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Surface Immobilization on Silica of Endoxylanase Produced from Recombinant *Bacillus subtilis*

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Abstract The plasmid, pJHKJ4, containing the endoxylanase gene, was introduced into *Bacillus subtilis* DB 104. The recombinant cells produced 587 unit/ml of endoxylanase at 33 h. The endoxylanase was immobilized covalently on the surface of silica for effective xylan hydrolysis. The activities of the immobilized and free endoxylanases were optimal at pH 6.5 and 10 mM $MnSO_4$. The optimal temperature of the immobilized endoxylanase was 60°C, whereas that of the free endoxylanase was 65°C. Under these optimal conditions, the activity of the immobilized endoxylanase was 1.7 times higher than that of the free endoxylanase. From microscope photographs, the immobilized endoxylanase was found to be bounded and evenly distributed on the surface of silica, a nonporous solid support. The enzyme kinetics between the immobilized and free endoxylanases was estimated to be uncompetitive, when plotting double-reciprocal plots against xylan concentrations and endoxylanase activities. These results suggest that the higher activity of the immobilized endoxylanase may be due to increased formation of enzyme-substrate complex, because of the easy accessibility of the immobilized enzyme to the polysaccharide-xylan as a high molecular weight substrate.

Key words: Endoxylanase, xylan, immobilization, silica

Xylan is a major hemicellulose component of the cell walls of monocots and hardwoods, representing up to 35% of the dry weight of these plants. The hydrolysis of xylan is of considerable interest for biotechnological applications in the pulp and paper, food, and animal feed industries [2, 39]. In the pulp and paper industry, xylanase enzymes enhance the bleaching of pulp, thereby decreasing the amount of chlorine-containing compounds in the process and the subsequent discharge of organochlorines in the effluent [35]. In the food industry, xylanase enzymes are used to accelerate the baking of cookies, cakes, crackers, and other foods by helping breakdown of polysaccharides in the dough [14]. In animal feeds, xylanase enzymes aid in the digestibility of wheat by poultry and swine, by decreasing the viscosity of the feed [14]. Several enzymes such as endo-β-1,4-xylanase, β-xylosidase, α-glucuronidase, α-arabinofuranosidase, and esterase are required for complete hydrolysis of xylan [2, 24, 36]. The xylanase enzymes are produced by *Trichoderma*, *Bacillus*, *Aspergillus*, *Penicillium*, *Aureobasidium*, and *Talaromyces* sp. [14].

Among these enzymes, endo-β-1,4-xylanase and β-xylosidase are known to be two main enzymes involved in the degradation of xylan into xylose. Endoxylanase is considered to be the most important enzyme, as it initiates the degradation of xylan backbone. It was previously reported that the endoxylanase gene from *Bacillus* sp. was cloned and expressed in *E. coli* and *B. subtilis* [16, 17], and a strong constitutive P_{JH} promoter was applied as an efficient expression system for overproduction of endoxylanase in *B. subtilis* [20]. The production of endoxylanase was increased by using the recombinant cells due to gene amplification.

A significant number of reports have been published concerning gene cloning, purification, and characterization of endoxylanase [5–7, 9, 13, 15, 18, 21, 23, 37]. However, there is little report on the immobilization of endoxylanase. The immobilization of endoxylanase has some disadvantages such as the decrease of the activity due to steric hindrance of polysaccharide-xylan as a high molecular weight substrate.

In the present study, endoxylanase was produced using recombinant *B. subtilis*, and the produced enzyme was immobilized on the surface of silica, a nonporous solid support, to enhance the accessibility of the enzyme to the polysaccharide-xylan.

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MATERIALS AND METHODS

Microorganism, Plasmid, and Media

B. subtilis DB 104 was used as the host cell for the production of endoxylanase [19]. The endoxylanase expression plasmid, pJHKJ4, was constructed as previously described [16]. The open reading frame of the endoxylanase gene (639 bp) in pJHKJ4 plasmid was transcribed using two promoters as its own endoxylanase promoter, P_B, and the strong constitutive *Bacillus* promoter, P_{JH}. The transformation of *B. subtilis* DB104 with the pJHKJ4 plasmid was carried out according to the method previously reported [8].

The growth medium, consisting of 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, and 25 mg/l kanamycin, was used to select transformants. The fermentation medium contained 20 g/l maltose, 10 g/l tryptone, 10 g/l yeast extract, and 5 g/l NaCl.

Culture Conditions

B. subtilis DB 104 cell harboring the pJHKJ4 plasmid was cultivated at 37°C in 10 ml growth medium to an optical density of 1.0 at 600 nm. The seed was inoculated into a 500-ml baffled flask containing 50 ml growth medium at 37°C with 240 rpm for 8 h. The culture broth was transferred to a 7 liter fermentor containing 5 liter fermentation medium (BioG-12, Biotron Co., Korea). The pH of the culture broth was controlled at 7.0 with 50% NH_4OH. The concentration of dissolved oxygen was maintained at above 20% air saturation by adjusting the agitation speed within a range of 300 to 800 rpm.

Enzyme Purification and Immobilization

After the cultivation, the supernatant of culture was collected by centrifugation (6,000 ×*g*, 4°C, 30 min). Protein was precipitated by adding ammonium sulfate to the supernatant at 70% saturation. The precipitate formed was collected by centrifugation (6,000 ×*g*, 4°C, 30 min) and then resuspended in 0.1 M phosphate buffer (pH 6.5). The solution was dialysed against the same buffer in a dialysis membrane with a molecular weight cut-off of 3,500 dalton. The endoxylanase was used for investigating the properties of free enzyme. The protein concentration used was 2.28 mg/ml and the reaction volume was 10 ml.

An aliquot of 10 ml of the dialysate containing endoxylanase (6 mg protein /ml) was mixed with 10 ml of 0.1 M Tris/HCl buffer at pH 8.0 and 10 g of silica (Silica-60, Merck, Germany). The mixture was stirred at 4°C for 3 h. The supernatant of silica was removed and the absorbed enzyme was treated with 10 ml of 0.2% glutaraldehyde at 4°C for 1 h. The covalently immobilized enzyme was filtered and then washed with 10 ml of the buffer [26]. The amount of immobilized enzyme was calculated by subtracting the amount of desorbed protein from the total protein added. The amount of protein immobilized on 10 g silica was 22.8 mg. This enzyme preparation was used for investigating the properties of immobilized enzyme with a 10 ml reaction volume.

Analytical Methods

The concentrations of maltose and xylan hydrolyzates were measured using the dinitrosalicyclic acid (DNS) method. The analyses of xylan hydrolyzates were performed by Bio-LC with an eletrochemical detector (Dionex ED-50, U.S.A.) using a Dionex Carbo PAC 100 column with 1.0 M sodium acetate and 0.25 mM sodium hydroxide at a flow rate of 0.25 ml/min. The standard materials used were xylose, xylobiose, xylotriose, and xylotetraose (Megazyme, Ireland). The activity of endoxylanase was determined by measuring the amount of reducing sugar liberated from the oat spelts xylan [25]. The intracellular activity of endoxylanase was determined with cell lysates obtained by lysozyme treatment [27]. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar per minute at 60°C and pH 6.5. Protein content was determined by the Bradford method using bovine serum albumin as a standard [4].

RESULTS

Endoxylanse Production by Batch Fermentation of Recombinant *B. subtilis*

The batch culture of *B. subtilis* harboring pJHK4 plasmid was performed in a fermentor (Fig. 1). The final concentration of cells was 6.9 g/l at 33 h. Maltose concentration was decreased from 20.6 g/l to 4.7 g/l by the cells. The endoxylanase production continued to increase as the batch fermentation proceeded, and the enzyme activity then reached a

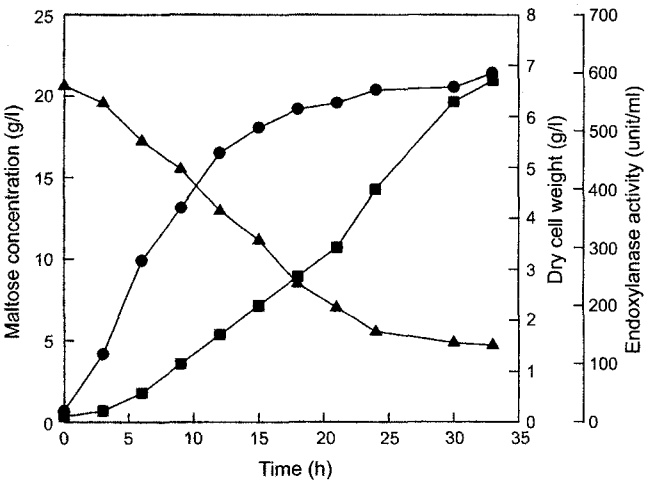

Fig. 1. Batch cultivation profiles of *Bacillus subtilis* DB104 harboring pJHK4 plasmid in a 7 liter fermentor.
Dry cell weight (●), residual maltose concentration (▲), and endoxylanase activity (■).

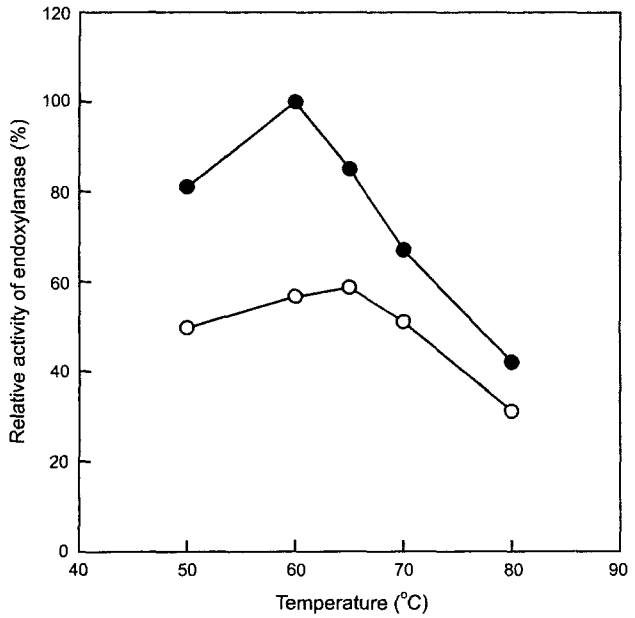

Fig. 2. Effect of temperature on the activities of free and immobilized endoxylanases.
Relative activities of free enzyme (○) and immobilized enzyme (●). The amounts of free and immobilized enzymes were the same at 22.8 mg.

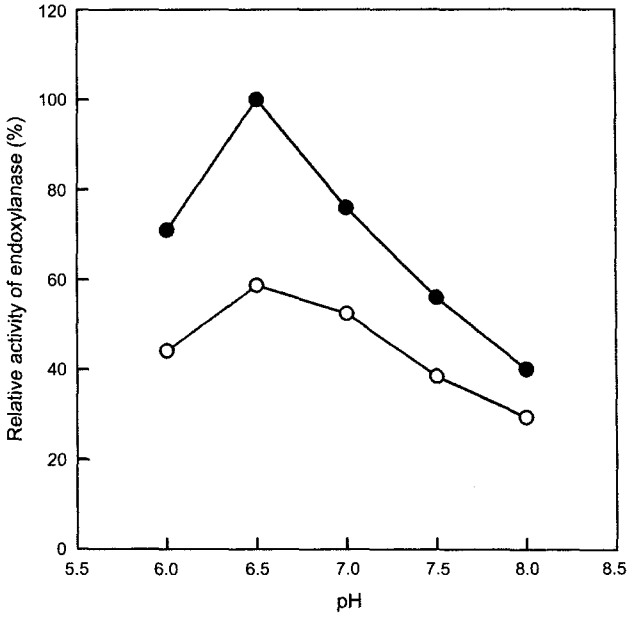

Fig. 3. Effect of pH on the activities of free and immobilized endoxylanases.
Relative activities of free enzyme (○) and immobilized enzyme (●). The amounts of free and immobilized enzymes were the same at 22.8 mg.

maximum level of 587 unit/ml at the end of culture time. The intracellular activity of endoxylanase was observed to be less than 25 unit/ml during the entire culture period. This result indicated that the majority of more than 95% of endoxylanase was efficiently secreted into the culture medium.

The fermentation parameters for the production of the endoxylanase by *B. subtilis* harboring pJHK4 plasmid were evaluated. The maximal specific growth rate of cells was 0.59 l/h, the overall growth yield from maltose was 0.416 g/g, the volumetric production rate of endoxylanse was 17.8 unit/ml·h, and the overall endoxylanse yield from maltose was 36.9 unit/mg.

Properties of the Immobilized and Free Endoxylanases

The effect of temperature on the activities of immobilized and free endoxylanases was investigated at 22.8 mg protein concentration (Fig. 2). The activities were measured for 15 min with 1.0% xylan solution. The optimal temperatures for the activities of immobilized and free endoxylanases were 60°C and 65°C, respectively. The activity of the immobilized endoxylanase was more temperature sensitive, and its optimal temperature was lower than that of the free endoxylanase.

The optimal pH for the activities of the immobilized and free endoxylanases was estimated to be 6.5 (Fig. 3). The activities were measured at 60°C for the immobilized enzyme and at 65°C for the free enzyme within 1.0% xylan solution for 15 min. The activity of the immobilized endoxylanase was also more pH sensitive, and higher than that of the free endoxylanase.

Effect of various metal ions on the endoxylanase activity was investigated (Table 1) at 60°C and pH 6.5 for the immobilized enzyme in 1.0% xylan solution for 15 min. Inhibition of endoxylanase was observed with copper and aluminum ions. Addition of magnesium and barium at 0.5 mM increased endoxylanase activity, whereas with a high concentration of 5.0 mM, the activity decreased. An increase in the activity under 5.0 mM concentration was observed with cacium, ferrous, cobalt, and manganese ions. The best effective metal ion for endoxyanase activity was found to be manganese ion. The activity of the

Table 1. Effect of metal ion on the activity of endoxylanase from *B. subtilis* DB104.

Concentration (mM)	$CaCl_2$	$CoCl_2$	$MgCl_2$	$CuSO_4$	$AlCl_2$	$BaCl_2$	$FeCl_3$	$MnSO_4$
0.0	100	100	100	100	100	100	100	100
0.5	102	120	116	95.8	100	104	108	128
5.0	104	108	95.3	84.0	95.3	95.3	102	130

Relative activity without metal ion is 100%.

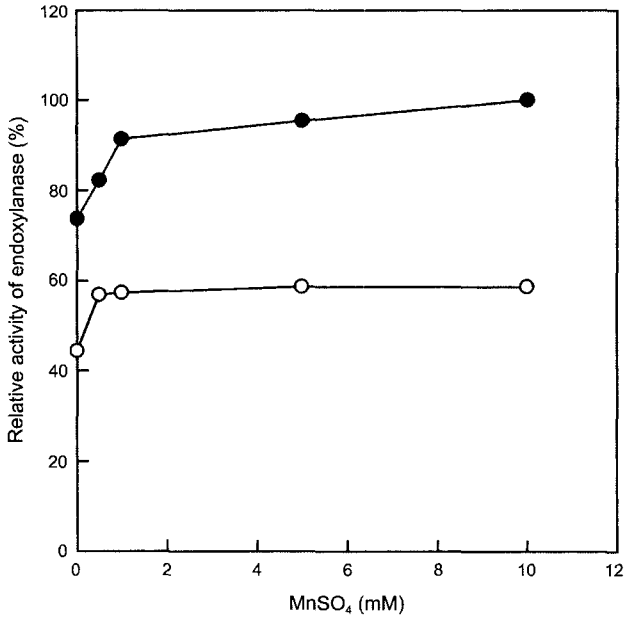

Fig. 4. Effect of $MnSO_4$ concentration on the activities of free and immobilized endoxylanases.
The free enzyme (○) and immobilized enzyme (●) in phosphate buffer (20 mM, pH 6.5) were incubated for 15 min at 65°C and 60°C, respectively. The amounts of free and immobilized enzymes were the same at 22.8 mg.

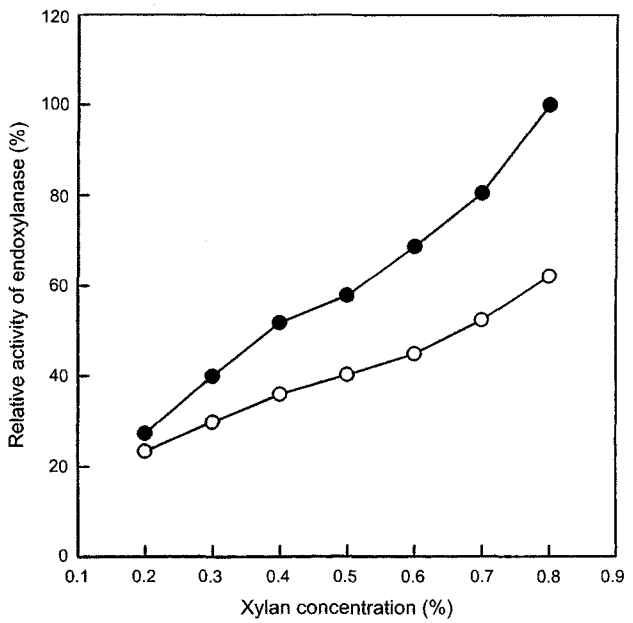

Fig. 5. Conversion of xylan to xylan hydrolysate by free and immobilized endoxylanases.
Relative activities of free enzyme (○) and immobilized enzyme (●). Xylan concentration was 1.0%. The amounts of free and immobilized enzymes were the same at 22.8 mg.

immobilized endoxylanase increased at a higher rate than that of free endoxylanase with manganese ion, when the manganese concentration ion was increased (Fig. 4).

The optimal conditions for the activity of the immobilized endoxylanase were determined to be 60°C, pH 6.5, and 10 mM $MnSO_4$. Those for the free endoxylanase activities were determined to be at 65°C, pH 6.5, and 10 mM $MnSO_4$.

The effect of the concentration of a high-molecular weight substrate, xylan, on the activities of the immobilized and free endoxylanases was investigated under the optimal conditions (Fig. 5). As the concentration of xylan was increased, the activities of the immobilized and free endoxylanases, and the differences in activity between the immobilized and free endoxylanases, increased due to steric hindrance of the high-molecular weight xylan.

Production of Xylan Hydrolysates from Xylan in the Immobilized and Free Endoxylanses

The enzyme reactions for xylan hydrolysis were performed with the immobilized and free endoxylanses under the optimal conditions in 1.0% soluble xylan (Fig. 6), using the DNS method to detect reducing sugar of xylan hydrolysates. The concentration of xylan hydrolysates proportionally increased up to 0.5 h, and the increased rate of xylan hydrolysates decreased markedly after 0.5 h as a saturation curve. The conversion yields of xylan hydrolysates from xylan by the immobilized and free endoxylanases were 23% and 13%, respectively. The production of xylan hydrolysates by the immobilized enzyme was 1.7 times higher than that of the free enzyme.

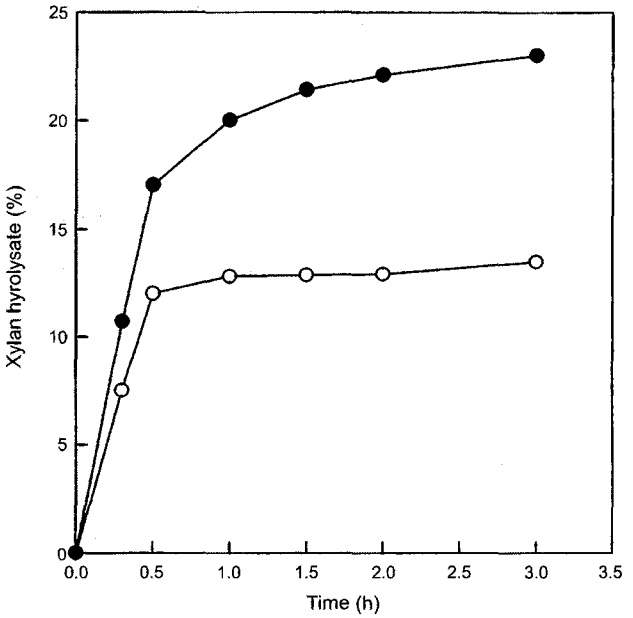

Fig. 6. Effect of xylan concentration on the activities of free and immobilized endoxylanases.
Relative activities of free enzyme (○) and immobilized enzyme (●). The enzyme reactions were performed with immobilized enzyme and free enzyme in 20 mM phosphate (pH 6.5) at 65°C and 60°C, respectively. The amounts of free and immobilized enzymes were the same at 22.8 mg.

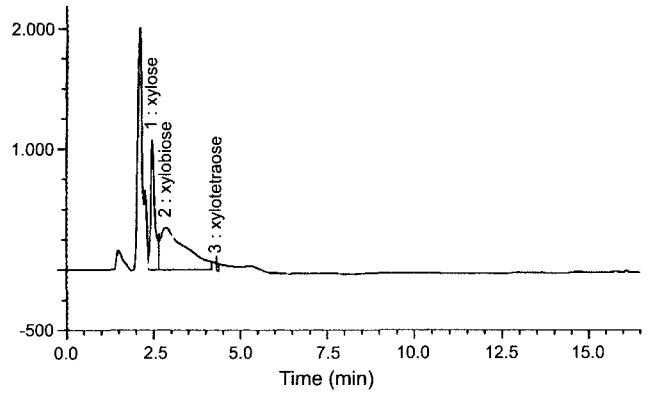

Fig. 7. Bio-LC analysis of the endoxylanase reaction product.

After hydrolysis of 1.0% xylan for 3 h by treating the immobilized endoxylanase, the major products of the hydrolysis were analyzed by Bio-LC. The major compounds were xylose and xylobiose, and trace amount of xylotriose was also found (Fig. 7). This result indicates that the enzyme is an endoxylanase which cleaves the internal backbone of xylan molecules.

DISCUSSION

The optimal conditions for the activity of the immobilized endoxylanase were determined to be 60°C, pH 6.5, and 10 mM $MnSO_4$ for 15 min in 1.0% xylan solution, whereas those of the free endoxylanase were at 65°C, pH 6.5, and 10 mM $MnSO_4$ for 15 min in 1.0% xylan solution. The activity of the immobilized endoxylanase under the optimal conditions was found to be 1.7 times higher than that of free endoxylanase as shown in Fig. 2–Fig. 6. In order to explain the results, the specific activities of endoxylanase during the immobilization steps were measured. The specific activity of the supernatant of the absorbed enzyme after absorbing to silica was the same as that of the free endoxylanase before the immobilization, indicating that the binding between silica and endoxylanase was not specific, and endoxylanase, among endoxylanase and crude proteins used in the immobilization, was not purified during absorption to silica.

Microscopic photographs of the enzyme immobilized by the silica, the silica, and the free enzyme were taken to investigate the structures of the immobilized and free endoxylanases (Fig. 8). The immobilized enzyme was bound and evenly distributed on the surface of the nonporous solid support, silica, and the free enzyme was shown as solid particles. The structure of the immobilized enzyme was more suitable to react to a high-molecular weight substrate, xylan, than that of the free enzyme due to easy accessibility of the immobilized enzyme to the substrate, resulting in a higher activity of the immobilized endoxylanase.

In order to investigate the mechanism involved in an increase of the endoxylanase activity by immobilization, the kinetic analysis of substrate-enzyme reactions, shown in Fig. 7, was performed using a double-reciprocal plot (Fig. 9). Apparent K_m and V_{max} values for the immobilized endoxylanase were 26.4 mg/ml and 673 unit/ml, respectively. For the free endoxylanase, the values were 6.89 mg/ml and 175 unit/ml. The values for the immobilized endoxylanase were 3.8 times higher than those for the free endoxylanase, respectively. The higher values of the immobilized endoxylanase indicate that the reaction rate increased by the surface immobilization and xylan was more specific to

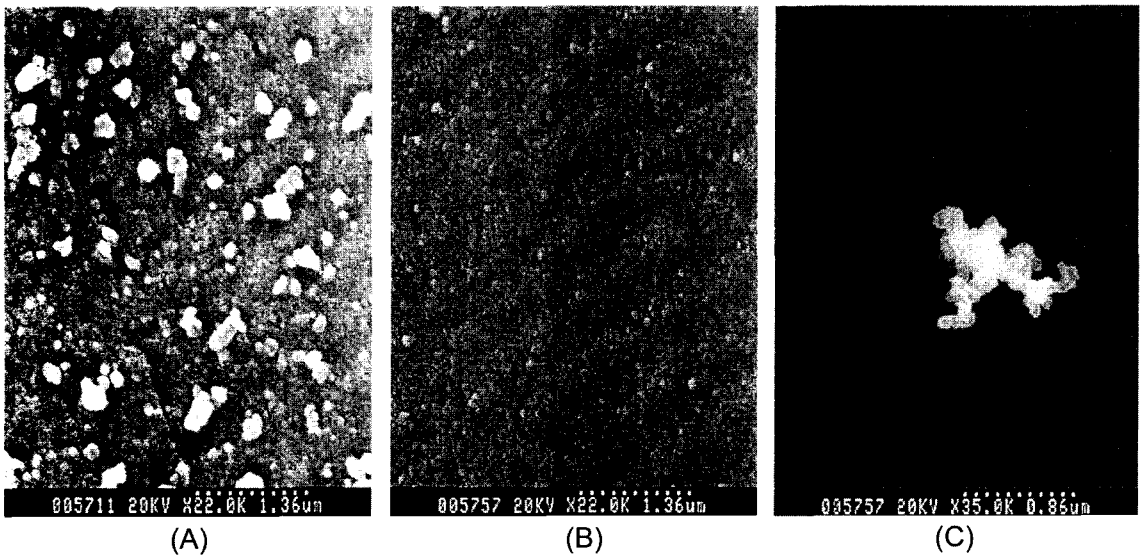

Fig. 8. Microscope photographs of the immobilized enzyme by the silica (A), the silica (B), and free enzyme (C).
This shows that the enzyme has been attached to the surface of the silica. Resolutions of A, B, and C are ×22k, ×22k, ×35k, resp ectively.

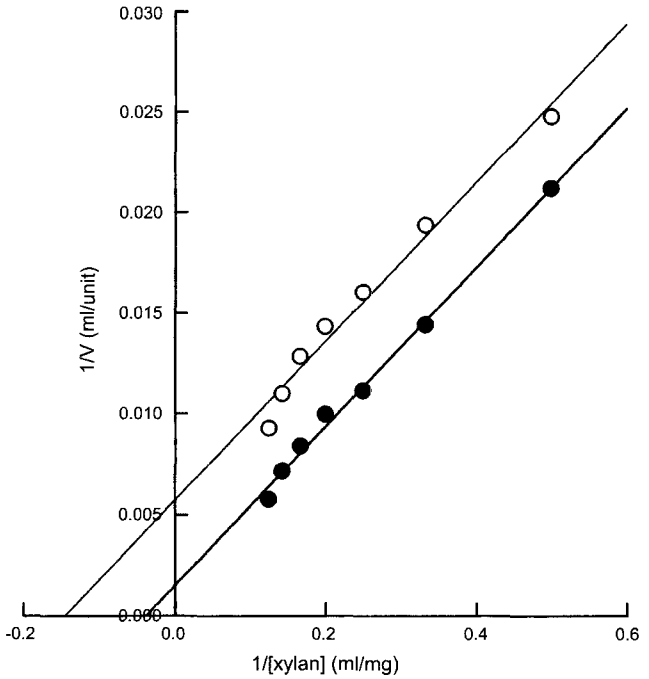

Fig. 9. Double-reciprocal plot for effect of xylan concentration on the activities of free and immobilized endoxylanases.
Relative activities of free enzyme (○) and immobilized enzyme (●). The enzyme reactions were performed with immobilized enzyme and free enzyme in 20 mM phosphate (pH 6.5) at 65°C and 60°C, respectively.

the immobilized enzyme than the free enzyme. The pattern of enzyme kinetics between the immobilized and free endoxylanase was shown to be uncompetitive. This result suggested that the formation of enzyme-substrate complex with the immobilized enzyme increased due to the accessibility of the immobilized enzyme to easy substrate, and the increased enzyme-substrate complex then resulted in a higher activity of endoxylanase.

The known methods of xylanase immobilization generally use two strategies: (i) adsorption on silica [30, 34], glass [26], or polymer [31, 32], and (ii) covalent binding with various polymeric matrices [26, 28, 33, 38]. In all of the reported cases, the activity of immobilized enzyme was 40–80% of that of the free enzyme. However, the enzyme activity obtained by immobilization of xylanase in xanthan/chitosan matrics was higher than that of free enzyme [12]. Fifty % increases in activity occurred within a wide range of enzyme loading. The structure of beads prepared from xanthan and chitosan was of fibrils, and the enzyme organized itself in globular substructures attached to the fibrils. These results suggest that the structure of immobilized enzyme is very important for the increase of enzyme activity by immobilization.

The structure of many xylanase enzymes and xylan substrate are known [1, 3]. The mode of action of several endoxylanases has been studied using both kinetic and end-product analysis techniques [10, 11, 23, 29], and the performance of various xylanase enzymes on a given substrate has been compared [22]. The paper suggests that different endoxylanases have different activities against various xylan structures and the key factors for influencing the rate of xylan hydrolysis are chain length and degree of substitution. However, there has been no report on immobilization of endoxylanase and modification to a suitable structure by immobilization. In this study, we developed an immobilization method to enhance production of xylan hydrolysates, which was due to easy accessibility of the silica-surface immobilized enzyme to the substrate.

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