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Molecular Cloning, Over-expression and Enzymatic Characterization of an Endo-acting β-1,3-glucanase from Marine Bacterium *Mesoflavibacter zeaxanthinifaciens* S86 in *Escherichia coli*

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Abstract – Glucanases are involved in degradation of glucans. Here, we report a new endo-β-1,3-glucanase Mzl86 identified in *Mesoflavibacter zeaxanthinifaciens* S86. The deduced amino-acid sequence of Mzl86 showed highest similarity (45.1%) with *Leeuwenhoekiella blandensi* and thus placed in glycosyl hydrolase family 16. Purified recombinant protein (rMz186) showed an optimum enzyme activity against laminarin at 50°C and pH 8. The enzyme was stable at 50°C for 1 hour (maintaining 80% of its maximum activity) and was strongly activated (187%) in the presence of 2.5 mM manganese. Substrate-specific activities of rMzl86 against laminarin, barley β-glucan and lichenan were 261, 128 and 115 unit/mg, respectively. rMzl86 degraded laminarioligosaccharides (lager than biose) and laminarin while producing mainly biose and glucose. Molecular and biochemical properties reveal that rMzl86 shares typical features of β-1,3-glucanase (EC 3.2.1.39) and thus is a potential candidate for use in agriculture, drug, chemical and bioethanol industries.

Key words – β-1,3-glucanase, *Mesoflavibacter zeaxanthinifaciens*, recombinant enzyme, laminarinase activity, biochemical properties

1. Introduction

Polysaccharides, including D-glucose monomers linked by β-1,3-glycosidic bonds, are essential cell wall components in fungi and are also widely distributed in plant and lichen. In the marine environment, β-1,3-glucan has been extracted from brown seaweed (Miyanishi et al. 2003a). There are four main types of endo-acting enzymes capable of depolymerizing β-1,3-1,4-glucan (Planas 2000; McCarthy et al. 2003): i) β-1,3(4)-glucanases (EC 3.2.1.6) which can randomly hydrolyze β-1,3 and β-1,4-glycosidic bonds in β-glucan; ii) β-1,3-glucanases (EC 3.2.1.39, also known as laminarinases) which can randomly hydrolyze only β-1,3-glycosidic bonds in β-glucan; iii) β-1,3-1-4-glucanases (EC 3.2.1.72) which can degrade only β-1,4-linkages adjacent to the reducing terminal side of β-1,3-linkage in mixed β-glucans; and iv) β-1,4-glucanases (EC 3.2.1.4) which hydrolyze β-1,4-glycosidic linkages, prominently present in cellulose.

The endo-β-1,3-glucanases are further classified into glycosyl hydrolase (GH) families, e.g. family 16, 17, 55, 64, and 81, based upon the structure and similarity of amino acid sequences (Krah et al. 1998; Song et al. 2010). These enzymes can be found in various organisms including plants, animals, fungi, archaea and bacteria (Wong 1979; Delacruz et al. 1995; Gueguen et al. 1997; Hong et al. 2002; Masuda et al. 2006; Genta et al. 2009; Kumagai and Ojima 2010; Shi et al. 2010b; Song et al. 2010). The bacterial β-1,3-glucanases and β-1,3-1,4-glcuanases are classified as glycosyl hydrolases family 16 (GH16) (Shi et al. 2010b). Endo-β-1,3-glucanases are widely used in diverse industrial applications. It can convert algal biomass to fermentable sugar for bioethanol production (Nisizawa K 1963). In agriculture, β-1,3-glucanases can be applied to control fungal diseases in combination with chitinase (Joosten and Dewit 1989; Castresana et al.

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1990). The hydrolytic products of the glucan polymer due to the action of endo-β-1,3-glucanases are useful in diverse processes, such as yeast extract production (Ryan et al. 1995), immuneactivation (Mohagheghpour et al. 1995; Akramiene et al. 2007), and wine extraction clarification (Pretorius 1991).

With the potential for a broader application, in this study we identified a new endo-β-1,3-glucanase from *Mesoflavibacter zeaxanthinifaciens* S86 (designated as Mzl86) using Next Generation Sequencing (NGS) technology. The newly identified gene encoding Mzl86 was cloned and expressed in *E. coli* BL21 (DE3) expression system. Subsequently, the hydrolytic activity and the biochemical properties of Mzl86 were characterized using the purified recombinant protein.

2. Materials and Methods

Experimental bacterial strain and identification of laminarinase coding sequence

Experimental bacterial strain was collected from seawater in Chuuk State of Micronesia and isolated following the previously described procedure (Oh et al. 2011). The bacterial strain was confirmed by 16S ribosomal DNA sequencing and designated as *M. zeaxanthinifaciens* S86. The draft genome (GenBank accession No. AFOE00000000) of *M. zeaxanthinifaciens* S86 was sequenced by Genome Sequencer-FLX System (GS-FLX^{TM}), a next generation sequencing (NGS) technology (Macrogen, Republic of Korea). Subsequently, a unique nucleotide sequence that encodes a protein with high homology to known laminarinases was identified by the Basic Local Alignment Search Tool (BLAST) algorithm, and the putative enzyme was designated as Mzl86.

Sequence analysis

The signal peptide of Mz186 was predicted by the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al. 2011) and the identity/similarity percentage values of Mzl86 sequence against other homologues were calculated by EMBOSS Pairwise sequence alignment tool (http://www.ebi.ac.uk/Tools/psa/) (Larkin et al. 2007; Goujon et al. 2010). The NCBI conserved domains database (CDD; http://www.ncbi.nlm.nih.gov/cdd/) (Marchler-Bauer and Bryant 2004; Marchler-Bauer et al. 2009; Marchler-Bauer et al. 2011) and Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de/) (Schultz et al. 1998; Letunic et al. 2009; Letunic et al. 2012) were used to identify active sites, catalytic sites and functional domains in Mz186.

Cloning of *mzl86* for overexpression

Genomic DNA of *M. zeaxanthinifaciens* S86 was isolated using *Accurprep*® Genomic DNA Extraction Kit (Bioneer, Republic of Korea) and was used as the template for PCR. The laminarinase coding sequence without signal peptide was amplified using primers (Lam-EF, 5′- gag aga CAT ATG ATG CAT CAT CAT CAT CAT CAT TGC CAA GAA GAA GAA CCA GAA-3′; and Lam-ER, 5′-gag aga GGA TCC TTA TTG ATA TAC GCG TAT ATA GTC CAC TTC-3′), which included restriction sites (*Nde*I and *BamH*I) and 6 histidine-coding sequence for cloning the amplicon into pET-11a expression vector (Novagen, USA). The PCR mixture (50 μL) consisted of 5 units of LA Taq polymerase, 5 μL of 10X LA PCR buffer II (Mg^{2+} free), 5 μL of 25 mM $MgCl_2$, 500 ng of gDNA, and 10 pmol of each primer. The thermal cycling condition included an initial denaturation step (94°C, 5 min), 25 PCR cycles of amplification (94°C, 30 s; 45°C, 30 s; 72°C, 150 s), and a final extension step (72°C, 5 min). The PCR product and pET-11b vector were digested with respective restriction enzymes and ligated together at 16°C for 30 min using T4 DNA ligase (Takara, Japan). The pET-11a-*Mzl86* expression plasmid was then transformed into *E. coli* DH5α competent cells, and putative recombinants were selected followed by sequencing confirmation. Finally, the recombinant pET11a-*mzl86* plasmid was transformed into *E. coli* BL21 (DE3).

Overexpression and purification of recombinant Mzl86 (rMz186)

The *E. coli* BL21 (DE3) containing pET11a-*mzl86* was cultured overnight at 37°C in 5 mL LB broth supplemented with 100 μL of ampicillin (100 mg/mL). The 2.5 mL culture was then inoculated into 250 mL of fresh LB broth at 37°C in a shaker until the culture reached a mid-logarithmic phase (OD_{600nm}=0.50-0.60). Isopropyl-β-D-thiolgalactoside (IPTG) was added to a final concentration of 1 mM and the temperature was adjusted to 25°C. After 16 h of induction, cultured pellet was collected by centrifugation at 11,000 rpm for 10 min. The cell pellet was re-suspended in 20 ml of binding buffer (Novagen, USA) and frozen at -20°C overnight. After thawing on ice, cells were disrupted by cold sonication and centrifuged (11,000 rpm, 15 min, 4°C) to obtain the supernatants. The rMzl86 was purified from the supernatant using the His·Bind

Kit (Novagen, Germany). Then the purified protein was analyzed by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of the purified recombinant protein was determined by the BCA protein assay reagent kit (Thermo Fisher Scientific Inc, USA).

Enzymatic characterization of rMzl86

To determine biochemical properties of rMzl86, laminarin was used as the substrate. The optimum temperature for rMzl86 enzyme activity was determined by monitoring its relative activity at temperatures ranging from 30-80°C with 5°C intervals at pH 7.0. The optimum pH for rMzl86 activity was determined using pH range of 3-11 at 50°C. Citrate-phosphate buffer, phosphate buffer and glycine-sodium hydroxide buffer were used to generate pH ranges 3-7, 7-8, and 9-11, respectively. The thermostability of rMzl86 was determined using pre-incubated enzyme at temperatures of 50 or 60°C for 10, 20, 30, 40, 50 and 60 min. The sensitivity of rMzl86 to various metal ions and chemical reagents was determined in 0.5% laminarin solution supplemented with 1 and 2.5 mM of KCl, $MgSO_4$, NaCl, $CaCl_2$, EDTA, $MnSO_4$, $FeSO_4$, $CuSO_4$, and $ZnSO_4$.

The specific activity of purified rMzl86 was determined by using the 3,5-dinitrosalicylic (DNS) method (Miller 1959) with 0.5% larminarin (Sigma, USA), 0.5% barley β-glucan (Sigma, USA), 0.5% lichenan (Megazyme, Ireland), 0.5% carboxymethyl cellulose (CMC; Sigma, USA), and 0.5% β-1,4-xylan (Tokyo Chemical Industry, Japan) as substrates.

Identification of hydrolysis products from rMzl86

To determine the hydrolytic pattern of rMzl86 upon its putative substrates, degradation products of different substrates, including laiminarin, barley β-glucan, lichenan and laminarioligosaccharides, were analyzed by thin layer chromatography (TLC) upon hydrolysis by rMzl86. The hydrolysates were applied to silica gel 60 plates (Merck, Germany), developed with ethyl acetate/acetic acid/water (2:2:1, v:v:v). Finally, the reducing sugars were visualized using 10% H_2SO_4 in ethanol and subsequent charring.

3. Results and Discussion

In this study, we investigated the molecular characteristics and biochemical properties of a new β-1,3-glucanase identified in *M. zeaxanthinifaciens* S86. This is the first report of such enzyme isolated from the genus *Mesoflavibacter*. The DNA sequence of *mzl86* was obtained from *M. zeaxanthinifaciens* S86 by NGS technology, and the nucleotide and amino acid sequences are shown in Fig. 1. The ORF comprised of 2,106 bp which encodes for a putative 703-amino-acid protein with a predicted molecular mass of 77 kDa and theoretical isoelectric point of 4.0. Sequence information was deposited in NCBI GenBank database under the accession no. WP_010517842.1. According to our *in silico* analysis the predicted signal peptide was observed at amino acid position 1-22. Hence, mature protein of Mzl86 consisted of 680 amino acids. Moreover, the predicted glycosyl hydrolase family 16 (GH16) laminarinase-like domain was observed in the C-terminal region. In addition, seven active sites were identified at GH16 domain, as per our predicted information. GH16 domain is generally observed in bacterial species, and has been classified into two subfamilies based on substrate specificity: endo-β-1,3-glucanases (EC 3.2.1.39) and β-1,3-1,4-glucanases (EC 3.2.1.73). The results of amino acid sequence comparison and motif prediction of Mz186 suggested that the enzyme can be classified as a β-1,3-glucanase (EC 3.2.1.39).

The amino acid sequence of Mzl86 had a low sequence identity with other bacterial β-glucanases. Furthermore, we observed a long sequence gap at ^{379}GAE - GPA^{536}, compare to other bacterial β-glucanases. The Mzl86 showed the highest identity (34.9%) and similarity (45.1%) with laminarinase counterparts from bacterial species *Leeuwenhoekiella blandensi* (data not shown). This observation convinced us that Mzl86 is a new laminarinase. Subsequently, IPTG-induced overexpression of His-tagged Mzl86 fusion protein was verified by SDS-PAGE (Fig. 2). A strong single band similar to the predicted molecular mass of rMzl86 (75 kDa, including the 6-his tag) was detected corresponding to the purified recombinant protein. This confirmed the successful overexpression of rMz186 along with sufficient purity and integrity for eluted fusion protein. Due to the detectable prominent stability of the percent activity of rMzl86 at 50°C we can logically assume that it is stable at lower temperatures. On the other hand, temperatures higher than 60°C probably make it highly unstable.

Optimum temperature for hydrolyzing activity of rMzl86 was determined under different temperatures (range 30-80°C, 5°C interval). The hydrolysis reaction was performed for 10 min. The activity of Mzl86 increased from 30 to 50°C, then gradually decreased along with temperature increase and no activity was observed over 80°C (Fig. 3). We observed

```
ATGAAAAGCATAAAAACATTAAGTTACCTTTTGGGCTTGCTTTGCTTGGTATTGGCTTGCCAAGAAGAAGAACCAGAATTACAAGCATTG   90
 M  K  S  I  K  T  L  S  Y  L  L  G  L  L  C  L  V  L  A  C  Q  E  E  E  P  E  L  Q  A  L    30
GTAACGCCAACAAATTTGGTGTTGACCACTAATGTGGCAGAAGACCAATCTGGTTTTGTTACGGTAACACCAACGGCAGAAAATGCCTTG   180
 V  T  P  T  N  L  V  L  T  T  N  V  A  E  D  Q  S  G  F  V  T  V  T  P  T  A  E  N  A  L    60
TATTATCATGTGTTTTTTAATCCAGGAGCAGAGCCTGTTGTCGTTTCAGCAGGGGAAACGGCAAGCTTTAGATATACCCGTTCTGGAGAA   270
 Y  Y  H  V  F  F  N  P  G  A  E  P  V  V  V  S  A  G  E  T  A  S  F  R  Y  T  R  S  G  E    90
TACCAACAGCCTATTACAGCCGTAGCCTTTGCCCGTGGCGGATTAAGTTCAAGCGCTACGGTGCTGGTAGACTTAAATGTGCGGTTGCGA   360
 Y  Q  Q  P  I  T  A  V  A  F  A  R  G  G  L  S  S  S  A  T  V  L  V  D  L  N  V  R  L  R   120
ATAGATGCGGCAACCTTAGGATTATTGGCAGGTGGTGATGGTACTACGCCTAGCAGTAAAAGATGGGTATGGGACCGTACCGTTGGTGGG   450
 I  D  A  A  T  L  G  L  L  A  G  G  D  G  T  T  P  S  S  K  R  W  V  W  D  R  T  V  G  G   150
CACTTTGGTGTAGGCCCTTTAACGAATGATTTTCCTGAGTTTTTTAGTGCAGGCCCTAATCAATTAAACAGTTGTTTGTATGATGATGTC   540
 H  F  G  V  G  P  L  T  N  D  F  P  E  F  F  S  A  G  P  N  Q  L  N  S  C  L  Y  D  D  V   180
TTAACTTTTGAGCACGATGGCAACGATAATTATACCTATTCTTTAGATGCAGGTGCGGATAATCTAGTATTCATTAACTGGACAGAGGTA   630
 L  T  F  E  H  D  G  N  D  N  Y  T  Y  S  L  D  A  G  A  D  N  L  V  F  I  N  W  T  E  V   210
AACCGTTTTTTTCCAGATGCTACGCCCCAACAATTTGCGGACGAGTGTAGGGATATTACGGATCAAGTGCCGTTTACCACCAATTATGCG   720
 N  R  F  F  P  D  A  T  P  Q  Q  F  A  D  E  C  R  D  I  T  D  Q  V  P  F  T  T  N  Y  A   240
GTAATAGAAAATACAGATGGTACCCAAACCTTGGATGTGGGTAGTAGTTTTTTAAGTTACTGGGCTGTGATTCCTGGGCAATATCAAATT   810
 V  I  E  N  T  D  G  T  Q  T  L  D  V  G  S  S  F  L  S  Y  W  A  V  I  P  G  Q  Y  Q  I   270
TTGGAACTGTCAGAAAACCGTTTGGCGGTACGCGGTATTAGTCAACCCTTTAACGGAGACGACCCACTGGCTTGGTATGCAGTTTTTGTA   900
 L  E  L  S  E  N  R  L  A  V  R  G  I  S  Q  P  F  N  G  D  D  P  L  A  W  Y  A  V  F  V   300
CCCGAAACGATGACAGATACGGGTAGCGAAATGCTAGAAACCCAATATGATACCTTGGTTTGGTCAGATGAGTTTGATGTAGACGGAGCG   990
 P  E  T  M  T  D  T  G  S  E  M  L  E  T  Q  Y  D  T  L  V  W  S  D  E  F  D  V  D  G  A   330
CCCAATCCAATTAATTGGACGTATGATCTAGGTACTGGAAATAATGGTTGGGGAAATAACGAATTGCAATCCTACACAGATGCTACTGAA  1080
 P  N  P  I  N  W  T  Y  D  L  G  T  G  N  N  G  W  G  N  N  E  L  Q  S  Y  T  D  A  T  E   360
AATGTACGAGTAGCCAATGATGTTTTACAGATAACAGCTAGAGCAACGGGTAGTACCGGTGCGGAGGTCTATTATTTTGATGATGTAACG  1170
 N  V  R  V  A  N  D  V  L  Q  I  T  A  R  A  T  G  S  T  G  A  E  V  Y  Y  F  D  D  V  T   390
GTTGCCGATGATGCAGGAACCGTAACGCAAACCGTCTCGGATTTTGAAGGAACGGCTCCGGAATTTACCGATTTTGAAGGTGCCAGTGCT  1260
 V  A  D  D  A  G  T  V  T  Q  T  V  S  D  F  E  G  T  A  P  E  F  T  D  F  E  G  A  S  A   420
ACGGTTGTAACCAATCCAGATACAAATGGGAATGCTTCGGGCAACGTGGTACAATTCACCAAAAATCCTGGTGCAGCTTTCTTTGCAGGA  1350
 T  V  V  T  N  P  D  T  N  G  N  A  S  G  N  V  V  Q  F  T  K  N  P  G  A  A  F  F  A  G   450
AGTTTTTATGAAGTAAGCACGCCAATAGATTTATCCGTCAATAAAAACATCAAACTAAAAACATGGTCGCCCAAGATAGGTGCGGTAGTG  1440
 S  F  Y  E  V  S  T  P  I  D  L  S  V  N  K  N  I  K  L  K  T  W  S  P  K  I  G  A  V  V   480
CGAGTTAAATTGGAGAACGCAGCCAATACTGATGAGTTTTATGAAGCCGATGCTACTACCACGGTGAACAATGCGTGGGAAGAATTAACA  1530
 R  V  K  L  E  N  A  A  N  T  D  E  F  Y  E  A  D  A  T  T  T  V  N  N  A  W  E  E  L  T   510
TTTGATTTTTCTGCCGCAGGTAATTTCAACTATAACCGCATTGTGGTATTCTTTGATTTTGGCGAAGTAGGCCCCGCTGCATCCGGGTAT  1620
 F  D  F  S  A  A  G  N  F  N  Y  N  R  I  V  V  F  F  D  F  G  E  V  G  P  A  A  S  G  Y   540
ACCTCCGCAAGAATAAAAACCGAAGGGTTACAAGAGTTTACCTACGGTAGGGTAGCGGCTAGGGCAAAACTGCCAACAGGTGGCGGTACA  1710
 T  S  A  R  I  K  T  E  G  L  Q  E  F  T  Y  G  R  V  A  A  R  A  K  L  P  T  G  G  G  T   570
TGGCCTGCCATTTGGATGCTAGGTGCAGATTACCAAACCAACCCTTGGCCAGCTGCTGGTGAAATTGATATTATGGAACATGTAGGCAAC  1800
 W  P  A  I  W  M  L  G  A  D  Y  Q  T  N  P  W  P  A  A  G  E  I  D  I  M  E  H  V  G  N   600
CAACAAGATATTATATTTGGTTCTACGCACGATCAAAATAATTCTGCCGGTAATGCCAGAACGGGGTCTACTTTGGTACCAGGCGTATCG  1890
 Q  Q  D  I  I  F  G  S  T  H  D  Q  N  N  S  A  G  N  A  R  T  G  S  T  L  V  P  G  V  S   630
GACGATTTTCATATTTATGAGGTAGAATGGACCAGTACGGAAATTCAATTTGCCGTAGATGGGGTAGTGTATCACACGGTGAGCAACGAT  1980
 D  D  F  H  I  Y  E  V  E  W  T  S  T  E  I  Q  F  A  V  D  G  V  V  Y  H  T  V  S  N  D   660
GGTAGTTTGCCCTTTAACAAAGATTTTTTCTTGATACTTAACGTGGCCATGGGCGGTACGTTTGGCGGAGAAGTAGATGCAGAATTTACG  2070
 G  S  L  P  F  N  K  D  F  F  L  I  L  N  V  A  M  G  G  T  F  G  G  E  V  D  A  E  F  T   690
GAATCTACCATGGAAGTGGACTATATACGCGTATATCAATAA                                                   2109
 E  S  T  M  E  V  D  Y  I  R  V  Y  Q  *                                                    703
```

Fig. 1. Complete nucleotide and amino acid sequences of Mzl86. The predicted signal sequence and glycosyl hydrolase family 16 laminarinase-like domain are double underlined and shaded in gray color, respectively. The active sites and catalytic sites are boxed and bolded, respectively

the highest activity of rMz186 at 50°C and more than 60% of its relative activity was retained at 45 and 60°C. Most of the reported β-1,3-glucanases have shown their optimal activity between 45-55°C (Pang et al. 2004; Monteiro and

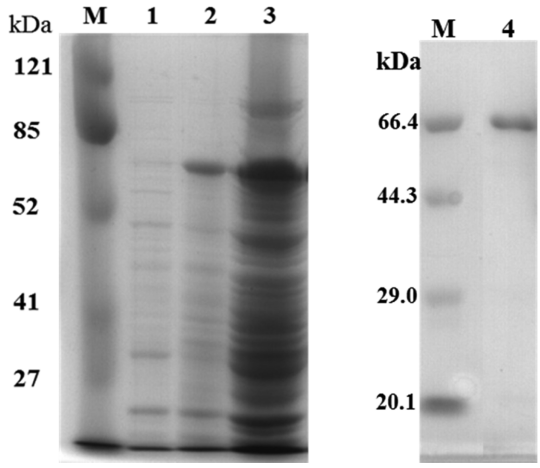

Fig. 2. SDS-PAGE analysis of the purified rMzl86. Lanes M, molecular weight maker; 1, whole cell lysate before induction; 2, whole cell lysate after induction; 3, soluble lysate after induction; 4, purified his-tagged fusion protein (rMzl86)

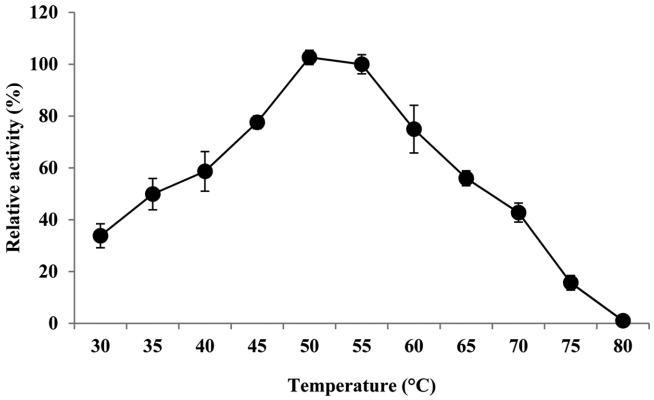

Fig. 3. Effect of temperature on rMzl86 activity

Ulhoa 2006; Kumagai and Ojima 2010; Song et al. 2010), and therefore a similar pattern was observed for rMzl86. On the other hand, thermostability of Mzl86 was determined at two different temperature values of 50 and 60°C. Mzl86 showed 80% of its relative activity at 50°C even when pre-incubation was carried out for 60 min. However the activity was significantly lost after pre-incubation at 60°C, even just for 10 min (Fig. 4). The rMzl86 showed maximum activity at pH 8, and the enzyme was stable and retained more than 80% of its activity after pre-incubation at 50°C for 1 h. However, the enzyme retained less than 20% of its activity after pre-incubation at 60°C, even for 10 min. While these results are similar to the report by Song et al. (Song et al. 2010), we could not find any detectable activity in media with pH 3 and 4 (Fig. 5).

The effects of metal ions and chemical reagents on rMzl86

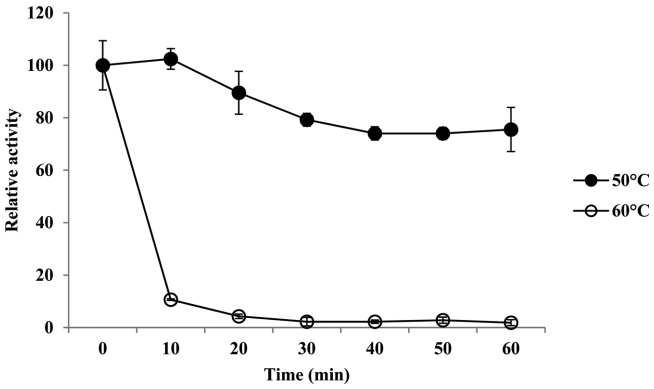

Fig. 4. Effects of thermostability on rMzl86 at different temperatures. Thermostability was evaluated by measuring the activity remaining after pre-incubation at 50 and 60°C for different time duration (10-60 min at 10 minute intervals)

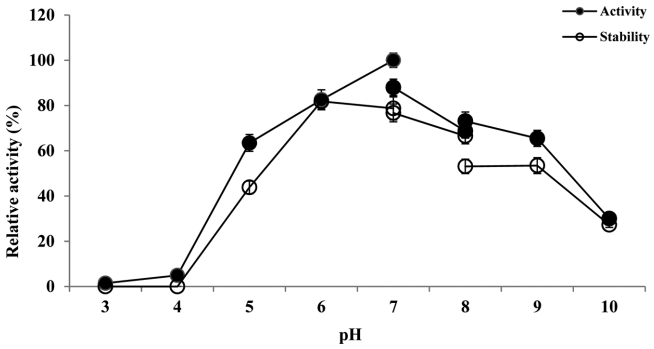

Fig. 5. Effects of pH on activity and stability of rMzl86. pH effects on rMzl86 activity at different pH ranges (pH 3-7: citrate-phosphate buffer, pH 7-8: phosphate buffer, pH 8-10: glycine-sodium hydroxide buffer). pH stability was assessed following 30 min incubation at different pH

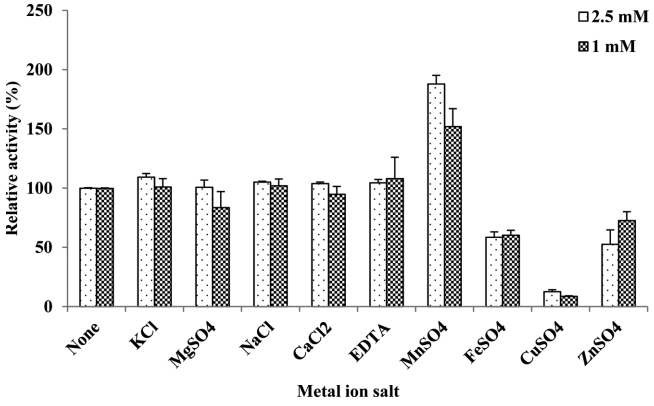

Fig. 6. Effect of metal ions and chemical reagents on rMzl86 activity. Enzymatic activity was measured in phosphate buffer (pH 7.0) containing 1 or 2.5 mM of different metal ions and chemicals reagents at 50°C for 10 min

activity are shown in Fig. 6. rMzl86 was not affected by the presence of potassium, magnesium, sodium ions or EDTA. However, rMzl86 activity was markedly enhanced by

Table 1. Substrate-specific activities and relative activities of rMzl86 against various substrates. Relative activity is expressed as % against laminarin

	Laminarin	Barley β-glucan	Lichenan	β-1,4-Xylan	CMC
Structure	1,3-β-Glucan contains some 1,6-β-linkages	1,3/1,4-β-glucan	1,3/1,4-β-glucan	β-1,4-xylan	β-1,4-glucan
Specific activity (unit/mg)	261	128	115	-	-
Relative activity	100 ± 3.5	49 ± 2.6	45 ± 2.0	-	-
Remark	Major structural and storage polysaccharide		Linear, ~30% 1,3-β and 70% 1,4-β; extended coil conformation		

manganese amendment. In contrast, iron, copper and zinc ions strongly suppressed the enzyme. The presence of 2.5 mM (187%) of manganese had a more prominent effect on rMz186 when compared to 1 mM manganese (151%). This result agrees with the previous reports on glucanases. *B. clausii* NM-1 β-1,3-glucanase was activated in the presence of Mn^{2+}, and strongly inactivated in the presence of Fe^{2+} and Cu^{2+} (Miyanishi et al. 2003b). The recombinant Agl9A of *Alicyclobacillus* sp. A4 also showed 123% activity in the presence of 2 mM Mn^{2+}, while strongly inactivated by Cu^{2+} (Bai et al. 2010).

The highest substrate specificity of rMzl86 was observed against laminarin (261 unit/mg) followed by barley β-glucan (128 unit/mg) and lichenan (115 unit/mg), thereby affirming the prominent potency of rMzl86 to hydrolyze β-1,3-glucans and β-1,3-1,4-glucans. In contrast, they could not hydrolyze β-1,4-glucans, e.g. CMC and β-1,4-xylan (Table 1). The substrate specificity of the enzyme was compatible with the proportion of β-1,3-glycosidic bonds present in their structure. According to our observation, rMzl86 catalyzed the hydrolysis of β-1,3-glycosidic bonds in β-1,3-glucan rather than in β-1,3-1,4-glucan. Thus Mzl86 can be clearly classified as an endo-β-1,3-glucanase. The β-glucanase from *Bacillus clause* NM-1 showed 100 and 32% of relative activity against laminarin and curdlan, respectively, but no activity against lichenan and barley β-glucan (Miyanishi et al. 2003b). However, several β-1,3-glucanses from bacterial species shared similar substrate candidates when compared to our enzyme, e.g. *Streptomyces* sp. S27 (Shi et al. 2010b), *Paenibacillus* sp. (Cheng et al. 2009) and *Streptomyces sioyaensis* (Hong et al. 2002). BglS27 from *Streptomyces* sp. S27 showed 100, 50.43 and 34.69% of its relative activity against laminarin, barley β-glucan and lichenan, respectively (Shi et al. 2010a). LamA from *Paenibacillus* sp. showed 3.7, 1.5 and 1.3 U/mol of substrate specificity against laminarin, β-D-glucan and lichenan, respectively.

We confirmed the degradation products of various

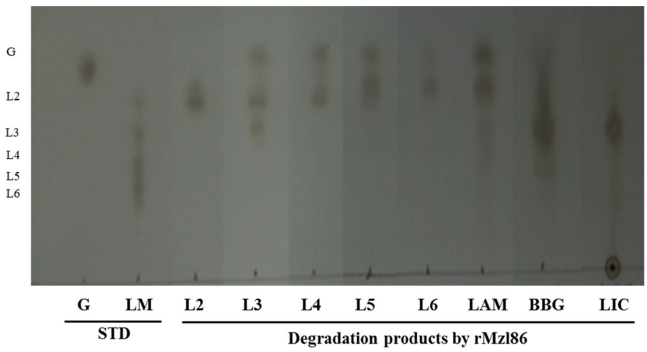

Fig. 7. TLC analysis of rMzl86 hydrolysis products with different substrates; D-glucose (G), laminaribiose (L2), laminaritriose (L3), laminaritetraose (L4), laminaripentose (L5), laminarihexose (L6), L2-L6 mixture (LM), laminarin (LAM), Barley β-glucan (BBG), lichenan (LIC). G and LM were used as standards

polysaccharides and oligosaccharides upon rMzl86 hydrolysis by thin layer chromatography (Fig. 7). The major products of laminarin hydrolysis were laminaribiose and glucose. Similarly the major products of laminarioligosaccharides hydrolysis were also found to be biose and glucose. While rMzl86 did not degrade laminaribiose and failed to produce glucose, the enzyme was able to degrade barley β-glucan and lichenan and mainly produced trisaccharide. Kumagai and Ojima (Kumagai and Ojima 2010) have already explained the catalytic mechanism of β-1,3-glucanase (HdLam; EC 3.2.1.39). Initially, the laminaribiose unit of laminaritriose is transferred to a laminaribiose producing laminaritetraose and glucose by transglcosylation. Then laminaritetraose is hybrolyzed to laminaritriose and glucose. Finally, laminaritriose is degraded to laminaribiose and glucose (Kumagai and Ojima 2010). HdLam33 from *Haliotis discus hannai* showed a similar degradation pattern as with rMzl86. HdLam33 degraded laminarin and laminarioligosaccharides and mainly resulting in biose and glucose. Further, the enzyme also degraded lichenan and produced mainly triose. Hence, Mzl86 also has the same enzymatic properties as HdLam33. Along with the

results obtained from our pairwise sequence alignment and deduced molecular properties of the enzyme, the hydrolytic activity against laminarioligosaccharides clearly affirmed that rMzl86 is an endo-acting β-1,3-glucanase enzyme.

4. Conclusion

In summary, we identified a laminarinase from *Mesoflavibacter*, a first such discovery from that genus. We isolated laminarinase-producing bacterium and the strain was designated as *M. zeaxanthinificiens* S86. A new laminarinase gene was identified by whole genome sequencing and *in silico* analyses. Mzl86 shared the standard characteristics of laminarinse homologues in its molecular characterization, biochemical properties and TLC analyses. All together, key features of Mzl86 will benefit the agriculture, drug, chemical and bioethanol industries.

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References

Akramiene D, Kondrotas A, Didziapetriene J, Kevelaitis E (2007) Effects of beta-glucans on the immune system. Medicina (Kaunas) **43**:597-606

Bai YG, Wang JS, Zhang ZF, Shi PJ, Luo HY, Huang HQ, Luo CL, Yao B (2010) A novel family 9 beta-1,3(4)-glucanase from thermoacidophilic *Alicyclobacillus* sp A4 with potential applications in the brewing industry. Appl Microbiol Biotechnol **87**:251-259

Castresana C, Decarvalho F, Gheysen G, Habets M, Inze D, Vanmontagu M (1990) Tissue-specific and pathogen-induced regulation of a *Nicotiana plumbaginifolia* beta-1,3-glucanase gene. Plant Cell **2**:1131-1143

Cheng YM, Hong TY, Liu CC, Meng MS (2009) Cloning and functional characterization of a complex endo-beta-1,3-glucanase from *Paenibacillus* sp. Appl Microbiol Biotechnol **81**:1051-1061

Delacruz J, Pintortoro JA, Benitez T, Llobell T, Romero LC (1995) A novel endo-beta-1,3-glucanase, Bgn13.1, involved in the mycoparasitism of *Trichoderma harzianum*. J Bacteriol **177**:6937-6945

Genta FA, Bragatto I, Terra WR, Ferreira C (2009) Purification, characterization and sequencing of the major beta-1,3-glucanase from the midgut of *Tenebrio molitor* larvae. Insect Biochem Mol Biol **39**:861-874

Goujon M, Mcwilliam H, Li WZ, Valentin F, Squizzato S, Paern J, Lopez R (2010) A new bioinformatics analysis tools framework at EMBL-EBI. Nucleic Acids Res **38**:W695-W699

Gueguen Y, Voorhorst WGB, Vanderoost J, Devos WM (1997) Molecular and biochemical characterization of an endo-beta-1,3-glucanase of the hyperthermophilic archaeon *Pyrococcus furiosus*. J Biol Chem **272**:31258-31264

Hong TY, Cheng CW, Huang JW, Meng MS (2002) Isolation and biochemical characterization of an endo-1,3-beta-glucanase from *Streptomyces sioyaensis* containing a C-terminal family 6 carbohydrate-binding module that binds to 1,3-beta-glucan. Microbiology **148**:1151-1159

Joosten MH, Dewit PJ (1989) Identification of several pathogenesis-related proteins in tomato leaves inoculated with *Cladosporium fulvum* (Syn Fulvia-Fulva) as 1,3-beta-glucanases and chitinases. Plant Physiol **89**:945-951

Krah M, Misselwitz R, Politz O, Thomsen KK, Welfe H, Borriss R (1998) The laminarinase from thermophilic eubacterium *Rhodothermus marinus* - Conformation, stability, and identification of active site carboxylic residues by site-directed mutagenesis. Eur J Biochem **257**:101-111

Kumagai Y, Ojima T (2010) Isolation and characterization of two types of beta-1,3-glucanases from the common sea hare *Aplysia kurodai*. Comp Biochem Phys B **155**:138-144

Larkin MA, Blackshields G, Brown NP, Chenna R, Mcgettigan PA, Mcwilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and clustal X version 2.0. Bioinformatics **23**:2947-2948

Letunic I, Doerks T, Bork P (2009) SMART 6: recent updates and new developments. Nucleic Acids Res **37**:D229-D232

Letunic I, Doerks T, Bork P (2012) SMART 7: recent updates to the protein domain annotation resource. Nucleic Acids Res **40**:D302-D305

Marchler-Bauer A, Anderson JB, Chitsaz F, Derbyshire MK, Deweese-Scott C, Fong JH, Geer LY, Geer RC, Gonzales NR, Gwadz M, He S, Hurwitz DI, Jackson JD, Ke Z, Lanczycki CJ, Liebert CA, Liu C, Lu F, Lu S, Marchler GH, Mullokandov M, Song JS, Tasneem A, Thanki N, Yamashita RA, Zhang D, Zhang N, Bryant SH (2009) CDD: specific functional annotation with the Conserved Domain Database. Nucleic Acids Res **37**:D205-D210

Marchler-Bauer A, Bryant SH (2004) CD-Search: protein domain annotations on the fly. Nucleic Acids Res **32**:W327-W331

Marchler-Bauer A, Lu SN, Anderson JB, Chitsaz F, Derbyshire MK, Deweese-Scott C, Fong JH, Geer LY, Geer RC, Gonzales NR,

Gwadz M, Hurwitz DI, Jackson JD, Ke ZX, Lanczycki CJ, Lu F, Marchler GH, Mullokandov M, Omelchenko MV, Robertson CL, Song JS, Thanki N, Yamashita RA, Zhang DC, Zhang NG, Zheng CJ, Bryant SH (2011) CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Res **39**:D225-D229

Masuda S, Endo K, Koizumi N, Hayami T, Fukazawa T, Yatsunami R, Fukui T, Nakamura S (2006) Molecular identification of a novel beta-1,3-glucanase from alkaliphilic *Nocardiopsis* sp strain F96. Extremophiles **10**:251-255

Mccarthy T, Hanniffy O, Savage AV, Tuohy MG (2003) Catalytic properties and mode of action of three endo-beta-glucanases from *Talaromyces emersonii* on soluble beta-1,4- and beta-1,3;1,4-linked glucans. Int J Biol Macromol **33**:141-148

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem **31**:426-428

Miyanishi N, Hamada N, Kobayashi T, Imada C, Watanabe E (2003a) Purification and characterization of a novel extracellular beta-1,3-glucanase produced by *Bacillus clausii* NM-1 isolated from ezo abalone *Haliotis discus hannai*. J Biosci Bioeng **95**:45-51

Miyanishi N, Matsubara Y, Hamada N, Kobayashi T, Imada C, Watanabe E (2003b) The action modes of an extracellular beta-1,3-glucanase isolated from *Bacillus clausii* NM-1 on beta-1,3-glucooligosaccharides. J Biosci Bioeng **96**:32-37

Mohagheghpour N, Abel K, Lapaglia N, Emanuele NV, Azad N (1995) Signal requirements for production of luteinizing-hormone releasing-hormone by human T-cells. Cell Immunol **163**:280-288

Monteiro V, Ulhoa C (2006) Biochemical characterization of a beta-1,3-glucanase from *Trichoderma koningii* induced by cell wall of *Rhizoctonia solani*. Curr Microbiol **52**:92-96

Nisizawa K YT, Handa N, Maeda M, Yamazaki H (1963) Chemical nature of a uronic acid-containing polysaccharide in the peritrophic membrane of the silkworm. J biochem **54**:419-426

Oh C, Heo SJ, Zoysa MD, Affan A, Jung WK, Park HS, Lee Y, Lee J, Yoon KT, Kang DH (2011) Whole-genome sequence of the xylanase-producing *Mesoflavibacter zeaxanthinifaciens* strain S86. J Bacteriol **193**:5557-5557

Pang Z, Otaka K, Suzuki Y, Goto K, Ohnishi M (2004) Purification and characterization of an endo-1,3-beta-glucanase from *Arthrobacter* sp. J Biol Macromol **4**:57-66

Petersen TN, Brunak S, Von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods **8**:785-786

Planas N (2000) Bacterial 1,3-1,4-beta-glucanases: structure, function and protein engineering. Biochem Biophys Acta **1543**:361-382

Pretorius I VDWT (1991) The impact of yeast genetics and recombinant DNA technology on the wine industry-a review. S Afr J Enol Vitic **12**:3-31

Ryan JM, Hoole ACF, Broers AN (1995) A study of the effect of ultrasonic agitation during development of poly(methylmethacrylate) for ultrahigh resolution electron-beam lithography. J Vac Sci Technol B **13**:3035-3039

Schultz J, Milpetz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: Identification of signaling domains. P Natl Acad Sci USA **95**: 5857-5864

Shi PJ, Li N, Yang PL, Wang YR, Luo HY, Bai YG, Yao B (2010a) Gene cloning, expression, and characterization of a family 51 alpha-L-arabinofuranosidase from *Streptomyces* sp S9. Appl Biochem Biotechnol **162**:707-718

Shi PJ, Yao GY, Yang PL, Li N, Luo HY, Bai YG, Wang YR, Yao B (2010b) Cloning, characterization, and antifungal activity of an endo-1,3-beta-d-glucanase from *Streptomyces* sp S27. Appl Microbiol Biotehcnol **85**:1483-1490

Song JM, Nam K, Sun YU, Kang MH, Kim CG, Kwon ST, Lee J, Lee YH (2010) Molecular and biochemical characterizations of a novel arthropod endo-beta-1,3-glucanase from the *Antarctic springtail*, *Cryptopygus antarcticus*, horizontally acquired from bacteria. Comp Biochem Phys B **155**:403-412

Wong MG (1979) 1,3-beta-D-glucnases from *Pisum sativum* seedlings. I. Isolation and purification. Biochim Biophys Acta **39**:861-874