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Evaluation of OxiOrganosolv pretreated hardwood and softwood lignocelluloses as substrates for the chemoenzymatic production of 5-hydroxymethylfurfural (HMF)

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Abstract

Furans, such as 5-hydroxymethylfurfural (HMF), are compounds of great importance that can serve as starting materials for the synthesis of polymers. Their production from lignocellulose-derived sugar streams offers a promising alternative to fossil fuels, while enabling biomass transformation to chemicals with higher value. In the present work, the production of HMF from OxiOrganosolv pretreated beechwood and pine was assessed by integrating a three-step process of enzymatic saccharification and isomerization followed by catalytic dehydration. The use of isobutanol in the pretreatment solvent and the addition of polyoxometallates (POMs) as oxidative catalysts were evaluated. The results showed that isobutanol leads to high delignification rates for both beechwood and pine, yielding cellulose-rich pulps with high susceptibility to enzymatic hydrolysis and isomerization. A fructose production up to 51.2 and 53.4 g/g of pretreated material was achieved for beechwood and pine, respectively, corresponding to 14 and 11.3 g of HMF/g of pretreated material. Regarding the use of POMs, the commercially available phosphomolybdic acid (HPMO) and POMs modified with oxidation metals (Fe-PMO, Cu-PMO) were tested, verifying their beneficial effect to lignin depolymerization and the composition of the final pulp. Hydrolysates produced from HPMo and Cu-PMo-assisted OxiOrganosolv pretreatment were efficiently used for the production of HMF, while severe inhibition of the dehydration reaction was observed with the hydrolysates from Fe-PMo pretreated biomass due to the presence of residual metals. This is the first systematic report comparing two lignocellulosic materials subjected to different pretreatment conditions for their potential to produce fructose and, subsequently, HMF.

Keywords 5-Hydroxymethylfurfural, Lignocellulosic biomass, OxiOrganosolv, Biocatalysis, Polyoxometallates

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Introduction

The potential of lignocellulosic biomass as a raw material has been extensively examined in the past in the field of biofuels, mainly for the production of bioethanol, biodiesel, and biogas [1]. However, since lignocellulose is comprised of many valuable compounds that can be used as starting materials for several industrial applications in different downstream processes, the interest has shifted towards other directions and uses in the recent years. Among popular fields are the synthesis of chemicals, food additives, pharmaceuticals, and polymeric materials [2, 3]. In particular, many biobased-polymers from lignocellulose have been developed, including nanocellulose, polylactic acid (PLA), and polyhydroxyalkanoates (PHAs), such as poly(3-hydroxypropionic acid) and polyhydroxybutyrate, by combining microbial fermentation and enzyme-mediated processes with chemical catalysis [4–8]. In this way, lignocellulosic biomass can serve as a substrate for the synthesis of green plastics, thus lowering the dependence of using petrochemicals and fossil fuels.

In seek of novel materials, furans are considered promising, because of their high chemical reactivity since they can participate in a plethora of reactions such as Diels–Alder, oxidative, and enantioselective cyclopropanation reactions [9]. Within this context, many furan monomers such as 5-hydroxymethylfurfural (HMF) and furfural (FF) can be exploited through biocatalytic and/or chemical processes for the synthesis of polyamines, polyesters, epoxides, and others [10]. These compounds can be easily produced from hexose and pentose-rich sugar streams obtained after fractionation of agricultural and forest residues, such as bleached birch kraft pulp, acid-treated corncob pulp, softwood hemicellulose, and corncob hydrolysates, thus rendering the use of lignocellulose an attractive alternative source compared to crude oil [11–13]. HMF can be used as a starting material, either chemically or through an enzymatic oxidative pathway for the production of 2,5-furandicarboxylic acid (FDCA). FDCA is a platform chemical whose polymerization leads to polyethylene furanoate (PEF) a substitute of polyethylene terephthalate (PET), thus paving the way to obtain biomass-derived polyesters. The application of FDCA is, however, not restricted to PEF and other polyesters, as it can also be used for preparation of polyamides, polyurethanes, and thermoset resins [14, 15]. Moreover, furans can be transformed into promising monomers through hydrogenation, it is possible to obtain HMF-derived diols such as 2,5-bis(hydroxymethyl)furan (BHMF) and 2,5-bis(hydroxymethyl)tetrahydrofuran (BHMTHF), which are strategic starting compounds for the synthesis of polyesters [16].

The conversion of glucose into HMF has been extensively studied with methods involving harsh experimental conditions such as high pressure and temperature, or corrosive and expensive solvents that are hard to extract from the end products [17]. Another issue of these processes lies in the fact that they suffer from low selectivity, since many side-products are also produced, with the main being humins and organic acids such as levulinic and formic acid derived from HMF. Although an increasing number of recent studies report the use of new heterologous Lewis–Brønsted acids in order to counteract low selectivity during the formation of HMF from pure sugar solutions, as reviewed by Megías-Sayago et al. [18], there is a need for more environmentally friendly processes. In parallel, within the frame of a biorefinery, after initial lignocellulosic biomass fractionation through pretreatment, it is possible to convert glucose to HMF by implementing mild procedures, including enzymatic saccharification, isomerization, and dehydration [19, 20]. The use of biocatalytic pathways shows great appeal due to the high selectivity towards the products combined with a green process profile that requires relatively mild reaction conditions. Although the overall process seems promising, there are few studies in the literature reporting the integrated procedure from real lignocellulosic biomass to HMF by applying enzymatic biocatalysis [20–22].

In our previous study [23], we have developed OxiOrganosolv pretreatment as an efficient process for the fractionation of many biomass feedstocks, including both hardwoods and softwoods. OxiOrganosolv leads to high delignification rates, thus producing a cellulose-rich solid pulp, while limiting the formation of inhibitors that could affect the subsequent biocatalytic processes. One step further, we have evaluated various solid pulps obtained from beechwood pretreatment with different organic solvents for the production of sugar-rich solution and its succeeding conversion to HMF under homogeneous catalysis [19]. Among the catalysts tested, the effectiveness of formic acid as an appealing alternative to strong inorganic acids was demonstrated by our group. When the dehydration reaction was conducted at 150 °C for 60 min, formic acid resulted in high HMF yield (44.6 wt.%), and selectivity (55.8 wt.%).

Following our previous work, we attempted to expand the range of materials used by testing not only OxiOrganosolv pretreated softwood (pine), but also beechwood solid pulps that are pretreated with isobutanol (iBuOH) as the organic phase of the liquid solvent. In addition, pulps obtained from pretreatment using various polyoxometallates (POMs) as catalysts were also included in an attempt to increase the process efficiency while keeping a relatively lower pretreatment temperature of 150 °C [24] and examine the impact of those catalysts

on saccharification, isomerization, and HMF production. The hydrolysates of the best performing materials were subsequently employed in dehydration reactions, whereas the resulting HMF yields were examined. Conducting a systematic comparison of utilizing solid pulps from different pretreatments will facilitate the evaluation of solid pulps' composition and the potential effect of the existing compounds on HMF production. This is the first report that examines two lignocellulosic materials that underwent a variety of different pretreatment conditions, and their potential to produce fructose and, subsequently, HMF.

Materials and methods

Organosolv pretreatment of lignocellulosic biomass

Two lignocellulosic feedstocks were used in this study, namely beechwood (Lignocel[®] HBS 150–500, JRS GmbH and Ko KG, Germany) and Southern yellow pine (*Pinus* sp.), both with a particle size of 150–500 μm . The biomass samples were subjected to oxidative pretreatment (OxiOrganosolv), as previously described [23], and four different parameters (organic solvent, temperature, time, and addition of POM catalysts) were examined among different runs. In all cases, an overpressure of 100% O₂ was continuously fed to the pretreatment vessel as the oxidative agent. In addition to acetone (ACO), ethanol (EtOH), tetrahydrofuran (THF), and isobutanol (iBuOH) were utilized as solvents with a solvent-to-water ratio of 50:50. The pretreatment was conducted at temperatures of 150, 160 and 175 °C, with residence times of 60 and 120 min. POMs were also introduced to the pretreatment mixture as catalysts at 150 °C, for 60 min. The first one was the commercially available phosphomolybdic acid (H₃PMo₁₂O₄₀ × H₂O, HPMo), while the other two were custom-made salts by replacing H₂ with metal ions Fe (Fe₃MO₁₂OP) and Cu (Cu₃MO₁₂OP) [24]. After pretreatment, the solid residue was vacuum filtered, washed with the respective solvent, and distilled water until pH 5, followed by air-drying. The compositional analysis of solid samples was performed according to NREL protocols [25]. The untreated pine composition was 29.5% lignin, 40.0% cellulose, and 18.4% hemicellulose, while the composition of untreated beechwood was 23.6% lignin, 40.1% cellulose, and 19.1% hemicellulose [23].

Enzymatic saccharification of pretreated pulps and isomerization towards fructose

In order to evaluate the susceptibility of cellulose-rich pretreated solid samples towards the production of glucose, hydrolysis experiments were conducted in 250 mL Erlenmeyer flasks. Each flask contained the respective sample in 10% solids loading, Cellic[®] CTec2 (Novozymes A/S, Denmark) at an enzyme loading of 15 mg/g_{solids} and

20 mM phosphate citrate buffer pH 5. Hydrolysis experiments were performed at 50 °C under 180 rpm agitation for 72 h. Cellic[®] CTec2 filter paper activity and protein content were determined 168 FPU/mL (FPU: filter paper units) and 137 mg/mL, respectively, under the methods introduced by Ghose [26] and Bradford [27]. Detection of the produced glucose was accomplished with glucose oxidase/oxidase (GOD/POD) assay [28].

Isomerization was performed on the hydrolysates by implementing the commercially available immobilized glucose isomerase Sweetzyme[®] (Novozymes A/S, Denmark). The thermodynamic equilibrium of the reaction was shifted towards fructose with the addition of sodium tetraborate at a molar ratio of 0.28 as it was determined by our previous work [19]. The difference in the pH optimum between Cellic[®] CTec2 and Sweetzyme[®] was adjusted upon the addition of NaOH 1 M. The hydrolysates were vacuum filtered and the liquid fraction along with the sodium tetraborate, NaOH, and Sweetzyme[®] were placed in 250 mL Erlenmeyer flasks at 50 °C for 24 h. Since glucose is isomerized to fructose with a molar ratio of 1:1, fructose concentration was assessed by the reduction of glucose before and after the reaction. After isomerization, the liquid fraction was also separated by vacuum filtration and was used for the dehydration experiments. Prior to the experiments, the possible occurrence of metals, which might have been released from the pretreatment catalysts and migrated into the hydrolysates during preceding process steps, was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES) analysis by using an Optima 4300 DV PerkinElmer spectrometer (USA).

Dehydration of fructose towards HMF

The hydrolysates of the best performing materials underwent conversion into furans by means of homogeneous catalysis. Following our previous study [19], the reaction was conducted under optimized conditions (150 °C for a duration of 60 min), employing 99 wt.% formic acid (Sigma-Aldrich) as the catalyst. The experiments were carried out in a batch, stirred, autoclave reactor (C-276 Parr Inst., USA) under N₂ gas. Prior to the reaction, the hydrolysates were acidified by the addition of concentrated formic acid until pH 1–2 and diluted with distilled water at a dilution factor of 1:4 [19]. Consequently, the mixture was introduced into the reactor and heated at 150°C for 60 min under continuous stirring. Zero time was recorded when the required temperature was reached. At the end of the reaction, the reactor was cooled down rapidly and the resulting solution was filtered to remove solids obtained as by-products (i.e., humins) [29]. The liquid fraction was then analyzed by Ion Chromatography (ICS-5000, Dionex USA). Standard

solutions employed for the product quantification comprised aqueous solutions containing sugars, sugars alcohols, HMF, and organic acids. The analysis of the sugars and HMF was conducted using a CarboPac PA1 column (10 μm , 4 \times 250 mm), a guard column (10 μm , 4 \times 30 mm), and a pulsed amperometric detector (PAD). The eluent used was 20 mM NaOH at 0.6 mL/min flow rate. The analysis of the organic acids was performed using an AS-15 (9 μm , 4 \times 250 mm) column, a pre-column (9 μm , 4 \times 30 mm), and a conductivity detector (CD), while the eluent used was 8 mM NaOH at 1 mL/min flow rate. The total analysis time was 75 min in both cases.

The conversion of sugars (glucose and fructose), the yields, and the selectivity of the products (weight based) were calculated according to the following Eqs. (1), (2), and (3):

$$\text{Conversion}_{\text{sugars}}(\%) = 100 \times \frac{\text{sugars}_{\text{reacted}}(\text{g})}{\text{sugars}_{\text{initial}}(\text{g})} \quad (1)$$

$$\text{Yield}_{\text{product}}(\%) = 100 \times \frac{\text{product}_{\text{produced}}(\text{g})}{\text{sugars}_{\text{initial}}(\text{g})} \quad (2)$$

$$\text{Selectivity}_{\text{product}}(\%) = 100 \times \frac{\text{Yield}_{\text{product}}(\%)}{\text{Conversion}_{\text{sugars}}(\%)} \quad (3)$$

Results and discussion

This work examines the effect of the OxiOrganosolv pretreatment process towards the production of HMF from beechwood and pine, by evaluating the use of four different organic solvents (EtOH, ACO, THF, and iBuOH), as well as the addition of POM catalysts. A total of 29 samples treated under different pretreatment conditions were tested regarding their susceptibility to enzymatic degradation and production of fructose, while the most promising pulps were selected to further assess HMF yield and selectivity through dehydration. The pretreatment conditions and the compositional analysis of the solid pulps are presented in Supplementary Table S1. By implementing enzymatic saccharification and isomerization, following a process already established in our previous work [19], it was possible to effectively produce fructose and, subsequently, HMF from real biomass samples. The evaluation of the pretreatment was performed by correlating the saccharification and the isomerization results for each sample with three different parameters: temperature, residence time, and type of solvent. In all cases, the initial pressure of pretreatment was 16 bar, and oxidation was achieved with the constant flux of oxygen in the chamber. The results from the saccharification and isomerization reactions were organized into

two groups depending on the biomass type (beechwood, pine). The first group includes beechwood samples (Lignocel[®]) which either underwent iBuOH pretreatment at three different temperatures (150, 160 and 175 °C) in the absence of any additional catalyst or were pretreated with different organic solvents at 150 °C upon the addition of POMs. POM catalysts were tested only at the lowest pretreatment temperature of those studied in this work, with the aim to evaluate their boosting effect in biomass delignification in suboptimal conditions. The second group includes pine samples which were pretreated with different organic solvents at 175 °C. Fructose yield expressed in g fructose/100 g pretreated biomass is depicted in Figs. 1 and 2 for beechwood and in Fig. 3 for pine.

Enzymatic saccharification of pretreated beechwood pulps and production of fructose

The results of the saccharification and isomerization experiments of biomass samples pretreated with different catalysts are shown in Table 1. The cellulose conversion of samples pretreated with iBuOH in the absence of catalyst was 52.36%, while the results for ACO and EtOH were 57.69% and 57.72% respectively. Pulps pretreated with THF showed a remarkably high cellulose conversion of 79.02%. The selection of the organic solvent seems to affect both lignin/hemicellulose removal and cellulose content in the resulting pulp, as well as the properties of the cellulose surface that may affect the subsequent hydrolysis yields, as shown in our previous studies [23, 24]. ACO, EtOH, and THF are all miscible with water; however, a local phase separation of the solution on the surface of polysaccharides, which is different for each solvent, takes place during pretreatment. This results in lignin dissolution and removal, depolymerization of hemicellulose, as well as cellulose swelling and partial degradation [23], which in turn affects not only the fractionation efficiency of the pretreatment, but also the properties of the remained cellulose, as reflected in the differences in saccharification efficiency. Among these solvents, THF is the most efficient [30], leading to better results at lower severity pretreatment which has been also shown in our previous work [23]. On the other hand, iBuOH is partly miscible at high temperatures employed during pretreatment; however, it turns immiscible upon temperature drop, which increases the efficiency of lignin removal but also affects the morphology of the surface and the subsequent saccharification. In addition, the differential polarity of the solvents affects their ability to stabilize acidic protons provided by the POMs catalysts, as will be discussed below.

Regarding the addition of polyoxometalates, as shown in Table 1, the cellulose conversion of samples pretreated with iBuOH was 48.79% when HPMo was added,

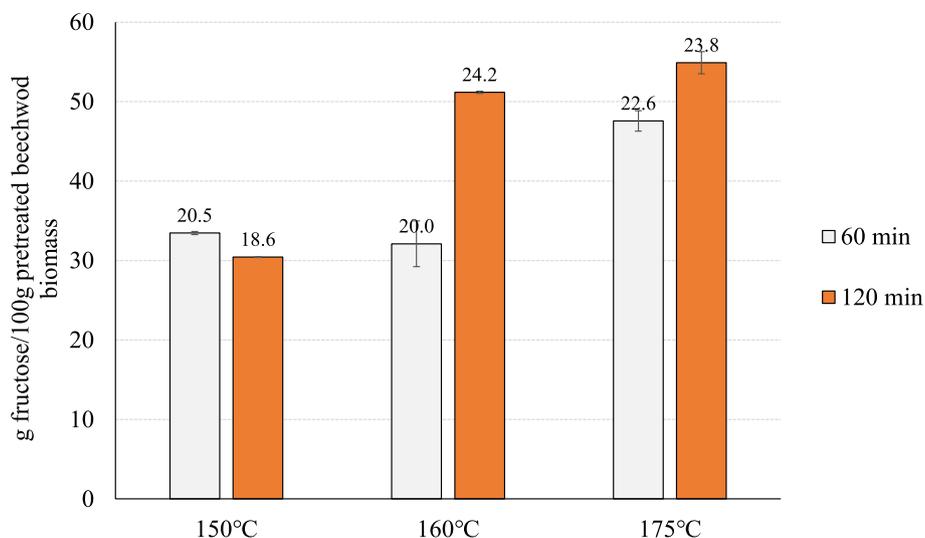


Fig. 1 Production of fructose per 100 g of pretreated solid pulp after enzymatic hydrolysis with Cellic® CTec2 and isomerization with Sweetzyme® of OxiOrganosolv pretreated beechwood samples with iBuOH at three different cooking temperatures (150, 160, 175 °C) and two residence times (60, 120 min). Labels indicate the production of fructose (g) per 100 g of initial untreated beechwood

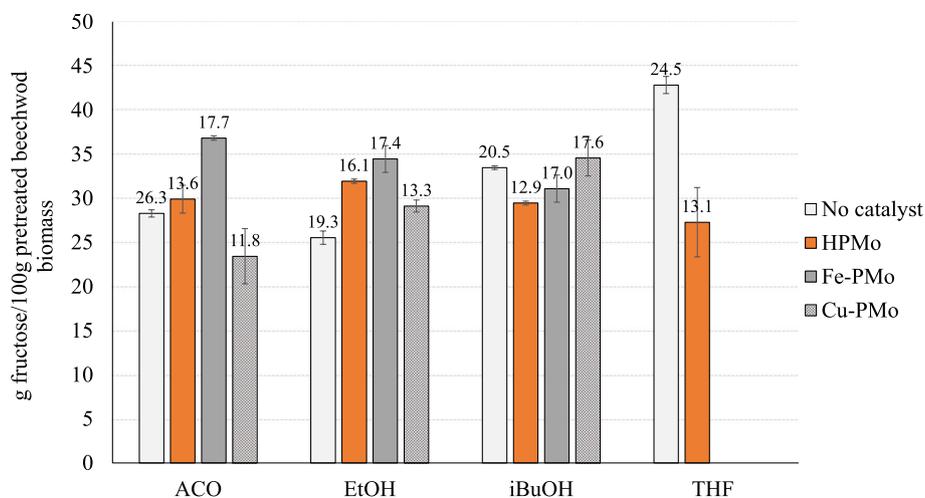


Fig. 2 Production of fructose (g) per 100 g of pretreated solid pulp after enzymatic hydrolysis with Cellic® CTec2 and isomerization with Sweetzyme® of OxiOrganosolv pretreated beechwood samples (150 °C, 60 min, O₂ 16 bar) with four different organic solvents and POMs catalysts at a loading of 5%. All experiments were run in duplicates and numbers represent the mean values. Labels indicate the production of fructose (g) per 100 g of initial untreated beechwood

followed by an increase to 53.06% and 53.43% for Fe-PMo and Cu-PMo, respectively. The corresponding results for ACO were 55.53% (HPMo), 58.22% (Fe-PMo), and 41.04% (Cu-PMo), while in the case of EtOH, cellulose conversion reached 56.95% (HPMo), 56.90% (Fe-PMo) and 46.95% (Cu-PMo). Finally, when THF was used as a solvent, a significant drop of cellulose conversion in the presence of HPMo at 49.11% occurred, thus discouraging further examination with the other two POM catalysts. The above results indicate that the use of POMs

and their metal-substituted salts, despite leading to the production of pulps with a higher cellulose content, does not necessarily contribute positively to enzymatic processes following pretreatment. The implementation of metal catalysts in the pretreatment process has been suggested in the literature due to their redox potential and their ability to interact with lignin as electron donors [32, 33]. As a result, the oxidation of lignin is sought to trigger its depolymerization and removal in the pretreatment liquor, thus achieving efficient delignification and

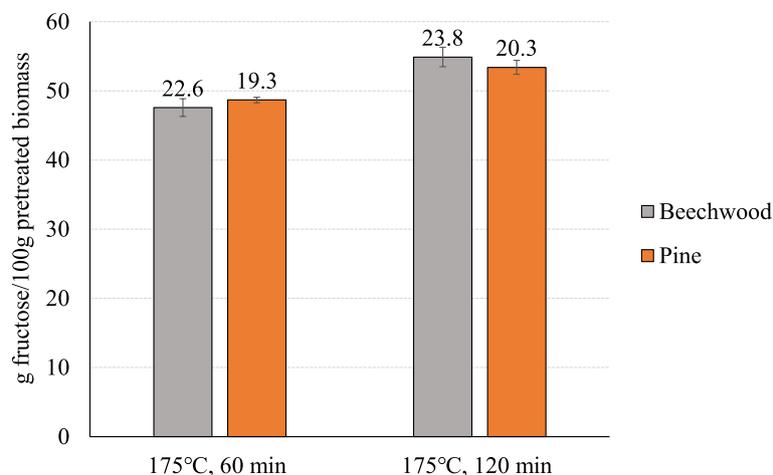


Fig. 3 Comparison of beechwood and pine regarding the production of fructose (g) per 100 g of pretreated solid pulp after enzymatic hydrolysis with Cellic[®] CTec2 and isomerization with Sweetzyme[®] with iBuOH at 175°C for two different residence times (60, 120 min). All experiments were run in duplicates and numbers represent the mean values. Labels indicate the production of fructose (g) per 100 g of initial untreated beechwood

Table 1 Description of pretreatment conditions, enzymatic digestibility of obtained pulps after treatment with Cellic[®] CTec2, and glucose isomerization to fructose yields with Sweetzyme[®] of OxiOrganosolv pretreated *beechwood* samples. All experiments were run in duplicates and numbers represent the mean values

Catalyst	Organic solvent	Pretreatment conditions	% cellulose content in pulp	% cellulose conversion to glucose	% glucose conversion to fructose
No catalyst	iBuOH	150 °C, 60 min	65.87 ^a	69.12 ± 0.86	73.51 ± 0.42
		150 °C, 120 min	65.87 ^b	62.78 ± 0	73.6 ± 0.1
		160 °C, 60 min	68.16 ^b	66.67 ± 3.14	70.7 ± 3.0
		160 °C, 120 min	87.75 ^b	73.39 ± 0	79.5 ± 0.2
		175 °C, 60 min	87.42 ^b	68.20 ± 1.47	79.8 ± 0.3
		175 °C, 120 min	92.6 ^b	70.07 ± 0.56	84.6 ± 1.2
		ACo	150 °C, 60 min	62.74 ^c	57.69 ± 2.39
EtOH	150 °C, 60 min		56.72 ^c	57.72 ± 0.29	78.11 ± 2.11
THF	150 °C, 60 min		73.09 ^c	79.02 ± 1.70	74.17 ± 0.01
HPMo	iBuOH	150 °C, 60 min	93.3 ^a	48.79 ± 2.44	64.71 ± 2.59
	ACo	150 °C, 60 min	89.13 ^a	55.53 ± 1.27	60.41 ± 2.36
	EtOH	150 °C, 60 min	86.63 ^a	56.95 ± 0.44	64.75 ± 0
	THF	150 °C, 60 min	90.39 ^a	49.11 ± 2.37	61.46 ± 6.59
Fe-PMo	iBuOH	150 °C, 60 min	78.48 ^a	53.06 ± 0.67	74.66 ± 1.8
	ACo	150 °C, 60 min	81.96 ^a	58.22 ± 0.8	77.17 ± 1.15
	EtOH	150 °C, 60 min	77.74 ^a	56.90 ± 1.86	77.9 ± 0.72
Cu-PMo	iBuOH	150 °C, 60 min	86.58 ^a	53.43 ± 2.12	74.72 ± 1.07
	ACo	150 °C, 60 min	83.3 ^a	41.04 ± 3.94	68.53 ± 2.34
	EtOH	150 °C, 60 min	89.9 ^a	46.95 ± 1.46	68.98 ± 0.51

^a Karnaouri et al. [24]

^b Karnaouri et al. [31]

^c Kalogiannis et al. [23]

high cellulose content in the residual pulps. As a matter of fact, in all runs of this study, the addition of POM catalysts increased the delignification degree of the

process and the cellulose content of the pulp, as depicted in Table 1. Nevertheless, POMs that are diluted in the pretreatment solvent or metal ions derived from their

partial degradation may have been absorbed and incorporated in the solid pulp. The presence of these residual ions that remain even after extensive washing has been confirmed in our previous study [24] and has been reported to affect the subsequent enzymatic processes due to interactions between POMs and enzymes [34, 35]. In their review, Lentink et al. [36] mention that these interactions are largely governed by factors such as pH, temperature, the ionic strength of the medium, the presence of other species such as surfactants and, in the case of metal-substituted POMs, the nature of the metal ion. In addition, the negative charge of POMs can interact with the positively charged amino acids of the enzyme, thus causing the hydrolysis of peptide bonds or the oxidation of amino acids and, in turn, enzyme inhibition [37]. While this can be very useful in fields demanding high-purity protein isolation such as crystallography or medicine (isolation of proteins from complex matrices such as human blood or tissue), in the case of our study POMs can have a detrimental effect to the overall yield of the process. For example, cellulose conversion, which is the means for saccharification evaluation, seems to drop with the use of HPMo, with THF exhibiting a drop in cellulose conversion of nearly 20%. This also seems to be the case for CuPMo as cellulose conversion remains on the same levels only for iBuOH as a solvent. In addition, the same pattern is also passed down to the isomerization experiments. While glucose conversion to fructose usually ranges between 70 and 80%, the use of HPMo and CuPMo seems to inhibit isomerization, resulting in yields below 70%. This can possibly be attributed to the nature of the metal catalyst utilized. Keggin-type POMs, such as the one used in this work, have been reported to demonstrate exceptional absorption affinity towards proteins [38], which may reduce the overall efficiency of the process. In addition, the presence of copper ions in metal POMs has been shown to produce reactive oxygen species in the vicinity of the protein binding site, thus resulting in protein fragmentation [39]. Hence, traces of Cu-PMo may induce cleavage of cellulases or the glucose isomerase lowering their activity. Nevertheless, Fe-PMo does not seem to affect enzymatic activity in these processes. In fact, in the case of iBuOH and ACO, cellulose conversion is slightly increased, a result that can be reinforced by studies reporting the upregulation of endoglucanase activities [40, 41].

The results presented in Table 1 and Fig. 1 offer insight into the dependence of beechwood hydrolysis and isomerization on pretreatment time and temperature for iBuOH. Firstly, it is apparent that, in any case, longer pretreatment times lead to higher cellulose content in the pulps and better cellulose conversion rates. In fact, harsher pretreatment conditions favor lignin and

hemicellulose dissolution, thus leading to higher cellulose content; in the case of 175 °C and 120 min, cellulose content in the pulp reaches 92.6%. This exhibits a difference of more than 30% between the harshest and the mildest pretreatment conditions, as the run of 150 °C and 60 min leads to 55.54% cellulose in the pulp. In addition, cellulose conversion follows the same trend with the highest conversion being 73.39% (160 °C, 120 min) followed by 70.07% (175 °C, 120 min). However, when isomerization results are considered, where the 175 °C, 120 min run shows the highest glucose to fructose conversion of 84.6%, it is evident that this sample also produced the highest amount of fructose with 54.9 g fructose/100 g pretreated material. Moreover, as depicted in Fig. 1, there is a sudden jump in the production of fructose from 32.1 g fructose/100 g pretreated sample (160 °C, 60 min) to 51.2 (160 °C, 120 min), 47.6 (175 °C, 60 min), and 54.9 (175 °C, 120 min). This can be partially attributed to the glucose concentration, which follows the same trend and is the substrate for fructose production; nevertheless, glucose-to-fructose conversion is also higher in runs with harsher pretreatment conditions as opposed to milder ones. These results are comparable to the work of Saikia et al. [42] who implemented a system for the production of fructose through the direct hydrolysis of inulin. In their study, the optimum fructose production was 65.7 g fructose/g inulin, albeit through the hydrolysis of a pure fructose polymer. Furthermore, present work shows significantly higher fructose yields than studies implementing custom-made chemical catalysts for the isomerization of fructose. For example, Zhang et al. [43] were able to reach 32.58% fructose yield from pure glucose using a custom Al-hydrochar catalyst for 160 °C for 20 min, while Yang et al. [44] implemented a similar Ca-hydrochar catalyst to reach 31% fructose yield at 120 °C for 45 min. Consequently, this study presents an effective way to produce fructose selectively from a complex system such as lignocellulose, while simultaneously achieving it in mild conditions, a prerequisite for enzymatic procedures.

Enzymatic saccharification of pretreated pine pulps and production of fructose

The results of pine hydrolysis indicate that, while being susceptible to hydrolysis, pine as a feedstock leads to lower fructose production when compared to beechwood. Firstly, beechwood biomass which was pretreated at milder conditions (150 °C, 60 min), exhibited cellulose conversions above 50% for every solvent (69.12% for iBuOH, 57.69% for ACO, 57.72% for EtOH, and 79.02% for THF). The respective cellulose conversion results for pine samples pretreated at 175 °C were 62.83%, 46.79%, 67.02%, and 31.71%, indicating that the use of EtOH at

high temperatures is preferable, contrary to ACO and THF, which appear to perform better at low temperatures, as it is also observed in our previous work [19]. In addition, cellulose conversion of pine samples at higher pretreatment durations seems to be on par with the corresponding beechwood samples for iBuOH and EtOH, but significantly lower for ACO and THF (Table 2). These low saccharification yields for THF can be attributed to the high lignin content of the pretreated pine sample, which works as a binding layer for the hydrolytic enzymes of cellulose, inhibiting their function [45]. When compared to hardwoods, softwoods, such as pine, typically have higher lignin content, while this mainly consisted of guaiacyl type units with many C–C bonds which are more recalcitrant than the ether linkages among the syringyl and guaiacyl type monomers of hardwoods [46]. As a result, lignin dissolution and removal during pretreatment in softwoods is often challenging. In addition, pine differs from beechwood regarding the type of hemicellulose and lignin they contain; hemicellulose in softwoods represents mainly gluco- and galactomannans, while in hardwoods it consists mostly of glucuronoxylans which can be hydrolyzed more easily, thus promoting lignin dissolution during pretreatment [47]. Differences in susceptibility to enzymatic saccharification between softwoods and hardwoods have been observed even for pulps having the same lignin content, which is apparently attributed to other factors that are not related to the composition of the solid fraction, such as lignin type and distribution, the nature of lignin-carbohydrate complexes and others [48].

In the case of ACO, the lower cellulose conversion yield compared to other solvents might be the result of enzyme inhibitors produced in the pretreatment process. These inhibitors include mainly degradation products formed

in different amounts depending on the nature of the pretreatment and have been found to affect negatively the ensuing downstream biocatalytic processes [49]. However, if the isomerization process is taken into consideration, it is evident that iBuOH as a pretreatment solvent not only assists the saccharification process, but also boosts the isomerase activity leading to higher fructose yields. In specific, while the pattern of glucose-to-fructose conversion fluctuates between the range of 70–80%, it increases above 80% in the iBuOH pretreated pine samples, effectively reaching 86.4% and 87.3% for 60- and 120-min residence time, respectively. This in turn translates to 19.3 and 20.3 g fructose/100 g of untreated biomass, respectively, which shows nearly a twofold increase than the majority of the rest of the pine samples. In addition, while in most cases beechwood appears to be better as a feedstock, the 60 min iBuOH pretreated pine sample exhibits a 48.7 g fructose production/100 g of pretreated biomass, which is higher than the corresponding beechwood sample (47.6 g fructose/100 g pretreated biomass) as depicted in Fig. 3. The above verifies the efficiency of the OxiOrganosolv process for the pretreatment of such a recalcitrant softwood feedstock such as pine, which has been also shown in our previous study [23].

Production of HMF

Optimum sugars-rich hydrolysates, which originated after enzymatic isomerization, were investigated as substrates for the production of HMF. Prior to the reaction, the samples were acidified by adding concentrated formic acid until reaching a pH of 1–2, since this particular acid was identified as the most appropriate among those tested for the dissociation of the borate species of sodium tetraborate in order to minimize its negative effect on the production of HMF [19]. Afterwards, the

Table 2 Description of pretreatment conditions, enzymatic digestibility of obtained pulps after treatment with Cellic[®] CTec2, and glucose isomerization to fructose yields with Sweetzyme[®] of OxiOrganosolv pretreated *pine* samples. All experiments were run in duplicates and numbers represent the mean values

Organic solvent	Pretreatment conditions	% cellulose content in pulp	% cellulose conversion to glucose	% glucose conversion to fructose
iBuOH	175 °C, 60 min	89.68	62.83 ± 0.49	86.4 ± 0.1
	175 °C, 120 min	83.64	73.13 ± 1.02	87.3 ± 0.4
ACO	175 °C, 60 min	82.75 ^a	46.79 ± 1.31	73.2 ± 1.1
	175 °C, 120 min	89.17 ^a	36.97 ± 0.57	79.6 ± 0.1
EtOH	175 °C, 60 min	77.6 ^a	67.02 ± 0.82	74.5 ± 0.5
	175 °C, 120 min	79.57 ^a	56.60 ± 0.5	75.7 ± 1.7
THF	175 °C, 60 min	60.8 ^a	31.71 ± 0.30	73.9 ± 0.4
	175 °C, 120 min	71.31 ^a	38.17 ± 0.89	76.1 ± 0.7

^a Kalogiannis et al. [23]

acidified hydrolysates containing mainly fructose and lower amounts of glucose (Table S3) were diluted with H₂O at a ratio of 1 to 4 and treated at 150 °C for 60 min. Under the reaction conditions employed, sugars were effectively converted to HMF in all cases, with respective values of selectivity and yield ranging between 13.1–49.9 wt.% (corresponding to 18.8–71.2 expressed in mol%) (Table 3) and 12.2–40.3 wt.% (corresponding to 17.4–57.6 expressed in mol%) (Table S4). Other detected products (Table S4) were traces of mannose (yield below 4% in any case), an isomer of glucose and fructose produced via epimerization, and organic acids levulinic and formic, formed through rehydration of HMF in a 1:1 molar ratio. The yield of levulinic acid remained below 15 wt.% in all cases (Table S4). Regarding formic acid, its concentration could not be directly determined, due to its role as the reaction catalyst and only an assumption could be made based on the reaction stoichiometry (nominal values in Table S4). Apart from the liquid products, unidentified solid by-products of dark brown color (humins), were also formed, revealing that HMF degradation side-reactions also occurred. These solids were removed from the reaction mixture through filtration, washed with water and, after drying at 100 °C overnight, were weighed and subjected to carbon analysis. According to experimental results, almost all of them consisted of 60–65% of carbon, while their yield was below 7.5% (weight based) in all cases (Table S4). However, the sum of humins and products detected by IC analysis did not complete the mass

balance, suggesting the presence of unidentified liquid products such as water-soluble oligomers (could not be measured by our methods) and water-soluble humins precursors as indicated by the dark brown color of the reaction mixtures [50].

The sugar-rich hydrolysates which were classified into two groups: beechwood hydrolysates and pine hydrolysates, depending on the biomass used as the initial feedstock for saccharification, were selected in order to infer conclusions regarding the impact of both the feed and the pretreatment method on HMF production. Starting from the first group, hydrolysates derived from beechwood pretreated with THF (Lign_THF) and iBuOH (Lign_iBuOH_1) at 150 °C in the absence of a catalyst yielded similar HMF production, with respective values of 8.7 and 8.3 g/100 g pretreated biomass. However, in the case of the Lign_iBuOH_1 hydrolysate, there was a higher sugar conversion (82.4 wt.%) and greater selectivity toward HMF (49 wt.% based on sugars, corresponding to 69.9 mol%) compared to the Lign_THF hydrolysate (74.7 wt.% sugar conversion, 38.9 wt.% selectivity corresponding to 55.7 mol%), without a simultaneous significant increase in byproducts (Table 3). Considering the better performance of the Lign_iBuOH hydrolysate, the impact of the pretreatment temperature was further examined by using samples obtained from beechwood after pretreatment at three different temperatures: 150 °C (Lign-iBuOH_1), 160 °C (Lign-iBuOH_2), and 175 °C (Lign-iBuOH_3). The results, presented in Table 3,

Table 3 HMF synthesis from sugars in the liquid product after dehydration with formic acid (reaction conditions: 150 °C, 60 min). All experiments were run in duplicates and numbers represent the mean values. The standard error deviation was $\leq 2.5\%$ in all measurements

Hydrolysate	Organic solvent	Catalyst	Pretreatment conditions	Sugars conversion (wt.%)	HMF selectivity (wt.%)	g HMF/100 g pretreated biomass
<i>Lignocel</i>						
Lign_iBuOH_1	iBuOH	No catalyst	150 °C, 120 min	82.4	49.0	8.3
Lign_iBuOH_2	iBuOH	No catalyst	160 °C, 120 min	75.9	47.8	13.0
Lign_iBuOH_3	iBuOH	No catalyst	175 °C, 120 min	71.7	39.3	14.1
Lign_THF	THF	No catalyst	150 °C, 60 min	74.7	38.9	8.7
Lign_EtOH_HPMo	EtOH	HPMo	150 °C, 60 min	81.2	32.4	5.9
Lign_iBuOH_HPMo	iBuOH	HPMo	150 °C, 60 min	84.1	33.3	5.8
Lign_ACO_FePMo	ACO	Fe-PMo	150 °C, 60 min	92.9	13.1	3.1
Lign_EtOH_CuPMo	EtOH	Cu-PMo	150 °C, 60 min	78.6	36.1	5.8
Lign_iBuOH_CuPMo	iBuOH	Cu-PMo	150 °C, 60 min	75.3	45.9	8.4
<i>Pine</i>						
Pine_EtOH	EtOH	No catalyst	175 °C, 120 min	77.6	49.9	9.2
Pine_ACO	ACO	No catalyst	175 °C, 120 min	60.3	43.5	5.0
Pine_iBuOH_1	iBuOH	No catalyst	175 °C, 60 min	73.7	39.5	9.9
Pine_iBuOH_2	iBuOH	No catalyst	175 °C, 120 min	74.1	40.8	11.3

indicate that with an increase in pretreatment temperature, there is a slight reduction in both sugar conversion and HMF selectivity. However, the production of HMF from the pretreated feedstock does not exhibit the same trend as its values increase from 8 to 14 wt.%. As shown above, the pretreatment temperature appears to exert some influence on the composition of the initial feedstock, increasing the delignification efficiency and the cellulose content of the pulps, thus consequently affecting the behavior of the hydrolysates during subsequent HMF production.

The effect of POM pretreatment catalysts on subsequent steps of the process was also evaluated by using optimal hydrolysates received after pretreatment of beechwood with HPMo (Lign_EtOH_HPMo, Lign_iBuOH_HPMo), FePMo (Lign_ACO_FePMo), and CuPMo (Lign_EtOH_CuPMo, Lign_iBuOH_CuPMo). Additionally, ICP-OES analysis was conducted to investigate the potential presence of metals that may have leached from the pretreatment catalysts and reached the hydrolysates during the prior process steps (Table S5). According to the ICP-OES analysis of the liquid samples, the molybdenum (Mo) content in Lign_EtOH_HPMo and Lign_iBuOH_HPMo hydrolysates was found to be negligible (Table S5), suggesting that the presence of this metal does not impact HMF production. Nonetheless, the presence of the catalyst during pretreatment appears to influence the feedstock, as the resulting hydrolysates exhibited lower HMF selectivity (32.4 and 33.3 wt.%, respectively, corresponding to 46.3 and 47.6 mol%), despite achieving higher sugar conversion (81.2 and 84.1 wt.%, respectively) when compared to those derived from feedstocks pretreated without the presence of a catalyst (Table 3). Furthermore, the production of HMF was 5.9 and 5.8 g/100 g pretreated feedstock respectively, values lower when compared to the ones received from Lign_iBuOH_1 and Lign_THF (Table 3). This trend was also observed by the Lign_ACO_FePMo hydrolysate, where sugar conversion reached its highest value of 92.9 wt.%, but with both the lowest selectivity (12.2 wt.%, corresponding to 17.4 mol%) and HMF yield based on the pretreated biomass (3.1 wt.%). In this case, the ICP-OES analysis revealed the presence of Mo and iron (Fe) in the reaction medium (Table S5), metals that promote side reactions leading to the formation of organic acids (levulinic and formic) and humins, as also indicated by the highest selectivity of these products (Table S4). Lign_EtOH_CuPMo and Lign_iBuOH_CuPMo appear to be more efficient in HMF production compared to the above-mentioned hydrolysates, especially the one with iBuOH, which exhibited high HMF selectivity (45.9 wt.%, corresponding to 65.5 mol%) and increased yield (8.4 g/100 g of pretreated feedstock), both

comparable to those achieved by Lign-iBuOH_1 (49 wt.% and 8.3 g/100 g of pretreated feedstock, respectively) (without any pretreatment catalyst). From the perspective of metal content, both hydrolysates were found to contain nearly the same amount of copper (Cu) but varying amounts of Mo (Table S5). Notably, the hydrolysate derived from feedstock pretreated with iBuOH exhibited a lower concentration of Mo, suggesting that iBuOH may enhance the catalyst's stability, resulting in negligible Mo leaching. It is likely that the presence of copper in low quantities contributes to the enhanced production of HMF, which contrasts with the roles of Fe and Mo, which further promote the reaction. The enhancement of HMF production in the presence of copper ions has been previously reported in the literature [51, 52] and can be possibly attributed to the stability of complexes that are formed between Cu^{2+} and fructose which facilitates the sugar dehydration and the subsequent formation of the furan compound [53].

In the case of the second group, which comprised of hydrolysates derived from pine, the selected samples underwent pretreatment without the use of a catalyst at 175 °C for 120 min, with EtOH (Pine_EtOH), ACO (Pine_ACO), and iBuOH (Pine_iBuOH_2) respectively, by applying the harsher reaction condition in order to increase the susceptibility of pine to saccharification. According to experimental data (Table 3), Pine_EtOH presented the highest HMF selectivity (50%) among the tested hydrolysates, converting sugars to HMF at 38.7 wt.%. Nevertheless, in this case, the overall yield of HMF was 9.2 g/100 g pretreated biomass, slightly lower compared to 11.3 wt.% obtained by Pine_iBuOH_2. Regarding the Pine-iBuOH hydrolysates, the one received after pine pretreatment at the same reaction temperature (175 °C), but in a lower reaction time (60 min), named Pine_iBuOH_1, was also examined for the production of HMF, presenting the exact same trend with the one observed in Pine_iBuOH_2. This fact implies that pretreatment time with iBuOH in pine does not have a significant effect on the subsequent production of HMF. On the other hand, Pine_ACO hydrolysate demonstrated a different behavior compared to the other hydrolysates leading to a reduced conversion of sugars (60.3 wt.%) and the lowest overall yield of HMF from the pretreated biomass (5 g/100 g pretreated biomass). In addition, the production of organic acids was enhanced, even though the selectivity of HMF reached 43.5 wt.%, corresponding to 62.3 mol%.

In summary, it was demonstrated that the production of HMF is favored by hydrolysates originating after the pretreatment of both feedstocks with iBuOH as an organic solvent. In a comparison between the beechwood and pine, in the absence of a pretreatment catalyst,

beechwood proved more efficient for the production of HMF resulting in the highest yield of 14.1 g/100 g pretreated biomass. Regarding the hydrolysates obtained from pulps pretreated upon the addition of POM catalysts, the highest HMF yield and selectivity were both observed by the hydrolysate obtained from beechwood pretreated with *i*BuOH upon the addition of Cu-PMo catalyst, achieving 45.9% selectivity, corresponding to 65.5 expressed in mol% and a total yield of 8.4 g/100 g of pretreated feedstock. The presence of Fe and Mo seems to suppress the production of HMF, most probably favoring the formation of degradation products, such as humins, organic acids, and other soluble compounds. As a result, hydrolysates obtained from beechwood biomass pretreated with Cu-PMo catalyst can serve as a starting material for HMF synthesis with relatively good yield and selectivity, while in our previous study [24], the presence of copper ions in the hydrolysates rendered this pulp cytotoxic and, thus not suitable for microbial fermentation. On the contrary, hydrolysates from beechwood biomass pretreated with Fe-PMo catalyst can be successfully used as carbon sources for microorganisms for the production of valuable products [24], however, in this study, it was shown that the formation of HMF is inhibited. These results suggest that the use of different POMs may affect not only the composition but also the quality of the pulp constituents and, consequently, determine the possible scenarios for its valorization.

Conclusions

In this work, the production of HMF from OxiOrganosolv pretreated beechwood and pine pulp through saccharification, isomerization, and dehydration reactions was demonstrated. The use of *i*BuOH as a pretreatment organic solvent yielded pulps susceptible to hydrolysis, which led to HMF production up to 14 and 11.3 g/100 g pretreated biomass for beechwood and pine, respectively. The addition of POM catalysts affected not only the composition of the pulps and the subsequent enzyme-catalyzed steps, but also the dehydration reaction, with pulps pretreated with Fe-PMO yielding lower amounts of HMF. Regarding the use of different feedstocks, although the formation of HMF was higher in the case of beechwood due to better saccharification yields which in turn resulted in higher production of fructose, the results of the study suggest a promising process for the valorization of the hexose sugar streams derived from the pretreatment of softwoods.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s44314-024-00002-5>.

Additional file 1: Table S1. Hydrolysis yields and glucose isomerization to fructose for different OxiOrganosolv pretreated beechwood samples. All experiments were run in duplicates and numbers represent the mean values. **Table S2.** Hydrolysis yields and glucose isomerization to fructose for different OxiOrganosolv pretreated pine samples. All experiments were run in duplicates and numbers represent the mean values. **Table S3.** Sugars composition of the diluted hydrolysate that was used for the production of HMF. **Table S4.** Sugars conversion to HMF and other side-products in the presence of formic acid as a catalyst (Reaction conditions: 150 °C, 60 min). All experiments were run in duplicates and numbers represent the mean values. Standard error deviation was $\leq 2.5\%$ in all measurements. **Table S5.** Molybdenum (Mo), iron (Fe) and copper (Cu) originating from POM catalysts during pretreatment, hydration and isomerization processes within the hydrolysates.

Acknowledgements

Assist. Prof. Konstantinos G. Kalogiannis from Department of Chemical Engineering, University of Western Macedonia is greatly acknowledged for providing the OxiOrganosolv pretreated pulps for this study. Authors would also like to thank Dr. Stamatia Karakoulia from the Centre for Research and Technology-Hellas for the synthesis of polyoxometalate-based catalysts.

Authors' contributions

GD participated in the design of the study, performed part of the experimental work (hydrolysis and isomerization reactions, production of fructose syrup) and participated in data interpretation and writing of the manuscript. AK participated in study conception and contributed to design of the study, data interpretation and manuscript writing, review and editing. AM performed part of the experimental work (homogeneous catalysis for the production of HMF) and participated in data interpretation and writing of the manuscript. AA provided lab and equipment resources for organosolv pretreatment and fractionation, as well as the dehydration experiments. ET participated in study conception, design of the study, supervision and he reviewed the manuscript. YZ participated in study conception and he reviewed the manuscript. The final manuscript was read and approved by all authors.

Funding

The research project was funded by National Technical University (NTUA) Basic Research Program, 2020–2022 (E. Topakas). This work was also supported by the Hellenic Foundation for Research and Innovation (H.F.R.I.) under the "3rd Call for H.F.R.I. Research Projects to support Post-Doctoral Researchers" (Project Number: 7315, A. Karnaouri).

Availability of data and materials

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applied.

Consent for publication

Not applied.

Competing interests

The authors declare no competing interests.

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Received: 10 January 2024 Accepted: 30 March 2024

Published online: 27 May 2024

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