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Improvement of ethanol production from birch and spruce pretreated with 1-H-3-methylmorpholinium chloride

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ABSTRACT

Background: Pretreatment is the critically important step for the production of ethanol from lignocelluloses. In this study, hardwood birch (*Betula pendula*) and softwood spruce (Norway spruce) woods were pretreated with a newly synthesized morpholinium ionic liquid, 1-H-3-methylmorpholinium chloride ([HMMorph][Cl]), followed by enzymatic hydrolysis and fermentation to ethanol.
Results: [HMMorph][Cl] was synthesized using inexpensive raw materials, i.e., hydrochloric acid and *N*-methyl morpholine, following a simple process. The influence of pretreatment time (2, 3, 5, and 8 h) and temperature (120 and 140°C) in terms of hydrolysis efficiency was investigated. Glucose yields from enzymatic hydrolysis were improved from 13.7% to 45.7% and 12.9% to 51.8% after pretreatment of birch and spruce woods, respectively, under optimum pretreatment conditions (i.e., at 140°C for 3 h) as compared to those from pristine woods. Moreover, the yields of ethanol production from birch and spruce were increased to 34.8% and 44.2%, respectively, while the yields were negligible for untreated woods.
Conclusions: This study demonstrated the ability of [HMMorph][Cl] as an inexpensive agent to pretreat both softwood and hardwood.
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1. Introduction

Softwoods, which is an abundant but the most recalcitrant feedstock, and hardwoods, which have more complex structures than softwoods, are two major types of lignocellulosic biomass in nature [1,2,3,4]. Spruce and pine are the dominant softwoods in the Northern Hemisphere. Birch, which covers approximately 10% of the forest in many countries such as Sweden, is the most popular hardwood [2,5]. Ethanol, one of the most important renewable liquid biofuels, can be produced from lignocellulosic materials consisting of cellulose, hemicellulose, and lignin [6,7,8,9,10].

Pretreatment is a necessary process to improve the performance of enzymatic hydrolysis and ethanol production yields from lignocelluloses [11,12]. Several pretreatment methods including physical, physicochemical, chemical, and biological pretreatments have been introduced for this purpose [2,4,13,14].Pretreatments using ionic liquids (ILs) are among the efficient chemical methods [15,16,17].

A number of ILs have been considered as green solvents, as they can be considered relatively nontoxic and nonvolatile and can be recycled several times [16]. Many imidazolium-based ILs are very powerful in the dissolution of lignocelluloses; however, they are not suitable for industrial applications because of their high prices [18]. Presently, morpholinium-based ILs have shown more promise in industrial

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applications because of their higher electrochemical stability and lower cost than imidazolium ILs [13,19,20]. Furthermore, detailed cost analysis revealed that ILs produced by simple acid–base reactions are cheaper than more complex ILs [13]. 1-H-3-methylmorpholinium chloride ([HMMorph][Cl]), a novel morpholinium-based IL, has a simple synthesis protocol.

The current work focuses on the pretreatment of softwood spruce and hardwood birch with the synthesized morpholinium-based IL, 1-H-3-methylmorpholinium chloride ([HMMorph][Cl]), followed by enzymatic hydrolysis and fermentation. The pretreatment time and temperature were optimized for best ethanol and hydrolysis yields. Subsequently, composition, crystallinity, and morphology changes of the woods were investigated.

2. Materials and methods

2.1. Lignocellulosic materials, microorganism, and enzyme

Native species of birch and spruce woods obtained from forests (Gothenburg, Sweden) were cut, debarked, milled, and screened to achieve a size between 177 and 841 μm (20–80 mesh). *Saccharomyces cerevisiae* Thermosacc (Lallemand, Duluth, GA, USA) was used for fermentation. The preparation of the yeast strain inoculum was performed according to the method described by Shafiei et al. [21,22]. Cellic®CTec2 (VCNI0013) provided by Novozymes A/S, Denmark, was used for enzymatic hydrolysis. Cellulase activity was measured using the filter paper method [23] as 149 FPU/g.

2.2. Synthesis of [HMMorph][Cl]

[HMMorph][Cl] was synthesized by simple acid–base neutralization. Briefly, a stoichiometric amount of hydrochloric acid (37%, Sigma–Aldrich) (24.64 g) was slowly added to *N*-methyl morpholine (99% purity, Geel, Belgium) (25.28 g) in a 100 mL laboratory bottle placed in a water-ice bath. After 1 h reaction time, the resulting aqueous IL (30 wt-%) was stored at 4°C for further use. Nuclear magnetic resonance (NMR) analysis (Bruker, Ultrashield 400 MHz model, Germany) was used to characterize the synthesized IL [HMMorph][Cl] NMR

^{1}H-NMR (400 MHz, DMSO-*d6*): 2.72 (s, 3H, CH3), 3.25 (m, 4H, NCH2), 3.87 (m, 4H,OCH_2), 11.72 (s, 1H, NH).

^{13}C-NMR (100 MHz, DMSO-*d6*): 42.13 (CH3), 52.11 (NCH2), 63.12 (OCH_2).

2.3. Pretreatment

Water solutions of [HMMorph][Cl] (50% water) were used for the pretreatment of birch and spruce woods on the basis of preliminary experiments (data not shown). Pretreatments were performed following the method presented by Shafiei et al. [21] with some modifications. Briefly, 5% (w/w) of the woods in the mixture (2.5 g wood + 47.5 g of the preheated solvent) was used. To investigate the influence of pretreatment time and temperature, the pretreatments were performed at 120°C for 5 and 8 h as well as at 140°C for 2, 3, and 5 h, respectively, in an oil bath with continuous stirring. Next, each suspension was poured gradually into 250 mL of boiling water, which acts as an antisolvent to regenerate the dissolved materials. Afterwards, the regenerated solids were filtered and washed with boiling water to remove the IL residues. The pretreated woods were kept at room temperature after drying. All experiments were conducted in duplicates.

2.4. Separate enzymatic hydrolysis and fermentation (SHF)

Enzymatic hydrolysis and fermentation were performed according to the protocol reported in Soudham et al. [24] with some modifications. Concisely, 0.3 g untreated or pretreated wood and 6 mL of sodium citrate buffer (50 mM, pH 4.8) in each sample were autoclaved separately at 121°C for 20 min. Then, 40 μL Cellic CTec2 enzyme and 20 FPU/g substrate were added to each tube. Enzymatic hydrolysis was performed at 45°C for 72 h in a shaking incubator at 200 rpm. After this, the suspensions were centrifuged. The liquid and solid fractions of the hydrolysates were stored at −18°C.

For ethanolic fermentation, the liquid hydrolysates were mixed with a nutrient-rich solution containing (g/L) 75 yeast extract, 1.88 $MgSO_4.7H_2O$, 37.5 $(NH_4)_2HPO_4$, and 119.1 $NaH_2PO_4.H_2O$. *S. cerevisiae* was inoculated in 20 mL of YPD medium (yeast extract, 10; peptone, 20; and D-glucose, 20 g/L) and incubated at 30°C under stirring (200 rpm) for 24 h. After that, the suspension was centrifuged (5 min at 1500 rpm). A cell density of 27 g/L based on dry weight of the new suspension in sterile water was used. Each liquid hydrolysate (2.3 mL) was poured into a screw-capped plastic tube (12 mL). Afterwards, 2.5 mL media containing 0.05 mL of the nutrient solution and 0.15 mL of yeast inoculum were prepared to achieve a yeast cell density of 1.6 g/L. All experiments were conducted in triplicates. The inoculated hydrolysates were incubated at 30°C for 24 h. Consequently, all samples were stored at −18°C.

Glucose yield and ethanol yield, based on the maximum theoretical ethanol yield (%), were calculated using [Equation 1]] and [Equation 2]], respectively.

$$\text{Glucose yield} = \frac{\text{Produced glucose (g/L)}}{\text{Glucan concentration(g/L)}^{\times}1.111}{}^{\times}100 \quad \text{[Equation 1]}$$

$$\text{Ethanol yield} = \frac{\text{Produced ethanol (g/L)}}{\text{Glucan concentration(g/L)}^{\times}1.111^{\times}(2.3/2.5)}{}^{\times}0.51^{\times}100 \quad \text{[Equation 2]}$$

where 2.3, 2.5, 1.111, and 0.51 are the volume of liquid hydrolysates, total volume of the fermentation samples, glucan hydration factor, and theoretical yield of ethanol from glucose (g/g), respectively.

2.5. Analytical methods

The concentrations of sugars and ethanol from separate enzymatic hydrolysis and fermentation (SHF) were measured using high-performance liquid chromatography (HPLC, Ultimate 3000, Thermo Scientific, Waltham, MA, USA). An ion-exchange column (AminexHPX-87P, Bio-Rad, USA) was used, with ultrapure water as the eluent, at 85°C and at a flow rate of 0.6 mL/min. This analytical technique was used to determine the sugar concentrations. Another column (Aminex HPX-87H, Bio-Rad) was used, with 5 mMH_2SO_4 as the eluent, at 60°C and at a flow rate of 0.6 mL/min to determine the ethanol concentrations.

The carbohydrate fraction of the untreated and the pretreated woods was determined using the procedure provided by National Renewable Energy Laboratory (NREL) [25]. According to the method, cellulose and hemicelluloses were hydrolyzed and the resulting monomeric sugars were analyzed by HPLC.

Fourier transform infrared spectra (FTIR) of the pretreated and untreated woods powder was recorded in the range of 600–4000 cm^{-1} with the resolution of 4 cm^{-1} to investigate the effect of pretreatment on the wood structures.

Morphology of the pretreated and untreated woods was investigated using a Carl Zeiss Merlin Field Emission Scanning Electron Microscope (FE-SEM) at 30 kV equipped with X-ray spectroscopy (EDS, Oxford Instruments X-MAX 80 mm2 detectors).

2.6. Statistical analysis

Statistical analysis of the results was performed by a two-way ANOVA using SAS system software (Version 9.1), and Student–

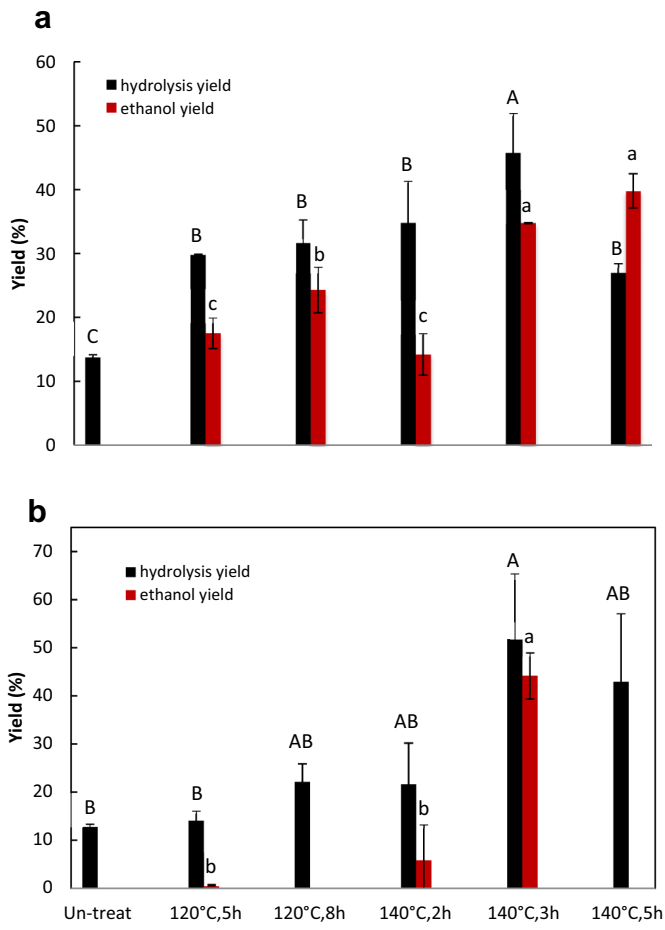

Fig. 1. Effects of different pretreatment time and temperature on the enzymatic hydrolysis yield and the ethanol yield based on the maximum theoretical ethanol yield of untreated and [HMMorph][Cl]-pretreated (a) birch and (b) spruce. The dissimilar letters indicate the significant differences at 95% confidence level, where there are no significant differences among the like-lettered groups. All experiments were performed in triplicate, and error bars indicate standard deviation.

Newman–Keuls (SNK) test was used to estimate the significant differences ($P < 0.05$).

3. Results and discussion

Birch and spruce woods were pretreated using a novel morpholinium-based ionic liquid, [HMMorph][Cl]. Pretreatment conditions were optimized to achieve the maximum yield of hydrolysis and fermentation. The influence of the pretreatment protocols was investigated in terms of morphology, crystallinity, and composition of the woods.

Fig. 2. FTIR spectra of untreated birch; [HMMorph][Cl]-pretreated birch at 140°C for 3 h; untreated spruce; and [HMMorph][Cl]-pretreated spruce at 140°C for 3 h.

3.1. Separate enzymatic hydrolysis and fermentation (SHF)

Hardwood birch and softwood spruce were pretreated with [HMMorph][Cl] at 120°C for 5 and 8 h as well as at 140°C for 2, 3, and 5 h, respectively. SHF was carried out by performing enzymatic hydrolysis for 72 h, followed by fermentation of the hydrolysates for 24 h. The results are presented in Fig. 1. Hydrolysis and ethanol yields were significantly improved after the pretreatment under all conditions used in this study. The best glucose and ethanol yields of 45.7% and 34.8%, respectively, were achieved after 3 h pretreatment of birch at 140°C, while ethanol was not obtained from untreated birch and glucose yield was only 13.7%. Moreover, pretreatment of spruce wood at 140°C for 3 h seemed to be the best conditions for the [HMMorph][Cl]-mediated pretreatment procedure. Using these pretreatment conditions, glucose yield was improved from 12.9% to 51.8% when compared with that of untreated spruce. The maximum ethanol yield was 44.2% obtained under similar conditions. Wang et al. [26] synthesized three different ILs, namely, 1-H-3-methylimidazoliumchloride ([Hmim][Cl]), N-methyl-2-pyrrolidonium chloride ([Hnmp][Cl]), and 1-hexylpyridinium chloride ([Hpy][Cl]), through a one-step process, and consequently, these ILs were used for the pretreatment of poplar and bamboo at 100°C for 30 min. The corresponding yield of enzymatic hydrolysis of poplar pretreated with these ILs was 50.7%, 53.8%, and 37.1%. The corresponding cellulose yield of bamboo was 53.4%, 53%, and 45.6%. In another study, Brandt

Table 1
Composition of untreated and [HMMorph][Cl]-pretreated birch and spruce woods.

Solvent	Temp (°C)	Wood type	Time (h)	Glucan (%)	Xylan (%)	Mannan (%)
[HMMorph][Cl]	120	Birch	5	52.31 ± 0.50	14.16 ± 0.14	2.06 ± 0.68
[HMMorph][Cl]	120	Birch	8	67.27 ± 0.24	4.02 ± 0.07	0.00 ± 0.00
[HMMorph][Cl]	120	Spruce	5	62.38 ± 0.03	2.29 ± 0.04	4.94 ± 0.48
[HMMorph][Cl]	120	Spruce	8	63.28 ± 0.53	1.06 ± 0.07	4.71 ± 0.03
[HMMorph][Cl]	140	Birch	2	60.21 ± 2.00	1.73 ± 0.04	0.00 ± 0.00
[HMMorph][Cl]	140	Birch	3	61.57 ± 0.41	0.81 ± 0.04	0.00 ± 0.00
[HMMorph][Cl]	140	Birch	5	57.92 ± 2.88	0.00 ± 0.00	0.00 ± 0.00
[HMMorph][Cl]	140	Spruce	2	57.46 ± 7.46	0.00 ± 0.00	0.00 ± 0.00
[HMMorph][Cl]	140	Spruce	3	45.72 ± 4.57	0.00 ± 0.00	0.00 ± 0.00
[HMMorph][Cl]	140	Spruce	5	32.23 ± 0.50	0.00 ± 0.00	0.00 ± 0.00
Untreated	–	Birch	–	38.46 ± 0.06	20.65 ± 0.07	1.86 ± 0.08
Untreated	–	Spruce	–	43.85 ± 0.47	4.33 ± 0.04	11.15 ± 0.14

Table 2
Variation of bands in FTIR spectra and crystallinity changes of untreated and [HMMorph][Cl]-pretreated birch and spruce.

Wavenumber (cm^{-1})	Assignment	Untreated birch	[HMMorph][Cl] –treated birch	Untreated spruce	[HMMorph][Cl]–treated spruce
2918	Cellulose	0.34	0.59	1.00	0.66
1730	Hemicellulose and lignin	0.32	0.42	0.49	0.43
1627	Lignin	0.11	0.35	0.09	0.38
1598	Lignin	0.22	0.49	0.31	0.56
1510	Lignin	0.20	0.55	0.49	0.76
1465	Lignin	0.28	0.62	0.46	0.67
1430	Cellulose	0.28	0.63	0.47	0.70
1423	Cellulose	0.30	0.63	0.48	0.69
1375	Cellulose	0.31	0.64	0.48	0.69
1335	Cellulose	0.29	0.69	0.44	0.69
1315	Cellulose	0.28	0.71	0.45	0.71
1158	Cellulose	0.35	0.77	0.59	0.81
896	Cellulose	0.14	0.22	0.23	0.16
CI	(A1430/A896)	2.00	2.86	2.04	4.38
TCI	(A1375/A2900)	0.91	1.08	0.48	1.05

et al. [27] pretreated miscanthus, grass type with 1-butyl-3-H imidazolium hydrogen sulfate ($[C_4Him][HSO_4]$). The glucose yield obtained was 44% after pretreatment at 120°C for 20 h. These results are in the line with the results obtained in the current work. According to our results, 3 h pretreatment at 140°C with [HMMorph][Cl] was chosen for the pretreatment of birch and spruce woods.

3.2. Gasoline equivalent

The amount of gasoline equivalent was calculated based on 1 ton of woods using the lower heating values of ethanol and gasoline (21.2 and 32.0 MJ/L, respectively) and the solid recoveries after pretreatment procedures [13]. These values were calculated for untreated birch and spruce as well as the woods pretreated under the optimum conditions. The amount of gasoline equivalent from one ton of untreated birch and spruce was negligible. This value increased to 48.5 and 56.3 L after pretreatment of birch and spruce with [HMMorph][Cl] at 140°C for 3 h, respectively. The values were in line with those of a previous study conducted by Kahani et al. [13]. They achieved the gasoline equivalent of the rice straw of 65.7 L per ton after pretreatment by morpholinium acetate ([Morph][Ac]) under the same condition (140°C for 3 h).

3.3. Influence of [HMMorph][Cl] pretreatment on the wood composition and structure

The composition, crystallinity, and morphology of birch and spruce pretreated with [HMMorph][Cl] were investigated under the optimum conditions (at 140°C for 3 h).

The composition of the wood species was determined before and after pretreatments (Table 1). Glucan and xylan contents of birch wood, the main carbohydrates of the hardwoods, were significantly influenced by the pretreatment. Glucan increased from 38.5% to 61.6% and xylan decreased from 20.6% to 0.8% after pretreatment under optimum conditions of 140°C for 3 h, as compared to those of the untreated birch. Further, glucan and mannan contents of spruce wood, the main carbohydrates of the softwoods, were clearly altered. Glucan increased from 43.8% to 45.7%, while mannan was completely removed (from 11.2%) after the pretreatment under optimum

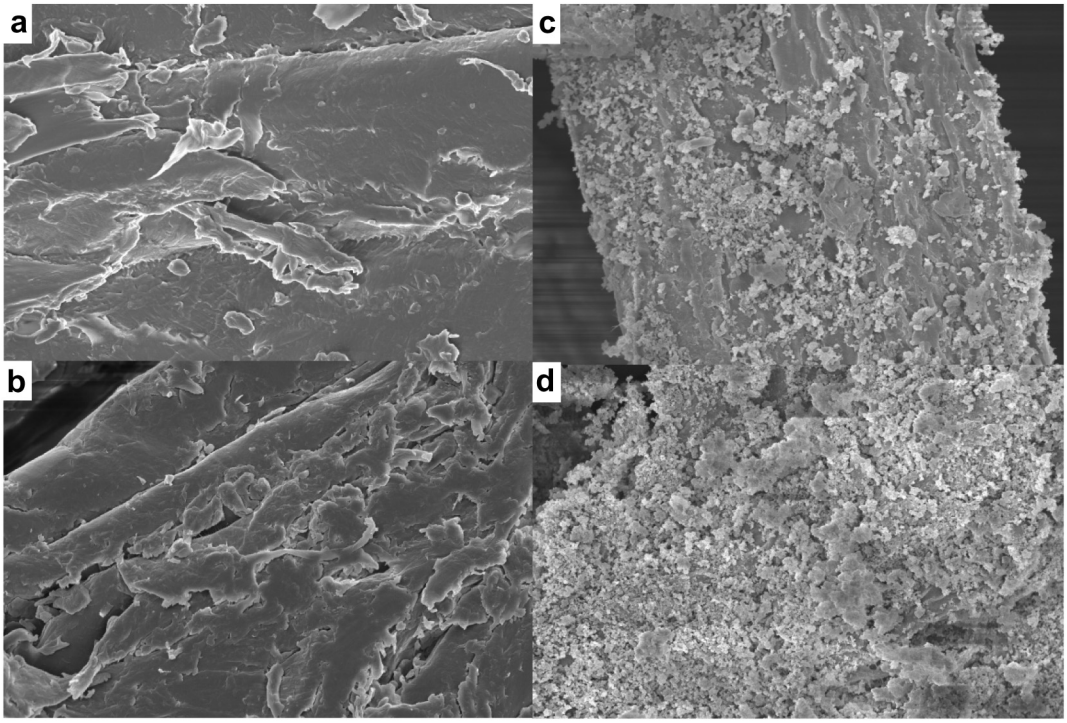

Fig. 3. SEM images of (a) untreated birch, (b) untreated spruce, (c) the birch, and (d) the spruce pretreated with [HMMorph][Cl] at 140°C for 3 h (5000× magnification).

conditions. Poornejad et al. [28] observed an increase in glucan content of rice straw pretreated with [BMIM][Ac] from 40.7% for untreated straw to 45.4% and 53.6% after 3 and 5 h pretreatment campaigns, respectively and the corresponding xylan content also decreased from 21.7% to 17.1% and 15.1%. Shafiei et al. [21] pretreated spruce with [BMIM][Ac] and [EMIM][Ac] and observed that the glucan content increased from 44.0% to 47.5% and 48.3%, respectively.

Changes in the chemical bands and crystallinity of birch and spruce after pretreatment with [HMMorph][Cl] were investigated by FTIR spectroscopy (Fig. 2 and Table 2). Crystallinity index (CI), which is related to crystalline cellulose I and cellulose II, was calculated as the ratio of (A_{1435}/A_{896}). The total crystallinity index (TCI) was calculated as the absorbance ratio of A_{1375} to A_{2900} [29]. The CI increased from 2.0 to 2.9 in case of birch and, 2.0 to 4.4 in case of spruce after pretreatment with [HMMorph][Cl] at 140°C for 3 h. Pretreatment with [HMMorph][Cl] increased the TCI value from 0.9 to 1.1 in case of birch and 0.5 to 1.0 in case of spruce. The reason could be the removal of amorphous cellulose and hemicellulose during the pretreatment campaigns.

SEM images (Fig. 3) were used to investigate the changes in the morphology of birch and spruce before (A and C) and after pretreatment with [HMMorph][Cl] (B and D). As depicted in the images, the organized structure of both woods was disrupted and changed to a sponge-like structure after pretreatment, which is the plausible reason for the enhanced enzymatic hydrolysis yields. Aid et al. [3] investigated the physical changes of wheat straw and cellulose after pretreatment with [BMIM][Ac]. The structure of wheat straw changed, while cellulose remained intact after 24 h pretreatment. Thus, the changes could be due to the presence of hemicellulose and lignin in the wheat straw structure.

4. Conclusions

[HMMorph][Cl] was synthesized through a neutralization reaction of hydrochloric acid with *N*-methyl morpholine, two inexpensive and commercially available raw materials, at ambient pressure and temperature. Subsequently, the influence of the pretreatment campaigns with [HMMorph][Cl] in terms of morphology, composition, and structure of hardwood, i.e., birch, and softwood, i.e., spruce, was investigated. The hydrolysis yields from the woods were improved by more than 3.3-fold after pretreatment under optimum conditions of 140°C for 3 h. The ethanol production yields were also increased by more than 34.8%. However, pretreatment was more efficient in case of the hardwood than the hardwood.

Conflict of interest

The authors declare no conflict of interest.

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