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Fruit pomace and waste frying oil as sustainable resources for the bioproduction of medium-chain-length polyhydroxyalkanoates

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ABSTRACT

Medium-chain-length polyhydroxyalkanoates (mcl-PHAs) are biobased and biodegradable alternatives to petrol-derived polymers, whose break-through has been prevented by high production cost. Therefore we investigated whether wastes from the food industry (nine types of fruit pomace including apricots, cherries and grapes, and waste frying oil) could replace the costly sugars and fatty acids typically used as carbon substrates for the bacterial fermentations. A selection of enzyme preparations was tested for converting the residual polysaccharides from the pomaces into fermentable monosaccharides. From the pomace of apricots, cherries and Solaris grapes, 47, 49 and 106 g L^{-1} glucose were recovered, respectively. Solaris grapes had the highest sugar content whereas apricots contained the fewest growth inhibitors. These two pomaces were assessed for their suitability to produce mcl-PHA in bioreactor. A 2-step fermentation was established with *Pseudomonas resinovorans*, hydrolyzed pomace as growth substrate and WFO as mcl-PHA precursor. Solaris grapes proved to be a very promising growth substrate, resulting in the production of 21.3 g PHA (L pomace)$^{-1}$ compared to 1.4 g PHA (L pomace)$^{-1}$ for apricots. Finally, capillary zone electrophoresis analyses allowed monitoring of sugar and organic acid uptake during the fermentation on apricots, which led to the discovery of reverse diauxie in *P. resinovorans*.

1. Introduction

Concerns about environmental and safety issues related to plastic usage and disposal has increased dramatically in recent years, yet the world demand for plastics is still steadily increasing. Extensive efforts have been made to look for bio-based, biodegradable and/or biocompatible alternatives to conventional petrol-derived plastics. As a result the total production of bioplastics amounted 1.4 million tons in 2012 and is expected to reach 6.2 million tons in 2017 [1]. Medium-chain-length polyhydroxyalkanoates (mcl-PHAs, composed of (R)-3-hydroxyalkanoates with 6–14 carbon units) represent a very promising family of biopolyesters in terms of versatility and material properties, with many applications foreseen especially in the biomedical field [2]. Nevertheless, their high production cost has been a hurdle for their break-through and further research and development is needed at this level.

Mcl-PHAs are produced intracellularly by *Pseudomonas* strains growing on carbohydrates (e.g. glucose) or fatty acids (e.g. nonanoic acid) [3]. However, these substrates are costly and there is also a need for more sustainable substrates that neither compete with the food chain nor rely on oil. In addition to agrowastes, waste residues from the food industry have a great potential as such [4] but require careful evaluation in the specific case of mcl-PHA production. Most of the recent studies dealing with wastes as PHA substrate have focused on the production of short-chain-length PHA (scl-PHAs, formed of (R)-3-hydroxyalkanoates with 4–5 carbon units) with mixed cultures [5]. However, this type of process cannot be applied for mcl-PHAs due to the small number of producing strains present in nature and the lack of selection pressure. Therefore, we decided to follow a different approach, targeting local waste streams to be upgraded into carbon substrates for aseptic mcl-PHA production processes with a single *Pseudomonas* strain. In addition, this strategy is expected to give rise to higher yields and allow better control of the polymer composition and properties.

Large amounts of fruit residues resulting from the pressing of grapes and the distillation of fruits such as apricots and cherries are available in Western Switzerland. These residues, called pomaces,

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are composed of fruit skins, pulp, stalks and seeds, and are considered as waste of no value. Their disposal, which mostly includes landfilling, incineration and composting, must be carried out carefully to prevent any environmental hazards [6]. Alternatively fruit pomaces can be used as a source of valuable products such as organic fertilizers, phenolic compounds (e.g. pigments, antioxidants, antimicrobials) or grape seed oil [7,8]. Another often overlooked feature of fruit pomaces is their high content in polysaccharides (cellulose, hemicellulose, starch and pectin) which can be turned into carbon substrates for fermentation [9]. The interest in this kind of low cost substrate has increased considerably in the last years and a few studies have reported pomace-based bioprocesses for the production of enzymes, organic acids and fuels. Most of these bioprocesses were performed as solid-state fermentations but, to the best of our knowledge, only two as submerged fermentations. The latter two relied on two different approaches: Korkie and coworkers isolated yeast strains able to both hydrolyze complex polysaccharides and produce ethanol [9] while Zheng and coworkers performed simultaneous saccharification and fermentations and added a mixture of cellulase, β-glucosidase and pectinase to hydrolyze the pomace in the culture broth [10].

Waste frying oil (WFO) also represents a promising source of carbon not only for scl-PHA [11–13] but also for mcl-PHA bioprocesses. Available in huge amounts it is typically incinerated for generating energy but since the 2000s it has attracted more and more interest for biodiesel production [14]. The triacylglycerides that constitute the oil must be converted into fatty acids in order to be metabolized in mcl-PHA by *Pseudomonas* strains. This can be achieved chemically by saponification [15–17], enzymatically with the help of lipases [18] or biologically by a few *Pseudomonas* strains such as *Pseudomonas* sp. strain DR2, *Pseudomonas aeruginosa* 42A [19] and *Pseudomonas resinovorans* [20,21] that possess the required extracellular lipases.

In this work, we evaluated for the first time the potential of pomaces from apricots, cherries and grapes to be used as cheap, sustainable carbon substrates for producing mcl-PHA with *Pseudomonas* strains. The pomaces were characterized with respect to sugar and acid contents, and an enzymatic saccharification step was established for the recovery of fermentable carbohydrates. In addition, the presence of cell growth inhibitors (e.g. polyphenols) in the different pomaces was investigated. A two-step fermentation process with *P. resinovorans* was designed in which hydrolyzed pomace (HP) of apricots or grapes was used as growth substrate and supplemented to a synthetic medium providing the other elements required for cellular growth (N, P, S, etc.) (Fig. 1). When growth was about to stop, WFO was added to serve as mcl-PHA precursor. Finally, an analytical method based on capillary zone electrophoresis (CZE) was implemented to monitor the uptake of monosaccharides and organic acids during the fermentation on apricot HP.

2. Materials and methods

2.1. Strains and substrates

Nine types of fruit pomace were collected by Changins from different locations in Switzerland and stored at −20 °C until use. The description of these pomaces is given in Table 1. WFO used for frying potatoes was obtained from a fast-food restaurant.

Pseudomonas putida KT2440 (ATCC 47054) was used for growth inhibition studies and *P. resinovorans* (DSMZ 21078) for mcl-PHA production bioprocesses. The strains were stored at −80 °C in the preculture medium (see Section 2.4) and 15 wt% glycerol.

2.2. Characterization of the fruit pomaces

The pomaces were thawed, stomached for 120 s after addition of sterile water (10 g of pomace + 40 g of sterile water), and filtered. The filtrate was centrifuged for 5 min at 13,000 rpm before analysis.

High pressure liquid chromatography (HPLC) was used for the quantification of sugars (glucose and fructose), ethanol and organic acids with a Grom Resin H+ IEX column (Grace Davison, Deerfield, USA, 8 μm, 388 mm × 8 mm ID). The eluent was H_2SO_4 with a flow rate at of 1 mL min^{-1} at 60 °C. The HPLC (Ultimate 3000, ThermoFischer, Waltham, USA) was connected to two detectors in series (UV at 210 nm and RI) [19]. UV detection was used for malic acid analysis and RI for succinic, lactic and acetic acid as well as for fructose, glucose and ethanol. Tartaric acid was quantified by the Rebelein method according to Lipka and Tanner for apricot and cherry pomaces, and by HPLC with RI detection for grape samples. Tannin power was estimated by a nephelometric method with a tannometer (Pfeuffer GmbH, Kitzingen, Germany) measuring the reactivity of tannin with polyvinyl pyrrolidone (PVP) as the amount of haze formed [22]. Tannin power was expressed in mg PVP L^{-1} and then converted to mg kg^{-1} of pomace.

2.3. Saccharification tests

2.3.1. Adjustment of dry matter and pH

After thawing, the pomaces (apricots and cherries) and grape residues were crushed in a Turmix CX 750 household blender (DKB Household Switzerland AG, Zurich, Switzerland) and a Retsch ZM 100 grinding mill (Retsch GmbH, Haan, Germany), respectively. The dry matter content of these materials was measured following overnight drying in an oven at 105 °C. Cherry and apricot pomaces were liquid enough and could be used directly, without addition of water, whereas 300 g of water were added to 100 g of grape residues prior to enzymatic treatment. The pureed material was passed through a kitchen strainer (mesh size 1.0 mm) to eliminate the largest particles. The pH of the suspension was adjusted with 30% NaOH to the optimal pH value for saccharification treatment (see Supplementary data, Table S1 for the detailed pH values).

2.3.2. Enzymatic treatment

A selection of enzyme cocktails were obtained from various suppliers. Ten milliliters of apricot pomace were heated to the optimal temperature and shaken at 1000 rpm in a Thermo Shaker Incubator MS-100 (Hangzhou Allsheng Instruments Co. Ltd, Hangzhou, China). The liquid enzyme preparation was added according to the recommended concentration and the solid/liquid suspension further shaken under the same conditions. The enzyme cocktails as well as their concentration and working conditions are described in Table S1 of Supplementary data. Samples of 1.0 mL were withdrawn at specific time intervals between 0 and 24 h and inactivated at 95 °C for 20 min. The samples were centrifuged 10 min at 20,000 × *g* and the supernatant analyzed by HPLC as described in Section 2.5.3.

The enzyme cocktail NS-22086 was found to be the most efficient enzyme (see Section 3.2) and therefore used for all subsequent saccharification experiments. Saccharification tests were carried out with the nine types of fruit pomace as described above. In addition, a control experiment was conducted with 6 g L^{-1} NS-22086 preparation at 50 °C, using 456 mg filter paper (pure cellulose) as substrate, dispersed in 5 mL acetate buffer at pH 5.

2.3.3. Preparation of the carbon substrates for growth experiments

Saccharification of the pomaces for the growth experiments with *P. putida* KT2440 and *P. resinovorans* was carried out similarly to as described in Section 2.3.2 but in a total volume of 1 L with an enzyme concentration of 6 g L^{-1} and a saccharification time of