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Sustainable Galactarate-Based Polymers: Multi-Enzymatic Production of Pectin-Derived Polyesters

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Dedicated to Salvatore Barone

Large amounts of agricultural wastes are rich in pectins that, in many cases, disrupt the processing of food residues due to gelation. Despite pectins being a promising sustainable feedstock for bio-based chemical production, the current pathways to produce platform molecules from this polysaccharide are hazardous and entail the use of strong acids. The present work describes a sequence of biocatalyzed reactions that involves 1) the extraction of pectin from sugar beet pulp and enzymatic recovery of galacturonic acid (GalA), followed by 2) the enzymatic oxidation of the GalA aldehyde and the recovery of galactaric acid (GA), and 3) the biocatalyzed polycondensation of GA to obtain fully bio-based polyesters carrying lateral hydroxy functionalities. The acid-free pectin extraction is optimized using enzymes and microwave technology. The conditions for enzymatic oxidation of GalA allow the separation of the GA produced by a simple centrifugation step that leads to the enzyme-catalyzed polycondensation reactions.

Evidence showing increasing pollution caused by single-use plastics is the driving force that leads companies around the world to develop novel materials derived from renewable resources that can comply with new legislation and regulations that will soon be enforced in several countries.[1]

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Pectin rich biomass by-products from the food industry represent a potentially important source of non-food residues suitable for sustainable bio-based chemical production.[2] Sugar beet is a major crop in northern Europe, with 8 million tonnes produced annually in the UK alone. For each kilogram of sugar produced from sugar beet, ≈0.85 kg of Sugar Beet Pulp (SBP) is produced after sucrose extraction. Pectins, particularly the ones extracted from SBP, are rich in C6 sugar acids (uronic acids, UA) that can be converted to aldonic or aldaric acids (AA) using biochemical routes, or to 2,5-furan dicarboxylic acid (FDCA) esters avoiding the unstable intermediate 5-hydroxymethylfurfural (HMF).[3,4] Although they have a range of potential applications, UAs have not been exhaustively exploited as platform molecules for the synthesis of high added-value products due to their high market price. Despite extensive efforts, no commercial scale chemical production of uronic acids or uronic acid derivatives exists at present.[3] Several methods have been investigated to replace the use of strong acids for pectin extraction from different biomasses.[5] Substantial progress has been made in the purification of UA from hydrolysis mixtures. Càrdenas-Fernàndez et al. reported the application of centrifugal partition chromatography (CPC) for simultaneous separation of all the pectin derived monosaccharides. CPC appears to be a promising separation technique for biorefinery applications, allowing a one-step recovery of the two main monosaccharides present in SBP, i.e., Ara and GalA, with high yields and adequate purity from crude hydrolysate.[6]

The conversion of UA to AA such as galactaric (GA) or glucaric acid (GlcA) is traditionally carried out via HNO_3-catalyzed oxidation, with the process usually limited by the yield and by the hazards associated with the use of concentrated strong acid.[7,8] Alternatives to this process are gold-catalyzed aqueous catalytic oxidation of AA under alkaline or base-free conditions, both providing efficient and highly selective reactions.[9,10]

An alternative strategy to oxidize UA to AA is via mild biochemical route using enzymes. Examples of biotransformation of GalA into GA by engineered microorganisms based on GalA metabolism have been reported.[11–13] The enzyme responsible for the oxidation in these studies is uronate dehydrogenase (UDH). The industrial in vitro exploitation of UDH is not viable due to the requirement of expensive NADH as a cofactor.

A pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH) immobilized on an electrode was used for the production of GA.[14] This biotechnological electrosynthesis is an appealing alternative because it does not require expensive cofactors or O_2, and can be easily applied for the oxidation of other bioderived monomers, showing a great potential for the development of low-cost and reagentless glucose-based sensors and biofuel cells.[15,16]

Using AA as the diacid component for making polymeric materials can represent a more environmentally friendly path for the synthesis of bio-based functional polymers fully derived from renewable resources. To the best of our knowledge, there are limited reports of AA-based polymerization strategies. These strategies involve two routes. The first strategy, reported by Rosu and co-workers, involves the esterification of GA with ethanol via Fisher esterification, followed by the protection of the hydroxy groups via the addition of 2,2-dimethoxypropane. The monomer is subsequently polymerized via aminolysis with a primary diamine and finally, the hydroxy groups are de-protected with the addition of trifluoroacetic acid (**Scheme 1**A).[17] A similar protocol was used for the synthesis of rigid polyamides having elevated glass transition temperatures (Scheme 1B) and for the dibutyltin (IV) oxide-catalyzed synthesis of acetalized galactarate polyesters (Scheme 1C).[18,19] The second strategy involves the conversion of GlcA to the glucaric acid chloride acetate and its polymerization with various diols (Scheme 1D) and diamines (Scheme 1E) in dimethylacetamide solution or by interfacial polymerization in water and chloroform solutions.[20,21]

In the present work, we describe a novel three step biocatalyzed strategy that involves: 1) the extraction of pectin from sugar beet pulp and the recovery of GalA; followed by 2) the laccase-catalyzed oxidation of the GalA aldehyde and the recovery of GA; and 3) the enzymatic polycondensation of GA to obtain fully bio-based functional polyesters carrying lateral hydroxy functionalities without the use of traditional protection chemistry (**Scheme 2**). The acid-free extraction of pectin was optimized using enzymes and microwave technology, while the use of laccases for the enzymatic oxidation of UA was investigated for the first time. The selected reaction condition allows easy recovery of the GA from the reaction mixture with a centrifugation step. GA is subsequently used in the polymerization step, after a simple Fisher esterification to improve its solubility.

SBP is the main by-product of sugar processing and is currently dried and sold as low value animal feed. In this work, SBP was selected as a biomass rich in pectin that might be used as a sustainable feedstock for biopolymer production. An innovative fully biocatalyzed route consisting of three steps was designed and assessed: i) recovery of GalA from SBP pectin, ii) enzymatic oxidation of GalA to GA, and iii) polymerization of GA to 100% bio-based polyesters carrying lateral functionalities (Scheme 2).

Pectin extraction from micronized SBP was performed using two different acid-free methodologies: a) enzymatic and b) microwave (MW) assisted. Three different enzymatic preparations: Viscozyme L, Celluclast 1.5, and Pectinase from *Aspergillus niger* were tested using two different loadings (50 and 100 µL, see Experimental Section for details on the used protocol). For the MW-assisted extraction instead, two solvents (H_2O and 50 mM 2-[[2-[Bis(carboxymethyl)amino]cyclohexyl]-(carboxymethyl) amino]acetic acid hydrate (CDTA) solution pH 6.5), two tem-

peratures (120 and 140 °C), two biomass:solvent (w v-1) ratios (1:6 and 1:10), and two extraction times (5 and 10 min) were tested. All reactions were precipitated with ethanol to collect the ethanol-insoluble pectin-enriched material (PEM) (**Figure 1**).[22]

The monosaccharide composition of PEM from different extractions was determined after trifluoracetic acid (TFA) hydrolysis with the optimized length and temperature of the hydrolysis to maximize the yield of UA (GalA is susceptible to degradation under some conditions).[23] For SBP the best conditions for GalA recovery via TFA-catalyzed acid hydrolysis were 2.5 h and 120 °C. Data obtained with the optimized TFA hydrolysis protocol was used as a benchmark for the sugar composition and the recovery of GalA using enzymes. The degree of esterification (DE), determined via ATR-FTIR, was significantly different for each extraction protocol (Table S1, Supporting Information).

The most efficient enzymatic extraction was performed with Viscozyme L. The Viscozyme L preparation includes a diversity of enzymes and the higher pectin yields compared to Pectinase treatments suggest that multiple activities are required for an efficient pectin extraction from SBP (Figure 1A).[24]

MW-assisted extractions produced higher PEM yields when higher temperatures were used (Figure 1B,C). Interestingly, the DE of the extracted material can be modulated by using different solvents. CDTA decreased the average DE (from ≈50% to ≈30% compared with the extractions in water) and increased the amount of extracted low esterified pectin. The likely reason for this is that CDTA is a powerful chelator and unesterified pectin is crosslinked into the cell wall through electrostatic interactions mediated by Ca^{++} ions bridging between carboxyl groups in the polygalacturonan. If the PGA is extensively methylated, there is less Ca crosslinking, so with CDTA more unesterified material is pulled out of the wall than when using just water. MW-assisted extraction in the presence of CDTA not only resulted in a significant increase in PEM, but also in GalA yields (Figure 1C). In contrast with extractions in water, where longer extraction time resulted in higher yields (Figure 1B), CDTA extraction for longer time only resulted in higher GalA yields when 1:10 biomass–solvent ratios were used (Figure 1C). With 1:6 biomass ratios, longer extraction times resulted in lower yields, probably due to degradation processes.

Pectin extracted at different temperatures showed different properties after precipitation and washes with ethanol. Pectin extracted at 120 °C is less dense in ethanol than pectin extracted at 140 °C (Figure S1, Supporting Information). No significant DE differences were observed in the extraction experiments performed at different temperatures using water or CDTA as solvent, suggesting that the different properties of pectins could be due to different molecular weights. Size exclusion chromatography (SEC-MALLS) revealed that extractions at 140 °C (both using water or CDTA) led to polymers with higher molecular weight with a bimodal size distribution with low molecular weight species (<500 Da) that were also observed for all samples (Figure S2, Supporting Information).

The highest yields of soluble GalA recovered were obtained using 100 µL of Viscozyme L and microwave-assisted extractions with CDTA (5 min, 1:6 ratio, 140 °C), which released 85 and 116 mg of GalA g−1 of biomass, respectively.

In the present work, we aim for a fully biocatalyzed process from feedstock to product. PEM derived from the two best
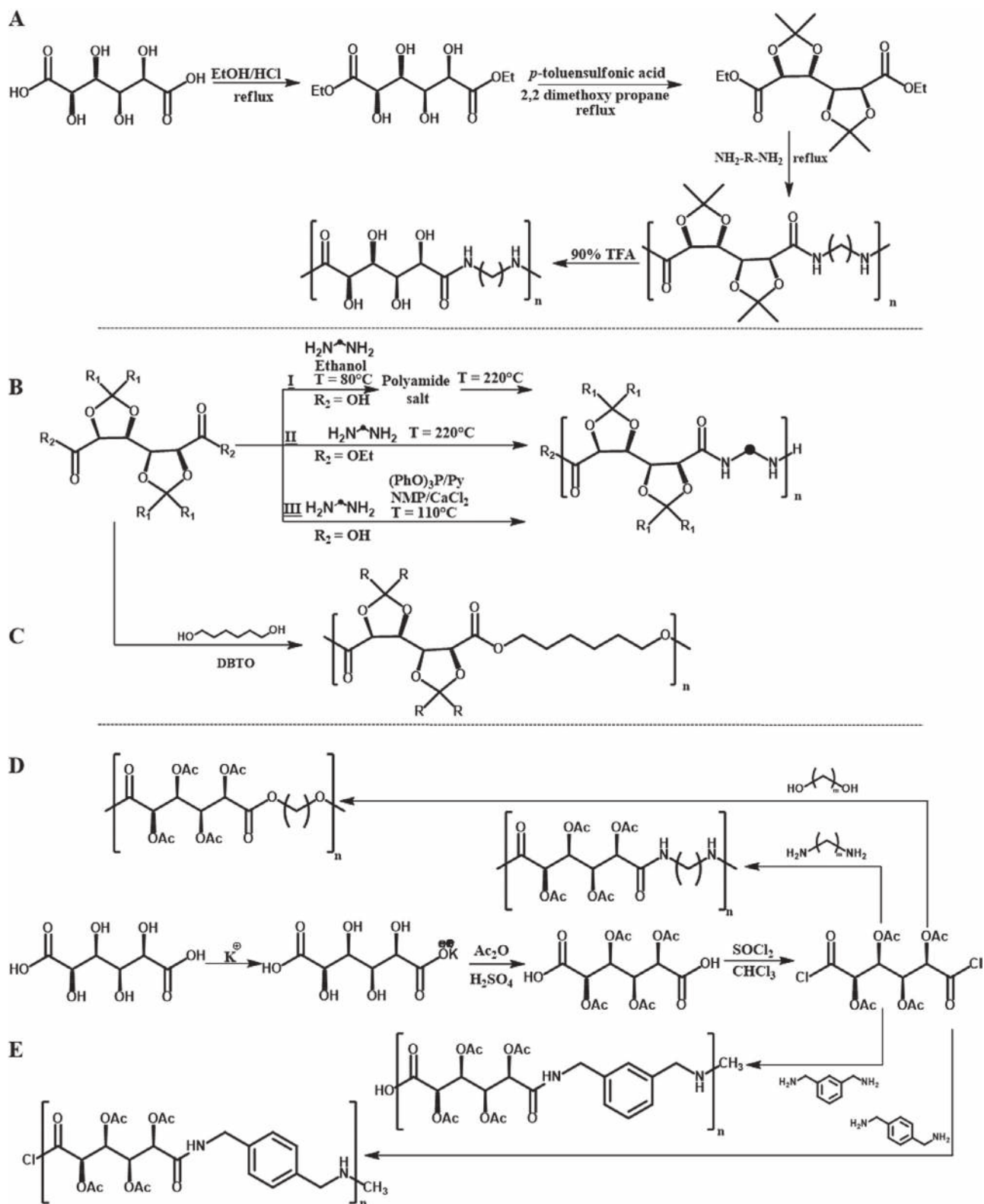
Scheme 1. GA polymerization strategies. A–E) AA-based polymerization strategies previously reported in the literature.

extractions of micronized SBP, were selected for enzymatic hydrolysis testing three different enzymatic preparations: Viscozyme L and Pectinase from *A. niger* (already used for pectin extraction) and an endo-polygalacturonase (e-PGA). Pectinase and e-PGA were used at 50 and 40 °C, respectively, while Viscozyme L was tested at both temperatures due to its broader
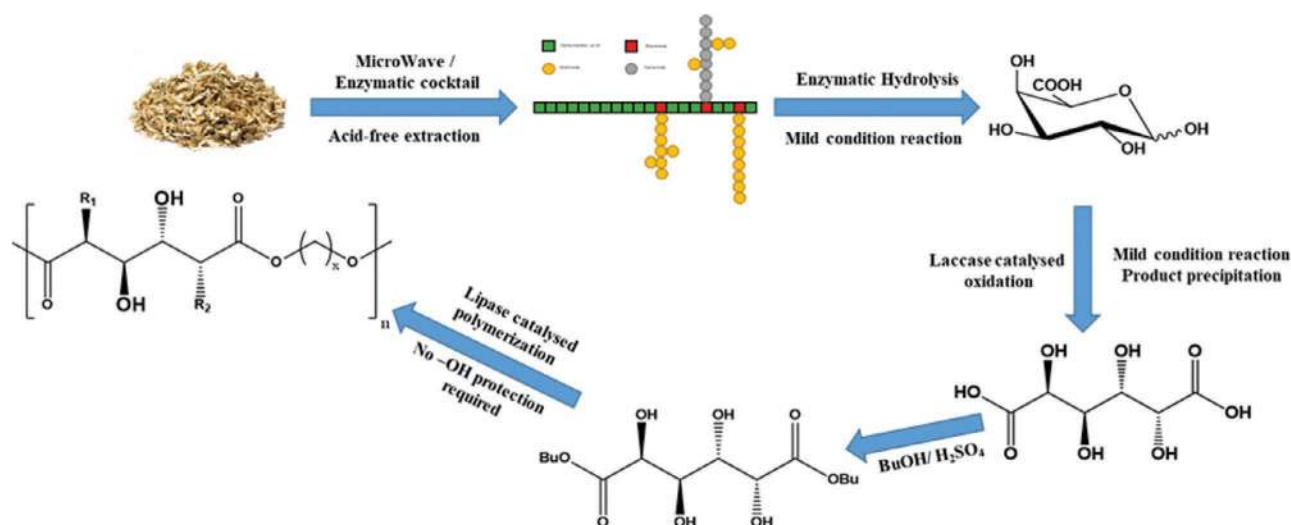
Scheme 2. Enzymatic GA polymerization strategy. The three-step biocatalyzed strategy used in this work for the extraction (1), oxidation (2), and polymerization (3) to bio-based GA-based semi-linear polyesters having lateral functionalities that could be readily used as anchor points for post-polymerization modifications.

temperature stability and activity in tested conditions. Hydrolysis combining a 1:1 mix of two enzymes was also tested at both temperatures (**Table 1**).

Table 1 shows that in pectin extracted with Viscozyme, the yield of GalA from enzymatic hydrolysis is similar to chemical hydrolysis. An enzymatic efficiency around 80% was achieved for all tested conditions, with exception of Pectinase.

For the MW-extracted PEM (Table 1), only Viscozyme L was effective in releasing GalA with an efficiency >80%, while e-PGA incubation produced very low amounts of GalA, most probably because of methyl esterification. The requirement of a more complex enzymatic cocktail to complete the hydrolysis of PEM extracted with MW reflects the higher molecular weight and structural complexity of the extracted pectin (Table S2, Supporting Information). The different results obtained in each extraction could be due to compositional differences in the PEM. In particular, Ara could be playing a crucial role in determining the yields. The amount of Ara is higher in the MW-extracted pectin (Figure S3, Supporting Information). Arabinan side ramifications in the pectin may hamper the activity of e-PGA and Pectinase enzymes. In pectin extracted with Viscozyme L, the hydrolysis starts during the extraction due to the side activity of this enzymatic cocktail. The esterase activity of the Viscozyme L cocktail may therefore make the pectin more susceptible to glycosyl hydrolases and lyases after de-esterification.[24] Although the chemical hydrolysis of microwave PEM showed the highest GalA yield, enzymatic hydrolysis produces large amounts of Ara that can be a valuable side-product while yielding >80% of GalA.

We investigated mild conditions for the oxidation of the GalA obtained as a second step in the production of polymers. Currently the main strategies for the UA oxidation require harsh conditions such as strong acid or strong alkali. To the best of our knowledge, there is only a single example of in vitro enzymatic oxidation of UA under mild conditions, based on a pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH) that uses Methylene Green (MG) as electron acceptor for the oxidation. This reaction, despite being novel and with potential application as a bioanode in a biofuel cell, has two main drawbacks: 1) it catalyzes the oxidation of GalA to GA, but also to its cyclic form (**Figure 2**), and 2) the PQQ-GDH is an expensive enzyme (≈20 USD kU^{-1}) and requires an MG cofactor, making the scale up of this reaction rather complicated.[15]

Laccases may represent a substitute for PQQ-GDH. Laccases are multicopper oxidases that catalyze the oxidation of an array of aromatic substrates with concomitant reduction of molecular oxygen to water. These enzymes are being evaluated for a variety of biotechnological applications due to their broad substrate range, which can be further expanded by using mediators for the electron transfer.[25]

Two laccases were selected: one from *Myceliophthora thermophila* (MtL) and one commercial laccase preparation (Amano Enzymes Inc). These laccases were selected due to their activity under conditions similar to the ones reported for the PQQ-GDH (phosphate buffer pH 7, 30 mM GalA). Laccases were tested in combination with two mediators: (2,2,6,6-tetramethylpiperidin-1-yl) oxyl (TEMPO) and *N*-hydroxyphthalimide (NHPI) in three different substrate:mediator ratios (100:1; 50:1; and 10:1). The reactions were performed in deuterated buffer to study the product distribution using ^{1}H-NMR spectrometry without any purification step (Figure 2A). The oxidation reactions were incubated for 2, 4, 8, and 12 days.

At 1:10 substrate:TEMPO ratio, we detected high amounts of GA obtained by laccase oxidation of GalA (≈40% of the reaction after 12 days). These results are similar to those observed using PQQ-GDH (Figure 2B). In reactions performed using TEMPO at 50:1 ratio, GA was found as product of the oxidation, but the conversion yield was lower than 10%, making the conditions not suitable for a large-scale exploitation (Figure S4, Supporting Information). Only very little, not quantifiable amounts of GA were obtained using the lowest amounts of TEMPO (100:1 ratio) and for all reactions using NHPI as the mediator. The oxidation probably proceeded in a different way according to the enzyme-mediator couple and TEMPO was the best mediator for GalA oxidation in all tested conditions (Figure S4, Supporting Information). The
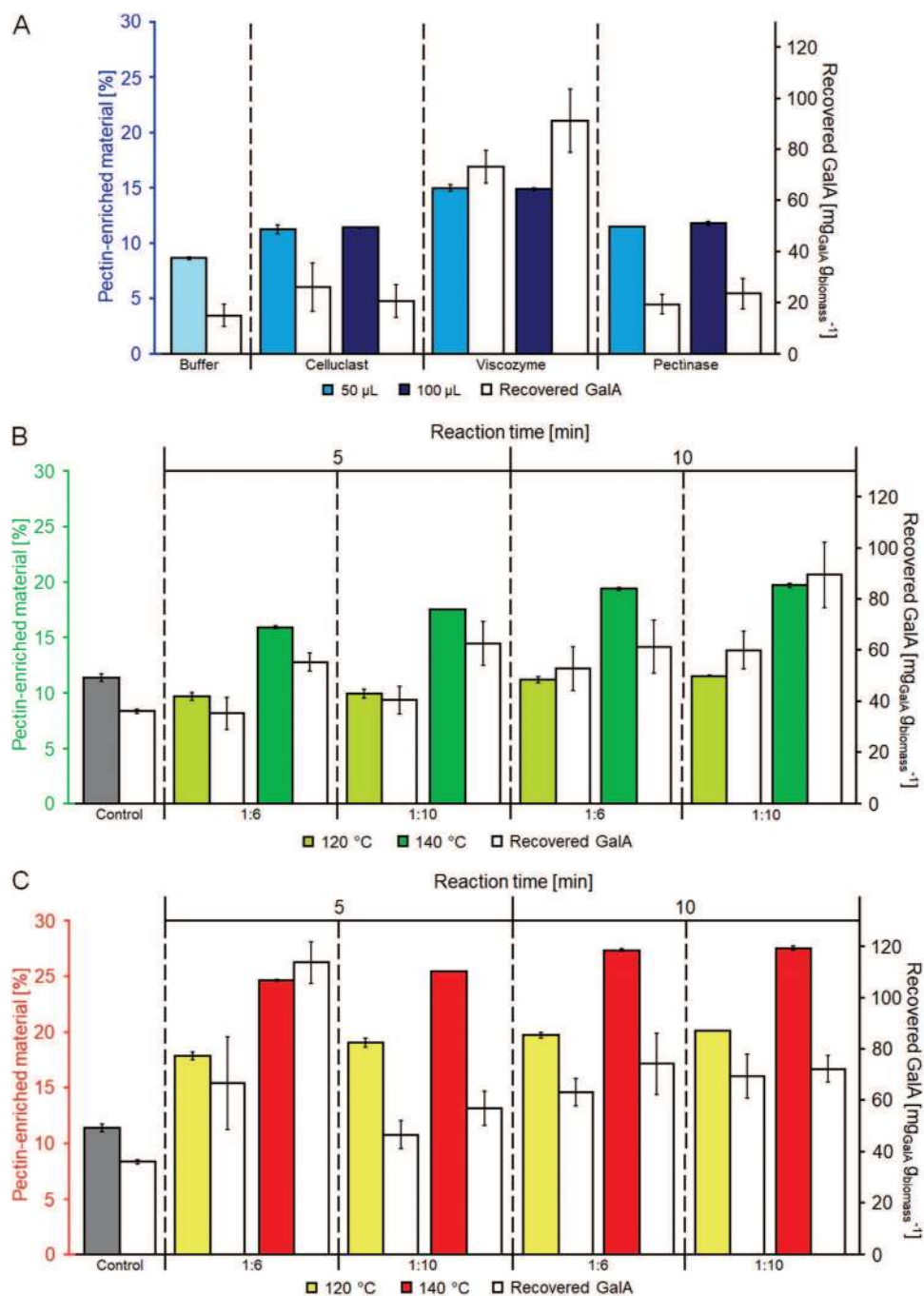
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Figure 1. Extraction of pectin from SBP. Pectin extractions from micronized SBP (colored bars) and relative GalA yield (white bars). Extractions were performed using A) enzymes, B) microwaves/water, and C) microwaves/CDTA.

results obtained with a 1:10 TEMPO ratio open the possibility for the exploitation of Laccase Mediator Systems (LMS) for convenient and sustainable enzymatic oxidation of uronic acid.

Since GA is only slightly soluble in acid solution (pH ≤ 3), lower pH was used to favor the purification of the desired product by precipitation. Acidity would also catalyze the hydrolysis of the lactone species increasing the yield in GA with the use of low pH laccases.

To explore this possibility, the laccase from *Trametes versicolor* (TvL) was tested for the oxidation of GalA at pH 3. The enzyme was tested in combination with two mediators, but due to the low solubility of NHPI in acid, it was replaced by 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). ^{1}H-NMR spectra shows that the selected TvL enzyme was effective for the selective oxidation of GalA to GA in combination with the mediator TEMPO.

Table 1. Comparison between MW-assisted and enzymatic hydrolysis of PEM. TFA was considered as the standard protocol.

Treatment	Catalyst	T [°C]	Recovered GalA [mg g$_{biomass}^{-1}$]	Efficiency [%]
Enzymatic	TFA*		91	100
	Viscozyme	40	77	84
	e-PGA		68	74
	Mix		74	81
	Viscozyme	50	70	76
	Pectinase		63	69
	Mix		69	75
MW-assisted	TFA*		117	100
	Viscozyme	40	102	87
	e-PGA		3	3
	Mix		103	88
	Viscozyme	50	77	66
	Pectinase		7	6
	Mix		72	62

To approach the first step of industrial scale-up and to evaluate the product precipitation, the TvL-TEMPO LMS method was tested using a substrate-enzyme ratio (mM:U) reduced from 15:1 to 30:1 and 100:1 for 150 and 500 mM GalA mixture, respectively. In all scaled up mixtures, we observed precipitation of GA (Figure 2C).

The LMS-catalyzed oxidation of GalA in acid environment represents the first example of in vitro enzymatic conversion of UA into an aldaric derivate in mild conditions with the simultaneous product precipitation. Although the method will require further process optimization, it opens new prospective paths for industrial exploitation of sustainable enzymatic production of this class of di-acid platform molecules.

Once GA was obtained and purified, we explored the enzymatic polymerization as an alternative to the protection strategies required in traditional chemo-catalytic methods. For this aim, a commercial immobilized lipase from *Candida antarctica* (CaLB) was selected due to its known stability to high temperatures and different solvents.[26,27] Solubility tests of GA in solvents suitable for the enzymatic polymerization highlighted the necessity of alternative strategies. The high melting temperature of GA (>140 °C) does not allow enzymatic polymerization in bulk. Several infrequently used solvents such as pyridine and DMSO were tested but only very limited (<5%) or no monomer conversion was observed. The approach of using the GA diacid as monomer was replaced using GA diesters and subsequently, diethyl (DEM) and dibutyl (DBM) esters of GA were prepared via Fischer esterification.

The solubility of the diester substrates drastically increased in several solvents suitable for enzymatic polymerization. In particular, two high boiling point solvents, namely diphenyl ether (DPE) and the levoglucosenone-derived Cyrene were selected for the reaction.[28] Using Hansen Solubility Parameters, the solubility values of DEM and DBM in DPE were predicted, indicating that DBM is the best monomer for the polycondensation reaction (Table S3, Supporting Information).
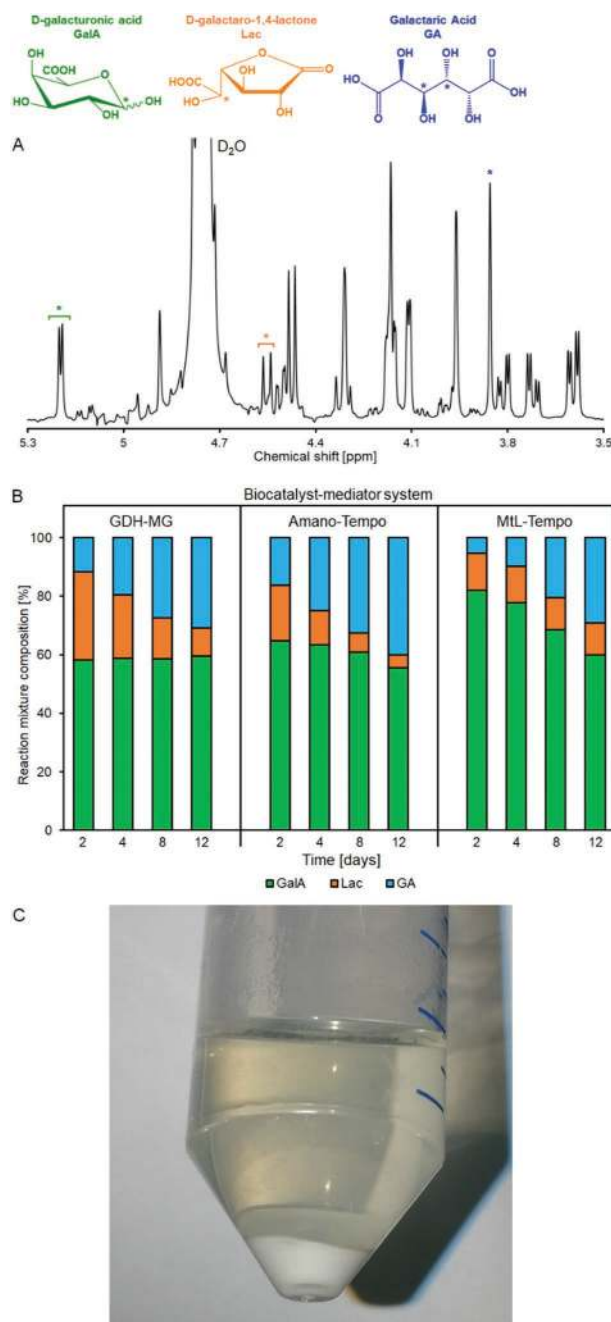

Figure 2. Enzymatic oxidation of GalA. A) ^{1}H-NMR of the reaction mixture with assignment of the components, B) time course of the reaction mixture composition, and C) photograph of the oxidation to GA and its easy separation via precipitation in acidic conditions.

DBM polymerizations were performed with two different diols, the polar 1,4-butanediol (BDO) and the more hydrophobic 1,8-octanediol (ODO) in DPE (100 °C) and Cyrene (100 and 70 °C). In all reactions performed at 100 °C, we recovered a methanol-insoluble polymer, which was further characterized using ATR FT-IR spectroscopy, MALDI-TOF–MS and TGA.

FT-IR analyses of the purified products confirmed the presence of bonds typical of polyesters for all reactions (**Figure 3**A). Although we did not observe a substantial shift of the
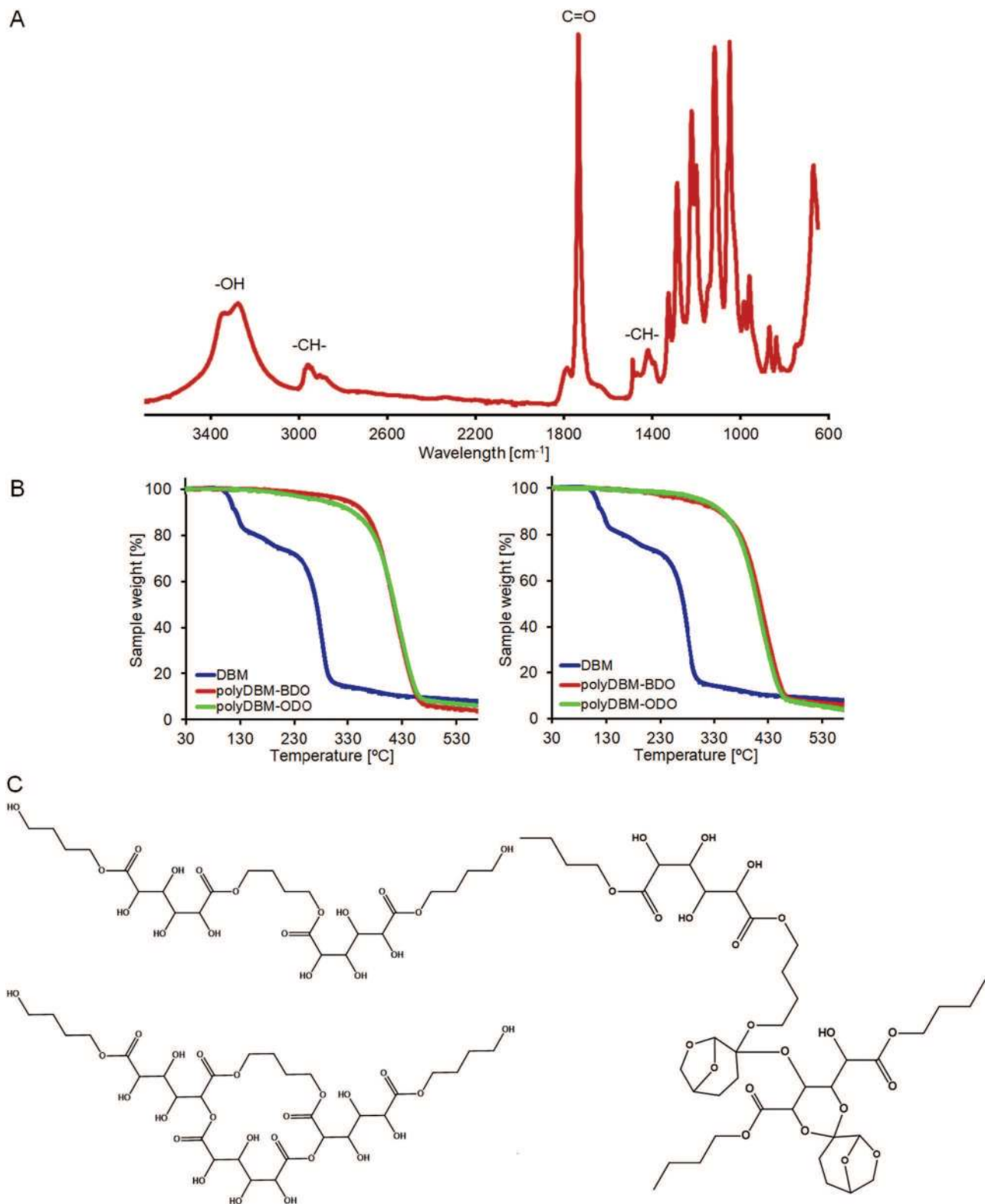
Figure 3. Enzymatic polymerization of DBM. A) Assigned FT-IR spectra of polyDBM-BDO, B) TGA of the polymers obtained in DPE (left) and Cyrene (right) at 100 °C using BDO (red) and ODO (green) as the diols and their comparison with the unreacted monomer DBM (blue), and C) main structures identified via solvent-free MALDI analysis of the reaction products.

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C=O signal between DBM (1735 cm^{-1}) and its polymerization products (1723–1735 cm^{-1}), a reduction of intensity of free −OH stretching around 3000 cm^{-1} observed for the polymer precipitated from the Cyrene reaction system suggests that the structure of the obtained polyesters is different from the polymers recovered from DPE (Figure 3A).

Thermogravimetric analysis (Figure 3B) revealed a significant increase in the degradation temperature for all the polymers compared to the monomeric diester (Table S4, Supporting Information for the complete set of TGA data). The T_d50 values of polymers purified from the reactions in Cyrene (410 and 419 °C for the polymers containing BDO and ODO, respectively) are similar to the products obtained in DPE (415 °C for BDO and 416 °C for ODO) supporting the hypothesis that the functional polymers obtained in the various solvents have only minor structural variations.

All the products synthesized at 100 °C were insoluble in the solvents used for NMR spectroscopy and gel permeation chromatography, making such analysis inapplicable. The molecular weight distribution was therefore evaluated using a solventless MALDI-TOF–MS. For reactions performed in DPE, polyesters up to 2000 and 1100 Da were identified for the reactions with BDO and ODO, respectively. From the analysis of the mass spectra, it was also possible to recognize the formation of branched species with a GA molecule establishing a bridge between two secondary hydroxyl groups of the same molecule (Figure 3C).

Comparing the obtained results with the ones previously reported in the literature, the work of which the reaction products are most similar to the ones reported here is the 2016 paper from Wu and co-workers that synthesized D-glucaric acid acetate derived polyesters and polyamides.[20] While all reported polyamides have $M_w > 6000$ Da, also in this case, the M_w of the obtained polyesters was rather low ($M_w < 1000$ Da) despite the applied protection strategy.

The present work describes an innovative, fully enzymatic strategy for the production of polyesters, from the recovery of GalA from SBP pectin to the polymerization of GA into 100% bio-based polyesters carrying lateral functionalities. This work has a potential technological and industrial impact as a streamline alternative to chemically catalyzed multistep processes proposed in the literature.[15]

The extraction of GalA from SBP derived pectins was optimized using an MW-assisted method. So far, it has been reported that using ultrasonic-assisted treatment combined with subcritical water (Us-Sc) and organic acids (OA) methods, it is possible to recover 24.6% (GalA 59%) from Us-Sc and 17.2% (GalA 75.8%) amount of PEM.[22,29,30] The MW-assisted extractions reported here produced higher PEM yields when higher operational temperatures were used. The DE of the extracted material was modulated by using different solvents with CDTA that decreased the average DE (from ≈50% to ≈30% if compared with the extractions in water). The MW-assisted extraction in the presence of CDTA also resulted in a significant increase in PEM as well as GalA yields (Figure 1). In all tested conditions, it was possible to recover pectin with a DE lower than the one of acid extracted pectins (>50% for pectin from orange or grapefruits). The MW of extracted pectin was higher than the one of microwave-extracted pectin from mango peels.[31]

The LMS-catalyzed oxidation of GalA in acid environment using a laccase, represents the first example of an in vitro enzymatic conversion of uronic acid into an aldaric derivate in very mild conditions (30 °C; pH 3) with the simultaneous GA precipitation, therefore allowing an easy and inexpensive recovery of the reaction product (Figure 2). So far, this process has been done using acids and metals while the here presented process represents a sustainable way of avoiding this using a fully biocatalyzed approach at mild conditions.[7–9]

The polymerization of the diester derivative of GA was also carried out using an enzymatic approach. In the literature, all works on the topic used various protecting strategies (Scheme 1A–E) together with traditional chemo-catalytic procedures. Here, we present a novel, biocatalyzed two-step one-pot protocol that allows the synthesis of GA-based linear polyesters keeping the secondary hydroxyl groups free for further functionalization (e.g., crosslinking to coating materials). The side reactions (reactivity of the secondary hydroxyl groups with the solvent or with the monomers) were contained due to the very mild conditions used to carry out the reaction (Figure 3). This process, as demonstrated in the present work, is easily scalable and does not require expensive biocatalysts or mediators like in the previously reported methods.

To further elucidate the structure of the polymers produced in Cyrene, we analyzed the nonpurified and methanol-soluble products of the reaction performed at 70 °C. Although a complete characterization of these products was not possible due to the amount of solvent present in the sample, MALDI-TOF spectroscopy suggests that some side reactions, such as ketal formation, occur with Cyrene. This side reaction leads to polymers similar to the ones obtained from Gavrila and co-workers that therefore lose all the side chain functionalities that are instead fully preserved using our synthetic method.[19] The formation of ketals and hemi-ketals between the −OH groups of the substrates (both DBM and diols) and the C=O of Cyrene may explain the peaks observed in the MALDI-TOF mass spectrum (Figure 3C). Molecules with this kind of bonds over 2000 Da can be in fact assigned to the MALDI-TOF mass spectra signals for the DBM-ODO reaction in Cyrene at 70 °C. This work demonstrates that it is possible to produce bio-based materials in an environmentally friendly way, starting from non-edible by-products of the food industry in a circular economy context.

Experimental Section

Chemicals and Enzymes: Efficiency Technologies Ltd provided SBP ground using their micronizing technology and SBP milled using a conventional milling process to 1 mm particles (see Table S5, Supporting Information). A benchmark compositional analysis of pectin extracted from SBP was established: Ash 1.02%; Moisture 11.97%; solubles 23.43%; matrix polysaccharides 19.65%; cellulose 13.48%; lignin 8.42%; pectin 12.37% (estimated on extraction of 10 mg of biomass); protein 9.5%; starch 11.9%; and total oil 2.9% (ether extract 2.23%). Methylene green (Basic Green 5) was purchased from La Santa Cruz Biotechnology, Inc. 2,2,6,6-Tetramethylpiperidine 1-Oxyl Free Radical (TEMPO) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from TCI. PQQ dependent Glucose dehydrogenase was purchased from Sorachim. Laccase M120 was kindly provided by Amano Enzyme Inc. All other chemicals, enzymes and solvents were purchased from Sigma–Aldrich and used as received if not otherwise specified.

Pectin Extraction: *Control*: Biomass (1 g) was extracted with water (6 mL) at 120 °C for 1 h. The supernatant was removed and the biomass was extracted with CDTA 50 mM pH 6.5 (6 mL) at 30 °C for 16 h. Both soluble fractions were pooled together.

Pectin Extraction: *Microwave Assisted Pectin Extraction*: Microwave-assisted pectin extraction was carried out on micronized dry powdered of sugar beet pulp, using an Anton Paar Monowave 300 microwave using a closed vessel. Biomass (1 g) was loaded into the reaction vessel along with a microwave safe stirrer bar and the prescribed amount of (6 or 10 mL) of extracting solvent added (water or CDTA solution 50 mM, pH 6.5). The microwave reaction temperature was set at 120 or 140 °C for the desired amount of time. This protocol was adapted from Matharu et al. 2016.[31]

Pectin Extraction: *Enzymatic Extraction*: Enzymatic extraction of pectin from sugar beet pulp (solid/liquid ratio 1 g/12 mL) was performed with the use of Celluclast 1.5L, Viscozyme L, and Pectinase from *Aspergillus niger* (prepared 15 mg mL⁻¹) at a dose of 50 and 100 µL g⁻¹ of biomass. All the enzyme preparations were desalted prior the use with a HiTrap Desalting Column in sodium acetate buffer pH 5, 100 mM. The extractions were performed in 50 mL plastic tube. The process was conducted for 24 h at 50 °C, in Sodium Acetate buffer pH 5 100 mM with constant shaking. This protocol was adapted from Wikiera et al.[23]

Pectin Extract Material Precipitation: After the extraction, the samples were cooled to 20 °C and centrifuged (4000 rpm, 30 min, 20 °C). From each reaction, 7 mL of the supernatant was collected and precipitated with 35 mL of cold ethanol (−20 °C). Samples were stored at 4 °C overnight in order to allow the complete precipitation of pectin. The precipitated pectin was collected by centrifugation (4000 rpm, 30 min, 4 °C), washed twice with fresh cold ethanol (vortex), centrifuged as done previously, and the pellets were dried at 42 °C for 24 h until the constant weight was achieved.

Pectin Hydrolysis: *Chemical Hydrolysis*: Hydrolysis was performed in Eppendorf tubes. Samples (4 mg) were treated with 2 M 0.5 mL of 2 M TFA. All samples were flushed with Argon, closed tightly, mixed, and incubated at 100 or 120 °C. Next, the hydrolyzed samples were evaporated, washed three times with isopropanol. Dried samples were dissolved in deionised (Milli-Q) water. Sugar composition determination was performed in an ion chromatograph system (ICS 2500, Dionex, California), equipped with pulsed amperometric detection and a CarboPac PA1 anion exchange column, using a 5 mM NaOH solution as the mobile phase (flow rate 0.25 mL min⁻¹).

Pectin Hydrolysis: *Enzymatic Hydrolysis*: Hydrolysis of pectin obtained from micronized SBP was done with three different enzymes.[32] Three different enzymes, i.e., Viscozyme L, endo PG-M2, and Pectinase from *Aspergillus niger* (15 mg mL⁻¹ in sodium acetate buffer 100 mM, pH 5) were used. All the enzymes used here are known for their diverse pectinase activity. All the enzyme preparations were desalted prior the use with a HiTrap Desalting Column in Sodium Acetate buffer pH 5, 100 mM. All the desalted commercial enzyme solutions were diluted 50×. The hydrolysis was then conducted at 10% v/v of the diluted enzyme/pectic solution 0.5% w/v. The sample was hydrolyzed for 24 h at 40 or 50 °C. The enzymes were inactivated by thermal treatment at 100 °C for 5 min, the supernatants were collected, filtered, and assayed for free monosaccharide composition on the Dionex system.

Pectin Hydrolysis: *Enzymatic Oxidation*: The pH values were adjusted to 6.7 (pD 7.1) for 0.2 M deuterated phosphate buffer and 2.7 (pD 3.1) for 0.2 M deuterated citrate buffer. All reactions were performed at 30 °C with 30 mM GalA in prepared buffer. Reactions with PQQ-GDH were performed with 30 mM methylene green (MG) as electron acceptor for the oxidation and 0.25 mg mL⁻¹ of enzyme as described by Sakuta et al.[15] Reactions with laccases were performed with 2 U mL⁻¹ of enzyme and different amounts of mediator: 0.3, 0.6, and 3 mM. ¹H-NMR measurements of the mixtures were conducted after 2, 4, 8, and 12 days.

Scale-up reactions were performed using 5 U mL⁻¹ of TvL in 0.2 M citrate buffer pH 3.1 with 150 or 500 mM of GalA with a substrate:mediator ratio 10:1. GA was collected after 8 days by centrifugation (4000 rpm, 10 min, 25 °C).

Enzymatic Assays: *PG Activity*: Polygalacturonase (PG) activity was assayed for 15 min with a 0.2% solution of polygalacturonic acid. The number of reducing groups, expressed as galacturonic acid released by enzymatic action was quantified by the DNS reagent assay and monitored by the absorbance of resulting colored mixture at 540 nm.[33] One unit (U) of PG activity was defined as the amount of enzyme releasing 1 µmol of galacturonic acid per min under assay conditions. The activity of Viscozyme, Pectinase, and endo PG-M2 expressed as PG units was determined to be 4350, 3370, and 2800 U mL⁻¹, respectively, at 40 °C.

Enzymatic Assays: *Laccase Activity*: Laccase activity against ABTS was assayed as reported by Pezzella et al. 2016.[34]

¹H-NMR Spectroscopy: ¹H-NMR spectroscopic analyses were performed on a JEOL JNM-ECS400A spectrometer at a frequency of 400 MHz. The oxidation reactions were monitored by collecting the ¹H-NMR spectra of the crude mixture. The product distribution was calculated using the ratio between the ¹H signals at δ 5.22 for the GalA (C1 α anomer) at δ 4.55 for D-galactaro-1,4 lactone and δ 3.86 for GA.[15]

Solubility Prediction: The solubility predictions for the investigated monomers were carried out using the Hansen Solubility Parameters following the procedures described in previous reports.[28,35,36]

Esterification of GA: Galactaric acid (2.5 g) was added to a mixture of ethanol or butanol (125 mL) and concentrated sulphuric acid (2 mL). The mixture was heated at reflux for 24 h. Upon cooling, the clear solution was stored for 24 h at 4 °C to allow the product to crystallize as a white solid. Product was filtered, washed with cold ethanol, and vacuum dried at 30 °C.

Biocatalyzed Polycondensation Reactions: The protocol was adapted from Pellis et. al.[26] A total of 8 × 10⁻⁴ mol of GA diester (0.2 M) and 8 × 10⁻⁴ mol of diol (BDO or ODO, 0.2 M) (diester:diol ratio = 1:1) were added together with 4 mL of DPE or Cyrene in a 25 mL round bottom flask. The mixture was then stirred at the desired operational temperature until complete dissolution of the monomers in the solvent. Note that 10% wt/wt (calculated on the total amount of the monomers) of CaLB was then added and the reaction was run for 6 h at 1000 mbar. A vacuum of 20 mbar was subsequently applied for additional 90 h. Warm chloroform was added to the reaction mixture to solubilize the polymer product and the biocatalyst was filtered off. The chloroform was then removed under vacuum. The polymer-solvent mixture was subsequently crashed out in ice-cold methanol achieving precipitation of the products. Three methanol washing steps were subsequently performed to remove the residual solvent. The reactions led to white powdery polymerization products.

ATR FT-IR Spectroscopy: ATR FT-IR spectra were recorded on a Perkin Elmer Spectrum 400 spectrometer. The ATR accessory (supplied by Specac Ltd., UK) was equipped with a diamond crystal. All spectra were recorded at 21 °C, wavelength interval between 4000 and 650 cm⁻¹. A total of 64 scans for each pectin sample were taken with a resolution of 2 cm⁻¹ for the determination of degree of esterification (DE) as described by Zainudin et al. 2018.[30] A total of 32 scans for each polymer sample were taken with a resolution of 2 cm⁻¹.

Differential Scanning Calorimetry: Modulated DSC analyses were performed on a TA Instruments Q2000 under nitrogen atmosphere. The used heating rate was of 5 °C min⁻¹ over a T range of −60 to 200 °C. Cooling rate was set at 5 °C min⁻¹ over the same T range. Sample mass was of 5–10 mg for all measured samples. The melting points of the polymers were calculated from the second heating scan.

Thermogravimetric Analysis (TGA): TGA was performed on a PL Thermal Sciences STA 625 thermal analyzer. Approximately 10 mg of sample was weighed in an aluminum pan. The sample was then placed in the furnace with N_2 flow of 100 mL min⁻¹ and heated from 25 to 625 °C at a heating rate of 10 °C min⁻¹. From the TGA profiles the temperatures at 5, 10 and 50% mass loss (T_d5, 10 and 50) were obtained.

Size Exclusion Chromatography: The molecular weight of pectin and its distribution were determined using the gel permeation chromatography technique using a Shimadzu HPLC system comprising a CBM-20A Controller, LC-20AD Pump with degasser, SIL-20A Autosampler and SPD-20A detector; and HELEOS-II light scattering and Optilab rEx refractive index detectors supplied by Wyatt. A PL aquagel-OH mixed column (7.5 mm × 300 mm, 8 µm particle size; Agilent Technologies, USA) and a PL aquagel-OH mixed guard column (7.5 × 50 mm, 8 µm

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particle size; Agilent Technologies, USA) were used as the stationary phase with 50 mM sodium nitrate dissolved in deionized water as mobile phase constituting. The flow rate of the mobile phase was 0.5 mL min^{-1}. The pectin solutions (1.5 mg mL^{-1}), dissolved in deionized water, were filtered through a nylon membrane (Whatman, UK) before analysis. Note that 0.146 was used as dn dc^{-1} value.

Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry: MS analyses were carried out by using a Bruker Solarix-XR FTICR mass spectrometer and the relative software package for the acquisition and the processing of the data. DHB and graphene were used as the matrix without any addition of solvent. The measurements were conducted in positive mode with the detector set in reflector mode.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

[1] https://ec.europa.eu/commission/news/single-use-plastics-2018-may-28_en.

[2] C. Chatterjee, F. Pong, A. Sen, *Green Chem.* **2015**, *17*, 40.

[3] T. Mehtiö, M. Toivari, M. G. Wiebe, A. Harlin, M. Penttilä, A. Koivula, *Crit. Rev. Biotechnol.* **2016** *36*, 904.

[4] F. van der Klis, J. van Haveren, D. S. van Es, J. H. Bitter, *ChemSusChem* **2017**, *10*, 1460.

[5] M. D. J. C. Sandarani, *J. Pharmacog. Nat. Prod.* **2017**, *03*, 3.

[6] M. Cardenas-Fernandez, M. Bawn, C. Hamley-Bennett, P. K. V. Bharat, F. Subrizi, N. Suhaili, D. P. Ward, S. Bourdin, P. A. Dalby, H. C. Hailes, P. Hewitson, S. Ignatova, C. Kontoravdi, D. J. Leak, N. Shah, T. D. Sheppard, J. M. Ward, G. J. Lye, *Faraday Discuss.* **2017**, *202*, 415.

[7] C. L. Mehltretter, C. E. Rist, *J. Agric. Food Chem.* **1953**, *1*, 779.

[8] V. Pamuk, M. Yılmaz, A. Alıcılar, *J. Chem. Technol. Biotechnol.* **2001**, *76*, 186.

[9] S. Rautiainen, P. Lehtinen, J. Chen, M. Vehkamaki, K. Niemela, M. Leskela, T. Repo, *RSC Adv.* **2015**, *5*, 19502.

[10] R. K. P. Purushothaman, F. van der Klis, A. E. Frissen, J. van Haveren, A. Mayoral, A. van der Benta, D. S. van Es, *Green Chem.* **2018**, *20*, 2763.

[11] J. Kuivanen, Y. M. J. Wang, P. Richard, *Microb. Cell Fact.* **2016**, *15*, 210.

[12] T. Paasikallio, A. Huuskonen, M. G. Wiebe, *Microb. Cell Fact.* **2017**, *16*, 119.

[13] Y. Li, Y. Xue, Z. Cao, T. Zhou, F. Alnadari, *World. J. Microbiol. Biotechnol.* **2018**, *34*, 102.

[14] R. Sakuta, K. Takeda, K. Igarashi, H. Ohno, N. Nakamura, *Energy Technol.* **2018**, *6*, 273.

[15] R. Sakuta, K. Takeda, K. Igarashi, H. Ohno, N. Nakamura, *J. Mol. Catal. B Enzym.* S76, **2016**, *133*, S76.

[16] D. Ivnitski, P. Atanassov, C. Apblett, *Electroanalysis* **2007**, *19*, 1562.

[17] C. Rosu, I. I. Negulescu, R. Cueto, R. Laine, W. H. Daly, *J. Macromol. Sci., Part A: Pure Appl.Chem.* **2013**, *50*, 940.

[18] A. A. Wroblewska, K. V. Bernaerts, S. M. A. De Wildeman, *Polymer* **2017**, *124*, 252.

[19] I. Gavrila, P. Raffa, F. Picchioni, *Polymers* **2018**, *10*, 248.

[20] Y. Wu, Y. Enomoto-Rogers, H. Masaki, T. Iwata, *ACS Sustainable Chem. Eng.* **2016**, *4*, 3812.

[21] Y. Wu, Y. Enomoto-Rogers, H. Masaki, T. Iwata, *J. Appl. Polym. Sci.* **2018**, 47255.

[22] H. Chen, X. Fu, Z. Luo, *Food Chem.* **2015**, *168*, 302.

[23] A. Wikiera, M. Mika, A. Starzynska-Janiszewska, B. Stodolak, *Food Chem.* **2015**, *172*, 675.

[24] A. Badhan, G. O. RibeiroJr., D. R. Jones, Y. Wang, D. W. Abbott, M. Di Falco, A. Tsang, T. A. McAllister, *Bioresour. Technol.* **2018**, *260*, 76.

[25] S. Giacobbe, C. Pezzella, V. Lettera, G. Sannia, A. Piscitelli, *Bioresour. Technol.* **2018**, *265*, 59.

[26] A. Pellis, F. P. Byrne, J. Sherwood, M. Vastano, J. W. Comerford, T. J. Farmer, *Green Chem.* **2019**, *21*, 1686.

[27] A. Pellis, J. W. Comerford, S. Weinberger, G. M. Guebitz, J. H. Clark, T. J. Farmer, *Nat. Commun.* **2019**, *10*, 1762.

[28] J. Sherwood, M. De Bruyn, A. Constantinou, L. Moity, C. R. McElroy, T. J. Farmer, T. Duncan, W. Raverty, A. J. Hunt, J. H. Clark, *Chem. Commun.* **2014**, *50*, 9650.

[29] S. Ma, S. Yu, X. Zheng, X. Wang, Q. Bao, X. Guo, *Carbohydr. Polym.* **2013**, *98*, 750.

[30] B. H. Zainudin, T. W. Wong, H. Hamdan, *Polym. Degrad. Stab.* **2018**, *147*, 35.

[31] A. S. Matharu, J. A. Houghton, C. Lucas-Torres, A. Moreno, *Green Chem.* **2016**, *18*, 5280.

[32] N. Babbar, S. Baldassarre, M. Maesen, B. Prandi, W. Dejonghe, S. Sforza, K. Elst, *Carbohydr. Polym.* **2016**, *146*, 245.

[33] S. Giacobbe, O. Pepe, V. Ventorino, L. Birolo, R. Vinciguerra, V. Faraco, *BioResources* **2014**, *9*, 4873.

[34] C. Pezzella, S. Giacobbe, V. G. Giacobelli, L. Guarino, S. Kylic, M. Sener, G. Sannia, A. Piscitelli, *J. Mol. Catal. B Enzym.* **2016**, *134*, 274.

[35] C. M. Hansen, *Hansen Solubility Parameters: A User's Handbook*, 2nd ed, CRC Press, Boca Raton, FL **2007**.

[36] F. P. Byrne, B. Forier, G. Bossaert, C. Hoebers, T.J. Farmer, J. H. Clark, A. J. Hunt, *Green Chem.* **2017**, *19*, 3671.