran	Deoiled	1:6	NaOH adjusted deionized water	11	60	60	15	13	38	[23]
22.	Corn germ		1:5	Na_2SO_4	9	room	60		8 mg/g solid	n.a	[48]
23.	Corn endosperm		1:5	Na_2SO_4	7	room	60		2 mg/g solid	n.a	[48]
24.	Corn wet distiller grain	Ammonia fiber exploded	1:20	0.1 M NaOH		30/60/70	60	30	7/12/13	n.a	[26]
		Ammonia fiber exploded	1:20	0.1 M NaOH + 0.2%SDS[c)]		70	60	30	32	n.a	
25.	Barley spent grain		1:6	0.1 M NaOH		60	60	25	41	60	[25]
26.	Barley mill run		1:10	0.55 N NaOH	10	25	1200	11	33	n.a	[20]

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Table 1. *Continued from previous page*

No.	Biomass	Pre-treatment	Biomass to solvent ratio	Solvent	pH	Temp. (°C)	Time (min)	Content in biomass (% w/w)	Yield (%)[a)]	Content in supernatant (%)[b)]	Refs.
27.	Barley rootlets		1:10	0.55 N NaOH	10	25	1200	26	35	n.a	[20]
28.	Wheat gluten		1:10	0.55 N NaOH	10	25	1200	78	85	n.a	[20]
29.	Wheat middling		1:10	0.55 N NaOH	10	25	1200	16	73	n.a	[20]
30.	Microalgae *Nannochloropsis*	Deoiled	1:20	NaOH adjusted deionised water	9 11 13 13	30/45/60 30/45/60 30/45/60 60	960 960 960 120		<2/<2/<2 <2/<2/<2 6/12/12 12	n.a n.a n.a n.a	[31]
31.	Microalgae *Dunaliella primolecta*	Freeze-dried	1:50	TCA [d)] followed by alkali (Lowry reagent D)		TCA[d)]: 95 Alkali: 55	TCA[d)]: 15 Alkali: 120			12	[49]
32.	Microalgae *Tetraselmis sp*	Freeze-dried	1:50	TCA [d)] followed by alkali (Lowry reagent D)		TCA[d)]: 95 Alkali: 55	TCA[d)]: 15 Alkali: 120			5	
33.	Microalgae *Chlorella ovalis*	Freeze-dried	1:50	TCA [d)] followed by alkali (Lowry reagent D)		TCA[d)]: 95 Alkali: 55	TCA[d)]: 15 Alkali: 120			11	
34.	Microalgae *Chlorella spaerckii*	Freeze-dried	1:50	TCA [d)] followed by alkali (Lowry reagent D)		TCA[d)]: 95 Alkali: 55	TCA[d)]: 15 Alkali: 120			7	
35.	Microalgae *Monodopsis subterraneae*	Freeze-dried	1:50	TCA [d)] followed by alkali (Lowry reagent D)		TCA[d)]: 95 Alkali: 55	TCA[d)]: 15 Alkali: 120			17	

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Table 1. *Continued from previous page*

No.	Biomass	Pre-treatment	Biomass to solvent ratio	Solvent	pH	Temp. (°C)	Time (min)	Content in biomass (% w/w)	Yield (%)[a]	Content in supernatant (%)[b]	Refs.
36.	Microalgae *Nannochloropsis oculata*	Freeze-dried	1:50	TCA[d] followed by alkali (Lowry reagent D)		TCA[d]: 95 Alkali: 55	TCA[d]: 15 Alkali: 120			13	
37.	Microalgae *Rhodella violaceae*	Freeze-dried	1:50	TCA[d] followed by alkali (Lowry reagent D)		TCA[d]: 95 Alkali: 55	TCA[d]: 15 Alkali: 120			2	
38.	Microalgae *Chlorella fusca*	Deoiled	1:10	NaOH adjusted deionized water	9.5/10/11	60/60/60	180	37	34/40/37	n.a	[12]
39.	Microalgae *Chlorella fusca*		1:14	NaOH adjusted deionized water	9.5/10/11	60/60/60	180	28	14/35/16	n.a	[12]
40.	Macroalgae *Palmaria palmata*		1:20	0.08 - 0.12 M NaOH + 5 % (w/v) N-acetyl-L-cysteine (NAC)	11.5 - 12.68	room	60		5-7 g/100 g biomass	n.a	[50]
41.	Green tea leaf residue		1:35	0.05-0.14 M NaOH		30	120		7-35	n.a	[51]
			1:35	0.06 M NaOH		25-50	120		27-41	n.a	
			1:35	0.06 M NaOH		30	30 - 360		17-38	n.a	
			1:20 - 1:50	0.06 M NaOH		30	120		13-38	n.a	
42.	Green tea leaf residue		1:40	0.1 N NaOH		25/40/60/80/95	240	26	24/30/45/78/95	n.a	[34]

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Table 1. *Continued from previous page*

No.	Biomass	Pre-treatment	Biomass to solvent ratio	Solvent	pH	Temp. (°C)	Time (min)	Content in biomass (% w/w)	Yield (%)[a)]	Content in supernatant (%)[b)]	Refs.
43.	Dwarf elephant grass	Ammonia treated	1:10	CaOH	10	60	5 – 60	12	34	n.a	[35]
			1:10	CaOH	11/12/ 12.6	60	30	12	35/38/ 46	n.a	
			1:10	CaOH	12.6	30-90	30	12	39-53	n.a	
			1:15	CaOH	12.6	30-90	30	12	41-53	n.a	
44.	Switchgrass		1:11	3% NH_3	10.5	25-70	3	7	37-57	n.a	[33]
			1:11	1-10% NH_3	10.5	50	3	26	52-55	n.a	
			1:11	3% NH_3	8-11.5	25	3	26	35-58	n.a	
45.	Ryegrass		1:10	0.55 N NaOH	10	25	1200	9	8	n.a	[20]
46.	Tomato seed	Deoiled hot break	1:30	NaOH adjusted deionised water	9	room	60	39	14	22	[52]
			1:30	0.5 M NaOH	11	room	60	39	18	22	
			1:30	1% NaOH	13	room	60	39	38	17	
47.	Tomato seed	Deoiled cold break	1:30	NaOH adjusted deionized water	9	room	60	41	38	64	[52]
			1:30	0.5 M NaOH	11	room	60	41	43	60	
			1:30	1% NaOH	13	room	60	41	45	21	

a) Yield is expressed as mass protein recovered in supernatant relative to mass protein in initial biomass. b) Content in supernatant is expressed as mass protein in supernatant relative to mass total solid recovered in supernatant.
c) SDS: sodium dodecyl sulphate. d) TCA : Trichloroacetic acid. n.a = not available.

4.1 Extraction time and temperature

Time and temperature have important effects on protein extractability. Most of the studies cited in Table 1 opted for less than 100 minutes extraction time and 50-60°C as the extraction temperature. Figure 1 shows a scatter plot generated from the data from Table 1. From this plot, no general optimal temperature or time could be found. Within experiments, an increase in either time or temperature generally increased protein yields as could be expected. An increase of extraction temperature from 25°C into 50°C, with a fixed extraction time (2 h), resulted in an increased yield from 25% into 40% for tea protein [51]. Even more protein is extracted when the extraction temperature was increased to 95°C. As much as 95% protein was extracted at this temperature, with 0.1M NaOH for 4 h, while only 20% was obtained at 25 °C [34]. Another study of the effect of temperature, ranging from 25, to 60, to 120°C, on extracting protein indicated that proteins from several biomass were already mostly extracted at 25°C [20]. In these experiments, protein extraction was performed overnight, extracting up to 85% biomass proteins, illustrating that also time can be used to increase yields. The long extraction time provided more time for diffusion of the protein into the solvent. Also other studies showed an increase of the yield from e.g. 12% to 38% in the case of tea when the extraction time was extended from 1 h into 6 h [51]. However, extremely long times of incubation (1000 or 1200 h, see Fig. 1) are not suggested due to the risk of microbial growth.

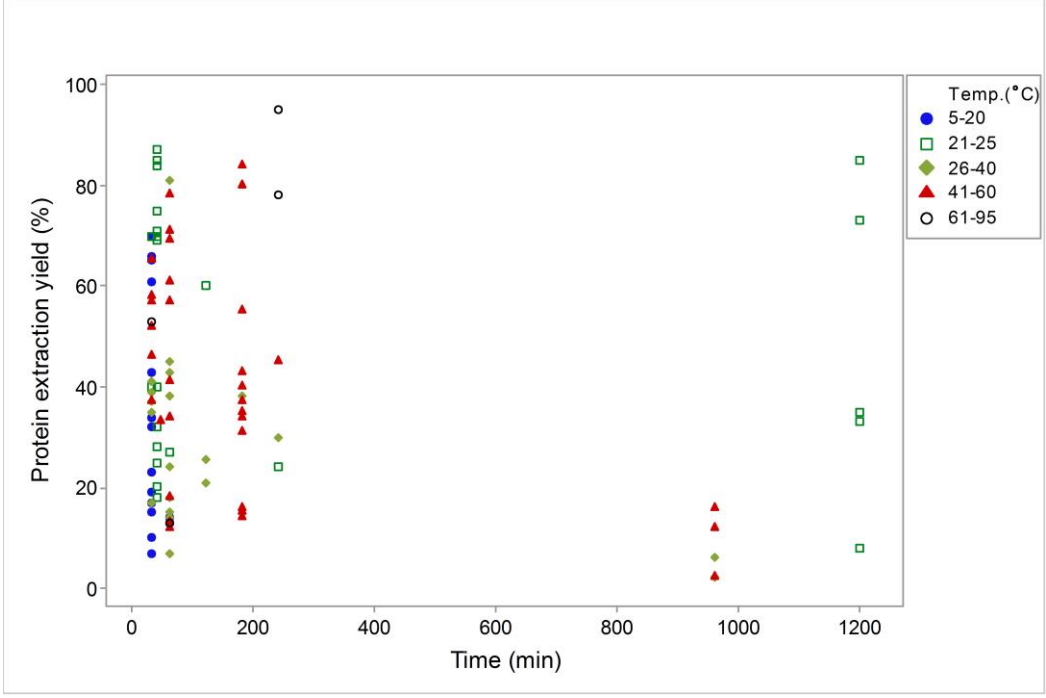

Figure 1. A scatter plot of time, temperature, and yield generated from data in Table 1.

4.2 Biomass to solvent ratio

A minimum amount of solvent is required to attain fast protein diffusion during extraction. Using an excess amount of solvent, protein concentration in the biomass will be higher than in the solvent, which is the driving force for the protein to go in solution, whilst maximal protein solubility in the liquid is not reached. A study on the influence on biomass to solvent ratio indicated that an increase of 20% protein yield was still obtained when biomass to solvent ratio was decreased from 1:20 into 1:40. Only an addition of 3% protein yield was obtained when more solvent was added, using a biomass to solvent ratio of 1: 50 [51], for tea leaves, showing that the maximal absolute yield was obtained.

Although more protein could be extracted, an extreme amount of solvent is unlikely to be used on industrial scale because of the high costs. Next to higher cost for chemicals, equipment, and energy, overall costs also increase due to an increase in cost for downstream processing for protein recovery that is now present in very low concentrations. An extreme amount of solvent also raises environmental issues such as the excess formation of salt, when a protein precipitation step is performed. A typical biomass to solvent ratio of 1:10 was selected in some studies (Table 1). This use of solvent can be reduced, while maintaining good protein extraction efficiency, by using counter current extraction [43].

4.3 pH

pH is one of most important factors determining protein extraction yield. The influence of pH on protein extraction may occur through two mechanisms, by altering cell wall and protein properties.

Cell wall provides a barrier for protein diffusion into the solvent. Thus, one approach to obtain a high protein yield is to degrade the cell wall. Solvent acidity influences the extent of cell wall degradation. Referring to the pre-treatment of lignocellulosic biomass for bioethanol, acid has been extensively tested to disrupt biomass cell wall, mainly aiming for cellulose degradation. Although acid has the capability to alter the cell wall, it does not aid in increasing protein extraction yield [12, 48]. Treatment of fibrous material at alkaline pH can also result in the disruption of cell wall surface properties. This involves a reduction in surface tension, partial removal of lignin, and complete removal of acetyl or uranic esters group of hemicellulose [53]. Also crystallinity of cellulose can be reduced following alkaline treatment [54].

Alkali does not only disrupt the cell wall, it also changes protein solubility. Most proteins have the lowest solubility at their isoelectric point, which mostly occurs around pH 4-5. Fig. 2 shows the dependence of soybean protein extraction on pH and the high recovery at alkaline pH. A recent study indicated that not only pH, but also the absolute amount of alkaline is of importance when aiming for protein extraction [34]. Solvent amount and pH can then vary. The importance of alkali amount is due to the fact that during alkaline protein extraction, other components in the biomass can react with the alkaline, such as lignin, thereby buffering the system [55]. Without these interfering components, only a limited amount may be needed for the actual protein extraction.

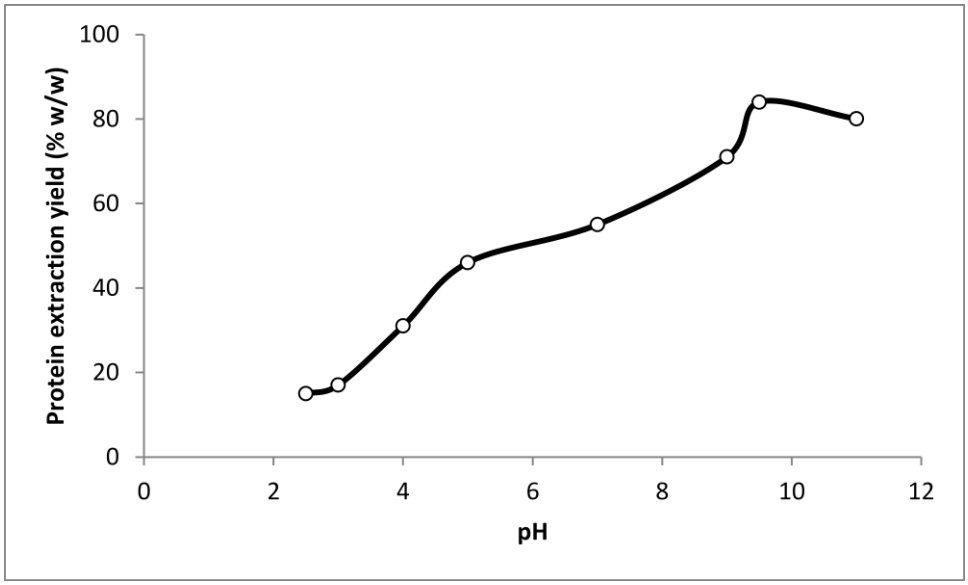

Figure 2. Soybean protein extraction yield obtained at various pH [12, 37, 56].

4.4 Combining high temperature and alkali

Protein properties are changed along the extraction process, particularly following severe heat or alkaline treatment [57]. These changes include denaturation, racemization, and lysinoalanine formation. The denaturation might result in poor protein functionality [58]. The racemisation from the naturally occurring L-form into D can reduce protein nutritional value and safety by creating non-metabolisable and biologically non-utilisable forms of amino acids through D-D, D-L, and L-D peptide bonds [59].

In addition to racemization, lysinoalanine is formed in alkali-treated protein. This cross linked unusual amino acid is formed concurrently with racemization. The presence of lysinoalanine also reduces protein nutritional value [59]. The poor protein nutritional value along with poor protein functionality resulting from changes in protein properties is not preferable for food or feed application. Thus relatively mild protein production is preferable for food applications.

To a certain extent, changes in protein properties are required for technical applications. Controlled denaturation of protein can be used to obtain useful protein for technical applications such as coating, adhesive, plastic, and surfactant [60]. Protein that is not used in food or feed can be used as feedstock for amino acid derived bulk chemical production [1] according to the "protein biorefinery for food, feed, and bulk chemicals" concept. When the amino acids are chemically converted to their end-product, it often doesn't matter what enantiomer is used.

5. Enzyme addition

Proteases and carbohydrolases are two types of enzymes that have been applied to assist in protein extraction (Table 2). Adding enzymes during protein extraction increased protein yield, as indicated by the Δ yield with the control experiment without enzyme in Table 2. Proteases assist through proteolysis while carbohydrolases assist by degrading (parts of) the cell wall. Since this article focuses on alkaline extraction, only alkaline proteases are listed in Table 2. Another study reported work on acidic proteases but without improvement in protein yield [12]. In contrast to proteases, most of studies involving carbohydrolases were performed at neutral to acidic pH conditions and therefore acidic examples are also included for carbohydrolases. Most of the studies used proteases and carbohydrolases separately. However, they may also be used in combination. Still, protease is more effective in extracting biomass protein as indicated in Fig. 3. A combination of protease and carbohydrolase, surprisingly does not extract more protein compared to the use of protease only.

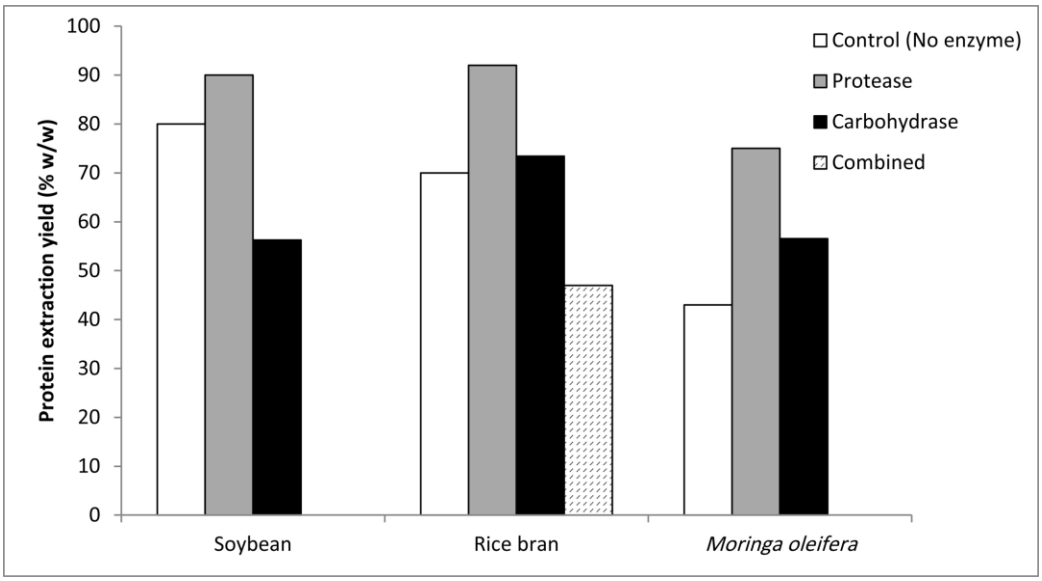

Figure 3. Effect of protease, carbohydrolase, combination of these enzymes, and control (without enzyme). The data presented are the maximum yield obtained by each type of enzyme for corresponding biomass as listed in Table 2. Protease data on soybean [12], rice bran [61], *Moringa oleifera* [62]. Carbohydrolase data on soybean [38], rice bran [63], *Moringa oleifera* [62]. Combined enzyme data on rice bran [64]. Control data on: soybean [12], rice bran [65], *Moringa oleifera* [62].

Table 2. Protein extraction with enzyme addition

No	Biomass	Pre-treatment	Biomass to liquid ratio	Enzyme				pH	Temp. (°C)	Time (min)	Yield (%)[a]	ΔYield with control (without enzyme) (%)	Content in supernatant (%)[b]	Refs.
				Name	Type	Amount	Unit							
1.	Soybean	Flaked	1:10	Protex 6L	Protease	0.50%	g enzyme/ g biomass	9	50	60	77	6	n.a	[11]
		Extruded	1:10	Protex 6L	Protease	0.50%	g enzyme/ g biomass	9	50	60	85	27	n.a	
2.	Soybean	Flaked	1:10	Multifect Neutral	Protease	0.50%	g enzyme/ g biomass	7	50	60	79	n.a.	n.a	[66]
3.	Soybean	Flaked	1:10	Protex 7L	Protease	0.50%	g enzyme/ g biomass	7	50	60	76	2	n.a	[56]
		Flaked and extruded	1:10	Protex 7L	Protease	0.50%	g enzyme/ g biomass	7	50	60	75	30	n.a	
		Flaked and Pressurised 200 MPa	1:10	Protex 7L	Protease	0.50%	g enzyme/ g biomass	7	50	60	81	0	n.a	
		Flaked and Pressurised 500 MPa	1:10	Protex 7L	Protease	0.50%	g enzyme/ g biomass	7	50	60	74	8	n.a	
4.	Soybean	Deoiled	1:11	Protex 5L	Protease	5%	ml enzyme/ g protein	9.5	60	180	91	7	n.a	[12]
			1:11	Protex P	Protease	5%	ml enzyme/ g protein	10	60	180	90	10	n.a	
			1:11	Protex 40XL	Protease	5%	ml enzyme/ g protein	11	60	180	88	5	n.a	
5.	Soybean	Flaked	1:10	Protex 6L	Protease	0.5%	g enzyme/ g biomass	9	50	60	85	n.a	n.a	[67]
			1:10	Protex 6L	Protease	1%	g enzyme/ g biomass	9	50	60	87	n.a	n.a	
			1:10	Protex 7L	Protease	0.5%	g enzyme/ g biomass	9	50	60	73	n.a	n.a	
6.	Soybean	Flaked	1:10	Multifect Neutral	Protease	0.50%	g enzyme/ g biomass	8	50	60	77	25	n.a	[68]
		Flaked	1:10	Cellulase	Carbo-hydrolase	1%	g enzyme/ g biomass	8	50	60	55	3	n.a	
7.	Soybean	Deoiled	1:20	Viscozyme L	Carbo-hydrolase	30 FBG		9	60	30	57	23	n.a	[38]

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Table 2. *Continued from previous page*

No	Biomass	Pre-treatment	Biomass to liquid ratio	Enzyme				pH	Temp. (°C)	Time (min)	Yield (%)[a]	ΔYield with control (without enzyme) (%)	Content in supernatant (%)[b]	Refs.
				Name	Type	Amount	Unit							
8.	Soybean	Deoiled and Flaked	1:6	Multifet GC	Carbo-hydrolase (Cellulase)	10%	g enzyme/ g biomass	4	50	180	35	5	n.a	[37]
			1:6	Multifect B	Carbo-hydrolase (Cellulase)	5%	g enzyme/ g biomass	5	50	180	48	2	n.a	
			1:6	Puradax HA	Carbo-hydrolase (Cellulase)	1/5/10%	g enzyme/ g biomass	7	60	180	60/59/ 60	5/4/5	n.a	
			1:6	IndiAge Super L	Carbo-hydrolase (Cellulase)	1/5/10%	g enzyme/ g biomass	7	45	180	58/62/ 62	5/9/10	n.a	
			1:6	Multifect Pectinase	Carbo-hydrolase (Pectinase)	1/5/10%	g enzyme/ g biomass	4	50		38/47/4 8	6/15/16	n.a	
9.	Rapeseed	Deoiled	1:15	Protex 5L	Protease	5%	ml enzyme/ g protein	9.5	60	180	64	49	n.a	[12]
			1:15	Protex P	Protease	5%	ml enzyme/ g protein	10	60	180	76	45	n.a	
			1:15	Protex 40XL	Protease	5%	ml enzyme/ g protein	11	60	180	75	36	n.a	
10.	Rapeseed		1:5	Multistep: Pectinase-cellulase-betaglucanase (4:1:1) (v/v/v)+ Alcalase 2.4L	Carbo-hydrolase + Protease	2.5 (Total carbohydrolase) + 1.4% (protease)	ml enzyme/ g biomass	Multi pH: 5-10-9	Multi temp.: 48-60	270+180	41-67	n.a.	62-74	[69]
11.	Sunflower	Deoiled	1:10	Alcalase 2.4L	Protease	0.01%	ml enzyme/ ml suspension	10			60	2	54	[70]
			1:10	Alcalase 2.4L	Protease	0.05%	ml enzyme/ ml suspension	10			76	18	52	
			1:10	Alcalase 2.4L	Protease	0.10%	ml enzyme/ ml suspension	10			87	29	61	

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Table 2. *Continued from previous page*

No	Biomass	Pre-treatment	Biomass to liquid ratio	Enzyme				pH	Temp. (°C)	Time (min)	Yield (%)[a]	ΔYield with control (without enzyme) (%)	Content in supernatant (%)[b]	Refs.
				Name	Type	Amount	Unit							
12.	Sunflower		1:6	Protex 7L	Protease		g enzyme/ g biomass		45	120	4	2	n.a	[71]
			1:6	Alcalase 2.4L	Protease		g enzyme/ ml biomass				3	1	n.a	
			1:6	Viscozyme L	Carbo-hydrolase (Multi-enzyme)		ml enzyme/ g biomass		45	120	4	2	n.a	
			1:6	Kemzyme	Carbo-hydrolase (Multi-enzyme)		g enzyme/ g biomass		45	120	2	0	n.a	
			1:6	Natuzyme	Carbo-hydrolase (Multi-enzyme) + Protease		g enzyme/ g biomass		45	120	3	1	n.a	
13.	Peanut	Roasted (130, 160, 190 °C)	1:4	Alcalase 2.4L	Protease	1%	ml enzyme/ g biomass	9.5	55	60	72	n.a.	n.a	[72]
		Roasted (220 °C)	1:4	Alcalase 2.4L	Protease	1%	ml enzyme/ g biomass	9.5	55	60	68	n.a.	n.a	
		Unroasted	1:4	Alcalase 2.4L	Protease	1%	ml enzyme/ g biomass	9.5	55	60	72	n.a.	n.a	
		Roasted (190 °C)	1:3	Alcalase 2.4L	Protease	1%	ml enzyme/ g biomass	9.5	55	60	67	n.a.	n.a	
		Roasted (190 °C)	1:5	Alcalase 2.4L	Protease	1%	ml enzyme/ g biomass	9.5	55	60	72	n.a.	n.a	
		Roasted (190 °C)	1:6	Alcalase 2.4L	Protease	1%	ml enzyme/ g biomass	9.5	55	60	74	n.a.	n.a	
		Roasted (190 °C)	1:7	Alcalase 2.4L	Protease	1%	ml enzyme/ g biomass	9.5	55	60	72	n.a.	n.a	
14.	Peanut	Deoiled	1:20	Alcalase	Protease	0.5-10	g enzyme/ g protein	7.5	60	135	30-44	17-31	n.a	[73]
		Deoiled	1:20	Papain	Protease	0.5-10	g enzyme/ g protein	7	50	135	13-35	0-22	n.a	
		Deoiled	1:20	Protamex	Protease	0.5-10	g enzyme/ g protein	7.5	50	135	18-25	5-12	n.a	

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Table 2. *Continued from previous page*

No	Biomass	Pre-treatment	Biomass to liquid ratio	Enzyme				pH	Temp. (°C)	Time (min)	Yield (%)[a]	ΔYield with control (without enzyme) (%)	Content in supernatant (%)[b]	Refs.
				Name	Type	Amount	Unit							
15.	Lupin	Flaking	1:10	Protex 6L	Protease	0.50%	g enzyme/ g biomass	9	50	60	74	4	n.a	[11]
		Extrusion	1:10	Protex 6L	Protease	0.50%	g enzyme/ g biomass	9	50	60	77	16	n.a	
16.	Rice bran	Deoiled	1:50	Alcalase 2.4L	Protease	0.50%	ml enzyme/ g protein	8	50		81	n.a.	28	[61]
		Deoiled	1:50	Flavour-zyme	Protease	0.50%	ml enzyme/ g protein	8	50		88	n.a.	30	
17.	Rice bran	Deoiled	1:40	Opticlean" L- 100"	Protease	4%	g enzyme/ g protein	8 and 10	40		90/93	20/23	n.a	[65]
		Deoiled	1:40	Opticlean" L- 100" with Na_2SO_3/SDS/Na_2SO_3 + SDS	Protease	4%	g enzyme/ g protein	8	50		79/81/85	11/13/7	n.a	
18.	Rice bran		1:5	Alcalase 0.6L	Protease	2%	ml enzyme/ g biomass	9	50	180	68	n.a.	n.a	[74]
19.	Rice bran	Deoiled	1:7.5	Finase S40	Phosphatase (Phytase)	400 PU/g bran		5	55	120	57	23`	80	[64]
			1:7.5	GC 140 xylanase	Carb-ohydrolase (Xylanase)	240 GXU/g bran		5	55	120	54	20	82	
			1:7.5	Finase S40 followed byGC 140 xylanase	Phosphatase + Carbo-hydrolase	400 PU/g bran + 240 GXU/g bran		5	55	120 + 120	73	39	89	
			1:7.5	GC 140 xylanase followed by FInase S40	Carbo-hydrolase + Phosphatase	240 GXU/g bran + 400 PU/g bran		5	55	120 + 120	70	36	90	

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Table 2. *Continued from previous page*

No	Biomass	Pre-treatment	Biomass to liquid ratio	Enzyme				pH	Temp. (°C)	Time (min)	Yield (%)[a]	ΔYield with control (without enzyme) (%)	Content in supernatant (%)[b]	Refs.
				Name	Type	Amount	Unit							
			1:7.5	Finase S40 + GC 140 xylanase	Phosphatase + Carbo-hydrolase	400 PU/g bran + 240 GXU/g bran		5	55	120	75	41	92	
			1:7.5	Finase S40 + GC 140 xylanase	Phosphatase + Carbo-hydrolase	400 PU/g bran + 240 GXU/g bran		5	55	240	72	38	91	
20.	Rice bran		1:7	Cellulast 1.5L + Alcalase	Carbo-hydrolase (Cellulase) + Protease	1% + 1%	g enzyme/ g biomass	4.5-5 +8 (carbo-hydrolase only)	50	60	48	38	n.a	[63]
								9 (carbo-hydrolase + protease)					n.a	
			1:7	Hemicellulase + Alcalase	Carbo-hydrolase + Protease	1% + 1%	g enzyme/ g biomass	4.5-5+8 (carbo-hydrolase only)	50	60	47	37	n.a	
								9 (carbo-hydrolase + protease)					n.a	
			1:7	Pectinex Ultra SP-L + Alcalase	Carbo-hydrolase (Pectinase) + Protease	1% + 1%	g enzyme/ g biomass	4.5-5 +8 (carbo-hydrolase only)	50	60	46	36	n.a	
								9 (carbo-hydrolase + protease)					n.a	

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No	Biomass	Pre-treatment	Biomass to liquid ratio	Enzyme				pH	Temp. (°C)	Time (min)	Yield (%)[a]	ΔYield with control (without enzyme) (%)	Content in supernatant (%)[b]	Refs.
				Name	Type	Amount	Unit							
			1:7	Viscozyme L + Alcalase	Carbo-hydrolase (Multi-enzyme) + Protease	1% + 1%	g enzyme/ g biomass	4.5-5 +8 (carbo-hydrolase only)	50	60	45	35	n.a	
								9 (carbo-hydrolase + protease)					n.a	
21.	Microalgae *Chlorella fusca*	Deoiled	1:10	Protex 5L	Protease	5%	ml enzyme/ g protein	9.5	60	180	67	33	n.a	[12]
			1:10	Protex P	Protease	5%	ml enzyme/ g protein	10	60	180	68	28	n.a	
			1:10	Protex 40XL	Protease	5%	ml enzyme/ g protein	11	60	180	74	42	n.a	
22.	Microalgae *Chlorella fusca*		1:14	Protex 5L	Protease	5%	ml enzyme/ g protein	9.5	60	180	63	49	n.a	[12]
			1:14	Protex P	Protease	5%	ml enzyme/ ml protein	10	60	180	69	34	n.a	
			1:14	Protex 40XL	Protease	5%	g enzyme/ g protein	11	60	180	60	56	n.a	
23.	*Moringa oleifera*		1:8	Protex 7L	Protease	2%	g enzyme/g biomass		45	120	75	32	n.a	[62]
			1:8	Multifect CX 13L	Carbo-hydrolase (Multi-enzyme)	2%	g enzyme/ g biomass		45	120	64	21	n.a	
			1:8	Viscozyme L	Carbo-hydrolase (Multi-enzyme)	2%	ml enzyme/ g biomass		45	120	68	25	n.a	
			1:8	Natuzyme	Carbo-hydrolase (Multi-enzyme)	2%	g enzyme/ g biomass		45	120	56	13	n.a	

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Table 2. *Continued from previous page*

No	Biomass	Pre-treatment	Biomass to liquid ratio	Enzyme				pH	Temp. (°C)	Time (min)	Yield (%)[a]	ΔYield with control (without enzyme) (%)	Content in supernatant (%)[b]	Refs.
				Name	Type	Amount	Unit							
			1:8	Kemzyme	Carbo-hydrolase (Multi-enzyme)	2%	g enzyme/ g biomass		45	120	52	9	n.a	
24.	Green tea leaf residue		1:30	Neutrase	Protease	2%		7	50	120	8	n.a.	n.a	[51]
			1:30	Alcalase	Protease	2/5%		7.5	55	120	18/18	n.a.	n.a	
			1:20 - 1:45	Alcalase	Protease	2%		7.5	55	120	10-22	n.a.	n.a	
			1:30	Alcalase	Protease	2%		7.5	55	60-360	10-24	n.a.	n.a	
			1:35	Alcalase	Protease	4%		6.5-8/8.5/9	55	240	25-38/ 37/35	n.a.	n.a	
			1:30	Protamex	Protease	2/5/6%		6.5	55	120	11/10/ 10	n.a.	n.a	
			1:20 - 1:35	Protamex	Protease	2%		6.5	55	120	7-12	n.a.	n.a	
			1:30	Protamex	Protease	2%		6.5	55	60-360	9-10	n.a.	n.a	
			1:35	Protamex	Protease	3%		5.5-7.5/8	55	240	15-25/ 23	n.a.	n.a	
			1:30	Flavourzyme	Protease	2%		6	50	120	8	n.a.	n.a	
			1:35	Alcalase/Protamex (1:3)	Protease	3%		7.5	55	240	42	n.a.	n.a	

[a] Yield is expressed as mass protein recovered in supernatant relative to mass protein in initial biomass. [b] Content in supernatant is expressed as mass protein in supernatant relative to mass total solid recovered in supernatant. n.a. = not available. Could not be calculated as there is no blank measurement without enzyme.

5.1 Protease

Alkaline extraction can be improved by protease addition [11, 12]. Due to the reduction in protein size through proteolysis, the proteins are more easily extracted. Moreover, the use of protease can also be used to enable lower processing pH, thus avoiding severe conditions that denature protein. In spite of those advantages, proteases have not yet been industrially applied in producing protein, due to high enzyme cost.

Studies have been conducted to find optimum conditions for protease-assisted protein extraction. A typical dosage of 1-5% g or ml enzyme/g biomass has been selected in some studies (Table 2). The enzymatic protein extractions are normally conducted at the optimal pH and temperature of the enzyme. For alkaline proteases this means that typically temperature and pH were between 45-60 °C and 8-10, respectively (Table 2). Longer incubation times may also be used to maximise extraction. However, a longer hydrolysis time will lead to a different product and may also not be preferable as it increases processing costs and the risk of microbial growth [37].

Protein extracted by protease-assisted extraction is likely to be hydrolysed. Without enzymatic treatment, soybean protein has a molecular weight ranging from 99 to around 7 kDa. Protex 7L and Protex 6L (0.5% g enzyme/g biomass) hydrolysed soybean to yield extracted protein with molecular weights below 54.1 kDa and 30 kDa [67]. The use of 0.5% Alcalase 2.4L and Flavourzyme gave a hydrolysate with 7.5% and 8.8% degree of hydrolysis, already extracting 81% and 88% rice bran protein respectively [61].

Microalgae proteins, which are hardly extracted by alkali [31] showed to be susceptible to protein hydrolysis by protease [12]. With proteolysis, almost 80% of microalgae meal protein was extracted by Protex 40XL [12]. The overall results show that proteases may be used to aid in hard to extract protein that can't be obtained by simple alkaline extraction.

Proteases are also used in the simultaneous co-production of oil and protein from oilseed that may be an alternative to the traditional two step extraction. Conditions (pH, temperature, particle size) that favour protein extraction may also favour oil extraction [9]. Protex 6L and Protex 7L have been used extensively to extract oil and protein from non-deoiled soybean. On average, as much as 90-96% oil and 73-87% of protein can be co-extracted with these proteases [11, 56, 67]. In general, Protex 6L extracted more protein (85%) compared to Protex 7L (73%). Doubling the Protex 6L dosage only extracted 2% additional protein [67]. Pressurization resulted in an increased protein yield from 75 to 81% when extracted by Protex 7L from non-pressurized and pressurized flaked soybean [56]. Next to an increase in protein extraction, addition of protease can also lead to increased oil extraction. Stabilisation of the oil emulsion can occur because of the hydrophobic interaction between the protease apolar side chain and the lipid, while free oil can increase because hydrolysed or denatured protein forms a weaker interfacial film, which makes it easier to disrupt and coalesce oil droplets. Therefore, addition of 5% (ml enzyme/g protein) Protex 40XL during co-extraction of rubber seed oil and protein did increase oil yield, but not protein yield [42].

5.2 Carbohydrolase

Carbohydrolases can be used to degrade biomass cell wall and so liberating intercellular constituents like protein. Carbohydrolases can be used independently, together with alkaline or protease treatment, or as a pre-treatment followed by alkaline or protease treatment. In all these cases the protein is the extracted component. Carbohydrolyses are normally mostly used under acidic conditions aiming to produce a fermentable sugar stream from the carbohydrates present. This low pH also enables e.g. ethanol producers to work in less than aseptic conditions. Since carbohydrolase experiments at alkaline pH were very limited, we also included some acidic examples. In general, carbohydrolase application increases protein recovery, but most were under acidic or mild alkaline conditions where protein recovery without enzymes is generally low. Acidic and neutral carbohydrolases have been tested to extract soybean protein with only a very small increase in protein recovery [37]. Mixtures of carbohydrolases [38] work better than the single use of cellulase [37], but the final yield is still lower than the use of protease only [11, 12, 56, 66-68].

In a few cases, the carbohydrolase was not used to dissolve the cell wall for protein release, but other non-proteinaceous compounds were dissolved into solution. More specifically, amylases have been used to treat rice flour and rice bran in order to produce rice protein isolate [75, 76]. Termamyl 120L effectively degraded starch in rice flour and rice bran while protein was maintained in the solid biomass. The recovery of the protein in rice flour and rice bran was up to 98% and 87%, respectively [75, 76]. The first example led to a concentrated protein extract, with 79% protein [76]. Cellulase AC and hemicellulase have also been tested and are capable to recover 65% and 54% of rice bran protein, respectively [76]. This example also illustrates that using these enzymes, protein is, at least partially, capable of leaving the cell.

5.3 Combined use of protease and carbohydrolase

Several studies tested the combination of proteases and carbohydrolases to maximize protein extraction. Avoiding carbohydrolase proteolysis, protease can be added after the carbohydrolase incubation. Alcalase, was added to rice bran suspensions that already contained a carbohydrolase; Celluclast 1.5L, Hemicellulase, Pectinex Ultra SP-L, or Viscozyme L. The protease addition increased protein yield in every suspension, yielding 30-40% more protein compared to the use of carbohydrolase alone. However, the result was not much better than the single use of Alcalase, without carbohydrolases [63].

In another study 0.5% (g enzyme per g biomass) Multifect Neutral, a protease, has been simultaneously combined with Cellulase 0.5% (g enzyme per g biomass) to soybean flakes. The combination increased soybean flake protein yield up to 75%. Also here, the use of Cellulases was apparently less effective as the maximum yield (77%) was comparable to the use of Multifect Neutral alone [68].

6. Concluding remarks and future trends on protein extraction

This review provides a literature study on alkaline plant protein extraction. Residues from plants that are grown and used for their main products such as oil or starch mostly end up as waste stream or animal feed. With biorefinery, it is expected that the whole plant can be used for spectrum of applications. The protein from agricultural by-products can still be used for their nutritional value to meet not only demands for feed, but also for food. Once the demand for both food and feed are met, the remaining protein or amino acids can also be used for chemical industry.

Oilseed residues are the most attractive agricultural by-products for further processing due to their high protein content and high protein extractability. Although lower in protein content, cereals are also attractive resources as they exhibit the same high protein extractability, and are produced in massive amounts, only mildly treated for starch production. The mild pre-treatment actually contributes to the ease of cereal-based protein extraction. Extractions at pH 9-10 at room temperature (25 °C) are sufficient to extract most of plant protein from cereal and oil seed residues. The use of low temperature minimises protein denaturation, amino acid racemization, and lysinoalanine formation.

In addition to oilseeds and cereals, several novel resources may also be considered such as microalgae and leaves. Yet, more research is still needed to optimally extract protein from these resources and more severe extraction conditions are recommended for high protein yields. In these cases, enzymes can be further investigated. Proteases that currently are intensively studied for their ability to co-extract protein and oil from oilseeds can also be used to extract protein from microalgae or leaves. Albeit, carbohydrolases show less improvement in extracting protein, they still may have a positive impact on protein extraction processes in a different way. Their capability to degrade the cell wall can be used to release components that otherwise buffer the reaction mixture. Having less buffering components present due to a pre-treatment with carbohydrolases, leads to lower alkali consumption during subsequent protein extraction.

After protein extraction, different methods for downstream processing can be used to effectively isolate soluble protein. In addition to protein, some other plant constituents will be present in the solvent as impurities. Acid precipitation is commonly used for further purification and isolation of protein. Protein has its lowest solubility at its isoelectric point (IEP), which commonly occurs at pH 4-5. Therefore, most proteins are precipitated when the pH of the solution is shifted towards this IEP. If required, the precipitated proteins can be re-solubilised by dissolving them in a solution with alkaline pH. The important drawback of acid precipitation is the generation of salt. Dialysis or washing can purify protein isolates from the salt, but the salt in the remaining solution is a liquid waste that may lead to high cost for removal. Filtration can be used as an alternative to acid precipitation, avoiding salt generation. The permeate can be supplemented with alkali and re-used for another protein extraction.

To complete the biorefinery concept, the solid residue following protein extraction should be used for other applications. The residue, which mainly consists of cellulose,

hemicellulose, and lignin, can be used as low protein containing animal feed for ruminants, or used for energy. The residue usage will reduce the overall processing cost for protein production, bringing protein biorefinery closer to industrial application.

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Chapter 3

How biomass composition determines protein extractability

Abstract

Biomass consists of a complex mixture of different components, of which protein potentially has a high added value for biorefinery. In this study, protein extractability of different types of biomass, mostly by-products, was analysed. Protein yield obtained from a three step alkaline extraction was correlated to biomass chemical composition through Partial Least Square (PLS) regression. The results showed that protein extractability depended crucially on the type of biomass used. Protein from cereals and legumes were highly extracted, compared to other materials. High protein extractability coincides with the biological function of protein as a storage protein, as opposed to functional protein. Protein extraction was furthermore correlated to the composition of the biomass. Especially cellulose and oil hamper extractability of protein whereas lignin has no significant influence, suggesting that alkaline treatment removed lignin sufficiently.

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1. Introduction

Agriculture provides food for human consumption and energy for the new bio-based society. However, it also generates side-streams that are currently only re-used in low economic value applications such as animal feed or as fuel for power generation. Examples are grain residues [1] and meal residues [2] from biodiesel production. However, in an efficient and ethically sound bio-based economy all parts of the biomass will have to be used at their highest value without competing for food and feed.

Due to the complex nature of biomass a wide variety of products can be extracted from it. Protein will be the focus point for this article, because of its relative high economic value [3]. Therefore, efficient extraction of protein from agricultural side streams can increase value in overall plant production chains. Proteins are also of interest because of the presence of functional side groups that makes protein and amino acids interesting for chemistry [4]. In addition, valorization of other components present in biomass is needed to increase overall sustainability and economic feasibility of bio-based processes. The use of cellulose from wheat straw instead of starch from sweet corn as a sugar feedstock may e.g. improve its valorization but also the sustainability of bioethanol farming [5]. Pulp industries already try to re-use their lignin containing waste stream for technical applications such as binders instead of just burning it for energy [6].

Selection of type of biomass for protein extractability may be based on technical, economic, environment or social aspects [7]. Here, we only consider the first two aspects. Technically speaking some biomass is easier to extract than others, as is also demonstrated in this article. Leafy biomass is e.g. less easily extracted than oil seed meals and still needs further technical optimisation to get to higher extraction yields [8, 9]. From an economic point of view biomass availability and prices are important. Oilseed meals may be one of the types of biomass to select, with their high protein content before and after de-oiling. However, because of their already high value for feed there is less added economic value in protein extraction. In addition to oilseed/legume meals and leaves, dry biomass such as hull, stover and stalks are also interesting resources due to their abundance. The biomass sources that were chosen for our experiment are all commonly used in agro-industries such as beer and vegetable oil production. These biomass sources contain different levels of the components and fall into different categories of biomass: legume, leafy, tuber or cereal.

When extracting biomass, different conditions can be used, with or without the addition of enzymes. pH is an important parameter, and it has already been shown that under alkaline condition more protein is extracted than at lower pH [10]. Alkali effectively extracts proteins, either by breakdown of e.g. cell wall components [11, 12] or by breakdown of the protein itself [13]. Addition of proteases can further increase yields [10, 14].

The efficiency of alkali in extracting biomass protein is influenced by several factors including the type of biomass and temperature. Soybean meal is one of the most easy types of biomass to extract protein by alkali [10]. Under similar conditions less protein was extracted from rapeseed meal [10], microalgae meal [10], rice bran [15], and dwarf elephant grass [16]. However, still little is known about the underlying mechanisms in alkaline protein extraction and the interactions that occur between protein and the other components present under these conditions. Therefore, protein extraction was performed

under alkaline conditions on sixteen biomass. Three sequential increasing temperatures were chosen. The lowest extraction temperature, 25°C, was chosen to avoid thermal energy input into the process. The second temperature, 60°C, was tested to see if an increase in temperature under alkaline conditions could increase protein extraction for some types of biomass. After that the residue was extracted at 120°C, without adding more alkaline. This was to see the influence of temperature at its most extreme. High temperature and pH combination is expected to severely damage protein and may therefore not be recommendable. However, it does give further insight the temperature dependence of protein extraction from biomass. The results were used to study the correlation between protein extraction and the chemical composition of the biomass at different temperatures.

Biomass sources with different chemical composition were selected and tested on protein extractability. Aiming for valorization of agricultural by-products, twelve out of the sixteen chosen biomass are by-products. In addition to these, barley grain, soybean, and microalgae were tested to compare extraction yields with their corresponding by-products; ryegrass was tested to represent leafy biomass. To get an insight of chemical composition on protein extractability, the selection of biomass comprised of biomass that was rich in protein (wheat gluten) and/or oil (untreated soybean), and/or cellulose (soybean hull) and/or lignin (palm kernel meal), and/or starch (barley grain). Also biomass with a more balanced chemical composition were selected, such as barley rootlets (balanced in protein and hemicellulose), microalgae (balanced in protein, oil, and cellulose), and palm kernel meal (balanced in protein and cellulose). The results from the extraction experiments are discussed in this paper and protein extractability was correlated to biomass chemical composition through regression analysis. In our case, the protein yield is identified as a function of eight chemical components. Thus, multiple linear regression is used in this study. For this, a Partial Least Square (PLS) method is selected. The Variable Importance Plot (VIP) scores obtained by PLS in this study are used as an importance measure of each explanatory variable [17]. The knowledge generated from this research is expected to aid in selecting biomass in biorefinery for protein.

2. Materials and Methods

2.1 Materials

Rapeseed meal, soybean hull, soybean meal, sunflower meal, and palm kernel meal were obtained from Schouten Ceralco (the Netherlands). Microalgae and microalgae meal (*Chlorella sp*.) were obtained from Ingrepro (the Netherlands). Wheat gluten, barley grain, barley mill run, barley rootlets, and malt by-products were obtained from Cargill (the Netherlands). Wheat middling was obtained from Meneba (the Netherlands). Ryegrass was obtained from a local farmer in Wageningen (the Netherlands). Sugar beet pulp was obtained from the sugar mill in Dinteloord (the Netherlands). For soybean without oil removal, we used the commercially available one (Heuschen & Schrouff, the Netherlands).

2.2 Protein content analysis

Protein content was determined as nitrogen content using DUMAS analysis (FlashEA 1112 series, Thermo Scientific, Interscience) using a nitrogen to protein conversion factor of 6.25. Methionine was used as a standard for the calibration. Protein extraction yield was determined as the ratio of nitrogen content in the supernatant to the nitrogen content of the raw material.

2.3 Protein extraction

Protein extraction was conducted at three ascending temperatures: starting with one day incubation at 25°C, followed by 1 h at 60°C, and ending with 1 h incubation at 120 °C. All experiments were performed in duplicate. Biomass (10 g) was mixed with 90 ml of 55 mM NaOH and the solution was kept stirring at 25 ºC for one day. After 5 h and 15 h the pH was measured and readjusted to pH 10 by addition of 2 M NaOH when needed. After 24 h, the mixture was centrifuged (Sorvall RC 6+ centrifuge at 10000 rpm; 4 °C; 15 min) and the supernatant (E25) was separated and kept for further analysis. The solid phase was re-mixed with 90 ml of 55 mM NaOH and the solution then heated to 60 ºC, shaking at 120 rpm in GFL 1086 water bath shaker for one hour. After that, the mixture was centrifuged to separate the supernatant (E60), which was kept for further analysis. Expecting that the final solid residue of the protein extraction should be usable for other applications (such as sugar production), instead of alkali, deionized water was used at the last temperature step. However, the pH of the slurry was still high (alkaline) due to preceding treatment with alkali. The solid phase from preceding extraction was mixed with 90 ml deionized water and heated to 120 °C in stainless steel reactors fitted with thermocouples. For temperature control, a Haake B silicon oil bath equipped with Haake N3 temperature controller (Thermofisher Scientific) was used. After 1 h, the reactors were cooled in ice water, and the mixture was subsequently centrifuged to obtain the supernatant (E120) and the remaining solid residue (R). All supernatants and the residue were kept for further analysis. Total protein yield obtained after three step protein extraction (Etotal) was defined as the sum of protein from E25, E60, and E120. As a control, protein extraction was also performed in deionized water with an identical experimental set up.

2.4 Statistical analysis

Correlation between eight components of chemical composition was analyzed through Pearson correlation analysis. As there was multicollinearity (strong correlation) between chemical composition components, Partial Least Square regression was used to predict the influence of chemical composition on protein extraction. Biomass chemical composition with eight components (protein, oil, ash, cellulose, hemicellulose, lignin, sugar, and starch) was used as a group of independent variable while protein extraction yield was used as a response variable. SAS Version 9.2 was used to run Pearson correlation analysis and PLS regression.

3. Results and discussion

3.1 Biomass composition

Biomass that commonly occur as side streams used in agro-industries were selected based on their differences in chemical composition and tested on protein extractability. The tested biomass had a wide variety in protein content, ranging from 8.6 – 77.5%. The chemical composition of the tested biomass (Table. 1) was retrieved from available literatures [18-22]. Eight major biomass chemical components were incorporated in this study, namely protein, oil, ash, cellulose, hemicellulose, lignin, sugar, and starch.

3.2 Protein extraction

The different types of biomass were used in three-step protein extraction using alkali. Protein content was measured for both supernatants and solid residues at different conditions: E25, E60, E120 and R. The three-step extraction method using alkali effectively extracted protein from biomass. From soybean, commonly used protein resource, as much as 73% of its protein was extracted. An even higher yield was obtained from its meal, 86%. This number is comparable with other research that extracted soybean meal protein using protease [14]. The reason for some other biomass types to have low protein yield might be due to the difficulty to break down the cell wall or the character of the protein, which is hardly soluble. Examples of biomass that fall into this category are microalgae and sugar beet pulp respectively. The overall protein mass balances for most types of biomass gave an over 95% closure. The 5 to maximum 10% protein loss is likely due to losses during transfer from one reactor to another. Protein extraction yields varied largely depending on the type of biomass (Fig 1). In the next sections these differences will be analysed and discussed.

Table 1.Biomass chemical composition (weight %)

No.	Biomass	Protein	Oil	Ash	Cellu-lose	Hemi-cellulose	Lignin	Sugar	Starch	Refs.
1	Barley grain	10.1	1.9	2.5	4.9	13	1.7	1.7	51.6	[18, 19]
2	Barley mill run	10.8	3.2	5.4	16.8	30	4.8	8.2	16.9	[18, 22]
3	Barley rootlets	26.3	2	6.3	12.9	24.3	1.7	12.7	6.2	[18]
4	Malt by-products	13.6	3	4.1	10	25	2	2	26	[18]
5	Microalgae	34.2	22	5.9	33	10.2	0	1	7	[18, 20, 21]
6	Microalgae meal	45.2	5.1	6.2	34	11.2	0	0.8	7.3	[18, 20, 21]
7	Palm kernel meal	17.2	1.7	3.9	17.1	24.5	9.9	1.6	1.5	[18]
8	Rapeseed meal	33.9	2.3	6.8	13.1	8.5	7	8.8	4.4	[18]
9	Ryegrass	8.6	2.3	8.5	22.1	25.6	4.7	6	3.8	[18]
10	Soybean	37	18.7	4.9	5.2	4.4	1.6	7.6	5.8	[18]
11	Soybean hull	14.1	2.2	5	40	17	2.2	1.6	6.3	[18]
12	Soybean meal	44.2	1.5	6	6.5	4.7	2	9.2	2.3	[18]
13	Sugar beet pulp	8.6	0.7	5.4	18.8	17.8	2.5	6.6	0.8	[18]
14	Sunflower meal	26.7	1.5	5.8	16.4	4.8	9.1	4.3	3.5	[18]
15	Wheat gluten	77.5	2.3	3.7	0	0	0	0	5	[18]
16	Wheat middling	16.2	4.1	4.9	1.9	26.5	3.3	5.3	24.2	[18]

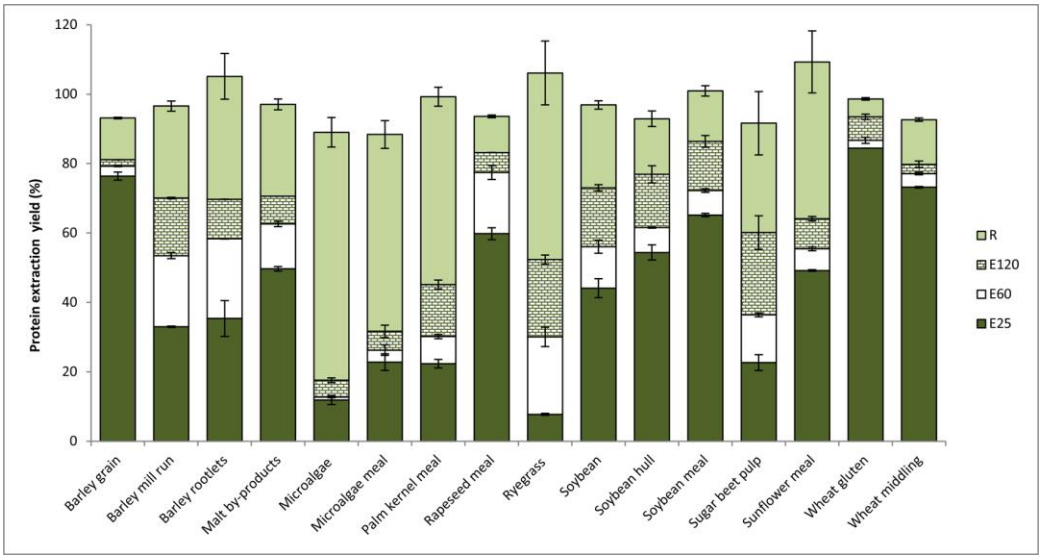

Figure 1. Protein extraction yield.

3.3 The influence of chemical composition on protein extractability

To determine the influence of composition on protein extractability, a PLS model based on total protein extraction yield (Etotal = E25 + E60 +E120) after three- step protein extractions was generated. The data set for PLS analysis was obtained without regarding biomass identity. The data set consisted of Etotal as response variable and chemical composition as independent variable. The PLS analysis works by reducing data dimensionality. The first two PLS factors explained 69% variability in protein extraction yield, but only 41% variability in chemical composition (Table 2) and could therefore not be used. An improved result was only obtained by using the first five PLS factors. This model explained 71 and 80% variability in protein extraction yield and chemical composition, respectively, and was used to build the model. The predictive capability of the model was evaluated by one-cross-validation method and a scatter plot between the experimental and prediction responses.

Cross validation using the Wold's R criterion identified the appropriate numbers of the independent variables, which in our case are the chemical compositions, to include in the model. For this the ratio of the PRedicted Error Sum of Squares (PRESS) was calculated and minimised [23]. Minimum PRESS was obtained using only the first two PLS factors (see Table 2). However, since these were unable to explain variance in chemical compositions, another approach was used. To check the predictive capability of the model, the obtained protein extraction yield from the model was plotted versus the one obtained by the experiment. This scatter plot (Fig. 2) shows that the model predictions and the experimental results are in a good agreement with 84% linear correlation and therefore, the five factor model was used.

Table 2. Percent variation accounted and cross validation for by PLS factors to model the influence of chemical composition on protein extractability

Number of PLS factors	Percent variation accounted (%)				Cross validation	
	Chemical composition		Protein extraction yield		Root Mean PRESS	Prob > PRESS
	Proportion	Total	Proportion	Total		
1	19.97	19.97	65.30	65.30	1.102	0.255
2	21.01	40.98	3.58	68.88	1.002	1.000
3	8.80	49.78	1.95	70.83	1.152	0.010
4	14.50	64.29	0.43	71.26	1.293	<0.001
5	18.10	82.39	0.19	71.45	1.358	<0.001

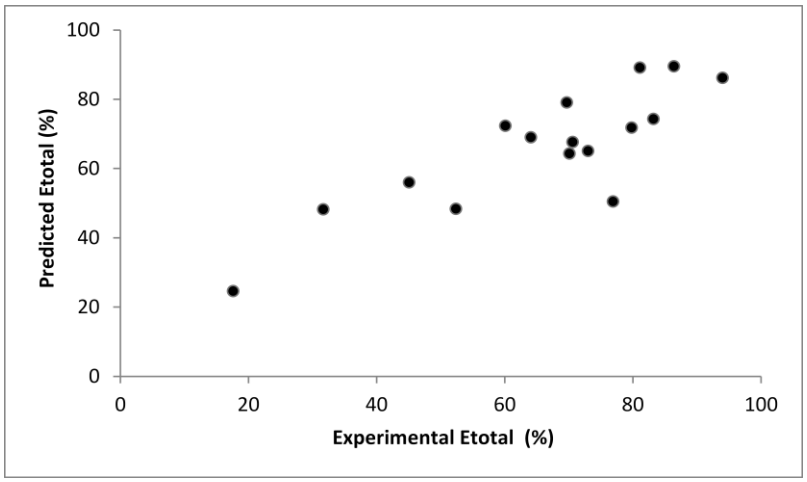

Figure 2. Scatter plot of predicted versus experimental Etotal.

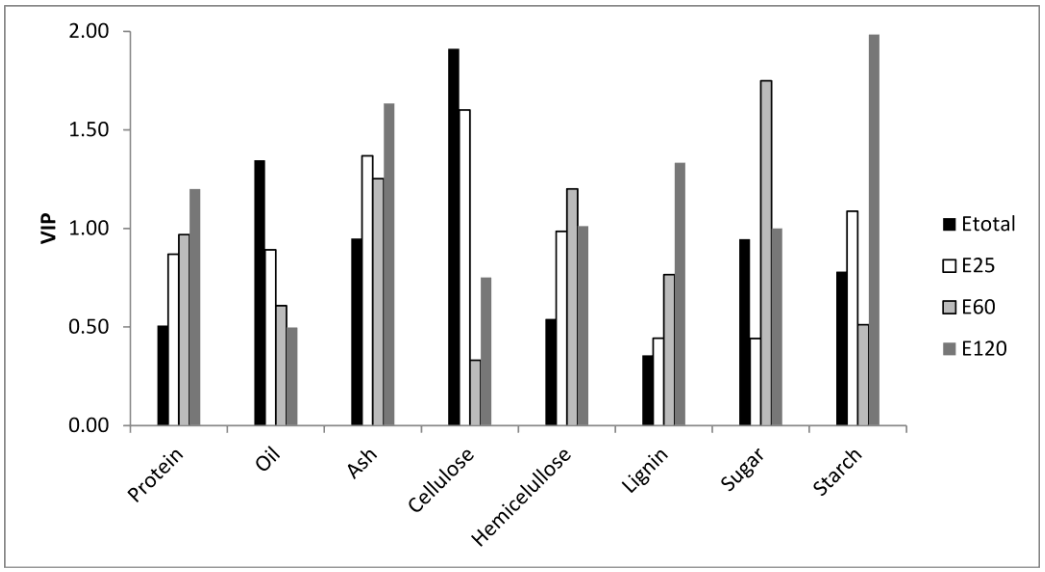

Figure 3. VIP of the PLS to model the influence of chemical composition on protein extractability.

PLS regression coefficient can be used to describe the relationship, either positively or negatively, of each chemical composition to protein extraction yield. However, the regression coefficient cannot be used directly to select which variables, chemical composition in our case, are the most important for modelling [24]. To select which variable is important, Variable Importance Plot (VIP) is considered to be more relevant than PLS regression coefficient [17]. For this purpose, VIP was calculated based on regression coefficient to evaluate the importance of chemical composition in determining protein extraction yield. A limit of 0.8 [25, 26] or 1 [17] can be used for VIP. In this study, a variable with VIP score higher than 1 was considered as an important variable. The combination of VIP scores and PLS regression coefficient was further used to infer the relationship between important predictors (chemical composition) and dependent variable (protein extraction yield). Of 8 variables tested, only 2 were considered important (Fig. 3). Cellulose showed the highest negative influence (Fig. 4) on protein extractability, followed by oil.

The high correlation between the presence of cellulose and protein extractability correlates to its natural appearance in plant. Cellulose is a crystalline matrix that builds up the rigid cell wall, together with hemicellulose and lignin. With its high degree of crystallinity, a considerable disturbance is needed to enable protein inside the cell to permeate to the outside. Alkaline treatment dissolves hemicellulose and lignin but not cellulose [27]. Therefore hemicellulose and lignin do not have high correlation with protein yield, as indicated by low VIP score. Contrary, cellulose has high VIP, suggesting that in this case cellulose is the limiting factor for protein extraction. When selecting resources for alkaline protein extraction, it is therefore suggested to prioritize on biomass with low cellulose content. In case it can't be avoided, a physical pre-treatment, such as ball milling [28], might help in reducing cellulose crystallinity to increase protein extraction yield.

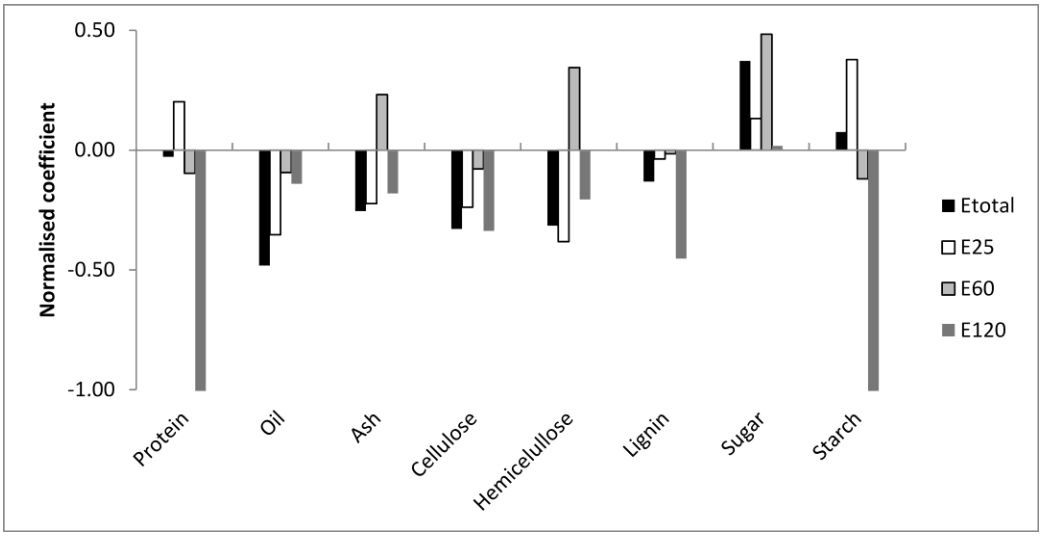

Figure 4. Normalised PLS regression.

Next to cellulose, oil also showed a negative correlation with protein extraction (Fig. 4). This is illustrated by comparing protein extraction yield from microalgae and soybean with and without oil. The soybean and microalgae give much lower yields (73% and 18%, respectively) that their respective meals (86% and 32%). Looking at cell distribution, oil fills the empty space within a cell while protein is distributed over the cell [29]. Thus oil provides physical barrier for protein to permeate. De-oiling will reduce this hindrance leading to higher protein extraction yields [10]. Chemically speaking, the use of alkaline in extracting protein may help reducing negative oil-effects through saponification.

3.4 Influence of temperature on protein extractability

Protein extraction was performed in three steps at different temperatures to see the influence of temperature. This alkaline extraction was performed subsequently at 25, 60, and 120 ºC. The first incubation at 25 ºC extracted considerable amounts of protein from most biomass sources, except from rye grass (E25 = 8%) and sugar beet pulp protein (E25 = 23%). The low E25 of rye grass confirms that high temperature is needed to extract protein from leafy biomass as was mentioned before [30, 31]. The low E25 of sugar beet pulp may be due to the high content of pectin. Although pectin was not included into the PLS model because it was not present in most materials, pectin represents 20% of sugar beet pulp and can form a complex with protein [32], making it more difficult to extract. In addition to the presence of pectin, the low E25 may relate to sugar beet pulp treatment during sugar production at which sugar beet is incubated at 72 °C with water. At this condition, some proteins may already have been extracted or modified leading to low protein extraction yields at 25 ºC.

The three highest E25s among all tested biomass were obtained from wheat gluten, barley grain, and wheat middling. The E25 of these biomass was 84, 76, and 73%, respectively. With that high E25, higher temperature is probably not needed for extracting protein from these types of biomass. Looking at the pre-treatment before protein extraction, no severe heating treatment was involved. Therefore, no protein was lost or denatured in pre-treatment, which could potentially have decreased protein extractability.

Protein of microalgae and their meal was also mostly extracted at 25 ºC. Although with very low yields, only 23 and 12% respectively, these were a considerable amounts compared to the total amount that was extracted (Etotal) with only 32 and 18%, respectively. These very low yields show that alkali is not effective in extracting protein from these biomass types. Instead enzymatic treatment may be much more effective, as it could extract 60% protein from microalgae and 71% from microalgae meal as shown in previous research [10].

In general, increasing temperature aided in protein extraction. However, barley grain, wheat gluten, wheat middling, microalgae and microalgae meals showed only very limited increases. To get more insight, PLS analysis with five first PLS factor components was used to model the influence of temperature in protein extractability. The percentage of variability is given in Table 3 and a scatter plot of the models for E25, E60, and E120 is given in Fig. 5. The linear correlation among predicted and experimental results is 86% for E25, E60, and E120. The VIP and PLS regression coefficient of these 3 stages are given in Fig. 3 and 4, respectively.

The effect of some chemical components during extraction may predominantly occur at a certain temperature. The modelled influence of chemical composition on protein extraction at 25 ºC (E25) shows that cellulose, ash, and starch (Fig. 3) are three important parameters determining the protein yield. Cellulose and ash both had negative correlations with protein extraction yield while starch had a positive correlation. The negative correlation of cellulose and ash to the protein extraction yield indicates that cellulose and ash were less disrupted by alkaline treatment at 25 °C. Thus, less protein would be extracted from biomass that contain high amount of cellulose and ash. The positive correlation of starch and protein extraction yield indicated that starch would be highly dissolved at this temperature thus enable more protein to be extracted.

Table 4. Percent variation accounted for by PLS factors to model the influence of temperature on protein extractability

Number of PLS factors	Response: E25				Response: E60				Response: E120			
	Chemical composition		Protein extraction yield		Chemical Composition		Protein extraction yield		Chemical composition		Protein extraction yield	
	Proportion	Total	Proportion	Total	Proportion	Total	Proportion	Total	Proportion	Total	Proportion	Total
1	24.25	24.25	68.98	68.98	28.90	28.90	69.39	69.39	26.92	26.92	40.80	40.80
2	19.66	43.91	4.47	73.45	16.07	42.97	4.48	73.87	10.75	37.67	19.39	60.19
3	13.77	57.68	1.46	74.91	19.65	62.62	0.33	74.20	19.07	56.74	7.75	67.94
4	18.64	76.32	0.49	75.40	13.95	76.57	0.15	74.35	7.28	64.02	3.96	71.90
5	7.89	84.21	0.21	75.61	11.70	88.27	0.07	74.42	7.80	71.82	2.18	74.08

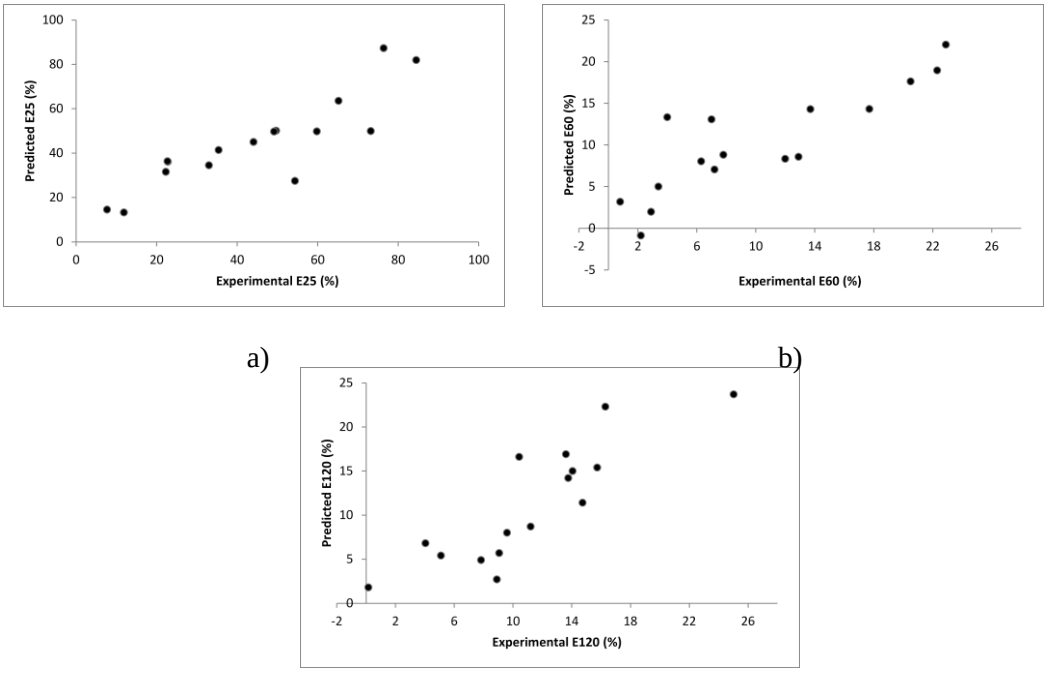

Figure 5. Scatter plot of predicted vs experimental of a) E25; b) E60; and c) E120.

Incubation at 60 ˚C yielded the least increase in protein compared all other incubation temperatures (Fig. 1). This might be due to the relatively moderate temperature and short incubation time. Sugar, ash and hemicellulose positively correlated with protein extraction at this temperature (Fig.3 and 4). Thus, extracting protein at least 60 °C is recommended for biomass with high content of those 3 components. Biomass resources that belong to this group in our studies are ryegrass and by-products from barley malting process, which are barley rootlets, and malt by-products. Looking at the malting process and comparing to the more mildly treated barley grain, wheat middling and gluten, it is further recommended to extract protein at 60 °C from biomass which has been pre-treated at high temperature.

At 120 ˚C incubation, only a minor fraction of protein was still available to be extracted since the majority of protein has been extracted at 25 and 60 ºC. At this temperature, protein showed high VIP, but negative correlation with protein extraction (Fig. 3 and 4). This negative correlation may be explained by the effect of preceding extraction and the disability of high temperature to further extract protein. Preceding extraction at 25 and 60 ºC already extracted most protein from high-content-protein biomass such as barley rootlets, rapeseed meal, soybean meal, sunflower meal, and wheat gluten. Microalgae and microalgae meal still contain a high content of protein, but only small amounts were extracted possibly due to the complexity of the protein of these type of biomass as discussed in the next section.

Starch and ash also showed high VIP and negative correlation with protein extraction at 120 ºC (Fig. 3 and 4). The negative correlation from starch most likely evolved from high E25 and E60 from high-content-starch biomass, particularly cereal type biomass. While the negative correlation from ash evolved from high-content-ash biomass as microalgae meal and rye grass.

In addition to protein, starch and ash, the sugar beet pulp protein extraction at 120 ºC is probably influenced by pectin. Pectin was not incorporated as a parameter when PLS model was built since it was not present in all types of biomass and therefore had too little data points. Most of sugar beet pulp protein was extracted at 120 ºC. This high yield can be explained by pectin degradation at high temperature. A temperature increase from 75 to 110 ºC can result in 3.5-fold increase of pectin degradation [33]. With this degradation, the pectin-protein complex is disturbed thus allowing protein to be extracted.

3.5 Influence of biomass type

As another explanation to the protein extraction differences, we looked into protein and cell wall properties of the different types of biomass. The sixteen tested biomass were classified as legume, cereal, tuber, and leafy biomass.

Protein properties of the tested biomass types can be seen in Table 4. In this table, alteration of protein properties due to pre-treatment was neglected. Cereals, particularly wheat gluten, barley grain, and wheat middling showed high extractability with protein extraction yields of 94, 81, and 80%, respectively. Up to one-third of cereal's amino acids is glutamine (Gln) [34]. Alkaline treatment of cereals leads to deamidation of Gln [35], thereby increasing the numbers of negatively charged amino acids in cereal's protein. As a consequence, the solubility of cereal protein increased resulting in a high protein extraction yield.

Protein extraction yields from legumes were varying (45 – 86%), but generally high. The variation is probably due to the variation in proteins present in legumes. Legume mainly consists of globulin and albumin. The globulin properties of legumes differ from each other. Soybean's globulin (named as glycinin) e.g. has lower surface hydrophobicity compare to rapeseed's globulin (named as cruciferin) [36]. With this, soybean's globulin is easier to solubilize than rapeseed's globulin, leading to higher protein extraction yields. From the legume types, soybean meal with 84% yield has the highest protein extraction yield. Together with rapeseed meal, sunflower meal, and palm kernel meal, soybean is a by-product of oil extraction from oil seed. Untreated soybean had 13% lower protein extraction yield from soybean meal. The different result can be due to structural changes resulting from pre-treatment [37-39] and indicates the benefit of oil extraction prior protein extraction of oil seed [10]. Images obtained from Scanning Electron Microscope and Transmission Electron Microscope of untreated and treated soybean showed that there was noticeable cell wall disruption [40, 41]. With adequate cell wall disruption in pre-treated soybean (soybean meal), its protein easily dissolves into alkaline solution [42].

Cell wall acting as a barrier in protein extraction also occurs in leafy biomass, particularly microalgae. Laser confocal microscope images of microalgae (*Chlorella sp*.) showed that part of their cell wall was still intact after alkaline treatment [43]. It indicated that microalgae have cell walls that are hardly disrupted by alkali. With this, microalgae with only 18% protein yield had the lowest extraction yield amongst the biomass tested in this study, even after treatment at 120 °C. Next to the presence of a cell wall, difficulty for extracting leafy biomass protein is strongly associated with the particular character and location of leafy biomass proteins. While cereal, legume, and tuber biomass have storage protein as their major protein, the major protein in leafy biomass is an enzyme named RuBisCo (Ribulose-1,5-bisphosphate carboxylase oxygenase). Storage protein in cereal, legume, and tuber biomass is located in vacuole, while RuBisCo is located in the chloroplast and has a function for carbon fixation [44]. The presence of a network of granum in the chloroplast increases the chloroplast structural complexity. With this, RuBisCo is difficult to extract from leafy biomass. In total, after the three treatments, only 52 % protein was extracted from ryegrass. Interestingly, an increase in temperature from 25 °C to 60 °C tripled the protein extraction yield in this case. This dependency on temperature might indicate the need of high temperatures in extracting leafy biomass protein [31]. However, even more extreme temperatures (120 °C) did not yield higher protein yields for ryegrass.

A major storage protein in sugar beet is globulin [45]. Sugar beet, together with other tuberous crops, contains relatively low amounts of protein [46]. Similar to other type of biomass, sugar beet protein extraction was influenced by the nature of its cell wall. Sugar beet cell wall shows its thermal stability even after heating at 100 °C for several hours [47]. The good mechanical properties of sugar beet fiber also makes the beet resistant to mechanical treatment [48]. The thermal and mechanical stability contributed to the low protein extraction yield of sugar beet although it has been mechanically and thermally pre-treated and thermally treated. Less than one-fourth of sugar beet pulp protein was extracted when it was dispersed at 25 °C for 24 h. More protein was extracted with an increase of temperature. A three step dispersion in alkali at 25, 60, and 120 °C extracted 60% of sugar beet pulp protein.

Table 5. Protein properties of tested biomass

Biomass	Category	Major protein component	MW	Main function	Solubility	Location
Soybean Soybean meal Soybean hulls	Legume	40% Glycinin (11S globulin) [illegible] [illegible]	Glycinin: Basic: 20 kDa Acidic: 10.5 and 36.4 kDa β-conglycinin: 50 kDa, 67 kDa, 71 kDa [48, 49]	Storage	Soluble in salt High at alkaline	Vacuole
Rapeseed meal	Legume	60% Cruciferin (2S globulin) 20% Napin (2S albumin) [50]	Cruciferin: Acidic 30-37kDa Basic 22-24 kDa [51] Napin: Light 4 kDa Heavy 10 kDa [52]	Storage	Cruciferin: soluble in salt Napin: soluble in water	Vacuole
Palm kernel meal	Legume	7S and 11 S Globulin [53]	14-141 kDa [53]	Storage	Globulin: soluble in salt High at alkaline Similar with SPI [54]	Vacuole [55]
Sunflower meal	Legume	Helianthinin (11S globulin, 11S) 10-30% Albumin (2S) [56]	Helianthinin: 300-350 kDa Albumin: 10 – 18 kDa	Storage	Globulin: soluble in salt Albumin : soluble in water	Vacuole

Continued on next page

Table 5. *Continued from previous page*

Biomass	Category	Major protein component	MW	Main function	Solubility	Location
Barley mill run Barley rootlets Barley malt byproducts	Cereal	Hordein (prolamin) Glutelin	Hordein 4 fraction [34] B (70-80%) 30-50 kDa (sulphur rich) C (10-20%) 55-80 kDa (sulphur poor) D 80-90 kDa A 15 kDa Glutelin: <20, 25-50, 55-70, 85-90 kDa [57]	Storage	Limited solubility pH 3-8 (< 30%) [57] Increased at pH 10 (60%) Limited solubility due to high content of Gln	Vacuole
Wheat gluten Wheat middling	Cereal	Gliadin (monomeric; prolamin) [51] Glutenin (polymeric; glutelin) [51]	Gliadin: 30-40 kDa [58] Glutenin [58]: HMW : 65-90 kDa LMW: 20-40 kDa	Storage	Gliadin: soluble in alcohol Glutenin: insoluble in most solvent Low at neutral pH (6-7) Increase with increasing pH or salt addition	Gliadin: vacuole [59] Glutenin: endoplastmic reticulum [59]
Sugar beet pulp	Tuber	11S globulin [44]	25-106 kDa [60]	Storage [44]	Globulin: soluble in salt Water insoluble [44]	Vacuole
Ryegrass hay	Leafy	60% Rubisco [61]	Large: 50-55kDa Small:12-18 kDa [62]	Carbon fixation	Low at pH 3-5 Increase towards pH neutral to alkali [63]	Chloroplast
Microalgae Microalgae meal	Leafy	Rubisco [62]	12-120kDa [64]	Carbon fixation	Low at pH 3-5 Increase towards pH neutral to alkali [63]	Chloroplast

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Chapter 4

Enzyme assisted protein extraction from rapeseed, soybean, and microalgae meals

Abstract

Oilseed meals that are by-products from oil production are potential resources for protein. The aim of this work is to investigate the use of enzymes in assisting the extraction of protein from different oilseed meals, namely rapeseed, soybean, and microalgae meals. In addition, microalgae without prior oil removal was also tested. The extraction was performed varying temperature, pH, and type of enzyme. More protein was extracted at alkaline conditions, compared to acidic conditions. At alkaline pH, 80% protein of soybean meal and 15-30% protein of rapeseed and microalgae meals was extracted without enzyme addition. The addition of enzyme under this condition increased protein extraction yield to 90% for soybean meal and 50-80% for rapeseed and microalgae meals. Here, Protex 40XL, Protex P, and Protex 5L that work at alkaline pH assisted protein extraction particularly for rapeseed and microalgae meals. Microalgae without prior oil removal had the lowest protein extraction yield, illustrating that oil removal prior to protein extraction is beneficial for protein recovery. In general, protein extraction was influenced by pH, the type of biomass, and the addition of enzyme, but not by the type of enzyme that was used. Besides the influence of pH, protein extraction was mostly influenced by the type of biomass, and the addition of enzyme, but not by the type of enzyme used.

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1. Introduction

Oilseed meals that are by-products from oil production can be potential sources of protein. Proteins can be used for food and feed, or hydrolysed to amino acids. These amino acids have been used in food and non-food industries as flavorant, animal feed, or can be used as building blocks for bulk chemicals in the chemical industry [1], where they replace products that are similar to those produced by petrochemical routes [2, 3]. When amino acids will be used as building blocks for chemical industries, there will be a high demand for cheap amino acids that encourage the development of new routes for production of amino acids. Protein extraction can then be combined with protein hydrolysis. Not only to yield single amino acids, but also to aid in protein extraction.

Methods have been developed for extracting plant protein based on acid, alkaline and enzyme assisted extraction. Acid aided extraction appears less promising as it e.g. only extracted a maximum of 20% of yellow pea protein [4]. A comprehensive study on salt extraction of *R. rubiginosa* protein gave 15% protein extraction yield at pH 4.5 and 90% at pH 11 [5]. Alkaline extraction shows better results and has amongst others been tested on several oilseeds. The high protein content combined with a low price of soybean triggered industries to apply its protein in food and feed applications [6-8]. Currently, soybean protein has been commercially extracted under alkaline conditions (pH 8-9) [6, 8, 9]. Rapeseed and other oil containing biomass also have high protein content. Counter current extraction in 0.02 N NaOH enabled 95% extraction of rapeseed protein [10, 11], multi stage counter current extraction in 55 mM NaOH extracted 74% *Jatropha curcas* protein [12] and two stage extraction at pH 10 extracted 70% of safflower protein [13]. Although alkaline conditions may improve extractability of oilseed meal protein, too severe alkaline extraction leads to several adverse effects such as racemisation of amino acids, reduced protein digestibility, and damage to some amino acids (lysine and cysteine) [14]. Moreover, the addition of concentrated alkali leads to salt formation, which preferably has to be avoided. Minimising side reactions and environmental problems, enzyme assisted extraction can be an alternative to alkaline extraction. Enzyme assisted extraction is considered to be a more mild extraction method and has lower impact on the environment [15].

A wide range of biomass could be used as a source for protein. Here, we limit ourselves to oilseed meals. Currently, oilseed meal is mainly used in animal feed [16]. Increased economic value can be obtained if the protein that is retained in the by-product is extracted. This valorization of protein from biomass does not only create direct added value, but will also add value to the overall chain, adding to the final economic feasibility of biofuel production [17].

The aim of this work is to investigate the possible use of enzymes in assisting the extraction of protein from rapeseed, soybean, and microalgae meal under mild conditions at different pH. To relate this research to other results, protein extraction from soybean meal was used as reference. We also tested untreated microalgae biomass to study the influence of de-oiling on the subsequent protein extraction. By applying mild conditions for protein extraction, we expect to reduce chemical usage and salt formation, leading to economic and environmental benefits.

2. Materials and methods

2.1 Materials

Milled rapeseed and soybean meals were obtained from Schouten Ceralco (Rotterdam, the Netherlands). Both microalgae meal (*Chlorella fusca*) and agricultural waste-water-cultured-microalgae (microalgae) (*Chlorella fusca*) were obtained from Ingrepro B.V., the Netherlands. Five enzyme mixtures; Protex 40XL, Protex P, Protex 5L, Protex 50FP, and Protex 26L- were obtained from Genencor (Danisco) International Oy, Denmark. We tested only Genencor enzymes due to the availability. All of the enzymes are classified as endoproteases, except for Protex 50 FP, which is a mixture of endo and exo proteases.

2.2 Dry weight determination

Dry matter content in hydrolysate was determined after oven drying at 95 ˚C to constant weight [18]. The protein content per gram dry matter (% w/w) in the hydrolysate was determined as the ratio of protein content to total dry matter in the hydrolysate.

2.3 Protein content analysis

Protein content in starting material and hydrolysate was determined using DUMAS analysis (FlashEA 1112 series, Thermo Scientific, Interscience). Methionine was used as a standard for the calibration. A constant of 5.53 [19], 5.66 [20], and 5.13 [21] was used as a nitrogen-protein conversion factor to calculate the protein content in the samples originating from rapeseed, soybean, and microalgae, respectively. Since the maximum contribution of enzyme is only 0.1% (for liquid enzyme: % volume of enzyme to volume of mixture; for solid enzyme: % weight of enzyme to volume of mixture) to the mixture, we did not correct the protein content for the enzyme added.

2.4 Degree of hydrolysis analysis

Degree of hydrolysis analysis of the hydrolysates was determined by a modified Trinitrobenzenesulfonic acid (TNBS) method [22]. The hydrolysate (15 µl) was mixed with 42 µl 0.21 M sodium phosphate buffer pH 8.2 in a microwell. Forty-five milliliter of 0.05% TNBS in ultrapure water was added and the microwell plate was covered with aluminum foil and incubated at 50 ºC for 60 min. After incubation, 90 µl 0.1 M HCl was added. Absorption was measured at 340 nm. Leucine (0.0 – 1.5 mM) was used to generate a standard curve.

2.5 Protein extraction

Protein extraction was performed at various conditions (Table 1). Temperature and pH were varied while the protein content was kept constant. Temperature and pH were varied based on the working pH of the enzymes. Biomass with a measured protein content of 2 g protein was left to dissolve in 50 ml deionized water for 30 min. Reaction mixtures were stirred and the pH was adjusted by adding 0.5 M NaOH for alkaline conditions or 0.5 M HCl for acidic conditions. Metrohm 718 STAT (Metrohm ion analysis) equipped with a water bath (± 0.1 °C) was used to maintain temperature and pH. After 30 min, 1% enzyme (for liquid enzyme defined as volume of enzyme per weight of protein (v/w) while for solid enzyme defined as weight of enzyme per weight of protein (w/w) was

added to the reactor. The reaction time was set as zero when the enzyme was added. After 2 h, another 4% enzyme was added to give a final enzyme concentration of 5%. Samples were taken in time at t = 0, 2 and 3 h. The enzymatic reaction was terminated by heating at 90 °C for 10 min. Samples were then centrifuged at 5000 rpm for 10 min in a Sigma 3-10 centrifuge to get a solid-liquid separation. The supernatant containing the proteins and peptides was filtered using Whatman filter unit UNIFLO 25/0.45 RC to remove small particles. Protein extraction was also performed in an identical experimental set up without enzyme addition.

Protex 40 XL was subsequently used to determine the effect of reaction time and enzyme dosage. The reaction (in triplicate) was performed in identical set up with previous experiment. The reaction was prolonged to 24 h. Only a single 1% enzyme addition was performed at t = 0. In the previous experiment, a high enzyme concentration (5%) was chosen to extract all or most of protein into the solution. Now, 1% is used to check feasibility to extract protein at lower enzyme concentrations to reduce production cost. The samples were taken in time at t = 0, 2, 3, 6 and 24 h.

2.6 Statistical analysis

Minitab Version 16 was used to obtain statistical analysis of data. Analysis of variance (ANOVA) was conducted using a General Linear Model procedure for the significance of differences amongst hydrolysates at a 5% significance level with three treatment replicates for most experiments, except for the protein content per gram dry matter content (only duplos).

Table 1. Enzymes characteristic and incubation conditions

Enzyme	Type	Optimum pH range as given by supplier	Activity as given by supplier	Microorganism	Incubation	
					pH	Temp (°C)
Protex 40XL	endoprotease	8 - 11.5	52 MPU/ml[a]	*B. subtilis*	11	60
Protex P	endoprotease	6 - 10	4240 APU/ml[b]	*B. subtilis*	10	60
Protex 5L	serine endoprotease	7 - 10	10000 MPU/ml[a]	*B. licheniformis*	9.5	60
Protex 50 FP	endo/exo protease	3 - 6	500000 HUT/g[c]	*A. oryzae*	3	50
Protex 26 L	endoprotease	2.5 - 6	2000 SAPU/ml[d]	*A. niger*	2.5	50

[a]MPU is defined based on the ability of a protease to cleave p-nitroanilide from a synthetic peptide, N-succinyl-ala-ala-ala-p-nitroanilide (suc-AAApNA), [b]APU = Alkaline Protease Units, [c]HUT = Hemoglobin Units on Tyrosine Basis, [d]SAPU = Spechtrophotometric Acid Protease Units

3. Results and Discussion

3.1 Protein content in starting materials

Rapeseed, soybean, and microalgae meals contain significant amount of protein (Table 2). The protein content of these meals ranges from 26 – 37% (w/w), depending on the type of meal. These values are in accordance with literature stating that de-hulled oilseed meals can contain protein in the range of 35 – 60% [23]. The slight difference between our result and the literature values might due to the difference of nitrogen to conversion factor. Untreated microalgae contain 28% (w/w) protein.

3.2 Influence of pH on protein extractability

An appropriate extraction method is required to get an optimal economic benefit of biomass protein. Protein extraction under acidic conditions only extracts a small fraction of protein (blanks with pH 2.5 and 3 in Fig. 1). Acid could just extract 15% of protein from rapeseed and 16% microalgae meal. More protein (17%) is extracted from soybean meal; considerably below the result with salt solution at pH 4.5 that gave 65% [24]. The low extractable protein yields in these experiments indicate little biomass cell wall degradation by acid, preventing protein diffusion to the medium. Moreover, the applied pH is closer to the protein isoelectric point than in the alkaline experiments; therefore, the protein has less net charge providing lower protein solubility. Protein solubility profiles clearly indicate the low solubility of rapeseed [25, 26], soybean [27] and microalgae [28] proteins at acidic condition.

Under alkaline conditions (blanks at pH 9.5, 10 and 11 in Fig. 1), more protein is extracted from selected biomass compared to the acidic conditions. The amount of extracted protein varies between 15-80%, depending on the type of biomass. Only small differences in protein extraction yield are observed when using different pH (9.5, 10, and 11).

Table 2. Nitrogen and protein content in biomass

Biomass	**Nitrogen content (% dm)**	**Protein content (% dm)**
Rapeseed meal	4.8	26.3[a]
Soybean meal	6.4	36.4[b]
Microalgae meal	7.3	37.3[c]
Microalgae	5.5	28.1[c]

Nitrogen to protein conversion factor for [a] = 5.53[19], [b] = 5.66 [20], [c] = 5.13 [21]

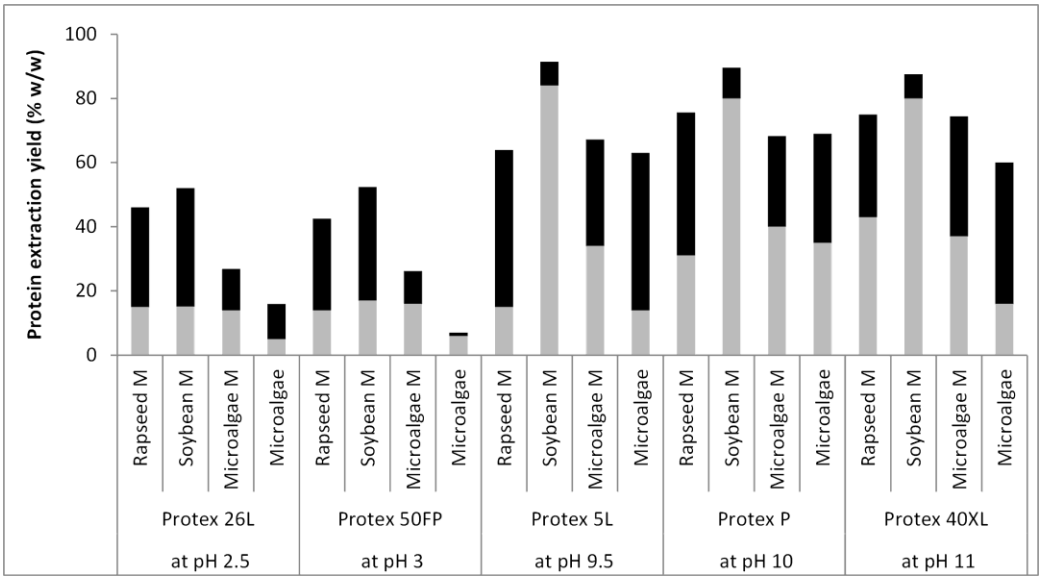

Figure 1. Protein extraction yield after 3 h incubation without enzymes (■) and an addition by the enzymes (■) (M = meal).

3.3 Influence of biomass source on protein extractability

Alkaline showed to be extremely effective in extracting soybean meal protein. As much as 80% of soybean meal protein was extracted, while the percentages of extractable protein for the other meals were much lower at about 15-30%, with untreated microalgae as the lowest. Several factors that may influence protein extraction include protein solubility, biomass cell wall structure and chemical composition. The high extractability of soybean meal compared to rapeseed meal and microalgae meal may be due to higher solubility of its protein and its low phenolic content.

The protein extraction is partly determined by the solubility of protein molecules [29]. For this, the alkali has to permeate into the cell. The biomass protein characteristics determine the protein solubility. Glycinin and β-conglycinin are the major protein in soybean, that represent 40% and 30% of total soybean protein, respectively [30]. Glycinin and β-conglycinin have different solubility properties. Their simultaneous presence in soybean contributes to high solubility of soybean protein under alkaline condition [27]. Rapeseed meal, mainly consists of cruciferin (60%) [31], which has a lower protein solubility compared to soybean protein [25, 26] resulting in lower rapeseed meal protein extraction.

Soybean meal furthermore has a low content of phenolic compounds compared to that of rapeseed meal, and microalgae meal, which can be twice as high. Total phenolic compounds per 100 g soybean, rapeseed, and microalgae (*Chlorella sp.*) are 693–780 mg [32, 33], 1166-1821 mg [32, 34], and 777-1946 mg [35, 36], respectively. Phenolic compounds are reactive to oxidation at alkaline pH [37]. The oxidized phenolic compounds can then reactively interact, via covalent or non-covalent bonding with protein. In the case of covalent linkage, the protein might even precipitate which will lower the protein solubility. The dark brown color that was observed in the alkaline

treatment of rapeseed meal, microalgae meals indicates the presence of phenolic compounds in combination with protein. This combination leads to lower protein extraction yields in rapeseed meal, microalgae meal, and microalgae compared to soybean meal.

Biomass pre-treatment also determines the protein extraction mechanism via alternations in the biomass cell wall structure. Out of the four biomass types tested, three were pre-treated by de-oiling. For the untreated microalgae, undisrupted cell and the presence of fat may inhibit higher protein extraction [38]. The de-oiling of rapeseed, soybean and microalgae ruptures their rigid cell wall. This facilitates solvent permeation followed by diffusion of protein inside the cell into the solvent.

The microstructure of oilseed cells shows that the protein spreads inside the cell and fat fills in the space between proteins [39]. The influence of fat on protein extraction is not only based on cell structure features but also chemical features. Chemically, fat will be hydrolyzed via saponification on alkaline conditions. When this happens, the protein can dissolve better into solution, partly explaining the better results under alkaline conditions. However, the negative effect of oil is better to be circumvented, and therefore, we recommend to de-oil prior to protein extraction.

3.4 Enzyme assisted protein extraction

The influence of enzymes in assisting protein extraction is reflected by the additional protein recovery percentage in Fig. 1. Enzymes assisted in protein extraction from all tested biomass.

Protex 5L, Protex P, and Protex 40XL that work at alkaline pH gave higher protein recovery yield compared to Protex 26L and Protex 50FP that work at acidic pH. After 3 h incubation, Protex 40XL, Protex P, and Protex 5L extracted most of biomass protein. These 3 enzymes gave comparable protein recovery. With 5% enzyme, we extracted 50-80% rapeseed and microalgae meals protein and 90% soybean meal protein. Fischer et al. [40] achieved 83% protein extraction yield by using a combination of Alcalase (2.5%) and Flavourzyme (5%) at pH 7.6 to extract soybean meal protein. With less total enzyme dosage, Protex 40XL, Protex P, and Protex 50FP gave higher protein recovery. The higher protein recovery can be due to a better extraction at slightly higher pH, or to the usage of other enzymes.

Protein recovery was also measured at t = 2 h (data not shown). Addition of 1% enzyme at t = 0 yielded additional protein after 2 h for all biomass. Meanwhile, subsequent addition of 4% enzyme at t = 2 h gave only little additional protein recovery at t = 3 h. To better estimate the effect of enzyme dosage and time, additional experiments were performed with Protex 40XL.

3.5 Enzyme dosage

Protex 40XL addition to biomass was studied in more detail. High amounts of extracted protein were obtained from all biomass (Fig. 2). However, the effectiveness of Protex 40XL in extracting protein is determined by the type of biomass. Protex 40XL significantly assisted protein extraction from all biomass except soybean meal when compared to the blank, in which no enzyme was added. Soybean meal protein was already extracted without the need for enzyme addition.

Additional protein recovery was obtained after 24 h incubation with 5% Protex 40XL, yielding 12, 23, and 36% protein for rapeseed meal, microalgae meal, and microalgae, respectively. It is likely that Protex 40XL assisted protein extraction for these biomass. However, that is not the case for soybean meal, in which 5% Protex 40XL was only able to provide an additional 6% soybean meal protein recovery.

Furthermore, increasing Protex 40XL dosage only significantly aided protein extraction for rapeseed meal and microalgae meal. While higher Protex 40XL dosage did not give added value on extracting microalgae. It may be explained by the presence of fat as aforementioned which contributed to chemical structural features that limit enzyme digestibility and accessibility, and therefore limit the rate of hydrolysis [41].

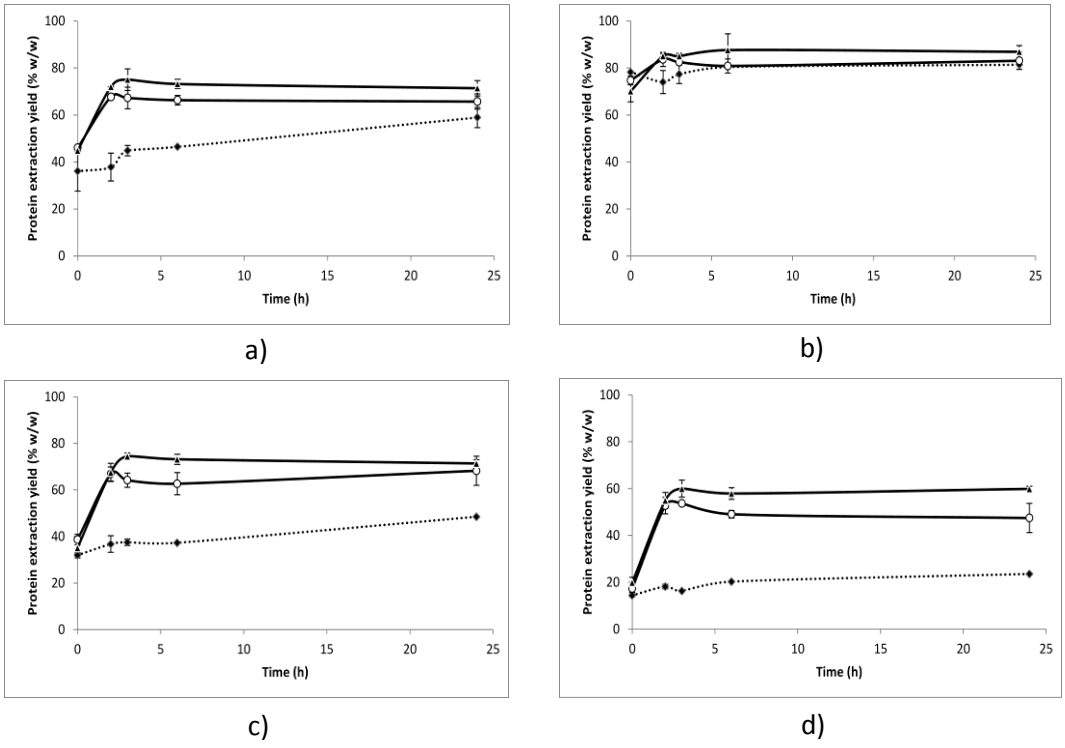

Figure 2. Protein extraction yield of (a) rapeseed meal, (b) soybean meal, (c)microalgae meal, (d) microalgae incubated by Protex 40XL for 24 h at 0, 1, and 5 % enzyme dosage. ····◆···· 0% —○— 1% —▲— 5%

3.6 Degree of hydrolysis (DH)

Figure 3 shows the primary amine concentration obtained in the hydrolysate. During protein hydrolysis peptide bonds are broken resulting an increase of primary amine concentration. It therefore corresponds to an increase in degree of hydrolysis. Referring to Fig 3, we could say that the enzyme hydrolyzed the protein, resulting in a (partially) hydrolyzed protein that dissolved into solution.

A certain amount of hydrolysis is needed, looking at the difference in protein extraction between the reactions with and without added enzyme. However, hydrolytic activity is not depending on the type of biomass (Fig. 3). The amine groups in the protein and its hydrolysates, all roughly go from 10 to 50 mM NH_2 groups, while protein extraction gives different numbers for each biomass type. Not only does the total hydrolytic activity fail in explaining the different protein extraction yield between types of biomass, it also fails in explaining the difference on protein extraction yield determined by enzyme dosage, particularly in rapeseed and microalgae meals. From this we conclude that extensive hydrolysis is less important for the protein extraction.

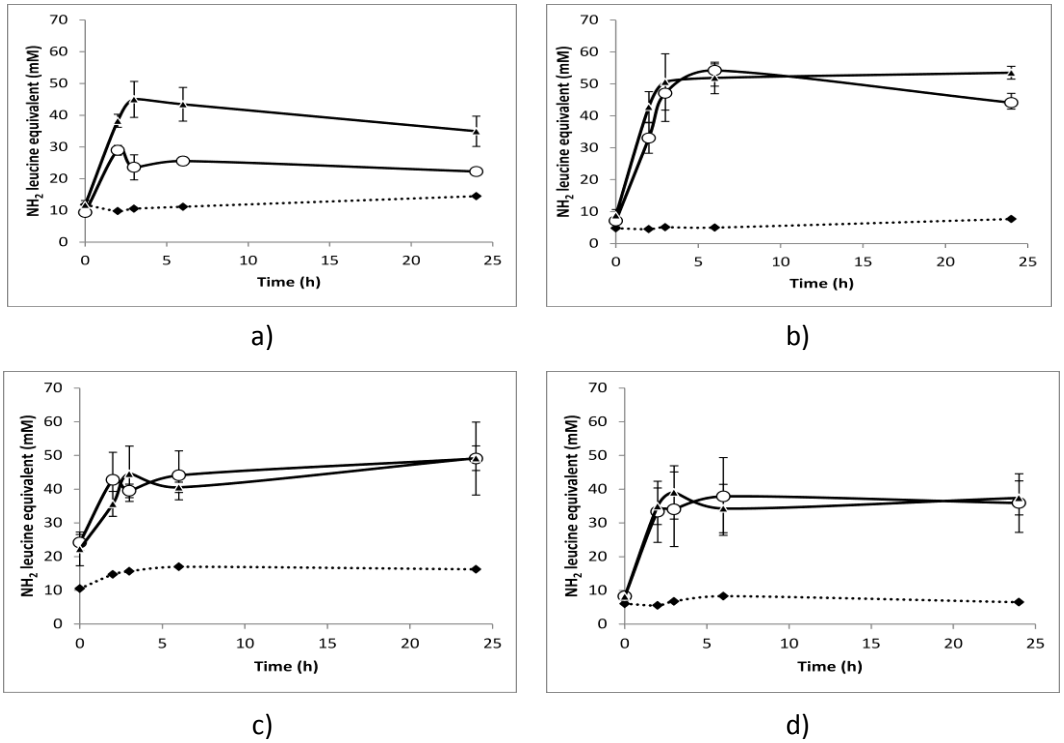

Figure 3. Primary amine concentration (determined as mM leucine equivalent).
(a) rapeseed meal, (b) soybean meal, (c) microalgae meal, (d) microalgae.

Table 3. Protein content per gram dry matter (% w/w) of supernatant obtained from 1% and 5% Protex 40XL (n = 2)

Time	Rapeseed meal		Soybean meal		Microalgae meal		Microalgae	
(h)	1%	5%	1%	5%	1%	5%	1%	5%
0	45 ± 2	40 ± 3	56 ±3	51 ±2	42 ± 0	37 ± 2	40 ± 6	40 ± 7
3	54 ± 5	57 ± 7	58 ±1	61 ± 9	57 ± 2	59 ± 4	68 ± 6	59 ± 3
24	58 ± 1	51 ± 1	52 ±2	61 ± 5	53 ± 5	60 ± 3	57 ± 3	73 ± 6

3.7 Protein content per gram dry matter (% w/w)

Next to protein, other components get extracted due to the alkaline treatment, therefore protein content per gram dry matter (% w/w) was also calculated. Table 3 shows the purity of extracted protein. Purity determines the difficulty of downstream processing of the extracted protein and the value of the material in case of feed. For the soybean meal case, enzyme treatment did not change protein content per gram dry matter (% w/w), since the enzyme did not aid in protein extraction. In all other cases protein content per gram dry matter (% w/w) increased by enzymatic treatment, due to a higher amount of protein that was extracted. However, the enzyme dosage did not significantly influence the protein content per gram dry matter (% w/w) in all cases. Only in microalgae meal the protein content was influenced by the addition of a different amount of enzyme.

4. Conclusion

We examined the feasibility of protein extraction from several biomass sources that originated from bio-oil production. Considered as bio-oil by-products, rapeseed, soybean, and microalgae meals are indeed good potential resources for protein production. Untreated microalgae showed its potential to a lesser extent and we therefore suggest to de-oil this biomass prior to its protein extraction. Soybean meal alkaline extraction already showed good yields without enzyme addition. However, with the other biomass sources, the use of enzymes improved protein extraction yields, compared to alkaline or acidic extraction as such. The alkaline enzymes that we used were all good potential candidates for protein extraction, hydrolysing part of the protein and thereby aiding in its solubilisation.

The enzyme assisted protein extraction showed its ability to extract protein particularly from rapeseed and microalgae meal. However for further application as bulk chemicals for chemical industries, we would recommend to use more active enzymes to breakdown the soluble protein to get to smaller peptide or better so amino acids that can be used directly as a starting material for further reactions.

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Chapter 5

Glutamic acid production from wheat by-products using enzymatic and acid hydrolysis

Abstract

Glutamic acid (Glu) has potential as feedstock for bulk chemicals production. It has also been listed as one of the top twelve chemicals derived from biomass. Large amounts of cheaper Glu can be made available by enabling its production from biomass by-products, such as wheat dried distillers grains with solubles (DDGS) derived from ethanol production. The aim of this study was to develop a new method for Glu production from biomass. Wheat gluten was used to represent wheat DDGS. To reduce chemicals usage, several methods were evaluated. These included enzymatic, dilute acid, and a combination of enzymatic and dilute acid hydrolysis. The separate enzymatic and dilute acid hydrolysis (1 M HCl; 95 ˚C) resulted in yields of 48% and 46% Glu, respectively. However, the combination of enzymatic and dilute acid hydrolysis resulted in a much higher yield of 70% Glu and 10% pyroglutamic acid thereby opening up new possibilities for the industrial production of Glu from biomass.

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1. Introduction

The biorefinery and biobased economy concepts promote the utilisation of biomass and by-products to produce bio-based products and create more value from plant production chains. With this, society will benefit ecologically and economically. Ecologically, the use of biomass will reduce the use of fossil raw materials. Economically, biomass has lower price compare to that of fossil raw material [1]. Recently there are numerous reviews or roadmaps exploiting the use of lignocellulose for producing biobased platform chemicals [2]. PNN/NREL (Pacific Northwest National/National Renewable Energy Laboratory) roadmap reported the top twelve chemicals derived from biomass sugar, which included glutamic acid (Glu) [3]. The possibility of valorizing Glu to bulk chemicals through its decarboxylation using Glu α-decarboxylase has been shown recently [4]. Another method of Glu decarboxylation, via electro-oxidative, could also be used for valorizing Glu [5]. Examples of chemicals that have been synthesized from glutamic acid include N-methylpyrrolidone, succinonitrile, pyrrolidone, and N-vinylpyrrolidone [6].

Glu production from sugar can be done via fermentation, but still needs improvement in microbial catalysis. Alternatively, Glu can be produced from biomass protein. A recent review on availability of protein-derived amino acids shows that Glu is abundantly available in protein from several biomass by-products, including wheat dried distillers grains with solubles (DDGS), corn DDGS, and sorghum DDGS [7]. Production of Glu from these types of materials will require less energy and chemicals compared to that of the sugar based route. In addition, DDGS is an important by-product of bioethanol production. With increasing bioethanol production, significant amounts of DDGS will become available. Currently DDGS is a low priced animal feed. Since Glu is categorized as non-essential amino acid, production of Glu from DDGS will generate additional value for use as a bulk chemical precursor without decreasing the animal feed value.

The Glu production from DDGS or other biomass by-products is still challenging. To start, protein from biomass has to be solubilized. The intrinsic hydrophobic nature of protein and the presence of disulphide bonds between protein subunits makes it difficult to completely solubilise protein at aqueous conditions [8-10].Hydrolysis, either chemically or enzymatically, may increase solubility by breaking down the protein into peptides and amino acids. Next, favourable hydrolysis conditions should be found, that not only liberate high amounts of Glu, but also should be more environmentally friendly. In this paper, different hydrolysis methods were evaluated for producing Glu from biomass by-product. Wheat gluten, which can be considered a by-product of wheat starch production, was chosen as a representative of DDGS.

2. Materials and Methods

2.1 Materials

Wheat gluten was obtained from Cargill (the Netherlands). Alcalase 2.4L FG and Flavourzyme 1000L were obtained from Novozyme (Denmark). Validase FP concentrate was obtained from DSM (the Netherlands). M Amano SD, Peptidase R, and Glutaminase SD-C100S were obtained from Amano (Japan). All other chemicals used were of analytical grade.

2.2 Acid hydrolysis of raw wheat gluten

Total acid hydrolysis of wheat gluten was done in duplicate. Therefore, wheat gluten (1 mg) was transferred to an analysis vial and placed in a reaction vial assembly from Eldex (Napa, CA, USA). A 500 µl 6M HCl containing 1% (w/v) phenol was added into the bottom of the reaction vial. The vial was flushed with nitrogen, sealed under vacuum, and subsequently heated up in an oven at 110 ºC for 24 h. The remaining acidic solution was vacuum dried applying a Waters Pico Tag workstation.

2.3 Dilute acid hydrolysis of wheat gluten

Acid hydrolysis of wheat gluten was done in duplicate at diluted conditions. In short, wheat gluten with a measured protein amount of 5 g protein was left to hydrated in 100 ml 1 M HCl at 95 ºC for 48 h. The reaction temperature was maintained by using a GFL 1086 water bath shaker. The water bath angular speed was set at 120 rpm. Samples were taken with time at t = 6, 24, and 48 h. Subsequently, samples were centrifuged at 5000 rpm for 10 minutes in a Sigma 3-10 centrifuge to obtain a solid-liquid separation. Next, the protein hydrolysate (supernatant) was neutralized by addition of NaOH. Samples were kept at -20 °C until further analysis.

2.4 Enzymatic protein hydrolysis

Wheat gluten was hydrolysed (in duplicate) using several enzymes (Table 1). Five different enzyme mixtures were evaluated. They were categorized as endo-proteases, exo-proteases, and mixtures of endo and exo-proteases. The hydrolysis conditions are given in Table 1. Temperature and pH were varied while the protein content was kept constant. Wheat gluten with a measured protein amount of 5 g protein was left to hydrate in 100 ml deionized water for 30 min. Subsequently, the pH was pre-adjusted by adding 2 M NaOH. The reaction temperature was maintained by using a GFL 1086 water bath shaker (120 rpm). After 30 min, 1% enzyme (v/w; enzyme volume (ml) per weight of protein (gram) or w/w for solid enzymes) was added to the mixture (t=0). The reaction time was set to zero after enzyme addition. After 2 h of incubation, another 1% enzyme was added to give a final enzyme concentration of 2%. Samples were taken at t = 0, 1, 3, 6, and 24 h. The hydrolysis was terminated by heating at 90 °C for 10 min. Samples were subsequently centrifuged at 5000 rpm for 10 min in a Sigma 3-10 centrifuge to get a solid-liquid separation. For some cases, another 1% additional enzyme dosage was added to this liquid part. As control, protein hydrolysis was also performed in an identical experimental set up without enzyme addition. The pH and temperature for control were selected based on the condition for enzymatic hydrolysis. pH 8.5 was the highest pH used in enzymatic hydrolysis. As higher pH normally solubilizes more protein, the highest pH

was selected as a control. With this, it was expected that the protein solubilized in the control would be the maximum protein yield that can be obtained without enzyme addition. Samples were kept in -20 °C until used for further experiment or analysis.

2.5 Enzymatic deamidation of wheat gluten protein hydrolysate

Glutaminase SD-C100S was used to catalyse the enzymatic deamidation (in duplicate) of glutamine (Gln) into Glu in hydrolysate of wheat gluten protein. A modified method of Yie et al. was used [11]. The pH of the wheat gluten protein hydrolysates was adjusted to 7 using 1 M NaOH. Deamidation was started by adding 0.08 unit/ml Glutaminase SD-C100S into 40 ml wheat gluten protein hydrolysate. The reaction temperature was maintained at 55 ºC by using a GFL 1086 water bath shaker (120 rpm). Samples were taken with time at t = 0, 3, 6, and 24 h. Deamidation was terminated by heating at 90 °C for 10 min. Deamidation with identical set up was also performed on pure Gln. Samples were kept at -20 °C until further analysis.

2.6 Combined enzymatic and dilute acid treatment of wheat gluten

Acid hydrolysis of wheat gluten hydrolysate (see Section 2.2) at diluted acid conditions was done in duplicate in wheat gluten protein hydrolysate obtained by enzymatic protein hydrolysis. Taking into account the water volume of the protein hydrolysate, 6 M HCl was added to the solution to obtain a final acid concentration of 1 M or 0.1 M. The reaction temperature was maintained at 95 ºC by using an Eppendorf thermal mixer equipped with 1.5 mL polypropylene Eppendorf tubes as reaction vessels. Samples were taken with time at t = 0, 6, 24, and 48 h. The reaction was stopped by cooling the samples to room temperature. Subsequently, the samples were neutralized to pH 7 by the addition of NaOH. Samples were kept in -20 °C until further analysis.

Table 1. Overview of enzymes. Enzyme type and experimental condition are indicated.

Enzyme	**Type**	**Activity as given by the supplier**	**Hydrolysis condition**	
			pH	**Temperature (°C)**
Alcalase 2.4L FG	Endo-proteases	2.4 AU/g[a]	8.5	55
Validase FP Concentrate	endo- and exo-proteases	400000HU/g[b]	6.0	55
M Amano SD	endo- and exo-proteases	5500 U/g[c]	7.0	40
Peptidase R	Exo-proteases	420 U/g[d]	7.0	40
Flavourzyme 1000L	Exo-proteases	1000 LAPU/g[e]	7.0	55
Glutaminase SD C-100S	Glutaminase	100 GTU/g[f]	7.0	55

[a]AU = Anson Unit, [b]HU = Hemoglobin Unit, [c]determined by Amano method, [d] determined by L-Leucyl-Glycyl-Glycine method, [e]LAPU = Leucine AminoPeptidase Unit, [f]GTU = Glutaminase Unit

2.7 Protein content analysis

Protein content in raw material and hydrolysate was determined using DUMAS analysis (FlashEA 1112 series, Thermo Scientific, Interscience). Methionine was used as a calibration standard.

2.8 Amino acid analysis

Dionex UltiMate 3000 U-HPLC (Ultra High Pressure Liquid Chromatography) was used to analyze amino acid based on Meussen, et al. [12]. For the analysis of free amino acids, acid hydrolysis prior to analysis was omitted.

Dionex UltiMate 3000 U-HPLC (Ultra High Pressure Liquid Chromatography) was also used to analyze pyroglutamic acid (p-Glu). Prior to detection, 500 µl samples were mixed with 500 µl valeric acid as an internal standard. p-Glu was detected at 210 nm on an UltiMate 3000 variable wavelength. A Phenomenex Rezex ROH-organic acid column (8 µm particle size, 300 x 7.8 mm) was used, with a column oven temperature of 60 ^{o}C, and 12 mM sulfuric acid as eluent.

2.9 Data analysis

The term "hydrolysate" used throughout this paper refers to the soluble hydrolysate that was obtained following solid-liquid centrifugation. Solubilised protein yield was determined as the ratio of protein (% w/w) in protein hydrolysate to protein in raw wheat gluten. Glx yield was determined as the ratio of the combined free Gln and Glu concentration in protein hydrolysate to total Glu concentration in raw wheat gluten. Glu yield was determined as the ratio of free Glu in protein hydrolysate to total Glu concentration in raw wheat gluten. Microsoft Excel version 2010 was used to calculate the average and standard deviation of the data (in duplicate). Minitab Version 16 was used to run a student t-test at 5% significance level.

3. Results

3.1 Acid hydrolysis of wheat gluten

The amino acid composition after total acid (6 M HCl) hydrolysis of wheat gluten is given in Table 2. This Table also includes the amino acid composition obtained from combined enzymatic and dilute acid hydrolysis that is discussed in the other sections. Asparagine, Gln, and tryptophan could not be detected due to the acidic conditions that were applied for hydrolysis. The nitrogen content of wheat gluten was 13.0 ± 0.9%, based on DUMAS measurement. The wheat gluten that was used in our research contained 37% (w/w) Glu, which is comparable with the material used in the other reported research [13]. The Glu content in wheat gluten obtained from a total acid hydrolysis was used as a reference for the calculation of the Glu yield in the experiments.

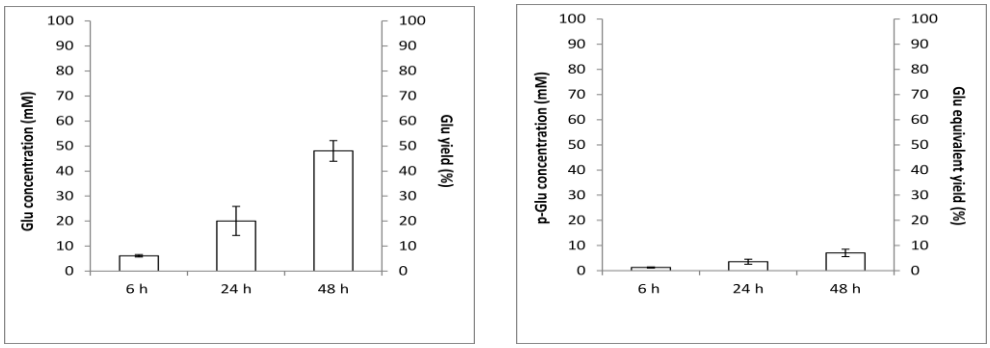
Table 2. Amino acid concentration in wheat gluten obtained after total acid hydrolysis with 6 M HCl and yield obtained after combined enzymatic and dilute acid (1 M HCl) hydrolysis of wheat gluten used in the experiments

Amino acid	Concentration in wheat gluten (6 M HCl hydrolysis)	Validase FP Concentrate + Peptidase R and 1 M HCl		M Amano SD and 1 M HCl	
	(mmol/g)	t = 24 h	t = 48 h	t = 24 h	t = 48 h
Aspartic acid	0.19 ± 0.00	68 ± 7	70 ± 7	48 ± 4	49 ± 3
Glutamic acid	1.86 ± 0.04	77 ± 11	84 ± 9	67 ± 4	79 ± 6
Histidine	0.06 ± 0.00	98 ± 17	108 ± 12	93 ± 9	99 ± 7
Serine	0.35 ± 0.01	18 ± 3	24 ± 6	24 ± 4	33 ± 6
Arginine	0.13 ± 0.01	9 ± 3	11 ± 2	5 ± 1	7 ± 0
Glycine	0.34 ± 0.02	59 ± 8	67 ± 7	54 ± 3	63 ± 3
Threonine	0.15 ± 0.01	59 ± 9	72 ± 17	45 ± 3	53 ± 4
Tyrosine	0.12 ± 0.00	54 ± 0	53 ± 9	57 ± 5	58 ± 10
Alanine	0.22 ± 0.00	68 ± 8	72 ± 6	39 ± 2	45 ± 3
Proline	0.83 ± 0.05	40 ± 6	43 ± 3	27 ± 2	40 ± 2
Valine	0.2 ± 0.00	82 ± 11	85 ± 9	63 ± 5	69 ± 5
Methionine	0.09 ± 0.00	67 ± 8	64 ± 6	44 ± 3	45 ± 4
Isoleucine	0.23 ± 0.02	94 ± 13	97 ± 10	75 ± 7	82 ± 7
Phenylalanine	0.15 ± 0.00	58 ± 10	61 ± 6	51 ± 3	56 ± 5
Leucine	0.39 ± 0.01	69 ± 10	73 ± 7	57 ± 6	61 ± 5
Cysteine	0.01 ± 0.01	n.a[a]	n.a[a]	n.a[a]	n.a[a]
Lysine	0.21 ± 0.04	22 ± 3	25 ± 3	19 ± 2	20 ± 1
Total amino acids	5.54 ± 0.34	57 ± 8	55 ± 6	47 ± 4	49 ± 4

[a] n.a = not available

3.2 Dilute acid hydrolysis of wheat gluten

The possibility to reduce the use of chemicals was evaluated by applying acid hydrolysis at dilute acid conditions. Therefore, wheat gluten was hydrolysed with 1 M HCl. In addition to Glu, application of this method also resulted in the production of p-Glu. Fig. 1a and b show the amounts of Glu and p-Glu formed during dilute acid hydrolysis of wheat gluten, respectively. The Glu concentration in initial biomass is 120 mM which is close to multiplication factor (100%), therefore, apparently the Glu yield and Glu concentration had almost similar value. For example at t = 48 h; Glu concentration was 48 mM which correlates to 40% yield. The concentration of p-Glu was 7 mM. Assuming that the p-Glu can be converted into Glu, a maximum of 46% Glu yield would be achieved. This value is still too low for an industrial feasible process.



a) b)

Figure 1. a) Free Glu concentration and Glu yield and b) p-Glu concentration and Glu-equivalent yield in protein hydrolysate after dilute acid hydrolysis with 1 M HCl of wheat gluten.

3.3 Enzymatic protein hydrolysis

Another option to reduce the use of chemicals in the (industrial) production of Glu is enzymatic hydrolysis. Fig. 2 shows the solubilised protein yield (%w/w), the amount of wheat gluten protein that was solubilised in the hydrolysate, after 24 h incubation with and without enzyme. Without enzyme addition, 35% protein was solubilised after 24 h incubation. More protein was solubilised as a result of the action of the added proteolytic enzymes, with a maximum of 94%, depending on the enzyme combinations.

To increase the solubilised protein yield, higher enzyme dosages and combinations of enzyme preparations were tested. With more solubilised protein, more Glu could be liberated. Enzyme combinations that gave the highest solubilised protein yield were Validase FP Concentrate + Peptidase R (94%), M Amano SD (91%), and Validase FP Concentrate + Validase FP Concentrate (89%). Therefore further investigation was carried out on these enzyme combinations.

The concentrations of free Gln and Glu in the protein hydrolysate obtained after incubation with these enzymes are given in Fig. 3. The results show large differences in the concentration of both free Gln and Glu compared to the control without enzyme. The increased hydrolysis is most probably the cause of the increased solubilised protein yield. Both an extra enzyme addition and prolonged incubation times resulted in an increase in free Gln and Glu yield. However, after a certain incubation time, proteolysis no longer aids in protein solubilisation. There was e.g. hardly any additional solubilised protein yield at t = 24 h compared to that at t = 6 h for most enzyme combinations (Fig. 2), although hydrolysis continued and more free Glu and Gln were liberated at t = 24 h compare to that at t = 6 h. Final % Glx yields, the ratio of the combined free Gln and Glu concentration in protein hydrolysate to total Glu concentration in raw wheat gluten, were 48% and 38% of their theoretical maximum yield, achieved by Validase FP Concentrate+ Peptidase R and M Amano SD, respectively.

Almost all protein was solubilised after a treatment with one or two enzyme combinations, but not all protein was hydrolysed to free Gln and Glu. Aiming increase free Gln and Glu concentrations, more enzymes were added during the incubation. They were added to the liquid protein hydrolysate mixture after removal of the solid (insoluble) part to exclude the effect of further protein solubilisation. However, even with an excess of enzymes, the concentration of free Gln and Glu concentration did not substantially increase (data not shown).

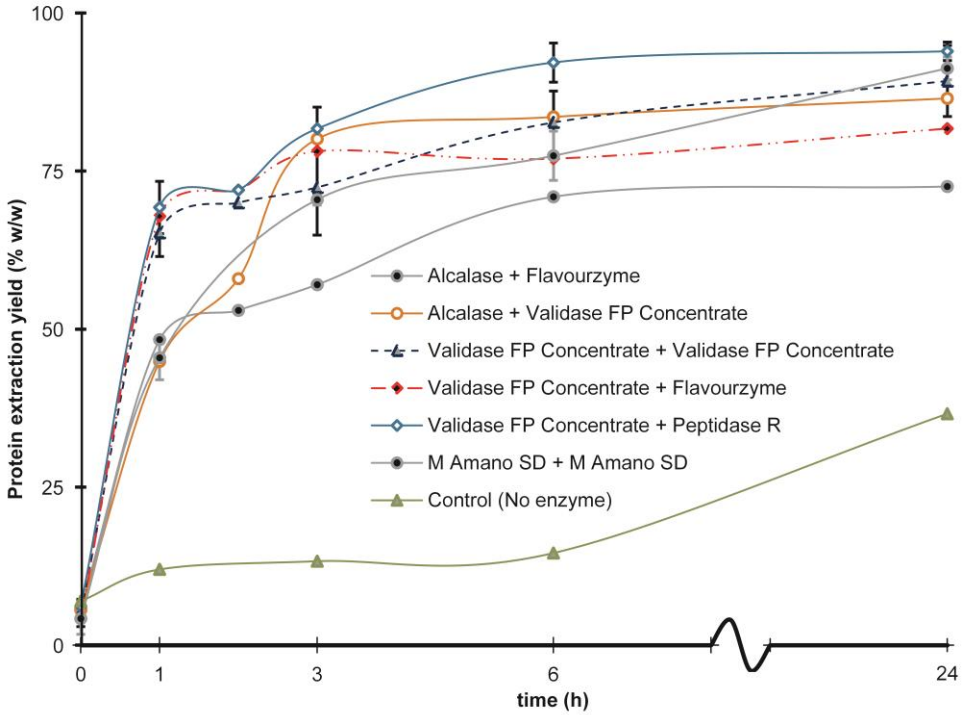


Figure 2. Solubilised protein yield obtained from several enzyme combinations. Lines are to guide the eyes of the reader.

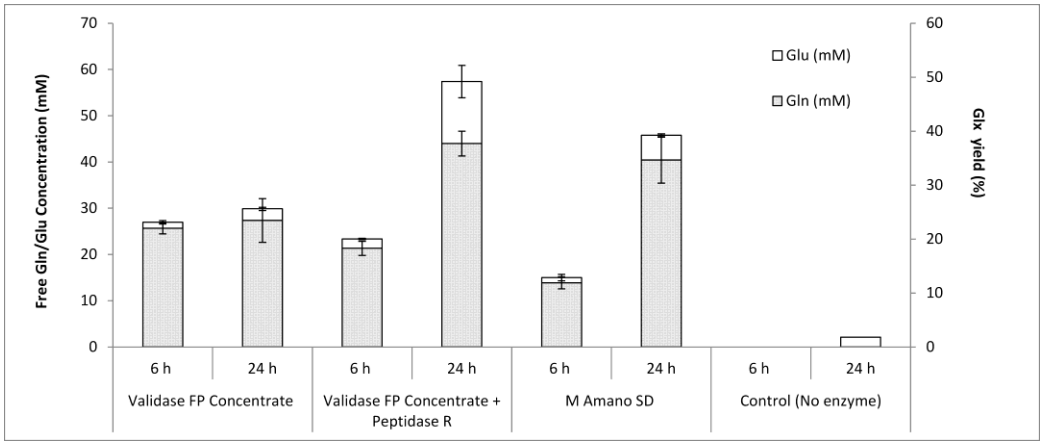


Figure 3. Free Gln Glu concentration and Glx yield after incubation with enzyme and without enzyme (control). Glx yield was determined as the ratio of the combined free Gln and Glu concentration in protein hydrolysate to total Glx concentration in raw wheat gluten.

3.4 Enzymatic deamidation of glutamine

One advantage of using acid during protein hydrolysis is the concomitant chemical conversion of Gln to Glu that is catalysed by acid. The challenge in enzymatic Glu production is the deamidation of Gln into Glu. Enzymatic deamidation of Gln that was present in three different protein hydrolysates obtained after protein hydrolysis with excessive amount of enzymes was done; using Glutaminase SD-C1000S.These hydrolysates might contain free amino acids, small peptides, and proteins. The amino acid analysis indicated that the protein hydrolysates, on average, contained 40 mM free Gln and 3 mM free Glu. Assuming that all Gln could be deamidated, this value would correspond to a 35% Glu yield at maximum. Fig. 4 shows a decrease in Gln concentration with time and an increase in Glu concentration, clearly indicating that deamidation occurred in the protein hydrolysate. However, after 24 h of deamidation, the hydrolysate only contained 30 mM Glu, which corresponds to a 26% Glu yield, much below the expected value of 35%.

However, the unexpected result was in line with a control experiment, in which the activity of Glutaminase SD-C1000S was tested with pure L-glutamine. The control started with 120 mM Gln that decreased in time. As expected, no Gln was left after 24 h deamidation. However, the deamidation only liberated 49 mM Glu. This value corresponds to 41% Glu yield, again much lower than expected.

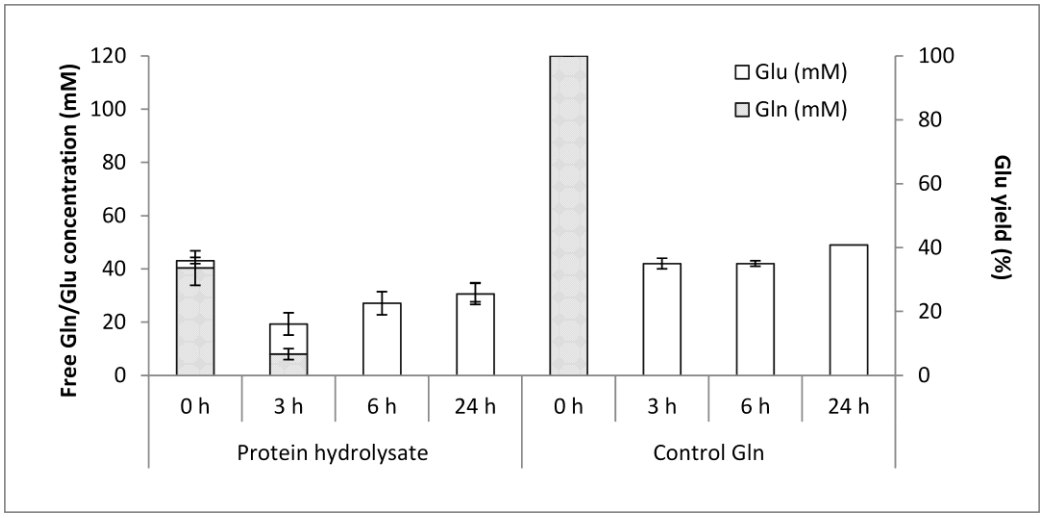


Figure 4. Free Gln /Glu concentration and Glu yield after deamidation with glutaminase. The deamination was performed on hydrolysate (average value) obtained after 24 h incubation with Validase FP concentrate + Flavourzyme; validase FP Concentrate + peptidase R; M Amano SD + Flavourzyme 1000L; and M Amano SD + Peptidase R. (% Glu yield relatives to 120 mM Glu).

3.5 Combined enzymatic and dilute acid treatment of wheat gluten

Dilute acid hydrolysis with 1 M and 0.1 M HCl was conducted on protein hydrolysates obtained after 24 h enzymatic incubation (Section 3.3). Two types of protein hydrolysates were tested: those obtained from a combination of Validase FP Concentrate + Peptidase R and those from M Amano SD. Fig. 5 shows the free Gln and Glu concentration after the combined enzymatic and dilute acid treatment with Validase FP Concentrate + Peptidase R and two concentrations of acid. Dilute acid hydrolysis with 0.1 M HCl on both hydrolysates (results not shown for M Amano SD) did not result in high Glu yields. A higher Glu yield was obtained through dilute acid hydrolysis by using 1 M HCl on both types of hydrolysates.

The concentration of free Gln decreased with time and resulted in an increased free Glu concentration. This phenomenon also occurred in our previous methods with either dilute acid or enzymes only. Yet, combining enzymatic and dilute acid liberated more Glu. Moreover, final free Glu concentration was higher than total free Gln and Glu in the initial protein hydrolysate, which was obtained from enzymatic hydrolysis.

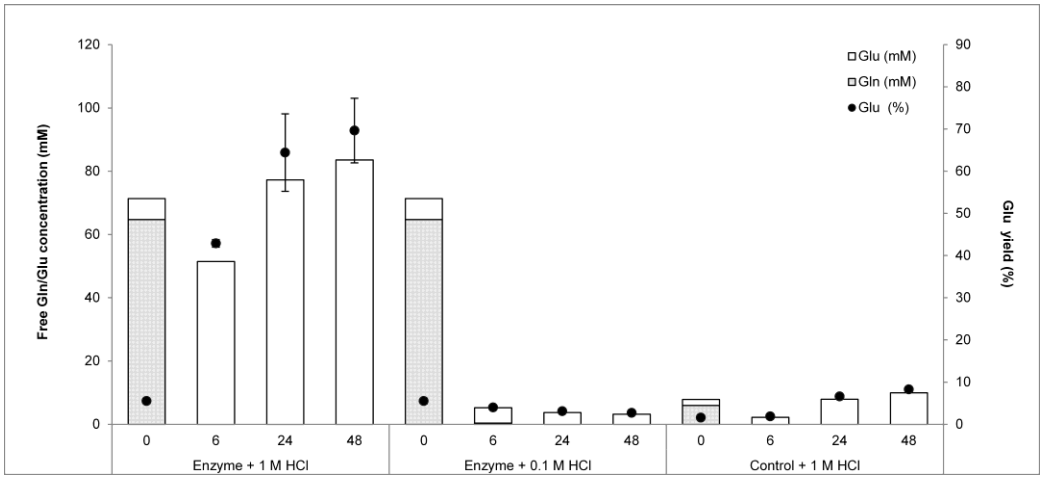


Figure 5. Free Gln /Glu concentration and Glu yield after 6, 24, and 48 h dilute acid hydrolysis. The acid hydrolysis was performed on protein hydrolysate obtained after 24 h incubation with Validase FP Concentrate + Peptidase R and control.

The total amounts of free Gln and Glu after treatment with M Amano SD and Validase FP Concentrate + Peptidase R (prior to dilute acid hydrolysis) were 46 mM and 70 mM, respectively. After subsequent 48 h hydrolysis with 1 M HCl at 95 ºC, a free Glu content in hydrolysates obtained from M Amano SD and Validase FP Concentrate + Peptidase R was 79 mM and 83 mM. These values corresponded to 66% and 70% Glu yield in hydrolysate obtained from M Amano SD and Validase FP Concentrate + Peptidase R, respectively (see the dots in Fig. 5). Dilute acid hydrolysis was also conducted on protein hydrolysate obtained from hot water solubilisation (Control + 1 M HCl in Fig. 5). However, this method only liberated 10% free Glu concentrations.

In the combined enzymatic and dilute acid hydrolysis, p-Glu was also liberated. The p-Glu concentration increased with hydrolysis time. After 48 h, 12 mM p-Glu was released. This value corresponded to 10 % Glu yield, assuming that the p-Glu can be converted into Glu.

3.6 Perspective on valorizing other amino acids

Table 2 lists other free amino acids that were liberated after the combined enzymatic protein hydrolysis and dilute acid hydrolysis. The relatively high yield of each amino acid shows the relatively extensive hydrolysis by this combined method. Potentially, this method could therefore be applied simultaneously to valorize wheat gluten by producing a wider variety of amino acids.

Overall, both combined methods showed comparable results. However, for certain amino acids, the combination with Validase FP Concentrate + Peptidase R liberated larger amounts of specific amino acids compared to the combination with M Amano SD. A t-student test with 5% significant level showed that these amino acids were aspartic acid, arginine, alanine, and methionine.

Strikingly, free cysteine was not liberated during the process. This might be related to the properties of cysteine. Wheat gluten has only a small amount of cysteine [14]. Most of the cysteine is present in the form of disulphide bridges. In order to obtain free cysteine residues, a disulphide reducing agent is needed in the process, which was not used in the current process. Therefore it is likely that the cysteine was still present in the form of disulphide bridges. Cysteine was also hardly recovered after acid hydrolysis with 6 M HCl, a standard acid hydrolysis (Table 2). Most of cysteine is degraded during standard acid hydrolysis. In order to precisely quantify cysteine, a pre-oxidation with performic acid is required [15]. This step was not included in this study.

4. Discussion

Wheat gluten, representing DDGS, was evaluated as potential source material for the production of Glu. Wheat gluten is relative rich in Glu and this amino acid was previously identified as a top value adding chemical building block, since it was shown that it could be utilized as a source material for bulk chemical via decarboxylation [4]. In 1866, wheat gluten was first used to produce Glu [16]. The method was based on evaporation of gaseous HCl at high acid concentrations. This method was discontinued because of an unsafe working environment and technical problems in maintaining the equipment from corrosion [17]. This study was focused on the optimization of the hydrolysis of wheat gluten aiming at a maximal liberation of Glu at an economically and environmentally feasible way. Acid hydrolysis with 6 M HCl at 110 ºC resulted in a total protein hydrolysis, and liberated all the Glu into the solution. The wheat gluten that was used in our research contained 37% (w/w) Glu. It is the apparent Glu content because Gln is deamidated during acid hydrolysis. Actual Glu content in wheat gluten is much lower. Considering economic and environmental issues, in this study it was aimed to develop mild(er) conditions for producing Glu.

Wheat gluten was hydrolysed at relative mild acidic condition, by mixing wheat gluten with 6 times lower acid concentration than normally applied for a total acid hydrolysis of proteins, 1 M HCl, and incubating it at 95 ºC for 48 h. With this method, 48 mM free Glu was obtained, which corresponds to Glu yield of 40%. This relative low yield (48 mM instead of the maximum amount of 120 mM free Glu) indicated that the dilute acid method did not totally hydrolyse wheat gluten protein. As a consequence, some Glu and or Gln residues were still present in the form of peptides or protein fragments. In addition, at the dilute acidic condition we demonstrated that the formation of p-Glu is promoted [18]. Fig. 1b indicates the formation of p-Glu during dilute acid hydrolysis of raw wheat gluten. Under more acidic conditions, it is possible to reconvert p-Glu into Glu [19]. In this case, we could include the formation of p-Glu in the total Glu yield, resulting in a yield of 46% (Table 3), which is still relatively low.

Another option for hydrolysis at more mild conditions is by using enzymatic treatment. Protease helps to catalyse the hydrolysis of protein, releasing peptides and amino acids [20]. Along with the hydrolysis, the nature of water-insoluble wheat gluten protein is altered. As a consequence of the hydrolysis, wheat gluten protein becomes soluble. A combination of Validase FP Concentrate + Peptidase R (Fig. 2) solubilised 94% wheat gluten protein. The resulting percentage solubilized protein is higher than another study where a maximum of 81% solubilised protein yield was obtained by Alcalase 2.4L [21].

To further increase the free Glu yield after enzymatic treatment, free Gln should still be converted into free Glu by a deamidation. Acid [22], alkali [22, 23], and enzymes [24] can be used to catalyse this reaction. Enzymatic deamidation applying glutaminase was expected to prevent p-Glu formation, which is a side product in the chemical reaction [18]. Using Glutaminase SD-C100S 40 mM of the free Gln in the protein hydrolysate was converted to 30 mM Glu. This percentage of deamidated Gln was comparable with other results at the same temperature [18]. However, compared to the initial Glu concentration in untreated wheat gluten, the deamidation of Gln only resulted in 26% Glu yield, which is much too low for industrial application.

In further process development, earlier findings from this study were combined. Enzymatic hydrolysis resulted in high solubilised protein yield; however the liberated free amino acids were mainly in the form of Gln instead of Glu. On the other hand, the dilute acid hydrolysis yielded only Glu instead of Gln. However, Glu yield was still relatively low.

A combination of enzymatic protein hydrolysis and dilute acid hydrolysis has been applied earlier [25]. Wheat gluten was enzymatically hydrolysed with Alcalase, Papain, Neutrase, and Pepsin. Next, dilute acid hydrolysis was performed with 0.1 M HCl at 70 ^{o}C for 7 h. However, the aim was not to produce Glu, but to improve wheat gluten functional properties related to food application. The result indicated that the hydrolysate consisted of peptides instead of free amino acids. Therefore, could not be adapted for Glu production.

In contrast, the combined enzymatic protein hydrolysis and dilute acid hydrolysis applied in the current study resulted in a relatively high free Glu liberation. The final Glu concentration also exceeded the free Gln and Glu concentration in the protein hydrolysate (prior to dilute acid hydrolysis). An increase in free Glu after dilute acid hydrolysis might be contributed by deamidation of free Gln and also an additional protein hydrolysis that liberated new free Glu. Taking into account the p-Glu that was also liberated, then the overall Glu yield after dilute acid hydrolysis on a hydrolysate obtained by the combination of Validase FP Concentrate + Peptidase R and M Amano SD were 80% and 73%, respectively (Table 3). With these high yields, Glu production comes close to industrial feasibility.

Valorizing other amino acids that were simultaneously liberated during the hydrolysis will also give additional benefit for industry. Interestingly, a combined method of enzymatic and dilute acid hydrolysis on wheat gluten liberated relatively high amounts of amino acids. Some non-essential amino acids such as aspartic acid, glycine, tyrosine, and alanine showed very high yields. As animals can synthesize these amino acids, they are not necessarily needed in their feed. Meanwhile, these amino acids have a potential to be used as building blocks for the synthesis of bulk chemicals [1]. Therefore, if the combined method of enzymatic and dilute acid hydrolysis will be applied on DDGS, then, Glu and other non-essential amino acids can be extracted for bulk chemicals while the essential amino acids can still be used for animal feed.

Table 3. Summary on final concentration and yield for all different methods

Treatment	Condition	Final concentration (mM)			Glu yield[c]
		Gln	Glu	p-Glu	
			(mM)		(%)
Acid hydrolysis	6 M HCl, 110 °C, 24 h	n.a[a]	120 ± 3	n.d[b]	100
Dilute acid hydrolysis	1 M HCl, 95 °C; 48 h	0 ± 0	48 ± 4	7 ± 0	46 ± 3
Enzymatic hydrolysis	Validase FP Concentrate + Peptidase R; 24 h	44 ± 3	13 ± 3	n.d[b]	48 ± 5
Enzymatic deamidation	Glutaminase SD-C100S; 55 °C; pH 7; 24 h	0 ± 0	31 ± 4	n.d[b]	26 ± 3
Combined dilute acid and enymatic hydrolysis		0 ± 0	84 ± 9	12 ± 0	80 ± 8

[a] n.a = not applicable, [b] n.d = not determined,
[c] Glu yield =(Free (Gln + Glu + p-Glu) in hydrolysate/total Glu in wheat gluten (120 mM))× 100%.

5. Conclusion and Recommendation

This study showed that Glu can be produced from biomass by involving two sequential hydrolysis steps. Enzymatic hydrolysis was selected as the first step to provide a solution with a high concentration of soluble biomass protein. Once available as soluble protein, this was then further hydrolysed with dilute acid. The dilute acid did not only concomitantly deamidate Gln into Glu, but it also provided additional hydrolysis on the soluble protein. The combination of enzymatic and dilute acid hydrolysis resulted in 70% Glu and 10% p-Glu thereby opening up new possibilities for the industrial production of Glu from biomass. The findings obtained from this study can be used as a basis for future research on new methods for economic and environmentally friendly Glu production. This may involve optimisation on deamidation step, either enzymatically or dilute acid. It also merits to observe the effect of altering the hydrolysis sequence; dilute acid deamidation followed by enzymatically hydrolysis.

Acknowledgement

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Chapter 6

General Discussion

1. Introduction

The aim of the work reported in this thesis was to explore the potential of biomass as protein resource. State of the art of protein extraction and application, types of biomass to be used as protein resources, protein extraction methods, and production of glutamic acid, an amino acid, from biomass have been discussed in Chapter 2-5. This chapter combines and discusses the previous chapters.

The research questions on technology that were posed in Chapter 1, and which are indicated in subtitles of this chapter subsections (Section 2.1-2.3), are answered in the subsequent paragraphs. We conclude that biomass, particularly by-products, are potential protein resources. Biomass with less cellulose and oil contents is preferred above others. Extraction under alkaline pH at room temperature is mostly sufficient to extract protein from most type of biomass. High temperature is required for leafy types of biomass or biomass which previously has been treated at high temperature. For microalgae, protein extraction can be enhanced using proteases. The use of protease combined with mild acidic hydrolysis provides a good, new method for amino acids production.

The economics of these processes have also been investigated and these results are presented in Section 3.1 – 3.4 together with an outlook on the applicability of protein products obtained after alkaline extraction.

2. Main findings and conclusion

2.1 How to extract biomass protein

The analysis on biomass composition and protein extractability in Chapter 3 demonstrated that biomass with high content of cellulose and oil is less preferable to be used as protein feedstock. Removal of these components from biomass prior to protein extraction will increase protein yield. De-oiling of soybean and microalgae, for example, yielded more protein compared to that without de-oiling (Chapter 3). Also cellulose removal by cellulose hydrolysis prior to protein extraction improved e.g. leaf protein extraction yield by 78% [1].

Following pre-treatment, appropriate extraction conditions will determine the optimal protein extraction yield. Various alkaline extraction conditions have been carefully reviewed in Chapter 2. In general, alkali has long been used to extract biomass protein. And also in our cases, more protein was extracted at alkaline pH compared to extraction at acidic pH (Chapter 4). The effectiveness of alkali in extracting biomass protein is highly influenced by biomass to solvent ratio, temperature, and pH. In Chapter

3, we describe alkaline protein extraction at 3 sequential temperatures. This study showed that up to 85% of biomass protein was already extracted when biomass was incubated overnight at 25 °C. An increased extraction temperature of 60 °C was only recommendable when protein is to be extracted from biomass that has been pre-treated at high temperature, such as malt by-products. Protein extraction at an even higher temperature of 120 °C is beneficial for extracting sugar beet pulp protein. This biomass contains up to 30% pectin [2]. With high extraction temperature, pectin is expected to degrade [3]. With this degradation, the pectin-protein complex is disturbed thus allowing proteins to be extracted. Pectin has shown to be a hindrance in protein extraction in leaf type materials, where it makes up part of the cell wall [4]. Although more protein can be extracted with an increase in temperature, in general, alkaline extraction at 25 °C is economically more efficient (Section 3.2).

Another approach to extract biomass protein was to use proteases. Proteases require a particular optimal pH to work. Therefore, both pH and protease activity contribute to overall protein yield. In our case, protease enhanced protein extraction yield by hydrolysing protein into peptides (Chapter 4). Since we used alkaline proteases, pH also aided to the overall yield. The additional effect of protease can be seen when the amount of additional extracted protein is calculated as shown in Fig. 1. It is clear that protease assisted protein extraction for all tested biomass with the largest protein yield increase in microalgae and microalgae meal. This finding can be an important step forward in the protein extraction of microalgae. Comparing enzyme dosage, Fig.1 shows that only 10-14% additional protein extraction yield was obtained when comparing a 1% (v/w) enzymes dosage with 5-fold dosage increase. It is therefore likely that 5% (v/w) enzyme dosage is beyond the optimum dosage, where 1% (g protein/ml protease) enzyme dosage has a higher effectiveness and may be preferred over 5% (g protein/ml protease) in practice. This is also supported by economic calculations on microalgae protein production (Section 3.3).

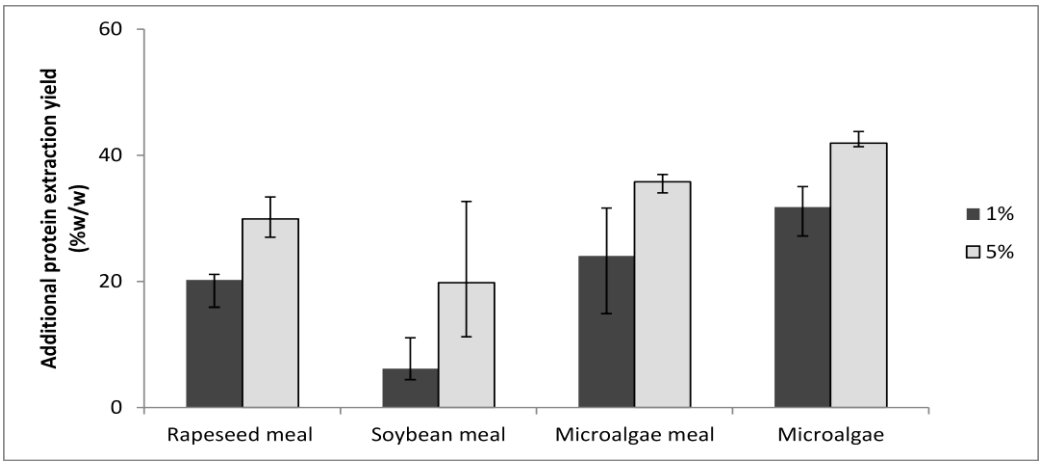


Figure 1. The effect of protease dosage on protein extraction: The absolute amount of additional protein extraction yield obtained compared to extraction without protease (at t = 6 h and pH 11 using Protex 40XL, Chapter 4).

2.2 Will it be technically feasible to produce amino acids from protein?

Biomass protein can be used for food [5, 6], feed [7, 8] and technical applications such as adhesive [9-11] and film [12, 13]. In addition, protein, from agricultural by-products, can also be used as feedstock for amino acid supplements for feed (Section 3.1) and bulk chemicals production [14]. For this, protein has to be hydrolysed to yield amino acids. Following hydrolysis, particular amino acids may be isolated from the mixture. Glutamic acid is the most abundant amino acid in biomass and can be used to produce bulk chemicals such as methylpyrrolidone, which has been demonstrated at laboratory scale [15]. The production of glutamic acid from by-products was investigated within this thesis. Using only alkaline proteases did not yield single amino acids (Chapter 5). More severe conditions are needed to cleave all peptide bonds.

Acid hydrolysis can be used to yield free amino acids. Protein total hydrolysis using 6 N HCl is commonly conducted prior to total amino acid analysis using chromatography. However, these extreme conditions are industrially disfavored and the use of enzymes may be an alternative. Therefore, industrially relevant alkaline proteases have been investigated to release glutamic acid from wheat gluten. Wheat gluten was selected, representing distillers grain (Section 3.4), due to the fact that it has high content of glutamic acid and glutamine, which can be up to a total of 40% [16-18]. Alkaline proteases showed their capability to hydrolyse wheat gluten, thereby increasing solubility. However, this method recovered less than 50% of the original glutamic acid and glutamine presence (Chapter 5). Further treatment by addition of 1N HCl after protease treatment was also tested. The already solubilized and partially hydrolysed wheat gluten was further hydrolysed, glutamine was converted to glutamic acid, and total glutamic acid recovery increased to 70 %. Using less concentrated HCl, with this method, it is expected that the process can be implemented by industry. The combined enzymatic and acid hydrolysis provides possibilities to produce amino acids from biomass. Next, further development on amino acids separation techniques such as electrodialysis [19, 20] and anti-solvent crystallization, which are not industrially applied yet, is required.

2.3 Biomass chemical composition and NaOH usage: guidance for selecting biomass as protein resource

After selecting biomass based on protein extractability, selection can be made according to the NaOH usage during extraction. Although section 3.2 indicates that NaOH is not the major processing cost, limiting NaOH usage should be considered, as it may increase cost for waste treatment and/or recycling. Salt formation will occur if acid precipitation is selected as downstream processing method to concentrate soluble protein from the supernatant. With salt formation, washing or dialysis is required during downstream processing to remove salt from protein [21].

Figure 2 illustrates the NaOH usage during overnight alkaline extraction at 25 °C (Chapter 3). This example will be discussed, as it was the most economical (Section 3.2). During extraction, the initial pH dropped from 12.5 to 9.5 – 10.5 in 15 h. Within this pH

range, no additional NaOH was added. In some of the experiments, the pH dropped below 9.5. This was the case when barley rootlets, malt by-product, palm kernel meal, rapeseed meal, and soybean hull were used. Extreme pH drop occurred in microalgae meal, to a pH of 6.5. For these types of biomass, more NaOH was added to maintain a pH of 10. As a consequence, these biomass have higher NaOH consumption than others (Fig. 2).

NaOH usage is a result of buffering by biomass components like cellulose [22], lignin [23], and organic acids [24]. NaOH uptake by cells may soften the cell wall by breaking the glycoside bonds and liberating the previously amorphous cellulose into solution [22]. This allows protein to diffuse into the alkaline medium outside the biomass cell. With the buffering characteristics of biomass, the NaOH usage in biomass is followed by a reduction in pH. NaOH may also react with oil through saponification. This probably did not occur in our case, as the NaOH usage by the biomass did not increase with the oil content, and soybean and soybean meal used the same amount of NaOH (% w NaOH/w dry biomass) (Fig. 2). Microalgae meal consumed even higher amounts of NaOH than microalgae, which contain more oil than microalgae meal. Similar phenomena occurred with cellulose. Although alkali treatment reduced cellulose crystalinity [25], still, cellulose was not completely dissolved by alkali [26]. Therefore, although oil and cellulose are important components determining protein extractability, it is most likely that these components provide physical barriers rather than hindrance through chemical interactions during protein extraction The NaOH usage may be explained by absorption of NaOH by other biomass components.

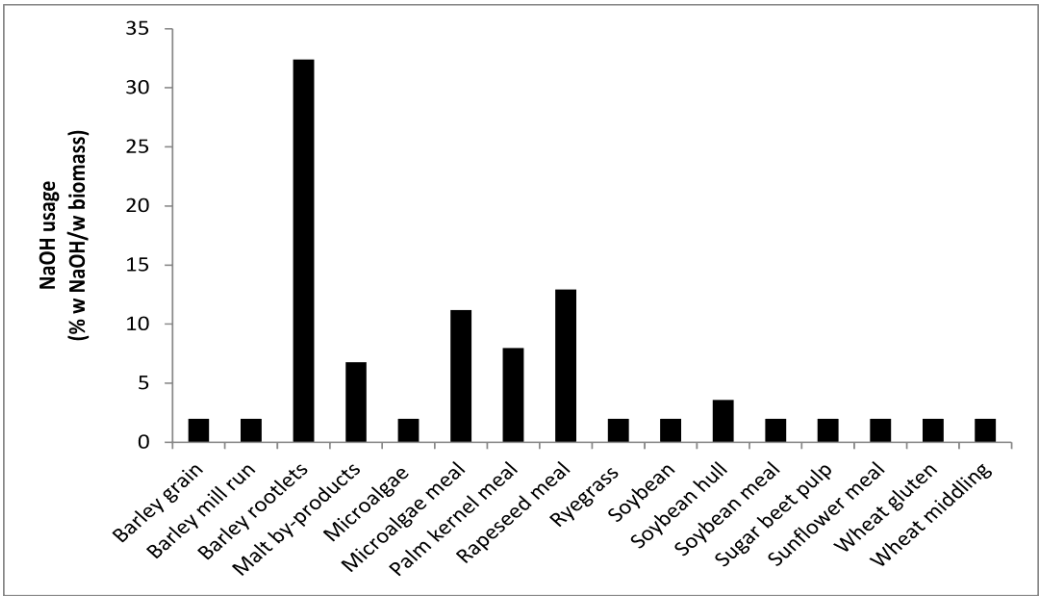


Figure 2. NaOH usage (% w NaOH/w dry biomass) by biomass during protein extraction at 25 °C for 24 h.

Looking at the composition of the types of biomass that required additional alkali during protein extraction at 25 °C, it is likely that lignin contributed to the high NaOH consumption for some biomass. NaOH use by alkali commonly occurs during early stages of biomass pulping that aims for removal of lignin [27]. Here, alkali is used to cleave aliphatic and aromatic carbon-oxygen bonds [23] resulting in a pH drop. With relatively high content of lignin, palm kernel meal and rapeseed meal required additional NaOH to keep the alkaline pH. For microalgae meal, the high NaOH usage may be due to the high mineral content, indicated as ash content. The microalgae meal cell wall is expected to be disrupted during oil production from microalgae. This decreases the diffusion barrier for the alkali, increasing chances of anion (OH^-) attraction by positively charged mineral ions (cations) or organic acids. The highest NaOH consumption occurred in barley rootlets. Barley rootlets are by-products of barley germination. During germination, barley releases amylase, which is used in following malting process. It is possible that following germination, the barley rootlets have acidic pH as amylase synthesis was triggered by the secretion of giberrilic acid [28]. With excessive cation (H^+), more NaOH is required to get alkaline pH.

Combining literature information on the possible OH^- reactions with chemical components from biomass with our experimental results (Chapter 3), leads to the conclusion that biomass with high content of lignin, minerals, or organic acids require more NaOH for protein extraction. This will increase processing cost. Not only because of higher chemicals usage, but also due to additional downstream processing cost for salt removal from the protein concentrate.

3. Outlook

This thesis has described several options for protein recovery from biomass, with acid or alkaline (Chapter 2 and 3), aided by proteases (Chapter 4), and at different temperatures (Chapter 3). Options for amino acid production have also been discussed (Chapter 5). Possible implementation of these scenarios will be discussed in this coming section. Economical evaluation is also provided based on the production of 1 ton protein product and limited to the processing cost.

3.1 Biomass refinery for feed

Global consumption of livestock and their products is growing along with the increase in world population and also with higher income earned by people living in the developing world. Current traditional livestock feed such as corn and soybean meal are forecasted to be unable to meet the future demands on feed. Alternatives are needed for feeding livestock. By-products can be used as alternative feeds, taking advantage of their protein and energy contents (examples in Table 1). Based on this, feed can be classified as protein source or energy source. Protein source feed is suitable for pig and poultry. Ruminants can be fed any type of feed; energy or protein source feed. Naturally ruminants only need energy source (low protein content) feed as they can synthesize protein in their rumen. However, for improved production of e.g. milk and meat, medium and high protein feed can be used.

Not only composition, but also digestibility is an important consideration for feed. Some alternative feeds, such as palm kernel meal, contain excessive amounts of fiber, which reduce feed digestibility. High digestibility is important for livestock since it determines the efficiency of livestock to convert biomass into available nutrient for growth, reproduction, etc [29]. For this purpose, sometimes treatments are required to improve by-products digestibility, especially for ruminants.

An approach to improve feed digestibility can be through pre-treating biomass with alkali. A study on the alkaline effect on digestibility has been conducted to corn, rapeseed silique, and rapeseed stem (Fig. 3). These biomass were incubated in alkali (40 g/L $Ca(OH)_2$) at 95 °C for 1 h and for 1 h and 2 weeks at 25 °C prior to digestibility testing. This test, performed by BLGG AgroXpertus, Oosterbeek, the Netherlands, indicates the amount of cell wall material comprised of cellulose, hemicellulose, and lignin that is digested in 48 h by cow's rumen intestine fluids. For all tested biomass, alkali treatment improved digestibility.

Next to a protein rich fraction, alkaline protein extraction (Chapter 3) yielded a residue that can be further used for several applications, including feed. The dry matter content of the residue following protein extraction is high (Fig. 4), and having been treated with alkali, it is expected that this residue has an improved digestibility. Having most protein extracted, this remaining solid is likely to have a low protein and high energy content, which suits ruminants.

Table 1. Agricultural by-products for animal feed, modified from [7, 30, 31]

Feed Characteristic	**Example of animal**	**Example feed source**
Protein source	Poultry, pig, duck, ruminant	Oilseed meal, cassava leaves, cereal-grain waste, coconut meal, rubber seed meal, palm kernel meal
Energy source	Ruminant	Cereal straw, corn stover, rice bran, cassava peel, cassava root meal

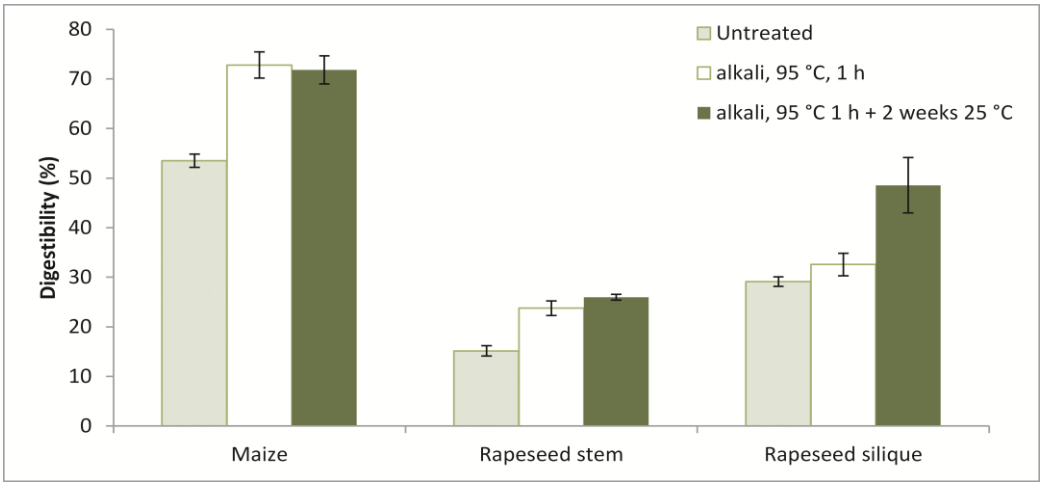


Figure 3. Digestibility of biomass following alkaline treatment [32].

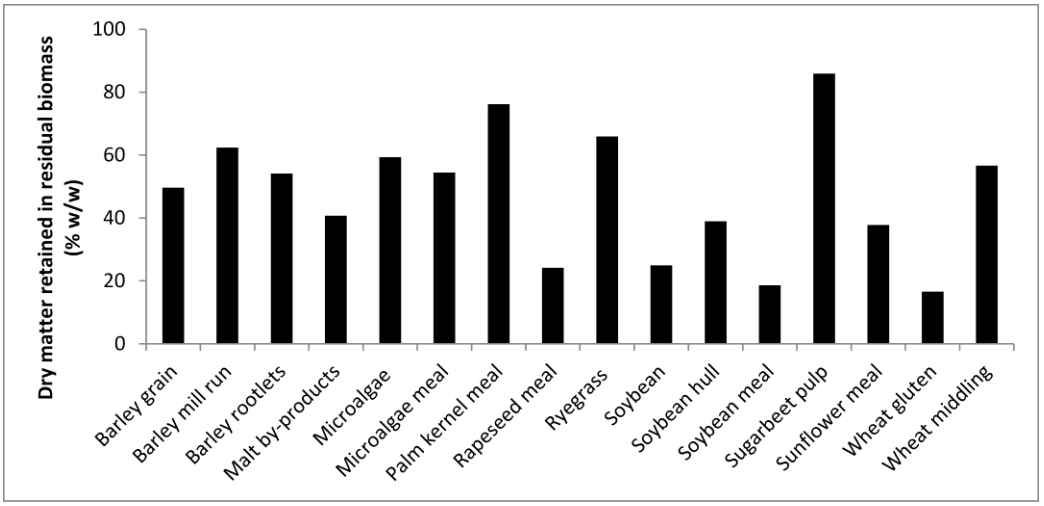


Figure 4. Dry matter retained in residue after protein extraction.

$$\text{Percentage dry matter retained in residue (\%)} = \frac{\text{mass dry matter residue(g)}}{\text{mass dry matter raw biomass (g)}} \times 100\%$$

Next to direct use of biomass after protein extraction for feed, biomass can be used to produce amino acids from the protein product after protein extraction (Fig. 5). Some of these amino acids may be useful for bulk chemicals production (Section 3.4), while others may be useful as feed supplement, particularly the essential amino acids. Essential amino acids for feed are arginine, lysine, methionine, threonine, tryptophan, isoleucine, leucine, histidine, phenylalanine, and valine. Results obtained from combined enzymatic and acid hydrolysis (Chapter 5) of wheat gluten show that these essential amino acids were partly presents as soluble free amino acids in the hydrolysate (Fig. 6). Arginine and lysine have the two lowest extraction yields. This might be due to arginine hydrolysis under acid condition [33] and the formation of lysinoalanine during alkaline treatment

[34]. With high extraction yield, essential amino acids can be used as feed supplements. For this, further development on amino acid hydrolysis and separation techniques is crucial.

Co-production of protein and feed is expected to reduce their production cost. Calculations in section 3.2 show that some residues of protein extraction still have feed value.

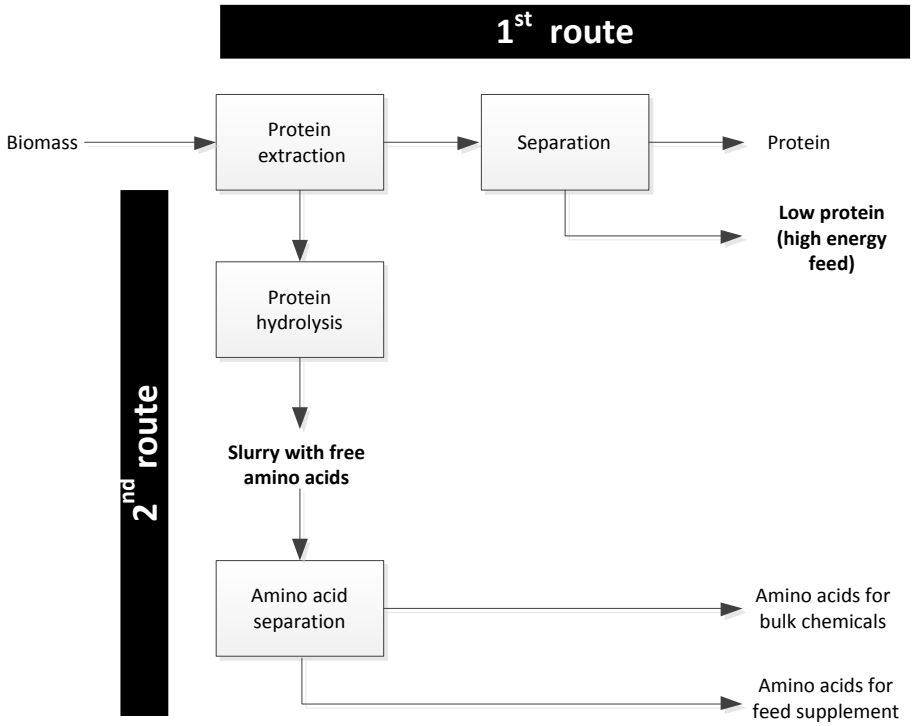


Figure 5. Two possible routes for biomass refinery for feed applications following protein extraction. The first route (horizontal) takes advantage of residue from protein extraction. This residue is used as low protein-high energy feed. The second route (vertical), protein extraction is followed by hydrolysis to yield amino acids. Parts of these amino acids can be used as feed supplement.

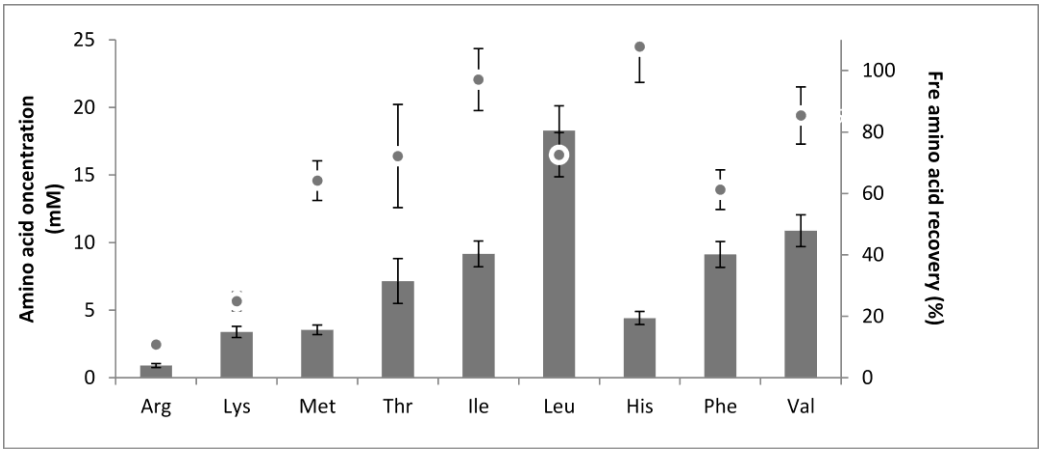


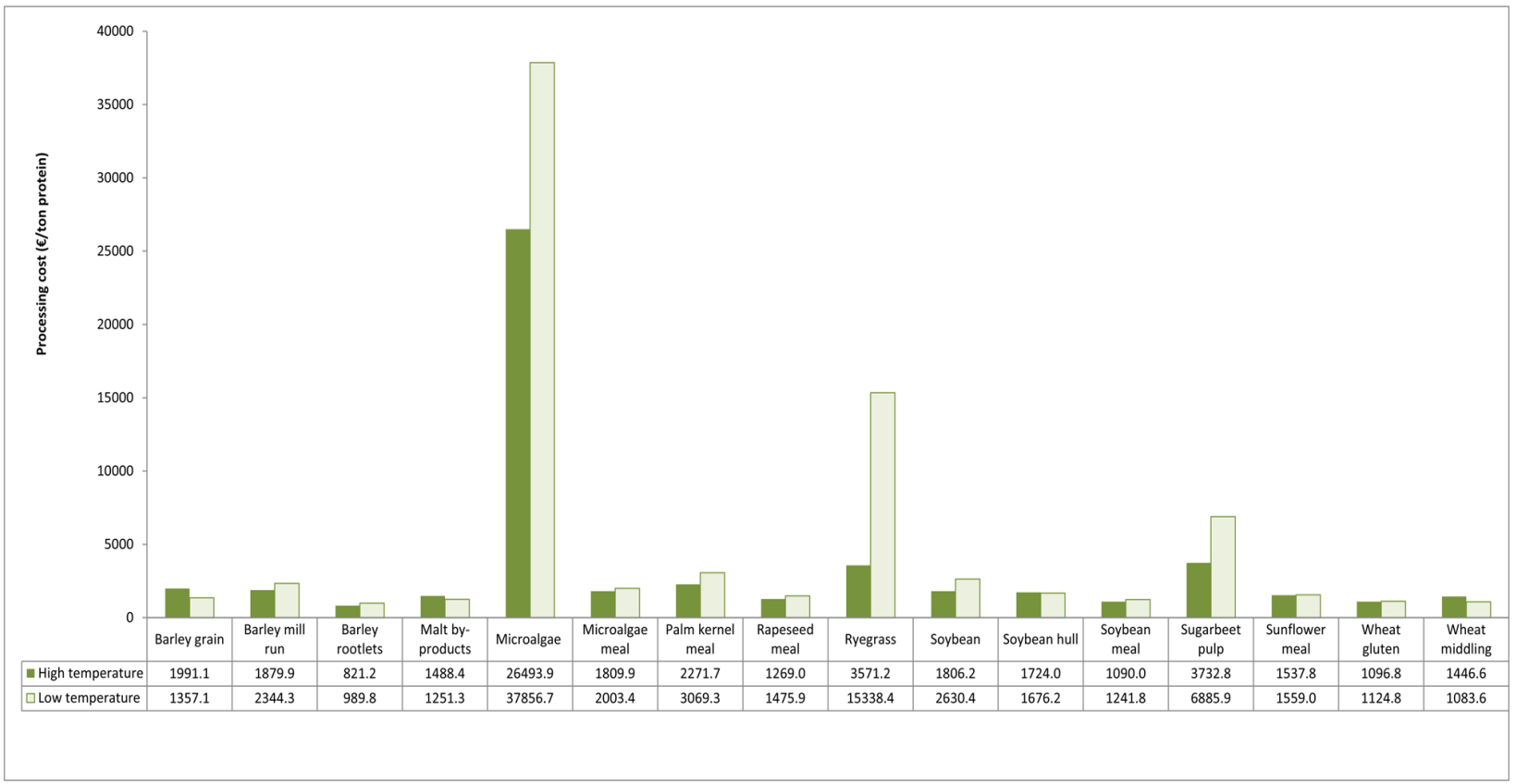
Figure 6. Concentration (■) and recovery (•) of essential amino acids extracted through enzymatic and acid hydrolysis. Enzymatic hydrolysis was performed for 48 h using Validase FP Concentrate (t = 2 h, T = 55 °C) + Peptidase R (added after 2 h, T = 40 °C). Acid hydrolysis was performed using 1 M HCl for 48 h.

3.2 Cost effective protein production

To gain insight in the cost effectiveness of alkaline protein production, an economic calculation on part of the processing cost (exclude capital and labor cost) is provided. This calculation compares some important processing cost for protein extraction at different scenarios; different starting raw material, low and high temperatures, and chemical usage (Fig 7) following the experimental set-up from Chapter 3. To simplify, the term "processing cost" is used in this section to indicate the cost for raw biomass, NaOH, and energy for heating. In the case of low temperature extraction, energy cost for heating is assumed to be zero. It was also assumed that all solubilised protein could be recovered following downstream processing. With further optimization, e.g. by increasing solid to liquid ratio, energy cost may be reduced, thus lowering the production cost. Data that were used to calculate these processing costs to produce 1 ton protein can be seen in the Appendix.

NaOH consumption contributes relatively small to overall processing cost, on average, only 0.7% of total processing cost (except for barley rootlets, which can be up to 10%). NaOH substitution with $Ca(OH)_2$ lowers the chemical cost to 0.1% of total processing cost. This substitution especially shows its significance only for barley rootlets where NaOH was highly consumed. For this biomass, substituting NaOH with $Ca(OH)_2$ lowers the relative chemical cost to total processing cost from 10 to 2%. Cost for NaOH or $Ca(OH)_2$ per ton dry biomass involved in extraction at low and high temperatures can be seen in Appendix (Table 2). Biomass cost is the major contributor to processing cost, where extraction at low temperature required more biomass costs than extraction at high temperature. This is due to the fact that less protein was extracted at low than at high temperature. As a consequence, more biomass is required to produce 1 ton protein. However, the exclusion of energy cost for heating when the extraction is performed at low temperature compensates the high biomass cost leading to similar total processing cost for both temperatures.

The remaining biomass after protein extraction still contains dry matter, and this residue may be further valorized for feed or for energy by burning it to give additional revenues. In the scenario with residues for feed, economic values of residues are obtained by interpolation of data for protein content and biomass price. This regression was performed on the data of protein content and biomass price that were involved in this study (Section 4 in the Appendix). In the scenario with residues for energy usage, the economical value of the residues is calculated based on their caloric content (Section 5 in the Appendix). For the residues, extraction at low temperature provides higher revenue than extraction at high temperature, as more dry matter is retained in residue (See Table 3 and 4 in Appendix) leading to a higher cost-effectiveness of the final overall process.



	Barley grain	Barley mill run	Barley rootlets	Malt by-products	Microalgae	Microalgae meal	Palm kernel meal	Rapeseed meal	Ryegrass	Soybean	Soybean hull	Soybean meal	Sugarbeet pulp	Sunflower meal	Wheat gluten	Wheat middling
High temperature	1991.1	1879.9	821.2	1488.4	26493.9	1809.9	2271.7	1269.0	3571.2	1806.2	1724.0	1090.0	3732.8	1537.8	1096.8	1446.6
Low temperature	1357.1	2344.3	989.8	1251.3	37856.7	2003.4	3069.3	1475.9	15338.4	2630.4	1676.2	1241.8	6885.9	1559.0	1124.8	1083.6

Figure 7. Processing cost required to extract 1 ton protein using alkaline extraction at high and low (room) temperatures. Processing cost in this calculation refers to cost for raw biomass, NaOH, and heating only.

Table 2 shows the processing cost and additional revenue for protein production from palm kernel meal, rapeseed meal and wheat middling. The first biomass is an important commodity for Indonesia. The latter two are important commodities for European countries. Detailed calculation for the other biomass can be found in the Appendix (Table 9 and 10). For palm kernel meal, protein production from this biomass requires € 3069/ton protein, which disfavors palm kernel meal as protein resources. However, combining protein and energy production, this valorization scenario gives additional revenue of € 2728/ton protein for the residue. Combining protein and feed is even better for palm kernel meal, giving additional revenue of € 3131/ton protein, showing that the value of the residue by itself is already enough to cover processing cost, before any revenue is made on the protein product. Rapeseed also has its highest revenue when protein and feed production are combined. However, wheat middling can be better used for protein and energy. Albeit combination of protein and feed productions gives additional revenue of € 260/ton protein, combination of protein and energy production provides additional revenue of € 580/ton protein. The low protein content in wheat middling residue after protein extraction contributed to its low feed value, leading to the suggested combination of protein and energy. Table 3 gives data on the revenue from the residue obtained after protein extraction at low temperature. Based on the highest residue provided, a scenario of residue valorization after protein extraction was suggested.

A special case is the microalgae and their meals for which no valorizing method is suggested in Table 3 as the estimated values for some of the costs are very uncertain. The extremely high microalgae processing cost are firstly a result of a high, and probably overestimated market price for microalgae that was included in the calculation, because a realistic market price for industrial scale is not yet available [35]. Secondly and probably equally important is the low protein extraction yield, with less than 10% protein extracted at room temperature. Further investigation on extraction parameters will be beneficial. The microalgae meal cost calculation has also used a predictive value [35] for the market price, which may again be too high or too low. This is the main reason to not suggest a valorizing method for microalgae meal. Section 3.3 will discuss further methods for utilizing microalgae as protein and/or amino acid resources.

Table 2. Processing cost and revenue from 1 ton protein production with alkaline protein extraction at low temperature. Processing cost in this calculation refers to cost for raw biomass, NaOH, and heating only

Biomass	Biomass needed to produce 1 ton protein	Residue obtained from production 1 ton protein	Total processing cost		Revenue per ton biomass		Revenue per ton protein	
					As feed	For energy usage	As feed	For energy usage
	(ton/ton protein)		(€/ ton biomass)	(€/ ton protein)	(€/ton biomass)		(€/ton protein)	
Palm kernel meal	26.01	20.50	118.0	3069.3	120	105	3130.9	2727.8
Rapeseed meal	4.94	2.39	298.9	1475.9	103	46	508.4	225.4
Wheat middling	8.44	4.92	128.4	1083.6	31	69	259.8	580.0

Table 3. Suggested valorizing method for residual biomass after protein extraction

Biomass	Revenue		Suggested valorization method
	Feed	Energy	
	(€/ton biomass)		
Barley grain	18	93	Energy
Barley mill run	64	95	Energy
Barley rootlets	165	91	Feed
Malt byproduct	60	93	Energy
Microalgae	253	67	n.s*
Microalgae meal	293	78	n.s*
Palm kernel meal	120	105	Feed
Rapeseed meal	103	46	Feed
Ryegrass	78	100	Energy
Soybean	174	87	Feed
Soybean hull	50	71	Energy
Soybean meal	141	79	Feed
Sugar beet pulp	56	126	Energy
Sunflower meal	144	69	Feed
Wheat gluten	98	29	Feed
Wheat middling	31	69	Energy

*n.s : no suggestion

3.3 Increasing protein yield from microalgae

Due to the unavailability of a reliable industrial microalgae market price, the processing cost for microalgae protein listed in Section 3.2 will not reflect the actual possible industrial protein production cost. The deviation on microalgae predictive market price is too high. In the calculation we cited € 1650/ton microalgae [36], which is already very high, while some also mentioned values of microalgae as high as € 25000/ton microalgae [35, 37].

With the current low 18% protein yield from microalgae, industrial microalgae protein production is not feasible. De-oiling increases protein yield to 32% from microalgae meal (Chapter 3 with high temperature method). Looking at the still low yield, it seems that further optimizations are required to extract microalgae protein. Protease-assisted-protein extraction has been developed for extracting protein from microalgae and microalgae meal. With protease addition, the protein yield for microalgae and microalgae meals increased from 18 to 58% and from 32 to 73%, respectively (Chapter 4). To estimate the feasibility of using proteases to industrially produce protein, economic calculations on production cost were performed (Table 4). The data given in Table 4 are for illustration only due to the limitation on available industrial microalgae market prices, as mentioned earlier. Yet, the overall trend can be used to compare the effectiveness of proteases in extracting microalgae protein.

The use of protease reduces the production cost from microalgae and their meals, most pronounced for microalgae. Without protease, the production cost will be € 39191/ton protein. Addition of 1% protease reduces the cost to € 13831/ton protein. Yet, this value is still too high for industrial production. The benefit of de-oiling microalgae brings protein production closer to industry. However, Adding 1% protease, only slightly reduces processing cost to € 2654/ton protein. This is true in most cases. Although we do see a higher protein yield and thus a reduction in costs for raw materials, the increase in enzyme costs cancels out this positive effect on the overall processing cost.

Table 4. Processing cost required to extract 1 ton protein from microalgae and microalgae meal using Protex 40XL, pH 11, 60 °C, 6 h (Chapter 4). Processing cost in this calculation refers to cost for raw biomass, NaOH, and heating only

Biomass	**Protease dosage (%)**	**Cost (€/ton protein)**				
		Biomass	**NaOH**	**Protease**	**Energy**	**Total**
Microalgae	0	36768	1088	0	1335	39191
	1	11970	426	1000	435	13831
	5	10141	338	5000	368	15847
Microalgae meal	0	1651	503	0	565	2719
	1	984	333	1000	337	2654
	5	858	308	5000	294	6460

3.4 Amino acids production from distiller grains

Distiller grains are by-products from ethanol production. European Commission's Agricultural and Rural Development department predicts an increase in ethanol production along with the target on 10% renewable energy by 2020 [38]. With this, more distiller grains will be available. The starch part of the grains is used to produce the ethanol, leaving remaining grain constituents such as protein, fat, and other nutrients to be used as livestock feed. Protein from distiller grains can be used as feedstock for amino acid production, using protein extraction techniques described in this thesis.

Wheat, corn, and sorghum are three grains that are commonly used in bioethanol production [39, 40]. Looking at the amino acid composition of these grains (Fig. 8), glutamine, proline, leucine, alanine are the most predominant amino acids. As most ethanol grains have a similar amino acid profile, without considering type of grain used in ethanol plant, availability of these 4 amino acids will rise and may be of industrial interest. The combined method of enzymatic and acid hydrolysis (Chapter 5) shows high recovery of these amino acids from wheat gluten, recovering as much as 80, 43, 73, 72% of glutamine, proline, leucine, alanine respectively. With development of amino acid separation through e.g. electrodialysis [19], producing amino acids from distiller grain will be at least technically feasible.

Nowadays, distiller grains are available in the form of wet distiller grain (WDG), distillers' dried grain (DDG), condensed distillers' solubles (CDS), and distillers' dried grain and solubles (DDGS) [41]. Distiller grains consist of the non-fermented grain components such as protein in WDG and oil and minerals in CDS [42]. These are components required in the animal diet. In practice, for animal feed purpose, WDG and CDS are normally mixed and dried (Fig. 4 in Chapter 1). The drying step is required due to the high moisture content in WDG and CDS. Without drying, WDG and CDS have shelf lifes of only four to five days. Thus, without drying, valorizing distiller grains is not economically feasible. Therefore, at the present time, DDG or DDGS is preferred over WDG or CDS and commonly used as animal feed.

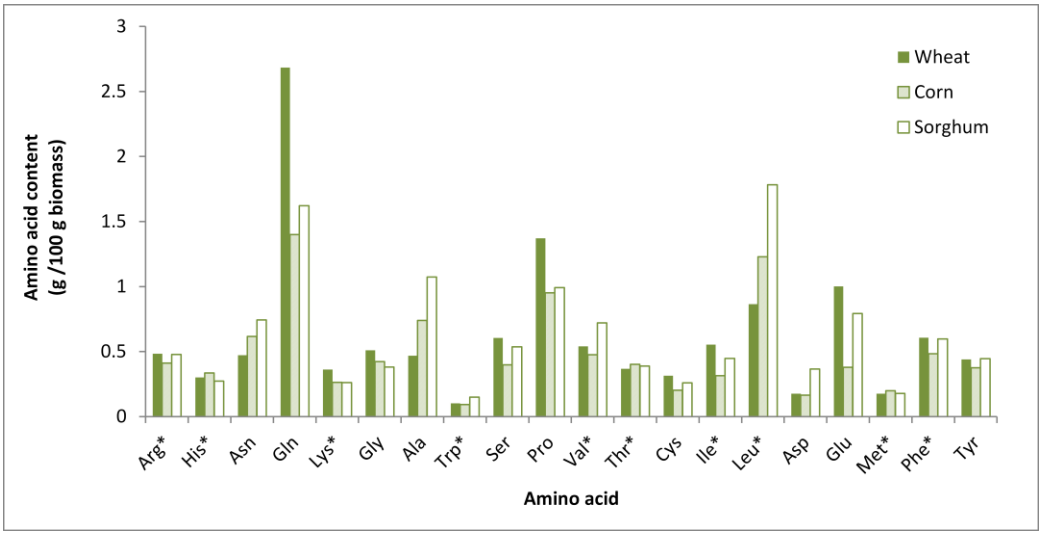


Figure 8. Amino acid composition of wheat, corn, and sorghum [43].*essential amino acid for feed.

As combined acid and enzymatic hydrolysis provided high glutamic acid yield (Chapter 5), but also essential amino acids (Section 3.1), DDG and DDGS may also be valorized as amino acid resources. In addition, this can also be done using WDG or CDS. If amino acids can be extracted from these by-products, processing cost will be less as it will eliminate the drying process from WDG to DDG or CDS to DDGS, which require high energy cost.

4. Concluding remarks

In this thesis we showed the applicability of alkaline protein extraction for several types of biomass, with emphasis on agricultural by-products. The process has been optimised for several parameters (temperature, pH, and type of biomass), and for some cases has been extended using proteases to get to even higher yields. Proteases were also used to further hydrolyse protein. Combined with a mild acidic treatment, this delivered a new method for the production of amino acids.

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Appendix

SUPPLEMENTARY INFORMATION TO CHAPTER 6

Cost calculation of alkaline protein extraction per ton dry biomass

1. Biomass cost

Biomass cost was calculated based on biomass price per ton biomass usage. The price of each biomass is given in Table 1. For some biomass, the price was an average from two reference values. Prices of barley mill run and malt by-product were assumed based on barley rootlets due to a lack of availability of reference prices.

Table 1. Biomass price (per ton dry biomass)

No.	Biomass	Price (€/ton dry biomass)	References	Remark
1	Barley grain	104.4	[1, 2]	
2	Barley mill run	82.8	[3]	
3	Barley rootlets	82.8		Assumed
4	Malt by-products	82.8		Assumed
5	Microalgae	1650	[4]	
6	Microalgae meal	230	[5]	
7	Palm kernel meal	115.7	[6, 7]	
8	Rapeseed meal	295.2	[8, 9]	
9	Ryegrass	100.8	[10]	
10	Soybean	428.4	[7, 11]	
11	Soybean hull	127.8	[8, 12]	
12	Soybean meal	357.2	[7, 8]	
13	Sugar beet pulp	133.2	[8]	
14	Sunflower meal	204.5	[7, 8]	
15	Wheat gluten	735	[5]	
16	Wheat middling	127.8	[3, 12]	

2. NaOH or $Ca(OH)_2$ cost

NaOH cost (Table 2) were determined based the amount of NaOH required to extract protein per ton dry biomass. The required NaOH mass was determined by lab scale experiments, as described in Chapter 3 and 6. Mass biomass used in the lab scale was 10 g. NaOH cost is determined as:

$$\text{Cost}_{\text{NaOH}} = m_{\text{NaOH}} \times p_{\text{NaOH}}$$

And

$$m_{\text{NaOH}} = \frac{m_{\text{NaOH lab scale}}}{m_{\text{biomass lab scale}}} \times m_{\text{biomass}}$$

with

$\text{Cost}_{\text{NaOH}}$ = cost NaOH (€)

m_{NaOH} = mass NaOH required (ton)

p_{NaOH} = price NaOH $(\frac{€}{\text{ton}})$

$m_{\text{NaOH labscale}}$ = mass NaOH required during lab scale experiment (g)

$m_{\text{biomass labscale}}$ = mass dry biomass during lab scale experiment (g)

m_{biomass} = mass dry biomass (ton)

For Scenario 2 and 3, calculations were based on $Ca(OH)_2$ price, to further reduce cost. NaOH and $Ca(OH)_2$ prices are assumed to be € 288 /ton [13] and € 53 /ton [14].

Table 2. Cost NaOH or $Ca(OH)_2$ per ton dry biomass in the single step extraction at low temperature, and in the three step extraction at elevated temperatures

Biomass	NaOH used		Cost NaOH		Cost $(CaOH)_2$	
	3 steps	1 step	3 steps	1 step	3 steps	1 step
	(ton/ton biomass)		(€/ton biomass)		(€/ton biomass)	
Barley grain	0.004	0.002	1.1	0.6	0.2	0.1
Barley mill run	0.004	0.002	1.1	0.6	0.2	0.1
Barley rootlets	0.034	0.032	9.9	9.3	1.8	1.7
Malt byproducts	0.009	0.007	2.5	2.0	0.5	0.4
Microalgae	0.013	0.011	3.8	3.2	0.7	0.6
Microalgae meal	0.004	0.002	1.1	0.6	0.2	0.1
Palm kernel meal	0.010	0.008	2.9	2.3	0.5	0.4
Rapeseed meal	0.015	0.013	4.3	3.7	0.8	0.7
Ryegrass	0.004	0.002	1.1	0.6	0.2	0.1
Soybean	0.004	0.002	1.1	0.6	0.2	0.1
Soybean hull	0.006	0.004	1.6	1.0	0.3	0.2
Soybean meal	0.004	0.002	1.1	0.6	0.2	0.1
Sugar beet pulp	0.004	0.002	1.1	0.6	0.2	0.1
Sunflower meal	0.004	0.002	1.1	0.6	0.2	0.1
Wheat gluten	0.004	0.002	1.1	0.6	0.2	0.1
Wheat middling	0.004	0.002	1.1	0.6	0.2	0.1

3. Cost on energy

The energy cost was calculated according to the thermal energy required to heat up the mixture of biomass and solvent. Specific heat capacity for water is 4.2 $J.g^{-1}.K^{-1}$ and for biomass is assumed to be 1.3 $J.g^{-1}.K^{-1}$ (at 25 °C)[15]. The ratio of biomass to water used in the calculation is 1:9.

The thermal energy required was calculated as:

$$Q = \left((m_{water} \times c_{water}) + (m_{biomass} \times c_{biomass})\right) \times \Delta T$$

The thermal energy in Joules is then converted to kWh values ($1\,J = 2.7 \times 10^{-7}$kWh). The price per kWh used in the calculations; € 0.041/kWh, is the price of natural gas for a medium-sized industrial consumer [16].

The final energy cost calculated for the high temperature extraction, the three step extraction, is € 58. For low temperature extraction, or the single step extraction, it is assumed that no energy cost is required as this is performed at room temperature.

4. Revenue from residue used as feed

Revenue obtained from residue after protein extraction if it is used as feed was calculated as:

$$\text{Price}_{\text{feed}} = m_{\text{residue}} \times p_{\text{feed}}$$

$$m_{\text{residue}} = \frac{m_{\text{residue lab scale}}}{m_{\text{biomass lab scale}}} \times m_{\text{biomass}}$$

with

$\text{Price}_{\text{feed}}$ = Total price of residue if it is used as feed (€)

m_{residue} = mass residue (ton)

p_{feed} = price residue as feed $\left(\frac{€}{\text{ton}}\right)$, extrapolated (see Fig. 1)

$m_{\text{residue lab scale}}$ = mass residue obtained during lab scale experiment (g)

$m_{\text{biomass lab scale}}$ = mass biomass required during lab scale experiment (g)

m_{biomass} = mass biomass (ton)

The price of the residue with low protein content (Fig. 1) was extrapolated from linear regression of the protein content and biomass prices of 11 tested biomass resources (Chapter 3). The other 5 biomass were not considered due to their high protein biomass (wheat gluten), or due to absence of current market price (microalgae, microalgae meal, barley mill run, and barley rootlets).

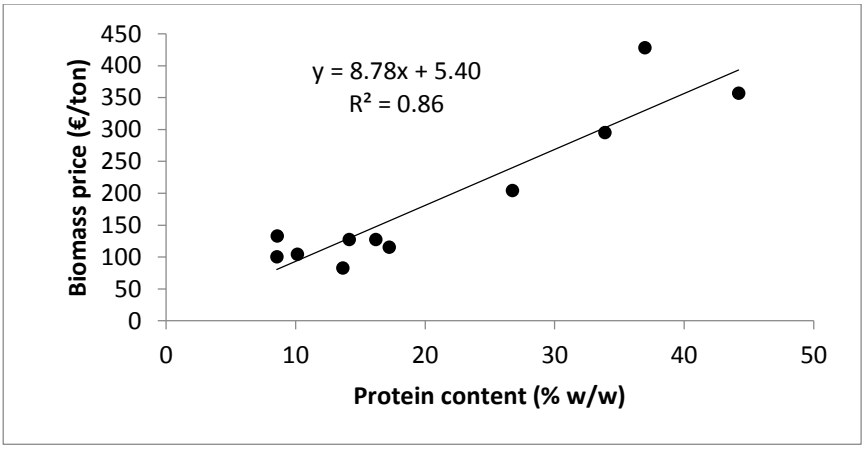

Figure 1. Plot biomass price vs protein content for biomass with protein content less than 50%.

The estimated residue revenues (per ton biomass) for application in feed are given in Table 3 and 4. In the experimental set-up with single step extraction at low temperature, the protein content in the residue was not measured. The protein content in this residue is then assumed to be equal to protein content in residue after three step extraction (C) and both supernatant at 60 and 120 °C (E60 and E120 in Chapter 3).

Table 3. Revenue from residue (€/ton dry biomass) used as feed. Residue was obtained after protein extraction at high temperature

Biomass	**Protein content in residue**	**Residue price as feed (per ton)**	**Mass residue**	**Revenue from residue as feed**
	(%)	**(€/ton)**	**(ton/ ton dry biomass)**	**(€/ton dry biomass)**
Barley grain	2.7	29	0.45	13
Barley mill run	5.1	50	0.56	28
Barley rootlets	19.1	173	0.49	84
Malt byproducts	9.8	92	0.37	34
Microalgae	49.2	437	0.53	233
Microalgae meal	58.4	517	0.49	254
Palm kernel meal	13.6	124	0.69	85
Rapeseed meal	16.2	148	0.22	32
Ryegrass	7.7	73	0.59	43
Soybean	39.4	351	0.22	79
Soybean hull	6.5	62	0.35	22
Soybean meal	38.4	342	0.17	57
Sugar beet pulp	3.5	36	0.77	28
Sunflower meal	35.5	317	0.34	108
Wheat gluten	26.8	240	0.15	36
Wheat middling	4.1	41	0.51	21

Table 4. Revenue from residue (€/ton dry biomass) used as feed. Residue was obtained after protein extraction at low temperature

Biomass	Protein content in residue	Residue price as feed (per ton)	Mass residue	Revenue from residue as feed
	(%)	(€/ton)	(ton/ton dry biomass)	(€/ton dry biomass)
Barley grain	2.6	28	0.66	18
Barley mill run	8.9	83	0.77	64
Barley rootlets	24.7	222	0.74	165
Malt byproducts	9.5	89	0.68	60
Microalgae	38.2	340	0.74	253
Microalgae meal	52.9	469	0.63	293
Palm kernel meal	16.8	153	0.79	120
Rapeseed meal	23.7	213	0.48	103
Ryegrass	11.7	108	0.72	78
Soybean	39.6	353	0.49	174
Soybean hull	11.1	103	0.49	50
Soybean meal	37.8	337	0.42	141
Sugar beet pulp	7.0	66	0.85	56
Sunflower meal	27.4	245	0.59	144
Wheat gluten	42.8	381	0.26	98
Wheat middling	5.4	53	0.58	31

5. Revenue from residue if it is used for energy use

For this scenario, the calorie content of biomass was calculated according to Association of American Feed Control Officials [17]. This is defined as the amount of protein, oil, and carbohydrate. Carbohydrate was not measured directly but estimated by calculating the "nitrogen free extract". Modified Atwater constants were used to calculate calorie content. Both protein and carbohydrate (as nitrogen free extract) have modified Atwater[7] constants of 3.5 kcal/g. Oil is denser than protein and carbohydrate, hence has value of 8.5 kcal/g. Nitrogen free extract and calorie content of residual biomass after protein extraction was calculated as:

$$\text{Nitrogen free extract (\%)} = 100\text{-}(\%\text{protein} + \%\text{oil} + \%\text{fiber} + \%\text{ash} + \%\text{moisture})$$

[7] Atwater constants are multiplying factors used to calculate the energy from food based on heat combustion of protein, oil, and carbohydrates. These values, calculated by Atwater, are 4, 9, 4 kcal/g, respectively. Based on a presumption that pet food is about 90% digestible than human foods, the modified Atwater constants of 3.5, 8.5, and 3.5 kcal/g for protein, oil and carbohydrate, respectively, are prescribed by the Association of American Feed Control Officials (AAFCO) to calculate feed energy value.

$$Calorie\ content\ \left(\frac{kcal}{kg}\right) = \begin{pmatrix}(3.5 \times \%\ protein) + (8.5 \times \%\ oil) + \\ (3.5 \times \%\ nitrogen\ free\ extract)\end{pmatrix} x\ 10$$

With

%protein, %oil, %fiber, %ash, %moisture
= percentage of protein, oil, fiber, ash, and moisture in residue (%)

The amount of protein used in the calculations corresponds to the protein content in the residue after protein extraction (Table 3). The residues have the same moisture content as the untreated biomass, which is 10%. Fractions of oil, fiber, and ash may be partially extracted during extraction. The mass percentage of these chemicals in the residue is assumed to be proportionally the same as the mass percentage in the untreated biomass. Therefore the percentage of these chemical components in residue can be calculated as:

$$\%\text{Chem} = \frac{\text{m}_{\text{chem}}}{\text{m}_{\text{residue}}} \times 100\%$$

$$\text{m}_{\text{chem}} = \frac{\text{m}_{\text{residue lab scale}}}{\text{m}_{\text{biomass lab scale}}} \times \text{m}_{\text{chem in biomass}}$$

with

%Chem = percentage of chemical (oil, fiber, or ash) in residue (%)

m_{chem} = mass chemical in residue (ton)

$\text{m}_{\text{residue}}$ = mass residue (ton)

$\text{m}_{\text{residue lab scale}}$ = mass residue obtained during lab scale experiment (g)

$\text{m}_{\text{biomass lab scale}}$ = mass biomass required during lab scale experiment (g)

$\text{m}_{\text{chem in biomass}}$ = mass chemical contained in untreated biomass (ton)

The calorie content in kcal/kg was subsequently converted to kWh/kg (1 cal = 1.16×10^{-6}kWh), and the energy content of residue (kWh) from 1 ton protein production was calculated as:

$$Energy\ content\ (kWh) = calorie\ content\ \left(\frac{kWh}{kg}\right) x\ mass\ residue\ (ton)$$

Finally, the price of residual biomass used for energy was calculated as:

$$Price\ of\ residual\ biomass\ used\ for\ energy\ usage(€) = 0.041\ \left(\frac{€}{kWh}\right) x\ energy\ content(kWh)$$

The energy price per kWh used in the calculation, € 0.041/kWh, is the price of natural gas for a medium-sized industrial consumer [16].

Estimated revenue from residue per ton dry biomass after protein extraction that is used for energy is given in Table 5 and 6.

Table 5. Revenue from residue (€/ton dry biomass) used for energy. Residue was obtained after protein extraction at high temperature

Biomass	Mass chemical component in biomass			Mass residue	Mass chemical component in residue			Percentage chemical component in residue					Nitrogen free extract	Energy content		Revenue from residue for energy usage
	Oil	Fiber	Ash		Oil	Fiber	Ash	Protein	Oil	Fiber	Ash	Moisture				
	(ton)			(ton/ton dry biomass)	(ton/ton dry biomass)			(%)					(%)	(kcal/ kg dry biomass)	(kWh/ ton dry biomass)	(€/ton dry biomass)
Barley grain	0.019	0.057	0.025	0.45	0.0085	0.03	0.011	2.7	1.9	5.7	2.5	10	77.2	2958	3431	63
Barley mill run	0.032	0.151	0.054	0.56	0.0180	0.08	0.030	5.1	3.2	15.1	5.4	10	61.2	2592	3007	69
Barley rootlets	0.02	0.129	0.063	0.49	0.0097	0.06	0.031	19.1	2	12.9	6.3	10	49.7	2578	2990	60
Malt by products	0.03	0.082	0.041	0.37	0.0110	0.03	0.015	9.8	3	8.2	4.1	10	64.9	2869	3329	50
Microalgae	0.022	0.336	0.05	0.53	0.0117	0.18	0.027	49.2	2.2	33.6	5	10	0.0	1909	2214	48
Microalgae meal	0.023	0.115	0.068	0.49	0.0113	0.06	0.033	58.4	2.3	11.5	6.8	10	11.0	2624	3044	61
Palm kernel meal	0.015	0.062	0.06	0.69	0.0103	0.04	0.041	13.6	1.5	6.2	6	10	62.7	2798	3246	91
Rapeseed meal	0.015	0.296	0.058	0.22	0.0033	0.06	0.013	16.2	1.5	29.6	5.8	10	36.9	1986	2304	21
Ryegrass	0.041	0.073	0.049	0.59	0.0243	0.04	0.029	7.7	4.1	7.3	4.9	10	66.0	2928	3396	83
Soybean	0.187	0.053	0.049	0.22	0.0419	0.01	0.011	39.4	18.7	5.3	4.9	10	21.7	3728	4324	40
Soybean hull	0.051	0.036	0.062	0.35	0.0179		0.022	6.5	5.1	3.6	6.2	10	68.6	3062	3552	51
Soybean meal	0.22	0.02	0.059	0.17	0.0368	0.00	0.010	38.4	22	2	5.9	10	21.7	3973	4609	32
Sugar beet pulp	0.023	0.003	0.037	0.77	0.0178	0.00	0.029	3.5	2.3	0.3	3.7	10	80.2	3125	3625	115
Sunflower meal	0.023	0.145	0.085	0.34	0.0078	0.05	0.029	35.5	2.3	14.5	8.5	10	29.2	2460	2854	40
Wheat gluten	0.007	0.177	0.054	0.15	0.0010	0.03	0.008	26.8	0.7	17.7	5.4	10	39.4	2377	2757	17
Wheat middling	0.017	0.177	0.039	0.51	0.0087	0.09	0.020	4.1	1.7	17.7	3.9	10	62.6	2479	2876	60

Table 6. Revenue from residue (€/ton dry biomass) used for energy. Residue was obtained after protein extraction at low temperature

Biomass	Mass chemical component in raw biomass			Mass residue per mass biomass	Mass chemical component in residue			Percentage chemical component in residue					Nitrogen free extract	Energy content		Revenue from residue for energy usage
	Oil	Fiber	Ash		Ooil	Fiber	Ash	Protein	Oil	Fiber	Ash	Moisture				
	(ton)			(ton/ton dry biomass)	(ton/ton dry biomass)			(%)					(%)	(kcal/ kg dry biomass)	(kWh/ ton dry biomass)	(€/ton dry biomass)
Barley grain	0.019	0.057	0.025	0.66	0.0126	0.04	0.017	2.6	1.9	5.7	2.5	10	77.3	2958	3431	93
Barley mill run	0.032	0.151	0.054	0.77	0.0246	0.12	0.042	8.9	3.2	15.1	5.4	10	57.4	2592.5	3007	95
Barley rootlets	0.02	0.129	0.063	0.74	0.0149	0.10	0.047	24.7	2	12.9	6.3	10	44.1	2578	2990	91
Malt by products	0.03	0.082	0.041	0.68	0.0204	0.06	0.028	9.5	3	8.2	4.1	10	65.2	2869.5	3329	93
Microalgae	0.022	0.336	0.05	0.74	0.0163	0.25	0.037	38.2	2.2	33.6	5	10	11.0	1909	2214	67
Microalgae meal	0.023	0.115	0.068	0.63	0.0144	0.07	0.043	52.9	2.3	11.5	6.8	10	16.5	2624.5	3044	78
Palm kernel meal	0.015	0.062	0.06	0.79	0.0118	0.05	0.047	16.8	1.5	6.2	6	10	59.5	2798	3246	105
Rapeseed meal	0.015	0.296	0.058	0.48	0.0073	0.14	0.028	23.7	1.5	29.6	5.8	10	29.4	1986	2304	46
Ryegrass	0.041	0.073	0.049	0.72	0.0295	0.05	0.035	11.7	4.1	7.3	4.9	10	62.0	2928	3396	100
Soybean	0.187	0.053	0.049	0.49	0.0920	0.03	0.024	39.6	18.7	5.3	4.9	10	21.5	3728	4324	87
Soybean hull	0.051	0.036	0.062	0.49	0.0250	0.02	0.030	11.1	5.1	3.6	6.2	10	64.0	3062	3552	79
Soybean meal	0.22	0.02	0.059	0.42	0.0919	0.01	0.025	37.8	22		5.9	10	22.3	3973.5	4609	
Sugar beet pulp	0.023	0.003	0.037	0.85	0.0195	0.00	0.031	7.0	2.3	0.3	3.7	10	76.7	3125	3625	126
Sunflower meal	0.023	0.145	0.085	0.59	0.0135	0.09	0.050	27.4	2.3	14.5	8.5	10	37.3	2460	2854	69
Wheat gluten	0.007	0.177	0.054	0.26	0.0018	0.05	0.014	42.8	0.7	17.7	5.4	10	23.4	2377	2757	29
Wheat middling	0.017	0.177	0.039	0.58	0.0099	0.10	0.023	5.4	1.7	17.7	3.9	10	61.3	2479	2876	69

6. Summary

Table 7 gives summary on processing cost of 1 ton raw biomass for protein production at high and low temperatures. Processing cost in this calculation refers to cost for raw biomass, NaOH, and heating only. Table 8 gives summary on revenue obtained if residue from protein extraction is used as feed or for energy usage

Table 7. Cost (€/ton dry biomass) for processing 1 ton biomass at high and low temperatures

Biomass	High temperature						Low temperature				
		Chemicals			Total			Chemicals		Total	
	Biomass	with NaOH	with $Ca(OH)_2$	Energy	with NaOH	with $Ca(OH)_2$	Biomass	with NaOH	with $Ca(OH)_2$	with NaOH	with $Ca(OH)_2$
	(€/ton biomass)						(€/ton biomass)				
Barley grain	104.40	1.14	0.21	57.92	163.47	162.54	104.40	0.57	0.11	104.97	104.51
Barley mill run	82.80	1.14	0.21	57.92	141.87	140.94	82.80	0.57	0.11	83.37	82.91
Barley rootlets	82.80	9.90	1.83	57.92	150.62	142.56	82.80	9.33	1.73	92.13	84.53
Malt by products	82.80	2.52	0.47	57.92	143.25	141.19	82.80	1.95	0.36	84.75	83.16
Microalgae	1650.00	3.79	0.70	57.92	1711.71	1708.63	1650.00	3.22	0.60	1653.22	1650.60
Microalgae meal	230.00	1.14	0.21	57.92	289.07	288.14	230.00	0.57	0.11	230.57	230.11
Palm kernel meal	115.71	2.87	0.53	57.92	176.50	174.16	115.71	2.30	0.43	118.00	116.13
Rapeseed meal	295.20	4.30	0.79	57.92	357.42	353.92	295.20	3.73	0.69	298.93	295.89
Ryegrass	100.80	1.14	0.21	57.92	159.87	158.94	100.80	0.57	0.11	101.37	100.91
Soybean	428.40	1.14	0.21	57.92	487.47	486.54	428.40	0.57	0.11	428.97	428.51
Soybean hull	127.80	1.60	0.30	57.92	187.33	186.02	127.80	1.03	0.19	128.83	127.99
Soybean meal	357.19	1.14	0.21	57.92	416.26	415.33	357.19	0.57	0.11	357.76	357.30
Sugar beet pulp	133.20	1.14	0.21	57.92	192.27	191.34	133.20	0.57	0.11	133.77	133.31
Sunflower meal	204.48	1.14	0.21	57.92	263.55	262.62	204.48	0.57	0.11	205.05	204.59
Wheat gluten	735.84	1.14	0.21	57.92	794.91	793.98	735.84	0.57	0.11	736.41	735.95
Wheat middling	127.80	1.14	0.21	57.92	186.87	185.94	127.80	0.57	0.11	128.37	127.91

Table 8. Revenue (€/ton dry biomass) from residue of processing 1 ton biomass at high and low temperatures

Biomass	High temperature		Low temperature	
	As feed	For energy usage	As feed	For energy usage
	(€/ton biomass)			
Barley grain	13.1	18.4	62.9	93.0
Barley mill run	28.1	64.2	69.3	94.8
Barley rootlets	84.4	165.0	59.7	91.1
Malt byproducts	33.6	60.3	50.1	92.8
Microalgae	233.3	252.8	48.5	67.5
Microalgae meal	253.5	293.4	61.2	78.1
Palm kernel meal	85.4	120.4	91.3	104.9
Rapeseed meal	32.1	103.0	20.5	45.7
Ryegrass	43.5	77.6	82.6	100.0
Soybean	78.7	173.8	39.7	87.3
Soybean hull	21.7	50.4	51.0	71.4
Soybean meal	57.3	140.9	31.6	78.9
Sugar beet pulp	27.8	56.4	114.9	126.3
Sunflower meal	107.8	144.1	39.8	68.7
Wheat gluten	35.8	97.8	16.9	29.0
Wheat middling	21.0	30.8	60.1	68.7

Data on processing cost per ton biomass are further used to calculate processing cost required to produce 1 ton protein product. Recalculation was performed by considering cost to process 1 ton biomass and the amount of biomass needed to produce 1 ton protein product. Table 9 and 10 give summary on processing cost for production 1 ton protein and the revenue obtained if residue is used as feed or for energy usage, respectively.

Table 9. Cost (€/ton protein) for production 1 ton protein at high and low extraction temperatures

Biomass	High temperature						Low temperature				
		Chemicals			Total			Chemicals		Total	
	Biomass	with NaOH	with $Ca(OH)_2$	Energy	with NaOH	with $Ca(OH)_2$	Biomass	with NaOH	with $Ca(OH)_2$	with NaOH	with $Ca(OH)_2$
	(€/ton protein)						(€/ton protein)				
Barley grain	1271.6	13.9	2.6	705.5	1991.1	1979.8	1349.7	7.4	1.4	1357.1	1351.1
Barley mill run	1097.2	15.1	2.8	767.6	1879.9	1867.5	2328.3	16.0	3.0	2344.3	2331.2
Barley rootlets	451.4	54.0	10.0	315.8	821.2	777.2	889.6	100.2	18.5	989.8	908.1
Malt by products	860.3	26.2	4.8	601.9	1488.4	1467.1	1222.5	28.8	5.3	1251.3	1227.8
Microalgae	25538.7	58.7	10.9	896.6	26493.9	26446.1	37783.0	73.7	13.6	37856.7	37796.6
Microalgae meal	1440.1	7.1	1.3	362.7	1809.9	1804.0	1998.5	5.0	0.9	2003.4	1999.4
Palm kernel meal	1489.3	36.9	6.8	745.5	2271.7	2241.6	3009.5	59.8	11.1	3069.3	3020.6
Rapeseed meal	1048.0	15.3	2.8	205.6	1269.0	1256.5	1457.5	18.4	3.4	1475.9	1460.9
Ryegrass	2251.8	25.5	4.7	1294.0	3571.2	3550.4	15252.1	86.3	16.0	15338.4	15268.1
Soybean	1587.4	4.2	0.8	214.6	1806.2	1802.8	2626.9	3.5	0.6	2630.4	2627.6
Soybean hull	1176.1	14.7	2.7	533.1	1724.0	1711.9	1662.8	13.4	2.5	1676.2	1665.3
Soybean meal	935.3	3.0	0.6	151.7	1090.0	1087.5	1239.8	2.0	0.4	1241.8	1240.1
Sugar beet pulp	2586.0	22.1	4.1	1124.6	3732.8	3714.7	6856.5	29.4	5.4	6885.9	6862.0
Sunflower meal	1193.2	6.7	1.2	338.0	1537.8	1532.4	1554.7	4.3	0.8	1559.0	1555.5
Wheat gluten	1015.3	1.6	0.3	79.9	1096.8	1095.5	1123.9	0.9	0.2	1124.8	1124.1
Wheat middling	989.3	8.8	1.6	448.4	1446.6	1439.4	1078.8	4.8	0.9	1083.6	1079.7

Tabel 10. Revenue (€/ton protein) from residue of production 1 ton protein at high and low temperatures

Biomass	High temperature		Low temperature	
	As feed	For energy usage	As feed	For energy usage
	(€/ton protein)			
Barley grain	159.4	224.2	812.8	1201.8
Barley mill run	371.8	850.3	1947.6	2666.1
Barley rootlets	460.1	899.6	641.8	978.8
Malt byproducts	349.3	626.6	739.0	1369.4
Microalgae	3610.7	3912.3	1110.3	1544.6
Microalgae meal	1587.5	1837.1	531.7	678.6
Palm kernel meal	1099.3	1549.3	2375.1	2727.8
Rapeseed meal	114.1	365.6	101.5	225.4
Ryegrass	971.7	1734.2	12501.2	15137.5
Soybean	291.6	644.0	243.6	535.1
Soybean hull	200.0	463.6	664.2	928.6
Soybean meal	149.9	368.8	109.7	274.0
Sugar beet pulp	540.6	1094.9	5914.4	6501.1
Sunflower meal	629.0	840.7	302.6	522.2
Wheat gluten	49.4	135.0	25.8	44.3
Wheat middling	162.4	238.2	507.3	580.0

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Summary

The use of biomass for industrial products is not new. Plants have long been used for clothes, shelter, paper, construction, adhesives, tools, and medicine. With the exploitation on fossil fuel usage in the early 20th century and development of petroleum based refinery, the use of biomass for industrial application declined. Since the late 1960s, the petroleum-based products have widely replaced the use of biomass-based products. However, depletion of fossil fuels, rising oil prices, and growing environmental awareness, push the attention and policy towards a transition from fossil into bio-based products. Bio-based products can also be obtained from protein. The amine group ($-NH_2$) in protein shows attractive functionality for nitrogen-containing chemicals production. In petroleum based conversion of crude oil into chemicals, co-reagents such as ammonia have to be used, and various process steps are involved. With the amine in protein, various co-reagent introducing process steps can be by-passed.

Biomass refinery for protein might not only be necessary for supplying feedstock for the chemical industry, before all, it is important to meet the world protein demand for food and feed. Chapter 1 illustrates the protein shortage in 2030 that we will encounter with the current uses of protein in the diet of both humans and animals. The worldwide protein production may provide this demand only if we consider the biomass refinery for protein and use the protein product in an effective and efficient way according the specific need of food, feed, and chemical industry. For this purpose, development in protein extraction technology from various types of biomass is essential. The thesis entitled "Biomass and its potential for protein and amino acids; valorizing agricultural by-products" describes possibilities for using agricultural by-products as protein and or amino acid resources.

An overview on alkaline plant protein extraction was first presented, in Chapter 2, including the potential of addition of different types of enzymes. Protein extraction from common resources such as soybean meal and other oilseed meals were reviewed. Also new protein resources, like microalgae, were discussed on the applicability of alkali based methods for protein extraction. Most of the experimental studies opted for less than 100 min and 50-60°C as extraction time and temperature, respectively. A typical biomass to solvent ratio of 1:10 was selected in some studies. Alkaline pH was selected over acidic pH, because it is far away from the isoelectric point (IEP). Most proteins have the lowest solubility at their IEP, which commonly occurs at pH 4-5. Adding proteases during protein extraction increased protein yield.

Two types of extraction methods were experimentally researched in this study; alkaline and combined alkaline and enzymatic. In Chapter 3, alkaline protein extraction method was used to extract protein from 16 types of biomass, mostly agricultural by-products. Aiming to maximise protein extraction yields, a three step extraction was performed at elevated temperatures; 25, 60, and 120 °C. Protein yield was correlated to biomass chemical composition through Partial Least Square (PLS) regression. The results showed that protein extractability depended crucially on the type of biomass used. Protein from cereals and legumes were highly extracted, compared to other biomass. High protein

extractability coincides with the biological function of protein as a storage protein, as opposed to functional protein. Protein extraction was furthermore correlated to the composition of the biomass. Especially cellulose and oil hamper extractability of protein, whereas lignin has no significant influence, suggesting that alkaline treatment removed lignin sufficiently.

In Chapter 4, the effect of proteolysis during protein extraction was studied. Based on their working pHs, both alkaline and acidic proteases tested. Oilseed meals from soybean, rapeseed, and microalgae were considered as protein resource. Proteases that worked at acidic pH assisted protein extraction; but, still, more proteins were extracted using proteases that work at alkaline pH. This finding is in line with the literatures study from Chapter 2 mentioning that more proteins can be extracted at alkaline pH. Protex 40XL, Protex P, and Protex 5L that work at alkaline pH assisted protein extraction, particularly for rapeseed and microalgae meals. To a lesser extent, these proteases also improved protein extraction yield of soybean meal and untreated microalgae.

Having shown that proteolysis aids in protein extraction, proteases were also used to solubilise wheat gluten at alkaline pH. Solubilising wheat gluten is one of the bottle necks for wheat gluten application. In this thesis, wheat gluten was used to represent wheat dried distillers grains with solubles (DDGS). From our perspective, more biomass by-products, such as wheat DDGS derived from ethanol production, will be available, also due to the target to replace 10% fossil fuel with bio-based fuel in 2050. With high glutamic acid content, wheat gluten provides possibilities to serve as an amino acid resource. Glutamic acid, which currently is microbial produced, has potential as feedstock for bulk chemicals production. Large amounts of cheaper glutamic acid can be made available by enabling its production from biomass by-products, such as wheat DDGS. Several methods for producing glutamic acid from wheat gluten were developed and the results were presented in Chapter 5. We found that a combination of enzymatic and mild acid hydrolysis opens up new possibilities for the industrial production of glutamic acid from biomass.

Finally, in Chapter 6, general knowledge obtained from this study is discussed and a perspective on biomass valorization for protein and/or amino acids is presented. It was concluded that biomass, and particularly agricultural residues, are potential resources for protein and/or amino acids. An outlook on protein and/or amino acids production from by-products was also provided in this chapter. For this, economic calculations were provided that focussed on the processing cost. Based on these calculations, overnight alkaline treatment at room temperature was most economical to extract protein from most types of biomass. Residual biomass following protein extraction can be used as animal feed or for energy usage to get to a more integrated biorefinery, thereby reducing protein production cost.

Samenvatting

Het gebruik van biomassa voor industriële producten is niet nieuw. Planten worden al lang gebruikt voor kleding, onderdak, papier, bouw, lijmen, gereedschappen en in geneeskunde. Met de exploitatie en het gebruik van fossiele brandstoffen in de vroege 20e eeuw en de ontwikkeling van olieraffinaderijen, is het gebruik van biomassa voor industriële toepassingen afgenomen. Sinds de late jaren 60, hebben de olie gebaseerde producten op grote schaal het gebruik van biomassa gebaseerde producten vervangen. Uitputting van fossiele brandstoffen, stijgende olieprijzen, en een groeiend milieubewustzijn, brengt echter de aandacht en het beleid weer naar een transitie van fossiel naar bio-based. Bio-based producten kunnen o.a. worden verkregen uit eiwit. De aminegroep (-NH_2) in eiwit toont namelijk een aantrekkelijke functionaliteit voor de productie van stikstofhoudende chemicaliën. De huidige, op petroleum gebaseerde omzettingen van ruwe olie in chemicaliën, gebruiken co-reagentia zoals ammoniak waarbij diverse processtappen betrokken zijn. Met gebruik van het amine uit eiwit, kunnen de verschillende stappen die de co-reagentia in het proces introduceren worden overgeslagen.

Eiwit bioraffinage kan niet alleen leiden tot nieuwe grondstoffen voor de chemische industrie. Belangrijker nog is dat er aan de wereldwijde vraag naar eiwitten voor levensmiddelen en diervoeders wordt voldaan. Hoofdstuk 1 illustreert het toekomstige eiwittekort uitgaande van de bestaande toepassing van eiwit in de voeding van mens en dier. De wereldwijde productie van eiwitten kan enkel aan deze vraag voldoen als we b.v. met behulp van bioraffinage eiwitproducten op een effectieve en efficiënte manier kunnen gebruiken volgens de specifieke behoeftes voor food, feed, en chemicaliën. Daartoe is de ontwikkeling van eiwit-extractie technologie van verschillende soorten biomassa noodzakelijk. Dit proefschrift beschrijft mogelijkheden voor het gebruik van agrarische bijproducten als eiwit en/of aminozuur bron.

Een overzicht van alkalische eiwit extractie uit plantaardig materiaal wordt gepresenteerd in hoofdstuk 2, inclusief de mogelijke verbetering van dit proces door toevoeging van verschillende enzymen. Eiwit extractie uit gangbare bronnen, zoals sojameel en residuen van andere oliehoudende zaden, of vanuit graan residuen is bediscussieerd. Ook nieuwe bronnen voor eiwit, zoals microalgen, zijn besproken op de toepasbaarheid van alkalische methoden voor eiwit extractie. De meeste experimentele studies kozen voor minder dan 100 minuten extractie tijd en 50-60°C als extractie temperatuur. Een typische biomassa tot oplosmiddel verhouding van 1:10 wordt vaak geselecteerd in de studies. De gebruikte alkalische pH werd geselecteerd omdat het ver ligt van het iso-elektrisch punt (IEP) van de eiwitten, dat gewoonlijk bij pH 4-5 ligt en waarbij de meeste eiwitten hun laagste oplosbaarheid hebben. Toevoegen van proteasen tijdens eiwit extractie verhoogde de eiwit opbrengst.

Twee extractiemethoden zijn experimenteel onderzocht in deze studie; alkalisch extractie en gecombineerde alkalische en enzymatische extractie. In hoofdstuk 3 staat beschreven hoe alkalische eiwit extractie wordt gebruikt om eiwitten te extraheren uit 16 soorten biomassa, voornamelijk agrarische bijproducten. In een poging de eiwit extractie

opbrengsten te maximaliseren, werd een drie-staps extractie uitgevoerd bij verschillende temperaturen; 25, 60 en 120°C. Eiwitrendement werd gecorreleerd aan de chemische samenstelling van de bijbehorende biomassa door middel van Partial Least Square (PLS) regressie. De resultaten toonden dat het gebruikte type biomassa doorslaggevend is voor de eiwit winbaarheid. Eiwit uit granen en peulvruchten werden zeer goed geëxtraheerd, vergeleken met andere biomassa. Deze goede extraheerbaarheid valt samen met de biologische functie van deze eiwitten als opslag eiwit, in tegenstelling tot functioneel eiwit (enzymen). Eiwit extractie werd verder gecorreleerd aan de samenstelling van de biomassa. Vooral cellulose en olie hadden een negatieve invloed op de winbaarheid van eiwit, terwijl lignine geen invloed van betekenis had, wat erop wijst dat de alkalische behandeling lignine voldoende verwijderde.

In hoofdstuk 4, werd het effect van proteolyse tijdens eiwitextractie bestudeerd. Zowel basische als zure proteasen zijn hiervoor getest bij hun optimale pH. Oliehoudende zaadresiduen als soja- en koolzaadmeel, en microalgen en hun meel, werden bestudeerd als potentiele eiwit bron. Proteasen die bij zure pH werken, hielpen bij de eiwit extractie, maar meer eiwitten werden geëxtraheerd met proteasen die werken bij alkalische pH. Deze bevinding is in overeenstemming met de literatuurstudie uit hoofdstuk 2, waar ook vermeld wordt dat er bij alkalische pH meer eiwit kan worden gewonnen. Protex 40XL, Protex P en Protex 5L, die werken bij alkalische pH, verhoogden de eiwit extractie, met name voor koolzaad- en microalgen meel. In mindere mate, verbeterden deze proteasen ook de eiwit extractie opbrengst bij sojameel en onbehandeld microalgen.

Na aangetoond te hebben dat proteolyse helpt bij eiwit extractie, werden proteasen ook gebruikt om tarwegluten oplosbaar te maken bij alkalische pH. Het oplosbaar maken van tarwegluten is één van de knelpunten bij tarwegluten toepassingen. In dit proefschrift werden tarwegluten gebruikt als modelsysteem voor tarwe DDGS (Dried Distillers Grains with Solubles). DDGS is afkomstig uit de bioethanol productie en zal in toenemende mate beschikbaar zijn, dankzij de 2050 doelstelling om 10% van de fossiele brandstoffen door bio-based brandstoffen vervangen te hebben. Tarwegluten kunnen mogelijk dienen als een aminozuur bron, door het hoge gehalte aan glutaminezuur. Glutaminezuur, dat momenteel op microbiële wijze wordt geproduceerd, heeft de potentie om als grondstof voor de productie bulkchemicaliën te dienen. Verschillende methodes voor de productie van glutaminezuur uit tarwegluten werden ontwikkeld, waarvan de resultaten zijn weergegeven in Hoofdstuk 5. De gevonden combinatie van enzymatische en mild zure hydrolyse opent nieuwe mogelijkheden voor de industriële productie van glutaminezuur uit biomassa.

Tenslotte wordt in Hoofdstuk 6, de algemene kennis die verkregen is uit deze studie besproken en wordt er een perspectief voor valorizatie van eiwit en/of aminozuren uit biomassa gepresenteerd. Biomassa, en in het bijzonder agrarische residuen, zijn potentiële bronnen voor eiwit en/of aminozuren en mogelijke processen daartoe voor productie worden bediscussieerd. Hiervoor werd de economische haalbaarheid ingeschat aan de hand van de operationele proceskosten. Op basis van deze berekeningen blijkt dat, voor de meeste soorten biomassa, het overnacht alkalisch behandelen bij kamertemperatuur het voordeligst eiwit extraheert. De resterende biomassa na eiwit extractie kan worden gebruikt als veevoer of voor energie, waardoor er een meer geïntegreerde bioraffinage ontstaat, dat de eiwitproductie kosten zal verminderen.

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-Yessie-

About the author

Yessie Widya Sari was born on April 14, 1980 in Jakarta, Indonesia. She obtained her bachelor degree in Physics and graduated in 2003 from Bogor Agricultural University. Her interest in biology related science inspired her to gain more biological knowledge by taking research on biophysics as her master thesis. During her master thesis, she conducted research that focused on hydroxyapatite, or bone mineral. She graduated in 2005 with honour from the University of Indonesia. After she obtained her master degree in 2005, Yessie joined the Biophysics Division of the Physics Department of Bogor Agricultural University as a research assistant. From 2005-2008, she was granted several research grants from The Indonesian Directorate General of Higher Education (DIKTI). She conducted research on development of bio-based bone substitute materials. As she wanted to pursue a future academic career, she started her PhD in Biobased Chemical Technology (formerly: Valorization of Plant Production Chains) at Wageningen University and Research Centre with a scholarship from the World Bank Institute through Joint Japan Indonesian Presidential Scholarship in 2008. Yessie conducted her research under the supervision of Prof. Dr. Johan P.M. Sanders and Dr. ir. Marieke E. Bruins. In the last years of her PhD, she was accepted as academic staff member at Biophysics Division, Physics Department, Bogor Agricultural University. She can be reached at yessie.sari@apps.ipb.ac.id

List of publications

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Sari YW, Bruins ME, Sanders JPM (2013) Enzyme assisted protein extraction from rapeseed, soybean, and microalgae meals. Industrial Crops and Products 43: 78-83.

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Overview of completed training activities

Discipline-specific courses

Study group on Spectroscopy and Theory, Netherlands Organisation for Scientific Research, the Netherlands, 2009

Renewable Resources for the Bulk chemical Industry, Wageningen University, the Netherlands, 2009

Advanced Downstream Processing Course, Institute Biotechnology Studies, Delft University of Technology and Leiden University, the Netherlands, 2010

Sustainable Process, Product, and System Design Course, OSPT, University of Groningen, the Netherlands, 2010

Sustainable analysis in food production, VLAG Graduate School, the Netherlands, 2011

Biorefinery processes course, Aalborg University, Denmark, 2011

Eighth International Conference on Renewable Resources & Biorefinery, University of Ghent, Belgium and University of Toulouse, France, 2012

2nd International Symposium on Technology for Sustainability, King Mongkut's Institute of Technology Ladkrabang Thailand, Institute of National Colleges of Technology Japan, Nagaoka University of Technology Japan, Toyohashi University of Technology Japan, Thailand, 2012

General courses

Knowledge Sharing Forum for the JJ/WBGSP Scholars in Continental Europe, World Bank Institute, France, 2009

Techniques for Writing and Presenting a Scientific Paper, Wageningen Graduate School, the Netherlands, 2009

Information Literacy and Introduction to EndNote, Wageningen Graduate School, the Netherlands, 2009

Presentation skill, Wageningen Graduate School, the Netherlands, 2010

Scientific Writing, Wageningen Graduate School, the Netherlands, 2010

Knowledge Sharing Forum for the JJ/WBGSP Scholars in Netherlands, Belgium, and Germany, World Bank Institute, the Netherlands, 2011

Scientific Meeting of Indonesian Student in Europe, the Embassy of Republic of Indonesia for Netherlands and Indonesian Student in Wageningen Association, 2012

Optional courses

Writing of research proposal, VLAG Graduate School, the Netherlands, 2008-2009

PhD trip to China, VLAG Graduate School, the Netherlands, 2009

Group meeting, Biobased Chemistry and Technology, Wageningen University, the Netherlands, 2008-2012

Theme meeting, Biobased Chemistry and Technology, Wageningen University, the Netherlands, 2008-2012

Chemistry of Life Science, Wageningen University, the Netherlands, 2008

Analytical Method on Organic Chemistry, Wageningen University, the Netherlands, 2008

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