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# Cloning and Expression of a Thermostable α-Galactosidase from the Thermophilic Fungus *Talaromyces emersonii* in the Methylotrophic Yeast *Pichia pastoris*

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**The first gene (*α-gal1*) encoding an extracellular α-D-galactosidase from the thermophilic fungus *Talaromyces emersonii* was cloned and characterized. The *α-gal1* gene consisted of an open reading frame of 1,792 base pairs interrupted by six introns that encoded a mature protein of 452 amino acids, including a 24 amino acid secretory signal sequence. The translated protein had highest identity with other fungal α-galactosidases belonging to glycosyl hydrolase family 27. The *α-gal1* gene was overexpressed as a secretory protein with an N-terminal histidine tag in the methylotrophic yeast *Pichia pastoris*. Recombinant α-Gal1 was secreted into the culture medium as a monomeric glycoprotein with a maximal yield of 10.75 mg/l and purified to homogeneity using His-binding nickel-agarose affinity chromatography. The purified enzyme was maximally active at 70°C, pH 4.5, and lost no activity over 10 days at 50°C. α-Gal1 followed Michaelis–Menten kinetics ($V_{max}$ of 240.3 μM/min/mg, $K_m$ of 0.294 mM) and was inhibited competitively by galactose ($K_m^{obs}$ of 0.57 mM, $K_i$ of 2.77 mM). The recombinant *T. emersonii* α-galactosidase displayed broad substrate preference, being active on both oligo- and polymeric substrates, yet had strict specificity for the α-galactosidic linkage. Owing to its substrate preference and noteworthy stability, α-Gal1 is of particular interest for possible biotechnological applications involving the processing of plant materials.**

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α-Galactosidases (E.C. 3.2.1.22) are glycoside hydrolases that catalyze the hydrolysis of terminal α-D-galactosyl groups from carbohydrates and glucoconjugates [23, 28, 31]. Some α-galactosidases are also capable of transglycosylation by transferring galactose from donor to suitable acceptor molecules [30, 36].

The classification of α-galactosidases has been based either on their substrate specificities or sequence similarities. The first criterion subdivides these enzymes into two groups; group I enzymes are active on oligosaccharides such as the raffinose-series sugars, whereas group II enzymes act on polysaccharide substrates such as galacto(gluco)mannans [9]. On the basis of their sequence and structural similarities, α-galactosidases have been assigned to glycosyl hydrolase (GH) families 4, 27, 36, and 57 (Carbohydrate Active Enzymes database, CAZy; http://www.cazy.org/) [3]. The majority of eukaryotic α-galactosidases belong to the monophyletic family GH27 [19]. Together, families GH27 and GH36 are classified into Clan D, which shares a common ancestry and mechanism of function [9]. Clan D enzymes catalyze cleavage of the glysosidic bond *via* a retaining, double-displacement mechanism, with two aspartic acids (Asp) acting as the catalytic residues. The first catalytic step consists of the formation of an enzyme–glycosyl intermediate, with release of the leaving group. In the second catalytic step, the intermediate is broken down with the help of an exogenous

nucleophile, a water molecule [25]. Eukaryotic GH27 α-galactosidases are functional in their monomeric form, whereas the GH36 enzymes (mainly prokaryotic origin) are organized as multimeric, high-molecular-weight complexes [4].

Research involving α-galactosidases has been propelled by various promising biomedical and biotechnological applications. Enzyme replacement therapy with modified human α-galactosidase A has been used in treating patients with the lysosomal storage disorder known as Fabry Disease, where the depletion of lysosomal α-galactosidase A leads to accumulation of glycolipids with consequent multi-organ symptoms. The disorder is X-chromosome-linked and results in premature death both in homo- and heterozygous individuals [24]. α-Galactosidase has also been used to modify blood group glycomarkers on erythrocytes. The removal of α-(1,3)-linked galactose units from blood group antigens on B blood group cells to yield type O cells (universal donors) has been safely conducted with recombinant α-galactosidase [20, 38] and used successfully in blood transfers with rhesus monkeys (*Macaca mulatta*) and gibbons (*Hylobates* sp.) [38]. α-Galactosidases have also found application in the sugar industry as crystallization aids in the hydrolysis of raffinose to sucrose [1]. The utilization of α-galactosidases in improving food and animal feed derived from plants of the Leguminoseae family has been evaluated. The hydrolysis of raffinose-type sugars and hemicellulolytic galactomannans, which are abundant in plants such as soy, would improve the digestibility and nutritional value of products made from these plant sources, as these generally induce gastrointestinal disturbance and flatulence [12, 33]. Furthermore, α-galactosidases have been utilized in softwood paper pulp bleaching [8] and in the production of biofuel from lignocellulosic biomass comprising both agricultural/wood waste and dedicated energy crops [15]. Of the α-galactosidases identified to-date, fungal enzymes are very attractive for biotechnological applications because of their extracellular nature, generally good stability profiles, and suitable pH optima [16].

*Pichia pastoris* is a methylotrophic yeast modified for efficient expression of recombinant proteins under the highly inducible promoter of alcohol oxidase (AOX). The expression of recombinant α-galactosidase in *Pichia pastoris* has been reported four times in the literature to-date; that is, for human (*Homo sapiens*) α-galactosidase A [6], coffee bean (*Coffea* sp.) α-galactosidase [38, 39], and extracellular α-galactosidase from *Penicillium* sp. F63 CGMCC 1669 [17]. On all occasions, the recombinant enzyme was successfully expressed and had similar characteristics to the native enzyme.

*Talaromyces emersonii* is a thermophilic filamentous fungus living in temperatures ranging from its optimum 40–45°C up to a maximum of 55°C, and it is a common microorganism involved in decomposition of decaying plant material [16]. Enzymes derived from thermophilic organisms, such as *T. emersonii*, are often used in biotechnology, since they can be purified by heat treatment, stored for longer periods at room temperature, and exhibit lower losses of activity at the high temperatures required for food/feed processing. Moreover, reactions conducted at higher temperatures are also less prone to microbial contamination [16, 32].

In this paper, we describe the cloning of the first α-galactosidase gene from *Talaromyces emersonii* (*α-gal1*), its overexpression in a heterologous host, *Pichia pastoris*, and the characterization of the recombinant protein. The main physicochemical and catalytic properties of the recombinant enzyme are presented.

## Materials and Methods

### Chemicals and Substrates

All chemicals were of analytical grade and purchased from Sigma-Aldrich (Dublin, Ireland). The water used throughout the research was Milli-Q $H_2O$ (Millipore Corporation, Ireland).

### Strains, Plasmids, Media, and Culture Conditions

*Talaromyces emersonii* IMI 393751 (laboratory stock) was routinely subcultured on Sabouraud Dextrose Agar (SDA) for 2–4 days at 45°C.

The pGEM-T easy plasmid (Promega, Mannheim, Germany) was used for subcloning of PCR products in *Escherichia coli* JM109 cells (Promega, Mannheim, Germany) grown on low-salt Luria–Bertani (LB) medium at 37°C with shaking at 300 rpm, prior to sequence analysis. Transformants were screened using LB agar plates with ampicillin/IPTG/X-Gal or Zeocin prepared according to the pGEM-T Easy Vector System manual (Promega).

The pPICZαA plasmid was used for expression of the *α-gal1* gene product in *Pichia pastoris* KM71H (Invitrogen, Paisley, UK) cells, which were grown at 30°C with shaking at 250 rpm. Expression transformants were screened on yeast potato dextrose sorbitol (YPDS)+Zeocin agar plates. Liquid media used in the cultivation of expression plasmids were MG/MM (minimal glycerol/methanol), BMG/BMM (buffered minimal glycerol/methanol, pH 6.0), MGY/MMY (glycerol/methanol-complex), and BMGY/BMMY (buffered glycerol/methanol-complex, pH 6.0). All *P. pastoris* media were prepared in accordance with the EasySelect *Pichia* Expression Kit manual (Invitrogen).

### Preparation of Genomic DNA and RNA from *T. emersonii*

Liquid cultures of *T. emersonii* were grown at 45°C, pH 4.5, with shaking at 220 rpm, in the mineral salts/inducing medium as described previously [18]. Glucose "starter" cultures were prepared by inoculating medium containing 2% glucose with segments of a mycelial mat removed aseptically from a SDA agar plate. The "starter" cultures were grown for 24 h and then used to seed 2 L Erlenmeyer flasks containing 2% lactose as a sole carbon source; the larger volume cultures were grown for 24 h prior to harvesting of fungal mycelia to recover RNA. Mycelia were collected by filtration through sterilized fine-grade muslin, washed with cold sterile distilled water, and frozen with liquid nitrogen.

Genomic DNA was isolated from *T. emersonii* mycelia cultivated on 2% glucose for 24 h [22]. Total RNA (1 μg) was isolated from lactose (2%)-induced cultures after 24 h [7] and used as a template for FirstChoice RLM-RACE (Ambion, Applied Biosystems, UK).

**PCR Amplification and Cloning of *α-gal1* Gene Fragments**

Genomic DNA from *T. emersonii* was used as the template in polymerase chain reaction (PCR) amplification, with degenerate primers designed from existing fungal α-galactosidase sequences present in GenBank. Two sets of degenerate primers were used to amplify the α-galactosidase gene fragments: 5'-CTI GGS TGG AAC TCI TGG AAY GC-3' and 5'-CGI CCR ATR GGR CGR GTI CA-3', and the second pair 5'-CAY TTY GCI CTK TGG GCR ATS ATG-3' and 5'-GGA TGC MTG GAC MGC AAA G-3' (I=Inosine; Y=C or T; S=C or G; R=A or G; and M=A or C). The reaction conditions for PCR amplification were as follows: initial denaturation at 94°C for 15 min, followed by 30 cycles of denaturation at 94°C for 30 s, primer annealing at 49–60°C (depending on the primer combinations) for 30 s, extension at 72°C for 1 min per kb, with a final extension of 10 min at 72°C. HotStarTaq DNA polymerase (Qiagen, Sussex, UK) was used in all reactions. PCR products were separated in a 1.2% (w/v) agarose gel (1× TAE) and products were purified using a Wizard PCR preps DNA purification system (Promega, Mannheim, Germany) and cloned into the pGEM-T easy vector (Promega, Mannheim, Germany) following the manufacturer's protocols. Plasmids were purified from *E. coli* cultures using the Qiaprep Spin Miniprep Kit (Qiagen, Sussex, UK) and sequenced (AGOWA Genomics, Berlin, Germany).

**Rapid Amplification of cDNA Ends**

Based on the gene fragment sequences obtained using the degenerate primers, oligonucleotide primers for rapid amplification of cDNA ends (RACE) were designed. Total RNA was used as the template for RACE following the FirstChoice RLM-RACE Kit protocol (Ambion, Applied Biosystems) unless otherwise stated. Reverse transcription was carried out in 20 μl reaction mixtures, each containing 6 U of *C. therm* polymerase (Roche Molecular Biochemicals, Basel, Switzerland), 1× RT buffer (Roche), 5% DMSO, 5 mM DTT, 200 μM of each deoxynucleotide triphosphate, 5 mM $MgCl_2$, and 1 μM 3' RACE adapter 5'-GCG AGC ACA GAA TTA ATA CGA CTC ACT ATA $GGT_{20}$ VN-3' (V=A, C or G; N=A,C,T or G), to make first-strand *c*DNA. Reactions were performed at 60°C for 1 h; 1 μl from individual reaction mixtures was then used as the template to perform 5' and 3' RACE PCRs using the outer and inner RACE primers supplied by the manufacturer, and outer and inner gene-specific primers designed from the *α-gal1* gene fragments. Reaction conditions for RACE PCR and subsequent cloning of products for sequencing were as described earlier.

**Isolation of the Full-Length cDNA and Genomic Copies of the *α-gal1* Gene**

The full-length *α-gal1* gene was amplified from *T. emersonii* first-strand lactose (24 h)-induced *c*DNA and genomic DNA by PCR using Phusion DNA proofreading Polymerase (Finnzymes, Espoo, Finland) with sense 5'-ATG CTT CAT CGT GCT ACT ACC-3' and antisense 5'-TCA ACA TTC CCC CGA CAC AAC-3' primers, corresponding to the regions containing the putative start and stop codons deduced from the 5' and 3' RACE PCR products. The PCR products were purified and cloned, creating the recombinant plasmid pTE*αgal1* containing the full-length *α-gal1* *c*DNA, and were sequenced as described previously.

**Heterologous Expression of *α-gal1* in *Pichia pastoris* Strain KM71H**

Full-length *α-gal1* cDNA was amplified from plasmid pTE*αgal1* using 5'-GGA ATT CCT CGT GAG G CC TGA TGG AG-3' and 5'-TTT CTA GAT TAC ATT CCC CCG ACA CAA CC-3' primers containing *Eco*RI (GGAATTC) and *Xba*I (TTTCTAGATT) restriction sites complimentary to the N- and C-termini of the mature α-Gal1 protein, but excluded the native N-terminal signal sequence for protein secretion (24 amino acids in length). The PCR product obtained was purified, digested with *Eco*RI and *Xba*I, and ligated to pPICZαA (also digested with the same restriction enzymes) using T4 DNA ligase, which yielded plasmid pTE*αgal1*X with the *α-gal1* gene in-frame with the N-terminal yeast secretory peptide and C-terminal polyhistidine tag. pTE*αgal1*X was transformed into *E. coli* JM109 cells; positive transformants were isolated on the basis of Zeocin resistance, purified, and sequenced. Purified pTE*αgal1*X DNA was linearized with *Sac*I and transformed into *P. pastoris* strain KM71H following the manufacturer's instructions (EasyComp Transformation, EasySelect *Pichia* Expression Kit, Invitrogen, Paisley, UK). Positive clones were selected on YPDS-Zeocin agar plates. Single colonies were used to inoculate 50 ml (MG or MGY) or 55 ml (BMG or BMGY) of a particular medium, in a 250 ml Erlenmeyer flask, and *P. pastoris* cultures were grown at 30°C with shaking at 250 rpm until the culture reached an $OD_{600}$ value of 2–6 (16–18 h). Yeast cells were harvested by centrifugation at 500 ×*g* for 5 min at 4°C and resuspended in 8 ml of the appropriate medium (MM, BMM, MMY, or BMMY) containing methanol at a concentration of 0.6% (v/v) to induce gene expression. The induction was maintained for 5 days by adding methanol at a final culture concentration of 0.6% (v/v) every 24 h. Culture samples were taken before induction (controls) and at 24 h intervals to analyze protein production and enzyme expression and determine the optimal time for post-inductional harvesting of the recombinant α-Gal1 protein. Supernatant samples, recovered after centrifugation of culture samples, were transferred to new sterile tubes and stored at −20°C. Supernatant samples were used for determining protein and enzyme activity without further purification of the α-Gal1 protein. Recombinant α-galactosidase (α-Gal1) was purified to homogeneity as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) from the *P. pastoris* KM71H supernatants using the Ni-NTA purification system (Invitrogen, Paisley, UK), which is designed for purification of $His_6$-tagged proteins. After purification, protein samples were dialyzed against 50 mM ammonium acetate buffer, pH 4.5, and total protein yield was measured as described below.

**Enzyme Activity Assay and Measurement of Total Protein Concentration**

α-Galactosidase activity was routinely measured spectrophotometrically using either 4-nitrophenyl-α-D-galactopyranoside (4NPG; λ=405 nm) or methylumbelliferyl-α-D-galactopyranoside (MUG; λ=365 nm) in 25 mM ammonium acetate (AmOAc) buffer, pH 4.5. Reaction mixtures containing 100 μl of 1 mM 4NPG or MUG and 10 μl of suitably diluted enzyme solution were incubated for 30 min at 50°C in triplicates, except where otherwise stated. The reaction was terminated by addition of 1 M sodium carbonate ($Na_2CO_3$) and absorbance was measured using a Wallac VICTOR Multilabel

Counter UV–VIS spectrophotometer (Perkin Elmer, Dublin, Ireland). Optimum pH values were determined in two constant ionic strength buffer systems: citrate phosphate buffer (McIlvaine buffers, pH 2.6–7.6) and 1 N KCl-HCl buffer (Clark–Lubs buffers, pH 1.0–3.0). Optimum temperatures for activity were determined in 25 mM AmOAc buffer, pH 4.5. Kinetic properties were investigated using the substrate 4NPG at concentrations of 0.1–2.0 mM under the standard assay conditions. The Michaelis–Menten constant ($K_m$) and maximal initial rate ($V_{max}$) values were calculated from a Lineweaver–Burk plot. The inhibition constant ($K_i$) was calculated, using the appropriate kinetic equations, with data determined under the standard assay conditions (0.1–1.0 mM 4NPG) in the presence of 2 mM galactose (concentration that yielded 50% inhibition). One unit of enzyme activity (IU) was defined as the amount of enzyme that catalyzed the release of 1 μmol of 4-nitrophenol per minute of reaction time and calculated with reference to the appropriate standard curve. Protein content was measured using the Bradford method [2] with reference to a bovine serum albumin (BSA) standard curve (0.1–10 μg/ml).

**Substrate Specificity**

The synthetic glycoside derivatives 4NP-α-L-fucopyranoside, 4NP-β-D-xylopyranoside, 4NP-β-galactopyranoside, 4NP-α-D-mannopyranoside, 4NP-α-D-glucopyranoside, 4NP-β-glucopyranoside, 4NP-β-cellobioside, 4NP-β-cellotrioside, and 4NP-α-arabinofuranoside (1 mM) were used in standard activity assays at 60°C for 60 min with purified α-Gal1. The natural polysaccharide substrates locust bean gum, carob galactomannan, and guar gum, and the oligosaccharides galactomannotriose, *di*-galactomannopentose, melibiose, raffinose, stachyose, verbascose, maltose, cellobiose, lactose, and sucrose (5 mg/ml) were incubated with purified recombinant α-Gal1 at 50°C. Samples were removed at timed intervals from each reaction mixture (*i.e.*, 3, 6, and 24 h) and reactions were stopped by boiling for 5 min to inactivate the enzyme. The reaction mixtures were stored at −20°C until analyzed. Transglycosylation activity was investigated by using higher concentrations (10 mg/ml and 20 mg/ml), respectively, of melibiose or galactose in the enzyme reactions (24 h at 50°C, pH 7.4). Total soluble carbohydrates were analyzed by high-performance anion-exchange chromatography on an ICS-3000 Ion Chromatography System from Dionex Corporation (Dublin, Ireland). Products of hydrolysis and standard sugars were fractionated on a CarboPac PA-10 column using a decreasing 200–18 mM sodium hydroxide (NaOH) gradient operating at a flow rate of 1 ml/min at ambient temperature. An ED40 electrochemical detector, in the integrated amperometry mode, facilitated detection of eluting product peaks. Data were collected and processed using Chromeleon Version 6.70 software. Products were positively identified by spiking samples with standard sugars.

**Protein Deglycosylation**

The presence of carbohydrate on the purified recombinant protein was investigated by deglycosylation with EndoF1 using a Native Protein Deglycosylation Kit (Sigma-Aldrich, Dublin, Ireland) according to the manufacturer's instructions.

**Analysis of Enzyme Purity by Electrophoresis and Isoelectric Focusing**

Crude and purified preparations of enzyme were analyzed for homogeneity under denaturing and non-denaturing conditions by polyacrylamide gel electrophoresis (PAGE). Enzyme purity was also evaluated by isoelectric focusing on ultrathin Ampholine PAG plates (GE Healthcare, Chalfont St. Giles, Buckshire, UK). α-Galactosidase activities in non-denaturing PAGE and isoelectric focusing gels were determined by immersing the previously run gels in 100 mM AmOAc, pH 4.5, containing 100 μM MUG for 20 min at 50°C, and visualizing methylumbelliferone released under ultraviolet light.

**Sequence Analysis, Secondary Structure Prediction, and Homology Molecular Modeling**

DNA sequencing was conducted in both directions by AGOWA Genomics (Berlin, Germany). A homology search was conducted with the translated α-galactosidase sequence from *T. emersonii* using protein BLASTP version 2.2.22+ (http://www.ncbi.nlm.nih.gov/BLAST/). Amino acid alignments were made with the program Clustal W version 2.0.12 (http://www.ebi.ac.uk/clustalw/) and GeneDoc (http://www.nrbsc.org/gfx/genedoc/). SignalP version 3.9 (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the location of a putative signal peptide cleavage site, and the presence of a potential α-galactosidase signature sequence was investigated using the ScanPROSITE tool (http://expasy.org/prosite/). In addition, an intergrated search in family and domain databases (*e.g.*, Pfam, PROSITE) for other potential domains in α-Gal1 was conducted using the InterPro Scan tool (http://www.ebi.ac.uk/Tools/InterProScan/). Putative consensus motifs for *N*- and *O*-glycosylation sites were identified using the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/), NetOGlyc 3.0 server (http://www.cbs.dtu.dk/services/NetOGlyc/), and the *O*-Glycosylation Prediction Electronic Tool (OGPET) version 1.0 (http://ogpet.utep.edu/OGPET/). Secondary structure prediction for the deduced amino acid sequence of *α-gal1* was performed with the program PHD on the Protein Predict server (http://cubic.bioc.columbia.edu/predictprotein) and the LOOPP server (http://folding.chmcc.org/loopp/loopp.html). Globe was used to predict protein globularity (http://cubic.bioc.columbia.edu/predictprotein). Structural classification of the sequences was based on SCOP (http://scop.mrc-lmb.cam.ac.uk/scop-1.61/index.html). Protein structure files were modified and manipulated using SwissPDB Viewer (http://www.expasy.org/spdbv/). The crystal structure of α-galactosidase from *Trichoderma reesei* (PDB 1SZN) was used as the template for homology studies for the deduced amino acid sequence of *α-gal1*.

**Nucleotide Sequence Accession Number**

The nucleotide sequence of the *T. emersonii* α-galactosidase gene (*α-gal1*) is deposited in GenBank under Accession No. EU106878.

## Results and Discussion

### Gene Cloning, Sequence Analysis, and Secondary Structure Prediction

Two PCR products of 240 and 300 bp in size were amplified from *T. emersonii* genomic DNA with degenerate primers designed from fungal α-galactosidase sequences present in GenBank. Sequence analysis of the products revealed similarity with other fungal α-galactosidases in glycosyl hydrolase (GH) family 27, indicating that parts of the *T. emersonii* α-galactosidase gene were successfully cloned. Based on these sequences, 5' and 3' outer and inner

RACE PCR primers were designed to amplify the 5' and 3' ends of the *α-gal1* gene from cDNA generated from RNA purified from lactose (24 h)-induced *T. emersonii* mycelia. Sequence analysis confirmed that the RACE products encoded part of the *α-gal1* gene, which included a 57 bp 5'-untranslated region and a 108 bp 3'-untranslated region, including a polyA tail. The full-length genomic and cDNA *α-gal1* clones were amplified from first-strand cDNA and genomic DNA, respectively, using 5' and 3' gene-specific primers based on the RACE products.

The *α-gal1* gene consists of a 1,795 bp long open-reading frame, interrupted by six introns (67, 71, 72, 73, 75, and 78 bp), with consensus 5' and 3' intron splice sites, and encodes a 452 amino acid protein. The N-terminal sequence of the α-Gal1 protein contains a putative signal peptide of 24 amino acids, suggesting that the protein is secreted into the culture medium by *T. emersonii*. The translated sequence has a theoretical pI of 4.65 and a calculated molecular mass of ~50 kDa. The signature pattern for α-galactosidases, GYEYVNIDDCWSVKSGRN, is located between amino acids 70 and 87. There are also seven predicted *N*-glycosylation sites in the α-Gal1 sequence (PHD server Prosite and NetNGlyc 1.0 server). Of the seven predicted *N*-glycosylation sites, one site has a very low probability of glycosylation, whereas three sites ($Asn^{138}$, $Asn^{184}$, and $Asn^{292}$) have a high probability of glycosylation. Database interrogation using protein BLAST (BLASTP 2.2.22+) and the *α-gal1* amino acid translation revealed high similarity with other fungal α-galactosidases from the genus *Aspergillus*. For example, identity with α-galactosidase from *A. fumigatus* (XP_748129.1) was 76%, whereas 74% identity was observed with α-galactosidases from both *A. fischeri* (XP_001266320.1) and *A. niger* (CAB46229.1). Similarity with the *Trichoderma reesei* α-galactosidase (CAA93244) was 58%.

The *T. emersonii* α-Gal1 was assigned to GH family 27, Clan D and is predicted to have a catalytic *N*-terminal domain and $(\beta/\alpha)_8$-barrel structure. The predicted secondary structure is composed of 24.5% helices, 17.3% strands, and 58.2% loops (Fig. 1). The structure of α-galactosidase

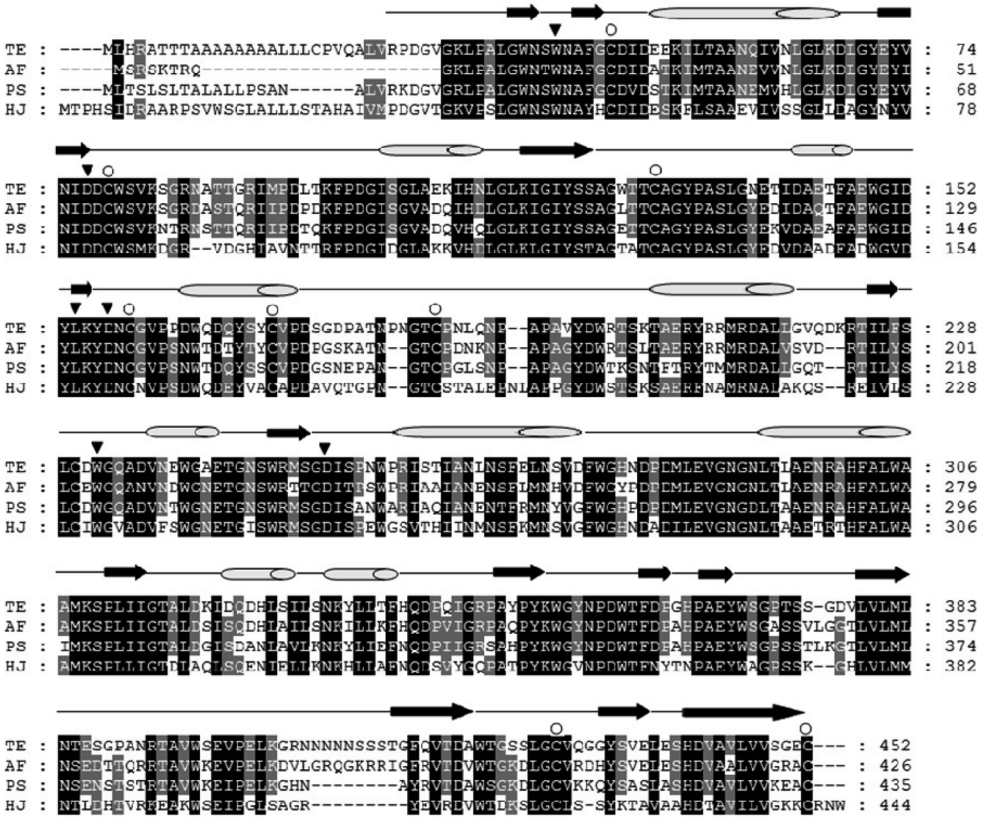

**Fig. 1.** Sequence alignment prepared with GeneDoc of the deduced amino acid sequences of *T. emersonii* α-Gal1 with α-galactosidase from *Aspergillus fumigatus* (AF, XP_748129.1), *Penicillium simplicissimum* (PS, CAA08915.1), and *Trichoderma reesei* [*i.e.*, *Hypocrea jecorina* (HJ, CAA93244.1)].

The single letter amino acid code is used. Residues that are identical or with a conservative substitution in three of the four sequences are printed in white on a grey background. Residues that are identical or with a conservative substitution in all sequences are printed in white on a black background. In the predicted secondary structure of α-Gal1, α-helices are presented as cylinders, β-sheets as arrows, and loops as lines. The catalytic residues predicted to act in the hydrolysis of the glycosidic bond are marked with inverted triangles and are in the order $Trp^{18}$, $Asp^{53}$, $Lys^{131}$, $Asp^{133}$, and $Asp^{229}$. The cysteines forming four disulfide bridges are highlighted with circles.

from *T. reesei* (PDB 1SZN) has been solved previously [14] and was used here as a template for structural interrogation of the *T. emersonii* enzyme. As mentioned earlier, Clan D enzymes catalyze hydrolysis of the glycosidic bond by a retaining double-displacement mechanism. This mechanism of hydrolysis involves a nucleophile in the formation of the substrate–enzyme intermediate and a general acid/base group that donates a proton to the leaving group during the intermediate formation and accepts a proton from a second nucleophile, a water molecule, in the hydrolysis of the intermediate [25, 35]. In *T. emersonii* α-Gal1, these catalytic residues appear to be $Asp^{133}$ (nucleophile) and $Asp^{229}$ (catalytic acid/base), as predicted from a comparison with the *T. reesei* enzyme (1SZN). Binding and recognition of substrate in 1SZN is mediated by $Trp^{19}$ and $Asp^{54}$ residues, which together form a hydrogen bond triad with $Lys^{130}$. The corresponding amino acids $Trp^{18}$, $Asp^{53}$, and $Lys^{131}$ are conserved in the deduced amino acid sequence of α-Gal1 from *T. emersonii*. The cysteine residues involved in the formation of four disulfide bridges in 1SZN are also conserved in the *T. emersonii* α-Gal1 (Fig. 1). Cysteine residues involved in the first two bonds are highly conserved in GH subfamily 27a enzymes, which are grouped together based on their amino acid sequence similarities [19]. The first pair of cysteine residues, $Cys^{104}$ and $Cys^{134}$ in 1SZN is present in all proteins possessing α-galactosidase activity, since the disulfide bridge between both residues is involved in formation of the catalytic pocket [14, 19]. The corresponding residues in α-Gal1 are $Cys^{105}$ and $Cys^{135}$.

### Expression of Recombinant α-Galactosidase in *Pichia pastoris*, and Enzyme Purification

*T. emersonii α-gal1* was successfully expressed as an extracellular protein in the *P. pastoris* strain KM71H. The host yeast strain was transformed with linearized pTE*αgal1*X harboring the *α-gal1* coding sequence, which was in-frame with an N-terminal native yeast secretory sequence and a C-terminal $His_6$ tag. Purified recombinant clones were cultivated in shake flasks, with methanol as the inducing carbon source in unbuffered and buffered (pH 6.0) minimal and complex media, for 5 days, with samples taken every 24 h for downstream analysis. Culture medium harvested from the yeast expression cultures was analyzed by SDS–PAGE and enzyme assay using 4NPG (Fig. 2) for secretion of active α-Gal1. α-Galactosidase activity was detected in samples harvested from all culture options. However, in complex media, protein expression was already high at the start of induction, whereas in minimal media the protein secretion was insignificant prior to methanol induction (T-0 h). This indicates that the host yeast was expressing its own proteins prior to induction in the complex media. Maximum levels of α-galactosidase activity were around 6.76 μmol/ml and these levels were reached earlier in both the buffered complex and minimal media cultures (Fig. 2). The activity levels reported in this study are comparable to values (6.05 μmol/ml after 48 h)

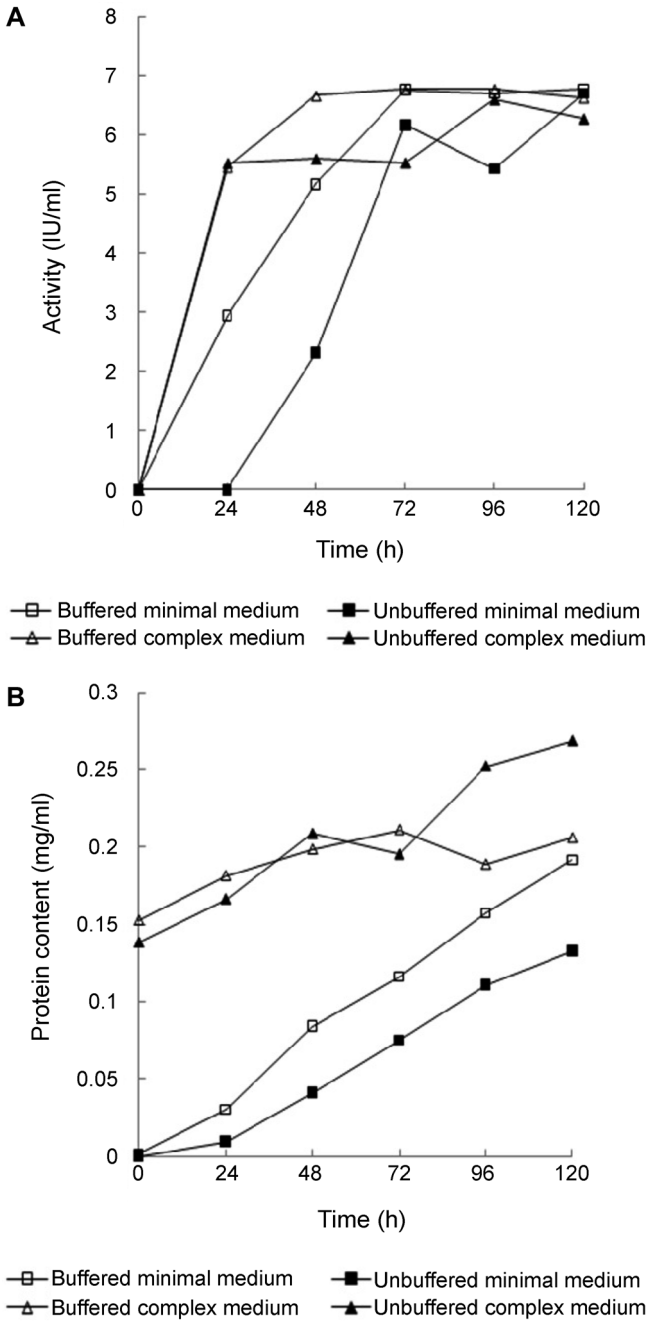


**Fig. 2.** Time course of α-galactosidase activity (**A**) and total protein (**B**) content of *P. pastoris* shake-flask cultures grown on different media.

**A**. Enzyme activity was measured using 1.0 mM 4PNG, pH4.5, as a substrate with 2.5 μl of specific culture supernatant. Reactions were incubated at 50°C for 30 min (datapoints were calculated from triplicate measurements). **B**. The total protein content in supernatant samples was measured (in triplicate) using the Bradford method. Protein secretion in complex media is already high prior to methanol induction.

**Table 1.** Purification of recombinant *T. emersonii* α-Gal1 expressed in *P. pastoris* strain KM71H.

| | Total protein (mg/ml) | Total activity (μmol/ml) | Specific activity (IU/mg) | Purification fold | Recovery (%) |
|---|---|---|---|---|---|
| Supernatant | 0.580 | 7.46 | 12.85 | 1.0 | 100.0 |
| Ni-NTA purification | 0.011 | 4.70 | 437.35 | 34.0 | 63.0 |

reported previously during expression of the *Penicillium* sp. F63 α-galactosidase Agl1 in *Pichia* sp. [17]. During expression of the *T. emersonii* α-Gal1 protein, it was clear that expression in the buffered media yielded a more stable α-galactosidase. Therefore, subsequent work concentrated only on the enzyme produced under these conditions. Recombinant α-Gal1 was purified from 5-day *P. pastoris* culture to apparent homogeneity as judged by SDS–PAGE using the ProBond nickel (Ni-NTA) affinity column purification system. A 34-fold purification of the enzyme was achieved and the specific activity of the purified α-Gal1 (4NPG as a substrate) was 437.3 IU/mg; the final enzyme recovery was 63.0%, with the yield 10.75 mg/l (Table 1).

## Characterization of Recombinant α-Gal1

**Relative molecular mass and pI.** Recombinant *T. emersonii* α-galactosidase was expressed in *P. pastoris* as a monomer with a relative molecular mass of 110 kDa (Fig. 3A) and an isoelectric point (pI) at pH 3.8 as judged by isoelectric focusing. In contrast, native *T. emersonii* α-galactosidase had a relative molecular mass of 70 kDa (unpublished data). A potential explanation for the apparent differences in molecular mass values of the native and recombinant α-Gal1 proteins is most likely due to differential glycosylation of the recombinant α-Gal1 in *P. pastoris*. To check this observation, the recombinant protein was deglycosylated using an endoglycosidase, EndoF1. A marked shift in the molecular mass was observed, in that the deglycosylated protein had a relative molecular mass of 75 kDa (Fig. 3A and 3B). The relatively high molecular mass of recombinant α-Gal1 in comparison with the deglycosylated form, or native enzyme, suggests a significant degree of *N*-glycosylation, most likely to be oligo- or hypermannose type based on yeast glycosylation patterns known to-date.

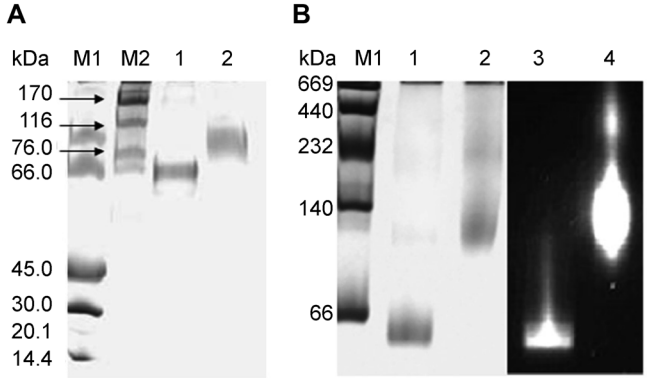


**Fig. 3.** Electrophoretic analysis of recombinant α-Gal1.
**A**. SDS–PAGE: M1, Low Molecular Weight marker; M2, High Molecular Weight marker; 1, deglycosylated recombinant α-Gal1; and 2, purified recombinant α-Gal1. **B**. Native PAGE: M, High Molecular Weight marker; 1, deglycosylated recombinant α-Gal1, and 2, purified recombinant α-Gal1. 3 & 4, Activity staining of the native PAGE gel with methylumbelliferyl-α-D-galactopyranoside; 3, deglycosylated recombinant α-Gal1, and 4, purified recombinant α-Gal1.

**Optimum reaction conditions and thermostability.** The optimum reaction conditions for recombinant α-Gal1 with 4NPG as substrate were measured in the temperature range 40–80°C and pH range 2.0–7.4. Highest activity was observed between 60 and 70°C and at pH 4.5 (Fig. 4). The preference for acidic pH values is typical of fungal α-galactosidases, which usually have optima between pH 4 and 5 [4, 5, 17, 34].

The optimum temperature for catalysis by α-Gal1 lay in the higher end of optimal temperatures reported (40–65°C) in the literature for fungal α-galactosidases [4, 5, 10, 17, 27, 33, 37]. A decrease in the temperature optimum value after *Pichia* expression has been reported also for *Penicillium* sp. F63 α-galactosidase, where the native enzyme had a 5°C higher optimum value than the recombinant enzyme [17]. Furthermore, recombinant α-Gal1 displayed good temperature tolerance over the tested range of 40–80°C. However, the thermotolerance of recombinant α-Gal1 was lower than the native *T. emersonii* enzyme, which had an optimum temperature for activity at 80°C and retained 90% of its activity after incubation at 75°C for 90 min (unpublished data); the half-life ($T_{1/2}$) of recombinant α-Gal1 at 70°C was 17 min (Fig. 4). Nevertheless, at 50°C, the recombinant enzyme displayed noteworthy stability and lost no activity after 10 days of incubation in the absence of substrate or protectants at this temperature. Relative few examples exist where a higher thermostability has been reported for fungal α-galactosidases. A polymeric α-galactosidase purified from the fruiting bodies of mushroom *Ganoderma lucidum* was maximally active at 70°C, and remained fully active after incubation for 30 min at this temperature [31]. The extracellular α-galactosidase from *Debaromyces hansenii* is optimally active at 60°C and pH 5.0, and has a half-life of 34.6 min at 70°C [33]. Thermostable α-galactosidase has also been purified from fungus *Aspergillus terreus*, with peak activity observed at 65°C and pH 5, and a t½ value of 25 min at 70°C when assayed with 4NPG [26].

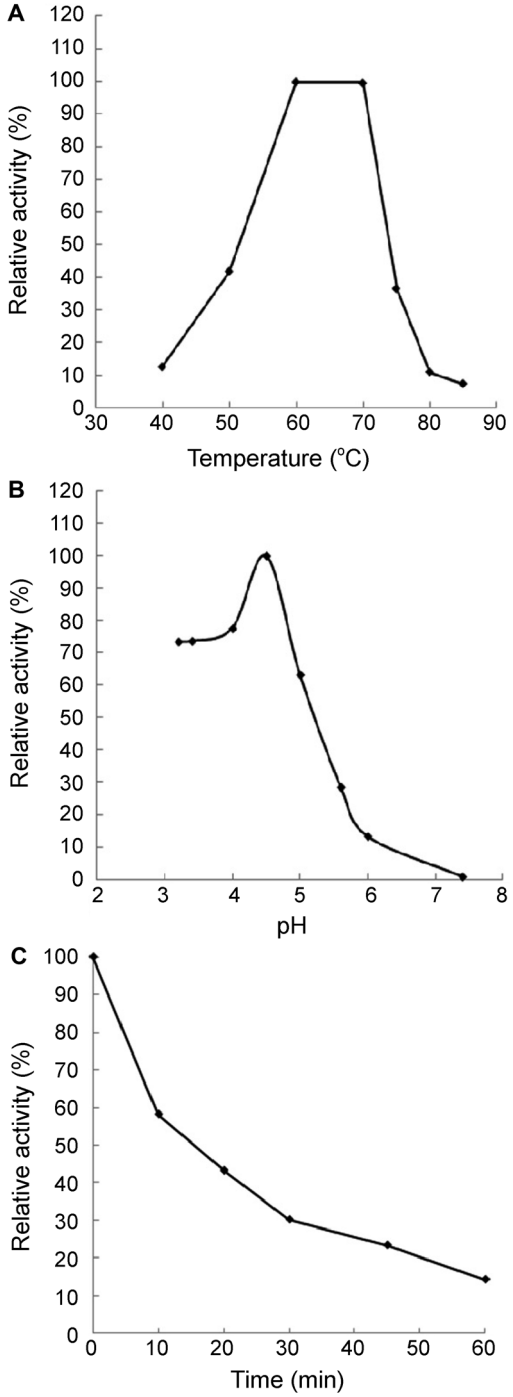


**Fig. 4.** Characterization of recombinant α-Gal1.
**A**. The effect of temperature on recombinant α-Gal1 activity determined at pH 4.5 (25 mM AmOAc as buffer) in duplicate experiments. **B**. The effect of pH on enzyme activity determined using constant ionic strength McIlvaine buffers and Clark–Lubs buffers at 50°C in duplicate experiments. **C.** Enzyme thermostability. Residual activity of recombinant α-Gal1 was measured using the standard assays with 4PNG after pre-incubation at 70°C for different timed intervals (0–60 min) in duplicate experiments. Examples of assays readings: pH 3.2, mean abs 1.100 with standard deviation (σ)±0.054; pH 3.4, mean abs 1.087, σ±0.002; pH 4, mean abs 1.136, σ±0.069; thermostability: pretreatment at 70°C 0 min, mean abs 1.281, σ±0.004; 10 min, 0.770, σ±0.070; 20 min, mean abs 0.587, σ±0.025.

**Enzyme kinetics and substrate specificity.** The recombinant α-Gal1 followed the Michaelis–Menten kinetics with respect to hydrolysis of the substrate, 4PNG. $V_{max}$ and $K_m$ values, with 4PNG as a substrate, were calculated from Lineweaver–Burk plot data and are presented in Table 2. Recombinant α-Gal1 was subject to competitive inhibition by galactose ($K_i$ of 2.77 mM, $K_m^{obs}$ of 0.57 mM). Galactose inhibition of α-galactosidases from other sources has been reported previously. The extracellular enzyme from *D. hansenii* was noncompetitively inhibited by galactose ($K_i$ of 2.7 mM) [33], whereas the intracellular enzyme from this yeast was competitively inhibited by galactose ($K_i$ of 0.70 mM) [34]. α-Galactosidase from *T. reesei* was competitively inhibited by galactose ($K_i$ of 0.6 mM) [37]. Comparison of the enzyme kinetic parameters obtained from the 4PNG reactions with corresponding figures for other fungal α-galactosidases showed that recombinant α-Gal1 is moderate in its ability to catalyze the hydrolysis of 4NPG in comparison with the other enzymes (Table 2).

The recombinant α-galactosidase specifically catalyzed the hydrolysis of α-(1,6)-linked galactose residues. α-Gal1 was active against synthetic 4NPG, but not on the β-linked galactopyranose derivative or against other synthetic glycosides tested, and also displayed activity against and preference for oligo- and polysaccharide substrates containing α-(1,6)-linked galactose. Recombinant α-Gal1 was found to be most active on the galactomannan oligosaccharide *di*-galactomannopentose ($G_2M_5$) and the oligosaccharide stachyose. Activities towards verbascose and polysaccharide substrates carob galactomannan (CGM), locust bean gum (LBG), and guar gum (GG) were notably lower. The amount of galactose released from $G_2M_5$ after 24 h was 1.67 mg/ml and with a specific activity of 30.68 IU/mg/min (Table 3). The substrate preference of recombinant α-Gal1 on the other substrates relative to $G_2M_5$ in terms of galactose released was as follows: $G_2M_5$>stachyose> raffinose>$GM_3$>melibiose>CGM=LBG>GG>verbascose (1:0.623:0.377:0.317:0.311:0.198:0.198:0.150:0.078). The enzyme did not display transglycosylation activity. The ability of α-Gal1 to release galactose both from oligosaccharides and polymeric carbohydrates could be due to its small size, which allows it to access galactose residues more easily. Small monomeric fungal α-galactosidases have been reported to have a wider substrate specificity when compared with their larger multimeric counterparts [4, 17, 34]. The ability of α-Gal1 to catalyze the hydrolysis of galactose from a broad range of substrates could also be a beneficial factor to *T. emersonii* in its natural habitat.

The *Aspergillus* fungi are a source of several enzymes used in the food and feed industries, in applications that require modification of plant cell wall polymers. These fungi produce a number of α-galactosidases with optimal reaction conditions between pH 4 and 6 and between temperatures of 50 and 60°C [11]. The generally higher

**Table 2.** Kinetics parameters of fungal α-galactosidases for synthetic substrate 4PNG.

| Fungal species | $V_{max}$ (μmol/ml) | $V_{max}$ (μmol/mg) | $K_m$ (mM) | Reference |
|---|---|---|---|---|
| *T. emersonii* | 2.58 | 240.3 | 0.29 | This study |
| *Aspergillus terreus* | 7.2 | Na | 0.11 | [26] |
| *Rhizopus* sp. F78 | 74.63 | Na | 0.61 | [5] |
| *Debaromyces hansenii* | 6.09 | Na | 0.3 | [33] |
| *Trichoderma reesei* | na | 30.1 | 1.2 | [37] |
| *Gibberella* sp. F75 | na | 0.18 | 1.06 | [4] |
| *Penicillium* sp. F63 | na | 1553.6 | 1.4 | [17] |
| *Thermomyces lanuginosus* | na | 2498 | 1.13 | [23] |

na: not available.

temperature optimum and thermal stability of *T. emersonii* α-Gal1 may prove advantageous in comparison with the α-galactosidases from *Aspergillus* species [11, 12, 29]. Enzymatic processing of legume-derived food products by α-galactosidase is so far the most efficient method in reducing the amount of raffinose-type oligosaccharides present in these materials. α-Galactosidase from *A. oryzae* has been used to decrease the concentration of these sugars in soy and chickpea milk [13, 21]. Since the hydrolysis conditions used were 50°C and pH 4.7, α-Gal1 with its noteworthy stability at 50°C and preference towards raffinose-type and galactomannan oligosaccharides could prove a particularly suitable alternative. Furthermore, as it possesses the ability to selectively remove galactose from galactomannans, recombinant α-Gal1 could have applications in food and cosmetic/pharmaceutical industries, where improving the gelling properties of these polysaccharides is desired [11].

We have successfully cloned and overexpressed in *P. pastoris* a thermostable α-galactosidase, the first gene coding for an extracellular α-galactosidase from the thermophilic fungus *T. emersonii*. The substrate preferences and catalytic properties of recombinant α-Gal1 in removing α-(1,6)-linked galactose residues from oligo- and polysaccharide substrates and its noteworthy thermal stability are of particular interest for possible biotechnological applications that involve the processing of plant material. Furthermore, unlike many microorganisms, *T. emersonii* has GRAS (generally regarded as safe) status.

**Table 3.** Specific activity of purified α-Gal1 on natural oligo- and polysaccharide substrates.

| Substrate | Specific activity (IU/mg/min) |
|---|---|
| *di*-Galactomannopentose ($G_2M_5$) | 30.68 |
| Stachyose | 19.11 |
| Raffinose | 11.68 |
| Galactomannotriose ($GM_3$) | 9.83 |
| Melibiose | 9.57 |
| Carob galactomannan (CGM) | 6.14 |
| Locust bean gum (LBG) | 6.12 |
| Guar gum (GG) | 4.59 |
| Verbascose | 2.48 |
| Maltose | 0.00 |
| Cellobiose | 0.00 |
| Lactose | 0.00 |
| Sucrose | 0.00 |

Activities were calculated after 24 h reaction.

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