Valorisation of sawdust through the combined microwave-assisted hydrothermal pre-treatment and fermentation using an oleaginous yeast

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Abstract

Oleaginous yeast, cultured on second generation lignocellulosic resources, have the potential to be a key part of the future energy sector. However, the multiple unit operations necessary to produce concentrated hydrolysates, with a minimum of fermentation inhibitors, limits the applicability to date. In this study, a simple microwave-assisted hydrothermal pre-treatment step of oak or beech sawdust was deployed to produce an oligosaccharide-rich hydrolysate. This was then catabolised by the oleaginous yeast, *Metschnikowia pulcherrima*, avoiding the need for costly enzymatic or further chemical steps in the processing. Up to 85% of the sawdust's hemicelluloses could be solubilised under these conditions, and 8 g/L DCW yeast with a 42% lipid content produced. While a number of studies have demonstrated that oleaginous yeasts possess high inhibitor tolerance, using this real lignocellulosic hydrolysate we demonstrate that lipid production is actually very sensitive to inhibitor and carbon availability, and the optimal system is not the one that gives the highest hydrolysate or cell biomass. Indeed, the yeast was shown to detoxify the inhibitors in the process, but at high inhibitor loading, this leads to very poor lipid production, especially at high furfural levels. These findings clearly highlight the importance of considering multiple variables when real, complex lignocellulosic media are involved, tuning processes conditions based on the desired fermentation outcomes.

Keywords

Microbial lipids

Metschnikowia pulcherrima

Microwave-assisted pretreatment

Fermentation

Sawdust

Abbreviations

OPEX:	Operating Expenses
M. pulcherrima:	*Metschnikowia pulcherrima*
5-HMF:	5-Hydroxymethylfurfural
Furf:	Furfural
2-PE:	2-Phenylethanol
DPs	Degree of Polymerization
XMG:	Sum of Xylose, Mannose and Galactose
DCW:	Dry Cell Weight
SEM:	Standard Error of the Means
SCC:	Shewart Control Charts
OOC:	Out Of Control
UCL:	Upper Control Line
LCL:	Lower Control Line
SD:	Standard Deviation
μ max:	Maximum growth rate

1. Introduction

In order to reduce the use of fossil resources, combined chemical and biological routes to produce fuels and chemicals from lignocellulosic biomass are needed. Due to the complex, recalcitrant nature of 2nd generation resources, the processing requires multiple stages, including mechanical pre-treatment, chemical treatment, enzymatic hydrolysis and eventually a final concentration step prior to the fermentation [1]. All of these stages are capital intensive and in the production of enzymes, account for up to 20% of the process OPEX [2]. We recently reported on an alternative system where we combined an efficient one-step microwave pre-treatment stage that can solubilise the hemicellulose, proteins and some of the cellulose from lignocellulose, with an oleaginous yeast (*M. pulcherrima*) that can catabolise the resulting complex hydrolysate [3]. This removes the need for enzymes, additive input and separations, prior to the fermentation.

One of the more abundant and sustainable sources of carbohydrate is sawdust produced from the timber and paper making industries [4]. Woody biomass is one of the most challenging feedstocks for the 2nd generation biorefinery and efficient solubilisation of the polysaccharides (e.g. hemicelluloses and cellulose). The use of microwave technology could offer the answer for more rapid, selective and controllable processing of woody biomass [5]. Previous work reported the higher efficiency of the microcrystalline cellulose depolymerisation using microwave heating compared to conventional. For example, at 220 °C, the microwave-assisted hydrothermal treatment generated a glucose yield 50 times higher than the conventional process [6]. Microwave irradiation offers a faster heating rate which allows the reduction of the exposure to harsh conditions, leading to lower production fermentation inhibitors such as furfural or 5-hydroxymethylfurfural [7].

However, the majority of the carbon is still present in oligosaccharides, requiring novel microbial conversions. To this end, we have developed the yeast *M. pulcherrima*, an ideal organism for industrial biotechnology, due to several processing advantages. For example, the yeast has a wide temperature range for cultivation (e.g. 15-35 ℃), [8] within a wide range of pH values, typically from pH 3 to pH 7, though lipid production from *M. pulcherrima* has been also reported at pH 1.9 [9]. *M. pulcherrima* is often used in wine fermentation and is regarded as a safe microorganism due to its non-existing pathogenic activity [10,11]. Like a lot of oleaginous yeasts, *M. pulcherrima* has higher tolerance towards different classes of lignocellulosic-derived inhibitors, such as furan-based (furfural and 5-HMF) and the most common organic acids (formic and acetic) [7,12]. Due to its wide carbon spectrum and little substrate bias, *M. pulcherrima* can easily metabolize the main saccharides found within lignocellulose, including C_5 and C_6 based sugars [9,7,8,12]. Finally, the yeast has the capability to produce high levels of secondary antimicrobial metabolites including 2-phenylethanol (2PE) and

pulcherrimin, as such *M. pulcherrima* can grow in non-sterile conditions, outcompeting other contaminant bacteria [13,14,11,15,16,8]. The yeast is oleaginous and can accumulate over 40% (w/w) of lipids in under 36 hours under optimal conditions [8].

While a number of oleaginous yeast have shown excellent inhibitor tolerance [12] and a handful have shown the ability to metabolise oligosaccharides [17], there are currently no investigations that show the complex interaction between all these factors together. In this paper, we demonstrate that an additive-free one-pot process for the pre-treatment of sawdust is plausible, and investigate the delicate interplay between the sugar concentration, inhibitor loading, growth rate and lipid production in a real-world fermentation system on a complex second-generation feedstock.

2. Material and Methods

2.1 Biomass and chemicals. Oak and Beech's sawdust was supplied from Norske Skoog (Norway), a paper making company. Sawdust was fractionated over 2 mm sieve and only particles below 2mm were used for the study. D-(+)-Glucose (+99%) was purchased from Fluka. Levoglucosan (98%) were purchased from Carbosynth. The 5-hydroxymethylfurfural (98%) were purchased from Acros. Lactic acid (+95%) was purchased from Wardle. Acetic acid (+99%) was purchased from Alfa Aesar. All the other chemicals were purchased from Sigma-Aldrich and Fisher Scientific, at standards analytical grade.

2.2 Pre-hydrolysis sawdust analysis. The water content in the sawdust was measured by drying the biomass at 105°C until constant weight. The ash content was measured after calcination of the dried biomass at 625 °C in air for 4h according to official NREL procedures [18]. The same NREL methodology was adopted to perform the two-step acid hydrolysis for the quantification of cellulose (glucan), hemicelluloses (xylan, arabinan, acetic acid) and Klason lignin (acid-insoluble lignin) in the sawdust [18]. Dry biomass samples were soaked in 72% H_2SO_4 for 2h at 40 °C and then autoclaved at 121 °C in 4wt.% of H_2SO_4 for 1h. The hydrolysate was analysed by HPLC and the residue was filtered in a ceramic porous crucible (porosity 8 µm), washed with distillate water and dried at 105 °C. The acid-insoluble lignin was determined after calcination of the dry residue at 500 °C for 6h.

2.3 Microwave-assisted hydrothermal solubilisation of woody sawdust. Beech and oak sawdust samples of 2 g or 4 g were added into 100mL PTFE vessel containing a magnetic stir bar. The vessels were then filled with 40 ml of distilled water in order to achieve feedstock to water ratio 1:10 wt/wt (for both biomasses) and 1:20 wt/wt (for oak only), respectively. Using Milestone FlexiWave laboratory microwave, the sample was irradiated under varying conditions using 15 min ramp time with a maximum power of 1800W. The hydrothermal reaction temperature ranged from 140 °C to 250 °C. The reaction was stopped once the temperature reached the selected value (no holding

time), and the vessel was air-cooled until 40 °C. The solid residues and hydrolysates were separated on a Buchner flask using filter paper (Fisherbrand, QL100). The hydrolysates of each tetraplicate were mixed and kept in the freezer for analysis. The solid residue was dried at 105 °C and stored in a sealed bag at room temperature. A 5 ml aliquot of the hydrolysate yield was dried in the oven at 105 °C for 2 days to measure the mass of soluble. 100 ml of each microwaved stream was cold-shipped to Bath University for the fermentation tests. Sterility was maintained adding to every stream, 12 mg/L of previously prepared tetracycline solution, kept as a sterile-filter 1000X stock in ethanol 70 % at -20 °C in a dark environment.

2.4 Post-hydrolysis oligosaccharides quantification. For full quantification of the oligosaccharides, the hydrolysates were autoclaved at 121 °C for 15 min in presence of 4% of H_2SO_4 in order to convert the oligosaccharides to monosaccharides. The hydrolysate was filtered over a 0.2 µm syringe filter and submitted to HPLC analysis. Values are reported in tables 1S-2S-3S (see Online Resource) as a total oligosaccharides content (g/L).

2.5 HPLC analysis of the hydrolysate. DP2 (as a sum of lactose, cellobiose and maltose), glucose, XMG (as a sum xylose, mannose and galactose), arabinose, formic acid, acetic acid and lactic acid content were determined by 1260 Infinity II LC HPLC system Agilent system (Agilent Tech. Inc., USA) equipped with a quaternary pump (G1311B), a 100-size infinity standard autosampler (G1329B), a thermostatted column compartment (G1316A), a RID – refractive index detector (G1362A), and a fully licensed Agilent ChemStation Software ®. Samples were briefly centrifuged at room temperature, 14.000 rpm for 5 minutes, filtered with 0.22 µm, (Millipore, UK), diluted 1-10 with Milli-Q water and injected (10 µl) onto a 300×7.8 mm Aminex HPX-87H column (BioRad, CA, USA) at 60 °C, with RID temperature set at 40 °C. Isocratic elution took place was over a 25 min analysis time at 0.6 ml min^{-1} using 0.2 µm-filtered and degassed 5 mM sulphuric acid as mobile phase. 5-Hydroxymethylfurfural (HMF) and furfural, were separated using an Agilent Hi-Plex H^+ (300 x 7.7 mm, 8 µm particle size) column. The operating conditions were an isocratic mobile phase of 0.005 M H_2SO_4, a flow-rate of 0.6 mL min^{-1}, a column temperature of 60 ^oC, a refractive index detector at 55 ^oC, a total run time of 52 minutes, and an injection volume of 5 µl.

2.6 ICP-OES. Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) was performed on Agilent 7700x fitted with a standard Ni sample and skimmer cones coupled to a Mass Spectrometer (MS).

2.7 Thermal gravimetric analysis. The thermal decomposition of the beech and oak sawdust was performed on simultaneous thermal analyser STA625 (Stanton Redcroft). 10 mg of sawdust was pyrolysed under nitrogen flow (60 mL min^{-1}) from room temperature to 625 °C at 10 °C min^{-1}.

2.8 Strain cultivation

M. pulcherrima NCYC2580 (National Collection of Yeast Cultures, Norfolk, UK) was evolved towards higher inhibitor tolerance (strain NCYC 4331) [9] and kept at −80 °C as 20 % (v/v) glycerol stocks in 0.5 ml cryovials. *M. pulcherrima* was cultivated at 20 °C on malt extract agar plates (MEA: agar 15 g L^{-1}; malt extract 30 g L^{-1}; mycological peptone 5 g L^{-1}) for 4 days, kept at 4 °C and refreshed every 4 weeks until necessary. A single colony was used to pre-inoculate soy-malt broth (SMB: soy peptone 30 g L^{-1}; malt extract 25 g L^{-1}; pH 5) in 100 ml in unbaffled Erlenmeyer flasks, incubated for 24 hr to an OD_{600} between 20 and 30 (preculture). Cultivation was carried out under sterile conditions using deionised water, maintaining a 1:5 media-to-air ratio and adjusted to pH 5 with HCl 6 M or NaOH 2M. Media were autoclaved at 121 °C for 20 min prior to use and sampled using aseptic techniques in a laminar flow hood. Further experimental details on the yeast cultivation are given in the supporting information.

2.9 Shake flask experiments

Shake flask (SF) tests were conducted in sterile 100 ml unbaffled Erlenmeyer flasks maintaining 1:5 as a media-to-air ratio with 0.5 OD_{600} nm as initial inoculum. Fermentation media were formed by 16 oak and beech sawdust stream microwave-hydrolysed at different temperature (140 °C-150 °C-160 °C-170 °C-180 °C-190 °C-200 °C-205 °C-210 °C-215 °C-220 °C-225 °C-230 °C-235 °C-240 °C-250 °C) without any external carbon or nitrogen supplementations. Prior autoclave, SF filled with the appropriate media were carefully brought to pH 5 as previously reported. Fermentations were carried out for 5 days, in orbital shakers (Unimax 2010, Heidolph) at 180 rpm in temperature-controlled cabinets (MLR-352-PE, Panasonic) and sterile-sampled daily. All cultivations were carried out at 25 °C, balancing cell growth and lipid production [8]. Yeast growth was assessed measuring the optical density at 600 nm was measured using an appropriately diluted sample, blanked with the original hydrolysate using a UV–vis spectrophotometer (Perkin-Elmer). Final dry cell weight (DCW) values at the end of the fermentation stage (120 h) were also estimated for every condition [19]. After the last sampling point (120 h), detailed images of *M. pulcherrima* (1:10 v/v dilution in deionised water) were taken and observed under an upright Olympus BX51 microscope with 40× magnification (Olympus ®, Tokyo, Japan). Ten microliters of 120 h-undiluted culture sample were aseptically plated on malt extract agar plates (MEA: agar 15 g L^{-1}; malt extract 30 g L^{-1}; mycological peptone 5 g L^{-1}) at 25°C for 3 days to check possible bacterial contaminations.

2.10 Kinetics calculations and lipid analysis

Starting from the growth profile of every stream analysed in this paper (plot OD_{600} VS time) – Online Resource figures S2, S3, S4 – the first 24h were set as exponential phase and used to calculate μ max

as the slope of the aforementioned plot (including appropriate standard deviation). The calculated μ max (h^{-1}) was also plotted versus the different substrate concentrations (g/L), reported as total sugars (g/L), resulting in a specific kinetic behaviour for every biomass (Fig.4) with an appropriate standard error of the means (SEM). Lipid production was evaluated through a slightly modified procedure given in [20] which freeze-dried yeast biomass (40 mg - 80 mg) was disrupted with 6 M HCl at 80 °C and subsequently extracted with chloroform/methanol (1:1, v/v), as previously reported [9]. The lipid production was calculated as a biomass-to-lipid ratio at 120 h of cultivation time, following the reported equations:

Equation 1: $$Lipid\ part\ (g/L) = \frac{DCW\ (g/L)}{100} * Lipids\ percentage\ (\%)$$

Equation 2: $$Non - lipid\ part\ (g/L) = DCW\ (g/L) - Lipid\ part\ (g/L)$$

Maximum lipid yield (expressed as mg_{lipid} / $g_{biomass}$), is reported following equation 3:

Equation 3: $$Lipid\ yield\ (mg/g) = \frac{Max\ lipid\ content\ (g) * 1000}{Max\ biomass\ (g)}$$

More detailed experimental method for lipid extraction is given in the supporting information.

2.11 Statistical data evaluation

All the samples were analysed in three independent replicates (including HPLC calibration curves), and the data are reported as means ± standard deviations unless stated otherwise. Graphs were plotted by using SigmaPlot version 14 (Systat Software, San Jose', California, USA). Single zero-order polynomial process control charts (Shewart control charts – SCC –) were used to monitor the HPLC reliability and OOC (Out of Control) signals. SCC charts derive from the assumptions that the variance of every independent analysis is random, and data points are randomly distributed around the nominal theoretical real value within two control lines (UCL – Upper Control Line, and LCL - Lower Control Line) proportional to the random variation of the process. Random variation is usually estimated as a classical standard deviation (SD) of a repeatability data set formed by 10 identical, independent, pure compounds injections at a chosen concentration. UCL and LCL are usually positioned at ± 2SD from the nominal value. Data within this line are acceptable, whereas data between ± 2SD and ± 3SD are considered borderline and no more than 1 data-point is allowed within this limit. Values that fall above ± 3SD are considered not acceptable [21]. The tested compounds are cellobiose, glucose, xylose, arabinose, acetic acid, formic acid, lactic acid and levulinic acid, and all data falls within ± 2SD, though none falls beyond the ± 2SD. Maximum SD (expressed as %) for the repeatability data set was estimated as no more than 17% of the nominal value.

3. Results and discussion

3.1 Beech and oak characterisation

The biomass compositional analysis showed that the beech sawdust contains higher amounts of cellulose and hemicelluloses compared to the oak (Table 1), with the Klason lignin (acid-insoluble lignin) being higher in the oak biomass. Comparably high amounts of xylan were quantified in the beech and oak sawdust with 15.0 and 13.2 % found respectively. The amount of acetate in the hemicellulose is not negligible in both biomass sources though higher in the beech sawdust with 5.7 % against 3.7 % in the oak samples. This is promising for bioprocessing as the acetate acetyl group in the hemicellulose is reported to auto-catalyse the biomass hydrolysis [22]. The ash content is low (0.4 wt.%) and nearly identical in both sawdust resources. However, the metal content of the sawdust is slightly different (Table 1). The main metals present in the beech and oak sawdust are calcium and potassium. The calcium is by far the most abundant in both samples with 810.77 and 1471.82 ppm for beech and oak sawdust, respectively. While the oak contains nearly two-fold of calcium, the amounts of the magnesium and manganese are above 100 ppm for the beech against 30 ppm for the oak. Metals contained in the biomass could have an influence in the microwave reaction by acting as a catalyst and microwave absorber [23]. For these reasons, both samples appear suitable for microwave-assisted hydrothermal treatment into a fermentable hydrolysate.

Table 1 Beech and oak sawdust constituents (triplicate test) and compositional ICP analysis (single test)

Sawdust composition								
ICP elemental analysis (ppm)						Constitutents (g/100g)		
	Beech	Oak		Beech	Oak		Beech	Oak
Al	3.38	9.18	Mn	119.49	28.74	Dry matter	91.30±0.20	89.40±0.30
Au	1.48	0.29	Na	41.35	41.1	Cellulose[a]	34.10±0.50	28.40±0.60
B	2.12	5.85	Ni	0.28	0.26	Hemicellulose[a,b]	20.80±0.70	17.10±0.40
Ba	25.95	16.34	P	22.86	19.47	Xylan	15.00±0.20	13.20±0.30
Bi	0.21	< 0.01	S	53.00	74.00	Acetyl groups	5.70±0.60	3.70±0.20
Ca	810.77	1471.82	Si	17.63	30.64	Klason lignin[a]	25.30±0.90	31.50±0.60
Cu	1.95	2.07	Sr	6.64	9.56	Ash[a]	0.40±0.10	0.40±0.10
Fe	8.85	18.1	V	0.05	0.18			
K	320.26	292.6	Zn	1.13	0.73			
La	0.85	1.55	Zr	0.19	0.41			
Mg	102.78	30.02						

[a] Calculated on a dry basis

[b] Includes xylan, arabinan and acetate

3.2 Microwave-assisted hydrolysate characterization

The beech and oak sawdust were submitted to microwave-assisted hydrothermal treatment in order to solubilise a portion of the biomass. The impact of the temperature, solid/liquid ratio and biomass type were investigated. For all three biomasses, the yields of hydrolysates were optimal around 210-220 °C. Above that temperature, the yield of oak hydrolysates decreased while the yield of beech hydrolysate remained approximately constant. The oak sawdust (Fig.1b-c) showed a more progressive release of products in hydrolysate than the beech sawdust. When comparing the solid loading (Fig.1a-c), the yield of solid residues showed the same trend while the higher yield of hydrolysate was achieved for oak 1-20 S/L ratio at 220 °C (Fig.1c). This could be explained by the dilution factor preventing product degradation. The dilution effect could be correlated to the fact that higher solid/liquid ratio increases the acid concentration (e.g. acetic acid) in the media leading to acid catalysed degradation of the solubilised products to furfural, reducing the overall hydrolysate yields [24]. Also, the methodology used for the solid-liquid separation could also explain the higher amount of solubles in a more dilute solution due to the absence of a solid residue washing step. The relative proportion of hydrolysates left in the solid residue was higher for high solid to liquid ratio [25]. For the beech sawdust, the yield of solid residue decreased from 86wt.% to 70wt.% from 215 to 250 °C while the hydrolysate yield remained constant, suggesting decomposition to more volatile products was occurring in the hydrolysates. Unlike the beech sawdust, the yield of solid residue from the oak remained constant between 220 to 250 °C while the decrease of the hydrolysate yields appeared to be due to the decomposition of solubilised products only. It has been reported that harsher

hydrothermal conditions promote the formation of 5-HMF, furfural, levulinic acid, formic acid and gases. Also, small organic acids and furfural are likely to evaporate during the evaporation of water at 105°C, underestimating the overall weight of solubilised products [24].

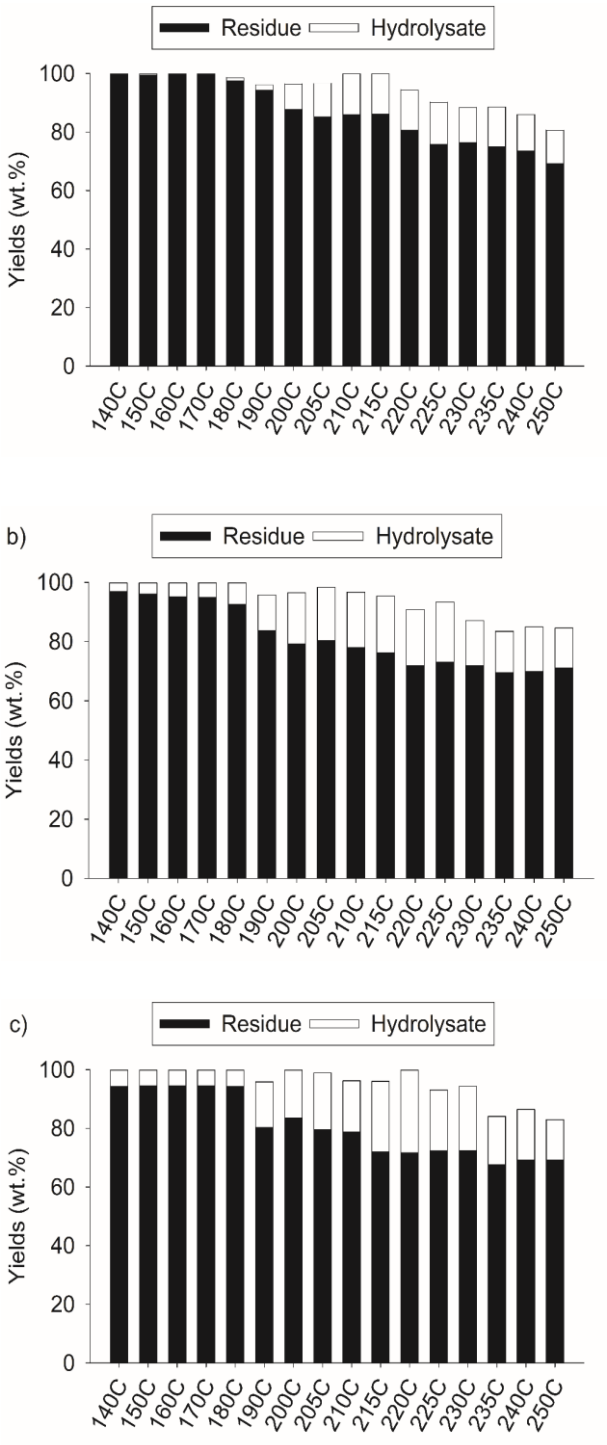


Fig. 1 Yields of hydrolysates (grey) and solid residues (black) after microwave-assisted hydrothermal solubilisation of beech 1-10 **(a)**, oak 1-10 **(b)** and oak 1-20 **(c)**

The full analysis of the sawdust hydrolysates is given in tables 1S-2S-3S (see Online Resource section), where mono and disaccharides (Dp1-Dp2), total inhibitors and as well as total saccharides (Dp1-Dpn) estimated after hydrolysates post-hydrolysis were calculated.

The hydrolysis of the hemicellulose (release of xylan) started immediately at 140 °C for all the biomass types. At 1-10 dilution factor (table 1S & table 2S – see Online Resource section), a higher amount of mono and disaccharides (DP1 and DP2) was measured in the hydrolysates with up to (8.54 ± 0.50) g/L at 225 °C for the oak against (2.73 ± 0.35) g/L at the same temperature for the beech. Indeed, an increase of 68.1%, in terms of DP1 and DP2 was observed in the oak compared to beech. By comparing the concentrations of monosaccharides to the concentrations of oligosaccharides present in the hydrolysates (table 1S & table 2S – see Online Resource section), the hydrolysis rate at 225 °C from solubilized xylan to xylose was 25wt.% and 70wt.% for beech 1/10 and oak 1/10, respectively. The hemicelluloses in the oak appeared more suitable for hydrolysis than the beech hemicelluloses. This is in correlation with the thermal behaviour of the oak and beech (see figure S1 in the Online Resource). The thermal degradation of both sawdust shows that the oak's hemicelluloses are decomposed at a lower temperature than the beech's hemicellulose fraction. The elemental analysis from ICP-OES analysis (Table 1), revealed that the oak sawdust contained nearly twice the amount of calcium of the beech sawdust. It is possible that the elevated Ca level could be catalysing the hydrolysis of the hemicelluloses. Even if there is no direct evidence that high level of calcium within the biomass could have a catalytic effect on the hydrolysis of hemicelluloses, previous works on the effect of salts for the microwave-assisted hydrolysis of cellulose and xylan has been reported in the past [26,27].

It has been reported previously that the release of acetic acid from biomass can autocatalyze the hydrolysis of the biomass,[22] and both the beech and oak's hemicelluloses are acetylated. At 225 ˚C, 2.23 ± 0.41 g/L of acetic acid was released for the oak 1-10 against 1.45 ± 0.10 g/L for the beech 1-10. This higher amount of released acetic acid could also explain the higher rate of hydrolysis of xylan to xylose in the oak biomass. Even in the presence of acetic acid generated *in situ*, it is worth noting that the additive-free hydrothermal conditions mainly hydrolysed the hemicellulose while the cellulose remained largely untouched. The maximum yields of glucose/glucan never exceeded 1g/L in both oak and beech sawdust. Comparing the effect of the dilution rate in the oak sawdust, it is evident that doubling the water content, from 1-10 to 1-20 solid-to-liquid ratio, the sugar concentration (DP1 and DP2) at 225 ˚C is almost halved with 8.54 ± 0.50 g/L for the oak 1-10 compared to 4.73 ± 1.13 g/L in the oak 1-20, presumably due to the double water amount for the same amount of biomass.

Regardless, for every biomass and every solid-to-liquid ratio, the best hydrolysis temperature to obtain the maximum sugar release is 225 ˚C. For temperature above 225 ˚C, Maillard reaction and sugar dehydration could play a determinant influence, reducing the obtained sugar yield. Based on the initial

amount of xylan in the beech and oak sawdust (Table 1), the maximum xylan recovery in the hydrolysates was 46% and 68% for beech 1-10 and oak 1-10. At S/L of 1/20, up to 85% of xylan in the oak sawdust was recovered in the hydrolysate at 220°C. This is higher than previous work on conventional hydrothermal solubilisation of softwood and hardwood where only up to 60% of hemicelluloses recovery in the hydrolysates for poplar, pine and grapevine at S/L ratio of 1/15 was observed [28]. In their work, lower temperature of 170 °C was associated with long holding times of 90-120 min and could explain the lower efficiency. Under these microwave conditions, higher temperature combined with no holding time was selected to maximise the hemicellulose recovery and minimize the inhibitors production. Under these conditions, the solid is predominantly comprised of cellulose and lignin, these fractions could be further valorised in a biorefinery concept. Providing a possible route to aromatic compounds and C6 sugars alongside the fermentation products [29].

Ultimately, the optimum temperature for hydrolysate yield was 225 °C, with no holding time for all biomass sources.

3.3 Effect of the temperature, S/L ratio and biomass type on the inhibitors production

The decrease of hydrolysate yields revealed that more volatile products were produced at higher temperatures. The dehydration of the monosaccharides such as xylose, arabinose or glucose to furfural and 5-hydroxymethylfurfural occurred at temperatures above 200°C for all biomass types. The higher amounts of xylose and glucose in the oak hydrolysate explained the higher inhibitor content of formic acid, acetic acid, lactic acid, furfural and 5-HMF at 225 °C, the maximum sugar yield. More than 4.5 g/L of inhibitors molecules were produced for oak 1-10 against 3.54 g/L for the beech 1-10. Previous work has shown that the concentration of furfural around 5.5g/L had a huge inhibitory effect on baker's yeast as well as a host of other microorganisms [7]. Furfural drastically affects the growth of most common oleaginous yeast at concentrations of 1 g/L or lower [30]. This behaviour is shared with different lactic-acid-bacteria strains. For example, less than 11% of relative growth in terms of DCW was observed in the presence of 5g/L of furfural as a single inhibitory molecule present in a well-defined synthetic medium [31].

As illustrated in table 1S and table 2S (see Online Resource section), the sum of inhibitors increased exponentially with the temperature, with a maximum content achieved for all the biomasses at 250 °C. This is not the case for sugar content where the maximum extraction yield appeared at different temperatures for each biomass. The maximum values reported for the beech sawdust 1-10 is 3.13 ± 0.19 g/L at 240 °C , whereas 8.54 ± 0.50 g/L was observed for the oak 1-10 at 225 °C. Regarding the oak biomass, at 250 °C, acetic acid and furfural are the compounds that showed a bigger increase of 23.5% and 49.7% respectively, compared to the beech at the same solid-to-liquid ratio. When this value is

increased from 1-10 to 1-20 in the oak biomass, acetic acid and furfural concentration decreased, as expected, from 3.16 ± 0.09 g/L to 2.01 ± 0.05 g/L and from 1.65 ± 0.10 g/L to 1.07 ± 0.14 g/L respectively.

At 220-225 °C, all the biomasses displayed the best biomass solubilization rate, with a maximum xylan recovery in the hydrolysates of 46.2%, 68.3% and 85.8 % for beech 1-10, oak 1-10 and oak 1-20 sawdust, respectively. At 225°C, the C_5-C_6 mono and disaccharides amounts accounted for 38.4%, 85.7% and 81.8% of the total solubilised oligosaccharides for beech 1-10, oak 1-10 and oak 1-20, respectively. Above 225 °C, the sugar content was stable, but the bioavailability was drastically reduced due to sugar dehydration. Furfural and 5-HMF, together with organic acids (acetic and formic) were the main inhibitors present in the streams, and their extraction is temperature-proportional, with total concentrations above 5.5 g/L for all the biomasses at 250 °C

3.4 Fermentation of the hydrolysates with *M. pulcherrima*

All sixteen hydrolysates, corresponding to each of the 16 different hydrolysis temperatures tested, for every biomass were fermented over 5 days with *M. pulcherrima*. The yeast strain (NCYC 4331) had been directly evolved to be able to handle higher inhibitor concentrations [19]. The growth curves reached the stationary phase after 48h irrespective of the hydrolysate composition, with final DCW values of (7.80 ± 0.72) g/L, (7.00 ± 2.65) g/L and (5.67 ± 2.08) g/L for beech 1-10, oak 1-10 and oak 1-20 respectively.

Regarding the beech 1-10 biomass, when the hydrolysis temperature was kept in the range between 140 °C and 180 °C, the maximum DCW values remained in the range between (3.17 ± 1.00) g/L and (4.67 ± 1.80) g/L, with total sugar content in the range of (0.35± 0.16) g/L and (0.47± 0.17) g/L. Between 190 °C and 225 °C a huge increase in the DCW values up to (7.80 ± 0.72) g/L at 215°C that correlates with the increasing amount of saccharides concentration, up to (1.69 ± 0.22) g/L at this temperature. This proportional increase suggests that in this range, there is no inhibitory effect from the acids and furan-based inhibitors derived from the hydrolytic treatment. Between 140 °C and 215 °C, in fact, μ_{max} values proportionally increase from (0.09 ± 0.01) h^{-1} and (0.28 ± 0.04) h^{-1}. Between 215 °C and 225 °C, even the total sugar concentration slightly increases from (1.69 ± 0.22) g/L to (2.73 ± 0.35) g/L, a DCW reduction to (4.77 ± 1.46) g/L was reported, whereas the growth velocity remained unchanged (Fig.2).

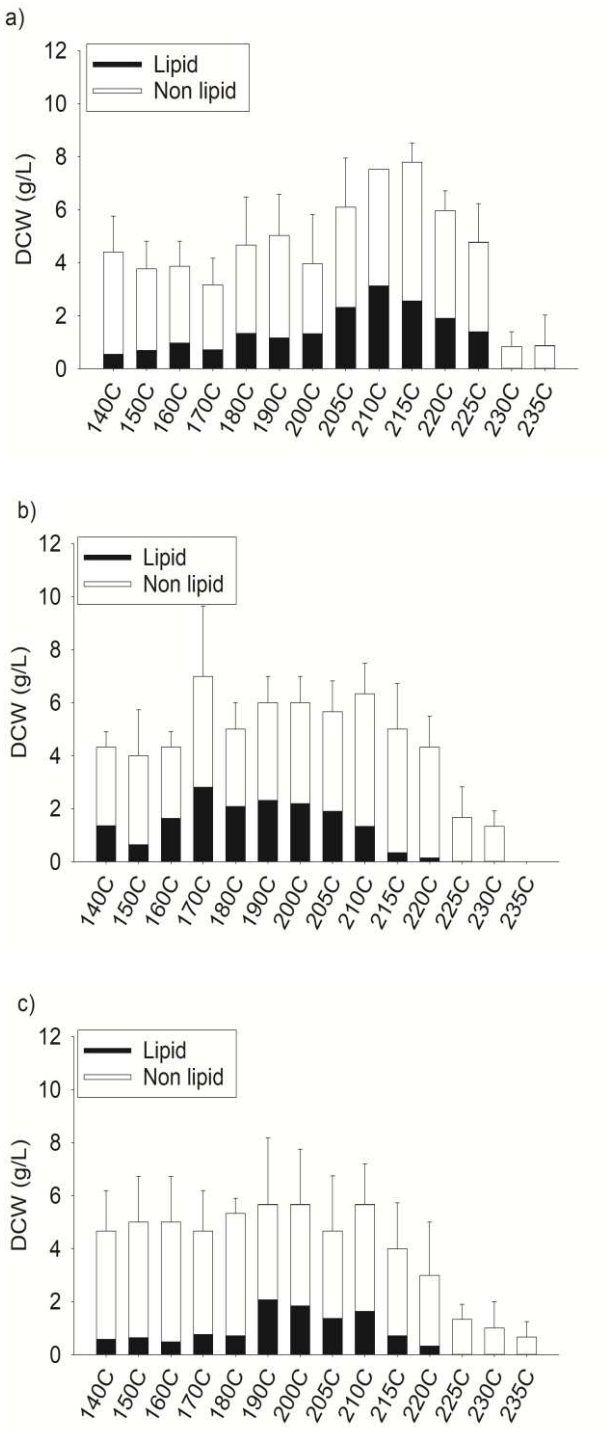


Fig. 2 Lipid-to-Biomass ratio as a function of different hydrolysis temperatures for beech 1-10 **(a)**, oak 1-10 **(b)** and oak 1-20 **(c)** sawdust. Black bars represent the lipid part of biomass, whereas white bars show the non-lipid part. The unreported temperatures did not sustain *M.pulcherrima* growth. Standard deviations are reported for total DCW values

Between 140 °C and 215 °C, the DCW reached the maximum observed value, supporting the idea of a negligible effect of the inhibitors on the yeast, (the concentration of the total inhibitors at 215 °C is 2.19 ± 0.26 g/L). In the 10 degrees range between 215 °C and 225 °C, an inhibitory effect from the organic acids and the furan-based molecules on growth is observed, reducing the capability of *M.*

pulcherrima to accumulate biomass, even if the total sugar values increased. Above 225 °C, negligible growth was observed, despite the presence of high sugar content in the stream, above 2.5 g/L, in each of them. This is probably due to the exponential increase in inhibitory compounds in the stream, up to 6.08 ± 0.36 g/L at 250 °C. In particular, a huge percentage increase of acetic acid and furfural of 40.2% and 74.3% respectively, was observed between 225 °C and 250 °C. Even a small hydrolysis temperature increase, from 225 °C to 230 °C, has a large impact on the yeast growth with the DCW reducing from 4.77 ± 1.46 g/L to 0.83 ± 0.57 g/L, despite a small increase in the concentrations of the inhibitory compounds from 3.54 ± 0.33 g/L to 3.60 ± 0.42 g/L. This strongly suggests that the inhibitory limit falls in this small range.

A similar trend was observed with the oak sawdust at the same solid-to-liquid ratio. Due to the high XMG content compared to the yeast cultured on beech hydrolysate, and the ability of *M. pulcherrima* to metabolize C5 sugars [12], the absolute DCW values are higher. Between 140 °C and 180 °C, the reached DCW lies in a range between (4.00 ± 1.73) g/L and (7.00 ± 2.65) g/L, with total sugar content in the range of (0.75± 0.33) g/L and (1.44± 0.34) g/L. However, not only the sugar but also the inhibitor content in the oak is higher, though this doesn't seem to have an effect in these range of temperatures.

Above 180 °C, although an increase in DCW values and sugar content was reported similarly to the beech hydrolysate. For the oak 1-10, the maximum dry cell weight for this range was reached immediately at 190 °C, 6.00 ± 1.00g/L, whereas for the beech 1-10 biomass the maximum was at 215 °C. That is probably due to the high level of inhibitors already present at 190 °C (more than 1 g/L), that is doubled compared to the content detected at the same temperature for the beech. Above 190 °C, the DCW is constantly kept steady, with minimal variations, in a range between 6.33 ± 1.1 g/L and 5.00 ± 1.73 g/L. For the beech sawdust, the interval was in the range between 190°C and 225°C. Between 220 °C and 225°C using the oak biomass, a drop in DCW was observed, similar to the one observed between 225 °C and 230 °C for the beech. This difference is again potentially related to the inhibitors content, especially the furfural and acetic acid. The furfural content in the beech stream treated at 225 °C was 0.21 ± 0.09 g/L, very similar to the one observed in the oak ones at 220 °C of 0.35 ± 0.05 g/L. The same concept could be applied to the acetic acid, with 1.45 ± 0.10 g/L for the beech sawdust at 225 °C and 1.68 ± 0.56 g/L for the oak at 220 °C.

Similar to the beech biomass, the growth of *M. pulcherrima* was impaired when cultured on oak streams hydrolysed at temperatures above 220 °C. DCW values dropped towards 0 g/L when the maximum total inhibitor value exceeded 8 g/L at 235 °C. Comparing the dilution effect between the oak 1-10 and 1-20, the trend reported for the oak 1-10 could be still detected. Between 140 °C and

180 °C, same DCW magnitude, between 4.67 ± 1.53 g/L and 5.33 ± 0.58 g/L, with total sugar content in the range of 0.79 ± 0.31 g/L and 1.14 ± 0.39 g/L were observed. The maximum DCW value of 5.67 ± 2.50 g/L was also reached at 190 °C, showing similar behaviour without any appreciable difference due to the dilution effect.

The optimal biomass production and lipid production differ from one another substantially demonstrating the effect of inhibitors on the yeast, and how joint chemical and biological processes must be optimised as a system rather than optimisation of each of the individual unit operations.

3.5 Effect of inhibitors on the growth rate of *M. pulcherrima*

The effect of inhibitors on the specific growth rate was assessed though the growth profile plot (OD 600 $_{nm}$ VS time; see supporting information) for each culture condition. μ_{max} was then plotted against each of the respective total monomeric sugar concentrations (Fig.3).

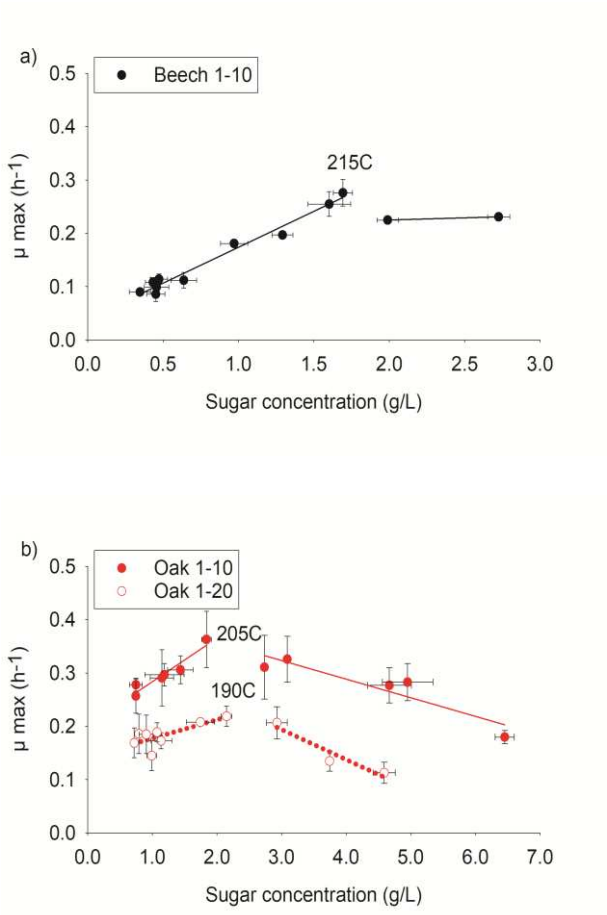


Fig. 3 *M. pulcherrima* kinetic details. Effect of sugar concentrations on specific growth rate. Black, red and white points represent beech 1-10, oak 1-10 and oak 1-20 experimental points respectively. Black and red solid lines show the beech 1-10 and oak 1-10 kinetic trends, while the red dotted line shows the oak 1-20 one. Error bars indicate the standard error of means (SEM). The reported temperatures correspond to the best specific growth rate achieved

Three optimal temperatures were identified (each one for every biomass type) that allows *M. pulcherrima* to achieve the most effective specific growth velocity. Regarding the beech sawdust 1-10, at 215 °C, a μ max value of (0.276 ± 0.025) h^{-1} was achieved when (1.693 ± 0.063) g/L of total sugar provided. Between 140 °C and 215 °C, a linear correlation between sugar concentration and μ max values were observed (R^2 = 0.976). Above 225 °C, increasing the sugar concentration doesn't change the specific grow velocity of *M. pulcherrima*, probably due to the inhibitory effect of the acid and the furan-based molecules present in the stream.

Applying the same concept to the oak 1-10 sawdust, a μ max value of 0.363 ± 0.053 h^{-1} was achieved with 1.838 ± 0.074 g/L of total sugar provided released at 205 °C. The higher μ max value of the oak sawdust 1-10 compared to the beech ones, was not a function of the more sugar released, in fact, the sugar content is quite similar (1.693 g/L vs. 1.838 g/L) but depends solely on the inhibitors content. At this temperature, the oak 1-10 biomass contains (1.05 ± 0.25) g/L of inhibitors, whereas the beech sawdust needs to be heated more to achieve a similar sugar content, and that leads to a higher inhibitors content (2.19 ± 0.26) g/L, that negatively affect the μ max value.

3.6 Lipid production and yield

In simpler systems, the total lipid yield is in direct correlation with the cell dry weight, where more cells with the same cellular lipid content give the highest overall lipid yield. To assess the system performance the lipid yields were calculated for each of the sawdust hydrolysates, to this end the cells were isolated at the end of the process, isolated from the broth, freeze-dried and the lipids were extracted using a standard literature procedure [20] (Fig.4).

Lipids are present in all the biomass grown at 140 °C for all the biomass analysed, even in low quantities. This value proportionally increased with the biomass, as a similar cellular lipid production is maintained, similar to other studies[32-34]. However, the temperatures at which the maximum lipid production was observed are different compared to the ones that show the best μ max. For the beech 1-10 biomass, the maximum specific growth rate was observed at 215 °C, whereas the maximum lipid production was identified at 210 °C of 3.12 g/L of lipid compared to 7.53 g/L of total DCW, that correspond to 34% of the cell. Similarly, with the oak 1-10 where the maximum specific growth rate was observed at 205 °C, the maximum lipid production was identified at 170 °C of 2.82 g/L of lipid compared to 7.00 g/L of total DCW, that correspond to 40% of the cell. Only for the oak 1-20, the two temperatures coincide (190 °C) with 2.08 g/L of lipid compared to 5.67 g/L of total DCW, which correspond to 37% of the cell. It is likely that the dilution of acids and furan-based molecules allow the cell to limit the interferences between these two biological pathways. It's possible that despite the lipid production being correlated to the growth, a strong influence of a non-growth-

related parameter e.g. metabolic maintenance effort, needs to be included in order to model the lipid production. Moreover, at elevated temperatures of 225 °C for the beech and oak 1-10, and above 220 °C for the oak 1-20, is possible to observe some growth without any lipid production. This also suggests than some specific compounds above a specific concentration could only affect the biochemistry of the lipid profile, but not the biomass growth. The maximum lipid yield as a function of hydrolysis temperature is given in figure 4.

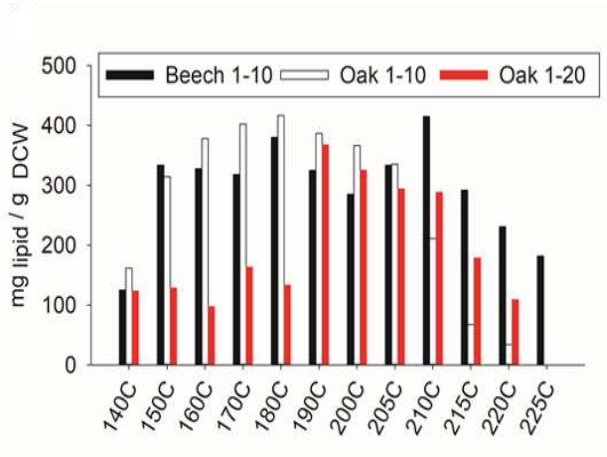


Fig. 4 Lipid yield as a function of different hydrolysis temperatures for beech 1-10 (black bars), oak 1-10 (white) and oak 1-20 (red bars). The unreported temperatures did not sustain *M.pulcherrima* growth. Values are obtained merging the dried biomass of the three replicates

A maximum yield cellular lipid content of 41.5% $_{DCW}$, 41.6% $_{DCW}$ and 36.7% $_{DCW}$, were found for the yeast cultured on the beech 1-10, oak 1-10 and oak 1-20 respectively. These values are similar to our previously reported values of *M. pulcherrima* cultured on macroalgae and other lignocellulosic hydrolysates [9]. This confirms that the hydrolysates derived from beech and oak sawdust are suitable for high-level lipid production using *M. pulcherrima* without any external addition of carbon or nitrogen sources.

Microscopic photographic images of *M. pulcherrima* were taken at the end of the fermentation using an upright Olympus BX51 microscope with 40× magnification (Online Resource figures 5Sa-c). The black halo around the cell body represents lipid accumulation. Sterility, (therefore maximum sugar efficiency) was ensured and controlled plating at 120h the culture on MEA plates. No bacterial contamination was detected in any cultures (Online Resource figures 6Sa-c). The high lipid content herein reported (between 34.37% and 40.22% depending from the sawdust stream type) is a remarkable result due to the utilization of sawdust as only carbon and nitrogen source, without any external addition.

Despite no additional additives to control the C/N ratio the lipid production was found to be up to 41% of the cell weight, however this was achieved on hydrolysate processed at lower temperatures than the optimal sugar release demonstrating dissociated growth and lipid production pathways.

3.7 Lipid inhibition by furfural and broth detoxification

These results indicate a partial-related trend between growth and lipid production. Especially at high sawdust hydrolysis temperature (where a complex plethora of inhibitory compounds are released). As such furfural concentration (in mmol) was plotted against the lipid production (in %) obtained from *M. pulcherrima* cultivation (Fig.5).

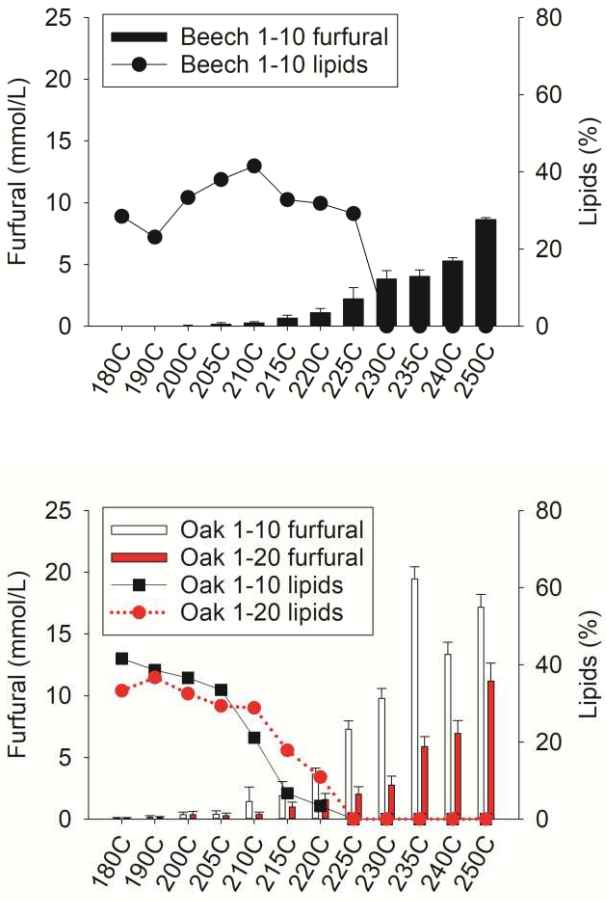
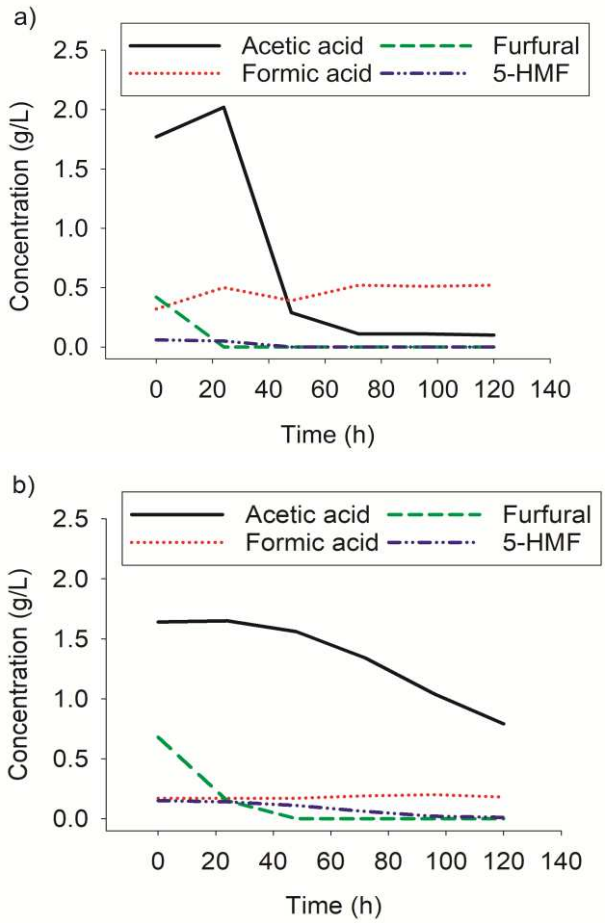


Fig. 5 Effect of furfural on the lipid content of *M. pulcherrima*. Black, white and red filled bars represent beech 1-10, oak 1-10 and oak 1-20 furfural concentration (mmol/L) respectively. Black continuous solid lines with white filled circles and white filled squares show the beech 1-10 and oak 1-10 lipid content (%), while the red dotted line with red filled hexagons shows the oak 1-20 one

With an increasing concentration of furfural in the hydrolysates, the lipid production drastically decreased before ceasing completely. Moreover, different furfural-limiting concentrations could be established for different biomasses. Regarding the beech sawdust 1-10, lipids drop to zero when the

furfural content is above (2.22 ± 0.90) mmol/L of furfural, corresponding to (0.213± 0.086) g/L. For the oak sawdust 1-10, instead, (3.65 ± 0.47) mmol/L of furfural, corresponding to (0.351 ± 0.046) g/L. Diluting the sample with a double amount of water, the furfural limit becomes (1.57 ± 0.49) mmol/L of furfural, corresponding to (0.151± 0.048) g/L. These differences are probably due to the different metabolic effects that different inhibitory molecules have on *M. pulcherrima* metabolism. It is possible that not only the furfural, but also the combined effect of furfural, and different organic acids levels could contribute to limit the lipid production without completely inhibiting the yeast growth, creating a more complex picture in order to understand the full mechanism.

One route that organisms can survive highly inhibitory cultures is through detoxification where compounds such as furfural are converted to less toxic molecules such as furfuryl alcohol [35]. To determine if this was also the case for *M. pulcherrima* the hydrolysates produced at 230 °C for beech 1-10, 225 °C for Oak 1-10 and 240 °C for Oak 1-20, which supported negligible growth of *M. pulcherrima* were analysed (Fig.6).



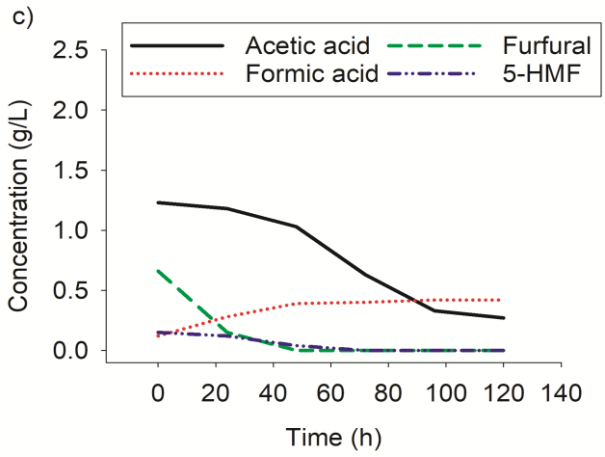


Fig. 6 Acetic acid (black continuous line), formic acid (red dotted line), furfural (green long dashed line) and 5-HMF (blue dash-dot-dot line) concentration in beech 1-10 **(a)**, oak 1-10 **(b)** and oak 1-20 **(c)** hydrolysed respectively at 230 °C, 225 °C and 240 °C respectively and inoculated with pure culture of *M. pulcherrima*. Single inhibitors HPLC analysis was performed daily and the result plotted as a function of time

Acetic acid, furfural and *5*-HMF were converted by the yeast at different rates. However, the level of formic acid was not reduced during the fermentation. For the other three inhibitors, it appears that the furfural was the quickest converted with no trace of it after 48h. Acetic acid was more slowly converted by *M. pulcherrima* with residual amount left after 120h fermentation. With lower initial concentration, the 5-HMF was more slowly consumed by the *M. pulcherrima* than the furfural. In the case of the formic acid, previous work on the *M. pulcherrima* tolerance to organic acid showed that the formic acid was less inhibitory than acetic acid [7], and a concentration of 60mM (2.8 g/L) of formic acid did not have any inhibition effect. This is a promising observation when considering a fed-batch fermentation strategy. The ability of the *M. pulcherrima* to consume the acetic acid, furfural and 5-HMF is in agreement with previous work pointing out that *in-vivo* detoxification is necessary for fed-batch fermentation strategy [35].

4. Conclusions

In this investigation a simple, additive-free, microwave-assisted hydrothermal pretreatment was used to solubilise sawdust biomass, resulting in an oligosaccharide rich stream where XMG was the main monosaccharide component (> 60%). Multiple fermentation tests were performed to identify the best growth conditions for *M. pulcherrima* using these streams as the only source of nutrients for the yeast. Best μ max values of 0.22 h^{-1} and 0.36 h^{-1}, and lipid accumulation (between 36.7 % and 41.6%) peaked in different temperature ranges compared to the best temperature for sugar extraction, suggesting how yeast can dissociate growth and lipid production pathways. Media detoxification was also reported where little yeast growth and no lipid accumulation was observed at 225 °C, 230 °C and 240 °C, confirming the yeast metabolic versatility responses, depending on media

complexity. Maximum acetic acid detoxification rate of 94% at the end of the fermentative stage (120h) and complete removal of the furfural and 5-HMF components were observed under these conditions. This approach demonstrates the potential of combined chemical and biological processing; however, multiple parameters affect the lipid production when real, complex lignocellulosic media are involved, and these parameters must be carefully tuned to achieve optimal system yields.

5. Acknowledgements

This work is financially supported by the Industrial Biotechnology Catalyst (Innovate UK, BBSRC, EPSRC) to support the translation, development and commercialization of innovative industrial Biotechnology processes (EP/N013522/1). The authors thank Dr Hannah Briers and Paul Elliott for expert technical assistance.

6. Conflict of interest

None of the authors listed has any conflict of interest with this work.

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