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Immobilisation of Endoinulinase on Polyhydroxybutyrate Microfibres

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Abstract

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Due to the health benefits associated with the consumption of prebiotic short-chain fructooligosaccharides (sc-FOS) and inulooligosaccharides (IOS), there is increased interest in the use of these compounds in food products. We have developed a new biocatalyst for the production of FOS and IOS by inulin hydrolysis. Endoinulinase from *Aspergillus niger* Inulinase® Novozym 960 (Novozymes) was immobilised on polyhydroxybutyrate (PHB) nanofibres and microfibres by hydrophobic interactions. The PHB fibres were prepared by centrifugal spinning. FOS and IOS profiles were determined by ion-exchange chromatography with the Rezex RSO-Oligosaccharide column. The biocatalyst had very good activity and stability after repeated applications. It can be used in biocatalytic membrane reactors for the production of prebiotic oligosaccharides.

Keywords: inulin; fructooligosaccharide; inulooligosaccharide; inulinase; nanofibres; PHB

Fructans (polyfructosylsucrose) are important storage carbohydrates in plants such as *Poaceae* (e.g. wheat and barley), *Asteraceae* (e.g. burdock, chicory, and Jerusalem artichoke), and *Liliaceae* (e.g. onion and asparagus) (Vijn & Smeekens 1999). Inulin-type fructan, which is a β-2,1 linked fructose oligomer or polymer terminated by glucose, is mainly accumulated in plants of the family *Asteraceae*. The degree of inulin polymerisation ranges from 10 to 60. Inulin can be degraded enzymatically or chemically to a mixture of oligosaccharides with the general structure Glu-Fru_n (GF_n), which are called fructooligosaccharides (FOS), and Fru_m (F_m), which are called inulooligosaccharides (IOS). This process also occurs in nature to some extent, and these oligosaccharides can be found in a large number of plants, especially in Jerusalem artichoke, chicory, and the blue agave plant.

FOS and IOS are low-molecular-weight materials with degrees of polymerisation up to 10. Inulin and fructooligosaccharides (FOS) are soluble prebiotic fibres that promote the gut and overall health through their fermentation by gut flora, to yield important metabolites, including lactic acid, and the short chain-fatty acids acetate, propionate and butyrate. FOS serves as a substrate for microflora in the large intestine, increasing the overall gastrointestinal tract health. It has also been tested as a supplement for preventing yeast infections. Several studies have found that FOS and inulin promote calcium absorption in both the animal and human gut. The intestinal microflora in the lower gut can ferment FOS, which results in a reduced pH. Calcium is more soluble in acids, and therefore, more of it comes out of food and is available to move from the gut into the bloodstream. FOS exhibit sweetness levels between 30 and 50% of saccharose (Singh & Tank 2014). Inulooligosaccharides from inulin have a very similar chemical structure to fructooligosaccharides. Although it has been reported that IOS show quite similar functional properties to FOS, and a few researchers have described their potential for alternative oligosaccharides (Norman & Hojer-Perderson

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1989), extensive experimental results have scarcely been presented. The main components of commercial prebiotic products are kestose (GF2), nystose (GF3), fructosylnystose (GF4), inulobiose (F2), inulotriose (F3), and inulotetraose (F4). Among FOS, the ones with low polymeric grade show better therapeutic properties than those with a high polymeric degree (Singh & Tank 2014).

Two different classes of FOS mixtures are produced commercially, based on inulin hydrolytic degradation or transfructosylation processes using fructosyltransferase (FTase) enzymes. FTase enzymes can be found in many plants and microorganisms. Inulin can be hydrolysed by either sole action of exoinulinase or synergistic action of exo-(β-D-fructanfructohydrolase, EC 3.2.1.80) and endoinulinase (2,1-β-D-fructanfructanohydrolase, EC 3.2.1.7) (Yun *et al*. 1997). Exoinulinases split the terminal units of inulin in sucrose and inulin with a lower degree of polymerisation and liberate fructose. Endoinulinases hydrolyse inulin by breaking the bonds between fructose units that are located away from the ends of the polymer network, to produce oligosaccharides (Baston *et al*. 2013). Inulinases can be used in a wide range of industrial applications: for obtaining an ultra-high fructose syrup from inulin, bioethanol production, FOS and IOS production, production of citric acid, alcohols, lactic acid, etc. During the years, many bacterial, filamentous fungi, and yeast strains were used for inulinase production. Among the various microbial strains, *Kluyveromyces marxianus* and *Aspergillus niger* were reported as the most common and preferred sources for inulinase production (Singh & Gill 2006; Chi *et al*. 2009). Inulinases are enzymes that have different catalytic properties: molecular weight, optimum pH, and temperature of action, stability, according to the sources (Baston *et al*. 2013).

Recent interest in nanotechnology has provided a wealth of diverse nanoscaffolds that could potentially support enzyme immobilisation due to their potential applications in biotechnology, immunosensing, and biomedical areas. Immobilisation of enzymes is advantageous for commercial application due to convenience in handling, ease of separation of enzymes from the reaction mixture and reuse, low product cost, and a possible increase in thermal and pH stability (Ansari & Husain 2012). Various nanostructures have been examined as hosts for enzyme immobilisation via approaches including enzyme adsorption, covalent attachment, enzyme encapsulation, and sophisticated combinations of methods (Kim *et al*. 2006).

Several carrier materials have already been examined for endoinulinase immobilisation, such as polystyrene, chitin and chitosan granules, activated alumina, anion-exchange resin (Yun *et al*. 2000), poly-D-lysine coated $CaCO_3$ micro-particles (Karimi *et al*. 2014b), functionalised silica nanoparticles (Karimi *et al*. 2014a), carbon nanotubes (Garlet *et al*. 2014), or alginate-chitosan beads (Missau *et al*. 2014).

Polyhydroxyalkanoates (PHAs) belong among the promising materials for enzyme immobilisation. PHAs are a group of water insoluble biodegradable biopolymers produced by a number of bacteria that naturally accumulate them as intracellular granules in response to environmental stress and nutrient imbalance. Among PHAs, polyhydroxybutyrate (PHB) is a short-chain-length polymer synthesised by bacteria and it is the most frequently studied among the PHA polymers (Deepak *et al*. 2009). PHB particles or beads have already been used for immobilisation of nattokinase (Deepak *et al*. 2009) and lipases (Mendes *et al*. 2012; Silva *et al*. 2014).

In this study we have developed a cheap, simple, and effective method of the preparation of a new biocatalyst for the production of FOS and IOS by inulin hydrolysis using a commercial endoinulinase from *Aspergillus niger*.

MATERIAL AND METHODS

Material. Commercial polyhydroxybutyrate Biomer microparticles, Lot T21, were purchased from Biomer (Krailling, Germany). Endoinulinase Inulinase® Novozym 960 (Sigma-Aldrich, Prague, Czech Republic) from *Aspergillus niger* and inulin Frutafit® (Sensus, Roosendaal, Netherlands) were used for the experiments.

Inulinase Novozym 960 from Novozymes A/S (Bagsvaerd**,** Denmark) is a liquid with the density of 1.17 g/ml and activity of 394 INU/g at pH = 6. One INU (inulinase unit) is equivalent to the amount of the enzyme that produces 1 μmole of reducing carbohydrate per minute under the conditions of 50°C and pH = 4.7.

FOS standards: 1-kestose, nystose, and 1-F-fructofuranosylnystose were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

PHB fibre production. A polyamide bell of 12 cm in upper diameter, 10 cm in bottom diameter, and

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8 cm in height was used for the centrifugal PHB fibre production. The bell rotated at a constant velocity of 2500 rpm using an electromotor. A 5% (w/w) PHB solution in chloroform was pumped to the bell bottom by a tube with a constant flow rate in the range of 1–5 ml/minute. The fibres formed at the edge of the rotating bell were collected on a scaffold made from stainless steel. The whole equipment for the centrifugal nanofibre and microfibre production was described in details elsewhere (Beran *et al*. 2015).

Immobilisation of endoinulinase onto PHB fibres. Initially, 100 mg of PHB fibres were soaked into 50 ml of anhydrous ethanol for 30 min at room temperature, following a previously published procedure (Mendes *et al*. 2012). After the swelling step, the excess of ethanol was removed and the fibres were washed thoroughly with demineralised water under vacuum filtration. The washed fibres were incubated in 50 ml of the Inulinase® Novozym 960 liquid under continuous agitation in an orbital shaker (200 rpm) at 25°C for 12 hours. Finally, the fibres were washed thoroughly with demineralised water, dried at room temperature, and weighed.

Scanning electron microscopy. The morphology of PHB fibres before and after inulinase immobilisation was evaluated by scanning electron microscopy (SEM). The samples of the fibres were fixed with a double-faced adhesive tape to the holders and evaluated in a Phenom G2 scanning electron microscope (Phenom-World BV, Eindhoven, Netherlands).

Inulinase assay. Inulinase assay was carried out by a modified method described by Baston *et al*. (2013). The assay was performed in a 2% (w/w) inulin solution in 0.1 M phosphate buffer, pH 6.0, temperature 60°C, under continuous agitation in an orbital shaker (200 rpm). The enzymatic reaction was stopped after 60 min by boiling the samples for 10 min in a water bath. After cooling, the amount of reducing sugars, expressed as fructose, was determined using the 3,5-dinitrosalicylic acid (DNSA) method based on a standard calibration curve. GF_n are non-reducing carbohydrates, F_m has a reducing fructose end group. The assay was performed in triplicate for each sample.

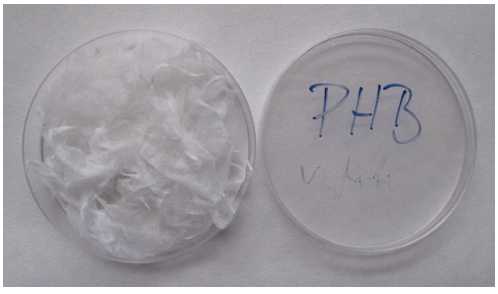

Figure 1. 3D fluffy structure of PHB fibres

Determination of product profiles of inulin hydrolysis with the immobilised Inulinase® Novozym 960 preparation. 0.25 g of the fibre mat with the immobilised Inulinase® Novozym 960 preparation was added into 100 ml of 15% (w/w) inulin solution in 0.1 M phosphate buffer, pH 6.0, temperature 60°C. The suspension was placed in an orbital shaker under continuous agitation at 200 rpm at 60°C. One ml samples were collected in given time intervals. The enzymatic reaction in the samples was stopped by boiling the samples for 10 min in a water bath. Inulin, FOS, IOS, saccharose, glucose, and fructose concentrations were determined chromatographically (see below).

Determination of reusability of the immobilised Inulinase® Novozym 960 preparation. To evaluate the reusability of the immobilised endoinulinase, the fibre mat with the immobilised enzyme was removed from the inulin solution and washed thoroughly with demineralised water in order to avoid any residual substrate on the fibre mat. The fibre mat was reintroduced into a fresh reaction medium.

Chromatographic determination of inulin, IOS, FOS, saccharose, glucose, and fructose. Product profiles of inulin hydrolysis were determined by HPLC chromatography using the Rezex RSO-Oligosaccharide Ag^+ (4%, w/w) column, 200 × 10.0 mm, with the flow rate of 0.3 ml/min of degassed and demineralised water, column temperature 50°C, refractometric detector temperature 35°C (Agilent 1100 HPLC system; Agilent Technologies, USA), and sample volume 20 µl. The FOS standards were used for determination of FOS peaks. IOS peaks were identified as they were eluted by the decreasing order of molecular weight, as described previously (Ronkart *et al*. 2007).

RESULTS AND DISCUSSION

The presence of the enzyme did not influence either fibre size or fibre morphology (Figure 2). Mainly microfibres with a diameter in the range of 1–3 µm were produced, but a small portion of submicron fibres was also observed.

Endoinulinase activity of the original Inulinase® Novozym 960 preparation was 394 ± 14 INU/g (mean ± average mean error). Endoinulinase activity of the immobilised endoinulinase preparation was 319 ± 12.

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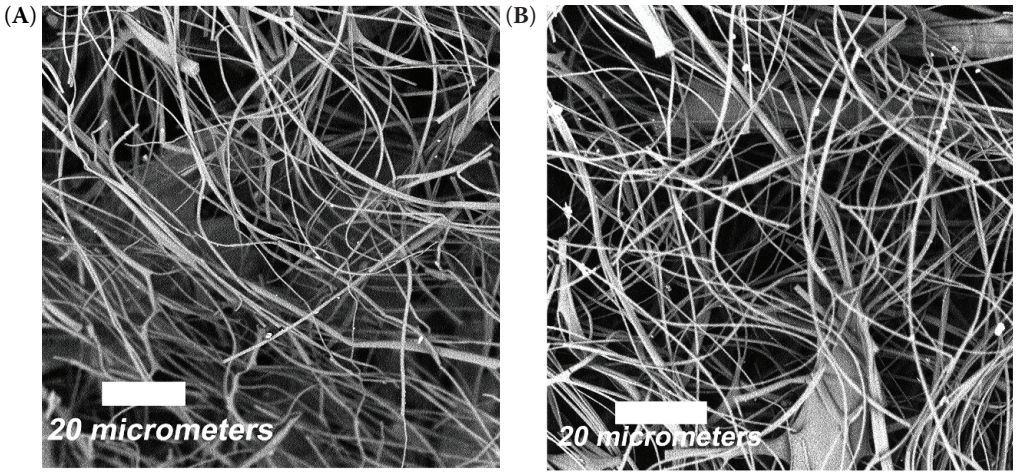

Figure 2. SEM photomicrographs of PHB fibres without (A) and with (B) immobilised endoinulinase

No significant decrease in endoinulinase activity was observed after 3-month storage of the immobilised enzyme preparation in dry conditions, when the activity was 312 ± 9 INU/g.

During the Ag^+ ion-exchange chromatography FOS and IOS peaks were eluted by the decreasing order of molecular weight (Figures 2 and 3). Thus, inulin was the first, then individual FOS, the disaccharide sucrose, and finally the monosaccharides glucose and fructose. Similar IOS and FOS product profiles were obtained with the free and immobilised endoinulinase (results for the free enzyme not shown). Inulotriose (F3), followed by inulobiose (F2), 1-F-fructofuranosylnystose (GF4), inulotetraose (F4), and xystose (GF3) are the main oligosaccharides produced by the inulin hydrolysis. GF4 accumulation was significantly faster with the immobilised enzyme than with the free one. No accumulation, but even a decline of GF2, originally contained in the Frutafit® inulin, was observed with both the free and immobilised enzyme. Yun *et al.* (2000) obtained a different product profile with endoinulinase extracted from *Pseudomonas* sp. and immobilised on different carrier materials, when inulobiose (F2) was the main product of inulin hydrolysis, followed by oligosaccharides with the degree of polymerisation = 3. The enzyme loading capacity was comparable with the results of Missau *et al.* (2014), and significantly higher in comparison with the results obtained by other authors (Garlet *et al.* 2014; Karimi *et al.* 2014a, b).

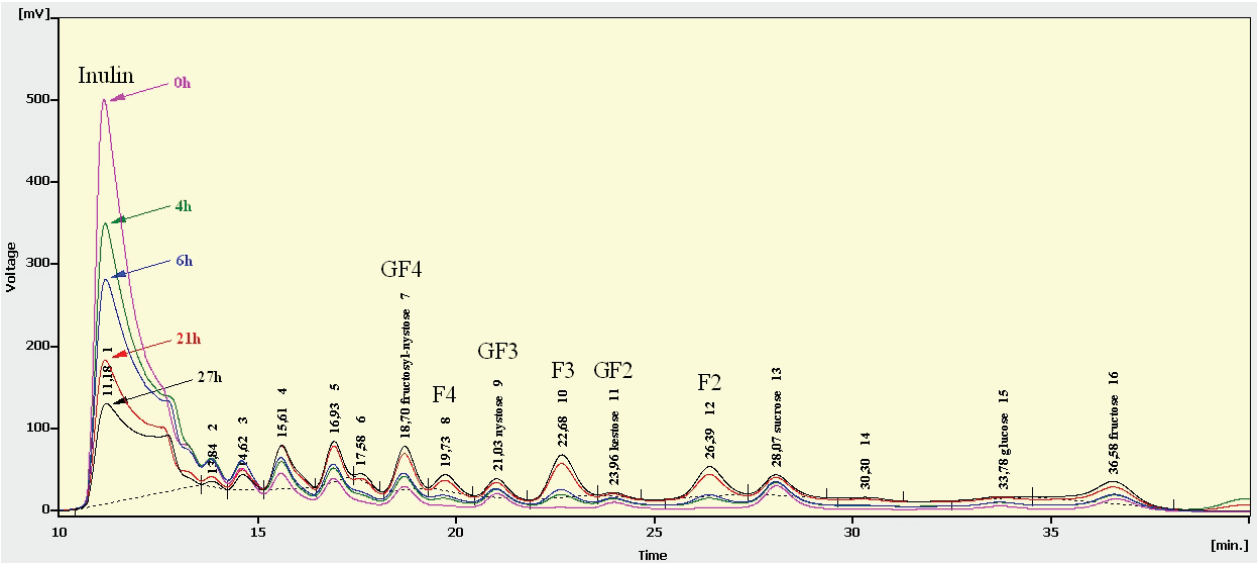

Figure 3. HPLC chromatograms of inulin hydrolysates obtained in different time intervals with an enzyme preparation consisting of endoinulinase immobilised onto PHB microfibres; the first experiment

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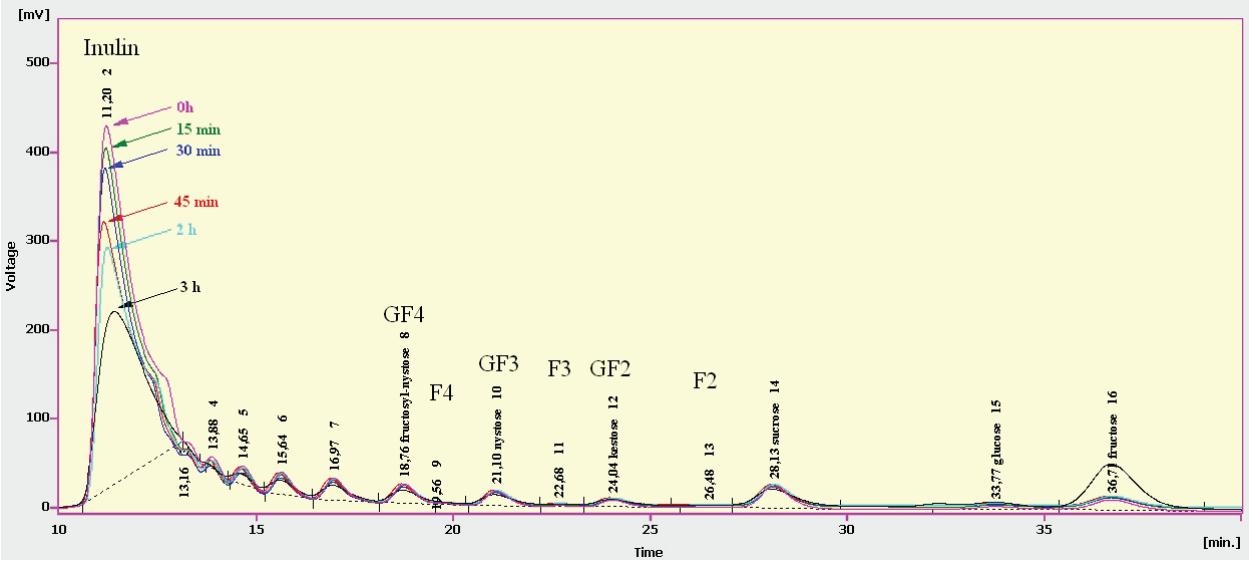

Figure 4. HPLC chromatograms of inulin hydrolysates obtained in different time intervals with endoinulinase immobilised onto PHB fibres; the second experiment with the same enzyme preparation, repeated after 93 days

CONCLUSION

Centrifugal production of PHB fibres is a simple and cheap process. PHB fibres can be used for the immobilisation of a wide range of different enzymes in an easy way, without necessity of chemical crosslinking. The biocatalyst described in this paper has very good activity and stability after repeated applications. It can be used for the production of prebiotic FOS and IOS oligosaccharides. Nanofibrous or microfibrous PHB membranes, hollow fibres, or tubes can be utilised in different kind of biocatalytic membrane reactors or biosensors.

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