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Sustainable protein and biogas production from green leafy biomass

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Sustainable protein and biogas production from green leafy biomass– A literature study

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Cover picture: Freeze-dried protein concentrate made from sugar beet leaves. Photo: Waleed Mlook

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Abstract

This paper explores the multifaceted potential of green leafy biomass as a sustainable resource for protein, energy, and biomass-derived chemicals, aligning with the goals of a circular bioeconomy and the United Nations' Sustainable Development Goals. Changing dietary trends, characterized by a shift towards plant-based protein alternatives, reflect evolving choices and sustainability concerns. The circular bioeconomy concept, focusing on renewable biogenic resources, is crucial for decarbonization and mitigating climate-negative effects.

Key points include the rising interest in plant-based proteins, the role of biorefineries in the circular bioeconomy, and the promise of green leafy biomass in addressing food security and protein demand. Examining by-products of the biorefinery process, including pulp, green juice, and brown juice, reveals opportunities for feed, biomaterials, fermentation, and biogas production. The valorization of green biomass, such as grass, ley crops, intermediate crops and leaves, is discussed in terms of raw material composition and potential applications, emphasizing local production to reduce environmental impact. The paper explores the significance of proteins, with a focus on RuBisCO from green leaves, in sustainable food production and biogas production. It provides insights into amino acid properties and protein structures, emphasizing the importance of RuBisCO, a key enzyme in carbon fixation. The paper discusses various protein extraction methods suitable for biorefinery processes, emphasizing the need to maximize protein yield. Biogas production from green biomass, particularly through anaerobic digestion, is highlighted, considering factors influencing production such as biomass composition.

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Background

Animal-based products are the major sources of protein-rich food, especially in the Western and South-American societies (Marcel et al., 2018). A high and decreasing demand for these products has been seen over the last 50 years, with a tripling of the meat production in the world and a doubling of the per capita consumption, from around 20 kg per person per year to slightly more than 40 kg per person and year (Ritchie et al., 2017). Also, a significant relationship exists between human wealth and the increase in meat consumption (York & Gossard, 2004). However, recently, the interest has increased for plant-based protein alternatives, which are gradually becoming more popular in some regions, reflecting evolving dietary choices and sustainability concerns (Coelho-Junior et al., 2020; Singhal et al., 2016). Predictions concerning global demand of protein for human food estimate a required doubling in amount in 2050 as compared to the current use (Henchion et al., 2017). Simultaneously, we see an escalating demand for renewable energy (Yang et al., 2021). In general, politicians and societal actors are striving towards the implementation of a circular bioeconomy, which will rely on renewable natural resources for food, products and energy, to secure a reduction of the human footprint when addressing food and energy challenges. Such an approach also aligns with several of the United Nations' 17 Sustainable Development Goals (Barrett et al., 2021; Cudlínová et al., 2017; Solarte-Toro & Alzate, 2021).

The SDGs are directives for global socio-economic and environmental improvement, countering the impacts of excessive fossil fuel use. The aim is that these goals should be achieved by 2030, which necessitates a national sustainable development framework across UN countries (Solarte-Toro & Alzate, 2021). One idea behind the circular bioeconomy concept is to replace conventional crude oil resources with renewable biogenic alternatives, thereby mitigating climate-negative effects through decarbonisation (House, 2012; Oborne, 2010).

Biorefineries stand out as a key to sustainability, representing the initial step toward a bioeconomy by converting biomass into valuable products and energy, thereby addressing issues related to waste and climate (Solarte-Toro & Alzate, 2021). In line with this, interest has grown in finding new protein-rich sources, such as agro-industrial side streams, to meet the demand for sustainable plant-based products (Boland et al., 2013). Furthermore, the development of clean energy is vital for a sustainable future (Chhandama et al., 2022). In order to reduce the negative consequences on the environment from food and especially meat production, there is an urgent need for a transition towards improved diets of more plant-based proteins, to which green biomass is an interesting source (Springmann et al., 2018). This introductory paper contributes an overview of the wide range of opportunities available for the utilization of green leafy biomass (GLBM) from agricultural production systems as a sustainable resource for production of protein, energy, biomass derived chemicals and value-added products. Also, methods and techniques suitable to be used in a biorefinery for fractionation of GLBM to increase the yield of plant-based proteins are presented.

The importance of green leafy biomass

The growing population, predicted to result in a total of 10 billion people in 2050, poses challenges to the environment, to food security and leads to an increased demand of food protein (Nadathur et al., 2017). This, together with environmental concerns from consumers has resulted in explorations from the food industry as related to novel sources of proteins that can be used in food applications (Day, 2013; Di Stefano et al., 2018). Already during the time of World War II, green biomass was proposed as a valuable protein resource to be used as human food (Pirie, 1942). GLBM holds significant promise as a protein and raw material source in biorefineries as it is one of the largest underutilized global nutrient sources (Balfany et al., 2023). Thus, it might be an appealing substitute for conventional protein sources in the food industry, and valuable compounds might be extractable from the process side streams (Balfany et al., 2023; Moller et al., 2021; Muneer et al., 2021).

Long transportation distances are one of the negative factors for the environment and therefore local production is desirable (Silva et al., 2010). Thus, the use of locally produced plant proteins is considered beneficial (Nynäs, 2018; Stodkilde et al., 2019), and such a use also reduces the environmental impact and water consumption compared to the production of animal proteins (Aiking, 2011; Day, 2013; Dijkstra et al., 2003). Grasslands have the potential to provide abundant green biomass, simultaneously delivering substantial ecological and dietary benefits. Perennial grasses offer advantages such as nutrient preservation, reduced pesticide needs, and soil carbon enrichment. (Jørgensen et al., 2022). Proteins from green biomass contribute a suitable amino acid composition for human consumption (Nynäs et al., 2023). Furthermore, these proteins have been shown suitable to replace soybean meal in feed for poultry and pigs without negative consequences on productive animal efficiency (Jørgensen et al., 2022). Lucerne has been valued as one of the most important sustainable resources for leaf protein concentrates as well as bio-organic fertilizers (El-Ramady et al., 2020).

Green biomass is abundant and widely available, the annual global production of dry biomass exceeds $2 \cdot 10^{11}$ t, which makes it a promising feedstock for any kind of product thereof, including the production of renewable energy (Kumar et al., 2008). Thus, residual grass, which is a byproduct of agricultural activities, can be effectively used for sustainable production of bioenergy in biorefineries (Nimmanterdwong et al., 2017). Biogas production from anaerobic digestion (AD) of agricultural biomass, such as grass, depends on various factors such as the content of cellulose, hemicellulose, lignin, and carbon-to-nitrogen (C/N) ratio (Karthikeyan & Visvanathan, 2013). However, previous studies have shown that the average methane production from the AD of residue grass is around 260-312 cubic meters/ton of volatile solids (VS) in the substrate (Mattioli et al., 2017).

From residual biomass to valuable products through biorefinery

The depletion of fossil fuels, coupled with environmental concerns, has led to a growing interest in using renewable resources as feedstock for the production a wide range of products (Figure 1), including biofuels, bioplastics, and other value-added chemicals (Cho et al., 2020). Proper management of various kinds of biomass, such as residues from forestry, agriculture, fruit and food processing, has the potential to decrease their negative environmental impact from biomass transportation by reducing costs and the footprint (Alatzas et al., 2019). A conversion of agricultural residues into valuable products and energy has the potential to contribute positively to sustainable development (Mechmech et al., 2015).

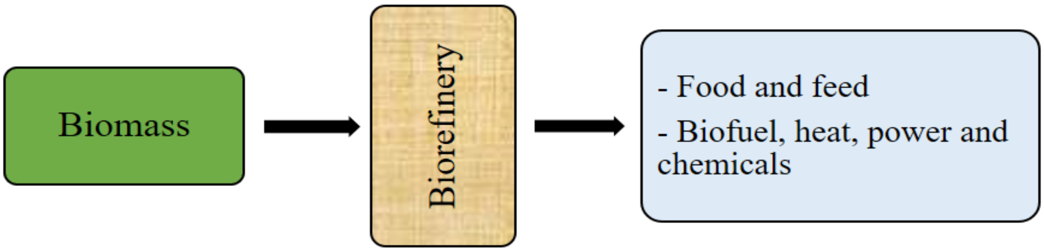


Figure 1 Schematic depicting of a sustainable processing of biomass into different value-added products in a biorefinery (Shim et al., 2018).

GLBM that is being composted or used for energy production, is also a promising resource for various valuable compounds (Langsdorf et al., 2021). Green biorefinery emerges as a highly promising and multifaceted pathway for the synthesis of a wide array of materials and energy resources, presenting an eco-friendly alternative to conventional fossil-derived commodities. The GLBM biorefinery generally consists of the following process steps: washing, juice pressing, precipitation of green fraction and precipitation of white fraction (Nynäs et al., 2024). Valorization of the streams (fibrous and water-soluble streams) generated from GLBM biorefinery processes have the potential to contribute to a more sustainable and economically viable process (Dale et al., 2009).

Fiber rich pulp

A side stream obtained in many of green biorefineries is a fiber rich pulp, which is the result of juice pressing to extract protein and other valuable water-soluble compounds. The fibers in the pulp fraction, comprise essential constituents such as cellulose (20-30%), hemicellulose (15-25%), and lignin (3-10%), which can be used for thermal insulation, biocomposite fabrication, packaging (Höltinger et al., 2014; Mandl, 2010), and several additional applications as summarized in Table 1. The pulp has also been suggested as a suitable feedstock for ruminants (Nynäs et al., 2024). Thus, fibrous pulp is a potential alternative forage source for ruminants, simultaneously as the green juice (GJ)/green protein can be used as a feed source for monogastric animals (Damborg et al., 2018). The pulp exhibits elevated concentrations of all vital amino acids compared to the original plant, as it retains a substantial portion of crude protein (Damborg et al., 2020). The content of methionine and lysine stands out as crucial in the feed of ruminants because they are commonly the first and second limiting amino acids in livestock feedings (Miller, 2004; Nynäs et al., 2024).

In the search for sustainable solutions and following consumer preferences, industry is increasingly adopting alternative and more sustainable packaging solutions, which include molded fiber products utilizing recycled and plant-based fibers. These products offer eco-friendly alternatives to plastic packaging and economic benefit to the company, simultaneously as protection during shipping is provided (Didone et al., 2017; Su et al., 2018). Thus, various companies are evaluating opportunities to use materials such as grass fibers and tomato leaves in their packaging solutions (https://production.huhtamaki.com/en/highlights/trends/beyond-recycled-paper/), thereby reducing production costs and greenhouse gas emissions. The use of recycled materials for cosmetic packaging was found to contribute both environmental benefits and creative designs (Zhang et al., 2022).

Opportunities to integrate GLBM residue conversion into lignocellulose biorefinery processes for diverse production applications have been widely investigated. The primary challenges identified are related to two main factors: the economic feasibility of the products and processes, and the specific structural properties of the lignin (Sathitsuksanoh et al., 2012).

However, functional UV-blocking cellulose/lignin composite films have been produced through the use of a dissolution-regeneration process. Thus, lignin residues (aspen, poplar wood, and corn stover) were found to enhance the UV-blocking performance, resulting in a reduced optical energy band gap from 4.31 to 3.72 eV (Yang et al., 2022). Poplar lignin, with a notable chromophore content, exhibited the highest UV-blocking improvement, simultaneously maintaining transparency, mechanical strength, and thermal stability, even at 4% lignin loading (Yang et al., 2022). When used as a soil improver, fibers from GLMB contributed also to structure and enriched the soil with organic matter upon decomposition, as has been reported for sugar beet, potato, and various grasses (Conijn et al., 2014; Kiskini, 2017; Skunca et al., 2021). The economic efficiency of a biorefinery depends on the economic returns from a multitude of streams and products, where fibers extracted from green leaves are an important part (Hulkko et al., 2023), making this a subject of keen interest among researchers.

Green juice

Green juice from GLBM holds promise as a nutritional source for both food and feed due to its rich nutritional content, including proteins, vitamins, organic compounds and minerals (Balfany et al., 2023). GJ can be used as protein source for animal feed (Nynäs et al., 2024; Santamaría-Fernández & Lübeck, 2020), although it also contains the white protein fraction suitable for human food applications (Nynäs, 2022). GJ contains chlorophyll, which can be used as natural pigment (Shahid et al., 2013) and chlorophyll rich extracts from leaves have been shown to have antioxidantproperties (Mehdipoor Damiri et al., 2020).

The chlorophyll's role as a remedy in modern medicine are becoming increasingly important, and has also been shown to have a potential as a modifier of genotoxic effects and a photosensitizer for cancer therapy (Mishra et al., 2011). Wheatgrass juice is noted for its high vitamin C content and nutrients, with potential to aid liver and kidney function, support detoxification, bolster the immune system, and influence fertility and sexual desire as magnesium has a role in the enzyme production linked to sex steroids (Khonsary, 2017). Furthermore, GJ derived from the grass miscanthus, has been evaluated as a growth substrate for yeast (Saccharomyces cerevisiae), with increased ethanol yield as a result (Boakye-Boaten et al., 2016).

Brown Juice (deproteinized leaf juice)

The brown juice (BJ) obtained after protein precipitation from green leaf juice is a nutrient-rich liquid that contains water-soluble carbohydrates, amino acids, and minerals (Martinez et al., 2018). The BJ contains a significant amount of macro- and micronutrients, and potentially abundant antioxidants, which contribute to opportunities for applications in microbiological media, plant nutrition, feedstock, and human dietary supplements or functional foods (Barna et al., 2020). The BJ has been investigated for its potential as a fermentation medium for microbial protein production (Mudgett et al., 1980), lactic acid fermentation (Andersen & Kiel, 2000), and biogas production (Feng et al., 2021).

BJ has successfully been acidified via lactic acid fermentation, and the fermented juice was utilized to produce volatile fatty acids (VFA) without sterilization or nutrient supplementation (Weimer & Digman, 2013). In addition, anaerobic mono-digestion of BJ from different green biomass has resulted in higher yields of methane compared to the mono-digestion of the corresponding fresh biomass (Santamaría-Fernández et al., 2018). Overall, BJ is a suitable fermentation medium for various products and has the potential to contribute to the economic feasibility of green biorefineries aiming at protein fractionation. However, further investigations are needed to expand the utilization and potential applications of the BJ (Moller et al., 2021).

The benefits of streams of biomass generated after pressing green leaves and their applications are shown in Table 1.

Table 1 Streams generated by protein fractionation from green leafy biomass, and their applications.

Side-streams	**Applications and benefits**	**Source**
Pulp	Feed for ruminants Biomaterials (e.g., insulation fiber) Fibers for food application	(Damborg et al., 2018; Tamayo Tenorio et al., 2017)
	Extraction of fibers for biorefinery	(Pirie, 1987)
	Source of cellulose, hemicellulose, and lignin	(Damborg et al., 2018)
	Protein source	(Damborg et al., 2020)
	Biogas/bioethanol	(Bruins & Sanders, 2012; S. Chiesa & E. Gnansounou, 2011; Simone Chiesa & Edgard Gnansounou, 2011)
	Packaging by molded fiber/pulp products	(Zhang et al., 2022)
GJ	Food and feed industry	(Santamaría-Fernández & Lübeck, 2020)
	White protein for human food	(Nynäs, 2022)
	Natural pigment	(Shahid et al., 2013)
	Vitamin C, B	(Khonsary, 2017)
BJ	Source of various nutrients (water-soluble carbohydrates, amino acids, and minerals)	(Martinez et al., 2018; Weimer & Digman, 2013)
	Fermentation medium for microbial protein production	(Mudgett et al., 1980)
	Lactic acid fermentation	(Andersen & Kiel, 2000)
	Biogas production	(Feng et al., 2021)

Green leafy biomass and valorization

The composition of GLBM depends on different factors such as the structural differences of GLBM (Nynäs et al., 2021), location, season (Boldrin & Christensen, 2010), plant species and maturity (Mohapatra et al., 2017). GLBM residues contain a variety of organic materials such as different types of carbohydrates (including fibers and various sugars), proteins, and bioactive phenolic compounds, constituting 25-35% of their dry matter (Aletor et al., 2002; Biondo et al., 2014; Prade et al., 2021; Smith, 1970).

Crop residues from leafy green plants are reported as a promising source of valuable bioactive compounds such as proteins (Berndtsson et al., 2019). The protein content in GLBM can vary significantly. For instance, grass has been reported to contain protein levels ranging from 1.5% to 4.5% (Juneja et al., 2011). In the case of elephant grass, the protein content is estimated to be around 5% to 6% (Menegol et al., 2016). In the case of broccoli, cabbage, lucerne, kale, sugar beet and spinach, the nitrogen content is estimated to be 3.2%, 2.1%, 2.8%, 3%, 3% and 4.8% respectively (Nynäs et al., 2021).

In terms of fats, yard waste, grass, and leaves typically contain approximately 2.5% of dry weight. The content of extractives, which are non-structural components in plants, can also vary among different types of herbaceous material. Miscanthus, for example, has been found to contain approximately 6.9% of extractives, while Switchgrass has a higher concentration of about 13.6% (Reza et al., 2013).

Table 2 Composition of different green leafy biomass based on their dry weight basis (% w/w).

Plant	**Common name**	**Protein**	**Carbohydrates**	**Ash**	**Fat**	**Source**
Medicago sativa L	Alfalfa	24.9*	22.5	9.6	4.8	(Smith, 1970)
Beta vulgaris L	Beetroot	28.7*	30.7	16.2	10.6	(Biondo et al., 2014)
Grass	Grass	6–25	39 - 67.5	5–20	1–2.5	(Grass, 2004)
Brassica Carinata	Abyssinian mustard	25.5	39.5	15.8	6.7	(Abuye et al., 2003)
Moringa Stenopetala	African Moringa	9	40.5 – 51.3	12.6	5.8	
Brassica oleracea, var. capitata	Cabbage	18.43	30-46	9.02	1.02	(Tanongkankit et al., 2012)
Brassica oleracea, var. italica	Broccoli	23.2	55.7	13.0	8.1	(Shi et al., 2019)

Protein is estimated based on nitrogen content using a protein conversion factor of 6.25.

Proteins

Proteins, often referred to as the "building blocks of life," exhibit remarkable structural diversity and functional versatility crucial for the functioning of living organisms. Their intricate three-dimensional structures allow for precise interactions with other molecules, enabling them to carry out a myriad of biological processes essential for life. Proteins play multifaceted roles in both biological and culinary domains, serving as essential components such as enzymes, structural frameworks, hormonal mediators, transport facilitators, immune defenders, and pivotal sources of sustenance (Damodaran, 2017). These foundational biomolecules are intricately composed of elongated chains of amino acids, intricately linked through peptide bonds, thus forming polypeptides (Figure 2). These molecular assemblies constitute the very foundation of life's intricate machinery. In the natural realm, a precise ensemble of "20 proteinogenic amino acids" exists, each bearing its distinctive set of side chains dictating their singular attributes and functions (Ayon et al., 2019). This diversified array of amino acids resembles a rich palette of colors, each contributing uniquely to the tapestry of biological processes. To attain a thorough comprehension of this intricate landscape, an exhaustive repository of information is available in Table 3 and Figure 3, providing meticulous details encompassing full names, 3-letter codes, 1-letter codes, chemical compositions of side chains, and noteworthy chemical characteristics of these amino acids.

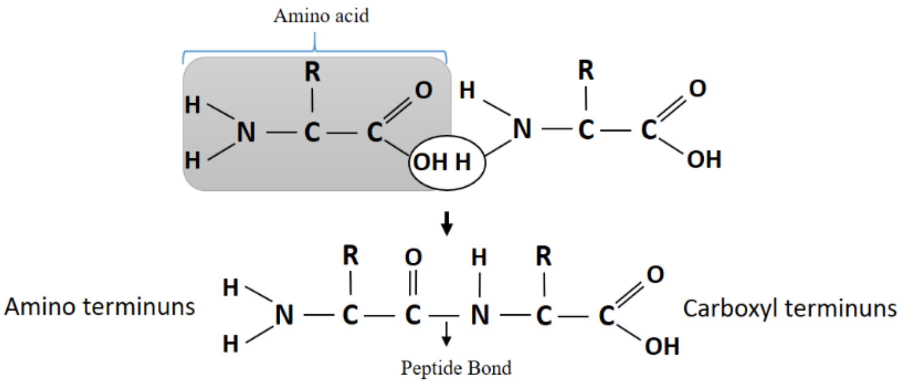


Figure 2 Molecular structure of an amino acid, R is a side chain.

The effectiveness of a protein depends on its unique arrangement at different levels primary, secondary, tertiary, and quaternary (Damodaran, 2017). Initially, proteins assume a linear sequence of amino acids, akin to beads strung on a necklace, forming the primary structure. Through subsequent folding, they undergo metamorphosis into intricate three-dimensional shapes, yielding secondary structures such as α-helices and β-pleated sheets, reminiscent of the art of origami. Further folding culminates in a tertiary structure, intricately held together by an array of bonds and interactions. Certain proteins harbor a quaternary structure, comprising interlinked units akin to pieces in a complex puzzle. These elaborate structural arrangements constitute the cornerstone of protein functionality and efficacy (Alberts et al., 2014; Berg et al., 2002; Levitt, 2009). Proteins' folded structure stability is influenced by various factors (Berg et al., 2015). Intra-protein amino acid interactions can be disrupted, leading to protein unfolding and denaturation. Commonly used compounds like urea and sodium dodecyl sulfate (SDS) break non-covalent bonds in proteins. Additionally, reducing agents like β-mercaptoethanol break disulfide bridges. Factors such as heat, detergents, and high salt concentrations also disrupt protein structure (Berg et al., 2015). Furthermore, when the pH is close to the isoelectric point, it changes how the protein is shaped.

Table 3. A guide to the 20 proteinogenic amino acids. The chemical formulas and various properties, such as side chain classification and charge status at pH 7.4. Essential amino acids for humans are marked with an asterisk (*).

Amino acid	**Abbreviation**	**R (Side chain)**	**R class**	**Charge at pH 7.4**
Alanine	Ala (A)	$- CH_3$	aliphatic	
Arginine *	Arg (R)	$-(CH_2)_3NH-C(NH)NH_2$	basic	positive
Asparagine	Asn (N)	$-CH_2CONH_2$	amide	polar
Aspartic acid	Asp (D)	$-CH_2COOH$	acid	negative
Cysteine	Cys (C)	$-CH_2SH$	S containing	polar
Glutamic acid	Glu (E)	$-CH_2CH_2COOH$	acid	negative
Glutamine	Gln (Q)	$-CH_2CH_2CONH_2$	amide	polar
Glycine	Gly (G)	$-H$	aliphatic	
Histidine*	His (H)	$-CH_2-C_3H_3N_2$	basic aromatic	positive
Isoleucine*	Ile (I)	$-CH(CH_3)CH_2CH_3$	aliphatic	
Leucine*	Leu (L)	$-CH_2CH(CH_3)_2$	aliphatic	
Lysine*	Lys (K)	$-(CH_2)_4NH_2$	aliphatic	positive
Methionine*	Met (M)	$-CH_2CH_2SCH_3$	S containing	
Phenylalanine*	Phe (F)	$-CH_2C_6H_5$	aromatic	
Proline	Pro (P)	$-CH_2CH_2CH_2-$	cyclic	
Serine	Ser (S)	$-CH_2OH$	-OH	polar
Threonine*	Thr (T)	$-CH(OH)CH_3$	-OH	polar
Tryptophan*	Trp (W)	$-CH_2C_8H_6N$	aromatic	
Tyrosine	Tyr (Y)	$-CH_2-C_6H_4OH$	aromatic	polar
Valine*	Val (V)	$-CH(CH_3)_2$	aliphatic	

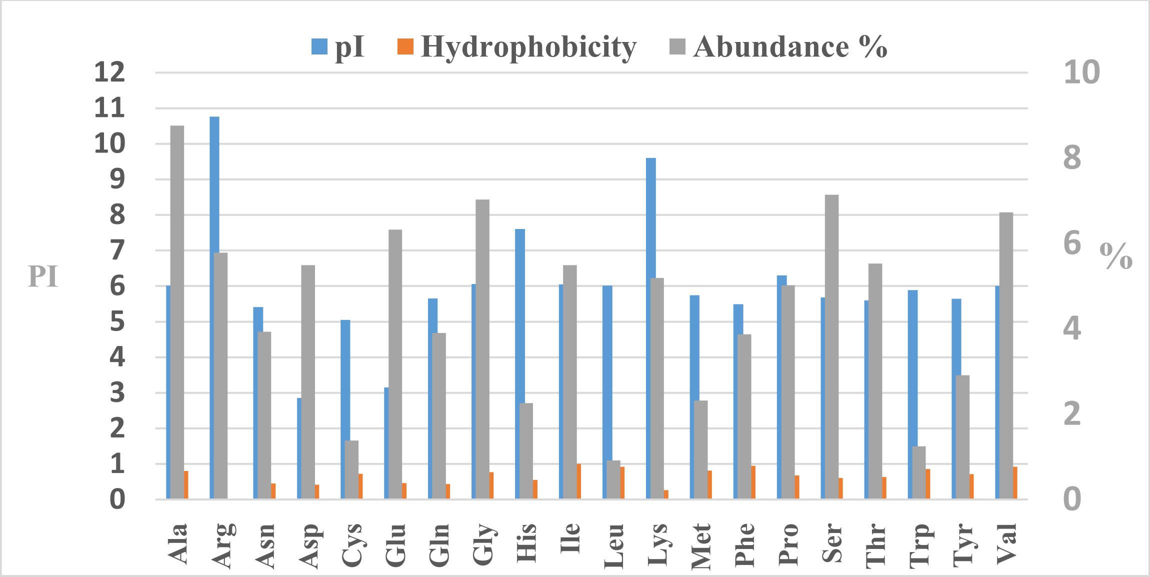


Figure 3. Properties (pI, hydrophobicity and abundance) of the 20 proteinogenic amino acids. The data is based on the NCBI Amino Acid Explorer (NCBI) and (Kozlowski, 2017).

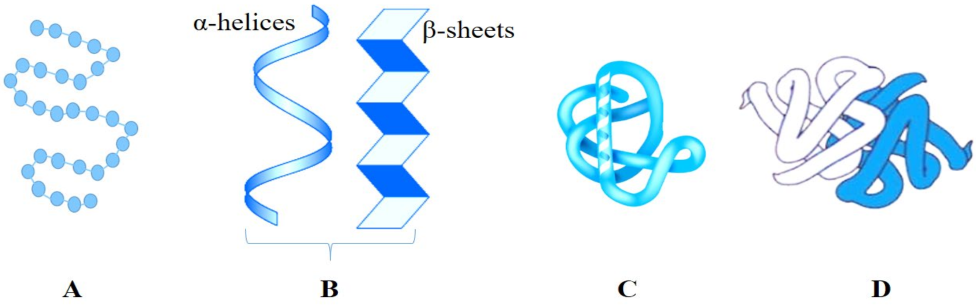


Figure 4. Protein structure. A: Primary structure refers to the sequence of amino acids in a polypeptide chain. B: Secondary structure involves local folded structures such as α-helices and β-sheets, formed by interactions between backbone atoms. Hydrogen bonds play a key role in maintaining these structures. C: Tertiary structure is the overall three-dimensional arrangement of a polypeptide, primarily determined by interactions between the amino acid R groups. The chain adopts a partially folded structure. D: Quaternary structure, multiple polypeptide chains or subunits that come together to form a complex.

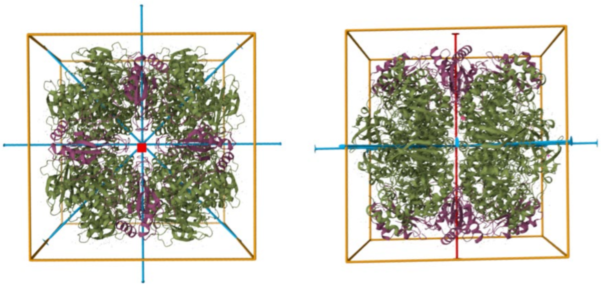
Proteins in green biomass

Considering the potential for enhancing the long-term sustainability of the global food production system, GLBM emerges as a promising protein source for various food applications. The extraction of soluble proteins from spinach leaves was first reported by Pirie in 1942, and since then, numerous researchers have delved into this topic (Barbeau & Kinsella, 1988; Hojilla-Evangelista et al., 2017; Martin et al., 2019; Martin et al., 2014). The GLMB differs both in degree of protein extractability and in level of undesirable compounds for animal and human consumption (Pérez-Vila et al., 2022). The quantity and quality of proteins are affected by several factors that must be taken into consideration if GLBM proteins should be extracted in industrial quantities (Nielsen et al., 2021; Pérez-Vila et al., 2022; Stodkilde et al., 2019). The protein content and composition, as well as their extractability, are affected by three main factors, i.e., the plant species, growing conditions, and stage of growth.

RuBisCO

One of the major proteins in GLBM is RuBisCO, an enzyme active in the carbon fixation cycle. RuBisCO is known to hold important functional and nutritional properties (Pérez-Vila et al., 2022). The plant species are divided into C3, C4 and CAM types depending on the ability and efficiency in carbon fixation (Maxwell, 2002). The C3 photosynthetic pathway is predominant among plant species which exhibit well-developed adaptations to cold climates, such as lucerne, rye, barley, sugar beet, soybean, and tobacco (Barbehenn et al., 2004). The leaves of C3 plants have been reported to hold a higher content of RuBisCO as compared to C4 plants (Ku et al., 1979). A RuBisCO content of 30% to 50% of the total plant protein has been reported in leaves of C3 plants and this high concentration is required to balance the photorespiration processes (Quick et al., 1991; Woodrow & Berry, 1988). The content of RuBisCO in C4 and CAM plants is significantly lower, with 8% to 23% reported for C4 plants (Ku et al., 1979). However, the lower contents are not necessarily related to a lower enzyme activity or photosynthetic capacity (Maxwell, 2002). For plants that exhibit facultative C3-CAM metabolism, significant variations in content of RuBisCO may not be the case, as their photosynthetic metabolism is changing rapidly as a result of variations in environmental conditions, as opposed to plants with stable metabolic pathways (Maxwell, 2002).

Life on Earth relies heavily on the capacity of photosynthetic organisms to convert atmospheric inorganic CO_2 into organic carbon through the Calvin-Benson-Bassham pathway. The key step in this process is the binding of CO_2 to ribulose-1,5 - bisphosphate (RuBP), facilitated by the enzyme RuBP carboxylase/oxygenase (EC 4.1.1.39), commonly known as RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase). RuBisCO is present in a wide range of autotrophic organisms, spanning from prokaryotes (such as photosynthetic and chemoautotrophic bacteria, cyanobacteria, and archaea) to eukaryotes (including various algae and higher plants) (Andersson & Backlund, 2008). RuBisCO is known as the most abundant protein on Earth and can account for up to 50% of the total soluble protein in plant leaves or microbial systems, (Ellis, 1979). In fact, the significance of RuBisCO extends beyond terrestrial environments, as it is also prevalent in phytoplankton found in the oceans. Phytoplankton is estimated to contribute over 45% of the global net primary production on an annual basis (Field et al., 1998). The plant RuBisCO protein exists in a hexadecameric form, composed of 8 large (L) and 8 small (S) subunits. The L subunits have a molecular weight of 55 kDa, while the small subunits weigh 12.5 kDa. The native structure of the protein is depicted in Figure 5 and consists of four L2 dimers that combine to form a barrel-shaped spherical structure (Andersson & Backlund, 2008). The S subunits are located at the top and bottom of this structure. Using X-ray scattering, researchers have determined that the outer radius of the protein structure measures 56.4 Å, while the inner radius is 14.3 Å (Donnelly et al., 1984). Each dimer-dimer interface involves eight salt links. When exposed to SDS, the integrity of these salt bridges between subunits is compromised, resulting in their separation during SDS-PAGE analysis. (Onaizi et al., 2007).



Figur 5. RuBisCO from spinach. Image from the RCSB PDB.

RuBisCO is a plant protein with an exceptional amino acid profile, surpassing other plant proteins in terms of nutritional quality for consumption (S. Chiesa & E. Gnansounou, 2011; Fiorentini & Galoppini, 1983a; Hermansen et al., 2017). Additionally, while most plant proteins suffer from low solubility, limiting their techno-functional properties, RuBisCO has proven to possess excellent functional characteristics when compared to well-established protein source like soy or whey (Barbeau & Kinsella, 1988; Martin et al., 2019). The amino acid sequence of RuBisCO shows high similarity across plant species (Fiorentini & Galoppini, 1983b; Hermansen et al., 2017; Stodkilde et al., 2019). Amino acid compositions of proteins extracted from green leaves demonstrate minimal variations, even under different harvesting times and fertilizer conditions (Gerloff et al., 1965). While variations between species mainly occur in the S subunit, the L subunit of RuBisCO exhibits almost identical amino acid compositions in all plants (Barbeau & Kinsella, 1988; Mangan, 2018). RuBisCO fulfills the essential amino acid requirements set by FAO/WHO (S. Chiesa & E. Gnansounou, 2011; De Jong & Nieuwland, 2011; Di Stefano et al., 2018; Hermansen et al., 2017). The GLBM has also been recognized as a potential source of bioactive compounds such as bioactive peptides (Di Stefano et al., 2018;

Kobbi et al., 2016; Udenigwe et al., 2017) and the techno-functional properties of RuBisCO, makes GLBM suitable for various food applications (Kobbi et al., 2016).

Figure 6 shows the essential amino acid content for cheese whey, egg white, RuBisCO of Spinach and protein requirement provided by FAO/WHO. RuBisCO, found in spinach, is a protein with excellent nutritional qualities. It has a balanced composition of essential amino acids, similar to egg protein. RuBisCO is rich in lysine, sulphur-containing amino acids, and tryptophan, making it beneficial for low-meat diets (Pouvreau et al., 2014) and compared to other plant proteins, it has a higher proportion of essential amino acids (Organization & University, 2007). The chemical index, comparing essential amino acids to egg protein, confirms the superior quality of RuBisCO, e.g., compared to soy protein. In the realm of nutritional evaluation, the FAO/WHO (2007) established an approach by adopting the amino acid pattern present of the egg white protein as the reference point for essential amino acids. A chemical index have been developed to describe the quality of a protein, as follows: “Chemical index = (mg of the limiting amino acid per g of the protein under analysis) / (mg of amino acid per g of the reference protein)” (Grácio et al., 2023). For calculation of the chemical index 1 an ideal protein is used while for the calculation of chemical index 2, egg white protein is used.

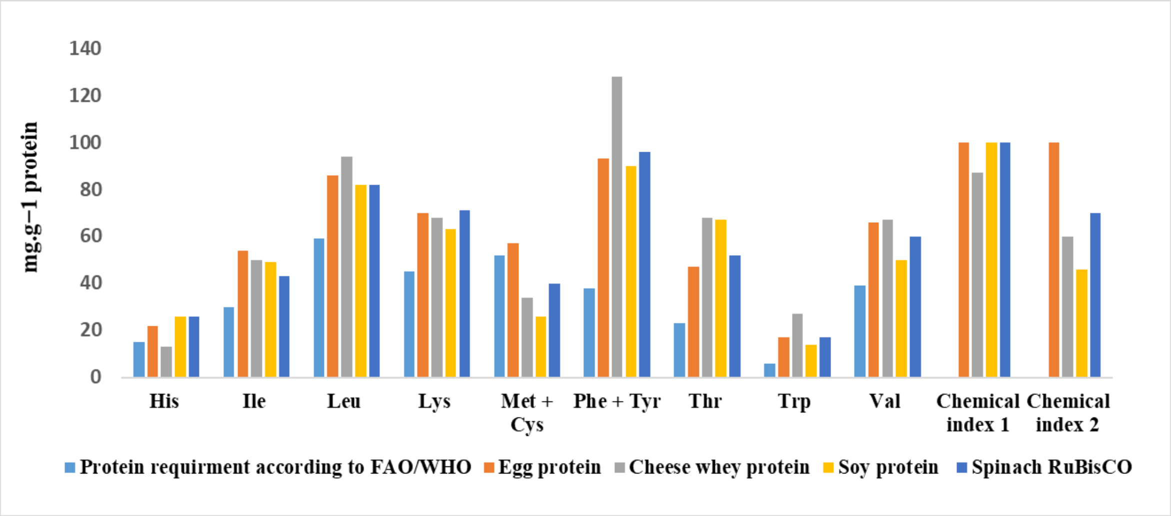


Figure 6. Essential amino acids of RuBisCO as compared to other types of proteins (Pouvreau et al., 2014).

In addition to its enzymatical functions, RuBisCO plays also a crucial role in nitrogen storage within plants, as it represents a substantial portion of leaf proteins. Thereby, RuBisCO is particularly important as a nitrogen storing unit in nutrient-poor environments compared to nitrogen-rich counterparts (Chapin et al., 1990; Millard & Thomson, 1989). Epiphytic plants, which thrive in nitrogen-poor habitats, utilize RuBisCO as a means of nitrogen storage in their leaves, despite having lower RuBisCO content (Maxwell, 2002). In addition to RuBisCO, photosynthetic pigments also serve as a nitrogen reservoir, and their production is directly linked to photosynthesis and RuBisCO activity (Evans, 1989).

Biogas

Biogas , a renewable gas comprising methane, carbon dioxide (CO_2), small amounts of water (H_2O) and hydrogen sulphide (H_2S), is produced through the anaerobic digestion (AD) of organic materials (Martinez-Alonso et al., 2023). With applications in mobility, heat, and power production, biogas serves as a potential substitute for natural gas (Mertins & Wawer, 2022). The use of biomass as a renewable energy source has gained considerable attention for its environmentally friendly characteristics. Researchers have been actively investigating different methods to convert biomass into renewable fuels or chemicals (Wang et al., 2019).

The sustainable management of biogas production through the AD process aims to tap into alternative biomass sources that do not compete with food production (Bedoić et al., 2019). Biogas production is a key aspect of Europe's bio-based economy development, as it not only produces energy, but also allow recycling of plant nutrients. Biomass is a valuable resource expected to meet future energy demands and replace fossil fuels (Kouassi-Kouadio et al., 2022; Szilagyi et al., 2021).

AD reactors are crucial for converting organic materials into biogas, serving as both energy and waste management solutions (Angelidaki & Sanders, 2004). These reactors come in various designs, each with unique advantages and challenges (Batstone et al., 2002). Plug-flow reactors (PFRs) offer improved retention times and reduced energy consumption but require careful management due to their sensitivity to shocks (Batstone et al., 2002). Anaerobic sequencing batch reactors (ASBRs) provide flexibility and versatility, suitable for small-scale operations and complex substrates (Angelidaki & Sanders, 2004).

Sustainable biogas production from green biomass in green biorefinery systems

Fractionation of green leafy biomass in a biorefinery system focuses on using it to produce valuable components and bioenergy, including biogas. The main streams with potential for biogas production are pulp, GJ, and BJ. These streams are rich in organic materials, making them suitable for the biogas production. Pulp is particularly notable for its high fiber content, including complex carbohydrates. Efficient biorefinery systems are being developed to convert biomass into various products, with a specific emphasis on increasing biogas production and the efficient use of green biomass (Clark et al., 2012).

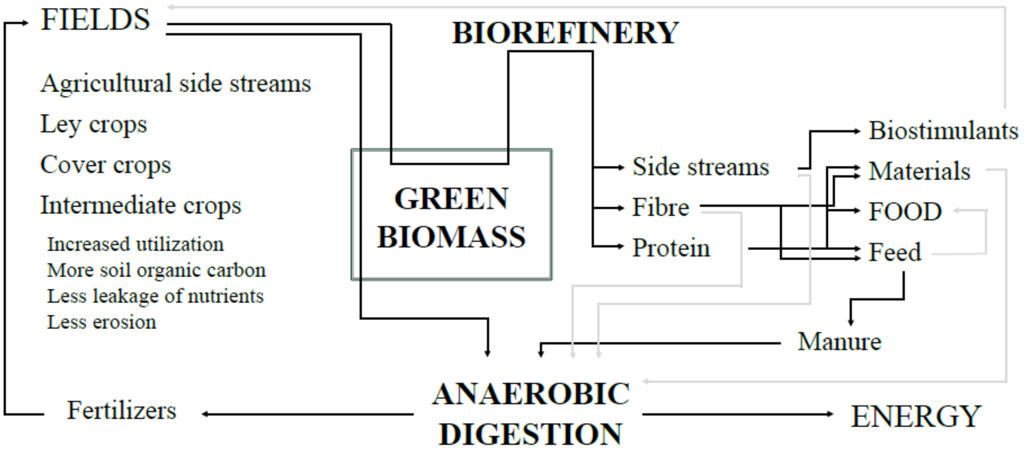


Figure 7. Scheme of the fractionation of green biomass in a biorefining process including the biogas production.

The composition of biomass plays a crucial role for the efficiency and stability of anaerobic digestion (AD). To address nutrient deficiencies in mono-digestion of energy crops, co-digestion with other substrates is commonly used. One successful approach involves co-digesting green biomass with manure, resulting in increased biogas production. However, due to the limited supply of organic farming manure as being a regional issue, it's important to develop manure-free AD processes that maintain optimal nutrient balance and dry matter concentration (Nges & Björnsson, 2012; Weiland, 2010). After protein extraction from green biomass, approximately 80 % dry matter of the organic matter remains in the pulp and BJ. These streams, namely pulp and BJ, offer potential for biogas production. The biomethane potential (BMP) of these fractions was evaluated in both mono-digestion and co-digestion scenarios, across various crops, and as compared with values from fresh biomass inputs. The analysis aimed to determine the feasibility of utilizing these fractions for efficient biogas production, thereby providing valuable insights into the potential of mono-digestion for biogas production (Santamaría-Fernández et al., 2018).

The BJ consists of accessible mono and oligosaccharides (Santamaría-Fernández et al., 2018). Biomethane production from BJ has generated interest for energy production through AD (Njakou Djomo et al., 2020). However, challenges arise due to the acidic nature and high “chemical oxygen demand (COD)” content of BJ. To enhance the biomethane yield, several factors have been investigated, including reactor configurations, mono digestion and substrate co-digestion. Using immobilized microorganisms in the “Up-flow Anaerobic Sludge Blanket (UASB) reactor” was proposed in order to offer process robustness against temperature, pH and substrate concentration fluctuation and to enable the possibility of shorter incubation times

(Martinez et al., 2018). However, the methane yield was lower than expected when compared to the biomethane potential test (Table 4). Thus, the incubation times used may not have allowed for the full degradation of organic load in the BJ. Additionally, the reduction in granule size of the activated sludge suggests process instability. To improve methane yields and stability, it is crucial to optimize the incubation times and reactor conditions (Martinez et al., 2018).

Table 4. Biomethane yields from varied streams from fractionation of green biomass in biorefinery.

Biomass	**Fraction**	**Methane yield**	**Mode**	**Duration (Day)**	**Reference**
Perennial rye grass	BJ (grass whey)	544^{a}	Batch	21	(Ravindran et al., 2022)
	BJ without FOS (whey)	520^{a}		21	
Grass	pulp	353^{a}		35	(Steinbrenner et al., 2021)
Rye 60 % grass + clover 40 %	BJ	409.6^{a}	Continuous	5,5	(Feng et al., 2021)
Clover grass	31 % pulp +69% BJ	238^{a}		20	(Santamaría-Fernández & Lübeck, 2020)
Alfalfa	Fresh crop	361.4^{a}	Batch	55	(Santamaría-Fernández et al., 2018)
	BJ	456.7^{a}			
	pulp	239.9^{a}			
Red clover	Fresh crop	330.6^{a}			
	BJ	428.7^{a}			
	pulp	218.6^{a}			
Clover grass	Fresh crop	343.6^{a}			
	BJ	464.4^{a}			
	pulp	464.4^{a}			
Oilseed radish	Fresh crop	452.2^{a}			
	BJ	475^{a}			
	pulp	374^{a}			
Red clover and clover grass	BJ	307^{a}	Continuous	3	(Martinez et al., 2018)
Grass biomass	Residue grass	0.275^{b}	Batch	42	(Bedoić et al., 2019)

^{a}L kgVS^{-1}, ^{b}Nm^{3}/kgTS

The anaerobic filter (AF) reactor has demonstrated stable biogas production after proper inoculum acclimation. The AF reactor, with a bacterial biofilm in porous media, facilitates high biological activity, organic loading rates, and shorter incubation times compared to conventional anaerobic digesters. Gradual adjustments of organic loading rates during initial testing of the AF reactor resulted in a stable biogas production at an organic loading rates of 3.8 kg chemical oxygen demand m^{-3} d^{-1}, by allowing the inoculum to adapt (Feng et al., 2021). In addition to inoculum adaptation, proper AD processes play a critical role when using BJ as the sole substrate (Martinez et al., 2018; Santamaría-Fernández et al., 2018). Co-digestion with pulp in a 1:1 ratio has been identified as a viable strategy for improving methane yield and stabilizing the process. Furthermore, the choice of feedstock from green biorefineries was found to affect the methane potential of BJ. However, AD of BJ from various green biomass sources showed similar methane yields per kilogram of volatile solids (VS) among the feedstocks, ranging from 429 to 475 L CH4 $kgVS^{-1}$ (Table 4) (Santamaría-Fernández et al., 2018).

The BJ may still contain dietary fibers in the form of “fructooligosaccharides (FOS)”, which can be extracted from it, leading to a BJ stream without FOS. The AD capability of the BJ compared to the BJ without FOS has been evaluated and showed that the FOS recovery process only caused a minimal 5% reduction in the biomethane potential (Table 4). These findings highlight that GLBM has the potential to contribute additional high valuable components while sustaining their capacity for renewable energy production (Ravindran et al., 2022).

The biomethane potential of pulp from fractionation of green biomass is subject to various factors, including fresh and ensiling conditions such as temperature and additives. Ensiling temperature (20 °C or 37 °C) under lab conditions and adding water and/or $CaCO_3$ to the grass during ensiling was found not to affect the biomethane potential of the pulp significantly. The exception was for the control treatment without additives, where ensiling at the higher temperature resulted in a slight increase in biomethane yield (Steinbrenner et al., 2021). In the mono-digestion process, the biochemical methane potential of the residual grass was measured, resulting in a value of 0.275 Nm^3/kg TS (Bedoić et al., 2019).

Fractionation and protein extraction methods

The challenges associated with RuBisCO extraction methods highlight the complexities inherent in achieving high yields (Valente et al., 2021). These challenges extend to the broader endeavor of large-scale extraction and purification of plant proteins, where the determination of optimal conditions becomes paramount (Pérez-Vila et al., 2022). However, this pursuit is compounded by the intricate nature of plant matrices, influenced by a myriad of factors that intricately shape the composition of plant tissues and subsequently impact the extraction process (Hermansen et al., 2017). Ensuring the preservation of functional properties and guarding against denaturation during extraction and purification processes emerge as critical considerations for the successful application of soluble plant proteins in food products (Barbeau & Kinsella, 1988; Jiménez-Munoz et al., 2021). his underscores the urgent necessity of comprehensively understanding the interplay between all plant components and their collective impact on protein nutritional value (Day, 2013). Despite the potential benefits, challenges related to extraction and purification, coupled with economic considerations, have unfortunately led to the underutilization of leaf proteins within the food industry (Di Stefano et al., 2018).

The extraction of RuBisCO and other intracellular leaf proteins typically follows a common procedure encompassing many steps: mechanical fractionation, protein precipitation, and protein concentration (Dotsenko & Lange, 2016; Nynäs et al., 2021; Tcherkez et al., 2013). These processes collectively aim to isolate and purify the desired proteins from the complex plant matrix. The fundamental steps of green leaf fractionation and protein extraction are visually depicted in Figure 8, while Table 5 provides an overview of several extraction methods employed in this context.

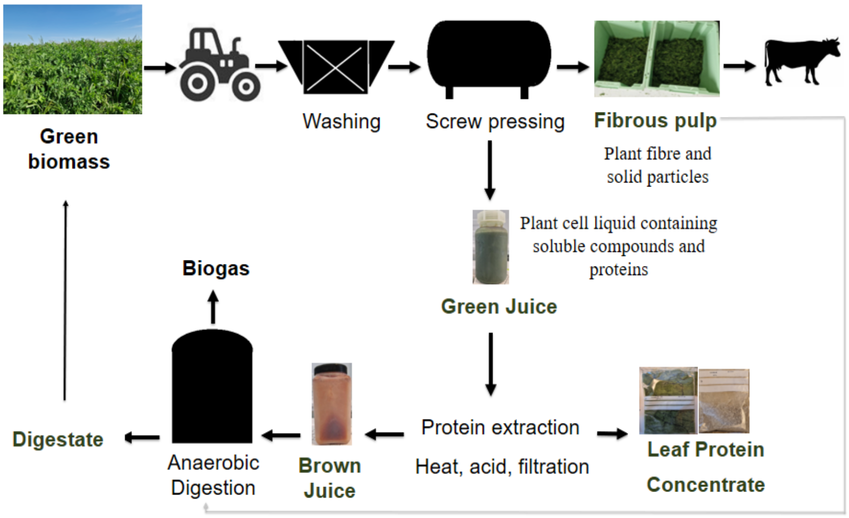


Figure 8. Illustration of basic steps for green leaves fractionation.

Proteins from leafy green plants can be extracted through various methods, including mechanical pressing, heating, or alkaline extraction. Mechanical pressing employs both historical approaches and technologies such as twin-screw press extrusion. Alkaline extraction involves the use of substances like sodium hydroxide and ammonia. Elevated temperature, while increasing juice extraction, can impact protein quality by reducing the amount of native protein in the extracted juice (Kerfai et al., 2011). Concentration methods include heat precipitation, acid precipitation, ultrafiltration, organic solvents, foam fractionation, and spray-drying (Table 5). Thermal precipitation performance differs according to the different plant species and the biomass. However, a range of different biomass sources showed a similar performance as to air-water interfacial behavior (Nynäs et al., 2021). Clear differences were noticed in pH value for protein precipitation between biomass types (Nynäs et al., 2021). Fractional precipitation methods, primarily differential heat precipitation, aim to produce a white protein fraction.

Table 5. Leaf protein extraction methods

Process	**Treatment**	**Method**	**Leaves**	**Source**
Protein extraction	Pretreatment	Rotary extrusion macerators, shredders, disc mills	Lucerne	(Nelson et al., 1983)
		Pulsed electric field		(Kerfai et al., 2011)
		Hydrolysis enzymes		(Mudgett et al., 1978)
	Mechanical fractionation	Hammer mills, screw expellers, sugar cane rolls, ball mills, and rod mills.		(Pirie, 1987)
		Twin and single-screw press		(Knuckles et al., 2002; Nynäs et al., 2021)
	Thermo-mechanical dewatering	Heat and pressure	Spinach	(Kerfai et al., 2011)
	Alkaline extraction	Sodium hydroxide, calcium hydroxide, or ammonia	Switchgrass	(Bals et al., 2007)
	Ultrasonic	macerated cauliflower leaves using ultrasonic	cauliflower	(Bals et al., 2007)
Precipitation/ Concentration	Heat	80–90 °C	-	(Kamm et al., 2009)
	Acidification	Acidify to pH 3.5–4.5	White clover, red clover, lucerne, and perennial ryegrass	(Damborg et al., 2020)
		fermentation by lactic acid producing bacteria	Red clover, clover grass, alfalfa and oilseed radish	(Santamaria-Fernandez et al., 2017)
	Ultrafiltration	Low cut off (1kD)	Ryegrass and alfalfa	(Koschuh et al., 2004)
	Polar solvent	Acetone or ethanol	Alfalfa, white clover, pea vines	(Huang et al., 1971)
	Dry	Spray dry	Alfalfa and pea vines	(Hartman et al., 1967)
Fractionating the green and white protein	Differential heat precipitation	55–60 °C 80–90 °C	Alfalfa	(Edwards et al., 2002; Nynäs et al., 2021)

The utilization of a standard protocol for protein production is favourable in an industrial context but contributes several challenges as related to the different types of green biomass and their biomass structures. Thus, a system needs to be developed that facilitates an easy change of parameters according to requirements by different types of green biomass (Nynäs et al., 2021). The choice of methods to be chosen for fractionation of green biomass depends on factors like efficiency, cost, and the desired protein product.

Conclusion

In addressing continuous challenges such as population increase, climate change, and resource scarcity, the exploration of GLBM as a sustainable resource for protein, energy, and biomass-derived chemicals provides a promising avenue. Aligned with the principles of a circular bioeconomy and the United Nations' Sustainable Development Goals, this approach emphasizes the need for efficient and sustainable solutions on a global scale.

Fractionation facilities of green biomass will play a pivotal role in this circular bioeconomy by converting abundant, cost-effective, and locally available GLBM into valuable products and energy. The multifaceted potential of green leaves is harnessed through applications in protein extraction, energy production, and the synthesis of value-added products. The exploration underscores the importance of maximizing protein yield to meet the rising demand for plant-based proteins, presenting various extraction methods suitable for biorefinery processes.

Biogas production from green biomass, particularly through anaerobic digestion, offers a sustainable solution influenced by factors like biomass composition and the carbon-to-nitrogen ratio of the biomass. Products from protein fractionation, including pulp, GJ, and BJ, have the potential to contribute to feed, biomaterials, fermentation, and biogas production, enhancing environmental sustainability and economic viability.

The valorization of GLBM, including grass, is crucial for local production to reduce environmental impact within the circular bioeconomy. In summary, this exploration highlights the significant potential of GLBM in addressing global challenges related to food, energy, and sustainability. Integrating knowledge from diverse disciplines positions green leaves as a valuable resource, providing insights for future advancements and innovations in sustainable practices and industrial applications.

Collaboration between producers of plant proteins and biogas emerges as a crucial aspect, facilitating the introduction of these products to the market with high resource efficiency and benefit. This synergy between sectors contributes to a more sustainable and impactful approach to addressing global challenges related to food, energy, and environmental resources.

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