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Immobilization of α-amylase on poly(vinylamine) and poly(vinylformamide) supports and its performance

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ABSTRACT

α-Amylase was immobilized on six poly(vinylamines) and three poly(vinylformamides) hydrogels polymerized using various techniques and crosslinkers. The enzyme was covalently bound to the supports using glutaraldehyde as a spacer. The immobilization procedure was optimized involving such factors as temperature, pH, time, sequence of reactions, and kind of carrier employed. Results of the immobilization were evaluated based on analyses of the enzyme activity and stability prior and after immobilization, as well as on the immobilization yield and stability. Highly active biocomposite preparations were designed which provided their multiple application for starch hydrolysis. The selection of a carrier was essential for the activity and stability of immobilized α-amylase. Poly(*N*-vinylformamide) crosslinked with divinylbenzene in form of spherical beads obtained in a suspension polymerization appeared to be a superior carrier for α-amylase.

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1. Introduction

Amylases play essential role as hydrolyzing enzymes widely used in food, fermentation, textile, and paper industry [1]. α-Amylase (1,4-α-D-glucan-glucanhydrolase, EC. 3.2.1.1) is an endo-acting, widely distributed enzyme that hydrolyzes the α-1,4-glycosidic bonds, by-passing α-1,6-glycosidic linkages in starch and related substrates. Amylases are available from various sources, including plants, animals and microorganisms [2–5]. Their immobilization on water insoluble carriers seems to be the most promising way to obtain more stable products for multiple use [6–14].

There are several methods for immobilizing insoluble enzymes. For that purpose, enzymes are enveloped into a gel matrix, encapsulated, incorporated into emulsions and membranes, bound to a support by either adsorption, coordination or covalent binding. In fact, the binding of an enzyme to a support is most common. Therefore, the selection of a suitable carrier for a given enzyme and a way of fixing enzyme to it are key problems. Glutaraldehyde renders the highest enzyme stability when bound to a support provided the support has amino moieties on its surface [15–18]. The amination introducing such groups to the support most commonly involved carcinogenic ethyleneimine. Use of ethylenediamine was an alternative approach. Fortunately, few years ago the precursor of polyvinylamine, PVAm, i.e. *N*-vinylformamide, NVF, became commercially available. Its polymer, poly(*N*-vinylformamide), PNVF, can be easily hydrolyzed to PVAm making the amination unnecessary. In this work, PVAm and PNVF have been applied as carriers for enzymes. These carriers have been produced in form of spherical beads suitable also for packing in columns where hydrodynamical factor should be considered.

Effect of the immobilization depends on several parameters such as surface area, accessibility of the surface for enzymes, number of activated functional groups on the support, availability for binding the functional groups on the protein, spacer used, distance between the bound enzyme and the surface of the support, and the steric orientation of the active centre. Mono- or multipoint binding of an enzyme to the support, the chemical affinity of protein to the material of the support are further factors influencing immobilization [19–21].

In this paper, immobilization of highly active, thermostable α-amylase on poly(vinylamine) (PVAm) and poly(*N*-vinylformamide) supports is described. Glutaraldehyde was used as a spacer.

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Table 1
Origin of immobilizing hydrogels.

Sample	Polymer	Monomer	Crosslinker	Polymerization type	Hydrolysing or rinsing agent
PVAm-1	PVAm	NVF	NMBA	Miniemulsion	KOH
PVAm-2	PVAm	NVF	NMBA	Miniemulsion	HCl
PVAm-3	PVAm	NVF	3A2H	In mass	KOH
PVAm-4	PVAm	NVF	DVB	In mass	HCl
PVAm-5	PNVF	NVF	DVB	In mass	NH_3[a]
PVAm-6	PNVF	NVF	DVB	Suspension	NH_3[a]
PVAm-7	PNVF	NVF	DVB	Suspension	None
PVAm-8	PVAm	NVF	DVB	Suspension	NaOH
PVAm-9	PVAm	NVF	DVB	Suspension	HCl

[a] Crosslinked poly(*N*-vinylformamide) was yielded.

2. Materials and methods

2.1. Materials

α-Amylase SPEZME® PRIME 107-05127-001 (Genencor International, USA) from a genetically modified strain of *Geobacillus stearothermophilus*, had 7163 U/g at pH 5.0–6.5 at 35–50 °C.

Hydrogels: poly(vinylamines) (PVAm) were prepared from *N*-vinylformamide (NVF) which was polymerized using three different crosslinkers. Six of so prepared poly(*N*-vinylformamides), PNVFs, were submitted to either base or acid hydrolyses, respectively, while in three cases the support was provided by PNVFs (Table 1). All the carriers were activated with glutaraldehyde (GA) functioning as a spacer between support and the enzymatic protein [22].

PVAm-1 and PVAm-2 supports were derived from PNVFs polymerized in microemulsion, crosslinked with *N*,*N*′-methylenebisacrylamide (NMBA), and hydrolyzed either by aqueous KOH or hydrochloric acid.

The PVAm-3 carrier represented a polyvinylamine crosslinked with (3-acryloyloxy-2-hydroxypropyl)methacrylate (3A2H). The respective PNVF was hydrolyzed in aqueous KOH [22].

PVAm-4 resulted from hydrochloric acid hydrolysis of with divinylbenzene, DVB, crosslinked PNVF, obtained in an "in mass" polymerization. It was rinsed in ammonia. The same procedure was applied to PVAm-5, and PVAm-6, which were polymerized in suspension. Unlike PNVF-5, PNVF-7 was not rinsed with NH_3 before treatment with GA. PVAm-8, and PVAm-9 supports were also prepared from poly(vinylformamide) crosslinked with divinylbenzene via free-radical polymerization in reversed suspension. PVAm-8 resulted from base-catalyzed hydrolysis with aqeous KOH, whereas PVAm-9 was hydrolyzed with hydrochloric acid [22].

In all experiments sago starch (Wah Chang International Group of Companies, Singapore) was used as a substrate.

2.2. Methods

2.2.1. α-Amylase immobilization

A support (0.1 ± 0.0001 g) was placed in 0.1 M phosphate buffer (pH 7) (25.0 mL), enzyme (1.0 mL) was added, the whole was gently agitated for 2 h followed by 24 h storage at 4–6 °C then filtered through the cellulose filter paper with 5 μm pores (Whatman). The support with immobilized enzyme was washed subsequently with 0.1 M phosphate buffer pH 7, 0.1 M phosphate buffer containing NaCl (30 g/L), 0.1 M acetate buffer pH 5.5 and, finally, with distilled water. Such product was stored in phosphate buffer pH 7 (25 mL). Prior to the use the mixture was repeatedly filtered through the cellulose filter paper whereupon in the filtrate activity of non-immobilized enzyme was determined in the enzymatic reaction.

2.2.2. α-Amylase activity

Activity of non-immobilized and immobilized enzymes was determined on enzymatic hydrolysis of starch (2 mg/1 mL buffer) carried out at 37 °C ± 1 °C and pH 7 (0.1 M phosphate buffer). Regardless either immobilized or non-immobilized, the same amount of enzyme (2 μL/1 mg starch) was applied.

At first, starch was 20 min gelatinized at 85–90 °C in phosphate buffer. After cooling to 37 °C enzyme was added on gentle stirring

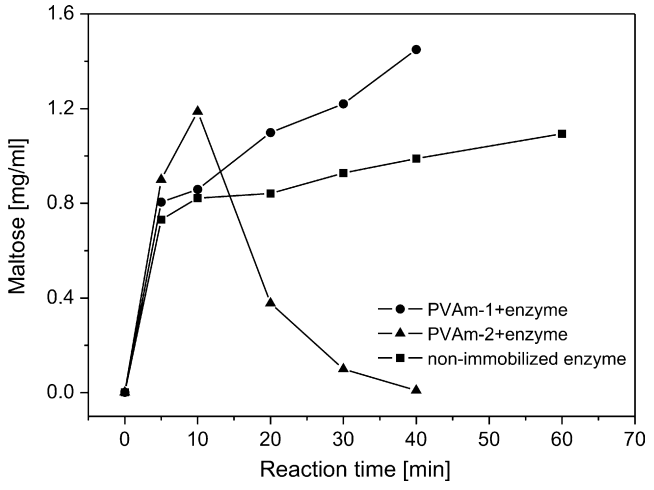

Fig. 1. Product output vs. time in starch hydrolysis catalyzed by α-amylase immobilized on PVAm-1 and PVAm-2 supports.

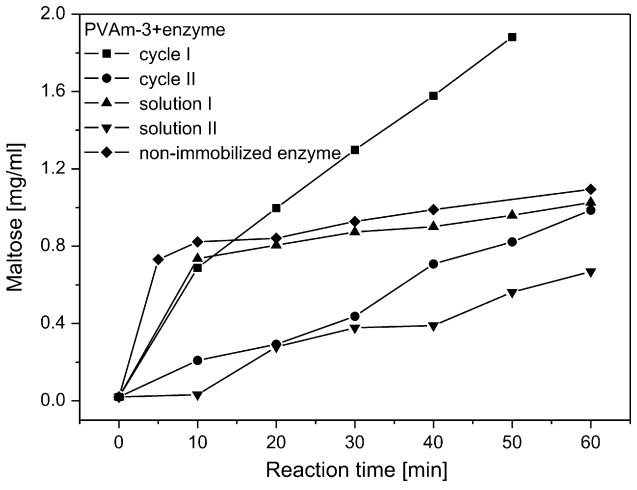

Fig. 2. Product output vs. time in starch hydrolysis catalyzed by α-amylase immobilized on PVAm-3 support.

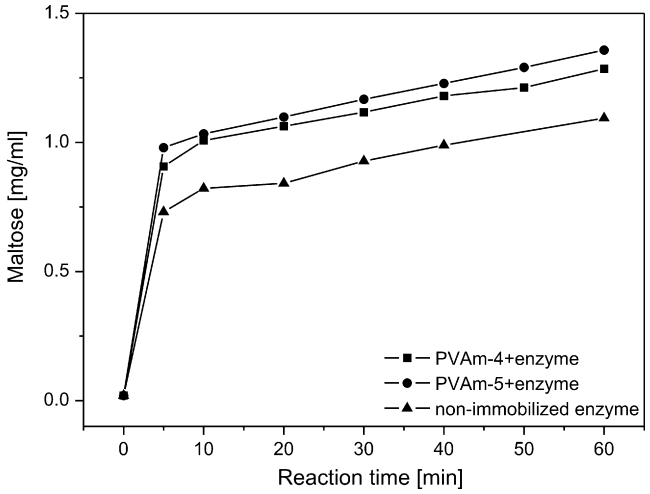

Fig. 3. Product output vs. time in starch hydrolysis catalyzed by α-amylase immobilized on PVAm-4 and PNVF-5 supports.

(30 rpm). After the first cycle of the enzymatic reaction with immobilized α-amylase, solutions were filtered trough a cellulose filter paper with 0.2 μm pores (Whatman) to recover the immobilized enzyme. Support with immobilized α-amylase was washed subsequently with 0.1 M phosphate buffer pH 7, 0.1 M, with phosphate buffer containing NaCl (30 g/L), 0.1 M acetate buffer pH 5.5 and, finally, with distilled water. The carrier with enzyme was transferred to a corresponding volume of phosphate buffer, stored at 4 °C in the dark for 4 weeks, and re-used. After second cycle with immobilized enzyme such enzyme was deactivated by heating the sample to 100 °C (7–10 min).

Concentration of reducing sugars was estimated spectrophotometrically [23] (2101PC, Shimadzu) at λ = 530 nm with solution of 3,5-dinitrosalicylic acid (DNS) in alkaline sodium potassium tartrate.

Enzyme activity was defined as the concentration of the hydrolysis product calculated for maltose. Calibration curve was designed using D-(+)-maltose monohydrate (Sigma–Aldrich, Poznan, Poland) as standard.

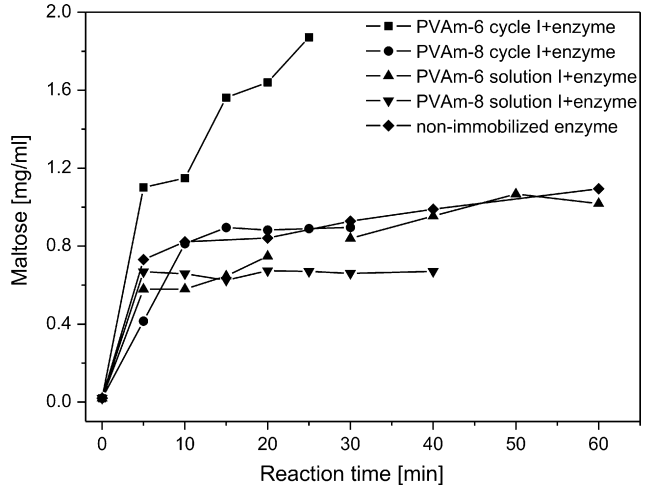

Fig. 4. Product output vs. time in starch hydrolysis catalyzed by α-amylase immobilized on PVF-6 and PVAm-8 supports in the first cycle and product concentration in the filtrate from washing after immobilization.

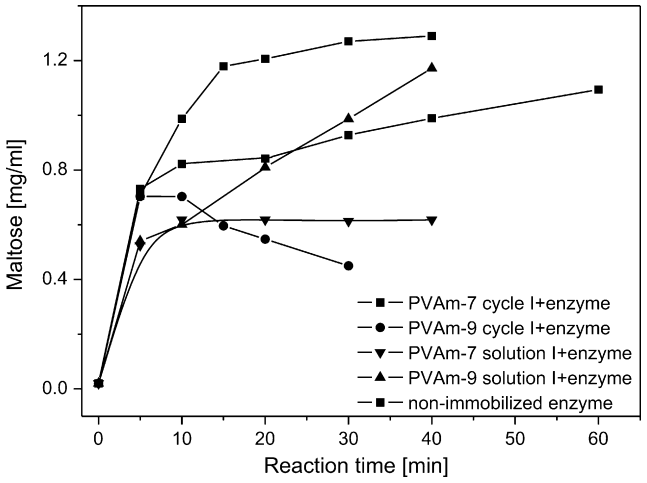

Fig. 5. Product output vs. time in starch hydrolysis catalyzed by α-amylase immobilized on PNVF-7 and PVAm-9 supports in the first cycle and product concentration in the filtrate from washing after immobilization.

3. Results and discussion

Because a product output vs. time relationship for the activity of α-amylase in its native, non-immobilized state was linear (Fig. 1) the enzyme hydrolysed starch following zero-order kinetics with $k = 6.7 \times 10^{-3}$ min^{-1} ($r = 0.996$ for 8 points).

3.1. Activity of α-amylase immobilized on various carriers

Only on the beginning of the process, activity of native and immobilized on PVAm-1 support α-amylase was similar. In the end of the process, immobilized enzyme provided by 46% higher product output. The same enzyme supported on PVAm-2, showed the 45% increase in its activity already at the onset period. However, shortly thereafter the enzyme totally lost its activity (Fig. 1).

Immobilization of the enzyme on PVAm-3 carrier resulted in an initial 21% relative increase in the activity which gradually rose up to 70%. The curve of the relationship only slightly changed in the second cycle (Fig. 2).

Immobilization of α-amylase on the PVAm-4 and PVAm-5 supports led to a relative increase in its activity from initial 22% up to 30% (Fig. 3).

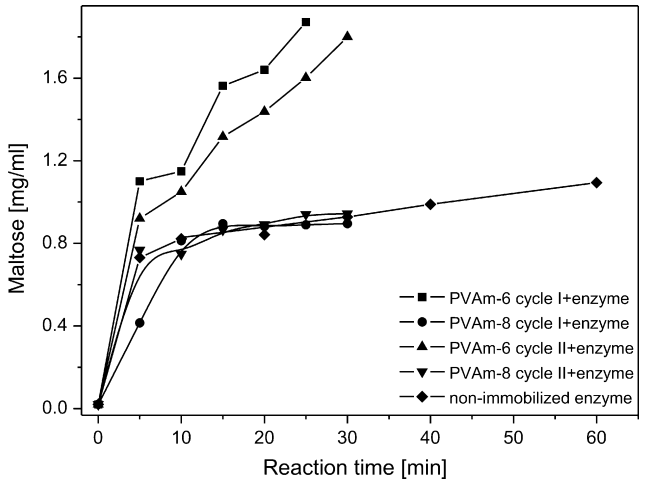

Fig. 6. Product output vs. time in starch hydrolysis catalyzed by α-amylase immobilized on PNVF-6 and PVAm-8 supports in the first and the second cycles.

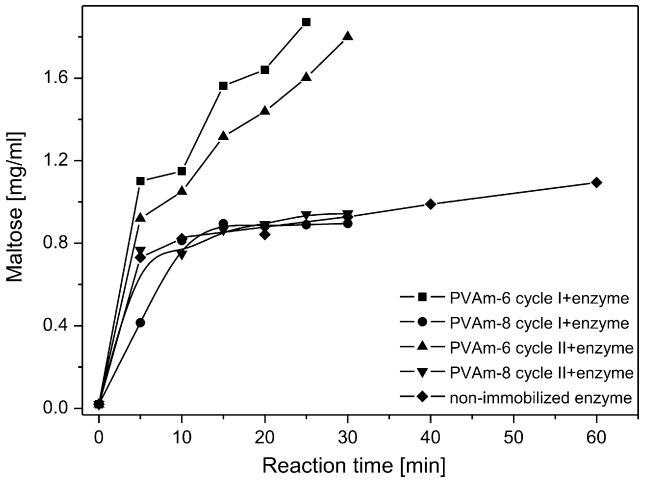

Fig. 7. Product output vs. time in starch hydrolysis catalyzed by α-amylase immobilized on PVAm-7 and PVAm-9 supports in the first and the second cycles.

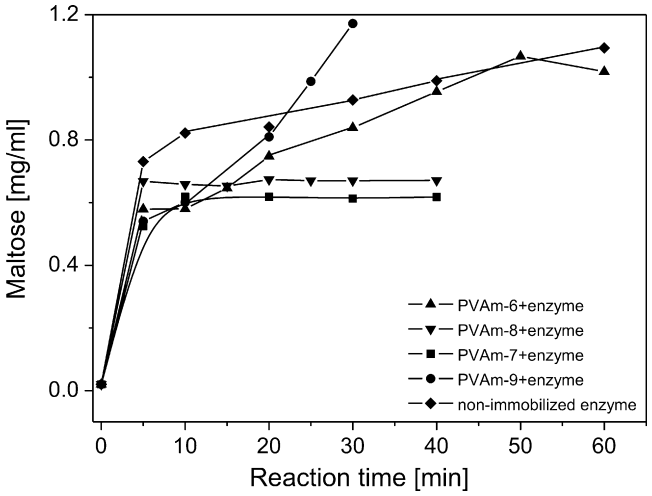

Fig. 9. Product concentration in starch hydrolysis catalyzed by α-amylase in filtrate from washing before the first reaction cycle.

Relative activity afforded by immobilization of the enzyme on the PVAM-6 carrier rose from initial 50% up to 95%. In that case, however, a slightly worse binding of the protein to the carrier was observed. The PVAm-8 carrier exhibited exceptionally low initial low activity of the immobilized α-amylase. Nevertheless, the relative activity of the enzyme rose in time to an acceptable level (Fig. 4).

Fig. 5 illustrates the influence of the PNVF-9 carrier on the activity of immobilized α-amylase. The relative increase in the activity reached from 50% to 70% (Fig. 5).

Although in the second cycle run after 4-week storage, α-amylase immobilized on PNVF-6 lost from 8% to 17% of initial activity its residual activity was still by 25–70% higher than that in its native state (Fig. 6).

The enzyme immobilized on the PNVF-7 support exhibited almost the same activity in the first and second cycle, whereas when supported on PVAm-9, its low activity in the first cycle increased in the second cycle up to 75% that is over the respective activity of the non-immobilized enzyme (Fig. 7).

Fig. 8 presents ratio of activity of the enzyme in the second and first cycle ($A_{I/II}$) after its immobilization on the PVAm-6, PVAm-7, PVAm-8, and PVAm-9 carriers. The ratio was defined by

$$A_{II/I} = \left(\frac{C_{II}}{C_I}\right) \times 100\% \quad (1)$$

where C_{II} and C_I were concentrations of maltose (mg/mL) after the second and first cycle.

Based on that criterion, among immobilized enzymes only that on PVAm-9 support appeared promising because its relatively high activity still increased with time. Activity of α-amylase immobilized on the PVAm-6 and PVAm-7 supports was much lower but stable in time whereas α-amylase immobilized on the PVAm-8 support dramatically and fast lost its activity to the level found for that enzyme immobilized on the PVAm-6 and PVAM-7 supports.

3.2. Efficiency of the immobilization process

Concentration of enzyme in a filtrate after washing the immobilized enzyme before the first cycle was low (Fig. 9) pointed to a high yield of immobilization with a strong covalent binding of the protein to the carrier. According to Gopinath and Sungunan [24] only enzymes bound by sorption can be washed out.

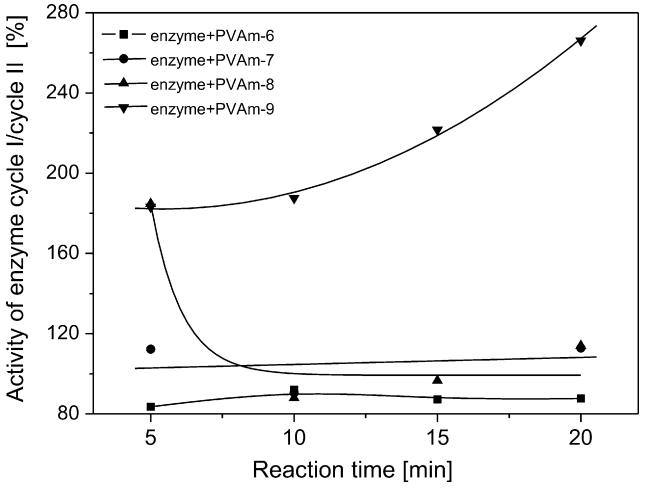

Fig. 8. Changes of product outputs in starch hydrolysis catalyzed by α-amylase immobilized on PNVF-6, PVAm-7, PVAm-8, and PVAm-9 supports in the indicated cycles of the enzymatic reaction.

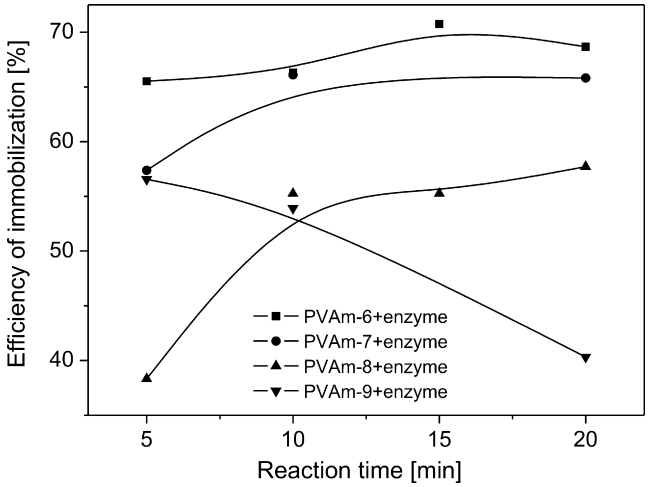

Fig. 10. Time-dependent decrease in efficiency of α-amylase immobilized on supports PNVF-6, PVAm-7, PVAm-8 and PVAm-9.

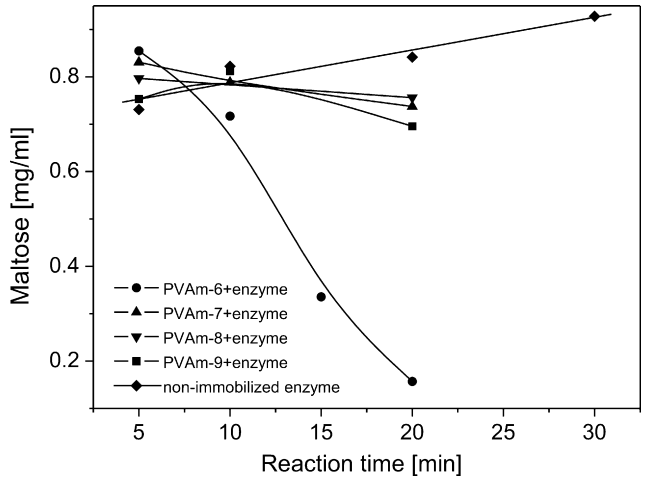

Fig. 11. Product concentration from starch hydrolysis catalyzed by α-amylase in filtrates from washing after the first reaction cycle.

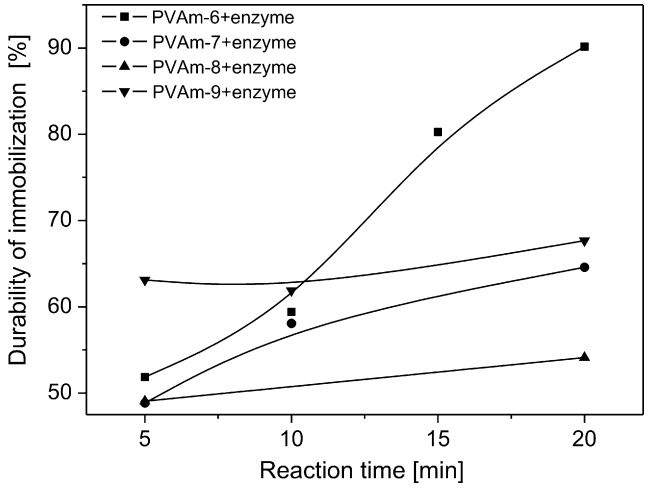

Fig. 12. Stability of the immobilization of α-amylase on the PNVF-6, PVAm-7, PVAm-8, and PVAm-9 supports after the first reaction cycle.

The immobilization yield, E (%), was defined as

$$E = \left(\frac{C_{nI}}{C_{nI} + C_{iI}}\right) \times 100\% \quad (2)$$

where C_{nI} and C_{iI} were concentrations of the products (mg/mL) yielded by enzymatic reactions in filtrates before and after the first cycle, respectively. Fig. 10 presents respective time-dependent yields of the enzyme immobilization on PVAm-6, PVAm-7, PVAm-8 and PVAm-9 carriers.

Except the PVNF-6 support which caused a fast deactivation of α-amylase all other carriers only slightly deactivated the enzyme in time as shown in Fig. 11 presenting the activity of α-amylase washed out after the first cycle.

3.3. Stability of immobilized enzyme

Stability of immobilization, S (%), of α-amylase was defined by means of Eq. (3).

$$S = \left[\frac{C_{fII}}{(C_{fII} + C_{cII})}\right] \times 100\% \quad (3)$$

where C_{fII} and C_{cII} were concentrations (mg/mL) of the product of the enzymatic reactions determined in filtrates from washing after the first and second cycle, respectively.

It was checked for PVAm-6, PVAm-7, PVAm-8, PVAm-9 (Fig. 12).

One could see from that figure that poly(*N*-vinylformamide), (PNVF-6), which was copolymerized with divinylbenzene in suspension and rinsed with ammonia, was superior carrier for α-amylase. Time dependent changes of activity of an enzyme immobilized on a carrier might result from blocking access to active centers of the supported enzyme [25] because of a multi-point immobilization.

NVF-based polymers crosslinked with divinylbenzene offer a possibility of production of the carrier in form of spherical beads. Additional amination with, for instance, either frequently used carcinogenic polyethyleneimine [14,16,26] or ethylenediamine [27] for fixation of glutaraldehyde, the most stable preparation for an enzyme [28] is unnecessary.

4. Conclusion

1. Both, activity and stability of immobilized α-amylase depend on the applied carrier
2. Poly(*N*-vinylformamide) crosslinked with divinylbenzene in form of spherical beads obtained in a suspension polymerization has proven to be an outstanding carrier for α-amylase.
3. PNVF-6 provides the highest activity and stability of the immobilized enzyme among all the other carriers based on *N*-vinylformamide and tested in the present work.

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