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Modulating the hydrophobicity of cellulose by lipase-catalyzed transesterification

Rahul Sharma [a], Kevin H. Putera [a], Mark M. Banaszak Holl [a,b], Gil Garnier [a], Victoria S. Haritos [a,*]

[a] Bioresource Processing Research Institute of Australia, Department of Chemical and Biological Engineering, Monash University, Melbourne, Victoria 3800, Australia
[b] Department of Mechanical and Materials Engineering, University of Alabama at Birmingham, USA

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ABSTRACT

The production of hydrophobic and oil resistant cellulosic fibers usually requires severe chemical treatments and generates toxic by-products. Alternative approaches such as biocatalysis use milder conditions; lipase-catalyzed methods for grafting nanocellulose with hydrophobic ester moieties have been reported. Here, we investigate the lipase-catalyzed esterification of cellulose fibers, in native form or pretreated with 1,4-β-glucanases, and cellulose nanocrystals (CNC) in solvent-free conditions. The fibers were compared for degree of ester formation after incubation with methyl myristate and lipase at 50 °C. After washing, the grafting of fatty esters on cellulose was confirmed by ATR-FTIR and the degree of substitution determined by ^{13}C CP/MAS NMR (from 0.04 up to DS 0.1) confirming successful esterification. Optical photothermal infrared (O-PTIR) spectroscopy showed strongly localized presence of ester moieties on cellulose. Functional properties mirrored the degree of substitution of the cellulose materials whereby cellulose esters made with glucanase-pretreatment produced the highest water contact angle of 117° ± 9 and esterified cellulose blended at 10 % w/w content in paper composites showed significant differences in hydrophobicity and lipophilicity compared to plain paper. The esterification of cellulose was completely reversed by lipase treatment in aqueous media. These ester-functionalized fibers show potential in a wide range of packaging applications.

1. Introduction

Polyolefin and oil-based plastic pollution has been highlighted as a major environmental concern across the world with an estimated 0.47–2.75 Mt. plastic/year globally entering the oceans [1]. Substitution of plastic by cellulosic fiber materials has already occurred for some packaging applications but is not yet at a stage to completely make plastic redundant. Increased use of renewable materials as sustainable replacements for plastics is a priority for chemistry and engineering in this century [1–3]. However, cellulose and plant biopolymers in general require improved characteristics and functionalities, such as greater hydrophobicity, for this to be realized. Furthermore, as the main driver for using renewable natural materials as plastic substitutes is to avoid environmental pollution, any approach to improve the physical and chemical characteristics of cellulose should be achieved using reactants and processes with least impact on the environment.

While natural cellulose is a bountiful organic material, it is recalcitrant to process and has a hydrophilic surface. Cellulose can be made more hydrophobic by covalently grafting alkyl chains; this is efficiently achieved through substituting some of the protons of the C6-alcohols of cellulose with the ester moiety. Grafting alkyl chains to surface residues also affects the functionality of the cellulosic fibers, especially their ability to form networks of hydrogen bonds between fibers resulting in weaker hydrogen bonding between fibers which lowers mechanical strength [4,5]. Many approaches to grafting lipophilic groups to cellulose have been investigated. These consist of reactions catalyzed either by enzymes or chemicals, delivered to the material in organic solvents or ionic liquids and yielding cellulose esters. Generally, the most reactive alcohol groups on cellulose i.e., C6 alcohol of the glucose ring are the initial group esterified in the reactions.

More specifically, chemical and enzymatic esterification reactions have utilized nano-sized cellulose as a substrate to take advantage of the large surface area to volume ratio of the nanocellulose fibrils. Boujemaoui et al. used acid hydrolysis and Fischer esterification with various carboxylic acids to simultaneously produce CNCs and graft ester groups on their surface for improved functionalities [6]. CNCs functionalized by 2-bromopropionic acid and 3-mercaptopropionic acid displayed higher degradation temperature than those prepared by sulfuric acid, beneficial

* Corresponding author at: Department of Chemical Engineering, Monash University, Clayton, Australia.
E-mail address: victoria.haritos@monash.edu (V.S. Haritos).

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for high temperature processing. Salam et al. and Guzman-Puyol used fluorination to make CNC esters which provided higher water contact angles and oil resistance on the cellulose surface using fluorinated aromatic and long chain alkyl groups, respectively. However, this conversion lowered the degradation temperature and tensile stress of the CNCs [7,8] and fluorinated molecules should be avoided plastic replacement materials due to environmental concerns. Takahashi et al. used ionic liquids to dissolve and functionalize cellulose with a phenolic ester in organic solvent at high temperatures; they reported that the selectivity for the primary alcohol was increased with ionic liquids [9]. Ionic liquids induced a high Degree of Substitution (DS) of up to 1.18 and a selectivity for esterification of the primary alcohol of up to 98 %, as calculated by ^{1}H NMR. Yin et al. employed a stepwise approach where microcrystalline cellulose (MCC) was first hydrolysed with sulfuric acid to produce CNC and then reacted with 2-bromoisobutyryl bromide catalyzed by triethylamine [10]. This resulted in the functionalized CNCs having an improved degradation temperature by 50 °C and better homogeneous dispersion into PMMA. However, the process involves toxic and corrosive reactants, against the principles of green chemistry.

Enzymes have also been widely explored for esterification of nanocellulose as they are renewable and biodegradable, and act as genuine catalysts to reduce the activation energy of reactions many-fold [11]. Cellulose surface esterification can effectively be catalyzed by lipases (EC 3.1.1.3) (serine hydrolase superfamily [12]) from different sources of organism to produce materials with enhanced hydrophobicity. These reactions are generally conducted in an organic/ionic liquid solvent system to partially solubilize cellulose. Recently, Bozic et al. completely solvent exchanged an aqueous nanofibrillated cellulose (NFC) suspension into DMSO before acetylating with lipase and acetic anhydride for 24 h [13], providing 0.18 DS on the cellulose compared to the chemical pathway only reaching 0.13 DS. Both treatments developed materials with wetting and moderate water contact angles (~80°). A binary ionic liquid system [BMIm]HSO_4 – [BmIm]BF_4 was chosen by Zhao et al. to esterify CNC with methyl laurate which provided DS as high as 0.14 [14]. The study showed that the DS of the cellulose esters increased with increase in [BmIm]BF_4 as the ionic liquid favoured the lipase catalysis. Lauric acid as a co-substrate was used by Yin et al. which found that the 12 h esterification was improved in a *tert*-butanol solvent with DS on CNC as high as 0.4 [15]. This was a different approach as it directly esterified cellulose instead of proton replacement of the primary alcohol of cellulose. It is evident that this is a higher energy esterification approach since the highest DS attained required the highest lauric acid concentrations. Cotton fabrics were esterified using polyesters synthesized from ethylene glycol, glycerol and adipate esters with lipase by Zhao et al. [16]. This "in-situ" method of esterifying cellulose provided very high hydrophobicity of fabrics with water contact angles as high as 137° and uses the co-substrates as solvents.

As these studies demonstrate, cellulose esters can be prepared using facile and solvent free esterification processing with lower water and oil spread on the composite paper surface. Furthermore, we demonstrate that lipase-catalyzed hydrolysis of cellulose esters to return the modified cellulose back to its original state and hydrophilic properties. We successfully achieved enzymatic esterification of nano- and fibrillated cellulosic fibers via transesterification with fatty acid methyl esters; cellulose esters were verified using a suite of analytical measurements including cellulose fiber analysis by Photothermal infrared (O-PTIR) spectroscopy and delivered remarkable increases in water contact angle, demonstrating changes in surface properties. Paper composites prepared with cellulose ester fibers blended at 10 % *w/w* were significantly more hydrophobic and oil retaining highlighting their potential applications in packaging and personal care products as plastic replacements.

2. Experimental

2.1. Materials and chemicals

Never-dried unrefined bleached eucalyptus Kraft (BEK) pulp fibers were supplied by Australian Paper Maryvale at a solids concentration of 10 wt% and composed of neutral sugars in the following range: xylose (8.4–18.9 wt%) and glucose (80–91.5 wt%) with negligible mannose, and will be referred to herein as cellulose fibers (Molecular weight ~ 271,000 g/mol for bleached hardwood fibers) [17]. Cellulose nanocrystals (137 nm particle size, 6.17 mmol AGU/g) [18] were purchased from CelluForce, Canada. 4-nitrophenyl butyrate (N9876), ethylene glycol (324558), Trizma base (T1503), methyl myristate (70129), ethanol (L4777), Brilliant Blue G (27815), Nile Red (N3013) and Sodium-carboxymethyl cellulose (419273) of molecular weight 90,000 g/mol and a degree of substitution (DS) of 0.7 carboxymethyl group/ anhydro glucose unit were purchased from Sigma-Aldrich, Australia.

2.2. Enzymes

Endoglucanase from *Trichoderma longibrachiatum* (C9748) and Lipolase 100 L from *Thermomyces lanuginosus* (L0777) were purchased from Sigma-Aldrich, Australia. Since the reaction was designed to be solvent-free, a non-immobilized enzyme was chosen to reduce mass constraints which is introduced by immobilized lipases [16] in non-aqueous heterogeneous enzyme reactions. Purified endoglucanase was chosen as the cellulolytic enzyme as random scission of cellulose chain was preferred to minimize degradation to monosaccharides, and to increase fibrillation. As was demonstrated in our prior research [19], the effect of endoglucase treatment is to increase surface area of cellulose and potentially reveal more C6 alcohol groups for reaction with esters. Protein contents were measured in Costar 96 flat bottom transparent plate adapted for absorbance changes in an Infinite 200 Pro multimode Technische Analysegeräte microplate reader (TECAN). The Bradford protein quantification method [20] was adapted to determine protein content in the enzyme solutions using Bovine Serum Albumin (BSA) as a calibration standard. The protein contents were measured to be 17.52 mg/mL, and 26.6 mg/mL respectively for endoglucanase and Lipolase 100 L.

Enzyme activities were determined via standard methods. Endoglucanase was tested for carboxymethyl cellulose (CMC) activity as described previously [19] and calculated to be 6031 U/mL. Lipolase activity was measured using an adaptation of Hernandez-Garcia's method [21] for plate size assay and provided an activity of 474 mU/L.

2.3. Glucanase and lipase treatments of cellulose fibers

2.3.1. Endoglucanase pretreatment of fibers

One set of cellulose fibers was pretreated with endoglucanase on a 300 μg protein /g dry fiber basis in deionized water in a 1 L, 2.4 wt% fiber solution for 1 h at 50 °C as previously described [19]. The reaction was performed in a 2 L flask placed in a Thermoline Incubator with no agitation. Post endoglucanase treatment, fibers were washed with deionized water to remove unreacted materials.

2.3.2. Alcoholysis of substrates

Cellulose fibers were first concentrated to 30 % solids using a Buchner funnel vacuum filtration system. Prior to attempting alcoholysis on all cellulosic substrates, an optimisation assessment of lipase loading was undertaken using endoglucanase-pretreated cellulose fibers and an enzyme protein range of 5.3 to 53.2 mg protein/ g dry fiber. The carbonyl peak height at 1700 cm^{-1} presented in FTIR spectra was chosen as a guide to best performing alcoholysis condition on the cellulose surface (Fig. S1). The carbonyl peak was the best estimate of surface action of enzyme due to the complexity introduced by insoluble substrates in kinetic studies.

Dry weight equivalent of 500 mg CNC (34 μmol) or cellulose fibers (1.85 μmol) were treated with 0.5 mL (2.1 mmol) methyl myristate (optimal amount to prevent enzyme inhibition) in 50 mL falcon tubes in an incubator for 48 h at 50 °C. For samples catalyzed enzymatically, 16 mg lipase protein/ g dry cellulose as Lipolase 100 L was added. This loading was demonstrated to be optimal in the study described above. The lipase from *Thermomyces lanuginosus* has been used at elevated temperatures in previous studies [16,22] and 50 °C was selected as a valid temperature to investigate the production of cellulose esters with extended incubation times as previously described [23]. Control reactions were performed with cellulose and methyl myristate in proportions described above but lacked lipase. At the end of the incubation period, samples were removed from the incubator, thoroughly washed with 30 mL ethanol and centrifuged at 10,000 rpm for 10 min. The supernatant consisting of ethanol and unreacted methyl myristate was removed and the remaining solids were dried for 48 h in a fume hood to evaporate any residual ethanol.

2.3.3. Hydrolysis of cellulose esters

Cellulose esters (500 mg) prepared from endoglucanase-pretreated fibers at an approximate DS of 0.1 were combined with 0.5 mL Milli Q water with Lipolase 100 L lipase representing 5.3 mg protein/g fiber dry fiber for 48 h at 50 °C. Post-reaction, the fibers were washed with ethanol (30 mL) and dried in a fume hood for 48 h before further analysis.

2.4. Composite preparation

Composite sheets were prepared by mixing 10 wt% addition of cellulose esters or endoglucanase treated fibers into raw cellulose fibers (90 wt%). Specifically, 120 mg (dry equivalent) hydrophobic material was taken and mixed with 1.08 g (dry equivalent) cellulose fibers in 2 L water and disintegrated for 15,000 revolutions using a 3 L Mavis Engineering (Model No. 8522) pulp disintegrator. The solutions were made into handsheets using an Automatic British Handsheet maker (diameter 16 cm) with a 150-size drainage mesh. Whatman Filter paper 541 was used to capture all the fibers from the solution. The sheets were pressed twice using an automatic L&W sheet press and conditioned for 24 h at 23 °C and 50 % relative humidity as per TAPPI 205 standard method [24]. Paper sheets were also prepared as described using 100 % raw cellulose fibers as control.

2.5. Chemical analysis performed per sample

2.5.1. ATR-FTIR

FTIR was performed on dried fibers with Agilent Technologies Cary 630 FTIR coupled with a diamond attenuated total reflectance (ATR) as previously described [25]. The resolution of the FTIR was set at 4 cm^{-1} and eight scans were performed per samples in the range of 4000–500 cm^{-1}.

2.5.2. ^{13}C Solid State CP/MAS NMR Spectroscopy

Solid state spectroscopy was performed on dry fibers. All cross-polarisation magic angle (CP/MAS) spectra were recorded on a Bruker Avance 300 spectrometer, operating at 75.4 MHz for ^{13}C at a Magic angle spinning rate of 5 kHz at room temperature. A double resonance H/X CP/MAS 4 mm probe was used. For ^{13}C CP/MAS, 24000 transients were acquired using a contact time of 2.0 ms, an acquisition time of 19.9 ms (1992 data points zero filled to 65 K) and a recycle delay of 4 s. Spectra were referenced using the carbonyl signal of glycine at 176.03 ppm and intensity was normalized for all samples peaks to account for received gain. The Degrees of Substitution was calculated using the method outlined by Jandura et al. [26]:

$$DS_{NMR} = \frac{n_{cel}\ X\ I_{ac}}{n_{ac}\ X\ I_{cel}}$$

where I_{ac} and I_{cel} are the integration of the aliphatic carbon peaks and cellulose carbon peaks respectively and n_{cel} and n_{ac} are the number of carbon atoms in cellulose ($n_{cel} = 6$) and carbon atoms in hydrocarbon chain ($n_{ac} = 13$).

2.5.3. Optical Photothermal Infrared (O-PTIR) Spectroscopy

O-PTIR experimentation was performed on a Photothermal mIRage Optical Photothermal Infrared (O-PTIR) system (Photothermal, Santa Barbara, CA, USA) equipped with a Quantum Cascade Laser (QCL) allowing data acquisition between 800 and 1880 cm^{-1} and an Optical Parametric Oscillator laser (OPO) which allows separate data acquisition between 2700 and 3588 cm^{-1}. When the IR light of the wavenumbers associated with the molecular vibrations of the carbonyl and alkyl chains, it results in a thermal response as the IR light is absorbed and causes local thermal expansion of the sample. Using mathematical relationships and Fourier transformation, the vibrational data is converted into an IR absorption measurement. All experiments were conducted in a 0 % relative humidity environment through N_2 gas purge. IR Spectra were randomly acquired on multiple cellulose fiber from the endoglucanase-treated cellulose sample using the QCL laser with 27 % IR laser power and 10 % Probe power, while using 12 % IR laser power and 10 % Probe power when using the OPO laser. Hyperspectral data collection with autofocus was used to collect arrayed spectra (800–1880 cm^{-1}) on a single cellulose fiber over a 2 × 20 μm region of interest at 1 μm spatial resolution.

IR spectra were processed and analysed using PTIR Studio 4.2 (Photothermal, Santa Barbara, CA, USA). All IR spectra were smoothed using Savitzky-Golay filter with 11 pt. smoothing and polynomial order of 3. Rubberband-correction filter was applied on IR spectra between 2700 and 3588 cm^{-1} to correct baseline for comparison. IR spectra between 800 and 1880 cm^{-1} were normalized to the 1420 cm^{-1} band assigned as methylene bending, while IR spectra between 2700 and 3588 cm^{-1} were normalized to the 3348 cm^{-1} band assigned as O—H stretching.

2.6. Materials characterisation

2.6.1. Water contact angle measurements

Cellulose esters and modified control cellulose fibers were pressed on to compression discs of 45 mm diameter using a L&W Press. The surface hydrophobicity of compressed cellulose discs were analysed by water contact angle measurements using Dataphysics OCA35 as described previously [18]. The static contact angle between a water droplet (5 μL) and the cellulose film was measured with five replicates.

2.6.2. Wettability and blot area estimation

A 1 % solution of Brilliant Blue G was prepared in Milli Q water and five independent 20 μL drops were pipetted on cellulose ester composite paper sheets and control sheets. In a similar fashion, Nile red was dissolved in Canola Oil to a concentration of 0.5 % and centrifuged to remove particulates. The supernatant solution was used to pipette five independent 10 μL drops on the sheets. The areas of each blot was estimated via measurement of the diameter of the circular blots.

Statistical analysis was performed using IBM SPSS Statistics 27.0.1. A significance level of 0.05 was chosen for one-way ANOVA analysis and the post hoc Tukey's Honestly significant difference (HSD) test.

3. Results and discussion

3.1. Lipase alcoholysis of cellulose: endoglucanase pretreatment and characterisation of cellulose esters

This study aimed to esterify cellulose fibers using low environmental impact processes catalyzed by enzymes with the objective of developing materials with significantly lower water and oil wettability for

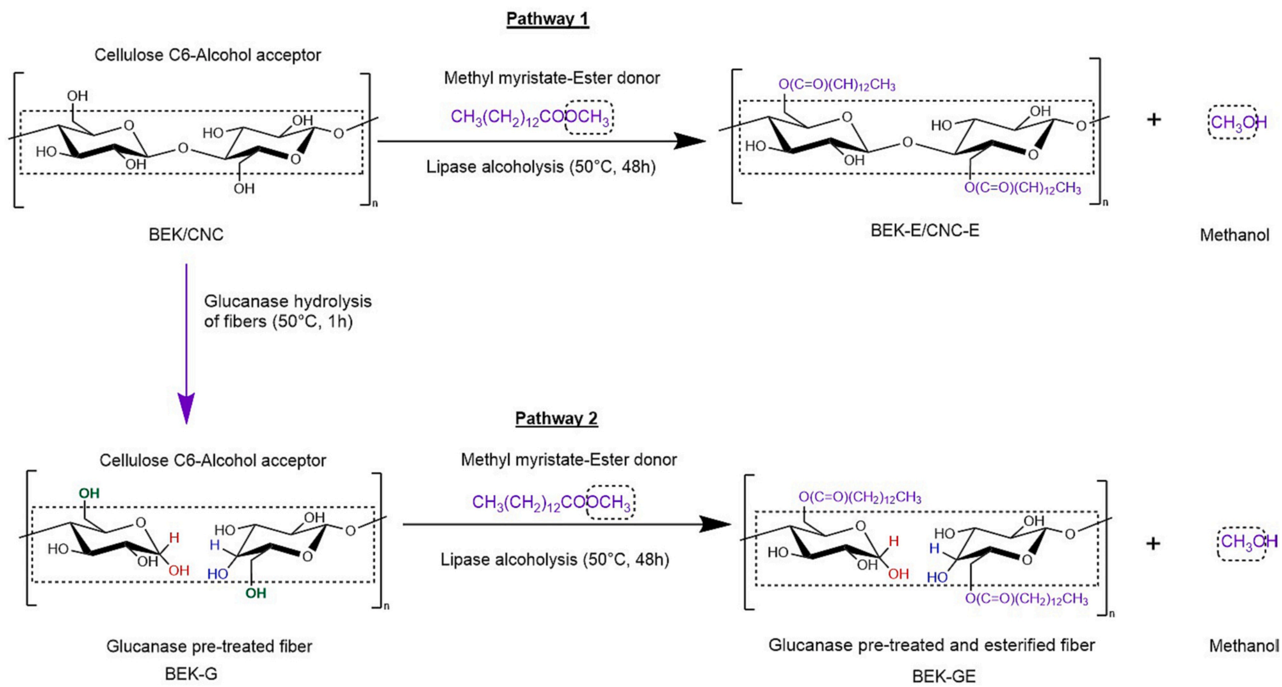

Fig. 1. Reaction schemes for cellulose alcoholysis on fibers by enzymatic pathways. Pathway 1 shows the reaction of native fiber or cellulose nanocrystals (alcohol acceptor) with methyl myristate donor directly catalyzed by lipase. Pathway 2 presents a two-step enzymatic process in which cellulose fibers are first treated with an endoglucanase, potentially exposing more reaction sites i.e., C6 hydroxyls, followed by lipase alcoholysis. Created with Chemdraw 21.0.0.

packaging and personal care applications. Following the concept of circular economy, our intention is to process fibers to engineer new functions and to return the material back to its original state via a facile process.

Lipase was investigated for the enzymatic and solvent-free alcoholysis of cellulose. Alcoholysis, which is a form of transesterification, was chosen to produce cellulose esters by two different pathways (Fig. 1). In previous studies, transesterification reactions have been shown to require lower activation energies than direct esterification reactions [27,28]; hence the transesterification pathway is an ideal method for cellulose ester production. Pathway 2 utilizes cellulose as a substrate which has been pretreated with endoglucanase and results in random scissions of the cellulose chain [29] hydrolyzing β-1,4 ether bonds within the chain (Fig. 1) and generally increasing enzyme accessible area [30–32] and reducing crystallinity. Lipase-catalyzed alcoholysis targets the primary C6 alcohol group of sugars [33,34] as these have higher reactivity and accessible conformations [35]; as it is difficult to specifically convert secondary alcohols using this approach due to steric hindrance and reduced rotation around the secondary carbons [36]. Endoglucanase treatment of cellulose likely generates additional reaction sites for lipase alcoholysis via increased fibrillation compared with intact fibers.

In comparison with cellulose fibers as substrates, we selected a common nanoscale substrate that is used for esterification with fatty esters namely, cellulose nanocrystals (CNC). Cellulose nanocrystals are ordered crystalline structures of high surface area whose morphology results from the removal of amorphous regions of the polymer by strong acid hydrolysis [37]. As substrates for esterification, highly crystalline CNC fibers contrast most with endoglucanase-treated cellulose which is highly fibrillated.

Another important requirement for the alcoholysis reaction is selection of an ester donor. Fatty methyl esters are benign, non-hazardous, and are not required in excess for the alcoholysis reaction to proceed. Long chain fatty methyl esters have previously been highlighted to improve the hydrophobicity of cellulose [14,38] and methyl myristate was selected as the ester donor due to its long hydrophobic aliphatic chain.

Optimisation of lipase loading was determined using glucanase-treated fibers in alcoholysis reactions with FT-IR (Fig. S1) used to reveal the highest degree of substitution as equated to the highest absorbance for C=O stretch at 1700 cm^{-1}. A lipase loading of 16 mg protein/g cellulose was identified to provide the best conversion and was selected to investigate alcoholysis on cellulose fibers and nanocrystals.

The FTIR spectra in Fig. 2 A confirm the presence of myristate esters on all three cellulose substrates. It is interesting to note that there is a 50 cm^{-1} shift in the carbonyl C=O stretch, from 1750 to 1700 cm^{-1}, when methyl myristate is converted to cellulose myristate demonstrating true grafting on the cellulose substrates. While the characteristic carbonyl (C=O) group can be seen clearly at 1700 cm^{-1} in all lipase-treated cellulose samples, the absorbance appears larger in endoglucanase pre-treated fiber. The degree of ester substitution is determined from solid-state NMR spectra (Fig. 2B) where the Degree of Substitution (DS) value for endoglucanase-treated fibers (0.10) exceeded that for cellulose fibers (0.08) and was lower for CNC (0.04). The carbonyl peak of the cellulose esters is clearly observed in all three substrates at 181 ppm and the aliphatic chain peaks of the myristate moiety are seen at 10–50 ppm. Overall, the NMR spectrum resembles the reported spectrum of cellulose conversion with methyl laurate in an ionic liquid environment [14]. Specifically, the current NMR spectrum for esterified CNC displayed a C6' peak at 62.06 ppm and C6 peak at 67 ppm which shows that substitution occurred at the C6 alcohol group, confirming successful alcoholysis of cellulose with the ester [14]. The calculation of DS for esterified cellulose nanocrystals was the lowest of all three substrates at 0.04 which suggests lipases had restricted access to C6 alcohol groups due to highly crystalline regions of CNC as lipases preferably target the amorphous regions of the cellulose structure.

3.2. Analysis of cellulose esters using Optical Photothermal Infrared Spectroscopy

The esterification of lipase catalyzed alcoholysis on cellulose

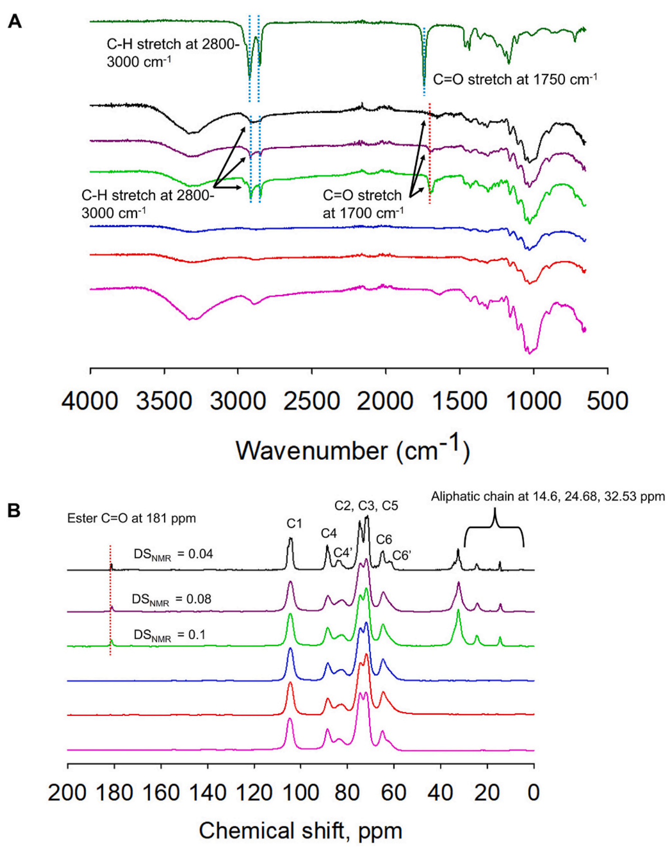

Fig. 2. Characterisation of unmodified cellulose and cellulose derivatives A. ATR-FTIR and B. ^{13}C CP/MAS NMR. All NMR spectra were referenced against glycine at 176.03 ppm. Legend for spectra: CNC (pink), BEK-G (red), BEK-G treated with methyl myristate, no catalyst (blue), BEK-GE (green), BEK-E (dark pink), CNC-E (black). ATR-FTIR only: Methyl myristate (dark green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

substrates was further explored using Optical Photothermal Infrared Spectroscopy (O-PTIR) with the mIRage instrument directed at four independent sample points. O-PTIR is an emerging technique using a continuous-wave visible-laser probe to detect thermal responses of mid-IR-absorbing regions without sample contact [39,40]. The mIRage has successfully detected localized photothermal expansion on the surface in a variety of materials [41–43] with up to 500 nm resolution and 0.4 pg sensitivity [44].

The O-PTIR spectra are illustrated for cellulose esters in Fig. 3A and B for the wavenumber range from 800 to 1800 cm^{-1} and 2700–3000 cm^{-1}, respectively. The carbonyl absorbance of the grafted ester (Fig. 3A) is apparent at 1700 cm^{-1}, indicating a degree of substitution on the esterified fiber surface that contrasts with the absence of signal in this range for non-esterified endoglucanase-treated fibers. The fatty chains of grafted myristate on cellulose are similar to those observed in FTIR (Fig. 2A), where two peaks at 2856 cm^{-1} and 2924 cm^{-1} are present in the esterified sample whereas the endoglucanase-treated fibers (red) show no intensity in this region (Fig. 3B). The analysis by O-PTIR aligns well with the DS of 0.1 as most conversion is expected on the surface and large amplitude response was observed in several locations. The complete O-PTIR acquisition spectra from 2700 to 3600 cm^{-1} is

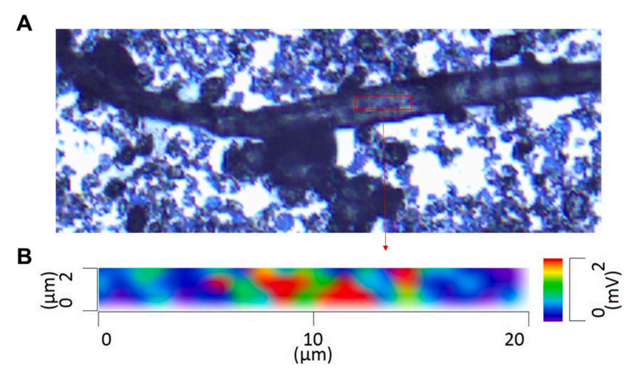

Fig. 4. Chemical maps acquired from the mIRage at 1700 cm^{-1} (carbonyl absorbance of cellulose ester) to quantify the surface distribution of grafted myristate esters on an endoglucanase pre-treated cellulose fiber. A. A sample fiber as viewed in mIRage B. Chemical distribution of grafted ester on the cellulose fiber mapped in colour over a 2 × 20 μm section of the fiber.

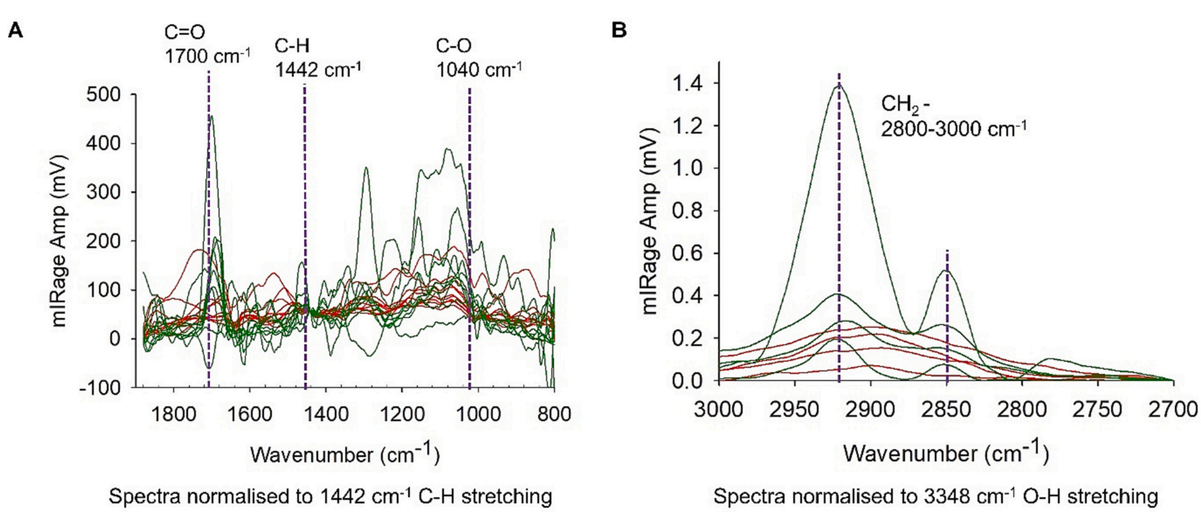

Fig. 3. Overlayed infrared spectra collected by mIRage O-PTIR at 4 independent samples sites per substrate. A. Spectra highlighting the 800–1800 cm^{-1} region with normalization to the 1442 cm^{-1} CH_2 bend and B. Spectra highlighting the 2700–3000 cm^{-1} region with normalization to the 3348 cm^{-1} OH stretch. Legend: BEK-G (red) and BEK-GE (dark green). The carbonyl absorbance of the grafted myristate ester on endoglucanase pre-treated fibers is detected at 1700 cm^{-1} (panel A) and aliphatic chains of the fatty acid are detected at 2800–3000 cm^{-1} (panel B). IR absorbance wavelengths corresponding to characteristic cellulose components are indicated above the spectra. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

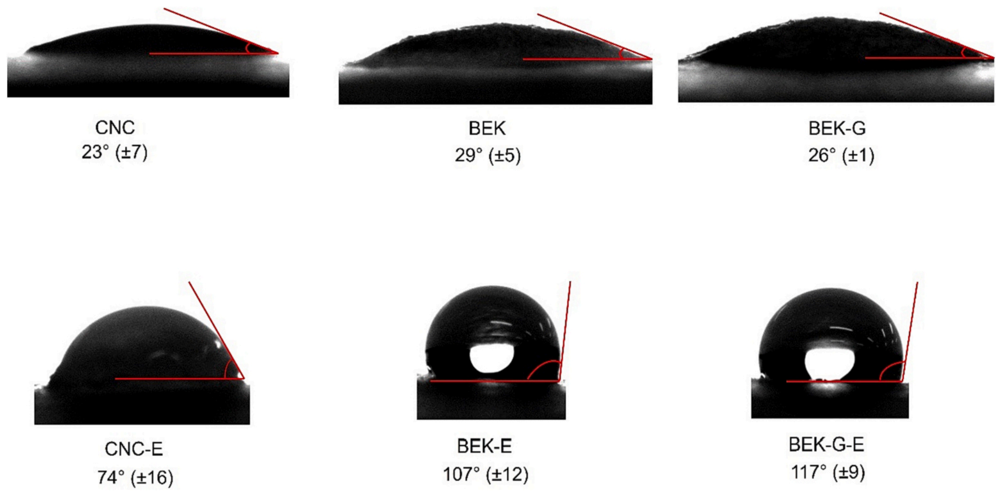

Fig. 5. Water contact angle measurements for untreated/modified cellulose substrates formed in a compression mould (data presented as mean ± standard deviation of 5 independent measurements).

displayed in Fig. S2.

The chemical map acquired at 1 μm spatial resolution from the mIRage on esterified endoglucanase pretreated fibers is displayed in Fig. 4. The voltage scale on the right corresponds to signal intensity from the cellulose esters ranging from nil (blue) to high (red). Domains of ester 1–2 μm in size are surrounded by untreated domains; each domain is surrounded by a thin corona of low concentration esters. The alcoholysis performed by the lipase is highly localized suggesting that the enzyme is first attracted to the surface of the fiber and then biocatalysis between the ester and C-6 primary alcohols is performed from these loci of adsorption.

3.3. Water and oil wettability

Surface hydrophobicity of cellulose is an important property which indicates the extent that a material will be a barrier to water transmission. Static water contact angle was measured for untreated and esterified cellulosic substrates and the results are shown in Fig. 5. Untreated CNC, raw cellulose fibers and endoglucanase treated fibers all displayed low water contact angles (~20–30°), as expected. Compressed samples of esterified cellulose showed a range of improvement in water contact angles from a low of 74° on esterified cellulose nanocrystal to an intermediate level of 107° for cellulose ester fibers while endoglucanase pre-treated cellulose esters had the highest value of 117°. Non-wetting conditions ($\theta > 90°$) were achieved with both esterified fibers. The values obtained for water contact angle are well correlated ($R^2 = 0.99$) to the estimated degrees of ester substitution from NMR spectra where endoglucanase pre-treated cellulose esters showed the highest substitution. The water contact angles determined for endoglucanase pre-treated and esterified cellulose here are comparable to those observed on cotton by surface functionalisation using poly(ethylene adipate) [16], on cellulose laurates [45] and many fold higher than those obtained from grafting of phenolic esters [18].

Hydrophobicity and oil resistance are important for paper packaging [46–48] especially in applications as a biodegradable alternatives to polyethylene terephthalate (PET). Composite paper handsheets of 60 gsm were prepared using raw cellulose fiber base (90 % w/w) and processed cellulose samples were blended at 10 % w/w, as commercially practiced. This proportion also allowed paper sheets to be formed without breaking and test the potential of small concentrations of esterified fibers to impart functional differences.

Hydrophobicity was estimated by the action of the paper composites to limit the spread of water droplets added to sheets (Fig. S3A) and areas of water droplet spread were compared with plain paper sheets; esterified endoglucanase pre-treated cellulose composites were the most effective at reducing the spread of water by almost 50 % (sample III,

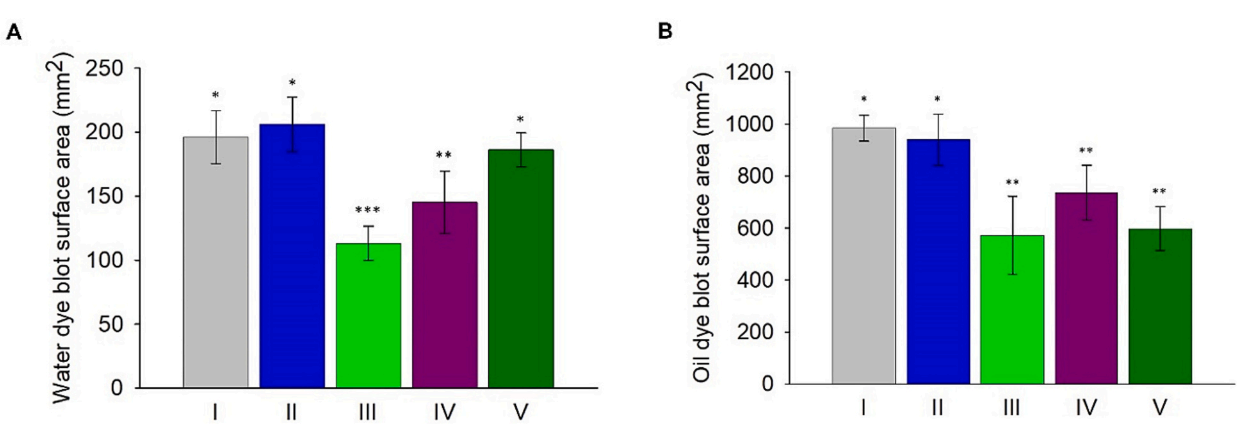

Fig. 6. Assessment of water and oil resistance of paper composites composed of cellulose ester or control cellulose added to raw cellulose at 10 % *w*/w. A. Surface area estimate of blots of Brilliant Blue dye made in water. B Surface area estimate of blots of Nile Red dye in Canola oil. All composites were prepared with the identical raw fiber base of 90 % *w*/w. Legend: I BEK-G; II BEK-G treated with methyl myristate no catalyst; III BEK-GE; *IV* BEK-E; V CNC-E. * Level 1 Tukey significance ** Level 2 Tukey significance *** Level 3 Tukey significance. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

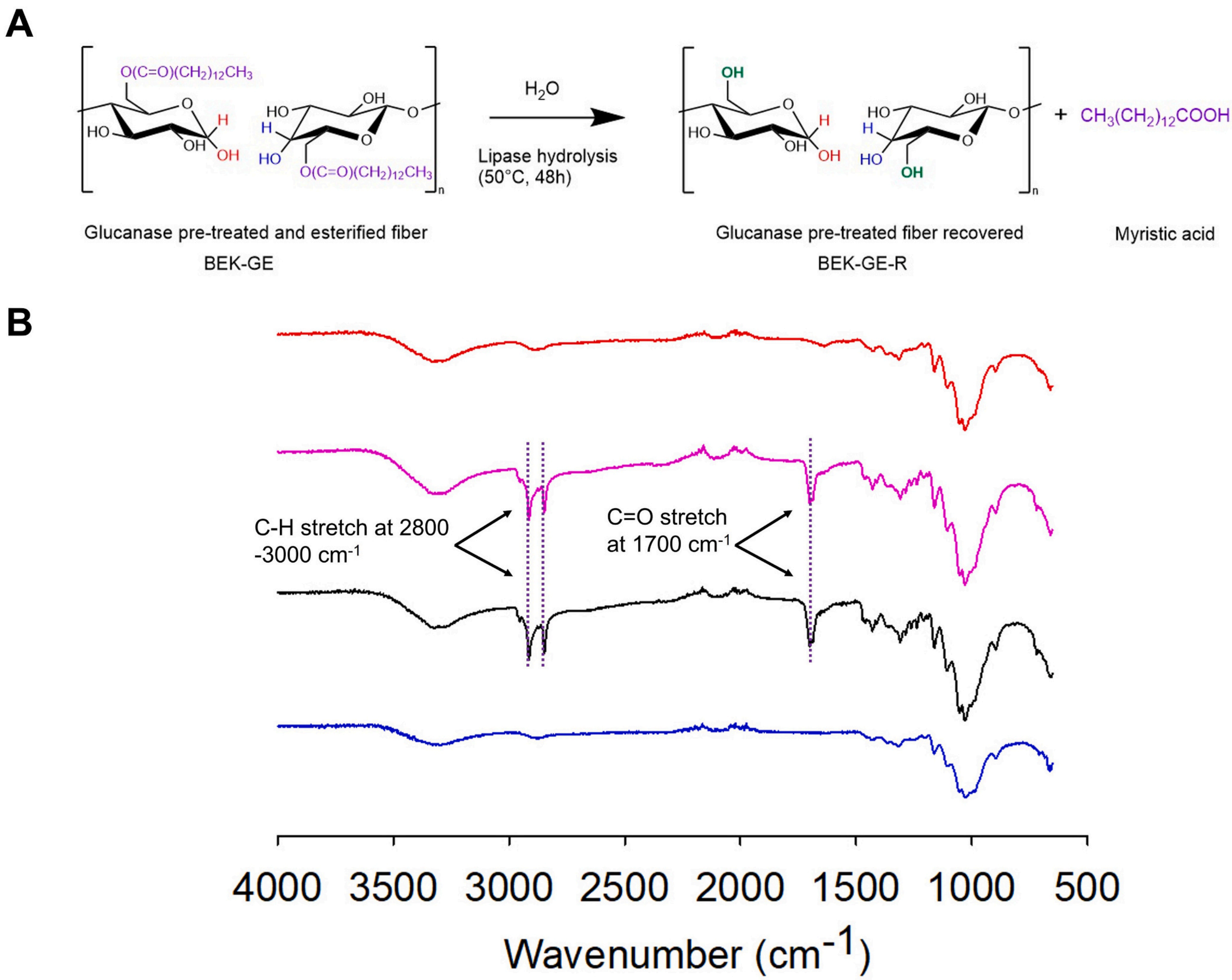

Fig. 7. A. Proposed reaction scheme of cellulose ester hydrolysis and B. FT-IR Spectra of lipase-treated hydrolyzed cellulose ester and initial materials. Legend: BEK-G (blue), BEK-GE (black); BEK-GE treated with water only (pink), BEK-GE-R (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6A). While all esterified cellulose composites reduced the spread of

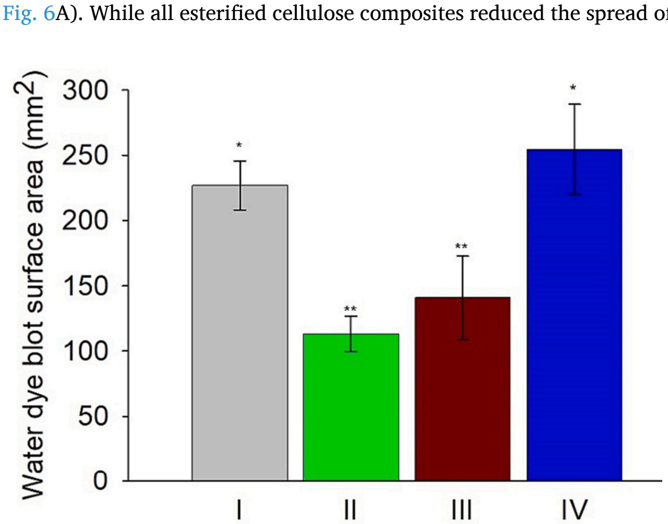

Fig. 8. Hydrophobicity assessment of paper composites prepared with hydrolysed cellulose ester (10 %). All composites are made with raw cellulose fiber base of 90 % w/w. I BEK-G; II BEK-GE; III BEK-GE treated with water only; IV BEK-GE-R. * Level 1 Tukey significance ** Level 2 Tukey significance.

water droplets (Fig. 6A), not all were statistically significant and cellulose nanocrystals with the lowest DS of 0.04 also showed the lowest hydrophobicity impact in composites (Fig. S3A). The hydrophobicity of cellulose esters in composites has good correlation with the DS calculations of NMR spectra and well within the range of previous work conducted on cellulose ester production [9] which is between 0.06 and 0.18. Additionally, analysis of esterified cellulose using other techniques (ATR-FTIR and O-PTIR) are in accordance with both the DS results and the functional tests.

For oil resistance, the spread of canola oil droplets was tested in a similar manner to water droplet as shown in Fig. S3B and Fig. 6B. The resistance of oil droplet spread was again most significant with endoglucanase-pretreated cellulose ester composite which reduced the oil droplet spread by ~40 % compared to control samples (Fig. 6B). All esterified fiber composites displayed significant reductions in spread. The resistance to oil droplet spread shown by the esterified fiber composites may be due to attractive hydrophobic interactions between the C14 aliphatic chains of the ester grafted to the cellulose and the long chain alkyl groups of the triglyceride oil which results in the net effect of restricting the movement of oil in the paper matrix. Preparation of paper composites is an effective practical method of assessing functional properties imparted by esterified cellulose such as hydrophobicity and oil resistance in a material that is representative of packaging material.

3.4. Enzymatic reversal of cellulose fibers esterification

The resistance of cellulose esters to water hydrolysis and the ability to recycle the original material to its original cellulose state was investigated using lipase catalysis. Cellulose esters showing the highest DS, that is, endoglucanase treated cellulose esters were processed with an aqueous lipase mixture as shown in the reaction scheme in Fig. 7A. Enzymatic hydrolysis of esters in excess aqueous media contrasts with the ester synthesis reactions which were performed in water-free media. The effectiveness of the enzyme hydrolysis is clearly shown by the FTIR spectra in Fig. 7B, where the grafted myristate ester peaks are absent both at the carbonyl stretch and the C—H stretch (red spectrum). By contrast, cellulose esters were resistant to uncatalyzed hydrolysis (pink spectra) which indicates the esters will be stable towards water and humidity for various packaging applications. For the first time, a green chemistry method to remove C6-substituted cellulose esters has been demonstrated. It is important to note that hydrolysis required only 1/3 the quantity of enzyme to remove the grafted myristate esters than to perform alcoholysis, confirming that the hydrolysis of esters is a facile natural pathway for lipases [49].

Furthermore, the hydrophobic functionality of cellulose esters was lost following lipase hydrolysis as shown in Figs. 8 and S4. Paper composites were prepared as previously described and blended with cellulose ester that had been hydrolysed by lipase (added at 10 %, Sample IV) and compared with composites made from cellulose fibers treated with water only (Sample III). Lipase was also employed for the hydrolysis of cellulose esters in an aqueous environment. The regenerated cellulose is more suitable for industrial recycling as general cellulose since it is difficult to separate modified materials. We expect aqueous lipase treatment would remove densely functionalised cellulose esters as well. The water droplet area estimates confirmed that hydrophobicity was reversed with aqueous lipase-treated esterified cellulose sample having the same lack of water resistance as the paper blended with endoglucanase pre-treated fibers (Sample I).

4. Conclusion

This study has demonstrated an enzyme-catalyzed process for the solvent-free production of cellulose esters that delivers ester substitutions of sufficient level to impart functional hydrophobic and oil resistance benefits to paper composites. The use of 1,4-β-endoglucanase for pretreatment of cellulose improved the degree of substitution presumably by increasing the amorphous content of cellulose and exposing some reactive C6 alcohol groups. Lipases preferentially catalyse alcoholysis at the C6 alcohol groups; this was confirmed in our system by the formation of a C6' peak for esterified cellulose nanocrystals by ^{13}C NMR. The formation of grafted myristate esters by lipase action on cellulose was demonstrated by key absorbance wavelengths for carbonyl and aliphatic C—H stretching evident in FTIR and O-PTIR spectroscopy. Furthermore, the grafted cellulose esters were shown to be stable in water but readily hydrolysed in the presence of lipase and water, at 2/3 lower loading of enzyme. Functional testing of esterified cellulose fibers showed significantly higher water contact angles indicating their potential in water barrier and controlled absorption applications. The hydrophobic qualities of esterified cellulose samples were translated to water retention characteristics in paper composites, especially for the endoglucanase pre-treated cellulose esters. Here, we have presented an elegant process for lipase-catalyzed cellulose esters via alcoholysis that imparts sufficient hydrophobic character to show significant improvement in water and oil retention in paper composites. This approach utilizes only biodegradable and renewable materials under mild conditions and can readily reverse esterification without use of chemicals to return the cellulose esters to native cellulose.

CRediT authorship contribution statement

Rahul Sharma and Victoria Haritos: Conceptualization, Methodology, Writing- Reviewing and Editing; **Kevin Putera and Mark Banaszak Holl**: Resources, O-PTIR mIRage, Formal analysis; Writing-Reviewing and Editing **Gil Garnier:** Methodology, Writing- Reviewing and Editing; All authors have contributed to writing the manuscript and approved the final draft.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The authors have no competing financial or personal interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2023.127972.

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