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Immobilization of Xylanase from *Bacillus pumilus* Strain MK001 and its Application in Production of Xylo-oligosaccharides

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Abstract Xylanase from *Bacillus pumilus* strain MK001 was immobilized on different matrices following varied immobilization methods. Entrapment using gelatin (GE) (40.0%), physical adsorption on chitin (CH) (35.0%), ionic binding with Q-sepharose (Q-S) (45.0%), and covalent binding with HP-20 beads (42.0%) showed the maximum xylanase immobilization efficiency. The optimum pH of immobilized xylanase shifted up to 1.0 unit (pH 7.0) as compared to free enzyme (pH 6.0). The immobilized xylanase exhibited higher pH stability (up to 28.0%) in the alkaline pH range (7.0–10.0) as compared to free enzyme. Optimum temperature of immobilized xylanase was observed to be 8 °C higher (68.0 °C) than free enzyme (60.0 °C). The free xylanase retained 50.0% activity, whereas xylanase immobilized on HP-20, Q-S, CH, and GE retained 68.0, 64.0, 58.0, and 57.0% residual activity, respectively, after 3 h of incubation at 80.0 °C. The immobilized xylanase registered marginal increase and decrease in K_m and V_{max} values, respectively, as compared to free enzyme. The immobilized xylanase retained up to 70.0% of its initial hydrolysis activity after seven enzyme reaction cycles. The immobilized xylanase was found to produce higher levels of high-quality xylo-oligosaccharides from birchwood xylan, indicating its potential in the nutraceutical industry.

Keywords *Bacillus pumilus* · Immobilization · Xylanase · Xylo-oligosaccharides

Introduction

Xylan is a major component of the hemicellulosic fraction in the plant cell walls. Xylanase (EC. 3.2.1.8 1,4-β-D-xylanase, xylanohydrolase) hydrolyzes β-1,4-glycosidic linkages present in xylan and, hence, is a crucial enzyme component of microbial xylanolytic system [1]. Xylanases are an important group of carbohydrases, with a worldwide market of approximately 200 million dollars, and are being routinely used in various industrial

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processes such as animal feed digestion, waste treatment, energy generation, production of chemicals, and paper manufacture [2–6]. One of the exciting applications of xylanases is the production of xylo-oligosaccharides [7]. Xylo-oligosaccharides have stimulatory effects on the selective growth of human intestinal *Bifdiobacteria* and are frequently defined as probiotics. They also show a remarkable potential for utilization in pharmaceuticals, in feed formulations, in agricultural applications, and as food additives [8].

In industrial applications, the free forms of enzymes pose difficulties, e.g., instability of enzyme structure and sensitivity under harsh process conditions, nonrecovery of the active form of enzyme from the reaction mixture for reuse, and contamination of the final product [9]. The strategies used for improving the stability of proteins include the use of additives, introduction of disulfide bonds [10], site-specific mutagenesis [11], and chemical modification or cross-linking [12]. However, in all these strategies, the yield and reusability of free enzymes as industrial catalysts is quite limited. Therefore, increased attention has been paid to immobilization techniques which offer advantages over free enzymes in choice of batch or continuous processes, rapid termination of reactions, controlled product formation, ease of enzyme recovery from the reaction mixture, and adaptability to various engineering designs [13]. The extent of stabilization depends on the enzyme structure, the immobilization method, and the type of support [9].

During the last decade, numerous supports for xylanase immobilization have been investigated [14–19]. *Bacillus pumilus* strain MK001 is a known hyper producer of xylanase under solid state fermentation (SSF) conditions [20]. The present investigation describes immobilization of *B. pumilus* strain MK001 xylanase and its application in the production of xylo-oligosaccharides.

Materials and Methods

Chemicals and Statistical Analysis

Birchwood xylan, chitin (CH), and chitosan were purchased from Sigma (St. Louis, MO, USA). Acrylamide and bis-acrylamide were purchased from BDH Chemicals (Dorset, England), and diethylamino ethyl (DEAE)-sepharose, Q-sepharose (Q-S), and CM-sepharose were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Silica, alginate, amberlite IR-120, and amberlite IR-440 were obtained from Himedia (Mumbai, India). All other media components and chemicals used were of the highest purity grade available commercially. Wheat bran was obtained locally. All the experiments were repeated at least four times and the results were reproducible. The data points represented the mean values within ±10.0% of the individual values.

Microbial Strain

An alkalophilic and cellulase negligible *B. pumilus* strain MK001 (Genbank accession no. AY389345) was isolated by enrichment culture technique from sanitary landfill at Bawana, New Delhi, India. The culture was cultivated and maintained by weekly transfers on Horikoshi-xylan agar slants, and xylanase production was carried out as described previously [20].

Preparation of Xylanase Sample

Five hundred milliliters of xylanase harvested after SSF of wheat bran [20] was concentrated by ultrafiltration (using a 50-kDa-cut-off molecular sieve) with a pellicon ultra filtration system at room temperature [flow rate of 1.5 l/h, pressure 0.8 bar (Millipore, Bedford, MA, USA)]. The ultrafilterate was desalted by five cycles of ultrafiltration. Finally, the ultrafiltered proteins were dissolved in 50.0 ml of 100.0 mM citrate–phosphate buffer (pH 6.0) and lyophilized. The xylanase activity and protein content of the lyophilized sample were determined.

Analytical Procedures

The xylanase activity was determined by measuring the release of reducing sugars from birchwood xylan (1.0% w/v) by dinitrosalicylic acid method [21]. Unless otherwise specified, the assay mixture consisted of 490.0 μl of substrate in citrate–phosphate buffer (100.0 mM; pH 6.0) and 10.0 μl of enzyme solution or weighed amount of the immobilized enzyme. The reaction mixture was incubated at 60 °C for 10.0 min. One unit of xylanase was defined as the amount of enzyme required to release 1 μmol of xylose from birchwood xylan in 1 min under the standard assay conditions [20]. The protein content was determined following the Lowry method [22]. The protein content of the immobilized enzyme was calculated by subtracting the amount of unbound protein from the protein originally added. Total sugars were estimated according to phenol-sulfuric acid method [23].

Immobilization Methods

Entrapment

Polyacrylamide Entrapment The gel was prepared as described elsewhere [24]. The polymerization mixture contained 10.0 ml of xylanase solution [30.0 mg (1,500.0 IU) lyophilized xylanase dissolved in 10.0 ml of 100.0 mM citrate–phosphate buffer pH 6.0] and 20 ml of 12.0% (w/v) acrylamide. The amount of cross-linking compound (*N*,*N*-methylene-bis acrylamide) added was at the level of 0.8% (w/w) of total monomer content. The catalyst system consisted of 0.1 ml of *N*,*N*,*N*,*N*-tetramethylenediamine and 0.1 g of ammonium persulfate dissolved in 0.2 ml of distilled water. The resultant mixture was stirred for 15 min and transferred to the gel-casting glass cassette for polymerization at 20 °C. The polymerized gel was washed with buffer until no activity or soluble protein was detected and cut into fragments (mean diameter 2.0 mm).

Ca-alginate Entrapment The alginate beads used as immobilization support were prepared by mixing sodium alginate (0.8% w/v) with 30.0 ml of xylanase solution [30.0 mg (1,500.0 IU) lyophilized enzyme dissolved in 30.0 ml of 100.0 mM citrate–phosphate buffer pH 6.0] and dropping the resultant mixture through a peristaltic pump into 100.0 ml of a 200.0-mM $CaCl_2$ solution with continuous stirring. The beads (mean diameter 3.3–3.5 mm) were washed with 100.0 mM citrate–phosphate buffer pH 6.0 until no enzyme activity or soluble protein was detected. The enzyme activity obtained in buffer after washing the beads was taken as unbound enzyme. Beads were stored at 4 °C in a 30.0-mM $CaCl_2$ solution prior to use [15].

Gelatin Entrapment Gelatin (GE) (1.0% w/v) [dissolved in 10.0 ml of distilled water at 50 °C, cooled (40 °C)] was mixed with 30.0 ml of xylanase solution [30.0 mg (1,500.0 IU) lyophilized xylanase dissolved in 30.0 ml of 100.0 mM citrate–phosphate buffer pH 6.0] under constant stirring. After 30 min, solidified GE was cut into small pieces (mean diameter 2.0 mm) and washed with the same buffer until no enzyme activity or soluble protein was detected. The enzyme activity obtained in buffer after washing GE was taken as unbound enzyme.

Physical Adsorption

Five hundred milligrams of support (CH, silica, alumina, and chitosan) was incubated with 10.0 ml of xylanase solution [10.0 mg (500.0 IU) lyophilized xylanase dissolved in 10.0 ml of 100.0 mM citrate–phosphate buffer pH 6.0] at 4 °C overnight. The support was then washed with 100.0 mM citrate–phosphate buffer pH 6.0 until no activity or soluble protein was detected. The enzyme activity obtained in buffer after washing the support was taken as unbound enzyme.

Ionic Binding

A series of ion exchangers (DEAE-sepharose, Q-S, CM-sepharose, Amberlite IR-120, and Amberlite IR-440) was used for immobilization of xylanase by ionic binding. Ion exchanger (500.0 mg) was equilibrated with 100.0 mM citrate–phosphate buffer (pH 6.0) or Tris–HCl buffer (100.0 mM, pH 8.0) and incubated with 10.0 ml of xylanase solution [10.0 mg (500.0 IU) lyophilized xylanase dissolved in 10.0 ml of 100.0 mM citrate–phosphate buffer pH 6.0 or 100.0 mM Tris–HCl buffer pH 8.0] at 4 °C overnight. The unbound enzyme was removed by washing with the same buffer until no activity or soluble protein was detected. The enzyme activity obtained in buffer after washing the support was taken as unbound enzyme.

Covalent Binding

Chitin and HP-20 (500.0 mg each) were shaken with 10.0 ml of 2.5% (v/v) glutaraldehyde (GA) for 1 h and thereafter washed three times with distilled water to remove excess GA. The washed matrices were mixed with 10.0 ml of xylanase solution [10.0 mg (500.0 IU) lyophilized xylanase dissolved in 10.0 ml of 100.0 mM citrate–phosphate buffer pH 6.0] for 2 h at 30 °C. The unbound enzyme was removed by washing with the same buffer until no activity or soluble protein was detected [25]. The enzyme activity obtained in buffer after washing the support was taken as unbound enzyme.

Physiochemical Properties of the Free and Immobilized Xylanase

Effect of pH The effect of pH on free and immobilized xylanase was studied in buffers of different pH (4.0–10.0). The pH stability of free and immobilized xylanase was determined by incubating the enzyme in 100.0 mM buffers at different pH values (4.0–10.0) for 10 h at room temperature. Buffers used were citrate–phosphate, pH 4.0–7.2; Tris–HCl, pH 7.2–9.2; carbonate–bicarbonate, pH 8.5–10.0; and glycine-NaOH, pH 9.0–10.0. The residual activity was assayed under the standard assay conditions.

Effect of Temperature The optimum temperature of free and immobilized xylanase was determined by carrying out enzyme reactions at different temperatures (40–80 °C) at pH 6.0 (free enzyme) and up to pH 7.0 (immobilized xylanase). Thermal stability studies were carried out by incubating free and immobilized enzyme samples with citrate–phosphate buffer (100 mM) (pH 6.0 for free enzyme and up to pH 7.0 for immobilized enzyme) at varied temperatures (50–70 °C) for 3 h followed by xylanase assay at respective optimal temperature.

Kinetics of Free and Immobilized Xylanase K_m and V_{max} for both free and immobilized enzymes were calculated from the Lineweaver–Burk equation under reaction conditions given earlier at different substrate concentrations between 0.1 and 2.5% (w/v).

Operational Stability of the Immobilized Xylanase The immobilized enzyme was assayed for 10 cycles of 10 min each. For each cycle, 10.0 ml of 1.0% (w/v) birch wood xylan in 100.0 mM citrate–phosphate buffer (pH 6.0 free xylanase; up to pH 7.0 for immobilized xylanase) was added to the immobilized enzyme and incubated for 10 min under continuous shaking at 60 °C (free enzyme), up to 68 °C (immobilized enzyme). At the end of reaction, the immobilized enzyme was collected by either centrifugation at 2,000×*g* for 10 min (Q-S, GE) or by filtration (HP-20, CH), washed with distilled water, and resuspended in freshly prepared substrate to start a new run. The supernatant was assayed for reducing sugars [21].

Production of Xylo-oligosaccharides Free and HP-20-immobilized xylanase were added separately to 100.0 ml of 100.0 mM citrate–phosphate buffer pH 6.0 and up to 7.0, respectively, containing 1.0% (w/v) birchwood xylan with a ratio of 600.0 IU of free and immobilized xylanase/g xylan. The mixture was incubated at 60 °C (free enzyme) or 68 °C (immobilized enzyme) for 10 h with mild agitation (100 rpm) and filtered thereafter. The filtrate obtained was subjected to high-pressure liquid chromatography (HPLC) (model no. CTO-10 ASVP), so as to analyze the concentrations of xylobiose, xylotriose, and xylose. Each freeze-dried supernatant sample (0.2 g) was dissolved in 1 ml of HPLC-grade water, filtered (0.2-μm-pore-size membrane disc filters; Bio-Rad, Hercules, CA, USA) and loaded on HPLC using BioRad Aminex HPX (87 H 300×7.8 mm) column with 5.0 mM sulfuric acid as the mobile phase at 0.6 ml/min flow rate. The retention times of hydrolytic products (xylose, xylobiose, xylotriose) were compared with known standards (Sigma) with a refractive index detector (Shimadzu, Kyoto, Japan; RID-10 A detector) at 50 °C column temperature.

Results and Discussion

Enzyme Immobilization

Xylanase from *B. pumilus* strain MK001 was immobilized on varied solid supports, and immobilization efficiencies of these supports were compared by determining the amount of xylanase expressed as shown in Table 1. Entrapment using GE (40.0%), physical adsorption on CH (35.0%), ionic binding with Q-S (45.0%), and covalent binding with HP-20 (42.0%) showed maximum xylanase immobilization efficiency. Therefore, these four carriers were chosen for further experiments. The reason for low immobilization

Table 1 Immobilization of *B. pumilus* strain MK001 xylanase.

Carrier	Enzyme added	Bound enzyme (A) (Theoretical)	Unbound enzyme	Expressed enzyme (B)	Specific activity of immobilized enzyme (IU/mg protein)	Efficiency [(B/A) ×100]
Entrapment	(IU/30.0 ml gel)	(IU/30.0 ml gel)	(IU/30.0 ml gel)	(IU/30.0 ml gel)		
Polyacrylamide (0.8[a])	1,500.0	729.0	771.0	145.8	120.0	20.0
GE 1.0% (w/v)	1,500.0	825.0	675.0	330.0	293.0	40.0
Ca-alginate (0.8[b])	1,500.0	1,050.0	450.0	315.0	125.0	30.0
Physical adsorption	(IU/500.0 mg carrier)	(IU/500.0 mg carrier)	(IU/500.0 mg carrier)	(IU/500.0 mg carrier)		
CH	500.0	300.0	200.0	105.0	87.0	35.0
Chitosan	500.0	300.0	200.0	76.4	26.0	17.0
Silica	500.0	395.4	104.6	38.0	20.0	9.6
Alumina	500.0	321.6	178.4	36.0	22.0	11.0
Ionic binding	(IU/500.0 mg carrier)	(IU/500.0 mg carrier)	(IU/500.0 mg carrier)	(IU/500.0 mg carrier)		
DEAE-sepharose	500.0	64.6	435.3	20.5	18.0	31.0
Q-S	500.0	79.1	420.8	35.5	28.0	45.0
CM-sepharose	500.0	31.2	468.7	2.5	ND	8.0
Amberlite IR-120	500.0	96.0	404.0	5.5	ND	5.7
Amberlite IR-440	500.0	68.6	431.3	26.0	19.8	33.0
Covalent binding	(IU/500.0 mg carrier)	(IU/500.0 mg carrier)	(IU/500.0 mg carrier)	(IU/500.0 mg carrier)		
CH	500.0	153.0	347.0	45.9	36.0	30.0
HP-20 beads	500.0	90.2	409.7	37.8	22.0	42.0

ND=Not detectable

[a] Percent (w/v) of cross-linking compound (*N*,*N*-methylene-bis acrylamide)

[b] Percent (w/v) alginate

efficiency may be the crowding of other proteins on the support with a direct effect on the accessibility of enzyme molecules to the adsorption material [26].

Out of four matrices selected for further studies, HP-20 [particle size 20–60 mesh, surface area (500 m^2/a) with polyaromatic rings] and CH (composed of *N*-acetyl-D-glucosamine residues, hydrophilic, porous, with highly reactive hydroxyl and amino groups) have high mechanical stability and rigidity, regenerability, and ease of preparation in different geometrical configurations that provide the system with permeability and surface area suitable for chosen biotransformation and are inexpensive. On the other hand, Q-S (particle size 45–165 μm, pore size 4,000,000 Da, cross-linked agarose with quaternary amine group) and GE (gel structure, heterogeneous mixture of single or multistranded polypeptides with carboxyl, amino, and hydroxyl groups) are rather fragile in nature. Immobilization by physical adsorption is one of the simplest and cheapest methods to prepare an immobilized enzyme, although adsorbed enzyme are susceptible to desorption from the carrier [27].

The immobilized enzymes retained 20–50% of the specific xylanase activity (Table 1). This drop of specific activity suggests diffusional limitation on substrate or product flux due to the association of the enzyme within the pores of the carriers [28].

Effect of pH on Activity and Stability

The behavior of an enzyme molecule may be modified by its immediate microenvironment. An enzyme in solution may have a different pH optimum from that of the same enzyme immobilized on a solid matrix, depending on the surface and residual charges of the solid matrix and the pH in the immediate vicinity of the active site. The pH optimum of free xylanase was found to be 6.0 with 60.0% activity limits within the pH range of 4.0–8.5 (Table 2). The GE-, CH-, Q-S-, and HP-20-immobilized enzyme exhibited optimum pH values of 6.5, 6.0, 6.5, and 7.0, respectively, with 60.0% activity limits at pH 4.0–9.5 (Table 2). The change in response to pH after immobilization onto carriers can be related to the functional groups located on the chains of different supports [29, 30]. The pH stability of immobilized enzyme also shifted towards an alkaline range. The immobilized xylanase was able to retain up to 28.0% more of its initial activity than free enzyme in alkaline pH range (7.0–10.0) after 10 h of incubation at room temperature (Fig. 1). The increased pH stability would favor the application of immobilized xylanase in pulp and paper industry [3]. In contrast to our results, *Streptomyces olivaceoviridis* E-86 xylanase immobilized on

Table 2 Properties and kinetic parameters (K_m and V_{max}) of free and immobilized xylanase from *B. pumilus* strain MK001.

Parameter	Substrate	Free enzyme	Immobilized enzyme			
			Q-S	HP-20	GE	CH
K_m	Birchwood xylan	7.9	8.2	8.2	8.3	8.4
	Oat spelt xylan	15.1	15.8	16.9	16.5	16.7
	Beech wood xylan	17.1	18.2	18.6	19.2	18.9
V_{max}	Birchwood xylan	1,450.0	1,320.0	1,250.0	1,278.0	1,224.0
	Oat spelt xylan	857.0	821.0	800.0	799.0	810.0
	Beech wood xylan	1,159.0	1,040.0	1,054.0	1,011.0	1,023.0
Optimum pH		6.0	6.5	7.0	6.5	6.0
Optimum temperature (°C)		60.0	63.0	68.0	63.0	65.0

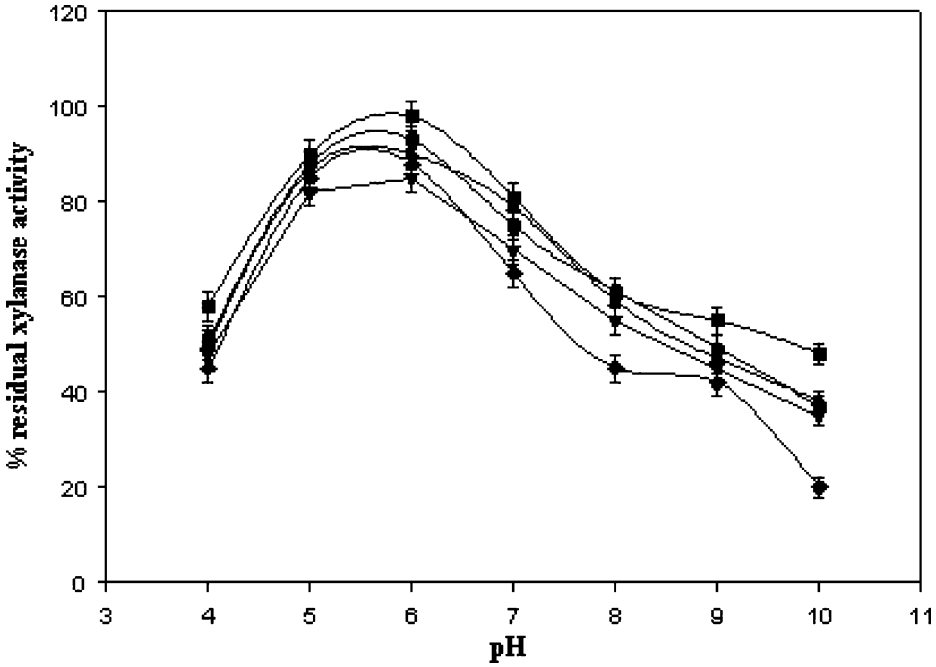

Fig. 1 pH stability of free (*diamonds*) and immobilized [GE (*upright triangles*), CH (*upside-down triangles*), Q-S (*circles*), HP-20 (*squares*)] xylanase from *B. pumilus* strain MK001

Eudragit S-100 showed the same pH stability as of free xylanase and had only a negligible shift in pH optima [19]. Similarly, *Aspergillus niger* xylanase immobilized on magnetic latex beads has an unchanged pH optima at 6.0 [16].

Effect of Temperature on Activity and Stability

Activity of free and immobilized xylanase was assayed over the temperature range of 40–80 °C. The maximum activity of free xylanase was observed at 60 °C (Table 2). However, the temperature/activity profiles of the immobilized xylanase preparations were different. Gelatin-, CH-, and Q-S-immobilized xylanase registered up to 5 °C rise in optimal temperature (65 °C), whereas HP-20-immobilized xylanase was maximally active at 68 °C (Table 2). Similar to our work, optimum temperature of *Streptomyces olivaceovirdis* E-86 xylanase after immobilization on Eudragit S-100 shifted to 65 from 60 °C [19]. The optimum temperature of *A. niger* NRC 107 xylanase was shifted from 50 to 52.5–65°C, whereas that of β-xylosidase was shifted from 45 to 50–60 °C. In general, the immobilization of enzyme protects the latter against heat inactivation [18, 31].

The free and immobilized (HP-20) xylanase retained 72.0 and 92.0% activity, respectively, at 60 °C after 3 h of incubation. Free xylanase retained 50.0% activity, whereas the immobilized enzyme on HP-20, Q-S, CH, and GE retained 68.0, 64.0, 58.0, and 57.0% of total activity, respectively, after 3 h of incubation at 80 °C (Fig. 2). The enhanced stabilization of immobilized xylanase may result either from the binding of xylanase at several places on the supports (GE, CH, Q-S) [30] or from restriction in unfolding process of protein as a result of introduction of both intra- and intermolecular

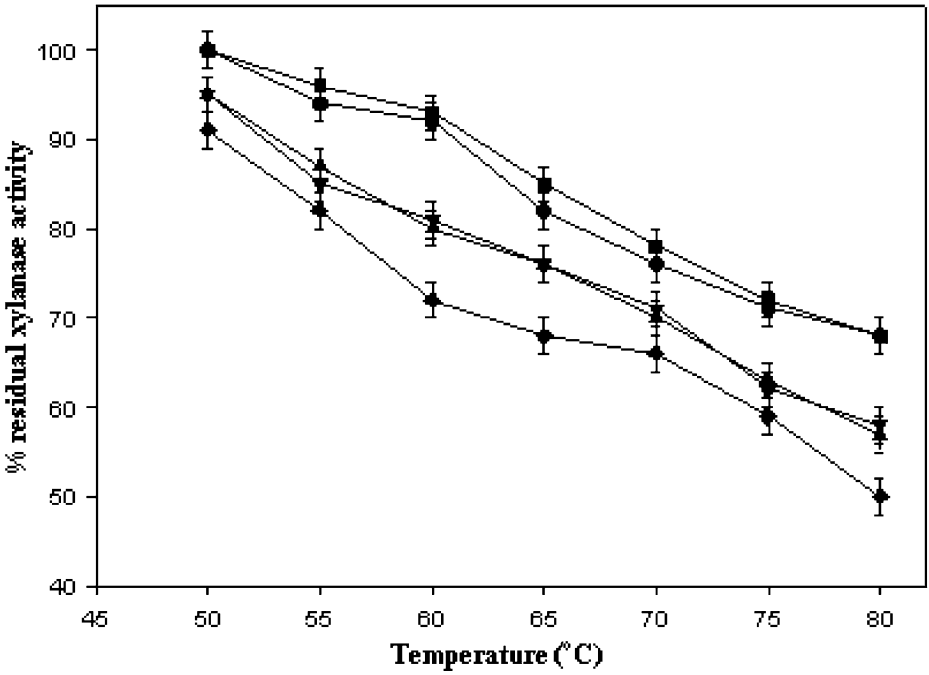

Fig. 2 Thermal stability of free (*diamonds*) and immobilized [GE (*upright triangles*), CH (*upside-down triangles*), Q-S (*circles*), HP-20 (*squares*)] xylanase from *B. pumilus* strain MK001 after 3 h of incubation at temperatures ranging between 50 and 80 °C

cross links (as in HP-20) [32]. Overall, these findings suggest that immobilization resulted in a more rigid structure of the protein, which is less easily unfolded and, consequently, more resistant to thermal inactivation.

Effect of Substrate Concentration on Activity of Free and Immobilized Xylanase

The K_m values of the immobilized xylanase for birchwood xylan, oat-spelt xylan, and beechwood xylan were increased marginally up to 8.4, 16.9, and 19.2 mg/ml, as compared to 7.9, 15.1, and 17.1 mg/ml for free xylanase (Table 2). This increased K_m value of immobilized xylanase may be partially due to the mass transfer resistance of the substrate into the immobilization matrices, like GE [33]. This is because the average diameter of a typical bead of enzyme-impregnated GE gel is much larger compared to the average diffusion length and does not allow macromolecular substrates like xylan to diffuse deep into the gel matrix. At the same time, the diffusional resistance encountered by the product molecules can also cause the product to accumulate near the center of the gel to an undesirable high level, leading to product inhibition. When the substrate molecules are larger then the pore size of the medium containing the enzyme, the substrate cannot diffuse to the catalytic sites. In the case of such molecular sieve effects, no reaction takes place even if the enzyme is fully active in the interior [34]. Blocking of the enzyme active sites due to chemical cross-linking, diffusional limitations, and confinement of the enzyme molecules within the support could be potential reasons for higher K_m for CH, Q-S, and HP-20 supports [29, 30]. K_m values can significantly vary from enzyme to enzyme and

even for different substrates of the same enzyme and thus explain the large range of K_m values reported for immobilized xylanases [15, 16, 18].

The V_{max} values of the immobilized enzyme for birchwood xylan, oat-spelt xylan, and beechwood xylan were observed to be slightly lower as compared to that of free enzyme. The marginally lower V_{max} values for immobilized enzyme suggest that the activity of immobilized xylanase was not affected drastically during the course of immobilization (Table 2). However, decrease in immobilized enzyme activity observed may be due to the binding of matrices to enzyme-active site, conformational change, or the new local environment formed [35]. The increase in K_m value and decrease in V_{max} following immobilization have also been reported earlier by many investigators [15, 25, 36, 37].

Retention of Activity by Immobilized Xylanase

The reuse of the immobilized enzyme is an important factor while considering its cost-effectiveness for commercial applications. A continuous assay of residual enzyme activity of xylanase immobilized on GE, CH, Q-S, and HP-20 was performed to find out the retention of xylanase activity by each support over 10 enzyme reaction cycles. In case of xylanase immobilized on GE and CH, more then half of the activity was lost after four cycles (Fig. 3). Xylanase immobilized on Q-S and HP-20 showed better retention and retained up to 70.0% activity after seven cycles (Fig. 3).

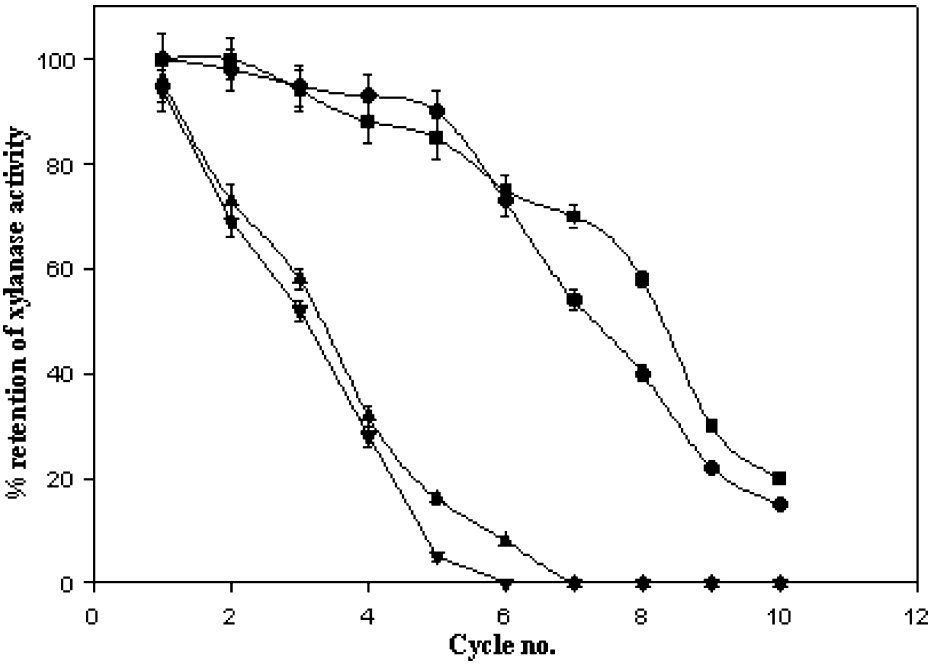

Fig. 3 Reusability of the *B. pumilus* strain MK001 xylanase immobilized on GE (*upright triangles*), CH (*upside-down triangles*), Q-S (*circles*), and HP-20 (*squares*). After one cycle of incubation of the immobilized xylanase and the substrate at respective optimum temperatures, immobilized enzyme was removed by centrifugation, as described in the "Materials and Methods" section. The immobilized xylanase was incubated again with fresh substrate to initiate the second cycle of hydrolysis. Each hydrolysis cycle lasted 10 min

Loss of enzyme immobilized on supports after several reuses could be due to (1) erosion of the support material; (2) thermal denaturation, inhibition, or inactivation, as the bonding pattern changes at higher temperatures; and (3) desorption causing severing of the chemical bonds or disturbing the attachment of the enzyme to the support [38]. The binding of immobilized xylanase with CH is mediated by physical interactions such as van der Waals forces, hydrogen bonding, or hydrophilic–hydrophobic effects. However, these binding forces are generally weak and do not prevent desorption of protein from the support under operational conditions [39–41]. The loss of xylanase activity from GE after repeated usage is due to the loss of gel structure, resulting thereby in enzyme leakage. The chemical and mechanical stability of GE largely depend upon the degree of cross-linking, as well as the rigidity of the support. Similarly, it is reported that tyrosinase from mushroom retained about 30% of its initial activity in GE gels after eight cycles of use [42]. The loss of operational stability by Q-S-immobilized xylanase might be due to change in bonding pattern at high temperatures [38], development of low positive charge resulting in elution of protein, presence of organic inhibitors, or clogging of carrier by macromolecular xylan fragments [43]. Although HP-20 did not bind maximum levels of xylanase in comparison to other matrices, it seemed to be a good candidate, as enzyme activity was sustained at high temperatures and, due to covalent binding, enzyme activity was retained for longer periods. HP-20 has been used as solid support for immobilization of various pharmaceutical enzymes in industries. In accordance to our work, *A. niger* NRC 107 xylanase covalently bound to chitosan showed the highest operational stability as compared to enzymes immobilized by physical adsorption or ionic binding [15]. Our results on retention of

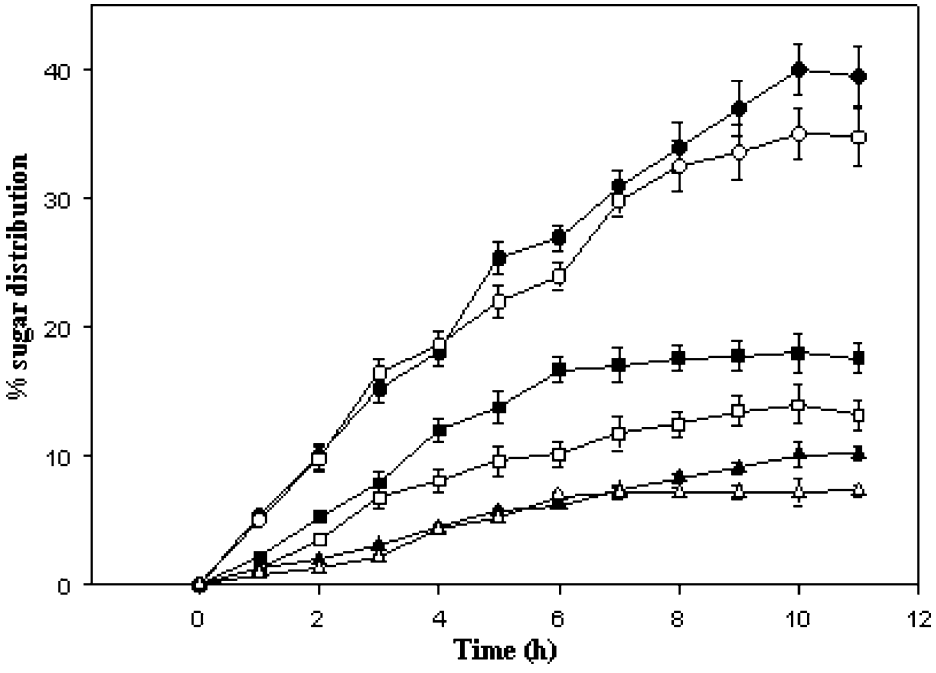

Fig. 4 Time course of the xylooligosacchride production from birchwood xylan by free and immobilized (HP-20) xylanase from *B. pumilus* strain MK001. Xylobiose [free (*white circles*), immobilized (*black circles*)], xylotriose [free (*white squares*), immobilized (*black squares*)], xylose [free (*white triangles*), immobilized (*black triangles*)]

activity by immobilized xylanase are better then earlier reports where significant losses in activity are reported after four [19, 44] and six [45] cycles.

Production of Xylo-oligosaccharides

Xylo-oligosaccharides (xylobiose, xylotriose, xylotetraose, etc.) prepared from various sources of xylans such as wheat bran, birchwood, or corncob can be utilized selectively by the beneficial intestinal microflora viz. *Bifdiobacteria* and are thus expected to be used as a valuable food additive. Hydrolysis of birchwood xylan by free and HP-20 immobilized xylanase produced xylobiose and xylotriose within 1 h of reaction time. Long-term incubation resulted in a steady increase in xylose content. After 10 h of incubation, immobilized enzyme released 40.0% xylobiose and 18.0% xylotriose; on the other hand, free enzyme released 35.0% xylobiose and 14.0% xylotriose from total sugar (9.8 mg/ml immobilized enzyme; 9.3 mg/ml free enzyme) released by the reaction products (Fig. 4). The xylo-oligosaccharides produced from xylan hydrolysis were purified by filtration of the hydrolyzed xylan (separating HP-20 beads immobilized with xylanase) followed by centrifugation at 5,000×*g* to remove any undegraded substrate. A 32.0% xylobiose and a 12.0% xylotriose were obtained from chemically (2.0% NaOH) pretreated corncob powder after 12 h of incubation with Eudragit S-100 immobilized xylanase from *S. olivaceovirdis* E-86 [19]. Similarly, cellulase-free xylanase produced by *Psedomomonas* sp. WLUNO24 has been reported to produce high-quality xylo-oligosacchride from xylan [46]. Immobilized xylanases from *Aspergillus* sp. strains 5 and 44 yielded 1.4 (7.6 vs 5.3 mg/ml) and 1.3 times (7.0 vs 5.2 mg/ml) more sugars as compared to free xylanase. In repeated batch saccharification studies, immobilized xylanase was recycled three times without loss of enzyme activity. However, significant decrease in its activity was observed in the fourth cycle [47]. A 5.0% increase in xylobiose and 4.0% increase in xylotriose production by *B. pumilus* strain MK001 immobilized xylanase over free enzyme may not be significant considering the cost of immobilization. However, reusability of immobilized enzyme appears to hold merit. Further studies for production of xylo-oligosaccharides from economical agricultural residues such as wheat bran and rice bran are currently underway.

Conclusions

The overall performance of the immobilized xylanase in terms of catalytic activity, thermal and pH stability, reuse, and higher xylo-oligosaccharide production are more promising than free enzyme. The higher/better efficiency observed for xylanase immobilized on cost-effective supports such as HP-20 or CH might favor its commercial utilization for high-quality xylo-oligosaccharide production.

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