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Increasing protein recovery from lucerne by a second pressing step: opportunities and challenges for process and feasibility

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ABSTRACT

Green leafy biomass is a promising source for extraction of protein for food and feed, but low yields in the process affects sustainability and feasibility. Here, opportunities and challenges concerning increased recovery of soluble protein were evaluated by chemical, enzymatic and mechanical treatments combined with re-pressing of pulp (P), whereafter the process was assessed environmentally and economically. The treatments affected the recovery of soluble proteins by impacting the nitrogen flow from P to green juice (GJ) and the recovery of soluble protein from GJ, with the latter most strongly correlating to the recovery of soluble protein. Treatments, such as freezing of P, addition of water, NaOH, SDS, or brown juice, and repeated ball milling, increased the nitrogen extraction from P to GJ significantly (>35% compared to control). However, several treatments caused protein degradation and co-extraction of cell wall components and particles containing insoluble protein, thus negatively affecting the recovery of soluble proteins. Treatments contributing to cell disruption was most beneficial for recovery of soluble protein, with the highest recovery (~14%) obtained by NaOH treatment at ≥0.1 M, corresponding to a five-fold increase as compared to water treatment. Assessment at demo-scale showed that re-pressing of P with 0.1 M NaOH was environmentally and economically more costly per protein unit than pressing original biomass, the same cost was seen for every additional re-pressing, but with reduced protein recovery. Thus, the initial pressing should be followed by maximum one re-press with 0.1 M NaOH, and produced heat should be recovered, if implementing the process industrially.

1. Introduction

The global consumption of protein is predicted to double until 2050 [1]. This surge is driven by the growing world population, placing heightened demands and pressures on the agri-food system, and sustainability issues call for solutions other than animal-based protein sources [2–4]. As a result, the exploration of alternative protein sources to be utilized in food production has increased over the years, including the use of plant-based protein derived from seeds, such as cereals and legumes [5,6], insect protein [7], and cultured meat [8]. Simultaneously, the request for locally produced protein to feed domesticated animals has increased [3]. Agricultural green leafy biomass, especially in the form of perennial ley crops and residual leaves, is a promising local protein source for both food and feed purposes, and overcoming the challenges related to protein extraction in a green biorefinery context is an important step towards sustaining both local and global agriculture [9–13].

The dominating protein in green biomass is ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), which constitutes 30-50% of the soluble leaf protein content [14], making it the most abundant protein globally [15]. This protein is present in all photosynthetic biomass, as it is the cornerstone of carbon fixation in the photosynthetic cycle [14]. RuBisCO offers exceptional nutritional value with a balanced amino acid profile comparable to egg protein [16]. Furthermore,

Abbreviations: BJ, Brown juice, here green juice deproteinized at 80 °C; BM, initial biomass; CO_2 eq., CO_2 equivalents; DMC, Dry matter content; DW, Dry weight; GJ, Green juice; GJ_R, Green juice from re-pressed pulp; $GP_{soluble}$, Soluble protein recovered by precipitation at pH4; GP_{RP}, Green pellet of suspended solids and recovered protein at pH 4; GP_{SS}, Green pellet of suspended solids; GWP, Global warming potential; LCA, Life cycle assessment; P, Pulp; PBS, Phosphate-buffered saline; P_R, pulp from re-pressed pulp; SDS, Sodium dodecyl sulfate.

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RuBisCO exhibits remarkable functional properties, *e.g.*, gelling properties rivalling those of egg white protein under certain conditions [17], and foam stabilizing properties comparable with egg white [18]. However, recent results [12,18] indicate that leaf protein concentrates, which normally contain a mixture of RuBisCO and other biomass proteins, show similar positive nutritional properties as pure RuBisCO isolates. Thus, the need to purify RuBisCO from other green biomass proteins in a biorefinery process is not obvious.

Previous studies have extensively examined the process applied to obtain protein concentrates from green biomass, and the methods commonly starts with screw pressing of biomass to obtain a protein rich green juice (GJ) and a press cake, the fibrous pulp (P) [13,19–21]. From these studies, a major draw-back has been identified – a large proportion (ca 50%) of the protein remains in P. Several different approaches have been suggested to increase protein recovery from the green biomass, *e.g.*, by aiming at more extensive cell and organelle disruption through enzymatic treatments using proteases or carbohydrases [22], mechanical by employing severe physical forces, often through methods like pressing, milling, or homogenization, to rupture cells and release intracellular proteins [23], or chemical treatments [24], by increasing the protein solubility by, *e.g.*, inhibiting polyphenol oxidases [25] or by reducing proteolytic activity impairing the protein recovery [26]. However, until now, and to the best of our knowledge, no concerted action comparing a range of various methods for their suitability to increase the protein recovery has been undertaken.

A significantly increased protein yield in the green biorefinery process is necessary for green biomass to be an economically and environmentally feasible protein source for food and feed [27], preferably in combination with further valorisation of the remaining P and compounds the deproteinized GJ, *i.e.*, brown juice (BJ). A more extensive protein recovery from the biomass might be reached by further treatment of P by mechanical, chemical, and biochemical methods. However, narrowly focusing on improving process performance may lead to negative outcomes for plant operators and the environment, as process innovations might result in increased emissions and costs. *Ex ante*, or preliminary, life cycle assessments are advantageous because they identify hotspots, impact drivers, and limitations of innovations at an early stage and thus guide process development towards more sustainable outcomes [28–30]. Until now, there are only a limited number of studies that has investigated the environmental [21,31–35] and/or economic [36–40] impact of protein extraction from green biomass and to our knowledge no study has evaluated method developments focusing on increased protein recovery from P. The assessments of emerging technologies are commonly limited by a lack of commercial data, and therefore, modelling methods need to be employed to estimate the commercial process data [29]. Thus, process calculation methods using existing models for upscaling chemical processes to extrapolate commercial data from experimental results has been developed [41,42].

The aim of the present study was to evaluate opportunities to improve protein recovery from green biomass, using lucerne (also known as alfalfa) as a readily available model biomass. To obtain an increased protein yield and quality, it is important to understand the challenges, such as, insufficient cell disruption and impaired protein solubility. Therefore, the impact of mechanical, chemical, and biochemical treatments for improving protein recovery were evaluated here. A second aim of the study was to evaluate the impact of these treatments on process sustainability through economic and life cycle assessments.

2. Materials and methods

To investigate opportunities for a sustainably and feasibly increased protein recovery from green biomass, the approach of the study was to target the insufficient protein extraction in the first screw pressing step. The present study was carried out in three parts as illustrated in Fig. 1: (A) lab-scale experiments to investigate effects on protein extraction and recovery from P using a wide array of treatments; (B) evaluation of the most promising treatment, based on results from the lab-scale

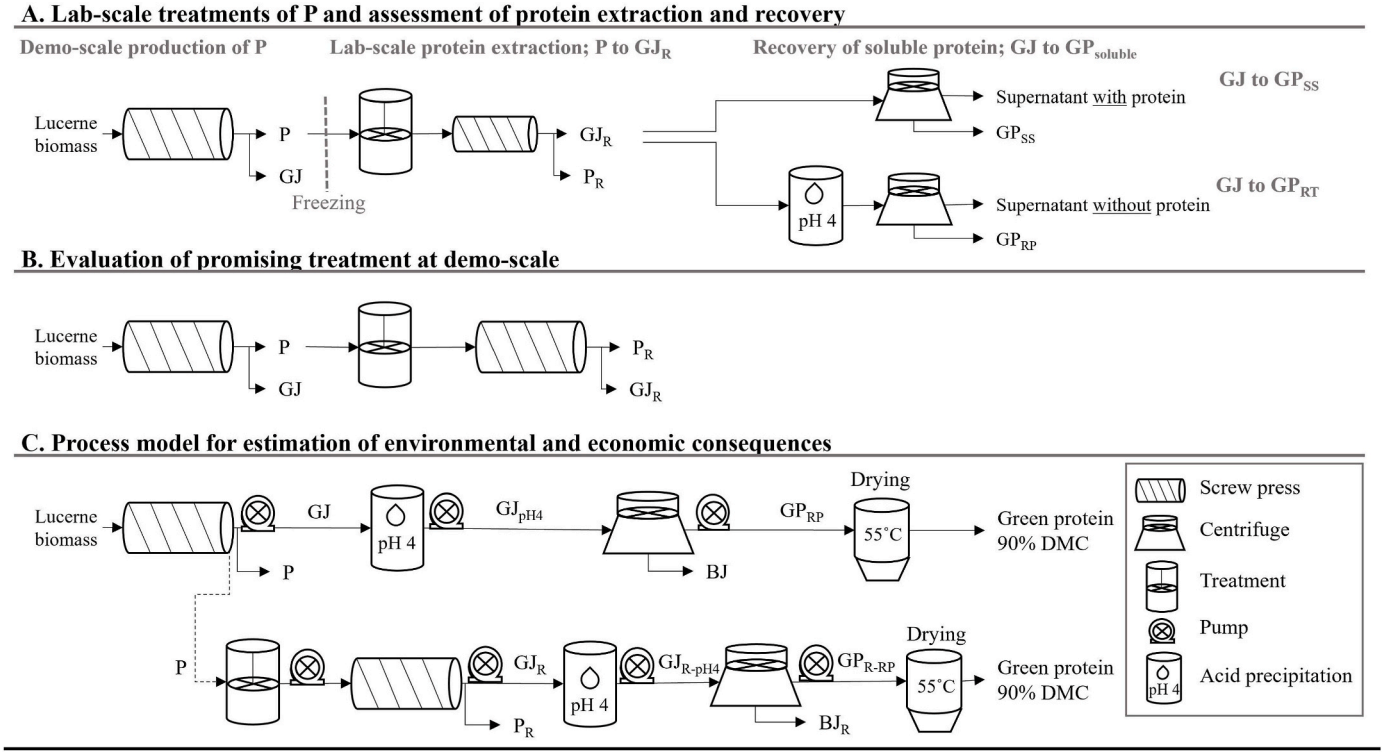

Fig. 1. Overview of the three parts of the study. The treatments included in the lab-scale experiments are described in Table 1. P: Pulp, Green juice: GJ, GJ from re-pressed P: GJ_R, re-pressed P: P_R, green particles: GP_{SS}, Green particles + protein precipitated at pH 4: GP_{RP}, Soluble protein, *i.e.*, the difference between GP_{RP} and GP_{SS}: $GP_{soluble}$, GJ at pH 4: GJ_{pH4}, GJ_R at pH 4: $GJ_{R\text{-}pH4}$, GP_{RP} from GJ_R: $GP_{R\text{-}RP}$, Dry matter content: DMC.

experiments, in a demo-scale facility; and (C) theoretical estimations of the environmental and economic consequences of implementing the treatments at a future commercial-scale biorefinery facility.

2.1. Agricultural biomass

Lucerne (*Medicago Sativa*, var. "Ludelis") was harvested at a research field in Lönnstorp, Sweden (N55.66917, E13.1027) using a Haldrup Harvester (Ilshofen, Germany) at three different occasions; one during 2022 and two during 2023. On November 10, 2022, lucerne corresponding to the third cut (previous cuts in late June and late August 2022), was harvested to be used for producing P for the lab-scale experiments evaluating opportunities for additional protein recovery. This biomass was stored overnight (~9 °C) until processing the following day as described below. The biomass dry matter content (DMC) was 25 ± 3% and the N content was 3.6 ± 0.2%. A sorting test of the biomass revealed that approximately 70% of the biomass was green lush lucerne, the rest was dry harvest residues from the previous cut. The dry harvest residues were not removed for any of the experiments. During the following growth season, lucerne for processing at demo-scale was harvested from different parts of the research field on 12 September, and October 3, 2023, both representing a third cut with previous cuts on 18 June and 10 August. At these occasions, no harvest residues were present. The maturity stage of the biomass was defined using the BBCH scale, a standardized phenological growth stage scale for describing the development of plants (*Biologische Bundesanstalt, Bundessortenamt and CHemical industry*). Biomass was harvested at BBCH stage 36 (mid-stem elongation) and BBCH stage 55 (mid-bud emergence), respectively. The biomass was harvested and processed on the same day. The DMC of the biomass at the respective occasions was 12.8 ± 0.5% and 15.2 ± 0.9%, and the N content was 4.6 ± 0.02% and 2.7 ± 0.01%. The more mature plants in the later harvest had a higher stem-leaf ratio, which gives a lower N content and higher DMC.

To produce comparable starting material in sufficient amounts for the lab-scale experiments, the lucerne biomass was processed at the demo-scale facility at the Swedish University of Agricultural Sciences in Alnarp, Sweden. In demo-scale, the biomass was washed in an industrial salad washer (Adria, Turatti, IT) to remove soil and other contaminants, and then the biomass was pressed in a single screw press (CP-10, Vincent, USA) at approximately 300 kg/h to obtain P and GJ [12]. Samples were taken of the biomass, P, and GJ for compositional analysis and from GJ for analysis of content of suspended solids and protein recovery (Fig. 1). Portions (~700 g) of homogenized P and some lucerne biomass was packed in sealed bags and immediately frozen at −20 °C and stored frozen until further processing at lab-scale. Fresh lucerne biomass and P were collected to be used as controls for the freezing, and these were stored at 4 °C until processing the following day. The amount of water entering the process from washing the biomass was estimated as the difference between the mass of ingoing biomass and the sum of P and GJ.

2.2. Lab-scale experiments

The major experimental part of this study was the lab-scale experiments, as illustrated in Fig. 1A, where a wide range of treatments was applied to P for increased protein extraction and recovery. The general method of the lab-scale experiments is described in section 2.2.1 below, and details of the applied treatments listed in Table 1 are described in section 2.2.2. The lab-scale experiments consisted of two main elements, *i.e.*, extraction of protein from P in a lab-scale screw press, followed by evaluation of the effects on protein recovery from the juice.

2.2.1. General method of the lab-scale experiments

Protein was extracted from P collected in the demo-scale facility (section 2.1) by applying a range of treatments (Fig. 1, Table 1). To evaluate the treatment effects on protein extraction (*i.e.,* the N flow from P to GJ_R), a lab-scale twin screw press (Angelia 5500, Angel Co. Ltd., Busan Korea) was used. For each treatment, the extraction was carried out in triplicates through soaking of 50 or 200 g fresh or thawed, but still cold, P in the chosen solution at concentrations, mass ratios, and times, as specified in Table 1, and described in detail in section 2.2.2. After each treatment, the triplicate samples were pressed in sequence. Before pressing the first replicate, the twin screw press was primed with 100 or 200 g of the corresponding material to ensure steady-state operation and prevent contamination from residual material. The pressing replicate

Table 1
Treatments for protein extraction from pulp (P) and biomass (BM). BJ: brown juice; SDS: sodium dodecyl sulfate; PBS: phosphate buffer.

A. Treatment to evaluate re-absorption effects

	Solution	Time and temperature	P:solution (w:w)	Material status	Material
Washing	Water	21 °C: 15 min	1:10	Fresh, Frozen	P
Washing + pressing	Water	21 °C: 15 min	Washing 1:10, pressing 1:1	Frozen	P

B. Treatments to evaluate increased solubility and/or cell disruption

	Solution	Time and temperature	P:solution (w:w)	Material status	Material
Control treatment	Water	21 °C: 15 min	1:1	Frozen	P
Soaking time & temperature	Water	4 °C: 1h, 5h	1:1	Frozen	P
		21 °C: 15 min, 5h	1:1	Frozen	P
		ice, 15 min	1:1	Frozen	P
Freezing[a]		21 °C: 15 min	1:0	Fresh, Frozen	BM
Freezing + dilution[a]	Water	21 °C: 15 min	1:0, 1:1, 1:2	Fresh, Frozen	P
Increased solubility/cell disruption	NaOH (0.05, 0.1, 0.2, 0.5, 1.0, 2.0 M)	21 °C: 15 min	1:1	Frozen	P
	SDS (0.5, 2.0%)	21 °C: 15 min	1:1	Frozen	P
	Sodium bisulfite (0.5%)	21 °C: 15 min	1:1	Frozen	P
	BJ	21 °C: 15 min	1:1	Frozen	P
	PBS[b] (0.05 M)	21 °C: 15 min	1:1	Frozen	P
Sequential pressing	Water	21 °C: 15 min	1:2	Frozen	P

C. Enzymatic treatments with Viscozyme® L at concentrations of 0%, 1%, and 2% in 0.05 M PBS[b] for improved cell disruption

	Mixing	Time and temperature	P:solution (w:w)	Material status	Material
Viscozyme®	None	25 °C: 1 h	1:1	Frozen	P
	Ball mill	25 °C: 1 h	1:6	Frozen	P
	Ball mill	25 °C: 3 h	1:6	Frozen	P
	Ball mill	25 °C: 3 h	1:6	Frozen	Ball milled P

[a] Priming portions of 100 g was used, instead portions of 200 g used for all other treatments.
[b] 0.05 M Na_2HPO_4 + 0.05 M NaH_2PO_4, pH 6.55.

was assumed complete 1 min after the last material had been fed to the screw press, and the following replicate was pressed. Samples were taken of re-pressed P (P_R) and resulting GJ (GJ_R) for DMC and N analyses and of GJ_R for analysis of content of suspended solids and acid precipitated protein (Fig. 1). Thereafter, the mass balances (section 2.3.1) and recovery of soluble protein were calculated.

The content of suspended solids and protein recovered by acid precipitation in GJ (in this section GJ refers to both GJ and GJ_R), were evaluated for all triplicates. For this, two portions of 35 mL of GJ were transferred to 50 mL conical tubes. The pH of one of the two portions was adjusted to 4.0 by addition of 1 M HCl approximately 30-45 min before centrifugation (3234 RCF, 30 min, room temperature), while the other one was centrifuged without any pH adjustments. Thereafter, 10 mL of each supernatant was collected for DMC determination, and the rest of the supernatant was discarded. The pellets were weighed before and after lyophilisation, and the N content of the pellets was analysed.

The content of suspended solids in GJ was determined as the dry weight of GP_{SS}, *i.e.*, the pellet of the samples in which the pH was not adjusted. The dry weight of the pellet from GJ adjusted to pH 4 (GP_{RP}), corresponds to both the content of acid precipitated protein and suspended solids. The recovery of soluble protein was determined as the flow of N from GJ to the acid precipitated soluble protein ($GP_{soluble}$), the latter obtained by comparing the N flows from GJ to GP_{SS} and GP_{RP} (see section 2.5 and Supplementary A for descriptions of the calculations).

2.2.2. Lab-scale experiments – detailed descriptions of P treatments

The effect of freezing of biomass and P was assessed, as storage of material in freezers was a prerequisite for the substantial experimental work in this study [13]. To assess the effect of freezing and subsequent thawing of samples on protein extraction and protein recovery, both fresh and frozen lucerne biomass and P from the demo-scale process were evaluated following the method in section 2.2.1. The lucerne biomass to be used for these comparisons was not soaked in any solution, while the P samples were soaked in water at mass ratios of 1:0, 1:1, or 1:2, to also allow assessment of how dilution affects the results. Throughout this first set of experiments, the screw press was primed with 100 g of sample, however, it became clear that 100 g of priming material was insufficient, hence for the following experiments 200 g were used. Thus, statistical analyses were done separately based on the amount of priming material.

Several treatments of P aiming at increasing the recovery of soluble protein was investigated. For these treatments, thawed, but still cold, P was mixed with various solutions before pressing in the juicer as described previously (for details, see Table 1). Sodium hydroxide (NaOH) and sodium dodecyl sulfate (SDS) solutions at different concentrations, as well as phosphate buffer (PBS; 0.05 M Na_2HPO_4 + 0.05 M NaH_2PO_4, pH 6.5), were used to either increase the protein solubility by increasing the pH, or to hydrolyze the cell walls to increase the cell wall disruption. Sodium bisulfite was used to prevent oxidation of the protein, retard protein-phytochemical interactions, and increase the protein solubility. An additional treatment was BJ, *i.e.*, GJ from the demo-scale facility deproteinized by heating GJ at 80 °C, followed by centrifugation [43]. The BJ had a DMC of 3.8% and N content of 4.0%. The N flow calculations for the process were adjusted for the additional N added with the BJ. The effect of different soaking times and temperatures were further evaluated using milliQ water as solvent.

To evaluate the effect of repeated mechanical treatment for cell disruption, P was pressed 10 sequential times in the lab-scale twin screw press following the description in section 2.2.1. The starting material of the sequential pressing consisted of portions of 600-700 g of P, which were soaked in water at a mass ratio of 1:2, soaked for 15 min on ice and fed through the screw press. To avoid excessive heating of the screws and the samples, a sealed plastic bag with crushed ice was placed on top of the screw barrel. The resulting P_{R1} and GJ_{R1} were weighed and sampled as described in section 2.2.1 above, and the P_{R1} was mixed with water at a ratio of 1:2, again soaked for 15 min and fed through the screw press. The procedure was repeated 10 times with 3 replicates. The recovery of soluble protein in GJ was not assessed in this set of experiments.

To evaluate the impact on protein extraction by GJ re-absorption of P in the demo-scale screw press, the material (both fresh and frozen P) was washed with water. The washing procedure was done in triplicates as follows: 20 g of P in a glass beaker was mixed with 200 g of MilliQ water for 20 min on a shaker at room temperature. The water was poured off and replaced with new water 5 times. After the last wash, the water was poured off, and the beaker was left in a down-wards tilted position for 5 min to allow complete drainage. The washed P was lyophilized and the N content was analysed. Another set of frozen P was washed according to the same procedure, and after washing, the protein extraction from P was evaluated as described in section 2.2.1 above, and the recovery of soluble protein was compared to frozen P not washed before processing.

Direct enzymatic treatment of P was performed utilizing the commercial enzyme mixture Viscozyme® L (Sigma-Aldrich, Sweden) and different treatment modes (Table 1C). Treatments with no mixing, and mixing with balls in a ball mill (Lortone Rotary Tumbler Model 33B, Detector Supply Company, USA) were compared. An additional treatment involved overnight ball milling at 4 °C prior to enzymatic treatment in a ball mill. The enzymatic treatments were conducted with varying enzyme concentrations (0%, 1%, 2%) and diluted in 0.05 M PBS (0.05 M Na_2HPO_4 + 0.05 M NaH_2PO_4, pH 6.5), adopting mass ratios of either 1:1 or 1:6 for different treatments at 25 °C. Different conditions were applied during the enzymatic treatments, including two distinct durations (1 or 3 h). The treated P was pressed as described in section 2.2.1, except only 50 g of treated P were used per replicate for ball mill treatments, and the screw press was primed with 100 g of P, treated with PBS but no enzymes. The following calculations were adjusted for N added in the form of enzymes.

2.3. Demo-scale processing – Re-pressing of P

Lucerne biomass was processed into P and GJ as described in section 2.1 at two separate occasions (12 September and October 3, 2023). The resulting P was collected in bins and soaked in 0.1 M NaOH (1:1 mass ratio) for 1 h and fed through the demo-scale screw press again. The masses of the resulting P_R and GJ_R were recorded and samples for compositional analysis were collected. The content of suspended solids and recovery of soluble protein in the GJ_R was not assessed. No control treatment with water was included in the experiment.

2.4. Compositional analysis

For DMC determination, samples of 10 g (P, GJ, and supernatants) or 50 g (original biomass) were dried overnight in a forced air oven at 60 °C. All pellets (GP_{SS} and GP_{RP}) were lyophilized to enable drying of the intact pellet. Lyophilized samples of biomass, GJ, P and pellets, were homogenized and the N content on dry matter basis was analysed in technical duplicates using the Dumas method (AOAC 990.03 Animal feed, Flash 2000 NC Analyzer, Thermo Scientific, USA). The content of N was assumed to be directly proportional to the content of protein, and a conversion factor of 5.8 was used to calculate the crude protein content when applicable as suggested by Ref. [44].

2.5. Calculations and data analysis

Equations used in the present study are all given in Supplementary A. The wet mass flows for each process stage were calculated as a ratio of mass fraction out to mass fraction in, as given by equation (A1). Dry matter and N content for each fraction (P, P_R, GJ, GJ_R, GP_{SS}, GP_{RP}) was calculated according to equations A2 and A3, respectively. Equation (A4) was used to calculate N flows, also referred to as N yield, by relating the amount of N in the product(s) of a process step to the amount entering. Equations A5 and A6 were used to calculate the N flow for GJ

to the pellet containing suspended solids, GP_{SS}, and from GJ to the pellet containing both suspended solids and protein recovered at pH 4, GP_{RP}. The recovery of soluble protein from GJ (GJ to $GP_{soluble}$), *i.e.*, the difference between the values from A6 and A5, was given by equation (A7). Recovery of soluble protein from P was further calculated according to A8.

All statistical analyses were performed in R studio, version 4.4.2 [45], using packages stats, multcomp, multcompview, and emmeans, while the packages ggplot2, ggpattern, and, ggpubr, were used for graphical presentations of the data.

2.6. Economic & environmental consequences of industrial implementation

To determine the most suitable treatment to improve recovery of soluble protein from P, the potential environmental and economic impact of applying a selection of the treatments at commercial scale was estimated. For that purpose, the experimental protocol presented in Fig. 1C and results from the lab-scale experiments were scaled up to a hypothetical commercial facility, assuming a 10 tons/h fresh biomass processing capacity, equivalent to between 1.3 and 3.6 tons dry matter/h, varying by harvest. Previous studies have evaluated potential commercial biorefineries sized between 0.2 and 5 tons or even larger facilities reporting processing capacities per year at 20,000 to 120,000 tons dry matter, with a functional hourly capacity of between 6.6 and 40 tons dry matter/h [40,46]. This data was then used as the basis for a life cycle assessment (LCA) and a cost assessment. In addition to the selected P treatments, the initial protein extraction process from biomass was modelled to estimate P flows into the re-pressing step of P. For comparison, the above method was applied also to the demo-scale processing. See Supplementary B for a summary of process steps and inputs included in each stage.

2.6.1. Up-scaling

The methodology suggested by Piccinno et al. [42] was applied here for up-scaling, which includes a simplified up-scaling to generate requisite life cycle inventory data from laboratory protocols for conducting *ex-ante* LCAs. For that purpose, each step of the process was scaled up individually, then linked to the overall system, before conducting the LCA and the cost assessment. See Supplementary B for a complete list of the process steps and corresponding inputs and outputs for the system. The treatments included in the scale-up are listed in Table 2. Cumulative results were also calculated using the data from the initial pressing of fresh lucerne and the secondary pressing of P for selected lab and demo-scale treatments. These cumulative cases were fresh lucerne combined with frozen P that underwent lab-scale (a) re-pressing 1:1 with water, (b) re-pressing with 0.1 M NaOH or with fresh P that underwent re-pressing with 0.1 M NaOH during the demo-scale runs of lucerne (c) from September 2023 and (d) October 2023.

Up-scaling of stirring, pumping, centrifuging and drying processes were performed using unaltered models suggested by Piccinno et al. [42]. For the pressing process, not modelled by Piccinno et al. [42], simple methodologies were developed. Mass flows as described in section 2.5 were used to determine relative co-product yields between process steps and overall system yields. Assumptions and constants used in these calculations are found in Supplementary C. The up-scaling method for process inputs is described in Supplementary D. Energy inputs for equipment were calculated using equation (D1) (pumping), D2 (stirring), D3 (drying). Acid input was calculated using equation (D5).

Table 2
Treatments of fresh lucerne biomass and pulp (P) included in the up-scaled LCA and cost assessments.

Treatments	Scale	Stage	Date
Fresh lucerne	Lab	Initial	2022-11-10
Frozen P 1:1 mixing with water	Lab	Re-pressing	2022-11-10
Frozen P 1:1 with 0.1 M NaOH	Lab	Re-pressing	2022-11-10
Sequential pressing of frozen P	Lab	Re-pressing	2022-11-10
Fresh lucerne	Demo	Initial	2022-11-10
Fresh lucerne	Demo	Initial	2023-09-12
Fresh P 1:1 with 0.1 M NaOH	Demo	Re-pressing	2023-09-12
Fresh lucerne	Demo	Initial	2023-10-03
Fresh P 1:1 with 0.1 M NaOH	Demo	Re-pressing	2023-10-03

2.6.2. Methodology

An attributional gate-to-gate LCA, *i.e.*, from the arrival of input lucerne to the production of dried green protein, corresponding to GP_{RP}, was performed following the LCA requirements of ISO (2006). The goal and scope of each assessment was limited to the extraction process and the corresponding protein yield. The functional unit of the evaluated system was 1 kg of crude protein in a protein powder with a 90% DMC. Full allocation of impacts was assigned to the protein product. The ReCiPe 2016 v1.03 (H) midpoint method was used to calculate the life cycle impact using activity data from the ecoinvent 3.10 database. See Supplementary E for the full list of impact categories and activity entries used.

The economic assessment was limited to estimating the cost of utilities and chemical inputs, *i.e.*, excluding equipment, overhead, packaging and labour. All economic data used in the assessment is presented in Supplementary F. The values for total utilities cost and unit utility cost, or cost per kg protein are given by Equations F1 and F2.

3. Results and discussion

3.1. Lab-scale experiments

3.1.1. Effects of treatments on recovery of soluble protein from P

The measurement *N flow from P to* $GP_{soluble}$ (eq (A8), Supplementary A) used in the present study, expresses how much of the soluble protein extracted from P during the screw pressing was further recovered by acid precipitation, and the results are presented in Figs. 2 and 3. The most obvious increase, as compared to the general control treatment of soaking P in water for 0.25 h at room temperature (2.4 ± 0.3%), was found when NaOH was used (14-15%). The recovery of soluble protein was found to increase with the NaOH concentration until 0.1 M NaOH, while with higher concentration no additional increase was seen. Other treatments that resulted in an increased recovery as compared to the control treatment were i) a prolonged soaking time, *i.e.*, 1 h instead of 15 min at cool temperature lead to recovery of 5 ± 1% instead of 3 ± 0.5% and ii) a treatment of soaking with 0.005% PBS for which 5 ± 1% of the soluble protein was recovered. Freezing of P did not have a significant effect on the recovery of soluble protein (Fig. 3), while a clear negative effect on the recovery was found when the original lucerne biomass was frozen and thereafter processed (15% recovery for frozen and 22% for fresh biomass).

For the enzymatic treatments, while the combined treatment (2% enzyme, 1:6 soaking ratio, and 3 h ball milling) showed higher soluble protein recovery than the control, similar increases were observed in non-enzymatic treatments, with no statistically significant differences. Therefore, the results do not demonstrate a distinct effect of enzyme addition under the experimental conditions (Fig. 2D, Figure SG1).

Recovery of soluble protein from P as reported here, is the result both of a variation in protein extraction during the screw pressing (*i.e.*, the N flow from P to GJ_R), and a variation in recovery of soluble protein from GJ during acid precipitation (*i.e.*, the N flow from GJ_R to $GP_{soluble}$) among the treatments. A Spearman rank correlation analysis of the data presented in Table 3 indicated a clearly significant effect for both N flow from P to GJ_R ($P < 0.05$) and from GJ_R to $GP_{soluble}$ ($P < 0.005$), with the recovery of soluble protein from P in the full process (as discussed below).

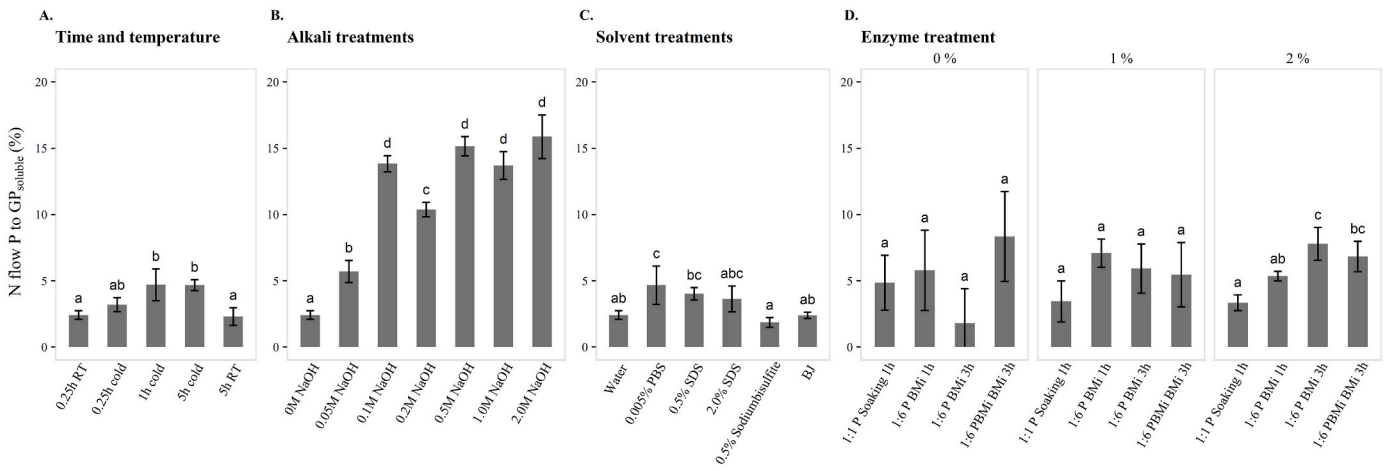

Fig. 2. N flow (%) from pulp (P) to soluble protein ($GP_{soluble}$) after P treatments. The treatments "0.25h RT", "0M NaOH" and "Water" in subfigures A, B, and C, are the same control data point. The subheadings in subfigure D indicate the enzyme (Viscozyme® L) concentration used, and the ratios indicate the mass ratio of substrate (P or ball milled P (P BMi)) and solution. Same letters above the bars indicate values not significantly different ($P < 0.05$, $n = 3$) within each subplot. PBS: Phosphate buffer, SDS: Sodium dodecyl sulfate, BJ: Brown juice, RT: Room temperature.

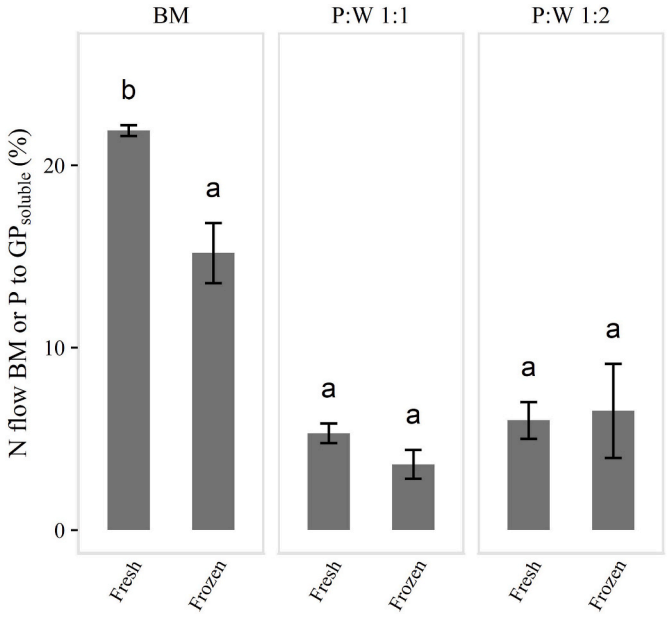

Fig. 3. N flow (%) from fresh and frozen lucerne biomass (BM), or fresh and frozen pulp (P) mixed with water at ratios of 1:1 or 1:2 (w/w), to soluble protein ($GP_{soluble}$). Same letters above the bars indicate values not significantly different ($P < 0.05$, $n = 3$) within each subplot.

3.1.2. Treatment effects on protein extraction (P to GJ_R)

An increased protein extraction in the screw pressing stage, here measured as *N flow from P to GJ_R*, is the first step to secure a high recovery of soluble protein [27]. Several of the treatments significantly increased this flow as can be seen in Table 3 (and partly illustrated in Figure SG2), among the most prominent was the addition of water, which resulted in a 140% increase for frozen P. Freezing P in combination with addition of water increased the N flow with 30% as compared to using fresh P, while using 2 M NaOH, 0.5% SDS, or BJ, increased the flow with 37%, 26%, and 51%, respectively, as compared to using only water. Ball milling P mixed with water, especially with two consecutive treatments, provided severe cell disruption and a 140% increase in N flow to GJ_R, as compared to soaking of P in water. Addition of water, or another liquid, prior to pressing was found essential for the protein extraction, as the added liquid dissolves water soluble compounds in P and flushes out the dissolved compounds, while it also acts to lubricate the screw press, which prevents heating due to friction.

As already stated, freezing of P notably affected the N flow to GJ_R, indicating a more extensive cellular disruption, higher solubility of protein in the frozen material, or a higher content of N containing particles in the resulting GJ. Differently to previous studies on, *e.g.*, sugar beet, pumpkin and amaranth leaves, reporting increased protein yield in GJ after freezing the biomass [47,48], the present study showed no significant effect of freezing on the N flow from the original biomass to GJ. The protein extraction in the screw pressing is greatly affected by the composition of the biomass, *e.g.*, by species, variety, maturity and weather conditions [13,49], as well as storage of the biomass [50]. As the current lucerne biomass was of a late harvest (mid-November) and with some dry harvest residues from the previous cut present, this likely affected the results.

Previous studies have shown that freezing of green biomass affects the cellular integrity, which causes release of intercellular liquid [51], and this might be the explanation to the increased N flow by freezing. This is also corroborated by the results from wash test presented in Table 4, with which almost a quarter of the N in frozen P was lost by washing, while no significant change as compared to the original material was seen for the fresh P. From the wash test, it is also clear that reabsorption of GJ by P is not responsible for low yields in the process. A clear visual difference in how the frozen material behaved in the screw press could be seen, as the frozen samples appeared to be pressed more easily, however there was no statistically significant differences in the flow of wet material (results not shown). For the present study, with its extensive experimental design, it was necessary to freeze P to ensure homogenous starting material for all treatments. However, as the present study also shows a significant effect of the freezing itself, results might have been different if fresh biomass had been used.

Treatment with 2 M NaOH resulted in a 37% increased protein extraction from P as compared to water (Table 3A). NaOH at high concentrations are known to hydrolyze cell wall components, such as cellulose and lignocellulose, thus aiding cell disruption [52], which might be the reason for the increase in N flow from P to GJ_R with this treatment. However, a high content of cellulosic and lignocellulosic compounds may dilute the total GJ dry matter, which is suggested as a possible explanation for the significantly lower N content of 3.3% observed in the GJ_R obtained from P treated with 2 M NaOH prior to pressing as compared to values of 4.6% with only 0.1 M.

Enzymatic cell wall degradation using Viscozyme® did not contribute to an increased protein extraction from P to GJ_R (Table 3B,

Table 3
N content (% of dry weight (DW)) in fractions resulting from re-pressing pulp (P) treated mechanically or chemically (A), or enzymatically (B), and subsequent separation of protein and suspended solids, and N flows (%) in these process steps. P was soaked in the solutions in a 1:1 (w/w) ratio for 15 min at room temperature (~21 °C) if nothing else is stated. BM: original biomass, GJ: Green juice, GP_{RP}: Protein recovered by acidification of GJ at pH 4, GP_{SS}: Suspended solids in GJ, PBS: Phosphate buffer, nd: Not determined, GJ to $GP_{soluble}$: Difference between GJ to GP_{RP} and GJ to GP_{SS}.

A.		N (% of DW)				N flow (%)		
Treatment		GJ_R	P_R	GP_{RP}	GP_{SS}	P to GJ_R	GJ_R to GP_{RP}	GJ_R to $GP_{soluble}$
1) Washing of P before pressing								
6 x washing	Frozen	5.0 ± 0.1	2.4 ± 0.1	5.2 ± 0.2	4.9 ± 0.2	19.8 ± 0.9	95.3 ± 2.9	11.6 ± 9.4
2) Freezing and dilution								
BM:water 1:0	Fresh	5.3 ± 0.2a	2.1 ± 0.1a	6.7 ± 0.4a	5.2 ± 0.4a	45±2a	83±3a	48±2b
	Frozen	5.84 ± 0.09b	3.9 ± 0.4b	7.8 ± 0.4b	6.2 ± 0.1b	45±2a	75±2a	34±2a
P:Water 1:0	Fresh	4.32 ± 0.08a	2.4 ± 0.1a	nd	4.68 ± 0.08a	18±1a	nd	Nd
	Frozen	5.17 ± 0.09b	3.66 ± 0.04b	nd	5.61 ± 0.03b	19±1a	nd	nd
P:Water 1:1	Fresh	4.19 ± 0.05a	2.13 ± 0.01a	5.6 ± 0.1a	5.01 ± 0.04a	35±2a	66±2b	15±2b
	Frozen	5.9 ± 0.3b	3.3 ± 0.2b	6.08 ± 0.06a	6.05 ± 0.02b	46±3b	45±3a	8±1a
P:Water 1:2	Fresh	4.24 ± 0.01a	1.95 ± 0.06a	5.6 ± 0.3a	4.9 ± 0.1a	37±6a	70 ± 10b	17±5a
	Frozen	5.4 ± 0.2b	2.9 ± 0.3b	6.6 ± 0.1b	6.12 ± 0.06b	49±3b	48±5a	13±6a
3) Soaking time and soaking temperature in water								
15 min	21 °C	4.25 ± 0.02a	2.57 ± 0.03bc	4.94 ± 0.05a	4.68 ± 0.04a	35±2a	59±1b	7±1a
15 min	Ice	4.34 ± 0.04b	2.43 ± 0.04a	5.09 ± 0.09a	4.7 ± 0.1a	35±1a	61±2b	9±1 ab
1 h	4 °C	4.48 ± 0.03c	2.48 ± 0.05 ab	5.8 ± 0.2c	5.25 ± 0.05bc	37±1a	61±1b	13±3b
5 h	21 °C	4.53 ± 0.03c	2.68 ± 0.09c	5.3 ± 0.2 ab	5.12 ± 0.07b	38±2a	53±2a	6±2a
5 h	4 °C	4.55 ± 0.02c	2.56 ± 0.01abc	5.8 ± 0.2bc	5.33 ± 0.09c	38±1a	61.5 ± 0.5b	12.2 ± 0.7b
4) Alkali treatment with NaOH								
0 M		4.25 ± 0.02b	2.58 ± 0.03a	4.94 ± 0.05a	4.68 ± 0.04b	35±2a	58.9 ± 0.8a	7±1a
0.05 M		4.34 ± 0.04bc	2.58 ± 0.02a	6.4 ± 0.1c	6.0 ± 0.1cd	35.9 ± 0.7 ab	69±2b	16±2b
0.1 M		4.63 ± 0.08d	2.49 ± 0.07a	7.73 ± 0.09f	6.1 ± 0.1d	38±2 ab	79±2d	36.6 ± 0.8de
0.2 M		4.45 ± 0.02cd	2.60 ± 0.02a	6.8 ± 0.1e	5.77 ± 0.08c	37.2 ± 0.6 ab	74±3c	28±1c
0.5 M		4.26 ± 0. 09bc	2.5 ± 0.1a	6.7 ± 0.1de	4.8 ± 0.1b	37.9 ± 0.3 ab	80±2d	40±2e
1.0 M		4.23 ± 0.02b	3.1 ± 0.1b	6.38 ± 0.05cd	4.8 ± 0.1b	39.5 ± 0.4b	77±1cd	35±2d
2.0 M		3.3 ± 0.1a	2.94 ± 0.02b	6.0 ± 0.2b	4.22 ± 0.03a	48±3c	62.3 ± 0.8a	33±2d
5) Protein solubility enhancing treatments								
Water		4.25 ± 0.02b	2.58 ± 0.03a	4.94 ± 0.05b	4.68 ± 0.04cd	35±2 ab	58.9 ± 0.8c	7±1abc
PBS	0.005%	4.23 ± 0.02b	2.35 ± 0.08a	5.3 ± 0.1c	4.81 ± 0.03d	38±3b	61±5c	12±4c
SDS	0.5%	5.4 ± 0.1d	3.02 ± 0.0.8b	5.63 ± 0.08d	5.15 ± 0.05e	44±2c	50±2bc	9.0 ± 0.7abc
	2.0%	4.52 ± 0.06c	3.4 ± 0.1c	4.53 ± 0.05a	3.88 ± 0.08a	31±2a	40 ± 10 ab	12±4bc
Sodiumbisulfite	0.5%	3.15 ± 0.04a	2.45 ± 0.04a	4.78 ± 0.02b	4.55 ± 0.04c	37±3b	51±1bc	5±1a
Brown juice		5.43 ± 0.06d	2.43 ± 0.09a	4.52 ± 0.06a	4.34 ± 0.09b	52.7 ± 0.6d	36±2a	5.3 ± 0.5 ab

B.				N (% of DW)				N flow (%)		
Mixing	Time	Substrate	Substrate:Solvent	GJ_R	P_R	GP_{RP}	GP_{SS}	P to GJ_R	GJ_R to GP_{RT}	GJ_R to $GP_{soluble}$
0% Viscozyme®rowhead										
Soaking	1h	P	1:1	4.04 ± 0.06a	2.7 ± 0.1c	5.0 ± 0.2a	4.71 ± 0.07 ab	27±2a	49±3 ab	18±8a
Ball mill	1h	P	1:6	4.03 ± 0.15a	2.1 ± 0.3b	5.27 ± 0.03a	4.85 ± 0.09 ab	45±2b	58±2b	13±7a
Ball mill	3h	P	1:6	4.37 ± 0.02a	1.83 ± 0.07 ab	5.1 ± 0.1a	5.18 ± 0.09b	54±2c	52±2 ab	13±5a
Ball mill	3h	$P_{Ball\ mill}$	1:6	4.13 ± 0.25a	1.5 ± 0.2a	4.7 ± 0.6a	4.3 ± 0.5a	65±2d	43±6a	13±5a
1% Viscozyme®rowhead										
Soaking	1h	P	1:1	4.1 ± 0.1b	2.56 ± 0.04c	5.2 ± 0.1b	4.7 ± 0.1b	28.9 ± 0.9a	49±3b	12±6a
Ball mill	1h	P	1:6	3.83 ± 0.09a	2.1 ± 0.1b	5.22 ± 0.09b	4.79 ± 0.06b	41±1b	62±1c	17±2a
Ball mill	3h	P	1:6	4.21 ± 0.03b	2.0 ± 0.2b	5.40 ± 0.07b	5.1 ± 0.2b	58.1 ± 0.7c	51 ± 5.b	10±3a
Ball mill	3h	$P_{Ball\ mill}$	1:6	3.7 ± 0.1a	1.5 ± 0.2a	4.2 ± 0.3a	3.9 ± 0.3a	64±4d	39±4a	9±4a
2% Viscozyme®rowhead										
Soaking	1h	P	1:1	4.15 ± 0.02c	2.60 ± 0.02b	5.13 ± 0.09b	4.55 ± 0.05b	29±2a	49±4b	12±3a
Ball mill	1h	P	1:6	3.80 ± 0.05b	2.1 ± 0.1 ab	5.13 ± 0.04b	4.73 ± 0.07bc	47±2b	56±3b	11.4 ± 0.4a
Ball mill	3h	P	1:6	4.19 ± 0.01c	1.9 ± 0.2a	5.39 ± 0.09b	5.01 ± 0.07c	60±4c	56±3b	13±2a
Ball mill	3h	$P_{Ball\ mill}$	1:6	3.5 ± 0.1a	1.8 ± 0.3a	4.3 ± 0.3a	3.6 ± 0.2a	64±5c	40±1a	10±2a

Same letters indicate values not significantly different ($P < 0.05$, $n = 3$) within each column of each data subset, except for A.1 which was not comparable to the other treatments, and A.2, where the statistical analyses are comparing the fresh and frozen material for each treatment.

Table 4
N content (% of dry weight) before and after washing fresh or frozen lucerne pulp.

Material status		N (% of DW)
Fresh	original	2.9 ± 0.1b
Fresh	washed	3.0 ± 0.1b
Frozen	washed	2.2537 ± 0.0005a

Same letters indicate values not significantly different ($P < 0.05$, $n = 3$).

Table SG1). Treatments without enzyme addition achieved comparable nitrogen recovery, indicating that enzyme application did not provide a measurable benefit. The observed increase in nitrogen flow was instead associated with ball mill mixing and additional soaking, which caused visible biomass disruption and increased surface area, thereby enhancing mass transfer rather than enzymatic action under the experimental conditions. Previous studies have shown that an enzyme-assisted extraction, combined with physical pretreatment techniques that enhance cell wall disruption, is leading to improved protein

extraction efficiency [53]. In a study by Acar et al. [54], the cellulose degrading enzyme Celluclast caused a 4-fold increase in protein yield from P as compared to water (9-14% extraction protein from P), while the protease Alloclast caused a 5-fold increment. These previous results show that enzymatic treatment has potential, further optimization was however out of scope for the current study.

The effect of mechanical biomass disruption on protein extraction was evident from ten sequential pressings of P with the lab-scale screw press with addition of water before each press. This harsh treatment enabled a total release of 84.2% of the initial N in the lucerne biomass to the GJs (Fig. 4, Table SG2). However, already after re-pressing P once in the lab, 63% of the N was extracted and after four presses in the lab, the effect plateaued at ~80%. These results corresponds well to previous findings by Hansen et al. [23], who subjected lucerne biomass to a similar sequential press, and concluded that only two consecutive pressings are legitimated based on the gained protein yield and additional costs. Roughly 33% of the total protein in lucerne biomass is assumed to be soluble protein [55]. When mechanically disrupting lucerne biomass as done here, particles containing insoluble protein are

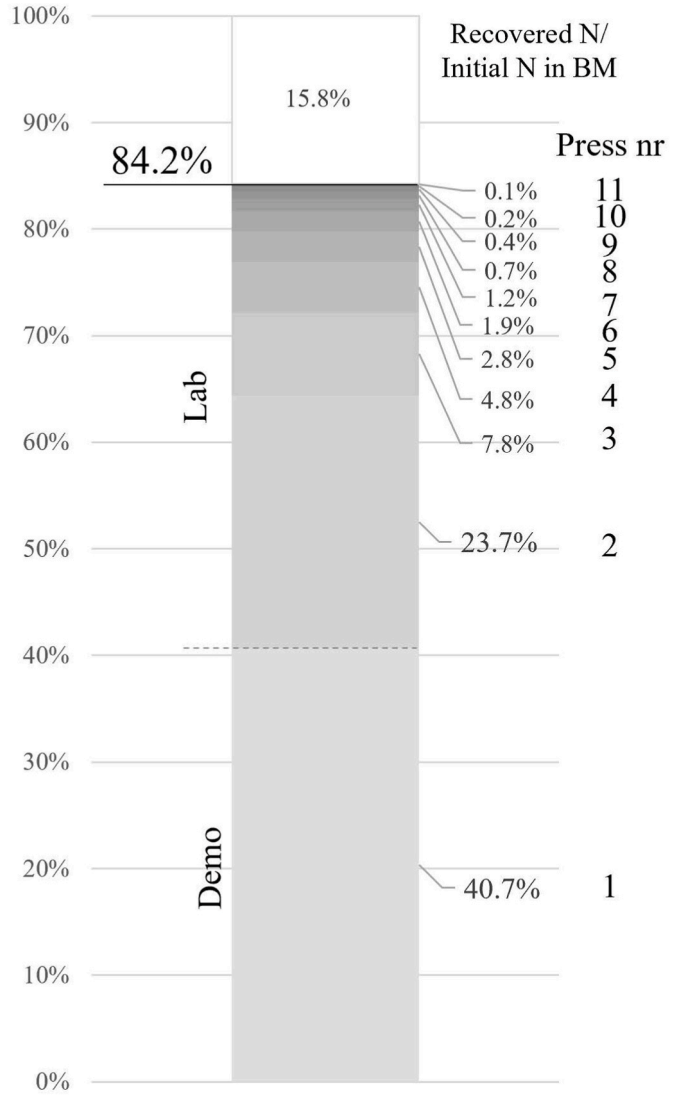

Fig. 4. Distribution of N to green juice during repeated pressing of pulp (P). "Demo" and "Lab" indicates the scale. The white section on top indicates the part of N in the initial biomass (BM) remaining in P after a total of 11 presses. Press number 1 is the average of three individual runs in the demo-scale facility and press 2-11 are calculated as average of three replicates. Standard deviations can be found in Table SH2.

transferred to the GJs together with the soluble protein. Thus, the extracted N in the GJs in the latter pressings, will mostly be bound in particles.

3.1.3. *Treatment effects on recovery of soluble protein from GJ_R (GJ_R to $GP_{soluble}$)*

As shown by correlation analyses of the N flows from *P to* GJ_R and GJ_R *to* $GP_{soluble}$ (Table 3) to the N flow from *P to* $GP_{soluble}$ (Fig. 2), the recovery of protein in the full process is significantly more correlated to the recovery of soluble protein from GJ_R than to the protein extraction from P to GJ_R. Thus, to understand the effect of the treatments on protein recovery from biomass, it is extremely important to evaluate both process steps. As an example from the current results, the protein extraction from frozen lucerne biomass did not differ from that of fresh biomass, while the recovery of soluble protein was severely impaired by the freezing as only 70% of the soluble protein was recovered. A similar pattern was obvious for NaOH treatments up to 0.5 M and varying soaking time and temperatures, as they all had similar values for P to GJ_R, while the values for GJ to $GP_{soluble}$ varied significantly.

Soaking time and temperature did not show any statistically significant effect on the N flow from P to GJ_R in the present study. However, at longer soaking times, recovery of soluble protein from GJ_R increased significantly at a decreased temperature, with 13% at 4 °C as compared to 7% at room temperature. The lower recovery of soluble protein at room temperature might be due to the presence of endogenous proteases, released from the cells in the first press of the biomass, which might be more active at room temperature than at colder temperatures. Proteases are known to degrade proteins into peptides and free amino acids, which are impossible to recover by isoelectric precipitation at pH 4 [26].

The treatments of P with NaOH, showed a positive impact on the recovery of soluble protein from GJ_R at all concentrations evaluated here (Table 3A), with a value of 16% for 0.05M and values ranging from 28% to 40% for the higher concentrations, as compared to 7% for water only (0 M). However, only the higher concentrations significantly influenced the protein extraction, while also the lower ones showed a significant effect on the recovery of soluble protein from GJ_R. Slightly elevated pH values are assumed to increase protein solubility, and previous studies (*e.g.*, Ref. [56]), have recommended adjusting the pH to 8 when extracting photosynthetic leaf proteins for proteomics analyses. Additionally, earlier green biorefinery systems were ammoniating the GJ to pH 8, and this was reported to generally improve the quality of the leaf protein concentrates [57]. Notably, the pH of the GJ obtained from treating P with 0.1 M NaOH in the current study was 8.0 (results not shown), and approximately 80% of the N in GJ was precipitated in GP_{RP}, and 46% of that N was soluble protein, while the rest of the N were either solids or small N containing compounds. Higher recovery of soluble protein was achieved for NaOH concentrations of 0.5 and 2 M, however, for up-scaling in the demo-scale facility, the treatment with 0.1 M was chosen as it was the lowest concentration giving a significant improvement on the total protein recovery and this concentration was also considered safe to work with at such scale. At elevated NaOH concentrations, *i.e.*, 2 M, the N recovery from P in the screw pressing was significantly higher, but the recovery of N to the GP_{RP} was only 62%, as compared to 74% to 80% for concentrations of 0.1 to 1.0 M. At 2 M NaOH (pH ~13.5), the N content of 4.2% in the GP_{SS} was the significantly lowest of all NaOH treatments, as the other concentrations, including 0 M, were ranging from 4.7% to 6.1% and water only. This reduction is likely due to solubilization of N containing phytochemicals in combination with an increased content of cell wall components.

The enzymatic treatments in the current set-up did not show any significant positive effect on the recovery of soluble protein, despite the high N flow from P to GJ_R (Table 3B). The concentration of the enzyme was found to not have a significant effect on the N flows in the different process steps (Table SG1), and this resulted in a focus to compare other parameters, *e.g.*, the effect of ball milling and different time settings, in

this experiment (Fig. 2, Table 3B). However, additional evaluation of the most proper use of enzymes might have resulted in additional effect of the enzymes in the process. Such an optimizing could have included enzyme-to-substrate ratio, pH, temperature, and hydrolysis time.

The present study clearly emphasized the low protein recovery from green biomass during screw pressing and the limited opportunities to sustainably increase these measures by additional treatment of P. The treatments were aiming to impact physical, chemical, and biological traits underlying insufficient cell disruption and/or impaired protein solubility and/or recovery. Among the >20 different treatments evaluated here, only few had positive impact on the recovery of soluble protein. Soaking P with 0.1 M NaOH prior to re-pressing was found to be the most promising treatment to sustainably increase the recovery of soluble protein from green biomass. Therefore, this treatment was evaluated in demo-scale. However, the following environmental and economic assessment indicated that as modelled, there is not sufficient evidence to recommend it as a sole solution for a process implemented at industrial scale. Extraction of additional components in the green biorefinery process might increase economic feasibility and decrease the environmental footprint.

Although this study cannot provide a final solution to the issues related to protein extraction from green biomass, it contributes a significant amount of novel knowledge useful in further research and development as presented and discussed above.

3.2. Demo-scale treatment of P

Demo-scale pressing of lucerne biomass, resulted in a P wet mass of approximately a quarter (24-29%) of the initial wet mass, corresponding to 61-73% of the initial dry matter and 49-74% of the initial N (Table 5A, Table SG3). The corresponding values for lucerne biomass from 2022 to 11-10, pressed in the lab-scale screw press, were 41% of total wet mass, 64% of dry matter, and 37% of N (results not shown), which emphasizes the higher de-watering capacity of the lab-scale press, while also showing the relation between the scales. In the lab-scale experiments, the biomass was not washed prior to the pressing, this would however not significantly affect the dry matter and N flows. Re-pressing of P after approximately 1 h of soaking in 0.1 M NaOH, resulted in a N extraction from P to GJ_R of 18-23%, equivalent to an additional 12-13% of initial biomass N (Table 5B). The composition and maturity level of the lucerne biomass varied between the harvests, which is likely the contributor to the variations seen in mass flows between the demo-scale experiments, as this has been proven in previous studies [49]. Storage of biomass prior to pressing has also been reported to significantly reduce the overall protein recovery and quality [50]. Further studies are of outmost relevance to reach an understanding of the impact of maturity and storage on protein yield in the process, with comprehensive knowledge optimized harvesting and processing routes can be developed.

3.3. Environmental & economic assessment

Based on previous assessments, which have shown limited economic and environmental feasibility from protein extraction of green biomass, additional extraction step(s) have been suggested for increased protein yield [27,38]. However, expanding such green biorefinery systems to include additional stages, or treatments, also result in extra "costs" as such treatments require additional consumption of energy, water, and other inputs [37,58].

3.3.1. LCA results & protein production costs

The global warming potential (GWP) per kg of extracted crude protein (corresponding to the crude protein content of GP_{RP}) was clearly lower for the initial pressing of lucerne biomass (BM to GJ) with 2.8 CO_2 eq. per kg, as compared to re-pressing of P (Fig. 5A). Re-pressing P treated with water showed a higher GWP than treatment with 0.1 M NaOH, due to the low amounts of additional protein recovered. However, even though the re-pressing had 74-115% higher impact as compared to the initial pressing, the cumulative result (initial + re-pressing after treatment) resulted in an impact per kg protein that was only 20-24% higher than for the initial pressing (Fig. 5A).

The major contribution to the environmental impact for the re-pressing of P comes from the electricity use, which is nearly as high as for the initial pressing but with only half the protein yield, thereby resulting in almost double the impact (see Supplementary H). Among the treatments evaluated, drying contributed most significantly to the GWP followed by, where applicable, treatment with NaOH or acidification. Thus, the results of this study correspond with previous studies indicating that emissions from drying contribute the major environmental impact (~50%) from biorefineries [32]. However, other studies have shown that physical maceration, especially if intensive physical disruption processes and heat recovery in drying are utilized, had similar or greater energy use per kg protein than the drying stage when processing fresh biomass [58]. The assessments in the present study are, for simplicity, built on 0% heat recovery. However, a production-scale biorefinery would invest in heat recovery, which would contribute to both economic feasibility and decreased GWP. A recent review of low temperature (<100 °C) waste heat recovery technology found that integrated heat recovery systems substantially improve energy efficiency and in, some cases reduce overall energy demand by over 50% [59]. Additionally, lab-scale protein DMC was 25% from the initial pressing while protein from repressing was between 16 and 18% DMC. Modest improvements to the centrifugation step or other upstream processing that result in more effective solid/liquid separation could increase protein dry matter content and decrease drying energy demands.

Table 5
Distribution (%) of wet mass (including water remaining on the biomass after washing), dry matter, and N flows in a demo-scale process, from A: lucerne biomass (BM) to green juice (GJ) and B: re-pressing of pulp (P) after treatment with 0.1 M NaOH. P_R: Re-pressed P, GJ_R: GJ from re-pressed P, nd: not determined.

A.	Wet mass flows					Dry matter flows				N flow	
Date	Input mass		Output mass		Proportion	Input mass	Output mass		Proportion		
	BM	Water[a]	P	GJ	Out/In	BM	P	GJ	Out/In	BM to P	BM to GJ
2022-11-10	63	37	28	72	100	100	61	30	91	49	38
2023-09-12	90	10	29	71	100	100	68	31	96	74	40
2023-10-03	71	29	24	76	100	100	73	31	104	55	51

B.	Wet mass flows					Dry matter flows				N flow			
Date	Input mass		Output mass		Proportion	Input mass	Output mass		Proportion				
	P	0.1 M NaOH	P_R	GJ_R	Out/In	P	P_R	GJ_R	Out/In	BM to P_R	P to P_R	BM to GJ_R	P to GJ_R
2022-11-10	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
2023-09-12	50	50	32	63	96	100	95	20	115	34	46	13	18
2023-10-03	50	50	38	48	86	100	95	12	107	42	76	12	23

[a] Estimated value.

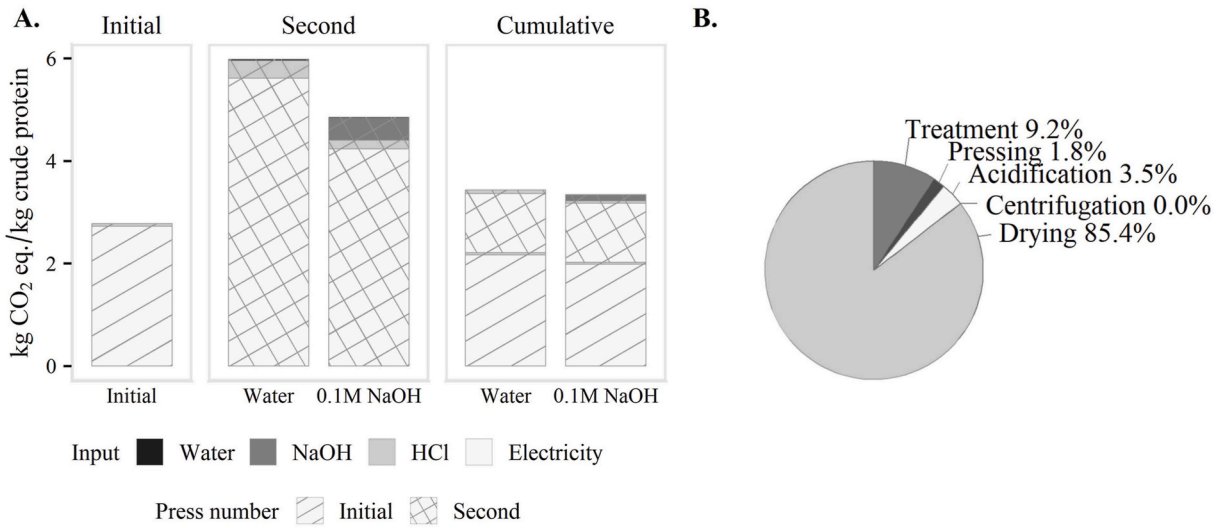

Fig. 5. A: Global warming potential (kg CO_2 eq. per kg of crude protein) for the initial and second press of pulp (P) treated with water or 0.1 M NaOH along with the cumulative process. B: Relative contribution of process stages to global warming potential (%) as caused by the secondary pressing of P treated with 0.1 M NaOH.

Previous studies on the sustainability of protein from green biomass have shown a lower environmental impact per kg protein than this work [10,31,32]. Such variance in results is potentially due to divergent methods, *e.g.*, functional unit, system boundaries, inventory data collection or estimation approach, and impact assessment, or differences in the technical system itself, *e.g.*, extraction methods and feedstock selection. The present study focused on processing alternatives and disregarded both credits from substituting products, decreasing overall emissions, along with emissions from cultivation and transportation as well as increasing overall emissions.

Similarly to the environmental impact, the economic assessment showed that the initial pressing resulted in the lowest cost, with 0.67 € per kg crude protein. Also, protein from re-pressed P showed a significantly higher economic cost, mostly as a result of electricity use, than the initial pressing, while the cost for the cumulative process was in between that for initial and re-pressing (Fig. 6A). However, different to the GWP impact (<1%), the protein cost was sensitive to the water use. The economic costs for the relative contribution of process stages also followed the pattern of the environmental impact results, *i.e.*, drying contributed the largest share, followed by treatments, that contributed 17-22% of the share (see example in Fig. 6B).

A previous study, assessing the economic threshold for leaf protein concentrate production, have identified a low feasibility price threshold of 2 €/kg and a high threshold of 10 €/kg corresponding to bulk protein products and specialty products, respectively [38]. Differently to that study, feedstock production cost, transportation costs, as well as capital investments for additional machinery were excluded in the present assessment. With that in mind, all the protein produced using the processing evaluated in this current work, would satisfy the low-price threshold. With the limited system boundaries of this study, only results over the thresholds are meaningful, as they signify no potential for economic feasibility in that product category. Results under the thresholds, however, can only be considered preliminary positive results requiring further study to investigate the impact of the excluded cost categories.

3.3.2. *Sequential pressing*

Evaluating the feasibility of sequential re-pressing (10 times) clearly showed that both the GWP and cost per kg protein were the lowest in the first re-pressing (which in turn were higher than in the initial pressing as

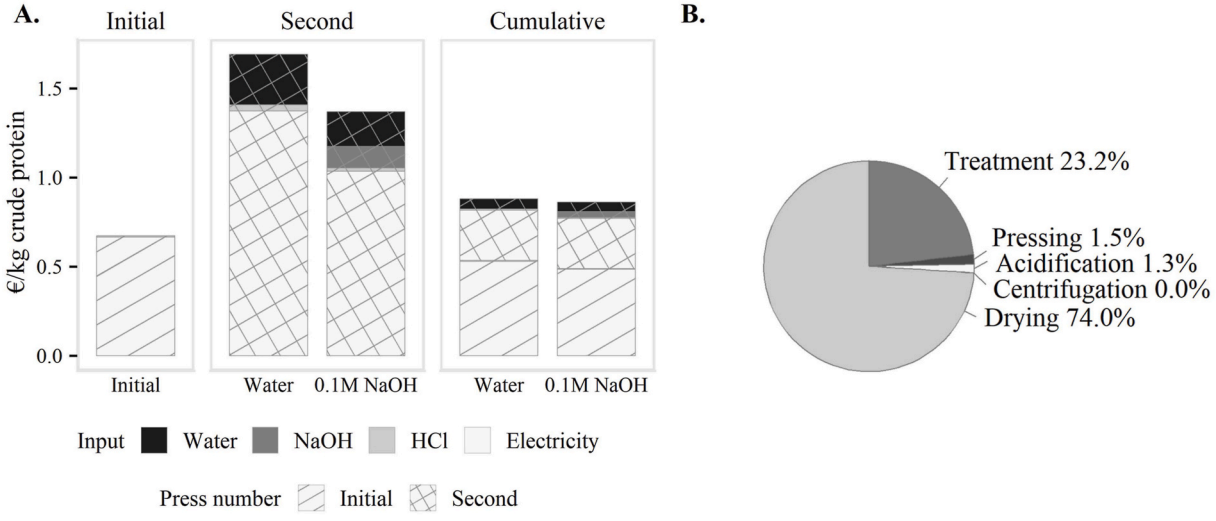

Fig. 6. A: Costs (€ per kg of crude protein) produced during the initial press of biomass and second press of pulp (P) treated with water or 0.1 M NaOH along with the cumulative process. B: Relative contribution of process stages to cost (%) as caused by the secondary pressing of P treated with 0.1 M NaOH.

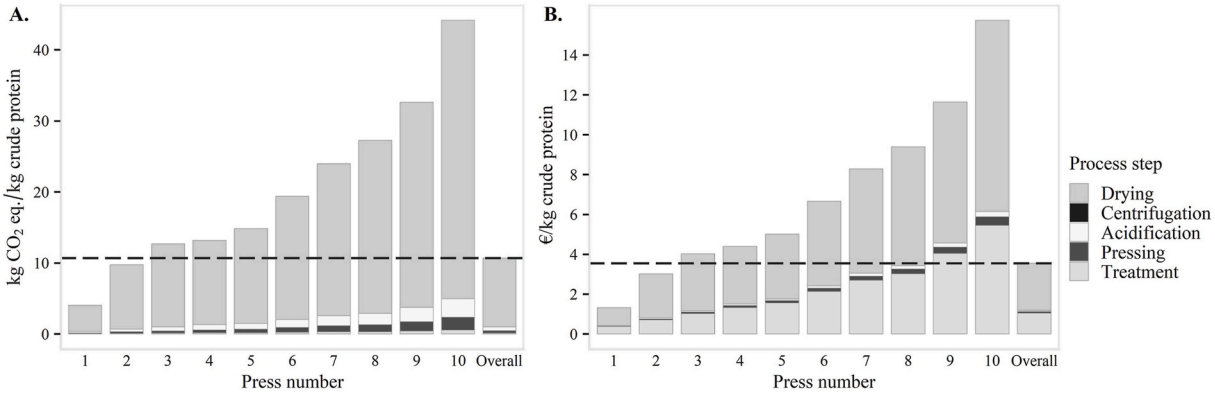

Fig. 7. A: Global warming potential (kg CO_2 eq.) per kg protein in green juice (GJ) per press in a 10x sequential pressing. B: Cost (€) per kg protein in GJ per press.

shown above) and thereafter increased significantly with every re-pressing (Fig. 7). The GWP increased from 4.0 to 44.1 kg CO_2 eq. per kg GJ protein from the 1st re-press to the 10th re-press (Fig. 7A) while the cost rose from 1.31 € to 15.73 € per press per kg GJ protein (Fig. 8B). However, the cumulative GWP and cost per kg protein in GJ, over all 10 sequential pressing steps, were 10.7 kg CO_2 eq. and 3.55 €, respectively. The cumulative values are driven by high marginal N extractions in the first re-presses and diminishing returns in subsequent re-presses, and thereby the impact of later re-presses is partially compensated by the high productivity of the initial few presses. Bals and Dale [37] compared the performance of a 2-press and a 3-press system and found that integrating a third press increased costs by 25%, although, the increase in protein yield and corresponding profit justified the addition. While this study did not consider revenues, and consequently profits, the cumulative unit cost surpassed the 2 €/kg protein threshold proposed by Muneer et al. [38] after the second re-press (which corresponds to the third pressing if the initial pressing is counted too).

3.3.3. *LCA and cost assessment results, demo-scale pressing*

Protein production from initial pressing of lucerne biomass in lab-scale and at demo-scale, and from demo-scale at different biomass harvest occasions, resulted in differences in GWP and economic costs, as illustrated in Fig. 8. Of the demo-scale results, the initial pressing of lucerne harvested in November 2022 ("initial, Nov-22") was the cheapest with less than 1€ per kg crude protein, and the least emitting, with ~3 kg CO_2 eq. per kg crude protein, followed by initial pressing of lucerne biomass harvested in September 2023 ("initial, Sept-23"). Corresponding with results above, protein from re-pressing had a higher GWP (~11-16 kg CO_2 eq. per kg crude protein) and higher cost (~3.5-4.5 € per kg crude protein) compared to initial processing, while the cumulative results were intermediate. Andrade et al. [58] found that for some crops and process configurations, more extensive processing (including a second pressing) resulted in a similar or better energy efficiency, kWh per kg crude protein, than a single press of pre-chopped biomass. In comparison with the lab-scale pressing ("initial, lab") of biomass harvested at the same date in November 2022, initial demo-scale pressing ("initial, Nov-22") resulted in protein with 34% higher GWP and cost. This result suggests that process efficiency might be overestimated when lab processes are scaled-up. However, the magnitude of difference between demo-scale data from 2022 ("initial, Nov. −22") and 2023 ("initial, Sept. −23, Oct. −23") is greater than the difference between lab and demo-scale pressing of the same biomass, suggesting that variation between biomass of different harvests might be of greater impact than the scale of the system utilized for the feasibility analysis. Hansen et al. [23] found that despite some differences the results of lab- and demo-scale pressing were similar enough to conclude that lab-scale experiments could be successfully scaled up, although this was a study on efficacy of protein extraction, not on efficiency, environmental impact, or cost. The main reason for the differences in feasibility of demo-scale pressing of the biomass from the two consecutive years was the higher use of electricity and water in 2022 than in 2023. However, the protein yield was also 1.8-2.1 times higher in 2022 compensating for the higher input use. As described above, patterns of GWP and cost per kg protein were similar, independent of pressing scale utilized. All the initial pressing and cumulative demo-scale cost results were under the 2 € threshold, however, the cumulative results leave very little remaining margin for costs excluded from this analysis, *i.e.*, capital, feedstock, labour, transportation, packaging, *etc*. Previous studies evaluating the economic feasibility of green biorefineries can give some indication of the relation of the excluded cost categories to the utilities

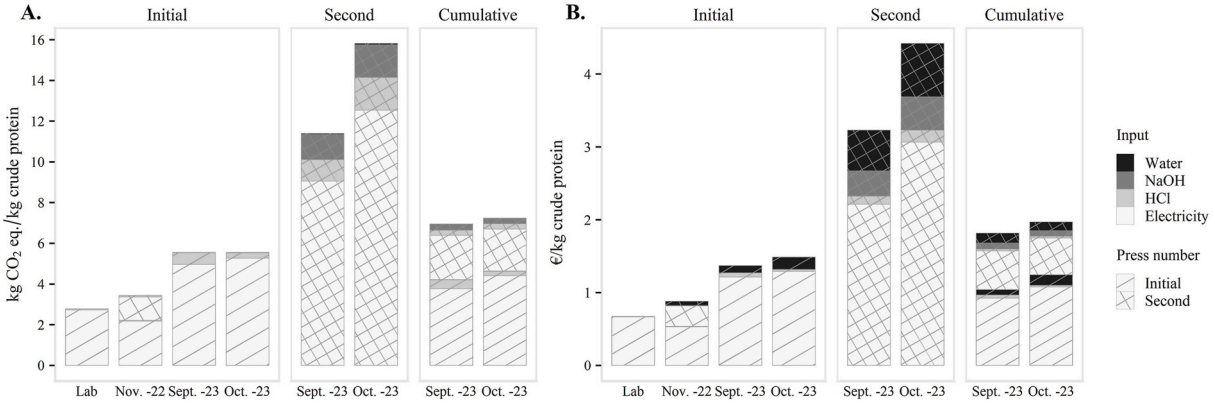

Fig. 8. A: Global warning potential (kg CO_2 eq. per kg crude protein) and B: cost (€ per kg crude protein) for demo-scale processing during the initial and second press, as well as cumulative results. Initial lab-scale pressing (Lab), included for comparison.

cost estimated here [40,46,60]. O'Keeffe et al., [40] found that capital costs were the largest cost category followed by labour then utilities, however, if the internal provision of energy via biogas production for use in CHP reactor and associated costs are excluded, energy costs are the largest cost category. Other studies have found that feedstock provision is responsible for over 50% of costs with capital costs a distant second [46,60]. Additionally, these studies have generally found that as refinery scale increases, per unit capital costs decreases following common assumptions on cost scaling of larger equipment capacity. For the secondary pressing of pulp, economies of scale are likely limited, as the processes as modelled here would require both additional equipment, i.e. a second screw press, and increased capacity, *e.g.* a larger centrifuge or dryer.

The limited scope of the economic and environmental assessment in the current study is sufficient for the preliminary screening of the proposed improvements. And, as currently modelled, a second pressing of P increased total protein yield but moderately increased GWP and cost per kg crude protein. Further investigations expanding the system are however required to make proper assessments of the biorefinery in full. Such assessments should include cultivation, further processing of the remaining P and BJ into secondary products, *e.g.*, textile production [61], feed [62], or bio energy [63], and the full area of inputs and costs to the biorefinery.

4. Conclusion

Protein extraction from green biomass in a green biorefinery context to produce food and feed is of great interest as the future will come with increased requirements of plant-based, locally/regionally produced protein products. However, such a biorefinery process is also challenging due to the low protein recovery obtained. An extra pressing step, *i.e.*, re-pressing of P, has potential to increase the overall protein yield of the process, but additional processing comes with additional environmental and economic costs. Re-pressing of P to obtain a protein concentrate includes two steps: extraction of proteins from P to GJ_R and recovery of proteins from GJ_R, the latter step having the highest impact on the overall recovery of soluble protein. An addition of a liquid, such as water, to P prior to re-pressing is necessary to obtain proper processing behavior. Using NaOH at concentrations of 0.1 M (resulting in GJ with pH 8), or higher, will contribute to increased protein extraction. However, at concentrations above 0.5 M, proteins might degrade into unrecoverable peptides, while hydrolysis of cell wall components will result in release of insoluble particles possibly impairing the quality of the protein products. Additionally, high NaOH concentrations are neither user-friendly nor environmentally friendly in an up-scaled process.

As GJ is not re-absorbed by P in screw press to any significant level, cell disruption is the most effective way to increase the recovery of soluble proteins from P, and for that either mechanical or chemical (*e.g.*, NaOH) treatments can be utilized, eventually followed by enzymatic treatments. However, too harsh treatments might lead to extraction of particles with insoluble proteins or other N containing compounds, which may decrease the quality of the resulting protein concentrates. During, and before, processing the plant material (*e.g.*, P, GJ, and other intermediate material and end products) should preferably be kept cold to avoid protease activity, which degrades proteins into peptides. Furthermore, freezing should be avoided as it influences cellular integrity, resulting in increased extraction of particles with insoluble proteins and lower product quality.

Protein extraction from green biomass for food and feed in a process including one screw pressing step and subsequent protein recovery is neither economically feasible, nor environmentally sustainable due to insufficient protein yield. However, implementing additional pressing of P to obtain a higher extraction and recovery of soluble protein is considered a way forward. Such additional processing does, nevertheless, imply considerable costs in terms of water and electricity use, costs that are not corresponding to the extra protein gained. Extra pressings, with additions of water in between, have approximately the same cost per press, but for each round, the amount of recoverable soluble protein will decrease, while the amount of insoluble particles will increase. Thus, in an industrial setting where the balance of protein yield and environmental and economic costs is highly relevant, only one re-pressing is sufficient and addition of 0.1 M NaOH can be advised.

Other solutions to consider are ways to increase the protein extraction already in the initial pressing, may that be addition of NaOH or preserving agents. Additionally, freezing of the plant material before processing should be avoided, as it potentially impairs the quality of the protein concentrate, and it will add to the environmental and economic costs associated to the process. Furthermore, costs associated with the production and transport of the biomass, and possibilities of heat recovery, as well as of circulation of BJ to replace some of the water utilized, need to be considered for industrial production of protein concentrates from green biomass. Also, differences in costs and yield for different crops and harvest occasions need to be taken into consideration in future assessments to evaluate feasibility for industrial scale protein extraction from green biomass.

CRediT authorship contribution statement

Anna-Lovisa Nynäs: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Waleed Mlook:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Cristian Wedgwood:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **William R. Newson:** Writing – review & editing, Visualization, Supervision, Funding acquisition, Conceptualization. **Thomas Prade:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Eva Johansson:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jafr.2026.102842.

Data availability

Data will be made available on request.

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