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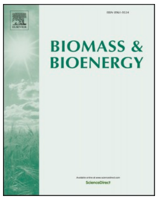

Pre-extraction affects the mycelial bioconversion potential of *Typha* spp. and *Salix* spp. biomass residues

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ABSTRACT

Mycelial bioconversion is a promising industrial avenue for transforming a wide array of bio residues and feedstocks into higher added value chains via application of filamentous fungi. Little research has been conducted on the bioconversion efficacy of pre-extracted biomass (PEB) from feedstocks suitable for paludiculture, a sustainable cultivation practice for degraded peat soils. This study investigated the bioconversion ability of five white-rot basidiomycete fungi on pre-extracted and unextracted biomasses derived from two mainstream paludicultural crops: short-rotation coppice willow (*Salix schwerinii* x *Salix viminalis*) and perennial cattail grass (*Typha latifolia*). Willow biomass fed through pressurised hot water extraction (PHWE) and hydrodynamic cavitation extraction (HCE) and cattail biomass via HCE were utilised. During a mycelial bioconversion incubation, *Trametes* sp. showed fastest hyphal extension rate (4.1 ± 0.35 mm/day) on willow PEB_{HCE} and *Pleurotus floridanus* hyphal extension was fastest on cattail PEB_{HCE} and unextracted biomasses (4.89 mm/day). Hyphal extension rates across all fungal species were generally faster on PEB_{HCE} compared to unextracted biomasses. Post-incubation analyses showed *Fomes fomentarius* yielded highest ergosterol content (marker of fungal biomass) across all plant biomasses compared to other fungal species (LSM = 64.0129 μg/g d.w., $p < 0.0001$). Pre-extraction via PHWE significantly inhibited mycelial bioconversion efficacy both in terms of hyphal extension and ergosterol content. In contrast, pre-extraction via HCE did not inhibit myceliation efficacy of willow and cattail biomass. These results highlight the novel value of PEB from large-scale paludiculture, and likely other cascaded lignocellulose biomasses, as viable bio-residue feedstocks towards industrial mycelial bioconversion applications.

1. Introduction

Paludiculture, the productive use of rewetted peatlands, provides an alternative mode of large-scale biomass production that can remediate degraded soils and restore carbon [1–3], improve water runoff quality and nutrient retention [4,5], reduce greenhouse gas emissions [3,4,6] and restore terrestrial biodiversity [2,5,7]. In the Nordics, two crops suitable for paludiculture are cold-tolerant short-rotation coppice (SRC) willow (*Salix* spp.) and cattail grass (*Typha* spp.). Though cultural and historical use of these crops for woven handicrafts, functional materials and for medicinal use are known [8–10], industrial applications utilising willow and cattail are needed to incentivise paludiculture as an effective economic land-management practice [11,12]. SRC willow stems are often harvested every 2–4 years due to rapid biomass growth rate

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[13–15]. Cattail grass is perennial and regenerates annually without the need for resowing [16,17]. Different modes of processing and extraction have been explored to yield valuable bioactive products derived from willow and cattail [13,18,19]. For both crops, the time of year of harvest will affect biochemical composition, such as protein content, which is important to consider in biorefinery processes. Reported C:N ratios (18.7–46.3), lignin (5–20.6 %), hemicellulose (21.8–30 %) and cellulose (22.4–68.1 %) for cattail biomasses [20–25] and C:N ratios (98–182), lignin (20.4–25.7 %), hemicellulose (18.5–21.8 %) and cellulose (42.5–44.1 %) for SRC willow biomasses [26–30], indicate their potential suitability as lignocellulosic feedstocks for mycelial bioconversion. Although cattail grass generally has higher total nitrogen levels, which are less favourable for basidiomycete cultivation, it has been successful in cultivation of *Pleurotus ostreatus* (oyster) mushroom [25,31,32]. To date, no research is available on basidiomycete mycelial bioconversion potential of different pre-extracted willow and cattail biomasses.

The process of extraction facilitates mass-transfer of compounds from a biomass to a solvent under a certain extraction method and its conditions, namely, solvent, liquid solvent-to-solid biomass ratio, temperature, time, pressure and pH [33,34]. Various types of extraction methods exist that are conventionally used to obtain polysaccharides, phenolics, terpenoids and other constituents depending on the extraction method, conditions and solvent polarity [34–37]. The process can also be employed as a pre-treatment to effectively enhance downstream fractionation, enzymatic digestion, pulping and ethanol fermentation [38–42] and may increase the bioavailability of sugars and nutrients in the substrate [43]. Extraction residues from agro-industrial processes are conventionally discarded, though may often be suitable as nutrient-rich growth media for microbial conversion [44,45]. So far, no research has investigated how mycelial bioconversion efficacy of a given biomass is affected by different pre-extraction treatments, nor the comparison with untreated biomass. Hydrothermal extraction is known to alter the structure of lignocellulosic biomasses and often involves hydrolysis of polysaccharides, where the degree of hydrolysis highly depends on the process temperature and pressure [46]. Pressurised hot water extraction (PHWE), also known as subcritical water extraction, has been shown to yield a higher degree of hydrolysis of wood-derived lignocellulosic biomass when compared to hydrodynamic cavitation extraction (HCE), likely due to PHWE's higher bulk temperature and pressure conditions that more effectively disrupt lignin-carbohydrate matrices, enhancing hemicellulose solubilization and cellulose accessibility [47,48]. HCE creates intense cavitation-induced temperature levels and pressure-gradients that facilitate mass-transfer of extractives and can alter the resultant biomass' surface area and porosity, which has been shown to enhance downstream biodegradability and digestion efficacy of pre-extracted lignocellulosic biomasses [49–51]. How these extraction techniques as a pre-treatment step alters the downstream mycelial bioconversion potential of cattail and willow biomass has not yet been studied.

Basidiomycete mycelial bioconversion, or myceliation, is the vegetative growth of fungi that is colonising a given substrate prior to the reproductive or fruiting stage that yields mushrooms ready for harvesting of edible or medicinal mushrooms in the functional food industry [52]. Growing industrial attention towards mycelial bioconversion due to its innovative production capabilities for fungi-derived protein [53], bio-based plastic packaging [54], building and insulation materials [55], and alternative leather [56] indicates the potential utility of untapped PEB and paludicultural willow and cattail as alternative, rapid-regenerative feedstocks. Important criteria for successful mycelial bioconversion in the solid-state are aseptic work conditions, strong performing fungal strain(s), suitable growth media that primarily consist of small size lignocellulosic biomass and often additional supplements, a minimally-aerobic bioreactor or growth chamber design that facilitates optimal moisture/humidity, low gas exchange and other tuneable conditions that promote basidiomycete metabolism and myceliation [57,58]. Fungal strains chosen for mycelium-based materials with structural integrity are often Basidiomycota of trimitic hyphal nature, consisting of all three generative, skeletal and binding hyphal traits [59]. After inoculating a sterile biomass with a chosen fungal candidate, the mycelial hyphae enmesh the biomass, transforming it physically and biochemically largely via enzymatic degradation of lignocellulose (cellulose, hemicellulose, and lignin). The deposition of chitinous hyphal networks function as a three-dimensional binding matrix that effectively converts disaggregated plant material into a structurally integrated mycelium-composite biohybrid material. High C:N ratio biomasses usually suit basidiomycete species that are inherent wood-decay specialists in nature, however, industries have found such fungi viable in metabolising a wide array of C:N varying substrates from agriculture [59], wastewater [60,61], lower C:N biomasses like coffee grounds, kitchen biowaste [62,63] and textiles [64]. Paludicultural biomasses have not been explored as much, though it is presumed that their compositional nature will suit basidiomycete mycelial bioconversion. To our best knowledge, there is no research output investigating pre-extracted paludicultural biomass as a feedstock for basidiomycete mycelial bioconversion, which this study set out to explore.

This study seeks to investigate the differences in mycelial bioconversion efficacy of unextracted and pre-extracted biomasses (PEB) derived from paludicultural willow and cattail. To accomplish this, we 1) screened compositional differences of unextracted and PEB of willow from pressurised hot water extraction (PHWE) and hydrodynamic cavitation extraction (HCE) as well as unextracted and PEB of cattail from HCE; 2) measured hyphal growth during mycelial bioconversion of biomass from all treatments using five basidiomycete species; and 3) measured final ergosterol content as an indicator of fungal biomass generated. We presume that differing pre-extraction conditions (PHWE or HCE) and hence PEBs will provide insight into lignocellulosic needs for mycelial bioconversion.

2. Materials & methods

2.1. Paludicultural feedstock sample collection

Willow variety Klara (*Salix schwerinii* L. *'Amgunskaja' x Salix viminalis* L. *'Ivar'*, frost-tolerant) stems (altogether 20) were harvested between 40 and 60 cm above ground from a paludiculture pilot site in Jokioinen, Finland, in November 2023 (60°54′15″N 23°30′44″E), operated by the Natural Resources Institute Finland. The length of the harvested stems was 190–200 cm, and the mean stem diameter was 1.66 cm at the base and 0.58 cm at the tip. Fresh willow stems were coarsely milled (Eliet Neo 2 garden shredder, Zwevegem, Belgium) into chips [47]. Chips of willow were then placed on frost cloth and air-dried at 40 °C on a grain table dryer for 36 h. Air dried chipped willow was pulverised (Fritsch Pulverisette P19, Idar-Oberstein, Germany) and stored at −30 °C in the dark to await further processing. The dry matter content of the willow crumb was 93.51 % (oven +105 °C).

Fresh cattail (*Typha latifolia*) was harvested from a paludicultural site in Kortenhoef, Netherlands, in June 2023. The fresh cut harvests were directly shredded on-site with a Loglogic shredder equipped with an ELHO double chopper. Rapid moisture content of fresh cut biomass was approximately 44 % (MA150, Sartorius AG, Göttingen, Germany). The bulk shredded cattail biomass was left to air dry on a concrete slab for 8 days, then fed through a conventional bailer (Welger AP 530, Wolfenbüttel, Germany) to form square bales for transport. Bales were received and stored in dry dark conditions prior to milling and extraction.

2.2. Extraction and separation

Two pilot-scale extraction methods utilised were PHWE and HCE. PHWE was conducted on willow biomass (D_{90} of 2260 μm) at the

Natural Resources Institute Finland using tap water as a solvent, according to Kilpeläinen et al. [65]. The extraction was conducted in two-phase with an increased temperature and pressure for phase 2 (Table 1). Due to limited resources, pilot-scale PHWE could only process SRC willow, which was retrieved also from HCE. For HCE, an additional reduction in biomass particle size was necessary to avoid clogging of the Venturi cavitation unit. HCE was conducted on additionally-milled willow (D_{90} of 1883 μm) and cattail (D_{90} of 1663 μm) using a 50 L pilot-scale hydrodynamic cavitation extraction system similar to Tienaho et al. [47]. The device consisted of a closed hydraulic circuit and a circular Venturi-shaped reactor as the key components, where the liquid-solid mixture was inserted and moved by a centrifugal pump. The pump was model HDM 25-19A (Salvatore Robuschi e C. S.r.l., Parma, Italy), nominal power 3.0 kW, open impeller with a diameter of 185 mm, adjustable frequency between 40 Hz (2320 rpm) and 60 Hz (3480 rpm) using the inverter ATV320U40N4C (Schneider Electric S.p.A., Stezzano, Italy) and AC Drive GA500 400V Class Three-Phase Input (Yaskawa, Orbassano, Italy). All the parts in contact with the circulating mixture were made of food-grade AISI 316 stainless steel. The processes were carried out at atmospheric pressure, with no active temperature control. In both HCE trials, 40 L of water were used. Power and energy consumption were measured using three-phase digital power meters with power resolution 1 W and energy resolution 10 Wh (model D4-Pd, IME, Milan, Italy) (see Supplementary Information for a schematic representation of the cavitation devices). Except for the different devices utilised, HCE was methodologically similar to Tienaho et al. [47]. Both extraction types and conditions employed are shown in Table 1 and were performed using tap water as a carrier solvent.

2.3. *Basidiomycete inoculum preparation*

For the mycelial bioconversion inoculum, we utilised five basidiomycete fungal cultures (Fig. 1) obtained from Microbial Domain Biological Resource Centre HAMBI (Finland). The species were *Fomes fomentarius* (FBCC 1298), *Ganoderma lucidum* (FBCC 665), *Trametes versicolor* (FBCC 564), *Trametes hirsuta* (FBCC 1239) and *Pleurotus floridanus* (FBCC 469). Mycelial cultures of each fungal strain were retrieved from cryopreservation (suspended in 10 % glycerol in liquid nitrogen) [66]. Vitality of each culture was observed on preliminary sterile substrate decay tests [67] and cultured on malt extract agar plates (MAE) (Malt extract, Neogen, Michigan, USA), Agar No. 1 Bacteriological (Neogen, Michigan, USA). Basidiomycete isolates of each species (10-day cultures on MEA plates at 25 °C) were used for inoculation.

2.4. *Mycelial bioconversion*

2.4.1. *Substrate preparation and inoculation*

PEB_{PHWE}, PEB_{HCE} and unextracted biomasses described above (approximately 500 g dried of each) were prepared for fungal inoculation by hydration to moisture content of 70 % (samples were oven dried at 105 °C to validate moisture content (70.5 %)), equilibrated in sealed containers for 24 h and autoclaved. Sterilised biomass of each type was aseptically distributed (20 g) and densely packed into labelled and weighed glass petri dishes (90 mm diameter, 15 mm depth) using a 85 mm-diameter steel press. Sterilised borer (5 mm diameter) was used to take plugs from the visible leading hyphal edge of the basidiomycete cultures and inoculated to the center of the biomass. Triplicates were made of each biomass-species combination. Dishes were randomised and placed into TP2000+TPD2000, #40 green filter microbox containers (SacO2, Deinze, Belgium) for incubation.

Table 1
overview of PHWE and HCE system conditions.

	Pressurised Hot Water Extraction (PHWE)	Hydrodynamic Cavitation Extraction (HCE)	
Extracted Biomass	Pulverised willow	Cattail	Pulverised willow
Solvent	Tap Water	Tap Water	
Solid-to-Liquid Ratio	1:5 (kg:L)	1:57 (kg:L)	1:20 (kg:L)
Total Extraction Time	60 min	28 min	42 min
Main Vessel Temperature	Phase 1: 121 °C Phase 2: 170–175 °C	25–50 °C[a]	25–60 °C[a]
Pressure	Phase 1: 6 bar; Phase 2: 10 bar	Atmospheric (1 bar)[a]	

[a] HCE temporarily creates extreme pressure and temperature gradients (up to 2500 - 20,000 °C and 2000 atm) at the nano-scale.

2.4.2. *Hyphal extension rate*

Microboxes containing all samples were kept in an incubation room fixed at +22 °C and darkened. Monitoring measurement was conducted on days 0, 2, 4, 6, 9, 11, 13, 16, 18, 20, 23, and 27 after inoculation. Hyphal extension was measured using a digital calliper (±0.5 mm) in x and y directions across two radii from the edge of the inoculum plug from above the sealed dishes (similar to Drais, 2023). Total weight of each dish was also measured with 0.01 g accuracy. Aerial photos (5 Mpixels) were captured using a Specim IQ hyperspectral camera (Spectral Imaging Ltd., Oulu, Finland). Although the total monitoring period was 27 days, plates that contained hyphae reaching the interior edge of the dish prior to day 27 were deemed complete and allowed to consolidate for an additional 7 days before being placed in −20 °C for one day followed by −60 °C awaiting further processing.

2.5. *Compositional analyses*

Frozen samples stored at −60 °C were lyophilised (HETO PowerDry FD8, Allerød, Denmark) for 72 h, weighed and sequentially milled thrice at 28 hz using one 25 mm diameter steel ball per 50 ml vessel (RETSCH MM 400 MM, Haan, Germany). The milled samples were homogenised and subsampled into 2 ml Eppendorf tubes and stored in desiccation chambers in darkness until compositional analyses.

2.5.1. *Chemical composition*

Extractives (including proteins, secondary metabolites and other non-structural components) were removed from the biomass samples via accelerated solvent extraction (ASE 350, Dionex, Sunnyvale, CA, US) using potassium hydroxide, hexane, acetone, and water as solvents, adapted from the NREL Laboratory Analytical Procedure [68]. Extractive-free biomass was then analysed for structural carbohydrate and lignin content. Amounts of cellulose and hemicelluloses (mannose, glucose, galactose, xylose, arabinose, rhamnose, galacturonic acid, 4-*O*-methyl glucuronic acid (4-O-Me-GlcA)) were determined using two-step acid hydrolysis and methanolysis [65,69] followed by gas chromatographic analysisGC-FID (Shimadzu GC-2010, Kyoto, Japan) with HP-1 Column (25 m × 0.2 mm I.d., film thickness 0.11 μm). Hydrogen was used as the carrier gas and the split ratio was 20:1. Lignin content was determined according to NREL procedure via fractionation of lignin into acid-insoluble lignin, and acid-soluble lignin. Acid-insoluble lignin was determined gravimetrically after two-step acid hydrolysis and acid-soluble lignin was quantified by UV–Vis spectroscopy at 205 nm. Cellulose content was determined as the difference between total glucan (determined by acid hydrolysis) and non-crystalline glucan (determined by acid methanolysis).

2.5.2. *Mineral composition*

Elements Mn, Mo, Ni, P, Pb, S, Zn, As, Se, Ca, Cd, Co, Cr, Cu, Fe, K, Mg and Na were measured either by inductively coupled plasma optical emission spectrometry (ICP-OES, PerkinElmer Optima 8300, Shelton, USA) or inductively coupled plasma mass spectrometry (ICP-MS, Agilent Technologies, Tokyo, Japan). Replicate measurements were conducted for extracted willow samples (PEB_{HCE} and PEB_{PHWE}). Single measurements of all cattail and unextracted willow samples were analysed due to

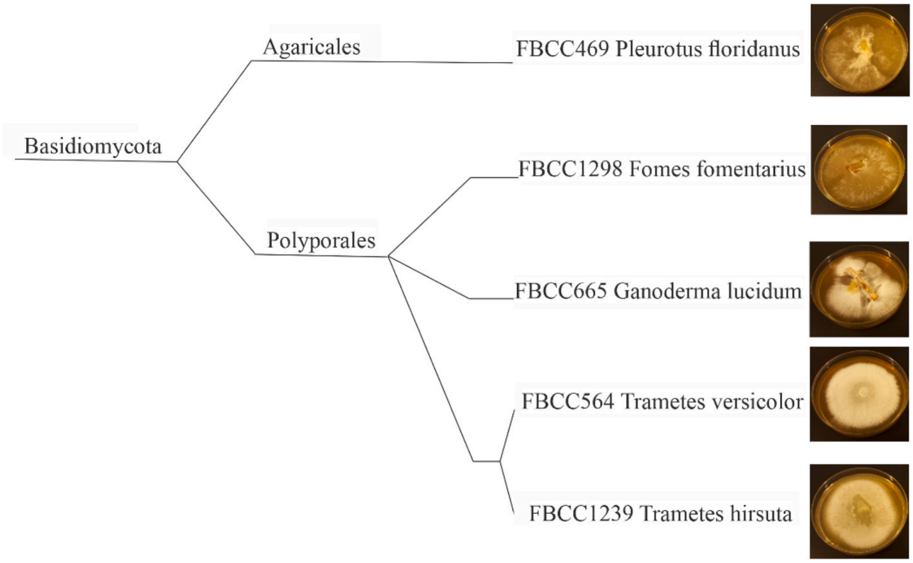

Fig. 1. Phylogenetic tree of the five basidiomycete strains utilised in this study sourced from HAMBI, Finland. (Image credit to Luomus Creative Commons Attribution Share-Alike 4.0 Luomus).

sample quantity limitations. Replicates of willow PEB were measured. Minerals including heavy metals are known for the importance in fungal metabolism and growth [70].

2.5.3. *Dry matter content and particle size measurement*

Dry matter content was determined gravimetrically by weighing samples before and after lyophilisation over 72 h. The particle size distribution percentiles D_{10}, D_{50} and D_{90} were measured in triplicate using a Mastersizer 3000 static light scattering device mounted with Aero S dry powder dispersion unit together with Mastersizer application software (Malvern Instruments Ltd, Malvern, UK). A refractive index representative of sawdust (1.47) was utilised.

2.5.4. *Total carbon, nitrogen and protein content*

For each freeze-dried sample, 0.1g was weighed in tin foil cups then carefully folded and compressed into compact balls (to minimize atmospheric contamination) before placement into the autosampler carousel of a LECO CN828 CN determinator (LECO Corporation, St. Joseph, Michigan, USA). Total C and N was determined by the Dumas total dry combustion method. Protein content was estimated using a nitrogen factor of 6.25 for biomass, according to Ref. [71].

2.5.5. *Ergosterol content*

Ergosterol is a unique product, and hence marker, of fungal biomass even in the presence of animal or plant tissue and thus it is commonly utilised as an indicator for estimating fungal biomass [72]. Ergosterol is generally more applicable than fungal chitin measurements. Ergosterol yields on myceliated biomass samples were measured using an established liquid chromatography-mass spectrometry (LC–MS) protocol described briefly below. Ergosterol was extracted from freeze-dried material with 1 ml of cyclohexane and 4 ml of 10 % (w/v) KOH in methanol. Ergosterol standard was obtained from Sigma-Aldrich (>99 % purity). The amount of ergosterol was measured with LC-MS (Arc Waters LC with qDa mass detector) using a C18 100A reverse-phase column (Phenomenex). Electrospray ionization (ESI+) and selected ion recording (SIR) is used to quantify ergosterol content and post-column ionization improvement is achieved via isocratic solvent manager (ISM) with acetonitrile (ACN) and formic acid (HCOOH). Electrospray ionization (ESI) in positive ion mode with mass spectrometry (Acquity qDa; Waters, MA, USA) was used. The LC–MS method has limit of detection (LOD) of 0.005 μg/ml and limit of quantitation (LOQ) of 0.0025 μg/ml. Further details can be seen as outlined in Ref. [73].

2.6. *Statistical analyses*

The results after mycelial bioconversion from total hyphal extension rate, ergosterol content and total C and N data were statistically modelled to evaluate the various biomass-pre-extraction-species combinations. Generalised linear mixed models (GLMM) were utilised separately for willow and cattail samples with the assumption of beta, gamma or Gaussian distribution depending on the dependent variable. The first is mentioned for percentages, the second for heavily skewed, and the last for normally distributed dependent variables. Fixed effects in the model were treatment (HCE, PHWE, untreated), fungal species (Ff, Gl, Pf, Th, Tv), and their interaction. Final hyphal extension day was used as a covariate for Ergosterol content. A block effect was used for the two unextracted willow biomasses to account for substrate variation derived from the additional pre-grinding required to avoid clogging of the HCE venturi device. The block enables both unextracted willow biomasses to be combined into one type and considered accordingly in the statistical model. Unequal variances were allowed for species, when necessary, based on a likelihood ratio test.

The residual pseudo-likelihood (RSPL) estimation method was used for the models having the assumption of beta or gamma distribution, and the residual maximum likelihood (REML) method for the Gaussian-distributed models. The normality of the residuals was studied and found adequate using diagnostic plots. Degrees of freedom were calculated using the Kenward-Roger method. Tukey's method was used for pairwise comparisons of means with a significance level of 0.05. The marginal and conditional R2-values were computed using SAS Macro % GOF [74], based on the variance components of fixed, random, and residual effects. The marginal R2-value quantifies the reduction in residual variance attributable solely to the fixed effects. In contrast, the conditional R2-value assesses the reduction in explained variance due to the combined influence of both fixed and random effects, compared to the variance explained by fitting an overall mean. Statistical analysis was conducted using the GLIMMIX procedure in SAS Enterprise Guide 8.3 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. *Effects of pre-extraction on biomasses*

Two phase PHWE significantly reduced the total hemicellulose content of willow biomass (50.2 ± 1.46 % reduction) Mannose and glucose were reduced to a lesser extent compared to galactose, xylose, arabinose, rhamnose, galacturonic acid, and 4-*O*-methyl glucuronic acid (Fig. 2b). PHWE reduced KOH-hydrolysis and water-extractives obtained via ASE compared to unextracted willow (−54 ± 4.72 % dw). Acid hydrolysis and methanolysis analyses showed that HCE had less of a reducing effect on total lignocellulose compared to PHWE for willow biomass (Fig. 2a). Hemicellulose fractions were slightly reduced following HCE when comparing to unextracted samples (Fig. 2b).

HCE had a negligible reducing effect on total hemicellulose content of the willow and cattail biomasses compared to PHWE. HCE was more effective in reducing total lignin in cattail (−39.54 ± 3.16 %) compared to willow (−13.98 ± 3.03 %). Main differences in chemical composition observed between willow and cattail biomasses were in acid insoluble lignin, nitrogen content and hemicellulose composition (primarily higher D-xylose, L-arabinose and D-glucuronic acid content and slightly lower D-galactose and D-galacturonic content in cattail compared to willow). Pre-extraction increased C/N ratio of willow $\mathrm{PEB_{PHWE}}$ (+13.1 ± 2.2), willow $\mathrm{PEB_{HCE}}$ (+7.2 ± 1.2) and cattail $\mathrm{PEB_{HCE}}$ (+11.3 ± 0.4), primarily via removal of nitrogenous extractives and total N reduction versus the unextracted biomass. Protein content in willow was reduced by −1.63 % ± 0.02 % for $\mathrm{PEB_{PHWE}}$ and −0.77 % ± 0.02 % for $\mathrm{PEB_{HCE}}$ and slightly more for cattail $\mathrm{PEB_{HCE}}$ (−3.16 % ± 0.06 %). HCE had a higher reducing effect on particle size for willow ($\Delta D_{10} = -84.7$ μm; $\Delta D_{50} = -124.7$ μm; $\Delta D_{90} = -200.0$ μm) compared to PHWE ($\Delta D_{10} = -11.2$ μm; $\Delta D_{50} = -30.0$ μm; $\Delta D_{90} = -45.0$ μm), with remarkable particle size reduction also for cattail ($\Delta D_{10} = -39.0$ μm; $\Delta D_{50} = -93.0$ μm; $\Delta D_{90} = -163.3$ μm). Key differences in low-resolution elemental composition between willow and cattail biomasses were higher Zn in willow and higher Mn and Fe in cattail (Table 2). Generally, both pre-extraction types reduced total mineral content apart from some minor trace metal variations.

3.2. Hyphal extension on PEB and unextracted biomasses

Basidiomycete hyphal extension rates were generally faster on $\mathrm{PEB_{HCE}}$ (for willow: 3.48 ± 0.37 mm/day; for cattail: 3.32 ± 0.18 mm/day) compared to unextracted biomasses (for willow: 3.31 ± 0.21 mm/day; for cattail: 2.86 ± 0.13 mm/day). $\mathrm{PEB_{PHWE}}$ had a significant inhibitory effect on mycelial bioconversion efficacy for willow requiring microscopy to verify presence of faint mycelial hyphae and fungal vitality (Fig. S5). Slower hyphal extension rates were also evident across all species for willow $\mathrm{PEB_{PHWE}}$ (2.03 ± 0.13 mm/day, see Fig. 3d). *T. versicolor* (4.1 ± 0.35 mm/day) and *T. hirsuta* (4.1 ± 0.36 mm/day) showed fastest hyphal extension rates on willow $\mathrm{PEB_{HCE}}$ (see Fig. 3b and e). *P. floridanus* showed fastest hyphal extension rates overall on cattail $\mathrm{PEB_{HCE}}$ and unextracted cattail biomass (4.89 mm/day). Across all basidiomycete species, hyphal extension rates were faster on cattail $\mathrm{PEB_{HCE}}$ (3.32 ± 0.18 mm/day) compared to unextracted cattail (2.86 ± 0.13 mm/day) (see Fig. 3c and f). All species showed promising mycelial conversion traits on both willow and cattail $\mathrm{PEB_{HCE}}$, indicating HCE as a

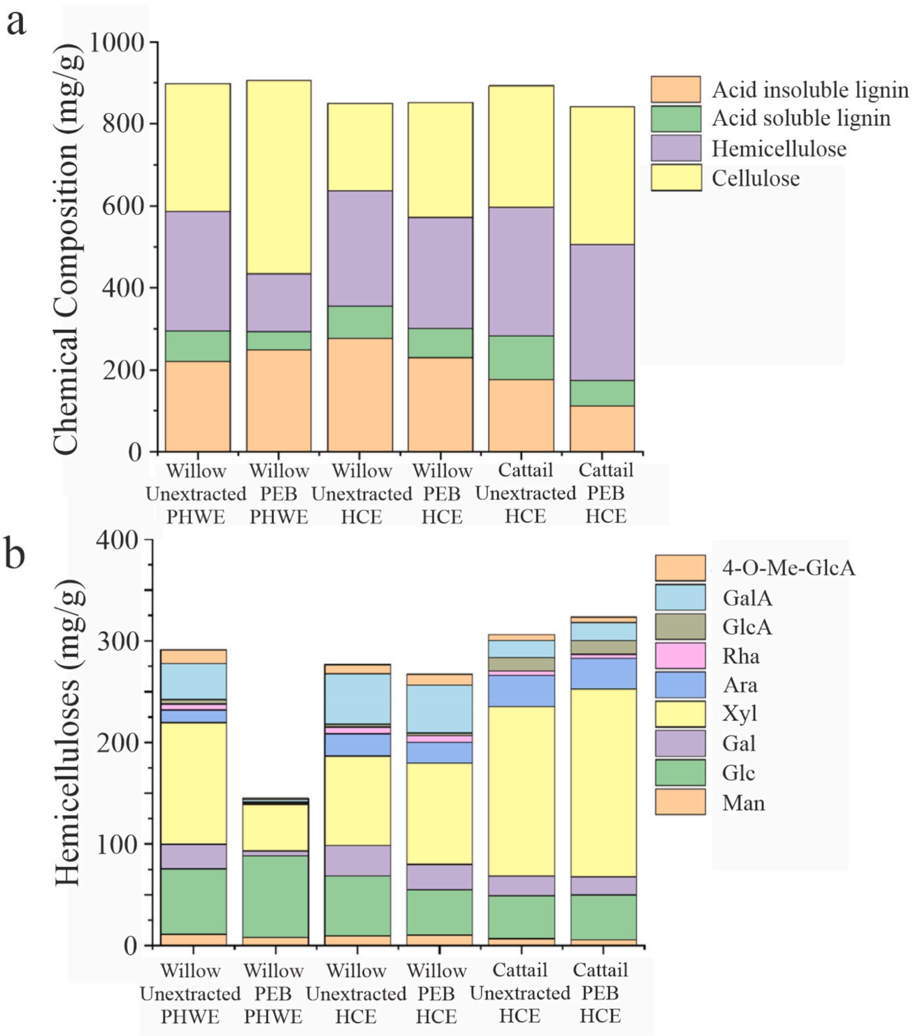

Fig. 2. a) comparison of acid insoluble and soluble lignin, hemicellulose and cellulose (N = 12), and b) hemicellulose composition across PEB and unextracted biomasses (N = 12).

Table 2

Elemental composition measured via ICP-OES and -MS across willow and cattail PEBs and unextracted biomasses. Colour scale red-to-blue indicating lowest-to-highest values, respectively (N = 8*).

	Elementals (mg/kg)																	
	Mn	*Mo*	*Ni*	*P*	*Pb*	*S*	*Zn*	*As*	*Se*	*Ca*	*Cd*	*Co*	*Cr*	*Cu*	*Fe*	*K*	*Mg*	*Na*
Willow ***Unextracted ****	292.5	0.21	0.385	0.97	0.045	0.695	104.35	0.035	0.01	3.65	1.66	0.4	1.925	11.6	45.8	2.56	0.845	0.085
Willow ***PEB$_{\text{PHWE}}$ ****	52.7	0.12	0.44	0.12	0.05	0.47	87.3	0.01	0.01	2.1	1.35	0.13	1.28	19.4	54	0.01	0.06	0.09
Willow ***PEB$_{\text{HCE}}$ ****	333	0.28	0.75	0.83	0.11	0.82	113	0.03	0.02	5.65	1.92	0.38	2.94	9.47	47.4	1.41	0.99	0.18
Cattail ***Unextracted***	645	0.84	0.86	1.65	1.26	1.77	39.3	0.65	0.03	11.7	0.07	0.13	15.3	7.41	387	16.1	1.41	2.17
Cattail ***PEB$_{\text{HCE}}$***	512	0.51	2.04	0.86	1.03	1.28	33.6	0.29	0.02	12.5	0.05	0.1	8.92	7.95	220	2.35	1.18	0.49

** indicates samples where replicates were measured*

* indicates samples where replicates were measured.

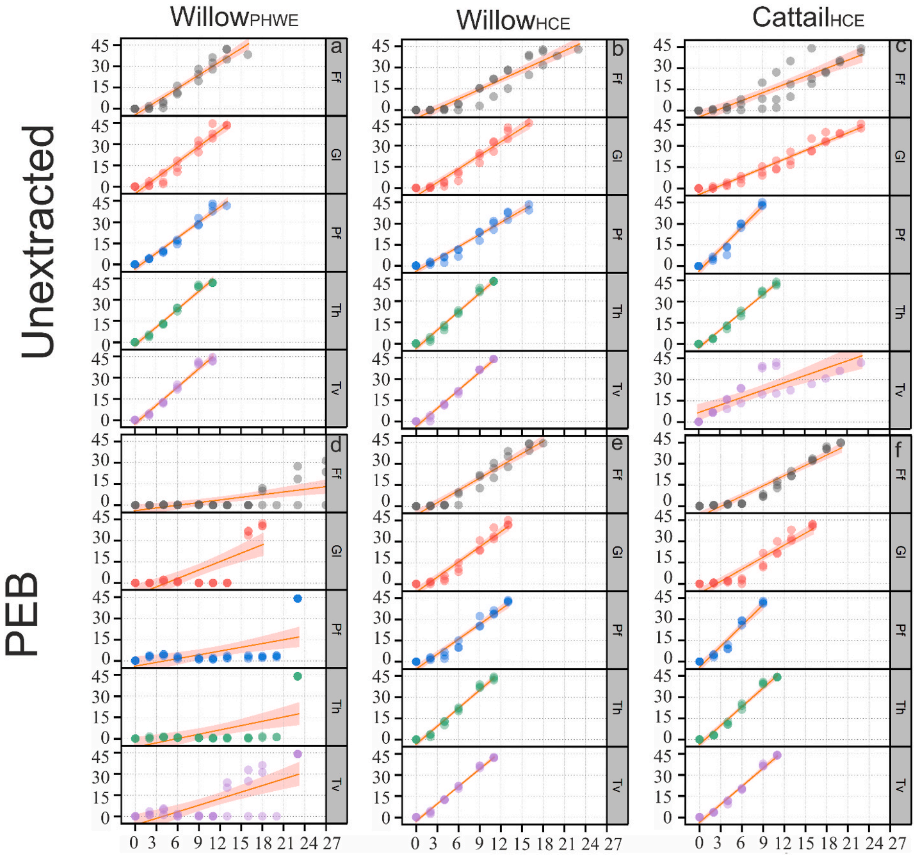

Fig. 3. Hyphal extension (mm) on y-axes and incubation time (day) on x-axes for unextracted biomasses (top row) and pre-extracted biomasses (PEB, bottom row) for **a) and d)** willow via PHWE **b) and e)** willow via HCE; **c) and f)** cattail via HCE. All samples studied with triplicates.

viable pre-extraction step prior to mycelial bioconversion.

3.3. *Effects of mycelial bioconversion on total C, N and ergosterol*

Mycelial bioconversion resulted in reduction of total C across all samples (−0.45 ± 0.12 % for willow, −1.22 ± 0.075 % for cattail). Total N analyses showed increase across all myceliated biomasses (+0.16 ± 0.005 % for willow and +0.27 ± 0.14 % for cattail), with the exception of willow PEB$_{\text{PHWE}}$. Mycelial bioconversion reduced C:N ratio of all biomasses compared to sterile controls, except for willow PEB$_{\text{PHWE}}$ which had negligible change in C:N compared to sterile controls (Fig. 4).

Pre-extraction via HCE increased ergosterol yields in both willow (48.32 ± 5.2 μg/g d.w.) and cattail (37.28 ± 3.2 μg/g d.w.) compared to their unextracted biomasses (44.16 ± 4.0 μg/g d.w. and 35.66 ± 3.2 μg/g d.w., respectively), suggesting that fungal biomass generation was not impeded by HCE, but slightly enhanced. Willow PEB$_{\text{PHWE}}$ samples revealed low amounts of ergosterol, indicative of the biomass's inhibition of significant mycelial growth (Fig. 5).

At the species level, *F. fomentarius* yielded highest ergosterol content for willow and cattail, followed by *G. lucidum*, *P. floridanus*, *T. hirsuta*

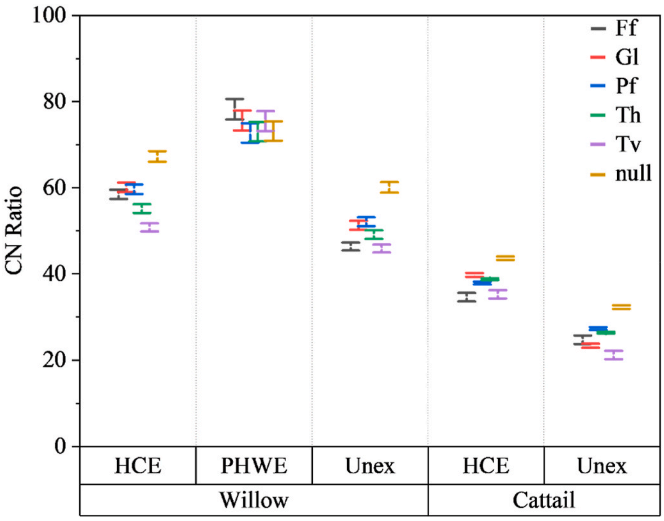

Fig. 4. Estimated C:N ratio (mean and standard error) measured across all biomasses and fungal species. The colour markers represent fungal species with the exception of the gold markers that represent sterile control (unmyceliated) samples. N = 30 for cattail and N = 60 for willow. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and *T. versicolor*, respectively (Fig. 6).

4. Discussion

This study aimed to investigate the efficacy of pre-extracted paludicultural willow and cattail biomasses as feedstocks for mycelial bioconversion. Unextracted paludicultural willow and cattail biomasses both showed promise as feedstocks for basidiomycete fungi, indicated by hyphal extension, mycelial growth and high ergosterol yields across all species. Interestingly, HCE had an enhancing effect on both hyphal extension (Fig. 3) and ergosterol yields compared to unextracted samples (Fig. 5). This may be largely due to hydrodynamic cavitation-induced disruption of the biomass' physical structure, which may have suited hyphal access to carbohydrates as lignocellulose-bound polysaccharides are transferred to more easily metabolizable locations [75]. The altered carbohydrate accessibility, microcrystalline cellulose digestibility and preservation of hemicelluloses (see Fig. 2b) via HCE are potential mechanisms by which fungal metabolism and hyphal formation are unimpeded [76–78]. Pre-extraction via two-phase PHWE significantly reduced the mycelial bioconversion efficacy of willow, indicating it's potential use as an alternative process to heat-treatment for enhancing microbial resistance in processed wood materials [79]. Higher hemicellulose content observed in PEB$_{\text{HCE}}$ compared to PEB$_{\text{PHWE}}$ likely result from significant differences in temperature and pressure during extraction, as more intense conditions (temperatures >160 °C) are needed to hydrolyse the glycosidic bonds in hemicellulose. Tienaho et al. (2025) found that HCE released more glucose from glucans and removed a higher degree of terpenes when compared to a similar PHWE setup, whereas PHWE removed greater yields of total sugars, oligosaccharides, polysaccharides and condensed tannins from Norway spruce bark [47]. HCE seemed to result in higher degradation of acid-insoluble lignin compared to PHWE (Fig. 2a), which again signifies different lignin degradation traits. Greater reduction in acid-insoluble lignin content in cattail (−39.54 ± 3.16 %) compared to willow (−13.98 ± 3.03 %) during HCE treatment may also reflect the fundamental differences in lignin structure of grassy cattail biomass (containing higher proportions of p-hydroxyphenyl (H) units) compared to woody SRC willow biomass (predominantly guaiacyl (G) and syringyl (S) units), whereby H units in grassy cattail biomass may be more susceptible to cleavage under mild HCE conditions. Further clarity in chemical composition could be gained by integrating analyses of the recovered liquid extracts from each PEB and comparing relative extractive content to infer what remains in the PEB.

Hyphal extension was generally faster across all PEB$_{\text{HCE}}$ samples compared to untreated biomasses and inhibited across all PEB$_{\text{PHWE}}$ samples (Fig. 3). Basidiomycete species with comparable hyphal extension rates on both PEB and unextracted biomass were *T. versicolor* and *T. hirsuta* on willow$_{\text{HCE}}$, and *P. floridanus* and *T. hirsuta* on cattail$_{\text{HCE}}$. Apart from minor initial phase growth for the first 3 days following plug inoculum (as described by Ref. [80]), mycelial growth on PEB$_{\text{PHWE}}$ was assumed terminal until microscopy on day 13 verified faint, but active hyphal extension with low resolution of hyphal branching and density (Fig. S5). Hyphal branching seemed more exploratory in nature (extension of sparsely-branched hyphal systems), but with very weak proliferation [78]. Ergosterol yields for bioconverted PEB$_{\text{PHWE}}$ were also very low in comparison to other biomasses (Fig. 5). PHWE treatment significantly reduced fungal biomass across all tested species compared to unextracted biomasses (5.7 ± 4.9 μg/g d.w.; p = 0.021), demonstrating a consistent inhibitory effect of pressurised hot water extraction on basidiomycete colonization. The weak growth may be due to lack of key nutrients in PEB$_{\text{PHWE}}$, either hemicelluloses (Fig. 2), minerals or other carbohydrates that are essential for mycelial growth [81,82]. L-arabinose was particularly low in PEB$_{\text{PHWE}}$ relative to the other treatments, which is an essential carbon source in induction of fungal enzymatic gene expression during cultivation [83–85]. It is known that absence of L-arabinose as a carbon-source may inhibit not only fungal L-arabinose regulators but also d-galactose catabolism [86]. PEB$_{\text{PHWE}}$ contained lower observable Mn, K and Mg compared to the other biomasses, which may also inhibit myceliation. Although Mn is necessary

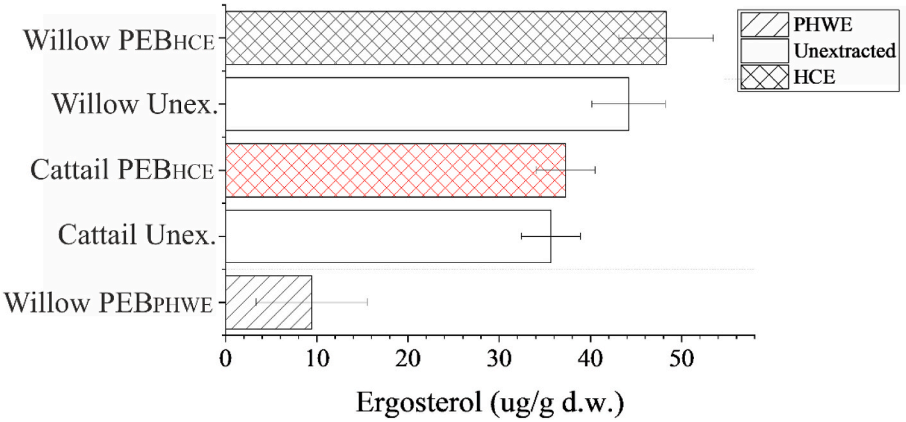

Fig. 5. Estimated ergosterol content (mean and standard error) measured across all biomass types for all fungal species. N = 30 for cattail and N = 60 for willow.

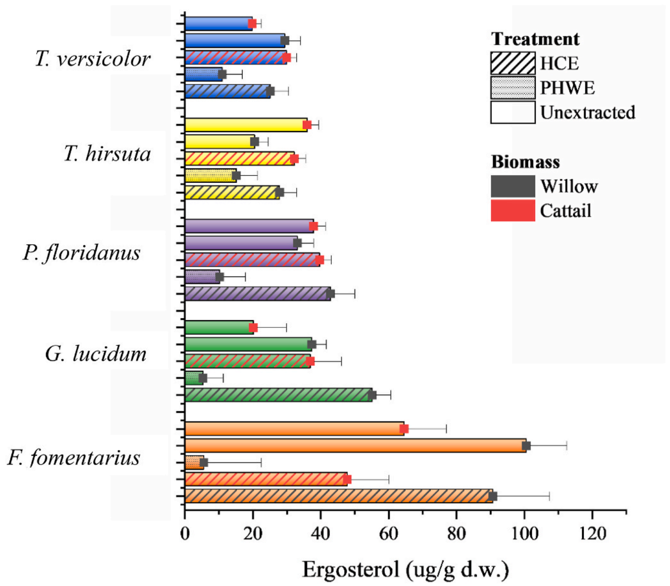

Fig. 6. Estimated ergosterol content (mean and standard error) for mycelial converted biomasses across all five species (Unex = unextracted; HC = hydrodynamic cavitation; PHWE = pressurised hot water extraction). N = 30 for cattail and N = 60 for willow.

for triggering fungal Mn-dependent peroxidases and critical for basidiomycete metabolism [87,88], even extremely small quantities of these minerals can induce vegetative growth [70]. Moreover, the presence of Cu in PEB_{PHWE} in similar quantities to other biomasses indicates that laccase and other multicopper oxidases should not be affected. The lack of phenolics and other extracted constituents in PEB_{PHWE} may have significantly reduced fungal enzymatic activity [88]. Extensive depletion of hemicellulose content, as seen in PEB_{PHWE}, can also increase moisture-accessibility in lignocellulose microfibrils [89,90] that can potentially negatively affect hyphal extension due to higher cellular moisture content. The high pressures in PHWE leads water to penetrate into the biomass, hydrolysing cellulose and possibly exposing more fermentation inhibitors [91]. Given that PHWE alters microfibril nanoscale architecture [92], the modified structural properties of PEB_{PHWE} samples may be incompatible with wood-decay basidiomycete metabolic requirements. We hypothesize that the significant depolymerization and disruption of lignocellulose microfibril alignment during PHWE treatment compromises the biomass' suitability for basidiomycete fungal colonization and enzymatic degradation [93].

Total C content of mycelial bioconverted samples reflects carbon that is innate (undigested, remaining in the plant biomass), assimilated (transformed into the mycelial biomass) and respired (given off as CO_2 due to fungal respiration), and correlates with fungal nitrogen utilisation, assimilation and storage [94]. One possibility of higher total N may be atmospheric N-fixation due to N-fixing symbionts that may have entered the samples during incubation [95]. Another possibility could be slight variation in N measurement via LECO combustion between the sterile lignocellulosic biomass and chitinous myceliated biomasses. Basidiomycete digestibility of lignocellulosic materials are particularly sensitive to C:N ratios, and these ratios differed slightly between willow and cattail across treatments. While willow PEB_{PHWE} significantly inhibited mycelial growth, PEB_{HCE} for both willow and cattail performed well cross all five species, indicating that hydrodynamic cavitation may serves as a viable upstream processing step for lignocelluloses cascaded into mycelial applications. These findings highlight the future added-value possibilities of channelling selective extraction residuals towards mycelial bioconversion applications, in this case paludicultural PEB_{HCE}. Further research is warranted to determine what other extraction types and conditions can provide synergises for industrial lignocellulosic PEB towards downstream mycelial applications.

5. Conclusion

Results demonstrated that pre-extraction of willow and cattail via hydrodynamic cavitation (HCE) did not inhibit mycelial growth, suggesting potential compatibility with mycelial bioconversion applications. In contrast, pressurised hot water extraction (PHWE) significantly inhibited mycelial bioconversion of pre-extracted willow biomass, suggesting its potential use in the prevention of myceliation or fungal growth in applications and that pre-extraction conditions could be tailored to facilitate optimal downstream mycelial bioconversion of cascading biomasses. Depending on the downstream applications desired, further studies are warranted to assess the necessary criteria such as structural integrity (towards functional materials) or nutritional value (towards functional foods) of myceliated pre-extracted biomasses. These findings also highlight that paludicultural biomasses are viable and sustainable feedstocks for generating myceliated products. Further studies could explore future strategies for fungi-based applications that valorise paludicultural biomasses in spent, extracted or unextracted forms to promote greater adoption of paludiculture as a healthier land management practice boasting ecosystem services and potential economic yield.

CRediT authorship contribution statement

Peter Petros: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pauliina Lankinen:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Sylwia Adamczyk:** Writing – review & editing, Methodology. **Francesco Meneguzzo:** Writing – review & editing, Methodology. **Luca Tagliavento:** Methodology. **Kalle Kaipanen:** Methodology, Formal analysis, Data curation. **Petri Kilpeläinen:** Writing – review & editing. **Janne Kaseva:** Software, Formal analysis. **Kristiina Lång:** Writing – review & editing, Methodology. **Kirsi S. Mikkonen:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Tuula M. Jyske:** Writing – review & editing, Supervision, Methodology, Conceptualization.

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.biombioe.2025.108675.

Data availability

Data will be made available on request.

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