RESEARCH ARTICLE

A low-temperature active endo-β-1,4-mannanase from *Bacillus subtilis* TD7 and its gene expression in *Escherichia coli*

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Abstract: A low-temperature active endo-β-1,4-mannanase (YBMan) from *Bacillus subtilis* TD7 was isolated, characterized and successfully expressed in *Escherichia coli* to enhance the yield of mannanase for a potential application as a gel-breaker in guar gum-based fracturing fluids in oilfields. YBMan showed good compatibility with a wide temperature range and retained about 70% relative activity at 20^{o}C compared to its optimal temperature (65^{o}C). This is the highest relative activity among reported low-temperature active mannanases against guar gum. The gene (1104 bp) of strain TD7 coding a protein with 367 amino acid residues was cloned and its expression generated two recombinant mannanases, TBMan-1 and TBMan-2. Compared to the wild type, the protein yield of TBMan-1 from a one-liter shake flask broth increased 5.6-fold, and the specific activity (crude enzyme) increased 6.4-fold. The total enzyme activity increased 35.8-fold with a total activity of approximately 79550 U. Moreover, TBMan-1 had at 20^{o}C still about 80% relative activity. The enzyme was evaluated also for its application as gel-breaker and showed excellent ability for viscosity reduction with guar gum at 20^{o}C. Low-temperature activity and high yield make the recombinant β-mannanase attractive for applications with guar-based hydraulic fracturing fluids and other biotechnological aspects.
Keywords: gel-breaker; guar gum; low-temperature active; β-mannanase; relative activity

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Received: August 19, 2018; **Accepted:** September 15, 2018; **Published Online:** September 27, 2018
Citation: Chen-Yang Li, Fang-Fang Liu, Jiang Ye, Jin-Feng Liu, Shi-Zhong Yang, Hui-Zhan Zhang and Bo-Zhong Mu., 2018, A low-temperature active endo-β-1,4-mannanase from *Bacillus subtilis* TD7 and its gene expression in *Escherichia coli*. http://doi.org/10.26789/AEB.2018.02.004.

1 Introduction

Mannanase (E.C 3.2.1.78, endo-β-1,4-mannanase) randomly hydrolyzes β-1,4-mannosidic linkages and is a crucial enzyme for biodegradation of mannans and heteromannans, such as glucomannan, galactomannan and galactoglucomannan (Zhao et al., 2011). It is widely found in softwoods, tubers, plant seeds, and beans (Saha, 2003; Dhawan and Kaur, 2007). The enzyme has been used in a number of fields, including delignification of kraft pulps, feed digestion for nutrient utilization improvement (Wang et al., 2016), detergent formulations for the removal of stains containing mannan gums (easily adsorbed onto cellulose fibers) (Srivastava and Kapoor, 2014), production of partially hydrolyzed guar gum (PHGG) with reduced viscosity for an alternative of guar gum as a food additive (Srivastava et al., 2015), conversion of lignocellulosic polysaccharides into fermentable sugars (can be subsequently used for ethanolic fermentation) (Gírio et al., 2010; Srivastava et al., 2016), and so on. In addition, mannanase is also applied for extraction of petroleum oil in low permeability oil and gas reservoirs, and thus, has received significant attention during the last decades.

Galactomannan-based guar gum has been applied widely to increase the viscosity of fracturing fluids. It is suspending and carrying proppant sands into the opened cracks of the fractured formation (Barati and Liang, 2014; Mudgil et al., 2014). To improve the oil and gas permeability and to make oil and gas flow to the wellbore in well stimulation processes, the viscosity of the hydraulic fluids in formation fractures must be reduced by gel breakers in order to be pumpable. Oxidizing agents were used traditionally as chemical gel breakers, such as potassium persulfate and ammonium persulfate. However, at low temperaturethe oxidation is poor, particularly at temperatures lower than 50^{o}C (You et al., 2016), and thus result in incomplete gel breaking. In most cases the chemical gel-breakers will lose their effect due to the low ground temperature.

Mannanase has been used as a gel-breaker to reduce the viscosity of the guar gum-based fracturing fluids. Tjonjoepin et al. reported an enzyme treatment, which attacked the β-1,4-mannosidic and α-1,6-D-galactomannosidic linkages in production operations and completions of oil and gas wells. A thermostable enzyme from *Thermotoganeapolitana* 5068 for the hydrolysis of guar gum (McCutchen et al., 1996), which is a commercial product named "Pyrolase enzyme" (Dang et al., 2010; Hu et al., 2014). A thermostable β-mannanase (DtManB) from *Dictyoglomusthermophilum* CGMCC 7283 demonstrated a great potential as an enzyme gel breaker for application in oil fields with a maximum activity towards hydroxypropyl guar gum at 80^{o}C (Hu et al., 2014). Many studies focused on thermophilic or mesophilic mannanases (Wang et al., 2015; Yang et al., 2015). However, up to date, only a few low-temperature or cold-active β-mannanases as gel-breakers are known (You et al., 2016; Zhou et al., 2012; Zakaria et al., 1998). There is still limited industrial application of mannanases at low temperature since the yield of these mannanases from wild-type strains is too low to match industrial requirements.

In this study, a low-temperature active β-mannanase (YBMan) from *Bacillus subtilis* strain TD7 (*B. subtilis* TD7) was isolated, characterized, and successfully expressed in *Escherichia coli* (*E. coli*). The highly expressed recombinant β-mannanase TBMan-1 was identified, and its effective performance as a gel-breaker for the viscosity reduction of guar gum was determined.

2 Materials and methods

2.1 Bacterial strains and plasmids

The low-temperature active β-mannanase producer *B. subtilis* TD7 was isolated from soil contaminated with crude oil and stored in our laboratory. It is a lipopeptide producer (Liu et al., 2012), but information about its production of mannanase is not available. *E. coli* JM83 and BL21 (DE3) were used for routine molecular cloning and protein expression experiments, respectively. Vector pET-28a(+) was used to construct a recombinant plasmid for gene expression.

2.2 Culture conditions

The wild-type strain *B. subtilis* TD7 was incubated at 37^{o}C in a sterilized medium containing 1% carbon source, 1% Na_2HPO_4•$12H_2O$, 0.245% KH_2PO_4, 0.1% NH_4NO_3, 0.01% $MgSO_4$, 0.05% yeast powder and 1% trace element solution. The trace element solution contained 0.20% $FeSO_4$•$7H_2O$, 0.03% $MnSO_4$•H_2O, 0.01% Na_2MoO_4•$2H_2O$, 0.006% $CuSO_4$ and 0.10% $CaCl_2$ (You et al., 2016). The effect of different carbon sources on the enzyme activity was tested by incubating *B. subtilis* TD7 on different substrates (guar gum, konjac, locust bean gum and xanthan gum) for 60 h. The substrates were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The culture broth was centrifuged at 8000 × *g* for 10 min at 4^{o}C to remove cells. The supernatant containing the exoenzyme was used for enzyme activity determination (Songsiriritthigul et al., 2010).

2.3 Enzyme assay

Endo-β-1,4-mannanase activity was measured by a modified 3,5-dinitrosalicylic acid (DNS) method (Songsiriritthigul et al., 2010). The substrate, 0.5% (w/w) guar gum (Sigma), was dissolved in 50 mM phosphate buffer (pH 7.5) and incubated at 37^{o}C for 3 h to form a homogeneous solution (You et al., 2016). The assays containing 0.050 ml of appropriately diluted enzyme solution and 0.450 ml of the substrate solution were incubated at 65^{o}C for 10 min. To measure the amount of reducing sugars released, the reaction liquid was mixed with 1.00 ml DNS solution, heated at 100^{o}C for 10 min, cooled on ice to 25^{o}C and then measured for the absorbance at 540 nm (Zhou et al., 2012). One unit (U) of β-mannanase activity was defined as the amount of enzyme producing 1.00 μmol of reducing sugar (using D-mannose as a standard) per minute under the assay conditions.

2.4 Biochemical analysis

The optimal pH for mannanase activity was measured in the pH range 2.5 to 12.0. Three buffer systems were used (each 50 mM): NaH_2PO_4 (pH 2.5-4.0), NaH_2PO_4-Na_2HPO_4 (pH 5.0-8.0), and NaH_2PO_4 (pH 9.0-12.0) (You et al., 2016). The pH stability of mannanase was estimated by measuring the residual enzyme activity after incubating the enzyme samples without substrate at various pH for 4 h at 50^{o}C. The optimal temperature for mannanase activity between 10^{o}C up to 100^{o}C was determined. The thermal stability of mannanase was determined by preincubating the enzyme solution at 50-80^{o}C for 5-60 min before measuring the enzyme activity.

Viscosity was determined by characterizing the degradation effect of mannanase with guar gum visually (You et al., 2016). The substrate, 0.80% (w/w) guar gum, was dissolved in 50 mM phosphate buffer (pH 6.5) and incubated at 25^{o}C for 30 min in a water bath to form a homogeneous solution. An appropriately diluted mannanase solution was added to a final concentration of 1.00%. Viscosity of the reaction solution was detected in 180 min at 20^{o}C and 65^{o}C, respectively.

2.5 Sequence and structure analysis

The total DNA of *B. subtilis* TD7 was extracted using Axygen Bacterial Genomic DNA Miniprep Kit (San Diego, CA, USA). *B. subtilis* TD7 gene sequences were obtained through whole genome sequencing, and the sequence encoding mannanase (namely YBMan) was determined using the ORF finder tool (https://www.ncbi.nlm.nih.gov/orffinder/). The signal peptide of YBMan was predicted using the SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) (Wang et al., 2016). The homology comparison was performed in NCBI

using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignment of protein primary structures between the TBMan and the other five β-mannanases was accomplished using the ClustalW program (http://www.ebi.ac.uk/ClustalW) (Zhao et al., 2011) and DNAMAN 6.0 software. Glycoside hydrolases (GHs) was performed according to the sequence and structure similarities of the hydrophobic cluster (http://www.cazy.org). The PI/MW of enzyme was predicted by proteomics computing tool (https://web.expasy.org/protparam/).

2.6 Cloning and expression of endo-β-1,4-mannanase from *B. subtilis* TD7 in *E. coli*

The coding sequence of the entire YBMan with native signal peptide, named *tbman*, was cloned by a PCR-based method. The primers ManTF: CCT CGC CAT ATG GGG GAG TTG C and ManR: TAC CGC GGA TCC TCA TTC AAC GAT T were used for PCR amplification of *tbman*. The primers were designed by the Primer 5.0 and synthesized by Genscript (Nanjing, China). They were compatible with the NdeI and BamHI cloning sites of pET-28a(+). To generate 6 × His tagged recombinant enzymes for further purification, DNA encoding a hexahistidine tag was incorporated at the *N*-terminus of *tbman* (Songsiriritthigul et al., 2010). The PCR reactionin a total volume of 100 μl consisted of 1.25 U of Primer Star (Takara), 0.200 mM dNTP Mixture (Takara), 0.500 μM primers, an appropriately diluted DNA sample and 5 × Primer Start Buffer (Mg^{2+} Plus) provided by the manufacturer. The PCR amplification was performed using the following conditions: 95^{o}C for 5 min, followed by 30 cycles of denaturation at 95^{o}C for 40 s, primer annealing at 56^{o}C for 60 s, extension at 72^{o}C for 70 s, and final extension at 72^{o}C for 10 min.

The purified PCR product was cut with the appropriate restriction enzymes (NdeI and BamHI, Thermo Scientific) and cloned into the pET-28a(+) cut with the corresponding enzymes. The ligation reaction was performed by T4 ligase (Thermo Scientific) at 37^{o}C. The resulting recombinant plasmid was transformed into *E. coli* JM83 for maintenance and propagation of the plasmid, and the recombinant plasmid of the positive strain was finally transformed into *E. coli* BL21 (DE3) for expression.

The clones harboring the recombinant plasmids were inoculated into 5 ml of LB broth containing 50 μg/ml of kanamycin and cultured at 37^{o}C for 12 h. One milliliter of overnight culture was then inoculated into 200 ml of LB broth containing 50 μg/ml kanamycin and grew at 37^{o}C (Zhou et al., 2012; Pongsapipatana et al., 2016). When the optical density of the culture broth reached 0.6-0.8 at 600 nm, IPTG was added into the culture medium to a final concentration of 0.50 mM followed by incubation for 16 h with shaking (190 rpm) at 16^{o}C. The culture broth was subsequently centrifuged at 8000 × *g* for 10 min at 4^{o}C to separate cells and supernatant. To extract the intracellular contents, the cells were washed for 3 times with sodium phosphate buffer (pH 7.5), resuspended to a final concentration of 10 OD/ml in the same buffer, and sonicated on ice (model W-380, Heat Systems-Ultrasonic, Inc., USA) for 2 min with a 30 s interval. The sonicated sediment and supernatant were collected after centrifuging at 8000 × *g*. The protein concentration of all fractions was determined according to the method of Bradford (Bradford, 1976) with BSA as a standard. Mannanase activities were determined according to the mentioned DNS method.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to analyze recombinant mannanase (Laemmli, 1970) in a 12% (w/v) polyacrylamide gel. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250. The molecular weight markers were from Takara (Dalian, China).

2.7 Purification of recombinant mannanase

The recombinant mannanase added 6 × His tag was purified by metal immobilization affinity chromatography using Ni-NTA Agarose. The collected supernatant was loaded onto a column and washed with increasing concentrations of imidazole from 10 mM to 400 mM (Songsiriritthigul et al., 2010) . When the concentration came to 200 mM, an appropriate volume (2-5 ml) of eluent buffer was required to obtain the purified recombinant mannanase. Finally, SDS-PAGE and enzyme assays were used to control the purification results.

2.8 Mass spectrometry analysis of the recombinant enzyme

Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) was used to obtain qualitative information about the protein after the corresponding protein bands on SDS-PAGE gel were cut off and digested to peptide fragments by trypsin (Pan et al., 2007).

2.9 Nucleotide sequence accession number

The nucleotide sequence for the endo-1,4-β-mannanase gene (YBMan) was deposited in GenBank under the accession number MG893206.

3 Results

3.1 Effect of different carbon sources on activity of the wild-type mannanase YBMan

The wild-type strain *B. subtilis* TD7, a lipopeptide-producing bacterium, produced extracellular mannanase. The effect of different carbon sources and incubation time on the activity of YBMan from *B. subtilis* TD7 are shown in Fig. 1. The enzyme exhibited the highest activity with guar gum, if the carbon source was konjac, followed by locust bean gum (LBG), and then guar gum. However, the enzyme showed

almost no activity, if the carbon source was xanthan gum. Compared to the highest activity of YBMan incubated with konjac, it was still about 90% and 80% relative activity, if the carbon source was LBG or guar gum, respectively. The optimal incubation time was 48 h for all carbon sources. In view of the extensive application of guar gum for different purposes such as petroleum extraction, guar gum was selected for incubation of *B. subtilis* TD7 and the time was set at 48 h.

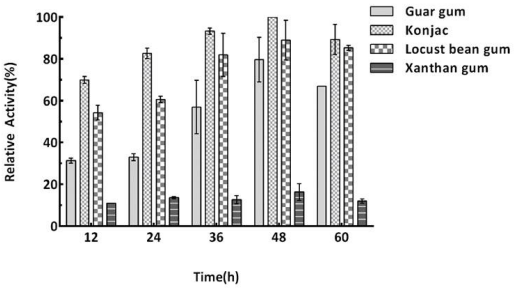

Figure 1. Effect of different carbon sources and incubation time on the activity of YBMan from *B. subtilis* TD7. Enzyme activities were measured based on the same volume of supernatant in different culture media and normalized to the maximum value (The relative activity with the maximum activity was defined as 100%).

3.2 Characterization of the wild-type mannanase YBMan

The optimal temperature for YBMan from *B. subtilis* TD7 was 65^{o}C with guar gum. The enzyme had still about 70% relative activity at 20^{o}C (Fig. 2A). The relative activity of YBMan decreased rapidly if the reaction temperature was set below 15^{o}C or above 75^{o}C. At these temperatures YBMan still had about 62% or 45% relative activity, respectively (Fig. 2A). The enzyme was stable up to 60^{o}C after incubation for 60 min (Fig. 2B) and retained its activity above 60% after incubation for 60 min at 70^{o}C (Fig. 2B). The optimal pH for YBMan was 6.5 and more than 80% relative activity remained in the pH range 5 to 7.5 (Fig. 2C). More than 60% of the maximum activity remained in the range pH 4.0-11.0 after incubation for 4 h at 50^{o}C (Fig. 2D). YBMan had approximately 2220 U activity for guar gum degradation from a one-liter shake flask culture with the specific activity for the crude enzyme of 35.1 U/mg (protein yield of 63.2 mg/l) after 48 h incubation of the wild-type strain *B. subtilis* TD7.

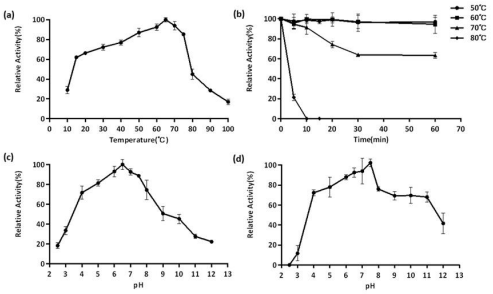

Figure 2. Effect of pH and temperature on the activity of the wild-type mannanase YBMan. (a): Effect of temperature on enzyme activity. (b): Thermostability assay of the enzyme. (c): Effect of pH on enzyme activity. (d): pH stability of the enzyme. The buffer system used was sodium phosphate buffer. Each value in the panel represents the mean ± SD (n = 3) and the relative activity (the maximum activity in each panel was defined as 100%).

3.3 Gene Cloning and sequence analysis

DNA sequence analysis showed that the target gene consisted of 1104 nucleotides coding for a mannanase with 367 amino acid residues. The theoretical molecular weight and *p*I value were predicted to be 41.4 kDa and 5.76, respectively. SignalP analysis indicated the presence of a putative signal peptide of 31 amino acid residues at the *N*-terminus. The deduced amino acid sequence of YBMan showed the highest identity (99%) with endo-1,4-β-mannosidase from *Bacillus subtilis* (strain 168) (NC_000964.3). However, published data on *Bacillus subtilis* (strain 168) about mannanase are limited. According to the sequence and structural similarities of the hydrophobic cluster, YBMan is a mannanase belonging to the Glycoside Hydrolase family 26 (GH26). The similarities of YBMan with other four β-mannanases from GH26 were 97%, 24%, 33% and 26%. These enzymes originated from *Bacillus subtilis* Bs5 (HM143944), *Aspergillus niger* CBS 513.88 (XM_001397260.1), *Bacillus licheniformis* DSM13 (NC006322) and *Sphingomonas sp.* JB13 (JF745875), respectively (Supplementary Fig. S1). Obviously, differences in the primary structures of mannanases from different species are existing.

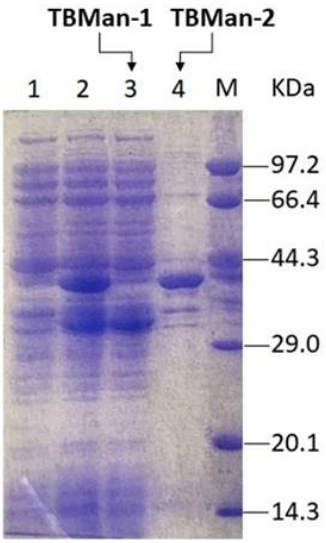

Figure 3. SDS-PAGE analysis of recombinant TBMan. Lane 1: whole-cell proteins of T-BL21 before IPTG induction; lane 2: whole-cell proteins of T-BL21 after IPTG induction; lane 3: supernatant of T-BL21 after ultrasonic processing; lane 4: sediment of T-BL21 after ultrasonic processing; M: protein molecular weight markers. Lane 1 and 2 were 10 OD/ml of T-BL21 by centrifuging the medium and resuspending the sediment (bacteria) with appropriate buffer. Lane 3 and 4 were ultrasonic products of the bacterial sediment resuspended to 10 OD/ml.

3.4 Expression of the recombinant mannanase in *E. coli*

The gene *tbman* coding sequence of the entire YBMan was amplified by PCR using the ManTF and ManR primers. The corresponding product was approximately 1100 bp in length (Supplementary Fig. S2). The recombinant vector pET28a-Man was constructed from the plasmid pET-28a(+) and the target mannanase gene (Supplementary Fig. S3). It was presumed that the recombinant protein including 6 × His tag and target gene *tbman* contained 1164 bp encoding 387 amino acids.

After induction of IPTG, the gene *tbman* was successfully expressed in *E. coli* BL21(DE3) (strain named T-BL21). Both, the sediment and the supernatant of strain T-BL21 after ultrasonic processing, were collected and analyzed by SDS-PAGE. As shown in Fig. 3, two different proteins were generated by strain T-BL21 after IPTG induction. The protein in the supernatant named TBMan-1 (shown in lane 3) was smaller than that from the sediment named TBMan-2 (shown in lane 4). Based on SDS-PAGE analysis, the molecular mass of TBMan-2 was estimated to be 43.6 kDa, matching the previously deduced TBMan protein. After resuspension and sonication of the bacteria in the culture medium, the supernatant activity (crude β-mannanase activity of TBMan-1) of strain T-BL21 was 339.7 U/ml with the specific activity of 225.0 U/mg. No β-mannanase activity was detected in the medium before sonication. In order to identify the two recombinant proteins, the corresponding protein bands of TBMan-1 and TBMan-2 on SDS-PAGE gel were cut out and analyzed by MALDI-TOF-MS(Rosengren et al., 2014; Egelhofer et al., 2000) . The data indicated that both TBMan-1 and TBMan-2 were endo-1,4-β-mannanases.

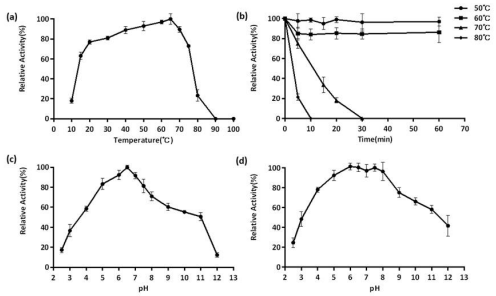

Figure 4. Effect of pH and temperature on activity of the recombinant mannanase TBMan-1. (a): Effect of temperature on enzyme activity. (b): Thermostability assay of the enzyme. (c): Effect of pH on enzyme activity. (d): pH stability of the enzyme. The buffer system used was sodium phosphate buffer. Each value in the panel represents the mean ± SD (n = 3) and the relative activity (the maximum activity in each panel was defined as 100%).

3.5 Characterization of the recombinant mannanase

The supernatant with the highest β-mannanase activity (TBMan-1, 339.7 U/ml) in shake flask culture was used in subsequent experiments. The effect of temperature and pH on TBMan-1 activities was investigated. The data are shown in Fig. 4. The optimal temperature for TBMan-1 was 65^{o}C with guar gum. The enzyme had about 80% relative activity at 20^{o}C and more than 60% relative activity even at 15^{o}C (Fig. 4A). TBMan-1 also showed a rapid decline in activity, if the reaction temperature fell below 15^{o}C or was increased above 75^{o}C (Fig. 4A). The activity remained above 80% after incubation for 60 min at 60^{o}C (Fig. 4B), but it lost half of the activity for 10 min at 70^{o}C and was completely inactivated for 10 min at 80^{o}C (Fig. 4B). The optimal pH of TBMan-1 was 6.5 with guar gum (Fig. 4C). Still more than 60% of the initial activity were maintained in the range pH 4.0-11.0 after incubation for 4 h at 50^{o}C (Fig. 4D).

The viscosity reducing effect of mannanase for guar gum was measured subsequently, as shown in Fig. 5. When TBMan-1 was diluted to 1 U/ml with a final concentration of 1.00% in the reaction system, the viscosity of the gel solution was reduced rapidly in the first 20 min. The guar gum viscosity was reduced approximately from 500 mPa•s to 10 mPa•s within 60 min at 65^{o}C. The viscosity reduction effect was also noticeable at 20^{o}C. Whether the temperature was 65^{o}C or 20^{o}C, TBMan-1 could reduce the guar gum viscosity below 5 mPa•s within 180 min. This indicates a potential application for fracturing fluids as enzyme gel-breaker, especially at low temperature (You et al., 2016).

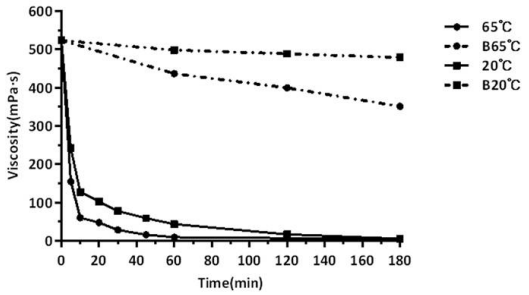

Figure 5. Viscosity reduction effect for guar gum by the recombinant mannanase TBMan-1 at 65^{o}C and 20^{o}C. B65^{o}C and B20^{o}C were blank control groups without enzyme at 65^{o}C and 20^{o}C, respectively.

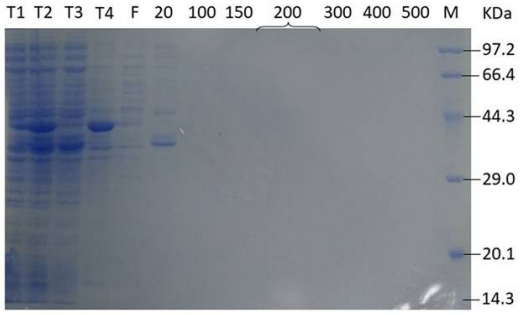

Figure 6. SDS-PAGE analysis of purified TBMan-1. T1: whole-cell proteins of T-BL21 before IPTG induction; T2: whole-cell proteins of T-BL21 after IPTG induction; T3: supernatant of T-BL21 after ultrasonic processing; T4: sediment of T-BL21 after ultrasonic processing; F: flow-through solution of the sample; 20/100: nonspecific proteins in the sample by washing with 20mM/100mM imidazole; 150-500: elution solution of the sample by washing with 150-500 mM imidazole.

The crude enzyme TBMan-1 from the sonication extract

was used for affinity purification on Ni-NTA Agarose. However, the SDS-PAGE of TBMan-1 showed that TBMan-1 could not be purified by Ni-NTA Agarose, as shown in Fig. 6.

4 Discussion

Industrial applications of guar gum increased rapidly during the last decades. Guar gum is applied in various food products for food stabilization as a novel food additive (Morris, 2010). It is also used in oil and gas well stimulation, specifically for hydraulic fracturing using high pressure applications to crack rocks (Mudgil et al., 2014). Consequently, a simple degradation of guar gum, especially biodegradation at medium and low temperatures, is required increasingly (You et al., 2016; Mclean et al., 2011; Wan et al., 2007). Different mannanases have different substrate specificity, and most studies focus on their optimal substrate like konjac or LBG (Zhou et al., 2012; Pongsapipatana et al., 2016). However, for applications in the oil industry, guar gum is important. Therefore, guar gum was selected particularly as the substrate for the enzyme assays in this study.

Most mannanases, especially those from *Bacillus subtilis*, are apparently stable at 40 to 60^{o}C, but inactive or nearly inactive at low temperature (Katrolia et al, 2012; Kim et al., 2011; Pan et al., 2011; Benech et al., 2007). Up to date, only about ten low-temperature or cold-active β-mannanases have been reported. Among these mannanases, GH26 covered six ones originated from *Sphingomonas* sp. JB13 (Zhou et al., 2012), *Sphingobacterium* sp. GN25 (Zhang et al., 2015), *Klebsiella oxytoca* KUB-CW2-3 (Pongsapipatana et al., 2016), *Aspergillus niger* CBS 513.88 (Zhao et al., 2011), *Bacillus subtilis* Bs5 (Huang et al., 2012) and *Bacillus licheniformis* DSM13 (Songsiriritthigul et al., 2010). Glycoside Hydrolase family 5 (GH5) covered three mannanases originated from *Cryptopygusantarcticus* (Song et al., 2008), *Gloeophyllumtrabeum* CBS900.73 (Wang et al., 2016) and *Enterobacter* sp. strain N18 (You et al., 2016). One mannanase originated from *Flavobacterium* sp. was family-unidentified (Zakaria et al., 1998) (Table 1). In addition, there have been a few reports about the biocatalytic characterization of low-temperature active mannanases against guar gum (only *Flavobacterium* sp. (Zakaria et al., 1998) and N18 (You et al., 2016). As shown in Table 1, the two mannanases from *Cryptopygusantarcticus* and *Flavobacterium* sp. exhibited a high relative activity at 20^{o}C with 80% and 70%, respectively. However, the mannanase from *Cryptopygusantarcticus* was characterized using LBG and had no activity with guar gum (Song et al., 2008). The specific activity of mannanase from *Flavobacterium* sp. was only 0.48 U/mg (Zakaria et al., 1998), and thus, inappropriate for an industrial application. YBMan produced by *B. subtilis* TD7 showed about 70% relative activity at 20^{o}C compared to its optimal activity at 65^{o}C (Fig. 2A). This enzyme exhibited almost the highest relative activity at 20^{o}C among the reported low-temperature active mannanases. YBMan has an optimum pH range between 5-7.5 with more than 80% activity. This is similar to the pH optimum of mannanases isolated from other *B. subtilis* strains (Mendoza et al., 1994; Jiang et al., 2006; Qiao et al., 2010). Although the activity of YBMan is affected by increasing acidity or alkalinity, the results indicate that YBMan has a broad pH stability range (pH 4.0-11.0), which was potential for industrial applications.

Table 1. Characterizations of the low-temperature active mannanases from different hosts.

Strain	Opt. temp.(°C)	Opt. pH	Activity (U/mg) at Opt. temp.	Relative activity (%) at 20°C	Reference
Sphingomonas sp. JB13	40	6.5	126.3 [b]	~ 55	(17)
Bacillus subtilis Bs5	35	5.0	1231.4 [UL;b]	40	(36)
Klebsiella oxytoca KUB-CW2-3	30-50	4-6	285.3 [b]	*NR*, 60 [30°C]	(21)
Aspergillus niger CBS 513.88	45	5.0	5069 [UL;a]	40	(1)
Sphingobacterium sp. GN25	35-40	6.5-7.0	*NR* [a]	44.8	(35)
Gloeophyllumtrabeum CBS 900.73	60	2.5	1356 [a]	*NR*, 40 [30°C]	(4)
Enterobacter sp. N18	50	7.5	4099 [a]	50	(11)
Bacillus licheniformis DSM13	50-60	6.0-7.0	1672 [a; nc]	~50	(20)
Cryptopygusantarcticus	30	3.5	416 [a; nc]	~ 80	(37)
Flavobacterium sp.	35	7	0.48 [c]	70	(18)

[a]: Mannanase activity against locust bean gum at optimal conditions;
[b]: Mannanase activity against konjac at optimal conditions;
[c]: Mannanase activity against guar gum at optimal conditions;
[nc]: Mannanase showed no activity against guar gum;
[UL]: Activity (U/ml) at Opt. temp.
30^{o}C: Relative activity (%) at 30^{o}C
NR: Not reported.

The gene for YBMan was successfully cloned and expressed in *E. coli*. The recombinant mannanase TBMan-1 produced by strain*E. coli* T-BL21 exhibited about 80% relative activity against guar gum at 20^{o}C (compared to its optimal temperature 65^{o}C). Compared to the mannanases shown in Table 1, the relative activity of TBMan-1 was the highest at both 20^{o}C and 30^{o}C. Moreover, we successfully obtained a total of approximately 79550 U of recombinant TBMan-1 enzyme from a one-liter culture with a 35.8-fold increase in total enzyme activity compared to the wild type. Recombinant mannanase TBMan-1 precisely increased by 5.6 times in the protein yield (from 63.2 mg/l to 353.5 mg/l) and 6.4 times in the specific activity (from 35.1 U/mg to 225.0 U/mg). Thus, TBman-1 has a good performance in activity at low-temperature and high yield, if compared to the reported low-temperature active mannanases (Table 1). To our knowledge, this is the first study to report a low-temperature active β-mannanase obtained from *B. subtilis* and expressed in *E. coli* with remaining activity of 80% for guar gum at 20^{o}C. This low-temperature active mannanase produced a high reduction for guar gum at 20^{o}C, which implies a potential applicability for viscosity reduction in fracturing fluids for low-temperature oil reservoirs and a thorough degradation in the ground as enzymatic gel breaker (You et al., 2016; You et al., 2016). It might also significantly help to expand the application range, if heating would cause increased cost and product denaturation such as in detergent additives, the textile industry, the food and feed industries and in aquaculture (Zhou et al., 2012; Gerday et al., 2013). Nevertheless, the recombinant mannanase could

not be secreted from *E. coli* with the native signal peptide of YBMan. To meet the commercial demands, a replacement of the native signal peptide with strong secretory signal peptide for expression to improve protein secretion is our future objective.

Interestingly, strain T-BL21 containing only one ORF encoding mannanase generated two proteins (TBMan-1 and TBMan-2) and both were mannanases, as identified by MALDI-TOF-MS. According to the difference in the molecular masses between TBMan-1 and TBMan-2 (Fig. 3) we speculate that the sequences (Ecoli-cut) were partially cut in *E. coli* due to the identification of the native signal peptide sequence and other sites by the *E. coli* system. The "Ecoli-cut" sequence included the signal peptide sequence and 6 × His tag, whose deficiency made a small protein (TBMan-1) with increased activity (Supplementary Fig. S4). Moreover, the deficiency of 6 × His-tag in TBMan-1 could possibly explain why it could not be purified by the Ni-NTA method. Due to the identification and cut out of the "Ecoli-cut" sequence, TBMan-1 was soluble expressed and retained mannanase activity. In this way it differed from TBMan-2, which was found in inclusion bodies. The gene without the native signal peptide sequence in YBMan, named pbman, was also cloned and expressed in *E. coli* BL21 (DE3) (stain named P-BL21) using the ManPF: CCA CGC CAT ATG CAT ACT GTG TCG C and ManR primers. The expression of pbmangenerated only one protein (Supplementary Fig. S5) named PBMan. The crude enzyme PBMan had an activity of 162 U/ml for guar gum less than TBMan-1. This indicated that a truncated mannanase may possess an increased activity (Pan et al., 2011). The purified PBMan (Supplementary Fig. S6) exhibited a specific activity of 1563 U/mg, which means that it showed a good reactivity among these low-temperature mannanases (Table 1).

5 Conclusion

In conclusion, the mannanase YBMan from *B. subtilis* TD7 belonging to the GH26 family is a novel low-temperature active mannanase. The recombinant mannanase TBMan-1 by expression in the *E. coli* system with the plasmid pET-28a(+) had a 35.8-fold increase in total enzyme activity compared to the wild type. Moreover, TBMan-1 had a relative activity at 20^{o}C of about 80%. The large temperature range makes this enzyme a good candidate for industrial application in oil exploitation, textile, food, and other industries.

Acknowledgements

The authors declare no conflict of interest. This work was supported by the National Natural Science Foundation of China (grant number 41530318); the Research Foundation of Shanghai (grant number 15JC1401400); and the Fundamental Research Funds for the Central Universities of China (grant number 222201817017). Our great thanks Professor Wolfgang Sand for providing language help of this article.

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