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Preparation of a sugarcane bagasse-based substrate for second-generation ethanol: Effect of pasteurisation conditions on dephenolisation

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Second-generation ethanol process uses lignocellulose, but a pre-treatment is required to degrade lignin before saccharification. Biological pre-treatment, using phenoloxidases, represents a cheap and eco-friendly option. Mould contamination can be overcome by heat pre-conditioning of substrates. Pasteurisation can be a mild and sustainable option. A 5-level response-surface experimental design was performed to test the effect of different time and temperatures on lignocellulolytic activities and substrate dephenolisation. Substrate microbial communities were characterised *via* catabolic profiles to determine functional diversity changes after pasteurisation. Temperatures ranging from 70 to 75 °C and time from 5 to 10 h led to the highest laccase activities and 30% of substrate dephenolisation was achieved while avoiding mould contamination. The per cent of O-Alkyl (polysaccharides) did not vary significantly, meaning that polysaccharides were not extensively consumed. Microbial communities were less catabolically diversified (H' = 1.2) but more active (AWCD = 0.9) and could degrade polysaccharides.

Keywords:
Heat pre-treatment
Lignocellulose transformation
Solid-state fermentation
White-rot fungi
Laccase

1. Introduction

Production of bioethanol from lignocellulose usually includes three major steps: pre-treatment of the substrate (dephenolisation), saccharification and fermentation [1]. Of all these steps, pre-treatment represents a bottleneck because of high-energy consumption and environmental concerns due to the use of hazardous compounds [2]. Many chemical, physical and biological approaches have been tested for pre-treatment [3]. Biological or enzymatic pre-treatments can be considered as a sustainable option, requiring no hazardous chemicals, low energy input and mild operating conditions [4]. Biological pre-treatment uses phenoloxidases like laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2), which are responsible for lignin degradation in soils and litters. These enzymes are mainly produced by white-rot fungi [5] and present some advantages: they catalyse a broad range of phenolic substrates (low specificity), do not need co-factors and have high catalytic efficiency. All these properties make them useful for a variety of industrial and environmental applications including aromatic compound transformation (bio-bleaching, bioconversion) or bio-detoxification (bioremediation and effluent treatment) [6,7]. Eventhough enzymatic pre-treatment presents some advantages, low rates of lignin oxidation become a challenge to obtain a sustainable process [8]. Interestingly, the white-rot fungus *Pycnoporus sanguineus* produces high amounts of laccases as the main ligninolytic enzymes, including induced and constitutive isoforms, making it interesting for industrial applications [9–11].

To enhance dephenolisation of lignocellulose, a heat-treatment has to be implemented to limit contamination by moulds. Moreover, heat-treatment can improve enzymatic digestibility of lignocellulose fibres by cellulases, phenol-oxidases and other enzymatic activities [12]. During heat-treatment, lignocellulosic biomass is heated from 150 to 180 °C to solubilize hemicelluloses and lignin [13]. Nevertheless, at these temperatures, inhibitory compounds like phenolic compounds, furfural or hydroxyl-methyl-furfural can be produced. These compounds can affect the fermentation of

monosaccharides into ethanol and reduce overall yields [14]. An alternative for this process is steam explosion treatment, which combines high temperature (250 °C) and pressure for a short period (from 2 to 5 min), limiting inhibitory compound production [12]. However, both methodologies require special equipments that endure high pressure and temperature and high-energy consumption increases production costs.

Pasteurisation represents a cheaper, milder and more ecological option compared to other thermal treatments [15,16]. Pasteurisation can open lignocellulose fibres thus enhancing substrate colonisation and degradation by white-rot fungi [16]. Different conditions of pasteurisation for lignocellulosic substrates have been reported with temperatures ranging from 60 to 80 °C for 2–24 h. Depending on pasteurisation conditions, different groups of microorganisms can be selected [17]. For instance, thermotolerant microbial communities can actively colonise lignocellulose after pasteurisation and contribute to polysaccharide degradation favouring lignin availability [18]. Moreover, substrate microbial communities can limit mould contamination and also have antagonistic interactions with *Pycnoporus sanguineus*, which can potentially induce laccase production [19,20]. White-rot fungi can indeed over-produce laccases during these interactions, which is considered as a mechanism of defence against antifungal metabolites produced by substrate microbial communities [21]. There are some reports of thermotolerant bacteria in lignocellulosic substrates that can enhance laccase production such as *Bacillus* spp. [19,22] This increase in laccase production can contribute to enhancing substrate dephenolisation.

The current study aims to determine adequate conditions of thermal treatment (temperature and time) which could limit contaminations, favour *Pycnoporus sanguineus* colonisation and thus eventually dephenolisation of the substrate. This study would thus help to define suitable conditions for pre-treatment of sugarcane bagasse (SCB). This lignocellulosic substrate is one of the most produced world wide, reaching 42.8 million tons in 2010 [11]. SCB is rich in cellulose (≈50%) and has high glucan digestibility (47%) [23]. Consequently, SCB is considered as a cheap source of polysaccharides that can be hydrolysed and then fermented into ethanol [11]. Moreover, this substrate has been complemented with coffee pulp which has been proved to induce laccase activities under such solid-state fermentation conditions [11]. To do so, a response-surface experimental design was set up to test the effects of time and temperature of pasteurisation on laccase and cellulase activities and transformation of lignocellulose *via* solid-state ^{13}C CP/MAS NMR (Cross Polarization at Magic Angle Spinning Nuclear Magnetic Resonance). Moreover, substrate microbial communities were characterised *via* catabolic profiles by Biolog EcoPlate™ to determine variations of functional diversity of the communities depending on pasteurisation conditions.

2. Materials and methods

2.1. White-rot fungal strain and inoculum preparation

The strain of *Pycnoporus sanguineus* used was isolated from a wild mushroom found in the sugar-cane fields of Jalcomulco, Veracruz, Mexico (19° 20 '00' 'N, 96° 46′ 00 'W), registered as F. Ramírez-Guillén 932 in the XAL herbarium (INECOL, A.C.) and identified by analysis of ITS fragments 1 and 4 (Access Genebank KR013138). Five agar pieces (1 cm diameter), after seven days of growth on malt extract agar (20 g/L), were used as inoculum of wheat grains in glass plates: 150 mL of water and 0.5 g of gypsum were added to 100 g of wheat grains and sterilized by autoclaving [24]. Then, the plates were incubated ten days at 30 °C [11].

2.2. Mesocosm preparation

SCB complemented with 10% of coffee pulp, used as an inductor of laccase [11,25], which was previously milled and sterilized twice by autoclave. Dried and milled SCB (particles from 0.5 to 3 cm) were received from Mahuixtlán sugar refinery, located at Mahuixtlán, Veracruz, Mexico. The composition of this by-product was analysed by ^{13}C CP/MAS NMR by Hernández et al. [24]: O-Alkyl-C 79.76%, aromatics 12.87%, COOH–C 3.57% and Alkyl-C 2.45%. The coffee pulp was obtained from a shade-grown commercial coffee culture with the dominant variety *Coffea arabica* [26], located in the Tlacontla farm, San Marcos de León, municipality of Xico, Veracruz, Mexico. Mesocosms were inoculated with *Pycnoporus sanguineus* with 5.0% of dry weight (DW). Each mesocosm was set up in 0.5 L crystal plates (10*14*5 cm^3), covered with an aluminium foil. For each treatment, three replicates were made, to obtain a total of 27 mesocosms which were incubated at 30 °C at 80% of the WHC in the dark for 60 days.

2.3. Experimental design

An experimental design was performed using, as independent variables, the pasteurisation time and temperature, using 5 levels each and a full-cubic model (Table 1). Time of pasteurisation ranged from 1 to 24 h (1, 4, 12, 20 and 24 h) and temperature from 55 to 80 °C (55, 58, 67, 76 and 78 °C). These conditions were selected according to previous studies on pasteurisation of lignocellulosic materials [20,22]. The dependent variables measured were laccase and cellulase activities, chemical properties of the substrate by solid-state ^{13}C CP/MAS NMR and catabolic profiles of bacterial communities by Biolog EcoPlate™.

2.4. Lignocellulolytic activities

Laccases were quantified according to Farnet et al. [27], with syringaldazine (15 μM in methanol) as substrate. Cellulase activity was assayed using Carboxy-Methylcellulose (CMC) at 1% as substrate according to the Somogyi-Nelson method modified by Farnet et al., [28]. One unit (U) of enzyme activities is defined as one μmole of the reaction product formed per h and per g of Dried Weight.

2.5. Solid-state ^{13}C CP/MAS NMR

The chemical composition of SCB, of coffee pulp and of the substrate at t0 was characterised by solid-state ^{13}C CP/MAS NMR, on a spectrophotometer Bruker DSX 400 MHz operating at 100.7 MHz. Samples (400 mg) were spun at 10 kHz at the magic angle. Contact times of 2 ms were applied with a pulse width of 2.8 μs and a recycle delay of 3 s. Chemical shift values were referenced to tetra-methyl-silane and calibrated to glycine carbonyl signal set at 176.03 ppm. The relative C distribution in ^{13}C NMR spectra was determined by integrating the signal intensity in different chemical shift regions [29], with an integration routine supplied with Dmfit 2003 software. Seven common chemical shift regions were defined according to Mathers and Xu [30]. The crystallinity of cellulose was measured according to Park et al. [31].

2.6. Microbial catabolic profiles

Microbial catabolic profiles were assessed using Biolog EcoPlate™. Each 96-well plate contained three replicates with 31 sole carbon sources and water blank for each one. Ten grams of mesocosm at 80% of humidity was vigorously shaken in 100 mL of a sterilized desorption-solution (NaCl, 0.08 g/L) for 1 h and then brought to a final OD = 0.005 at 595 nm. A 150 μL aliquot of the

Table 1
Experimental design testing the effects of temperature and time of pasteurisation (5 levels for each factors) and the responses obtained: lignocellulolytic activities,^{13}C NMR signals and H', Shannon-Weaver index of catabolic diversity, AWCD, Average Well Colour Development, R, Richness, number of substrates used from Biolog data.

Pasteurisation conditions			Enzymatic activities		^{13}C NMR						Microbial catabolic profiles		
Conditions	Temperature (°C)	Time (h)	Laccase (U)	Cellulase (U)	Total aromatics	O-Alkyl	Aromatic ratio	Alkyl/COOH	Crystalline rate	Alkyl-C	H′	AWCD	Richness
1	**76**	**20**	0.65	28.21	10.26	79.65	0.11	1.28	0.23	3.68	2.86	0.26	10.0
1	**76**	**20**	0.60	35.24	9.89	79.11	0.10	1.36	0.24	4.47	3.08	0.27	15.0
1	**76**	**20**	0.55	31.52	8.30	81.22	0.09	1.57	0.23	4.44	2.76	0.42	18.0
2	**67**	**24**	0.38	160.43	10.07	77.78	0.10	1.54	0.24	4.99	2.88	0.24	14.0
2	**67**	**24**	0.16	195.37	7.42	80.89	0.08	1.80	0.25	5.50	2.50	0.29	30.0
2	**67**	**24**	0.24	173.51	8.61	78.98	0.09	1.61	0.22	5.49	2.48	0.49	25.0
3	**80**	**12**	0.01	32.50	9.73	77.79	0.10	1.48	0.23	5.23	2.54	0.30	11.0
3	**80**	**12**	0.11	42.18	8.32	77.07	0.09	2.39	0.22	7.05	2.16	0.12	6.0
3	**80**	**12**	0.02	45.86	8.82	77.35	0.09	1.71	0.24	6.26	2.58	0.32	13.0
4	**58**	**4**	0.01	10.42	9.53	78.72	0.10	1.48	0.20	4.73	1.76	0.07	1.0
4	**58**	**4**	0.02	11.81	9.81	79.02	0.10	1.52	0.20	4.47	1.87	0.04	1.0
4	**58**	**4**	0.10	12.84	9.34	79.45	0.10	1.80	0.20	4.74	1.96	0.03	1.0
5	**76**	**4**	0.32	53.00	7.39	80.70	0.08	1.55	0.23	5.42	2.55	0.43	28.0
5	**76**	**4**	0.32	53.25	7.64	80.01	0.08	1.44	0.26	5.57	2.00	0.65	29.0
5	**76**	**4**	0.32	53.00	7.39	80.27	0.08	1.50	0.26	5.79	1.45	0.92	27.0
6	**67**	**12**	0.65	69.03	8.51	79.50	0.09	1.37	0.25	5.14	2.49	0.42	29.0
6	**67**	**12**	0.56	87.49	8.93	79.48	0.09	1.52	0.23	4.85	1.94	0.52	27.0
6	**67**	**12**	0.77	75.00	7.63	81.08	0.08	1.36	0.25	4.83	1.45	0.25	29.0
7	**55**	**12**	0.07	8.23	9.36	79.70	0.10	1.39	0.22	4.38	1.63	0.05	2.0
7	**55**	**12**	0.08	10.61	9.12	79.96	0.09	1.69	0.21	4.42	1.71	0.30	8.0
7	**55**	**12**	0.09	27.28	9.28	77.91	0.10	2.13	0.20	6.25	1.35	0.05	6.0
8	**67**	**1**	0.27	18.02	9.69	78.95	0.10	1.37	0.22	4.41	2.30	0.51	20.0
8	**67**	**1**	0.27	22.67	9.78	78.96	0.10	1.54	0.20	4.58	1.84	0.52	28.0
8	**67**	**1**	0.20	20.91	10.75	79.19	0.11	1.01	0.20	3.40	1.18	0.27	10.0
9	**58**	**20**	0.10	6.98	10.84	78.18	0.11	1.32	0.20	4.17	2.30	0.16	15.0
9	**58**	**20**	0.14	4.84	10.61	79.01	0.11	1.13	0.22	3.95	1.78	0.38	27.0
9	**58**	**20**	0.14	7.28	9.57	80.65	0.10	1.30	0.21	3.70	1.45	0.24	13.0

extracted solution was added to each of the 96 wells in a Biolog® EcoPlate (Biolog, California, USA). The plates were incubated at 30 °C for 24 h and the absorbance was read at 590 nm using a microplate reader (Metertech ∑960, Avantec, France). The functional diversity was assessed by Shannon's diversity index (H′) [32,33] from H' = −∑pi (ln pi), where pi is the ratio of colour development of well "i" to the sum of colour development of all positive wells. This index gives information about the functional diversity of substrate microbial community. The global activity of these communities was assessed as the average well-colour development (AWCD) calculated as follows: AWCD = ∑ODi/31, where ODi is the optical density for each well (i). Richness, R, was calculated as the number of substrates used, which informs about the catabolic potential of the substrate microbial communities.

2.7. Statistical analyses

Correlations between variables and the factors tested were determined using UNSCRAMBLER-X 10*3 (CAMO). To find out whether the independent factors have significant effects on each response, a two-way ANOVA was carried out (Table 2). Homoscedasticity and normality were analysed previously. The interaction effects of time and temperature of pasteurisation were analysed by response surface methodology [34]. Pearson's correlation coefficients were calculated among all the NMR peaks from the 27 mesocosms after 60 days of incubation.

Table 2
Two-way ANOVA testing the effect of temperature and time of pasteurisation on lignocellulosic activities, chemical properties and catabolic diversity.

Response	Model check	P-value	F ratio
Laccase activity	Cubic model	<0.001	18.45
Cellulase activity	Cubic model	<0.001	23.14
Crystallinity	Cubic model	<0.005	7.01
O-Alkyl	Cubic model	<0.05	3.4
Total aromatics	Cubic model	<0.001	10.91
Aromatic ratio	Cubic model	<0.001	9.81
Alkyl-C	Cubic model	<0.005	6.87
H′	Interaction	<0.05	0.14
AWCD	Interaction	<0.002	2.84

3. Results

3.1. Effect of different pasteurisation conditions on dephenolisation of sugarcane bagasse

Results obtained for laccase and cellulase activities are shown in Table 1. Analysing the surface plot for laccase activity ("Cubic model" $P < 0.001$, Table 2), we observed that the range of temperatures from 70 to 75 °C and time from 10 to 18 h of pasteurisation favoured laccase activities (up to 0.8 U/g DW) of *Pycnoporus sanguineus* (Fig. 1A). On the other hand, it is worth noticing that pasteurisation at low temperatures whatever the time used and pasteurisation at high temperatures (76–80 °C) for short time (1–5 h), limited laccase activities of *Pycnoporus sanguineus*. Thus, there are very specific conditions of pasteurisation that enhance laccase activities during solid-state fermentation.

Cellulase activity surface plot ("Cubic model" $P < 0.001$, Table 2) showed that there were two conditions of pasteurisation that enhanced such activities. The first conditions which led to higher cellulase activities (up to 160 U/g DW) were 24 h of pasteurisation, no matter the temperature used. The second condition which favoured cellulase activities but to a lesser extent (around 98 U/g DW), were temperatures ranging from 64 to 73 °C and time ranging from 5 to 10 h (Fig. 1B).

Substrate chemical modifications were followed by solid-state ^{13}C CP/MAS NMR and results obtained are shown in Table 1. Total aromatics decreased up to 30%, compared to control at t0, for

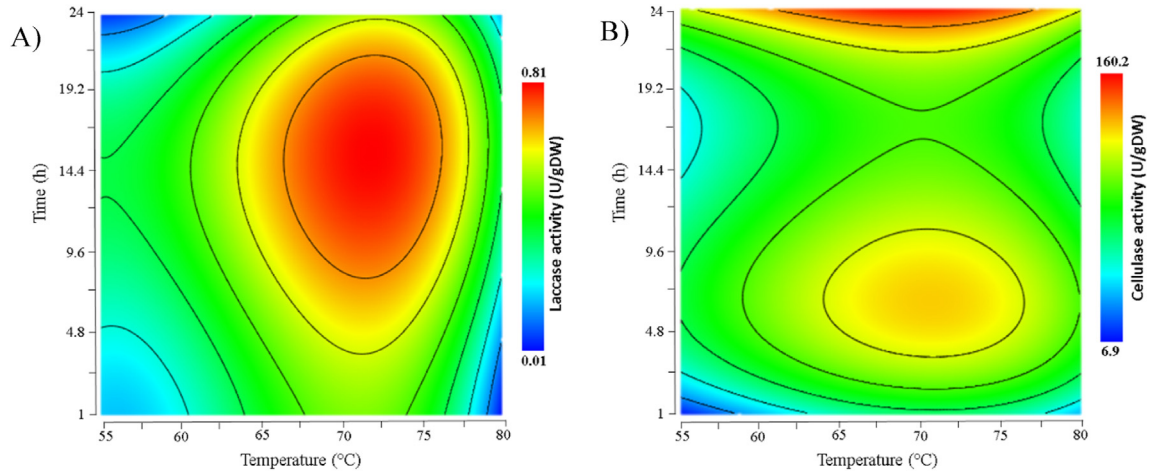


Fig. 1. Surface plots of the interaction between temperature and time of pasteurisation of the substrate for A) Laccase activities and B) Cellulase activities responses after 60 days of incubation.

temperatures ranging from 70 to 75 °C and for time ranging from 5 to 10 h ("Cubic model" $P < 0.001$, Table 2, Fig. 2A). Under these pasteurisation conditions, total aromatics were reduced from 11.66 to 6.97%. This tendency was also observed for the Aromaticity ratio ("Cubic model" $P < 0.001$, Table 2) for the same ranges of temperatures and time. It has to be noted that this range of temperatures (from 65 to 75 °C) overlapped with those where high laccase activities were observed (Fig. 1A). On the other hand, a high proportion of aromatics was found for low temperatures and short time of pasteurisation (Fig. 2A) and this also corresponded to pasteurisation conditions where laccase activities were low.

For O-Alkyl signal (a marker of polysaccharides), the surface plots revealed significant differences ("Cubic model" $P < 0.05$, Table 2, Fig. 2B). Low O-Alkyl percentages were observed for high temperatures (80 °C) whatever the time of pasteurisation. On the other hand, high O-Alkyl percentages were found for temperatures ranging from 70 to 75 °C whatever the time of pasteurisation. Interestingly, the ranges of temperature and time (from 70 to 75 °C and from 5 to 10 h) favouring cellulases were similar to those where a higher crystallinity ratio (crystalline vs amorphous cellulose) around 26% ("Cubic model" $P < 0.005$, Table 2) was found (Fig. 3A). This reveals that for these conditions, the more available fraction of polysaccharides ('amorphous cellulose') was consumed.

Thus, temperatures from 70 to 75 °C and time from 5 to 10 h favoured high lignocellulolytic activities (laccases and cellulases), led to a low amount in total aromatics and a high crystallinity index. This corresponds to the responses required for a suitable substrate pre-treatment. Moreover, no visual contaminations by moulds were observed in the mesocosms using these conditions of pasteurisation.

At high temperatures (80 °C) and short time of pasteurisation (5–10 h), Alkyl-C surface plots ("Cubic model" $P < 0.005$, Table 2, Fig. 3B) showed higher amounts of these compounds (6.65%) compared to other pasteurisation conditions (Fig. 3).

3.2. Variation in catabolic diversity of bacterial communities of the substrate depending on pasteurisation conditions

The Shannon-Weaver diversity index (H′), Richness, R, (number of substrates used) and average well colour development (AWCD) were calculated from Biolog data to analyse the catabolic diversity, the potential of substrate utilisation and global activity of substrate microbial communities after different pasteurisation conditions. All the results are shown in Table 1. H′ surface plot ("Interaction model" $P < 0.05$, Table 2) indicated that microbial communities in the substrate exhibited a high catabolic diversity when high

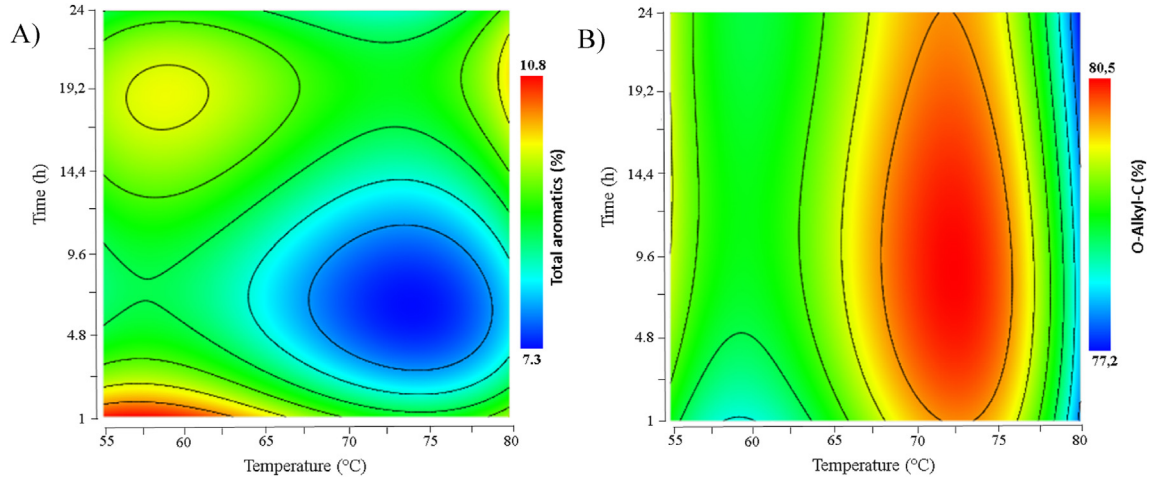


Fig. 2. Surface plots of the interaction between temperature and time of pasteurisation of the substrate for A) Total aromatics and B) O-Alkyl responses after 60 days of incubation.

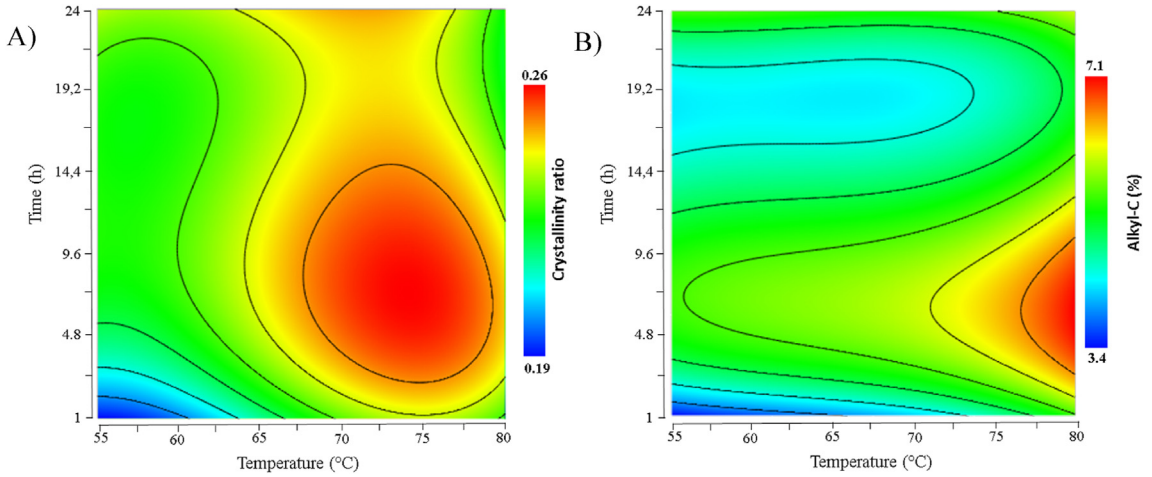


Fig. 3. Surface plots of the interaction between temperature and time of pasteurisation of the substrate for A) Crystallinity and B) Alkyl-C responses after 60 days of incubation.

temperatures (80 °C) and long time of pasteurisation (24 h) were used (H' = 3.6) (Fig. 4A). On the other hand, low levels of catabolic diversity (from 1.54 to 2) were obtained for low temperatures (55 °C) and pasteurisation time ranging from 6 to 20 h. Thus, for those ranges of temperature and time where dephenolisation was high (i.e. 70–75 °C and 5–10 h), H′ was low (H' = 2.0). Moreover, for these conditions and though substrate consumption richness was high (28 of 31 substrates consumed), the main carbon sources catabolised (OD > 0.3) were specifically related to cellulose and hemicellulose decomposition (D-cellobiose, D-mannitol and D-xylose), or pectin (D-galacturonic acid) and other types of sugar (L-erythritol).

Conversely, the surface plot of AWCD showed an opposite tendency compared to H' ("Interaction model" $P < 0.002$, Table 2) (Fig. 4B). AWCD was relatively high (OD = 0.73) for time ranging from 1 to 5 h of pasteurisation and for temperatures ranging from 70 to 75 °C. It is noteworthy that a relatively high AWCD (OD around 0.53) was found under the same pasteurisation conditions as those favouring laccase activities and dephenolisation of the substrate. It seems that long time of pasteurisation and high temperatures increased H′ value while leading to relatively low AWCD. On the other hand, for short time of pasteurisation and high temperatures, the index of diversity, H' was lower, and AWCD was high.

4. Discussion

Our results showed that high temperatures (70–75 °C) and short time (5–10 h) of pasteurisation enhanced laccase activities and favoured a decrease in the aromatic fraction of the substrate, revealing that these specific pasteurisation conditions were suitable for dephenolisation. High laccase activities, observed after these pasteurisation conditions, could be related to two different phenomena. First, the thermotolerant microflora of the substrate selected after pasteurisation may develop an antagonist interaction with *Pycnoporus sanguineus*. There is evidence that, in lignocellulosic material like sugarcane bagasse, high temperatures of pasteurisation can select thermotolerant microorganisms like *Bacillus* sp. [35–37]. According to literature, this microbial selection could be beneficial for dephenolisation, since this bacterial genus has been proved to interact with white-rot fungi and to enhance laccase activities *via* antagonism [19,21]. Secondly, phenolic compounds from coffee pulp can enhance laccase activities [11]. During pasteurisation, phenolic compounds, commonly found in coffee pulp, such as ferulic, caffeic, coumaric or chlorogenic acids can be released into the substrate [37]. Temperatures around 80 °C can enhance the extraction of phenolic compounds [38], and since these molecules are like-laccase substrates, they can induce the

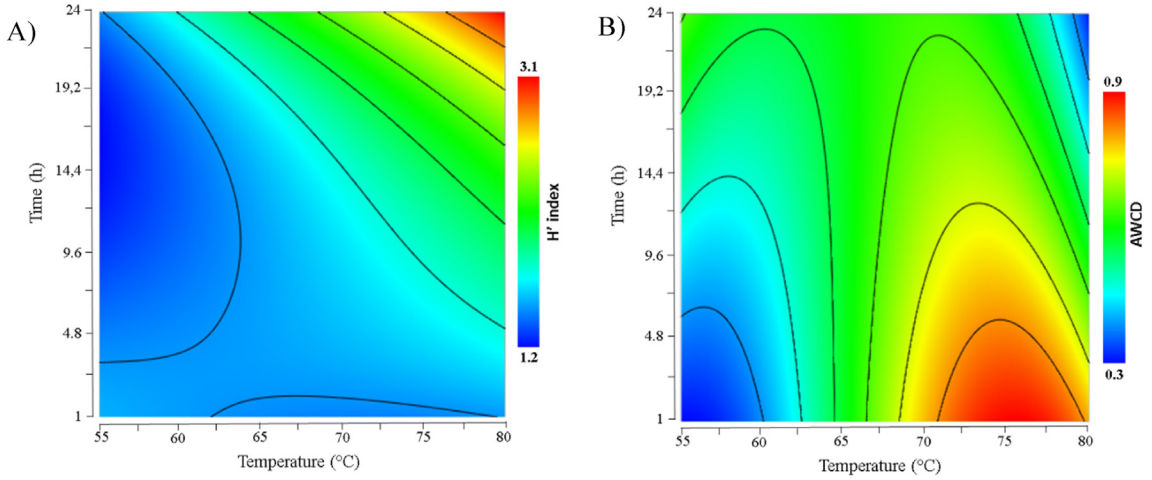


Fig. 4. Surface plots of the interaction between temperature and time of pasteurisation of the substrate for A) Shannon-Weaver index and B) AWCD responses after 60 days of incubation.

production of these enzymes [39,40]. On the other hand, certain pasteurisation conditions (mainly low temperatures) did not favour laccases. Low laccase activities can be linked to low fungal growth of *Pycnoporus sanguineus.* The production of these enzymes is indeed characteristic of white-rot fungi and thus, these enzymes can be considered as a biological marker of white-rot fungus development [41]. Moreover, decrease in laccase activities at low temperature and short time of pasteurisation, can be linked to a weaker extraction of aromatics from coffee pulp that can act as laccase inducers as described above.

Pasteurisation conditions linked to more intense dephenolisation (high temperature, short time) also led to an increase in cellulase activities together with high crystallinity index. High cellulase activities can favour lignin oxidation since hydrolysis of polysaccharide fibres makes lignin more available [42,43]. Synergetic mechanisms between cellulases and laccases have indeed been described. The intricate matrix of cellulose, hemicellulose and lignin requires a multi-enzymatic action to be degraded significantly [44]. Crystallinity index increase can be explained by the fact that cellulose degradation has been proven to begin with consumption of amorphous regions, where polysaccharides are more available for enzymatic hydrolysis [45–48]. Hernández et al. [24], also observed similar results: crystallinity index increased after solid-state fermentation of SCB using *Pycnoporus sanguineus* and the authors also concluded that the amorphous cellulose fraction of substrate was first consumed by fungal enzymes.

On the other hand, low crystallinity was observed at lower temperatures and short time of pasteurisation and this can be related to low lignocellulolytic activities and high aromatic proportions. Thus, all these chemical and enzymatic markers indicate that these pasteurisation conditions led to a weak degradation of lignocellulosic substrate [49].

High cellulase activities were also observed for other pasteurisation conditions characterised by a long time of pasteurisation (22–24 h) and a wide range of temperatures (61–78 °C). This can be linked to the observed contamination of the substrate by moulds after these pasteurisation conditions. This group of fungi is known to actively produce cellulases, which can explain the increase in such activities [50]. Moreover, during long-lasting pasteurisation, high temperatures probably provoked modifications in fibre structure, increasing the surface of colonisation, making the fibres more available for degradation by cellulolytic microorganisms [51]. Moulds are fast-growing fungi that can overpass the potential of colonisation of white-rot fungi such as *Pycnoporus sanguineus* [19]. For example, mesocosms pasteurized at 76 °C for 20 h or at 80 °C for 12 h were contaminated by moulds around 15 days after incubation, inhibiting the growth of the white-rot fungus (Table 3). On the other hand, mesocosms pasteurized at 76 °C for 4 h were colonized by *Pycnoporus sanguineus* only, requiring up to 45 days for complete coverage of the substrate. This means that short pasteurisation time at high temperatures has crucial effects to avoid contaminations by fast-growing moulds and favour the white-rot fungus growth. This goes in line with previous reports where high temperature (80 °C) and short time of pasteurisation (2 h) were used [52], to limit substrate contaminations.

Alkyl-C signal is usually related to lipids and thus to phospholipids from fungal biomass under solid-state fermentation conditions [53]. However, here, an increase in Alkyl-C at high temperatures (80 °C) and a short time of pasteurisation (5–10 h) was not connected with markers of fungal growth such as lignocellulolytic activities, which decreased for those conditions of pasteurisation [11].

To characterise the catabolic potential of microbial communities of the substrate, H'index, AWCD and catabolic richness were determined. H′ index increased at high temperature and long time of pasteurisation, indicating that microbial communities were functionally diversified. This can be related to low selectivity of thermotolerant microorganisms like *Bacillus* spp. after pasteurisation under these conditions. Vieira and Pecchia [20] observed that relative abundance of *Bacillae* -in wheat straw-based substrate-pasteurized at low temperature (57 °C), decreased. Gram-positive bacteria such as *Bacillus* spp. and *Actinobacteria* spp. became dominant after high-temperature pasteurisation (68 °C) [17]. Moreover, increasing temperature and time of pasteurisation can make the substrate more accessible to the hydrolysis potential of diverse microorganisms, which proliferate in the substrate [12]. On the other hand, low H′ observed at low temperature and time between 6 and 20 h of pasteurisation, strengthened the fact that temperature is a determinant factor that controls the diversity of substrate microbial communities after pasteurisation [20].

Conversely, AWCD was low when H′ was high, showing that eventhough microbial communities were functionally diverse, they were less metabolically active. On the other hand, high AWCD but low H′ were observed for pasteurisation conditions where laccases, cellulases and dephenolisation were favoured (from 5 to 10 h and temperatures from 70 to 75 °C). Similar results were obtained by Feng et al. [54], who found a negative correlation between catabolic diversity index, H′ and lignin degradation from lignocellulosic materials (wheat straw). Phenol oxidation leads to the release of polysaccharide-derived products from the lignocellulosic matrix [55] and thus selects microorganisms with specific enzymatic activities like cellulases or xylanases [56]. Thus, it seems that, for these pasteurisation conditions, certain microorganisms specialised in sugar oxidation were selected, leading to a low index of catabolic diversity H'. Moreover, this specialization of substrate bacterial communities can be beneficial for the degradation of polysaccharides during the further saccharification step.

5. Conclusion

High temperature (70–75 °C) and short time (5–10 h) of pasteurisation limited contaminations by fast-growing moulds,

Table 3
Mould contaminations and *Pycnoporus sanguineus* growth observed in the mesocosms during the fermentation after different pasteurisation conditions.

Condition	Temperature (°C)	Time (h)	Mould contamination	*Pycnoporus sanguineus* growth
1	76	20	xx	x
2	67	24	xxx	x
3	80	12	xx	x
4	58	4	No contamination	x
5	76	4	No contamination	xxx
6	67	12	No contamination	x
7	55	12	No contamination	x
8	67	1	No contamination	x
9	58	20	No contamination	xx

Low (X) medium (XX) and high (XXX) mould contamination or white-rot fungus growth (visual observations).

meanwhile, 30% of substrate dephenolisation was achieved. Under these pasteurisation conditions, laccase activities of *Pycnoporus sanguineus* were enhanced, which can be linked to the reduction observed in total aromatics. Moreover, these pasteurisation conditions selected thermotolerant bacterial communities from the substrate. These bacterial communities were able to actively produce specific enzymes related to degradation of lignocellulose polysaccarides, which can be beneficial for lignin availability and also for the further saccharification step and overall bioethanol production. Further studies should aim at screening and identifying thermotolerant bacteria with antagonistic effects against *Pycnoporus sanguineus*, which may enhance laccase production of the white-rot fungus.

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