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Immobilization of the β-fructofuranosidase from *Xanthophyllomyces dendrorhous* by Entrapment in Polyvinyl Alcohol and Its Application to Neo-Fructooligosaccharides Production

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Abstract: The β-fructofuranosidase (Xd-INV) from the basidiomycota yeast *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*) is unique in its ability to synthesize neo- fructooligosaccharides (neo-FOS). In order to facilitate its industrial application, the recombinant enzyme expressed in *Pichia pastoris* (pXd-INV) was immobilized by entrapment in polyvinyl alcohol (PVA) hydrogels. The encapsulation efficiency exceeded 80%. The PVA lenticular particles of immobilized pXd-INV were stable up to approximately 40 °C. Using 600 g/L sucrose, the immobilized biocatalyst synthesized 18.9% (*w*/*w*) FOS (59.1 g/L of neokestose, 30.2 g/L of 1-kestose, 11.6 g/L of neonystose and 12.6 g/L of blastose). The operational stability of PVA-immobilized biocatalyst was assayed in a batch reactor at 30 °C. The enzyme preserved its initial activity during at least 7 cycles of 26 h.

Keywords: glycosidases; fructooligosaccharides; prebiotics; enzyme entrapment; immobilization; bioreactors; hydrogels; neokestose

1. Introduction

Fructooligosaccharides (FOS) are fructose oligomers linked to a sucrose skeleton by different β(2→1) or β(2→6) glycosidic bonds [1]. In addition to their prebiotic properties—which promote the development of bifidobacteria and lactobacillus in the gastrointestinal tract [2]—and their low glycemic index, FOS may exert other benefits in human health, including a better gut absorption of Ca^{2+} and Mg^{2+}, a reduction of blood lipid levels and a reduced risk of suffering colon cancer [3,4]. FOS can be synthesized from sucrose by a transfructosylation reaction [5]. Commercial FOS possess an inulin-type structure containing β(2→1) linked fructose units [6]. However, it has been reported that neo-FOS, in which one fructosyl moiety is β(2→6) linked to the glucose unit of sucrose, could display improved prebiotic and physicochemical properties with regard to inulin-type FOS [7,8].

The β-fructofuranosidase Xd-INV from the yeast *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*) is a dimeric glycoprotein with a molecular mass of 320–380 kDa, which belongs to the glycoside hydrolase (GH) family 32 [9,10]. Like other β-fructofuranosidases, Xd-INV

catalyzes both the hydrolysis of sucrose and the synthesis of FOS [11,12]. However, Xd-INV is unique in its ability to catalyze the transfer a fructosyl moiety to the 6-OH hydroxyl of glucose unit in sucrose. In fact, this is the most efficient enzyme reported for the production of neo-FOS (basically neokestose and neonystose) [11,13]. Xd-INV is an attractive enzyme not only for the production of neo-FOS, but also for the preparation of novel fructosylated derivatives [13]. Recently we successfully expressed this enzyme in *Pichia pastoris* (pXd-INV) yielding a significant volumetric activity [12].

Despite the enormous potential of biocatalytic processes [14], the industrial application of enzymes is often hampered by a lack of long-term operational stability and the difficulties to recover and reuse the biocatalysts [15]. Enzyme immobilization can help to overcome these drawbacks, since it allows the easy separation of the biocatalyst facilitating product recovery, which is commonly accompanied by the stabilization effect towards denaturation by high temperatures, extreme pHs or organic cosolvents [16,17].

Immobilization methodologies for industrial biotransformations should be relatively simple, inexpensive and provide active biocatalysts with substantial stability [15]. The strategies for enzyme immobilization are commonly classified into three groups [18]: support binding (by adsorption or covalent linkages), entrapment and cross-linking. For reactions involving the transformation of carbohydrates, covalent binding is preferred over adsorption to avoid enzyme leakage [19], but most of the commercial activated carriers are expensive [20–22]. Cross-linking gives rise to biocatalysts with highly concentrated enzyme activity, significant stability and low production costs due to the absence of carrier, although the recovery of activity is commonly low [14,15]. Entrapment is an efficient and inexpensive technique, which is very useful when substrates and products have low molecular sizes and high diffusion rates, as occurs with simple sugars [14,23,24]. The entrapment in hydrogels can be combined with cross-linking in order to provide more resistant biocatalysts [25,26].

In this work, we have investigated the immobilization of recombinant pXd-INV to facilitate its industrial application in the production of neo-FOS and other fructosylated derivatives. Considering that the size of Xd-INV is significantly large (it is a dimeric enzyme with an average molecular mass of 360 kDa and dimensions 135 × 75 × 45 Å [10]), we believed that entrapment methodologies could be appropriate for this enzyme as the leakage through pores should be restricted Our focus was to evaluate polyvinyl alcohol (PVA) entrapment as an immobilization strategy. PVA is cheap, mechanically robust and nontoxic to organisms [27]. The efficiency of this methodology was assessed in terms of the recovered activity and operational stability. The resulting biocatalysts were applied to the production of neo-FOS.

2. Results and Discussion

2.1. Immobilization of pXd-INV in PVA Hydrogels

The recombinant β-fructofuranosidase pXd-INV from *X. dendrorhous* was immobilized by entrapment in lenses of polyvinyl alcohol (PVA), as represented in Figure 1. The enzyme was mixed with a solution of PVA, and the gelification was accomplished by dripping the mixture onto a surface (96-well microplate) followed by partial drying at 50 °C [28]. The gelification can be also promoted by freezing and thawing [29,30] or by UV radiation [31]. The entrapment in highly elastic and stable hydrogels formed by PVA has given excellent results with glycosidic enzymes [26,30,32]. The gelation takes place by the formation of hydrogen bonds between the hydroxyl groups of the PVA, resulting in a noncovalent spatial network [33]. These hydrogels are also very useful in controlled drug release, artificial tissues, bioseparations or biosensors [34,35]. PVA-based biocatalysts have proven remarkable operational and mechanical stability in different types of bioreactors, including shaken microtiter plates, batch stirred tanks and packed-bed reactors [32].

Table 1 summarizes the main immobilization parameters using 10% (*w*/*v*) PVA in 100 mM sodium acetate (pH 5.0), the optimum buffer for this enzyme. Two different enzyme loadings were assayed. Lens volume ranged 52–56 µL. An increment of the initial enzyme activity did not result in a significant increase of the volumetric activity of the biocatalyst. In contrast, the recovered activity was substantially diminished compared with lower enzyme loadings. We checked by successive washings that pXd-INV was not released from the PVA hydrogels. Considering that this enzyme is quite stable under the immobilization conditions, we believe that a high enzyme concentration inside the PVA lenses may cause some diffusional limitations, thus explaining the results obtained when increasing the enzyme loading. Starting from 35.5 enzyme units (measured in the DNS assay) and 5 mL PVA solution, the encapsulation efficiency was satisfactory (>80%).

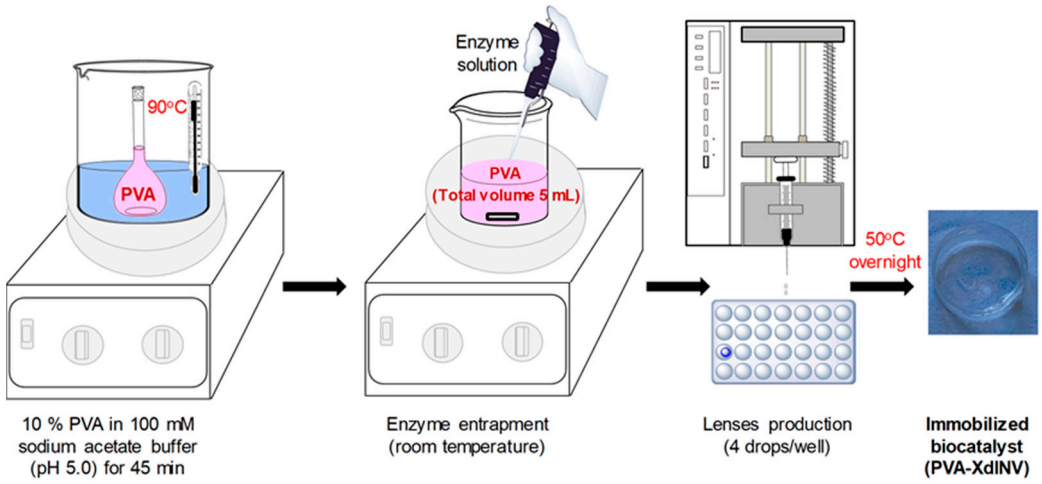

Figure 1. Immobilization of pXd-INV by entrapment in polyvinyl alcohol (PVA).

Table 1. Effect of pXd-INV enzyme loading on the activity and recovery upon immobilization by entrapment in PVA hydrogels.

Total Initial Activity (U) [a]	Volume of Biocatalyst (mL)	Lens Volume (µL) [b]	Activity of the Biocatalyst (U/lens) [a]	Activity of the Biocatalyst (U/ml) [a]	Recovered Activity (%) [c]
35.5	4.8	56.3	0.34	5.96	80.6
84.5	4.7	52.6	0.33	6.20	34.5

[a] Measured by the DNS assay; [b] Lens volume = (volume dispensed/number of drops dispensed) × number of drops in each lens; [c] (Activity of the biocatalyst × Volume of biocatalyst obtained × 100)/Total activity introduced.

The operational stability of the biocatalyst obtained with the lowest enzyme loading (35.5 U) was assayed following a micro-scale procedure previously described by our group [36]. We performed 10 reaction cycles of 20 min with 100 mg/mL sucrose as substrate at 50 °C, measuring the amount of reducing sugars by the DNS method (Figure 2). Between cycles, the biocatalyst was thoroughly washed with 100 mM sodium acetate buffer (pH 5.0). As shown, the operational stability of PVA-lens shaped particles was very satisfactory during at least 10 short cycles.

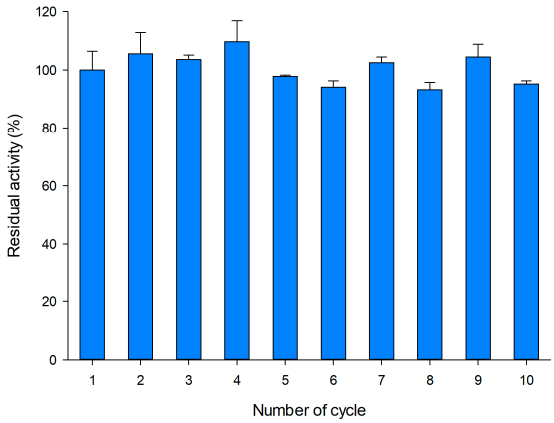

Figure 2. Operational stability of entrapped pXd-INV in PVA. Conditions for each cycle: 100 mg/mL sucrose, 50 °C, 20 min reaction.

2.2. Production of FOS by Entrapped pXd-INV

Using 600 g/L substrate, we analyzed the production of fructooligosaccharides catalyzed by immobilized pXd-INV. A high concentration of sucrose favours transglycosylation reaction in detriment of hydrolysis of the disaccharide [37]. The profile of the reaction products was characterized by High Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) (Figure 3). The products pattern correlated well with that obtained using the soluble enzyme expressed in *P. pastoris* [12]. Using neo-FOS standards purified and characterized as described in previous works [11,38], we were able to identify neokestose (the major product), 1-kestose and neonystose. Peak 5 was blastose [Fru-β-(2→6)-Glc], a sucrose isomer member of the neo-FOS series, which is produced by the hydrolysis of neokestose. We demonstrated in previous publications that Xd-INV was unable to transfer the fructosyl moiety to free glucose [11,12]. The structure of the FOS synthesized by immobilized pXd-INV is depicted in Figure 4.

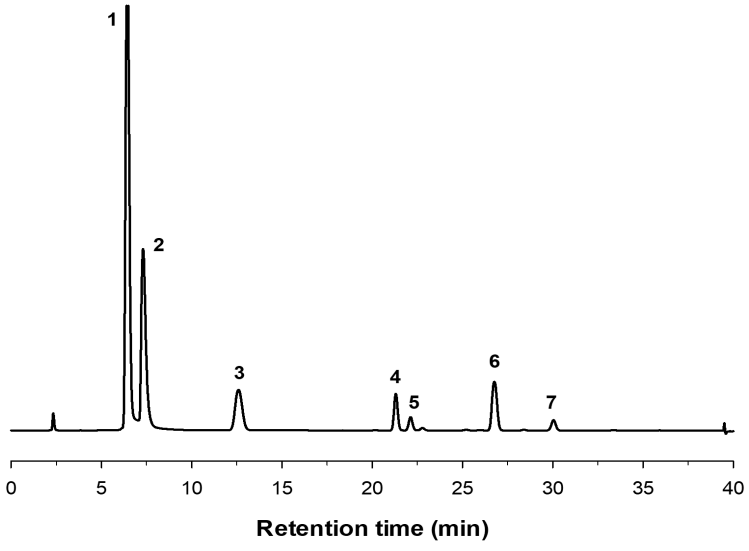

Figure 3. HPAEC-PAD chromatogram of the reaction of 600 g/L sucrose at 50 °C with pXd-INV entrapped in PVA. (1) Glucose; (2) Fructose; (3) Sucrose; (4) 1-Kestose; (5) Blastose; (6) Neokestose; (7) Neonystose.

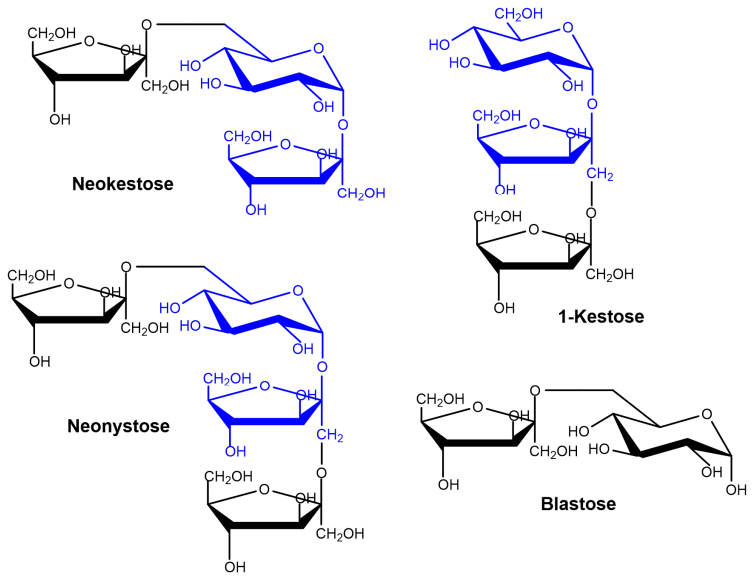

Figure 4. Structure of the FOS synthesized by immobilized pXd-INV. The sucrose skeleton is represented in blue.

2.3. Thermal Stability of PVA Lens-Shaped Particles

The operational stability represented in Figure 2 corresponded to short reaction cycles (20 min) at 50 °C. The optimum temperature for both native and recombinant Xd-INV is in the range 50–60 °C [11,12]. However, in order to assess the potential of the immobilized pXd-INV biocatalysts for the synthesis of neo-FOS, we should consider the thermostability of the PVA lens-shaped particles in long-term operation. Thus, the immobilized biocatalyst was incubated at different temperatures (4–60 °C) for 24 h at pH 5.0, and the residual activity was measured by the DNS assay (Figure 5). As shown, the activity of the PVA-lens shaped particles was maintained up to approximately 40 °C. At higher temperatures, more than half of the activity was lost in one cycle. However, the soluble enzyme is very stable up to 60 °C during at least 24 h (data not shown). In this context, it is well reported that PVA hydrogels become unstable at temperatures above 50 °C [39,40]. The loss of 3D structure of the lenses may facilitate enzyme leakage thus resulting in a decrease of the activity. Based on the above data, we selected 30 °C to assess the operational stability of the biocatalyst for neo-FOS synthesis, as a compromise between enzyme activity and stability.

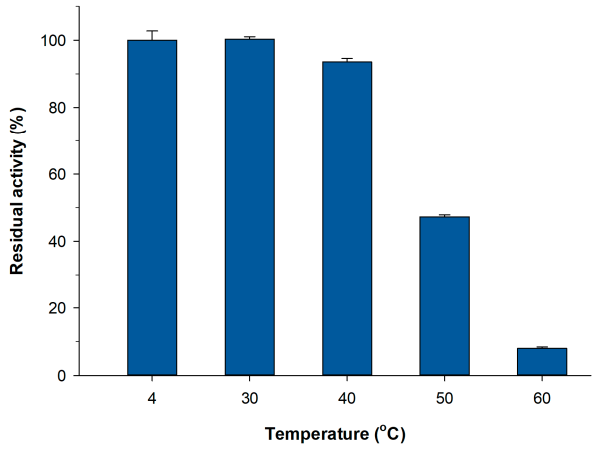

Figure 5. Thermostability of immobilized pXd-INV in PVA. Incubation conditions: 100 mM sodium acetate buffer (pH 5.0), 24 h.

2.4. Kinetics of Neo-FOS Formation by Entrapped pXd-INV

The kinetics of FOS formation with the PVA-entrapped enzyme was analyzed using 600 g/L sucrose as substrate at 30 °C. Figure 6 represents the formation of the different products as a function of sucrose consumption. Maximum FOS production was reached when approximately 86% of the initial sucrose had disappeared, as occurred with the soluble enzyme [12]. At this point, the immobilized pXd-INV yielded 18.9% (*w*/*w*) FOS (113.5 g/L), of which 59.1 g/L corresponded to neokestose, 30.2 g/L to 1-kestose, 11.6 g/L to neonystose, and 12.6 g/L to blastose.

We confirmed that blastose was formed by hydrolysis of neokestose, as the concentration of this disaccharide increased up to 30.5 g/L concomitant with the sharp decrease of neokestose at the end of the reaction. This value represents a notable concentration of blastose considering that by now the largest production reported of this disaccharide was 34 g/L using the *Cladosporium cladosporioides* mycelium-bound transfructosylation activity [38]. The sharp decrease of FOS concentration observed after 85–90% consumption of sucrose was also observed with the soluble native enzyme [12] and is typical in the preparation of FOS with other β-fructofuranosidases [41].

It is worth noting that the maximum FOS concentration (18.9%) was significantly lower than the obtained with the soluble enzyme (29%) [42]. This fact might be related with the hydrophilic microenvironment of the PVA lenses, which could favour the hydrolysis in detriment of the transfructosylation. The effect of the microenvironment of the carrier on the transferase to hydrolysis ratio has been also reported in previous works [30,43–46]. In this context, the amount of blastose synthesized by the immobilized pXd-INV was higher than the reported with the recombinant soluble enzyme (30 g/L vs. 8 g/L), which is in accordance as it is obtained in a hydrolytic process.

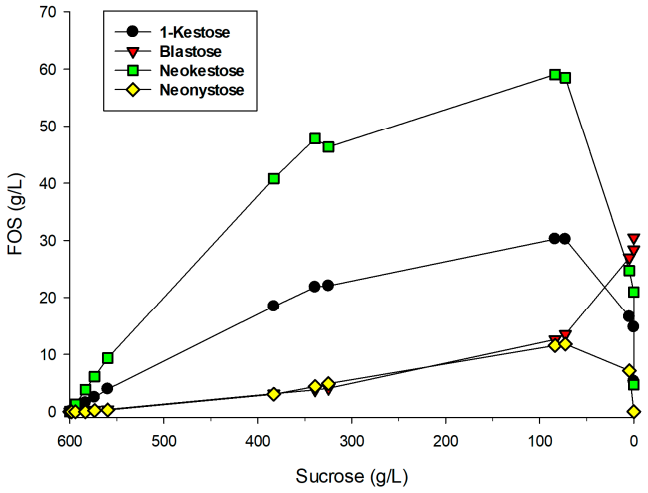

Figure 6. Kinetics of FOS formation with entrapped pXd-INV in PVA. Reaction conditions: 600 g/L sucrose, 100 mM sodium acetate buffer (pH 5.0), 30 °C.

2.5. Operational Stability of Immobilized pXd-INV for Neo-FOS Production

We performed seven cycles of 26 h at 30 °C using 600 g/L as substrate in a batch reactor. Between cycles, the lenses were thoroughly washed with the reaction buffer to remove any remaining sugars. The production of the different FOS is represented in Figure 7. Under the assayed conditions, the conversion of sucrose was approximately 23%, which implies that it was a bit far from the optimum FOS yield (Figure 6). However, the present study was very valuable to determine if these PVA biocatalysts could be reused for the industrial production of neo-FOS a. As shown in Figure 7, the concentration of the main reaction products was maintained nearly constant during the seven reaction cycles.

The excellent operational stability of the lens-shaped PVA biocatalysts seems to be related with the fact that the lenses apparently maintained their original shape during the study. The operational stability of immobilized enzymes for FOS synthesis has been studied by different groups [47]. Satisfactory results have been obtained, among other methods, by entrapment in alginate followed by a drying process [48], cross-linking in presence of chitosan [49], glutaraldehyde-activated chitosan [50] or adsorption onto niobium ore [51].

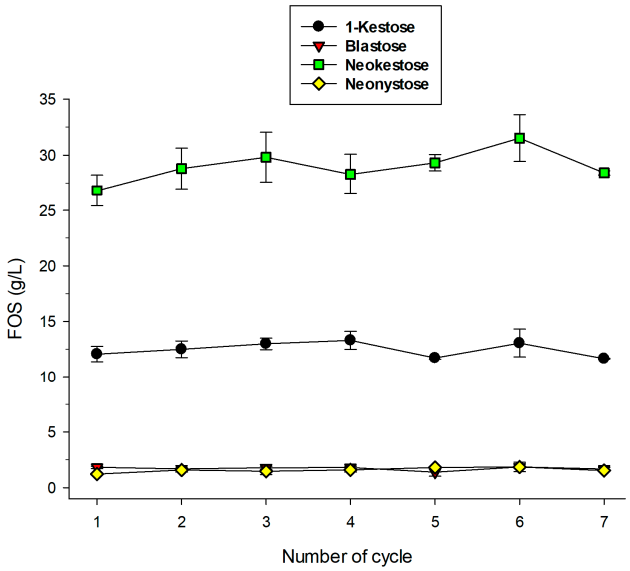

Figure 7. Operational stability of immobilized biocatalyst. Reaction conditions per cycle: 600 g/L sucrose in 100 mM sodium acetate buffer (pH 5.0), 26 h, 30 °C.

3. Materials and Methods

3.1. Materials

Sucrose was from Scharlau. Polyvinyl alcohol (PVA) (99% hydrolyzed, average MW 130,000) was purchased from Sigma Aldrich (Madrid, Spain). Fructose was from Merck and 1-kestose was from TCI. Neokestose, 6-kestose, neonystose and blastose were synthesized according to previous works [9,38,52]. All other reagents and solvents were of the highest purity available.

3.2. β-Fructofuranosidase Activity Source

The β-fructofuranosidase from *Xanthophyllomyces dendrorhous* ATCC MYA-131 (Xd-INV) was expressed in *Pichia pastoris* as previously reported [12]. Basically, the gene *Xd-INV* (GenBank accession no. FJ539193.2) fused to the *Saccharomyces cerevisiae* MFα secretion signal sequence was cloned in plasmid pIB4 (construction QDNS-pIB4) and included in *P. pastoris*. Transformants were grown in 50 mL of Buffered Minimal Glycerol (BMG), yeast nitrogen base w/o amino acids 1.34%, biotin 4×10^{-5}%, glycerol 1%, 50 mM potassium phosphate buffer, pH 6.0) during 24 h and then in 400 mL of Buffered Minimal Methanol (BMM) the same as BMG but containing 0.5% methanol instead of glycerol) for 35 h, giving approximately 21 U/mL of β-fructofuranosidase activity per mL of culture. The extracellular β-fructofuranosidase activity (pXd-INV) was purified by tangential concentration followed by DEAE-Sephacel chromatography. Active fractions were concentrated using Microcon YM-10 (Amicon) filters (0.7 mL; 4220 U/mL; 5.8 mg/mL) and stored at −70 °C.

3.3. Entrapment of β-Fructofuranosidase in PVA Lenses

The PVA solution (10% *w*/*v*) was prepared in 100 mM sodium acetate buffer (pH 5.0) at 90 °C under magnetic stirring for 45 min [53]. Enzyme entrapment was carried out at room temperature

by directly adding the enzyme to the PVA solution under magnetic stirring. Two different enzyme loadings were assayed (7.1 and 16.9 enzyme units per mL of PVA solution). The amount of enzyme was adjusted by diluting the enzyme stock solution (in 20 mM Tris-HCl pH 7.0) with 100 mM sodium acetate buffer (pH 5.0) and measuring the activity by the 3,5-dinitrosalicylic acid (DNS) assay. Lenses were produced by pumping the mixture through a syringe pump volume dispenser (NewERA model NE-300) into a 96-well microplate. Lenses were made by dripping 4 drops of the PVA solution in each well and then dried overnight at 50 °C. After that, lenses were hydrated in 100 mM sodium acetate buffer (pH 5.0) until constant weight. The average volume of each lens was calculated according to the total number of drops dispensed, the drops used for the production of each lens and the total volume of solution dispensed. Lens volume was determined using the following equation:

$$\textit{Lens volume } (\mu L) = (\textit{volume dispensed/number of drops dispensed}) \times \textit{number of drops in each lens}$$

3.4. Enzyme Activity Assay

β-Fructofuranosidase activity was determined by detection of reducing sugars with a modified 3,5-dinitrosalicylic acid (DNS) method adapted to a 96-well microplate scale [37]. The reaction mixture contained 45 µL of a 100 mg/mL sucrose solution in 100 mM sodium acetate buffer (pH 5.0) and 5 µL of a conveniently diluted enzyme solution. The reaction was incubated at 50 °C for 20 min, and then stopped by adding 50 µL of 3,5-dinitrosalicylic acid (DNS). The quantification of reducing sugars was carried out with a calibration curve of D-glucose, and one unit of activity (U) corresponded to the release of one µmol of reducing sugars per minute. The apparent activity of the immobilized biocatalysts was determined using a methodology developed in our group [36]. Basically, the lens-shaped PVA particles was incubated with 500 µL of 100 g/L sucrose solution in a micro-centrifuge filter tube (Spin-X®, 0.45 µm, Costar, Corning Inc., Corning, NY, USA) at the desired temperature for 60 min under vigorous agitation (900 rpm). The reaction mixture was separated from the biocatalyst by centrifugation at 2000× *g*. Inactivation of the possible lixiviated enzyme was carried out by adding 500 µL of 0.4 M sodium carbonate. Finally, reducing sugars were measured by the DNS assay as described above.

3.5. Microscale Assay for the Operational Stability of Immobilized pXd-INV

The operational stability of the immobilized biocatalysts was assayed following a previously described microscale assay [36]. One PVA lens was placed in a filtered micro-centrifuge tube (Spin-X®, 0.45 µm, Costar, Corning Inc., Corning, NY, USA) with 500 µL of a 100 mg/mL sucrose solution. Reactions were carried out at 50 °C and pH 5.0 for 20 min. Centrifugation was carried out at 2000× *g* to separate the lens from the reaction medium. The amount of reducing sugars was measured by the DNS method, as described before. The biocatalysts were washed three times with 100 mM sodium acetate buffer (pH 5.0) between cycles. Experiments were performed in triplicate to calculate the standard deviations.

3.6. Thermostability of the Immobilized PVA Particles

The thermostability of the lens-shaped PVA particles was analyzed by incubating the immobilized biocatalyst at different temperatures (4–60 °C) for 24 h in 100 mM sodium acetate buffer (pH 5.0). Residual activity was measured using the DNS assay under standard conditions (50 °C, pH 5.0). Experiments were performed in triplicate to calculate the standard deviations.

3.7. Analysis of Fructooligosacharides by HPAEC-PAD

The identification and quantification of FOS was carried out by High Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD, Dionex ICS3000 system, Sunnyvale, CA, USA) and a CarboPack PA-1 column (4 × 250 mm) connected to a PA-1 guard column. The method was adapted from Campbell et al. [54]. Initial mobile phase was 100 mM NaOH at 1 mL/min and it was maintained for 8 min. Then, a gradient from 100

to 88% 100 mM NaOH and from 0 to 12% 100 mM NaOH/600 mM sodium acetate was performed in 22 min. These conditions were kept for 6 min and then the eluents concentration was changed to 50% 100 mM NaOH and 50% 100 mM NaOH/600 mM sodium acetate. Eluents were degassed by flushing with helium and peaks were analyzed using Chromeleon software.

3.8. Production of Fructooligosacharides by Immobilized pXd-INV

Lenses of immobilized pXd-INV were added to a 600 mg/mL sucrose solution in 100 mM sodium acetate buffer, pH 5.0, until reaching a final activity of 1 U/mL. The reaction mixture was incubated at 30 °C in an orbital stirrer, and aliquots (100 µL) were taken out at different times and inactivated with 0.4 M Na_2CO_3. The formation of the different FOS was analyzed by HPAEC-PAD.

3.9. Operational Stability of Immobilized pXd-INV for Neo-FOS Production

The operational stability of the immobilized biocatalysts was assayed following neo-FOS production. One lens-shaped PVA particle (0.3 U) was placed in a filtered micro-centrifuge tube (Spin-X®, 0.45 µm, Costar, Corning Inc, Corning, NY, USA) with 340 µL of a 600 mg/mL sucrose solution in 100 mM sodium acetate buffer (pH 5.0). The mixture was incubated at 30 °C for 26 h in a Thermoshaker (model TS-100, bioSan, Nebikon, Switzerland) at 900 rpm. The tube was then centrifuged at 2000× *g* for 2 min to separate the supernatant. To inactivate any possible lixiviated enzyme, the supernatant was diluted with 340 µL of 0.4 M sodium carbonate solution. Samples were diluted 1:500 before analyzing the FOS composition by HPAEC-PAD as described before. Experiments were performed in triplicate to calculate the standard deviations. Between cycles, the lens-shaped biocatalysts were washed three times with 100 mM sodium acetate buffer (pH 5.0) followed by centrifugation at 2000× *g* for 2 min.

4. Conclusions

The PVA-entrapped biocatalysts of pXd-INV displayed a notable recovery of activity and excellent operational stability at 30 °C during at least 180 h. These immobilized biocatalystscould be employed for the high-scale production of neo-FOS as well as for the fructosylation of different bioactive compounds.

Author Contributions: M.H.R. and F.J.P. conceived and designed the experiments; N.M. performed most of the experiments; D.F.-P. contributed operational stability of biocatalysts; F.V.C. contributed HPAEC-PAD analysis; M.G.-P. and M.F.-L. contributed preparation and characterization of pXd-INV; A.O.B. contributed discussion of results; F.J.P. wrote the paper.

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