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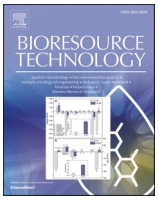

Spent mushroom substrates for ethanol production – Effect of chemical and structural factors on enzymatic saccharification and ethanolic fermentation of *Lentinula edodes*-pretreated hardwood

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HIGHLIGHTS

- Spent shiitake mushroom substrate (SMS) was studied for potential ethanol production.
- Enzymatic saccharification resulted in 80–90% glucan digestibility in SMS.
- The relative ratio of guaiacyl units and SMS crystallinity did not affect hydrolysis.
- The high nitrogen content in SMS hydrolysates ensured efficient fermentation by yeast.
- Phenolics and acetic acid in the hydrolysates had only a minor inhibitory effect.

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ABSTRACT

Spent mushroom substrates (SMS) from cultivation of shiitake (*Lentinula edodes*) on three hardwood species were investigated regarding their potential for cellulose saccharification and for ethanolic fermentation of the produced hydrolysates. High glucan digestibility was achieved during enzymatic saccharification of the SMSs, which was related to the low mass fractions of lignin and xylan, and it was neither affected by the relative content of lignin guaiacyl units nor the substrate crystallinity. The high nitrogen content in SMS hydrolysates, which was a consequence of the fungal pretreatment, was positive for the fermentation, and it ensured ethanol yields corresponding to 84–87% of the theoretical value in fermentations without nutrient supplementation. Phenolic compounds and acetic acid were detected in the SMS hydrolysates, but due to their low concentrations, the inhibitory effect was limited. The solid leftovers resulting from SMS hydrolysis and the fermentation residues were quantified and characterized for further valorisation.

1. Introduction

With the increasing concerns on environmental issues, cellulosic ethanol, which is produced by enzymatic saccharification and fermentation of lignocellulosic biomass, has received considerable attention as an alternative to fossil-based transportation fuels. Since lignocellulose is recalcitrant to the action of enzymes, pretreatment is needed for improving susceptibility to enzymatic saccharification of cellulose (Jönsson and Martín, 2016; Shirkavand et al., 2016). Pretreatment effect is related to removal of lignin and hemicelluloses, among other factors, and high temperature and expensive materials are typically required (Jönsson and Martín, 2016). To be relevant, pretreatment methods should be environmentally friendly, resource efficient and cost effective. Biological pretreatment with lignin-degrading fungi is a low-cost alternative, but its slow rate and the preferential consumption of carbohydrates of many fungi have so far limited its viability as a stand-alone option (Shirkavand et al., 2016; Wan and Li, 2012). Using edible fungi can open new perspectives for biological pretreatment of lignocellulose. During the last 15 years, the global consumption of edible mushrooms is doubled and has reached near 40 million tons per year (Royse et al., 2017). The rapid growth of the mushroom market results in generation of large quantities of spent mushroom substrate (SMS). For example,

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production of shiitake (*Lentinula edodes*), the most cultivated edible mushroom (Royse et al., 2017), results in up to 12.5 million tons SMS dry mass worldwide per year (Wei et al., 2020).

Recent studies have shown that the SMS resulting from cultivation of shiitake on birch is a potential feedstock for production of fermentable sugars through enzymatic saccharification (Chen et al., 2020; Xiong et al., 2019). The SMS resulting from shiitake cultivation is delignified to a high degree, which implies a potential reduction of the substrate recalcitrance towards the action of cellulolytic enzymes. In a recent study in parallel with this paper (Chen et al., 2022), a considerable degradation of lignin and hemicelluloses, up to 67.6% and 61.3% respectively, together with a preservation of around 80% of the initial cellulose, were observed in SMSs, where shiitake grew in different combinations of three hardwood species, namely white birch (*Betula pubescens* Ehrh.), alder (*Alnus incana* (L.) Moench), and aspen (*Populus tremula* L.). Although previous studies have shown that shiitake cultivation can be an effective biological pretreatment for facilitating enzymatic saccharification of cellulose (Chen et al., 2020; Xiong et al., 2019), the issue regarding the suitability of different SMSs for downstream biochemical processing to ethanol have not been well investigated (but see Asada et al., 2011 for oak based SMS; Hiyama et al., 2016 for salix based SMS). Investigating the enzymatic saccharification of the SMSs from the above mentioned cultivations, and the ethanolic fermentation of the resulting hydrolysates is important for assessing the potential of using shiitake cultivation as a pretreatment for production of cellulosic ethanol.

An issue to be understood is how SMS chemical and structural differences, either caused by the fungal growth or intrinsic to the nature of the used wood species, influence the enzymatic saccharification. For example, the share of guaiacyl (G) lignin units and the substrate crystallinity in SMS differed from those of the initial substrates due to removal of lignin and xylan during fungal pretreatment (Chen et al., 2022). Changes of lignin content, composition and structure, and crystallinity have often been associated with the improvement of susceptibility to enzymatic saccharification observed after many pretreatment methods. For example, Santos et al. (2012) reported that kraft pretreatment increased the S/G ratio of lignin in wood chips, which increased enzyme adsorption and resulted in higher enzymatic hydrolysis efficiency. While the changes of S/G ratio and cellulose crystallinity did not affect the hydrolysis of sulfuric-acid pretreated birch, spruce and aspen (Wang et al., 2018a). It would be of interest to know whether the fungal-pretreated materials behave differently.

In fermentation of cellulosic hydrolysates, nutrient supplementation is required for ensuring the metabolic activity of the ethanologenic microorganism, usually baker's yeast (*Saccharomyces cerevisiae*), and avoiding sluggish fermentations. Nitrogen is required, and urea, ammonium sulphate and peptone are some of the sources typically used as nitrogen-rich nutrient supplements (Liu et al., 2014; Martín et al., 2002). The situation might be different if SMS hydrolysates are used, since they can already contain nitrogen due to nutrient supplements, such as wheat bran and straw, which are added initially during substrate preparation. In previous study, it was found that more than 70% of initial nitrogen remained in the SMS after shiitake mushroom harvesting (Chen et al., 2022). Nitrogen is used in fungal metabolic reactions and it is likely incorporated in the mycelia remaining in the SMS (Koutrotsios et al., 2014; Parchami et al., 2021). The SMS nitrogen might be released to the liquid phase during hydrolysis, and serve as nutrient in the fermentation of the hydrolysate. That would minimize external nutrient supplementation, thus resulting in decreasing the fermentation cost and environmental impact. However, differently from nitrogen contained in the nutrient supplements, which is effectively solubilised in fermentation media, the solubilisation of SMS nitrogen and the forms of solubilised nitrogen, as well as its use during fermentation process is still an open question worth being explored.

The potential inhibition of cellulolytic enzymes and fermenting microorganisms by lignocellulose degradation by-products is another issue of interest. By-products, such as furan aldehydes, formic acid and levulinic acid, exerting toxic effects on ethanol fermentation have been investigated for hydrothermal pretreatment methods (Bolado-Rodríguez et al., 2016; Martín et al., 2018), but they are not relevant for biological pretreatment (Chen et al., 2022). On the other hand, acetic acid and phenolic compounds can be relevant inhibitors in fungal pretreatment. Acetic acid resulting from splitting of xylan acetyl groups, and phenolic compounds formed as a consequence of lignin degradation, and both of them are known to be inhibitory (Jönsson and Martín, 2016). Previous study revealed the presence of phenolics in water extracts of spent substrates after shiitake mushroom cultivation (Chen et al., 2022). Meanwhile, even if acetic acid was not detected in the SMS extracts (Chen et al., 2022), there is concern about its formation during enzymatic saccharification due the splitting of acetyl groups in the remaining hemicelluloses. Since the potential toxicity of by-products of fungal pretreatment is poorly understood, investigating whether and to what extent by-products affect bioconversion is a relevant question to be answered, especially considering that SMSs from different tree species might have divergent characteristics regarding inhibitory compounds.

In this work, the enzymatic digestibility of SMSs resulting from growing shiitake on three hardwood species (birch, alder and aspen) was assessed by small-scale analytical enzymatic saccharification trials. Additionally, preparative enzymatic hydrolysis in shake-flask experiments was performed, and the fermentability of the resulting hydrolysates with *S. cerevisiae*, both with and without nutrient supplementation, was evaluated. Furthermore, a preliminary characterization of the hydrolysis leftovers and fermentation residues was performed in order to evaluate their suitability for further valorisation using biorefinery processes.

2. Materials and methods

2.1. Materials

Six initial substrates (non-treated woody substrates) and their SMSs after shiitake cultivation were collected from a parallel research (Chen et al., 2022). Each initial substrate contained 80% hardwood sawdust (in dry mass, DM) and 20% wheat bran (*Triticum aestivum* L.). White birch, alder, and aspen, as well as their combinations (50–50%), were the sources of hardwood sawdust. Four replicates were used for each substrate treatment. Before the chemical analysis, the replicated samples of each treatment were proportionally pooled (20% of every replicate by weight) into one mixed sample (Chen et al., 2022). The characteristics of the initial substrates and SMSs were cited from Chen et al. (2022) and shown in Table 1 of this study.

2.2. Enzymatic saccharification

2.2.1. Analytical enzymatic saccharification

The susceptibility of the mushroom substrates to enzymatic hydrolysis was determined by analytical enzymatic saccharification (Gandla et al., 2018). The commercial enzyme preparation Cellic CTec2, which is a blend of cellulases, β-glucosidases and hemicellulases acquired from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), was added at a load of 100 CMCase units/g biomass. Details of the protocol were described in a previous study (Xiong et al., 2019).

2.2.2. Preparative enzymatic saccharification

Preparative enzymatic saccharification of the SMSs was performed in order to produce hydrolysates to be used in the fermentation experiment. Fifty g (DM) of SMS were suspended in sodium citrate buffer (50 mM, pH 5.2) at 10% solids content in 2 L Erlenmeyer flasks. The enzyme preparation Cellic CTec2 was added at a load of 200 CMCase units/g biomass. The reaction mixtures were incubated for 72 h, at 50 °C and 130 rpm in an Infors Ecotron orbital incubator (Infors AG, Bottmingen, Switzerland). At the end of hydrolysis, the slurry was vacuum filtered for

Table 1
Characteristics of non-treated and biologically-treated (SMS) woody substrates (data are reproduced from Chen et al. (2022)).

		Tree species					
		Birch	Alder	Aspen	Alder/Aspen	Birch/Aspen	Birch/Alder
Glucan, %	Non-treated	28.3	30.6	37.8	32.7	30.2	29.2
	SMS	37.0	36.7	39.3	41.0	39.7	38.3
Xylan, %	Non-treated	14.1	14.4	12.1	12.0	13.8	14.7
	SMS	8.9	8.1	5.5	6.6	7.7	7.3
KLL, %	Non-treated	16.0	17.9	15.6	17.0	16.1	17.2
	SMS	8.4	10.6	8.5	9.1	8.3	8.8
ASL, %	Non-treated	8.1	6.8	6.4	6.2	7.0	7.7
	SMS	4.4	4.2	4.0	4.0	4.1	4.0
S/G	Non-treated	2.9	2.3	2.4	2.1	2.6	2.9
	SMS	1.6	1.2	1.3	1.2	1.4	1.3
Extractive, %	Non-treated	10.9	12.1	9.6	9.8	10.6	11.0
	SMS	31.2	29.8	28.8	29.3	30.8	30.9
Nitrogen, %	Non-treated	0.53	0.64	0.50	0.64	0.55	0.58
	SMS	0.74	0.76	0.74	0.70	0.67	0.73
CrI, %	Non-treated	70.0	72.4	72.3	70.4	66.6	68.9
	SMS	72.0	71.0	72.3	76.8	73.6	72.0

KLL, Klason lignin; ASL, acid-soluble lignin; S/G, Ratio of lignin syringyl and guaiacyl units; CrI, Crystallinity index.

separating the hydrolysate from the hydrolysis residue. The hydrolysate was centrifuged at 10 000 rpm (~12 000 g-force) for 15 min for removing small particles, adjusted to pH 5.5, and filter-sterilized through 0.22 μm sterile filtration unit under vacuum (Corning, Darmstadt, Germany). The solid leftover of enzymatic saccharification was air-dried until 90% DM content, and stored for compositional analysis.

2.3. Nitrogen solubilisation test

The solubilisation of nitrogen contained in the initial substrates and SMSs was examined. Aliquotes of 5 g (DM) of the substrates were suspended in sodium citrate buffer (50 mM, pH 5.2), at 10% solids content, for 72 h, mimicking the environment of the preparative enzymatic saccharification, but without adding enzymes. After that, the liquid phase was separated by centrifugation. Samples of the supernatants, in parallel with samples of SMS preparative enzymatic hydrolysates, were analysed by an accredited laboratory (EUROFINS, Sweden) for the contents of ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), nitrite nitrogen (NO_2^--N) and total nitrogen.

$$\text{Nitrogen solubilisation}\% = \frac{\text{Total nitrogen of liquid phase (g)}}{\text{Total nitrogen of substrate (g)}} \times 100$$

2.4. Fermentation of SMS hydrolysates and reference media

2.4.1. Inoculum and media

For preparing the inoculum, freeze-dried yeast (*S. cerevisiae* Ethanol Red, Fermentis Ltd., Marcq-en-Baroeul, France) was suspended in sterile deionized water at 35 °C and for 30 min. The cell concentration in each fermentation flask was 1 g/L. The fresh yeast suspension was prepared right before inoculation.

The fermentation media consisted of 92.4% (v/v) filter-sterile SMS hydrolysate, 5.6% (v/v) yeast inoculum and nutrient solution in four different doses (2%, 1%, 0.5% and 0%; Table 2). The raw nutrient solution, corresponding to total nitrogen content of 30.9 g/L, contained 150 g/L yeast extract, 75 g/L $(NH_4)_2HPO_4$, 3.75 g/L $MgSO_4{\cdot}7\ H_2O$ and 238.2 g/L $NaH_2PO_4{\cdot}H_2O$ as previously described (Ilanidis et al., 2021; Martín et al., 2018). The 2% dosage corresponded to the full nutrient supplementation reported previously in fermentation of lignocellulosic hydrolysates (Ilanidis et al., 2021; Martín et al., 2018), while the 1% and 0.5% corresponded to half and one quarter of the full nutrient charge. Two reference fermentations, one with full nutrient charge (Reference 1) and one with no nutrients (Reference 2), of a glucose medium with concentration ~ 35 g/L were also included. The pH of all the media was adjusted to 5.5.

Table 2
Overview of fermentation experimental set-up.

Medium	Fermentation media × 100% (v/v)				
	Glucose	Hydrolysate	Yeast inoculum	Nutrient solution	Water
Birch-based SMS hydrolysate	–	92.4	5.6	2.0	0
				1.0	1.0
				0.5	1.5
				0	2.0
Alder-based SMS hydrolysate	–	92.4	5.6	2.0	0
				1.0	1.0
				0.5	1.5
				0	2.0
Aspen-based SMS hydrolysate	–	92.4	5.6	2.0	0
				1.0	1.0
				0.5	1.5
	–			0	2
Reference 1	92.4	–	5.6	2	0
Reference 2	92.4	–	5.6	0	2

2.4.2. Fermentation

Filter-sterile SMS hydrolysates and reference media were mixed under aseptic conditions with the required amount of nutrient solution (Table 2) in 25-mL bottles with a working volume of 18 mL. One mL of the yeast inoculum was added, and the flasks were sealed with cotton plugs to allow the release of CO_2 formed during fermentation. The fermentation media were incubated in an Ecotron orbital incubator at 35 °C and 180 rpm under oxygen-limited conditions. Samples were taken at the beginning of the fermentation, and after 4, 8, 12 and 16 h. The fermentations were monitored by regular OD (optical density) measurements at 600 nm using a spectrophotometer (Shimadzu, Kyoto, Japan), and by glucose determination using an Accu-Chek Aviva glucometer (Roche Diagnostics, Basel, Switzerland). Ethanol and glucose concentrations were also determined by HPLC and used for calculating the ethanol volumetric productivity and yield. The volumetric productivity (g/L h) was based on grams of ethanol produced per litre of culture medium per hour during the first 8 h of the fermentation because the maximum volumetric productivity in the reference was obtained after 8 h as previously reported (Martín et al., 2002). The ethanol yield (g/100 g) was calculated as the maximum amount of ethanol formed per 100 g of initial glucose. Duplicate experiments were run for each condition.

2.5. Analytical methods

Total nitrogen was determined in solid biomass using an elemental analyzer-isotope ratio mass spectrometer (DeltaV, Thermo Fisher Scientific, Germany). The substrates crystallinity and the relative ratios of guaiacyl and syringyl units of lignin were determined by X-ray diffraction (XRD) and Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS), respectively, according to the methods described previously (Chen et al., 2022). Determination of the extractives in the substrates, as well as solid leftover after preparative enzymatic saccharification, was performed by extraction according to an NREL protocol (Sluiter et al., 2005). Extractive-free materials were air-dried and used for the analysis of structural carbohydrates and lignin by analytical acid hydrolysis combined with high performance liquid chromatography (HPLC) (Sluiter et al., 2008).

The concentrations of glucose, xylose, levulinic acid, formic acid and acetic acid in the enzymatic hydrolysates and in fermentation samples, as well as ethanol in fermentation samples, were determined by HPLC, using an Aminex HPX-87H column and an RI detector. Elution was performed with isocratic flow of a 5 mM aqueous solution of sulfuric acid. The flow rate was 0.6 mL/min and the column temperature was set to 55 °C. Total phenolics compounds were determined using Folin Ciocalteu's method (Singleton et al., 1999) using vanillin as calibration standard. Residues dry weight in the remained media after fermentation was determined by drying 1 mL aliquots of the broth at 105 °C.

2.6. Data analysis

Principal component analysis (PCA) was performed using SIMCA 14.0 (Umetrics, Sartorius Stedim Biotech, Umea, Sweden) to gain an overview of the data and multivariate relations. Each experiment treatment was carried out in duplicate (triplicates if too much variation) and mean values and standard error (SE) are reported. One-way ANOVA followed by Post Hoc Multiple Comparisons (Tukey) was conducted using SPSS software (IBM SPSS version 26.0) to analyse statistical significance of differences of examined variable between samples.

3. Results and discussion

3.1. Characterisation of substrates

3.1.1. Characteristics of the SMSs

Glucan, with a content ranging between 36.7 and 41% (w/w), was the main component of the SMSs, followed by lignin (12.4–14.8%), which was calculated as the sum of the Klason and acid-soluble fractions, and xylan (5.5–8.9%) (Table 1). In general, the SMSs had higher glucan content and lower content of lignin and xylan than the non-treated woody substrate, which is a result of selective degradation on lignocellulose during fungal pretreatment (Chen et al., 2020; Xiong et al., 2019). Among the wood species, aspen contained a higher glucan but lower xylan and lignin than birch and alder in both non-treated woody substrate and SMS (Table 1). The ratio of syringyl to guaiacyl units of lignin (S/G ratio) was lower in the SMSs than in the initial woody material, while the crystallinity was generally higher in SMSs than in wood. The nitrogen content in the SMSs ranged from 0.67% to 0.76%, and it was remarkably higher than that of the initial woody substrates (0.50–0.64%). The content of extractive compounds was around 30% of the SMS.

3.1.2. Nitrogen solubilisation in non-treated wood substrates and SMSs

Solubilised nitrogen in the supernatants of the suspensions of SMSs in sodium citrate buffer (without enzyme preparation) is presented in Table 3. Woody substrate not subjected to fungal treatment was also included. The solubilised nitrogen in initial substrates was only 10–20% of the total nitrogen in the solid sample, while in the SMSs it was 48–55%. The highest value of solubilised nitrogen was achieved for alder SMS. Remarkably, the solubilisation of SMS nitrogen was higher than that previously reported for nitrogen recovered from brewer's spent grain after a hydrothermal pretreatment (Parchami et al., 2021). Shiitake mushroom cultivation not only resulted in nitrogen accumulation in the woody substrate (Table 1), but it also increased the solubility of nitrogen sources contained in the spent substrate. As consequence, the availability of nitrogen for downstream processes increased considerably. Meanwhile, inorganic nitrogen (NH_4^+, NO_3^- and NO_2^-) forms represent a minor proportion (on average 1.8%) of the total solubilised nitrogen in SMSs (Table 3), suggesting that the major part of the solubilised nitrogen was organic, likely as protein existing in remaining fungal mycelia.

Table 3
Solubilised nitrogen forms in non-treated woody substrates and SMS.

Parameters	Unit		Tree species		
			Birch	Alder	Aspen
NH_4^+-N	mg/L	Non-treated	0.03	0.04	0.09
	mg/L	SMS	5.3	5.8	7.1
NO_3^--N	mg/L	Non-treated	< 0.10	< 0.10	< 0.10
	mg/L	SMS	< 0.10	< 0.10	< 0.10
NO_2^--N	mg/L	Non-treated	< 0.002	< 0.002	< 0.002
	mg/L	SMS	0.3	0.41	0.33
Total N	mg/L	Non-treated	98.3	60.5	94.2
	mg/L	SMS	350	390	330
Nitrogen solubilisation	% of substrate	Non-treated	20.0	10.2	20.3
	% of substrate	SMS	49.3	55.4	47.8

3.2. Enzymatic hydrolysis of the SMSs

3.2.1. Analytical enzymatic saccharification

As indicated by the results of the analytical enzymatic saccharification (Fig. 1a), the enzymatic digestibility of glucan in the non-treated woody was rather low for the different tree species. Around 16–27% of initial glucan was hydrolysed for the different materials, and it was slightly higher for aspen, either alone or mixed, than for the other wood species.

Fungal pretreatment resulted in an enhancement of the susceptibility of the substrate to enzymatic saccharification (Fig. 1a). Glucan digestibility of the studied SMSs reached values averaging 80.3%, which corresponds to an increase of 3.5 times compared with the non-treated materials. For the SMSs based on a single wood species, the aspen-based one displayed a significant higher ($p < 0.05$) glucan digestibility (88%) than the birch- and alder-based ones (75–76%). For the SMSs based on wood mixtures, no remarkable differences were observed between the enzymatic digestibility of different samples. These results confirm previous reports on shiitake cultivation as pretreatment method for enhancing the enzymatic convertibility of birch cellulose (Chen et al., 2020; Xiong et al., 2019). Furthermore, the current results show that the method is suitable also for other hardwood species, and that for some species, for example, aspen, it can even be more effective than for birch.

Based on a 12 × 7 data matrix including the twelve analytical enzymatic hydrolysis experimental runs and seven characteristic variables of substrates, a PCA was performed (Fig. 1b) to identify multivariable relations. The biplot, composed of the first two PCA components and explaining 95.9% of the total variation, shows that the observations were visually clustered into two groups that were separated from left to right by the non-treated woody substrates and SMSs. The negative effect of lignin and xylan on enzymatic digestibility is anticipated (Jönsson and Martín, 2016; Shirkavand et al., 2016), which is in line with the highest enzymatic digestibility of the aspen-based SMS, which had the lowest content of both lignin and xylan (Table 1). The negative effect of S/G ratio and the positive effect of the crystallinity index were less expected, since they have often been assumed to affect the enzymatic saccharification in a different way to what our results

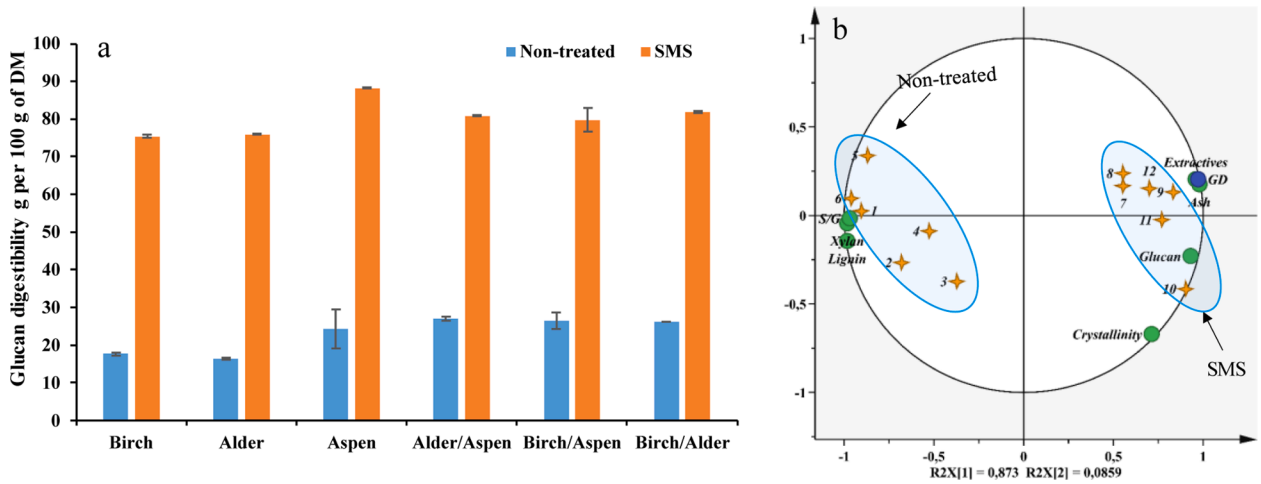

Fig. 1. Enzymatic digestibility of glucan contained in the non-treated woody substrates and SMSs (a); PCA biplot showing major chemical components of substrates and glucan digestibility (GD) (b). Each star represents an observation.

show (Guo et al., 2014; Hall et al., 2010).

It has previously been reported that low S/G ratio is deleterious for enzymatic digestibility. That has been associated with lignin guaiacyl units having higher adsorption capacity than syringyl ones, thus causing non-productive adsorption of cellulases onto lignin (Guo et al., 2014), which is a key problem affecting enzymatic hydrolysis of cellulose (Oliva-Taravilla et al., 2020). However, in the current study, an opposite trend, *e.g.*, a good correlation between low S/G ratios and enzymatic digestibility, was observed. As a possible explanation, one might hypothesize that the reduced amount of syringyl units, which are more voluminous than guaiacyl ones, can weaken steric impediment limiting lignin role as barrier blocking the access of enzymes to cellulose. It has been shown that in some pretreated biomass, lignin inhibits the enzymatic hydrolysis by acting as a physical barrier rather than by inducing non-productive adsorption of cellulases (Djajadi et al., 2018).

Cellulose crystallinity has traditionally been considered one of the main factors behind the poor enzymatic convertibility of cellulose (Hall et al., 2010). However, although the role of crystallinity is unambiguous for pure cellulose, it is not that straightforward for pretreated lignocellulose. There are many examples, where improved enzymatic convertibility of pretreated biomass and its crystallinity index are directly correlated, or even when no relationship at all is found between digestibility and crystallinity (Karimi and Taherzadeh, 2016). We have previously found that acid pretreatment of de-starched cassava stems led to enhancement of the enzymatic hydrolysis of cellulose in spite of the observed increase of the crystallinity index (Martín et al., 2017). Other authors have also stressed that ultrastructural and compositional changes caused during pretreatment are more important than cellulose crystallinity for explaining different enzymatic hydrolysis of pretreated materials (Agarwal et al., 2013; Pardo et al., 2019).

3.2.2. *Preparative enzymatic saccharification*

Preparative enzymatic saccharification was performed for generating hydrolysates to be used in fermentation experiments. Larger volumes, and higher biomass load and enzyme dosage were used compared to those of the analytical enzymatic saccharification. The experiment was performed with only three SMS samples, namely, those based on wood from individual tree species. The resulting hydrolysates contained glucose concentrations roughly between 30 and 35 g/L (Table 4), which are equivalent to glucose yields of 350–412 g/kg SMS. Aspen hydrolysate had the highest glucose content, which is consistent with the results of the analytical enzymatic saccharification (Fig. 1a). It is noteworthy that the glucose yield upon preparative hydrolysis was higher than previously reported values for acid pretreated aspen and birch (274–312 g/kg) (Wang et al., 2018a). The glucan digestibility in the preparative enzymatic hydrolysis (80.3–90.2%) was, in general, higher than the range of values achieved in the analytical enzymatic saccharification (75–88%) (Fig. 1a). That can be attributed to the higher enzyme dosage used in the preparative enzymatic hydrolysis (200 CMCase units/g biomass) compared with analytical enzymatic saccharification (100 CMCase units/g biomass).

Table 4
Composition and pH of the SMS hydrolysates.

Parameters	Unit	SMS hydrylysates		
		Birch	Alder	Aspen
Glucose	g/L	29.6	31.4	34.6
Xylose	g/L	11.7	10.4	9.9
Acetic acid	g/L	2	1.8	1.8
Formic acid	g/L	ND	ND	ND
Levulinic acid	g/L	ND	ND	ND
Phenolic compounds	g/L	2	1.8	1.8
NH_4^+-N	mg/L	0.37	0.42	0.36
NO_3^--N	mg/L	< 0.10	< 0.10	< 0.10
NO_2^--N	mg/L	1.5	1.5	1.5
Total N	mg/L	840	890	810
Glucose/Total N		35.2	35.3	42.7
pH		4.2	4.2	4.2

ND, Not detected.

The hydrolysis resulted in xylose concentrations between 10 and 12 g/L (Table 4). The high formation of xylose was due to the hydrolysis of SMS hemicelluloses. In the used enzyme preparation (Cellic CTec 2), in addition to cellulases, contains also different hemicellulases. The xylanase activity of that enzymatic preparation has been reported before (Yang et al., 2017). Acetic acid was another product of the hydrolysis of hemicelluloses. Although in a previous study, acetic acid was hardly detectable in SMSs (Chen et al., 2022), it was found in the hydrolysates in a range between 1.8 and 2.0 g/L (Table 4) and the concentration trend correlated well with that of xylose, which can be explained because both of them are products of the hydrolysis of hardwood hemicelluloses (Fengel and Wegener, 1989). Acetic acid, as well as formic and levulinic acids, is a known inhibitor of ethanolic fermentation (Jönsson and Martín, 2016). However, formic acid and levulinic acid are not a problem in SMS hydrolysates because they cannot be formed under the low temperatures typical of biological pretreatment process (Chen et al., 2022).

The hydrolysates contained also phenolic compounds with concentration of 1.8–2.0 g/L (Table 4), which were formed by lignin degradation during fungal cultivation (Chen et al., 2022). Phenols ended up in the liquid fraction when the SMS was suspended in the buffer solution. A

part of the phenolics was released right away before the hydrolysis (Table 4), while some additional amount was released during the incubation (Data not shown). The concentration of phenolic compounds, which are inhibitors of cellulolytic enzymes and fermenting microorganisms (Jönsson and Martín, 2016), was the highest in birch-based SMS hydrolysate. Their higher concentration in the initial reaction mixture of the birch-based SMS used in the analytical enzymatic saccharification might have been another reason behind the lower digestibility of that SMS compared with those of the aspen and alder.

The total content of solubilised nitrogen in the SMS hydrolysates ranged between 810 and 890 mg/L (Table 4). The highest value was detected in the hydrolysate of alder-based SMS, while the lowest one was found in that of aspen-based SMS, which is in agreement with the amounts released in the solubilisation test (Table 3). Nitrogen comes mostly from the fungal mycelium retained in SMS, but some contribution by the enzyme preparation is also possible since the amount detected in the hydrolysates was higher than the values released in the solubilisation test. The inorganic nitrogen in forms of ammonium (0.36–0.42 mg/L) and nitrate (1.5 mg/L) represented a minor proportion. To facilitate the understanding of the fermentation of the SMS hydrolysates, a ratio of glucose to total nitrogen in hydrolysates was calculated. For the hydrolysates of birch- and alder-based SMSs, the glucose-to-nitrogen ratio was the same, 35.2, while for the hydrolysate of aspen-based SMS, that value was higher (42.7).

3.3. Fermentation of hydrolysates to ethanol

It is hypothesized that the high nitrogen content and the possible presence of other substances originated from fungal biomass remnants in SMS hydrolysates could contribute to the nutrient requirements of fermenting organisms grown on them. That is a strength of SMS hydrolysates if they are going to be used as substrates for ethanolic fermentation. In order to assess that feature of SMS hydrolysates, a fermentation experiment was included in this study.

The fermentation experiment (Table 2) was first directed to assess the effect of addition of different amounts of nutrients on yeast growth and ethanol formation. A nutrient mixture, typically used for yeast fermentation, was added in three different dosages based on the supplementation reported previously for lignocellulosic hydrolysates (Ilanidis et al., 2021; Martín et al., 2018). Fermentations with non-supplemented hydrolysates, and with glucose reference media, either with no nutrients or fully-supplemented were also run. Based on the data presented in Tables 2 and 3, at the highest nutrient dosage, the expected nitrogen contents, including the nitrogen contained in the hydrolysate and that from the nutrient solution, were ~ 1394, 1440 and 1367 mg/L, respectively, for the hydrolysates of birch-, alder- and aspen-based SMSs. For the non– supplemented SMS hydrolysate, the corresponding total nitrogen values were ~ 776, 822 and 748 mg/L, respectively. That were higher than that in the glucose reference fermentation medium with full nutrient charge (2% dosage), which had nitrogen content of 618 mg/L.

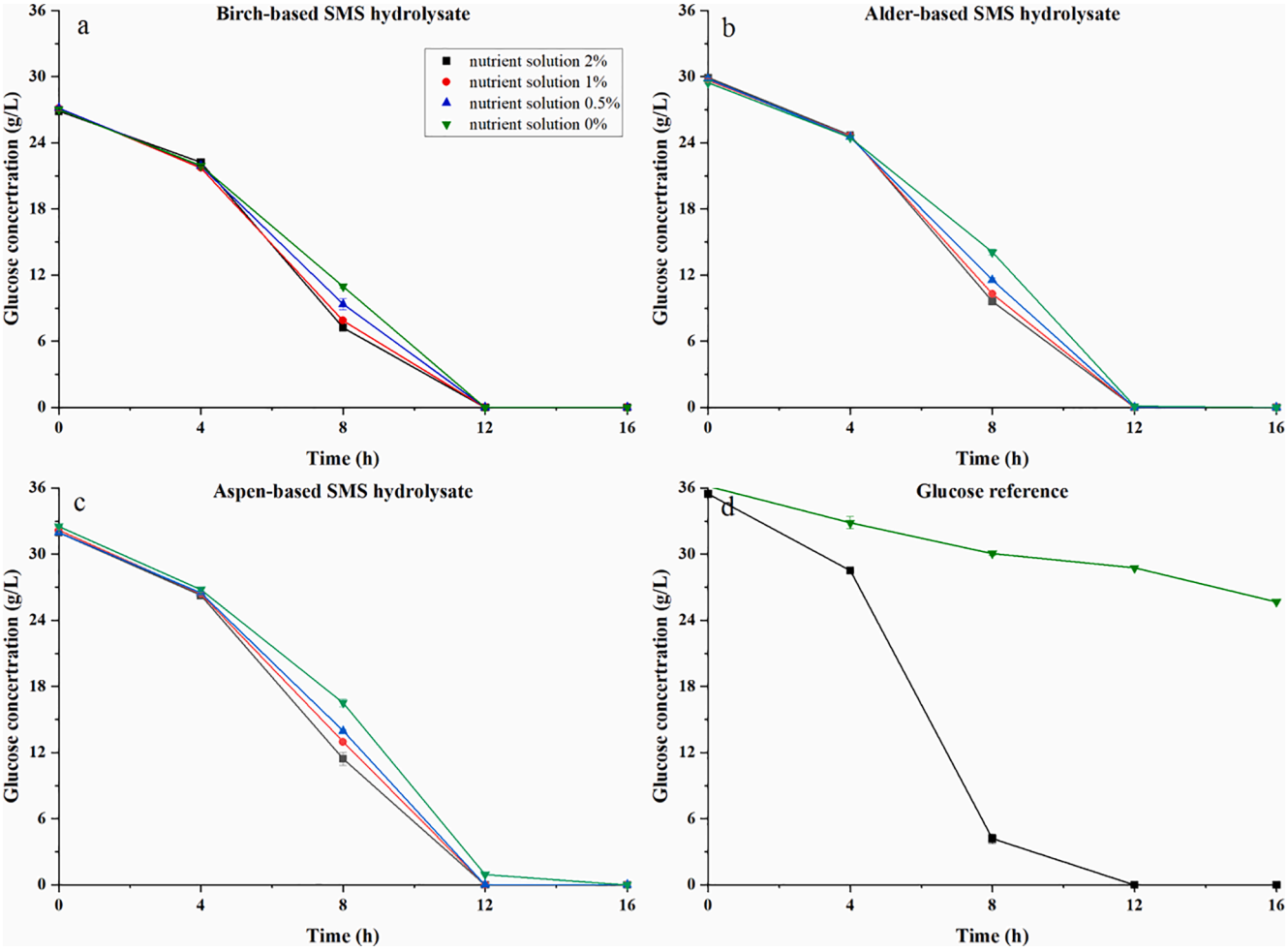

Fig. 2. Glucose consumption during *S. cerevisiae* fermentation of SMS hydrolysates (a-c) and reference media (d) with different nutrient loadings. The data refer to mean values of duplicate experiments, and the error bars represent the standard errors.

3.3.1. Glucose consumption

During the first 4 h of fermentation, around 19, 17 and 18% of the initial glucose was consumed by yeast in the hydrolysates of birch-, alder- and aspen-based SMS, respectively, and the values were comparable between media with different nutrient loadings (Fig. 2a, b and c). The consumed glucose in the hydrolysates after 8 h ranged between 50 and 73% of the initial amount, and a significant slower consumption was observed in non-supplemented hydrolysates than in those containing externally-added nutrients ($p < 0.05$) and in the fully-supplemented reference (88%) (Fig. 2d). After 12 h, glucose was depleted as in that reference as in nearly all the hydrolysates independently of the nutrient supplementation, and no clear differences were observed. The only exception was the fermentation of non-supplemented hydrolysate of aspen-based SMS, where 2.9% of the initial glucose was still unconsumed after 12 h (Fig. 2c). Compared with the birch- and alder-, aspen-based SMS hydrolysate had a higher glucose-to-nitrogen ratio (42.7; Table 4), and it is reasonable that the large amount of glucose required longer time to be consumed by yeast, especially considering a lower amount of nitrogen available for fermentation. Anyway, the fermentation behaviour of all the non-supplemented hydrolysates, including that from aspen-based SMS, was visibly better than that of the non-supplemented glucose reference. In the fermentation of the non-supplemented reference, glucose consumption was clearly restricted by the nutrient deficiency, and only 29% of the initial glucose was consumed after 16 h (Fig. 2d).

3.3.2. Yeast growth

The effect of nutrient limitation was more visible for yeast growth than for glucose consumption as can be seen from the faster OD increase in the fermentations with higher nutrient dosage than in that with no nutrient addition (Fig. 3). The OD measurements of the fermentation samples indicated that yeast growth started to be affected by the nutrition deficiency after 4 h, and the effect was more visible for the rest of the fermentation. Cell growth was more affected in the hydrolysates of birch-based and aspen-based (Fig. 3a and c) SMSs than in that of alder-based SMS (Fig. 3b). Anyway, the cell growth pattern for all the hydrolysates was comparable with that of the fully-supplemented reference medium, and it was significantly higher than that of the non-supplemented reference (Fig. 3d). Evidently, nutrient deficiency caused the inhibition of yeast metabolic activity and sluggish fermentation.

3.3.3. Formation of ethanol and acetic acid

For the fermentations of SMS hydrolysates, ethanol formation was comparable for the different loadings of the nutrient solution during the first 4 h, but it was different after 8 h (Fig. 4a, b and c). With the reduction of the nutrient amount, there was significant decrease ($p < 0.05$) of the volumetric productivity of ethanol, calculated with concentration values corresponding to the first eight hours of fermentation (Fig. 5a). The fully supplemented SMS hydrolysates resulted in ethanol productivities ranging between 0.99 and 1.09 g/L h, while the corresponding values were 0.77–0.84 g/L h in non-supplemented fermentation. The poor performance of the hydrolysate of aspen-based SMS (productivity 0.77 g/L h) can be attributed to its lower nutrient richness, since it had the lowest nitrogen content (810 mg/L) among the different non-supplemented hydrolysates (Table 4). Compared with the volumetric productivity of ethanol in the fermentation of the fully-supplemented reference medium (1.51 g/L h), the values in the

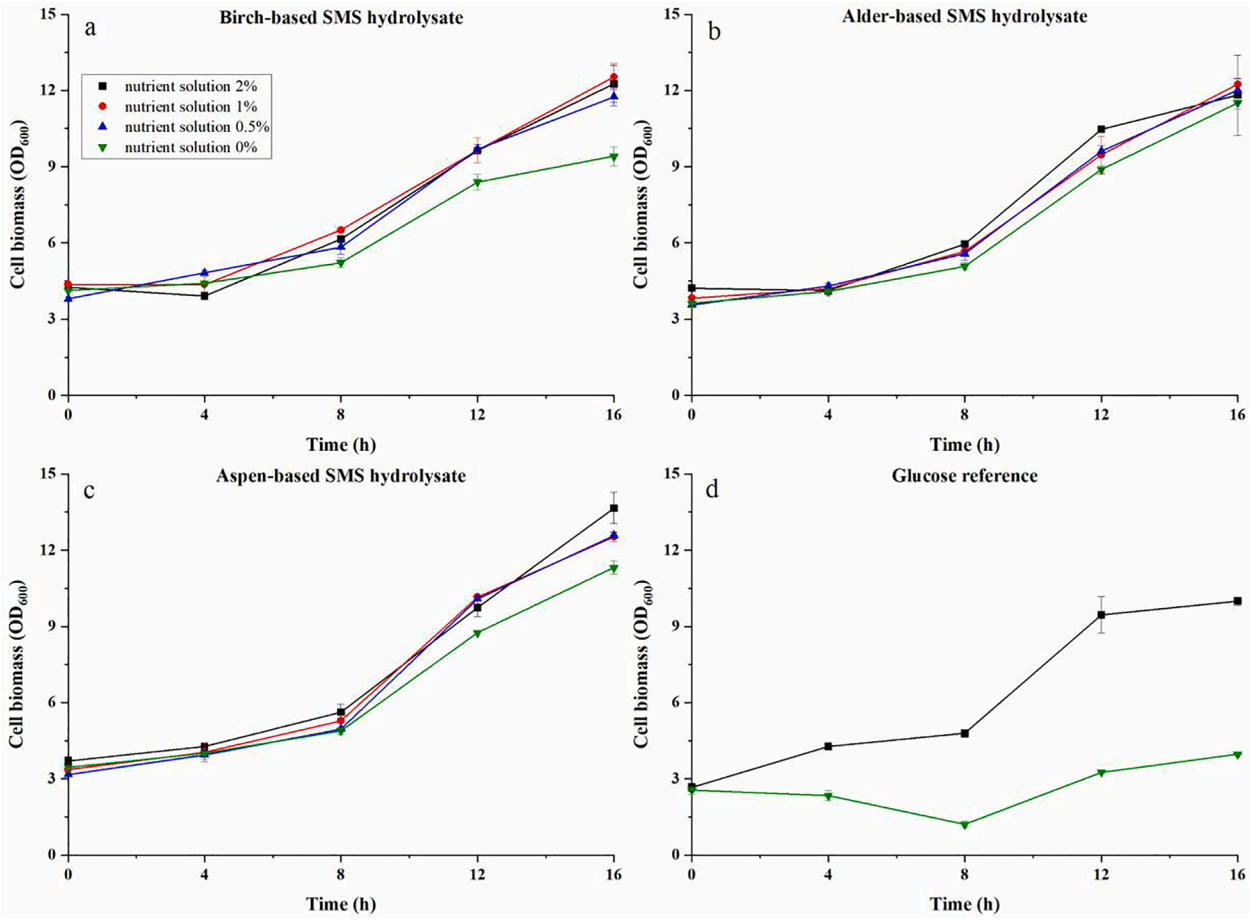

Fig. 3. Cell growth of *S. cerevisiae* during fermentation of SMS hydrolysates (a-c) and reference media (d) with different nutrient loadings. The data refer to mean values of duplicate experiments, and the error bars represent the standard errors.

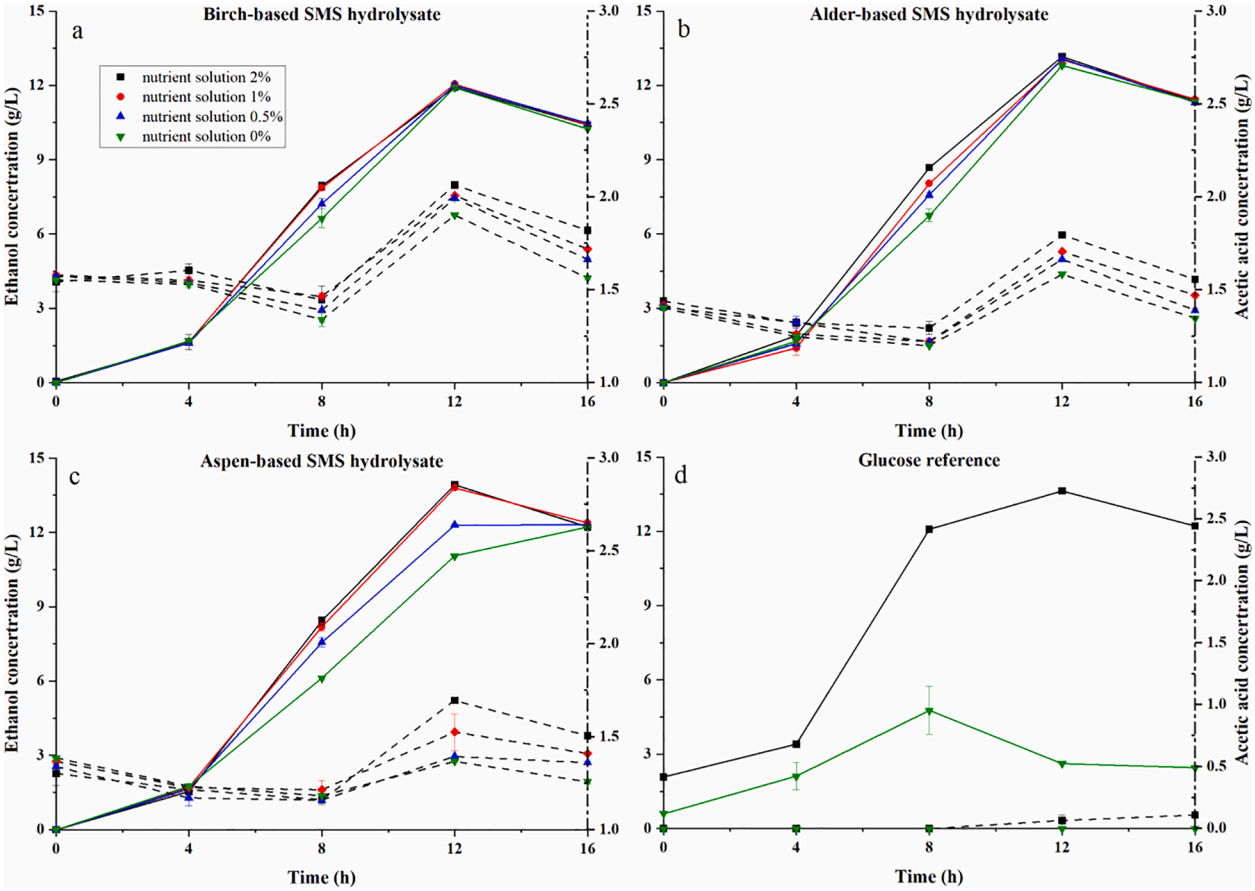

Fig. 4. Ethanol (solid line) and acetic acid (dotted line) production during *S. cerevisiae* fermentation of SMS hydrolysates (a-c) and reference media (d) with different nutrient loadings. The data refer to mean values of duplicate experiments, and the error bars represent the standard errors.

fermentation of hydrolysates was significantly lower, but they were obviously higher than that of the non-supplemented reference (0.6 g/L h).

Although ethanol concentrations were slightly different in fermented hydrolysates of birch- and alder-based SMS between nutrition loadings during the first 8 h, the values became comparable at the end of the fermentation (Fig. 4a and b). At 12 h, the concentrations of ethanol in the hydrolysate of birch-based SMS reached the maximum (11.9–12.1 g/L) (Fig. 4a), which corresponds to yields of 44.1–44.4 g/100 g glucose (Fig. 5b), and then decreased due to evaporation. For the hydrolysate of alder-based SMS, 12.6–13.2 g/L ethanol (Fig. 4b), corresponding to yields of 43.5–44.0 g/100 g glucose (Fig. 5b), was achieved. In the fermentation of the hydrolysate of aspen-based SMS, ethanol formation was clearly affected ($p < 0.05$) by differences in nutrient dosages. Supplementation with the full nutrient loading (2%) and half loading (1%) resulted in comparably high ethanol formation after 12 h (13.3–13.9 g/L) (Fig. 4c), corresponding to yields of 42.9–43.6 g/100 g glucose (Fig. 5b), whereas for the fermentation with 0.5% nutrient supplement and for the non-supplemented one, ethanol formation was remarkably lower, and it decreased proportionally with the decrease of the nutrient dosage. The higher glucose-to-nitrogen ratio in the aspen-based SMS hydrolysis (42.7) compared with the other two hydrolysates (35.2) might be behind reason for the difference. Anyway, aspen-based hydrolysate performed better than the non-supplemented glucose reference, which displayed and ethanol yield of only 7.3 g/100 g glucose (Fig. 5b).

For most hydrolysates, independently on the nutrient supplementation, the ethanol yield was high, and corresponded to 84–87% of the theoretical maximum yield (51.1) (Krishnan et al., 1999). Only the hydrolysates of aspen-based SMS with the lowest nutrient supplementation or with no supplementation at all behaved differently. Ethanol yields were higher in the hydrolysates than in the fully-supplemented reference (38.5 g/100 g glucose) (Fig. 5b). The explanation might be that in fully-supplemented reference, glucose was consumed faster and the fermentation was probably completed soon after 8 h, and no ethanol concentration value was measured at that point. In general, although increased addition of nutrients resulted in some minor increase of glucose consumption rate and volumetric productivity of ethanol for the hydrolysates, it did not affect the final ethanol yields.

The dynamics of acetic acid during the fermentation is shown in Fig. 4. There was a clear increase of acetic acid in the hydrolysate media after 12 h of fermentation. The increases of acetic acid above its initial amount can be attributed to its transient accumulation in the medium as it typically happens during glucose fermentation by yeasts. That is generally associated with the presence of contaminant microbes, other environmental issues, and it is species- and strain-dependent (Dzialo et al., 2017). Acetic acid formation increased proportionally with the increase of loading of nutrients, and the increase was more significant ($p < 0.05$) in the media with the full nutrient charge than in those with less or no nutrients (Fig. 4a, b and c). Acetic acid concentration decreased by the end of the fermentation. That can be attributed to its consumption by yeast when no more sugars were available, since acetate use as carbon source in *S. cerevisiae* is repressed by glucose (Palma et al., 2018). Although formation of acetic acid was also observed in the fermentation of fully-supplemented glucose reference (Fig. 4d), it was negligible (0–0.1%) compared with those in SMS hydrolysates.

3.3.4. SMS hydrolysates as nutrient-rich fermentation media

The good fermentation behaviour of hydrolysates supplemented with low nutrient dosages or not supplemented at all can be attributed to

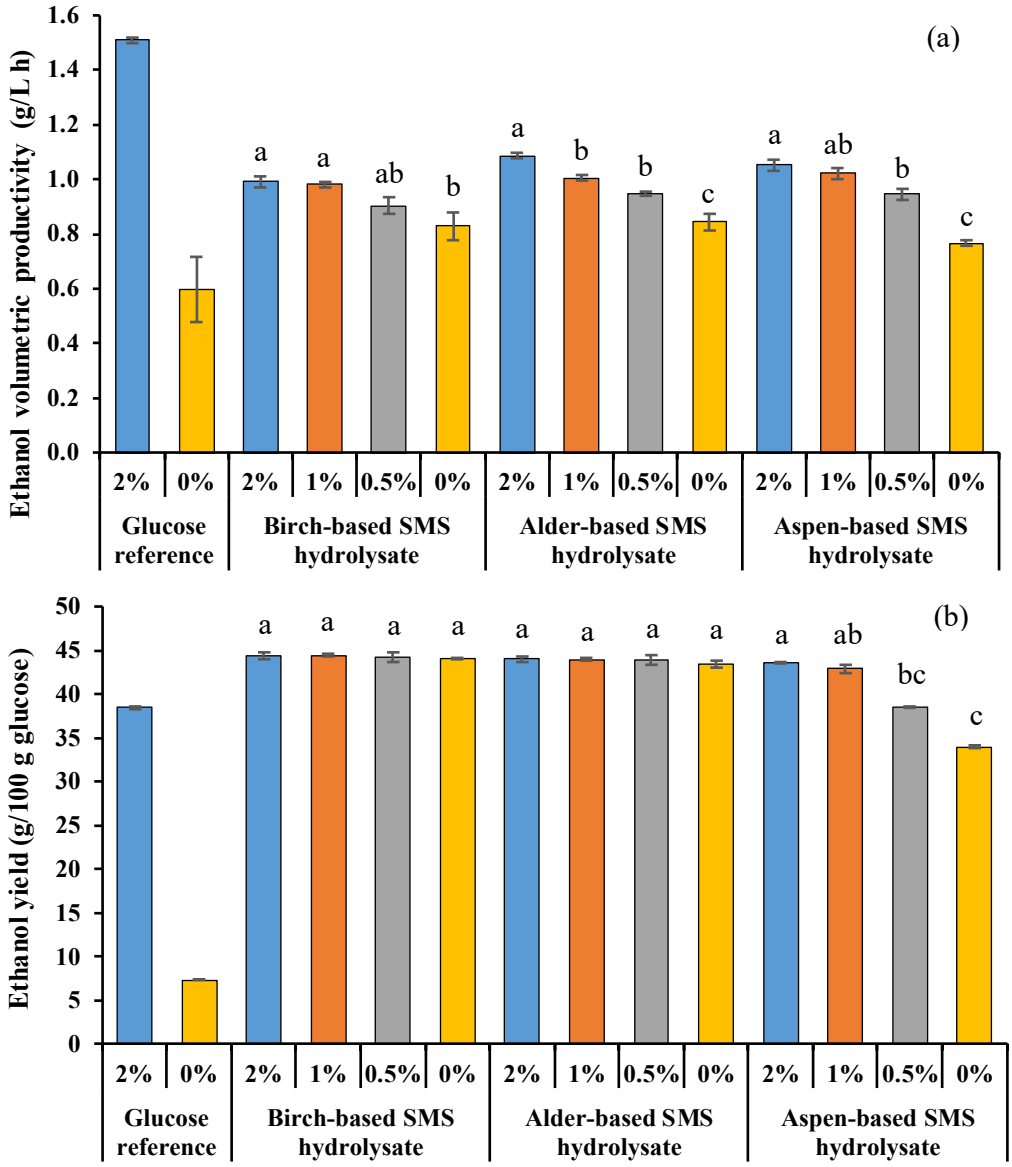

Fig. 5. Ethanol volumetric productivity (a) and yield (b) in *S. cerevisiae* fermentation of SMS hydrolysates and reference media with different nutrient loadings. The data refer to mean values of duplicate experiments, and the error bars represent the standard errors. Values within one fermentation type followed by different letters (a, b and c) indicate significant differences ($p < 0.05$) between nutrient loadings.

the presence of nutritional substances of fungal origin. Components of fungal biomass remnants contained in the SMS might have been solubilised during the enzymatic saccharification, and might be suitable as nutrient sources for yeast. Therefore, differently to other lignocellulose-based media, SMS hydrolysates can provide not only carbon sources, but also nutrients required for fermentation. The glucose-to-nitrogen ratios in SMS hydrolysates seems to be enough for ensuring the carbon–nitrogen balance that is required for ensuring an efficient fermentation by yeast.

Apparently, the nitrogen sources contained in fungal biomass remnants in the SMS (Table 3) are suitable to be used by yeast as nutrients. Among other nitrogen forms, ammonia, glutamine, and asparagine have long been considered the main sources of yeast assimilable nitrogen (YAN) (Gobert et al., 2019). In the current work, all soluble inorganic nitrogen accounted for < 0.3% DM of the total nitrogen contained in SMS hydrolysates (Table 4). In spite of that, fermentation of non-supplemented birch- and alder-based SMS hydrolysates resulted in excellent fermentation performance. That points towards involvement of other unidentified nitrogen forms, probably organic components, such as amino acids, originated from fungal mycelium. For example, glutamic and aspartic acids, which make up 4–10% of the fruit bodies, are important component of edible mushrooms (Bach et al., 2017). They might be also expected to account for a high proportion in the fungal mycelium. This might be associated with the increase of pH of the SMS hydrolysates (from 5.5 to 6.2) during fermentation (Fig. 6a), and which was not observed in the reference media, whose final pH was below 4.1. The increase of the pH during the fermentation of the SMS hydrolysates might be linked with the dissociation of amino acids in slightly acidic conditions, which results in the protonation of the amino group with formation of an alkylammonium cation (Russo and Casazza, 2012). The pH increase was inversely proportional to the amount of added nutrients. An explanation might be that high nutrient supplementation provided more direct nitrogen source, and thus reduced use of amino acid, resulting a significant lower pH ($p < 0.05$).

3.3.5. Potential effect of inhibitors on fermentation performance

Regardless of nutrient supplementation, *S. cerevisiae* growth during fermentations of SMS hydrolysates was inhibited in comparison with

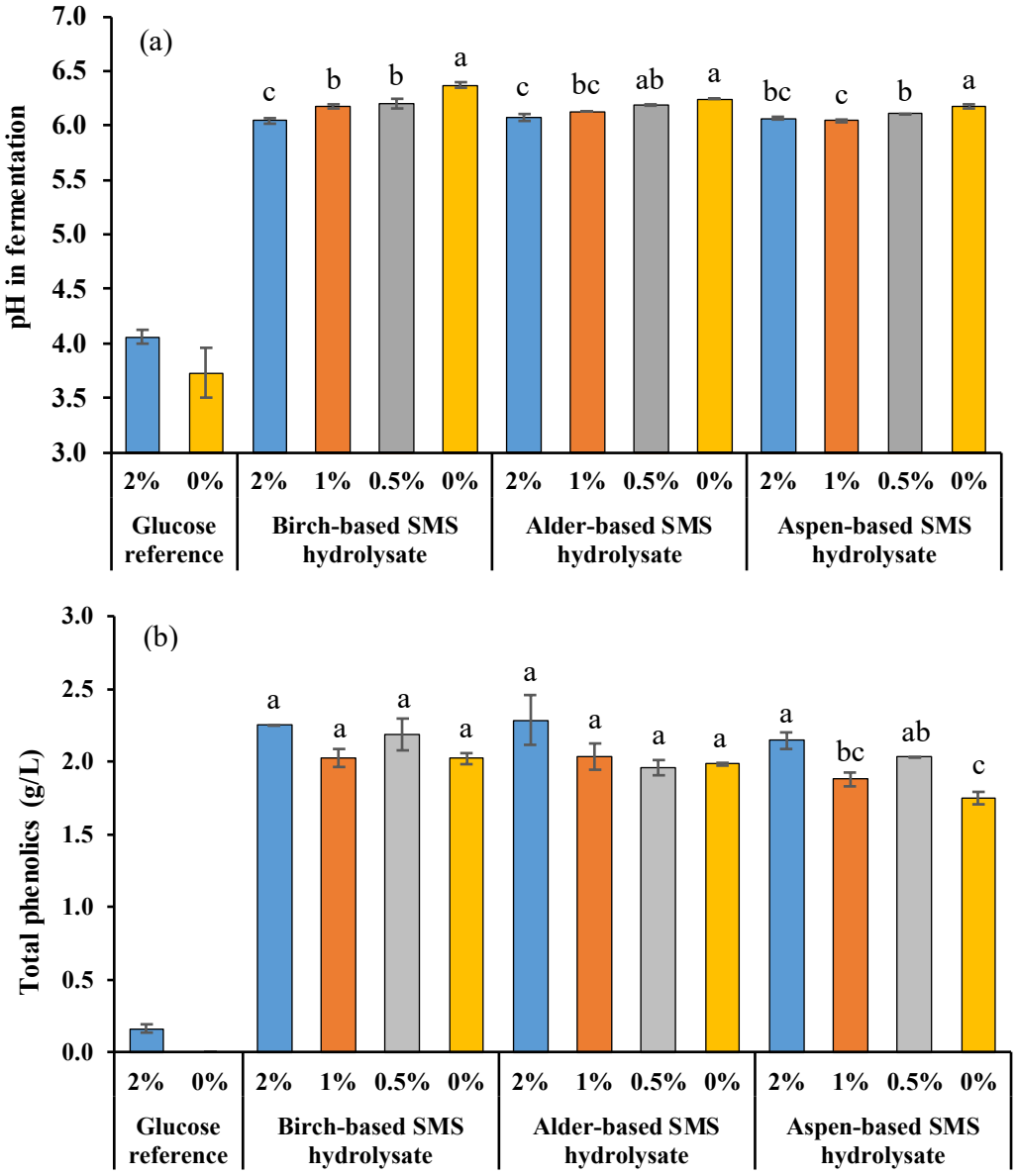

Fig. 6. Final pH (a) and concentrations of total phenolic compounds (b) in *S. cerevisiae* fermentation of SMS hydrolysates and reference media with different nutrient loadings. The data refer to mean values of duplicate experiments, and the error bars represent the standard errors. Values within one hydrolysate type followed by different letters (a, b and c) indicate significant differences ($p < 0.05$) between different nutrient loadings.

that of the reference medium with full nutrient charge. In the reference fermentation, where the initial nitrogen concentration was 618 mg/L, the cell growth, measured as optical density, increased by 60, 80 and 254% of the initial value after 4, 8 and 12 h, respectively (Fig. 3d). In contrast, the non-supplemented SMS hydrolysates (Fig. 3a, b and c), which had higher nitrogen concentration than the reference (greater than748 mg/L), resulted in lower increases of cell growth, namely up to 12, 36 and 134% after 4, 8 and 12 h, respectively. The lower cell growth in SMS hydrolysates can be attributed to inhibition caused by toxic compounds, such as acetic acid and phenolic compounds, which are known to inhibit microbial processes (Jönsson and Martín, 2016), and were contained in all the hydrolysates (Table 4).

When comparing ethanol production in the SMS hydrolysates and in the reference fermentation, some inhibition was observed for the volumetric productivity (Fig. 5a), while the yield was not affected (Fig. 5b). The inhibition of the productivity might has been caused by phenolic compounds and acetic acid in the hydrolysates. However, the concentration of those inhibitors was low; their effect on fermentation was weak and did not last long. The situation is different from the stronger inhibition reported in fermentation of hydrolysates produced by methods resulting in high formation of inhibitors. Acetic acid concentration in SMS hydrolysates was up to 2 g/L (Table 4), while in hydrolysates of acid pretreated corn cob, spruce and oak it is typically above 4 g/L (Du et al., 2020; Ilanidis et al., 2021; Ko et al., 2016). In addition, it had been reported that inhibition by acetic acid is pH dependent, i.e., high toxicity towards *S. cerevisiae* is observed when the extracellular pH is below the pKa of acetic acid (4.7), but at pH above that value, no severe inhibitory effect occurs (Ko et al., 2016; Taherzadeh et al., 1997; Wei et al., 2013). During the fermentation, the pH of the media was always above acetic acid pKa, since it was 5.5 at the beginning, and then it increased to up to 6.2, especially at low nutrient supplementation (Fig. 6a). That might have further weakened the inhibition effect of acetic acid. Furthermore, differently from hydrolysates produced by conventional pretreatment methods (Bolado-Rodríguez et al., 2016; Martín et al., 2018), SMS hydrolysates do not contain other inhibitors, such as furan aldehydes, formic acid and levulinic acid. Phenolic

compounds are the only type of inhibitors that was detected in comparable amounts in this study and in acid pretreated sugarcane bagasse and spruce hydrolysates (Ilanidis et al., 2021). The dynamics of acetic acid during fermentation is shown in Fig. 4, and it is discussed in 3.3.3, while the concentration of phenolic compounds in SMS hydrolysates remained rather similar before (Table 4) and after the fermentation (Fig. 6b).

3.4. Implication of using shiitake SMS for biorefinery

Table 5 summarizes the mass balance of converting shiitake SMSs (100 g) to ethanol, with an emphasis of lignocellulose fractions. After enzymatic saccharification, about 40–50% of SMS was solubilised and recovered as monosaccharides in the hydrolysates. The obtained glucose, around 35–41 g of 100 g SMS, was then fermented to ethanol yielding 14–17 g. The amount of produced ethanol from SMSs is comparable or higher to what is produced from pretreated solids from other pretreatment methods (Du et al., 2020; Qureshi et al., 2015; Wang et al., 2018b). This reveals the high potential of fungal pretreatment using shiitake as a biorefinery approach producing ethanol and edible mushrooms with high value as food and source of nutraceuticals and pharmaceuticals.

During the fermentation without nutrient supplementation, except for the hydrolysate of aspen-based SMS, glucose was depleted after 12 h. There was some apparent xylose consumption, which is unexpected since *S. cerevisiae* cannot ferment xylose. That might be attributed to consumption of mannose, resulting from mannan contained in SMS, for example, it represented around 2% of the weight of birch-based SMS in a previous study (Wei et al., 2020). Mannose and xylose elute as a single peak with the used HPLC method. Anyway, it was found that per each 100 g of SMS, 9–12.4 g of xylose, corresponding to 77–90% of the initial amount, remained unconsumed in the fermentation broth. Such a remarkable amount should be considered for conversion to other products in future research.

Approximately 49–57 g out of 100 g SMS remained as a solid residue after enzymatic saccharification (Table 5). That residue was composed of 33–40% extractives, 21–25% lignin and 20% carbohydrates. It is noticeable that Klason lignin was completely recovered after the hydrolysis, while acid-soluble lignin was only partially (50%) retained in the solid leftovers (Table 1 vs. Table 5). The explanation of that phenomenon is beyond the scope of the current investigation, and a deeper understanding remains to be studied. Anyway, the solid leftover, a major side stream of enzymatic hydrolysis, implies an important potential resource for producing additional bio-based products, probably alongside the same biorefinery chain. Although a previous study showed its potential for thermal energy production (Chen et al., 2021), alternative valorisation routes, for example, can be direct its lignin or polysaccharide components to advanced biofuels or other bio-based products. Assesment of the technical feasibility and systems analysis studies on those expectations have to be carried out in future studies.

4. Conclusions

It was found that using shiitake cultivation as pretreatment for facilitating lignocellulose bioconversion results in a good enhancement of the enzymatic saccharification of cellulose and provides easily-accessible nitrogen sources for supporting ethanolic fermentation. The initial nitrogen contained in the SMS hydrolysates ensured good ethanol production even without supplementing additional nutrients. The presence of phenolic compounds and acetic acid was detected in the enzymatic hydrolysates, but their concentrations were low and caused only some limited inhibitory effect on ethanol production. Mass balance analysis of the presented fermentation system revealed important amounts of xylose and hydrolysis leftover that require being valorised.

Table 5
Mass balance during enzymatic hydrolysis and fermentation.

	Units	Substrate species		
		Birch	Alder	Aspen
SMS	g	100	100	100
Enzyme preparation	g	13.6	13.6	13.6
Buffer chemicals	g	23.4	23.4	23.4
After preparative enzymatic saccharification [1]				
Hydrolysates				
Glucose	g	34.9	37.6	41.2
Xylose [2]	g	13.8	12.5	11.8
Solid leftover				
Glucan	g	7.3	8.3	7.8
Xylan	g	3.1	3.2	2.4
KLL	g	8.5	10.3	10.3
ASL	g	2.3	2.1	2.2
Extractives	g	19.0	23.1	16.4
Ash and other [3]	g	9.5	10.1	10.3
After fermentation of hydrolysates [4]				
Ethanol	g	15.4	16.4	14.0
CO_2 loss	g	14.7	15.7	13.4
Residuals				
Glucose	g	0	0	1.2
Xylose [2]	g	12.4	9.6	9.1
Others [5]	g	43.1	45.2	45.5

[1]With addition of 200 CMCase units/g biomass; [2] Includes other hemicellulosic sugars; [3] Includes unidentified components; [4] Without nutrient supplementation; [5] Estimated by differences.

CRediT authorship contribution statement

Feng Chen: Investigation, Data curation, Writing – original draft, Writing – review & editing. **Shaojun Xiong:** Conceptualization, Project administration, Funding acquisition, Writing – original draft, Writing – review & editing. **Madhavi Latha Gandla:** Investigation. **Stefan Stagge:** Investigation. **Carlos Martín:** Conceptualization, Data curation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

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

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