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The orange gold: Biotechnological production of PLA/P(3HB)/limonene based polyesters from orange peel waste

Sophia Mihalyi[a,1], Annika Putz[a,1], Manuel Draxler[b], Andreas Mautner[a,c], Marion Sumetzberger-Hasinger[a], Filippo Fabbri[d], Alessandro Pellis[e], Markus Neureiter[a], Felice Quartinello[a,d,*], Georg M. Guebitz[a,d]

[a] *University of Natural Resources and Life Sciences, Department of Agrobiotechnology, IFA-Tulln, Institute of Environmental Biotechnology, Konrad Lorenz Strasse 20, 3430, Tulln an der Donau, Austria*
[b] *Fachhochschule Wiener Neustadt, Biotech Campus Tulln, Konrad-Lorenz-Straße 10, 3430 Tulln an der Donau, Austria*
[c] *University of Vienna, Institute of Materials Chemistry, Währinger Straße 42, 1090 Wien, Austria*
[d] *acib GmbH, Konrad-Lorenz-Straße 20, 3430 Tulln an der Donau, Austria*
[e] *Department of Chemistry and Industrial Chemistry, Università degli Studi di Genova, Via Dodecaneso 31, 16146 Genova, Italy*

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A B S T R A C T

Globally, vast amount of food-derived waste is generated including residues from fruit processing, which requires innovative strategies to avoid problematic disposal of useful resources. Orange peels contain a variety of valuable compounds such as limonene, enzymes, and carbohydrates that exhibit interesting properties for various applications. In this work, a biorefinery concept is presented to generate versatile bioproducts from orange peel waste. First, limonene and peroxidase enzymes were extracted from orange peels by solvent extraction and three phase partitioning, respectively. The remaining solids, containing mainly cellulose, were enzymatically hydrolyzed, and soluble monosaccharides converted into lactic acid (LA) by *Weizmannia coagulans* and the biopolyester polyhydroxybutyrate (P(3HB)) by *Priestia megaterium.* 8 g L^{-1} limonene and peroxidases with remarkable specific activity of 426 U mg^{-1} were extracted. Utilization of the sugars in batch fermentations resulted in a LA concentration of 17 g L^{-1} as well as a P(3HB) content up to 43 % in cell dry weight without the need for further medium components. By combining these bioproducts, fully biobased polymer blend films of P(3HB) with PLA and limonene as plasticizer were successfully fabricated by thermoplastic processing, i.e., extrusion. In conclusion, the tested concept has shown very promising results and thereby emphasize the potential of the presented valorization strategies for orange peel waste.

1. Introduction

Large amounts of food waste and by-products generated have become a serious issue nowadays, with almost one third of the total food production going to waste resulting in over 58 million tons each year in the EU alone [1]. In a biorefinery concept, biomasses are utilized to generate value-added products, aiming for zero-waste [2,3] where fruit wastes are cheap and carbon-rich residues that are highly interesting [4]. Reduction of food waste is a critical measure to decrease the environmental impact of the food industry and should be preferentially aimed for [5]. Yet, finding solutions to valorize food waste and side streams also constitutes a strategy to mitigate the impact of food waste [6]. Even more so, residues from food processing industry should be considered a valuable resource rather than as waste.

For instance, whereas the global production of oranges accounts for 48 million tons annually [7], almost 50 % of the total orange fruit ends up as waste after processing, which results in approx. 20 million tons that are discarded as peel residues [2,8]. At its end of life, orange peels

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* Corresponding author at: University of Natural Resources and Life Sciences, Department of Agrobiotechnology, IFA-Tulln, Institute of Environmental Biotechnology, Konrad Lorenz Strasse 20, 3430, Tulln an der Donau, Austria.

E-mail addresses: sophia.mihalyi@boku.ac.at (S. Mihalyi), annika.putz@boku.ac.at (A. Putz), andreas.mautner@boku.ac.at (A. Mautner), marion.sumetzberger-hasinger@boku.ac.at (M. Sumetzberger-Hasinger), filippo.fabbri@students.boku.ac.at (F. Fabbri), alessandro.pellis@unige.it (A. Pellis), markus.neureiter@boku.ac.at (M. Neureiter), felice.quartinello@boku.ac.at (F. Quartinello), guebitz@boku.ac.at (G.M. Guebitz).

[1] Sophia Mihalyi and Annika Putz equally contributed to this work.

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represent a voluminous waste, which traditionally is managed as animal feedstock, by landfilling, or incineration [9]. All these solutions are viable but leave a lot of space for improvement, in particular taking into account environmental aspects, energy demand as well as low nutritional value [8]. Considering the chemical composition of orange peel waste (OPW), which still contains valuable components such as essential oils (limonene), carbohydrates (pectin, cellulose) as well as soluble sugars and enzymes [2,10], there is great potential for valorization that is awaiting exploitation [11–13].

Essential oils are volatile substances, with heterogeneous properties, that are synthesized in plants as secondary metabolites functioning either as compounds for defense against parasites or to attract pollinator insects [14]. Limonene is one of the most ubiquitous terpenes found in nature and can reach up to 98 % of total essential oil composition in orange peels. It can be applied in many industrial sectors such as the food or cosmetic industry as fragrance as well as biobased additive in thermoplastic processing. Moreover, it is an interesting molecule due to its antimicrobial properties [15–17]. Limonene can be extracted from OPs with different solvents and under various conditions. Most commonly, the petrochemical and toxic solvent hexane is applied in this process, however to reduce the environmental footprint, other solvents can perform successfully as well [18]. Another defense mechanism in the plant kingdom against pathogens is represented through the presence of oxidative enzymes, i.e. peroxidases. Peroxidases act as a major component in catalyzing oxidation reactions of phenols or aromatic amines in the presence of H_2O_2. Furthermore, peroxidases are enzymes that are exploited on an industrially relevant scale for their catalyzing ability of different types of redox reactions for a broad variety of substrates [19]. The conventional source for plant peroxidase is horseradish, however, the available quantity is limited, and it also exhibits limitations based on isoenzyme variability as well as high costs associated with the extraction process. Another method for obtaining peroxidase is via heterologous expression in bacteria or yeast, still, low yields and production costs represent challenges obstructing mass production of recombinant peroxidases [20]. Overcoming these obstacles, OPW could provide an attractive alternative in acquiring plant-based peroxidase, due to their abundance in the food processing industry and low resource cost [21]. The structural components of OPW comprise cellulose together with pectin, hemicellulose, and lignin [3]. Due to its high share of polysaccharides, OPW is a considerable candidate to obtain soluble sugars through enzymatic hydrolysis. These waste stream derived monosaccharides could be used for various fermentation processes and to produce valuable bio-based compounds such as bioethanol or lactic acid [2,22].

Besides the direct issue of disposal of food and food processing waste-streams, biobased and biodegradable alternatives to conventional fossil-derived plastics are needed to reduce the environmental impact of (food) packaging [23]. Sustainable production of building blocks for biobased polymers or biopolymer production by microbial action is of high interest opening up a wide range of applications specifically for food packaging [24,25]. Applying residues from agriculture or the food processing industry as substrates in microbial production is particularly attractive since it enhances the economic competitiveness and prevents competition with food and feed supply [26]. In general, substrates should preferably be of constant quality and composition, which makes orange peels a great source for microbial fermentation [4,26].

In this study, two compounds were primarily targeted by different microbial fermentation processes, lactic acid (LA), the precursor of poly (lactic acid) (PLA), and the biopolymer polyhydroxybutyrate (P(3HB)). LA is a natural, optically active organic acid that serves as the main building block for PLA synthesis. It can be produced in optically pure form through microbial fermentation [27]. In the present study, the organism *Weizmannia coagulans* (formerly *Bacillus coagulans*) was used for pure L-LA production [22,24]. Polyhydroxyalkanoates (PHAs) are produced by several microorganisms as intracellular granules serving as carbon and energy storage compounds [28]. In this study, *Priestia megaterium* (formerly *Bacillus megaterium*) was used for the production of P(3HB), a short-chain-length PHA [29]. This organism is able to form P(3HB) from residues such as desugarized beet molasses [30] or various agro-industrial by-products [31]. Eventually, the obtained bio(based) products from extraction and microbial fermentation were intended to be blended into PLA/P(3HB)/limonene films with mechanical and thermal properties as well as oxygen permeability facilitating application as packaging material. In this blend, limonene acts as a plasticizer to allow production of films, as unplasticized blends of PLA and PHB are commonly too rigid [15].

The aim of this work was to develop a full valorization approach for OPW by subsequent extraction of essential oils, mostly containing D-limonene, and peroxidase. The peel residue is then treated by enzymatic hydrolysis to break down the cellulose and obtain glucose, that is used for fermentation with *W. coagulans* to acquire LA or *P. megaterium* to produce P(3HB), which both represent valuable biobased building blocks or biopolymers, respectively. Ultimately, a blend of PLA/P(3HB)/limonene was formed to assess the application of obtained valuable products in food packaging.

2. Material & methods

2.1. Chemicals and materials

All chemicals and solvents were purchased from Sigma-Aldrich (Vienna, Austria) or Carl Roth (Germany) and used without further purification unless stated otherwise. Cellic CTec3® cellulase cocktail was provided by Novozymes (Copenhagen, Denmark). Pectinases from *Aspergillus aculeatus* (Pectinex® Ultra SPL) were purchased from Sigma-Aldrich. Oranges were purchased from local supermarkets in Vienna and Lower Austria (Origin: Italy).

2.2. Limonene extraction, purification, and quantification

After collecting the fruit juice from the oranges, the peels, which were frozen at −20 °C (Beko, Austria-Germany, RFSE200T30WN) for storage, were treated in a blender (Bosch, Austria, Serie 6 Stabmixer ErgoMaster 1200 W Edelstahl) for homogenization and as well to increase accessibility for the subsequent steps of limonene extraction, enzyme purification, and assessing cellulolytic enzyme activity towards cellulose fibers.

Limonene extraction was performed on an EDGE solvent extraction device (CEM, Kamp-Lintfort, Germany) using methanol or ethanol as a solvent (Table S1) to obtain the orange oil, majorly containing limonene. In total 100 g of blended orange peels were filled in the Q-Cups (10 g per cup) and inserted in the extraction device. To remove excess solvent and impurities, distillation of the orange oil extract was performed on a Rotavapor (Büchi, Switzerland, R-300) at 185 mbar, 50 °C, and 100 rpm.

The distillate was analyzed by gas chromatography mass spectrometry (GC–MS) for limonene quantification. External standards of commercial limonene (Sigma-Aldrich) with concentrations of 50, 100, 250, 500, 800 and 1000 μg L^{-1} were prepared for quantification of the extracted samples on an Agilent Technologies 78890 A GC-System with MSD 5975C Tripel-Axis-Detector. A DB-17MS (30 m × 250 μm × 0.25 μm) column operated at a flow of 1.2 mL min^{-1} with helium as carrier gas was applied. The temperature program was (i) starting at 40 °C (upon 1 min hold), then (ii) ramping at 20 °C per min to 250 °C, and (iii) holding for 1 min. MSD was operated in scan and SIM mode with the source kept at 230 °C and the quadrupol at 150 °C. Linear calibration was performed with SIM mode data and identification was performed by comparison with NIST20 library.

2.3. Peroxidase extraction and characterization

2.3.1. Peroxidase extraction

Three phase partitioning (TPP) was applied to extract the protein from the peel. 20 g of blended orange peels were added to 200 mL of a 100 mM phosphate buffer at pH 7. This mixture was treated in a blender and residual solid peels were removed by centrifugation at 4 °C with 3700 rpm for 20 min (5920 R, ThermoScientific, Austria). The supernatant was collected and filtered through filter paper (Whatman grade 595 1/2, Cytiva). Subsequently, 4 g of ammonium sulfate were added to 10 mL of filtered extract together with 10 mL of *t*-butanol and mixed with a Thermomixer (Eppendorf) for 100 min at 50 °C and 900 rpm. This mixture was centrifuged for 20 min at 4 °C and 3700 rpm. After centrifugation, the mixture was transferred into a 50 mL separatory funnel and rested for 100 min to encourage separation into three distinct phases. Each phase was collected individually for further analysis [21].

2.3.2. Protein concentration determination (Bradford assay)

The protein concentration was determined by the Bradford assay with bovine serum albumin (BSA) as a reference for calibration from 0.025 to 1 mg mL^{-1}. 200 μL of 1:5 diluted Bradford reagent (Coomassie brilliant blue G-250 dye, Bio-Rad) were added to 10 μL accordingly diluted sample in a 96 well plate and the absorbance was measured at 595 nm on a Tecan Reader (Tecan, Grödig, Austria) after 5 min incubation at room temperature (21 °C). All measurements were performed in triplicates [32].

2.3.3. Peroxidase activity assay

To remove smaller impurities, the peroxidase sample was filtered through a 30 kDa MWCO membrane (VivaSpin) by centrifugation for 15 min at 4 °C and 3700 rpm. The activity assay was performed using a 10 mM ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) solution as substrate together with 1 mM H_2O_2 as previously described with slight modifications [33]. 150 μL of phosphate buffer, 20 μL of protein sample, and 2 μL of 1 mM H_2O_2 solution were added to a 96-well plate in triplicates. Shortly before the measurement, 50 μL ABTS was added to start the peroxidase reaction. Together with H_2O_2 and peroxidase the ABTS is oxidized resulting in a radical cation indicated by a change in absorbance. The volumetric activity [U mL^{-1}] was measured with a Tecan Reader (Tecan, Grödig, Austria) at 420 nm for 15 cycles and calculated according to *Eq. 1.* Vf represents the total volume [mL], Ve the volume of the enzyme solution [mL], ε the extinction coefficient [12.07 L $mmol^{-1}$ cm^{-1}], d the layer thickness [0.611 cm], Df the dilution factor and ΔA the change in absorbance [min^{-1}].

$$Volumetric\ activity = \frac{\Delta A}{\varepsilon \bullet d} \bullet \frac{Vf}{Ve} \bullet Df \qquad (1)$$

2.3.4. Sodium dodecyl sulfate – Polyacrylamide gel electrophoresis (SDS-PAGE)

To confirm the presence of extracted peroxidase, SDS-PAGE was performed as previously described [34]. Mini-PROTEAN TGX precast stain-free SDS-PAGE gels (BioRad, USA) were used without further treatment. A Protein marker IV (pre-stained), peqGOLD (Avantor, VWR, Germany) was applied for protein size determination. The samples were prepared by adding 20 μL of sample to 20 μL of Laemmli buffer and heating to 100 °C for 5 min. Gels were stained with Coomassie Blue R-250 (0.125 % in 30 % ethanol and 10 % acetic acid) for 1 h and de-stained with a de-staining solution (30 % ethanol, 10 % acetic acid) for 1 h and then with fresh solution overnight. Finally, the gels were scanned using a ChemiDoc (ChemidocTM MP Imaging System, Bio-Rad).

2.4. Orange peel hydrolysate (OPH) valorization

2.4.1. Enzymatic hydrolysis of orange peels

After the extraction processes and prior to hydrolysis the residual peels were dried at 60 °C (Memmert, Germany, Universalschrank U). Enzymatic hydrolysis was performed either in a simple buffer or cultivation medium (described below) to obtain glucose and other monosaccharides (fructose, xylose, galacturonic acid) from the orange peels for further valorization [35].

For lactic acid production, 75 g L^{-1} OPs were enzymatically hydrolyzed using 2 % Cellic CTec3 Cellulase at pH 5, 50 °C, 150 rpm (Infors HT Multitron Triple Incubator Shaker), and for 72 h in the cultivation medium for *Weizmannia coagulans* as previously described by Mihalyi et al. [22]. Cultivations in standard media are referred to as “control” throughout the manuscript in comparison to OPH as carbon source.

For polyhydroxybutyrate production, 100 g L^{-1} OPs were enzymatically hydrolyzed in 50 mM potassium phosphate buffer using 2 % Cellic CTec3 Cellulase and 0.5 % Pectinase solution at pH 5, 50 °C, and 150 rpm for 72 h. The remaining solids were removed by centrifugation (Thermo scientific, Sorvall Lynx 6000) and the supernatant filtered through a nylon fabric (20 DEN) and a nylon membrane filter (0.45 μm pore size, Cytiva, Whatman™), following a sterile filtration step with a nylon filter membrane (0.20 μm pore size, Graphic Controls, DIA-Nielsen GmbH &Co KG).

2.4.2. Chemical analysis of orange peel hydrolysate

The concentration of glucose within the hydrolysate was determined by HPLC measurements (1260 Infinity II Agilent technologies, USA, with Transgenomic IC SEP-ION-300 coupled with a refractive index detector) as previously described with slight modifications [34]. Calibration curves were prepared with concentrations from 0.1 to 20 mM to quantify glucose in the samples. The mobile phase was 0.01 M H_2SO_4 with a flow rate of 0.325 mL min^{-1} at 45 °C for 45 min. The total Kjeldahl nitrogen (TKN) content was determined after hydrolysis with sulfuric acid and a Kjeltab within a Digest Automat K-438 (Buechi, Flawil, Switzerland) and ammonium nitrogen was measured directly with a AutoKjeldahl Unit K-370 (Buechi, Flawil, Switzerland). Total phosphates within the OPH were determined with rapid test kits (Hach® Lange LCK350) after hydrolysis with a high temperature thermostat HT200S.

2.4.3. Lactic acid production

LA was produced by cultivating the wildtype organism *Weizmannia coagulans* (formerly *Bacillus coagulans*) strain M-39 purchased from DSMZ (Germany, DSM No. 2314) in batch mode using a vertical stirred-tank reactor with a maximum working volume of 2 L for 48 h in the cultivation medium described above. Precultures were first grown in 100 mL Lennox LB medium overnight supplemented with 2 % of glucose (*w/v*) at 50 °C and 150 rpm. The pre-cultured cells were collected by centrifugation (5 min, 3700 rpm, 4 °C), re-suspended in cultivation media, and added to the bioreactor to start cultivation at an initial OD of 0.2. The pH was adapted with KOH (1 M) to 5.0 ± 0.2 and temperature was maintained at 50 °C. Samples were centrifuged for 5 min at 4 °C and 3700 rpm and the supernatant was filtered through a 0.2 μm nylon filter and stored at 4 °C until further analysis. Lactic acid was quantified as described above in section 2.4.2 for glucose.

2.4.4. P(3HB) production

Priestia megaterium [36] (formerly *Bacillus megaterium,* type: uyuni S29 CECT 7922; [29]) cultures were incubated from glycerol stocks at −80 °C in 100 mL CECT1 medium containing 5 g L^{-1} beef extract, 5 g L^{-1} sodium chloride, and 10 g L^{-1} peptone, in shake flasks at 35 °C, pH 7, and 130 rpm overnight.

Bioreactor fermentations were carried out in batch mode at 35 °C in a benchtop system (DASGIP, Eppendorf, Jülich, Germany) with four fermentation vessels equipped with various probes as previously described by Schmid et al. [37]. 5 % inoculum was applied at a working volume of 400 mL. 5 M H_2SO_4 and 10 M NaOH were added automatically to maintain pH 7. A minimum of 20 % dissolved oxygen (DO) level was achieved by varying stirrer speed (400–1600 rpm) and by supplying pressurized air at a constant flow of 38 L h^{-1}. 100 % DO was defined at saturation with air during maximum aeration and maximum stirrer speed. Fermentations with OPH as medium were repeated in duplicates (four fermentations in total) and control medium cultivations were performed in duplicates.

As control, mineral medium (modified) described by Kulpreecha et al. [38] was used. Modified mineral medium contained [g L^{-1}]: glucose 25, KH_2PO_4 2, $(NH_4)_2SO_4$ 1, $MgSO_4 \cdot 7\ H_2O$ 1, Na_2HPO_4 0.6, citric acid 0.75, KCl 12.76, $CaCl_2 \cdot 2\ H_2O$ 0.02, and $FeSO_4 \cdot 7\ H_2O$ 0.025. Ammonium sulfate was supplied as nitrogen source and provided at low concentration to ensure nitrogen limitation. The trace element solution (SL-6; DSMZ) composed of [g L^{-1}]: $ZnSO_4 \cdot 7\ H_20$ 0.1, $MnCl_2 \cdot 4\ H_2O$ 0.03, H_3BO_3 0.3, $CoCl_2 \cdot 6\ H_2O$ 0.2, $CuCl_2 \cdot 2\ H_2O$ 0.01, $NiCl_2 \cdot 6\ H_2O$ 0.02, and $Na_2MoO_4 \cdot 2\ H_2O$ 0.03 and was added to the medium (1 mL L^{-1}).

Cell dry weight (CDW) and glucose content, respectively, were determined as described in detail by Schmid et al. [30]. The P(3HB) content during fermentation was determined by hydrolysis of dried cells with concentrated sulfuric acid and subsequent analysis of crotonic acid content (modified method of Karr et al. [39], procedure described by Schmid et al. [37]). For glucose and crotonic acid determination the HPLC system reported by Schmid et al. [30] was used. Additionally, the P(3HB) content of harvested biomass was quantified by gas chromatography according to an adapted method from Furrer et al. [40]. Volume of solutions for transesterification (methylene chloride and 20/80 (*v*/v) mixture of HCl (37 %) and 2-propanol) was increased to 2 mL, respectively. The gas chromatography system applied was: Agilent 6890 N with capillary column J&W 122–3232 (30 m × 0.25 μm), 1 μL injection volume, carrier gas helium; flame ionization detector, make up gas: nitrogen, and a split ratio of 100:1. The temperature program started at 80 °C and was increased to 250 °C with a ramp of 25 °C per min.

For cell harvest, the biomass was centrifuged at 10000 x*g* (Sorvall™ Lynx™ 6000 centrifuge, Thermo Scientific, Thermo Fisher Scientific Inc., Massachusetts, United States) for 20 min. The cell pellet was washed two times with double distilled water, frozen at −80 °C and subsequently lyophilized (Alpha 2–4 LSCplus, Christ, Osterode am Harz, Germany). Extraction was performed as described by Haas et al. [41], with increased volume of ice-cold ethanol (Chem-Lab, Belgium) for precipitation (6× surplus). Filtration was done with a nylon filter membrane (0.20 μm pore size, Graphic Controls, DIA-Nielsen GmbH&CoKG) after ethanol extraction and with a qualitative filter paper (401, particle retention 12–15 μm, VWR) after chloroform extraction. Precipitated P(3HB) was separated via a glass fiber filter (Osmonics Presep TCLP Filter, 0.7 Micron) and dried on air.

The volumetric productivity and product yield, respectively, were calculated at maximum P(3HB) concentration ($P(3HB)_{max}$ [g L^{-1}]). $P(3HB)_{max}$ was calculated from the P(3HB) content [g (100 g $CDW)^{-1}$], determined by GC analysis from harvested biomass at the end of fermentation and from CDW at the time of ($P(3HB)_{max}$. The product yield was calculated by P(3HB) formed per glucose consumed at the time of $P(3HB)_{max}$.

The molecular weight of extracted polymers was determined by Gel Permeation Chromatography (GPC) as previously described with slight modifications [42]. Calibration was done using linear polystyrene standards (0.5–2500 kDa) purchased from Merck (Sigma-Aldrich). Results obtained from the GPC include the number-average molecular mass (M_n), weight-average molar mass (M_w) as well as the dispersity ($Đ = M_w/M_n$,). ^1H NMR spectroscopy was performed using a JEOL ECZ400R/S3 at a frequency of 400 MHz with $CDCl_3$ as the solvent at room temperature.

2.5. PLA/P(3HB)/limonene blended films

Blends of limonene, PLA, and P(3HB) were produced on a micro twin-screw extruder (DSM, The Netherlands) with an Xplore Film Device 35 mm (Xplore Instruments B·V, Sittard, The Netherland) in a PLA/P(3HB) ratio of 3:1 by weight with 15 % limonene added at 180 °C.

2.5.1. Characterization

To evaluate the successful blending into films of bioproducts obtained from OPW, Fourier Transformed Infrared (FT-IR) Spectroscopy (ATR accessory, Perkin Elmer, Traiskirchen, Austria) was performed. A total of 40 scans between wavenumbers of 4000 cm^{-1} and 650 cm^{-1} were recorded and compared to spectra of pure PLA or P(3HB). ^1H NMR spectroscopy was performed as described in section 2.4.4 for P(3HB) and thermogravimetric analysis (TGA) was performed using a Mettler Toledo "TGA/DSC1 STARe System®" operating in a temperature range from 30 to 800 °C with a heating rate of 10 °C/min. In the first segment (30–700 °C), a nitrogen flow of 80 mL min^{-1} was used. Subsequently, the second phase (700–800 °C) was performed under oxidizing atmosphere by switching to an O_2 flow of 80 mL min^{-1}. All thermograms were corrected by subtracting the blank curve of the empty crucible obtained under the same analysis conditions. Differential scanning calorimetry (DSC) analysis was performed with a Mettler Toledo "DSC1 STARe System®" using 40 μL aluminium pans with a singular central perforation of the lid. An empty aluminium pan with a perforated lid served as a reference. The measurements were performed under dry N_2, with a heating phase from −60 to 200 °C, followed by a cooling phase to −60 °C, and another heating phase up to 200 °C.

3. Results and discussion

3.1. Limonene extraction

Limonene is the main component of the essential oils present in orange peels accounting for around 4–5 % of the dry mass [43] and was initially extracted assessing two different solvents. The limonene content was 8.2 g L^{-1} and 7.0 g L^{-1}, for methanol and ethanol extraction, respectively, in the distillate which corresponds to 2.18 % and 1.86 % (*w*/w) in relation to initial wet weight of orange peels. Extraction with commonly applied hexane at 30 °C for 120 min resulted in around 0.5 % limonene yield (g/g DM) and a maximum of 1 % under elevated temperatures (up to 90 °C) [18]. In another study 1.2 % (w/w) limonene extraction yield was reached at 70 °C and solid/liquid ratio of 1:2 [44]. Even higher temperature (150 °C) reached an extraction yield of 3.56 % from lemon peels with lower extraction time of 30 min [45]. The results obtained in this study indicate that methanol and ethanol are suitable solvents for extracting limonene at higher temperatures (120 °C) and short extraction time (4 min). The highest concentration of limonene was detected in the distillate from the methanol extraction (8.2 g L^{-1}, 2.18 %), which exceeds previously obtained results with methanol (4.6 g L^{-1}, 0.4 % *v*/v), which could be explained by lower temperature during extraction [46].

3.2. Peroxidase extraction

Peroxidases are abundant in the orange peel as well and were extracted through three phase partitioning (TPP) yielding three separate phases (Fig. S1). The upper phase contained nonpolar molecules, while the aqueous bottom phase contained polar compounds. Upon addition of ammonium sulfate, the protein equilibrated in the middle phase between the polar and nonpolar phases with a concentration of 1.03 mg mL^{-1}. After concentration by ultrafiltration (30 kDa MWCO), 1.22 mg mL^{-1} of protein were obtained. The presence of peroxidase was confirmed through SDS-PAGE, where it showed a clear band at about 35 kDa (Fig. S2), which was in the expected range [47]. The specific peroxidase activity was 426 ± 15 U mg^{-1}, which is comparable to a specific activity of 505 ± 101 U mg^{-1} reached in *Pichia pastoris* cultivation supernatant [20] and higher than 126 U mg^{-1} expressed in *E. coli* after refolding [33]. The extraction efficiency could still be optimized and orange peels therefore offer a promising source for peroxidases.

3.3. Orange peel hydrolysate valorization

In a next step, glucose was recovered through enzymatic hydrolysis of the OPW remaining after extractions and used as a carbon source in different fermentation processes. The objective was to produce building blocks for biopolymers or biopolymers directly, respectively, that can be applied for example as food packaging material. Enzymatic hydrolysis was conducted under mild conditions, where no toxic compounds are formed and no aggressive chemicals are needed opposed to conventional chemical pre-treatment methods [48].

To optimize the hydrolysis yield of cellulosic components of the orange peel waste, different conditions were tested. The released glucose concentration was higher when hydrolysis was performed in the presence of pectinases (Fig. 1) resulting in a maximum of 15.09 ± 0.24 g L^{-1}, which also facilitated downstream processing (centrifugation and filtration) of the hydrolysis supernatant. Synergistic action of cellulases and pectinases was described previously to enhance hydrolysis yield [49]. As an increase in cellulase concentration did not result in an increased glucose release, enzymatic hydrolysis of orange peels was performed in the presence of 6 FPU mL^{-1} of cellulolytic enzymes as well as 19 U mL^{-1} of pectinase activity. After scaling up to a 4 L system and under the identified optimized conditions, a weight loss of 80.4 ± 0.1 % of remaining orange peels was reached, which indicated remaining 20 % of non-hydrolyzed compounds, such as lignin and recalcitrant cellulose [2].

3.3.1. Lactic acid production

W. coagulans was cultivated on OPH and control medium containing commercial glucose, respectively, for production of optically pure L-LA. Glucose was entirely consumed in both media and converted into 17.1 ± 0.1 g L^{-1} LA after 48 h on OPH, which was similar to 16.4 ± 0.3 g L^{-1} reached in the control medium (Fig. 2). Thus, the yield was slightly higher for the OPH, which indicated consumption of additional sugars, such as xylose [50], or other carbon-containing compounds that are present in the OPH. This showed that OPH can serve as a valuable carbon source for LA production and subsequent PLA synthesis. The advantages of microbial production of LA for PLA synthesis include optical purity, homolactic fermentation as well as application of waste materials as substrate. However, LA purification after cultivation can represent up to 60 % of the production cost, which is usually performed by membrane separation, distillation, precipitation, adsorption, or extraction [51]. Therefore, process optimization towards high LA concentration during fermentation is essential in future research.

3.3.2. P(3HB) production

P. megaterium was cultivated on OPH and control medium containing commercial glucose, respectively, for assessing an additional OPW valorization pathway by production of a biopolymer. After optimization of enzymatic hydrolysis, 25.1 ± 0.30 g L^{-1} glucose in phosphate buffer were available from OPH for P(3HB) fermentation. Total phosphate analysis resulted in 1.74 ± 0.00 g L^{-1} and the TKN content was 1.23 ± 0.05 g L^{-1}, which included 0.12 ± 0.00 g L^{-1} ammonium nitrogen. TKN also contains bound nitrogen that might not be available as a nitrogen source for the organism during fermentation. Low ammonium nitrogen concentration of OPH was applicable for P(3HB) production under nitrogen limited conditions.

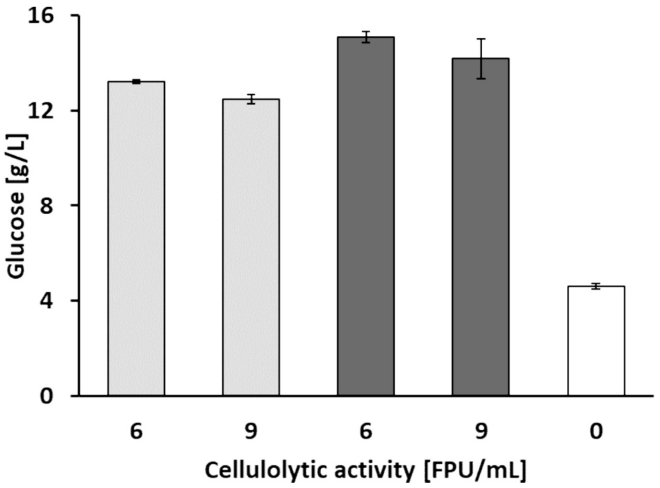

Fig. 1. Concentration of released glucose after enzymatic hydrolysis of cellulosic fibers from orange peels with cellulase and pectinase enzymes at different concentrations. Light grey bars indicate sole addition of cellulolytic enzymes, dark grey bars indicate additional presence of 19 U mL^{-1} pectinase and the white bar indicates sole presence of 19 U mL^{-1} pectinase activity.

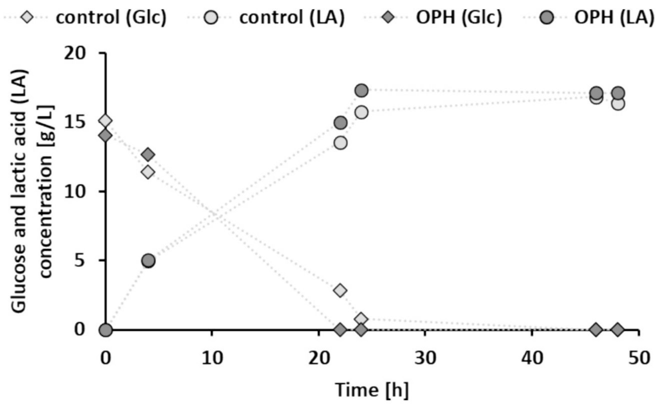

Fig. 2. Glucose consumption (diamonds) and LA production (circles) by *W. coagulans* during cultivation on OPH in cultivation media and commercial glucose as control.

P. megaterium showed strong metabolic activity in OPH, which is clearly visible by decreasing DO after a short lag phase (Fig. 3). Concurrently, CDW increased, and glucose was depleted within 16 to 18 h reaching 15.1 ± 0.4 g L^{-1} CDW, which remained constant until the time of harvest (15.5 ± 0.0 g L^{-1} after 23 h). This was accompanied by P(3HB) formation within the first 18 h (Fig. 3), with a maximum of 6.5 ± 0.1 g L^{-1} (Table 1). In the control fermentation on mineral medium microbial growth was slower with 3.7 ± 0.0 g L^{-1} CDW after 40 h and a remaining glucose concentration of 6.9 ± 0.6 g L^{-1} (Table 1). For glucose consumption was reduced in the mineral medium, lower CDW and P(3HB) concentrations at the time of $P(3HB)_{max}$ (3.7 ± 0.0 g L^{-1} and 1.7 ± 0.1 g L^{-1}, respectively) were determined. With the same strain, Schmid et al. [30] reached higher growth and glucose consumption from modified mineral medium. However, phosphate-limited conditions were present, whereas with the control medium nitrogen limitation could be responsible for reduced growth [37].

Within this study, *P. megaterium* produced P(3HB) from OPH in phosphate buffer without any additional nutrients, where in previous studies utilizing fruit residues as carbon source often yeast extracts or other medium components were supplemented. For instance, Sukan et al. [52] obtained 1.24 g L^{-1} P(3HB) with a recombinant strain of *B. subtilis* from orange peel as sole carbon source supplemented with yeast extract. A *B. subtilis* strain was able to generate 19.39 g L^{-1} CDW and 9.68 g L^{-1} PHA from orange peel powder as carbon source in mineral salt medium [53]. Utilization of aqueous extracts or powders of peels might differ from enzymatically pre-treated fruit residues. Enzymatic hydrolysis of OP increases the overall concentration of carbon sources such as glucose within the liquid fraction compared to untreated aqueous extracts [49]. Changing the C:N ratios of substrates, achieved for example by releasing glucose after enzymatic hydrolysis of cellulose, can be used to further optimize the PHA production process [54].

Here, from OPH, a product yield of 0.29 g P(3HB) per g glucose consumed was obtained together with a volumetric productivity of 0.36 g P(3HB) L^{-1} h^{-1}. The same strain reached 0.25 g P(3HB) L^{-1} h^{-1}, when grown on modified minimal mineral medium with regular glucose addition and without nitrogen limitation [55]. In this work, OPH as sole medium resulted in 42.8 ± 1.4 g P(3HB) in 100 g CDW, which is very promising in comparison to results from literature discussed above. The

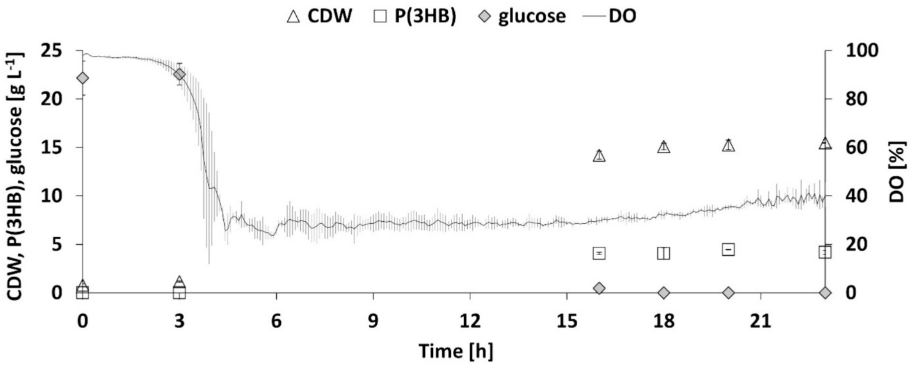

Fig. 3. Batch fermentation with *P. megaterium* on OPH: mean values including error bars = standard deviation of repeated fermentations. DO online data shown in 5 min intervals. CDW = cell dry weight; P(3HB) = Polyhydroxybutyrate; DO = dissolved oxygen.

Table 1
Results of batch fermentations with OPH and mineral medium as control. Volumetric productivity ($Q_{\text{P(3HB)}}$) and product yield ($Y_{\text{P(3HB)}}$) were calculated at the time of P(3*HB*)$_{max}$.

		OPH	Control
	Unit	± *SD; n* = *2*	± *SD; n* = *2*
Time (at P(3*HB*)$_{max}$)	[h]	18	40
Glucose$_{start}$	[g L^{-1}]	22.3 ± 1.5	22.6 ± 0.1
Glucose$_{residual}$ (at P(3*HB*)$_{max}$)	[g L^{-1}]	–	6.88 ± 0.63
CDW (at P(3*HB*)$_{max}$)	[g L^{-1}]	15.1 ± 0.35	3.70 ± 0.00
P(3*HB*)$_{max}$	[g L^{-1}]	6.45 ± 0.05	1.68 ± 0.08
$Y_{\text{P(3HB)}}$ at P(3*HB*)$_{max}$	[g P(3HB) (g glucose$_{consumed}$) $^{-1}$]	0.29 ± 0.01	0.11 ± 0.00
$Q_{\text{P(3HB)}}$ at P(3*HB*)$_{max}$	[g P(3HB) L^{-1} h^{-1}]	0.36 ± 0.00	0.04 ± 0.00
Time harvest	[h]	23	67
P(3*HB*)$_{harvest}$ by GC	[g P(3HB) (100 g CDW) $^{-1}$]	42.8 ± 1.4	45.3 ± 2.2

amount of P(3HB) per g CDW after cultivation on OPH was similar to mineral medium with glucose (Table 1). However, CDW and the corresponding product yield were remarkably higher on OPH. These results are comparable with a study by Zhang et al. [56], where *B. megaterium* showed improved growth and P(3HB) production on hydrolysates of oil palm empty fruit bunch compared to pure sugars. Also, Kulpreecha et al. [38] reported that molasses as a carbon source enhanced biomass and P(3HB) formation compared to pure sugars.

Finally, characterization through GC, GPC, and ^{1}H NMR analysis helped to gain a more detailed insight into produced P(3HB). Exclusively hydroxybutyrate monomers were identified by GC, which is consistent with characterization of P(3HB) produced from glucose by this strain [55]. Additionally, ^{1}H NMR spectrometry from extracted polymer showed characteristic signals for P(3HB) at 5.2 ppm, 2.5 ppm, and 1.2 ppm [30,55] (Fig. S3). M_w of extracted P(3HB) was 57 ± 4 kDa with a Đ of 5.9. A wide range of M_w of PHA is reviewed in literature (50 to 10,000 kDa) with Đs between 1.1 and 6.0 [57], commonly >2 for the same *P. megaterium* strain [58]. Process parameters such as carbon source, nutrient supply as well as extraction method can affect the molecular weight of the polymer [59]. *P. megaterium* uyuni S29 was previously described to produce a P(3HB) homopolymers from glucose with different molecular mass fractions: 600 kDa and 125 kDa [55] or 795, 190, and 39.6 kDa with a Đ of 1.12 to 1.47 [29]. Comparably high Đ of P(3HB) obtained from OPH fermentation could be explained by the influence of pre-treatment or extraction procedure on the molecular masses of the polymer [60]. 27 % (*w*/w) of P(3HB) were recovered by the applied chloroform extraction method, which represented a similar recovery rate of P(3HB) to previous results (30 %) from the same *P. megaterium* strain after chloroform extraction [58]. Additionally, an extraction yield of 31 % from chloroform extraction was reported from P(3HB) produced by *B. cereus* [61].

3.4. PLA/P(3HB)/limonene blended films

The obtained bioproducts from the OP biorefinery approach were blended into PLA/P(3HB)/limonene blend films by twin-screw extrusion in a ratio based on previously tested optimal characteristic improvements [15,62]. Thereby an application for the combination of obtained valorization products of OPW could be demonstrated (Fig. S4). The films were characterized by FTIR as well as NMR spectroscopy. The spectra of the blend clearly showed the typical P(3HB) as well as PLA peaks confirming the successful presence of both polymers (Fig. 4). The limonene content (15 %), however, was too low to display clear bands in the blend spectra.

In Addition, TGA analysis showed the typical degradation profile of a polyester including the evaporation of limonene (Fig. 4C). DSC analysis showed that in comparison to PLA (Tg = 55 °C) the blended material has a decreased Tg of 45 °C, that is in line with the expected plasticization effect of the added limonene (Fig. 4D).

3.5. Biorefinery concept for orange peel waste

The present biorefinery concept includes pre-treatment of biomass followed by hydrolysis to solubilize substances such as monosaccharides or organic acids that can provide input for subsequent biotechnological processes [3,26]. OPW was previously discussed as valuable input to biorefineries for sustainable production of various bioproducts [2,63,64]. In the present study, a holistic valorization approach of OPW is presented and important process steps such as peroxidase extraction, limonene extraction, and fermentative LA and P(3HB) production, respectively, from OPW and hydrolysate are accomplished and evaluated (Fig. 5). Combining three of the obtained products, a transparent film by blending PLA/P(3HB)/limonene was produced. PLA is commonly used as a biobased alternative to fossil resource derived packaging materials as it can be produced from renewable resources and at the same time presents biodegradable properties. However certain characteristics such as water vapor and oxygen permeability as well as mechanical and thermal properties could be optimized through blending with other biopolymers and plasticizers [15]. Therefore, P(3HB) was considered as additional biobased and biodegradable polymer that finds application in short-term food packaging and is commonly blended with PLA thereby not only improving mechanical properties but as well increasing the biodegradability of the polymer [62]. For improving the

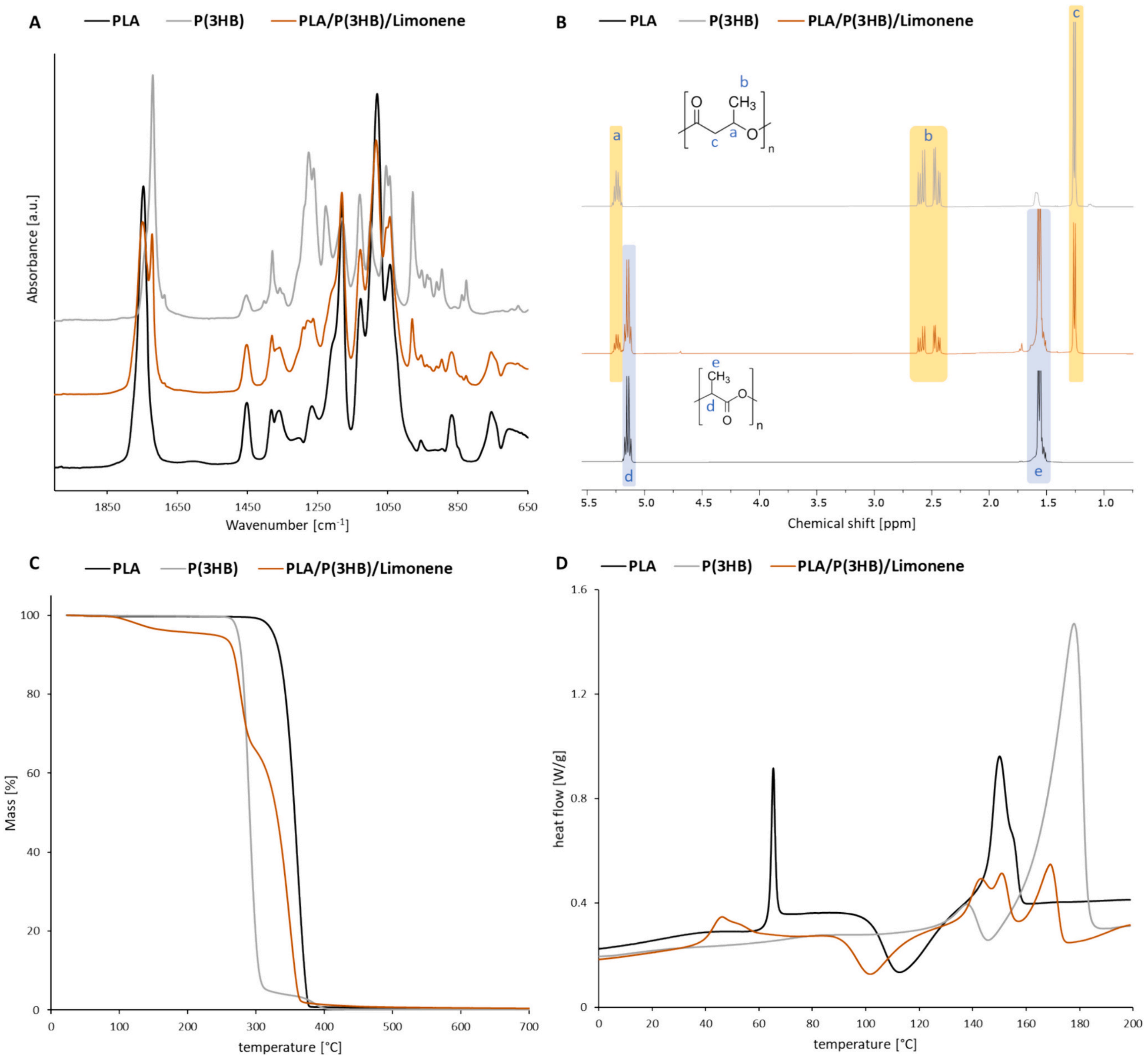

Fig. 4. A) FTIR spectrum from pure PLA and P(3HB) as well as blended PLA/P(3HB)/limonene films. Bands at 1720 cm^{-1} and 1750 cm^{-1} correspond to the aliphatic ester stretching of P(3HB) and PLA, respectively, with both bands also being present in the blend spectrum (full FTIR spectra Fig. S5). B) ^{1}H NMR spectra of PLA, P(3HB) and PLA/P(3HB)/Limonene blend. In yellow and blue the P(H3B) and PLA peaks are highlighted which could be observed in the PLA/P(3HB)/Limonene blend, respectively. $CDCl_3$ was used as the deuterated solvent. C) TGA curves. D) DSC curves.

flexibility of such polymer films, further addition of limonene as plasticizing agent has been described as a natural alternative suitable for food applications [15].

Residual streams generated within the biorefinery process can be treated [3], for instance, via anaerobic digestion [44,65]. While this study focuses on glucose for microbial fermentation, there are other potential carbon sources present in OPH, which could also be used in fermentation processes and contribute to the proposed biorefinery concept [3,63].

Overall, the results of our study constitute a proof of concept for extraction of the valuable bio compound limonene and peroxidase enzyme as well as production of building blocks and biopolymers, LA and P(3HB), respectively, from OPH. This offers an important option to reduce the overall costs of PLA and P(3HB) production [66] and concomitantly helps to exploit new valorization routes for OPW. In addition, utilization of sustainable and biodegradable polymers will help to make the packaging industry more environmentally friendly.

4. Conclusion

This study presents a holistic valorization route for OPW through initial extraction of the value-added products limonene and peroxidase and subsequent enzymatic hydrolysis yielding fermentable sugars. 8.2 g L^{-1} of limonene and high levels of specific peroxidase activity (426 ± 15 U mg^{-1}) were obtained. Limonene extraction was performed with the alternative solvents methanol and ethanol in contrast to conventionally applied hexane that raises environmental concerns. Besides 17.1 g L^{-1} ± 0.1 of LA from *W. coagulans*, 42.8 ± 1.4 % P(3HB) in CDW from *P. megaterium* were produced, showing the great potential of OPW as carbon source for microbial fermentation processes. Ultimately, a fully biobased PLA/P(3HB)/limonene blend was formed with limonene acting as a plasticizing agent for possible application in food packaging.

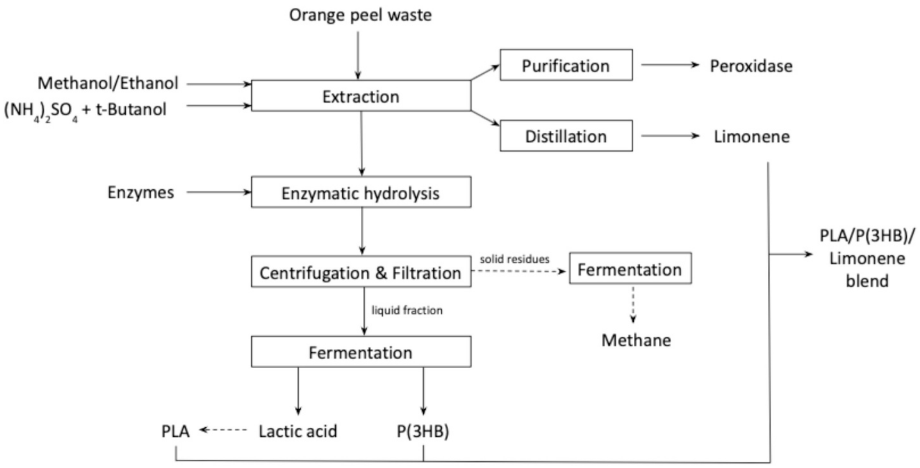

Fig. 5. Flow chart of proposed biorefinery concept for the valorization of orange peel waste with full arrows indicating processes performed in the current work.

Taken together, the presented concept was successfully tested, and the achieved results highlight the potential of OPW as a valuable input material for biorefineries. Thereby, this work helps to realize additional valorization routes for OPW and to reduce the loss of valuable resources.

CRediT authorship contribution statement

Sophia Mihalyi: Writing – original draft, Visualization, Investigation, Formal analysis. **Annika Putz:** Writing – original draft, Visualization, Investigation, Formal analysis. **Manuel Draxler:** Investigation. **Andreas Mautner:** Writing – review & editing, Resources. **Marion Sumetzberger-Hasinger:** Methodology, Formal analysis. **Filippo Fabbri:** Investigation, Formal analysis. **Alessandro Pellis:** Writing – review & editing, Investigation. **Markus Neureiter:** Writing – review & editing, Supervision, Resources. **Felice Quartinello:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Georg M. Guebitz:** Writing – review & editing, Supervision, Resources, Project administration.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.susmat.2024.e01110.

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