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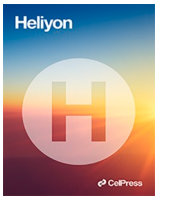

Optimizing monosaccharide production from liquid hot water pretreatment and enzymatic hydrolysis of grass-clover press cake

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ABSTRACT

In the present study, clover-grass press cake was treated by liquid hot water at temperatures of 180–200 °C for a reaction time of 5–10 min. Evaluation of pretreatments was based on the monosaccharide yield after enzymatic hydrolysis of the pretreated slurry and solid fraction, respectively. Extraction of up to 48% hemicellulose and 4% cellulose was observed during pretreatment. The optimal pretreatment conditions were identified as 190 °C and 10 min resulting in monosaccharide yields of 90% and 73% of the theoretical maximum by slurry and solid conversion, respectively. At optimal conditions, the C6 monosaccharide yield (83–90%) was fairly equal compared to the C5 monosaccharide yield (56–89%), which increased by slurry conversion due to near-complete monomerization of soluble xylo-oligosaccharides. In this study, we showed that clover-grass press cake possesses considerable potential as feedstock for production of fermentable sugars in a biorefinery context.

1. Introduction

In the search for alternative and sustainable sources of food, energy, chemicals and materials, agro-industrial residues have been regarded as promising feedstocks [1,2]. One of the new bio-based industries in Europe is the green biorefinery, which aims to exploit 'green biomasses' including grasses, clover and alfalfa for the production of valuable bio-products such as protein concentrates [3,4]. In green biorefining, press cake (PC) is an abundant residue, which is produced in an initial mechanical pressing of the green biomass [5,6]. The press cake fraction represents a significant part of the original plant material (395–401 g/kg) [7] and has a high content of fiber (529–694 g/kg DM) [8], which makes it an attractive source of monosaccharides for microbial bioconversion to high-value products including enzymes, materials and single cell protein.

To expose polysaccharides (i.e. cellulose and hemicellulose) of press cake fibers to enzymatic hydrolysis, several pretreatment methods can be applied including dilute acid, ammonia soaking, alkaline peroxide, steam explosion (SE) and liquid hot water (LHW) [9–12]. Among these, the LHW pretreatment uses compressed hot water to weaken the fiber structure, mainly by hemicellulose removal, with the advantages of low cost and minimum by-product formation [13,14]. However, to the best of our knowledge, no investigations have attempted to optimize the LHW pretreatment of press cake and only little attention has been paid to enzymatic hydrolysis of the 'whole' pretreated slurry.

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This study investigates liquid hot water pretreatment and hydrolysis of a grass-legume press cake as a novel feedstock for production of monosaccharides. The effect of pretreatment conditions is studied with respect to monosaccharide yields from hydrolysis of the pretreated solid and whole slurry fraction. The results presented provide a systematical overview of pretreatment conditions for obtaining fermentable sugars to use in biotechnological production of food, feed, chemicals and bio-fuels.

2. Materials and methods

2.1. Feedstock

Press cake was produced from a mixture of grass (*Festulolium*) and red clover (*Trifolium pratense*) harvested from a 2 nd year field (1st cut) on June 23, 2021 provided by BioRefine A/S (Janderup, Denmark). The press cake fraction had a dry matter (DM) content of 23% (w/w) and the composition on a DM basis was as follows: 34.5% ± 1.6% (standard deviation, SD) cellulose, 19.1% ± 1.3% hemicellulose (84% xylan and 16% arabinan), 11.1% ± 0.3% lignin, 20.7% ± 2.8% H_2O extractives, 1.8% ± 0.3% ethanol extractives, 6.2% ± 0.7% crude protein and 5.4% ± 0.5% ash. The H_2O extractives were primarily fructose (2.5% ± 0.8% of DM) and lactic acid (1.4% ± 0.7% of DM) but glucose, arabinose and acetic acid were also detected. The press cake was stored at −20 °C until use.

2.2. Liquid hot water pretreatment

Pretreatments were carried out using demineralized water in stainless steel batch reactors (20 mL volume) at a fixed solid loading of 5% (w/w). Before each experiment, the residual air was removed by purging the reactor with N_2 at 100 bars. The reactors were heated at temperatures of 180 °C, 190 °C and 200 °C for 5 min and 10 min, respectively, using a preheated sand bath (SBL-2D, Techne, USA). After pretreatment, the reactors were cooled in a water bath and the whole slurry ('LHW slurry') was separated into a solid ('LHW solid') and liquid ('LHW liquid') fraction by filtration. All experiments were carried out in duplicate and the resulting fractions were stored at −20 °C until use. Total weight and DM measurements of each solid and liquid fraction were used for mass balance calculations.

The severity (log R_0) of the investigated pretreatments was calculated according to Eq. (1) originally proposed by Overend and Chornet (1987) [15]. The severity is a key parameter in hydrothermal pretreatment relating the impact of temperature and time in a single factor.

$$\log(R_0) = \log\left[t \cdot exp\left(\frac{(T-100)}{14.75}\right)\right] \quad (1)$$

R_0: severity factor, t: pretreatment time [min] and T: pretreatment temperature [°C].

2.3. Enzymatic hydrolysis

The enzyme preparations used were Cellic® CTec3 HS containing cellulolytic and hemicellulolytic activities and Viscozyme® Wheat FG containing arabanase and xylanase. Both preparations were provided by Novozymes, Bagsvaerd, Denmark.

Enzymatic hydrolysis was performed with Cellic® CTec3 HS at 50 °C, pH 5.0, with a constant loading of 24 mg enzyme preparation per g DM. 0.1 g DM of LHW solid and raw press cake were diluted with 0.1 M citrate buffer, pH 5.0, in 50 mL Greiner tubes for a total DM of 5% and 2% (w/w), respectively. Tubes with raw press cake contained 0.4 mL of 5% (w/w) sodium azide solution to prevent microbial growth. For hydrolysis of LHW slurries, 0.1 g DM of solid was mixed with the corresponding pretreatment liquid. The LHW slurries contained between 2.5% and 5.3% (w/w) of DM. The hydrolysis reactions were performed in a thermomixer (MHR 23, DITABIS, Germany) at 600 rpm for 72 h and terminated by heating to 95 °C for 15 min.

Pretreatment liquids were analyzed for oligomeric saccharides by enzymatic hydrolysis with Cellic® CTec3 HS and Viscozyme® Wheat FG used in a 4:1 ratio (w/w) and a total dosage of 30 mg enzyme preparation per g DM. Samples of LHW liquid fractions were diluted 1:1 (v/v) in 0.1 M acetate buffer, pH 5.0, and incubated at 50 °C at constant shaking (600 rpm) for 40 h. The reactions were stopped by heating to 95 °C for 15 min. Full degradation was confirmed by HPLC analysis.

Enzymatic hydrolysates were filtered through Advantec GC-50 glass fiber discs and analyzed for monosaccharides (D-glucose, D-xylose and L-arabinose) by HPLC (see section 2.4.1). The yield of C6 monosaccharides (glucose) and C5 monosaccharides (sum of xylose and arabinose) were calculated as C6 and C5 monosaccharides in hydrolysates relative to the amount of C6 and C5 monosaccharides in raw press cake, respectively. The total monosaccharide yield was based on the sum of released C5 and C6 monosaccharides. All yields are presented in weight percentages and were corrected for saccharides in enzyme preparations. The hydrolysis of raw press cake was done in triplicate and the hydrolysis of pretreated fractions was done in duplicate.

2.4. Chemical analysis

2.4.1. Quantitative analyses of saccharides, furanic aldehydes and organic acids

Contents of saccharides and organic acids were quantified by HPLC (Waters Corporation, USA) using a Rezex™ ROA-Organic Acid H^+ column (8%, 300 mm × 7.8 mm) (Phenomenex Inc., USA) at 25 °C and refractive index (RI) detection. Eluent (5 mM H_2SO_4) was applied at 0.6 mL/min. Furanic aldehydes were analyzed with UV detection at 284 nm.

2.4.2. DM and ash determination

Analyses of DM and ash were done according to standard laboratory analytical procedures made by the National Renewable Energy Laboratory (NREL) (Colorado, USA) [16,17]. Samples of raw and pretreated material were dried at 105 °C until constant weight. Ash was determined after the combustion of dried samples at 540 °C for 24 h.

2.4.3. Water and ethanol extraction

Extractions were based on NREL standard procedures and were performed in a Soxhlet apparatus with 215 mL of ELGA-H_2O and 99% ethanol, respectively [18]. 2 g of press cake (94% DM) was wrapped in a filter disc (Filtrak™, 3w) and extracted for 24 h with 1–2 siphon cycles per hour. Saccharides and organic acids in water extractives were analyzed by HPLC as described above. The amount of extractives in press cake, on a DM basis, was determined from oven-dry weights of samples with water and ethanol extractives, respectively.

2.4.4. Structural carbohydrates and lignin

A two-step acid hydrolysis was used to quantify structural carbohydrates and lignin in extractives-free press cake according to NREL standard procedures [19]. 300 mg of extracted press cake (96% DM) was hydrolyzed in 3 mL 72% (w/w) H_2SO_4 at 30 °C for 1hr followed by a dilution to 4% (w/w) H_2SO_4 and a second hydrolysis at 125 °C for 1hr. After hydrolysis, hydrolysates were separated by filtering with Whatman® 43 (ashless) discs and analyzed for saccharides by HPLC (2.4.1). The cellulose content was calculated as the glucose content divided by 1.11 and the hemicellulose content was calculated as the sum of xylose and arabinose contents divided by 1.136. The lignin content was determined from the DM loss of solid residue after combustion at 540 °C for 24 h. Contents were corrected for the DM loss due to extraction and are presented per DM in raw press cake.

2.4.5. Determination of crude protein

The nitrogen content of raw press cake (94% DM) was analyzed using an elemental analyzer (2400 series II, CHNS/O, PerkinElmer, USA) and corrected for the moisture content. The crude protein content of samples was determined by multiplying the nitrogen content with 6.25 (standard Jones' factor) [20].

2.5. Statistical analysis

Statistical differences between the means of parameters were analyzed with Tukey tests based on results from one-way ANOVA in open-source "RStudio" software (version 2022.7.1). Significance was accepted at $p < 0.05$.

3. Results and discussion

3.1. Effects of liquid hot water pretreatment

Grass-clover press cake was subjected to liquid hot water pretreatment at different severities (log R_0 = 3.05–3.94) followed by enzymatic hydrolysis. LHW dissolved 15–22% of press cake dry matter (Table 1), which was mainly recovered as saccharides (43–52% of DM). As expected, the extraction yield of hemicellulose (23–48%) varied according to the severity, while the cellulose-extraction yield was consistently low (approx. 4%) (Fig. 1). An increase in hemicellulose extraction was observed as severity increased for log $R_0 \leq 3.65$ (190 °C and 10 min), whereas it decreased at the highest severity of 3.94 (200 °C and 10 min). Hence, the 3.94 severity presumably caused over-degradation of soluble saccharides. Notably, a high difference in hemicellulose-extraction yield was observed between the 3.05 and 3.35 severity condition, specifically a 1.8-fold enhancement of extraction yield, which reflected a considerable increase in fiber disruption. No significant difference in hemicellulose extraction was seen between similar severity conditions of 3.35 and 3.36, as well as the similar 3.64 and 3.65 conditions, which indicated that the severity function (log R_0) was an appropriate predictor of the LHW effect on extraction of saccharides.

A feature of the LHW pretreatment was the release of oligosaccharides (Table 2). These, in particular xylo-oligosaccharides, constituted 32–72% (w/w) of released saccharides and became dominant with increasing severity. On the other hand, a significant decrease in monosaccharide contents (glucose and arabinose) was observed at high severity. Low levels of by-products such as furfural (the degradation product of pentoses) (up to 133 mg/L) and 5-HMF (the degradation product of hexoses) (up to 158 mg/L) were

Table 1
Experimental conditions and corresponding dry matter recoveries. Values are mean ± standard deviation (n = 2). The pretreatment severity was calculated according to Eq. (1). DM recovery in solid fraction (%) = [Wet Weight (recovered solids) · DM% (solids)/Raw press cake DM] · 100. DM recovery in liquid fraction (%) = [Wet Weight (recovered liquid) · DM% (liquid)/Raw press cake DM] · 100. DM, dry matter.

Experiment no.	Temperature (°C)	Reaction time (min)	Severity, log (R_0)	DM recovery in solid fraction (%)	DM recovery in liquid fraction (%)
1	180	5	3.05	87.4 ± 0.2	14.9 ± 1.6
2	180	10	3.36	74.4 ± 1.5	20.5 ± 1.9
3	190	5	3.35	67.9 ± 4.5	20.7 ± 1.3
4	190	10	3.65	68.7 ± 3.8	21.5 ± 0.5
5	200	5	3.64	63.7 ± 5.8	21.3 ± 3.4
6	200	10	3.94	72.7 ± 7.9	15.9 ± 1.3

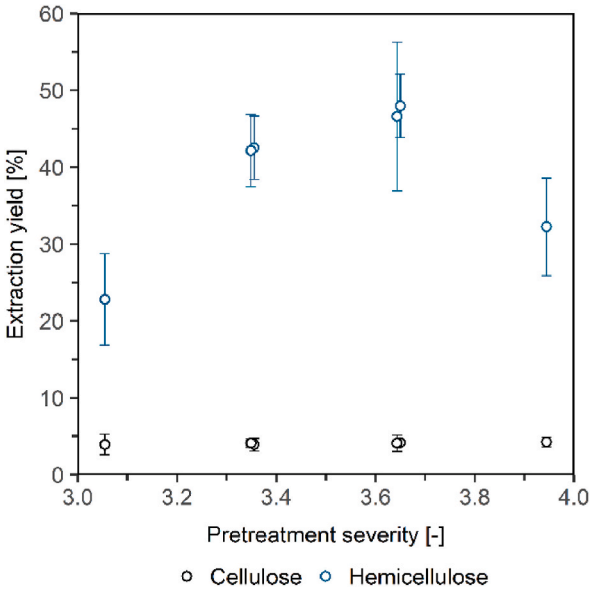

Fig. 1. Extraction yields of cellulose and hemicellulose as a function of pretreatment severity (log R_0). Hemicellulose-extraction yield (%) = [hemicellulose in LHW liquid fraction (g)/hemicellulose in raw PC (g)] · 100. Cellulose-extraction yield (%) = [cellulose in LHW liquid fraction (g)/cellulose in raw PC (g)] · 100. Values are mean ± standard deviation (n = 2).

Table 2
Characteristics of LHW liquids at different pretreatment conditions. Values are mean ± standard deviation (n = 2). Different letters indicate values that are significantly different (p < 0.05), row-wise (A > B > C). Values with similar letters have no significant difference. 5-HMF, 5-hydroxymethylfurfural.

Parameter/variable	Unit						
Temperature	°C	180		190		200	
Reaction time	min	5	10	5	10	5	10
Severity (log R_0)	–	3.05	3.36	3.35	3.65	3.64	3.94
pH	–	4.4 ± <0.1 A	4.1 ± 0.1 AB	4.1 ± 0.1 AB	4.0 ± 0.1 BC	4.0 ± <0.1 BC	3.7 ± 0.1 C
Saccharides							
Glucose	g/L	0.5 ± 0.1 A	0.4 ± 0.2 A	0.4 ± 0.1 A	0.3 ± <0.1 AB	0.4 ± 0.1 A	<0.1 ± <0.1 B
Gluco-oligosaccharides	g/L	0.7 ± 0.2 A	0.7 ± <0.1 A	0.8 ± 0.1 A	0.8 ± <0.1 A	0.7 ± 0.2 A	1.1 ± 0.1 A
Xylose	g/L	1.9 ± 0.6 A	1.3 ± 0.5 A	1.3 ± 0.3 A	1.6 ± <0.1 A	1.5 ± 0.3 A	1.3 ± 0.4 A
Xylo-oligosaccharides	g/L	1.1 ± 0.1 C	4.2 ± 0.3 A	4.5 ± 0.2 A	4.7 ± 0.2 A	4.6 ± 0.1 A	2.9 ± 0.2 B
Arabinose	g/L	1.1 ± 0.1 A	1.1 ± 0.2 A	1.2 ± 0.1 A	0.9 ± <0.1 A	1.1 ± <0.1 A	0.4 ± <0.1 B
Arabino-oligosaccharides	g/L	<0.1 ± <0.1 A	0.3 ± 0.2 A	0.2 ± <0.1 A	0.2 ± 0.3 A	<0.1 ± 0.1 A	0.5 ± <0.1 A
Acids							
Acetic acid	g/L	0.5 ± <0.1 A	0.7 ± <0.1 A	0.7 ± 0.2 A	0.7 ± 0.1 A	0.7 ± 0.1 A	1.1 ± 0.3 A
Lactic acid	g/L	1.9 ± 0.1 A	1.5 ± 0.4 A	1.8 ± 0.4 A	1.8 ± 0.5 A	1.8 ± 0.1 A	1.6 ± <0.1 A
Formic acid	g/L	0.2 ± <0.1 A	0.3 ± 0.2 A	0.4 ± 0.1 A	0.6 ± 0.2 A	0.6 ± 0.2 A	0.6 ± 0.2 A
Furanic aldehydes							
5-HMF	mg/L	26 ± 10 A	88 ± 46 A	70 ± 33 A	115 ± 27 A	93 ± 47 A	158 ± 31 A
Furfural	mg/L	6 ± 1 C	35 ± 6 C	52 ± 13 BC	92 ± 11 AB	44 ± 12 BC	133 ± 19 A

detected. A higher content of 5-HMF was likely related to degradation of furfural to formic acid. As expected, an increase in severity facilitated higher by-product formation, although the increase in 5-HMF content was not statistically significant.

3.2. Enzymatic saccharification of the LHW solid and slurry fraction

Pretreated fractions (i.e. the LHW solid and LHW slurry fraction) were hydrolyzed with Cellic® Ctec3 HS (24 mg/g DM) at pH 5.0 and 50 °C for 72 h. The C6 monosaccharide yield showed an increasing trend with increasing severity and reached the highest values at severities of 3.65 (190 °C and 10 min) (82% yield) and 3.94 (200 °C and 10 min) (92% yield) for LHW solid and LHW slurry hydrolysis, respectively (Fig. 2A). These values fall within the interval of reported glucose yields (62–104%) obtained from LHW pretreatment and enzymatic hydrolysis of grass and legume press cakes [12]. Notably, the C6 yield was higher (about 10%) at a severity of 3.65 (190 °C and 10 min) compared to a close severity of 3.64 (200 °C and 5 min), which indicate that pretreatment time was a crucial factor for efficient hydrolysis. The C5 monosaccharide yields peaked at intermediate severities (3.36–3.65) and showed a significant decrease at

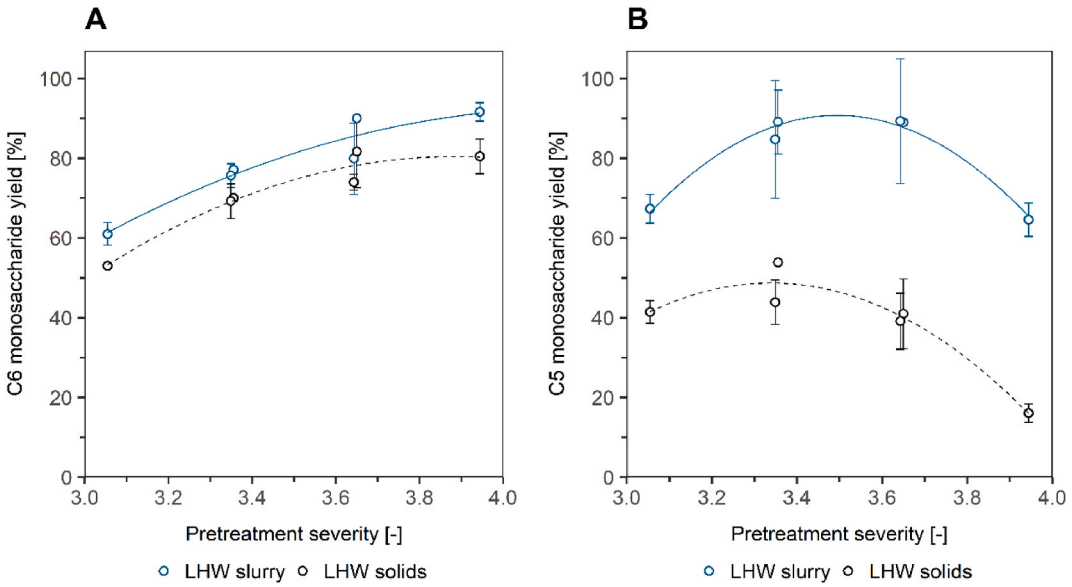

Fig. 2. Monosaccharide yield from enzymatic hydrolysis of LHW slurry and LHW solid fractions as a function of pretreatment severity (log R_0). Hydrolysis was performed with Cellic® CTec3 HS (50 °C, pH 5.0) for 72hr. (A) C6 monosaccharide yield. (B) C5 monosaccharide yield. Values are mean ± standard deviation ($n = 2$). Lines are quadratic fits to mean values. LHW, liquid hot water.

the highest severity partly explained by over-degradation of saccharides as already discussed (Fig. 2B). The C5 yield was clearly affected by the type of fraction, compared to the C6 yield, varying from 16 to 54% by hydrolysis of the LHW solid fraction and from 65 to 89% by hydrolysis of the LHW slurry fraction. This observation could be ascribed to Cellic® Ctec3 HS enzymes acting on oligosaccharides in the liquid phase of LHW slurries and explains the consistently higher monosaccharide yields by LHW slurry hydrolysis. In fact, the difference in monosaccharide yields between LHW solid and LHW slurry hydrolysis corresponded to 94–100% conversion of oligosaccharides in the pretreatment liquids.

3.3. Total available monosaccharides

To summarize on the saccharification process, released monosaccharides (glucose, xylose and arabinose) obtained in LHW liquids and hydrolysates were added together (Table 3). A total monosaccharide yield of 56–73% and 63–90% was achieved by LHW and enzymatic hydrolysis of the LHW solid and LHW slurry fraction, respectively. Hence, slurry hydrolysis improved the monosaccharide yield by 7–21%. By enzymatic hydrolysis of untreated press cake, the monosaccharide yield was reduced significantly to 30%. Interestingly, the highest monosaccharide yields were obtained under the same severity of 3.65 (190 °C and 10 min). At this optimal condition, a fairly equal C6 yield (83–90%) could be obtained while the C5 yield (56–89%) varied considerably owing to oligosaccharide hydrolysis by slurry conversion.

4. Conclusion

This study showed that polysaccharides in clover-grass press cake can be efficiently converted to monosaccharides – up to 90% – by LHW and enzymatic hydrolysis. Based on the monosaccharide yield, pretreatment conditions of 190 °C and 10 min were identified as optimal. It was demonstrated that hydrolysis of the whole pretreated slurry improves the monosaccharide yield considerably. The high recovery of monosaccharides obtained in this study suggests clover-grass press cake to be a promising feedstock in a biorefinery context. For a comprehensive evaluation of the technology, experiments on solid loading, enzyme loading, scale-up and fermentability of the hydrolysates need to be carried out.

Author contribution statement

Nicolai Sundgaard Bekker: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper., Saqib Sohail Toor: Performed the experiments; Contributed reagents, materials, analysis tools or data., Kamaldeep Sharma, Thomas Helmer Pedersen: Contributed reagents, materials, analysis tools or data., Lars Haastrup Pedersen: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Table 3
Total monosaccharide yield (sum of glucose, xylose and arabinose) after LHW and enzymatic hydrolysis of the LHW solid and LHW slurry fraction. Values are presented in weight percentages of monosaccharides in raw press cake and are mean ± standard deviation (LHW fractions, $n = 2$; untreated press cake, $n = 3$). Different letters indicate values that are significantly different ($p < 0.05$), row-wise (A > B). LHW, liquid hot water.

Parameter/variable	Unit							
Temperature	°C		180		190		200	
Reaction time	min		5	10	5	10	5	10
Severity (log R_0)	–		3.05	3.36	3.35	3.65	3.64	3.94
LHW slurry hydrolysis	%	–	63.3 ± 0.5 B	81.5 ± 2.8 AB	79.0 ± 7.3 AB	89.7 ± 0.1 A	83.4 ± 11.4 AB	81.9 ± 0.1 AB
LHW solid hydrolysis	%	–	56.0 ± 1.0 A	70.9 ± 1.0 A	66.3 ± 6.5 A	73.2 ± 8.9 A	68.1 ± 0.5 A	60.9 ± 3.2 A
Untreated press cake	%	30.3 ± 0.4	–	–	–	–	–	–

Data availability statement

Data will be made available on request.

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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