RESEARCH ARTICLE

ScienceAsia 35 (2009): **17–23**

doi: 10.2306/scienceasia1513-1874.2009.35.017

Over-expression and characterization of the alkalophilic, organic solvent-tolerant, and thermotolerant endo-1,4-β-mannanase from *Bacillus licheniformis* isolate THCM 3.1

Pornpimon Kanjanavas[a], Paisarn Khawsak[a], Arda Pakpitcharoen[a], Supatra Areekit[a], Thayat Sriyaphai[a], Khajeenart Pothivejkul[b], Somchai Santiwatanakul[c], Kenji Matsui[d], Tadahiko Kajiwara[d], Kosum Chansiri[a,*]

[a] Department of Biochemistry, Faculty of Medicine, Srinakharinwirot University, Bangkok 10110, Thailand
[b] Department of Biology, Faculty of Sciences, Srinakharinwirot University, Bangkok 10110, Thailand
[c] Department of Pathology, Faculty of Medicine, Srinakharinwirot University, Bangkok 10110, Thailand
[d] Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi, Japan

[*] Corresponding author, e-mail: kosum@swu.ac.th

Received 29 Apr 2008
Accepted 6 Feb 2009

ABSTRACT: Gene encoding for endo-1,4-β-mannanase (EC 3.2.1.78) from *Bacillus licheniformis* THCM 3.1 was cloned and over-expressed in pET 100/D TOPO vector. The molecular weight of the purified enzyme was about 40 kDa. This enzyme had an optimum pH of 9 and an optimum temperature of 45 °C and retained up to 77% of its activity after incubation for 48 h. The activity of the enzyme was inhibited by 10 mM of Pb^{2+}, Ag^{+}, Fe^{3+}, Sn^{2+}, Cu^{2+}, and EDTA. Although partially inhibited, the enzyme retained much of its activity when the reaction solution was mixed with 15% (v/v) of the organic solvents acetone, toluene, benzene, dimethyl sulphoxide, 2-propanol, acetonitrile, or cyclohexane.

KEYWORDS: glycosyl hydrolase enzyme, hot spring, thermotolerant bacteria, cloning

INTRODUCTION

Mannans function as carbohydrates storage in the bulbs and endosperm of some plants. Galactoglucomannans and glucomannans in softwoods and hardwoods are both branched heteropolysaccharides requiring several enzymes for their complete degradation[1]. β-Mannanase (1,4-β-D-mannan mannanohydrolase; mannan endo 1,4-β-mannosidase; EC 32.1.78) is the enzyme that cleaves the β-1,4-mannosidic linkages of mannans, galactomannans, glucomannans, and galactoglucomannans[1,2]. The β-mannanases have been grouped into two families, glycosyl hydrolase 5 (GH5) and glycosyl hydrolase 26 (GH26). The protein folding, catalytic mechanism, and mechanism of glycosidic bond cleavage are conserved in both enzyme families[3].

β-Mannanase, required for the utilization of various β-mannans, occurs in certain endosperms such as copra and ivory palm nuts, in the beans of guar, locust, and coffee, and in the roots of konjak[2]. This enzyme has found several industrial applications such as the liquefaction and extraction of fruit and coffee beans[4]. An important industrial application of mannanases is likely to be seen in pulp and paper industry. Treating the pulp with β-mannanase alone[5] or in combination with cellulase-free xylanase improves lignin extraction[6]. In doing so, one can save on bleaching chemicals and at the same time reduce wastes which may harm the environment[7]. The bleaching process requires a high temperature and alkaline conditions, but in industrial applications β-mannanases treatment is done under milder conditions[8]. There are numerous commercial and agricultural uses for *Bacillus* sp. and their extracellular products. They have been used for decades in the manufacture of industrial enzymes including several proteases, β-amylase, penicillinase, pentosanase, cycloglucosyl transferase, and several pectinolytic enzymes[9].

In this study the over-expression and characterization of thermotolerant endo-1,4-β-mannanase from *Bacillus* spp. THCM 3.1 collected from a hot spring in Chiang Mai, Thailand were investigated.

MATERIALS AND METHODS

Isolation and identification of endo-1,4-β-mannanase producing bacteria

Thermotolerant *Bacillus* spp. were isolated from hot spring water in the Chiang Mai and Ranong provinces of Thailand. Soil and water samples were cultured in Luria-Bertani (LB) medium at 45 °C for 24 h and spread on LB agar. Screening of the bacterial isolates producing mannanase was performed by cultivation in LB media at 45 °C for 14 h. The cells were separated from the culture medium by centrifugation at 8000g for 10 min. The supernatant was collected and assayed for endo-1,4-β-mannanase activity according to the Somogyi-Nelson assay[10,11]. The strain with highest enzyme activity was selected and identified by morphology under a microscope and by biochemical activity (API 50 kit, Biomerilux). To confirm the identification, a 16S rDNA sequence analysis was performed as described by Goto et al[12]. A phylogenetic tree was constructed with PAUP 4.0[13] using *Clostridium perfringens* EF153891 as the outgroup.

PCR amplification of manBL3.1

The β-mannanase encoding gene from *B. licheniformis* THCM3.1 (manBL3.1) was amplified from chromosomal DNA by using specific oligonucleotide primers. The pair of primers were designed based on the nucleotide sequences of the *B. licheniformis* gene (GenBank accession no. AE017333)[14]. The forward and reverse primers were 5′-CACCTAGTGAAAAAAAACATCGA-3′ and 5′-TTCCACGACAGGCGTCAAAGA-3′, respectively. The reaction was carried out in a 25 µl volume containing 100–200 ng of genomic DNA. The PCR mixture consisted of 1× PCR buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4), 1.5 mM $MgCl_2$, 10 mM dNTPS, 20 µM of each primer, and 3 units of *Taq* DNA polymerase (Invitrogen). Sterile distilled water was used to complete the volume to 25 µl and the reaction was performed using the Peltier Thermal Cycler (MJ Research, PTC-200). The PCR mixture was incubated at 95 °C for 3 min prior to amplification for 30 cycles. Each cycle consisted of denaturation at 95 °C for 1 min, annealing at 45 °C for 1 min, and extension at 72 °C for 1 min. The PCR product was subjected to 1.2% agarose gel electrophoresis and observed under ultraviolet light. The PCR fragment containing amplified endo-1,4-β-mannanase gene was purified by using the Qiagen PCR purification kit.

Cloning and sequencing of manBL3.1

Directional cloning of double-strand DNA was accomplished using pET 100/D TOPO vector (Invitrogen). The transformation was performed according to the instructions from the company in *Escherichia coli* OneShot TOP10 competent cells. DNA sequencing was achieved using the Big Dye Terminator Cycle Sequencing procedure and analysed using an ABI PRISM 3100 (Perkin Elmer).

Expression of *manBL3.1* and purification of its enzyme

For expression, 5–10 ng of purified recombinant DNA was added to a vial of *E. coli* BL 21 star (DE3) OneShot cells. The transformed cells were grown in 500 ml of LB containing 50 µg/ml ampicillin and 1% glucose at 37 °C until the optical density at 600 nm reached 0.5. Then the production of ManBL3.1 was induced by incubating with 1 mM IPTG for 6 h. After that, the cells were disrupted using two passages through a French pressure cell at 16 000 lb/in^2. Cell debris was removed by centrifugation at 10 000g for 20 min at 4 °C. The solution was brought to 20% saturation with ammonium sulphate and centrifuged at 4 °C and 10 000g for 15 min. All subsequent fractionation steps involving ammonium sulphate were carried out at 4 °C. After centrifugation, the pellet was discarded and the solutions were brought to 60% saturation with ammonium sulphate. Then, the suspension was centrifuged at 10 000g for 15 min and the pellet was dissolved in 10 ml of 20 mM phosphate buffer pH 7. The crude enzyme was dialysed in 10 mM Tris-HCl, pH 8 prior to application on a Toyopearl DEAE 650 column (1.7 × 12.5 cm) with a linear KCl gradient (0 to 0.5 M) in 10 mM Tris-HCl, pH 8 as eluent. All eluted fractions were stored at 4 °C prior to analysis by 15% SDS-PAGE. Protein concentrations were determined according to the Micro BCA Protein Assay (Pierce) with bovine serum albumin as standard.

Enzyme assay

β-Mannanase activity was determined by monitoring the hydrolysis of azo-carob galactomannan (Megazyme) substrate as described in Ref. 15. This assay was used to determine the optimal pH, temperature optima, and stability of the enzyme, and to study the effect of organic compounds and metal ions on its activity. The blank had the same compositions as in the assay reaction except that the enzyme solution was absent.

Optimum pH, optimum temperature, and enzyme stability

To determine the optimum pH, the enzyme activity was examined at 45 °C using azo-carob galactomannan as a substrate in the presence of the following buffers: 100 mM glycine-HCl (pH 2 and 3), 100 mM sodium acetate (pH 3–5), 100 mM NaH_2PO_4 (pH 5–7), 100 mM Tris-HCl (pH 7–9), and 100 mM glycine-NaOH (pH 9–11). To determine the temperature optima, the enzyme activity was determined at temperatures ranging from 30 to 85 °C in 5 °C intervals at the previously determined optimum pH. The enzyme stability was determined at pH 9. The enzyme was incubated at temperatures of 4, 45, and 60 °C after incubation, and the residual enzyme activities were examined at 0, 3, 6, 12, 24, 36, 48, and 72 h.

Effect of chemical reagents, metal ions and organic solvents

Each chemical reagents solution was added into the enzyme solution up to a final concentration of 10 mM and incubated at room temperature for 30 min prior to the residual enzyme activity assay at 45 °C and pH 9. Each organic solvent (final concentration, 15% v/v) was added to the enzyme solution and incubated with agitation at room temperature for 30 min prior to the enzyme assay for the residual activity at 45 °C and pH 9. Note that the enzyme was stored at 4 °C for 6 months prior to performing the assay.

Kinetic parameters determination

The enzyme activity was estimated by monitoring the release of reducing sugar from locust bean gum (LBG, Sigma). Substrate solutions containing LBG from 0.45 to 2.7% (w/v) solutions were prepared, and the activity of enzyme was measured using Somogyi-Nelson assay[10,11]. A standard curve for reducing equivalents was generated using mannose as a substrate. The Michaelis-Menten kinetic parameters K_m and $V_{\max}$ were calculated using a Lineweaver-Burk plot.

Substrate specificity

Substrate specificity of the enzyme was performed using the Somogyi-Nelson assay at 45 °C using 0.5% (w/v) of konjak galactomannan (Wako) locust bean gum, guar gum (Sigma), spino gum (Wako), gum arabic (Wako), gellan gum (Wako) and carboxymethyl cellulose (CMC, Wako) as substrates.

Table 1 Biochemical tests using API 50 CHB kit of *Bacillus* sp. THCM3.1.

Characteristics	CM3.1	Characteristics	CM3.1
Glycerol	+	Esculin	+
Erythritol	-	Salicin	+
D-Arabinose	-	Cellobiose	+
L-Arebinose	+	Melibiose	+
Ribose	+	Sucrose	+
D-Xylose	+	Trehalose	ND
L-Xylose	-	Inulin	-
Adonitol	-	Melezitose	-
β-methyl-D-Xyloside	-	Raffinose	-
Galactose	ND	Starch	-
Glucose	+	Glycogen	-
Fructose	+	Xylitol	-
Mannose	+	Gentiobiose	-
Sorbose	-	D-Turanose	-
Rhamnose	-	D-Lyxose	-
Dulcitol	-	D-Tagatose	+
Inositol	+	D-Fucose	-
Manitol	+	L-Fucose	ND
Sorbitol	+	D-Arabitol	-
α-Methyl-D-Manoside	-	L-Arabitol	-
α-Methyl-D-Gluoside	ND	Gluconate	+
N-Acetyl-Glucosamine	+	2-keto-Gluconate	-
Amygdalin	+	5-keto-Gluconate	-
Arbutin	+	Lactose	-
Maltose	+		

ND = not detected

RESULTS

Isolation and identification of endo-1,4-β-mannanase producing bacteria

Biochemical and morphological analysis revealed that all the thermotolerant bacterial isolates were gram-positive and rod-shaped. Among them, *Bacillus* sp. THCM3.1 showed the highest production of endo-1,4-β-mannanase. The biochemical tests (Table 1) and 16S rDNA sequence word identified the *Bacillus* sp. THCM 3.1 as *B. licheniformis* of GenBank accession no. EU304962.

Analysis of PCR amplification of *manBL3.1*

During the β-mannanase gene amplification from genomic DNA, a PCR product of 1080 bp in size was observed. The nucleotide and deduced amino acid sequences revealed that the putative gene belonged to the endo-1,4-β-mannanase family (glycosyl hydrolase family) when compared to the those of other endo-1,4-β-mannanases from *Bacillus*. The molecular weight of the protein, estimated by 15% SDS-PAGE, was 40 kDa.

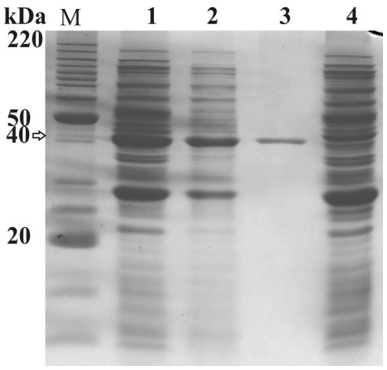

Fig. 1 SDS-PAGE analysis of purified ManBL3.1 enzyme from *B. licheniformis* THCM3.1. Lane M, protein markers; lane 1, crude extract; lane 2, the ManBL3.1 protein precipitated with 60% ammonium sulphate; lane 3, purified recombinant mannanase with AEC; lane 4, proteins from *E. coli* BL21 (DE3 inserted with pET100/D-TOPO vector).

Purification of expressed *manBL3.1*

The enzyme expressed from a recombinant clone containing *manBL3.1* was purified with ammonium sulphate and anion exchange chromatography (AEC). The endo-1,4-β-mannanase (ManBL3.1) from each purification step was analysed on 15% SDS-PAGE and showed a single band of protein (Fig. 1) after AEC (Toyopearl DEAE 650 column). The specific activities of the crude enzyme and the enzyme after 60% ammonium sulphate precipitation and AEC were 88.29, 98.52, and 625 U/mg, respectively. After AEC, the crude enzyme had been purified by a factor of 7 with a yield of 62.8% (Table 2).

Optimum pH, optimum temperature, enzyme stability and kinetic analysis

ManBL3.1 was active from pH 5–10 with the optimum at pH 9 (Fig. 2a). The ManBL3.1 showed high activity from 30 °C to 55 °C with the optimum temperature at 45 °C (Fig. 2b). The stability test revealed that ManBL3.1 was stable at pH 9 after incubation for 48 h with a residual activity of approximately 71%, 77%, and 42% at 4 °C, 45 °C, and 60 °C, respectively. Furthermore, the enzyme was still active after incubation for 72 h with a residual activity of approximately 46%, 37%, and 23% at 4 °C, 45 °C, and 60 °C, respectively (Fig. 2c). The K_m and $V_{\max}$ determined for LBG were 2.52 mg/ml and 69.5 U/mg, respectively.

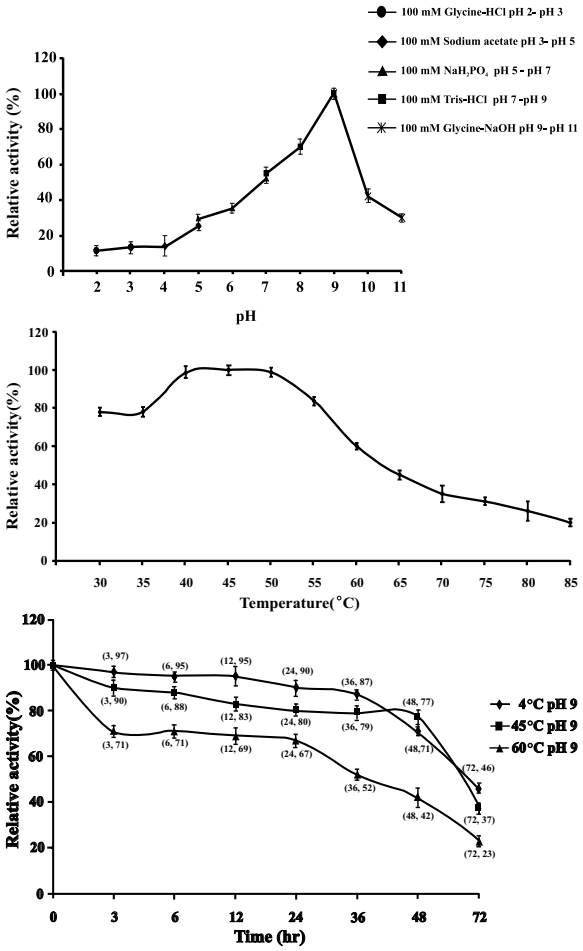

Fig. 2 Activity of β-mannanase as a function of pH, temperature, and time at pH 9 at various temperatures.

Effect of chemical reagents, metal ions and organic solvents

The effects of chemical reagents, metal ions and organic solvents on ManBL3.1 activity were determined using azo-carob galactomannan as substrate. The activity of ManBL3.1 was partially inhibited by Zn^{2+} (70% residual activity) and its residual activity dropped to 20–33% in the presence of EDTA, Pb^{2+}, Ag^{3+}, Fe^{3+}, Sn^{2+}, or Cu^{+} (Table 3). The activity of ManBL3.1 in organic solvent is an important characteristic of biocatalysts used in organic synthesis reactions. Storage of the enzyme at 4 °C for 6 months and incubation with organic solvent prior to enzyme assay revealed that ManBL3.1 was effective and stable in a solution in equilibrium with n-hexane. The residual activities of the enzyme were reduced to 75–85% after incubation with acetone, toluene, benzene, dimethyl sulphoxide, 2-propanol, acetonitrile, or cyclohexane.

Table 2 The fold purification and specific activities of the β-mannanases enzyme from each purification step.

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Recovery (%)	Fold purification
Crude enzyme	519	5.9	88.3	100	1
60% $(NH_4)_2SO_4$	335	3.4	98.5	64.5	1.1
Toyopearl DEAE 650	326	0.5	625.7	62.8	7

Table 3 Effect of metal ions (final concentration, 10 mM) on the activity of purified β-mannanases enzyme. The data are mean values of triplicate measurements with SD values.

Agents (10 mM)	Enzyme activity (U mg^{-1})	Relative activity (%)
None	956 ± 58	100
NaCl	1058 ± 66	111
KCl	1012 ± 19	106
NH_4Cl	978 ± 56	102
$MgCl_2 \cdot 6\,H_2O$	973 ± 41	102
$Pb(CH_3COO)_2 \cdot 3\,H_2O$	164 ± 33	17
$CaCl_2 \cdot 6\,H_2O$	1004 ± 87	105
$AgNO_3$	187 ± 22	20
$ZnCl_2$	675 ± 101	71
$MnCl_2 \cdot 4\,H_2O$	963 ± 80	101
$FeCl_3 \cdot H_2O$	163 ± 26	17
$CoCl_2 \cdot 6\,H_2O$	897 ± 51	94
$NiCl_2 \cdot 6\,H_2O$	932 ± 83	98
$SnCl_2 \cdot 2\,H_2O$	190 ± 32	20
$CuSO_4 \cdot 5\,H_2O$	182 ± 4	19
EDTA	316 ± 30	33

Table 4 Effect of organic solvents (final concentration, 15%(v/v)) on the activity of purified β-mannanases enzyme.

Agents (15% v/v)	Enzyme activity (U mg^{-1})	Relative activity (%)
none	461 ± 11	100
n-hexane	471 ± 71	102
Chloroform	215 ± 22	47
Acetone	405 ± 26	88
Toluene	393 ± 21	85
Benzene	411 ± 18	89
N, N-dimethylformamide	301 ± 2.9	65
dimethyl sulphoxide	378 ± 2.2	82
2-propanol	360 ± 43	78
Acetonitrile	358 ± 23	78
Cyclohexane	387 ± 17	84

Compared to the initial activity, chloroform and N, N-dimethyl formamide reduced the enzyme activity to 50% and 65%, respectively (Table 4).

Substrate specificity

The substrate specificity of ManBL3.1 was determined using several of gums as substrates. The highest hydrolysis rates were obtained with LBG and konjak glucomannan, which exhibited relative activities of 100% and 85%, respectively. The enzyme could partially digested spino gum (70%) but barely hydrolysed guar gum (7%). There was no enzymatic activity when gum arabic, gellan gum, or CMC was used as a substrate (Table 5).

Table 5 Substrate specificity test of the β-mannanases.

Substrates	Enzyme activity (U mg^{-1})	Relative activity (%)
Guar gum	0.6 ± 0.1	7
Gum arabic	ND	ND
Carboxymethyl cellulose (CMC)	ND	ND
Gellan gum	ND	ND
Konjak glucomannan	7.0 ± 1.2	85
Spino gum	5.8 ± 0.4	70
Locust bean gum	8.2 ± 0.2	100

ND = not detected

DISCUSSION

Expression of recombinant β-mannanase enzyme from *B. licheniformis* has not previously been reported. This study reports the expression and characterization of an alkalophilic and thermostable β-mannanase (ManBL3.1) which was active in organic solvents. Protein expression of *manBL3.1* was successfully achieved. Ni-NTA affinity columns failed to purify the enzyme, presumably because the six histidine C-terminus added by the TOPO system was hidden inside the structure. This is why AEC was used for enzyme purification. The characteristics and properties of ManBL3.1 enzyme were compared to those of other organisms (Table 6). According to its molecular weight, optimum pH and temperature optimum, ManBL3.1 was similar to the β-mannanase from *Bacillus* sp.[16–18] whereas the β-mannanase enzyme from other bacteria reported so far showed highest activity at neutral or slightly acidic pH. Moreover, ManBL3.1 was more stable than those of the other microorganisms shown in Table 6 at each optimum pH and temperature[15, 17, 19–21].

In the presence of metal ions, the enzyme was

Table 6 Comparison of β-mannanases from *Bacillus* sp. THCM3.1 with those of various organisms.

Species	Specific activity (U mg^{-1})	M_r (kDa)	pH optimum	Temp. optimum (°C)	pH stability	Thermal stability (°C)	Inhibitors	References
Bacillus licheniformis THCM3.1 (ManBL3.1)	625.7	40	9	45	7 (48 h) 9 (48 h)	45 (48 h) 60 (48 h)	Cu^{2+}, Pb^{2+}, Sn^{2+}, Ag^{+}, Fe^{3+}, EDTA	This study
Alkaline *Bacillus* sp. N16-5 (ManA)	5065	55	9.5	70	9 (1 h)	60 (2 h)	Hg^{2+}, Ag^{+}	Ma et al (2004)[16]
Alkaline *Bacillus* sp. (M1)	312	58	9	60	8–9 (30 min)	60 (30 min)	Ag^{+}, NBS[a]	Akino et al (1988)[22]
Alkaline *Bacillus* sp. (M2)	312	59	9	60	8–9 (30 min)	60 (30 min)	Ag^{+}, NBS[a]	Akino et al (1988)[22]
Alkaline *Bacillus* sp. (M3)	470	42	8.5	65	7–9 (30 min)	65 (30 min)	Ag^{+}, NBS[a]	Akino et al (1988)[22]
Bacillus subtilis KU-1	407.7	39	7	50–55	4.5 (48 h) 9 (48 h)	60 (1 h)	Hg^{2+}, Cr^{2+}, Mn^{2+}, Ag^{2+}, Cu^{2+}	Zakaria et al (1998)[23]
Bacillus subtilis 5H	1900	37	7	55	6–7.5 (24 h)	45 (1 h)	Hg^{2+}, Ag^{2+}, Fe^{+}	Khanongnuch et al (1998)[19]
Bacillus subtilis	-	38	5	55	4–9 (30 min)	55 (10 min)	Hg^{+}	Mendoza et al (1994)[17]
Bacillus sp. W-2	710	40	7	70	5–10 (1 h)	60 (1 h)	Hg^{2+}, EDTA	Ooi et al (1995)[18]
Rhodothermus marinus (Man26A)	-	113	5.4	85	-	70 (1 h) 90 (1 h)	-	Politz et al (2000)[20]
Thermotoga neapolitana 5068	3.8	65	7.1	92	-	90–92 (34 h)	-	Duffaud et al (1997)[15]
Dictyoglomus thermophilum Rt46B.1 (ManA)	3590	40	5	80	-	80 (16 h)	Cu^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}	Gibbs et al (1999)[24]
Enterococcus casseliflavus (M-I)	-	142	6	50	4–7 (30 min)	40 (30 min)	Hg^{2+}, NBS[a]	Oda et al (1993)[21]
Enterococcus casseliflavus (M-II)	-	137	6	50	4–7 (30 min)	40 (30 min)	Hg^{2+}, NBS[a]	Oda et al (1993)[21]

[a] NBS: N-Bromosuccinimide

strongly inhibited by Ag^{+}, Fe^{3+}, or Cu^{2+} which was similar to other β-mannanases[22–24]. The enzyme activity was inhibited with EDTA (70% of enzyme activity), as occurred with a β-mannanase of *Bacillus* sp. W-2 (50%)[18]. These observations indicate that trace divalent metal ions seem to enhance the enzyme activity.

The ManBL3.1 enzyme retained its activity by more than 65% after 30 min in most of the water-immiscible organic solvents. However, slightly water-miscible organic solvents such as chloroform could have affected the function of enzyme. The effect of solvents on enzymatic activity could result from direct interaction with the essential water surrounding the enzyme molecule. Highly polar solvents are capable of rapidly absorbing the essential water from the enzyme, depriving it of catalytic properties[25].

Acknowledgements: We are especially thankful to the Thailand Research Fund Royal Golden Jubilee PhD Programme (Grant No. PHD/0202/2546) and Japan Student Services Organization for financial support of Pornpimon Kanjanavas. Parts of this work was done through the cooperation programme "Development of Thermotolerant Microbial Resources and Their Application" of the Core University Programme between Yamaguchi University and Kasetsart University supported by the Japanese Society for Promotion of Science and the National Research Council of Thailand and Srinakharinwirot University (Grant Nos. 216/2550 and 190/2551).

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