# Investigating the technical and economic potential of solid-state fungal pretreatment at nonsterile conditions for sugar production from poplar wood

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## Abstract

Pretreatment is crucial for the conversion of lignocellulose to biofuels. Unlike conventional chemical/physicochemical methods, fungal pretreatment uses white-rot fungi and mild reaction conditions. However, challenges including substrate sterilisation, long duration and low sugar yields associated with this method contribute to lower techno-economic performance, an aspect that has rarely been investigated. This study aimed to evaluate the feasibility of fungal pretreatment of non-sterilised poplar wood. Various factors, including inoculum types, fermentation supplements and cultivation methods, were investigated to optimise the process. A techno-economic assessment of the optimised processes was performed at a full biorefinery scale. The scenario using non-sterilised wood as substrate, pre-colonised wood as inoculum and a 4-week pretreatment showed a 14.5% reduction in sugar production costs (€2.15/kg) compared to using sterilised wood. Although the evaluation of non-sterilised wood pretreatment showed promising cost reductions, fungal pretreatment remained more expensive than conventional methods due to the significant capital investment required.

## 1 Introduction

Lignocellulose is the most abundantly available source of carbohydrates for the sustainable fermentative production of biochemicals and biofuels[1,2]. However, it is highly recalcitrant to enzymatic saccharification and therefore requires a pretreatment step to enhance the accessibility of cellulose to hydrolytic enzymes[3,4]. The commonly used chemical and physicochemical pretreatment technologies need harmful chemicals and/or high energy input and often generate by-products that inhibit fermentative microorganisms[5,6].

47 Fungal pretreatment is a biological alternative that uses white-rot fungi in a solid-state
48 fermentation system to effectively degrade lignin and reduce biomass recalcitrance [7].
49 Compared to the conventional chemical/physicochemical pretreatment methods, solid-
50 state fungal pretreatment is operated under mild reaction conditions, with low wastewater
51 generation, no or limited addition of chemicals and no production of inhibitors, reducing
52 the need for washing and/or detoxification prior to enzymatic hydrolysis[3,8]. However, it
53 also suffers from potential weaknesses, including feedstock sterilisation requirement,
54 long pretreatment time and relatively low saccharification yield due to the inefficient and
55 non-selective lignin degradation[9–11]. The latter is caused by the cellulase enzyme
56 production by the white-rot fungi, which results in the undesired degradation of cellulose
57 with a lower glucose yield after the enzymatic hydrolysis process step. The low
58 delignification selectivity is indicated by the selectivity value (SV), which is defined as
59 the ratio of lignin degradation and cellulose consumption[11,12]. These drawbacks
60 associated with fungal pretreatment result in lower techno-economic performance, an
61 aspect that has only been investigated in a few studies. The work by Baral and Shah [13] on
62 a corn stover-to-butanol biorefinery showed significantly higher capital investment and
63 sugar production costs compared to conventional chemical and physicochemical
64 methods. These higher costs were attributed to the greater amount of feedstock required
65 to produce an equivalent amount of fermentable sugars, and the longer pretreatment time,
66 both of which contribute to the need for larger reactors.
67 In the study by Vasco-Correa and Shah[9], the production of fermentable sugars from
68 various feedstocks in a fungal pretreatment-based biorefinery resulted in significantly
69 higher sugar production costs compared to previous studies using chemical and
70 physicochemical pretreatment methods. The authors identified several key challenges

71 associated with the fungal pretreatment process, including the long duration, low sugar
72 yields, low feedstock bulk density and the need for feedstock sterilisation. Therefore, the
73 optimisation of operating parameters plays a crucial role in improving the economic
74 viability of fungal pretreatment. One important parameter is the elimination or reduction
75 of substrate sterilisation requirements. This approach has the potential to improve the
76 techno-economic feasibility of the process, which has only been evaluated using sterilised
77 feedstock in previous studies.
78 Most fungal pretreatment studies have been performed on sterilised feedstock to prevent
79 indigenous microbial communities from competing with the white rot fungi. Steam
80 sterilisation is the routinely used sterilisation method with a sterilisation time typically
81 ranging from 15 to 60 minutes[14–16]. In research, only a few attempts have been made on
82 the fungal delignification of non-sterilised feedstock. The direct inoculation of the
83 feedstock with the fungal mycelium, which was proven to be effective on sterilised
84 feedstock, often led to low pretreatment efficiencies when used on non-sterilised
85 feedstock [17–19]. However, a few studies have reported effective fungal pretreatment using
86 an inoculation strategy that is uncommon in the field of fungal pretreatment, i.e., first
87 growing the white rot fungi on sterilised feedstock and then using the pre-colonised
88 substrate as inoculum during fungal pretreatment.
89 In the study by Zhao et al.[20], the fungal pretreatment of non-sterilised yard trimmings
90 using yard trimmings pre-colonised with *Ceriporiopsis subvermispora* as an inoculum
91 resulted in comparable degradation of lignocellulose components to that obtained in the
92 sterilised system inoculated with fungal mycelium. In the work of Vasco-Correa et al.[21],
93 the use of pre-colonised feedstock as inoculum resulted in an effective fungal
94 pretreatment of non-sterilised miscanthus, softwood (*Pinus sp.)* and hardwood (*Fraxinus*

*Americana)* but was unsuccessful for corn stover. Indeed, it is well known from the literature that the effectiveness of fungal pretreatment is highly dependent on the applied substrate and microorganism[17,22]. The inoculation technique of applying pre-colonised feedstock has not yet been studied for the fungal pretreatment of non-sterilised poplar wood by *Phanerochaete chrysosporium.*

In a previous study by Wittner et al.[23] fungal delignification of poplar wood was optimised using sterilised poplar wood as a substrate. The inoculum consisted of a spore suspension of *P. chrysosporium*, and a complex fermentation medium supplemented with optimal concentrations of $Mn^{2+}$ and $Cu^{2+}$ was used to enhance delignification.

The present research aims to improve the economic feasibility of this process by reducing the sterilisation requirement through the use of non-sterilised poplar wood as a substrate. The main research objectives include the comparison of two inoculation techniques, namely spore inoculation and pre-colonised wood inoculation, and the evaluation of different fermentation supplements (metal ions with or without glucose and sodium nitrate) and cultivation methods (sterile vented bottles and open trays) to optimise the non-sterile fungal pretreatment process.

The degradation of lignocellulose in non-sterilised wood, the resulting enzymatic saccharification yield and the economic feasibility of the process are compared with those obtained from sterilised wood.

## 2 Materials and Methods

### 2.1 Lignocellulose substrate

Poplar wood sawdust was obtained from Sawmill Caluwaerts Willy (Holsbeek, BE) and used as a substrate for solid-state fungal pretreatment. Particle size distribution was determined by sieve analysis, with 86.1% w/w of wood particles lying between the 2 mm

and 0.075 mm sieves [23]. The composition of the raw feedstock was 45.1 ± 1.0% w/w glucan, 30.8 ± 0.8% w/w total lignin and 17.1 ± 0.8% w/w xylan (Table S1) determined by the standard NREL protocol (NREL/TP-510-42618)[24] as described in Section 2.5.1. The sawdust was used with or without sterilisation, depending on the solid-state fermentation (SSF) set-up. Sterilisation was performed by autoclaving at 121 °C for 20 min.

## 2.2 Fermentation media

Five fermentation media, including metal salts alone (M), with glucose (M+G), with $NaNO_3$ (M+N), with glucose and $NaNO_3$ (M+G+N) and with a complex medium (M+CM), were examined in this study. The metals salts were used in 3.69 mM $CuSO_4$ and 1.41 mM $MnSO_4$ concentrations, creating the optimal metal ion dosages of 2.01 µmol $Cu^{2+}$ and 0.77 µmol $Mn^{2+}$ $g^{-1}$ dry weight (DW) wood for enhanced delignification [23]. The glucose and $NaNO_3$ were added at concentrations of 20 g/L and 3 g/L, respectively. The complex medium, i.e., the standard medium used in the optimisation study of Wittner et al.[23], contained 20 g/L glucose, 3 g/L $NaNO_3$, 0.5 g/L $FeSO_4 \cdot 7H_2O$, 0.5 g/L KCl, 1 g/L $KH_2PO_4$, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.1% v/v Tween 80, 0.34 g/L veratryl alcohol, 3.69 mM $CuSO_4 \cdot 5H_2O$ and 1.41 mM $MnSO_4 \cdot H_2O$. All media were sterilised by autoclaving at 121°C for 20 min.

## 2.3 Inoculum preparation

*Phanerochaete chrysosporium* (MUCL 19343) was purchased from the Mycothèque of the Catholic University of Louvain (Belgium). Two different inoculation strategies, i.e., inoculation with spore suspension and with pre-colonised poplar wood, were tested. Spore suspension of $5 \cdot 10^6$ spores/mL was prepared in distilled water using 5 days old *P. chrysosporium* cultures grown on potato dextrose agar plates at 39°C. Pre-colonised

poplar wood was obtained by performing SSF in sterile vented Schott bottles (DURAN®), as described in the study of Wittner et al.[23]. In brief, the bottle (1 L) contained 36.7 g dry-weight sterilised poplar wood, 20 mL sterile M+G+N media, 37 mL spore suspension ($5 \cdot 10^6$ spores/g DW wood) and sterile distilled water creating a moisture content of 75% w/w on a wet basis. The wood was sterilised by autoclaving directly in the Schott bottle at 121°C for 20 min. The SSF bottles were rolled on a bottle roller (88881004 Bottle/Tube Roller, Thermo Scientific™) at 4 rpm and incubated (TC 255 S, Tintometer Inc.) at 37°C for 4 weeks. The duration of fermentation was chosen as 4 weeks SSF is required for increased ligninase enzyme production [23]. The obtained pre-colonised wood contained 4.49 ± 0.09 mg fungal biomass/g dry wood as determined by ergosterol measurement[25]

## 2.4 Solid-state fungal pretreatment

Solid-state fermentations were carried out at varying fermentation conditions. The pretreatments differed in the applied substrate sterilisation (none or autoclaving at 121 °C for 20 min), medium composition (Section 2.2), inoculation strategies (Section 2.3) and fermentation set-up (rolling bottles or trays).

Bottle fermentation (B) was carried out in 100 mL Scott bottles closed with sterile vented screw caps with a welded-in 0.2 µm PTFE membrane (DURAN®) as described in the study of Wittner et al.[23]. The spore-inoculated bottle SSFs contained 3.67 g DW poplar wood, 2 mL sterile medium, 3.7 mL spore suspension ($5 \cdot 10^6$ spores/g DW wood) and sterile distilled water creating a moisture content of 75% w/w on a wet basis. The bottle SSFs inoculated with pre-colonised wood contained 0.9 g DW of freshly harvested pre-colonised wood and 2.8 g DW untreated wood (mixing ratio of 1:3), 2 mL sterile medium and sterile distilled water to obtain the 75% moisture content. The bottles were rolled at

4 rpm on a bottle roller (88881004 Bottle/Tube Roller, Thermo Scientific™) and incubated (TC 255 S, Tintometer Inc.) at 37°C for 4 weeks.

Tray fermentations (T) of non-sterilised poplar wood were carried out in 500 mL glass crystallizing dishes (flat bottom without spout, LABSOLUTE®) containing 0.9 g freshly harvested pre-colonised wood as inoculum, 2.8 g DW untreated non-sterilised wood in a layer of approximately 1 cm, 2 mL sterile medium and sterile distilled water were added to obtain a final moisture content of 75%. Tray fermentations were carried out for up to 4 weeks at 37°C in a humidified chamber placed in a Heratherm IMH 100 incubator (Thermo Scientific™). The humidified chamber was built by bubbling compressed air through an aeration stone at an aeration rate of 3 L per minute into the water bath placed at the bottom of the chamber.

The bottle and tray fermentations were performed in triplicate and duplicate, respectively. Negative controls consisted of untreated non-sterilised wood without fungal inoculation under the same conditions as the pretreatment.

## 2.5 Analytical methods

### 2.5.1 Compositional analysis

Before compositional analysis, the pretreated biomass samples were thoroughly washed to remove the produced lignocellulolytic enzymes and other water-soluble substances. One washing cycle included the shaking of the pretreated biomass (400 rpm, 20 min) with 50 mM acetate buffer (pH 4.5) using a solid-to-liquid ratio of 1:80, followed by centrifugation (Sigma 3-16KL) for 15 min at 4500 rpm and 4°C. After removing the supernatant, the rinsing cycle was repeated once with acetate buffer and twice with distilled water to remove the traces of acetic acid. The rinsed solid was freeze-dried

(ALPHA 1-2 LDplus, Martin Christ Gefriertrocknungsanlagen GmbH) and used for compositional analysis.

The compositions of the wood samples were determined by the standard NREL protocol (NREL/TP-510-42618)[24]. Briefly, glucan, xylan, acid-soluble (ASL) and acid-insoluble lignin (AIL) content of the samples were measured by two-stage acid hydrolysis with sulfuric acid. ASL was determined at 240 nm ($\varepsilon_{240\ nm}$=25 L/(g·cm)). The lignin content discussed in this paper refers to the sum of AIL and ASL. The glucan and xylan contents in the samples were calculated from the monomeric sugars glucose and xylose, respectively, using the anhydro coefficient of 0.88 for xylose and 0.9 for glucose. The sugar analysis was carried out with an HPLC system (1260 Infinity II LC system, Agilent Technologies) with the detailed procedure described in the study of Wittner et al. (2021). In brief, a Coregel ORH 801 6.5 ID x 300 mm column (Concise separations) equipped with a refractive index detector (55°C) was used. As a mobile phase, 8 mM $H_2SO_4$ was applied at a flow rate of 0.6 mL/min at 75°C.

After fungal treatment, the total dry content of the fermentation bottle/tray was measured and the degradation of the different lignocellulose components (lignin, glucan and xylan) was calculated as a percentage of the initial content of these components in the bottle/tray [23]. Total solid degradation was calculated as the degradation of all components, including lignin, glucan and xylan resulting in the formation of $CO_2$ and water [26,27].

### 2.5.2 Enzymatic saccharification

Enzymatic saccharification of the biomass was performed using the commercial enzyme mixture of Cellic® CTec3 (Novozymes) with a total cellulase activity of 474 FPU/mL determined by the NREL/TP-510-42628 protocol[28] and a total protein content of 130.9 mg/mL determined by Bradford Assay Kit (TCI Europe N.V). Hydrolysis reaction

contained 0.5 g dry biomass (10% w/w solid loading), 0.5 mL of 0.5 M citrate buffer (pH 4.6), 40 µL of cellulase enzyme mixture (38 FPU/g dry biomass equivalent to 10.5 mg protein/g dry biomass), 20 µL of tetracycline solution (10 mg/mL in 70% ethanol), 15 µl of cycloheximide solution (10 mg/mL in distilled water) and 4.425 mL distilled water. Enzymatic saccharification was carried out at 50°C, 240 rpm for 72 hours. The obtained hydrolysates were boiled for 15 min in a water bath to denature the enzymes and centrifuged at 21,500 g for 3 min. The supernatant was filtered through a syringe filter (0.2 µm, PES) and was analysed for its glucose and xylose content by HPLC as described in the study of Wittner et al.[23]. The glucose and xylose yields were calculated relative to the theoretical yield from the raw feedstock[29,30].

In contrast, the enzymatic digestibility is calculated based on the amount of glucose and xylose released relative to the theoretical yield from the pretreated feedstock entering the enzymatic hydrolysis unit; thus, it does not take into account the carbohydrate consumption occurring during the pretreatment process.

When it was applied, the added glucose in the media was subtracted from the glucose yield and enzymatic digestibility.

### 2.6 Statistical analysis

Statistical comparisons were made to assess lignocellulose degradation and saccharification yields. The normality of data distribution was confirmed using the Shapiro-Wilk test, while the homogeneity of variances was assessed using Levene's test. Analysis of variance (ANOVA) followed by Tukey's post hoc test was used. Specifically, one-way ANOVA was applied to evaluate the effect of medium composition or pretreatment time within a given fermentation set-up. In addition, two-way ANOVA was used to examine the effects of the medium composition along with factors such as the

ratio of pre-colonised to untreated wood, cultivation environment or sterile/non-sterile conditions. Jamovi 2.3.26 software was used for all statistical analyses. Statistical significance was set at a level of $p < 0.05$.

## 2.7 Techno-economic assessment

### 2.7.1 Process design and modelling

The production of fermentable sugars from poplar wood was simulated in Aspen Plus® v.12.1 (Aspen Technology Inc.), based on the National Renewable Energy Laboratory (NREL) report on the biochemical conversion of lignocellulosic biomass to bioethanol [31]. Scenario I included feedstock sterilisation, inoculation with fungal mycelium and supplementation with M+G+N medium. Scenario II used non-sterilised wood as substrate, pre-colonised wood as inoculum and M+N supplemented medium. Both scenarios produce 135,000 tonnes of fermentable sugar per year to be comparable to the techno-economic study of Vasco-Correa and Shah[9]. The unit operations were grouped into three units, feedstock preparation through sterilisation, solid-state fungal pretreatment and enzymatic hydrolysis.

### 2.7.2 Data sources

Feedstock properties, fungal pretreatment and enzymatic hydrolysis conditions, degradation of lignocellulosic components during pretreatment and enzymatic saccharification yields were based on the small-scale bottle and tray experiments described in this study (Section 2.1–2.5). Where additional data were required, recently published studies on the fungal pretreatment of lignocellulose were consulted, and the data sources were indicated in the text.

### 2.7.3 Process description

Poplar sawdust is assumed to be delivered ready for pretreatment with 51.9 ± 3.2% dry matter content [32] and no storage is required. Details on the feedstock properties can be found in Table S1. When applied, the raw feedstock was transferred to the horizontal batch autoclaves for 20 min sterilisation with saturated steam at 121°C using a screw conveyor. No dry matter loss was observed during autoclavation, and the alteration in composition was insignificant ($p > 0.05$)[23].

After sterilisation, the feedstock was transferred from the autoclave to the solid-state fungal pretreatment unit, composed of packed bed reactors operating at 37°C for 28 days. This reactor type is recommended for solid-state fungal pretreatment because its static operation is advantageous for microorganisms sensitive to shear stress [16,33] and because it provides sterile conditions during the fungal pretreatment[9]. Similar to the study by Vasco-Correa and Shah[9], inoculation with mycelium suspension grown in yeast mould (YM) broth in an air-lift fermenter was assumed for the SSF of sterilised wood due to insufficient literature data on the large-scale production of white-rot fungi spores. Pre-colonised wood was prepared under the same conditions as SSF of sterilised wood and used to inoculate non-sterilised poplar wood. Cooling water was used to manage the heat generated by the metabolism of the white-rot fungi and maintain the operating temperature during inoculum preparation and fungal pretreatment.

After fungal pretreatment, the pretreated feedstock is transferred to the stirred tank reactor, where the enzymatic hydrolysis takes place, based on the applied laboratory-scale conditions (Section 2.5.2) but without antibiotics. The sugar yields and enzymatic digestibility values were assumed to be identical to those obtained in laboratory-scale conditions (Table S2). The model did not include the utilisation of the lignin-rich solid

residue after enzymatic hydrolysis, which typically means incineration for heat and power generation[31,34]. Detailed process conditions can be found in Table S2 for both Scenarios.

### 2.7.4 Economic analysis

The levelized cost of sugars is calculated for both scenarios, according to Moomaw et al.[35]. The main economic assumptions are shown in Table 1. Capital Expenditure (CAPEX) was determined based on the NREL report of Humbird et al.[31] and ASPEN Capital Cost Estimator. The Operational Expenditure (OPEX) was calculated as the sum of the variable and fixed operating costs. The variable operating cost is calculated based on the mass and energy balances obtained from the simulation models and raw materials prices, while the fixed operating cost is according to Table 1. Cost estimates (in EUR 2022) were adjusted to the scale of the process using the Chemical Engineering Plant Cost Index[36].

**Table 1** Main techno-economic assessment assumptions. (FCI: Fixed Capital Investment; ISBL: Inside the Battery Limits)

| Parameter | Value | Reference |
|---|---|---|
| **Economic parameters** | | |
| Plant lifetime (years) | 30 | Assumption |
| Year of analysis | 2022 | Assumption |
| Discount rate (%) | 15 | [37] |
| Annual operating hours (h) | 8000 | Assumption |
| Working capital | 5% of FCI | [31] |
| Land cost | 2% of FCI | [38] |
| Operator wages (EUR/y) | 33511 | [39] |
| Biomass cost (EUR/t) | 98.27 | Sawmill Caluwaerts Willy (Holsbeek, BE) |
| Enzyme cost (EUR/kg) | 9.64 | [40] |
| **Fixed operating costs** | | |
| Supervision | 25% of operating labour | [41] |
| Direct salary overhead | 50% of operating labour and supervision | [41] |
| Maintenance | 3% of ISBL | [31] |
| Property taxes and insurance | 0.7% of FCI | [31] |

### 2.7.5 Economic global sensitivity analysis

The combined influence of various economic parameters (i.e., fixed capital investment (FCI), discount rate and costs for biomass, enzyme, utilities and chemicals) on the sugar production cost is evaluated by conducting a global sensitivity analysis for both scenarios. This consists of Monte Carlo simulations with 5000 model evaluations by sampling economic parameters simultaneously from a triangular distribution with $\pm$15% deviation from the base case values.

## 3 Results and discussion

### 3.1 Spore-inoculated solid-state fermentation

A comparison was made between the fungal pretreatment efficiency of sterilised and non-sterilised poplar wood using spore suspension as inoculum, five different fermentation media and sterile vented fermentation bottles as cultivation environment. The five media included the metal salts $CuSO_4$ and $MnSO_4$ alone (M), with glucose (M+G), with $NaNO_3$ (M+N), with both of these compounds (M+G+N) and with a complex medium (M+CM) containing glucose, $NaNO_3$, $KH_2PO_4$, KCl, $MgSO_4$, $FeSO_4$, veratryl alcohol and Tween 80 as described in Section 2.2.

In the case of non-sterilised poplar wood, less than 2% of the total solids were degraded for each fermentation medium used (<0.5% in negative controls), see Fig. S1A. These results indicate unsuccessful fungal pretreatment, probably caused by the presence of indigenous microorganisms outcompeting the applied *P. chrysosporium*.

In contrast, solid-state fermentation of sterilised wood showed degradation values ranging from 1.49% to 17.64%. The highest degradation values of 17.64 ± 0.33% and 16.86 ± 0.23% were achieved in the M+G+N and M+CM supplemented fermentations, respectively. The medium composition significantly influenced the degradation of the

individual lignocellulose components, i.e., lignin ($p < 0.001$), glucan ($p < 0.001$) and xylan ($p = 0.021$) (Fig. 1A), and consequently the glucose ($p < 0.001$) and xylose ($p < 0.001$) yields (Fig. S1B).

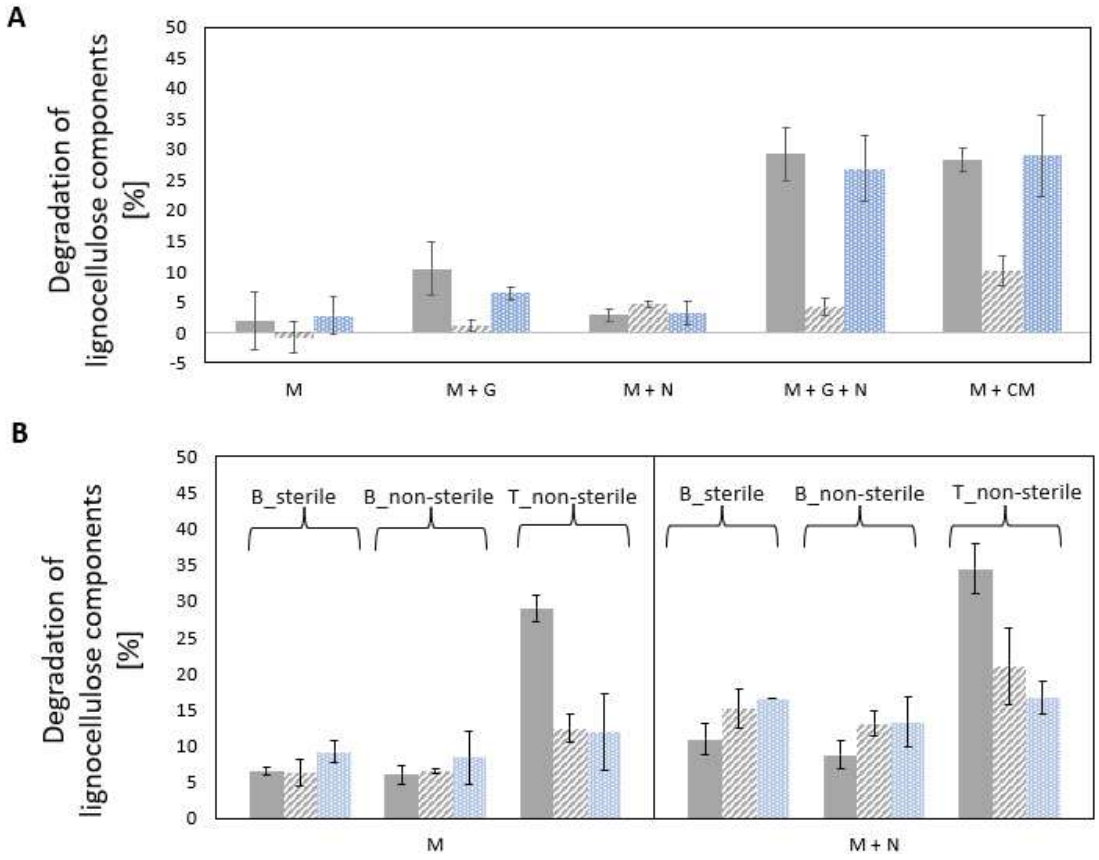


**Fig. 1** Degradation of ( ) lignin; ( ) glucan and ( ) xylan (A) after spore-inoculated pretreatment of sterilised wood in bottles and (B) after pre-colonised wood-inoculated pretreatment of sterilised wood in bottles (B_sterile), non-sterilised wood in bottles (B_non-sterile) and in trays (T_non-sterile) in the presence of metal salts (M), metal salts with glucose (M+G), metal salts with $NaNO_3$ (M+N), metal salts with glucose and $NaNO_3$ (M+G+N) and metals salts with a complex medium (M+CM)

The fermentation using a complex medium (M+CM) resulted in the degradation of 28.29 ± 1.92% lignin, 10.09 ± 2.41% glucan and 28.99 ± 6.63% xylan, giving a delignification selectivity value (SV) of 2.91 ± 0.88. In comparison, in the simplified M+G+N medium,

337 a comparably high lignin and xylan degradation (29.16 ± 4.41% and 26.76 ± 5.41%,
338 respectively) but a significantly reduced ($p = 0.024$) glucan consumption (4.10 ± 1.44)
339 was obtained, resulting in a 2.2 times higher delignification selectivity value (SV = 8.84
340 ± 3.02) and the highest glucose and xylose yields (28.84 ± 2.26% and 27.28 ± 2.72%,
341 respectively) after enzymatic saccharification of the pretreated biomass (Fig. S1B). These
342 results indicate that the addition of both glucose and sodium nitrate is crucial for the SSF
343 process when spores are used for inoculation and that one or more components present
344 exclusively in the complex medium, enhance the degradation of cellulose over lignin. The
345 presence of glucose supports spore germination and mycelial growth[42]. A nitrogen source,
346 i.e., sodium nitrate in this study, plays a complex role in the fungal pretreatment system.
347 Nitrogen is used for fungal growth and enzyme production but must be present in an
348 optimal concentration since nitrogen limitation is important for the production of lignin-
349 degrading enzymes by *P. chrysosporium*[43].
350 Further investigation aiming to reveal which component(s) in the complex fermentation
351 medium (i.e., $KH_2PO_4$, KCl, $MgSO_4$, $FeSO_4$, veratryl alcohol and Tween 80) are
352 responsible for the increased cellulose degradation is out of the scope of this research.
353 In conclusion, the sterilisation of poplar wood prior to fungal pretreatment is essential
354 when spores are used as inoculum. In addition, the use of a simplified medium containing
355 only glucose, $NaNO_3$, $Cu^{2+}$ and $Mn^{2+}$ provides the most selective delignification and
356 highest sugar yields after the enzymatic saccharification of the pretreated and previously
357 sterilised wood.

358 **3.2 Pre-colonised wood as inoculum**

359 The possibility of eliminating substrate sterilisation was also tested with the use of pre-
360 colonised wood as inoculum instead of fungal spores. The main advantage of this

inoculation technique is the presence of the already-grown white-rot fungi and their lignin-degrading enzymes, which promote the faster colonisation and utilisation of the lignocellulosic substrate. An additional benefit of using pre-colonised wood as inoculum is that it eliminates the need for glucose supplementation, which is critical for spore germination when spores are used as inoculum. This hypothesis was also confirmed in our preliminary experiments, where the addition of glucose to the fermentation medium did not improve the pretreatment efficiency and, consequently, the achievable glucose yield after the enzymatic saccharification of the pretreated biomass (Fig. S2). Therefore, in further experiments, only metal ions with or without $NaNO_3$ were investigated as fermentation supplements. In addition to sterile aerated Schott bottles, open trays were also investigated as a cultivation environment for the SSF of the non-sterilised wood, as the omission of the sterilisation step eliminated the need for sterile aeration. The SSF of sterilised wood was tested in Schott bottles for comparison.

The degradation of the individual lignocellulose components, i.e., lignin, glucan and xylan, in the sterilised and non-sterilised wood, is shown in Fig. 1B. In the Schott bottles, the SSF of sterilised and non-sterilised wood resulted in a comparable lignocellulose degradation profile. Compared to the M medium, the supplementation with sodium nitrate (M+N medium) significantly increased the lignin degradation ($p = 0.008$), with a 1.7-fold increase (6.45 ± 0.55% → 10.91 ± 2.20%) in the sterilised wood, and 1.5-fold increase (5.97 ± 1.22% → 8.76 ± 1.86%) in the non-sterilised wood. However, the consumption of carbohydrates, especially the one of cellulose, increased to a greater extent ($p < 0.001$), i.e., 4.6-fold (6.28 ± 1.88% →15.13 ± 2.78%) in the sterilised poplar and 2.0-fold (6.55 ± 0.36% →13.00 ± 1.73%) in the non-sterilised wood. This resulted in a significant

384 reduction ($p = 0.005$) in glucose yield (Fig. S3A) and no significant effect on xylose yield
385 (Fig. S3B).
386 When the results were compared with the use of spores (see Section 3.1), inoculation with
387 pre-colonised wood improved the fungal pretreatment of non-sterilised wood. However,
388 it provided lower lignin degradations and saccharification yields in the sterilised wood.
389 This might be caused by an easier distribution of spores in the heterogeneous SSF, leading
390 to better fungal colonisation or by a different inoculum size. However, it is difficult to
391 make a quantitative comparison for the latter due to the different types of inoculum used.
392 The applied fungal inoculum size was $5 \cdot 10^6$ spores/g DW wood in the case of the spore-
393 inoculated SSF, and 1.12 mg fungal biomass/ g DW wood in the SSF inoculated with pre-
394 colonised wood, as determined by ergosterol measurement[25]. Based on our earlier
395 research, increasing the spore concentration to $10^7$ spores/g DW did not improve the
396 pretreatment efficiency and saccharification yield (data not shown); however, the
397 minimum inoculum size was not researched. Increasing the proportion of pre-colonised
398 wood or adding spores together with pre-colonised wood could potentially further
399 improve pretreatment in both sterilised and non-sterilised wood. However, it should be
400 noted that using a higher proportion of pre-colonised wood will increase the sterilisation
401 requirement of the inoculum preparation. Conversely, the energy requirement for
402 sterilisation could be further reduced by using a lower ratio of pre-colonised to untreated
403 wood for inoculation. However, in this study, the use of a lower mixing ratio (1:6 or 1:9)
404 had a statistically significant negative effect on glucose yields ($p < 0.001$), but no
405 significant effect on xylose yields (Fig. S4).
406 Compared to the bottle experiments with a 1:3 mixing ratio, SSF in trays resulted in
407 significantly higher lignin degradation ($p < 0.001$), i.e., $28.97 \pm 1.82\%$ and $34.45 \pm 3.43\%$

408 in the M and M+N systems, respectively (Fig. 1B). This can be attributed to the higher
409 oxygen availability in the open trays which is markedly important for the ligninase
410 activity of the white-rot fungi[16]. There was no statistically significant difference in
411 lignocellulose degradation between the M and M+N systems. However, the nitrogen
412 supplementation had a significant positive effect on both the glucose ($p = 0.002$) and
413 xylose ($p = 0.006$) yields, probably due to the higher reproducibility of sugar yield
414 determination compared to the compositional analysis which uses a smaller sample size
415 than the enzymatic saccharification (300 mg vs 500 mg) and is very laborious. The
416 increased glucose (28.51 ± 0.28%) and xylose (24.49 ± 1.41%) yields obtained in the
417 M+N-supplemented trays (Fig. S3A–B) indicate that nitrogen supplementation has a
418 positive effect on the pretreatment efficiency when the fermentation conditions are
419 favourable (e.g. adequate colonisation and oxygen availability), and substantial lignin
420 degradation (>20%) can occur, requiring a higher nitrogen availability for ligninase
421 production. Pretreatment durations of less than 4 weeks were also investigated for the
422 M+N-supplemented tray SSFs. Although the glucose yield increased steadily with
423 prolonged pretreatment compared to 3 weeks of SSF, 4 weeks of pretreatment did not
424 significantly increase the saccharification yields (Fig. S3C). These results indicate that
425 the fungal pretreatment went fast and that the gain in saccharification yield was limited
426 after more than 3 weeks of pretreatment in trays.
427 In conclusion, 28.51 ± 0.28% glucose yield was obtained for the 4 weeks of tray
428 fermentation of non-sterilised wood, i.e., a similar value to the one measured in the sterile,
429 spore-inoculated system (28.84 ± 2.26%). However, tray fermentation using only $NaNO_3$
430 and a small amount of metal ions, but no glucose supplementation, eliminated the need
431 for sterile aeration and required sterilisation of only 28.83% of the feedstock. The latter

was necessary to produce pre-colonised wood given the 1:3 mixing ratio and the loss of dry matter (17.70 ± 0.26%) during inoculum preparation.

Although the glucose yield obtained in this optimisation study is significantly lower than that already achieved by chemical pretreatment (>90%), it is comparable to the average glucose yield (~30%) obtained from fungal pretreatment of sterilised hardwood [9]but with the advantage of reduced sterilisation requirements.

### 3.3 Techno-economic assessment

#### 3.3.1 Economic performance of process scenarios

Following the laboratory-scale optimisation studies, a techno-economic analysis was carried out considering a sugar production scale of 135,000 tonnes per year (16.9 tonnes per hour). This production scale is capable of feeding a biorefinery producing approximately 76,500 $m^3$ of ethanol per year, assuming 90% and 80% of the theoretical conversion of glucose and xylose to ethanol, respectively[31]. Two process scenarios were compared differing primarily in substrate sterilisation and subsequently in inoculation strategy and nutrient input. Fig. 2 shows an overview of the two process scenarios and overall material balances. The economic analysis resulted in a levelised sugar production cost of €2.51/kg in Scenario I, which was reduced by 14.5% (€2.15/kg) in Scenario II. The latter was approximately 17% lower than previously reported for the fungal pretreatment-based biorefinery of hardwood at the same sugar production scale[9]. However, it was still 7-13 times higher than estimated in wood biorefineries using conventional chemical and physicochemical pretreatment[44].

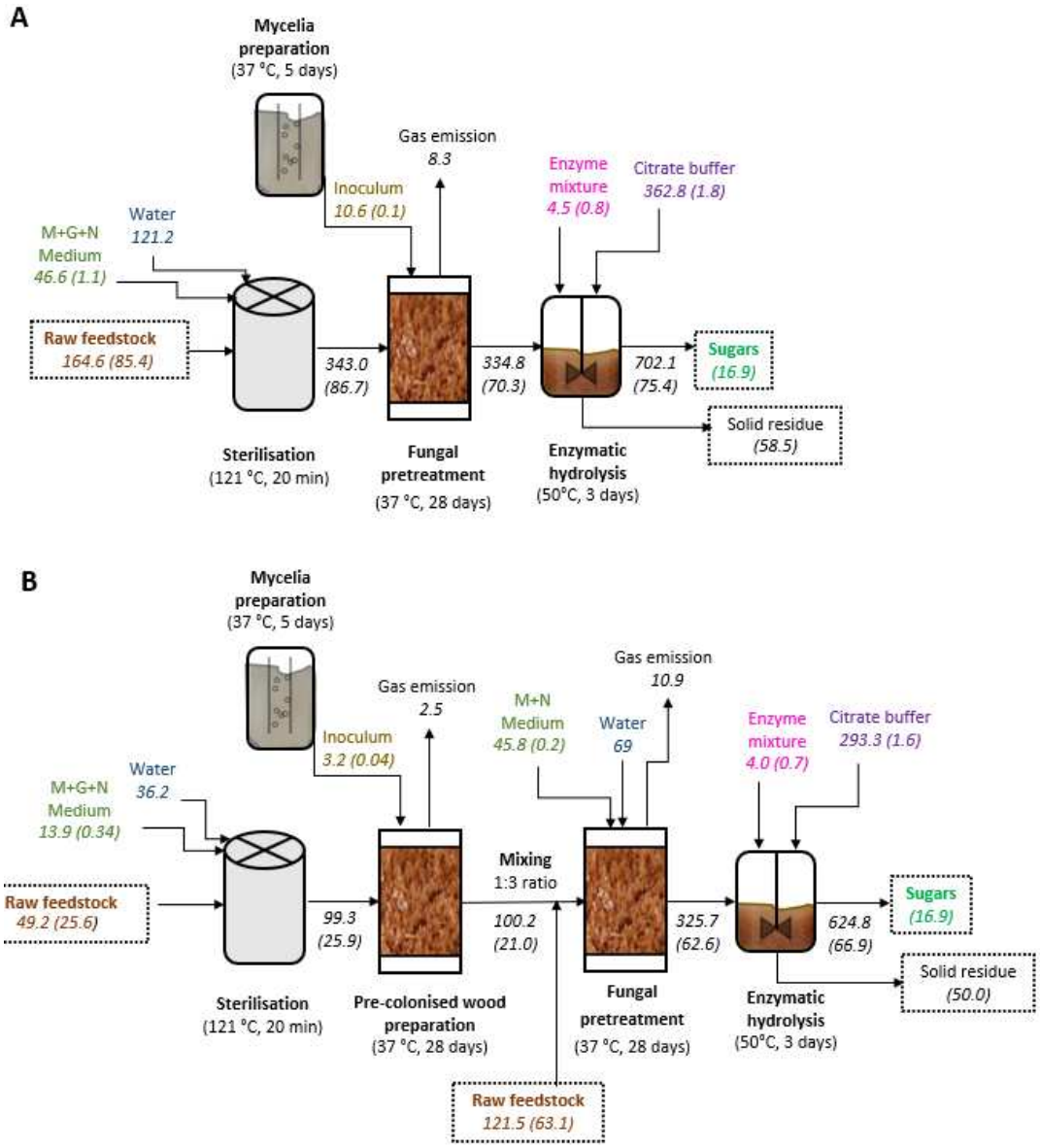


**Fig. 2** Process flow diagrams and mass balances are presented for a fungal pretreatment-based wood biorefinery producing 135,000 tonnes of sugar per year (16.9 tonnes per hour) in two scenarios: (A) Scenario I and (B) Scenario II. The feedstock is sterilised by autoclaving. Fungal mycelium is prepared in an air-lift fermenter. Solid-state fungal pretreatment and pre-colonised wood preparation are performed in a packed bed bioreactor. Wet mass flows are shown in italics, with the equivalent dry mass values in brackets. Please note that values may not add up exactly due to rounding. Aeration flows are not included in the graphs.

462 The annual sugar production costs were further broken down into capital expenditure
463 (fixed capital, working capital and land costs) and operating expenditure (fixed and
464 variable) in order to identify the main cost drivers and to explain the reduced production
465 costs in Scenario II (Fig. 3A). The two scenarios showed a similar trend in terms of cost
466 drivers, with some notable findings. In both scenarios, the largest cost contributor was the
467 annual variable operating costs (Fig. 3A), with utilities, raw materials and enzymes being
468 the main drivers of these costs (Fig. 3B). However, a 26.8% lower annual operating cost
469 (€183 M) was obtained in Scenario II, compared to Scenario I. This is mainly due to the
470 2.7 times lower (€30 M) annual utility costs as a result of the reduced sterilisation
471 requirements in Scenario II, leading to lower steam and cooling water consumption (Fig.
472 3B). In addition, the enzyme and nutrient inputs required (YM broth and glucose) were
473 also lower in Scenario II. The lower nutrient input is due to the use of pre-colonised wood
474 as inoculum instead of mycelia grown in YM broth, which allows fungal pretreatment
475 with $NaNO_3$ and metal ions as the only supplements without the need for glucose. The
476 decreased enzyme cost can be explained by the increased enzymatic digestibility (38.44%
477 vs 35.49% for glucose) measured in Scenario II compared to Scenario I (Table S2). In
478 Scenario I, raw material and enzyme costs accounted for 26.9% and 30.9% of the variable
479 operating costs, respectively, while in Scenario II, they represented 27.9% and 27.5%,
480 respectively. Despite advances in technology, the cost of feedstock and enzymes remains
481 a significant challenge for lignocellulose-based biorefineries as it represents a large
482 proportion of the product cost[45]. As commercial hydrolytic enzymes continue to advance,
483 enzyme dosages and costs may decrease, thereby reducing the overall enzyme cost as a
484 proportion of the sugar production cost in fungal pretreatment plants. In addition, on-site
485 enzyme production can play a key role in leveraging low-cost sugar production[46].

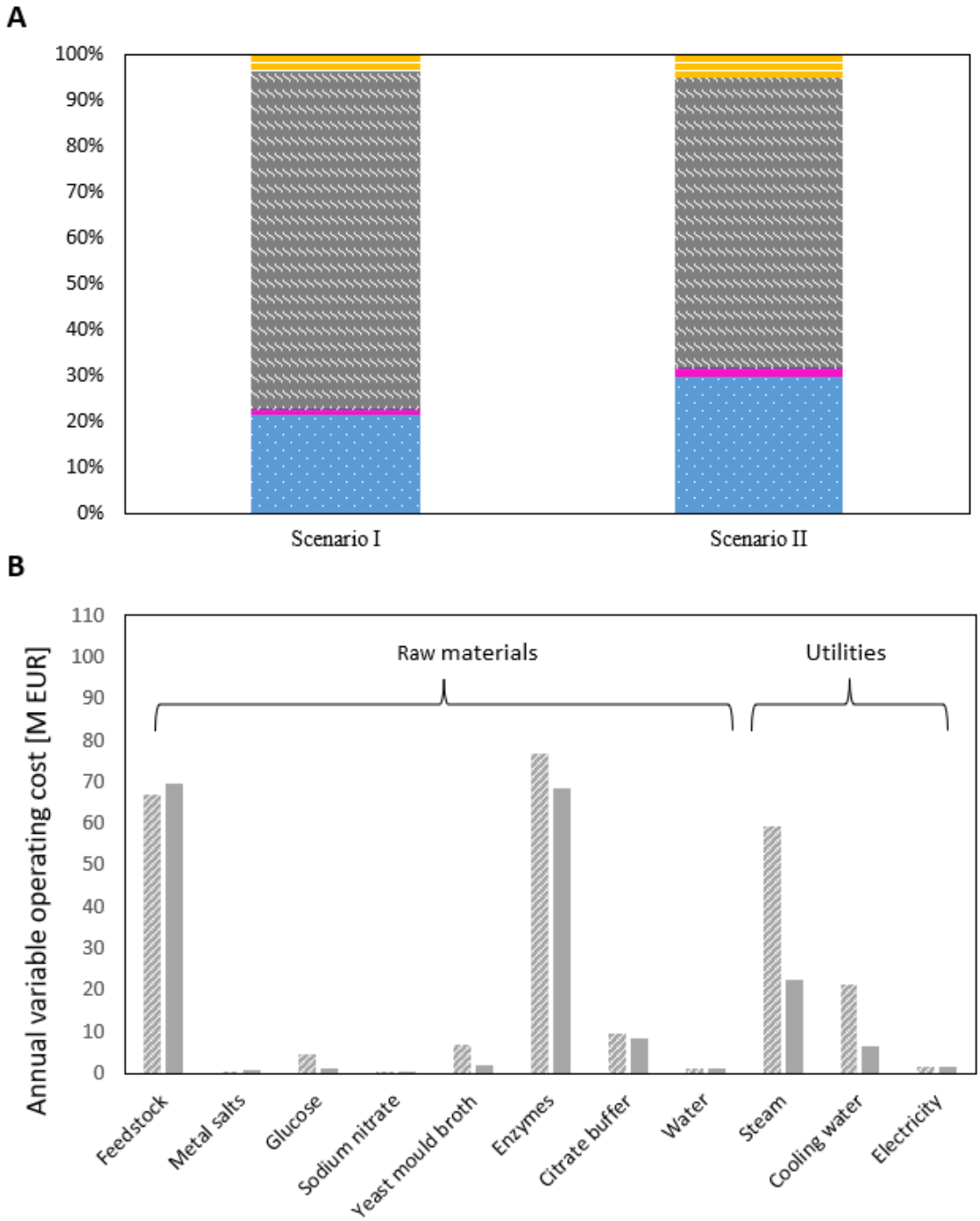


**Fig. 3** (A) The contribution of ( ) fixed capital investment, ( ) working capital, ( ) land cost, ( ) variable operating and ( ) fixed operating cost to the levelised sugar production cost. (B) Annual variable operating costs in ( ) Scenario I and ( ) Scenario II.

The second largest cost contributor was the fixed capital investment (Fig. 3A) which was €476 M and €569 M in Scenario I and II, respectively. These FCIs were approximately

493 2-3 times lower than estimated in other studies evaluating fungal pretreatment-based
494 facilities[9,13] but 4-6 times higher than those found for chemical and physicochemical
495 pretreatment technologies at a comparable sugar production scale[13]. The higher capital
496 investment required for biological pretreatment can be attributed to the need for large
497 equipment in high quantities (Table 2) to produce the same amount of fermentable sugars
498 as conventional pretreatment methods, which are generally faster and offer higher
499 saccharification yields[13]. The 1.2 times higher FCI in Scenario II compared to Scenario I
500 can be explained by the additional equipment used to prepare pre-colonised wood (Table
501 2).

502 **Table 2.** Size, quantity and installation cost of major equipment used in Scenario I and II

| Process | Major equipment | Equipment size [$m^3$] | Number of vessels [-] | | Installation cost in 2022 [M EUR] | |
|---|---|---|---|---|---|---|
| | | | Scenario I | Scenario II | Scenario I | Scenario II |
| **Mycelia preparation** | Airlift fermenter | 303 | 5 | 2 | 4.51 | 1.80 |
| **Sterilisation** | Autoclave | 34 | 6 | 2 | 4.67 | 1.35 |
| **Pre-colonised wood** | Packed bed reactor | 3000 | | 21 | | 61.26 |
| **Fungal pretreatment** | Packed bed reactor | 3000 | 69 | 67 | 204.86 | 201.44 |
| **Enzymatic hydrolysis** | Stirred tank reactor | 3785 | 14 | 13 | 26.62 | 24.72 |

503

504 The cost of these additional units offsets the reduction in installation costs achieved by
505 the less equipment required for autoclaving, mycelial preparation and enzymatic
506 saccharification in Scenario II. A cost-saving approach could be to use a tray reactor or a
507 pretreatment hall similar to a composting facility since fungal pretreatment of non-
508 sterilised wood does not require a packed bed reactor with sterile conditions. However,
509 due to the lack of relevant reference studies on scale-up and reactor design, the evaluation

of these configurations was outside the scope of this study. Further methods of cost reduction include the evaluation of shorter inoculum preparation times and the implementation of a sequential fungal pretreatment process where the pretreated feedstock from one pretreatment step is used to inoculate the subsequent fungal pretreatment cycle. This sequential process was investigated in the study of Vasco-Correa et al.[21] on four different non-sterilised feedstocks, including miscanthus, corn stover, white ash and softwood pine. However, the sequential fungal pretreatment was ineffective for all feedstocks in the second and third pretreatment cycles. Further research is needed to evaluate the feasibility of a sequential process for the fungal pretreatment of poplar wood with *P. chrysosporium*.

Since the experimental results of tray SSF of non-sterilised wood suggested that the improvement in saccharification yield is limited beyond 3 weeks of pretreatment (Fig. S3C), the economic feasibility of Scenario II using 3 weeks of pretreatment was also evaluated and compared to 4 weeks of pretreatment. By using the shortened pretreatment time, there was a 14% reduction in capital expenditure (€609 M→€526 M) due to the reduced number of parallel reactors required to produce the same amount of sugar per year. However, more feedstock and enzymes were required to achieve the desired sugar production scale resulting in slightly higher annual operating expenditure (€197 M→€203 M). As a result, the final cost of sugar production was at €2.1/kg, only 2.3% lower than in Scenario II.

### 3.3.2 Global sensitivity analysis

The sensitivity of sugar production cost to several economic parameters was investigated by performing a global sensitivity analysis for both scenarios (Fig. 4). The FCI was found to have the highest impact for both scenarios. However, a contribution of 29% was

calculated for Scenario I, while a significantly higher contribution of 41% was obtained for Scenario II, due to its higher equipment costs. The most significant difference between the two scenarios is observed for the cost of utilities, with a contribution of 22% and 3% for Scenarios I and II respectively. This is attributed to the remarkable difference in energy consumption between the two scenarios, mainly related to the need for sterilisation. Discount rate, feedstock and enzyme costs also have a considerable influence on both scenarios, although Scenario II is more sensitive to the discount rate due to its high CAPEX. On the other hand, the sugar production cost is the least sensitive to the cost of chemicals, as the variable operating cost is mainly dominated by the cost of feedstock, enzymes and utilities (for Scenario II).

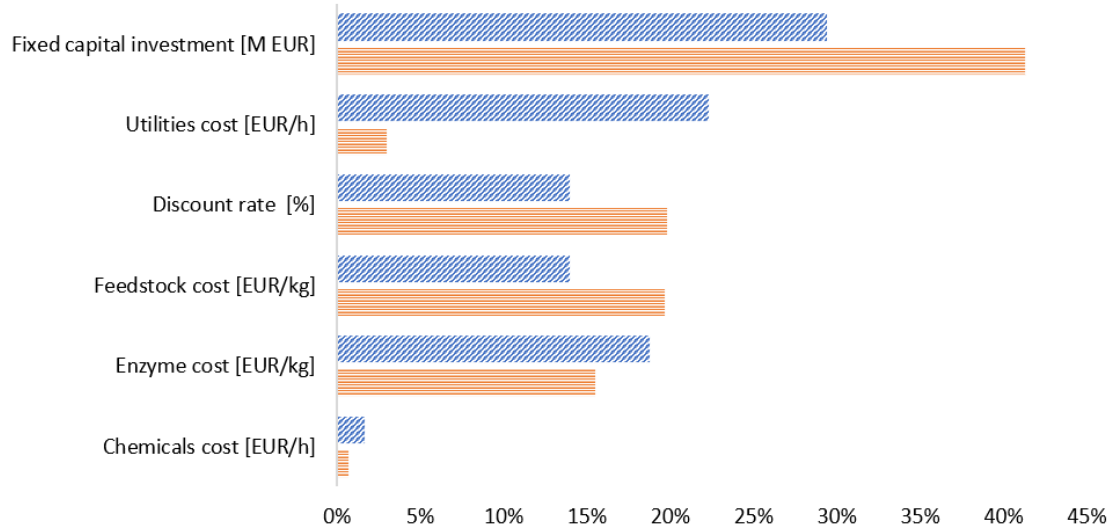


**Fig. 4** Global sensitivity analysis results for ( ) Scenario I and ( ) Scenario II

## 4 Conclusions

Fungal pretreatment needs further development to improve its economics. In this study, a simple solid-state fermentation process was developed using pre-colonised wood to inoculate unsterilised wood in trays without sterile ventilation. Based on this method, a

large-scale process scenario was defined and subjected to a techno-economic evaluation. While a reduced sugar production cost (€2.15/kg) was achieved compared to other fungal wood pretreatment literature, it remains significantly higher than conventional pretreatment methods due to the significant capital investment required. Further research is required to increase sugar yield and reduce pretreatment time for large-scale implementation of fungal pretreatment.

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**Supporting Information**

The following Supporting Information is available free of charge at the ACS website

**Fig. S1** (A) Lignocellulose degradation after spore-inoculated pretreatment of sterilised and non-sterilised wood. (B) The obtained glucose and xylose yield after the enzymatic saccharification of the pretreated, sterilised wood.

**Fig. S2** Glucose yield after the enzymatic saccharification of pretreated, non-sterilised wood inoculated with pre-colonised wood in bottles

**Fig. S3** (A) Glucose and (B) xylose yield after the enzymatic saccharification of sterilised wood pretreated in bottles and non-sterilised wood pretreated in bottles or in trays

**Fig. S4** The obtained glucose and xylose yield after the enzymatic saccharification of wood pretreated in bottles applying different mixing ratios

**Table S1** Feedstock properties

**Table S2.** Fungal pretreatment conditions, lignocellulose degradation and enzymatic digestibility in Scenario I and II

## Abstract Graphics

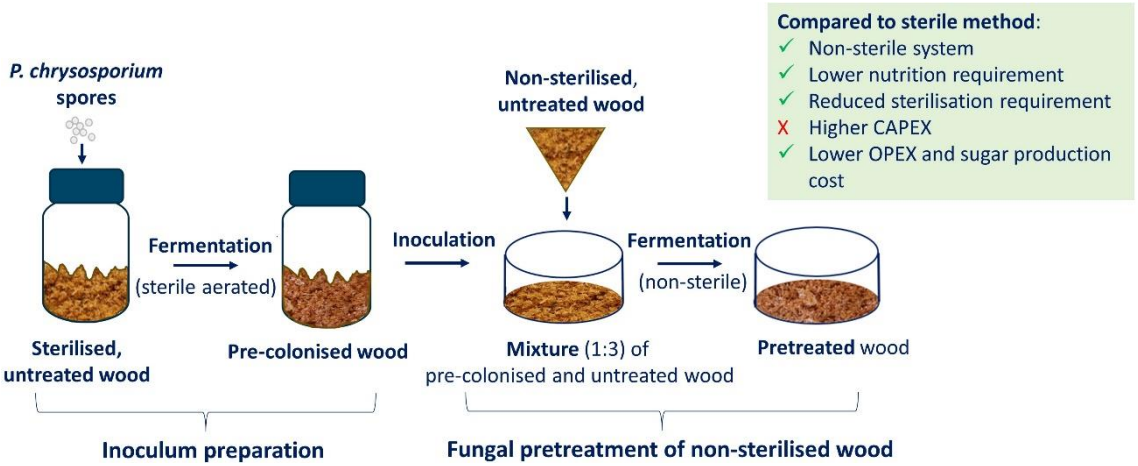

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**Figure captions**

**Fig. 1** Degradation of ( ) lignin; ( ) glucan and ( ) xylan (A) after spore-inoculated pretreatment of sterilised wood in bottles and (B) after pre-colonised wood-inoculated pretreatment of sterilised wood in bottles (B_sterile), non-sterilised wood in bottles (B_non-sterile) and in trays (T_non-sterile) in the presence of metal salts (M), metal salts with glucose (M+G), metal salts with $NaNO_3$ (M+N), metal salts with glucose and $NaNO_3$ (M+G+N) and metals salts with a complex medium (M+CM)

**Fig. 2** Process flow diagrams and mass balances are presented for a fungal pretreatment-based wood biorefinery producing 135,000 tonnes of sugar per year (16.9 tonnes per hour) in two scenarios: (A) Scenario I and (B) Scenario II. The feedstock is sterilised by autoclaving. Fungal mycelium is prepared in an air-lift fermenter. Solid-state fungal pretreatment and pre-colonised wood preparation are performed in a packed bed bioreactor. Wet mass flows are shown in italics, with the equivalent dry mass values in brackets. Please note that values may not add up exactly due to rounding. Aeration flows are not included in the graphs.

**Fig. 3** (A) The contribution of ( ) fixed capital investment, ( ) working capital, ( ) land cost, ( ) variable operating and ( ) fixed operating cost to the levelised sugar production cost. (B) Annual variable operating costs in ( ) Scenario I and ( ) Scenario II.

**Fig. 4** Global sensitivity analysis results for ( ) Scenario I and ( ) Scenario II