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Preparation and Characterization of Aspergillus niger B 03 Endo-Xylanase Immobilized on the Smart Polymers Eudragit L-100 and S-100.

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ABSTRACT

An endo-xylanase produced by Aspergillusniger B 03 was immobilized on the smart polymers Eudragit L-100 and S-100. Solubility of the two types of Eudragit was determined by changing pH from 2 to 7 and measuring the % T at 600 nm. Maximum immobilization and activity yield was 67.6 % and 44.7 % for Eudragit L-100 and 60.5 % and 59.7 % for Eudragit S-100, respectively. The degree of immobilized xylanase was studied using different concentration of the polymer. Maximum immobilization yield achieved with 2 % polymer concentration was 44.7 % for Eudragit L-100 and 59.7% for Eudragit S-100. The basic biochemical characteristics of immobilized xylanase were investigated. Immobilized enzyme exhibited maximum activity at pH 4.0–4.5 and at 50–55 ºC. Thermal stability at 40 ºC and 50 ºC was also determined. Continuous enzymatic degradation of oat spelt and birchwoodxylan was performed and the extent of polysaccharide hydrolysis was analyzed. Immobilized enzyme preparation was also examined for reusability in 7 consecutive cycles at 40 ºC. These results indicatethatfungal xylanaseimmobilized onEudragit L-100 and S-100is a promising and efficient biocatalyst for xylooligosaccharides (XOS) production and could be also used as a feed additive in animal feeding.

Keywords: Endo-xylanase; Aspergillusniger; Immobilization; Eudragit L-100 and S-100; Xylooligosaccharides

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INTRODUCTION

Xylan is the second most abundant polysaccharide in nature, next to cellulose involved in the composition of secondary plant cell wall. Xylan is the major component of hemicellulose accounting for 5-50 % of the dry weight of plant materials [1-3]. Most of xylan types are branched polysaccharides which backbone consists of β-D-xylose units linked with β-1,4-glycosidic bonds [4].

Extensive degradation of xylans to monosaccharides is achieved by the cooperative action of multi-enzymes including: endo-β-1, 4-xylanase (EC 3.2.1.8), usually called xylanase and β-xylosidase (EC 3.2.1.37) [5].Xylanase degrades polymer's chain into smaller XOS, while β-xylosidasecatalyzes the hydrolysis ofxylobiose and short chain xylooligosaccharides from the non-reducing end to xylose.Xylanolytic enzymes are produced by numerous fungal, bacterial and yeast strains [2-4].

Xylanases have been the focus of research interest because of their industrial potential in many fields. Xylanases are used commercially in the pulp and paper, food and animal feed industries. Another application of endo-xylanases is the production of low molecular weight xylo-oligosaccharides that are prebiotics and stimulate intestinal Bifidobacteria growth [2-11].

Immobilized enzymes are widely used since immobilization allows multiple reuses, easy separation from the product, and 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 [12, 13].

The aim of the present paper is to study the conditions for immobilization of Aspergillusniger B 03 endo-xylanaseon the smart polymers Eudragit L-100 and S-100 and to determine the basic characteristics of the immobilized preparation in terms of efficiency of immobilization, pH andtemperature optimum, thermal stability and reusability.

MATERIALS AND METHODS

Chemicals

The strainAspergillusniger B 03 was a gift from Biovet JSC (Peshtera, Bulgaria), Eudragit L-100 and S-100were a gift from DegussaAG (Düsseldorf, Germany).Oat spelt and birchwoodxylan were purchased from Sigma and 3,5-dinitrosalicylic acid (DNSA) was purchased from Merck. Xylanase from Aspergillusniger B 03 was produced and purified according to procedures described by Dobrev et al.[14]. All other chemicals used were of analytical grade.

Enzyme assay

Xylanase activity was determined by the method of Miller [15] with dinitrosalicylic acid (DNSA) using 1% solution of oat speltxylan as substrate.One unit xylanase activity was defined as the amount of enzyme required to liberate 1 µmol reducing sugars (measured as xylose) per minute at 50 °C, pH 5.0. The activity of xylanasewas 43 U/mL.The quantitative determination of protein was made by the method of Lowry [16].

Immobilization of endo-xylanase

4 mL 2 % Eudragit L-100 and S-100 were mixed with 1 mL xylanase solution (1 mg/mL). The reaction mixture was incubated 2 h at room temperature and the polymer was precipitated by decrease of pH with 0.1 M citric acid solution to pH 4.0 for Eudragit L-100 and to pH 4.5 for Eudragit S-100. The test tubes were centrifuged at 4000 rpm for 30 min and the precipitate containing the immobilized enzyme was washed with citrate-phosphate buffer, pH 4.0. The same procedure was repeated and after removal of the buffer solution, immobilized xylanase was stored after addition of citrate-phosphate buffer to final volume 5 mL.

The enzyme activity yield after immobilization was expressed as follows:Immobilization yield, % = [(A-B)/A]×100 and Activity yield, % = [C/A] ×100, where A are the total units added for immobilization, B are the unbound units, (A-B) represents theoretical immobilized units, and C the actual immobilized units.

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Additional stabilization of immobilized xylanase with glutaraldehyde (GA)

In order to increase thethermal and operational stability of the immobilized enzyme preparation, 1mL immobilized xylanase solution was incubated with 1 mL 0.5 % solution of glutaraldehydein citrate-phosphate buffer, pH 4.0 at25 °C for 30 min. Reaction mixture was washed with buffer and the test tubes were centrifuged at 4000 rpm for 15 min.

Enzyme hydrolysis of oat spelt and birchwoodxylan with immobilized xylanase

Enzyme hydrolysis of oatspelt and birchwoodxylan was carried out at 40 ˚C. The temperature was chosen considering the higher thermal stability of xylanase at 40 ˚C with regard to the experiment continuance. The reaction mixture contained 10 mL 1 % xylan solution and 12 U of free and immobilized xylanase. Aliquots were withdrawn at every 60 min and the concentration of reducing sugars (expressed as xylose) was measured by the method of Miller [15]. The extent of xylan hydrolysis was calculated as follows:

$$\text{Xylan hydrolysis, \%} = \frac{\text{mg xylose produced x } 0.9}{\text{mg initial xylan}} \text{ x } 100$$

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

Reusability of the immobilized xylanase

Reusability of immobilized xylanase was examined in 7 consecutive cycles at 40 ºC and each cycle lasted 30 min.Xylanase activity was analysed using 1 % solution of oat spelt xylan as substrate. After each cycle immobilized xylanase was separated by decrease of pH with 0.1 M citric acid solution to pH 4.0 for Eudragit L-100 and to pH 4.5 for Eudragit S-100. The test tubes were centrifuged at 4000 rpm for 30 min and the precipitate containing the immobilized enzyme was added to a new portion of substrate.

RESULTS AND DISCUSSION

Eudragit L-100 and EudragitS-100 are anionic copolymers based on methacrylic acid and methyl methacrylate. Eudragit L-100 is a methacrylic acid - methyl methacrylate copolymer (1:1). The polymer is completely soluble at pH 4.5-5.0 and insoluble at pH below 4.0. EudragitS-100 is a methacrylic acid - methyl methacrylate copolymer (1:2) and dissolves at pH above 5.5.

The immobilization of Aspergillus niger B 03 endo-xylanase on the two types of Eudragit was studied and the extent of immobilized enzyme was determined (Table 1 and 2). Maximum immobilization and activity yield was 67.6 % and 44.7 % for Eudragit L-100 and 60.5 % and 59.7 % for Eudragit S-100, respectively (Table 1).

Table 1: Immobilizationof xylanase on Eudragit L-100 and S-100

Eudragit	Added units (A)	Unbound units (B)	Active immobilized units (C)	Immobilization yield, % [(A-B)/A]x100	Activity yield, % [C/A]x100
L-100	30	14.6	11.6	51.3	38.7
	60	30.8	26.8	48.7	44.7
	300	176.4	80.5	41.2	26.8
	600	194.6	168	67.6	28.0
	1200	454	353	62.2	29.4
S-100	30	17	11.6	43.3	38.7
	60	23.7	35.8	60.5	59.7
	300	180.3	113.4	39.9	37.8
	600	420.4	143.7	29.9	24.0
	1200	600	304.6	50.0	25.4

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The degree of xylanase immobilization was investigated using different concentration of the polymer from 0.1 to 2.0 %. We found that the highest activity yield was achieved with 2 % Eudragit and it was 44.7 % for L-100 and 59.7 % for S-100, respectively (Table 2).

Table 2: Effect of Eudragit concentration on xylanase immobilization

Eudragit concentration, %	Added units	Active immobilized units	Immobilized enzyme,%	Immobilized protein, %
L-100				
0.1	60	2.4	4.1	3.6
0.2	60	3.9	6.5	10.7
0.4	60	4.4	7.3	17.9
0.8	60	4.6	7.7	21.0
1.0	60	5.2	8.7	25.0
1.5	60	26.4	44.0	44.6
2.0	60	26.8	44.7	50.4
S-100				
0.1	60	1.6	2.7	15.7
0.2	60	3.5	5.8	28.2
0.4	60	15.6	26.0	36.4
0.8	60	29.6	49.3	35.7
1.0	60	29.4	49.0	42.9
1.5	60	28.5	47.5	80.4
2.0	60	35.8	59.7	88.6

The biochemical properties of the native and immobilized xylanase were compared. We observed a change in the pH-activity curve as a result of enzyme immobilization (Fig. 1). Both free and immobilized xylanase exhibited optimal activity in the pH interval 4.0-4.5. Other authors report optimum pH values of free and immobilized xylanaseon Eudragit L-100 at pH 5.8[10].

Figure 2 shows the temperature dependance of the activity of native and immobilized xylanase. The effect of temperaturewas studied in citrate phosphate buffer, pH 5.0. No shift of the temperature optimum was observed after immobilization. The two forms of xylanase had optimum temperature interval at 50-60 ºC. In other studies the authors report an increase in temperature optimum of the immobilized xylanase compared to the free enzyme[10,17].

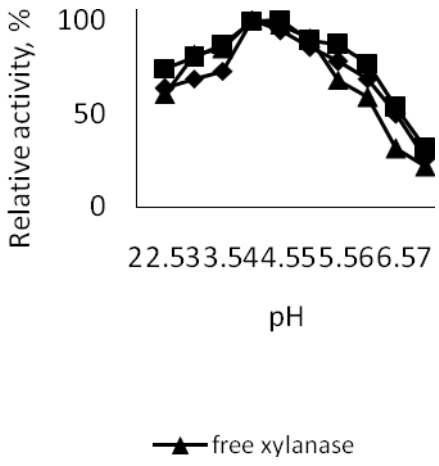

Fig 1: Effect of pH on the activity of free and immobilized xylanase

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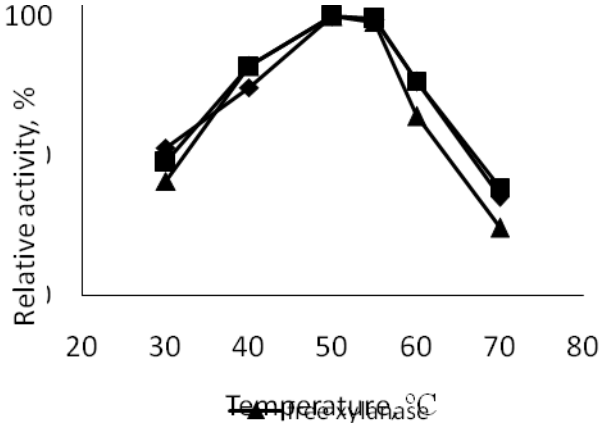

Fig 2: Effect of temperature on the activity of free and immobilized xylanase

Figure 3 shows the thermal stability of the immobilized xylanase at 40°C and 50 °C, pH 4.0. At 40°C both free and immobilized enzyme retained 90 % of their initial activity after 4 hours incubation. The enzyme preparation pretreated with GA retained about 30 % of its original activity after 2 hours. At 50°Cfree xylanase retained 55 % of its activity after 2 hours incubation whereas theenzyme immobilized on Eudragit L-100 preserved about 30 % of its initial activity. The activity of the enzyme pretreated with GA declined to 10 % after one hour at the same conditions.

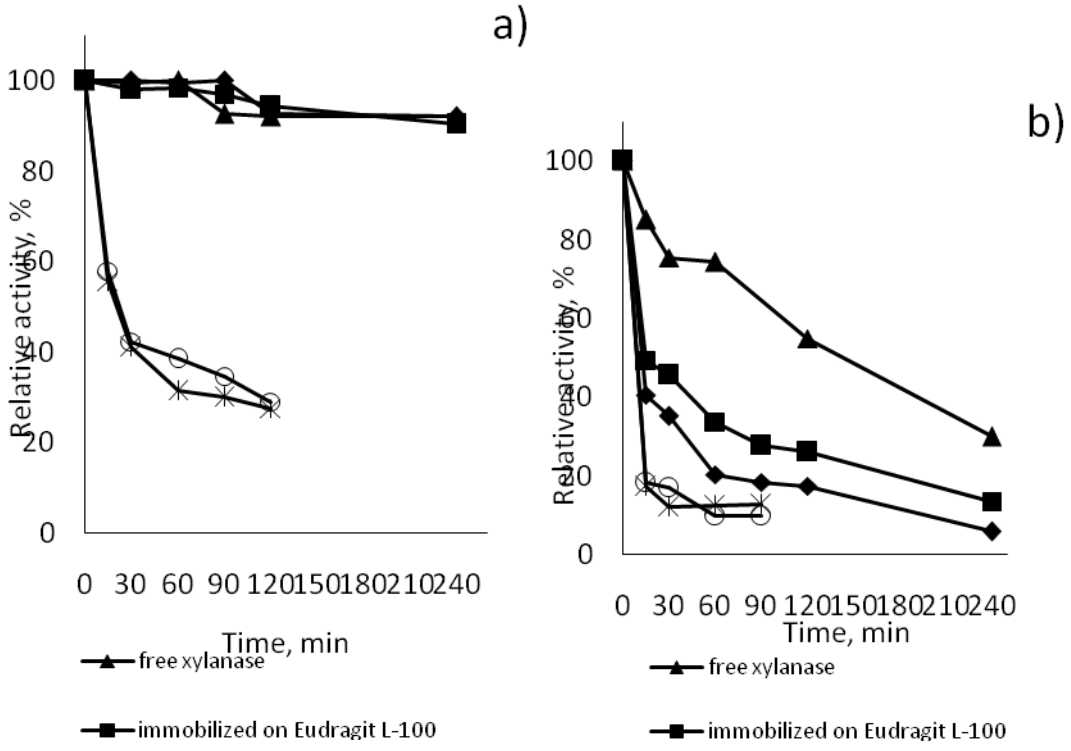

Fig3: Thermal stability of free and immobilized xylanase at 40 °C(a) and 50 °C (b), pH 4.0

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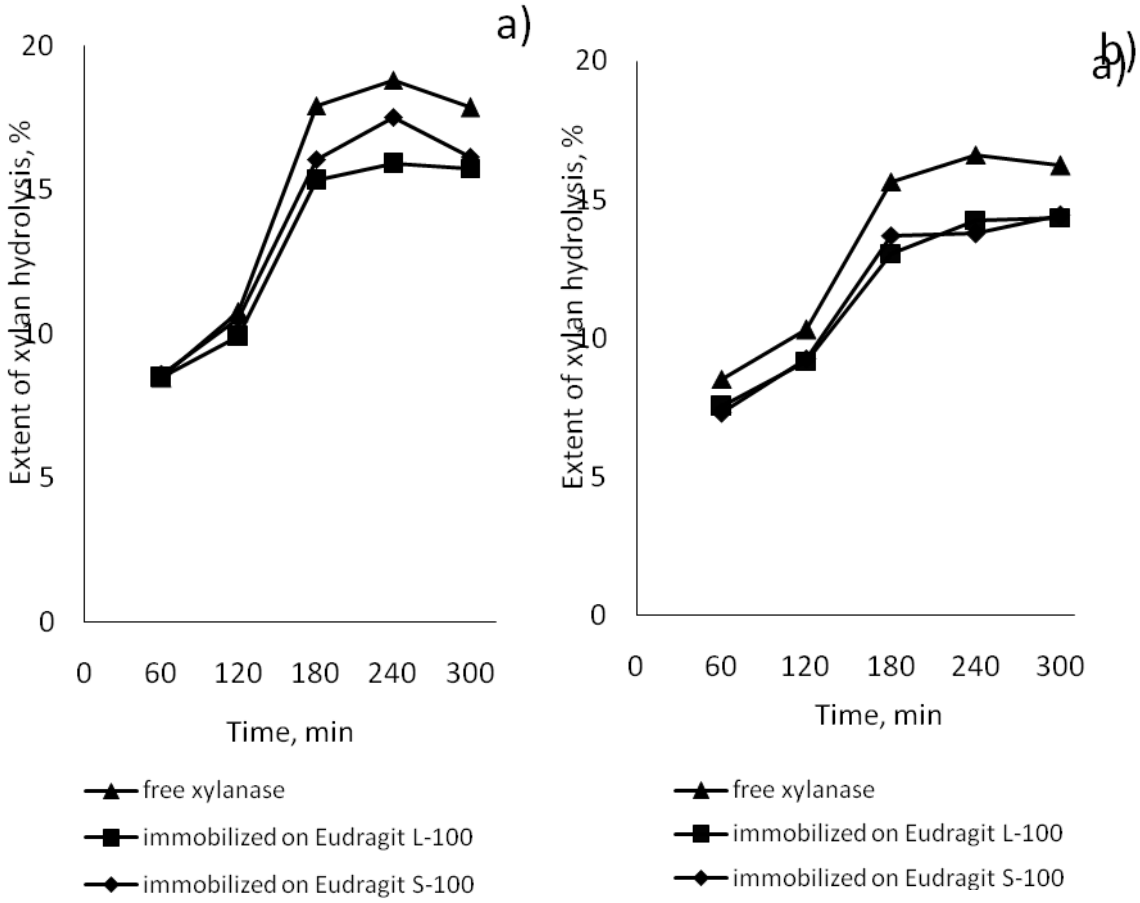

Fig 4: Enzyme hydrolysis of oat spelt (a) and birchwood (b)xylan with free and immobilized xylanase

Figure 4 shows the extent of oat spelt and birch wood xylan fragmentation after continuous enzymatic hydrolysis with immobilized xylanase calculated as described in Methods and Materials section. Maximum extent of oat spelt xylan hydrolysis was achieved after 4 hours with xylanase immobilized on Eudragit S-100 and it was 17.5 %. With the Eudragit L-100 preparation the extent of polysaccharide hydrolysis was 15.9 %. When birchwoodxylan was used as a substrate, the extent of xylan hydrolysis measured after 5 hours using the immobilized enzyme preparation was 14.4%.

The reusability of enzymes is one of the major advantages of immobilization since it provides cost reduction which is a factor of economical importance [18].Figure 5 shows the operational stability of xylanase immobilized onEudragit L-100 and S-100.Reusability of immobilized xylanase was examined in 7 consecutive cycles at 40 ºC.Xylanase immobilized on Eudragit S-100 was more stable after multiple reuse compared to Eudragit L-100 preparation. The immobilized enzyme was repetitively used,retaining about 12 % of its initial activity.

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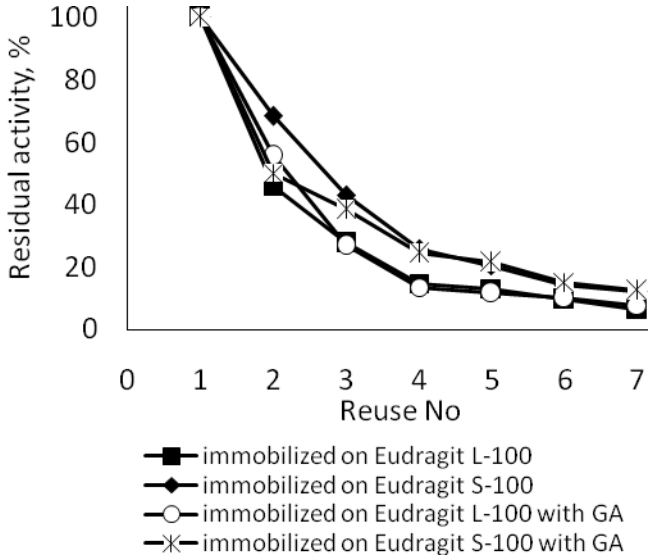

Fig 5: Reusability of immobilized xylanase at 40 °C

Roy et al. [19] report that xylanase immobilized on Eudragit L-100 was reused in 10 cycles at 60 ºC whereas in other studies fungal xylanase immobilized on Eudragit S-100 was reused in 4 cycles at 45 ºC [20] or in 3 cycles at 50 ºC and pH 6.0 [21]. Bacterial Streptomyces rameusxylanase immobilized on the same polymer retained over 5 cycles of multiple reuse more than 88 % of its activity [22].

CONCLUSION

Our results indicate that Aspergillusniger B 03endo-xylanase, immobilized on the reversible soluble polymers Eudragit L-100 and S-100is a promising and efficient biocatalyst for xylooligosaccharides (XOS) production. This enzyme preparation could also find other industrial application and could be used as a feed additive in animal feeding for the treatment of digestivedisorders caused bynonstarch polysaccharides in feed.

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