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Immobilization of maltogenase onto polyurethane microparticles from poly(vinyl alcohol) and hexamethylene diisocyanate

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ABSTRACT

A series of porous polyurethane (PU) microparticles from poly(vinyl alcohol) (PVA) and hexamethylene diisocyanate (HMDI) using different ratios of components were obtained by one step method. Molar compositions of PU microparticles were estimated by determination of nitrogen, isocyanate and hydroxyl groups. PU carriers which were synthesized using optimal initial molar ratios of PVA and HMDI were applied for immobilization of maltogenase (MG) from *Bacillus stearothermophilus*. Immobilized enzyme exhibited higher catalytic activity and enhanced temperature stability in comparison with the native MG. Maximal loading 7.78 mg/g wet carrier was reached when PU microparticles with initial molar ratio of PVA and HMDI = 1:3 was used as a carrier for immobilization. The high efficiency of immobilization (EI) was obtained using PU microparticles when initial molar ratio of HMDI and PVA was 1:1–1:10. High stability of MG immobilized onto PU microparticles during storage was demonstrated. Immobilized starch hydrolyzing enzyme was successfully tested in batch and column type reactors for hydrolysis of potato starch. MG immobilized onto PU enables easy separation from the reaction medium and reuse of the immobilized preparation over seven reaction cycles in bath operation and at least three cycles in column type reactor.

1. Introduction

Starch hydrolysis is essential in many aspects of the application of starch [1]. It is carried out primarily by the use of enzyme or chemicals (acid or alkaline hydrolysis), or in combination (starch is treated under conditions of high pressure, shear and moisture). Starch hydrolysis is the cleaving of the starch polymer to short chain fragments such as dextrins and maltose, or to glucose monomers [2]. Depending on the composition of the mixture two widely used products, such as maltose and glucose syrup, are obtained [3]. These products are characterized by the dextrose equivalent (DE) value. The DE is a percentage measure of the extent of starch hydrolysis, which is expressed as the reducing power of the substance, i.e. glucose has a DE of 100%, maltose 50%, whereas starch at the other extreme has a DE of 0% [3,4]. DE value gives only a rough idea of reaction yield but no information on the hydrolysate profile. It means that at a same DE value may be associated very different products [5]. Chemical hydrolysis of starch has had widespread use in the past. It is now largely replaced by enzymatic processes, as it required the use of corrosion resistant materials, gave rise to high colour and saltash content (after neutralisation), needed more energy for heating and was relatively difficult to control [6]. Enzymatic hydrolysis of the starch is a two-step (named liquefaction and saccharification) process involving different catalysts. Usually, concentration of starch for enzymatic hydrolysis is varied 10–30% [7–9]. In the liquefaction step, suspended in aqueous medium starch is partially hydrolyzed using α-amylase. After that, saccharification of liquefied starch is proceeded using exo-amylases such as β-amylase, glucoamylase and fungal α-amylase. As a result of saccharification final low-molecular weight hydrolysates such as glucose or maltose are produced. One of the important products of starch hydrolysis is maltose syrups. Maltose syrups are characterized by their low viscosity, low hygroscopy, low sweetness and good heat stability. Thus they are essential as a regulator of moisture, inhibitors of crystallization and stabilizers. They may also be used as nutrient for children [5]. They have wide application in many areas such as beer, soft drinks, sweets and desserts productions. Mainly, three types of syrups containing maltose are produced: (1) high maltose syrups with DE 35–50%. Such syrups consist of 45–60% maltose, 2–7% glucose, 10–25% maltotriose; (2) super high maltose syrups, DE 45–60% (70–85% maltose, 1.5–2%

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glucose, 8–21% maltotriose); (3) high conversion syrups, DE 60–70% (30–37% maltose, 35–43% glucose, 10% maltotriose) [10].

β-Amylase which hydrolyzes starch specifically to β-maltose is the most commonly used exo-enzyme in maltose syrup production. However, this enzyme alone does not lead to high maltose concentration because it is not able to bypass 1,6-α-linkages thus adding debranching enzyme such as pullulanase is recommended [11]. Using Novo Nordisk isolated exo-enzyme, maltogenic α-amylase, named maltogenase (MG), for hydrolysis of starch leads to production of α-maltose [5,12]. MG is able to cleave maltotriose to maltose and glucose, moreover, compared to β-amylase, this enzyme may bypass 1,6-α-linkages [11]. The saccharification of starch to maltose and dextrin was carried out in Simulated Counter-Current Chromatographic Reactor-Separator system by using an eluent of dilute MG solution, starch conversions of up to 60% were achieved [13,14]. MG was used for saccharifying liquefied starch to maltose in a continuous rotating annular chromatograph. For soluble potato starch, maltose conversions of up to 79% were achieved at feed flow rates of up to 400 cm^3/h and 15–5% w/v concentrations [8]. The use of ultrafiltration membrane reactor with various cut-offs for hydrolysis of cassava starch (30% of solids) by pullulanase (Promozyme®) and MG was studied in [15]. Native MG withal fungal α-amylase (Fungamyl®) or Promozyme® is used for high maltose syrup production from partially hydrolyzed starch solution (DE 10–20%). A high starch concentration is processed, usually 30–40% solids. Hydrolysis is carried out in the tank at 333 K, pH 6 for 24–48 h or at 338 K, pH 5 for 18–42 h using MG withal fungal α-amylase and pullulanase, respectively. Obtained product is containing 50–55% of maltose [7].

However, enzymatic hydrolysis of starch has also disadvantages. Enzymes are expensive and in classical batch processes, due to denaturation, they must be replaced after each run [5]. Immobilization is a very effective alternative in overcoming problems of instability and repetitive use of enzymes. Only two immobilized enzymes such as glucoamylase and glucose isomerase are industrially used for hydrolysis of starch. However, only immobilized glucose isomerase is commercial important for production of high fructose syrups. Most of immobilized preparations use either the adsorption or the crosslinking technique [16].

PUs as a functional materials have a wide application in many areas. PUs are considered as a suitable carrier for enzyme immobilization. As immobilization carriers, PU-based materials are used in various forms: foam, microspheres and microparticles, powder, layer and coatings [17]. An enzyme can be attached to PU carrier covalently, by a physical adsorption, by an entrapment or by a coupled chemical and physical binding. A number of enzymes (e.g. phytase, endoglucanase, cellulase [18], β-galactosidase [19] and chloroperoxidase [20]) were immobilized onto PU, however they were all immobilized onto PU foams. PU microspheres (1–100 μm) synthesized from 4,4′-diphenylmethane diisocyanate and 1,4-butanediol or mixture of 1,4-butanediol and polyether glycol (M_r 1400) were applied for immobilization of maltogenase in our previous work [4]. PVA is a polymer with unique physical and chemical properties. Since its discovery in 1915 by Klatte it has found many applications and new areas are still being added. The polymer properties include reactivity of the numerical hydroxyl groups with other substances such as reactive resins, and aldehyde [21,22]. Only few reports were found where PVA was used instead of polyols in synthesis of PU [23]. Crosslinked PVA found promising application in the preparation of biomedical materials as films [24] and magnetic-field-sensitive gels [25]. Herein, synthesis of PU microparticles from PVA and hexamethylene diisocyanate (HMDI), considered as a suitable carrier for MG immobilization is presented. Suitability of immobilized enzyme for production of maltose syrups was studied. This study is part of the Program for Development of Industrial Biotechnology in Lithuania, which covered the area of 3.5. "Carrying out investigations of biocatalysts immobilization, development of technologies of production and applications of native and immobilized biocatalysts".

2. Materials and methods

2.1. Materials

The reagents poly(vinyl alcohol) (FLUKA), hexamethylene diisocyanate (ALDRICH), dimethylsulphoxide (DMSO) (ALDRICH), acetone, diethyl ether were used as received, except DMSO, which was distilled from CaH_2 before using.

PU microparticles were obtained from HMDI and PVA according to our previous work, only PVA was used instead of 1,4-butanediol [4].

2.2. Enzyme

MG (Novozymes, Denmark) from *Bacillus stearothermophilus* (glucan-1,4-α-maltohydrolase, E.C.3.2.1.133) recombinant exo-acting maltogenic amylase, removes maltose units (through 1,4-α-D-glucosidic linkages) from the non-reducing chain ends in maltooligosaccharides and polysaccharides such as amylose and amylopectin [26]. Commercial enzyme preparation was used as purchased without additional purification. Enzyme was diluted with citrate buffer (pH 5.0) before immobilization or use for saccharification reaction.

2.3. Preparation of liquefied starch solution

100 cm^3 of 5% potato starch suspension in 0.1 M citrate buffer (pH 5.0) was stirred with 0.5 cm^3 (250 U) of α-amylase from *Bacillus subtilis* solution for 10 min at 313 K and after that enzyme was inactivated by heating solution for 20 min in boiling water bath. DE of so prepared solution was 2–4%.

In the case of preparation of 20% liquefied starch solution, 100 cm^3 of 20% starch suspension was vigorously mixed with 1 cm^3 (2500 U) of α-amylase solution at room temperature and then the heating was started up to 348 K. Reaction was carried out at 348 K for 60 min, and after that enzyme was inactivated by heating solution for 20 min in boiling water bath. DE of prepared liquefied starch was 2.5%.

Quantity of α-amylase and incubation time could be varied for obtaining much or less liquefied starch solution.

DE was estimated from amount of reduced sugars in starch solution after liquefaction. DE was calculated as follows:

$$DE = \frac{RS}{TC} \cdot 100\%$$

where RS is reducing sugars, expressed as glucose (μmol) and TC is total carbohydrate, (μmol).

2.4. Enzyme assays

2.4.1. Determination of enzymatic activity of maltogenase and content of protein

The catalytic activity of immobilized MG was determined by reaction with a 5% liquefied potato starch solution and incubating the mixture at 313 K for 20 min. Activity of native and immobilized MG as well as protein content in native enzyme solution or left in solution after immobilization was assayed by Somogyi [27] and bicinchinonic acid method (BCA kit, Sigma, Germany). Activity unit of native or immobilized MG was defined

as the amount of enzyme which under standard conditions (at 313 K, pH 5.0) produced 1 μmol of reduced sugars per minute. Four separate measurements of the native and immobilized MG were performed to check the reproducibility of the data.

2.4.2. Determination of temperature and storage stability

The temperature stability of immobilized MG was checked by incubation it for 60 min in the temperature range of 313–343 K and compared with that of an identical amount of native enzyme in 0.1 M citrate buffer under similar conditions.

The storage stability of native or immobilized MG was assayed after 35 days storage in citrate buffer, pH 5.0 at 277 K.

2.4.3. Immobilization of maltogenase

Immobilization of MG was carried out in 0.1 M citrate buffer (pH 5.0). The mixture of 0.25 cm^3 (22.2 mg of protein) of the MG, 10 cm^3 of buffer and 6.4 g of PU carrier (immediately after synthesis) was stirred at 310 K for 30 min and then left at 277 K overnight. Next day the immobilized enzyme was thoroughly washed with buffer. Loading of immobilization was defined as a mg protein per grams of wet microparticles and depending on carrier was varied from 3.00 to 7.78 mg/g. EI was defined as the total units of enzyme activity obtained with the immobilized preparation divided by enzyme activity of the immobilized enzyme molecules when present in the free state that is an activity of immobilized MG in % from the activity of native enzyme used for immobilization. Yield of immobilization was defined as the protein quantity of immobilized enzyme in % from the quantity of protein of native enzyme used for immobilization.

Adsorption of MG onto PU microparticles was carried out at the same conditions, except carrier before adsorption was rinsed with distilled water in order to inactivate free isocyanate groups in PU.

2.5. Hydrolysis of starch in batch operation

Maltose syrups are usually produced using initial starch concentration in the range of 5–30% [9]. Liquefied starch solution with concentration of 5% was used for experiments due to low viscosity and better control of reaction. Higher concentration of liquefied starch (20%) was used for experiments on purpose to set process relevant to industrial and for comparison reason. Hydrolysis of liquefied 5% (DE 4.3%) or 20% (DE 2.5%) potato starch solution was carried out in flasks in the presence of MG immobilized onto PU microparticles under stirring for 180 min at 313 K or 343 K, respectively. 1 g (100 U) of immobilized MG was added to 100 cm^3 of 5% liquefied starch solution. In order to obtain comparable DE of starch hydrolysate after similar reaction time, higher amount (7 g, 700 U) of immobilized MG was used for hydrolysis of 20% of liquefied starch solution. MG immobilized onto PU microparticles with protein content of 7.78 mg protein per grams wet carrier was used. Excess amount of immobilized MG in the case with hydrolysis of concentrated starch was used due to higher viscosity of substrate solution and slower reaction rate.

2.6. Hydrolysis of starch in column type reactor

Hydrolysis of 5% liquefied potato starch solution was carried out at 313 K in column, which volume was 12.5 cm^3 (thickness of column was 0.8 cm, height was 25 cm). Starch solution was added by dropwise with different rates through column, filled with immobilized MG (total activity of enzyme in column was 100 U/cm^3).

Table 1
Molar composition of PU microparticles.

No.	[PVA]:[HMDI], initial molar ratio	Molar composition of PU (%)		
		I	II	III
1	1:1	81.0	1.6	17.4
2	1:3	55.1	14.0	40.9
3	1:5	26.4	20.8	52.8
4	1:7	25.5	21.6	52.9
5	1:10	21.4	35.4	43.2

3. Results and discussion

3.1. Immobilization of maltogenase onto PU microparticles

PU microparticles synthesized from PVA and HMDI consist of macromolecules with three types of monomeric units: non-reacted vinyl alcohol unit (I type), monomeric unit with one urethane group after reaction of vinyl alcohol monomeric unit with one isocyanate group of HMDI (units with one free isocyanate group, II type) and monomeric unit with two urethane groups after crosslinking reaction of two vinyl alcohol monomeric units with two isocyanate groups of HMDI (crosslinked units, III type). PU microparticles synthesized using different initial molar ratios of PVA and HMDI (Table 1) were applied as carriers for immobilization of MG from *B. stearothermophilus*. Liquefied potato starch solution (5%) was used for activity determination of native and immobilized onto PU enzyme.

MG is one of the most important enzymes in starch industry, which is used for saccharification of starch for obtaining of high-maltose syrups [5,12,26]. In the case of immobilization of enzymes onto PU microparticles mostly covalent bonds between amino and hydroxyl groups of the enzyme and isocyanate groups of PU may cause immobilization [19] differently from immobilization onto PU foams where without covalent binding entrapment into foam pores could cause immobilization. Reaction of free NCO groups with primary amines is much faster than reaction with OH groups and water [28]. Whereas immobilization procedure followed in aqueous media remained free NCO groups reacted with water by formation of CO_2 and they do not have any inactivation effect on enzyme latter [4]. Adsorption of MG onto PU carrier was negligible and was reached up to 10%. Moreover, leaching of MG adsorbed onto PU was observed after few days.

Hydrolysis of 10 cm^3 of 5% liquefied potato starch solution was carried out using 0.1 g of immobilized MG for 20 min at 313 K. For comparison, the catalytic activity of identical amount of native enzyme was determined under similar conditions. It was observed that specific activity, which is defined as an activity of enzyme per milligram of total protein, of immobilized enzyme is higher in some cases (Table 2). However, yield of immobilization in those cases was 92–95%. It is known that high retention of activity (more than 100%) can be achieved by change of enzyme conformation or orientation, as a result of disturbance of the forces involved in maintaining the

Table 2
Specific activity of immobilized and native MG.

[PVA]:[HMDI] initial molar ratio	Amount of NCO-groups in PU (%)	Immobilization efficiency (%)	Specific activity of enzyme (U/mg)
–	–	–	30.41[a]
1:1	0.8	58.7	19.37
1:3	1.8	133.2	43.96
1:5	4.6	152.9	50.46
1:7	4.8	152.1	50.20
1:10	7.7	159.5	52.54

[a] Native enzyme in solution.

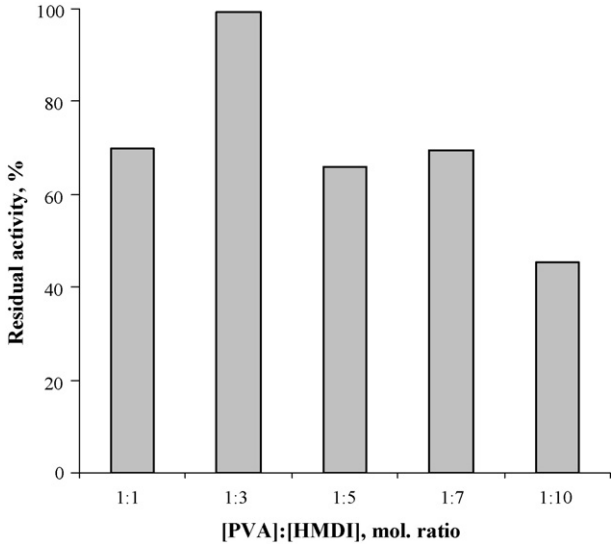

Fig. 1. Stability of immobilized MG onto PU microparticles after 35 days storage (in citrate buffer, pH 5.0, at 277 K) as a function of molar ratio of components used for synthesis of PU.

native structure [29]. It is supposed that some additives, which may be in commercial enzyme were eliminated by immobilization. An increase of enzyme activity after immobilization into PU foams was found previously with phytase [18] and glucose isomerase [30].

The EI over 100% was achieved, when PU microparticles with initial molar ratios of PVA and HMDI = 1:3–1:10 were used and it is in correlation with specific activity of immobilized MG. EI correlated with quantity of isocyanate groups in PU when [PVA]:[HMDI] was from 1:1 to 1:5 (Table 2). Further increasing of quantity of HMDI during synthesis of PU was not influenced on EI. It is supposed that all amine and hydroxyl groups of the enzyme, which could react with NCO groups, were expended.

Good mechanical properties of PU microparticles were observed. No volume change of immobilized onto PU MG was observed after storage in citrate buffer. Those properties provided potential for use of PU microparticles as a carrier for immobilization of enzyme for industrial processes.

Storage stability of immobilized MG was investigated after 35 days storage in citrate buffer, pH 5.0, at 277 K (Fig. 1). Four separate measurements were performed for each storage measurements to check the reproducibility of the data. Storage stability of native enzyme solution in citrate buffer (pH 5.0) was estimated at similar conditions. It was observed that about 90.6% of native MG was remained after 35 days storage. The highest residual activity of immobilized enzyme (99.4%) was noticed when PU microparticles with initial molar ratio of PVA:HMDI = 1:3 was used as a carrier. These PU microparticles were used for further immobilizations and investigation of starch hydrolysis.

The results in Table 3 show the influence of temperature on catalytic activity of native and immobilized enzyme determined at pH 5.0.

Four separate measurements were performed at each temperature to check the reproducibility of the data. It was determined that the native enzyme is less stable to increasing temperature of reaction medium than immobilized. The catalytic activity of immobilized MG was fallen only 4.5% after hydrolysis of 5% liquefied starch at 343 K. The maximal specific activities of immobilized as well as native MG were achieved at 333 K and they were 102.23 and 73.10 U/mg, respectively, when 5% liquefied starch solution was used for hydrolysis and 94.61 and 79.56 U/mg, respectively, in the case with 20% of substrate. It was found that optimal pH (data are not shown) and optimal temperature of immobilized enzyme was corresponded to obtain for native enzyme and was 5.0 and 333 K, respectively. Relative activity of immobilized enzyme was higher than obtained with native enzyme at similar conditions and it was correlated with immobilization efficiencies (Table 2). However, specific activity of immobilized MG was lower when hydrolysis of 20% starch was carried out at 313 and 323 K. It is supposed that reduced activity was obtained due to insufficient amount of immobilized MG used for hydrolysis of this amount of substrate. Leaching of enzyme during hydrolysis was negligible and reached less than 2%.

3.2. Hydrolysis of starch in batch operation

MG immobilized onto PU microparticles showed high stability and was tested for the hydrolysis of starch. It was found that liquefied starch (30%) hydrolysis was carried out using MG in the range of temperatures 333–338 K for 18–48 h [7]. Herein, most experiments of this work were performed in mild conditions at 313 K temperature and low concentrations of substrate in order to better control the process and understand regularities of hydrolysis. However, in industrial scale low concentration of substrate is undesirable due to large volume of reaction mixture and as a result evaporation of high amount of water in obtained syrup product is needed. In compliance with industrial processes higher concentration of substrate (20%) was used for investigation. Using higher temperature for hydrolysis of starch leads to reduce viscosity of substrate and protect it from microbial contamination. Whereas activity of immobilized MG at higher temperature was high, hydrolysis of 20% starch was performed at 343 K, despite the fact that maximal activity of native as well as immobilized MG was achieved at 333 K (Table 3).

The saccharification of starch by immobilized MG was studied as a function of reaction time (Fig. 2).

Two liquefied starch solutions—5% and 20%, were used for hydrolysis in the batch reactor at 313 and 343 K, respectively. 100 U of immobilized enzyme for hydrolysis of 100 cm^3 of 5% starch was used, while 700 U for 100 cm^3 of 20% starch was used. Higher amount of immobilized MG was used for hydrolysis of 20% of liquefied starch considering to higher viscosity of substrate and in order to reduce reaction time. After hydrolysis for 180 min of 5% and 20% starch at 313 and 343 K, DE was reached 22% and 25%, respectively. Repeated batch starch hydrolysis by MG, immobilized onto PU microparticles, allowed at least seven 180 min cycles without sensible decrease in starch saccharification. It was also noticed that hydrolysis of starch in the first cycle using immobilized preparation

Table 3
Relative activity of immobilized and native MG at different temperature of hydrolysis.

Temperature (K)	Hydrolysis of 5% starch		Hydrolysis of 20% of starch	
	Relative activity of immobilized MG (%)	Relative activity of native MG (%)	Relative activity of immobilized MG (%)	Relative activity of native MG (%)
313	43.0	41.6	35.5	88.4
323	71.0	61.7	94.1	99.4
333	100.0	100.0	100.0	100.0
343	95.5	71.6	96.1	60.9

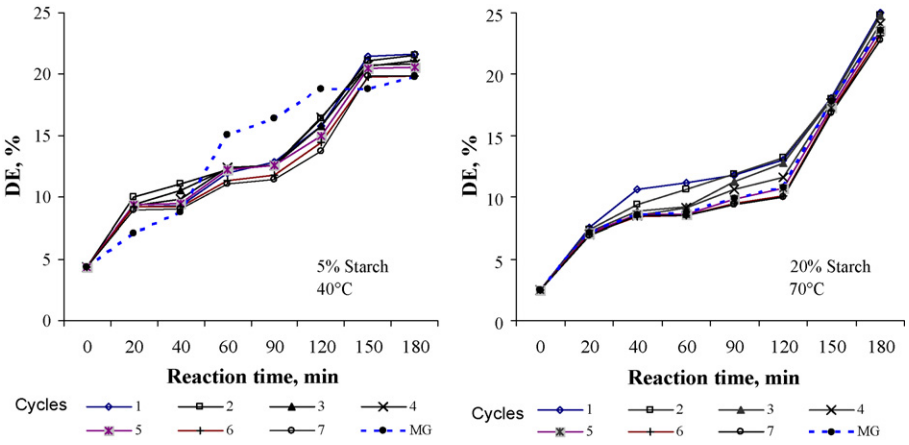

Fig. 2. Dextrose equivalent as a function of liquefied starch hydrolysis time in batch operation (pH 5.0).

Table 4
Dextrose equivalent of saccharified starch solution after hydrolysis in column type reactor by immobilized MG.

No.	Rate of dropping (cm^3/h)	Dextrose equivalent (%)
1	Initial substrate	13.2
2	1.5	40.9
3	5	39.2
4	30	34.5

was higher than using native enzyme (Fig. 2). As in previous cases, obtained results were correlated with immobilization efficiencies and specific activities of immobilized MG (Table 2).

3.3. Hydrolysis of starch in column type reactor

PU microparticles were used for starch hydrolysis in fluidized vertical column type reactor as well. Liquefied starch solution was injected into the column with different dropping rate for 5–6 h. After collection of eluate, column with immobilized enzyme was washed with citrate buffer (first cycle). Next day substrate was injected into column with higher dropping rate (second cycle). Third cycle was proceeded with highest dropping rate. It was estimated that DE of hydrolyzed starch did not depended on dropping rate of substrate into column (Table 2). As a result, amount of immobilized MG in column will be enough to hydrolyze higher amount of starch. Immobilized MG remained all initial catalytic activity after three cycles of starch hydrolysis and washing after each cycle (Table 4).

According to received results, it could be proposed that MG immobilized onto PU microparticles can be used for fabrication of continuous column type reactor for production of high-maltose syrups via saccharification of starch.

4. Conclusion

In this study, we have demonstrated the immobilization of MG onto PU microparticles based on PVA and HMDI. PU microparticles with different initial molar ratios of components were used as carriers for immobilization of MG. Maximal enzyme loading (7.78 mg/g) was reached when PU microparticles with initial molar ratio of PVA and HMDI = 1:3 was used as a carrier for immobilization. Catalytic activity of immobilized MG was slightly higher in some cases in comparison to native enzyme. The high EI was obtained using for immobilization PU microparticles, when excess of HMDI in initial molar ratios of PVA and HMDI was used. The highest stability of MG immobilized onto PU microparticles, which were obtained when initial molar ratio of HMDI and PVA was 1:3, during storage was demonstrated. Immobilized enzyme remained all initial catalytic activity after at least seven cycles of starch hydrolysis in batch or three cycles in column type operation.

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