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# Chemical and Biological Pretreatments on Sugarcane Bagasse to Enhance its Enzymatic Hydrolysis.

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Alkaline and biological lignocellulose pre-treatments are commonly used to enhance enzymatic hydrolysis and improve ethanol production. In this study, <sup>13</sup>C CPMAS NMR spectroscopy was used to describe changes in sugarcane bagasse (SCB) pre-treated with NaOH, Ca(OH)<sub>2</sub> and with *Pycnoporus sanguineus*. Changes in the contents of alkyl C, Carboxyl C, Aromatic C (tertiary, quaternary and p-hydroxyphenyl C), O-alkyl C, amino acids, ergosterol and chitin, as well as in the crystallinity index of cellulose were observed. Through a multivariate analysis, relations between changes in the chemical composition of SCB and enzymatic hydrolysis were established. *P. sanguineus* promotes better lignin decay, glucose release and hydrolysis yields than chemical pre-treatments, and increases the amount

of amino acids and ergosterol in SCB, while NaOH increases the cellulose crystallinity index. The hydrolysates were fermented with *Saccharomyces cerevisiae* for 96 h, and analysed through HPLC. The initial composition of the hydrolysates [mg.ml<sup>-1</sup>] and biomass production (cells.ml<sup>-1</sup>) were then related to the ethanol production and fermentation yields. We found that ethanol production and fermentation yields were negatively correlated with cell growth in *Saccharomyces*, but positively correlated with glucose consumption in the *P. sanguineus* pre-treatment. We conclude that the biological pre-treatment using *P. sanguineus* in the conditions hereby described, has a potential to increase ethanol productivity.

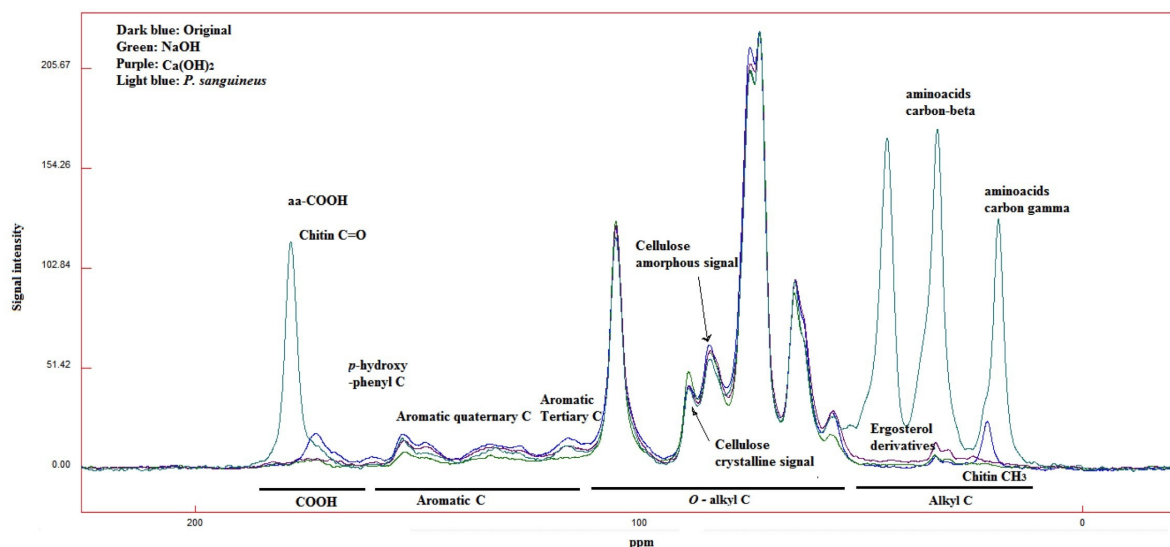
## Introduction

Sugarcane bagasse (SCB) is a promising source of raw material for bioethanol production<sup>[1]</sup> through enzymatic hydrolysis. The efficiency of this method depends on a pre-treatment aimed at reducing the crystallinity of the raw material, as well as its hemicellulose and lignin content, lignin being a cellulase adsorbent.<sup>[2,3]</sup> Thus, for an efficient lignocellulose hydrolysis, a pretreatment is necessary in order to decrease lignin, the cellulose-crystallinity index, and the hemicellulose content.

A good pre-treatment includes alkaline agents like NaOH, Ca(OH)<sub>2</sub> and KOH.<sup>[4]</sup> Alkali attacks lignin via saponification of ester-linkages between cellulose and hemicellulose with lignin,<sup>[5]</sup> although it may generate alkaline-cellulose by ion exchange.<sup>[6]</sup> Also, as opposed to acids, alkaline agents for biomass delignification rarely generate compounds that are harmful to microorganisms.<sup>[7]</sup>

Biological pre-treatments using live fungi, on the other hand, have been found to enhance enzymatic hydrolysis.<sup>[8]</sup> Fungi (mainly white-root fungi) can oxidize lignin via phenoloxidase enzymes (i.e., laccases, Mn-peroxidases, lignin peroxidases) and produce cellulases and hemicellulases (e.g. xylanases), decreasing the stock of polysaccharides and, consequently, the production of bioethanol. And since fungi also transform some lignocellulose material into fungal biomass, the outcome is a different raw material altogether. The structural characterization of SCB fibres pre-treated with live fungi is key to the understanding of how fungal enzymes affect fibre composition, and how this structural composition affects in turn the hydrolysis/fermentation processes downstream. Classical methods for fibre analysis based in subsequent

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**Figure 1.**  $^{13}\text{C}$  CPMAS NMR spectra of sugarcane bagasse before after pretreatments (indicated with colours). Important regions are shown.

detergent hydrolysis (e.g. Van Soest and Wine<sup>[9]</sup>) are not enough to explain the complex modifications of lignocellulose fibre by fungi or chemical pre-treatments. Thus, for lignocellulose characterization, the use of other techniques, such as near infrared spectroscopy (FTIR) and  $^{13}\text{C}$  solid-state nuclear magnetic resonance (NMR), are preferred.

$^{13}\text{C}$  CPMAS NMR spectroscopy is a powerful technique of analysis, successfully used on soil litter,<sup>[10]</sup> the lignification process,<sup>[11]</sup> and the extent of condensation in lignin characterisation,<sup>[12]</sup> and can also be used to evaluate changes in fungal biomass.<sup>[13]</sup> Solid-state NMR allows for chemical analysis to be carried out in a natural state, and is an excellent choice for samples with restricted solubility, as are lignin residues, or when the physical structure of cellulose is to be examined.<sup>[14]</sup> Therefore, the aim of this study was to characterize SCB fibre by  $^{13}\text{C}$  CPMAS NMR spectroscopy after a biological pre-treatment, using the fungus *Pycnoporus sanguineus* and two alkaline pre-treatments with NaOH and  $\text{Ca}(\text{OH})_2$ . For more details see SI. After that, pre-treated SCB was hydrolysed with *Aspergillus niger* cellulases and fermented with *Saccharomyces cerevisiae*, and the yields of both steps were related to their structural composition using multivariate statistical methods.

## Results and Discussion

### Chemical shifts in SCB submitted to different pre-treatments

Both chemical and biological pre-treatments lead to changes in the chemical composition of SCB. The O-alkyl C signal (45–110 ppm) corresponds to the carbohydrate (i.e., holocellulose) content of SCB, indicating that the original composition of SCB had 79.76% holocellulose, and that after an alkaline pre-treatment the proportion increased to 88.86% with NaOH, and to 84.13% with  $\text{Ca}(\text{OH})_2$  (Figure 1). Conversely, biological pre-

treatment decreased the amount of holocellulose to 50.65%. The aromatic C region, which corresponds to lignin, had an original proportion of 12.87%, and the pre-treatments decreased this value as follows:  $\text{Ca}(\text{OH})_2$  to 10.26%, NaOH to 7.03%, and *P. sanguineus* to 6.53%. All treatments led to a decrease in aromatic C content, but the highest lignin decay was achieved with *P. sanguineus* (Table 1).

The values of the regions Carboxyl C and Alkyl C are associated to proteins and lipids, and their peaks correspond to the acetate groups in the hemicellulose that appears in this region. Taking them together, they were very low in the original composition of SCB (3.57% and 2.45%, respectively). Alkaline pre-treatments diminish the amount of Carboxyl C to 1.48% (NaOH) and 1.60% [ $\text{Ca}(\text{OH})_2$ ], and in general they did not affect the Alkyl C region. On the other hand, the pre-treatment with *P. sanguineus* strongly increased both regions: Carboxyl C increased to 9.31%, and Alkyl C to 33.49% (Figure 1).

The signals corresponding to the  $\text{C}\alpha$ ,  $\text{C}\beta$  and  $\text{C}\delta$  of amino acids increased with the treatments with *P. sanguineus* from 5.61% to 18.38% (as did the signals corresponding to chitin and the  $\text{C}\gamma$  of amino acids, from 3.47% to 8.49%), whereas alkaline treatments diminished or did not change those signals (Figure 1). The ergosterol signal was near to 0 in the original composition of SCB, and remained the same after the alkaline pre-treatments. However, when *P. sanguineus* was used, ergosterol increased from 0.24% to 4.33%. Thus, *P. sanguineus* transformed a portion of SCB into fungal biomass, as was hypothesized at the beginning.

The proportion of amorphous cellulose and crystalline cellulose, estimated by the signal position of the C4 of pyranoglucose,<sup>[20]</sup> indicates that SCB has an original crystallinity index (CI) of 0.29. This value changed slightly according to the pre-treatments employed: SCB pre-treated with NaOH increased its CI to 0.32, decreased to 0.27 when  $\text{Ca}(\text{OH})_2$  was used, and

Table 1. Regions of $^{13}\text{C}$ CPMASNMR spectra utilized for assess chemical shifts in SCB		
Component	Position (ppm)	Reference
Alquil-C region	0-45	[15]
Aminoacids carbon $\gamma$	16.1-24.7	[15]
Aminoacids carbon $\beta$	28-37.6	[15]
$\text{CH}_3$ of quitin	23.32	[16]
Ergosterol and derivatives C4, C24, C20, C13, C10	37.05-42.85	[17,18,19]
O-alkyl C Region	45-110	
Cellulose crystallinity index	Is calculated by dividing the total area of the crystalline peak (87 to 93 ppm) by the total area assigned to the $\text{C}_4$ peak (80 – 93 ppm).	[20]
Aminoacids carbon $\alpha$	53.5-61.3	[15]
Aminoacids carbon $\delta$	47.5-49.2	[15]
Xylane C4	81.2 – 81.7	[21]
Aromatic C Region	110 – 160	
Aromatic tertiary C	110-123	[22]
Aromatic quaternary C	123-160	[22]
<i>p</i> -hydroxyphenyl	157-162	[22]
	160-190	
Carboxil-C Region	173.7	
Quitin $\text{C}=\text{O}$	170.9-174.5	[16]
Carbon of $\text{COOH}$ from aminoacids		[15]

remained unchanged with the *P. sanguineus* pre-treatment. This indicates that NaOH promotes amorphous cellulose removal, which may affect the performance of hydrolysis. Finally, the signals of aromatic carbons decreased in all treatments, *P. sanguineus* being the treatment that obtained the highest decrease of aromatic carbons, followed closely by the SCB pre-treated with NaOH, and then by the SCB pre-treated with  $\text{Ca}(\text{OH})_2$ .

### Hydrolysis step

In all treatments, cellulase activity decreased rapidly within the first 24 hours, while total sugar content increased within the first 24 hours, remaining almost unchanged afterwards (Figure 2).

The area under the curve of FPU activities showed that when SCB pre-treated with *P. sanguineus* (99.21 FPU for 96 h) and NaOH (115 total FPU for 96 h) was hydrolysed, the enzymatic stability was higher. Conversely, less FPU activity was registered in treatments with SCB pre-treated with  $\text{Ca}(\text{OH})_2$  (56.91 total FPU in 96 h), and with untreated SCB (57.33 total FPU for 96 hours). Cellulase stability was positively correlated with O-alkyl C, Xylan C4 and Cellulose crystallinity, and negatively correlated with Alkyl C, Carboxyl C, carbon amino acids, chitin and ergosterol (Table 2).

Like total reducing sugars release, glucose production was faster within the first 24 h of hydrolysis. SCB pre-treated with *P. sanguineus* produced the highest amount of glucose ( $29.76 \pm$

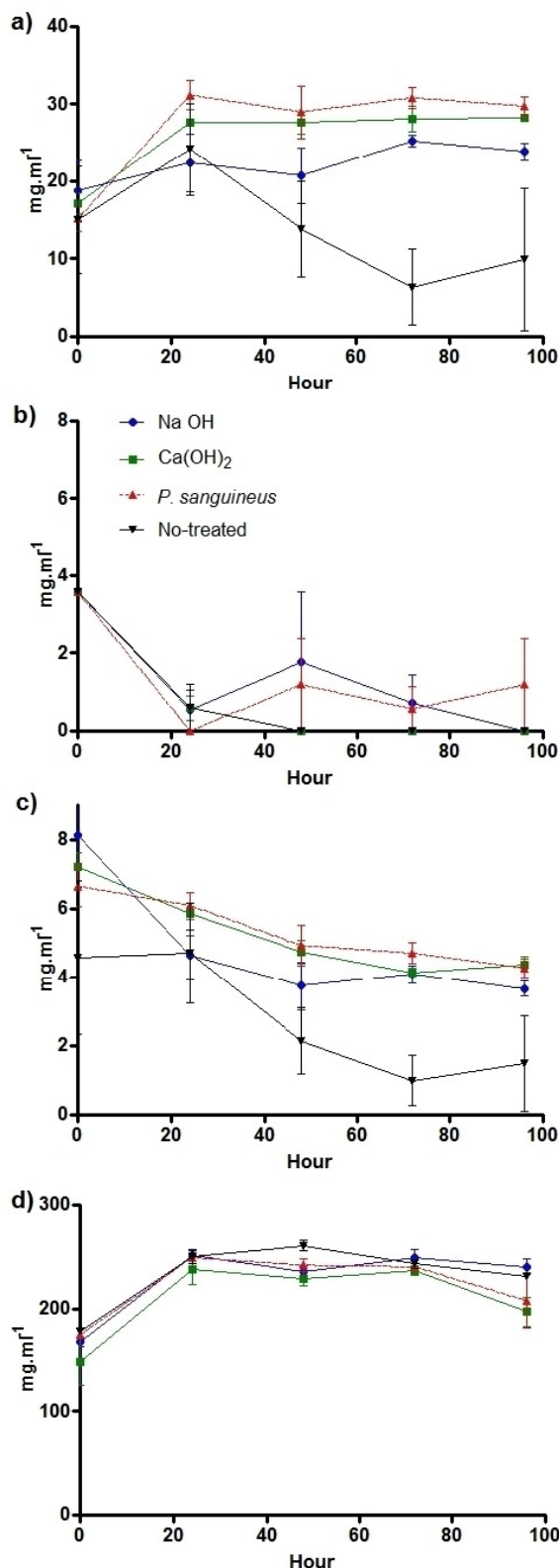


Figure 2. Kinetics of the different parameters measured during the hydrolysis phase. a) glucose released, b) cellulase activity (FPU), c) saccharose released, d) total reducing sugars released. Different colours indicate different pretreatments. Middle points represent the means, and bars the standard error.

Compounds	Cellulase stability	Glucose released	Hydrolysis yield	Saccharose final content
Alkyl C	-0.592	0.746	0.923	0.435
Carboxyl C	-0.500	0.545	0.786	0.158
Aromatic C	0.117	-0.653	-0.668	-0.739
O-alkyl C	0.597	-0.634	-0.847	-0.254
Aαγ + chitin	-0.478	0.489	0.742	0.087
aaCβδα	-0.675	0.797	0.950	0.483
Ergosterol	-0.99	0.762	0.932	0.459
Xylane C4	0.497	-0.593	-0.823	-0.232
Cellulose CI	0.793	-0.311	-0.268	-0.036
Tertiary aromatic C	0.257	-0.757	-0.740	-0.833
Quaternary aromatic C	0.057	-0.604	-0.632	-0.694
p-hydroxyphenyl aromatic C	0.247	-0.736	-0.633	-0.908

2.2 mg.ml<sup>-1</sup>), followed by SCB pre-treated with Ca(OH)<sub>2</sub> (28.19 ± 0.45 mg.ml<sup>-1</sup>), NaOH (23.84 ± 2.0 mg.ml<sup>-1</sup>), and the untreated SCB (9.95 ± 15.93 mg.ml<sup>-1</sup>) (Figure 2). Saccharose content decreased slightly during SCB hydrolysis in all treatments (Figure 2), reaching a final concentration of 4.38 ± 0.36 mg.ml<sup>-1</sup> when pre-treated with Ca(OH)<sub>2</sub>, 4.26 ± 0.4 mg.ml<sup>-1</sup> when pre-treated with *P. sanguineus*, 3.66 ± 0.3 mg.ml<sup>-1</sup> when pre-treated with NaOH, and 1.48 ± 2.4 mg.ml<sup>-1</sup> when untreated (Figure 2).

The glucose released was positively correlated with alkyl C, carboxyl C, amino acids content, chitin and ergosterol. These variables positively affected the hydrolysis yield too (Table 2). Meanwhile, cellulose crystallinity, aromatic C, and tertiary, quaternary and p-hydroxyphenyl aromatic C, negatively affected glucose release and the hydrolysis yield. Saccharose was positively correlated with the variables associated to the presence of fungal biomass, and negatively correlated with lignin compounds (aromatic carbons) (Table 2).

The treatment that promoted the highest glucose and hydrolysis yield was *P. sanguineus*.

The highest hydrolysis yield was achieved when SCB was pre-treated with *P. sanguineus*, followed by pre-treatments with Ca(OH)<sub>2</sub> and NaOH. The lowest yield was obtained with untreated SCB. The fungal biomass gain did not negatively affect enzymatic hydrolysis. In fact, the highest hydrolysis yield was obtained with SCB pre-treated with *P. sanguineus*. The effect of proteins or lipids on cellulase catalysis is not well documented, but it is known that some lipids can act as surfactants, and avoid the absorption of cellulases by cellulose, increasing the hydrolysis yield.<sup>[23,24]</sup> Some proteins, like bovine serum albumin, can perform the same role.<sup>[25]</sup>

Thus, the enrichment of proteins and ergosterol by the pre-treatment with *P. sanguineus* lead to a decrease in the absorption of cellulase by the SCB fibre, improving enzymatic hydrolysis. The decay in the aromatic C and O-alkyl C regions in this pre-treatment are mainly due to the production of laccases

and cellulases (as previously reported<sup>[27]</sup>). Many authors suggest that this fungus is a good producer of laccase,<sup>[26,28]</sup> and therefore an excellent choice for delignification processes.<sup>[29]</sup>

According to our Principal Component Analysis, the hydrolysis yield was mainly affected by the content of aromatic C and xylan C4, which, as previously reported,<sup>[2]</sup> corresponds to the content of lignin and hemicellulose. The cellulose crystallinity index (CI) was negatively related to the hydrolysis yield, according to Mussato et al.<sup>[3]</sup> In this study, the pre-treatment with NaOH increased CI, which is related to lower hydrolysis, compared to the SCB pre-treated with *P. sanguineus*. Pre-treatments with NaOH and Ca(OH)<sub>2</sub> (of less effect) increased cellulose crystallinity, but also increased the O-alkyl region and cellulase stability. Untreated SCB had the highest amounts of lignin compounds, and was negatively associated to the release of glucose and the hydrolysis yield (Figure 3).

### Fermentation step

As with enzymatic hydrolysis, SCB pretreated with *P. sanguineus* had the highest fermentation yield and the second highest production of ethanol. Differences in the hydrolysis yield led to differences in the initial glucose content of the fermentation cultures (Figure 3). Glucose decreased near to 0 mg.ml<sup>-1</sup> in all treatments after 48 h of culturing with *S. cerevisiae*, while saccharose metabolized more slowly (Figure 3).

On the other hand, *S. cerevisiae* biomass (Figure 4) and ethanol increased after 96 h. *S. cerevisiae* cell growth (biomass) was positively correlated with the glucose consumed, but negatively related with the fermentation yield and ethanol production (Table 3).

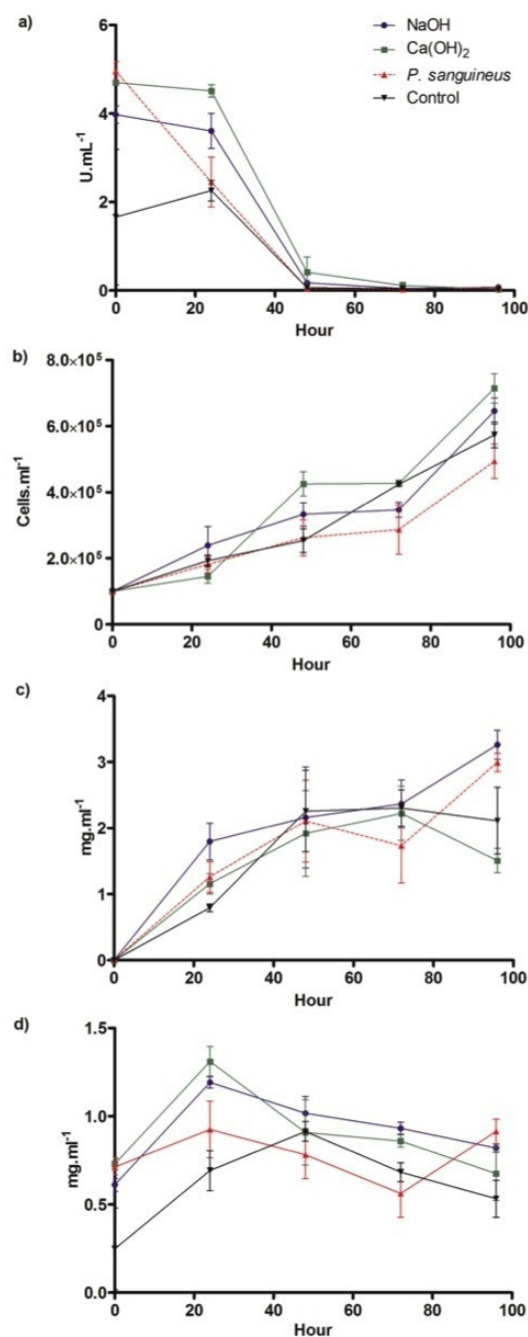
A negative effect of hydrolysates pretreated with *P. sanguineus* on *S. cerevisiae* biomass was observed, which could be related to a secondary metabolite produced by fungus during the pretreatment. Cinnabarin and poliporin, produced by *P. sanguineus*, have antimicrobial properties;<sup>[30,31]</sup> however, non-antifungal metabolites have been reported for this fungus. In fact, hydrolysed SCB pre-treated with *P. sanguineus* does not completely inhibit the growth of *S. cerevisiae*, although it does induce cell compilation. This interesting phenomenon needs further study.

SCB hydrolysates pre-treated with NaOH (3.26 ± 0.3 mg.ml<sup>-1</sup>) and *P. sanguineus* (2.99 ± 0.2 mg.ml<sup>-1</sup>) were the ones that produced more ethanol, followed by untreated SCB (2.11 ± 0.8 mg.ml<sup>-1</sup>) and SCB pre-treated with Ca(OH)<sub>2</sub> (1.50 ± 0.3 mg.ml<sup>-1</sup>) (Figure 3). The highest fermentation yield was attained from the hydrolysate of SCB pre-treated with *P. sanguineus*, followed by untreated SCB, SCB pre-treated with NaOH, and, finally, SCB pre-treated with Ca(OH)<sub>2</sub>.

No strong relations were observed between those variables measured with ethanol production and fermentation yields. Nevertheless, a PCA biplot indicates that the hydrolysed composition resulting from SCB pre-treated with *P. sanguineus* reached better ethanol and fermentation yields (Figure 4), while the least efficient pre-treatment was that with Ca(OH)<sub>2</sub>.

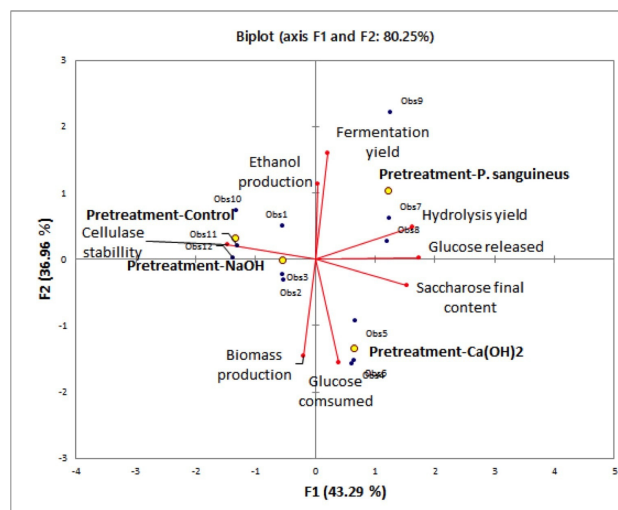
Having examined the effects of alkaline and biological pre-treatments on the downstream process, we suggest that when





**Figure 3.** Kinetics of the different parameters measured during fermentation. a) glucose consumption, b) *S. cerevisiae* cell growth, c) ethanol production, d) saccharose consumption. Different colours indicate different pretreatments. Middle points represent the means, and bars the standard error.

the aim is to produce ethanol, the use of *P. sanguineus* is preferable to the use of  $\text{NaOH}$  or  $\text{Ca(OH)}_2$ , since it leads to higher hydrolysis and fermentation yields than alkali treatments. Further studies will focus on the effect of ergosterol and fungal proteins on cellulase catalysis, and the use of other fungus in the SCB pre-treatment.



**Figure 4.** Principal component analysis. The biplot shows the relationship between the different pre-treatments (qualitative variables) with all the parameters measured during hydrolysis and fermentation.

## Conclusions

In summary, both alkaline and biological pre-treatments were able to decrease the intensity of aromatic C (lignin) signals. However, biological pre-treatments converted a portion of the O-alkyl C (carbohydrates) into fungal biomass (lipids and proteins). The ergosterol and protein gained by the SCB fibre by the pre-treatment with *P. sanguineus* might be a positive factor to enhance the hydrolysis yield. These beneficial effects include better fermentation yields than the ones obtained by hydrolysates from SCB pre-treated with alkaline solutions.

## Supporting Information Summary

For experimental details, such as chemical and biological treatments, enzymatic hydrolysis and fermentation, and analytical methods ( $^{13}\text{C}$  CPMAS NMR), and statistics, please refer to the Supporting Information (SI).

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## Conflict of Interest

The authors declare no conflict of interest.

**Keywords:** alkaline pretreatment • bioethanol • cellulose crystallinity • *Pycnoporus sanguineus*

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