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Cloning, expression, and biochemical characterisation of a novel endomannanase from *Thermobifida alba*

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ABSTRACT

Thermobifida alba is the mesophilic member of the *Thermobifida* genus, the genome and enzyme sets of which have not been described and published yet. *Thermobifida* strains are thermotolerant actinomycete, which possess wide sets of cellulose and hemicellulose hydrolysing enzymes. Previously, three endomannanases (Man5ATh, Man5ATc, and Man5AThf) of thermobifidas were cloned and investigated, and hereby the endomannanase of *T. alba* DSM 43795 is described. All four endomannanases belong to the glycoside hydrolase family 5, their sizes are around 50–55 kDa. Their structure consists of a catalytic domain and a carbohydrate binding module, while there is an interdomain linker region in-between consisting repetitive tetrapeptide motifs (eg.: PPTEPTD-Ta, PTDP-Tc, TEEP-Tf, DPGT-Th). The pH optima of Man5A enzymes from *T. alba*, *Thermobifida halotolerans*, *Thermobifida cellulosilytica*, and *Thermobifida fusca* are slightly different (6.5, 7.0, 7.5, and 8.0, respectively), however, the temperature optima of the enzymes were detected within a wider range of 65–75 °C. In this research, Man5ATa

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exhibited the lowest Michaelis-Menten constant (KM) (0.13 mM) on LBG-mannan substrate, while others shared similar kinetic parameters: 0.9–1.7 mM of KM. Despite the high sequence similarity of the investigated mannanases, they exhibit different temperature stability parameters. These different functional characteristics can be advantageous for industrial applications producing biologically active, oligomannan prebiotics under different conditions.

KEYWORDS

endomannanase, hemicellulase, LBG, protein expression, *Thermobifida alba*

1. INTRODUCTION

The main components of plant cell wall are cellulose and hemicellulose. Hemicelluloses are heteropolymers with varying degrees of branching and are classified based on their sugar residues attached to the backbone of the structural polymer. Hemicelluloses include different sugar monomers such as xylose, mannose, galactose, rhamnose, arabinose and polymers like xylan, heteromannan, and mannan (de Vries and Visser, 2001). The second most abundant hemicelluloses in plant cell wall are galacto(gluco)mannans, which consist of a backbone of ß-1,4-linked D-mannose and D-glucose residues with D-galactose side chains (Stoll et al., 2000). Mannan biopolymers play roles in storing carbohydrates in the seed of leguminous plants and beans, or as being structural carbohydrates that cross link cellulose fibrils and lignin in plants (Eriksson et al., 1991; Puls, 1997; Várnai et al., 2011; Barros et al., 2015; von Freiesleben et al., 2018).

Enzymatic degradation of plant materials to fermentable monomeric sugars is demanding due to their complex composition and inhomogeneous structure (Várnai et al., 2012). In addition, hemicelluloses like β-1,4 mannan and β-1,4 xylan prevent enzymatic hydrolysis of cellulose in the absence of relevant accessory enzymes as at least nine different enzyme activities are required to completely degrade the hemicellulose substituents (van den Brink and de Vries, 2011; Hu et al., 2013; Østby et al., 2020).

The degradation of hemicellulose is performed by the action of ß-endomannanases (EC 3.2.1.78) and ß-mannosidases (EC 3.2.1.25), ß-glucosidases (EC 3.2.1.21), and α-galactosidase (EC 3.2.1.22), commonly expressed by fungi and bacteria.

Endomannanases are necessary enzymes for the release of mannan from the hemicellulose substrate matrix (Tenkanen et al., 1997; Várnai et al., 2012). Endomannanases are classified based on CAZY database into four glycosyl hydrolase (GH) families: 5, 26, 113, and 134 (http://www.cazy.org/Glycoside-Hydrolases.html) (Lombard et al., 2013). According to certain studies of mannanases of *Cellvibrio* spp., GH5 enzymes primarily attack insoluble mannans, while GH26 enzymes may primarily attack soluble mannans (Tailford et al., 2009; Zhang et al., 2014). About the other two GH families much less is known, as only three GH113 mannanases were described (*Aa*ManA, Man113A, and *Ax*Man113A) so far, and the exact catalytic mechanism and substrate-enzyme complex structure is still unclear. Substrate specificity experiments showed that all these GH113 mannanases show higher affinity toward konjac powder than LBG, which is preferable for GH5 and GH26 enzymes (Xia et al., 2016; You et al., 2018). On the contrary, from the group of GH134 only one β-1,4-mannanase was described from bacteria,

SsGH134 from *Streptomyces* sp. NRRL B-24484, which showed higher affinity toward insoluble microcrystalline cellulose and insoluble mannans similarly to GH5 and GH26, but further studies are needed to confirm these observations due to lack of similar enzymes (Sakai et al., 2018).

Cellulolytic bacteria can be found in different genus such as *Acetivibrio, Bacillus*, *Caldicellulosiruptor*, *Cellulomonas, Clostridium*, *Erwinia, Streptomyces, Thermobifida, Thermotoga, Fibrobacter, Cytophaga,* and *Sporocytophaga* (Lynd et al., 2002). These bacteria possess the so-called plant cell wall-degrading enzymes (CWDEs), which have a wide range of industrial applications throughout the food and feed industry and for the sustainable production of chemicals and biofuels like bioethanol. Most widely used enzymes are endo-acting CWDEs, such as mannanases, xylanases, galactanases, and exo-acting CWDEs, such as β-galactosidases, β-mannosidase, β-xylosidase, and L-α-arabinofuranosidases. Mannanases are used for the production of prebiotic oligosaccharides, namely manno-oligosaccharides (MOS) retrieved from the digestion of low-cost agro-industrial by-products such as copra, carob pods, coconut residues, sugar beet molasses, and palm kernel cake (Gibson et al., 2005; Rastall et al., 2005; Ozturk et al., 2010; Zurmiati et al., 2017; Bello et al., 2018). These mannan-rich by-products can be cheap alternatives of feed ingredients in animal feeding instead of maize and soybean. Apart from MOS, β-mannanases are also used in feed industry to improve digestibility, nutrient utilisation of meals by hydrolysing mannan and thus, enhancing the performance and growth of poultry. Feed ingredients contain high levels of β-mannans, which cause higher viscosity in meals, therefore, negatively affect the growth of animals due to their indigestive nature. This mannan component can be hydrolysed by the addition of β-mannanases to the feed (Shastak et al., 2015; Caldas et al., 2018; Cano et al., 2020; Norizan et al., 2020).

Mannanases are also used in other parts of food industry like the extraction of coffee and coconut oil. Mannan is present as the main polysaccharide in coffee extract and increases the viscosity of the extract, which negatively affects the preparation of instant coffee. β-mannanase hydrolyses mannan into simple sugars, thus, it reduces the viscosity of coffee extract. This kind of enzymatic hydrolysis procedure is a substitute to the applied thermal procedure as it produces smaller amount of secondary compounds like furfural, acetaldehyde, and 5-HMF, and also needs less energy for the process of hydrolysis (Murkovic and Bornik, 2007; Soni and Kango, 2013; Baraldi et al., 2016; Favaro et al., 2020).

In addition, treatment of drinks like raw fruit juice is another application area for β-mannanases. Natural fruit juice is viscous and turbid mainly due to its content of pectin, starch, cellulosic and hemicellulosic compounds, so for consumers it must be clarified. These polysaccharides need to be hydrolysed mainly by microbial enzymes, which results in a juice that is more suitable for the consumer's taste, moreover, has good storage stability. In studies, β-mannanase isolated from *Weissella viridescens* LB37 was effective in juice clarification from different fruits such as grape, peach, orange, pomegranate, kiwi, and apple, as compared to control (Adiguzel et al., 2015). Furthermore, not only clarity but increased yield of juice can be achieved. It was reported that treatment of crude β-mannanase of *Lactobacillus casei* HDS-01 resulted higher yield of apple, orange, and pear juices with a yield ratio of 150.96%, 188.20%, and 172.62%, respectively, compared to control (Zhao et al., 2020).

Over the years, the demand for enzymes with higher thermal stability (cellulases, amylases, xylanases, lipases, proteases, and pectinases) was growing compared to their mesophilic homologues. These are commonly used in high temperature biotechnology processes,

as at higher levels of temperature the reaction rates are accelerated, thus, reactions are shortened, the process needs reduced energy input, and there is a lower contamination risk. The largest producers of cellulases are within the genus of *Streptomyces*, which have the ability to ferment cellulose. It was reported in a study that cellulases produced by *Streptomyces* spp. are highly thermostable (Jang and Chang, 2005). The other important genus for the production of thermostable enzymes within the Actinobacteria phylum is the genus *Thermobifida,* including four strains: *Thermobifida fusca* and *Thermobifida alba* (Zhang et al., 1998), *Thermobifida cellulosilytica* (Kukolya, 2002), and *Thermobifida halotolerans (*Yang et al., 2008). *Thermobifida* strains are mostly compost-inhabiting strains that are well-known for their compost-decomposing and lignocellulolytic activities. Thermobifidas are mostly thermophilic bacteria, thus, able to grow under high temperature conditions, except *T. alba,* which is rather mesophilic and slightly thermotolerant. Among the four species, the genomes of *T. fusca* and its isolate *Thermobifida fusca* YX (ASM1240v1) (Lykidis et al., 2007) have been completely, while *Thermobifida fusca* TM51 (ASM40191v1) (Tóth et al., 2013) have been partially sequenced. By the analysis of these genomes, 39 glycoside hydrolases belonging to different GH-families were determined. During the years, the cellulolytic enzyme system of *T. fusca* has become well-characterised, and the strain became a model organism for the study of thermostable cellulases due to their thermostability, broad pH range (pH 4–10), and high activity (Wilson, 2004).

There are not so many studies and publications on the hemicellulolytic enzyme systems of the *Thermobifida* spp. yet. From the most well-known *Thermobifida fusca* TM51 (ASM40191v1), two GH5 hydrolases - endomannanase (man5A) and endoglucanase (cel5A) genes - (Yan et al., 2013) and one GH2 beta-mannosidase (ManB) (Béki et al., 2003) were identified. Moreover, xynA, a recombinant xylanase from *T. fusca* was also confirmed with its ability to degrade xylan (Zhao et al., 2015). *T. cellulosilytica* TB100 (ASM151797v1) enzyme set shows similar homology in the xylanases, mannanases, and xyloglucanases, which was identified in the *T. fusca* genome project. Among the hemicellulases, the mannanase enzymes consist of an intracellular GH2 mannosidase and an extracellular GH5 endomannanase (Tóth et al., 2017). So far, from *T. halotolerans* YIM90462 (ASM166038v1) one endoglucanase (Thcel9A) and one GH5 endomannanase (Man5-ATh) were described (Zhang et al., 2011).

Hereby, in this report, we are presenting and characterising the only endomannanase (Man5ATa) that has not yet been described from the *Thermobifida* genus, *T. alba,* and also comparing it with the other three previously described endomannanases from the *Thermobifida* species, namely *T. fusca*, *T. cellulosilytica,* and *T. halotolerans*.

2. MATERIALS AND METHODS

2.1. Microorganism and culture conditions

T. alba DSM 43795 type strain was used in this study, retrieved from DSM collection, originally isolated from garden soil (Locci et al., 1967). The strain was initially plated onto 1% saccharose-containing plates and incubated at 42 °C for 3–4 days, when sporulation phase started. Stand-alone sporulating colonies were inoculated into LB medium in baffled Erlenmeyer flask, and the cultivation continued with agitation at 180 r.p.m., at 42 °C.

2.2. Cloning of endomannanase-encoding gene in accordance with genome sequence data

Prior to genomic DNA isolation, the strain was streaked on Luria-Bertani (LB) agar plate to produce discrete colonies. Using a discrete colony LB liquid medium was inoculated for producing biomass for isolation of genomic DNA. For the isolation of genomic DNA from *T. alba*, a modified method by Nagy et al. (1997) was used. The data of the genome sequence of *T. fusca* TM51 (ASM40191v1) (Tóth et al., 2013) and the previously described three endomannanase gene sequences (Man5ATf, Man5ATc, and Man5ATh) were used as references for partial sequencing in the *T. alba* DSM 43795 genome. Based on sequence similarities, the hypothetical endomannanase gene could be located, making it possible to manually design primers on that certain DNA segment (outside the gene and also at the start of the gene) and to amplify it by PCR. For amplification of the endomannanase gene primers, man5ATa forward (5′-ATGAGAA-AACGCCTCACGGTCGCGGCAGC-3′) and man5ATa reverse (5′-TCAGTCGGTCGTG-CAGCTCAGCACCA-3′) were used with underlined sequences harbouring *Nde*I and *Xho*I restriction sites. PCR reactions were carried out by using *Pfu* DNA polymerase (Thermo Fisher Scientific Inc., USA) for 32 cycles of 10 s at 98 °C, 20 s at 68 °C, and 45 s at 72 °C, preceded by incubation for 30 s at 98 °C. After the PCR reaction, the PCR products were separated by gel electrophoresis in 1% agarose gel and were resected directly from the gel. After dissolution of the gel and re-purification of the PCR products, the fragments were digested with *Nde*I and *Xho*I enzymes (Thermo Fisher Scientific Inc., USA) and ligated into pET28 and pRSF plasmid vectors by using T4 DNA ligase (Thermo Fisher Scientific Inc., USA). Then *E. coli* DH5α competent cells (NEB, USA) were transformed to isolate proper clones, which were used for protein expression in *E. coli* BL21 (DE3) cells (NEB, USA).

2.3. Phylogenetic analysis of *Thermobifida* strains and their endomannanase

For the molecular identification of investigated *Thermobifida* strains, PCR amplification of 16S rDNAs were performed as described by Rainey et al. (1996). The sequences of the 16S rDNA were initiated with the conservative eubacterial primer 27f and 1492r (Baker et al., 2003). 16S rDNA sequence reads and amino acid sequences of the cloned endomannanases were assembled by MEGA X, respectively (Kumar et al., 2018), then aligned by ClustalW algorithm. Neighbour-joining trees were also constructed in MEGA X software, performing 1000 bootstrap replicates.

2.4. Expression and purification of *T. alba* endomannanase

The recombinant His-tagged endomannanase was over-expressed in *E. coli* BL21 (DE3) cells. Transformants were grown in 500 mL of LB medium containing 50 μg mL^{-1} of kanamycin until OD600 reached 0.6–1.0, at 37 °C with 180 r.p.m. aeration. By the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM final concentration) the protein expression was induced and followed by an overnight agitation at 18 °C with same parameters. After incubation, cells were harvested and disrupted by sonication in lysis buffer (pH 7.5), the lysate was centrifuged at 16,000×*g* for 20 min at 4 °C, and the supernatant was loaded onto a 5 mL HiTrap column (GE Healthcare, USA) for immobilised metal-ion affinity chromatography (IMAC) by ÄKTA Start purification system (GE Healthcare, USA). Protein elution was performed with a gradient of 0–500 mM imidazolein, 300 mM NaCl, and 20 mM sodium phosphate buffer, pH 7.5.

The purified protein concentration of the pooled fractions was determined by Bradford method using BSA as protein standard (Bradford, 1976).

The molecular mass of the enzyme was estimated by SDS-PAGE analysis.

2.5. Biochemical characterisation of the endomannanase

Substrate specificity of the endomannanase was assayed using different substrates, such as low viscosity carboxymethyl cellulose (CMC), crystalline and microcrystalline cellulose (MN300, Avicel), beechwood xylan, and locust bean gum (LBG-mannan) as polysaccharides and 4-nitrophenyl β-D-mannopyranoside (pNP-βM) as an artificial aryl-mannoside substrate.

Endomannanase activities were determined on polysaccharide substrate by the Somogyi-Nelson method (Somogyi, 1952), in which the liberated reducing sugars were measured. For the calibration curves, dilution series of mannose stock solution was used for the determination of the reducing sugar content.

Michaelis-Menten kinetic parameters of the endomannanase was estimated for LBG-mannan substrate in 10 different concentrations between 0 and 5 mg mL^{-1} (0, 0.2, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 5.0 mg mL^{-1}) at 50 °C, in 50 mM sodium phosphate buffer, pH 7.5. The final volume of the enzyme reactions was 50 µL containing 1.2 µg mL^{-1} of Man5ATa. LBG-mannan substrate solutions were pre-incubated at 50 °C prior the initiation of the reactions by adding enzyme samples to the solutions, then the incubation continued for further 5 min during the enzyme reaction.

The pH dependence of endomannanase activity was measured in the presence of 5 mg mL^{-1} LBG substrate with different buffers in the pH range of 4–10 at 50 °C, when the enzyme reactions contained 1.2 µg mL^{-1} of Man5ATa in 50 µL reaction volumes. The following buffers were used (pH ranges are indicated in brackets): 100 mM citrate-phosphate (pH 4.0–6.5), 100 mM sodium phosphate (pH 6.5–7.5), 200 mM triethanolamine/HCl (pH 7.5–9.0), and 100 mM CAPS/HCl (pH 9–10.0).

The effect of temperature on endomannanase activity was determined in 50 mM sodium phosphate buffer, pH 7.5 at different temperatures ranging from 25 to 90 °C and using 5 mg mL^{-1} LBG-mannan substrate in 50 µL reaction volume. Enzyme concentrations were the same as in case of pH optimum determination. For both pH and temperature dependence studies the Somogyi-Nelson method was applied for the determination of the liberated reducing sugar concentration. Then, initial rates were calculated and converted into relative rates and plotted against pH and temperature.

All measurements were carried out in triplicates. The initial rate of the enzyme catalysis was expressed in mM of reducing sugar liberated in one minute as it was determined from the calibration curve.

Thermal stability study of Man5ATa enzyme was performed in 0.5 mL of 100 mM sodium phosphate buffer, pH 7.5, incubated at 60 °C. From the incubated endomannanase-substrate solutions, time course aliquots (10 µL) were withdrawn and then assayed for endomannanase activity determinations. Reaction was performed at 50 °C for 10 min in the presence of 2 mg mL^{-1} LGB-mannan in 50 mM phosphate buffer, followed by the addition of DNA reagent and 10 min of boiling according to DNS (3,5-dinitrosalicylic acid) method by Miller (1959). After cooling the mixture to room temperature, the absorbance of the supernatant was measured at 540 nm. The residual activity was calculated as a fraction of the initial activity and plotted against time.

2.6. Nucleotide sequence accession numbers

GenBank accession numbers of determined endomannanase nucleotide sequences are as follows: man5ATf: KF684964, man5ATc: KF684965, and man5ATh: KF684966. GenBank accession numbers of the three identified endomannanase amino acid sequences are: Man5ATf: AHB89702, Man5ATc: AHB89703, and Man5ATh: AHB89704.

16S rDNA sequence accession numbers are: *T. alba* DSM 43795: NR_037093, *T. cellulosilytica* TB100: NR_025438, *T. halotolerans* YIM 90462: NR_044446, and *T. fusca* TM51: AOSG01000000.

The nucleotide and amino acid sequences of the endomannanase of *T. alba* DSM 43795 has not been published on GenBank yet.

3. RESULTS AND DISCUSSION

3.1. Biochemical characterisation of Man5ATa

3.1.1. Substrate specificity measurements. The GH5 family includes several types of hydrolases, among other endo-β-1,4-glucanase/cellulase (EC 3.2.1.4), exo-β-1,4-glucanase/cellodextrinase (EC 3.2.1.74), cellulose β-1,4-cellobiosidase (EC 3.2.1.91), β-glucosidase (EC 3.2.1.21), β-mannosidase (EC 3.2.1.25), mannan endo-β-1,4-mannosidase (EC 3.2.1.78), β-1,3-mannanase (EC 3.2.1.-), and xylanases like endo-β-1,4-xylanase (EC 3.2.1.8) and arabinoxylan-specific endo-β-1,4-xylanase (EC 3.2.1.-). Substrate specificities of expressed endomannanases were tested with carboxymethyl-cellulose (CMC), crystalline and micro-crystalline cellulose (MN300, Avicel), beechwood xylan, pNp-mannopyranoside, and locust bean gum (LBG). All investigated Man5A exhibited activity only on LBG mannan. This narrow substrate specificity indicates that the axial OH group at C2 on the pyranose ring is essential in ligand binding at the active site and suggests a potential biotechnological application of the enzymes in the production of oligomannan prebiotics. Microbial mannanases can be used to break down mannan-rich residues to yield manno-oligosaccharides (MOS), known as functional oligosaccharides, which are non-digestible food ingredients that have health-promoting benefits. In general, commercially available MOS can be derived from yeast cell wall mannan or from other mannan-rich sources and is widely used as prebiotic in feed supplements for poultry and aquaculture (Benites et al., 2008; Dimitroglou et al., 2010; Torrecillas et al., 2014; Zhou et al., 2019). Beside the stimulation of the growth of probiotic microbiome, MOS might possess anticancer and immunomodulatory effects by inducing different gene markers in colon cells (Jana and Kango, 2020). According to a scientific study, acute colitis was induced by dextran-sulphate-sodium (DSS) in mice, which was then fed with yeast cell wall manno-oligosaccharide (MOS). The results were promising, as MOS reduced DSS-induced clinical- (weight loss, diarrhea) and histological scores (mucosal damage) as well as sickness-related anxiety. Moreover, due to the DSS treatment, the colon microbiome has changed and the number of coliform bacteria increased, while the addition of MOS attenuated colitis-related increase of coliforms and also decreased the local expression of pro-inflammatory cytokines. These inflammatory bowel disease animal models are similar to IBD in human patients, thus, the outcome suggests that the application of MOS in human can have similar protective, anti-inflammatory results and direct effect on the

composition of the gut microbiome (Ferenczi et al., 2016). Interestingly, in the human microbiome, one of the dominant members is the *Bacteroides thetaiotaomicron*. After sequencing its whole genome, it was revealed that it encoded 36 proteins, which predicted to possess α-mannosidase or α-mannanase activities. By this mechanism it is presumed that the human gut can also utilise yeast mannan by its own adapted microbiota, by the evolution of the incorporation of eukaryotic microorganisms into the human diet (Xu, 2003; Cuskin et al., 2015).

3.1.2. Kinetic studies. Endomannanase activities of the four GH5 glycoside hydrolases were investigated on LBG-mannan substrate. The applied substrate consisted of a β-(l,4)-linked mannan backbone with single α-(l,6)-linked galactose side chains. Kinetic studies were performed in 50 mM phosphate buffer, pH 7.5, using LBG-mannan as substrate in the concentration range of 0–5 mg mL^{-1} at 50 °C.

The working temperature of the endomannanase of *T. alba* is in the range of 50–75 °C at given assay conditions, while the optimum for the enzyme activity is at 65 °C (Fig. 1). Thus, this enzyme possesses the lowest temperature optimum of all other *Thermobifida* endomannanase enzymes, which is 70 °C for Man5ATc and Man5ATh and 75 °C for Man5ATf (Tóth et al., 2016). This result could be expected as *T. alba* strains are mesophilic and more like thermotolerant bacteria with low growth temperature conditions between 37 and 55 °C similarly to *T. halotolerans* (37–55 °C), while the other thermobifidas are characterised as thermophilic bacteria with a higher temperature optimum in the range of 65–75 °C. In accordance with these results, thermophilic actinobacteria grow at relatively high temperatures ranging from 40 °C to 80 °C (Shivlata and Satyanarayana, 2015). There are two group types based on temperature conditions: strictly thermophilic and moderately thermophilic actinobacteria. The thermophilic actinobacteria grow in the temperature range between 37 °C and 65 °C, and the optimum growth temperatures are between 55 and 60 °C. While moderately thermophilic actinobacteria like *T. alba* are able to grow at 28–60 °C and require 45–55 °C for cultivation (Jiang and Xu, 1993).

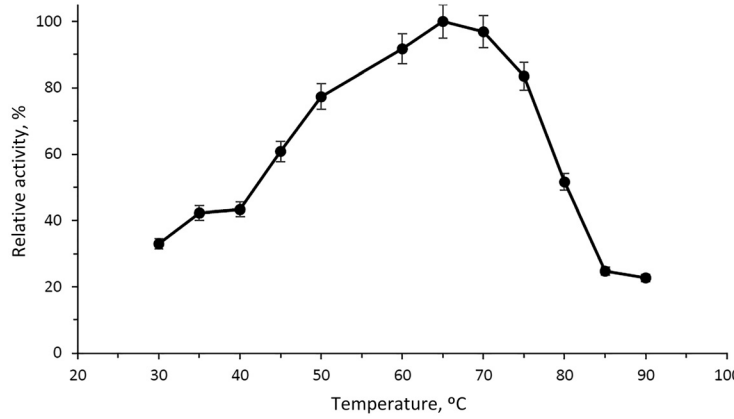

Fig. 1. Temperature optimum of Man5ATa. The study of determination of thermal optimum was done in the presence of 5 mg mL^{-1} LBG-mannan, in 50 mM Na-phosphate buffer (pH 7.5). The temperature optimum of Man5ATa is at 65 °C

The functional pH range of Man5ATa endomannanase is between 4.5 and 7.5 (Fig. 2). It is slightly moved to the lower acidic range compared to Man5ATc and Man5ATh that both have values in the overlapping range of 5.5–9.0 and Man5ATf with the highest pH range of 5.5–9.7. The optimum for *T. alba* endomannanase is pH 6.5, while it is 7.0 and 7.5 for Man5ATc and Man5ATh, respectively, and pH 8.0 for Man5ATf.

Michaelis-Menten kinetic parameters determined at pH 7.5 and 50 °C are listed in Table 1. Kinetic performances expressed in catalytic constants (k_{cat}) are similar for the three investigated mannanases with the value of 100 ± 20 s^{-1}. Slight difference was found in the individual Michaelis-Menten constants (KM) among the enzymes, Man5ATf had the lowest affinity toward the LBG-mannan substrate with KM value of 1.65 mg mL^{-1}, while Man5ATa had the highest affinity toward the LBG-mannan substrate with the lowest KM value of 0.13 mg mL^{-1} (Table 1).

Similarly to *T. alba,* the β-mannanase from *Aspergillus niger* had a higher affinity towards LBG (Km, 0.11 mg mL^{-1}) than other mannans like guar gum and copra mannan (Naganagouda et al., 2009).

3.1.3. Thermostability studies. *T. alba* β-(l,4)-endomannanase exhibited the lowest thermostability among all investigated enzymes of thermobifidas with a life-time of 30 min at 60 °C (Fig. 3). Man5ATa loses its complete activity after 5 min at 70 °C. On the contrary, based on recent studies, the half-life of Man5ATf, Man5ATh, and Man5ATc were 123 min, 36 min, and 21 min, respectively (Tóth et al., 2016). The differences in the thermal stability of mannanases can be due to the diverse environmental conditions of niches that are populated by *Thermobifida* strains. *T. alba* can grow at the lowest temperature (37–55 °C), thus, its endomannanase exhibits the lowest thermal stability. Similarly, *T. halotolerans* and *T. cellulosilytica* have low temperature optima (50–55 °C). While *T. fusca* TM51, the endomannanase of which exhibits the highest stability, was isolated from compost and its temperature optimum is 60 °C (Kukolya et al., 1997).

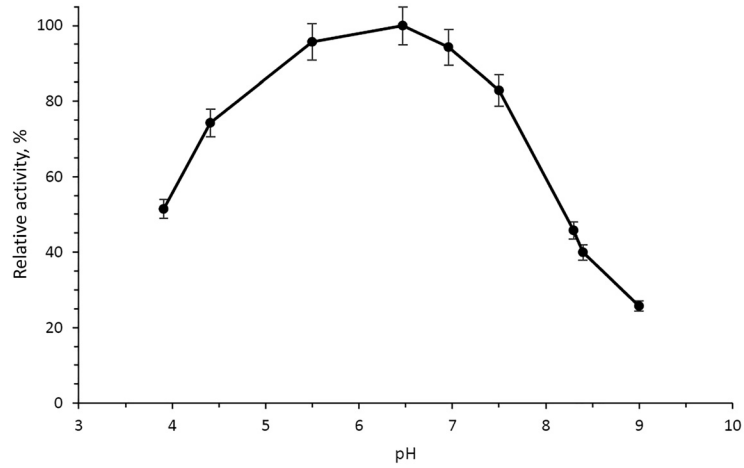

Fig. 2. pH effect on enzyme activity of *Thermobifida alba* endomannanase. The pH optimum determination study was performed in the presence of 5 mg mL^{-1} LBG-mannan. The functional pH range of Man5ATa is in between 4.5 and 7.5 and the pH optimum is at 6.5

Table 1. Michaelis-Menten kinetic parameters of endomannanases for LBG-mannan substrate. Kinetic studies were performed in 50 mM phosphate buffer, at pH 7.5, in the concentration range of 0–5 mg mL^{-1} of LBG-mannan at 50 °C. Man5ATa exhibited the highest affinity toward the mannan substrate with KM value of 0.13 mg mL^{-1}

Endomannanase	KM (mg mL^{-1})	k_{cat} (s^{-1})	k_{cat}/KM (mL s^{-1} mg^{-1})
Man5ATa	0.13 ± 0.02	4.28	33
Man5ATf	1.65 ± 0.40	122 ± 11	74
Man5ATh	1.30 ± 0.30	78 ± 9	60
Man5ATc	0.84 ± 0.15	89 ± 5	106

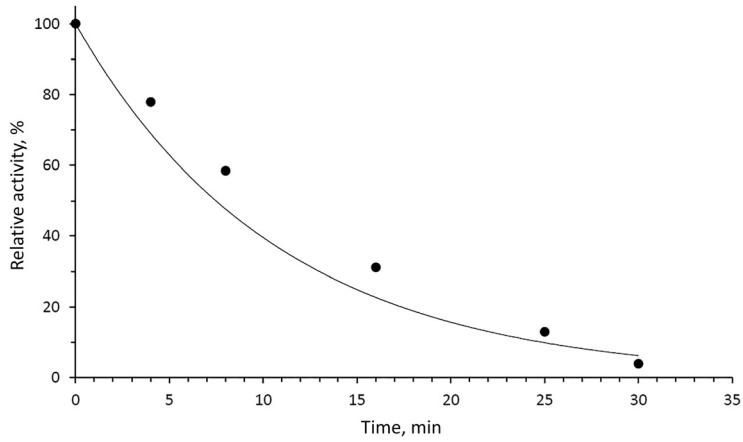

Fig. 3. Thermostability of *Thermobifida alba* endomannanase at 60 °C measured under optimal conditions. Man5ATa was measured in 50 mM Na-phosphate (pH 7.5) in the presence of 2 mg mL^{-1} LBG mannan. The half-life of Man5ATa under these conditions is around 30 min

The thermal stability of Man5ATf (half-life 123 min at 70 °C) put this enzyme among the most stable enzymes, such as mannanase of *Caldibacillus cellulovorans* (half-life 48 min at 85 °C and detectable activity even after 24 h at 70 °C) (Sunna et al., 2000).

3.2. Phylogenetic analysis of *Thermobifida* strains and their endomannanases

The four investigated strains were taxonomically characterised by 16S rDNA sequence analysis and represented on a phylogenetic tree based on sequence similarities. The obtained phylogenetic tree indicates that *T. alba* shows higher homology with *T. halotolerans* than with the other two *Thermobifida* strains (Fig. 4).

The lengths of the man5ATa, man5ATc, man5ATh, and man5Tf genes are 1364, 1362, 1320, and 1368 bp, respectively. In the case of endomannanases, terminal signal peptides were determined by using SignalP software (http://www.cbs.dtu.dk/services/SignalP/). The endomannanases were cloned containing 425 (Man5ATf), 424 (Man5ATc), and 423 (Man5ATh) amino acids without signal peptides (Tóth et al., 2016). The phylogenetic analysis of AA sequences

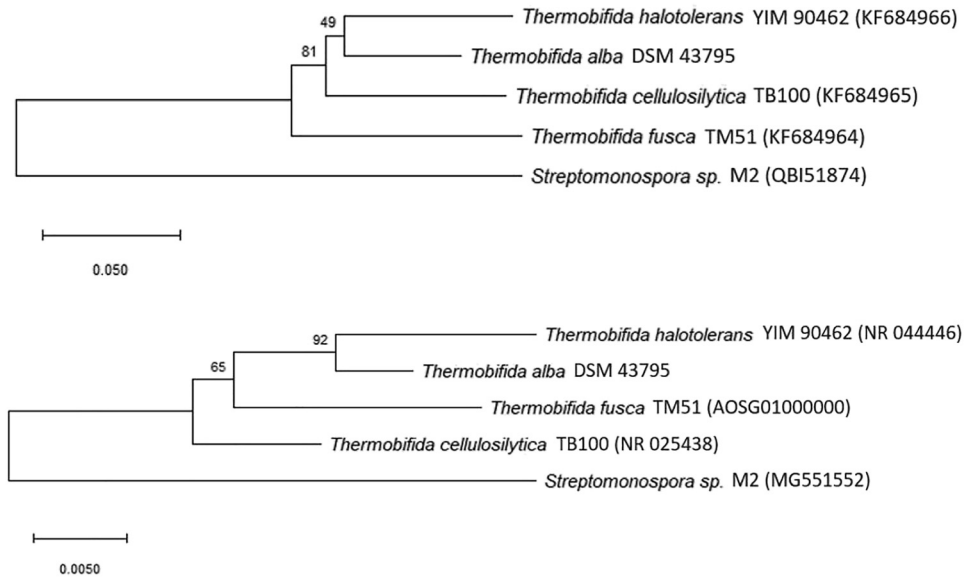

Fig. 4. Phylogenetic representation of *T. alba* and Man5Ata. The upper tree represents the phylogenetic relation of *T. alba* DSM 43795 and *T. fusca* TM51 compared to other thermobifida type strains as *T. cellulosilytica* TB100 and *T. halotolerans* of the genus using 16S rDNA sequences. While the lower phylogenetic tree represents the phylogenetic relations of Man5A enzymes of *T. fusca* TM51, *T. halotolerans* YIM 90462, *T. alba* DSM 43795, and *T. cellulosilytica* TB100 based on their AA sequences. *Streptomonas* sp. M2 serves as an outgroup strain showing the direction of the root on both trees

revealed that endomannanases of *T. alba* and *T. halotolerans* were much closer to each other and even *T. cellulosilytica* showed higher similarities to these two than to *T. fusca*, which was located on a separate branch (Fig. 4). The identities between Man5ATa and Man5ATc, Man5ATa and Man5ATh, Man5ATa and Man5ATf are 89, 89, and 88%, respectively (Fig. 3). The structure of mature proteins is composed of an N-terminal GH5 catalytic domain and a C-terminal carbohydrate binding module (CBM2). The identity between catalytic domain pairs is between 87 and 89%, while we found lower homology values of 76–83% among the CBM domains. Between the two domains there is a so-called inter-domain region, which shows great variability. The 23–25 AA linker sequences show a variation of proline, threonine, and aspartate/glutamate rich, 3–6 times repetitive tetrapeptide motives: 3xPPTEPTD-Ta, 4xPTDP-Tc, 3xTEEP-Tf, and 6xDPGT-Th (Fig. 5).

The role of linker regions is not totally clear yet, but there are already some investigations of cellulases of *T. fusca* YX. In a study of Cel9A, cleaving the endoglucanase by a protease changed the substrate specificity of the enzyme, thus its activity without the CBM domain increased towards shorter oligosaccharide fractions (Lao and Wilson, 1996). This result coincides with other studies, where it was reported that the length of the linker sequence had a role in the activity of processive cellulases, as shortening or deleting the linker region decreased the enzymatic activity on crystalline cellulose (Srisodsuk et al., 1993). Linker length also can play a role in the thermal adaptation of cellulase Cel9A (Batista et al., 2011).

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Man5ATa ----MRKRLTVAAATVLALLASVFVIAQPAGAATGLHVQNGRLHEANGQEFVIRGVSHPH 56
Man5ATf ----MRKRLAVAAATVLALLASVFALTQPANAATGLHVKNGRLYEANGQEFIIRGVSHPH 56
Man5ATh MRKAMRKRLTVAAATILALLASVFVFAQPANAATGFYVDNGRLYEAGGQEFVIRGVSHPH 60
Man5ATc ----------------MLLASVFVTAQPASAATGIHVRNGRIYEANGQEFVMRGVSHAH 43
                         ******. :***.****::* ***::**.****::***** *

Man5ATa NWYPQHTGAFADIKAHGANTVRVVLSNGVRWAKNGPSDVAGVISLCKQNRLICMLEVHDT 116
Man5ATf NWYPQHTQAFADIKSHGANTVRVVLSNGVRWSKNGPSDVANVISLCKQNRLICMLEVHDT 116
Man5ATh AWYAQETDSFAGIKSHGANTVRVVLSNGVRWTKNDAADVANVISLCKQNRLICMLEVHDT 120
Man5ATc TWYPQHTRAFADIKSHGANTVRVVLSNGVRWSKNGPSDVADIISLCKQNRLICMLEVHDT 103
          ** *.* :**.**:****************:**. :***.:******************

Man5ATa TGYGEEGAASTLDQAVDYWIELKSVLQGEEDYVLINIGNEPYGNDAATVADWAPDTSAAI 176
Man5ATf TGYGEQSGASTLDQAVDYWIELKSVLQGEEDYVLINIGNEPYGNDSATVARWASDTSAAI 176
Man5ATh TGYGEQSGASTLDQAVDYWIEIQSALEGQEDYVLINIGNEPYGNDSATVADWASDTSAAI 180
Man5ATc TGYGEQSGAATLDQAVDYWIELKSVLQGQEDYVLINIGNEPYGNNAATVANWASDTSAAI 163
        *****:..*:***********::*.*:*:**************::**** ** ******

Man5ATa QRLRGAGFDHTLVVDAPNWGQDWSHTMRDNAAGVYASDPTGNTVFSIHMYGVYAQGATVT 236
Man5ATf QRLRAAGFEHTLVVDAPNWGQDWTNTMRNNADQVYASDPTGNTVFSIHMYGVYSQASTIT 236
Man5ATh QRMRDAGFDHTLVVDAPNWGQDWSHTMRDNAGSVYTSDPTGNTVFSIHMYGVYEQGSTVT 240
Man5ATc RRLRAAGFEHAIVVDAPNWGQDWSHTMRTNAADVYASDPTGNTIFSIHMYGVYSQGSTVT 223
        :*:* ***:*::**********::*** **  **:*******:********* *.:*:*

Man5ATa SYLEHFVNAGLPIVIGEFGHDHSDGNPDEDTIMAEAERLGLGYIGWSWSGNSGGVEYLDM 296
Man5ATf SYLEHFVNAGLPLIIGEFGHDHSDGNPDEDTIMAEAERLKLGYIGWSWSGNGGGVEYLDM 296
Man5ATh SYLEHFVNAGLPIMIGEFGHDHSDGNPDEDTIMAEAERLGLGYIGWSWSGNGGGVEYLDM 300
Man5ATc SYLEHFVNAGLPLVIGEFGHDHSDGNPDEDTIMAEAERLGLGYIGWSWSGNGGGVEYLDM 283
        ***********::************************ **********.********

Man5ATa VDDFDGDSLTPWGQRIFYGPDGIADTAQEATVFGPPTE--PTDPPTEPT-DPPTEPTDPP 353
Man5ATf VYNFDGDNLSPWGERIFYGPNGIASTAKEATIFGGSQP----GPTEEPTEEPTEEPTPTP 352
Man5ATh VNNFDADSLTSWGQRIFYGANGISSTAEEATVYGGEPPTDPTDPPTDP----TDPP--TD 354
Man5ATc VDNFDGDSLTSWGRRIFYGPNGISGTAREATVYSGDPGTDPG---TDPGTDPGTDP--GT 338
        * :**.*.*: **.***** :**:.**.***::.           :*       *

Man5ATa VEPTGDCTAAYATIGSWGGGFQGEVTVTAGDSAISNWTVSWTFPGGQTVSQGWNASFSGS 413
Man5ATf PPAEGDCTATYATIGSWGGGFQGEVTVTAGDSAISSWQVSWTFPGGQSVAHGWNASFSGT 412
Man5ATh PPAEGDCSADYTTVGSWGGGFQGEVTVTAGDSAISNWTVSWTFPGGQSVSHGWNASFGGG 414
Man5ATc DPGTGACSAAYTTVGDWGSGFQGEVTVTAGSSAISHWKVTWTFPGGQSVSHGWNAAFNGS 398
            * *:* *:*:*.**.***********.**** * *:*******:*::****:*.*

Man5ATa SSVTAGNVSYNGQLGAGQSTAFGFIGSGSAPDSLVLSCTTD 454
Man5ATf STVTASNLSYNGQLGAGQSATFGFIGSGDAPSSLTLSCTAR 453
Man5ATh SPVTASNAPYNGQLGAGQSTTFGFIGSGSAPGSLTLTCTTD 455
Man5ATc SSVTATSLSYNGQLGAGQSTAFGFIGSGSAPGALTLTCSVD 439
        * *** .  **********::*******.**.:*.*:*:.
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Fig. 5. The alignment of AA sequences of Man5ATa, Man5ATc, Man5ATf, and Man5ATh endomannanases from *T. cellulosilytica*, *T. fusca*, *T. halotolerans*, and *T. alba*. N-terminal GH5 catalytic and C-terminal polysaccharide binding modules (CBM2) are separated by the linker sequences (boxed region). Symbols: '*' – indicates positions that have a single, fully conserved residue, ':' – indicates conservation between groups of strongly similar properties, '.' – indicates conservation between groups of weakly similar properties

4. CONCLUSIONS

Endomannanase gene from *T. alba* DSM 43795 strain was isolated, expressed as a recombinant protein, and has been partially characterised. All investigated endomannanases from *T. alba*, *T. fusca*, *T. cellulosilytica,* and *T. halotolerans* belong to GH5 hydrolases. Enzymes have modular structure and possess a polysaccharide binding site at the C-terminus. The AA homology between them is 87–89% and their characteristics are similar regarding the kinetic parameters, however, the pH and temperature optima are slightly different. Man5ATh of *T. halotolerans* is moderately salt tolerant, its thermal stability is preserved up to 0.8 M of NaCl concentration. Despite the high sequence similarity of the investigated mannanases, they exhibit different temperature stability, so this can be a starting point for further structural-functional investigations and for industrial applications to produce biologically active, oligomannan prebiotics.

Conflict of interest: The 9th author, I. Bata-Vidács, is a member of the Editorial Board of the journal. Therefore, the submission was handled by a different member of the editorial board, and she did not take part in the review process in any capacity.

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REFERENCES

Adiguzel, G., Sonmez, Z., Adiguzel, A., and Nadaroglu, H. (2015). Purification and characterization of a thermostable endo-beta-1,4 mannanase from *Weissella viridescens* LB37 and its application in fruit juice clarification. *European Food Research and Technology*, 242(5): 769–776. https://doi.org/10.1007/s00217-015-2584-x.

Baker, G., Smith, J., and Cowan, D. (2003). Review and re-analysis of domain-specific 16S primers. *Journal of Microbiological Methods*, 55(3): 541–555. https://doi.org/10.1016/j.mimet.2003.08.009.

Baraldi, I.J., Giordano, R.L.C., and Zangirolami, T.C. (2016). Enzymatic hydrolysis as an environmentally friendly process compared to thermal hydrolysis for instant coffee production. *Brazilian Journal of Chemical Engineering*, 33(4): 763–771. https://doi.org/10.1590/0104-6632.20160334s20140028.

Barros, J., Serk, H., Granlund, I., and Pesquet, E. (2015). The cell biology of lignification in higher plants. *Annals of Botany*, 115(7): 1053–1074. https://doi.org/10.1093/aob/mcv046.

Batista, P.R., de Souza Costa, M.G., Pascutti, P.G., Bisch, P.M., and de Souza, W. (2011). High temperatures enhance cooperative motions between CBM and catalytic domains of a thermostable cellulase: mechanism insights from essential dynamics. *Physical Chemistry Chemical Physics*, 13(30): 13709. https://doi.org/10.1039/c0cp02697b.

Béki, E., Nagy, I., Vanderleyden, J., Jäger, S., Kiss, L., Fülöp, L., Hornok, L., and Kukolya, J. (2003). Cloning and heterologous expression of a β-d-mannosidase (EC 3.2.1.25)-encoding gene from *Thermobifida*

fusca TM51. *Applied and Environmental Microbiology*, 69(4): 1944–1952. https://doi.org/10.1128/aem.69.4.1944-1952.2003.

Bello, B., Mustafa, S., Tan, J.S., Ibrahim, T.A.T., Tam, Y.J., Ariff, A.B., Manap, M.Y., and Abbasiliasi, S. (2018). Evaluation of the effect of soluble polysaccharides of palm kernel cake as a potential prebiotic on the growth of probiotics. *3 Biotech*, 8(8). https://doi.org/10.1007/s13205-018-1362-4.

Benites, V., Gilharry, R., Gernat, A., and Murillo, J. (2008). Effect of dietary mannan oligosaccharide from bio-MOS or SAF-mannan on live performance of broiler chickens. *Journal of Applied Poultry Research*, 17(4): 471–475. https://doi.org/10.3382/japr.2008-00023.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1–2): 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Caldas, J.V., Vignale, K., Boonsinchai, N., Wang, J., Putsakum, M., England, J.A., and Coon, C.N. (2018). The effect of β-mannanase on nutrient utilization and blood parameters in chicks fed diets containing soybean meal and guar gum. *Poultry Science*, 97(8): 2807–2817. https://doi.org/10.3382/ps/pey099.

Cano, M.E., García-Martin, A., Comendador Morales, P., Wojtusik, M., Santos, V.E., Kovensky, J., and Ladero, M. (2020). Production of oligosaccharides from agrofood wastes. *Fermentation*, 6(1): 31. https://doi.org/10.3390/fermentation6010031.

Cuskin, F., Lowe, E.C., Temple, M.J., Zhu, Y., Cameron, E.A., Pudlo, N.A., Porter, N.T., Urs, K., Thompson, A.J., Cartmell, A., Rogowski, A., Hamilton, B.S., Chen, R., Tolbert, T.J., Piens, K., Bracke, D., Vervecken, W., Hakki, Z., Speciale, G., and Gilbert, H.J. (2015). Human gut Bacteroidetes can utilize yeast mannan through a selfish mechanism. *Nature*, 517(7533): 165–169. https://doi.org/10.1038/nature13995.

de Vries, R.P. and Visser, J. (2001). Aspergillus enzymes involved in degradation of plant cell wall polysaccharides. *Microbiology and Molecular Biology Reviews*, 65(4): 497–522. https://doi.org/10.1128/mmbr.65.4.497-522.2001.

Dimitroglou, A., Merrifield, D.L., Spring, P., Sweetman, J., Moate, R., and Davies, S.J. (2010). Effects of mannan oligosaccharide (MOS) supplementation on growth performance, feed utilisation, intestinal histology and gut microbiota of gilthead sea bream (*Sparus aurata*). *Aquaculture*, 300(1–4): 182–188. https://doi.org/10.1016/j.aquaculture.2010.01.015.

Eriksson, K.E.L., Blanchette R.H., and Ander, P. (1991). Microbial and enzymatic degradation of wood and wood components. *International Journal of Biochemistry*, 23(11): 1333. https://doi.org/10.1016/0020-711x(91)90236-g.

Favaro, C., Baraldi, I., Casciatori, F., and Farinas, C. (2020). β-Mannanase production using coffee industry waste for application in soluble coffee processing. *Biomolecules*, 10(2): 227. https://doi.org/10.3390/biom10020227.

Ferenczi, S., Szegi, K., Winkler, Z., Barna, T., and Kovács, K.J. (2016). Oligomannan prebiotic attenuates immunological, clinical and behavioral symptoms in mouse model of inflammatory bowel disease. *Scientific Reports*, 6(1). https://doi.org/10.1038/srep34132.

Gibson, G.R., McCartney, A.L., and Rastall, R.A. (2005). Prebiotics and resistance to gastrointestinal infections. *British Journal of Nutrition*, 93(S1): S31–S34. https://doi.org/10.1079/bjn20041343.

Hu, J., Arantes, V., Pribowo, A., and Saddler, J.N. (2013). The synergistic action of accessory enzymes enhances the hydrolytic potential of a "cellulase mixture" but is highly substrate specific. *Biotechnology for Biofuels*, 6(1). https://doi.org/10.1186/1754-6834-6-112.

Jana, U.K. and Kango, N. (2020). Characteristics and bioactive properties of mannooligosaccharides derived from agro-waste mannans. *International Journal of Biological Macromolecules*, 149: 931–940. https://doi.org/10.1016/j.ijbiomac.2020.01.304.

Jang, H.D. and Chang, K.S. (2005). Thermostable cellulases from *Streptomyces* sp.: scale-up production in a 50-l fermenter. *Biotechnology Letters*, 27(4): 239–242. https://doi.org/10.1007/s10529-004-8356-5.

Jiang, C. and Xu. L. (1993). Actinomycete diversity in unusual habitats. *Actinomycetes*, 4:47–57.

Kukolya, J. (2002). *Thermobifida cellulolytica* sp. nov., a novel lignocellulose-decomposing actinomycete. *International Journal of Systematic and Evolutionary Microbiology*, 52(4): 1193–1199. https://doi.org/10.1099/00207713-52-4-1193.

Kukolya, J., Dobolyi, C., and Hornok, L. (1997). Isolation and identification of thermophilic cellulolytic actinomycetes. *Acta Phytopathologica et Entomolica Hungarica*, 32: 97–107.

Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, 35(6): 1547–1549. https://doi.org/10.1093/molbev/msy096.

Lao, G. and Wilson, D.B. (1996). Cloning, sequencing, and expression of a *Thermomonospora fusca* protease gene in *Streptomyces lividans*. *Applied and Environmental Microbiology*, 62(11): 4256–4259. https://doi.org/10.1128/aem.62.11.4256-4259.1996.

Locci, R., Baldacci, E., and Petrolini, B. (1967). Contribution to the study of oligosporic actinomycetes. I.-Description of a new species of *Actinobifida: Actinobifida alba* sp. nov. and revision of the genus. *Giornale di Microbiologica*, 15: 79–91.

Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P.M., and Henrissat, B. (2013). The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Research*, 42(D1): D490–D495. https://doi.org/10.1093/nar/gkt1178.

Lykidis, A., Mavromatis, K., Ivanova, N., Anderson, I., Land, M., DiBartolo, G., Martinez, M., Lapidus, A., Lucas, S., Copeland, A., Richardson, P., Wilson, D.B., and Kyrpides, N. (2007). Genome sequence and analysis of the soil cellulolytic actinomycete *Thermobifida fusca* YX. *Journal of Bacteriology*, 189(6): 2477–2486. https://doi.org/10.1128/jb.01899-06.

Lynd, L.R., Weimer, P.J., van Zyl, W.H., and Pretorius, I.S. (2002). Microbial cellulose utilization: fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews*, 66(4): 739. https://doi.org/10.1128/mmbr.66.4.739.2002.

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31(3): 426–428. https://doi.org/10.1021/ac60147a030.

Murkovic, M. and Bornik, M.A. (2007). Formation of 5-hydroxymethyl-2-furfural (HMF) and 5-hydroxymethyl-2-furoic acid during roasting of coffee. *Molecular Nutrition & Food Research*, 51(4): 390–394. https://doi.org/10.1002/mnfr.200600251.

Naganagouda, K., Salimath, P.V., and Mulimani, V.H. (2009). Purification and characterization of endo-beta-1,4 mannanase from *Aspergillus niger* gr for application in food processing industry. *Journal of Microbiology and Biotechnology*, 19(10): 1184–1190. PMID: 19884778, https://doi.org/10.4014/jmb.0901.029.

Nagy, I., Schoofs, G., de Schrijver, A., Vanderleyden, J., and de Mot, R. (1997). New method for RNA isolation from actinomycetes: application to the transcriptional analysis of the alcohol oxidoreductase gene thcE in *Rhodococcus* and *Mycobacterium*. *Letters in Applied Microbiology*, 25(1): 75–79. https://doi.org/10.1046/j.1472-765x.1997.00179.x.

Norizan, N.A.B.M., Halim, M., Tan, J.S., Abbasiliasi, S., Mat Sahri, M., Othman, F., and Ariff, A.B. (2020). Enhancement of β-mannanase production by *Bacillus subtilis* ATCC11774 through optimization of medium composition. *Molecules*, 25(15): 3516. https://doi.org/10.3390/molecules25153516.

Østby, H., Hansen, L.D., Horn, S.J., Eijsink, V.G.H., and Várnai, A. (2020). Enzymatic processing of lignocellulosic biomass: principles, recent advances and perspectives. *Journal of Industrial Microbiology & Biotechnology*, 47(9–10): 623–657. https://doi.org/10.1007/s10295-020-02301-8.

Ozturk, B., Cekmecelioglu, D., and Ogel, Z.B. (2010). Optimal conditions for enhanced β-mannanase production by recombinant *Aspergillus sojae*. *Journal of Molecular Catalysis B: Enzymatic*, 64(3–4): 135–139. https://doi.org/10.1016/j.molcatb.2010.02.009.

Puls, J. (1997). Chemistry and biochemistry of hemicelluloses: relationship between hemicellulose structure and enzymes required for hydrolysis. *Macromolecular Symposia*, 120(1): 183–196. https://doi.org/10.1002/masy.19971200119.

Rainey, F.A., Ward-Rainey, N., Kroppenstedt, R.M., and Stackebrandt, E. (1996). The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of *Nocardiopsaceae* fam. nov. *International Journal of Systematic and Evolutionary Microbiology*, 46(4): 1088–1092. https://doi.org/10.1099/00207713-46-4-1088.

Rastall, R.A., Gibson, G.R., Gill, H.S., Guarner, F., Klaenhammer, T.R., Pot, B., Reid, G., Rowland, I.R., and Sanders, M.E. (2005). Modulation of the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human health: an overview of enabling science and potential applications. *FEMS Microbiology Ecology*, 52(2): 145–152. https://doi.org/10.1016/j.femsec.2005.01.003.

Sakai, K., Kimoto, S., Shinzawa, Y., Minezawa, M., Suzuki, K., Jindou, S., Kato, M., and Shimizu, M. (2018). Characterization of pH-tolerant and thermostable GH 134 β-1,4-mannanase SsGH134 possessing carbohydrate binding module 10 from *Streptomyces* sp. NRRL B-24484. *Journal of Bioscience and Bioengineering*, 125(3): 287–294. https://doi.org/10.1016/j.jbiosc.2017.10.009.

Shastak, Y., Ader, P., Feuerstein, D., Ruehle, R., and Matuschek, M. (2015). ß-Mannan and mannanase in poultry nutrition. *World's Poultry Science Journal*, 71(1): 161–174. https://doi.org/10.1017/s0043933915000136.

Shivlata, L. and Satyanarayana, T. (2015). Thermophilic and alkaliphilic *Actinobacteria*: biology and potential applications. *Frontiers in Microbiology*. 6(1014): 8–36. https://doi.org/10.3389/fmicb.2015.01014.

Somogyi, M. (1952). Notes on sugar determination. *Journal of Biological Chemistry*, 195(1): 19–23. https://doi.org/10.1016/s0021-9258(19)50870-5.

Soni, H. and Kango, N. (2013). Microbial mannanases: properties and applications. *Advances in Enzyme Biotechnology*, 41–56. https://doi.org/10.1007/978-81-322-1094-8_4.

Srisodsuk, M., Reinikainen, T., Penttilä, M., and Teeri, T. (1993). Role of the interdomain linker peptide of *Trichoderma reesei* cellobiohydrolase I in its interaction with crystalline cellulose. *Journal of Biological Chemistry*, 268(28): 20756–20761. https://doi.org/10.1016/s0021-9258(19)36847-4.

Stoll, D., Boraston, A., Stalbrand, H., McLean, B.W., Kilburn, D.G., and Warren, R.J. (2000). Mannanase Man26A from *Cellulomonas fimi* has a mannan-binding module. *FEMS Microbiology Letters*, 183(2): 265–269. https://doi.org/10.1111/j.1574-6968.2000.tb08969.x.

Sunna, A., Gibbs, M.D., Chin, C.W.J., Nelson, P.J., and Bergquist, P.L. (2000). A gene encoding a novel multidomain β-1,4-mannanase from *Caldibacillus cellulovorans* and action of the recombinant enzyme on kraft pulp. *Applied and Environmental Microbiology*, 66(2): 664–670. https://doi.org/10.1128/aem.66.2.664-670.2000.

Tailford, L.E., Ducros, V.M.A., Flint, J.E., Roberts, S.M., Morland, C., Zechel, D.L., Smith, N., Bjørnvad, M.E., Borchert, T.V., Wilson, K.S., Davies, G.J., and Gilbert, H.J. (2009). Understanding how diverse β-mannanases recognize heterogeneous substrates. *Biochemistry*, 48(29): 7009–7018. https://doi.org/10.1021/bi900515d.

Tenkanen, M., Makkonen, M., Perttula, M., Viikari, L., and Teleman, A. (1997). Action of *Trichoderma reesei* mannanase on galactoglucomannan in pine kraft pulp. *Journal of Biotechnology*, 57(1–3): 191–204. https://doi.org/10.1016/s0168-1656(97)00099-0.

Torrecillas, S., Montero, D., and Izquierdo, M. (2014). Improved health and growth of fish fed mannan oligosaccharides: potential mode of action. *Fish & Shellfish Immunology*, 36(2): 525–544. https://doi.org/10.1016/j.fsi.2013.12.029.

Tóth, Á., Baka, E., Luzics, S., Bata-Vidács, I., Nagy, I., Bálint, B., Herczeg, R., Olasz, F., Wilk, T., Nagy, T., Kriszt, B., Nagy, I., and Kukolya, J. (2017). Plant polysaccharide degrading enzyme system of *Thermobifida cellulosilytica* TB100T revealed by *de novo* genome project data. *Acta Alimentaria*, 46(3): 323–335. https://doi.org/10.1556/066.2016.0014.

Tóth, K., Barna, T., Nagy, I., Horváth, B., Nagy, I., Táncsics, A., Kriszt, B., Baka, E., Fekete, C., and Kukolya, J. (2013). Draft genome sequence of the lignocellulose decomposer *Thermobifida fusca* strain TM51. *Genome Announcements*, 1(4): e00482–13. https://doi.org/10.1128/genomea.00482-13.

Tóth, K., Barna, T., Szabó, E., Elek, R., Hubert, G., Nagy, I., Nagy, I., Kriszt, B., Táncsics, A., and Kukolya, J. (2016). Cloning, expression and biochemical characterization of endomannanases from *Thermobifida* species isolated from different niches. *Plos One*, 11(5): e0155769. https://doi.org/10.1371/journal.pone.0155769.

van den Brink, J. and de Vries, R.P. (2011). Fungal enzyme sets for plant polysaccharide degradation. *Applied Microbiology and Biotechnology*, 91(6): 1477–1492. https://doi.org/10.1007/s00253-011-3473-2.

Várnai, A., Huikko, L., Pere, J., Siika-aho, M., and Viikari, L. (2011). Synergistic action of xylanase and mannanase improves the total hydrolysis of softwood. *Bioresource Technology*, 102(19): 9096–9104. https://doi.org/10.1016/j.biortech.2011.06.059.

Várnai, A. (2012). *Improving enzymatic conversion of lignocellulose to platform sugars*. VTT Science.

von Freiesleben, P., Spodsberg, N., Stenbæk, A., Stålbrand, H., Krogh, K.B.R.M., and Meyer, A.S. (2018). Boosting of enzymatic softwood saccharification by fungal GH5 and GH26 endomannanases. *Biotechnology for Biofuels*, 11(1). https://doi.org/10.1186/s13068-018-1184-y.

Wilson, D.B. (2004). Studies of *Thermobifida fusca* plant cell wall degrading enzymes. *The Chemical Record*, 4(2): 72–82. https://doi.org/10.1002/tcr.20002.

Xia, W., Lu, H., Xia, M., Cui, Y., Bai, Y., Qian, L., Shi, P., Luo, H., and Yao, B. (2016). A novel glycoside hydrolase family 113 endo-β-1,4-mannanase from *Alicyclobacillus* sp. strain a4 and insight into the substrate recognition and catalytic mechanism of this family. *Applied and Environmental Microbiology*, 82(9): 2718–2727. https://doi.org/10.1128/aem.04071-15.

Xu, J., Bjursell, M.K., Himrod, J., Deng, S., Carmichael, L.K., Chiang, H.C., Hooper, L.V., and Gordon, J.I. (2003). A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis. *Science*, 299(5615): 2074–2076. https://doi.org/10.1126/science.1080029.

Yan, P., Su, L., Chen, J., and Wu, J. (2013). Heterologous expression and biochemical characterization of an endo-β-1,4-glucanase from *Thermobifida fusca*. *Biotechnology and Applied Biochemistry*, 60(3): 348–355. https://doi.org/10.1002/bab.1097.

Yang, L.L., Tang, S.K., Zhang, Y.Q., Zhi, X.Y., Wang, D., Xu, L.H., and Li, W.J. (2008). *Thermobifida halotolerans* sp. nov., isolated from a salt mine sample, and emended description of the genus *Thermobifida*. *International Journal of Systematic And Evolutionary Microbiology*, 58(8): 1821–1825. https://doi.org/10.1099/ijs.0.65732-0.

You, X., Qin, Z., Yan, Q., Yang, S., Li, Y., and Jiang, Z. (2018). Structural insights into the catalytic mechanism of a novel glycoside hydrolase family 113 β-1,4-mannanase from *Amphibacillus xylanus*. *Journal of Biological Chemistry*, 293(30): 11746–11757. https://doi.org/10.1074/jbc.ra118.002363.

Zhang, F., Chen, J.J., Ren, W.Z., Nie, G.X., Ming, H., Tang, S.K., and Li, W.J. (2011). Cloning, expression and characterization of an alkaline thermostable GH9 endoglucanase from *Thermobifida halotolerans* YIM 90462T. *Bioresource Technology*, 102(21): 10143–10146. https://doi.org/10.1016/j.biortech.2011.08.019.

Zhang, X., Rogowski, A., Zhao, L., Hahn, M.G., Avci, U., Knox, J.P., and Gilbert, H.J. (2014). Understanding how the complex molecular architecture of mannan-degrading hydrolases contributes to plant cell wall degradation. *Journal of Biological Chemistry*, 289(4): 2002–2012. https://doi.org/10.1074/jbc.m113.527770.

Zhang, Z., Wang, Y., and Ruan, J. (1998). Reclassification of *Thermomonospora* and *Microtetraspora*. *International Journal of Systematic Bacteriology*, 48(2): 411–422. https://doi.org/10.1099/00207713-48-2-411.

Zhao, D., Zhang, X., Wang, Y., Na, J., Ping, W., and Ge, J. (2020). Purification, biochemical and secondary structural characterisation of β-mannanase from *Lactobacillus casei* HDS-01 and juice clarification potential. *International Journal of Biological Macromolecules*, 154: 826–834. https://doi.org/10.1016/j.ijbiomac.2020.03.157.

Zhao, L., Geng, J., Guo, Y., Liao, X., Liu, X., Wu, R., Zheng, Z., and Zhang, R. (2015). Expression of the *Thermobifida fusca* xylanase Xyn11A in *Pichia pastoris* and its characterization. *BMC Biotechnology*, 15(1). https://doi.org/10.1186/s12896-015-0135-y.

Zhou, M., Tao, Y., Lai, C., Huang, C., Zhou, Y., and Yong, Q. (2019). Effects of mannanoligosaccharide supplementation on the growth performance, immunity, and oxidative status of partridge shank chickens. *Animals*, 9(10): 817. https://doi.org/10.3390/ani9100817.

Zurmiati, W., Abbas, H., and Mahata, M.E. (2017). Production of extracellular β-mannanase by *Bacillus amyloliquefaciens* on a coconut waste substrate. *Pakistan Journal of Nutrition*, 16(9): 700–707. https://doi.org/10.3923/pjn.2017.700.707.