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Laccase pretreatment for agrofood wastes valorization

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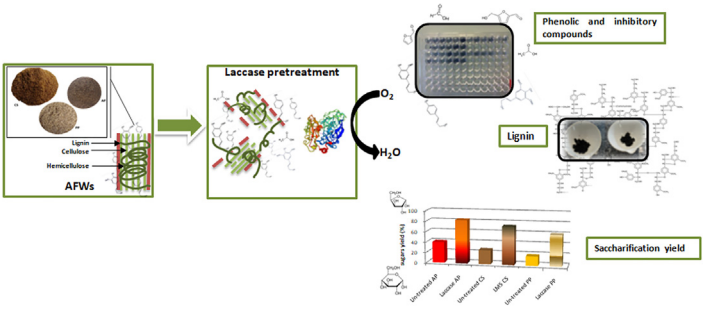
GRAPHICAL ABSTRACT



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ABSTRACT

Apple pomace, potato peels, and coffee silverskin are attractive agrofood wastes for the production of biofuels and chemicals, due to their abundance and carbohydrate content. As lignocellulosic biomasses, their conversion is challenged by the presence of lignin that prevents hydrolysis of polysaccharides, hence demanding a pretreatment step. In this work, the effectiveness of *Pleurotus ostreatus* laccases (with and without mediator) to remove lignin, improving the subsequent saccharification, was assessed. Optimized conditions for sequential protocol were set up for all agrofood wastes reaching delignification and detoxification yields correlated with high saccharification. Especially noteworthy were results for apple pomace and coffee silverskin for which 83% of and 73% saccharification yields were observed, by using laccase and laccase mediator system, respectively. The herein developed sequential protocol, saving soluble sugars and reducing the amount of wastewater, can improve the overall process for obtaining chemicals or fuels from agrofood wastes.

1. Introduction

In recent years, the concept of linear economy is started to switch towards a circular economy (CE) one, since the linear model is mainly based on the use of fossil resources. The main problem related to the fossil resources is their limitation in supply due to their non-renewability. Moreover the linear economy is also based on "take-make-dispose" linear flow, making it nowadays unsustainable. In contrast, the circular economy model endorses the "reduce-reuse-recycle" approach closing the loop of product lifecycles (Hennig et al., 2016; Liguori and Faraco, 2016; Nizami et al., 2017).

In this contest the utilization of wastes, mainly agrofood feedstocks, in biotechnological processes for the production of value added chemicals and fuels represents an application of the CE concept, linked to the bio-based economy (Pleissner et al., 2016).

Many industrial food wastes, rich in cellulose and hemicellulose,

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represent secondary raw materials to be valorized through biorefineries, as reported by different studies in literature (Amore et al., 2014; Mirabella et al., 2014). Nevertheless, as lignocellulosic materials, they contain also a variable amount of lignin, and their biochemical conversion into chemicals and fuels requires a pretreatment step to remove/modify lignin with consequently increase of (hemi)cellulose accessibility and reduction of hydrolytic enzyme adsorption to residual lignin. An ideal pretreatment should be cheap, effective against various substrates without increase of inhibitors, and should prevent loss of polysaccharides. Several pretreatment methods have been developed, including steam explosion, ammonia fiber expansion (AFEX), dilute acid, biological, enzymatic treatment and ionic-liquid pretreatment (Kumar et al., 2009; Woiciechowski et al., 2013; Amore et al., 2014). Among all reported processes, the enzymatic pretreatment, by ligninolytic enzymes, represents an attractive method to both delignify and detoxify lignocellulosic materials, such as agrofood wastes (AFWs), preventing the occurrence of side reactions or formation of by-products.

Laccases (EC 1.10.3.2) are ligninolytic enzymes, which can oxidize phenolic substrates with a concomitant reduction of oxygen to water (Giardina et al., 2010) and are suitable for industrial applications thanks to their broad substrate specificity (Pezzella et al., 2015). In particular, laccase enzymes play an important role in lignin degradation and modification processes offering the possibility to increase the yield of both hydrolysis and fermentation phases due to alteration of lignin hydrophobicity and porosity (Giardina et al., 2010; Piscitelli et al., 2011; Plácido and Capareda, 2015; Fillat et al., 2017). Several bacterial and fungal laccases have been used for detoxification and/or delignification of various pretreated and milled un-pretreated feedstocks, alone or in the presence of a mediator, the laccase mediator system (LMS) (Fillat et al., 2017). All previous studies have reported “separate” laccase delignification and saccharification (Kuila et al., 2011a, 2011b; Mukhopadhyay et al., 2011; Moreno et al., 2016a; Moreno et al., 2016b) in which filtration and washing steps are applied between the two phases to remove inhibitory compounds and mediator, and to change the operative conditions. Laccases alone have resulted able to delignify materials with a lignin content ranging from 17 to 24%, reaching a delignification yield of up to 89% with a consequential increase of saccharification (Kuila et al., 2011a, 2011b; Mukhopadhyay et al., 2011; Rico et al., 2014; Rencoret et al., 2016; Rajak and Banerjee, 2016). When the LMS has been tested, different results have been reported, depending on the nature of the used mediator (natural or chemical one) (Fillat et al., 2017).

The use of laccases on already pretreated feedstocks have yielded high detoxification, although contrasting results on delignification have been observed, due to the occurrence of the covalent coupling of phenolic radicals to the aromatic lignin fibers, the so-called grafting phenomenon (Moreno et al., 2016a; Oliva-Taravilla et al., 2015).

In this study the ability of two laccase preparations from *Pleurotus ostreatus* to delignify and detoxify three different un-pretreated AFWs was evaluated. The treatment was applied on apple pomace (AP), potato peels (PP) and coffee silverskin (CS), selected on the basis of their European availability and carbohydrate content. To the best of our knowledge, no study has been reported regarding laccase pretreatment of these AFWs. Moreover, to the aim of reducing cost and environmental impact of the process, the effect of LMS was investigated exploiting a lignin-derived natural mediator, supposed to not produce toxic side-products (Cañas and Camarero, 2010; Rico et al., 2014).

2. Materials and methods

2.1. Materials

Reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO), unless otherwise specified.

The commercial enzymes used in this study were:

- Commercial cellulolytic enzyme cocktail Cellic® CTec2 (kindly supplied by Novozyme);
- *endo*-1,4-β-Xylanase M1 from *Trichoderma viride* (purchased from Megazyme);
- α-amylase from *Bacillus licheniformis* (purchased from Megazyme).

AP, CS and PP used in this study were kindly supplied by Spanish and Italian companies, in the frame of the Waste2fuels project. The supplied biomass was oven-dried at 40 °C and milled. Solids collected in the range 1–0.5 mm were stored under dry conditions at room temperature until further use.

AFWs characterizations were performed following the National Renewable Energy Laboratory (NREL) Laboratory Analytical Procedures (LAPs) standard protocols (Laurens, 2013; Sluiter et al., 2008a, 2012).

2.2. Laccase enzymes

Two different preparations of laccases from *P. ostreatus* were used in this work: rPOXA1b laccase recombinantly expressed in *Pichia pastoris* (Pezzella et al., 2017) and a mix of native laccases ($mix_{P.o.}$) produced after 10 days of growth in PDY supplemented with 150 μM $CuSO_4$ and 2 mM ferulic acid (Pezzella et al., 2013).

2.2.1. Assay of laccase activity

Laccase activity against ABTS was assayed as reported by Macellaro et al. (2014).

2.2.2. Stability at pH and temperature

The pH and temperature stabilities of both laccase preparations were evaluated at two different pH by using McIlvaine buffer adjusted at pH 5.0 and in 50 mM sodium phosphate buffer adjusted at pH 6.5 at 28 °C, 40 °C and 50 °C. The activity was assayed as described above (Section 2.2.1).

2.2.3. Effect of laccase on cellulase activity

The effect of laccases on cellulases was analysed by measuring endo- and eso-cellulolytic activity against two different substrates, carboxymethyl cellulose-Remazol Brilliant Blue R (AZO-CM-Cellulose) (Megazyme Co., Bray Ireland), and *p*NP-β-D-glucopyranoside (pNPG), respectively.

In both assays laccases were added at time zero (active laccase), after incubation at 50 °C for 5 h (partially deactivated laccase), and after incubation at 50 °C for 24 h (deactivated laccase).

Endo-Cellulase activity was determined spectrophotometrically utilizing the soluble chromogenic substrate AZO-CMC following the manufactories instructions with some modifications. The assay mixture, containing 500 μL of 0.2% (w/v) substrate in 50 mM sodium acetate buffer pH 4.6, 250 μL of properly diluted cellulase enzyme solution and 250 μL of laccase enzymes, was incubated for 30 min at 50 °C. The reaction was stopped by adding 2.5 mL of 96% ethanol to the mixture, followed by incubation at room temperature for 10 min and centrifugation. The activity was measured at 590 nm. The β-glucosidase activity of the cellulases was evaluated as reported by Marcolongo et al. (2014) with some modifications. 25 μL of diluted enzyme solution and 25 μL of laccase enzymes were added to 450 μL of 50 mM sodium acetate buffer pH 5.0 containing 2 mM *p*NPG. After heating the assay mixture at 50 °C for 10 min, the reaction was stopped by adding 1 mL of 1 M sodium carbonate and the release of *p*-nitrophenol was measured at 405 nm.

Cellulase activity reduction was estimated as percentage respect to control samples containing only cellulase enzymes.

2.2.4. Laccase pretreatment

The pretreatments were carried out at 10% (w/v) in case of AP and CS, while at 5% (w/v) in the case of PP, due to its high water

absorption, in 50 mM sodium citrate pH 5.0 at 28 °C for 24 h. The two laccase preparations were used in different combination and concentration as reported below:

- 10U/mL rPOXA1b:mix$_{P.o.}$ ratio 1:0; 1:1; 2:1; 0:1
- 50U/mL rPOXA1b:mix$_{P.o.}$ ratio 1:0; 0:1.

Moreover, the effect of 2.5% (w/w) vanillic acid, was investigated.

Control assays were performed under the same conditions without the addition of laccase. All the experiments were carried out in triplicate.

2.3. Klason lignin evaluation

The Klason lignin content of untreated and pretreated AFWs was determined according to NREL LAPs (Sluiter et al., 2012). Lignin reduction was estimated as percentage respect to control samples.

2.4. Determination of total phenolic compounds and other inhibitors

The total phenolic content of AFWs after pretreatment was analyzed by using Folin–Ciocalteu assay as reported by Lettera et al. (2015). Gallic acid stock solution (1 mg mL^{-1}) was used as standard. Phenol reduction was estimated as percentage respect to control samples.

Acetic acid, formic acid, furfural, hydroxymethyl-furfural (5-HMF) concentrations were also analyzed according to NREL LAPs (Sluiter et al., 2008b, 2012) after the pretreatment.

2.5. Determination of protein concentration

Protein concentration was determined with the BioRad Protein Assay (Bio-Rad Laboratories, Segrate (MI), Italy) using bovine serum albumin (BSA) as standard.

2.6. Enzymatic hydrolysis and sugar concentration

Enzymatic hydrolysis of pretreated and un-treated AFWs (used as control) was carried out at 10% (w/v) in case of AP and CS, while at 5% (w/v) in case of PP in 50 mM citrate buffer (pH 5.0), in presence of 0.5 mM sodium azide to prevent microbial and fungal growth. After laccase pretreatment, an incubation step of 6 h at 50 °C was performed, than the saccharification with commercial enzymes was performed in a shaking incubator at the same temperature, at 250 rpm. Sampling was done every 24 h and sugar composition was analyzed by following NREL LAPs (Sluiter et al., 2008a, 2012).

Enzymatic cocktails were optimized for each AFWs based on their carbohydrate content. The enzymatic cocktail was composed by 90% of Cellic® CTec2 and 10% of xylanase for AP and CS. In the case of PP, the enzyme mixture contained 80% of amylase, 10% of Cellic® CTec2 and 10% of xylanase. For all saccharification experiments, 15 mg of enzyme mixture for gram of initial glucan present in AFWs was used, as previously described by Giacobbe et al. (2016).

3. Results and discussion

3.1. AFWs composition

Analyses of the AFWs composition were performed according to the NREL protocol and reported in Table 1. Data revealed that, among the three AFWs, AP presents the highest sugar content (52%) with a high percentage (16%) of soluble carbohydrates. Therefore for AP a mild pretreatment, without washing step, is required to avoid loss of soluble sugars. The CS contains the lowest sugar amount (28%) with a balance in glucan (10%), xylan (8%) and starch (7%) content. As regarding PP, as expected, the 23% of total carbohydrates is related to starch, while glucan and hemicellulose are present in equal amount (about 8%).

Table 1
Chemical analysis of the three selected agrofood wastes (AFWs). Standard deviation less than 1% for sugars, 5% for lignin and phenols.

	Apple pomace (% dry basis)	Coffee silverskin (% dry basis)	Potato peel (% dry basis)
Total carbohydrates	52	27	38
Soluble carbohydrates	16	< 1	< 1
Glucan	21	10	8
Xylan	13	8	6
Arabinan	2	2	1
Starch	–	7	23
Lignin	18	30	33
Moisture content	6.5	4.8	5.3
Total phenolic compounds (gL^{-1})	2.6	1.7	0.4

As regards lignin, PP showed the highest lignin content (33%) followed by CS (30%) and AP (18%). The high lignin content of PP is due to the presence, in potatoes wall, of suberin, a complex polymer composed of polyaromatic and polyaliphatic domains (Liang and McDonald, 2014).

3.2. Reaction conditions for AFWs pretreatment

The *P. ostreatus* laccases adopted were the recombinant POXA1b (rPOXA1b) (Pezzella et al., 2017), and the native mix$_{P.o.}$, containing 99% of POXC and 1% of POXA1b/POXA3, as reported by Pezzella et al. (2013). Preliminarily, the possibility to develop a sequential enzymatic pretreatment and hydrolysis was verified, with the aim to avoid the loss of soluble carbohydrates (in particular for the AP), and reduce costs linked to the filtering step and wastewater disposal. Indeed, the pH and temperature stability of the two laccase preparations were evaluated, along with the effect of laccases on the activity of cellulase enzymes.

As regards the pH stability (Table 2), both enzymatic preparations were stable at pH 5.0 and 6.5 for over 24 h at 28 °C, but their half-life at 40 °C and 50 °C was drastically reduced at both tested pHs. Considering that enzyme stability is comparable at both pHs, the pH selected for the pretreatment was the same of that needed for the enzymatic hydrolysis, therefore pH 5. In summary, pretreatment experiments were performed at 28 °C and pH 5.0, thus avoiding the need of buffer exchange.

Fig. 1 shows the cellulase activity of commercial Cellic® CTec2 in presence of rPOXA1b and mix$_{P.o}$ laccases. In presence of active laccase, the Cellic® CTec2 was drastically inhibited, indeed about 9% and 20% of residual cellulase activity was observed for rPOXA1b and mix$_{P.o.}$, respectively. On the other hand, in presence of partially deactivated laccases, the commercial mix retained about 50% of the initial activity. When Cellic® CTec2 worked in presence of deactivated laccase enzymes, no significant alteration in its activity was observed. These results confirm that, as already reported (Wang et al., 2013), fungal laccases inhibit cellulase enzymes.

Taking together, these data let to envisage a sequential protocol for AFWs valorization, avoiding filtering and washing steps provided that laccases are deactivated. Beside economic and environmental advantages, it is worth of note that this optimized protocol, skipping the washing step, could allow achieving high sugars yield, in particular for AFWs with high soluble sugars content, such as AP.

Table 2
Laccase preparations stability at pH and temperature.

	t ½ pH5 (h)			t ½ pH6.5 (h)		
	28 °C	40 °C	50 °C	28 °C	40 °C	50 °C
rPOXA1b	> 24	3	1.5	> 24	1.5	1
Mix$_{P.o.}$	> 24	3	3	> 24	6.5	3

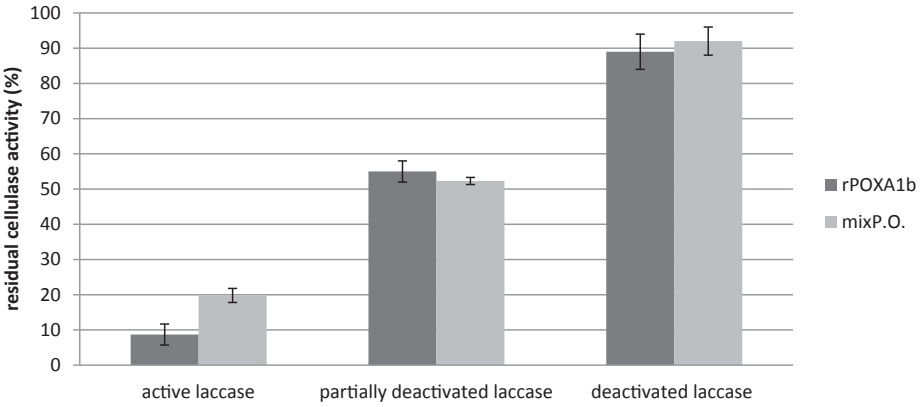

Fig. 1. Effect of laccase enzymes on cellulase activity. Residual cellulase (both endo- and eso-) activity of commercial Cellic® CTec2 in presence of active or deactivated laccases.

3.3. Effect of laccase pretreatment on phenol and inhibitor contents

Enzymatic pretreatment of the three AFWs was carried out by using 10 U/mL and 50 U/mL of the two laccase preparations combining them in different ratios (1:0; 1:1; 2:1; 0:1) as reported in Section 2.2.4. Moreover, LMS was also investigated using vanillin as natural mediator.

The use of 50 U/mL of laccases did not reduce phenol contents proportionally (data not shown), therefore 10U/mL of laccase enzymes were selected for all the experiments. Fig. 2 summarizes the reduction of phenols by using the different laccase ratios. The obtained results suggest a synergistic action of rPOXA1b and $mix_{P.O.}$ in the case of AP and PP, whilst LMS was not very efficient for detoxification of these AFWs. The CS pretreatment revealed a different scenario, as a fact no improvement was observed in presence of coactions of the two laccase preparations and LMS is needed to achieve high detoxification yield.

In the case of AP, the rPOXA1b:$mix_{P.O.}$ 2:1 ratio gave the highest reduction of phenols (~53%). As regards the CS, the highest detoxification (~70%) was achieved by using LMS (rPOXA1b:$mix_{P.O.}$ 1:0 ratio). The rPOXA1b:$mix_{P.O.}$ 1:1 and 2:1 ratios allowed achieving comparable and high reduction of phenols content (~50%) in case of PP.

For the following investigations, a threshold of phenolic compounds ≥ 30% was defined. The conditions satisfying this value were reported in Table 3, with the exception of the rPOXA1b:$mix_{P.O.}$ 0:1 ratio for CS (phenols reduction 20%) to allow a comparison with the corresponding LMS system. As expected, the mild condition of pretreatment did not cause sugars degradation, as a fact no formation of furfural and 5-hydroxymethylfurfural (HMF) was observed (Table 3), thus no additional detoxification strategy is required after laccase pretreatment.

3.4. Effect of laccase pretreatment on delignification

Klason-lignin (KL) content was evaluated on the above mentioned conditions.

When analyzing KL content, it has to be considered that laccase enzymes possess both polymerizing and depolymerizing activities (Claus, 2004). Hence, soluble phenols present in the sample and/or derived from lignin degradation are oxidized by laccase to reactive radicals, which may be covalently coupled to the aromatic lignin fibers, with consequently increase of the lignin content (grafting process) (Moreno et al., 2016a; Oliva-Taravilla et al., 2015).

The obtained results suggest that, in the case of AP and CS, the main action of rPOXA1b is the lignin oxidation resulting in lignin degradation and delignification of AFWs, whilst $mix_{P.O.}$ is involved in the oxidation of phenols, which in turn generates radicals coupling reactions, thus increasing lignin content. The scenario obtained for PP pretreatment is different, because no coupling reactions occurred. This different enzyme reactivity could be due to the presence of suberin, a rubbery polyester material composed of polyaromatic and polyaliphatic domains, found in the cell walls of potatoes together with lignin (Liang and McDonald, 2014).

As showed in Table 3, the AP pretreated with laccase rPOXA1b:$mix_{P.O.}$ 1:0 ratio with and without LMS showed an interesting delignification (15% and 16% KL reduction, respectively). It is worth to note that, as supposed, the presence of $mix_{P.O.}$ (rPOXA1b:$mix_{P.O.}$ 1:1 and 2:1) affected lignin reduction. These delignification results are lower than those reported (63%) by Procentese et al., (2018) by using deep eutectic solvent (DES) pretreatment, however this pretreatment causes decrease of both pentose and soluble sugars.

In the case of CS, the highest delignification (48%) was observed by using the LMS with rPOXA1b:$mix_{P.O.}$ 1:0 ratio, while the use of rPOXA1b:$mix_{P.O.}$ 0:1 ratio always increased the lignin content.

As regards as PP, the AFW with the highest lignin content (Table 1), a delignification yield of about 50% was observed when rPOXA1b:$mix_{P.O.}$ 2:1 or LMS with 1:1 ratio were used. The delignification yields obtained on CS and PP by laccase pretreatment were higher than those reported by DES pretreatment (35%) on the same AFWs (Procentese et al., 2018).

Interestingly, fungal laccase enzymes, alone or in combination with natural mediator, were able to simultaneously detoxify and delignify two out of the three analyzed AFWs with yields higher than those obtained by other reported pretreatments for the same AFWs using harder conditions.

It has to be highlighted that in this study for the first time, laccases were used to pretreat AFWs with a high lignin content (29 and 33% for CS and PP, respectively) reaching an interesting delignification yield (about 50%) by using a low amount of *P. ostreatus* laccases. In fact, *P. ostreatus* laccases have already been used to pretreat feedstock characterized by a lignin content of about 20% obtaining a high delignification yield (about 90% Kuila et al, 2011b; Mukhopadhyay et al., 2011), although using up to 50-fold higher amount of enzymes.

3.5. Effect of laccase pretreatment on saccharification

The conditions showing the best delignification yields for each AFW were selected for the enzymatic hydrolysis studies. In particular: *i)* the laccase rPOXA1b:$mix_{P.O.}$ 1:0 ratio with and without the mediator for AP; *ii)* two different mixes were analysed for PP with or without the mediator; *iii)* the presence of the mediator was necessary in the case of CS.

The hydrolytic cocktail mixtures were optimized to maximize saccharification yields for each pretreated AFW based on their composition. The commercial hydrolytic enzymes cocktail was designed including Cellic® CTec2 and β-xylanase and, in the case of PP and CS,

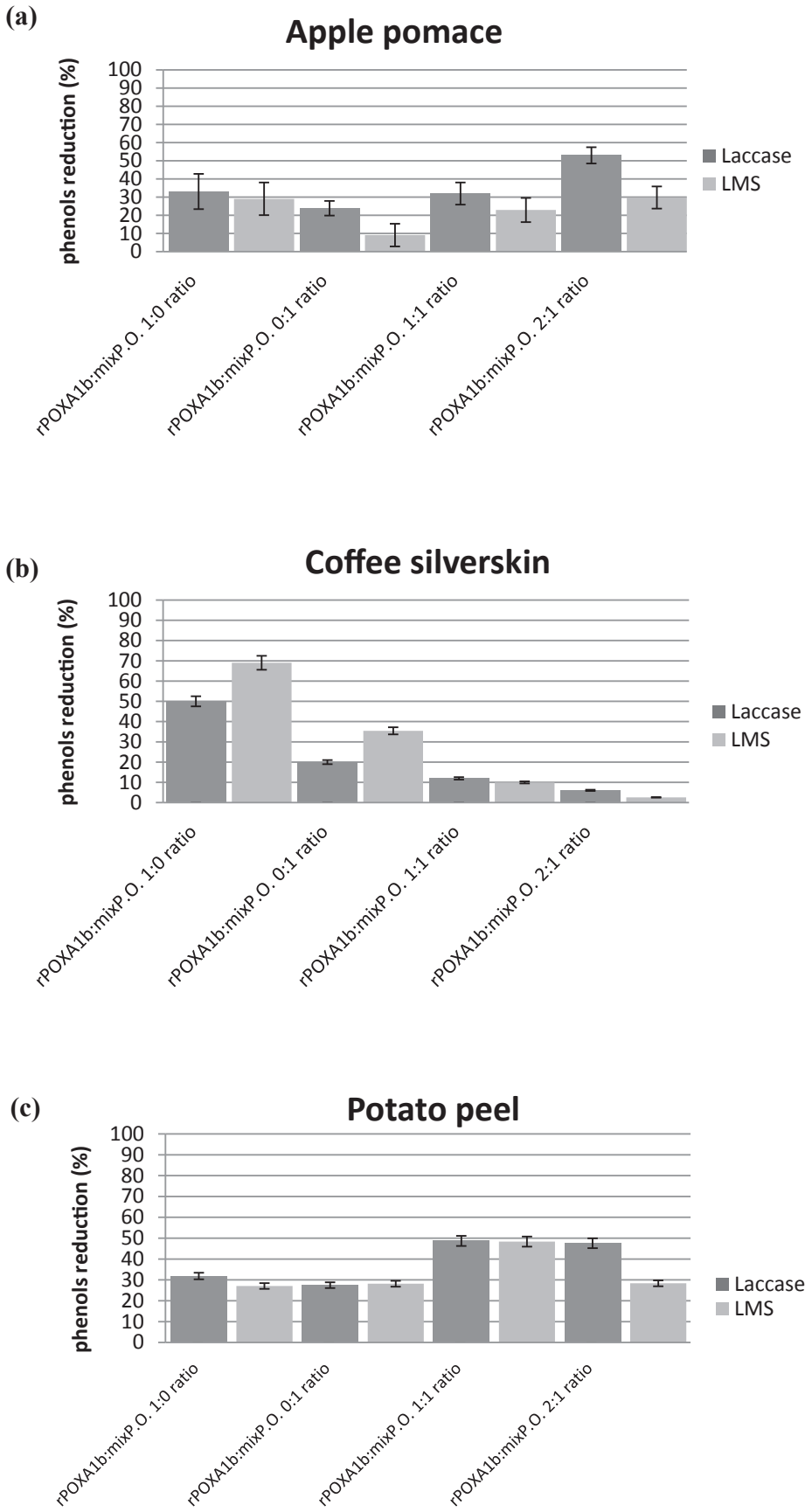

Fig. 2. Reduction of phenols content. Effect of laccase and laccase mediator system (LMS) pretreatment on phenols content of (a) apple pomace, (b) coffee silverskin and (c) potato peels.

Table 3
Analysis of AFWs after laccase and LMS (reduction of Klason lignin and phenolic compound) and sugars conversion after 72 h of enzymatic hydrolysis. Standard deviation less than 1%.

AFWs	Laccase enzymes	Acetic acid (gL^{-1})	Formic acid (gL^{-1})	Furfural (gL^{-1})	5-HMF (gL^{-1})	Phenols reduction (%)	Lignin reduction (%)	Sugars conversion (%)
Apple pomace	Control	1.4	0.5	n.d.	n.d.			40
	rPOXA1b:$mix_{P.o.}$ 1:0 ratio	1.6	0.3	n.d.	n.d.	33	16	83
	rPOXA1b:$mix_{P.o.}$ 1:1 ratio	1.8	0.3	n.d.	n.d.	33	−12	n.a.
	rPOXA1b:$mix_{P.o.}$ 2:1 ratio	1.3	0.4	n.d.	n.d.	53	1	n.a.
	LMS							
	rPOXA1b:$mix_{P.o.}$ 1:0 ratio	1.4	0.5	n.d.	n.d.	30	15	79
	rPOXA1b:$mix_{P.o.}$ 2:1 ratio	1.4	0.4	n.d.	n.d.	30	11	n.a.
Coffee silverskin	Control	1.3	0.2	0.09	0.04			27
	rPOXA1b:$mix_{P.o.}$ 1:0 ratio	1.3	0.2	n.d.	n.d.	50	15	n.a.
	rPOXA1b:$mix_{P.o.}$ 0:1 ratio	1.4	0.3	n.d.	n.d.	20	−5	n.a.
	LMS							
	rPOXA1b:$mix_{P.o.}$ 1:0 ratio	1.4	0.2	n.d.	n.d.	69	48	73
	rPOXA1b:$mix_{P.o.}$ 0:1 ratio	1.4	0.2	n.d.	n.d.	36	−8	n.a.
Potato peel	Control	0.9	0.3	n.d.	n.d.			18
	rPOXA1b:$mix_{P.o.}$ 1:0 ratio	0.9	0.4	n.d.	n.d.	32	35	n.a.
	rPOXA1b:$mix_{P.o.}$ 1:1 ratio	1.1	0.3	n.d.	n.d.	49	36	n.a.
	rPOXA1b:$mix_{P.o.}$ 2:1 ratio	1	0.3	n.d.	n.d.	48	50	60
	LMS							
	rPOXA1b:$mix_{P.o.}$ 1:1 ratio	1	0.3	n.d.	n.d.	49	49	47

n.d. not detected.
n.a. not analysed.

amylase enzyme was also added. The sequential protocol (no filtration and washing steps before enzymatic hydrolysis) was performed: before the enzymatic hydrolysis, laccase pretreated AFWs were incubated for 6 h at 50 °C in order to deactivate laccases and avoid their interference with cellulase enzymes (see Section 3.2). Results of sugars conversion after 72 h of hydrolysis are shown in Table 3.

In the case of AP, a very relevant sugars yield of about 83% was achieved, albeit with a modest delignification. A comparable result was achieved in the presence of vanillin as mediator, thus its presence is not necessary to improve the sugar conversion. This sugar yield was higher than that reported by the same AFW pretreated with soft physico-chemical pretreatment (Hijosa-Valsero et al., 2017) and DES (Procentese et al., 2018).

As regard as CS, a sugars yield of 73% was obtained. These results were higher than those achieved after DES (Procentese et al., 2018) or ultrasound (US) assisted dilute acid pretreatment (Niglio et al., 2017). Interestingly, the presence of 2.5% vanillin did not inhibit cellulase activity, differently to what reported by Ximenes et al. (2011) on other cellulases. Hence, also for LMS the sequential protocol can be applied, obtaining high saccharification yield.

As for the PP, differences in sugars conversion were observed for the two analyzed conditions, even if starting from the same lignin content. As a fact rPOXA1b:$mix_{P.o.}$ 2:1 ratio showed 60% of sugars yield, whilst LMS with rPOXA1b:$mix_{P.o.}$ 1:1 ratio 47%. This different behavior could be explained by an inhibiting effect of the mediator on this specific hydrolytic mixture, characterized by a high percentage of amylase.

4. Conclusions

Laccase pretreatment of AFWs was successfully performed obtaining effective removal of lignin and resulting in efficient (hemi)cellulose hydrolysis, reaching up to about 80% of sugar yield. In particular, the pretreatment benefits of the mediator only in the case of valorization of CS. The herein described pretreatment led to both detoxification and reduction of inhibitory compounds avoiding the necessity of filtration and washing steps. The developed sequential protocol, saving soluble sugars and reducing the amount of wastewater, can improve the overall process for obtaining chemicals or fuels from AFWs with different lignin content. In conclusion, these results suggest *P. ostreatus* laccases represent a specific, effective and environmental friendly tool for AFWs valorization.

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