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Order matters: Methods for extracting cellulose from rice straw by coupling alkaline, ozone and enzymatic treatments

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ABSTRACT

Rice straw is a widely produced residue that can be converted into value-added products. This work aimed at using greener processes combining mild alkali (A), ozone (O) and enzymatic (engineered xylanase) (E) treatments to extract cellulose and other value-added compounds from rice straw and to evaluate the effects of the order of the treatments. Solid (S) and liquid (L) fractions from the process were collected for physicochemical characterization. AOE treatment showed the best capacity to extract high purity cellulose and other valuable compounds. The lignin content was significantly decreased independently of the order of the treatments and, its content in the extract obtained after the AOE process was lower than the one obtained after the OAE process. Moreover, thermal stability of the samples increased after the enzymatic process, being higher in SAOE. The alkaline treatment increased the hemicellulose and polyphenol content (antioxidant activity) in the liquid fractions (LA and LOA). In contrast, the ozonized liquid fractions had lower polyphenol content. Therefore, alkali was fundamental in the process. In conclusion, the AOE strategy could be a more environmentally friendly method for extracting cellulose and other valuable compounds, which could be used to develop active materials in the future.

1. Introduction

Rice straw is one of the most abundant, renewable and low-cost agricultural waste that remains in the fields after harvest, with close to 600 million tons generated annually. In particular, between 75.000 and 90,000 tons of rice straw are generated over only a few weeks in the Spanish region of Valencia (Ribó et al., 2017). The environmental implication of rice straw cultivation has caused controversy in recent years since this residue has been traditionally burned, thus producing greenhouse gases and having huge effects on the environment. To avoid this problem, other rice straw management strategies involve its use as raw material for mushroom cultivation, animal feed, biofuel or conversion into high-value products (Ismail & Mohamad, 2021; Sander et al., 2019). Similar applications include utilizing rice straw as animal feed, using it as raw feedstock for paper and pulp and producing organic fertilizers through bio-production. It also has potential uses as bedding for animals, for fungal cultivation and packaging. Nevertheless, the proportion currently utilized for these applications remains low compared to the millions of tons generated (Rathour et al., 2023, b). Previous studies have demonstrated that rice straw is rich in phenolic compounds, mainly phenolic acids and tannins (Zheng et al., 2017). These could be used in different applications due to their antioxidant and antimicrobial properties (Elzaawely et al., 2017). For instance, phenolic acids derived from rice straw strongly inhibited *Staphylococcus aureus* due to the induction of peroxide which causes cell death (Cui et al., 2019). In addition, phenolic acids can be incorporated into polymeric systems to improve their properties and produce active materials.

Thus, rice straw biomass could be considered an attractive residue for upcycling, producing fuels, chemicals and bioactive compounds which could be generated using a green biorefinery approach. A product-driven biorefinery, specifically a biochemical platform using

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pretreatments and hydrolysis, can facilitate the extraction of biomaterials and chemical compounds from rice straw (Abraham et al., 2016).

The possible by-products obtained using rice straw as raw materials are wide. Some of the processes and their respective products include the fermentation (biofuels), enzymatic hydrolysis (hexose and pentose sugars), hydrothermal carbonization (nano-crystalline cellulose or lignin), chemical hydrolysis (furfurals – flavoring agents), composting (fertilizers), mechanical compression (bio-composites), gasification (bioelectricity) and anaerobic digestion (biogas) (Rathour et al., 2023, b). With the aim to extract value added products biorefining of rice straw has been studied, using the biochemical (hydrolysis and fermentation) or thermochemical (gasification, pyrolysis, liquefaction or combustion) pretreatments. Related to the material composition the recalcitrant character of the rice straw requires pretreatments that disrupt the main structure and increase the accessibility for subsequent hydrolysis. Some processes include utilizing rice straw hydrolysate that is abundant in xylose to synthesize polyglutamic acid, co-producing ethanol and xylitol with *Saccharomyces cerevisiae* and *Candida tropicalis*, separating and concentrating hydroxycinnamic acid from alkaline-pretreated hydrolysate, and using anaerobic digestion of biomass to generate biogas (Abraham et al., 2016).

As around one third of the total biomass of rice straw is cellulose, rice straw has also been used by several pulp and paper industries. With conventional plastics being partially replaced by cellulose-based materials in many applications, purification strategies to recover cellulose from different biomass sources is a focus of both research and industrial interest. Recently, cellulose-based polymers are gaining attention due to their versatility as they can be transformed into packaging films, food packaging, and tridimensional printing materials, among other applications (Nanda et al., 2021).

Nevertheless, most lignocellulosic biomass treatments are based on chemical/enzymatic processes, which are aimed at the valorization of one of the main components from the feedstock, neglecting the possibility of an integrated biorefinery approach. In this sense, the pulp paper industries use extreme high temperature conditions and some chemical compounds during the delignification process, such as sodium sulfide, which generate toxic effluents that can cause several damages to aquatic flora and fauna (Singh & Chandra, 2019). In addition, enzymatically assisted cellulose purification methods require highly concentrated chemicals and high-energy processes that could harm the environment. For example, sulfuric acid 1 % (*w*/w) was used as a pretreatment to effectively improve the enzymatic hydrolysis and the delignification of the rice straw (Hsu et al., 2010). Consequently, greener pretreatments, extraction and purification methods should be proposed to obtain high-quality cellulose and other bioactive compounds with a lower environmental impact compared to traditional methods.

Ozone can be used as a pretreatment during the cellulose purification process, increasing lignin and hemicellulose solubilization. This may in turn facilitate the enzymatic hydrolysis of carbohydrates before fermentation. The ozonolysis process has been reported to promote high delignification for diverse types of biomass, increasing the sugar release during enzymatic hydrolysis, enzyme activity yields and even bleaching the biomass (Lee et al., 2010; Travaini et al., 2016).

The coupling of this greener pretreatment with enzymatic treatments opens up the possibility of producing more selective reactions with lower energy requirements and less aggressive or polluting chemicals. Xylan is the main hemicellulose present in the type-II cell wall of cereal plants, such as in rice. Xylanases are key enzymes that degrade the xylan backbone of the biomass. Some of the studied xylanases come from thermophilic microorganisms. Therefore, they present great thermostability and are compatible with the processing temperatures of the biomass (Basit et al., 2020). For example, Talens-Perales et al. (2021) described a novel engineered xylanase Xyn11 that showed extraordinary xylanolytic activity under high temperature 90 °C and alkalinity pH 10.5. Xyn11, a xylanase from *Pseudothermotoga thermarum*, has been integrated into an industrial elemental chlorine-free bleaching sequence of eucalyptus Kraft pulp with the aim of increasing pulp bleachability and reducing the need for ClO_2 as a bleaching agent. The enzyme was validated at laboratory and pilot scale saving 15 % and 25 % of ClO_2 used to obtain the reference material (Almeida et al., 2022). In addition, these processes can be combined with a traditional alkali mild treatment to release the hemicelluloses from the lignocellulosic matrix occur and purify cellulose (Sun et al., 2000).

To the best of our knowledge, the effect of alkaline and ozone pretreatments coupled with enzymatic hydrolysis, as less environmentally aggressive techniques for valorizing rice straw to obtain added-value industrial products, has not yet been explored. Therefore, this work was aimed at evaluating not only the effect of the combined treatments but also at understanding how the order of pretreatments affected yields, composition, and side-streams, being this information key to optimize rice straw green biorefinery schemes. The present study was based on the hypothesis that it is possible to develop a more environmentally friendly method to extract a cellulose-rich fraction from rice straw by combining alkali, ozone, and enzymatic treatments. It is also hypothesized that the order of treatments is a key factor in the extraction of cellulose-rich fractions.

2. Materials and methods

2.1. Materials

Rice straw was collected in September of 2021 (Sueca, Spain). The rice straw was milled using an industrial mill with 3 mm, and then, 1.5 mm mesh and was stored in plastic bags until processing. The reagents used in this study were of analytical grade. Sodium hydroxide NaOH (PanReac, 98.9 %), TRIS-HCl (Roche, >99 %), sulfuric acid H_2SO_4 (96 %). In addition, ABTS (>98 %), potassium persulfate $K_2S_2O_8$ (>99.0 %), phosphate buffer solution PBS, (*R*)-Trolox (98 %), Folin-Ciocalteu (2 N), sodium carbonate Na_2CO_3 (>99.0 %), gallic acid (>98.0 %), trifluoroacetic acid (99 %) and microcrystaline cellulose (CAS number 9004-34-6) were acquired from Sigma-Aldrich.

2.2. Alkaline hydrolysis

A proportion of 1:10 of rice straw: distilled water was used, and the pH was adjusted to 13 using NaOH 1 M. Based on preliminary trials, the mixture was autoclaved for 3 h at 121 °C, then centrifuged for 45 min, 4 °C at 10000 rpm. After that, the solid fraction was dried in an oven for 48 h at 60 °C. The dried extract was milled and stored in a desiccator at 0 % relative humidity and the liquid fraction was frozen and lyophilized.

The extraction yield was calculated through the following equation:

$$Extraction\ yield\ (\%) = \frac{W0}{Wt} x\ 100$$

where W_0 refers to the dry weight of solid fraction and W_t to the initial weight of the raw material.

2.3. Enzymatic hydrolysis

The enzyme Xyn11 (GH10 xylanase) was used for the enzymatic hydrolysis step (Talens-Perales et al., 2021). For each gram of the dry treated material, 12.5 mL of TRIS-HCl and 0.5 mL of xylanase were incorporated. First, the solid fraction was manually homogenized in TRIS-HCl, and then, the pH was adjusted to 9 with NaOH 1 M. Later, the enzyme was added and manually homogenized. The mixture was incubated in a water bath in agitation at 250 rpm for 5 h at 90 °C. Subsequently, the mixture was centrifuged for 45 min, 4 °C at 10000 rpm. The supernatant or liquid fraction was collected, frozen and lyophilized for further analysis. The solid fraction was washed consecutively with distilled water, ethanol and acetone, homogenized with the solvent on

each washing step and then centrifuged for 30 min, 4 °C at 10000 rpm. Finally, the solid fraction was first air-dried in a fume hood and then dried in an oven for 48 h at 60 °C. Then, the solid fraction was milled and stored in a desiccator.

2.4. Ozone treatment

The ozonation process was performed on the ozone platform at UniLaSalle (LaSalle O3, UniLaSalle, France). The equipment consists of a 250 mL glass reactor connected to an ozone generator, analyzer and destructor as shown in Fig. S1. To generate ozone, 99.5 % pure oxygen was injected into an electrical corona discharge (OZATCFS3-2G, Ozonia, France). The ozone concentration at the inlet and outlet was continuously monitored with an ozone analyzer (BMT 964, Germany). The remaining ozone was destroyed at 350 °C using a thermal destruction device (ORV/ODT-020, Ozonia, France).

7.5 g of the materials were placed in the 250 mL glass reactor with 200 mL of distilled water. The initial pH was adjusted to 13 with NaOH 1 M, based on preliminary assays which showed higher delignification degree without affecting the carbohydrate fraction at higher pH. The ozone was injected into the reactor with an initial concentration of 100 $g.m^{-3}$ and a gas flow rate of 0.1 $m^3.h^{-1}$. The ozone was introduced into the aqueous solution through a porous ozone diffuser inside the reactor. Throughout the 120 min ozonation process, the pH and ozone inlet and outlet concentrations were carefully monitored (recorded every 2 min). Finally, the solid fraction was dried at 45 °C and the liquid extract was lyophilized. Ozone consumption was calculated according to the equation:

$$O_3\ Consumption\ (g\ O_3) = [O_3\ inlet - O_3\ outlet] \times FR \times (t_i - t_{i-1}) + O_3 consumption\ at\ t_{i-1}$$

where the ozone consumption was based on ozone inlet content, ozone outlet content, *FR* the flow rate and the reaction time *t* according to Beauvais (2013).

2.5. Soluble and insoluble lignin determination

Klason lignin determination method was used to characterize the solid fractions according to TAPPI T222 om-06 and Yasuda et al., 2001 with some specifications mentioned below. 100 mg of the samples were treated with sulfuric acid 72 % (*v*/v) and incubated for 1 h at room temperature. Then, 27 mL of distilled water were added and the solutions were autoclaved at 125 °C for 3 h. Later, the samples were filtered with glass fiber filters, and the filters were dried in an oven at 103 °C for 24 h. The glass filters were weighted to determine the acid-insoluble lignin. On the other hand, the samples absorbance (205 nm) was measured to determine the soluble lignin (TAPPI 222). Sulfuric acid 72 % (*v*/v) was used as the negative control. Three technical replicates were performed. The soluble lignin was calculated according to the equation:

$$ASL\left(\frac{mg}{g}\right) = \frac{ABS*dil*V}{110*b*m}$$

where *ASL* is the acid soluble lignin, *ABS* is the absorbance, *dil* is the dilution factor, 110 is the extinction coefficient, *b* is the cuvette length in cm, and *m* is the initial mass of the sample. Total lignin content was calculated as the addition of soluble and insoluble lignin.

2.6. Antioxidant capacity quantification

Antioxidant capacity was quantified according to Fontes-Candia et al., 2019. Briefly, 0.192 g of ABTS and 0.033 g of potassium persulfate were dissolved in 50 mL of PBS to homogeneity. The solution was stored at −20 °C until further use. The ABTS solution was diluted in PBS until it reached the range of 0.7 ± 0.02 a.u. at a wavelength of 748 nm. In addition, a solution of Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was used as a calibration curve and PBS was used as a blanc. The Trolox solution was prepared by dissolving 0.006 g in 5 mL of 99 % ethanol and mixing it up with 20 mL of PBS. Finally, 20 μL of samples, calibration standards or blanc were added to a microplate with 230 μL of the ABTS solution. The plate was incubated in the dark for 6 min and the absorbance was read at 748 nm. Three technical replicates were made. The antioxidant capacity of the test extracts was expressed as mg Trolox equivalents (TE)/g extracts.

2.7. Polyphenol quantification

The phenolic content was determined using a modified method described by Singleton et al., 1999. Briefly, a Folin-Ciocalteu (FC) solution was prepared at 1:10 FC: distilled water. The samples were prepared at 1–5 mg/mL, and 200 μL of the sample was placed on a tube with 1000 μL of FC solution. They were incubated for 8 min, and then 800 μL of the sodium carbonate solution (75 g/L) was added. A calibration curve was built by using gallic acid as the standard. Finally, the samples were incubated statically in a water bath at 40 °C for 30 min and then, the absorbance (760 nm) was recorded using the CLARIOstar ((BMG LABTECH, Germany) equipment. The total phenolic content was expressed as mg of gallic acid per gram of the extract. Three technical replicates were performed.

2.8. Monosaccharide composition

The sugar composition was determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC_PAD) after either sulfuric or trifluoroacetic hydrolysis, separately, according to Saeman et al. (1954), as to discern the cellulose or mixed linkage beta-glucan contribution from the total glucose in the samples. Microcrystalline cellulose was used as a control. The analysis was performed using the equipment Dionex ICS-6000 (Thermo Scientific, USA). Three technical replicates were performed and to simplify only the average was plotted.

2.9. Fourier-transform infrared spectroscopy

FTIR analyses were analyzed in attenuated total reflectance (ATR) mode using the Thermo Nicolet Nexus (GMI, USA) equipment. Samples were placed on the ATR crystal for analysis at room temperature (22 ± 2 °C), and the scans were performed with 4 cm^{-1} resolution in the spectra range of 4000–600 cm^{-1} and averaging a minimum of 32 scans, similarly to de Oliveira et al., 2019. Duplicates were made, and those that showed the most significant changes were selected to be shown in the graph.

2.10. Thermogravimetric analysis (TGA)

Thermogravimetric curves (TG) were recorded using the Setaram Setsys 16/18 (SETARAM Instrumentation, France) equipment. The samples were heated from 30 °C to 700 °C with a heating rate of 10 °C/min under an oxygen atmosphere. Derivative thermogravimetric curves (DTG) were used to express the derivative of weight loss as a function of temperature. The analysis was performed in duplicate. One of the duplicates is shown graphically.

2.11. Optical microscopy and macrophotography

Macrophotographs and optical/fluorescence microscopy techniques were used to evaluate the general appearance of the material during the process, the nature of the particles, and the integrity of the solid fractions. For the microscopic images, 100 μg of each sample was stained with 200 μL of Calcofluor white stain 0.01 %. The solution was re-suspended and added directly to the glass slide. For epifluorescence

microscopy, incident light was used with an external ring USB-LED and the filter block UV-2E/C was used. Images were acquired with a Nikon Eclipse 90i wide-field microscope (Nikon Corporation, Japan) equipped with a cooled 5 MP digital color camera (DS -5Mc; Nikon Corporation, Japan). For the macrophotography images, 20× and 65× magnifiers were used. These macrophotography digital images were acquired using an EVOCAM-II macroscope (Vision engineering Woking, UK). Images were acquired using ViPlus v1.00.82 software (2018, Vision Engineering) and analyzed using Nis Elements BR 3.2 software (Nikon Corporation, Japan). Processing operations were performed using Fiji (ImageJ

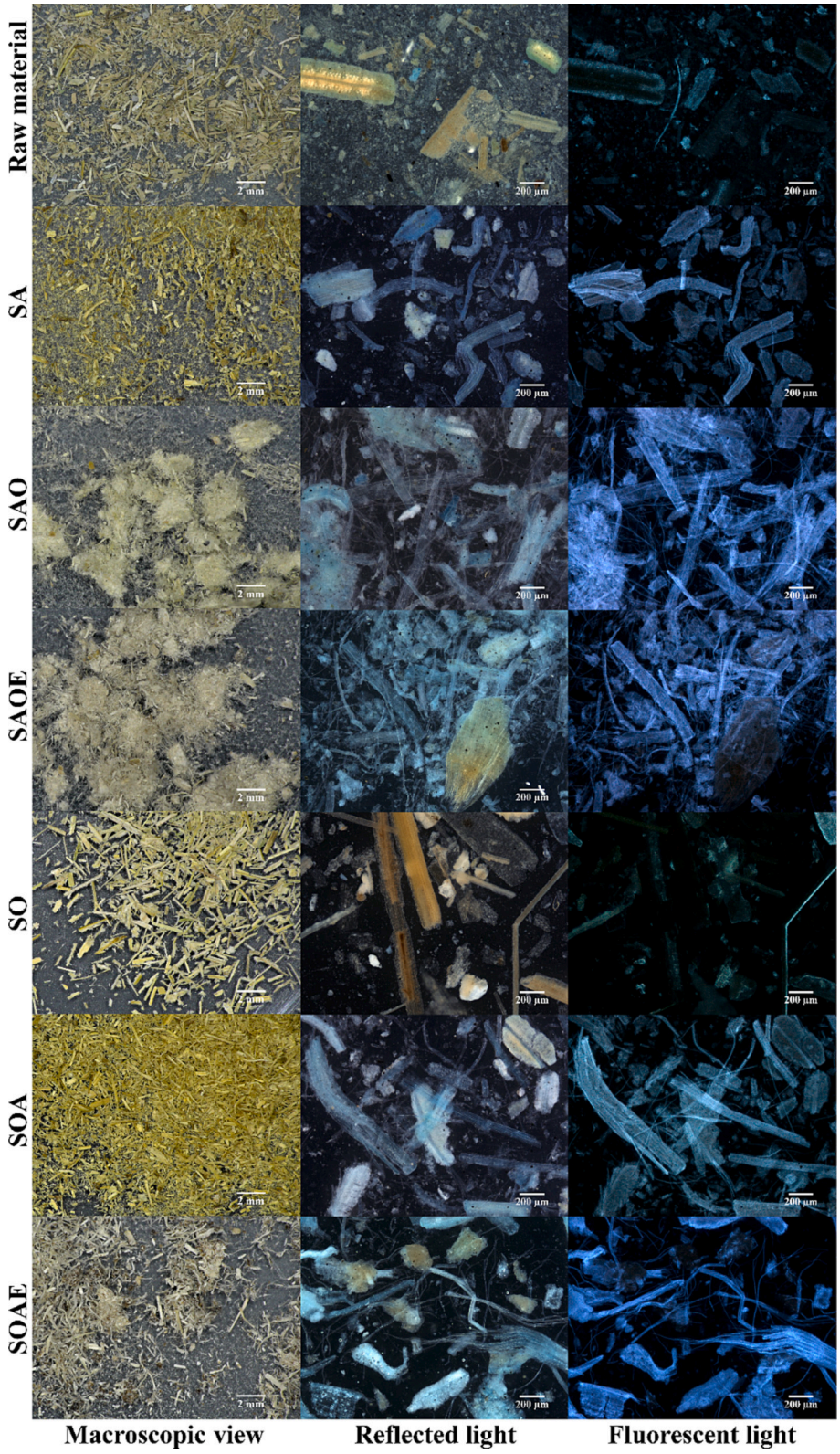

Fig. 1. From left to right: macroscopic characterization, reflected light microscopy and fluorescent light microscopy and of the samples through the processes AOE and OAE. The scale bar for microscopic and macroscopic pictures corresponds to 200 µm and 2 mm, respectively. (S) Is the solid fraction abbreviation.

1.49q software, National Institutes of Health, USA).

2.12. Abbreviations and clarifications

For a better understanding, it is important to highlight that the A-O-E process means alkali-ozone-enzymatic treatments consecutively performed in that order, while the O-A-E process refers to ozone-alkali-enzymatic order; (S) has been used for solid fractions and (L) for the supernatants or liquid fractions obtained on each step of the process.

Abbreviation	Meaning
A	Alkaline hydrolysis
O	Ozonation
E	Enzymatic hydrolysis
S	Solid fractions
L	Liquid fractions / Supernatants
A-O-E	Specify the sequence of the treatments: alkaline – ozonation – enzymatic
O-A-E	Specify the sequence of treatments: ozonation - alkaline - enzymatic.

3. Results and discussion

In order to develop a greener strategy for cellulose extraction while recovering other valuable by-products from rice straw, coupling different techniques helps to build a robust process that improves the separation and purification of the building blocks in rice straw. The strong base NaOH significantly dissolves hemicellulose and lignin under certain conditions. In addition, the alkali process was selected because it selectively removes lignin without degrading carbohydrates and increases porosity and surface area, improving enzymatic hydrolysis (Kim et al., 2016). In addition, NaOH pretreatment reduces biomass recalcitrance by breaking the bonds between lignin and hemicellulose, degrading β-O-4 bonds, cleaving ester bonds, and partially breaking down the lignin structure (Yao et al., 2022). Ozone pretreatment was chosen for its ability to convert lignin into soluble products and to take advantage of its decolorizing ability (Binder et al., 1980). Finally, the enzymatic treatment not only allowed the selective digestion of xylan, increasing cellulose purity in the solid fraction, but also showed the ability to modify the mechanical properties of the materials obtained. Nevertheless, it was important to determine the best possible sequence of these strategies in order to optimize their application with the aim of obtaining purer materials for different applications.

3.1. Morphological characterization

The morphology of the raw material and the obtained solid fractions was studied by means of both macrophotography and optical microscopy. Fig. 1 shows the appearance of the solid fractions using macrophotography and micrographs taken with bright light and fluorescence filters, in which fluorescent characteristics of material components can be observed. The images evidence that raw materials presented particles with cylindrical, circular, and irregular morphologies and a great difference in the visual appearance of the resulting material was observed depending on the order of the treatments. While in the AOE process, the extracts showed cellulose fiber-like particles with a cottony appearance after the ozone (AO) and enzymatic treatments (AOE), the solid fractions obtained after the OAE process were similar in shape to the initial material, even after the enzymatic treatment. It should be noted that the bleaching effect ascribed to the ozonation treatment was also more clearly observed when ozonation was performed after the alkaline treatment. This can be related to the lower lignin content (mainly soluble lignin) in the resulting material (as it will be described below).

According to the visual images, the microstructure of the solid fractions obtained with bright and fluorescence lights after each treatment revealed significant differences depending on the procedure used. In order to qualitatively evaluate the structural organization of rice straw cell wall, the cellulose was stained with calcofluor-white stain, which in previous studies showed the ability to bind oriented cellulose fibrils (Peretz et al., 2019). As clearly observed in the fluorescent light panel, shown in Fig. 1, cellulose fibers were stained in SA samples and they were observed in all the extracts obtained after each step of the AOE process, being the bright blue fluorescent regions increased after the ozone and enzymatic treatment. However, the cell walls from the rice straw treated with ozone (SO) did not lose their integrity and cellulose fibers were not easily discerned because they might be still tightly packed and surrounded by a hemicellulose-lignin matrix. Once it was treated with the alkaline treatment (SOA), a faint bright blue fluorescent region ascribed to the cellulose fibers appeared, thus evidencing that the order of the treatments played an important role on the extraction process. Although the additional enzymatic treatment (SOAE) produced a higher fluorescence and fibers were visibly affected, the samples were substantially different (cylindrical particles, less cottony-like appearance) compared to SAOE samples, which was again an indicator that the order of the process effectively affects the resulting solid fraction. To confirm this hypothesis, the composition of solid (S) and liquid (L) fractions was deeply characterized in order to ascertain the main differences attributed to the extraction procedure.

3.2. Compositional analysis

3.2.1. Starting material

It is well known that biomass composition can vary depending on location, harvesting method, and other environmental factors. Therefore, the composition of rice straw was characterized and the results are gathered in Table 1. As expected, structural carbohydrates represented the major components, with contents of ca. 51 % and lignin was the second main component (ca. 22 %), in line with previous works. For example, Abraham et al. (2016) reported similar values for lignin, carbohydrate, and ash composition. The lignin content was also in the range of 17–20 % in previous reports with grasses (Schutyser et al., 2017). The structural carbohydrates mainly consisted of cellulose and arabinoxylan, followed by β-glucan, with only minor amounts of pectin. The high concentration of cellulose ϵ 32 %) in the rice straw biomass, comparable to wood biomass, confirmed that it is a valuable source of this polysaccharide. The high content in arabinoxylan (xylose and arabinose sugar units) could also be an interesting source of building blocks for other industries.

Table 1
Chemical composition of rice straw.

Rice straw composition (dry wt%)	
Lignin	22.17 ± 0.71
Lipid content	4.90 ± 0.58
Ashes	15.62 ± 0.71
Protein	6.02 ± 0.13
Carbohydrates[a] of which (μg/mg)[b]	43.97 ± 5.76
Mannose	0.00
Rhamnose	0.84
Fucose	0.62
Galactose	7.63
Arabinose	15.54
Xylose	93.84
Galacturonic acid	1.59
Glucuronic acid	2.25
Glucose [c] of which	317.41
Cellulose [c]	288.21
β-glucan [c]	29.20

Mean value ± standard deviation;
[a] As the sum of all detected monosaccharide constituents;
[b] Monosaccharide composition determined by HPAEC-PAD after sulfuric hydrolysis.
[c] Determined by HPAEC-PAD using acid hydrolysis with trifluoroacetic acid.

3.2.2. Solid fractions

Once the rice straw was subjected to the extraction protocols described in Section 2 to obtain cellulose, the yields and compositional analysis of the solid fractions obtained in each step were also characterized in order to better evaluate the efficiency and the effect of the order of the treatments (AOE vs. OAE) in the extraction process. The solid yields obtained for the different treatments were SA (43.47 %), SAO (25.85 %), and SAOE (22.29 %); SO (57.39 %), SOA (39.17 %), and SOAE (27.23 %) for the AOE and OAE processes, respectively. A significant yield mass decrease was observed after the alkaline, ozone and enzymatic treatments, indicating removal of lignin and hemicelluloses accompanied by a cellulose enrichment after each step in the solid fraction. It is interesting to observe that the final solid fraction after the three consecutive treatments was similar in yield regardless of the order. However, a higher yield was observed in the first two treatments when the ozone treatment was applied first, thus indicating that the extraction was less efficient. The carbohydrate composition of these samples, including glucose arising from cellulose (Fig. 2A) and other carbohydrate constituents (Fig. 2B), such as arabinoxylan or pectin, clearly showed a cellulose enrichment in the solid fractions with each treatment. However, clear differences can be observed comparing AOE or OAE, with AOE showing higher cellulose purity than OAE. AOE samples displayed the highest cellulose purity (>60 %), with <7 % of other carbohydrate components.

On the other hand, applying ozonation as first treatment (SO) resulted in the cellulose content not significantly differing from the raw material. Furthermore, the application of an alkaline treatment to the ozonated samples did also not substantially increase cellulose purity (SOA). Ozonation is an oxidative process which was expected to oxidize and degrade lignin, enriching cellulose in the solid fraction (SO). It is known that ozone produces high delignification rates but it can also degrade carbohydrates in the presence of hydroxyl radicals, whose formation and half-life highly increases with pH (Valls et al., 2022). Hydroxyl radicals produced from lignin during ozone treatment at high pH (pH 13) may result in extensive degradation, e.g. carbonyl groups being introduced into the glucose units of the cellulose chain, which would prevent them to be detected in any of the solid or liquid fractions. The absence of a real enrichment of cellulose after the SO or SOA process is an indication of cellulose oxidation, as this fraction could not be found in the liquid fraction. The observation of a significant drop in pH during ozonation of the raw material (O) compared to ozonation of the alkali treated samples (AO; see Fig. S2 in the supplementary material) also aligns with a more aggressive degradation process taking place during this ozonation treatment. This puts the AOE process forward as a better process design to purify native cellulose. Nevertheless, further research is needed to ascertain the oxidation products, molecular weight and potential changes in the technological properties of this partially oxidized cellulose. The presence of higher contents of soluble and insoluble lignin in the raw material could have increased the rate of formation of carbonyl groups due to the formation of hydroxyl radicals. On the contrary, if the alkali extraction occurs before ozonation (AOE), the concentration of radicals derived from lignin oxidation during ozone might be lower and cellulose might be less affected.

Regarding the application of enzymatic treatments, it should be highlighted that both processes starting either with the alkaline or ozone treatment showed the capacity to reduce the hemicellulose content as deduced from the monosaccharide composition of the obtained solid fractions. Despite the much milder conditions of the enzymatic treatment, xylanase was able to reduce the xylose content in the solids >50 % (see SAOE and SOAE extracts), compared to the previous solid ozonated and alkali-treated fraction.

Looking at the lignin contents (see Table 2), considered as one of the major barriers for enzymatic hydrolysis, a significant decrease was observed during the extraction process. The alkali pretreatment contributed to delignification to a greater extent than the ozone treatment, although it did not result in reduced lignin content in SAO or SAOE samples compared to SOA or SOAE samples. A significant decrease in soluble lignin after either alkaline or ozone treatment is expected, as this is removed with the liquid fraction. The increase in soluble lignin after enzymatic hydrolysis can be probably ascribed to the cleavage of

Table 2
Insoluble, soluble, and total lignin in the raw material and in the solid fractions.

Sample	Insoluble lignin (mg / g)	Soluble lignin (mg / g)	Total lignin (mg/ g)
Raw	184.78 ± 10.90 [a]	36.90 ± 4.50 [a]	221.68 ± 7.09 [a]
SA	119.72 ± 38.10 [b]	22.07 ± 0.33 [b]	141.89 ± 38.29 [b]
SAO	113.57 ± 10.13 [b]	15.60 ± 0.03 [c]	129.17 ± 23.10 [b]
SAOE	90.47 ± 8.09 [c]	41.65 ± 2.37 [d]	132.12 ± 15.76 [b]
SO	146.30 ± 8.33 [a.]	26.05 ± 1.08 [e]	172.35 ± 7.55 [a. b]
SOA	103.71 ± 9.54 [b]	20.73 ± 0.87 [b]	124.43 ± 13.30 [b]
SOAE	94.65 ± 7.75[b]	43.18 ± 1.84 [d]	137.83 ± 6.98 [b]

Values with different letters in the same column are significantly different ($p \leq 0.05$).

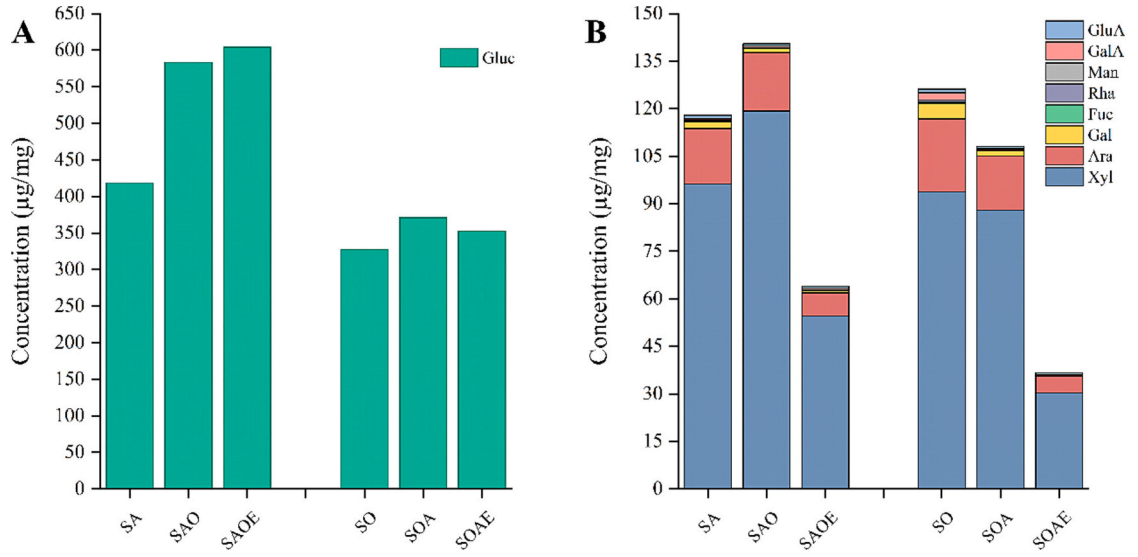

Fig. 2. Monosaccharide concentration in mol% from the solid (S) fractions obtained from AOE and OAE processes. (A) Glucose concentration and (B) glucuronic acid, galacturonic acid, mannose, rhamnose, fucose, galactose, arabinose and xylose concentrations.

hemicellulose favoring the exposure of lignin to be solubilized in the basic conditions at which the enzymes were used (cf. Section 2.3). Thus, these results showed that coupling both processes promotes the delignification of rice straw, which in turn enhances the enzymatic activity as the hemicellulose is exposed to a greater extent.

3.2.3. Liquid fractions

The relative aggressiveness of the treatments and their combinations can result in the partial degradation of the hemicelluloses or other valuable compounds, such as antioxidants, which could be further valorized within the biorefinery. A less aggressive approach would theoretically allow for the lignin-hemicellulose network to be transformed into soluble lignin, with potential antioxidant properties, as well as into arabinoxylooligo- or monosaccharides, depending on the treatment. Therefore, liquid fractions were also analyzed for the carbohydrate content (suppl. Fig. S3). The effect of the alkaline treatment showed higher extraction capacity of intact structural sugar units (mainly glucose but also xylose and arabinose as part of arabinoxylan) when it was applied before the ozone treatment (LAO vs.LOA extract). Nevertheless, the carbohydrate content of the liquid fractions was <100 mg/g in all cases pointing at a higher contribution of soluble lignin or lignin degradation products in these fractions. It has been reported that the alkaline pretreatment selectively removes lignin without degrading carbohydrates and increases porosity and surface area (Kim et al., 2016), thereby enhancing the efficiency of the subsequent enzymatic hydrolysis. Therefore, the ozonation treatment preceded by alkali hydrolysis can be a good strategy before the enzymatic hydrolysis. In addition, the liquid fractions from both processes showed glucose concentrations below 100 μg/g, indicating a small amount of glucans was solubilized during the extraction process.

Therefore, considering the compositional analysis of the solid and liquid fractions obtained from the extraction processes, they were further characterized depending on their potential application. Liquid fractions were characterized in terms of antioxidant capacity and polyphenol content due to their higher lignin content, whereas thermal stability of solid fractions was deeply analyzed for their potential use as a cellulosic additives/fillers for materials applications.

3.3. Structural, thermal and functional properties of the fractions obtained

3.3.1. Antioxidant capacity and polyphenol content of liquid fractions

Lignin has received considerable attention for its antioxidant activity owing to its polyphenol structure. In fact, An et al. (2017) stated that soluble lignin (with lower molecular weight) and high total phenolics content exhibited significant antioxidant activity. Therefore, the antioxidant activity and total phenolic content of the liquid fractions obtained after the sequential cellulose extraction process were evaluated and the results are compiled in Table 3. The first thing to highlight is the highest antioxidant activity found for the liquid fractions consecutively obtained from the alkaline treatment (LA and LOA), evidencing that these alkaline conditions had an important role on the release of polyphenol compounds. In fact, base reaction at alkali treatment consists in cleavage of aryl-ether bonds, which brings about an increase of phenolic hydroxyl content (Derkacheva & Sukhov, 2008). In contrast, the ozonized liquid fractions (LAO and LO) showed a significantly lower antioxidant capacity. Finally, the LAOE and LOAE samples showed similar values being half in magnitude with respect to LA and LOA, respectively. In line with the antioxidant results, higher polyphenols content was observed in the liquid fractions obtained after the alkaline treatment (LA and LOA), followed by LOAE and LAOE samples, as compiled in Table 3. Similarly, Elzaawely et al., 2017 stated that rice straw released different phenolic acids such as p-coumaric, ferulic, caffeic, syringic, rosmarinic, protocatechuic and cinnamic after an alkaline hydrolysis.

In contrast, lower polyphenol content was evidenced in the LAO and LO liquid fractions probably because of phenolic compounds being degraded during the ozonation process. It has been stated that the ozone exposure is harmful to phenolic compounds due to direct reactions between the free radicals produced by the auto-decomposition of the ozone molecule and the selected components or its derivatives (Kaur et al., 2022). Overall, alkali liquid fractions showed a higher phenolic concentration with respect to the other treatments and, ozonization significantly decreased the polyphenol content in the resulting liquid fractions.

Table 3
Polyphenol concentration of the liquid fractions obtained from A-O-E and O-A-E processes.

Sample	Antioxidant capacity (Equivalent mg Trolox/g)	Polyphenol content (Equivalent mg gallic acid/g)
LA	77.53 ± 6.80 [a]	69.20 ± 1.98 [a]
LAO	1.28 ± 0.82 [a]	8.82 ± 1.19 [a]
LAOE	21.91 ± 4.86 [b]	31.21 ± 1.52 [b]
LO	4.08 ± 0.44 [c]	8.16 ± 0.40 [c]
LOA	70.50 ± 1.60 [d]	60.81 ± 2.65 [d]
LOAE	34.58 ± 5.60 [d]	44.37 ± 7.86 [d]

Values with different letters are significantly different ($p \leq 0.05$).

3.3.2. FTIR of solid fractions

To better understand the compositional differences between the extracted solid fractions, they were analyzed by means of FTIR and the obtained spectra are gathered in Fig. 3. Several differences were observed in the spectra from the different samples. Firstly, the broad band at 3600–3100 cm^{-1}, characteristic from O—H stretching (Zheng et al., 2018), present very similar shapes, except in enzymatically treated samples (SAOE and SOAE) in which a sharper peak was observed. Moreover, the pattern and intensity of several bands characteristic of hemicelluloses, such as those located 1735 and 1533 cm^{-1} (corresponding to esters and acetyl groups; Sun et al., 2005), and lignin, such as those centered at 1620 and 1510 cm^{-1} (corresponding to C═O and C═C stretching, respectively; Binod et al., 2012; Lionetto et al., 2012), were seen to strongly depend on the treatment. Briefly, the shoulder at 1735 cm^{-1} and the homogeneous peak at 1620 cm^{-1} were similar in the raw material and the ozonized samples (SO and SAO), which indicated that there were mild changes in the vibration of C═O

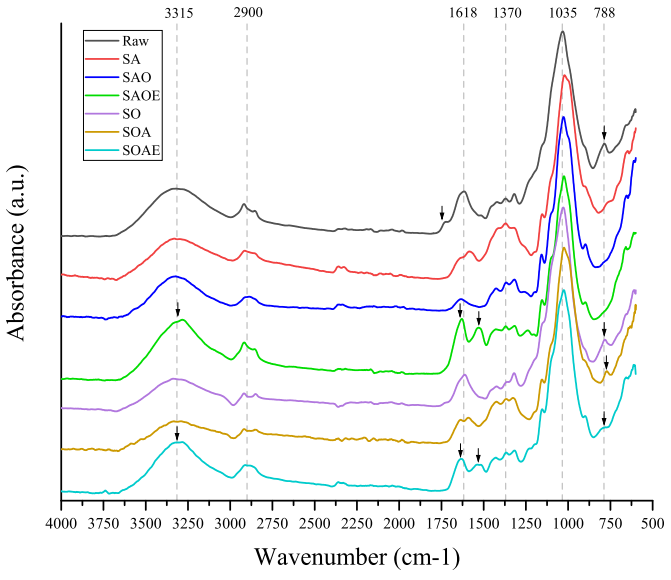

Fig. 3. FT-IR spectra of the solid fractions obtained from AOE and OAE processes. The spectrum of the raw material was included as a reference. This image shows one of the duplicates analyzed for each material. The arrows indicate the differences.

groups present in lignin. In contrast, the alkali-treated samples (SA and SOA) showed a different pattern in this region, suggesting that there might be breakage of lignin macromolecules producing changes in C═O groups (Ghasemi et al., 2013). Moreover, after enzymatic treatment, two peaks centered at 1620 and 1533 cm^{-1} were clearly discerned, evidencing the presence of residual lignin and hemicelluloses in the free form, in agreement with the compositional analysis (Fig. 2). Thus, FTIR analysis indicated the breakage of lignin and lignocellulosic constituents due to alkaline and ozone pretreatment of rice straw.

The bands around 1035 cm^{-1} and 897 cm^{-1} present in all spectra of raw and treated samples are assigned to the e C-O-C and C—O stretching at the β- glycosidic linkage (Dinh Vu et al., 2017; Han et al., 2016), indicating that the cellulose was preserved throughout the different treatments. The intensity of these bands increased in treated samples, evidencing an increase in cellulose content after the chemical treatment. Interestingly, the peak located at 790 cm^{-1} in the raw material, ascribed to the presence of silica (Nyquist et al., 1996; Sócrates, 1994), disappeared in SAOE sample but it remained in the SOAE sample. This finding supports the greater efficiency of using the alkaline treatment before the ozonisation (SAOE process) that also remove the silica (SiO_2) present in the raw material.

3.3.3. Thermogravimetric analysis (TGA) of solid fractions

Fig. 4 shows the TGA first derivative (DTGA) graphs from the raw material and the solid fractions obtained after each alkaline or ozone treatment. The raw material showed a three-step degradation profile, having the most intense peak at 280 °C, which corresponds to the cellulose degradation processes such as depolymerization and decomposition of glycosyl units (Ahuja et al., 2017; Benito-González et al., 2018; Miranda et al., 2013; Zhao et al., 2015). The results evidence that the thermal stability of the different fractions depended on their degree of purity. It should be noted that all fractions presented multiple-step degradation processes evidencing that pure cellulose was not obtained in any case, in agreement with compositional analysis (Section 3.2) and FTIR results. However, the most purified fractions (SAOE and SOAE) showed a sharper degradation peak and shifted towards higher temperatures. SAOE fraction presented a narrower and more defined peak compared to its counterpart SOAE. It is worth mentioning that the degradation peak of cellulose was shifted to lower temperatures in alkaline-treated samples (SA and SOA), which could be related to the removal of hemicelluloses, from the complex cell wall structure, or to a reduction of cellulose molecular weight.

Another degradation step, occurring at temperature between 350 and 500 °C, was observed in all the samples, which has been previously ascribed to the degradation of lignin (Ahuja et al., 2017). As stated by Freitas et al. (2022), lignin degradation can occur in a broad spectrum from around 220 °C to >400 °C. This means that lignin is still present in all the samples, in agreement with the compositional analysis (Section 3.2). Interestingly, an additional degradation peak was observed in the range of 500–700 °C in SA and SOA samples which can be related to the formation of sodium silicate in alkaline conditions. Rice straw contains a significant amount of silica (10 to 15 %), which originates from the soil and enters the roots of the rice plant as mono silicic acid and it is hypothesized to interact with cellulose and lignin (Bakar & Carey, 2020; Le et al., 2015). Accumulation of silica in rice straw via polymerization of water-soluble silicic acid absorbed from the soil into insoluble polysilicic acids, followed by precipitation as amorphous silica and deposition in exterior plant cell walls (Bakar & Carey, 2020). In fact, during the alkaline pretreatment, the linkages between silica and other lignocellulose components can be broken down, being silica released to the alkaline medium. Indeed, alkali pretreatments have been applied to extract silica from rice straw (Bakar & Carey, 2020). Thus, sodium silicate can be formed and it has been reported to degrade at higher temperatures (>600 °C) (Felsche et al., 1985).

3.4. Advantages of the proposed valorization strategy

In the present study, two strategies have been studied with the aim of proposing an alternative biorefinery process. In this regard, it is crucial to discuss the environmental impact that this technology could have. It is important to mention that the use of an agricultural residue (rice straw) as raw material is different from the use of on purpose planted species to obtain cellulosic materials. The use of these agricultural residues as materials reduces greenhouse gas emissions caused by the controlled burning in the fields. Likewise, this process uses lower temperatures and fewer chemicals than other technologies and involves the use of a targeted enzyme, which reduces random reactions. The strategy demonstrated for the extraction of different compounds can be adapted to different biomasses and by-products of interest.

As mentioned by Lehto and Alén (2015) and pursued in the current investigation, the integrated forest biorefinery approach focused the process not only on the main product (cellulose/pulp fiber), but also on value/added green chemicals characterized by a low volume/high value lignin extractives/based fine chemicals. This results in improved feedstock utilization, increased profitability, and reduced greenhouse gas emissions. For example, the liquid fraction obtained during the alkaline process is one of the most important by-products due to the high concentration of polyphenols and its antioxidant activity, which could be used in various applications such as in the production of food packaging, biomaterials engineering, among others (Shavandi et al., 2018).

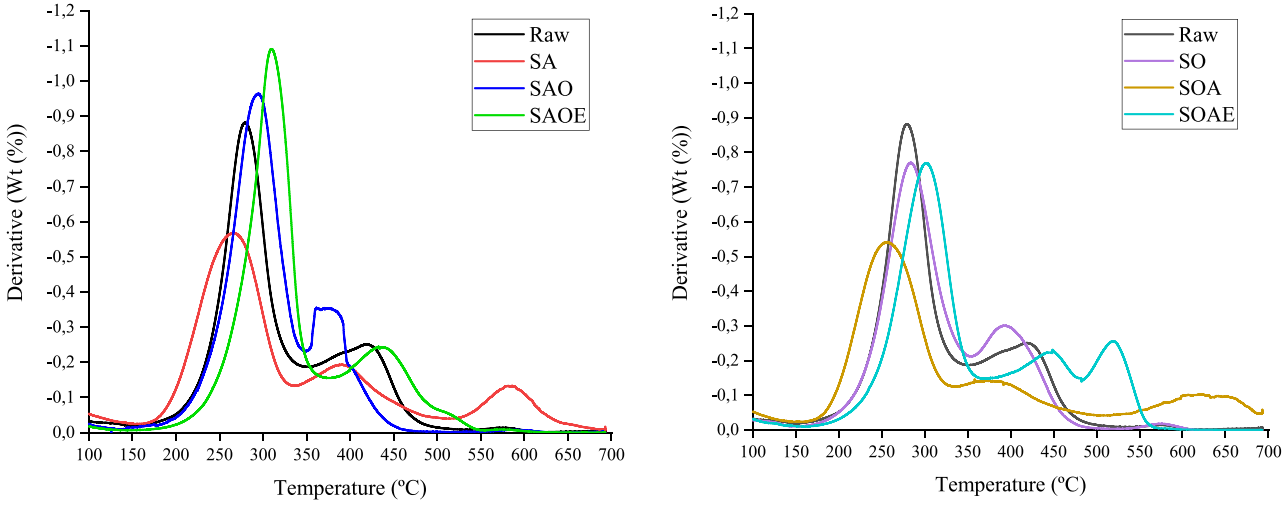

Fig. 4. Derivative thermogravimetric curves of the solid fractions obtained from the A-O-E and O-A-E processes. The raw material curve was included as a reference.

On the other hand, pulp production generates a number of critical emissions: Odor (methyl mercaptans, H_2S), atmospheric emissions caused by combustion processes (dust, $SO_2/CO_2/NO_x$), wastewater pollution (mainly organic), chlorine-organic compounds (AOX) produced by certain process configurations (Weiss, 2008). Using the methods presented in this work, there were no bad/odor emissions from the processes because no sulfur-containing compounds were used. In addition, no chlorine compounds were added and ozonation allowed bleaching of the solid extracts. Wastewater was treated as a by-product due to its richness in different compounds and bioactivity. On the other hand, the enzymatic process showed the capacity to extract even more hemicelluloses from the solid extracts, increasing the extraction yield of these compounds. Allowing that in one step thermal and enzymatic mediated hydrolysis were effectively carried out. In addition, xylanases are used as biobleaching agents, which is an environmental advantage over chemical alternatives (Kumar et al., 2016).

4. Conclusions

With the aim of developing greener strategies for rice straw biorefinery, the coupling of alkali, ozone and enzyme treatments was carried out. Therefore, the effect of the combined alkali-ozone-enzymatic (A-O-E) and ozone-alkali-enzymatic (O-A-E) treatments was compared. As a result, the A-O-E and O-A-E processes reduced the hemicellulose and lignin concentration of the biomass.Remarkably, the A-O-E process showed a better ability to remove arabinoxylan and lignin, yielding a more purified cellulose than the O-A-E process, indicating that the order of treatments is critical in rice straw processing. In addition, the alkaline liquid fractions from the A-O-E process showed greater potential for valorization due to its antioxidant capacity and high recovery of arabinose and xylose. Thus, these results confirmed the hypothesis related to the order of the treatments which was critical in the outcome biomass. These results provide the basis for a potential greener valorization of rice straw and the obtention of added-value by-products useful for food and packaging industry.

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CRediT authorship contribution statement

Laura Andrea Cabrera-Villamizar: Writing – original draft, Methodology, Investigation, Formal analysis. **Mahrokh Ebrahimi:** Methodology. **Antonio Martínez-Abad:** Writing – review & editing, Methodology, Data curation, Conceptualization. **David Talens-Perales:** Writing – review & editing, Methodology, Investigation. **Amparo López-Rubio:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **María José Fabra:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2023.121746.

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