Faraday Discussions

Cite this: *Faraday Discuss.*, 2026, **262**, 414

PAPER

Determining the biodegradation of functionalised cellulose esters

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Received 17th March 2025, Accepted 15th August 2025
DOI: 10.1039/d5fd00040h

Recently, there has been an increased interest in developing functionalised carbohydrates, such as cellulose palmitate, as novel replacements for petroplastics. The functionalisation gives the materials excellent water barrier properties, as well as processability and mechanical properties akin to PET, while potentially having superior biodegradability to conventional first-generation biopolymers. However, the true biodegradability of these novel polymers is still unknown with some recent reports suggesting that it is limited. In this study, we investigated the potential of cellulose palmitate to biodegrade under controlled laboratory conditions, comparing the polymer to cellulose acetate. To this end, studies using specific enzymes, targeted whole cell fungal degradation and model edibility experiments were devised to study the biodegradability at end-of-life. On an enzymatic level, a combination of cellulase and lipase enzymes were found to hydrolyse the fatty acid linkages, allowing the cellulases to access the carbohydrate chain and release glucose. Under optimal conditions the biopolymer was completely hydrolysed within 6 hours. A soil fungi was then isolated from a compost heap that had been loaded with the functional material, to establish the most suitable species for whole cell degradation. This common soil fungi, *Mucor* sp., was then grown successfully under lab conditions on the functional material as a 95% carbon source. Finally, an edibility experiment was designed, using pepsin and pancreatic enzymes at precise pH concentrations found in the gastrointestinal tract to mimic real life conditions of ingestion by birds. While cellulose acetate broke down under just the acidic conditions, with no enzymes, the cellulose palmitate was found to be stable at the acidic conditions, but hydrolyse over 7 days when the enzymes were present. To the best of our knowledge this is the first study to confirm the biodegradability of functionalised cellulose highlighting the large promise of functionalised carbohydrates as a sustainable alternative to petrochemical plastics within the packaging industry.

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1. Introduction

Plastics are synthetic polymers, predominantly obtained from hydrocarbons derived from crude oil. These polymers are fundamental to the food system with sturdy water barriers, making them ideal as packaging, extending the shelf life of food and facilitating the global food system.[1] However, plastic pollution, when they are deposited in the environment, has extremely detrimental effects causing harm to both humans and animals. Worldwide, it is estimated that only 10% of plastics are recycled, 14% incinerated whilst 76% ends up in landfills or the natural environment.[2] When placed in landfills these materials do not break down fully. Rather they produce smaller particles over time, termed microplastics that can leach into the environment potentially damaging our terrestrial and marine ecosystems.[3] The main issue is that the chemical bonds in the majority of synthetic plastics are not recognised by microorganisms found in nature, therefore they lack the specific enzymes required to carry out biodegradation.[4]

Annually approximately up to 23 million tonnes of plastic waste pollutes the marine ecosystem.[5] Plastics in the marine environment are of increasing concern due to persistence and damage to oceans, wildlife and humans. Weathering of plastic debris appears to cause fragmentation into particles that even small marine invertebrates may ingest.[6] It's estimated more than 267 species have ingested plastics.[7] Adsorption of persistent organic pollutants onto plastic can be transferred into tissues and organs of the animals as well as their predators and ultimately into humans.[8] Achieving substantial reductions in global plastic emissions to the environment requires an urgent transformative change.[9] Removal of these products from our world is not plausible, however we need to ensure their production is sustainable and harmless to wildlife.

One potential solution to this problem is the development of polymers sourced from biomass. Bioplastics that are made from natural resources, yet mimic the properties of petroplastics have been developed over the last few decades. Two of the main first generation biopolymers produced are polylactic acid (PLA) and thermoplastic starch (TPS). PLA is derived from lactic acid produced through the fermentation of starch derived from corn, whereas TPS is manufactured through plasticisation of starch through high temperature extrusion with water.[10] These bioplastics mimic the superior water barrier of fossil fuel derived plastics and have been termed as 'biodegradable/compostable'. However, this can be misleading, as effective degradation typically requires industrial composting conditions, which involve high temperatures (around 58 °C) and are highly energy intensive with such facilities limited to only a handful of locations. Since first-generation bioplastics do not readily break down in natural environments, it is evident that advancements are needed to develop next-generation bioplastics.

Recently, various commercial and academic research has focussed on utilising carbohydrates from nature as biopolymers, such as cellulose and alginate, which rapidly biodegrade in the environment, reducing plastic pollution.[11] Although effectively biodegradable, these carbohydrates lack the water barrier necessary to replace current plastics, and even with mechanical processing only retain a barrier for a very short period of time.[12]

To increase the water barrier properties, chemical functionalisation of these carbohydrates is necessary. However, building in hydrophobic bonds affects their

biodegradability. It is essential that these functionalised materials can still break down in soil and marine environments, creating a sustainable product helping to solve this plastic pollution crisis. Furthermore, with the tendency of plastics to enter the food chain (90% of all seabirds ingest plastics)[13] it is critical for these functionalised materials to not only break down in the environment, in a reasonable timeframe, but also to be broken down in the gastrointestinal tract, to ensure the safety of our marine wildlife.

The two leading functionalised carbohydrates are cellulose acetate, and more recently cellulose functionalised with fatty acids, such as cellulose palmitate.[14] Cellulose acetate is a popular functionalised material constructed *via* the acetylation of cellulose. Ref. 15 evidenced that early research reported limited biodegradability, however more recent experiments identified the key mechanism of biodegradation occurs firstly *via* deacetylation by acetyl esterases, allowing cellulases to then degrade the cellulose backbone.[16] Cellulose palmitate has been reported more recently and contains excellent bulk water barrier properties akin to PET. However studies into the biodegradability of these functionalised polymers including 12 weeks in compost conditions and 2 days of enzymatic hydrolysis have reported that cellulose palmitate completely lacks biodegradability.[17]

The work presented herein aims to fully investigate the biodegradability of functionalised carbohydrates, with particular focus on cellulose palmitate. To test biodegradability we looked on an enzymatic level, whole cell and edibility scale.

2. Materials and methods

2.1 Materials

DNS reagents (distilled water, 3,5-dinitrosalicylic acid, sodium hydroxide, rochelle salts (sodium potassium tartrate), phenol, sodium metabisulfite, HCl), lipase from *Candida rugosa*, pepsin (from porcine gastric mucosa powder), pancreatin (from porcine pancreas), hexane, $NaHCO_3$ and CTEC2 were all purchased from Sigma-Aldrich. The citrate buffer (citric acid monohydrate, DI water, NaOH), sodium carbonate, bile bovine powder were purchased from Thermo Scientific. Cellulose acetate and filter paper were purchased from Merck. *Mucor* sp. was purchased from DMSZ and cultured using standard fungal techniques. MEA/PDA and potato dextrose broth were purchased from Fisher and agar plates prepared using standard aseptic protocols. FT-IR was used for bond analysis, GC-MS used for palmitic acid concentration. OPUS software was used for analysis of FT-IR data and MassHunter for analysis of GC-MS data.

2.2 Methods

2.2.1 Cellulase assay. This protocol was adapted from ref. 18; briefly, before carrying out the experimental assay a 'measurement of cellulase activity' was used to determine the correct FPU and enzyme dilution required for the study. To this end, Whatman filter paper was used as the substrate. 1 × 6 cm per samples (approximately 50 mg) were rolled into 50 ml falcon tubes, using 2 repeats for each glucose concentration.[19]

For the glucose standards, a stock solution of 10 mg ml^{-1} was made and then further diluted.

Three controls were used: reagent blank (1.5 ml citrate buffer), enzyme control (1 ml citrate buffer + 0.5 ml enzyme dilution) and a substrate control (1.5 ml citrate buffer + filter paper).

To each of the glucose standard tubes, 1 ml of 0.05 M citrate buffer was added, saturating the filter paper strip. Citrate buffer was also added to the blank and controls tubes in the amounts provided above. In the enzyme control tube 0.5 ml of the CTEC2 enzyme was used (diluted in citrate buffer, a stock solution was prepared first, 1 ml enzyme & 19 ml citrate buffer, further diluted 1800 μl buffer and 200 μl enzyme). To each of the glucose standard tubes, 0.5 ml of each dilution standard was placed into the corresponding tube, ensuring the filter paper was covered by the solution. All the samples were placed into a 50 °C water bath shaking at 150 rpm for 1 hour. After the incubation period, the tubes were removed from the water bath and placed into a fume hood. 3 ml of DNS was added and the tubes placed into a vigorously boiling beaker of water for 5 min. After boiling, the tubes were transferred to a bucket of ice for 5 min, followed by an incubation period of 20 min at room temperature. Absorbance was measured at 540 nm using a spectrophotometer (Thermo Scientific, Spectronic 200E) 200 μl of the sample was mixed well with 2.5 ml water in a cuvette (see supplementary information (SI)).[20]

2.2.1.1 Assay of glucose release via cellulase enzyme. From the 'measurement of cellulase activity' assay a CTEC2 dilution of 0.00130 was calculated (as per below) and this concentration was used to set up the experimental assay.

$$\text{Enzyme releasing 2 mg glucose per 0.5 ml} = (2 \times 0.0004) + 0.0005 = 0.00130$$

$$\text{Filter paper activity} = 0.37/0.00130 = 284.62 \text{ FPU ml}^{-1}$$

To determine the glucose release of the samples, a 14 day incubation period was used, with sampling once every 2 or 3 days. Samples were initially placed in petri dishes. Filter paper was chosen as a positive control, and spectro zero along with enzyme blank as the negative controls. The functionalised carbohydrate, cellulose palmitate, was the material under investigation. Two repeats of 0.1 g of cellulose palmitate were placed into seven petri dishes (day 0, 3, 5, 7, 10, 12 & 14). The same procedure was done for filter paper. A total volume of 5 ml solution was placed into the petri dishes containing 0.00130 dilution of CTEC2 enzyme in citrate buffer. The sample and control dishes were incubated at 25 °C, removing the samples at the intervals mentioned above. Enzyme blank and spectro zero controls were also incubated at 25 °C. On each of the sample days, the glucose concentration was measured using a DNS assay as described previously.

2.2.2 Lipase assay

2.2.2.1 Breakdown of ester bond via lipase activity. A 2.5% aqueous solution of lipase, 0.001 M of aqueous sodium carbonate solution and 5% solution of bile salts were prepared. To 1 g of cellulose palmitate, 1 ml of 5% bile salts solution, 2 ml of 0.01 M sodium carbonate and 2 ml of the lipase solution were added and the sample incubated at 37 °C for 3 hours with shaking at 130 rpm.[21,22]

2.2.2.2 Weight change. Samples were dried and weighed pre- and post-treatment. Hexane was used as a solvent to dissolve the palmitic acid. % Weight change was then calculated through gravimetric measurements.

2.2.2.3 GC-MS. Hexane was used as a solvent to dissolve the palmitic acid and samples analysed *via* Thermal Desorption-Gas Chromatography Mass Spectrometry (TD-GC-MS, Agilent/Markes 8890 GCMS with TDX100R) to measure the palmitic acid concentration. MassHunter was used for data processing. A calibration curve was produced using known palmitic acid concentrations and samples analysed against this graph to estimate concentration.

2.2.2.4 Fourier transform infrared spectroscopy (FT-IR). Fourier Transform Infrared Spectroscopy (FT-IR) measurements were carried out using a FT-IR Spectrometer INVENIO (Bruker, Germany) with an attenuate total reflectance (ATR) diamond. Resolution 4 cm^{-1}, sample scan time 4, background scan time 4. The spectra were scanned between 4000 and 400 cm^{-1}. Dry films and 1–2 drops of liquid samples were placed on ATR crystal and data processed using OPUS software (Bruker, Germany).

2.2.3 Cellulase and lipase assay. The lipase assay was carried out as previously described (see Section 2.2.2). CTEC2 enzyme was then added to the sample also as previously described (see Section 2.2.1). This experiment was carried out over 96 hours. Previously we used a 2-week timeframe, however, as no change in glucose concentration was observed beyond day 3, we terminated the experiment at 96 h. Samples were taken every hour in the first seven hours and then at 24 and 96 hours. A DNS assay was carried out as previously described (see Section 2.2.1) to measure glucose concentration. In addition, GC-MS was used to measure palmitic acid concentration.

2.2.4 GI tract assay. The gastrointestinal (GI) tract assay was adapted from Ref. 23. Briefly, to test the ability of avian digestive enzymes to degrade biopolymers, pepsin from the stomach and pancreatin from the small intestine were used. A pH of 2.8 was used to mimic the stomach environment and a pH of 6.1 to mimic that of the small intestine. Fourier transform-infrared spectroscopy (FT-IR) was used to analyse the structure of the biopolymers before and after the experiment.

2.2.4.1 Degradation in digestive tract of birds. A three-step assay was used to mimic crop, stomach, small intestine of the avian digestive tract. The assay conditions paid particular attention to water content, temperature, retention time, pH and proteolytic enzymes. Before each test, the amount of HCl & $NaHCO_3$ necessary to achieve the target pH values for each step (step 1 'crop': pH 5.8, step 2 'stomachs': pH 2.8, step 3 'small intestine': pH 6.1) was determined. Approximately 50 mg of each sample condition (exact weight was noted) were placed in a 50 ml falcon tube. Step 1 of the assay was intended to simulate the 'crop'. 1.5 ml dH_2O added and the pH adjusted to 5.8 using HCl. Tubes were vortexed well to achieve homogeneity. The samples were incubated at 40 °C in a water bath (shaking at 130 rpm) for 24 hours. After incubation, 3000 U of pepsin was added to the sample, with the pH adjusted to 2.8 using HCl – this step was employed to mimic the conditions of the stomach. The tubes were again placed into a 40 °C water bath (shaking at 130 rpm) for 24 hours. After the incubation period, 3.7 mg per ml pancreatin was added, adjusting the pH to 6.1 using $NaHCO_3$. This third step was used to mimic the conditions found within the small intestine. After mixing, tubes were again placed into a 40 °C water bath (shaking at 130 rpm) for 24 hours. Immediately following the incubation period, all samples were placed on ice.

To test the pH effect, the experiment was also repeated without the presence of any enzymes, at the low pH 2.8 this time for 7 days.

2.2.5 Growth of microorganisms

2.2.5.1 Fungal reactivation and growth. Mucor sp. fungal species was purchased from DMSZ, Germany. The yeast arrived in a powder form within a glass vial and required reactivation upon arrival under aseptic conditions. 0.5 ml of the appropriate medium was added to the vial and the pellet allowed to rehydrate. After rehydration, the content was mixed with an inoculation loop and about half mixed with 5 ml of the appropriate culture medium. The other half was streaked onto the specific agar plate. Both the liquid and agar cultures were incubated at 25 °C.

2.2.5.2 Fungal growth on agar plates. To investigate the ability of common soil fungi to grow on functionalised carbohydrates as the primary carbon source, we used the polymers as the 95% carbon source on an agar plate. *Mucor* sp. was streaked onto the plate and incubated at 25 °C. Potato dextrose agar and sorbitan stearate were used as positive control plates, the negative control plate did not contain the 95% carbon source. The objective of these experiments was to determine the ability of a common soil fungus (*Mucor* sp.) to grow on functionalised carbohydrates as their primary carbon source under controlled laboratory conditions.

3. Results and discussion

3.1 Enzymatic degradation of cellulose palmitate

A standard cellulase mixture (CTEC-2) was used to examine the ability of the polymers to be degraded by a carbohydrate degrading enzyme. The cellulose control showed a high level of degradation (Fig. 1a) demonstrating the effectiveness of the enzymes used. However, no glucose was observed in the degradation of the cellulose palmitate. This is presumably due to the fatty acid functionality protecting the carbohydrate framework from attack by the enzymes. As such, to gain access to the β-1,4 linkages, the sugar fatty acid ester bond must be cleaved first.

Fungi that produce lipases which can cleave β-1,4 linkages can be found in various environments including soil, industrial waste sites, oil contaminated soil & dairy waste.[24] Fungal lipases are responsible for catalysing the hydrolysis of triglycerides into fatty acids and glycerol.[25] To study the cleavage of the sugar–fatty acid ester linkage in cellulose palmitate, a lipase from *Candida rugosa* was used on both cellulose palmitate films and powdered samples. The resulting degradation was assessed by measuring the weight change, after washing with hexane to remove any evolved palmitic acid and directly examining the evolved palmitic acid concentration (Fig. 1b and c). In both films and powdered samples, the palmitic acid concentration increased over time and the % weight of the remaining polymer reduced, before plateauing. The weight change in both the powdered and solid samples were indicative of samples losing up to 50% of the mass. Palmitate constitutes 57% of cellulose palmitate by weight, suggesting that the weight change corresponds to the almost complete removal of all palmitate linkages. The hexane used to wash the polymers was then assessed through GC-MS. As expected, the degradation byproduct palmitic acid was present, and despite the large experimental error, the measured value corresponds with the weight change in the original samples, further confirming the effectiveness of the lipase in cleaving the palmitate bond.

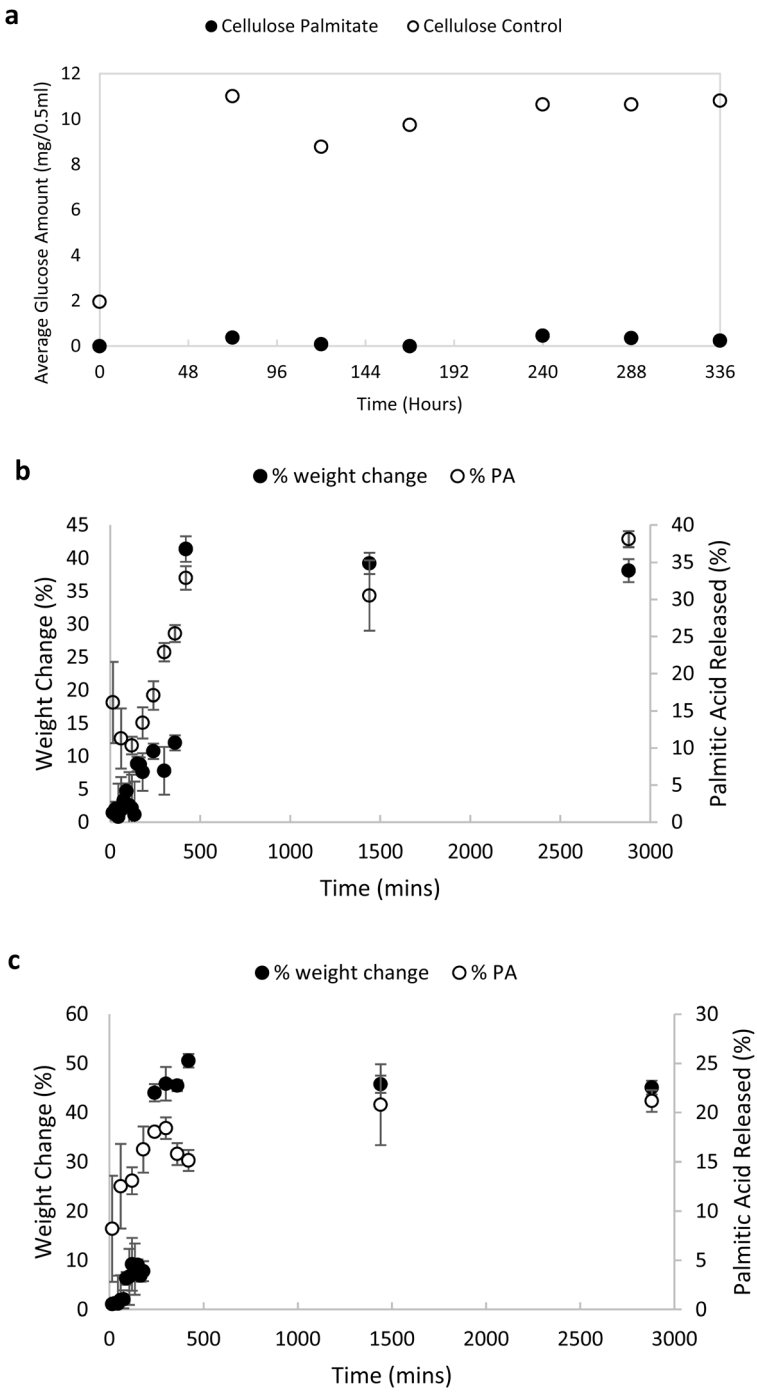

Fig. 1 Enzymatic breakdown of cellulose palmitate: (a) glucose concentration after cellulase activity. Palmitic acid concentration measured *via* GC-MS and weight change (%) of cellulose palmitate after lipase activity on solid films (b) and powdered sample (c).

Cellulose acetate has been extensively studied in the literature. Ref. 26 showed that microorganisms containing naturally occurring esterases had the ability to deacetylate the cellulose ester. Removing the acyl group allowed cellulases to naturally degrade cellulose. Ref. 27 proved cellulolytic enzymes alone do not have the ability to attack cellulose acetate. A similar mechanism is therefore present in degrading cellulose palmitate.

Following the lipase treatment the material was also analysed by FT-IR. A spectrum of the cellulose starting material is given in Fig. 2a, the spectrum of the cellulose palmitate is given in (Fig. 2b). The OH stretch, typical of cellulose, appears in the region of 3300–3500 cm^{-1} (Fig. 2a) for the cellulose control, and is absent in the cellulose palmitate sample pre-treatment (Fig. 2b), as these OH groups are bound to the palmitic acid. However, cellulose palmitate treated with lipase shows strong degradation over the 3-hour period, as evidenced by the appearance of the OH stretch indicating the presence of cellulose in the sample and confirming polymer breakdown.[28] The data confirms bond cleavage by a common lipase, making it possible for cellulase to gain access to the active site on cellulose palmitate. Release of cellulose, in turn, can be a carbon source for fungal growth, making the production of lipases a desirable feature of common soil fungi.[29,30]

Ref. 16 demonstrated that acetyl esterases can be responsible for the first step of biodegradation of cellulose acetate. The cellulose backbone is then available for utilisation *via* cellulase enzymes. We have evidenced that a lipase is necessary for the first step of cellulose palmitate biodegradation (Fig. 2c) and we next investigated the addition of cellulase enzymes after lipase treatment.

When using a combination of lipase enzyme and the CTEC2 enzyme mix, glucose is clearly released from the cellulose palmitate sample (Fig. 3a) as seen by increase in glucose concentration with time. The palmitic acid concentration in the mixture also increases over time as the enzymes hydrolyse the ester bond (Fig. 3b).

These results show that only a small amount of ester cleavage is required before glucose begins to be released and glucose concentration increases over time as more fatty acid is released. The initial rate is very similar to the control, suggesting that the lipase hydrolysis is rapid, and faster than the cellulase catalysed reaction. The palmitic acid concentration then plateaus and glucose release is faster in the cellulose control most likely due to the absence of the ester bond, allowing immediate access of the cellulolytic enzymes to the active site. However, glucose is still released from the polymer sample as the cellulases access the carbohydrate backbone. This data confirms that the lipase can break the fatty acid bond, allowing the cellulolytic enzymes to access the cellulose. This mechanism has also been demonstrated for cellulose acetate.[31]

3.2 GI tract – edibility

While the action of lipases suggests that the material will readily degrade in the soil environment, one of the key issues with petroplastics is the ingestion by animals, especially seabirds, polluting the food chain. In the experimental design, the conditions found within the digestive system of birds was replicated to investigate the ability of these specific enzymes and pH to degrade the polymers. This is of particular interest as plastic waste is frequently ingested by birds,

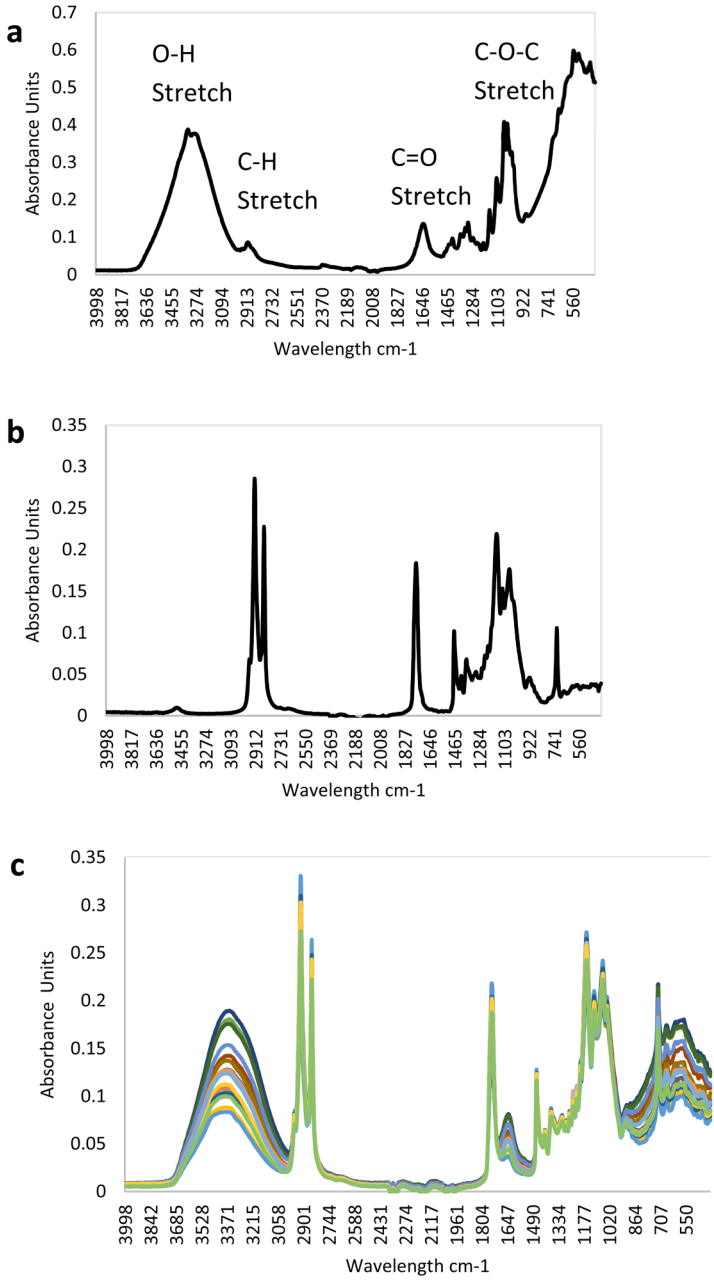

Fig. 2 Further assessment of the lipase catalysed breakdown of cellulose palmitate with (a) cellulose FT-IR spectra. (b) Cellulose palmitate FT-IR spectra pre-experiment. (c) Time series FT-IR spectra of cellulose palmitate after lipase activity, sampling every 15 minutes over 3 hours.

causing unnecessary death. Edible biopolymers would be a solution to this plastic pollution issue. To simulate the gut environment pepsin (gastric enzyme) and pancreatin (small intestine enzyme) were used. Two stages were used with an

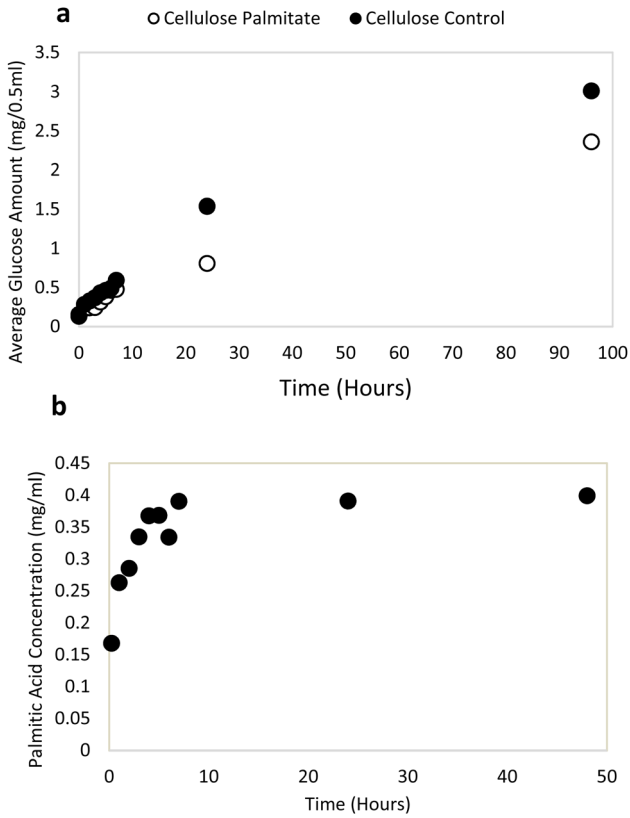

Fig. 3 The degradation of cellulose palmitate in the presence of both lipase and cellulase enzymes where (a) glucose concentration of cellulose control and cellulose palmitate after lipase and cellulase activity experiment. (b) GC-MS data of palmitic acid concentration after lipase and cellulase activity on cellulose palmitate.

initial gastric pH of 2.8 followed by a small intestine pH of 6.1. Each of the following steps were carried out for 24 hours: crop step, lower pH (stomach) and higher pH (intestine). FT-IR analysis was used to examine the structure of the polymers pre- and post-experiment, with cellulose used as the control.

Initially the polymers were subjected to the low pH conditions (pH 2.8 over 7 days) without additional enzymes to establish whether the acidic environment was enough to hydrolyse the polymer. Interestingly, these acidic conditions are enough to break down cellulose acetate with a large OH band observed indicating the presence of free cellulose (Fig. 4a). However cellulose palmitate was found to be unchanged under these conditions, with no free cellulose observed in the sample (Fig. 4c).

One of the key factors in the biodegradable nature of substituted polymers is the degree of substitution. To garner the high-water barrier, a high degree of substitution is needed, however, this increases the substituent's ability to prevent enzymatic attack of the cellulose backbone and therefore reducing degradation rate.[17] Cellulose acetate has a lesser degree of substitution, and it is likely that this influences degradation making it highly susceptible to degradation in just an

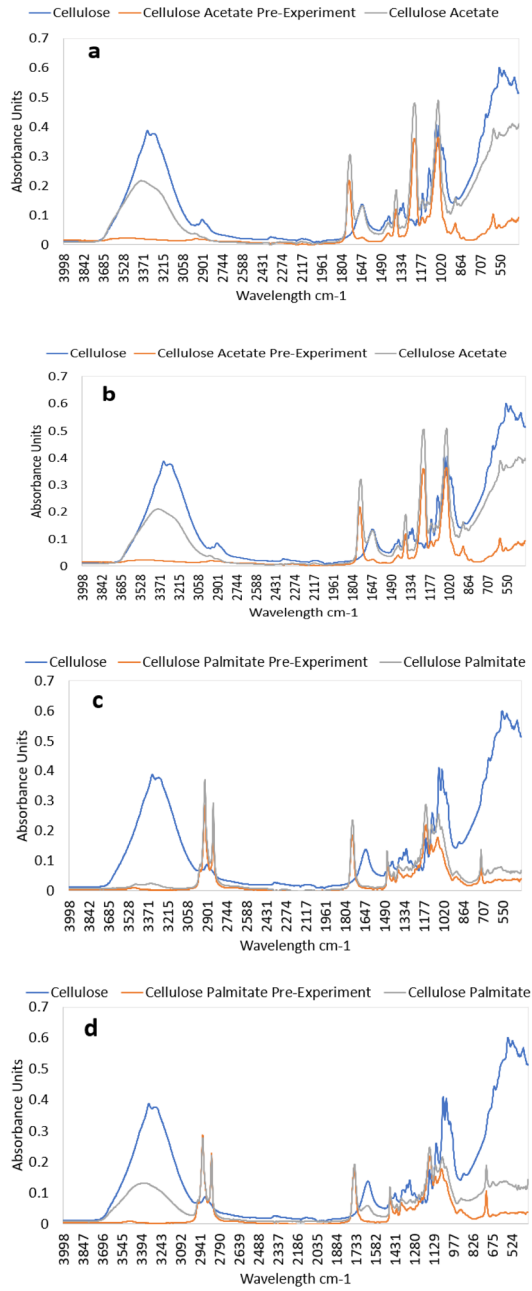

Fig. 4 The degradation of cellulose acetate and palmitate under conditions typical of a seabird's gastrointestinal tract (GI): (a) FT-IR spectra of cellulose acetate, at low pH for 7 days, no enzymes. (b) FT-IR spectra of cellulose acetate under conditions of seabird's GI tract, gut enzymes at specific pH values. (c) FT-IR spectra of cellulose palmitate, at low pH for 7 days, no enzymes. (d) FT-IR spectra of cellulose palmitate under conditions of seabird's GI tract, gut enzymes at specific pH values.

acidic environment. However, cellulose palmitate, which has a higher degree of substitution did not break down demonstrating that the degree of substitution and size of the ester group could determine the polymer degradation rate.[15] The chemical hydrolysis of cellulose acetate can be catalysed by strong acids, as the acetate group is susceptible to deacetylation in acid environments.[28,32,33] This is evidenced in our findings, as cellulose acetate broke down in just an acidic environment, whereas cellulose palmitate remained intact. The findings suggest that although cellulose acetate has a reasonable water barrier, it is not acid resistant, questioning its use as a packaging film in the food industry.

Conversely, cellulose palmitate has an excellent resistance to acid degradation. However, in the gut environment when the pancreatic enzymes were also present, extensive degradation of the cellulose palmitate is observed (Fig. 4d), with both free palmitic acid and cellulose observed in the FT-IR spectra (Fig. 4d). This provides evidence that cellulose palmitate is potentially edible and could be readily digested by animals in the food chain. Supporting the claim that cellulose palmitate can be used as a structurally sound packaging film whilst displaying biodegradability through enzymatic degradation rather than through chemical break down.

3.3 Whole cell

For polymer films to degrade effectively in the soil, microorganisms that secrete extracellular enzymes capable of degrading the polymer need to be present. This in turn means that the polymers need to be able to support soil microbial growth, without inhibiting the complex ecology. The biopolymer then acts as a substrate, causing the microbes to produce specific enzymes to break down the material, providing a carbon source.[15] To determine suitable microbial consortia to break down the cellulose palmitate, a cellulose palmitate film was buried in a compost pile, and the microbial community collected after 180 days. Samples from the film inoculated onto potato dextrose agar (PDA) and malt extract agar (MEA) plates confirmed that a diverse number of microorganisms were growing on the surface of the biopolymer, suggesting its ability to support soil microbial growth (Fig. 5a and b).

Gene sequencing was carried out on these plates to determine the most dominant fungal species growing on the surface of cellulose palmitate and could potentially be responsible for the polymer break down (Fig. 5c). The predominant species was found to be *Mucor* sp. These fungi produce various enzymes including cellulases, lipases, amylases, polygalacturonases and proteases. This wide spectrum of enzyme production, in particular, their ability to produce lipases, is of particular importance to our studies as these could potentially break down the ester bonds present within the functionalised carbohydrates.[34,35]

To determine the effectiveness of *Mucor* sp. to degrade the biopolymers, the defined species was cultured on cellulose acetate and cellulose palmitate on agar plates to specifically determine the ability of the species to use the biopolymer as a carbon source. To this end *Mucor* sp. (DMSZ 1222) was used to ensure specific fungal isolation and consistency. PDA and sorbitan stearate (a sugar source containing the functionalised ester bond) were used as positive controls displaying fungal growth and a negative control plate eliminating the 95% carbon source was used (Fig. 6). Good coverage of microbial growth on the plates

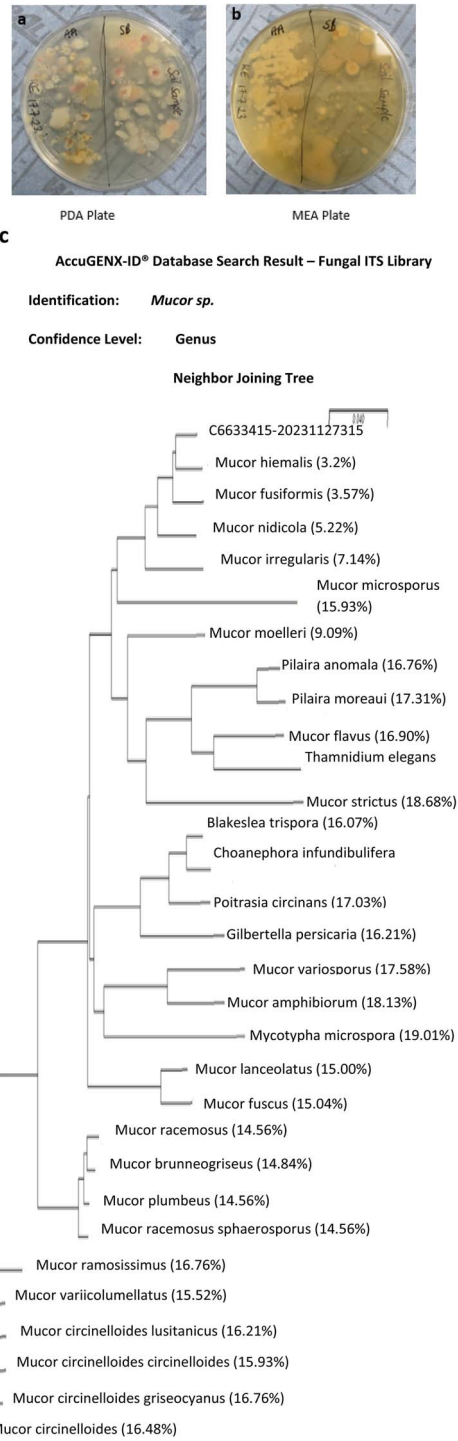

Fig. 5 Scrapings taken from the surface of a buried cellulose palmitate sample onto a PDA plate (a), MEA plate (b), incubated at 25 °C for 3 days under lab conditions. (c) Gene sequencing data from samples (a and b).

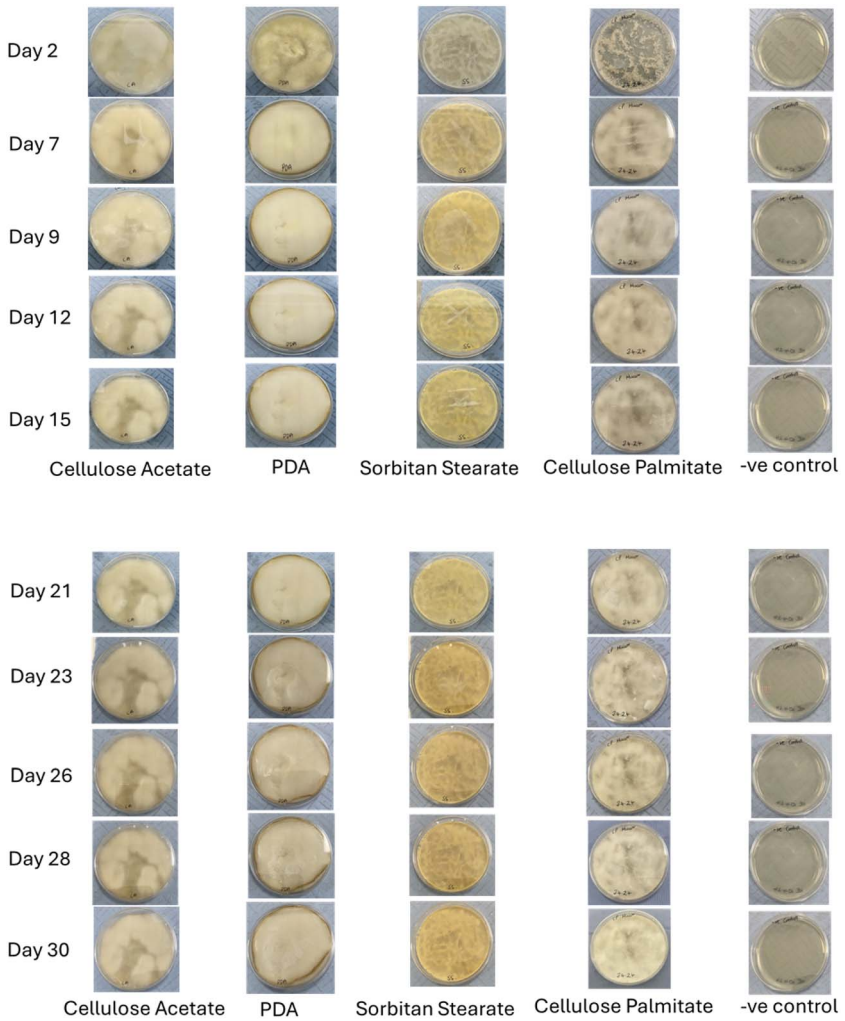

Fig. 6 Time series of *Mucor* sp. growth on cellulose acetate and cellulose palmitate, PDA & sorbitan stearate used as positive controls, negative control omitting 95% functionalised carbohydrate as carbon source on a single agar plate.

confirms the ability of this fungal species to break the ester bond present within these functionalised carbohydrates, under controlled laboratory conditions, and to use the cellulose as a carbon source for further growth. Fungi, in nature, have been shown to secrete extracellular lipases to break down materials to obtain carbon and nitrogen for their energy source.[30] The lipid substrates are typically triacylglycerols and many oils such as olive, palm, canola, coconut and corn. In addition, studies have shown that the presence of nitrogen could influence fungal lipase production.[36] Other factors influencing lipase production, under natural conditions, could include moisture content, surfactants, and temperature.[30] At the end-of-life, if these biopolymers are discarded in nature where fungal conditions are optimal, then it is apparent that they can be broken down by soil microbes and not persist in that environment.

4. Conclusions

While a number of carbohydrate packaging materials have been developed, these unfunctionalized carbohydrates lack sufficient water barrier properties to replace the vast majority of petroplastics. Functionalisation of the carbohydrate with a fatty acid chain improves the water barrier, however this hydrophobicity has been shown to delay break down in nature. In this study the biodegradation of functionalised cellulose was examined under controlled conditions, examining the effect of specific enzymes, whole cell and a modelled bird gut.

Our results show that the cellulase CTEC2 enzyme mixture was unable to break down the functionalised carbohydrates in isolation. However, lipase enzymes acted on the fatty acid ester bond, releasing palmitic acid and exposing the carbohydrate backbone. On the dual use of lipase and cellulase together, palmitic acid and glucose are released rapidly. This finding suggests a combination of enzymes commonly found in nature can break down functionalised carbohydrates.

Similarly, in a model avian digestive tract, the cellulose palmitate was broken down into cellulose and palmitic acid by the combination of pH and the pancreatic enzymes. It is likely that these materials can indeed be broken down under conditions typical of a seabird's gut. This is a significant finding, suggesting that there will be little bioaccumulation of the packaging material if it does enter the food chain.

Finally, a suitable microorganism for catalysing the breakdown of the polymer was recovered from a compost heap. Under controlled laboratory conditions, the common soil fungus *Mucor* sp. could grow on both cellulose acetate and cellulose palmitate as a 95% carbon source. The *Mucor* grew well, demonstrating its ability to break the bonds and use the carbohydrate and palmitic acid as carbon sources.

Our findings are a major step towards proving functionalised carbohydrates not only have superior polymer properties but are also susceptible to break down in the environment, especially through fungi commonly found in the soil. This work demonstrates that functionalised carbohydrates are an extremely promising future material for replacing petrochemical-derived non-biodegradable plastics within food packaging industry.

Author contributions

Katrina Entwistle: investigation, formal analysis, writing – original draft. Sandhya Moise: resources, supervision, writing – review and editing. Christopher J. Chuck: conceptualisation, project administration, resources, supervision, writing – review and editing. Fatma Guler – investigation. Katherine A. Smart – review. Matthew Crow – review.

Conflicts of interest

The authors declare no competing financial interest.

Data availability

The data supporting this article have been included as part of the supplementary information. See DOI: **https://doi.org/10.1039/d5fd00040h**.

Acknowledgements

The authors gratefully acknowledge the sponsorship by Diageo.

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