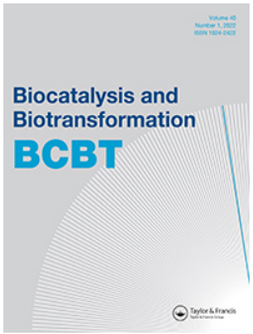

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RESEARCH ARTICLE

Inulinase immobilisation in PAA/PEG composite for efficient fructooligosaccharides production

Mishela Temkov[a,b], Darko Dimitrovski[a], Elena Velickova[a] and Albert Krastanov[b]

[a]Faculty of Technology and Metallurgy, Ss. Cyril and Methodius University in Skopje, Skopje, Republic of North Macedonia; [b]Faculty of Technology, University of Food Technologies, Plovdiv, Bulgaria

ABSTRACT

Inulinase was immobilised by entrapment method in polyacrylamide/polyethylene glycol composite and evaluated for its efficiency for short-chain fructooligosaccharides (3–6 degrees of polymerisation) production in batch hydrolysis system. Aqueous two-phase polymerisation technique was used to synthesise the composite, where aqueous polyethylene glycol 1000 containing the enzyme was used as dispersant with ammonium persulfate as initiator. The characteristics of free and immobilised inulinase were investigated and compared, and the results showed shift of pH and temperature optimum and change in stability caused by the immobilisation material. The immobilised preparation retained 50% of its initial activity after 20 successive batch cycles of 1 h each. The conversion degree of highly polymerised inulin to fructooligosaccharides (3–6 degrees of polymerisation) was 36% when using 30% PAA/PEG, w/v.

HIGHLIGHTS

- ATPS extracted inulinase was effectively immobilised in PAA/PEG composite.
- Immobilised enzyme showed good pH and thermal stability.
- Immobilised catalyst presents good retention of activity after 20 reuses.
- The entrapped enzyme is effective in producing FOS with the DP from 3 to 6 with proved prebiotic activities.
- The optimum conditions for batch operating mode were: 2% (w/v) inulin, 30% (w/v) PAA/PEG composite, pH = 4.4 and $T = 40^{\circ}C$.

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KEYWORDS
Inulinase; immobilisation; fructooligosaccharides; polyacrylamide/polyethylene glycol composite; characterisation

Abbreviations: ATPS: aqueous-two phase systems; APS: ammonium persulfate; DP: degree of polymerisation; FFN: fructofuranosyl nystose; FOS: fructooligosaccharides; Mw: molecular weight; PAA: polyacrylamide; PAH BAH: 4-hydroxybenzoic acid hydrazide; PEG: polyethylene glycol; TLC: thin-layer chromatography

Introduction

Fructooligosaccharides (FOS) are carbohydrates consisting of 2–20 polymerised fructose units linked by β-2,1-glycosidic bonds with or without a terminal glucose residue. They have a low degree of polymerisation (DP), thus low molecular weight (Mw), whereas inulin has the same chemical composition but high DP and therefore high molecular weight (Roberfroid and Slavin 2000). Due to the lack of hydrolysing enzymes in the human upper gastrointestinal tract, these compounds are non-digestible (Mao et al. 2019). However, they become hydrolysed and extensively fermented by the microbiome in the large intestine to short-chain fatty acids like butyrate, propionate, and acetate (Bali et al. 2015). Because of this, their caloric value is estimated at 25–35% of a fully digested fructose (Roberfroid 1999). FOSs act as prebiotics by selective stimulation of growth and sustainability of beneficial microbiota in the gut, especially bifidobacteria and lactobacilli (Oliveira et al. 2011). In addition to low caloric value and prebiotic effect, these oligosaccharides can induce plenty of other physiological effects after their consumption (Flamm et al. 2001). They can improve gut absorption of calcium and magnesium; reduce the levels of serum cholesterol, phospholipids, and triglycerides; increase faecal weight, act

CONTACT Mishela Temkov mishela@tmf.ukim.edu.mk Department of Food Technology and Biotechnology, Faculty of Technology and Metallurgy, Ss. Cyril and Methodius University in Skopje, Rudjer Boskovic 16, 1000 Skopje, Republic of North Macedonia

Supplemental data for this article can be accessed here.

as a dietary fibre and maintain glucose homeostasis (Jackson et al. 1999; Bali et al. 2015; Roberfroid 2005). These effects are revealed in a number of health benefits such as: reducing gastroenteritis, colon cancer, allergies, obesity, cardiovascular disease, and osteoporosis (Kleessen et al. 2001; Coxam 2005; Fernandes et al. 2017; Nobre et al. 2018). Moreover, in recent studies the symbiotic effects of *Lactobacillus* strains and FOS have been used to modulate the immunity and prevent or treat neonatal sepsis (Panigrahi et al. 2017; Schüller et al. 2018). Due to above mentioned health benefits and physiological effects, FOS and inulin have been extensively applied in the food industry as prebiotic and dietary fibres, sugar or fat replacers, texture modifiers and stabilisers (Aidoo et al. 2014; Salvatore et al. 2014; Krasaekoopt and Watcharapoka 2014; Tiengtam et al. 2015; Liu et al. 2016). FOS can be obtained by enzymatic synthesis when several fructose molecules are added to the substrate sucrose (transfructosylation) using the fungal enzymes fructosyltransferase (FTase; 2.4.1.9) and β- fructofuranosidase (FFase 3.2.1.26) with maximum theoretical yield from 0.55 to 0.60 $g_{FOS}/g_{Sucrose}$ (Bali et al. 2015; Han et al. 2017). The resulting solution is mainly composed of 1-kestose (GF_2), nystose (GF_3), and fructofuranosyl nystose (GF_4), all of which have terminal glucose. On the other hand, FOS can be also produced with a high yield by inulin hydrolysis using endo-inulinases (EC 3.2.1.7), where the product may not contain terminal glucose (Mao et al. 2019). In both cases, glucose, fructose and sucrose are produced as non-prebiotic by-products, which reduce FOS's prebiotic activity, and they should be removed from the process by nanofiltration, activated charcoal system and ion-exchange chromatography or microbial treatment (Nobre et al. 2018; Picazo et al. 2018; Jiang et al. 2019). FOS with DP 3-9 have been shown to be the most suitable source of carbon for bacterial growth compared to FOS with DP 10-17 and Inulin DP $\geq$ 23 (Fu et al. 2018). The increased use of fructooligosaccharides in the food industry resulted in exploring "new" microbial enzymes for their production. However, industrial production of FOS requires stable, highly active enzyme with low-priced immobilisation preparation under industrial conditions.

Nobre et al. (2018) synthesised FOS from sucrose with 0.64 $g_{FOS}/g_{initial\ sucrose}$ yield after 38 h of fermentation, whereas Han et al. (2017) hydrolysed inulin to FOS by using engineered *Yarrowia lipolytica* strain Enop56, in which an optimised endo-inulinase gene from *Aspergillus niger* was over-expressed. They obtained a high 0.91 g_{FOS}/g_{Inulin} yield from 6% initial substrate concentration and only 4.97% of non-prebiotic saccharides in the fermentation broth. In another study, Park et al. (1999) reported a maximum yield of about 86% of FOS on a total sugar basis with 1% initial inulin concentration, using partially purified enzyme from *Xanthomonas* sp. High yield of 84.8% FOS was also achieved by Singh et al. 2016 (Singh et al. 2017) when they hydrolysed 5% inulin solution by yeast inulinase immobilised on chitosan beads after 5 h of reaction. Purified endoinulinase isolated from *A. arachidicola* CBS 117610 was also used for the efficient production of inulooligosaccharides from inulin. The inulooligosaccharides yields with DP_3 and DP_4 as major products reached 80% when 5% of inulin was used as substrate (Jiang et al. 2019).

Immobilisation protocols have to ensure good retention of enzyme activity, long term stability and desired physical properties for a large scale operation. A number of matrices and methods have been used for inulinase immobilisation applied for FOS production in batch systems. Enzymes were physically adsorbed on porous support, covalently attached to the activated surface, crosslinked into crystals or entrapped in polyacrylamide gels (Whitesides et al. 1979; Tumturk et al. 2007; Singh et al. 2017; Temkov et al. 2019).

In this work, previously developed composite, consisting of polyacrylamide (PAA) and polyethylene glycol (PEG) (Guorong and Zhihai 2009; Xu et al. 2010), was for the first time employed for inulinase immobilisation by entrapment. The two components polymerised in an aqueous two-phase system using ammonium persulfate (APS) as initiator. Inulinase was extracted from newly isolated *Bacillus* sp. 11/3 from thermal water samples from the region of Velingrad, Bulgaria (Gavrailov and Ivanova 2015) and was partially purified in PEG rich phase from the ATPS system, as described in our previous study (Temkov et al. 2018). The separation of the enzyme from the PEG phase is a time-consuming step that can be omitted by crosslinking the PEG phase (containing the enzyme) with PAA, yielding an immobilised system entrapping the entire amount of the enzyme. The immobilised enzyme was characterised for its pH and temperature optimum, its stability, as well as storage stability and reusability. At the same time, the characteristics were compared to those of the free enzyme. The immobilised enzyme was used for FOS production in a batch system. The distribution of FOS with different DP's was evaluated with specific attention to the production of FOS with DP between 3 and 6.

Materials and methods

Materials

Acrylamide (AA), N,N′-methylene-bis-acrylamide (MBA), N,N,N′,N′-tetramethyl-ethylenediamine (TEMED) and ammonium persulfate (APS) were purchased from Sigma Aldrich, USA. Polyethylene glycol (PEG) (M_w = 1000 g/mol) was supplied by Merck, Germany. Highly polymerised inulin (DP > 25) extracted from Jerusalem artichoke was donated by Beneo, Belgium. 4-Hydroxybenzhydrazide (PAH-BAH) was obtained from Alfa Aesar, Germany. Bradford reagent and bovine serum albumin (BSA) were purchased from Bio-Rad, USA. All chemicals were of analytical grade. Extracellular inulinase was produced by submerged cultivation of *Bacillus sp.* 11/3 and purified in an aqueous two-phase system.

Inulinase production and purification

The enzyme used in the study was produced by submerged cultivation of *Bacillus* sp. 11/3 in a medium composed of (g/L): inulin 4.0; yeast extract, 4.0; peptone, 4.0; $MgSO_4$, 0.16; K_2HPO_4, 0.8 in a flask at 50 °C for 24 h at 200 rpm (Gavrailov and Ivanova 2015). The medium was centrifuged in order to remove the microbial cells (14,000 rpm, 3 min) and stored at −18 °C as a crude enzyme solution.

Further, the enzyme was purified by the aqueous two-phase system (ATPS) described in our previous study (Temkov et al. 2018). Briefly, the enzyme was purified in the PEG phase in a system composed of 26% PEG (M_w = 1000 g/mol) and 26% $MgSO_4$, whereas the bulk proteins were partitioned in a salt phase. After partitioning, the PEG phase containing the enzyme was concentrated in a vacuum evaporator at 40 °C, until 115 μg protein/mL in the final solution was reached.

Inulinase immobilisation in PAA/PEG composite

A blend of acrylamide (AA), and N,N′-methylene-bis-acrylamide was polymerised in the presence of PEG (containing the enzyme) in ratio 1:1. In fact, 3 mL of 50% w/v diluted PEG, enriched with the enzyme at a concentration of 97.5 U/mL, was added to 3 mL of acrylamide/bis-acrylamide (AA). Ammonium persulfate (APS) and tetramethyl-ethylenediamine (TEMED) were used as crosslinking agents to induce AA polymerisation. The prepared solution, in a total volume of 6 mL, was mixed thoroughly for even distribution of the enzyme and was poured immediately into a Petri dish for solidification. The composites were left to air dry for 48 h and ground prior to further tests. Taking into consideration the densities of the used solutions, the total amount of obtained solid biocatalyst was 6.38 g. The composite was washed with distilled water twice to remove unbound protein and the supernatant was examined for protein and activity recovery. The obtained immobilised biocatalyst had an enzyme load in a concentration of 45.84 U/g. This biocatalyst was subjected to further characterisation and used in batch inulin hydrolysis in different amounts.

Inulinase activity assay of free and immobilised enzyme

Inulinase activity assay of the free enzyme was performed by incubating 1 mL partially purified enzyme solution from the PEG phase (ATPS purification method) with 2% (w/v) inulin prepared in phosphate (H_2PO_4–/HPO_4^{2-}) buffer pH 8.0 at 50 °C for 20 min. After incubation, the reaction tubes were kept in a boiling water bath for 10 min to stop the enzyme reaction and then cooled to room temperature. In addition, the inulinase activity assay of the immobilised enzyme was performed by incubating 0.25 g PAA/PEG-inulinase composite with 2.5 mL 2% (w/v) inulin dissolved in acetate (CH_3COO^-/CH_3COONa) buffer (pH 4.4) at 50 °C for 20 min. The reaction was stopped by boiling the reaction mixture for 10 min. After cooling to room temperature both samples were analysed for reducing sugars with a method by Lever (1972) with some modifications. For this method, 250 μL of the reaction mixture were mixed with 1750 μL solution of PAH BAH dissolved in 10% 0.5 M HCl and 90% 0.5 M NaOH and were boiled for 10 min. The absorbance was measured at 410 nm by using a microplate reader SPECTROSTAR–Nano (BMG LABTECH). The concentration of fructose was calculated using a calibration curve. One unit of enzymatic activity was defined as the amount of enzyme needed to obtain 1 μmol of fructose per minute under the assay conditions. Assays were performed in triplicate and the average value was used in calculations. The efficiency of immobilisation at different enzyme dosages was calculated by using Equation (1) for the immobilisation activity recovery:

$$\text{Activity recovery } (\%) = \frac{\text{Total activity of immobilised enzyme}}{\text{Total activity of enzyme in PEG phase}} \times 100 \quad (1)$$

Determination of optimal pH and temperature

Optimal pH of the inulinase was determined by incubating the reaction mixture at different pH in the range between 3.6 and 10.7 at 50 °C and expressed as enzymatic activity. To achieve desired pH conditions: acetate (CH_3COO^-/CH_3COONa), phosphate (H_2PO_{4-}/HPO_4^{2-}), and carbonate-bicarbonate buffers (H_2CO_3/HCO_{3-}) were used, prepared and adjusted at room temperatures. The optimal temperature of immobilised inulinase was measured by incubating the reaction mixture for 20 min at temperatures ranging from 20 to 90 °C at optimal pH. Enzymatic activity was determined by the method described in the Section "Inulinase activity assay of free and immobilized enzyme".

Determination of pH and temperature stability

For the pH stability test, the free and immobilised inulinase were pre-incubated for 120 min at 50 °C in the same pH buffer solutions ranging from 3.6 to 10.7. Temperature stability of the immobilised preparation was measured by pre-incubating the reaction mixture for 120 min in the range of 20−90 °C at optimal pH. For both tests, after the pre-incubation, the residual inulinase activity was measured under standard assay conditions. The recovered activity was normalised to the same amount of free and immobilised enzyme. In order to estimate the inactivation rate constant of the immobilised enzyme at a certain temperature, the activity was measured at appropriate time intervals during pre-incubation (Karimi et al. 2014). The inactivation constant k_d was determined according to Equation (2), whereas half-lives ($t_{1/2}$) and D values of the free and immobilised enzyme were estimated according to Equations (3) and (4).

$$\ln\frac{A_0}{A} = k_d \cdot t \tag{2}$$

$$t_{1/2} = \frac{\ln 2}{k_d} \tag{3}$$

$$\text{D value} = \frac{ln10}{k_d} \tag{4}$$

where A_0 and A are the initial and the residual enzymatic activities, respectively.

Determination of kinetic parameters

Kinetic parameters K_m and V_{max} for the immobilised inulinase were calculated from a Hanes-Woolf plot, using concentrations of inulin as substrate ranging from 0.5% to 3.0% with an increment of 0.5%. The reaction was performed in acetate (CH_3COO^-/CH_3COONa) buffer (pH 4.4) at 40 °C for 20 min.

Operational and storage stability of immobilised inulinase enzyme

0.25 g of PAA/PEG composite was added to 10 mL 2% inulin and the mixture was placed on a rotary shaker and incubated at optimum pH and temperature as determined in the Section "Determination of optimal pH and temperature" for 1 hour. After each cycle, the composite was removed by filtration, washed with distilled water and re-suspended in a new portion of 2% inulin solution for the next cycle. After each 1 h lasting cycle, the remaining activity was measured. The activity of the immobilised enzyme after the first cycle was set as a control and assumed to possess a relative activity of 100% (Mubarak et al. 2014). The experiment was repeated 20 times using the same conditions described above. For determining the storage stability, the immobilised enzyme in PAA/PEG composite was stored at 4 °C and the residual activity was determined after 6 months of storage.

FOS batch production

The enzymatic reaction was performed in Erlenmeyer flasks on a shaker at optimum pH and T (defined in the Section "Determination of optimal pH and temperature") for 24 hours. In this experiment, the amount of immobilised preparation, initial substrate concentration and time of hydrolysis were optimised. Different amounts of PAA/PEG/enzyme composite (1, 2 and 3 g) obtained according to the procedure described in the Section "Inulinase immobilization in PAA/PEG composite" were added to 10 mL total volume of inulin used as a substrate, resulting in total enzyme activity of 47, 92 and 138 U, respectively. Three different concentrations of inulin (2, 4 and 6% w/v) were tested for initial substrate optimisation. Samples were taken at 0, 1, 3, 5, 7, 9, 12 and 24 h. To determine the distribution of FOS in the reaction mixture at different times, the collected samples were analysed by thin-layer chromatography (TLC). The conversion degree of inulin (%) was calculated by subtracting the concentration of the product FOS (DP 3–6) from the initial substrate concentration.

FOS analysis by thin-layer chromatography (TLC) coupled with ImageJ

Standard solution or samples (5 μL) from the batch reaction were applied on chromatographic silica gel

60 F_{254} plates by using an Agilent HPLC syringe. The chromatographic plates were placed in a TLC chamber containing a mixture of *n*-butanol, *i*-propanol, acetic acid, H_2O in a ratio of 7:5:4:2 as mobile phase. After 6 hours the plates were dried and incubated in an oven at 100 °C for 20 minutes. The spots of separated compounds were visualised with diphenylamine, aniline, acetone and H_3PO_4 in a ratio of 1:1:50:5 (Petkova and Denev 2013). The plates were heated to 100 °C until the colour spots became visible. The concentrations of produced FOS DP 3-6 during hydrolysis were calculated from the standard curves by using the ImageJ software (National Institutes of Health and LOCI, University of Wisconsin, USA). For this purpose calibration curves of fructose, sucrose, kestose (DP3), nystose (DP4) and 1,1-fructofuranosylnystose (DP5) in concentrations of 0.075, 0.1, 0.25, 0.5, 0.75 and 1.0 mg/mL were prepared separately (see SI, Figure S1).

Results and discussion

Bacterial inulinase partially purified in the PEG phase (from a system composed of polymer/salt) is immobilised in PAA by the so-called "aqueous two-phase polymerisation", which can be carried out separately and also in two aqueous phases. This method for immobilisation where PAA can be polymerised in the presence of PEG carrying the enzyme involves one step to obtain the biocatalyst, while leaving out the time-consuming and costly ultrafiltration for enzyme purification. The enzyme is captured within the polymeric network that allows the substrate and products to pass through, while the diffusion of the enzyme molecules is constrained.

pH and temperature optimum

Different pH caused a significant alteration in the activity of the immobilised preparation due to changes in the ionisation state of the free enzyme or the enzyme-substrate complex. For both, free and immobilised enzyme, the test was performed at different pH in the range of 3.6–10.7 and at a constant temperature of 50 °C. The pH profile of the free as well as the immobilised inulinase in the PAA/PEG composite demonstrated two peaks of optimal pH suggesting that the inulinase is present in two distinct forms (Figure 1(a)). Compared to the free enzyme with pH optimums at 8.0 and 5.8, in this type of immobilisation the optimal pH shifted to more acidic values. The maxima of the two enzyme isoforms were at pH 4.4 with a maximum activity of 1.6 μmol/g·min and at pH 7.6 with a maximum activity of 1.4 μmol/g·min. The pH shift is probably due to the presence of polyvalent ions in the immobilisation matrix (Costa et al. 2001). In addition, highly alkaline conditions cause stretching of the protein molecule influenced by the electrostatic repulsion which will lead to distortion of enzyme's active centre (Mubarak et al. 2014). Gonzalez-Saiz and Pizarro (2001) reported a maximum activity at pH 8.5 for the alkaline phosphatase immobilised in PAA, which is in a more alkaline region than the maximum activity reported for the same enzyme in free form. In this regard, they discussed the degree of carrier's protection over the enzyme activity which is connected to the external – environmental pH and internal – carrier's pH. Since it is difficult to quantify the pH of the polyacrylamide matrix internal microenvironment, thus it is hard to estimate the matrix protection degree over pH optima. Sattar et al. (2018) reported the same optimum pH, 5.0, for both free and immobilised protease entrapped in PAA beads. The pH optimum reported by Ohmiya et al. (1975) for β-galactosidase immobilised in PAA was 4.5, while for *Fusavium oxysporum* inulinase immobilised in a PAA gel, the pH was found to be 6.0 (Gupta et al. 1990).

Concerning the effect of temperature, both free and immobilised inulinase exhibited different temperature optimums as shown in Figure 1(b). From the figure, it can be noticed that the optimum temperatures of the immobilised enzyme in PAA/PEG composite are 40 and 80 °C for the two isoforms of the enzyme as opposed to those of the free enzyme, which were 30 and 70 °C. The change of the enzyme activity with increasing temperature was as expected: the activity rose until the critical temperature was reached and irreversible deactivation occurred. As seen from the data, the carrier has a mild protective effect at the high temperatures at which the enzyme is inactivated, due to the temperature-mediated conformational changes within the catalytic structure of the enzyme molecule. This protection probably comes from the increased ability of the enzyme to renature in the immobilised preparation, whereas the free form in the solution is highly susceptible to damages. This was also noticed by other authors as well. Upon immobilisation of alkaline phosphatase in a polyacrylamide gel, the optimal temperature was increased by 5 °C compared to the optimal temperature for the free enzyme (Gonzalez-Saiz and Pizarro 2001). The optimum temperature of immobilised inulinase in polyacrylamide gel increased from 37 °C to 45 °C in comparison to the free inulinase isolated from *Fusavium oxysporum* (Gupta et al. 1990). Sattar et al. (2018) showed 5 °C higher temperature optimum for the immobilised enzyme in polyacrylamide beads.

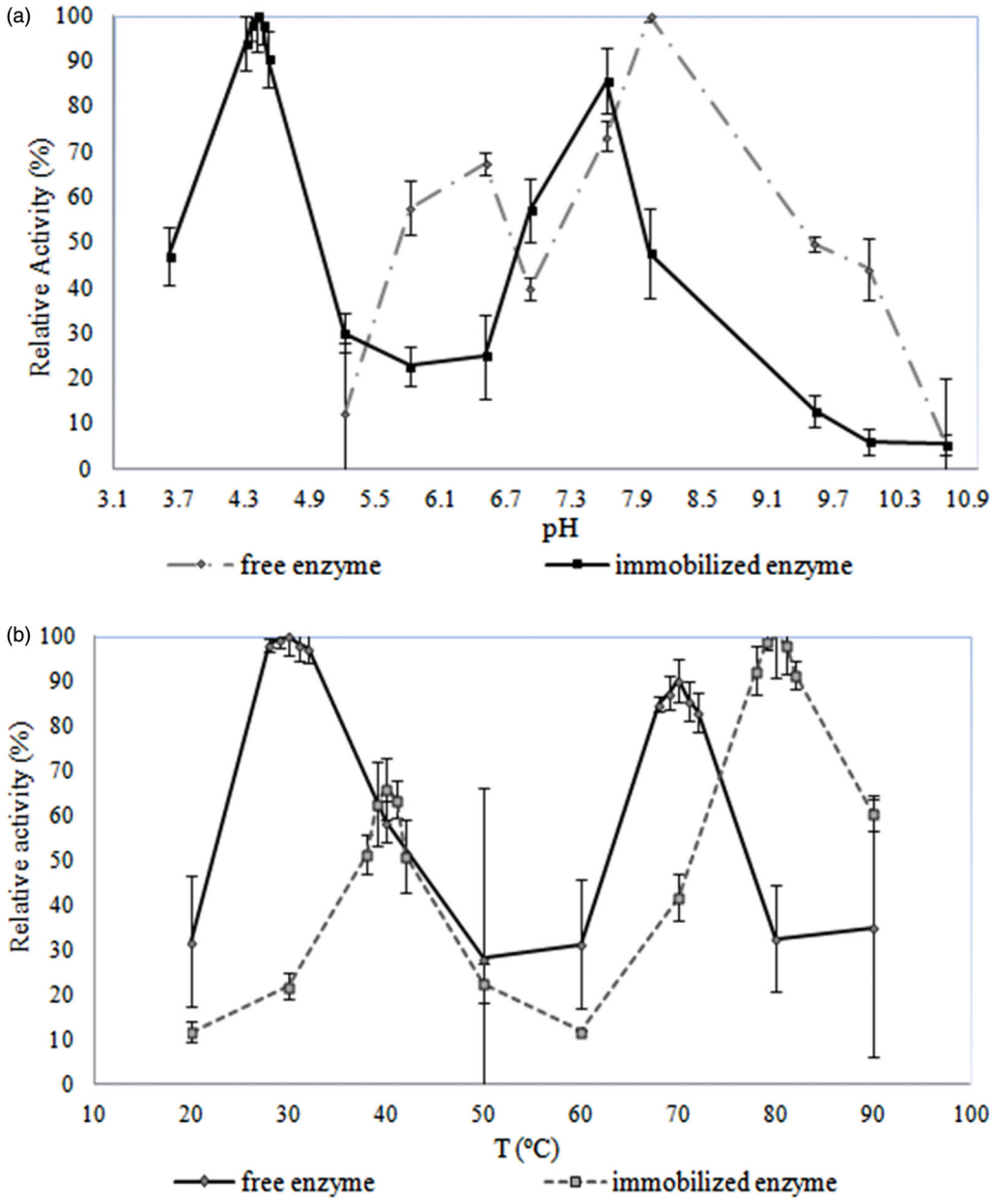

Figure 1. Effects of different pHs (a) and temperatures (b) on the activity of the free and entrapped inulinase in PAA/PEG composite. The highest activities were taken as 100%. Optimal pH was determined by incubating the reaction mixture at different pH in the range between 3.6 and 10.7 at 50 °C for 20 min. Optimal temperature was measured by incubating the reaction mixture for 20 min at temperatures ranging from 20 to 90 °C at pH 4.4. Free inulinase had pH optimums at 8.0 and 5.8 of both isoforms with maximum activity of 68 U/mL and 46 U/mL respectively. The pH optimum in the immobilised form shifted to pH 4.4 with a maximum activity of 1.6 U/g and at pH 7.6 with a maximum activity of 1.40 U/g. Optimum temperatures of the free enzyme were 30 and 70 °C with their maximum activities of 47 U/mL and 43 U/mL for both isoforms, respectively. The immobilised enzyme had optimum temperatures at 40 and 80 °C with maximum activities 1.4 U/g and 0.9 U/g for both isoforms, respectively. Values are the means of three replications ± standard deviation.

These results imply protection of the enzyme against temperature variation by the polymer matrix.

pH and thermal stability

The stability of an immobilised preparation to the extreme pH and temperature conditions is an important factor for scaling up the process and extending the life cycle of the preparation. pH stability of the free and immobilised preparation in PAA/PEG composites was examined by their pre-incubation in the same pH range of 3.6–10.7 and at a constant temperature of 50 °C for 2 h. After 2 h, the residual activity was measured according to the standard inulinase

assay. Free inulinase showed good pH stability in the range between 4.6 and 7.6, retaining 96% of its initial activity, followed by a steep decrease for higher pH values (Figure 2(a)). On the other hand, the immobilised enzyme showed greater pH stability in the alkaline range up to pH 9.6 in which the activity sustained over 90% of the initial activity. For higher pH values up to 10.8 the activity diminished rapidly to only 15–30% of its initial activity.

The data for the thermal stability is presented in Figure 2(b). As seen, in the range between 20 and 50 °C both free and immobilised inulinase were very stable with relative activity varying from 90 to 99% of its initial activity. The free enzyme showed high thermal sensitivity for temperatures above 50 °C, retaining only about 9% of the initial activity at 60 °C. Subsequently, at 60 °C the relative activity of the immobilised enzyme was 78%, at 70 °C it dropped to 60% and 50% remained at 80 °C. The superior thermal stability of the entrapped inulinase could come from the protective property of the PAA/PEG composite towards the conformational changes of the enzyme's tertiary structure (Sattar et al. 2018). The immobilisation of inulinase in PAA/PEG has not been reported; therefore our results were compared with enzymes immobilised in matrices with similar composition. β-galactosidase immobilised in polyacrylamide gel has been reported to be stable at 37 °C, but the relative activity markedly decreased to 40% at 50 °C (Ohmiya et al. 1975). Sattar et al. (2018) also reported an increased thermal stability for the protease immobilised in polyacrylamide beads.

Figure 2(c) presents the residual activities of the free and entrapped enzyme at optimal temperatures over the course of time. The deactivation rate constants (k_d) are calculated as inclinations of the logarithms of the residual activities as a function of time at 40 °C and 80 °C (Figure 2(d)). The results in Figure 2(c) show that under the same conditions, the deactivation rate of free inulinase is much higher than that of the entrapped enzyme. The free enzyme lost more than 50% of its initial activity after 1 h of incubation at 80 °C, while the entrapped enzyme in PAA/PEG composite retained almost 100% under the same conditions. The deactivation rate (k_d), half-life ($t_{1/2}$) and decimal reduction time constants (D values) for both temperatures of free and immobilised inulinases are given in Table 1. We can conclude that the thermal stability of inulinase significantly increases after the entrapment in the PAA/PEG composite with the $t_{1/2}$ increasing from 130 min (2.2 h) to 6930 min (115.5 h), and the D-value increasing from 415 min (6.9 h) to 22,030 min (367 h). This makes enzymatic hydrolysis at high temperatures possible which prevents microbial contamination and enable inulin to dissolve better, thus making the process more suitable for industrial applications (Awad et al. 2017). In the literature, there are several reports on the thermal kinetic parameters, but almost none for the enzyme entrapped in PAA composites. The thermal stability reported by Awad et al. (2017) for the inulinase immobilised in alginate-CMC beads with k_d, $t_{1/2}$ and D values 0.0064 min^{-1}, 108 min and 359 min, respectively, was significantly lower than the one obtained in our study.

Kinetic parameters

The apparent values or the kinetic parameters K_m and V_{max} for the immobilised enzyme in the PAA/PEG composite were calculated from the Hanes-Woolf plot (see SI, Figure 2S). K_m was calculated to be 3.1 μM, while V_{max} was 64 μM/min compared to 26 μM and 526 μM/min for the free enzyme. These data show that the substrate affinity significantly increases when the enzyme is entrapped; however, its maximum rate was reduced by around 8 times. The increased substrate affinity was probably due to the greater distribution of the substrate in the enzyme microenvironment than in the external solution. On the other hand, low availability of the enzyme's active centres and the poor substrate diffusion within the polymer matrix may be the reason for the reduced rate. Ohmiya et al. (1975) reported results for β-galactosidase immobilised in polyacrylamide gels, where K_m for immobilised and free enzyme did not change and was calculated as 20 mM at pH 4.5.

Moreover, the binding rate constant (k_{cat}) was calculated for both (free and immobilised enzyme) to determine the number of molecules of product made per enzyme per unit time. The specificity constant (k_{cat}/K_m) was also calculated to compare the relative rates of the enzyme. The immobilised enzyme had k_{cat} 0.54×10^4 s^{-1}, while this constant for the free enzyme was calculated as 2.8×10^4 s^{-1}. This means that the free enzyme can turn over more molecules of the substrate into a product per second, i.e., the reaction is faster. The catalytic potential expressed by the ratio of k_{cat}/K_m was higher for the immobilised enzyme with a value 1.8×10^3 $\mu M^{-1}s^{-1}$, rather than for the free enzyme which was calculated as 1.1×10^3 $\mu M^{-1}s^{-1}$. These results show that the immobilised enzyme has greater affinity towards the substrate of the enzyme which is also confirmed by the lower K_m.

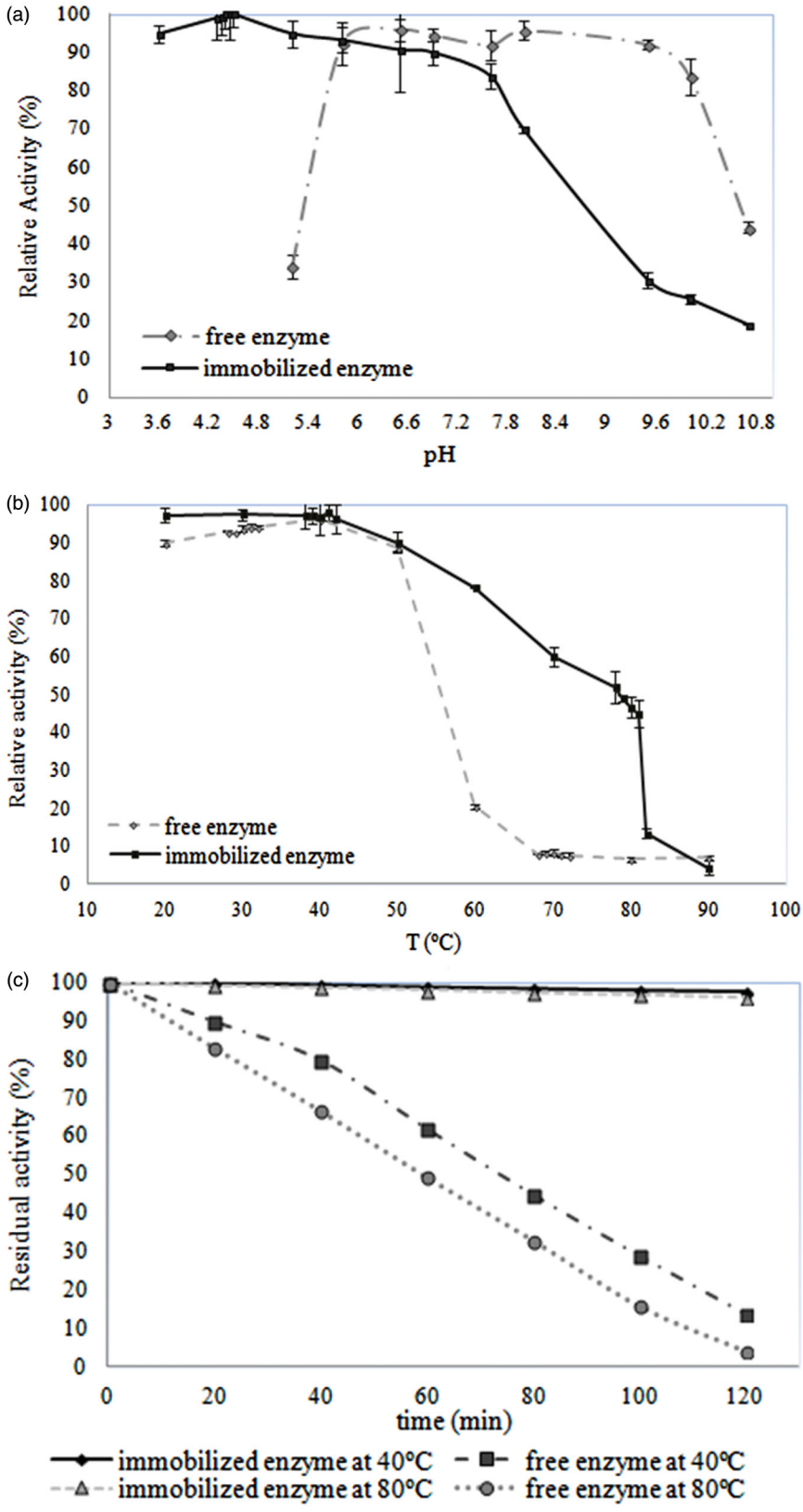

Figure 2. Effects of different pHs (a) and temperatures (b) on the stability of the free and immobilised inulinase in PAA/PEG composite, c) Thermal deactivation of free and immobilised inulinase at 40 °C and 80 °C; d) Determination of k_d of immobilised inulinase in PAA/PEG composite. The effect of pH was measured by pre-incubating the enzymes (free and immobilised) at 50 °C for 120 min in the pH buffer solutions ranging from 3.6 to 10.7. For the temperature stability test, the reaction mixture was pre-incubated for 120 min in the range 20–90 °C at optimal pH. The activities of the free and immobilised enzymes that correspond to 100% were measured as 47 U/mL and 1.4 U/g respectively. The inactivation rate constant was estimated by measuring the activity at appropriate time intervals during pre-incubation. Values are the means of three replications ± standard deviation.

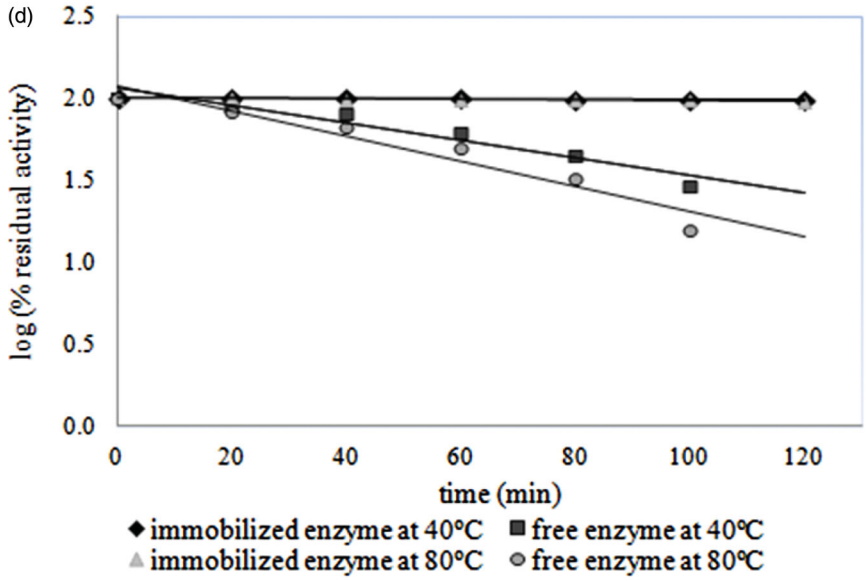

Figure 2. Continued.

Table 1. Kinetic parameters for thermal inactivation of free and immobilised inulinases.

	k_d (min^{-1})	$t_{1/2}$ (h)	D value (h)
Immobilised enzyme at 40 °C	0.0001	115.5	367.1
Immobilised enzyme at 80 °C	0.0001	115.5	367.1
Free enzyme at 40 °C	0.0053	2.2	6.9
Free enzyme at 80 °C	0.0076	1.5	4.8

Operational and storage stability of immobilised inulinase

The operational stability of the immobilised preparation is one of the most important factors in the processes of bioconversions based on enzymes. It determines the possible applications of the enzyme in large processes for reducing operating costs and for practical purposes. Therefore, that aspect is widely studied in the present work. From the results presented in Figure 3, it can be seen that inulinase entrapped in PAA/PEG composite has high operational stability. With this method of immobilisation, the enzyme maintained up to 50% of its initial activity after 20 consecutive cycles. This significant result can be due to the multipoint attachment of the enzyme to the polyacrylamide gel, which creates a large number of bonds. The decrease in activity and stability after successive cycles, on the other hand, could be due to enzymatic denaturation or leaching out of entrapped enzyme from the matrix. Mansour and Dawoud (2003) (Mansour and Dawoud 2003) immobilised invertase on polyacrylamide gel by an absorption procedure and reported an exceptional 18 cycles without loss of activity. They started to observe a decrease in the initial activity of 2.85% only after the 20th cycle. Kikani et al. (2013) immobilised α-amylase from *Bacillus amyloliquifaciens* TSWK1-1 in a polyacrylamide gel and reported a 65% loss after 20 cycles. In contrast, Sattar et al. (2018) (Sattar et al. 2018) observed approximately 72.0% retention of protease's initial activity entrapped in polyacrylamide beads up to the 4th and around 24.0% activity retention at 8th cycle. The immobilised inulinase was tested after 6 months of storage at 4 °C and displayed only 43% loss of the initial activity.

Optimisation of FOS production in a batch mode using immobilised inulinase

The initial enzyme amount and substrate concentration were optimised for the best batch production of FOS with DP 3-6. Three different enzyme amounts were examined: 10%, 20% and 30% (w/v) PAA/PEG composite with the entrapped inulinase with total enzyme activities of 46, 92 and 138 U. In addition, three different inulin concentrations: 2%, 4% and 6% (w/v) were examined. The FOS production after 24 h is shown in Figure 3S and 4S (see Supplementary material).

The concentrations of FOS with different DP at the end of the batch hydrolysis are presented in Figure 4. Figure 4(a–c) shows the data when different amounts of immobilised preparation were used, while Figure 4(d–f) shows the data with different initial substrate concentration. Kestose, nystose, fructofuranosyl nystose (FFN) and FOS with DP = 6 which have prebiotic activity were interest of our research and represent

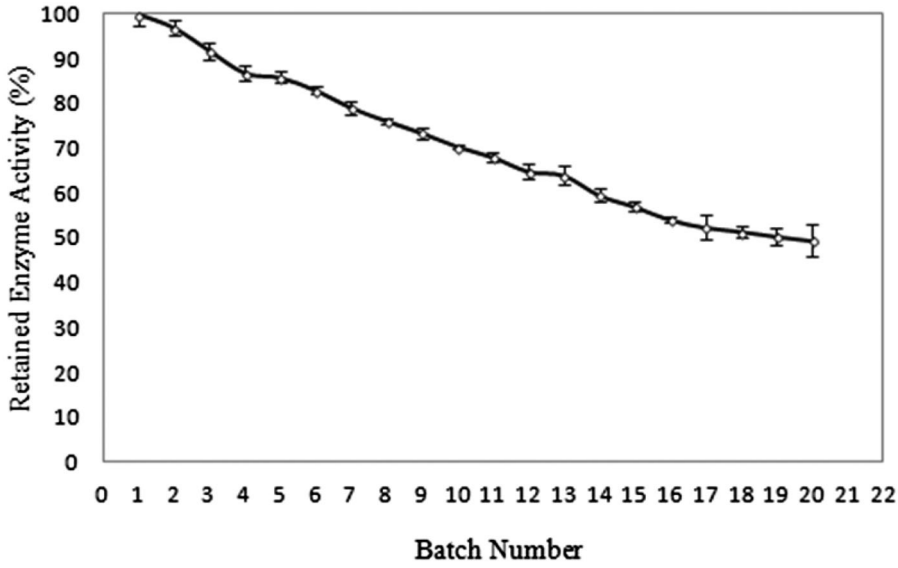

Figure 3. Operational stability of immobilised inulinase in PAA/PEG composite. The reaction mixture composed of 0.25 g immobilised enzyme and 10 mL 2% inulin solution was incubated at optimal pH and temperature (4.4 and 40 °C) for 1 h. After each cycle the catalyst was removed by filtration and the remaining activity was measured under standard assay. The activity of the immobilised enzyme after the first cycle (1.7 U/g) was set as a control and assumed to possess a relative activity of 100%.

part of FOS with DP = 3–6. Fructose and sucrose are byproducts.

The total amount of obtained prebiotic FOS was 22% when 10% (w/v) of immobilised preparation was applied (Figure 4(a)), while the unconverted inulin in the final mixture, that is composed of FOS with DP> 6 was 68%, along with the amount of the byproducts fructose and sucrose that were 10% together. By raising the amount of immobilised enzyme to 20% (w/v), the percentage of products was increased to 25% (Figure 4(b)). However, in this system, a higher total amount of fructose and sucrose of 14% was produced. The highest conversion of inulin (36%) to FOS with DP = 3-6 occurred when hydrolysis was performed with 30% (w/v) immobilised preparation (Figure 4(c)). The figure shows the distribution of sugars: kestose 6%, nystose and fructofuranosyl 9%, FOS with DP = 6 12%, while fructose and sucrose are 4 and 7%, respectively. The unconverted inulin composed of FOS with DP > 6 remained about 53% in final the mixture.

In the experiment performed to study the effect of the initial concentration of the substrate on the degree of its conversion similar trends with other published results were observed. The highest conversion of 30% of the inulin to prebiotic FOS occurred when 4% inulin (w/v) were used (Figure 4(e)). Fructose and sucrose were also obtained in high percentages of 13 and 6%, respectively. In contrast, the degree of conversion of inulin to useful FOS was 16%, when the initial concentration of the inulin solution was 6%. This can be seen in Figure 4(f). A large percentage of unconverted inulin, 77% remained in this system, while the fructose and the sucrose were produced in total amounts of 7% and 2%.

In conclusion, the best degree of conversion of highly polymerised inulin is obtained using a 2% solution of inulin and 30% (w/v) immobilised inulinase in PAA/PEG composite, where the yield of useful FOS is high, while the yield of non-prebiotic monosaccharide and disaccharide is small.

Comparing the efficiency of the hydrolysis with free enzyme, added to the reaction mixture in the same amount as the biocatalyst, it can be seen that the concentration of prebiotic FOS increased from 30% to 57% as the amount of enzyme increased. More precisely it was about 1.5 times higher than the product obtained with immobilised inulinase (see SI, Figure 5S). Despite the better productivity of FOS, non-prebiotic sugars were also produced in higher total amount of 30%. However, when higher initial substrate concentrations were used (4 and 6%), the amount of FOS with DP = 3-6 substantially decreased to 17 and 19%, respectively. These quantities are comparable or even less than the products obtained with immobilised enzyme. The production of by-products – fructose and sucrose in these systems was negligible.

There are many articles that deal with inulin hydrolysis using inulinase extracted from different microorganisms, immobilised on various supports. Jiang et al. (2019) used endoinulinase in free form

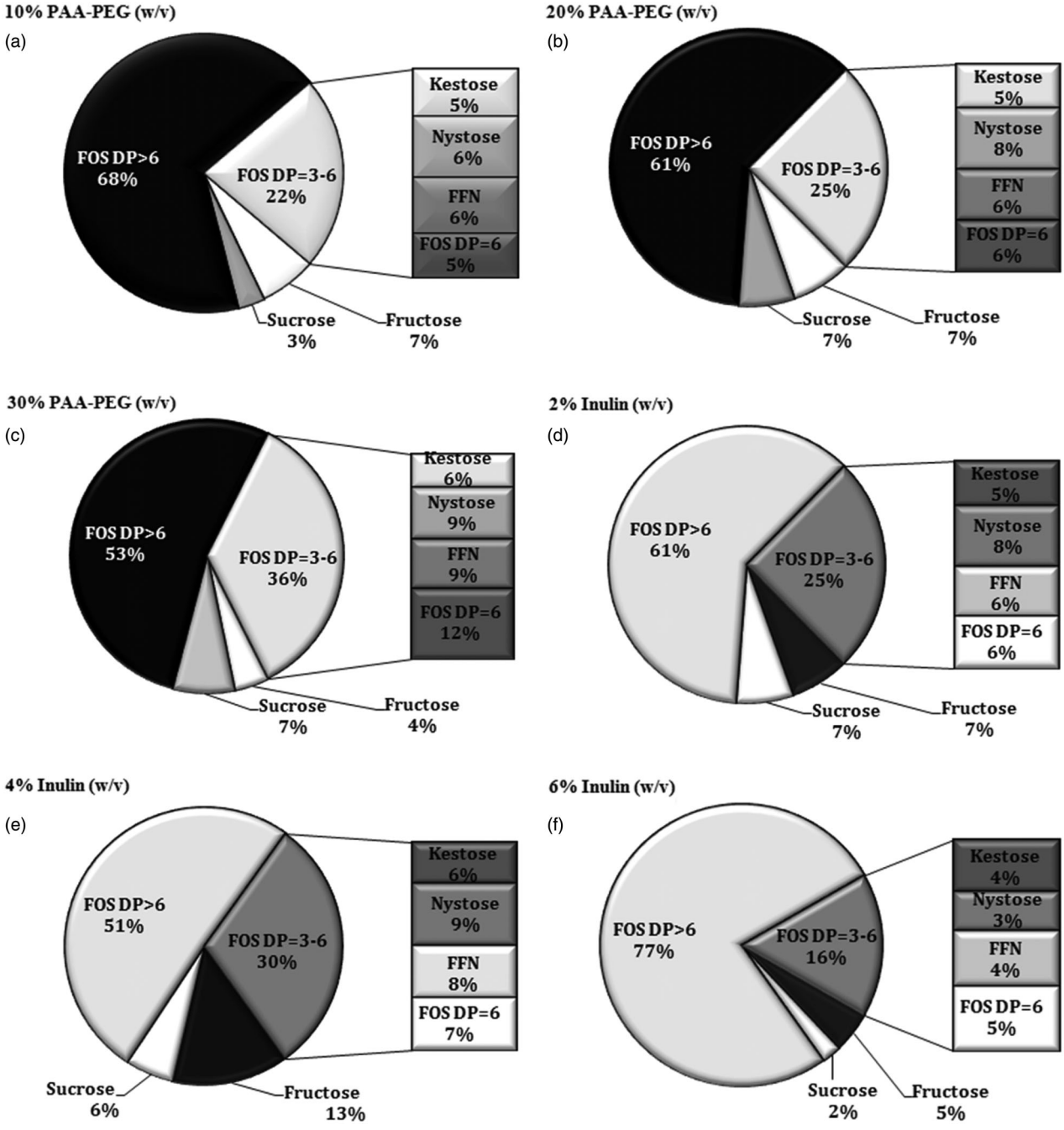

Figure 4. Composition of the final mixture of the hydrolysis of inulin to fructose, sucrose, kestose, nystose, fructofuranosyl nystose and FOS DP $>$ 6 with addition of different amounts of immobilised preparation (w/v): a) 10% PAA/PEG with enzyme activity of 46 U, b) 20% PAA/PEG with enzyme activity of 92 U, c) 30% PAA/PEG with enzyme activity of 138 U, and different initial substrate concentrations (w/v) d) 2% inulin, e) 4% inulin, f) 6% inulin. The enzymatic reaction was performed on a rotary shaker at optimum pH and temperature (4.4 and 40 °C). The samples were withdrawn from the reaction mixture at 0, 1, 3, 5, 7, 9, 12 and 24 h and analysed with TLC coupled with ImageJ.

from *A. arachidicola* at different inulin concentrations of 1, 3, 5 and 10%, with final inulin conversion to total inulooligosaccharides of 88.4%, 83.5%, 80.2% and 60.1% respectively. The highest conversion was obtained when the lowest initial concentration of substrate was used, which match our results. Decrease in total yields of oligosaccharides was observed by Yun et al. (1997) as the concentration of inulin increased from 5% to 20% using endoinulinase from *Pseudomonas* sp. No.65 for inulin hydrolysis. Their maximum yield of 75.6% was observed when the initial inulin concentration of 5% was used with FOS DP $=$ 2, DP $=$ 3 obtained as main products. The highest initial substrate concentration of 20% gave 67.6% of

the product. Wang et al. (2016) (Wang et al. 2016) optimised the production of FOS with endoinulinase from *A. ficuum* and produced the highest yield of FOS of 94.4% using 15% (w/v) inulin. When the inulin concentration of 10% was used for the reaction, endoinulinase from the wild microorganism *Pseudomonas* sp. immobilised on ion exchange resin converted 77.6% of it (Yun et al. 1997). Compared to our results, the yields obtained in these studies are much higher which might be related to the initial degree of polymerisation of the inulin used as a substrate. Regarding the distribution of FOS, DP = 3 and DP = 4 as major products were reported by Chen et al. (2012) (Chen et al. 2012), using endoinulinase from *A. arachidicola* in free form and recombinant endoinulinase from *A. ficuum* JNSP5-06. In comparison, endoinulinase from *A. niger* produces FOS with DP = 2-5 as main products, while inulinase from *Pseudomonas* sp. produces FOS with DP = 2 and DP = 3 as main products (Yun et al. 1997; He et al. 2014). Interestingly, endoinulinase from *Xanthomonas* sp. produces only FOS with DP = 5 and DP = 6 as sole products. The enzyme produced 86% of total FOS when 8% of inulin was used as a substrate and 57% total FOS when the concentration of the substrate was increased to 10% inulin (Park et al. 1999). Recombinant endoinulinase from *Arthrobacter* sp. S37 produces only FOS with DP = 2 as the main product (Li et al. 2012), but interestingly the native enzyme produced by the wild strain of *Arthrobacter* sp. S37, produces FOS with DP = 3, DP = 4 and DP = 5 as main products (Kang et al. 1998). Sucrose and mono-saccharides are also produced in the process which reduces the quality of FOS products. The production of these by-products is observed in the hydrolysis of inulin by some other endoinulinases. For example, endoinulinase from *A. ficuum* generates 5.1% sucrose (Wang et al. 2016), endoinulinase from *P. mucidolens* produces 20.1% glucose and 5.1% fructose (Yun et al. 1997), whereas endoinulinase from *Pseudomonas* sp. produces 7.4% glucose, 5.4% fructose and 3.1% sucrose (Yun et al. 1997). These findings are in accordance with our results.

Conclusion

In the present study, an inulinase was successfully immobilised in PAA/PEG composite. Immobilised enzyme had two pH optima of 4.4 and 7.6 and two temperature optima of 40 and 80 °C. The immobilised preparation retained almost 100% stability after 2 hours of incubation in the pH range of 3.6 to 6.9 and a temperature range from 20 to 50 °C. The half-life $t_{1/2}$ at the optima was 115 h, while the D-value was 367 h, indicating that the produced catalyst had enhanced thermal stability and is suitable for industrial applications. Immobilised inulinase in PAA/PEG can be reused 20 times until the initial activity decreases to its half, thus reducing operational costs and widening the possible applications. Finally, the prepared catalyst is effective in producing FOS with the DP from 3 to 6 with proven prebiotic activities. In addition, the yield of FOS from the enzymatic hydrolysis was 36% when initial inulin concentration was 2% (w/v) and the activity of the enzyme was 138 U. The composition of the final mixture was composed of: kestose 6%, nystose and fructofuranosyl 9%, FOS with DP = 6-12% thus reducing operational costs and widening the possible applications.

Disclosure statement

The authors have declared neither conflicts of interest nor competing financial interests. All authors have approved the manuscript.

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