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Synthesis and characterization of poly(urethane-urea) microparticles from poly(vinyl alcohol) and binary blends of diisocyanates and their application for immobilization of maltogenic α-amylase

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Novel poly(urethane-urea) (PUU) microparticles were synthesized from poly(vinyl alcohol) (PVA) and a blend of 1,6-hexamethylene diisocyanate (HMDI) and 2,4-toluene diisocyanate (TDI) in a dimethyl sulfoxide / water (99/1 vol.%) solution. Influence of the initial molar ratio of PVA and the blend of HMDI and TDI, reaction time and temperature on the yield of PUU and contents of functional groups have been studied. The structure of PUU was verified by chemical analysis and infrared spectrometry. Thermal stability of PUU in an inert atmosphere was studied by simultaneous thermal analysis. The size of PUU microparticles was evaluated by optical microscopy. Maltogenic α-amylase (EC 3.2.1.133) from *Bacillus stearothermophilus* was immobilized onto PUU microparticles. The effect of synthesis parameters of PUU on the efficiency of immobilization (EI) of maltogenic α-amylase and the yield of immobilization of protein (YP) were studied. The high yield of PUU microparticles (72%) and EI of maltogenic α-amylase (96%) was obtained when the initial molar ratio of PVA : (HMDI:TDI) was 1 : (0.75 : 0.25). The residual activity of immobilized maltogenic α-amylase onto PUU microparticles after storage for 28 days remained 95%. Repeated batch starch hydrolysis by maltogenic α-amylase, immobilized onto PUU microparticles, allowed at least seven cycles without a sensible activity decrease in starch saccharification. Immobilized maltogenic α-amylase maintained all initial catalytic activity after seven cycles.

Key words: poly(urethane-urea), poly(vinyl alcohol), thermogravimetric analysis, immobilization, maltogenic α-amylase

INTRODUCTION

Polyurethane (PU) and poly(urethane-urea) (PUU) as functional materials have a wide application in many areas. PU and PUU particles are used for coatings, adhesives, paints, powder moulding, biomedical and biotechnological purposes [1–9]. Usually, PU and PUU materials are synthesized in the same way. The PU chain extender is diol and the PUU chain extender is diamine. PU and PUU particles could be synthesized in the organic or aqueous medium. One of the most popular methods of PU synthesis is the one-step polyaddition polymerization of diols or polyols and diisocyanates in the organic medium using steric stabilizers, such as amphiphilic block copolymer poly(butadiene)-block-poly(ethylene oxide) [1–3], polycondensable macromonomer prepared from trimethylolpropane with carboxyl

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terminated poly(lauryl methacrylate) [3], copolymer poly(vinylpyrrolidone-1-hexadecene) [4], poly(lauryl methacrylate) macrodiol [5], poly(1,4-isoprene)-block-poly(ethylene oxide) [6], and using the catalyst, such as organometallics [1–6] or tertiary amines [3, 7], that are used for synthesis of PU particles. During the synthesis of PUU in the organic medium, diamines were added at the end of synthesis and they reacted with free isocyanate groups [10, 11]. Another way of synthesis of PU particles is one-step or two-step procedures in the aqueous medium [8, 9]. According to the one-step method [8], the reaction mixture consists of diol or polyol, diisocyanate, water, surfactant and a co-stabilizer (hydrophobe). For the successful generation of particles, the following requirements must be fulfilled: a) the reactants (diisocyanate and diol) require low water solubility; b) the reaction between diisocyanate and diol has to be slower than the time needed for the miniemulsification step; c) the side reaction of diisocyanate with water in the dispersed state has to be slower than the reaction with diol. According to the two-step method [9], in the first step, PU prepolymers were prepared by polyaddition in bulk or in inert solvent. In the second step, the PU prepolymer was dispersed in aqueous medium. In this case all diisocyanates and diols can be used. Aqueous PU dispersions are widely used for applications such as adhesives, coatings of various materials and as vehicles for sustained delivery of active agents in biomedical and pharmaceutical fields [8, 9]. In the case of synthesis of PUU, PU prepolymer in bulk or organic medium was obtained in the first step. In the second step, PU prepolymer was dispersed in water and then free isocyanate groups of PU reacted with water to form urea linkages. Diamines could be added at this stage, too. Sometimes synthesized PU materials were cured with laboratory humidity to form urea linkages which produce the branch structure [12–16].

Only few reports were found where poly(vinyl alcohol) (PVA) was used instead of polyols in the synthesis of PU in the form of films or membranes [17–19]. PVA is a polymer which contains secondary hydroxyl groups. According to the article [17], a film of PVA was modified with 1,6-hexamethylene diisocyanate (HMDI). Residual isocyanate groups were hydrolysed to primary amino groups to increase the positive charge on the film surface. The modified film of PVA could be used for binding of biomolecules such as fibrinolytic enzymes in an attempt to achieve biocompatible materials. The method developed for enzyme membrane preparation is based on crosslinking PVA with triisocyanate in the presence of enzyme [18]. M. Krumova et al. [19] investigated the effect of crosslinking PVA and HMDI on the mechanical and thermal properties of PVA. In our previous work [20], PU microspheres were synthesized from 4,4'-diphenylmethane diisocyanate and 1,4-butanediol or a mixture of 1,4-butanediol with poly(tetrahydrofuran)-terathane 1 400 polyether glycol in one-step polyaddition polymerization without stabilizers and catalysts. According to our recent work [21], PU microparticles were synthesized from PVA and HMDI using different molar ratios of components.

Usually, native enzymes are expensive and sensitive for environment. Immobilization of enzymes is a very effective alternative in overcoming problems of instability and repetitive use of enzymes [22]. The use of an immobilized enzyme permits the control of the reaction: the simple filtering of the enzyme to stop the reaction. The immobilized enzyme could be used several times or reused for long times in reactors and, in addition to that, some critical enzyme properties have to be improved, like stability, activity, and inhibition by reactions products [21, 22]. Enzymes could be immobilized by two methods: physical and chemical [23]. PU-based materials were used as immobilization carriers in various forms: foam, microspheres, microparticles, powders, layers, membranes and coatings [21]. A number of enzymes (e. g. phytase, endoglucanase, cellulase [24], β-galactosidase [25], chloroperoxidase [26], invertase [27], lipase [28], alcohol oxidase [29]) were immobilized onto the PU foam and film. PU membrane was used for preparation of enzyme electrode and all tested enzymes (alkaline phosphatase and alcohol, cholesterol and glucose oxidases) retained sufficient activity for use in electrochemical sensors [18]. According to the articles [28, 30], PU microspheres have been used for immobilization of enzymes by physical forces or covalent binding. The authors S. Phadtare et al. synthesized PU microspheres from diols and 2.4-toluene diisocyanate (TDI) which have been covered with colloidal gold [30, 31]. Polyurethane-gold microspheres were used for immobilization of pepsin [30] and endoglucanase [31]. These PU biocomposite materials exhibited outstanding reuse capability and temperature / pH stability.

Maltogenic α-amylase was used as a model enzyme. It is an exo-acting enzyme and clips off 1,4-α-glucosidic linkages in starch molecules. Hydrolysis starts from the non-reducing end of the substrate molecule and releases maltose units in the α configuration. Very high maltose syrups can be used to obtain pure maltose which is used in the pharmaceutical industry for producing maltitol, antibiotics, vaccines, and intravenous nutrients [32].

The aim of the present study was to synthesize PUU microparticles from PVA and a blend of HMDI and TDI in a dimethyl sulfoxide / water (99/1 vol.%) solution and to apply PUU as carriers for immobilization of maltogenic α-amylase. Effects of the reaction time, temperature and initial molar ratio of PVA : (HMDI : TDI) on the yield of PUU microparticles, quantity of functional groups, structure, size, thermal properties and immobilization parameters of maltogenic α-amylase onto PUU were attempted to ascertain.

EXPERIMENTAL

Materials

1,6-hexamethylene diisocyanate (HMDI), 2,4-toluene diisocyanate (TDI), poly(vinyl alcohol) (PVA) were purchased

from Fluka, Switzerland, dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich, France, acetone was purchased from Reachem Slovakia, Slovakia, and diethyl ether was purchased from Lach-Ner, Czech Republic. All reagents were used as received.

Maltogenic α-amylase (EC 3.2.1.133) from *Bacillus stearothermophillus* and α-amylase (EC 3.2.1.1) from *Bacillus subtilis* were obtained from Novozymes, Denmark.

BCA kit was used for determination of the content of protein (Sigma, Aldrich, Germany).

Synthesis of PUU microparticles

PUU microparticles from PVA and the blend of HMDI and TDI were synthesized by one-step method in the dimethyl sulfoxide / water (99/1 vol.%) solution according to our previous work [20]. The initial concentration of PVA was 0.1 M. The initial molar ratio of PVA and the blend of diisocyanates was varied from 1.0 : 0.5 to 1.0 : 1.0. The initial molar ratio of HMDI : TDI was also varied. The obtained PUU microparticles were dried in vacuum at 70 °C.

Characterization of PUU microparticles

Determination of the functional groups

The amounts of amino, isocyanate and hydroxyl groups were determined by chemical methods [33]. Determination of the amount of isocyanate groups was performed immediately after synthesis of PUU microparticles and it was recalculated with respect to weight loss in drying process.

FT-IR analysis

The structure of PUU was characterized by using the FRONTIER FT-IR spectrometer (Perkin Elmer).

Thermal analysis

The TGA and DSC investigations were carried out by using a Simultaneous Thermal Analyzer STA 6000 (Perkin Elmer) in the temperature range between 30–600 °C in 20 mL min^{-1} flow of nitrogen and heating rate of 10 °C min^{-1}.

Determination of the microparticle size of PUU

The microparticle size was investigated by using the optical microscope Olympus BX51.

Enzyme assays

Immobilization of maltogenic α-amylase

Immobilization of maltogenic α-amylase was carried out in a 0.1 M citrate buffer (pH = 5.0). The mixture of the enzyme, buffer and PUU microparticles (immediately after synthesis) was stirred at 40 °C for 30 min and then left at 4 °C overnight. Next day the immobilized enzyme was thoroughly washed with the buffer.

Determination of the enzymatic activity of maltogenic α-amylase and the amount of protein

The catalytic activity of native and immobilized maltogenic α-amylase was determined by reaction with a 5% of liquefied potato starch solution and incubating the mixture at 40 °C for 20 min. Liquefied starch was prepared according to this procedure: 100 mL of 5% potato starch suspension in the 0.1 M citrate buffer (pH = 5.0) was stirred with 0.5 mL (250 U) of α-amylase from a *Bacillus subtilis* solution for 5 min at 40 °C and after that enzyme was inactivated by heating the solution for 30 min in a boiling water bath. Dextrose equivalent of liquefied starch was 6%. Dextrose equivalent is a measure of the amount of reducing sugars present in a sugar product, relative to glucose, expressed as a percentage on a dry basis. Activity of the native and immobilized maltogenic α-amylase as well as the protein content in the native enzyme solution or left in the solution after immobilization was assayed by the Somogyi-Nelson method [34] and bicinchinonic acid method (BCA kit), respectively. Activity unit of the native or immobilized maltogenic α-amylase was defined as the amount of enzyme which under standard conditions (at 40 °C, pH = 5.0) produced 1 μmol of reduced sugars per minute. Four separate measurements of the native and immobilized maltogenic α-amylase were performed to check the reproducibility of the data.

Efficiency of immobilization (EI) was defined as the activity of the immobilized maltogenic α-amylase in percentage from the activity of native enzyme used for immobilization.

Yield of immobilization of protein (YP) was defined as the protein quantity of the immobilized enzyme in percentage from the quantity of the protein of the native enzyme used for immobilization.

Determination of the storage stability of immobilized maltogenic α-amylase

Stability of the immobilized maltogenic α-amylase was indicated by the residual activity of immobilized maltogenic α-amylase after 14 and 28 days storage in the citrate buffer, pH = 5.0 at 4 °C. The initial activity after the immobilization was expressed as 100%. Others were expressed as the relative activity to the activity after the immobilization.

Hydrolysis of starch in batch operation

The hydrolysis of liquefied starch was carried out in a flask in the presence of 10 mL liquefied starch and maltogenic α-amylase immobilized on PUU microparticles under stirring for 20 min at 40 °C. 2.2 U of the immobilized maltogenic α-amylase was used for the hydrolysis of 1 mL starch solution.

RESULTS AND DISCUSSION

Synthesis and characterization of PUU microparticles

PUU microparticles were synthesized from PVA and the blend of diisocyanates HMDI and TDI in DMSO/H_2O (99/1 vol.%) solution. The reaction between isocyanates and water leads to production of gaseous carbon dioxide and an amino group which can react with a free isocyanate group

to form urea linkages. Reactivity of primary amine in reaction with isocyanate is much faster than in reaction with primary alcohol or water. Primary hydroxyl groups or water in reaction with isocyanate groups are around three times more reactive than secondary hydroxyl groups [35–37], which are present in PVA. In this study, PUU microparticles were obtained from PVA and diisocyanates without using diamines.

Polyurethane microparticles were obtained from PVA and HMDI in our previous work [21], but unsuccessful attempts to synthesize PUU microparticles from PVA and TDI were done following the same procedure. Nanoparticles were obtained in the case when the initial concentration of PVA was 0.1 M and molar ratios of PVA and TDI were from 1 : 1 to 1 : 3. Gel formation was observed at higher PVA concentrations. The reactivity of diisocyanates toward active hydrogen compounds diols or diamines is different. HMDI is a flexible, linear, symmetrical molecule with two primary aliphatic isocyanate groups of equal reactivity. TDI is a rigid asymmetrical molecule with two secondary isocyanate groups of different reactivity [38]. The isocyanate group of TDI in the *para* position is approximately four times the reactivity of the *ortho* position and at least two orders higher than the isocyanate group in HMDI [35]. As a general rule, the isocyanate groups of diisocyanate have different reactivities, in spite of the perfect symmetry of the molecule. The explanation of this effect is simple: after the reaction of the first molecule of the hydrogen active compound, diisocyanate first transforms into urethane isocyanate. The second isocyanate group has a much lower reactivity than the first isocyanate group because the urethane group, due to its electron releasing effect, decreases the reactivity. The different reactivity of isocyanate groups in HMDI and TDI against hydroxyl groups is $K_1 \cdot K_2^{-1} = 2$ and $K_1 \cdot K_2^{-1} = 12$ [37], respectively. The *ortho* isocyanate in TDI may not react until the concentration of the *para* isocyanate has been sufficiently depleted. However, at very high temperature (T ~ 125 °C) both *ortho* and *para* isocyanate groups react almost simultaneously as observed by the authors [36].

Preparation of PUU microparticles from PVA and blend of HMDI and TDI depends on many factors including the ratio of initial components, their reactivity, reaction temperature and time. The initial molar ratio of PVA and diisocyanates in the reaction mixture was varied from 1 : 0.5 to 1 : 1 and the initial molar ratio of HMDI and TDI in the blend of diisocyanates was also varied (Table 1). Increasing the molar amount of diisocyanates in the initial molar ratio of PVA and (HMDI : TDI) from 1 : 0.5 to 1 : 1.0 and the molar amount of HMDI in the blend of diisocyanates resulted in increasing yield of PUU, quantity of isocyanate groups, slightly increasing quantity of amino groups from 0.3% to 1.0% and decreasing quantity of hydroxyl groups. The highest yield of PUU microparticles and the highest quantity of isocyanate groups were in the case when the initial molar ratio of PVA : (HMDI : TDI) was 1 : (0.75 : 0.25) (Table 1, No. 5). Prolongation of the reaction time from 60 to 120 min resulted in slightly increasing yield of PUU microparticles from 64.0% to 67.5% and decreasing quantity of isocyanate groups from 8.0% to 5.2% (Fig. 1a). In-

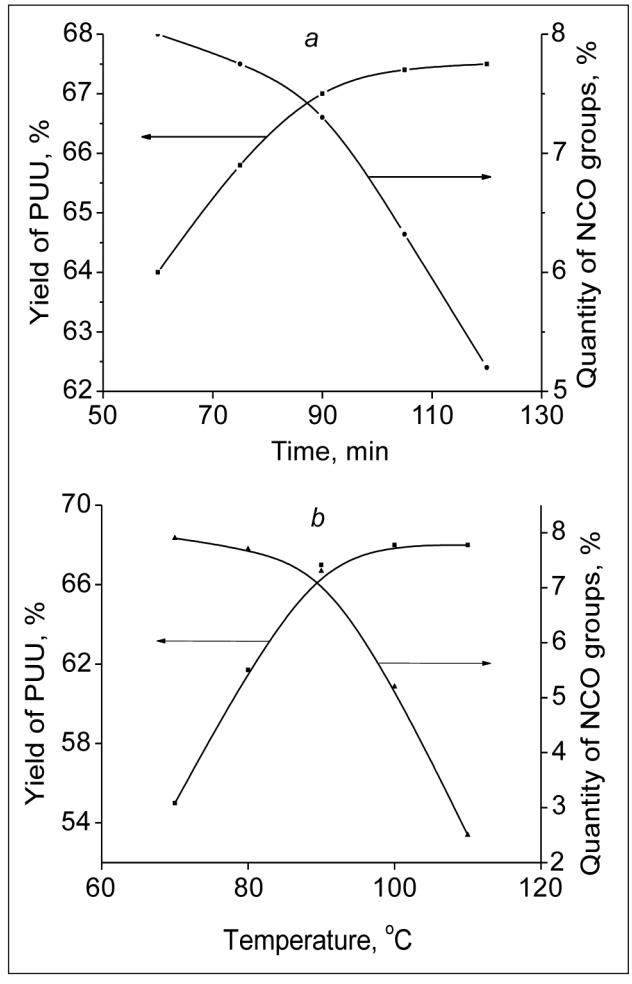


Fig. 1. Yield of PUU and quantity of NCO groups in PUU as a function of time (T = 90 °C) (*a*) and temperature (t = 90 min) (*b*) ([PVA] : ([HMDI] : [TDI]) = 1 : (0.50 : 0.50))

Table 1. **Yield of PUU and quantity of isocyanate and hydroxyl groups in PUU (PUU were synthesized using various initial molar ratios of PVA and blend of diisocyanates)**

No.	PVA : (HMDI : TDI) initial molar ratio	Yield of PUU, %	Quantity of NCO groups, %	Quantity of OH groups, %
1	1 : (0.25 : 0.25)	47.1	2.8	6.6
2	1 : (0.35 : 0.35)	50.3	3.2	5.2
3	1 : (0.50 : 0.50)	67.2	7.3	2.7
4	1 : (0.40 : 0.60)	55.4	5.4	4.0
5	1 : (0.75 : 0.25)	72.2	9.1	2.6

creasing reaction temperature from 70 °C to 110 °C resulted in increasing yield of PUU from 55.0% to 68.0% and decreasing quantity of isocyanate from 7.9% to 2.5% (Fig. 1b).

The structure of PUU has been proven by attenuated total reflectance (ATR) FT-IR spectra (Fig. 2). FT-IR spectra of the PUU microparticles, prepared using various molar ratios of PVA and the blend of HMDI and TDI show the same band at 3 319 cm^{-1} related to NH, bands at 2 933 cm^{-1} and 2 856 cm^{-1} related to C-H from alkyl groups, band at 1 615 cm^{-1} related to C=O of the urea group, band at 1 570 cm^{-1} is assigned to amide II (δ N-H, ν C=N), band at 1 250 cm^{-1} is assigned to amide III (another type of δ N-H, ν C=N) and band at 1 076 cm^{-1} (ν C-O-C) is assigned to ether linkage and a small shoulder at 1 691 cm^{-1} is related to C=O of the urethane group [39]. FT-IR spectra of PVA show typical bands at 3 301 cm^{-1} related to OH, at 2 916 cm^{-1} related to C-H from alkyl groups, at 1 733 cm^{-1} related to C=O, at 1 240 cm^{-1} related to ester C=O and at 1 086 cm^{-1} related to C-O-C [40].

Scheme 1 shows the expected structure of PUU. PUU microparticles consist of macromolecules with three types of constitutional units: a non-reacted hydroxyethylene constitutional unit of PVA (type I), a constitutional unit with one urethane group after reaction of a hydroxyethylene monomeric unit with one isocyanate group from either of diisocyanates (HMDI or TDI) and a free isocyanate or amino group (type II) and a constitutional unit with two urethane groups after crosslinking reaction of two hydroxyethylene monomeric units with two isocyanate groups from either of diisocyanates (type III). The poly(urea) segments could present in constitutional units of type II and III.

The non-isothermal stabilities of PVA and PUU microparticles were studied using simultaneous TGA and DSC measurements (Fig. 3, Table 2). According to DSC results, the melting point of PVA is 189.3 °C. According to the article [41], a similar melting point of PVA (191.2 °C) was obtained. Melting point of PUU was not detected because of its

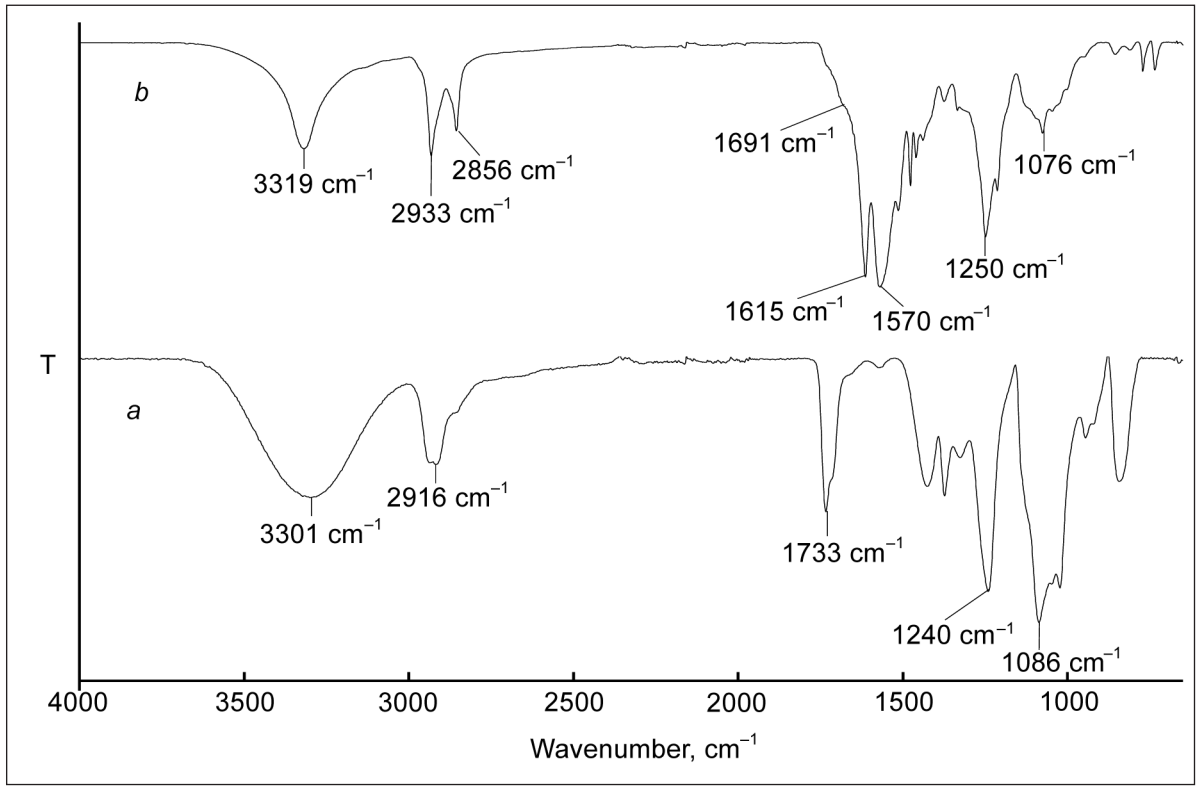
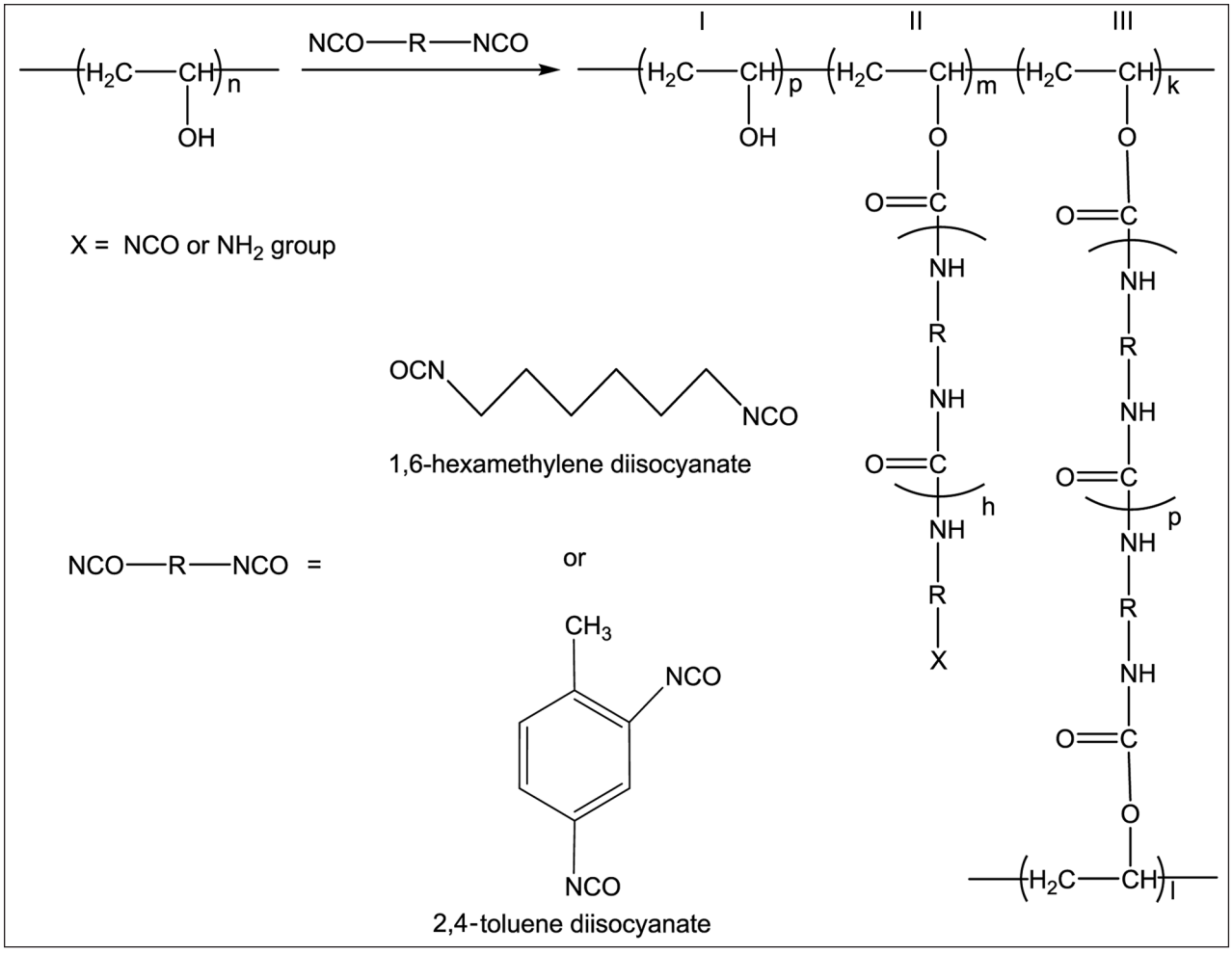


Fig. 2. FT-IR spectra of PVA (*a*) and PUU ([PVA] : ([HMDI] : [TDI]) = 1 : (0.75 : 0.25), $t = 90$ min, $T = 90$ °C) (*b*)

Table 2. **Results of TGA of PVA and PUU**

No.	PVA : (HMDI : TDI) initial molar ratio	First decomposition step			Second decomposition step		Third decomposition step		
		T_{init} [a], °C	T_{max} [b], °C	Δm[c], %	T_{max} [b], °C	Δm[c], %	T_{max} [b], °C	T_{end} [d], °C	Δm[c], %
1	1 : (0.00 : 0.00)	249	309	78	–	–	427	481	11
2	1 : (0.25 : 0.25)	221	282	72	–	–	443	482	18
3	1 : (0.35 : 0.35)	214	299	78	–	–	452	486	14
4	1 : (0.40 : 0.60)	200	293	77	–	–	450	486	12
5	1 : (0.50 : 0.50)	202	301	66	328	15	464	504	11
6	1 : (0.75 : 0.25)	212	291	56	334	26	466	506	10

[a] – initial decomposition temperature, [b] – maximum decomposition temperature, [c] – weight loss, [d] – end decomposition temperature.



Scheme 1. Structure of PUU (p and $h \geq 0$)

crosslinked structure. Decomposition of PVA proceeds in two stages according to TGA and DTGA results (Table 2, Fig. 3). The first stage with a maximum of decomposition temperature at 309 °C mainly involves dehydration accompanied by the formation of some volatile products. In the second stage, the polyene residues are further degraded at 427 °C to yield carbon and hydrocarbons [41]. According to the article [42], PUU materials mainly have two or three peaks of thermal decomposition. The first group of peaks were related to the decomposition of rigid segments formed by urethane and

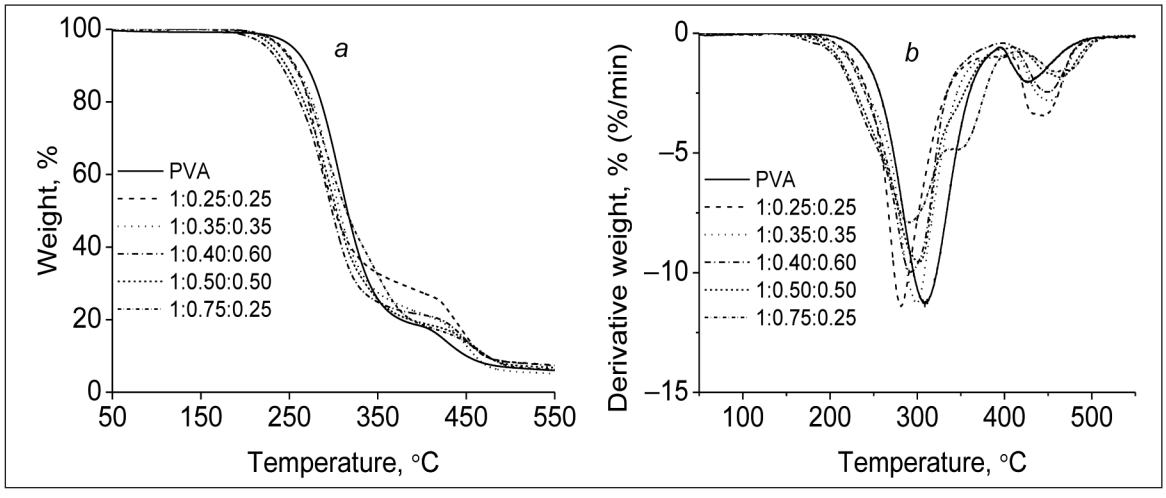
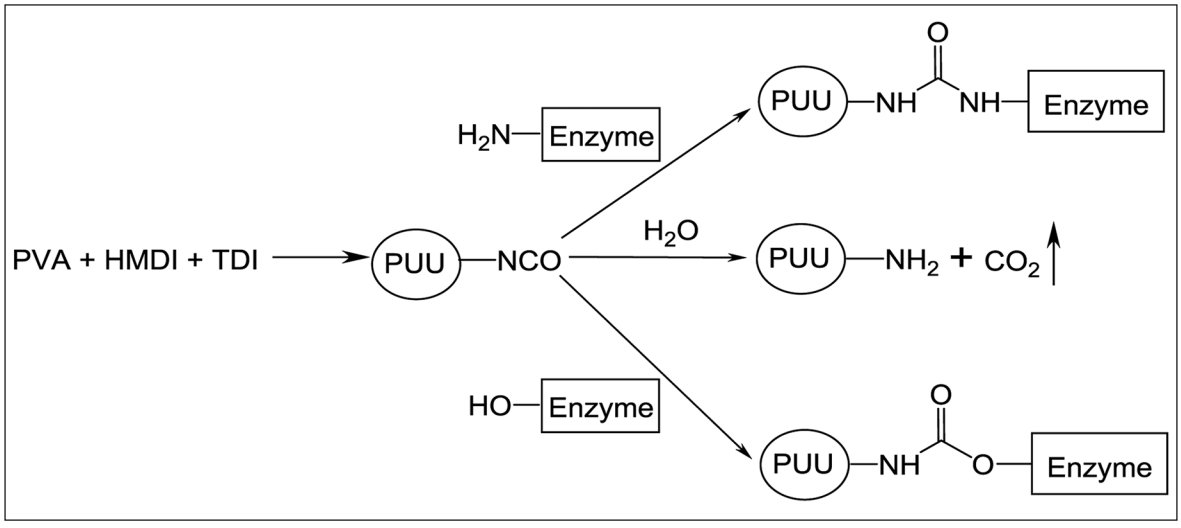


Fig. 3. TGA (*a*) and DTGA (*b*) curves of PVA and PUU (PUU were synthesized using various initial molar ratios of PVA : (HMDI : TDI), reaction time = 90 min and temperature $T = 90$ °C)



Scheme 2. Covalent immobilization of maltogenic α-amylase

urea linkages and the subsequent peaks were related to the decomposition of flexible segments, derived from polyols. The authors [42] observed that urethane-urea materials have better thermal stability than urethane materials, probably because of higher hydrogen-bonding capacity of the urea group compared with that of the urethane group. In the case of PVA-based PUU, TGA and DTGA curves show two step decomposition profile of PUU microparticles, which were obtained when initial molar ratios of PVA : (HMDI : TDI) were 1 : (0.25 : 0.25), 1 : (0.35 : 0.35) and 1 : (0.40 : 0.60). The first stage maximum decomposition temperature of PUU microparticles was around 282 °C–299 °C and it was lower than the first step decomposition temperature of PVA. The first step involves probably two overlapping peaks, which are difficult to distinguish from results. The second stage maximum decomposition temperature was more than 443 °C and it was 16 °C–23 °C higher than the second step decomposition temperature of PVA. TGA and DTGA curves of PUU microparticles, which were obtained when initial molar ratios of PVA : (HMDI : TDI) were 1 : (0.50 : 0.50) and 1 : (0.75 : 0.25), show 3 steps of thermal decomposition (Fig. 3, Table 2). The first two steps are decomposition of urethane and urea linkages, respectively. The third step is decomposition of PUU main chains.

Size of PUU microparticles was evaluated by optical microscopy. PUU microparticles have relatively narrow size distribution in the range of 10–30 μm. Microparticles are tended to agglomeration. Decreasing amount of HMDI in the initial blend of diisocyanates resulted in increasing size of PUU agglomerates from 50–150 μm to 100–300 μm.

Immobilization of maltogenic α-amylase onto PUU microparticles

Maltogenic α-amylase is one of the most important enzymes in starch industry, which is used for saccharification of starch for obtaining of high-maltose syrups [21, 32]. Enzyme, which has amino and hydroxyl groups, can be immobilized onto PUU microparticles, which have unreacted NCO groups and form urea or urethane linkages (Scheme 2).

NCO groups of PUU microparticles react faster with amino than with primary alcohol groups or with water [35]. Whereas the immobilization procedure followed in the aqueous media, the remaining free NCO groups reacted with water by formation of CO_2 and they did not have any inactivation effect on enzyme [21]. The results (Table 3) show that the initial molar ratio of PVA : (HMDI : TDI) during the synthesis of PUU has an obvious influence on the EI and YP of maltogenic α-amylase. This implies that the structure and porosity of PUU and the quantity of isocyanate groups in PUU microparticles have a significant effect on the binding of maltogenic α-amylase. EI and YP of maltogenic α-amylase increased when the amount of diisocyanates in the initial molar ratio of PVA and the blend of diisocyanates increased from 1 : 0.5 to 1 : 1 (Table 3, Nos. 1–3). Increasing initial molar amount of HMDI in the blend of diisocyanates from 0.40 to 0.75 resulted in increasing quantity of NCO groups and, as a result, increasing EI and YP of maltogenic α-amylase. The highest EI was 96% when initial molar ratio of PVA : (HMDI : TDI) = 1 : (0.75 : 0.25) was used for synthesis of carrier.

EI of maltogenic α-amylase on PUU microparticles depends on the reaction time (Fig. 4a) and temperature (Fig. 4b) of synthesis of PUU microparticles because the structure of PUU and quantity of NCO groups depend on these criteria,

Table 3. **Results of immobilization of maltogenic α-amylase onto PUU microparticles (Different initial molar ratios of PVA and blend of diisocyanates were used for synthesis of carrier)**

No.	PVA : (HMDI : TDI) initial molar ratio	EI, %	YP, %
1	1 : (0.25 : 0.25)	45.3 ± 1.4	47.0 ± 0.6
2	1 : (0.35 : 0.35)	51.1 ± 1.2	52.4 ± 0.4
4	1 : (0.40 : 0.60)	60.2 ± 1.6	62.4 ± 0.5
3	1 : (0.50 : 0.50)	74.0 ± 1.1	76.3 ± 0.8
5	1 : (0.75 : 0.25)	96.0 ± 2.0	100.0 ± 0.3

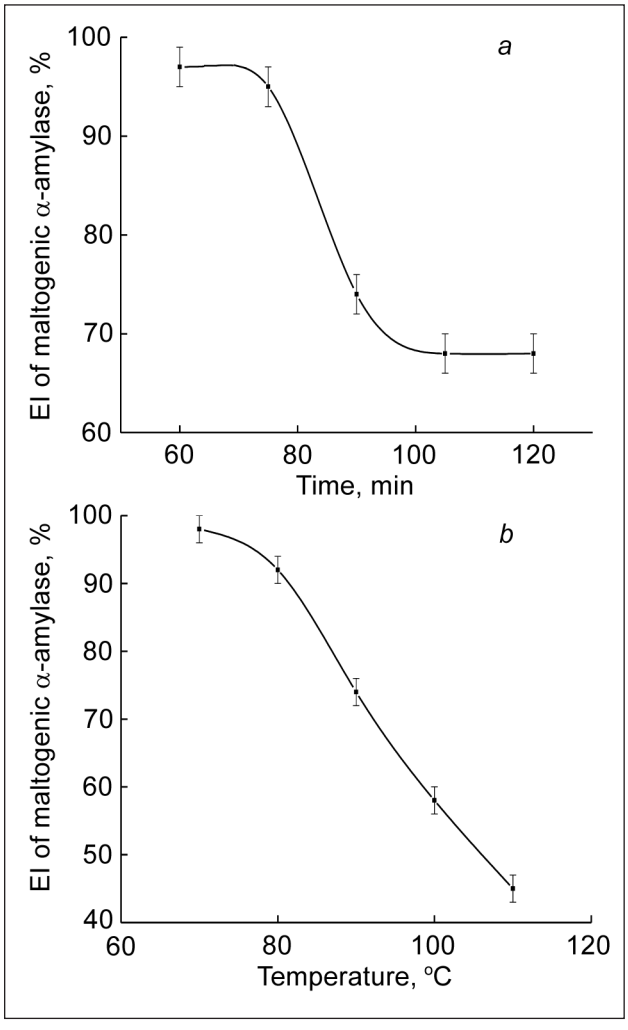


Fig. 4. Influence of the reaction time ($T = 90$ °C) (*a*) and temperature ($t = 90$ min) (*b*) of synthesis of PUU on EI of maltogenic α-amylase onto PUU (initial molar ratio PVA : (HMDI : TDI) = 1 : (0.5 : 0.5))

too. Prolongation of the reaction time of synthesis of PUU from 60 min to 120 min resulted in decreasing quantity of NCO groups and EI of maltogenic α-amylase (Fig. 4a). Increasing reaction temperature from 70 °C to 110 °C of synthesis of PUU resulted in decreasing quantity of NCO groups and EI of maltogenic α-amylase from 98% to 45% (Fig. 4b).

The immobilized maltogenic α-amylase preparations were stored in the citrate buffer (0.1 M, pH = 5.0) at 4 °C. In these storage conditions, the residual activity of immobilized maltogenic α-amylase remained from 42% to 54% after 14 days and from 6% to 16% after 28 days when initial molar ratios of PVA : (HMDI : TDI) were from 1 : (0.25 : 0.25) to 1 : (0.40 : 0.60) (Fig. 5).

Immobilization of maltogenic α-amylase in pores of PUU microparticles by physical adsorption which is caused by hydrogen bonds and Van der Wall's forces predominates in these cases. High storage stability exhibited the immobilized maltogenic α-amylase onto PUU microparticles, which were obtained when initial molar ratios of PVA : (HMDI : TDI) were 1 : (0.50 : 0.50) and 1 : (0.75 : 0.25). The residual activity of immobilized maltogenic α-amylase after 28 days remained 72% and 95%, respectively. The generated multipoint covalent attachment between enzyme amino and hydroxyl groups and isocyanate groups of PUU microparticles prevails in these cases. In comparing carriers which were synthesized from PVA and mixture of HMDI and TDI with carriers which were synthesized from PVA and HMDI [21], it was found that EI of maltogenic α-amylase and stability of immobilized maltogenic α-amylase were similar. Immobilized maltogenic α-amylase onto microparticles, which were synthesized from PVA and HMDI, showed better stability in pH and temperature range than native maltogenic α-amylase [43]. Repeated batch starch hydrolysis by maltogenic α-amylase, immobilized onto PUU microparticles, which were obtained when the initial molar

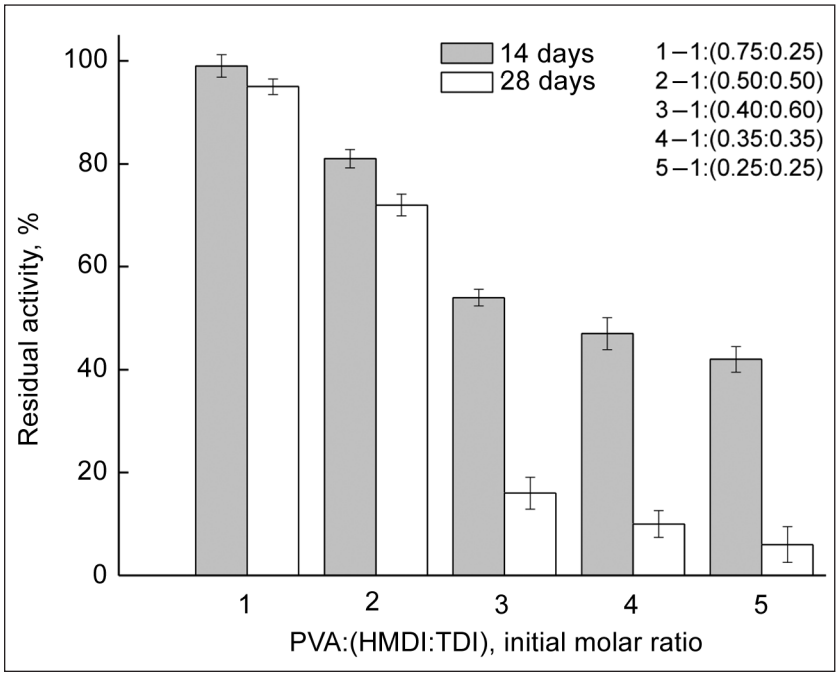


Fig. 5. Residual activity of immobilized maltogenic α-amylase onto PUU microparticles after 14 and 28 days of storage (in citrate buffer, pH = 5.0 at 4 °C) as a function of initial molar ratio of PVA : (HMDI : TDI) used for synthesis of PUU

ratio of PVA : (HMDI : TDI) was 1 : (0.75 : 0.25), allowed at least seven cycles without a sensible decrease of activity in starch saccharification. Immobilized maltogenic α-amylase remained in all initial catalytic activity after seven cycles. Dextrose equivalents were 21% and 19% when the native and immobilized maltogenic α-amylase was used. The similar results of repeated batch starch hydrolysis were obtained when maltogenic α-amylase was immobilized on microparticles which were synthesized from PVA and HMDI [21]. In all these cases maltogenic α amylase was immobilized onto PUU microparticles via reaction of amino and hydroxyl groups of the enzyme with active isocyanate groups of PUU. Any activation step of the carrier was applied in these cases. The obtained results of immobilization and stability of maltogenic α amylase were found to be comparable to the results obtained when CPC-silica particles were used as a carrier [44]. During immobilization of maltogenic α amylase onto CPC-silica particles, an additional activation step of carrier should be applied. Moreover, using a glutaraldehyde activated carrier, the enzyme was attached only via amino groups by formation of Schiff bases.

According to the received results, it could be proposed that maltogenic α-amylase immobilized onto PUU microparticles could be used for saccharification of starch for obtaining maltose syrups in food industry.

CONCLUSIONS

PUU microparticles were synthesized from PVA and the blend of diisocyanates of HMDI and TDI by one-step method in DMSO / water (99/1 vol.%) solution for the first time and were used for immobilization of maltogenic α-amylase. Structure of PUU microparticles has been proven by chemical analysis and FT-IR spectra. TGA analysis of PUU microparticles shows two-step decomposition. The range of PUU microparticles size distribution was from 10 μm to 30 μm. The highest yield of PUU microparticles (72.0%), quantity of NCO groups (9.1%) and high efficiency of immobilization of maltogenic α-amylase (96.0%) were obtained when the initial molar ratio of PVA : (HMDI : TDI) was 1 : (0.75 : 0.25). The residual activity of immobilized maltogenic α-amylase remained 95.0% after 28 days of storage at 4 °C in the citrate buffer in this case. Repeated batch starch hydrolysis by maltogenic α-amylase, immobilized onto PUU microparticles, allowed at least seven cycles without sensible decrease of activity in starch saccharification. Immobilized maltogenic α-amylase remained in all initial catalytic activity after seven cycles.

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References

1. P. G. Shukla, S. Sivaram, United States patent application 5814675, 1998.09.29.
2. P. G. Shukla, S. Sivaram, United States patent application 5859075, 1999.01.12.
3. L. S. Ramanathan, P. G. Shukla, S. Sivaram, *Pure Appl. Chem.*, **70**, 1295 (1998).
4. J. J. McGarr, United States patent application 4032516, 1977.06.28.
5. L. S. Ramanathan, S. Sivaram, United States patent application 6123988, 2000.09.26.
6. L. S. Ramanathan, D. Baskaran, P. G. Shukla, S. Siwaram, *Marcomol. Chem. Phys.*, **203**, 998 (2002).
7. J. Blahak, H. Hubner, J. Koster, H. J. Meiners, H. Thomas, United States patent application 4348536, 1982.09.07.
8. F. Tiarks, K. Landfester, M. Antinietti, *J. Polym. Sci., Part A: Polym. Chem.*, **39**, 2520 (2001).
9. E. Jabbari, M. Khakpour, *Biomaterials*, **21**, 2073 (2000).
10. M. Sadeghi, M. A. Semsarzadeh, M. Barikani, B. Ghalei, *J. Membr. Sci.*, **354**, 40 (2010).
11. S.-J. Choi, J.-H. Lee, Y.-H. Lee, D.-Y. Hwang, H.-D. Kim, *J. Appl. Polym. Sci.*, **121**, 3516 (2011).
12. A. K. Mishra, R. Narayan, K. V. S. N. Raju, T. M. Aminabhavi, *Prog. Org. Coat.*, **74**, 134 (2012).
13. S. Turri, M. Levi, T. Trombetta, *J. Appl. Polym. Sci.*, **93**, 136 (2004).
14. J.-K. Yun, H.-J. Yoo, H.-D. Kim, *J. Appl. Polym. Sci.*, **105**, 1168 (2007).
15. A. K. Mishra, D. K. Chattopadhyay, B. Sreedhar, K. V. S. N. Raju, *J. Appl. Polym. Sci.*, **102**, 3158 (2006).
16. Y. Shi, X. Zhan, Z. Luo, Q. Zhang, F. Chen, *J. Polym. Sci., Part A: Polym. Chem.*, **46**, 2433 (2008).
17. S. V. Caro JR., C. S. Paik Sung, E. W. Merrill, *J. Appl. Polym. Sci.*, **20**, 3241 (1976).
18. C. Galiatsatos, K. Hajizadeh, J. E. Mark, W. R. Heineman, *Biosensors*, **4**, 393 (1989).
19. M. Krumova, D. Lopez, R. Benavente, C. Mijangos, J. M. Perena, *Polymer*, **41**, 9265 (2000).
20. S. Budriene, T. Romaskevic, K. Pielichowski, J. Pielichowski, *Polym. Adv. Technol.*, **18**, 67 (2007).
21. T. Romaskevic, E. Viskantiene, S. Budriene, A. Ramanaviciene, G. Dienys, *J. Mol. Catal. B: Enzym.*, **64**, 172 (2010).
22. B. Krajewska, *Microb. Technol.*, **35**, 126 (2004).
23. C. Mateo, J. M. Palomo, G. Fernandez-Lorente, J. M. Guisan, R. F. Lafuente, *Enzyme Microb. Technol.*, **40**, 1451 (2007).
24. M. Bakker, F. van de Velde, F. van Rantwijk, et al., *Biotechnol. Bioeng.*, **70**, 342 (2000).
25. S. Budriene, N. Gorochovceva, T. Romaskevic, et al., *Centr. Eur. J. Chem.*, **3**, 95 (2005).
26. F. van de Velde, M. Bekker, F. van Rantwijk, R. A. Sheldon, *Biotechnol. Bioeng.*, **72**, 523 (2001).
27. P. G. Cadena, R. A. S. Jeronimo, J. M. Melo, R. A. Silva, J. L. Lima Filho, M. C. B. Pimentel, *Bioresour. Technol.*, **101**, 1595 (2010).
28. M. Guncheva, E. Tashev, D. Zhiryakova, T. Tosheva, N. Tzokova, *Process Biochem.*, **46**, 923 (2011).

29. A. Kakoti, A. K. Kumar, P. Goswami, *J. Mol. Catal. B: Enzym*., **78**, 98 (2012).
30. S. Phadtare, A. Kumar, V. P. Vinod, et al., *Chem. Mater.*, **15**, 1944 (2003).
31. S. Phadtare, S. Vyas, D. V. Palaskar, et al., *Biotechnol. Prog.*, **20**, 1840 (2004).
32. O. Gaouar, C. Aymard, N. Zakhi, G. M. Rios, *Starch / Staerke*, **49**, 231 (1997).
33. R. Makuska (ed.), *Synthesis and Characterization of Polymers* (In Lithuanian), University Press, Vilnius (2006).
34. M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).
35. D. Randall, *The Polyurethane Book*, Whiley, New York (2002).
36. S. Ajithkumar, S. S. Kansara, N. K. Patel, *Eur. Polym. J.*, **34**, 1273 (1998).
37. M. Lonescu, *Chemistry and Technology of Polyols for Polyurethanes*, Rapra Technology, Shropshire (2005).
38. D. M. Simons, R. G. Arnold, *Am. Chem. Soc.*, **78**, 1658 (1956).
39. S. Zhang, Z. Ren, S. He, Y. Zu, C. Zhu, *Spectrochim. Acta, Part A*, **66**, 188 (2007).
40. H. S. Mansur, C. M. Sadahira, A. N. Souza, A. P. Mansur, *Mater. Sci. Eng. C*, **28**, 539 (2008).
41. K. Lewandowska, *Thermochim. Acta*, **493**, 42 (2009).
42. F. M. B. Coutinho, M. C. Delpech, T. L. Alves, A. A. Ferreira, *Polym. Degrad. Stab.*, **81**, 19 (2003).
43. R. Romaskevic, Ph. D. Thesis, Vilnius University (2008).
44. G.-J. Kang, M.-J. Kim, J.-W. Kim, K. H. Park, *J. Agric. Food Chem*., **45**, 4168 (1997).

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POLI(URETANKARBAMIDO) MIKRODALELIŲ IŠ POLI(VINILALKOHOLIO) IR DIIZOCIANATŲ BINARINIŲ MIŠINIŲ SINTEZĖ, TYRIMAS IR PANAUDOJIMAS MALTOGENINEI α-AMILAZEI IMOBILIZUOTI

S a n t r a u k a

Susintetintos naujo tipo poli(uretankarbamido) (PUU) mikrodalelės naudojant įvairius pradinius poli(vinilalkoholio) (PVA) bei binarinio 1,6-heksametilendiizocianato (HMDI) ir 2,4-toluendiizocianato (TDI) mišinio molinius santykius DMSO/H_2O (99/1 tūrio %) tirpale. Ištirta PVA ir diizocianatų mišinio molinio santykio, reakcijos trukmės ir temperatūros įtaka PUU išeigai ir funkcinių grupių kiekiui. Didžiausia PUU išeiga (72,0 %) ir didžiausias izocianato grupių kiekis (9,3 %) gauti esant PVA : (HMDI : TDI) pradiniam moliniam santykiui 1 : (0,75 : 0,25). PUU sandara nustatyta iš cheminės analizės duomenų ir patvirtinta IR spektrais. Remiantis vienalaikės terminės analizės duomenimis, nustatytas terminis polimerų stabilumas. PUU destrukcija vyksta per 2 stadijas: pirmojoje stadijoje – uretano ir karbamido ryšių, o antrojoje – pagrindinės grandinės skilimas. PUU mikrodalelių dydis įvertintas optinės mikroskopijos metodu. Susintetintos dalelės panaudotos maltogenazei imobilizuoti. Ištirta PUU dalelių sintezės parametrų įtaka fermento imobilizavimo efektyvumui ir išeigai. Didžiausias maltogenazės imobilizavimo efektyvumas (96 %) gautas ant PUU mikrodalelių, susintetintų esant pradiniam PVA:(HMDI:TDI) moliniam santykiui 1 : (0,75 : 0,25). Imobilizuoti preparatai yra stabilūs: po 28 laikymo parų, esant 4 °C temperatūrai, liko 95 % pradinio fermento aktyvumo. Imobilizuota maltogenazė išlieka aktyvi po 7 bandymų ciklų periodinio tipo reaktoriuje.