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Performance of *Aspergillus niger* B 03 β-xylosidase immobilized on polyamide membrane support

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Abstract

The dynamics of β-xylosidase biosynthesis from *Aspergillus niger* B 03 was investigated in laboratory bioreactor. Maximum xylosidase activity 5.5 U/ml was achieved after 80 h fermentation at medium pH 4.0. The isolated β-xylosidase was immobilized on polyamide membrane support and the basic characteristics of the immobilized enzyme were determined. Maximum immobilization and activity yield obtained was 30.0 and 6.8%, respectively. A shift in temperature optimum and pH optimum was observed for immobilized β-xylosidase compared to the free enzyme. Immobilized enzyme exhibited maximum activity at 45 °C and pH 4.5 while its free counterpart at 70 °C and pH 3.5, respectively. Thermal stability at 40 and 50 °C and storage stability of immobilized β-xylosidase were investigated at pH 5.0. Kinetic parameters K_m, V_{max} and K_i were determined for both enzyme forms. Free and immobilized β-xylosidase were tested for xylose production from birchwood xylan. The substrate was preliminarily depolymerized with xylanase to xylooligosaccharides and the amount of xylose obtained after their hydrolysis with free and immobilized β-xylosidase was determined by HPLC analysis. Continuous enzyme hydrolysis of birchwood xylan was performed with xylanase and free or immobilized β-xylosidase. The maximum extent of hydrolysis was 25 and 30% with free and immobilized enzyme, respectively. Immobilized preparation was also examined for reusability in 20 consecutive cycles at 40 °C.

Keywords: β-Xylosidase; Biosynthesis; Immobilization; Xylan; *Aspergillus niger*

1. Introduction

Xylan is the major hemicellulose component in wood and is the second most abundant polysaccharide in nature, next to cellulose. Its backbone consists of β-(1,4) linked D-xylopyranose units substituted with acetyl, L-arabinofuranosyl, galactosyl and glucuronyl groups [1–3]. The complete hydrolysis of xylan requires several enzymes. Endo-β-1,4-xylanase or xylanase (EC 3.2.1.8) degrade polymer's chain into smaller xylooligosaccharides [4]. β-Xylosidase (EC 3.2.1.37) is the other key enzyme responsible for complete degradation of xylan since it hydrolyzes xylobiose and short chain xylooligosaccharides from the non-reducing end to xylose. Affinity usually decreases with increasing the degree of polymerization. Thus β-xylosidase is almost completely inactive against polymeric xylan [4–8]. β-Xylosidases are produced by different bacteria and fungi and may be extracellular or cell-bound [5]. Xylosidases from *Aspergillus* have been assigned to glycosidase family 3 and have a retaining mechanism. Their molecular mass range from 60 to 110 kDa, temperature optimum from 50 to 75 °C and pH optimum from 2.5 to 6.5 [4].

Because of the great abundance of xylan in nature it offers different possibilities to be utilized after its bioconversion into xylooligosaccharides and xylose. Xylan degradation has the potential to be an energy resource in future [1]. With the proper fermentative microorganisms xylose can be converted in fuel ethanol, xylitol, butanediol, etc. Xylitol finds wide application as a natural food sweetener, a dental caries reducer and a sugar substitute for diabetics [9].

The use of β-xylosidase for xylose production is constantly increasing [9,10]. The enzyme has found application also for synthesis of alkyl-glycosides which can be used as non-ionic surfactants [11] and for synthesis of specific oligosaccharides from xylose [12].

The use of enzymes for industrial purposes has several disadvantages: high cost required for their isolation and difficult

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recovery of the active enzyme from the end product [10]. To overcome these limitations immobilized enzymes can be used. Immobilization is achieved by fixing the enzyme to or within a water-insoluble support, as a result of which a heterogeneous system is obtained. The immobilized enzyme allows multiple reuse, easy separation from the product, continuous operation of the enzymatic process and rapid termination of the reaction. The reusability of enzymes is one of the major advantages of immobilization since it provides cost reduction which is a factor of economical importance [13–17].

In literature there are very few reports on immobilization of β-xylosidase on different supports [10,11,18–20]. Morana et al. [10] used entrapment into sodium alginate for immobilization of thermostable β-xylosidase to obtain a reusable biocatalyst. The immobilized enzyme was used for hydrolysis of xylooligosaccharides prepared from birchwood and oat-spelt xylan. In other work fungal β-xylosidase was immobilized on different supports as Duolite, Amberlite, Celite, DEAE-sepharose and polyacrylamide gel [11]. Immobilized enzyme was used for alkyl-β-xyloside synthesis by trans-glycosylation reaction. Abdel-Naby reported for immobilization of *A. niger* β-xylosidase via different methods—physical adsorption on tannin–chitosan, ionic bindinig onto Dowex, covalently on chitosan and entrapment in polyacrylamide [18]. Operational stability of immobilized enzyme was evaluated in packed-bead column-type reactors. In other work Duenas and Estrada [19] immobilized fungal β-xylosidase covalently on nylon powder and investigated the operating conditions for reaching highest substrate conversion in reactor loaded with immobilized enzyme.

The aim of the recent work was to investigate *A. niger* B 03 β-xylosidase biosynthesis in laboratory bioreactor and immobilize the isolated enzyme on polyamide membrane support. Immobilized β-xylosidase was characterized in terms of efficiency of immobilization, kinetic parameters, temperature and pH optimum, thermal and storage stability and reusability. It was applied for xylose production from birchwood xylan hydrolyzate.

2. Materials and methods

2.1. Chemicals

A. niger strain B 03 was a gift from Biovet JSC (Peshtera, Bulgaria), birchwood xylan and *p*-nitrophenyl-β-D-xylopyranoside (*p*NPXP) were from Sigma, polyamide membrane (PAM) with pore size 0.22 μm was purchased from Ekofilter Ltd. (Bourgas, Bulgaria) and xylose from Fluka. Xylanase from *A. niger* B 03 was produced and purified according to procedures described by Dobrev [21]. All other chemicals used were of analytical grade.

2.2. Methods

2.2.1. β-Xylosidase biosynthesis

The production of β-xylosidase by strain *A. niger* B 03 in submerged fermentation was examined. The strain was grown on optimized nutrient medium containing corn cobs, wheat bran, malt sprout, $(NH_4)_2HPO_4$ and urea [22]. Cultivation was performed at 28 °C for 95 h in 15 l laboratory bioreactor Aplikon. The enzyme was purified from the culture filtrate by ultrafiltration with membrane 10 kDa (Millipore) followed by gel chromatography with Sephadex G 100 (Pharmacia) [23,24]. The column (Ø26 mm × 700 mm) was eluted with 0.05 M NaCl. The specific activity of purified β-xylosidase was 3.4 U/mg.

2.2.2. Immobilization of enzyme

Purified β-xylosidase was immobilized via physical adsorption by the following procedure: 20 cm^2 membrane was immersed in 5 ml enzyme solution containing from 0.5 to 8 U. The mixture was mild stirred for 16 h at room temperature. After that the membrane was separated from the solution and washed with distilled water, 0.5 M NaCl and C-P buffer, pH 5.0 until no protein is detected in the washings by measuring the absorbance at 280 nm.

When investigating the effect of immobilization time on the activity of immobilized β-xylosidase the process was performed for different times-from 2 to 24 h. In all other experiments immobilization lasted 16 h.

The enzyme activity yield after immobilization was expressed as:

Immobilization yield (%) = $[(A - B)/A] \times 100$ and activity yield (%) = $[C/A] \times 100$, where A is the total units added for immobilization, B is the unbound units, $(A - B)$ represents theoretical immobilized units and C the actual immobilized units.

2.2.3. Enzyme assays

Xylanase activity was determined by the method of Miller [25] with dinitrosalicylic acid (DNSA) using 1% solution of birchwood xylan as substrate.

One unit xylanase activity was defined as the amount of enzyme required to liberate 1 μmol reducing sugar (measured as xylose) per minute at 50 °C, pH 5.0. The activity of xylanase was 43 U/ml.

β-Xylosidase activity was determined as described by Ponpium et al. [26]. The reaction mixture containing 1 ml 1 mM *p*NPXP in C-P buffer, pH 5.0 and 0.1 ml properly diluted enzyme was incubated at 50 °C for 15 min. Reaction was stopped by adding 2 ml 0.4 M Na_2CO_3 and the amount of free *p*-nitrophenol was measured at 405 nm.

One β-xylosidase unit was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute under the conditions described above.

The activity of immobilized enzyme was determined by adding 1 cm^2 membrane with immobilized enzyme to 1 ml of 1 mM *p*NPXP and 0.1 ml buffer. The reaction was carried out under the same conditions described above for the free enzyme.

2.2.4. Effect of pH and temperature on the activity and stability of β-xylosidase

Activity of free and immobilized β-xylosidase was assayed at different temperatures (30–75 °C) and pH (2.0–7.0) by the enzyme assay described above. The effect of temperature on the stability was determined at 40 and 50 °C. Free and immobilized enzyme preparations were incubated in C-P buffer, pH 5.0 at the desired temperature. Aliquots were withdrawn at regular inter-

vals and the remaining activity was determined at the standard assay conditions.

Storage stability of immobilized β-xylosidase was determined at 4 °C in C-P buffer, pH 5.0. The residual activity of immobilized preparation was determined every 2 weeks at the standard assay conditions.

2.2.5. Determination of kinetic parameters

Kinetic parameters K_m, V_{max} and K_i of free and immobilized β-xylosidase were determined by measuring the rate of *p*NPXP hydrolysis at various concentrations ranging from 0.18 to 0.91 mM. K_m and V_{max} were calculated from Lineweaver–Burk plot. K_i was determined using different concentration of the inhibitor xylose ranging from 3.03 to 13.64 mM. The inhibition constant K_i was calculated by Sigma Plot software program.

2.2.6. High performance liquid chromatography (HPLC)

The identification of xylose obtained after enzyme hydrolysis of birchwood xylan with xylanase and β-xylosidase was performed by HPLC system Waters, equipped with differential refractometer R401. Separation was achieved by high performance carbohydrate analysis column, Waters. The mobile phase was composed of CH_3CN:H_2O in ratio 87:13. The flow rate was 0.6 ml/min. Xylose was used as a reference standard.

Reaction mixture containing 10 ml 1% birchwood xylan and 35 U xylanase was incubated 4 h at 40 °C. After that 0.44 U free or immobilized β-xylosidase were added and hydrolysis left to proceed for 2 h.

2.2.7. Hydrolysis of xylan from birchwood by xylanase and β-xylosidase

Enzyme hydrolysis of birchwood xylan was carried out with xylanase and free or immobilized β-xylosidase at 40 °C. The temperature was chosen considering the higher thermal stability of xylanase and β-xylosidase at 40 °C with regard to the experiment continuance. The reaction mixture contained 5 ml 1% birchwood xylan in C-P buffer pH 5.0 and 5.5 U purified xylanase from *A. niger* B 03. To the same preparation 1 U of free or immobilized β-xylosidase were added. Aliquots were withdrawn at regular intervals for 6 h. The amount of reducing sugar produced (measured as xylose) was determined by the method of Miller with DNSA [25]. The extent of xylan hydrolysis was calculated as follows:

$$\text{xylan hydrolysis } (\%) = \frac{\text{xylose produced (mg)} \times 0.9}{\text{initial xylan (mg)}} \times 100$$

the coefficient 0.9 estimates water elimination from xylose molecules for the formation of glycoside bonds in xylan.

2.2.8. Reusability of immobilized preparation

The reusability of immobilized β-xylosidase was examined at 40 °C for 20 consecutive cycles. Each cycle lasted 30 min. The substrate used was 1 mM *p*NPXP in C-P buffer with pH 5.0. After each cycle of hydrolysis the immobilized preparation was removed, washed with distilled water and added to fresh portion of substrate.

3. Results

3.1. β-Xylosidase production

The dynamics of growth of strain *A. niger* B 03 and β-xylosidase biosynthesis in submerged cultivation in 15 l stirred and aerated laboratory bioreactor were studied (Fig. 1).

The period within 15–35 h is the log phase of the strain growth and it was characterized with sharp increase of biomass production and outlet CO_2. β-Xylosidase biosynthesis started after 35 h of fermentation and according to biomass and CO_2 production increased biosynthesis was observed at the end of log phase and continued during the stationary phase of strain growth. Considerable increase in β-xylosidase activity was observed after 55 h.

After 20 h of fermentation a tendency of decrease in culture medium pH was observed. The most significant decrease in pH was at 30 and 35 h and it was 5.2 and 3.6, respectively. After 60 h of fermentation pH slowly increased from 3.1 to 4.0 at the end of the process.

Significant increase in β-xylosidase activity was observed at medium pH within 3.0–3.5. Maximum enzyme activity 5.5 U/ml was achieved after 80 h of fermentation at medium pH 4.0.

The amount of reducing sugar in the culture medium was determined during the cultivation process. The initial concentration of reducing sugar in the nutrient medium was 20 g/l and during the fermentation it decreased gradually. Slow increase of β-xylosidase activity and medium pH started after the complete

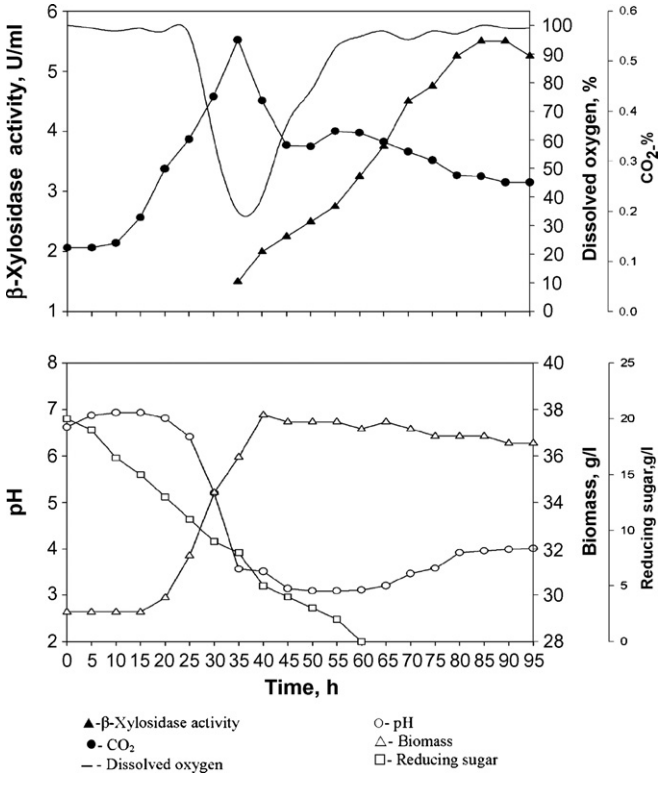

Fig. 1. β-Xylosidase biosynthesis at submerged cultivation of *Aspergillus niger* B 03 in 15 l bioreactor.

Table 1
Immobilization of β-xylosidase on polyamide membrane support

Added units (*A*)	Unbound units (*B*)	Active immobilized units (*C*)	Immobilization yield (%) $[(A-B)/A] \times 100$	Activity yield (%) $[C/A] \times 100$	η^*
0.5	0.4	0.02	20.0	4.0	0.20
1	0.8	0.04	20.0	4.1	0.20
2	1.5	0.10	25.0	5.0	0.20
4	2.8	0.27	30.0	6.8	0.23
8	6.3	0.31	21.3	3.9	0.18

η^* (effectiveness factor) = (active immobilized units (*C*)/theoretical immobilized units $(A-B)$).

run out of reducing sugar, which was probably due to the use of the obtained organic acids as carbon source.

3.2. Immobilization of β-xylosidase on PAM

Different amounts of enzyme were added to 20 cm^2 membrane. Immobilization and activity yields obtained and the efficiency of immobilization are shown in Table 1. Maximum activity yield obtained was 6.8% which represents the actively bound enzyme. In all cases immobilization yield is higher than activity yield. This fact is usually explained with difficult accessibility of the substrate to the immobilized enzyme due to steric restrictions or unfavorable orientation of the immobilized enzyme molecules. The result of these effects is blocked interaction between the substrate and the active site of the immobilized enzyme [27].

Activity yield below 10% is reported also by Gargouri et al. for the immobilization of fungal β-xylosidase when using different resins (Amberlite and Duolite) as supports [11].

The effect of duration of immobilization is shown in Fig. 2. Immobilized xylosidase activity was affected significantly by immobilization time. The activity of immobilized enzyme increased gradually with time and maximum activity was achieved after 16 h lasting immobilization. The longer contact between the enzyme and the support allowed more effective adsorption of β-xylosidase which resulted in higher amount of actively immobilized enzyme.

3.3. Effect of pH and temperature on the activity and stability of immobilized β-xylosidase

The properties of immobilized β-xylosidase were compared with those of the free form. The effect of temperature on the enzyme activity is shown in Fig. 3. The temperature optimum shifted from 70 °C for the free form to 45 °C for the immobilized enzyme. Such significant change of temperature optimum after immobilization of β-xylosidase has not been reported by other authors. Compared with the free enzyme immobilized xylosidase exhibited higher activity in the interval 30–50 °C and lower activity at temperature above 55 °C. Probably upon immobilization conformational changes in the enzyme molecules occurred that favored xylosidase activity at temperatures below 50 °C. On the other hand, this changes caused decreasing of the immobilized enzyme catalytic activity in the interval 55–70 °C compared to the native enzyme.

Abdel-Naby [18] reported a shift of the optimal temperature for immobilized *A. niger* β-xylosidase on different supports from 45 to 50–60 °C while Morana et al. [10] did not observe any

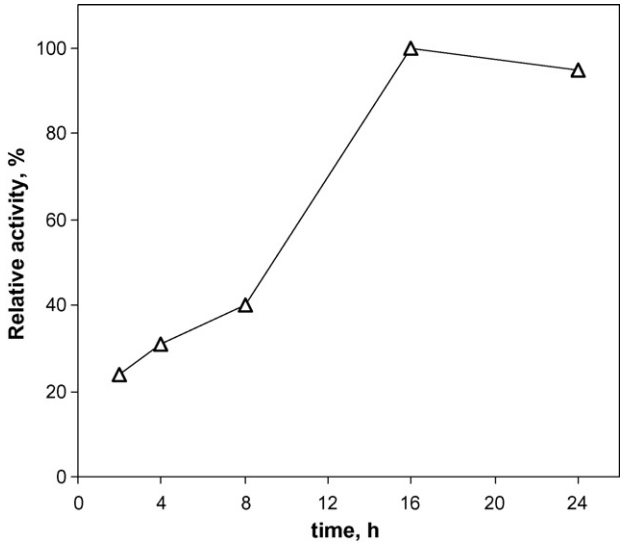

Fig. 2. Effect of immobilization time on the activity of immobilized β-xylosidase. The enzyme activity was measured at 50 °C with 1 mM *p*NPXP at pH 5.0.

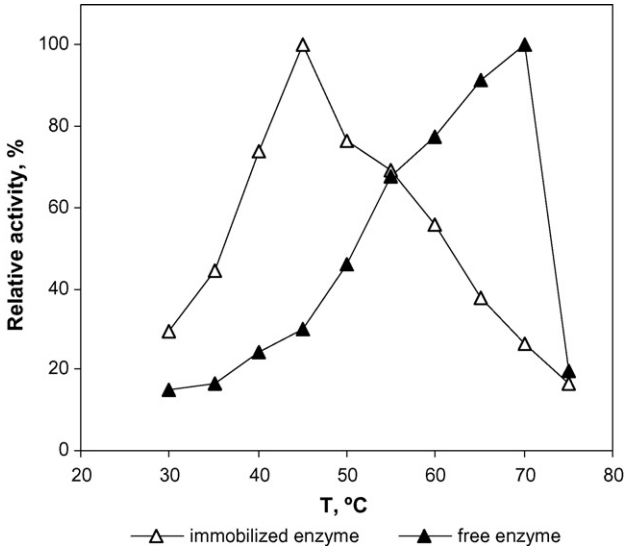

Fig. 3. Effect of temperature on the activity of free and immobilized β-xylosidase. Enzyme activity was measured at different temperatures with 1 mM *p*NPXP at pH 5.0. Experiments were run in duplicate and the difference was less than 5%.

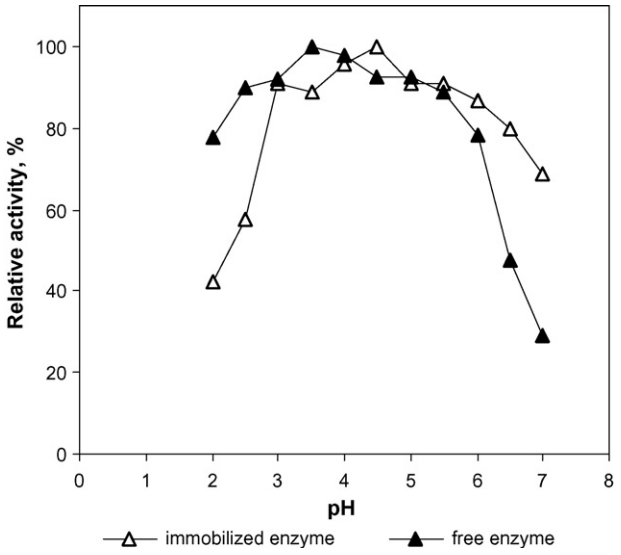

Fig. 4. Effect of pH on the activity of free and immobilized β-xylosidase. Enzyme activity was measured at 50 °C with 1 mM *p*NPXP buffered at different pH. Experiments were run in duplicate and the difference was less than 5%.

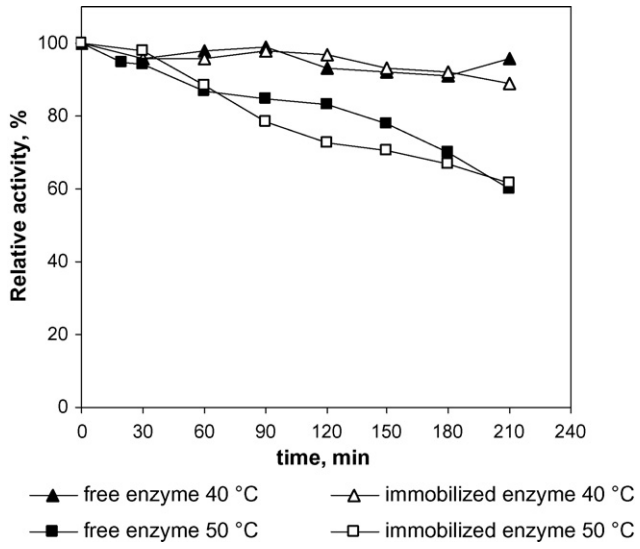

Fig. 5. Thermal stability of free and immobilized β-xylosidase at 40 and 50 °C, pH 5.0. Aliquots were withdrawn every 30 min and the residual activity was measured at 50 °C with 1 mM *p*NPXP at pH 5.0.

change in temperature optimum of thermostable β-xylosidase when being immobilized in alginate.

As a result of immobilization a change in pH optimum and pH-activity curve was also observed. The enzyme pH optimum shifted from 3.5 for free xylosidase to 4.5 for the immobilized enzyme (Fig. 4). In the interval pH 3.0–6.0 the pH profile for both enzyme forms is very similar. Immobilized xylosidase exhibited higher activity compared to the free enzyme at pH above 6.0 and lower activity at pH below 3.0. A possible explanation of the results obtained is that immobilization may cause conformational changes in the enzyme molecules resulting in changes of protein's charge. Usually the shift of pH optimum to the alkaline side is explained with increase of negative charge. As a result of that the concentration of H^+ in the microenvironment of the immobilized enzyme increases which means that the pH of the immobilized enzyme region is more acidic than that of the external solution. Since the effect of pH on the enzyme activity is expressed based on the pH of the external solution, which in this case increases, the pH optimum shifts to the alkaline side [13].

Thermal stability of free and immobilized enzymes was investigated at 40 and 50 °C, pH 5.0 (Fig. 5). For 210 min both preparations showed similar stability at 40 °C retaining about 90% from their initial activity. At 50 °C immobilized and free β-xylosidase retained about 70% activity after 3 h incubation. No increase in thermal stability was observed after immobilization of xylosidase on polyamide membrane. Improved thermal stability of immobilized β-xylosidase from *Sulfolobus solfataricus* was reported by Morana et al. [10] when using entrapment into alginate. Probably the membrane used as support in the present study did not protect the enzyme against heat. Considering the method of immobilization which is physical adsorption another possible explanation for the thermal stability of immobilized xylosidase is the easier desorption of the enzyme from the support surface with increasing of temperature.

3.4. Storage stability

Storage stability is an important parameter for immobilized enzymes. The immobilized xylosidase preparation was very stable at 4 °C, pH 5.0 retaining 100% of its initial activity for 12 weeks of storage. After 18 weeks only about 20% loss of activity was observed. Morana et al. [10] reported 16% loss of the initial activity of β-xylosidase immobilized in alginate after 4 months storage at 4 °C.

3.5. Kinetic parameters

Kinetic parameters of immobilized β-xylosidase K_m, V_{max} and inhibition constant K_i were determined and compared to the free enzyme (Table 2). *p*NPXP was used as substrate. No change of maximum reaction velocity of immobilized enzyme was observed. Little change occurred in Michaelis constant K_m which was a result of decreased affinity between the immobilized enzyme and the substrate.

Xylose acts as a competitive inhibitor of β-xylosidase. The inhibition process was examined using different concentrations of xylose as described in Materials and methods section. The immobilized enzyme had higher K_i than the free form. This means that xylose inhibited immobilized enzyme in lower extent than the native enzyme. Probably this fact is due to steric

Table 2
Kinetic parameters of free and immobilized β-xylosidase

	K_m (μmol/ml)	V_{max} (μmol/h)	K_i (mM)
Free enzyme	0.35	0.33	1.857
Immobilized enzyme	0.41	0.34	4.935

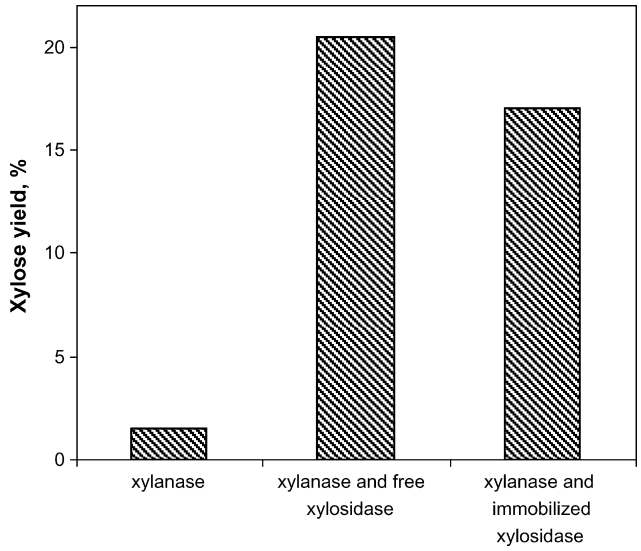

Fig. 6. Xylose yield from initial xylan—(mg xylose/mg initial xylan) × 100. The amount of xylose produced was determined by HPLC analysis. The hydrolysis of 1% birchwood xylan was performed at 40 °C with 35 U xylanase and 0.44 U free or immobilized β-xylosidase.

restrictions for the interaction between the inhibitor and the immobilized enzyme.

3.6. HPLC analysis of xylose

Xylose obtained after enzyme hydrolysis of birchwood xylan with xylanase and β-xylosidase was determined by HPLC analysis. Since β-xylosidase hydrolyzes only short chain xylooligosaccharides to xylose and is inactive against polymeric xylan [4,5] the substrate was preliminarily incubated 4 h with purified xylanase and then 2 h with free or immobilized β-xylosidase at 40 °C. The amount of xylose produced as a result of these experiments was determined (Fig. 6). Free β-xylosidase produced about 20% more xylose (2.1 mg/ml) from xylan hydrolyzate than immobilized enzyme (1.7 mg/ml). This was probably due to restricted access of the substrate to the support bound enzyme which resulted in lower extent of hydrolysis and lower amount of xylose obtained.

3.7. Hydrolysis of xylan from birchwood by xylanase and β-xylosidase

In order to examine the cooperative action of xylanase and β-xylosidase in degradation of xylan, continuous enzyme hydrolysis of 1% birchwood xylan solution at 40 °C was performed (Fig. 7). The extent of hydrolysis was estimated by measuring the reducing sugar amount and calculated as described in Section 2. Maximum extent of hydrolysis achieved with xylanase was 16%. In the case of using both xylanase and β-xylosidase the process proceeded further. The final xylan hydrolysis increased to 25% for free xylosidase and to 30% for immobilized enzyme. Immobilized β-xylosidase has higher inhibition constant ($K_i = 4.935$) than free enzyme ($K_i = 1.857$)

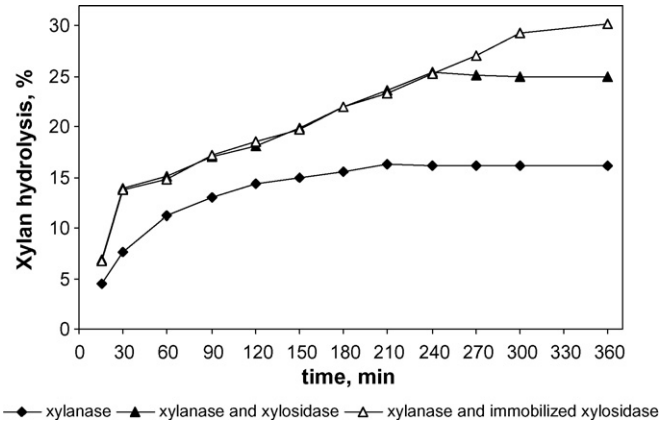

Fig. 7. Enzyme hydrolysis of birchwood xylan. The extent of hydrolysis was calculated by measuring the amount of reducing sugar at regular intervals. The experiment was performed at 40 °C with 5.5 U xylanase and 1 U free or immobilized β-xylosidase.

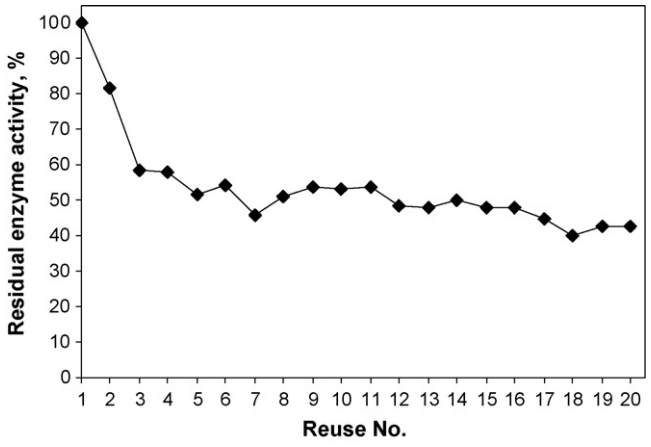

Fig. 8. Reusability of immobilized β-xylosidase. The immobilized enzyme was incubated at 40 °C with 1 mM *p*NPXP. After each cycle (30 min) the membrane with immobilized β-xylosidase was removed, washed with distilled water and added to fresh portion of substrate for a new cycle.

which can be a possible explanation for the increased extent of xylan hydrolysis.

3.8. Reusability of the immobilized enzyme

One of the most significant benefits of immobilized enzyme preparation is their multiple reuse. Immobilized β-xylosidase was reused repeatedly in 20 consecutive batch cycles at 40 °C, pH 5.0. Each cycle lasted 30 min (Fig. 8). The immobilized preparation showed very good operational stability retaining about 50% of its total immobilized activity after being reused 16 times using 1 mM *p*NPXP as substrate.

4. Conclusions

β-Xylosidase is one of the enzymes responsible for the complete hydrolysis of xylan to xylose. Microorganisms able to degrade xylan synthesize xylanolytic enzymes mainly endo-xylanase and β-xylosidase that degrade polymer's chain

synergistically [23]. In the present study we investigated β-xylosidase biosynthesis in submerged fermentation of *A. niger* B 03. This strain was cultivated on nutrient medium rich in xylan, containing agricultural wastes such as corncobs and wheat bran. According to the results obtained for dynamics of strain growth the enzyme biosynthesis started at the end of log phase and increased considerably during the stationary phase. Maximum β-xylosidase activity 5.5 U/ml was achieved after 80 h of fermentation.

Considering the advantages of immobilized enzymes especially the possibility for recovery and reuse of the enzyme we decided to immobilize β-xylosidase after its isolation and purification. Polyamide membrane was chosen as water-insoluble support with high thermal and pH stability and porous surface suitable for the enzyme attachment. The enzyme was immobilized via physical adsorption under very mild conditions that did not affect the enzyme activity after immobilization. The highest activity yield obtained for immobilized β-xylosidase was 6.8%. It exhibited very good thermal and storage stability and retained high catalytic activity after its multiple reuse in 20 cycles.

Immobilized and free β-xylosidase were tested for hydrolysis of xylooligosaccharides to xylose. Satisfactory results were obtained showing that the immobilized enzyme can be used as an effective biocatalyst for xylose production.

Acknowledgement

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