CATALYTIC HYDROGELS

Ângela Tomaz Francisco Neves*

*Master Student in Biotechnology at Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1, 1049-001 Lisboa, Portugal

The advantages of enzyme immobilization in biotechnology impelled scientific community to search for suitable immobilization matrices. Hydrogels have been playing an important role in enzyme entrapment, due to their chemical and biological properties. Within such scope, this work aimed to produce and characterize resilient polyvinyl alcohol and chitosan hydrogels, displaying high operational and thermal stability for inulinase entrapment. Chitosan beads and polyvinyl alcohol films were obtained, yielding an immobilization efficiency of roughly 98% and 97%, respectively. The optimum pH of the immobilized enzyme in both matrices was slightly more basic (5.0) than the one observed for the free enzyme (4.5). An optimum temperature of 50ºC was displayed for free inulinase, whereas chitosan and polyvinyl alcohol immobilized inulinase exhibited at 65ºC and 55ºC, respectively. The Michaelis constant (K_M) of inulinase for inulin had a 1.5 and 1.6 fold-increase after immobilization in chitosan and polyvinyl alcohol, respectively, suggesting an apparent decrease in affinity of inulinase towards inulin. Nonetheless, the maximum reaction rate (V_{max}) did not significantly change after immobilization in chitosan, whereas 2-fold decrease was observed upon immobilization in polyvinyl alcohol. Also, thermal stability increased considerably after immobilization in both matrices. Moreover, encouraging results were obtained for continuous operation. After 47 days at 55ºC a product yield of 91% was achieved for inulinase immobilized in chitosan. Continuous operation of inulinase immobilized in polyvinyl alcohol obtained 63% of product yield after 31 days at 50ºC.

Keywords: enzyme immobilization, inulinase, polyvinyl alcohol based hydrogel, chitosan based hydrogel, hydrolytic activity, continuous operation.

INTRODUCTION

The number of applications of immobilized enzymes is increasing steadily. The immobilization of enzymes using entrapment technique can lead to increase enzyme stability, one of the key properties for industrial applications [1], [2].

Hydrogels has shown suitable characteristics for enzyme immobilization, due to their water holding capacity and permeability, biocompatibility and low coefficient of friction [3]. Nonetheless, low mechanical stability of most hydrogels and enzyme leakage trough the pores of the gel lattice impair their use in a more comprehensive way. Nowadays, hydrogels are used in several areas, among them food, pharmaceutical, biomedical and biotechnology industries [3].

Polyvinyl alcohol (PVA) and chitosan (CS) are synthetic and natural polymers, respectively used for immobilization matrices. Both polymers display a mechanical and chemical stability, facility of processing, hydrophilicity, elastic nature and high degree of swelling in water. Therefore several successful immobilizations have been reported [4]–[10]. Additionally, these polymers are excellent candidates for biomaterials, since they are biologically safe, non-toxic, biocompatible and biodegradable, also they display antimicrobial activity.

PVA hydrogels are generated by freeze-thawing, irradiation or by chemical cross-linking using aldehydes such as glutaraldehyde (GA) [11]. GA is one of the most common cross-linker, as it is less expensive, readily available, and highly soluble in aqueous solution [12]. The high reactivity of the aldehyde groups, which readily form imine bonds (Schiff's base) with amino groups and acetal bonds with hydroxyl groups provides the efficiency of GA on cross-linking. However, due to it proved toxicity, other cross-linking techniques are preferable to pharmaceutical or medical applications. Also, the reaction between polymer chains and GA has to be in acidic medium (pH 1-2), which in some cases leads to enzyme denaturation.

Chitosan hydrogels can be produced by chemical crosslinking or physical crosslinking. In order to obtain the chitosan particles through chemical crosslinking, two groups of cross-linkers are usually employed. One group, such as

glutaraldehyde, cross-links through covalent bonds leading to quite stable matrices. The second group is composed of ionic cross-linkers that cross-links through ionic gelation and electrostatic interactions between the positively charged chitosan chains and polyanions. The polyanion most commonly used for the ionic cross-linking is tripolyphosphate (TPP) [13]. In order to obtain a more stable and rigid hydrogel, some authors reported the use of GA after the cross-linking with TPP [4]. Solvent evaporation method, neutralization method and ionotropic gelation method can also be employed to prepare chitosan gels [14].

Inulinases are classified as endoinulinase and exoinulinase, depending on their mode of action. Endoinulinases (EC 3.2.1.7) are specific to inulin and hydrolyze it by breaking the bonds between fructose units that are located away from the ends of the polymer network to produce oligosaccharides, similar to fructooligosaccharides (FOS). Exoinulinases (EC 3.2.1.80) split terminal fructose units from the non-reducing end of the inulin molecule to liberate fructose [15].

The purpose of this study, using as model system the hydrolysis of inulin to fructose with inulinase, is to produce and characterize resilient PVA and CS hydrogels, displaying high operational and thermal stability. A relatively simple, robust and low-cost procedure is intended, as to make the process appealing for commercial application and, alongside being competitive to the existing ones, namely Lentikats®. The resulting enzyme formulation is characterized. In particular, the kinetic parameters, temperature and pH profiles as well as the storage and thermal stability were established. Operational stability was also tested in continuous flow in a miniature, custom-made, packed-bed reactor.

MATERIALS AND METHODS

Materials

Fructanase Mixture (batch number 121001), a commercial mixture of endo and exoinulinase from *Aspergillus niger*, was provided by Megazyme (Bray, Ireland). The enzyme was kept at -20°C as suggested by the manufacturer. Chitosan (CS) (average MW 200 kDa) was from Acros Organics (Geel, Belgium). Polyvinyl alcohol (PVA) (98.0%-98.8% hydrolyzed, average MW 50 kDa) from Acros Organics. Mowiol® 28-99 (PVA, average MW 145 kDa) and Mowiol® 10-98 (PVA, average MW 61 kDa) were from Sigma-Aldrich (St Louis, USA). Glutaraldehyde (GA), as 24% (w/w) aqueous solution, and sodium tripolyphosphate (TPP), as 85% pure, were purchased from Acros Organics. Inulin (Fibruline® S-20 with an average polymerization degree of about 10-12 units) was an offer by Induxtra (Moita, Portugal). All solutions were prepared in distilled water. All other chemicals were of analytical grade from various suppliers.

The continuous flow reactor was designed and assembled in acrylic at Acrilicos Fernando Gil. The reactor has 3 cm height for 1 cm width with a pre-chamber at both ends with 3 mm height and 9 mm width.

Methods

Enzyme immobilization in polyvinyl alcohol (PVA) film

In order to entrap inulinase in PVA film, a PVA solution (5% w/v in aqueous solution) was prepared by heating at 100ºC and then cooled to 40ºC. Before the addition of enzyme suspension, the pH was adjusted to 4.5, with hydrochloric acid 5 M or sodium hydroxide 2 M. The enzyme suspension (500 µL of a 10-fold diluted preparation in acetate buffer 100 mM pH 4.5) was added to the PVA solution and mixed under mild magnetic stirring. Then, pH was again adjusted to 4.5 and 2 mL of this enzyme enriched solution was added to each of the four screw-capped vessels. After, different volumes of GA (50 µL, 100 µL, 200 µL and 400 µL) were added to the corresponding vessel. The solutions were incubated for 16 hours at 35ºC to promote a controlled dehydration. After film formation, it was rinsed with distilled water and the excess of water was removed with filter paper. The film was weighed and stored in acetate buffer 100 mM pH 4.5 at 4°C until use.

The immobilization procedure was performed for three different PVA molecular weights, 50 kDa, 61 kDa and 145 kDa.

To evaluate the effect of different volumes of cross-linker added as well as temperature on immobilized biocatalyst, enzymatic hydrolysis assays were performed in 10 mL screw-capped vessels at different incubation temperatures (45ºC, 50ºC, 55ºC, 60ºC and 65ºC) with a stirring rate of 1000 rpm. Approximately, 100 mg of each PVA film, with different MW and volume of GA, were weighted. For each temperature, 1 mL of 5% (w/v) inulin substrate solution in acetate buffer 100 mM pH 4.5 was added to the vessel. Samples (10 µL) were collected at 2.5, 3.5, 6.5 and 24 hours after

the beginning of incubation and immediately assayed for reduced sugars.

Enzyme immobilization in chitosan (CS) beads

For the CS immobilization inulinase protocol, a methodology adapted from Wentworth *et al.* (2003) [16] was used. Briefly, a chitosan solution of 68.3 g.L^{-1} was prepared during 2 hours. 100 µL of enzyme suspension (10-fold diluted preparation in acetate buffer 100 mM pH 4.5) was added to 900 µL of CS solution and mixed under magnetic stirring. The resulting solution was extruded through a 1 mL needle (Therumo Neolus, 20 G×2'', Therumo Corp., Leuven, Belgium) into a solution of 15% (w/v) sodium tripolyphosphate (pH 5.0) at a vertical distance of approximately 15 cm, under mild stirring. After extrusion and curing for 10 minutes the beads were removed from the sodium tripolyphosphate solution and rinsed with distilled water. Excess of water in the beads was removed with filter paper. CS beads were weighted and divided into three parts. One portion for stabilization in acetate buffer 100 mM pH 4.5 at 4°C (absence of treatment), a second portion of the beads were dehydrated in desiccator during 15 minutes and the remaining beads were reinforced with 500 µL of GA during 1 hour under magnetic stirring (850 rpm). Then, they were weighted and stored to stabilize in acetate buffer 100 mM pH 4.5 at 4°C until use.

To study the influence of each applied treatment and temperature on the immobilized biocatalyst, enzymatic hydrolysis assays were performed in screw-capped vessels (10 mL) at different incubation temperatures (50ºC, 55ºC, 60ºC and 65ºC) with a stirring rate of 1000 rpm. 100 mg of each type of CS matrix (absence of treatment, dehydrated or reinforced with GA) were weighted. For each temperature, 1 mL of 5% (w/v) inulin substrate solution in acetate buffer 100 mM pH 4.5 was added to the vessel. Samples (10 µL) were collected at 2.5, 3.5, 6.5 and 24 hours after the beginning of incubation and immediately assayed for reduced sugars.

The procedures described henceforth were only performed with the matrices selected, namely PVA film 50 kDa reticulated with 400 µL of GA and CS beads reinforced with GA.

The immobilization efficiency was defined in the Equation 1:

$$Immobilization\ efficiency\ (\%) = \frac{Total\ IA\ added - IA\ \ in\ the\ supernatant}{Total\ IA\ added} \times 100 \quad \textbf{(1)}$$

whereas IA stands for inulinase activity.

Protein entrapment was determined as follows:

$$Protein\ entrapment\ (\%) = \frac{Total\ protein\ added - Protein\ in\ the\ supernatant}{Total\ protein\ added} \times 100 \quad \textbf{(2)}$$

The immobilization yield (%) was determined as defined in the Equation 3:

$$Immobilization\ yield\ (\%) = \frac{Specific\ activity\ of\ immobilized\ enzyme}{Specific\ activity\ of\ free\ enzyme} \times 100 \quad \textbf{(3)}$$

Enzyme activity (U) is defined as the amount of protein needed to convert 1 µmol of reducing sugars in 1 minute.

Bioconversion studies

Inulinase bioconversion studies were carried out in magnetically stirred (850 rpm) 10 mL screw-caped vessels at 50ºC (free enzyme), 55ºC (enzyme immobilized in PVA) and 65ºC (enzyme immobilized in CS). A 2 mL of a 5.0% (w/v) inulin prepared in acetate buffer 100 mM pH 4.5 (free enzyme) or pH 5.0 (immobilized enzyme) with either: 50 µL of a 100-fold diluted preparation of Fructanase; 60 mg of immobilized enzyme in CS beads or 100 mg of immobilized enzyme in PVA film. When assessing inulinase activity in supernatants, 500 µL of supernatant were mixed with 3 mL of a 5.6% (w/v) solution of inulin in acetate buffer 100 mM pH 4.5 containing. The bioconversion runs were performed at 50ºC in 10 mL screw-capped, magnetically stirred (850 rpm) reactors. Samples were collected: every 5 minutes until 40 minutes for the immobilized enzyme; every 3 minutes until 15 minutes for the free enzyme. Then, samples (10 µL) were immediately quenched in DNS reagent and assayed for quantification of reducing sugars. Enzyme activity was determined through the initial reaction rates. Each bioconversion run was performed in duplicate, at least.

pH and temperature profiles

The effects of pH and temperature on enzyme activity were evaluated in batch runs by incubating either form of the enzyme in inulin solutions, 5% (w/v) in acetate buffer 100 mM, in a pH range of 3.6 to 5.5, and in a temperature range of 45ºC to 70ºC. The conditions of enzymatic assays were

performed according to bioconversion studies. Also, samples (150 µL) were collected from supernatant for protein detection by Bradford method. All runs were performed in triplicate.

Determination of kinetic parameters

The effect of substrate concentration in the free and immobilized inulinase activity was tested with different concentrations of inulin: 1.5%, 2.5%, 5.0%, 7.5%, 10.0%, 12.0% and 15.0% (w/v) in acetate buffer 100 mM pH 4.5 (for the free enzyme) or pH 5.0 (for the immobilized enzyme). For each run, 60 mg of CS beads and 100 mg of PVA film were weighted and 50 µL of 100-fold diluted preparation of free enzyme were used. Initial reaction rates were carried out as described in Bioconversion studies. Determination of kinetic parameters, V_{max} and K_M, was carried out through Hyper32® software and confirmed by excel using non-linear regression analysis.

Thermal stability

The thermostability of the free and immobilized inulinase was determined under different temperatures (40ºC, 50ºC, 60ºC and 70ºC) for different time spans, namely: 48 hours for the 40°C assay; 30 hours for the 50°C assay; 10 hours for the 60°C and 5 hours for 70ºC. 1.5 g of PVA film and 800 mg of CS beads were weighted and incubated in 25 mL screw capped vessels, filled with 10 mL of acetate buffer 100 mM pH 5.0, with agitation promoted by magnetic stirring (850 rpm) at the determined temperature. Also, free inulinase as 100-fold diluted preparation in acetate buffer 100 mM pH 4.5 was incubated at defined temperatures with the same agitation. Biocatalyst, either in immobilized or free form was recovered at different times depending on the temperature and enzymatic assays were performed as described in Bioconversion studies. Simultaneously, samples from the supernatant were taken to assess protein leakage. The results were then analyzed and processed in order to determine the deactivation pattern according to the three-parameter bi-exponential model [17]. All the assays were performed in duplicate.

Storage stability

Given amounts of immobilized biocatalyst PVA film and CS beads were stored (100 mg and 60 mg for each independent run, respectively) at 4ºC in 25 mL screw capped vessels filled with 10 mL acetate buffer 100 mM pH 5.0. Also, 1 mL of free enzyme (100-fold diluted preparation in acetate buffer 100 mM pH 4.5) was stored. The storage stability was evaluated by determining the enzyme activity up to 180 days. Bioconversion studies and initial activity assays were performed as described in Bioconversion studies. At the same time, a supernatant sample was collected to access protein leakage from immobilization matrices. The data was analyzed through the to the three-parameter bi-exponential model [17]. All the assays were performed in duplicate.

Operational stability

Continuous hydrolysis o inulin was carried out in the miniature tubular bioreactor. The reactor volume was determined as described by Lilly *et al.* [18]. The reactor was immersed in a temperature-controlled bath and feeding was provided through the peristaltic pump.

Continuous production of fructose

Continuous production of fructose was performed during 31 and 47 days at 50ºC and 55ºC for immobilization in PVA film and CS bead, respectively. The reactor was filled with 3.450 g of PVA film, cut into cubes with dimensions of 2×2×2 mm in average, and 1.206 g of CS beads. A 5% (w/v) inulin solution in acetate buffer 100 mM pH 5.0 was fed to the reactor at a flow rate of 0.0053 $mL.min^{-1}$. Samples were collected on a daily basis and assayed for reducing sugars as well as for protein detection.

Analytical methods

The quantification of reducing sugars was performed by the DNS method [19] using as reference a calibration curve from fructose standards. The protein quantification was performed according to the Bradford method [20]. Both methods were adapted to microplate scale [21].

Protein characterization by SDS-PAGE

The buffer sample was prepared with 62.5 mM Tris–HCl pH 6.2, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue, and 10% (v/v) glycerol. All samples were denaturated in reducing conditions with 100 mM dithiothreitol (DTT) (Sigma-Aldrich) at 100°C for 5-10 minutes. Samples were loaded in a 12% acrylamide gel, prepared from a 40% acrylamide/bis stock solution (29:1) (Bio-Rad), and ran at 90 mV using a running buffer composed by glycine 192 mM, Tris 25 mM, and 0.1% SDS pH 8.3. Gels were stained with Coomassie PhastGel (Pharmacia AB Laboratory Separations®) (Azevedo et al. 2010). Gels were destained using

a 30% ethanol/10% acetic acid solution. The molecular markers used were Precision Plus Protein Dual Color (BioRad).

RESULTS

Immobilization in polyvinyl alcohol (PVA) film

The formation of PVA films were effectively accomplished, however an exception was observed for PVA of 61 kDa with 50 µL which did not solidified. The feasibility of the proposed methodology for enzyme immobilization in PVA was assessed primarily by evaluating the physical stability of PVA films as well as the product concentration over 24 hours of enzymatic hydrolysis. These assays were performed at temperatures ranging from 40ºC to 65ºC (Table 1). The selection of the PVA matrix was based on the concentration of product and if the film remained at solid state during all the assays. Taking into account this consideration, the film with the best performance was 50 kDa with 400 µL of GA.

Table 1. Concentration of fructose ($g.L^{-1}$) over 24 hours of enzymatic hydrolysis at different temperatures. The symbol (-) means that the film was not considered for further studies due to the poor hydrolytic capacity.

PVA MW	Volume of GA	40ºC	45ºC	50ºC	55ºC	60ºC	65ºC
50 kDa	50 µL	Liquid	-	-	-	-	-
	100 µL	Liquid	-	-	-	-	-
	200 µL	50.7	48.4	51.7	6.3	-	-
	400 µL	47.0	48.4	50.0	39.5	41.05	44.0
61 kDa	50 µL	-	-	-	-	-	-
	100 µL	39.0	-	-	-	-	-
	200 µL	35.0	-	-	-	-	-
	400 µL	45.0	45.0	49.0	40.3	32.5	-
145 kDa	50 µL	36.0	35.0	43.0	30.0	-	-
	100 µL	34.0	-	-	-	-	-
	200 µL	31.0	-	-	-	-	-
	400 µL	28.0	-	-	-	-	-

Immobilization in chitosan beads (CS)

After the successfully formation of CS beads, the effect of different treatments (stabilization, dehydration and reinforcement with GA) on the enzymatic hydrolysis was assessed, under temperatures ranging from 50ºC to 65ºC. The choice of the best treatment was based on the concentration of fructose ($g.L^{-1}$) over 24 hours of reaction (Table 2). Through the analysis of Table 2, the beads reinforced with GA exhibited the best performance. This may be a consequence of the effect of stabilization promoted by glutaraldehyde.

Therefore, the matrices chosen for further characterization were PVA film of 50 kDa with 400 µL of GA and CS beads that were reinforced with GA. Henceforth, these matrices will be denoted as PVA or CS only.

Table 2. Concentration of fructose ($g.L^{-1}$) over 24 hours of enzymatic hydrolysis at different temperatures.

Treatment applied to CS beads	50ºC	55ºC	60ºC	65ºC
Stabilization	40.0	45.6	35.0	26.9
Dehydration	42.4	46.3	40.7	34.6
Reinforcement	47.2	48.6	48.4	46.5

The feasibility of the proposed methodology for enzyme immobilization was also assessed by evaluating the immobilization yield and efficiency and protein entrapment for the immobilization procedures previously selected (Table 3). The fact that the immobilization efficiency achieved a value slightly higher than the protein entrapment may be a consequence of the inulinase leakage but also due to the lack of specificity of the Bradford method. Fructanase Mixture also contains other enzymes with low MW which are susceptible to leakage.

Table 3. Immobilization of inulinase in PVA and CS based matrices.

Matrix	Protein entrapment (%)	Immobilization efficiency (%)	Immobilization yield (%)
CS	96 ± 4	98 ± 6	29 ± 3
PVA	94 ± 5	97 ± 3	25 ± 4

The SDS-PAGE analysis revealed the presence of a more evident band between 50 and 70 kDa, suggesting approximately 60 kDa as molecular weight for inulinase. Since the commercial inulinase studied is a combination of endo and exo-inulinase, it was expected to see more than one band. This may be a consequence of the similar molecular weight of both inulinases.

Effect of pH on the activity of free and immobilized inulinase

The effect of pH on the initial activity of free and immobilized inulinase was assessed at 50ºC, in the pH range of 3.6 to 5.5 (Figure 1). Free inulinase showed its maximum activity at pH 4.5 whereas the optimum pH of the immobilized enzyme in PVA film or CS beads was shifted to a less acidic pH value of 5.0. For pH values below 4.5 no visible difference between free and immobilized forms was observed, however the enhancement of activity following immobilization was observed only for higher pH values (above 5.0). Therefore,

immobilization slightly altered the enzymatic pH-activity profile, as compared with the free form.

Such pH shift upon immobilization (0.5 pH units variation), either in PVA or CS, are suggested to be because of secondary interactions between the inulinase and the matrix, which could lead to conformational changes in inulinase [22]. More specific, glutaraldehyde displays conformational changes depending on the pH of the aqueous solution, suggesting different modes of cross-linking the amino groups in enzymes and the functional groups in matrices [23]. Moreover, negatively charged groups of the matrix, namely OH^- from sodium tripolyphosphate, will tend to concentrate protons (lowering the pH) around the enzyme. Therefore, the pH around the enzyme will be lower than that of the bulk phase from which the measurement of pH is carried out. Boliver *et al.* (2013) have reviewed some methodologies to measure more precisely the pH inside the matrix, giving a powerful tool to understand how the pH is affected by immobilization [24].

Concerning PVA immobilized inulinase, Anes and Fernandes (2013) reported no shift in pH upon immobilization of inulinase mixture in PVA particles using Lentikat® liquid [25]. Similar patterns, where optimum pH profile is not significantly altered with immobilization, were reported previously by Cattorini *et al.* (2009) [26] and Fernandes *et al.* (2009) [27].

Effect of temperature on the activity of free and immobilized inulinase

The influence of temperature on the initial activity of free and immobilized inulinase was evaluated at pH 4.5, in the range of 45°C to 70°C (Figure 1). In this study, the optimum temperature for the immobilized enzyme increased to 65°C for CS immobilized enzyme and 55ºC for PVA immobilized enzyme, compared to 50°C for the free enzyme. Therefore, the enzymatic temperature-activity profile displayed great differences with the immobilization.

According to the literature, the optimum temperatures for free inulinase could range from 30ºC to 60ºC and they are generally higher for bacteria and yeasts than for fungi [15]. The inulinase optimum temperature obtained herein is consistent with reports by many other authors [28].

The temperature shift of 5ºC and 15ºC, when compared to the optimal temperature observed for the free enzyme, could be due to the formation of a molecular cage around the enzyme, which protected the enzyme molecules from the temperature, hence denaturation.

Additionally, chitosan beads are robust even at 80ºC unlike some other synthetic hydrogels e.g. PVA (Lentikat® liquid) which lack physical stability even at 60ºC [25], [26].

Recently, Paripoorani *et al.* (2015) reported an optimum temperature of 70ºC when using soluble inulinase in magnetite nanoparticles entrapped in chitosan [29]. Yewale *et al.* (2013) found 60°C as the best temperature for the immobilized inulinase on chitosan [30].

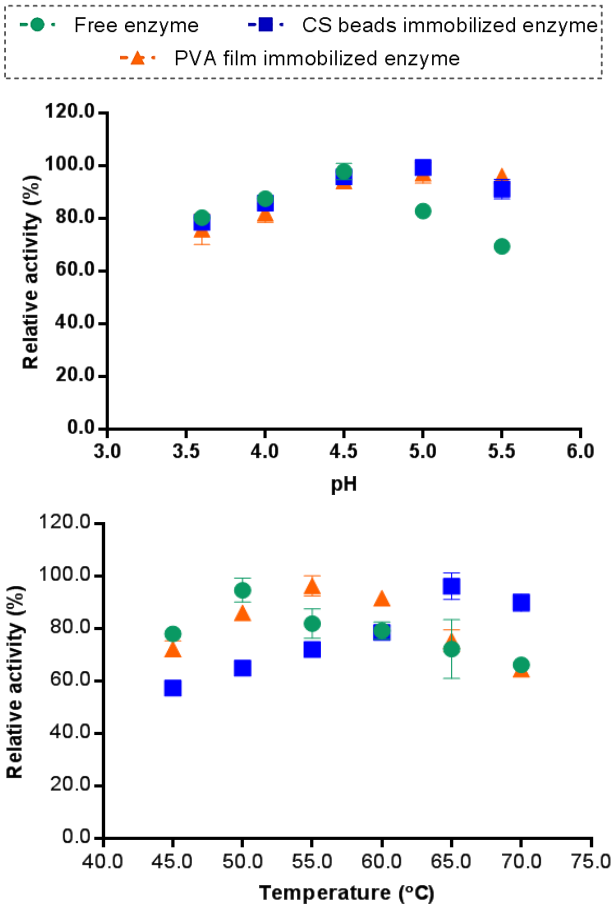

Figure 1. pH and temperature profiles of free inulinase and inulinase immobilized in CS beads and PVA film. Bioconversion runs were performed in 5% (w/v) inulin solution at different temperatures (45ºC – 70ºC) and pH values (3.6-5.5). 60 mg of immobilized inulinase in CS beads, 100 mg of immobilized inulinase in PVA film or 50 µL of a 100–fold diluted preparation of free inulinase were used as biocatalyst.

Kinetic study of enzymatic inulin hydrolysis

Kinetic parameters, V_{max} and K_M, were obtained using Hyper32® Software, assuming Michaelis-Menten kinetics hydrolysis (Table 4). According to Table 4, a 1.5 and 1.6-fold increase in K_M were obtained for the immobilized inulinase in CS and PVA, respectively, when comparing with the free inulinase. However, given the similarity of V_{max}

values for the free and immobilized biocatalyst in CS, such immobilization effect is not considered significant. Nonetheless, a 2-fold decrease in V_{max} was outstanding for PVA immobilized inulinase.

The increase in K_M suggests less affinity of enzyme for inulin, which can be ascribed to the impaired accessibility of the bulky inulin molecule to the active site of the immobilized enzyme, as a result of structural changes of the enzyme upon immobilization or steric limitations created by the immobilization matrix [31].

Comparing K_M values between PVA and CS immobilization no difference was notable, suggesting that K_M was not affected by differences in polymers or matrix shape. Lower value of V_{max} observed after PVA immobilization may also result from loss of enzyme active during immobilization procedure, caused by new interactions between enzyme and matrix [32]. The insignificant difference on V_{max} value after immobilization in CS was not unusual, a behavior also reported previously by Gill *et al.* (2006) [33].

The K_M of free inulinase from the commercial preparation Fructanase Mixture is not reported in literature. However when comparing the results obtained with the free enzyme from Fructozyme L, the K_M achieved is lower [34][27]. The increase in K_M values and decrease in V_{max} values is a commonly feature reported when inulinase is entrapped in hydrogels for inulin hydrolysis. Anes and Fernandes (2013) reported also a 1.6-fold increase in K_M when inulinase is entrapped in PVA beads cross-linked with GA [25]. Moreover, a 1.8-fold increase was cited by Fernandes *et al.* (2009), when inulinase was entrapped using PVA-based hydrogel particles [27]. Altun and Cetinus reported a 1.6-fold increase in K_M when chitosan beads immobilized pepsin [4]. Yewale *et al.* (2013) entrapped inulinase in chitosan beads, however no significant variation on K_M and V_{max} was observed [30].

Table 4. Kinetics constants obtained for inulin hydrolysis with free and immobilized inulinase. Data were processed through Hyper32® software.

Enzyme	V_{max} ($g_{fructose}.min^{-1}.mg_{enzyme}^{-1}$)	K_M ($g.L^{-1}$)	r^2
Free	1.1 ± 0.1	12.8 ± 4.8	0.92
CS beads	1.0 ± 0.1	19.6 ± 6.9	0.88
PVA film	0.57 ± 0.04	20.4 ± 4.1	0.78

Thermal stability

The effect of immobilization on thermal stability was evaluated at four different incubation temperatures (40ºC, 50ºC, 60ºC and 70ºC) (Figure 2 A, B and C).

In order to predict the effect of temperature in the stability of the biocatalysts, a three parameter bi-exponential model (Equation 4) was used. Here, K_i stands for the initial activity, K(t) stands for the activity at a given time, A stands for a complex function of individual rate constants, and α and β are apparent first-order rate constants.

$$K(t) = K_i\left[Ae^{(-\alpha t)} + (1-A)e^{(-\beta t)}\right] \quad \textbf{(4)}$$

Constant rates and predicted half times for each temperature are given in Table 5. Through the analysis of Figure 2 A, B and C and Table 5 are possible to conclude that immobilization enhanced the thermal stability. At 50ºC around 71% of relative activity was observed for either immobilization. For temperatures between 40ºC and 60ºC no significant differences in relative activity of both immobilizations, at the end of the assay, were observed.

However, at 70ºC a pronounced decay in the activity of PVA immobilized inulinase was observed where 70% of the initial activity was lost. Below 70ºC no visible structural changes in the films or beads were observed, however at 70ºC the PVA film melting becomes noticeable. This melting behavior occurred at a higher temperature than Lentikats®, which start to melt at 55ºC [26]. In the case of CS immobilization, the relative activity remained above 50% even at 70ºC. This result is higher than the one reported by Yewale *et al.* (20% of residual activity at 70ºC) [30]. Hence, the activity of immobilized inulinase in PVA or CS displayed an improved thermal stability when compared to free enzyme. Anes and Fernandes (2013) were able to immobilized inulinase from Fructozyme L in PVA lenses (Lentikats®) with a half time of 50 hours at 40ºC, 23 hours at 50ºC and 0.52 hours at 60ºC [25]. Therefore, the immobilized inulinase in PVA film proved to have better stability.

Storage stability

During storage of immobilized biocatalyst in solution the enzyme activity may decay along time [27]. This behavior can be ascribed to the decay of the intrinsic activity to the biocatalyst or to enzyme leakage.

The storage stability of free and immobilized biocatalyst, stored at 4ºC in acetate buffer 100 mM pH 4.5 or 5.0 during 180 days, was assessed.

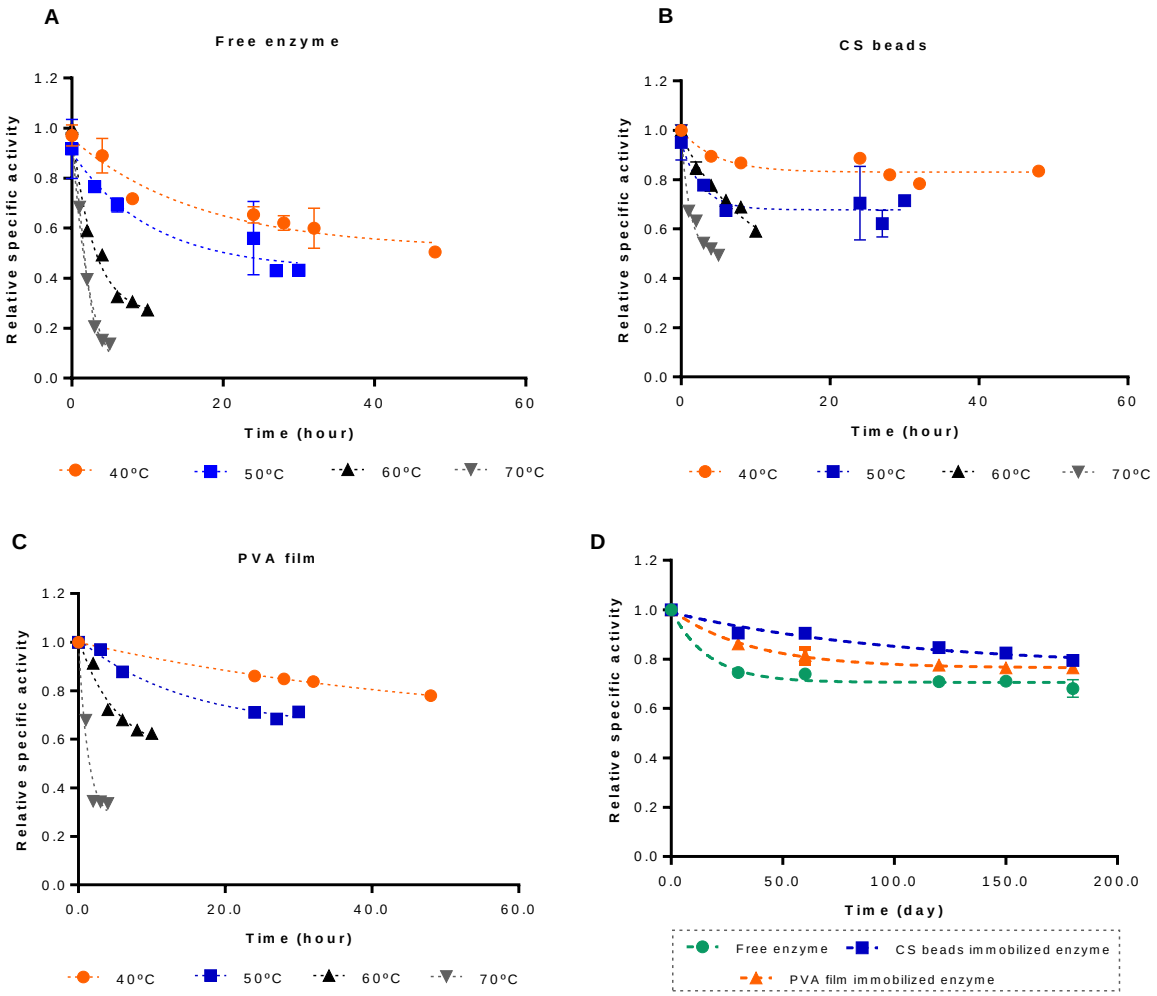

Figure 2. Thermal (A, B, C) and storage stability (D) of free and immobilized inulinase. Incubation was performed at 40ºC, 50ºC, 60ºC and 70ºC. The lines represent the trend of the three-parameter bi-exponential model. Bioconversion runs for initial activity were performed at optimum conditions using 50 µL of 100-fold diluted enzyme preparation, 60 mg of CS beads or 100 mg of PVA film as biocatalyst.

Table 5. Calculated parameters for the thermal and storage stability deactivation of free and immobilized inulinase using a three-parameter bi-exponential model.

Thermal Stability						
Enzyme	**Temperature (ºC)**	**A (-)**	**α (h^{-1})**	**β (h^{-1})**	**r**	**$t_{1/2}$ (h)**
Free	40	0.89	1.16×10^{-2}	9.15	0.972	49
	50	0.88	2.00×10^{-2}	9.15	0.987	28
	60	0.73	1.09×10^{-1}	9.15	0.995	3.5
	70	1.13	5.15×10^{-1}	16.63	0.997	1.5
Immobilized in CS	40	0.89	2.00×10^{-3}	17.23	0.901	285
	50	0.74	3.09×10^{-3}	17.23	0.920	124
	60	0.92	4.13×10^{-2}	17.23	0.994	15
	70	0.73	8.37×10^{-2}	17.23	0.997	5
Immobilized in PVA	40	0.95	4.18×10^{-3}	16.85	0.999	155
	50	0.98	1.22×10^{-2}	16.85	0.982	54
	60	0.95	4.86×10^{-2}	16.85	0.964	13
	70	0.82	0.29	16.85	0.966	1.7
Storage Stability						
Free	-	0.76	5.61×10^{-4}	16.83	0.998	740
Immobilized in CS	-	0.94	9.01×10^{-4}	16.83	0.994	700
Immobilized in PVA	-	0.87	8.00×10^{-4}	16.83	0.991	685

Again, the three-parameter bi-exponential model was used to predict the deactivation behavior under storage of each immobilized form of inulinase (Equation 4). The data estimated for the parameters of the bi-exponential model, as well as the predicted half time, are presented in Table 5.

Regarding the results obtained for the free enzyme, the activity decays to values around 70% after 60 days and seemed to be stable until 180 days of storage. This initial activity decay may be caused by enzyme adaptations to the storage environment. Nonetheless, in the immobilized inulinase the initial decay was not so noticeable, may be due to the matrix protection.

CS immobilization retained about 80% of the initial activity, whereas the immobilized inulinase in PVA retained about 76%, which is a promising result. Furthermore, no protein was detected during the storage time and no contamination was observed.

Continuous production of fructose using CS and PVA immobilized inulinase

The feasibility of using inulinase immobilized in PVA and in CS for full inulin hydrolysis under continuous flow was then assessed. The reaction took place for 47 days at 55°C for CS and 31 days at 50ºC for PVA, where 5% (w/v) inulin solution pH 5.0 was being fed to the reactor at a flow rate of 0.0053 mL.min^{-1} (Figures 3 and 4). DNS analyses were performed on a daily basis for fructose quantification. The reactor had a void volume of 0.78 mL and 0.87 mL and the correspondent residence time was 147 and 164 minutes for CS and PVA continuous operation, respectively.

For CS immobilized inulinase the product yield was not under 90% during the first twenty four days and a final product yield of 91% was achieved. The initial volumetric activity was 22.6 g.L^{-1}.h^{-1}. Regarding PVA immobilized inulinase, the product yield was not under 90% during the first eight days and a final product yield of 63% was achieved.

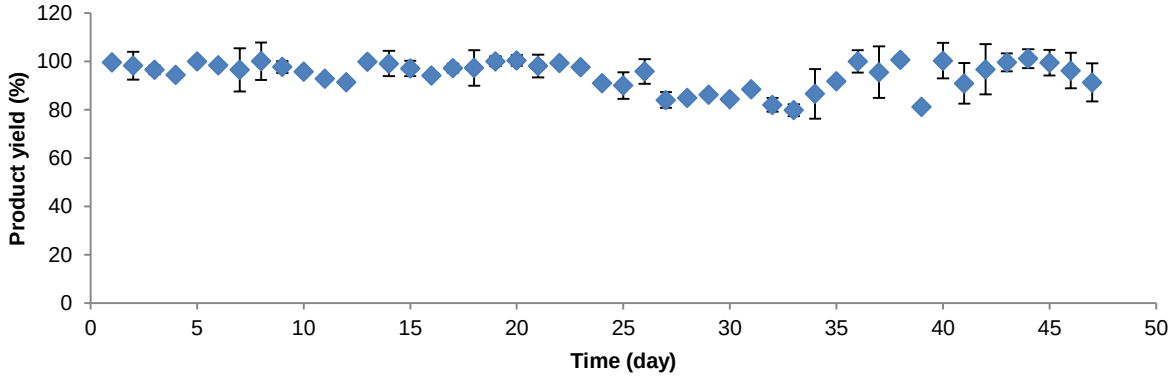

Figure 3. Operational stability at the packed bed reactor for inulin hydrolysis, based on the relative product yield. The 1.206 g of immobilized inulinase in CS were used for the hydrolysis of 5% (w/v) inulin solution in acetate buffer 100 mM pH 5.0 at 55°C. At day one the initial concentration of fructose was 55.5 ± 0.37 g.L^{-1}, with volumetric productivity of 22.6 g.L^{-1}.h^{-1}. Moreover, no deactivation model fit to the experimental data.

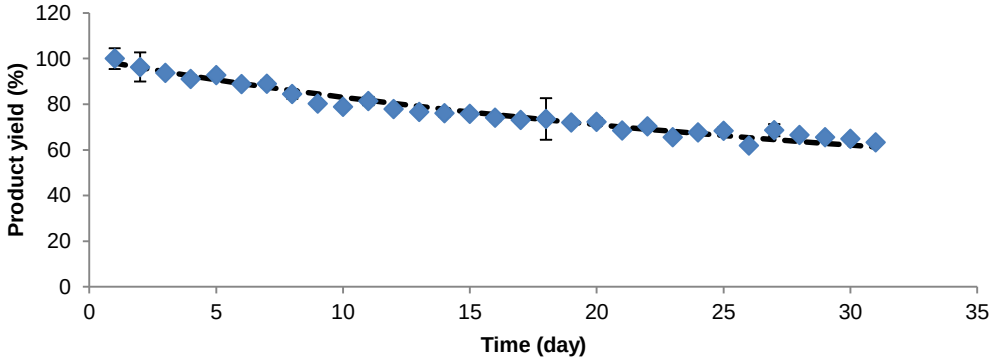

Figure 4. Operational stability at the packed bed reactor for inulin hydrolysis, based on the relative product yield. Predicted values (line) were estimated assuming a linear inverted model. The 3.450 g of inulinase in immobilized in PVA were used for the hydrolysis of 5% (w/v) inulin solution in acetate buffer 100 mM pH 5.0 at 50°C. At day one the initial concentration of fructose was 50 ± 2.5 g.L^{-1}, with volumetric productivity of 18.3 g.L^{-1}.h^{-1}.

The initial volumetric activity was 18.3 $g.L^{-1}.h^{-1}$. Using as reference a linear inverted model for enzyme decay (Equation 5), a deactivation constant of 0.02 day^{-1} and a half time of 49 days were calculated.

$$K(t) = \frac{K_i}{1+(K_d \times t)} \quad \textbf{(5)}$$

CONCLUSIONS

Inulinase from was efficiently immobilized on chitosan beads and polyvinyl alcohol. The best compromise between polyvinyl alcohol molecular weight and volume of cross-linker was achieved, with the film of 50 kDa and 400 µL of glutaraldehyde, yielding 97% of immobilization efficiency. The chitosan beads reinforced with glutaraldehyde were the ones with more stability under a range of different temperatures, with an immobilization efficiency of 98%.

Free inulinase displays an optimum pH at 4.5 and an optimum temperature of 50ºC. Shifts of the optimum pH and temperature to 5.0 and to 65ºC or 55ºC were observed for enzyme immobilized in chitosan or polyvinyl alcohol, respectively, were assayed.

The K_M values of free and immobilized inulinase for inulin were 13 $g.L^{-1}$ and 20 $g.L^{-1}$, approximately suggesting lower affinity towards substrate. No difference was detected of the V_{max} value concerning immobilization in chitosan, whereas a 2-fold decrease was detected for polyvinyl alcohol immobilization. Thermal stability was enhanced after immobilization on both matrices. This improvement was mostly noticed at high temperatures. Nonetheless, immobilization seemed not affect storage stability during the 180 days of storage.

Operational stability highlights the potentials of the matrices selected, since a product yield of 91% was achieved for inulinase immobilized in chitosan, after 47 days at 55ºC. Also, the continuous operation of inulinase immobilized in polyvinyl alcohol obtained 63% of product yield, after 31 days at 50ºC. In the case of chitosan no deactivation model was possible to fit to the experimental data.

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