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Purification and characterization of β-1,3-xylanase from a marine bacterium, *Alcaligenes* sp. XY-234

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A β-1,3-xylanase-producing bacterium, *Alcaligenes* sp. XY-234, was isolated from the marine environment. The organism produced endo-1,3-β-xylanase at a high level in the culture fluid. The enzyme was purified 292-fold by ammonium sulfate precipitation and several column chromatographies. The final enzyme preparation appeared to be homogeneous on disc gel electrophoresis and SDS-PAGE with a molecular mass of 59 kDa, and the p*I* was 4.0. The enzyme hydrolyzed β-1,3-xylan and larger xylooligosaccharides than xylobiose to give several xylooligosaccharides, but it could not hydrolyze xylobiose, *p*-nitrophenyl-β-D-xyloside, and β-1,4-xylan. The K_m of the enzyme was 4.0 mg/ml. Optimal pH and temperature were 7.5 and 40°C, respectively. It was stable from pH 6.0 to 10 and at a temperature of less than 40°C. The enzyme was strongly inhibited by 1 mM $HgCl_2$, $AlCl_3$, $CuCl_2$, $FeCl_3$, $HgCl_2$, $Pb(CH_3COO)_2$, and N-bromosuccinimide.

Key Words——*Alcaligenes*; marine bacterium; β-1,3-xylan; β-1,3-xylanase

Based on the structure, xylan can be divided into β-1,4-xylan and β-1,3-xylan. β-1,4-Xylan is found mainly in the hemicelluloses of the cell walls of land plants (Horton and Wolfrom, 1963; Whistler and Richards, 1970), and β-1,3-xylan constitutes the cell wall polysaccharides of seaweeds (Iriki et al., 1960). Because of their wide industrial applications, β-1,4-xylan-degrading enzymes have been extensively studied (Okazaki et al., 1985; Tan et al., 1987; Tsujibo et al., 1990; Viikari et al., 1994). In contrast, β-1,3-xylan-degrading enzymes are not well studied. One of the key β-1,3-xylan-degrading enzymes, endo-1,3-β-xylanase (EC 3.2.1.32), until now has been isolated from only three microorganisms, *Aspergillus terreus* A-07 (Chen et al., 1986), *Vibrio* sp. AX-4 (Aoki et al., 1988), and *Pseudomonas* sp. PT-5 (Yamaura et al., 1990). We recently found that in conjunction with agarase and β-1,4-mannanase prepared from the other bacteria, β-1,3-xylanase from *Alcaligenes* sp. was very useful for the isolation of protoplasts from a red alga, *Bangia atropurpurea* (Araki et al., 1994). In this paper, we report the purification and characterization of the β-1,3-xylanase from *Alcaligenes* sp. XY-234.

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Materials and Methods

Materials. β-1,3-Xylan was prepared from a green alga, *Caulerpa racemosa* var. *laete-virens*, according to the method of Iriki et al. (1960). β-1,4-Xylan and *p*-nitrophenyl-β-D-xyloside were purchased from Seikagaku Kogyo (Tokyo, Japan) and Sigma (St. Louis, MO, USA), respectively. β-1,3-Xylooligosaccharides (xylobiose to xylopentaose) were prepared by fractionation of the products released from β-1,3-xylan by the β-1,3-xylanase isolated from *Alcaligenes* sp. XY-234 by using charcoal column, Sephadex G-10 column, and thin-layer chromatographies.

Identification for isolate. Identification of the isolate was made according to the criteria described in Bergey's Manual of Systematic Bacteriology, Vol. 1 (Kersters and Ley, 1984). The mol% G+C of the DNA range was measured by thermal denaturation (Marmur and Doty, 1962).

Enzyme assay. The reaction mixture contained 2 ml of 0.5% β-1,3-xylan suspension in 50 mM 2-morpholinoethane sulfonic acid (Mes)-NaOH buffer, pH 7.0 and 0.5 ml of the enzyme solution. After incubating at 37°C for 10 min the reducing sugar generated was determined by the Somogyi-Nelson method (Somogyi, 1952) and expressed as xylose. One unit of β-1,3-xylanase activity was defined as the amount of enzyme producing 1.0 μmol of xylose from the substrate per

min under the above conditions. Protein concentration was determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard. The absorbance at 280 nm was used for monitoring protein in the column effluent.

Preparation of crude enzyme from culture supernatant of Alcaligenes sp. XY-234. The organism was inoculated into two 100 ml cotton-plugged Erlenmeyer flasks containing 20 ml of the peptone medium supplemented with 0.3% β-1,3-xylan and incubated at 25°C for 24 h. The peptone medium was composed of 0.5% Polypepton, 0.1% yeast extract, 2% NaCl, 0.05% $MgSO_4$, 0.2% K_2HPO_4, and 0.04% KH_2PO_4 (pH 7.5). Each seeding culture was transferred to 700 ml of the same medium in a 2,000 ml cotton-plugged flask and incubated with shaking (120 strokes/min) at 25°C for 2 days. The culture was centrifuged at 9,000×*g* for 20 min and the culture supernatant was used to purify β-1,3-xylanase according to the following scheme.

Purification of β-1,3-xylanase. All operations for purification were performed at 4°C unless otherwise stated. The last three steps of chromatography were run by a fast-protein liquid chromatography (FPLC) system (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Step 1: The culture supernatant (1,300 ml) of *Alcaligenes* sp. XY-234 was adjusted to 75% saturation with solid ammonium sulfate and allowed to stand overnight. The precipitate was collected by centrifugation, dissolved in a small volume of 50 mM sodium acetate buffer (pH 6.0), and dialyzed against the same buffer overnight.

Step 2: The enzyme preparation obtained from step 1 was applied to a Q Sepharose FF column (2.0×30 cm, Pharmacia LKB Biotechnology), previously equilibrated with 50 mM sodium acetate buffer (pH 6.0). After being washed with the same buffer until no protein was detected, the column was eluted with a linear gradient of NaCl from 0 to 1.5 M in the same buffer (total 600 ml) at a flow rate of 30 ml/h. The active fractions appearing around 0.4 M NaCl were pooled and concentrated by dialyzing against saturated ammonium sulfate solution reverse salting-out. The precipitate collected by centrifugation was dissolved in a small volume of 50 mM sodium acetate buffer (pH 6.0) containing 0.3 M NaCl.

Step 3: The enzyme preparation from step 2 was applied to a Toyopearl HW-55S column (2.5×100 cm, Tosoh Co., Tokyo, Japan) previously equilibrated with 50 mM sodium acetate buffer (pH 6.0) containing 0.3 M NaCl. Elution was performed with the same buffer at a flow rate of 20 ml/h. The active fractions were concentrated with a Diaflo membrane ultrafiltration system (UHP-43K, Advantec, Tokyo, Japan) and dialyzed against 50 mM Tris-HCl buffer (pH 7.3).

Step 4: The final ammonium sulfate concentration of the dialyzed enzyme solution obtained from step 3 was adjusted to 1.7 M by the addition of an equal portion of 3.4 M ammonium sulfate solution. The enzyme solution was then applied to an Ether-Toyopearl 650S column (1.0×10 cm, Tosoh Co.) equilibrated with 50 mM Tris-HCl buffer (pH 7.3) containing 1.7 M ammonium sulfate. The column was washed with 30 ml of the same buffer containing 1.7 M ammonium sulfate and eluted with a reverse linear ammonium sulfate gradient from 1.7 to 0 M in 50 mM Tris-HCl buffer (pH 7.3) at a flow rate of 30 ml/h. β-1,3-Xylanase activity appeared around 0.05 M ammonium sulfate.

Step 5: The active fraction obtained from step 4 was dialyzed against 1 mM sodium phosphate buffer (pH 6.8). The dialysate was applied to a hydroxyapatite column (1.0×10 cm, Wako, Ltd., Tokyo, Japan) equilibrated with the same buffer. After the column was washed with the same buffer, the enzyme was eluted with a linear gradient from 1 to 200 mM phosphate buffer (pH 6.8) at a flow rate of 30 ml/h. The active fractions appeared around 150 mM phosphate buffer.

Step 6: The active fraction obtained from step 5 was dialyzed against 50 mM Tris-HCl buffer (pH 7.3) and applied to a Mono Q column (0.5×5.0 cm, Pharmacia LKB Biotechnology) equilibrated with the same buffer. After it was washed with the same buffer, the column was eluted with a linear NaCl gradient from 0 to 1 M in the same buffer at a flow rate of 30 ml/h. The active fractions that appeared at about 0.67 M NaCl were pooled and used as the final enzyme preparation.

Analytical methods. Native polyacrylamide disc gel electrophoresis (native PAGE) was performed on 7% gel in a Tris-glycine buffer (pH 8.3) (Davis, 1964). Sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970), using 12.5% acrylamide. The Pharmacia low-molecular-weight standards (MW: 14.4, 20.1, 30, 43, 67, and 94 kDa) were used as molecular weight markers. The gel was stained by Coomassie brilliant blue R-250. Zymography was achieved after the electrophoresis was completed. SDS was removed from the gel by washing twice for 1 h in 0.1 M phosphate buffer (pH 6.8); then the gel was placed on a 1.5% agarose gel sheet (10 mm thick) containing 0.3% β-1,3-xylan and 0.1 M phosphate buffer (pH 6.8) in the Petri dish. The polyacrylamide gel and agarose gel were incubated for 1 h at 37°C. Isoelectric focusing was carried out in a 110 ml column by the method of Vesterberg and Svensson (1966). Ampholines with pH ranges from 3.5 to 10 and 2.5 to 5.0 were used. The N-terminal amino acid sequence of the purified β-1,3-xylanase was determined by a gas-phase peptide sequencer (Model 476; Applied Biosystems, Branchburg, NJ, USA). Thin-layer chromatography of xy-

looligosaccharides was performed on a precoated Silica Gel 60-plastic sheet (Merck & Co. Inc., Rahway, NJ, USA), developed with *n*-butanol-acetic acid-water (10 : 5 : 1), and the saccharides were visualized by spraying the plate with diphenylamine-aniline-phosphate reagent (Bailey and Bourne, 1960).

Results and Discussion

Identification of β-1,3-xylanase-producing bacterium strain no. XY-234

Strain no. XY-234, which produces β-1,3-xylanase, was isolated from sea bottom mud in 1990. The strain belonged to *Alcaligenes*: Gram negative, rod shape, peritrichate flagella, positive on catalase and oxidase tests, nonproduction of acid and gas from glucose, nonproduction of indole, and G+C content of 61.5%.

Culture conditions for β-1,3-xylanase-productivity of Alcaligenes sp.XY-234

β-1,3-Xylanase of *Alcaligenes* sp. XY-234 productivities were compared with peptone media containing 0.5% concentration of various carbohydrates (β-1,3-xylan, dry powder of *Caulerpa racemosa* var. *laetevirens*, β-1,4-xylan, D-xylose, pectin, wheat flour, soybean powder, and refined rice flour). The organism produced β-1,3-xylanase only when grown in the culture medium containing β-1,3-xylan. It was cultured in peptone media containing β-1,3-xylan of various concentrations. The high activity was detected in the concentration of above 0.3%, but no activity in the medium without inducer (Fig. 1). The bacterial β-1,3-xylanase was therefore found to be an induced enzyme. When the organism was cultured in peptone media containing NaCl concentration of 0, 0.2, 0.5, 0.75, 1.0, 2.0, 3.0, 5.0, and 10%, the concentrations of 1 and 2% were most effective on β-1,3-xylanase production. The organism could not grow in media having a concentration of NaCl under 0.5% and above 7%. The test was carried out in media of initial pH 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5. The suitable pH was 7.5. When the enzyme activity at the culture temperature of 25°C was arbitrarily set at 100, those at 15, 20, 30, and 35°C were 45, 95, 60, and 0, respectively. When incubated at 25°C for 2 to 5 days, the organism produced with a high level of β-1,3-xylanase into the culture fluid. Based on these results, *Alcaligenes* sp. XY-234 was cultured under the condition described in MATERIALS AND METHODS, and the culture supernatant was used to purify β-1,3-xylanase.

Purification of β-1,3-xylanase

According to the purification scheme, the enzyme was purified 292-fold with a recovery of 9%. The final enzyme preparation was found to contain one protein

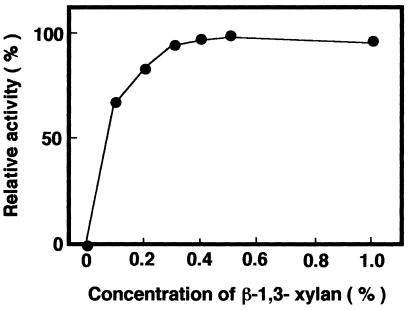

Fig. 1. Effect of the concentration of β-1,3-xylan in culture medium on β-1,3-xylanase-production of *Alcaligenes* sp. XY-234.

The organism was inoculated into 100 ml cotton-plugged Erlenmeyer flasks containing 20 ml of the peptone medium supplemented with a different concentration of β-1,3-xylan and incubated at 25°C for 2 days. After centrifugation, the enzyme activity in the culture supernatant was detected.

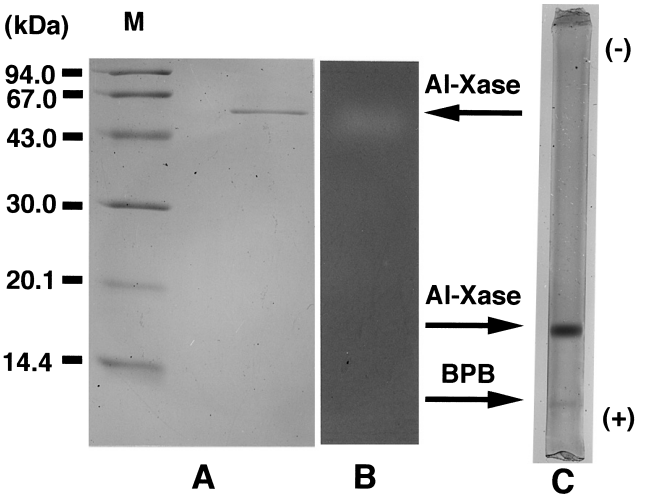

Fig. 2. SDS-PAGE (A), zymography (B), and native PAGE (C) of purified β-1,3-xylanase from *Alcaligenes* sp. XY-234.

Al-Xase, purified β-1,3-xylanase; BPB, bromophenol blue; M, standard proteins: phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), α-lact albumin (14.4 kDa). The clear band exhibited on zymogram was formed by the degradation of β-1,3-xylan mixed in agar plate.

band by both native PAGE (Fig. 2C) and SDS-PAGE (Fig. 2A), and formed a clear zone by the degradation of β-1,3-xylan mixed in agar gel (Fig. 2B). Table 1 summarizes the recovery of β-1,3-xylanase from 1,300 ml of the culture fluid of *Alcaligenes* sp. XY-234.

Molecular mass and isoelectric point

The molecular mass of *Alcaligenes* β-1,3-xylanase was estimated to be 59 kDa by SDS-PAGE (Fig. 2A). The molecular mass of β-1,3-xylanase of *Alcaligenes* sp. XY-234 was compared between the β-1,3-xylanases of three reported organisms; thus it was found to be larger than those of *Vibrio* sp. AX-4 (53 kDa),

Table 1. Purification of β-1,3-xylanase from the culture fluid of *Alcaligenes* sp. XY-234.

Steps	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Culture fluid	65.2	1,094	0.06	1	100
$(NH_4)_2SO_4$ precipitate	40.3	57.1	0.71	12	62
Q Sepharose FF	30.1	7.6	4.0	67	46
Toyopearl HW-55	16.7	2.1	8.0	133	26
Ether Toyopearl 650S	9.0	0.7	12.9	215	14
Hydroxyapatite	7.3	0.45	16.2	270	11
Mono Q	5.6	0.32	17.5	292	9

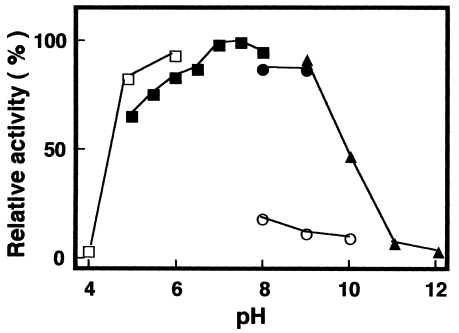

Fig. 3. pH-activity curve of β-1,3- xylanase from *Alcaligenes* sp. XY-234.

□, sodium acetate-acetic acid (pH 4.0–6.0); ■, Mes-NaOH (pH 5.0–8.0); ●, Hepes-NaOH (pH 8.0–9.0); ○, Ches-NaOH (pH 8.0–10.0); ▲, glycine-NaOH (pH 9.0–12.0).

Pseudomonas sp. PT-5 (35 kDa), and *Aspergillus terreus* A-07 (six enzymes; 11 to 20 kDa). By isoelectric focusing, the p*I* was estimated to be 4.0.

Effect of pH and temperature

As shown in Fig. 3, the enzyme exhibited pH optimum of about pH 7.5, using 50 mM buffers of sodium acetate-acetic acid (pH 4.0–6.0), Mes-NaOH (pH 5.0–8.0), 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (Hepes)-NaOH (pH 8.0–9.0), 2-N-cyclohexylamino ethanesulfonic acid (Ches)-NaOH (pH 8.0–10), and glycine-NaOH (pH 9.0–12.0). The enzyme activity was strongly inhibited by Ches-NaOH buffer in comparison with Hepes-NaOH buffer. The optimum pH of *Pseudomonas* xylanase, as well as *Alcaligenes* xylanase, was 7.5. However, *Vibrio* xylanase and six *Aspergillus* xylanases showed maximal activity on the acidic side, pH 6.0 to 6.5 and 4.0 to 5.5, respectively.

The pH stability of *Alcaligenes* xylanase was studied by placing the enzyme in the buffers of various pHs described above at 4°C for 24 h. The residual activity was then assayed under standard conditions at pH 7.5. The enzyme was found to be stable in the pH range of 6.0 to 10, retaining 90–100% of the activity. Even at pH 4.0 and 12, the enzyme retained 65 and 70% of the activity, respectively.

The enzyme showed the maximum activity at 40°C for 10 min incubation at various temperatures. When the enzyme dissolved in 50 mM Mes-NaOH buffer (pH 7.5) was exposed to different temperatures for 10 min, it retained full activity below 40°C, but it rapidly lost its activity at a temperature higher than 40°C. At 50 and 60°C, the enzyme lost 70 and 99% of its activity, respectively. It was found to be extremely sensitive to temperatures above 40°C.

Kinetics and inhibitors

The enzymatic hydrolysis was performed in the presence of various concentrations of β-1,3-xylan (0.05–1.0 mg/ml). The K_m value obtained from Lineweaver-Burk plot analysis was 0.40 mg/ml.

The effect of metal ions and chemical reagents at 1 mM concentration against *Alcaligenes* xylanase activity was determined. The enzyme activity was almost completely inhibited by 1 mM $CuCl_2$, $FeCl_3$, and $HgCl_2$, and AgCl, $AlCl_3$, $Pb(CH_3COO)_2$, and dithiothreitol gave 55–90% inhibition at the same concentration. $BaCl_2$, $CaCl_2$, KCl, $MgCl_2$, $MnCl_2$, NaCl, $ZnCl_2$, ethylenediaminetetraacetic acid, N-ethylmaleimide, iodoacetic acid, and *p*-chloromercuribenzoic acid had very little effect on the enzyme activity. *Alcaligenes* xylanase was also inhibited perfectly by 1 mM N-bromosuccinimide (NBS) solution, which is the modification reagent for tryptophan residue in protein. NBS is reported to give the activities of *Pseudomonas* xylanase and six *Aspergillus* xylanases 66% and 82 to 97% inhibition, respectively. In this way, the β-1,3-xylanases described above were inhibited strongly with NBS reagent. It can therefore be presumed that tryptophan plays an important role in β-1,3-xylanase catalysis.

N-Terminal amino acid sequence

The first 13 N-terminal amino acid sequences of β-1,3-xylanase from *Alcaligenes* sp. XY-234 were determined and found to be Leu-Asp-Gly-Lys-Leu-Val-Pro-Asn-Glu-Gly-Val-Leu-Val. The N-terminal amino acid sequences were compared with those of *Pseudomonas xylanase* (Val-Ser-Gly-Thr-Leu-Val-Pro-Asp-Asp-Gly-Val-Met-Leu), of which the N-terminal amino acid sequence had been the only one reported until now. For this region a 46% sequence homology was between them.

Substrate specificity

As shown in Fig. 4, *Alcaligenes* xylanase released β-1,3-xylotriose and β-1,3-xylobiose as the main products from β-1,3-xylan prepared from the green alga, *Caulerpa racemosa*. It also produced xylotetraose, xylopentaose, xylohexaose, and xylose as minor prod-

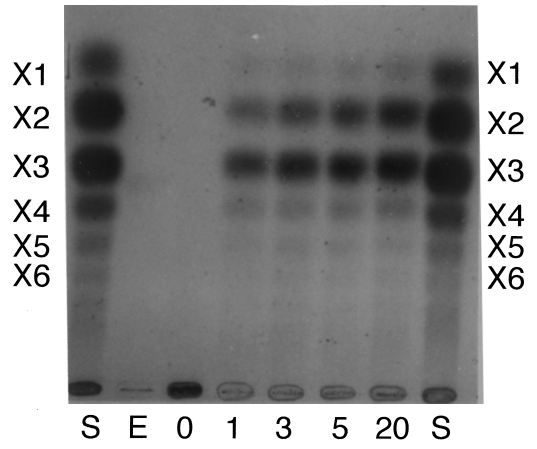

Fig. 4. Thin-layer chromatogram of hydrolysis products of β-1,3-xylan with β-1,3-xylanase from *Alcaligenes* sp. XY-234.

E, enzyme; S, standard; X1–X6, xylose–xylohexaose; 0, 1, 3, 5, 20, reaction times (h) of the enzyme with β-1,3-xylan at 37°C. The reaction mixture consisted of 100 μl of 2 mM sodium acetate buffer, pH 6.0, containing 1% β-1,3-xylan and 100 μl enzyme solution (0.2 unit).

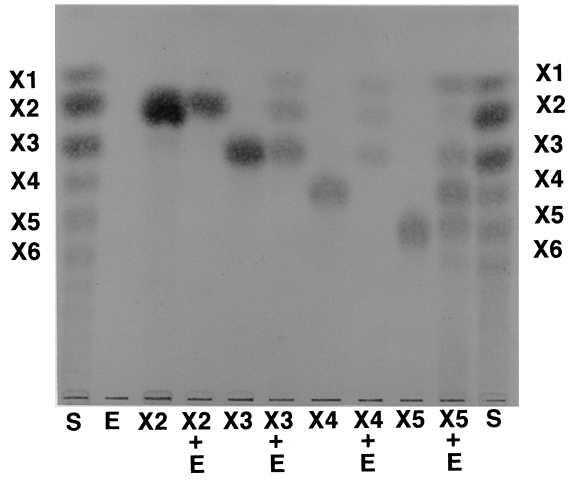

Fig. 5. Thin-layer chromatogram of hydrolysis products of several xylooligosaccharides with β-1,3-xylanase from *Alcaligenes* sp. XY-234.

E, enzyme; S, standard; X1–X6, xylose–xylohexaose. The reaction mixtures consisted of 50 μl of 2 mM sodium acetate buffer, pH 6.0, containing different xylooligosaccharides, and 50 μl enzyme solution (0.1 unit) and were incubated at 37°C for 20 h.

ucts. Therefore the enzyme was found to be an endo-1,3-β-xylanase.

The enzyme hydrolyzed xylotriose to form xylose and xylobiose. The hydrolysis products of the enzyme from xylotetraose were xylotriose, xylobiose, and xylose, and those from xylopentaose were xylotetraose, xylotriose, xylobiose, and xylose (Fig. 5). The enzyme did not cleave xylobiose, *p*-nitrophenyl-β-D-xyloside, or β-1,4-xylan.

Alcaligenes xylanase exhibited a similar action pattern to *Vibrio* xylanase, but a different pattern from *Aspergillus* xylanases on xylooligosaccharides. That is, the three enzymes among six β-1,3-xylanases from *A. terreus* A-07 hydrolyzed xylotetraose to give xylobiose, xylotriose, and isomeric xylotetraose, and the other scarcely acted on xylotetraose. The action pattern of β-1,3-xylanases from *Pseudomonas* sp. PT-5 for xylooligosaccharides is not published.

The cell wall of a red alga, *Bangia atropurpurea*, is composed of three kinds of polysaccharides (β-1,3-xylan, β-1,4-mannan, and porphyran). We tried to produce protoplasts from the alga by using *Alcaligenes* xylanase addition to the β-1,4-mannanase and agarase capable of degrading porphyran so that we could obtain a large number of protoplasts (Araki et al., 1994). β-1,4-Mannanase (Araki et al., 1992) and agarase were prepared from marine bacteria stocked in our laboratory. However, the yield of protoplasts from the alga by treatment with the mixture of β-1,4-mannanase and agarase without β-1,3-xylanases was very poor. *Alcaligenes* xylanase was therefore found to be a superior enzyme for protoplast isolation from *B. atropurpurea*.

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