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Xylan Hydrolysis by Treatment with Endoxylanase and β-Xylosidase Expressed in Yeast

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Abstract The endoxylanase (642 bp; 213 amino acids) and β-xylosidase (1,602 bp; 533 amino acids) genes from *Bacillus* sp. were amplified by PCR and separately inserted into the downstream of the yeast *ADH1* promoters, resulting in the pAEDX-1 (7.63 kb) and pAEX (8.47 kb) plasmids, respectively. When the yeast transformants, *S. cerevisiae* SEY2102 harboring pAEDX-1 or pAEX, were grown on YPD medium, the total activities of the enzymes were approximately 9.8 unit/ml for endoxylanase and 2.9 unit/ml for β-xylosidase. When the three kinds of xylan from oat spelts, birch wood, and corncob were hydrolyzed by treating with recombinant endoxylanase and β-xylosidase, it was found that xylose, xylobiose, and xylotriose were produced. To efficiently hydrolyze xylan, various reaction conditions such as amount of enzymes, substrate type, substrate concentration, temperature, and reaction time were examined. The optimized conditions for the hydrolysis of xylan were as follows: amount of endoxylanase, 10 units; amount of β-xylosidase, 10 units; temperature, 50°C; substrate type, oat spelts xylan; substrate concentration, 6%; reaction time, 1 h. Under the optimal condition, xylose was mainly produced from oat spelts xylan by cooperative action of endoxylanase and β-xylosidase.

Key words: Endoxylanase, *S. cerevisiae*, xylan, β-xylosidase, xylose

Xylan is a major component of the cell walls of monocots and hardwoods, and it represents up to 35% of the dry cell weight of these plants. Hydrolysis of xylan draws considerable interest for various biotechnological applications (biobleaching, food, and animal feed). Unlike cellulose, xylan is a complex polymer consisting of a β-D-1,4-linked xylopyranoside backbone substituted with acetyl, arabinosyl, and glucuronosyl side chains [2, 23]. Hydrolysis of the xylan backbone is catalyzed by endo-β-1,4-xylanases (EC 3.2.1.8) and β-D-xylosidases (EC 3.2.1.37). Endo-β-xylanases act on xylans and xylooligosaccharides, producing mainly mixtures of xylooligosaccharides. In addition, β-D-xylosidases hydrolyze xylooligosaccharides to D-xylose [2, 24, 26].

Many bacterial and fungal species are able to utilize xylan as a carbon source. There is considerable interest in their degradation properties due to their possible application to waste treatment, fuel and chemical production, and paper manufacturing [3, 19, 23]. However, an enormous amount of xylan is mostly wasted all over the world. Consequently, if xylan can be converted into industrially applicable and value-added material, the value of xylan will be highly enhanced.

Saccharomyces cerevisiae is an excellent host for the production of recombinant proteins, including the proteins derived from medicinal and food substances with important characteristics, since it is nonpathogenic and free of endotoxins for man and has been grown on an industrial scale for centuries. To increase the ability of yeast to hydrolyze different polysaccharide substrates present in plant raw materials, several heterologous genes coding for hydrolytic enzymes such as cellulose, amylase, pullulanase, and xylanase have been expressed in this organism [1, 4, 10, 12, 15]. Among them, the biodegradation of xylan would be of considerable economic importance. In this paper, we describe the expression of *Bacillus* endoxylanase and β-xylosidase genes in *S. cerevisiae*, and process optimization of the hydrolysis of xylan.

Materials and Methods

Yeast Strain, Plasmids, and Media

Yeast host strains used in this work were *S. cerevisiae* SEY2102 (*MATα ura3-52 leu2-112 his4-519 suc2-Δ9*) [6].

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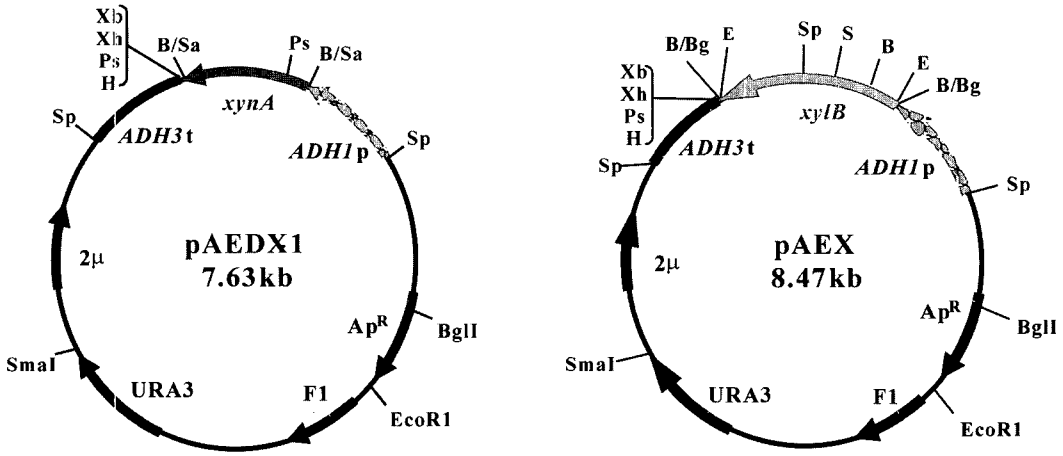

Fig. 1. Schematic diagram of recombinant plasmids, pAEDX-1 and pAEX, used in this work.

The ORFs of endoxylanase (642 bp; 213 amino acids) [8, 9] and β-xylosidase (1,602 bp; 533 amino acids) [5] genes were constructed under the control of yeast *ADH1* promoter [25], respectively. *S. cerevisiae* transformants harboring pAEDX-1 or pAEX were grown on YPD medium (1% yeast extract, 2% peptone, 2% dextrose). *Escherichia coli* DH5α was used for all bacterial transformations and plasmid preparations.

Measurement of Cell Growth and Residual Glucose

The yeast cell growth was monitored by measuring the optical density at 600 nm (OD_{600}). The periplasmic and cytoplasmic fractions of yeast were obtained by treating with Zymolyase 100T (Seikagaku Kogyo, Japan) and glass beads [18]. The residual concentration of glucose was measured by applying the dinitrosalicylic acid method [17].

Determination of Enzyme Activity

The endoxylanase activity was determined by measuring the reducing sugar released from oat spelts xylan [16]. One unit of endoxylanase activity was defined as the amount of enzyme that liberates 1 μmol of xylose equivalent per minute at 60°C. β-Xylosidase activity was assayed by measuring the released *p*-nitrophenol (*p*NP) from 5 mM *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX). One unit of β-xylosidase activity was defined as the amount of enzyme releasing 1 μmol of *p*NP from *p*NPX per min at 40°C.

Preparation of Corncob Xylan

A 20 g of corncob was mixed with 3 N of NaOH and reacted for 4 h. After filtration of this reaction mixture, it was washed 3 times with 100 ml of warm water and then was filtered. This filtrate was neutralized with 10 N of HCl in pH 6.0. Following addition of 1 l of ethanol, the filtrate was reacted for 5 min, and then filtered again in the filter paper (90 mm). This residue was dried for 12 h and used in the subsequent experiment.

Preparation of Crude Enzyme Solutions

S. cerevisiae SEY2102 cells harboring pAEDX-1 or pAEX plasmids were precultured at 30°C in 10 ml of SD (0.67% Bacto-yeast nitrogen base without amino acids, 0.5% casamino acids, 2% dextrose) medium. The second preculture was completed in a 50-ml baffled-flask containing 50 ml of SD medium, and inoculated into fermentor containing 2 l of YPD medium. The recombinant endoxylanase in the culture supernatant of SEY2102/pAEDX-1 was collected at the early stationary phase by centrifugation (6,000 ×g, 10 min). Extracellular proteins were precipitated by adding ammonium sulfate at 70% saturation. The solution was stood overnight and the resulting precipitate was collected by centrifugation (6,000 ×g, 10 min). The precipitates were dissolved in 20 mM of phosphate buffer (pH 6.5) and dialyzed against the same buffer in a dialysis membrane with 5 changes of buffer at 2 h intervals. The dialysate was ultrafiltrated through an ultrafiltration membrane (MW cut-off=10000, Millipore, Bedford, MA, U.S.A.).

The yeast transformant, SEY2102/pAEX, was harvested by centrifugation (6,000 ×g, 15 min), washed once, and suspended in 10 mM of NaN_3 and 1.2 M of sorbitol. The cells were disrupted by Tunicase FN (Daiwa, Japan) and the resulting cell debris was removed by centrifugation (10,000 ×g, 15 min). The cell-free extract was precipitated with ammonium sulfate (70% saturation) and dialyzed against 20 mM of phosphate buffer (pH 6.5).

TLC and HPLC Analyses

Hydrolysis products of xylan by the endoxylanase and β-xylosidase were analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). First, enzyme solution (10 units of endoxylanase and 10 units

of β-xylosidase) was added to 30 ml of 4% (v/v) oat spelts, birchwood, and corncob xylan that was dissolved in 20 mM of phosphate buffer (pH 6.5) in a 250-ml beaker. The volume was adjusted to 60 ml with the same buffer and the enzyme reaction was carried out in the temperature range of 40°C to 60°C. Then, the enzyme reaction was stopped by heating the reaction mixture at 100°C for 5 min. Products from xylan and xylose hydrolysis were analyzed on the silica gel 60 F_{254} plates (Merck KGaA, Darmstadt, Germany). The solvent used was chloroform-acetic acid-water (6:7:1). Saccharides were detected by spraying the plates with an ethanol-concentrated sulfuric acid mixture (95:5). Hydrolysis products of xylan by the endoxylanase and β-xylosidase were analyzed by HPLC (Alliance 2690, Waters, Milford, MA, U.S.A.) by using TSK gel Amide-80 (Tosoh, Japan) with 65% acetonitrile as a mobile phase. One ml of the reaction mixture was sampled and then filtered through a membrane filter (ADVANTEC MFS, Inc., Japan) to remove the unhydrolyzed xylan. The xylanase reaction was stopped by heating at 100°C for 5 min. The sample was concentrated by using a centrifugal evaporator (Bioneer, Taejon, Korea) and the pellet was resuspended in 100 μl. Resuspended sample (5 μl) was injected into the column and xylose was detected by RI detector at 65°C. Xylose (Sigma, St. Louis, U.S.A.), xylobiose, and xylotriose (Megazyme, Ireland) were used as standards.

RESULTS AND DISCUSSION

Expression of Endoxylanase and β-Xylosidase Genes in *S. cerevisiae*

S. cerevisiae transformants were cultivated on YPD medium and the results were shown in Fig. 2. The total activities of the enzymes reached about 9.8 unit/ml for endoxylanase and 2.9 unit/ml for β-xylosidase. The major activity of endoxylanase was found in the extracellular medium, whereas most of the activity of β-xylosidase was detected in the periplasmic space of yeast. The expression of endoxylanase was accelerated in proportion to cell growth after cultivation for 6 h to 12 h, and then it leveled off. The expression of β-xylosidase was enhanced together with cell growth from 6 h to 30 h and then it reached a stable level of 2.5 unit/ml. The β-xylosidase activity in the cytoplasm was maintained around 0.5 unit/ml until the end of cultivation. The β-xylosidase activity was not detected in the extracellular medium and the cytoplasmic β-xylosidase activity was kept at a constant level. Since the constitutive *ADH1* promoter was employed, the expression of both genes occurred in a growth-associated manner, *i.e.*, the gradual decrease of expressions of endoxylanase and β-xylosidase might be caused by the growth retardation after the fast-growing phase. The yeast cell lysis was not detected

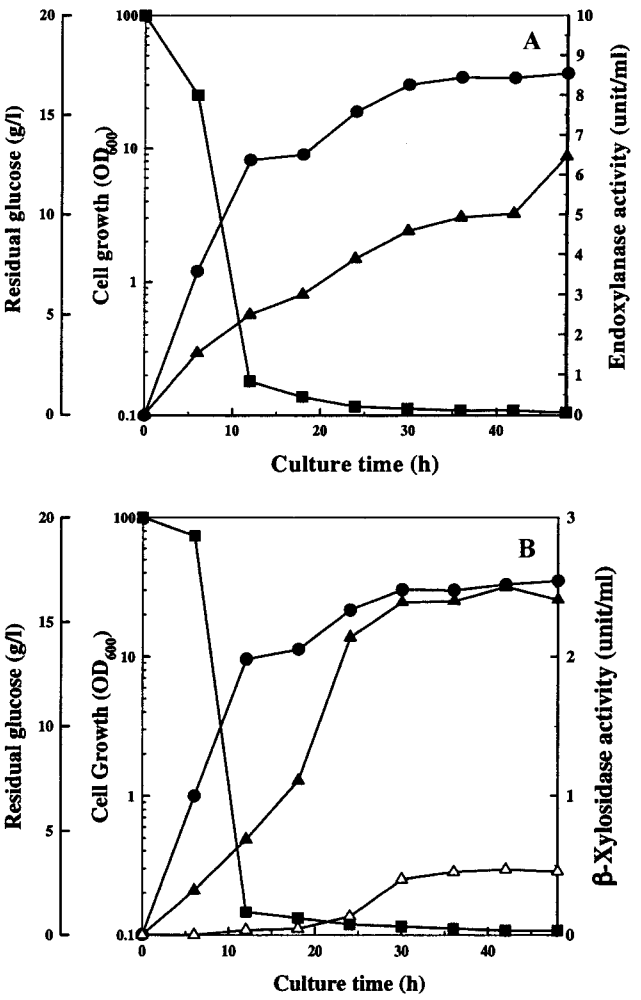

Fig. 2. Time profiles of cell growth, glucose consumption, and endoxylanase and β-xylosidase expression in the flask cultures of *S. cerevisiae* SEY2102/pAEDX-1 (A) or SEY2102/pAEX (B).
Symbols: (●), Cell growth; (■), Residual glucose; (▲), Extracellular endoxylanase activity; (▲), periplasmic β-xylosidase activity; (△), cytoplasmic β-xylosidase activity.

during the late period of growth. Therefore, the decrease of periplasmic β-xylosidase activity seems to be caused by proteolytic degradation by unknown protease(s) located within the yeast periplasmic space.

The accumulation of β-xylosidase in the yeast periplasmic space was also observed in the expression of the galactose-inducible system [13] and expression of the *B. pumilus xynB* gene in *S. cerevisiae* [14]. The retention of foreign proteins in yeast periplasmic space might arise from saturation of the secretion machinery, malfolding of heterologous proteins, or physical structure of the yeast cell wall [21].

Effect of Amount of Endoxylanase and β-Xylosidase

When only the endoxylanase (10 unit) was used in the hydrolysis of oat spelts xylan, xylose was produced after 8- 10 h reaction time, and this indicates that the complete

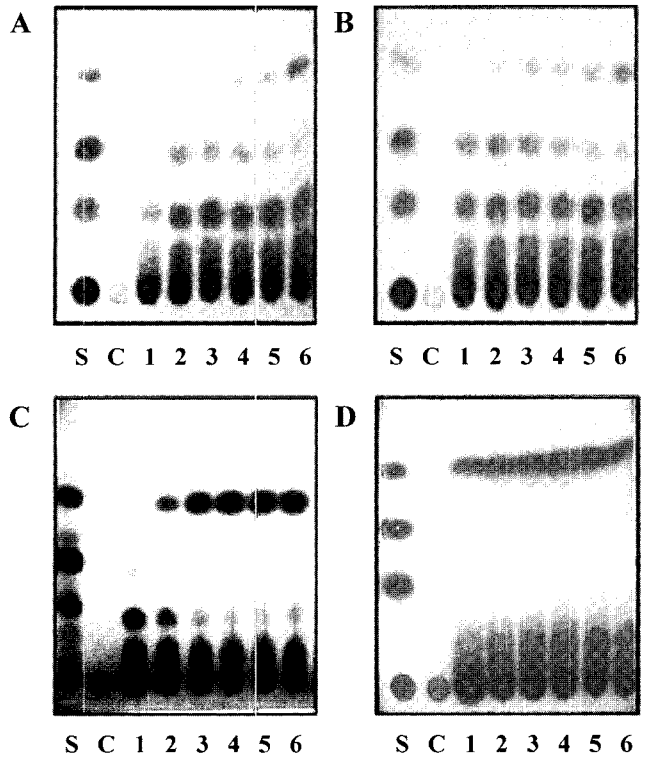

Fig. 3. Effect of amounts of enzymes on the hydrolysis of oat spelts xylan.
S, standard; C, xylan without enzymes. Reaction time: lane 1, 10 min; lane 2, 30 min; lane 3, 1 h; lane 4, 4 h; lane 5, 5 h; lane 6, 12 h. Amount of enzymes: A, 2.5 units; B, 5 units; C, 10 units; D, 25 units.

hydrolysis of xylan actually takes a long time. Among the examined xylan-degrading enzymes, the β-xylosidase was more efficient for the xylan hydrolysis than acetyl xylan esterase. Therefore, two enzymes such as endoxylanase and β-xylosidase were used to react with xylan. To investigate the effect of amount of enzymes on the hydrolysis of xylan, 2% oat spelts xylan was hydrolyzed at 40°C for 12 h with different amounts of enzymes (2.5–25 units of endoxylanase and β-xylosidase). When 2.5 and 5 units of enzyme were added to oat spelts xylan, xylobiose and xylotriose were produced after 10 min and xylose was formed after 1 h (Fig. 3). Ten units of enzymes produced the highest yield of xylose. When over 10 units of enzymes were added, xylan hydrolysis occurred after 10 min. The production of xylose was greatly increased, compared to the production of xylobiose and xylotriose. With 25 units of enzymes, the hydrolysis rate seems to be faster than that with 10 units, but the hydrolysis was almost completed after 10 min. Therefore, it is thought that the optimal amount of enzyme for the hydrolysis of xylan was chosen as 10 units of endoxylanase and 10 units of β-xylosidase. The synergic hydrolysis of xylan by these two enzymes was also observed in the birchwood and oat spelts xylans [22, 23].

Effect of Substrate Type

To study the effects of substrate type on the xylan hydrolysis, 4% (v/v) of xylans was incubated for 12 h with enzyme solutions (10 unit endoxylanase, 10 unit β-xylosidase). When the three kinds of xylan from oat spelts, birchwood, and corncob were hydrolyzed, xylose, xylobiose, and xylotriose were produced at different amounts. Xylotriose was the major product with birchwood xylans, whereas xylose was the main product with the corncob and oat spelts xylans (Figs. 4–6). It seems that as time went by, xylotriose in oat spelts xylan changed to xylose, while xylotriose and xylobiose in birchwood xylan were still left even after time. This result shows that unknown inhibitor(s) prevented the action of those enzymes that exists in birchwood xylan other than in oat spelts xylan [14, 22, 23]. Therefore, since xylobiose and xylotriose were produced with a very little amount and xylose was formed with a

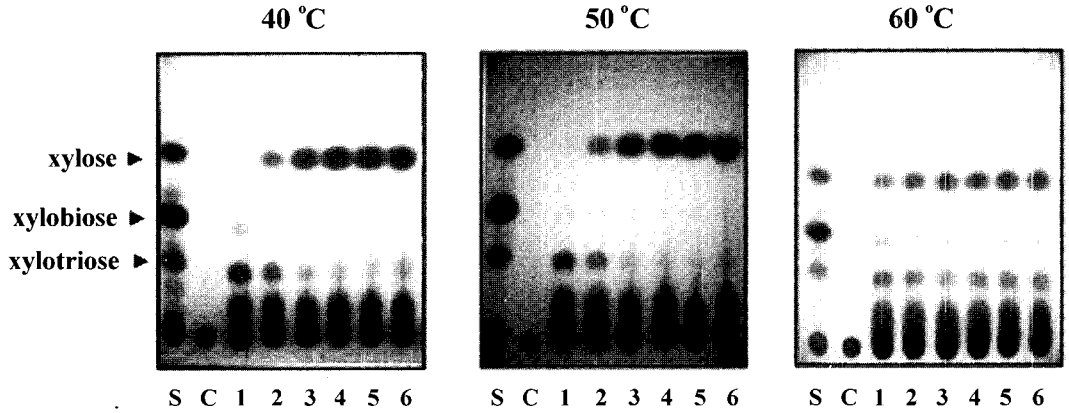

Fig. 4. Effect of reaction temperature on the hydrolysis of oat spelts xylan.
S, standard; C, xylan without enzymes. Reaction time: lane 1, 10 min; lane 2, 30 min; lane 3, 1 h; lane 4, 4 h; lane 5, 8 h; lane 6, 12 h. Each 10 units of endoxylanase and β-xylosidase was used.

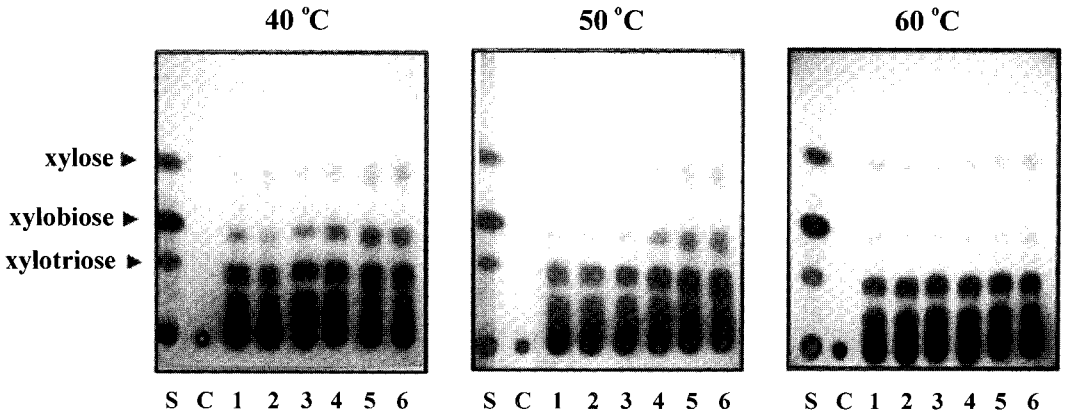

Fig. 5. Effect of reaction temperature on the hydrolysis of birchwood xylan.
S, standard; C, xylan without enzymes. Reaction time: lane 1, 10 min; lane 2, 30 min; lane 3, 1 h; lane 4, 4 h; lane 5, 8 h; lane 6, 12 h. Each 10 units of endoxylanase and β-xylosidase was used.

very high amount, it is suggested that oat spelts xylan is suitable for making a complete hydrolysis and for the production of xylose to take place by these two enzymes.

Effect of Reaction Temperature

Presumedly due to the N-linked glycosylation, recombinant endoxylanase produced in *S. cerevisiae* was not inactivated for 1 h at 60°C. In order to determine the optimal temperature in the xylan hydrolysis, enzyme reaction was carried out for 0.1-12 h at different temperatures; 40°C, 50°C, 60°C. As shown in Figs. 4, 5, and 6, a remarkable amount of xylose was produced even at 60°C, irrespective of xylan type. This result indicates that the thermostable endoxylanase can be used for the complete hydrolysis of xylan at high temperature. The hydrolysis of birchwood and oat spelts xylans occurred after 10 min of reaction, while the hydrolyzates from corncob xylan started to appear after 30 min of reaction. This result indicates that the higher the temperature becomes, the faster the rate of hydrolysis occurs. Therefore, 50°C was chosen as an optimal reaction temperature for achieving a complete hydrolysis of oat spelts xylan.

Effect of Substrate Concentration

In order to determine the optimal concentration of xylan, oat spelts xylan with different concentration levels (2-8%) was hydrolyzed at 50°C. The hydrolyzates of 2% of xylan started to appear after 30 min reaction, while the xylooligosaccharides from 6% of xylan were shown after 1 h of reaction (Fig. 7). This result indicates that the higher the substrate concentration becomes, the slower the rate of hydrolysis is obtained. When 6% of oat spelts xylan was used, the highest production of xylose was observed. At 40°C, low concentration (1-2%) of xylan was usually employed in the xylan hydrolysis [22, 23]. Our result demonstrates that high concentration (6-8%) of xylan can

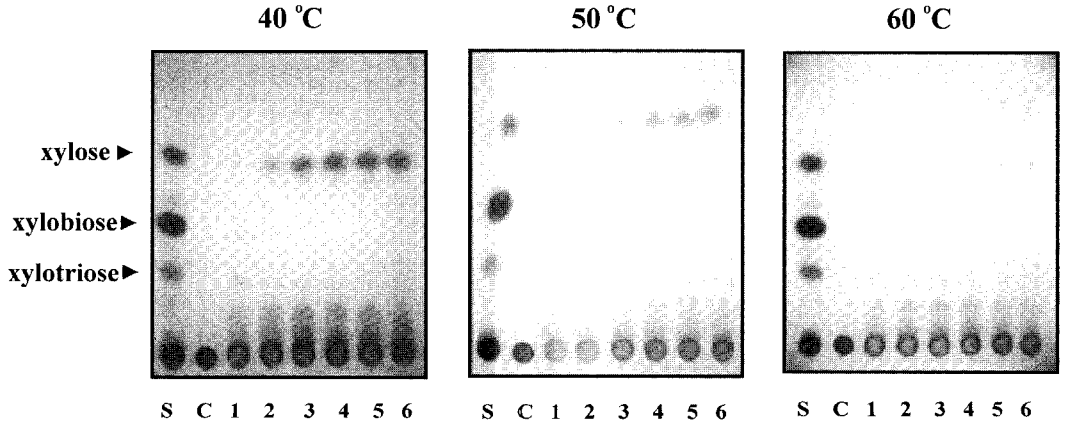

Fig. 6. Effect of reaction temperature on the hydrolysis of corncob xylan.
S, standard; C, xylan without enzymes. Reaction time: lane 1, 10 min; lane 2, 30 min; lane 3, 1 h; lane 4, 4 h; lane 5, 8 h; lane 6, 12 h. Each 10 units of endoxylanase and β-xylosidase was used.

be used and enhanced production of xylooligosaccharides and xylose may be obtained. When the xylan hydrolyzates were analyzed by HPLC under the optimal conditions, the major compound was xylose, and xylobiose with xylotriose were also observed as minor products (Fig. 8).

Previously, La Grange *et al.* [15] also showed that the degradation of xylan to D-xylose by recombinant β-xylanase (endoxylanase) and β-xylosidase expressed in *S. cerevisiae*. When the transformed yeast coexpressing both enzymes was cultivated on YPD medium containing 5% of birchwood xylan, breakdown of xylan to D-xylose was visible after a 72 h incubation period. However, in our enzymatic degradation systems, D-xylose was produced from 6% of oat spelts xylan even after 1 h of reaction with less amount of both enzymes. Therefore, an efficient D-xylose production process with high productivity may be established by further optimizing xylan hydrolysis conditions.

Consequently, in our studies, the optimized conditions for the complete hydrolysis of xylan were determined and the conditions are as follows: 10 units of endoxylanase, 10 units of β-xylosidase, temperature at 50°C, 6% of oat spelts xylan, 1 h of reaction time. In addition, the resulting xylan-hydrolyzates such as xylose, xylobiose, and xylotriose may be used as xylitol production or food additives.

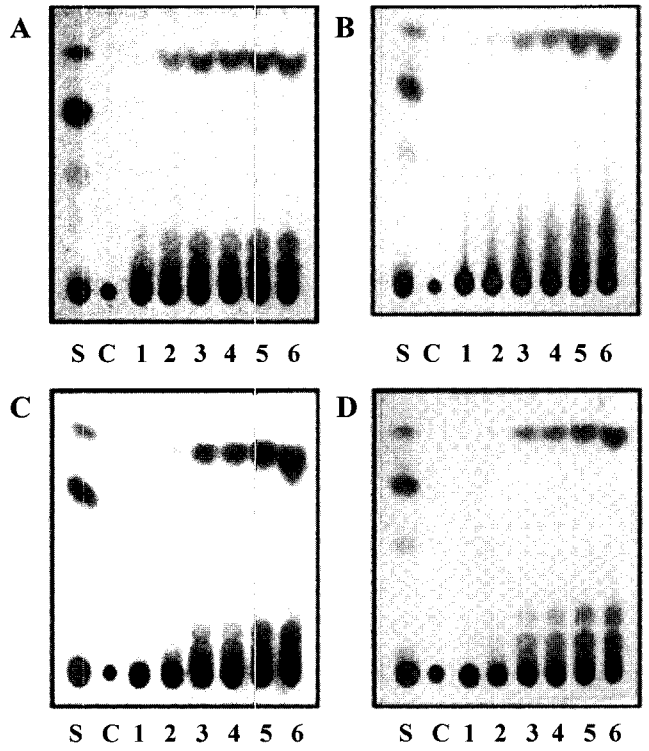

Fig. 7. Effect of substrate concentration on the hydrolysis of oat spelts xylan.
S, standard; C, xylan without enzymes. Reaction time: lane 1, 10 min; lane 2, 30 min; lane 3, 1 h; lane 4, 4 h; lane 5, 8 h; lane 6, 12 h. Amount of xylan: A, 2%; B, 4%; C, 6%; D, 8%.

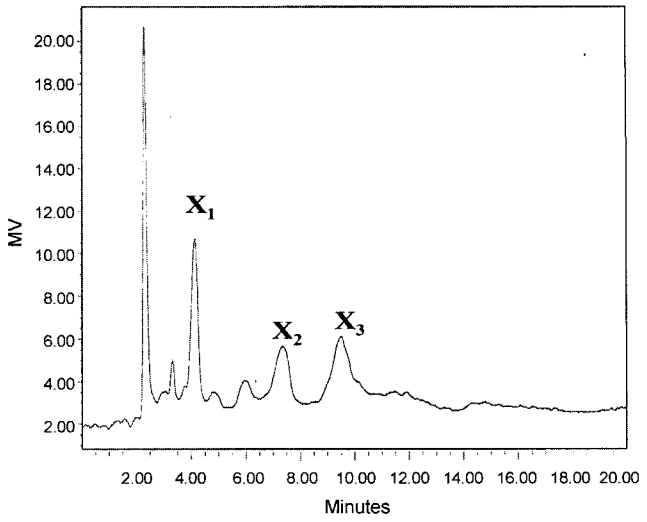

Fig. 8. HPLC analysis of the hydrolyzates of oat spelts xylan by treatment with endoxylanase and β-xylosidase.
X_1, xylose; X_2, xylobiose; X_3, xylotriose. Six percent of oat spelts xylan was incubated at 50°C for 1 h with 50 units of endoxylanase and 50 units of β-xylosidase.

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