Optimization for β-Mannanase Production of a Psychrophilic Bacterium, Flavobacterium sp.

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We found that a psychrophilic bacterium, Flavobacterium sp., characterized in this study, has a β-mannanase (EC 3.2.1.78) activity in the culture medium. The mannanase activity was the highest in the culture medium, containing 1.0% (w/v) guar gum as a carbon source, 0.3% (NH₄)₂SO₄ as a nitrogen source, and 0.06% (w/v) yeast extract, of five-days cultivation at 4°C. No mannanase activity was found in the medium containing a monosaccharide or a disaccharide as a carbon source, although the psychrophile could use them. The enzyme activity was found only when the bacterium was cultured in the medium containing a polysaccharide. The enzyme preparation showed a single activity band on a washed gel of SDS-PAGE. The optimal temperature for the enzyme activity was 35°C. When the reaction was done at 10°C, the enzyme showed 25% of the optimal activity. The β-mannanase preparation efficiently hydrolyzed guar gum, locust bean gum, and glucomannan as well as β-mannan.

Key words: β-mannanase; psychrophile; Flavobacterium sp.; optimized enzyme production

β-Mannanase(1, 4-β-D-mannan mannanohydrolase, EC 3.2.1.78), which catalyzes the random hydrolysis of 1,4-β-mannosidic linkages in 1,4-β-mannan, glucomannan, and galactomannan, is an important enzyme for an effective utilization of various plant β-mannans as bioresources. Mannanases have been found in many organisms including plants,(2,3) bacteria,(4) fungi,(5-9) actinomycetes,(10) and mollusks(11) and characterized. Bacterial mannanases are mostly obtained from mesophiles.(4,12,13) Although a few mannanases of thermophiles(14) and alkalophilites(15) are reported, few enzymes produced by psychrophiles have been found.

While mesophilic β-mannanases generally show the maximum activity at comparatively high temperatures of more than 50°C and relatively low activity at a low temperature, psychrophilic mannanases may have significant activity under low temperature conditions: it is well known that psychrophilic enzymes show significant activity under low temperature conditions. The psychrophilic β-mannanases should be useful for decreasing the viscosity of food materials, which causes some trouble in food processing, at low temperatures. The food processes at low temperatures are preferable to prevent deterioration of the food materials, especially disruption of thermolabile nutrients.

This paper describes the isolation of a β-mannanase-producing psychrophilic bacterium, Flavobacterium sp., and the optimum conditions for the enzyme production. In this study, we used the definitions of Ingraham and Stokes(16) for the classification of cold-adapted bacteria.

Materials and Methods

Materials. Glucomannan (GM) of Amorphophallus konjac was purchased from Wako Pure Chemical Industries. β-Mannan of Codium fragile prepared by the method of Love and Percival(17) was offered by T. Araki, Mie University. Locust bean gum (LBG), guar gum (GG), and xylan (from birch wood and oat spelt) were obtained from Sigma Chemical Company. α- and β-cyclodextrins from Hayashibara Biochemical Industries Inc., cellulose from Toyohashi Co. Ltd. and pectin from Nacalai Tesque Inc. were purchased. A silica gel 60-glass sheet (HPTLC Fertigplatten Kieselgel 60) was purchased from Merck & Co. Inc. All other chemicals were of analytical grade.

Microorganisms. Three psychrophilic bacteria (strain Anti-300, strain P1, and Bacillus psychrophilus), which were gifts from the Institute for Chemical Research, Kyoto University, and four GM-using psychrophilic bacterial strains that had been isolated and stored in our laboratory were used.

Media. The isolation and selection media for β-mannanase-producing bacteria consisted of 0.3% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.15% NaH₂PO₄, 0.02% MgSO₄·7H₂O, 0.01% yeast extract, and 1.0% GM. GM was replaced with other carbon sources when an effect of carbon sources on the enzyme production was examined. Various nitrogen compounds (0.1% each) were added to the isolation medium to examine their effect on the enzyme productivity.

Selection and isolation of β-mannanase-producing psychrophilic bacteria. The three psychrophilic bacteria obtained from Kyoto University were inoculated in 100 ml of the isolation medium and cultured at 4°C for 72 h. A bacterium grown in the medium was selected as a β-mannanase-producing psychrophilic bacterium. The four GM-using psychrophilic bacteria were isolated from various sources such as milk and soil with the isola-
tion medium. The selected and isolated bacteria were stored on agar plates of the isolation medium containing 1.8% (w/v) agar.

*Growth and enzyme preparation.* The bacterium was grown aerobically at 6°C for 72 h in 200 ml of the isolation medium. Cell growth was measured by the turbidity of culture broth at 600 nm. Cells were removed by centrifuge at 9500 × g for 20 min at 4°C. The supernatant was adjusted to pH 7.0 with potassium phosphate buffer (KPB) (in a final concentration of 20 mM). The solution was used as the enzyme solution.

*Enzymatic hydrolysis and thin-layer chromatography.* The polysaccharides, GM, LBG, GG, and mannan (25 mg each) were incubated in 5 ml of 100 mM KPB (pH 7.0) containing 200 μg of the crude enzyme preparation at 30°C for 6 h. The reaction mixtures (1.5 ml) containing hydrolyzed products were used for thin-layer chromatography, which was done on a silica gel sheet developed with a solvent of n-butanol-pyridine-water (6:4:3). Products were made visible with a diphenylamine-aniline-phosphate reagent.18

*Enzyme and protein assays.* The reaction mixture (1 ml) consisted of 5 mg of GM, 100 μmol of KPB (pH 7.0), and enzyme, was incubated at 30°C for 10 min. Reducing sugars formed were measured by the Somogyi-Nelson method20 with β-mannose as the standard sugar. One unit of enzyme activity was defined as the amount of the enzyme that released 1 μmol of reducing sugar per min.

Protein was measured by a dye binding method20 with bovine serum albumin as a standard.

*Electrophoresis and activity staining.* Native and SDS polyacrylamide slab gel electrophoresis were done by the methods of Davis21 and Laemmli,22 respectively. Samples were prepared without a heat treatment in both the electrophoreses. Crude culture filtrate was concentrated 10-fold by an ultrafiltration with an Ultrafree C3LG (Millipore) and then used as the samples. The molecular weight standards used in the SDS-PAGE were a kit of Bio-Rad consisting of phosphorylase b (Mr 97,400), serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500), and lysozyme (14,400). After the electrophoresis, the gels were washed three times with 200 ml of 20 mM potassium phosphate buffer (pH 7.0) containing 0.01% 2-mercaptoethanol and 5 mM mannitol. Then the washed gels were activity-stained with Congo Red by the method of Beguin.23

**Results**

*Selection and isolation of a β-mannanase-producing psychrophile*

We first examined growth of three psychrophilic bacteria (Ant 300, P1 and Bacillus psychrophilus) in the isolation medium (pH 7.0) under the low temperature conditions, and found that only strain P1 grew in the medium. The growth curve, decrease in viscosity, and extracellular β-mannanase activity were measured for the strain P1 at 4°C as shown in Fig. 1. After a week, the growth of the strain P1 reached the maximum, while the viscosity of the culture medium decreased to about 20% of the initial viscosity after only 2 days of cultivation. β-Mannanase activity was the highest in the culture medium of 5 days of cultivation: the maximum specific activity was 0.48 U/mg. We have isolated four β-mannanase producing bacteria from various natural sources. However, the β-mannanase activities in the culture medium of the bacteria were lower than that of the strain P1. Thus, we selected the strain P1 as the object of our study.

*Characterization of strain P1*

Table I shows the morphological and physiological characteristics of the strain P1. The strain P1 was a Gram-negative, aerobic, catalase-positive, oxidase-positive, and non-sporing bacterium. It was a non-motile rod shaped cell (0.5–0.7 × 1.5–4.0 mm). The strain produced yellow pigments. The profile of the cellular fatty acids was typical for members of the Flavobacterium/Cytophaga-complex. The sequence of 16S ribosomal DNA of the strain showed 98% similarity to that of Flavobacterium columnare. Thus, we decided that the strain P1 is a species of Flavobacterium.

*Optimization of the β-mannanase production*

To discover the optimal conditions for the β-mannanase production by Flavobacterium sp., we investigated effects of pH, temperature, and carbon and nitrogen sources on the production. The bacterium was cultured
at 4, 15, 30, and 60°C (Fig. 1). The best temperature for the rate of cell growth was 15°C. However, it grew maximally at 4°C as shown in Fig. 1: the final cell concentration of the culture medium when grown at 4°C was 2.8-fold higher than that of the medium grown at 15°C. The cell growth was very slow and limited at 30°C. The β-mannanase activity was also the highest in the medium when grown at 4°C: the maximum values of the specific activity (U/mg) of the culture medium at 4, 15, and 30°C were 0.46, 0.24, and 0.16, respectively. The culture medium of 120-, 60-, and 60-h cultivation at 4, 15, and 30°C, respectively, showed the maximum value.

The effect of the initial pH of the medium on the β-mannanase production were examined. When the strain was cultivated at 6°C for 5 days in the medium adjusted to various pHs, the relative activities of the enzyme in the culture supernatants were 0, 27, 56, 100, 96, and 42% at pH 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0, respectively.

Effect of natural nitrogenous compounds on the activity in the cultured medium was examined. The bacterium could grow in the medium containing (NH₄)₂SO₄ as a sole nitrogen source and showed a basic β-mannanase activity (0.14 U/mg). Addition of natural nitrogenous compounds increased the final yield of the bacterial cells and the enzyme activity (Table II). The activities in the media containing yeast extract and Bacto casitone were higher than those of other nitrogenous compounds. The highest activity was obtained by the addition of yeast extract. Then, concentration of yeast extract was changed from 0.01 to 0.2% to examine the best concentration. The enzyme activity was shown to be the highest in the medium containing 0.06% yeast extract: the specific activity (U/mg) of the culture medium added 0.00, 0.01, 0.02, 0.06, 0.08, 0.10, 0.15, and 0.20% of yeast extract was 0.14, 0.40, 0.43, 0.47, 0.42, 0.35, 0.30, and 0.20, respectively. The turbidity of the media containing various concentrations of yeast extract was almost identical. Thus, the bacteria seemed to use the ingredients contained in the yeast extract such as amino acids as a nitrogen and carbon sources with limit use of GM as a carbon source when the high concentrations of yeast extract were added. The result indicated that GM induced the β-mannanase activity.

Then, the effects of carbon sources on the enzyme activity were examined. The strain could grow in the medium containing 1% of a monosaccharide such as glucose, mannose, or galactose, or a disaccharide such as cellulose, but no mannanase activity was found by the mono- and disaccharides. The bacteria showed the β-mannanase activity when they were grown in a medium containing a polysaccharide (Table III). The enzyme activity in the media containing guar gum (GG) was the highest, followed by locust bean gum (LBG), and then those in the media containing GM and pectin. The enzyme activities in the medium with other polysaccharides were lower.

Thus, the best induction medium consisted of 1.0% (w/v) guar gum, 0.3% (NH₄)₂SO₄, 0.06% (w/v) yeast extract, 0.1% KH₂PO₄, 0.15% Na₂HPO₄, and 0.02% MgSO₄·7H₂O, in which the specific activity of β-mannanase increased to 1.46 after 5-days of cultivation.
Effects of Temperature and pH on the β-Mannanase Activity.

The reaction was done at different temperatures with 100 mM KPB (pH 7.0) (▲). Carmody buffer (an universal buffer) was used for determination of an optimal pH (●). Substrate (GM) concentration was 0.5% (w/v) as described in Materials and Methods.

Temperature Stability of the β-Mannanase.

Different samples of β-mannanase preparations were treated at different temperatures for 10 min and then used for reaction with 0.5% (w/v) GM as a substrate under standard conditions as described in Materials and Methods.

Properties of the β-mannanase preparation from Flavobacterium sp.

The supernatant of culture medium showing 0.46 specific activity was used as a crude β-mannanase preparation. To characterize the β-mannanase preparation, effects of pH and temperature on the enzyme activity were examined. Optimal pH and temperature for the activity were 7.0 and 35°C, respectively (Fig. 2). When the reaction was done at 20°C and 10°C, the β-mannanase showed 75% and 25% activities of the maximum at 35°C, respectively. On the contrary, a change in temperature from 35 to 45°C gave 70% lower activity. Then, effects of temperature on stability of the β-mannanase from Flavobacterium sp. were investigated (Fig. 3). The enzyme retained full activity at 20°C and over 90% at 40°C. The temperature at which half of the activity was lost by 10-min incubation was 48°C.

Fig. 4. Hydrolysis of Various Mannans.

The reaction mixture (1 ml) consisted of 5 mg of glucomannan (▲), locust bean gum (●), guar gum (■) or mannnan (♦), 100 μmol of potassium phosphate buffer (pH 7.0) and 30 μg of the crude enzyme protein, was incubated at 30°C for 150 min. Reducing sugar concentration was determined as described in Materials and Methods.

To find whether the β-mannanase preparation contained two or more different β-mannanases, the preparation, concentrated 10-fold by ultrafiltration, was put on native PAGE and SDS-PAGE, and then β-mannanase activity and protein were stained. The native gel showed neither activity or protein band: some viscous materials, which were not stained with Coomassie Brilliant Blue, in the applied sample hindered normal electrophoretic migration of proteins. On the contrary, the washed SDS-PAGE gel showed several well-separated protein bands and a single activity band, the molecular weight of which was estimated to be 46,000. The enzyme activity was stable in the sample buffer of SDS-PAGE. However, when the sample of SDS-PAGE was heat-treated, no activity or activity band was observed.

Reactivity of the enzyme toward various substrates were examined. The initial courses of the enzymatic hydrolysis for GM, LBG, GG and mannan are shown in Fig. 4. The hydrolytic rate for GG was the highest among the polysaccharides. The rate for GM was similar to that for LBG. Both activities for GM and LBG were higher than that for β-mannan from Codium fragile.

To analyze the products of the enzymatic hydrolysis, the enzymatic hydrolysates were put on a thin-layer chromatograph (Fig. 5). Several spots corresponding to the products were found in all lanes, proving that the mannanase catalyzed hydrolysis of GM, LBG, GG, and mannan. The chromatogram for the reaction products by the enzyme was similar to those by typical man-
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Fig. 5. Thin-layer Chromatogram of the Enzymatic Hydrolysates of Glucosanmannan (GM), Locust Bean Gum (LBG), Guar Gum (GG), and Mannan.

The standard sugars and 150-min hydrolysates of Fig. 4 were put on TLC. Lane 1, D-glucose; lane 2, D-mannose; lane 3, D-galactose; lane 4, authentic GM; lane 5, hydrolyzed GM; lane 6, authentic LBG; lane 7, hydrolyzed LBG; lane 8, authentic GG; lane 9, hydrolyzed GG; lane 10, authentic mannann, and lane 11, hydrolyzed mannan.

These showed that oligosaccharides are formed from GM, LBG, GG, and mannann during the enzyme reaction.

Discussion

We found a bacterium, strain P1, that produced a large amount of \(\beta\)-mannanase at low temperature in the culture medium containing GM. The strain P1 was a Flavobacterium. The optimal temperature for the growth rate was 15°C. The yield of cells, however, were the largest at 4°C. The bacterial strain grew well on a solid medium containing beef extract (0.3%), peptone (0.5%), guar gum (0.5%), and agar (2.0%) at -1.0°C in 7 days, but not at 35°C (data not shown). The results showed that the Flavobacterium sp. is classified as a psychrophilic bacterium according to definitions for the classification of cold-adapted bacteria.\(^{15}\) Bacterial strains of the genus Flavobacterium together with those of Pseudomonas are principal members of psychrophilic bacteria so far found in nature.\(^{16}\)

Psychrophilic bacteria generally show a high activity of extracellular enzyme when they are cultured at low temperature rather than at high temperature. In this study, the \(\beta\)-mannanase activity in the culture supernatant was stabilized by addition of 1 mM phenylmethylsulfonyl fluoride (an inhibitor of proteases) (data not shown). Thus, the highest activity found in the culture supernatant grown at 4°C may be attributable to a lower reactivity of some proteases, which probably coexisted in the culture supernatant, at the low temperature.

It may be pointed out that the mannanase activity is preferably induced by polysaccharides containing mannose or galactose as a monomeric unit, and the induction is strongly increased by heterogeneous polysaccharides containing mannanose, galactose or glucose. Thus, GM consists of glucose and mannanose; both LBG and GG of mannose and galactose; mannan from Codium fragile of mannan only;\(^{17}\) and pectin of 1,4-\(\alpha\)-linked galacturonyl residues.

The curve of optimal pH of the \(\beta\)-mannanase preparation had a shoulder at pH of 10.0. This may be attributable to either the presence of different \(\beta\)-mannanase in the preparation or some change in reactivity of the substrate depending on change of pH of the reaction mixture. In view of the presence of a single activity peak on the washed SDS-PAGE gel and the fact that the homogeneous \(\beta\)-mannanase from Bacillus sp. KK01\(^{12}\) had such a shoulder, we consider the latter may be the case.

The optimal temperature (35°C) of the \(\beta\)-mannanase activity in the preparation was the lowest among those of \(\beta\)-mannanases so far studied.\(^{2-13}\) Thermodependence of the \(\beta\)-mannanase activity in the culture medium of Flavobacterium sp. showed a characteristic pattern for psychrophilic enzyme;\(^{26}\) the activity decreased dramatically and gradually above and below 35°C, respectively. On the contrary, mesophilic enzymes from Bacillus sp. KK01\(^{12}\) and B. subtilis NM-39,\(^{13}\) which show higher (around 50°C) optimum temperatures, lose their activity to a higher extent below their optimal temperatures. These results suggested that the \(\beta\)-mannanase in the culture medium of Flavobacterium sp. is among the enzymes with low activation energy thus minimizing the decelerating effect on the reaction rate of a lowering of temperature. However, detailed studies with homogeneous preparations of the enzyme are required to test this suggestion. We are now investigating the purification of the psychrophilic \(\beta\)-mannanase from this strain to identify its structure and function, and the cloning of the mannanase gene to develop a high expression system for its technical use.

References