ISSN 0026-2617, Microbiology, 2014, Vol. 83, No. 3, pp. 227–234. © Pleiades Publishing, Ltd., 2014.

EXPERIMENTAL ARTICLES

# A Novel Type I Thermostable Pullulanase Isolated from a Thermophilic Starch Enrichment Culture[1]

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Received August 6, 2013

**Abstract**—In this paper we report identification, cloning and characterization of a novel thermostable pullulanase type I. Pullulanase AmyA1 was detected in a sample of extracellular proteins of thermophilic enrichment culture, growing on starch. The zone of enzymatic activity in zymogram was aligned with the corresponding band on the equivalent gel without substrate. The band was excised from SDS/polyacrylamide gel and subjected to liquid chromatography/mass spectrometry (LC/MS) analysis. LC/MS-based analysis identified thermostable pullulanases, homologues to type I pullulanases of *Geobacillus thermodenitrificans* NG80-2 and *Geobacillus* sp. G11MC16. Nucleotide sequences of these two pullulanases were used for design of primers for PCR with DNA from enrichment culture, leaded to 2181 bp PCR product, coding a 726 amino acids protein, named pullulanase AmyA1. Molecular weight of AmyA1 was calculated to be 81.7 kDa. AmyA1 was cloned and expressed in *Escherichia coli*. Recombinant pullulanase was purified by two chromatographic separation steps. Pullulanase AmyA1 was active against pullulan, glycogen and soluble starch. It was active in the temperature range of 4–95°C, optimum temperature was determined to be 60°C. The highest activity of the recombinant pullulanase was observed at pH 6. Divalent cations $Mg^{2+}$ and $Mn^{2+}$ as well as dithiothreitol, Brij 35 and Brij 58 had a stimulating effect on the enzymatic activity. Pullulanase AmyA1 was stable during incubation in the presence of 4 M urea. After removal of the His-tag, addition of $Ca^{2+}$ stimulated activity of the enzyme suggesting the native pullulanase activity to be dependent on $Ca^{2+}$. Thermostability of AmyA1 was not enhanced by the addition of $Ca^{2+}$.

*Keywords*: pullulanase type I, thermostable pullulanase, *Geobacillus*, thermostable, stability in surfactants

**DOI:** 10.1134/S0026261714030084

Pullulanases (EC 3.2.1.41) are divided into two classes based on substrate specificity: (1) type I pullulanases or debranching enzymes, which specifically hydrolyze the α-1.6 linkages in pullulan as well as in branched oligosaccharides, such as starch, amylopectin, and glycogen, forming maltotriose and linear oligosaccharides, respectively; and (2) type II pullulanases or amylopullulanases, which cleave α-1,6 glycosidic linkages in pullulan and branched substrates in addition to the α-1,4 glycosidic linkages in polysaccharides [1]. Thermoactive and thermostable pullulanases are very useful for industrial applications.

A number of thermostable pullulanases have been identified. Thermostable type I pullulanases have been characterized from *Anaerobranca gottschalkii* [1], *Bacillus acidopullulyticus* [2], *Bacillus flavocaldarius* [3], *Caldicellulosiruptor saccharolyticus* [4], *Fervidobacterium pennivorans* [5], *Geobacillus stearothermophilus* [6], *Geobacillus thermoleovorans* [7, 8], *Thermotoga maritima* [9], *Thermotoga neapolitana* [10], *Thermus aquaticus* [11], *Thermus caldophilus* [12], and *Thermus thermophilus* [13]. There is a low overall sequence conservation among type I enzymes, and this reflects on the diverse biochemical characteristics of these enzymes.

In this paper we report identification, cloning, and characterization of the novel thermostable pullulanase type I. This pullulanase was detected in the sample of extracellular proteins of thermophilic enrichment culture, growing on starch. The pullulanase showed unique characteristics including the wide pH and temperature ranges of activity, high stability during incubation in the presence of high concentrations of urea, as well as stimulating effect of different surfactants on its activity, that could be useful in industrial applications of this enzyme.

## MATERIALS AND METHODS

**Preparation of extracellular proteins sample from thermophilic enrichment culture.** Thermophilic enrichment culture AmyA1 was grown on starch in the medium described by Al-Qodah [14]. 1 g of compost was suspended in 100 mL of growth medium. Enrichment culture AmyA1 was cultivated for 24 h at 60°C at 180 rpm. 1 mL of the enrichment culture was transferred into the fresh medium and cultivation was

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repeated for 24 h. Cells were removed by centrifugation (7000 *g*, 20 min, 4°C), and solid ammonium sulphate was slowly added to the supernatant with constant stirring to achieve 80% saturation. The precipitate was recovered by centrifugation (12000 *g*, 20 min, 4°C), then dissolved in 50 mM Tris-HCl buffer (pH 7/60°C). Extracellular proteins were subjected to SDS-PAGE and zymographic analysis.

**SDS-PAGE preparation and zymography.** SDS-PAGE was performed by the method of Laemmli [15]. Concentration of the separating gel was 12%, and that of the stacking gel was 5%. Non-reducing four-fold sample buffer (250 mM Tris-HCl, pH 6.8, 4% SDS, 40% glycerol, 0.001% bromophenol blue) was used for sample preparation. 5 μL of this buffer was mixed with 15 μL of the sample of extracellular proteins. The mixture was applied to the gel. Electrophoresis was carried out in a Tris–glycine–SDS buffer at 20 mA. Protein bands were visualized by staining with the PageBlue™ Protein Staining Solution (Thermo Fisher Scientific).

For starch zymography, soluble starch (from potato, Sigma-Aldrich) in a final concentration of 0.1% was added to the separating gel. Sample preparation and electrophoresis were carried out as described above for SDS-PAGE. After electrophoresis, the gel was washed for 20 min in 1% Triton X-100. Then the gel was rinsed thrice with water and incubated in 50 mM Tris-HCl buffer (pH 7/60°C) supplemented with 10 mM $CaCl_2$ at 60°C overnight. After incubation, starch zymogram was stained with iodine (1% iodine, 2% potassium iodide in water) and destained for 10 min in water. The zone of enzymatic activity in zymogram was aligned with the corresponding band on the equivalent SDS/polyacrylamide gel without substrate. The band was excised from the SDS/polyacrylamide gel without starch and subjected to liquid chromatography/mass spectrometry (LC/MS) analysis.

**LC/MS analysis.** LC/MS was performed in Mass Spectrometry Laboratory of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences. Prior to the analysis, proteins were subjected to in-gel digestion procedure. Peptide mixtures were analysed by LC-MS/MS (liquid chromatography coupled to tandem mass spectrometry) using nanoAcquity (Waters) LC system and Orbitrap Velos mass spectrometer (Thermo Electron Corp., San Jose, CA). Acquired raw data were processed by Mascot Distiller followed by Mascot Search (Matrix Science, London, UK) against NCBInr 20121126 database. Search parameters for precursor and product ions mass tolerance were 15 ppm and 0.6 Da, respectively. Peptides with Mascot Score exceeding the threshold value corresponding to <5% False Positive Rate, calculated by Mascot procedure were considered to be positively identified.

**Cloning and sequence analysis of thermostable pullulanase AmyA1.** In order to amplify thermostable pullulanase AmyA1 gene, the primers AmyA1-F (5'-ATG CTT CAT ATT AAC CGA ACG TTT GTC G-3') and AmyA1-R (5'-TCA TCC CGC ATT TTC ATC GGC ATT C-3') were designed. Pullulanase type I gene sequences extracted from the genomic sequences of *Geobacillus thermodenitrificans* strain NG80-2 and *Geobacillus* sp. strain G11MC16 were used for the construction of these primers. The primers were designed using the PRIMERSELECT component of LASERGENE 6 (DNASTAR). Genomic DNA was extracted from the enrichment culture using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific). Pullulanase gene was amplified (AmyA1-PCR) in 50 μL of reaction mixture containing DreamTaq Green PCR Master Mix (2×) (Thermo Fisher Scientific), 0.25 μM each primer, and 10 ng of genomic DNA. AmyA1-PCR was conducted under the following conditions: initial denaturation at 95°C for 2 min followed by 29 cycles each consisting of 95°C for 1 min, 60°C for 2 min, and 72°C for 3 min with a final extension step at 72°C for 7 min. PCR amplicons for the cloning of pullulanase gene were prepared using the primers GEOPUL-F (5'-CAT ATG GCT AGC ATG CTT CAT ATT AAC CG-3') and GEOPUL-R (5'-AGT GCG GCC GCT CAT CCC GCA TTT TCA TC-3'). A *Nhe*I site (underlined) was incorporated into the forward primer and a *Not*I site (underlined) was incorporated into the reverse primer for cloning into pET-28c(+). As a consequence of this cloning strategy, the His-tag as well as additional amino acids were added to the N-terminal end of the recombinant protein. GEOPUL-PCR conditions were the same as stated for AmyA1–PCR. AmyA1 PCR product served as a template for GEOPUL-PCR. GEOPUL-PCR products were digested with *Nhe*I and *Not*I (Thermo Fisher Scientific) according to the manufacturer's instructions. The pullulanase gene was ligated into the pET-28c(+) vector, and the products were transformed into *Escherichia coli* strain BL21 (DE3). Positive clones were confirmed by colony PCR using T7 promoter/T7 terminator as well as AmyA1 primers. Transformants were grown in Luria-Bertani medium containing 30 μg kanamycin $mL^{-1}$. Plasmid DNA was isolated from clones with inserts using the GeneJET™ Plasmid Miniprep Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Plasmids containing appropriately sized inserts were verified by sequencing. The sequences were aligned and analysed using MEGA 5.05 program [16].

**Expression and purification of the recombinant pullulanase.** Transformants were grown in Luria-Bertani medium containing 30 μg kanamycin $mL^{-1}$. Protein expression was induced with 0.7 mM of isopropyl-β-D-thiogalactopyranoside when $OD_{600}$ reached 0.6, and the incubation was continued for another 2.5 h at 37°C. Cultures were harvested by centrifugation (7000 *g*, 20 min, 4°C), washed twice and resuspended in buffer A (20 mM Tris-HCl, 250 mM NaCl, 10 mM imidazole, pH 7/20°C). Cells were disrupted by soni-

cation, and then the cell debris was removed by centrifugation at 12000 *g* for 20 min at 4°C. The resulting supernatant was subjected to affinity chromatography. The supernatant was loaded onto Bio-Scale™ Mini Profinity™ IMAC column (40 mm × 5.6 mm; Bio-Rad, USA) equilibrated in the buffer A. The column was washed with 25 mM imidazole, proteins were eluted with a linear 25 to 250 mM imidazole gradient in the same buffer at a flow rate of 1.0 mL $min^{-1}$. Active fractions were pooled and dialysed for 12 h at 4°C against buffer B (20 mM Tris-HCl, 5 mM EDTA, pH 8.6/20°C). The resulting protein solution was subjected to ion exchange chromatography. The solution was loaded onto UNO Q6 column (12 × 53 mm; Bio-Rad, USA) equilibrated in the buffer B. Pullulanase AmyA1 was eluted with a linear 0 to 1 M NaCl gradient in the same buffer at a flow rate of 3.0 mL $min^{-1}$. Active fractions were pooled and dialysed for 12 h at 4°C against 50 mM MES buffer (pH 6/60°C). The molecular weight and the purity of the recombinant pullulanase AmyA1 were estimated by gel electrophoresis on a 12% running gel by the method of Laemmli [15] under non-reducing conditions. Protein concentration was determined by Bradford's assay using bovine serum albumin as a standard [17].

**Removal of N-terminal His-tag from recombinant pullulanase.** N-terminal His-tag was cleaved by thrombin using the Thrombin Cleavage Capture Kit (Novagen) according to the manufacturer's recommendations. Both His-tagged and tag-free recombinant pullulanase were used for activity analysis. As the results were highly similar, only those for the His-tagged pullulanase are shown. The only exception was the substrate specificity analysis.

**Pullulanase activity and substrate specificity assay.** The assay mixture (200 μL) consisted of 50 mM MES buffer (pH 6/60°C), 0.24% (w/v) pullulan (from *Aureobasidium pullulans*; Sigma-Aldrich) and 2.0 μg of the purified recombinant pullulanase. The amount of released reducing sugars was determined by the dinitrosalicylic acid (DNS) method [18] at 60°C for 10 min unless otherwise specified. One unit of pullulanase activity was defined as the amount of enzyme that released 1 μmol reducing sugars (with maltotriose as the standard) per min under the assay conditions specified. The hydrolytic activity against 0.24% (w/v) pullulan, glycogen (from bovine liver, Sigma-Aldrich) and soluble starch (from potato, Sigma-Aldrich) was determined to evaluate the substrate specificity of recombinant pullulanase. In order to evaluate the influence of $Ca^{2+}$ on substrate specificity, calcium chloride was added to achieve final $Ca^{2+}$ concentration 1 and 10 mM. All assays were performed in duplicate.

**Effect of pH, temperature and metal ions on the enzyme activity.** In order to establish the effect of temperature and pH, recombinant enzyme assay was carried out at different temperatures (4–95°C) and pH (4–10). The effect of pH was tested using 50 mM acetate (pH 4/60°C), 50 mM MES (pH 5–6/60°C), 50 mM Tris-HCl (pH 7–9/60°C) and 50 mM glycine-NaOH (pH 10/60°C) buffers. The effect of temperature, metal ions and other reagents was determined using 50 mM MES (pH 6/60°C). Reaction mixtures were incubated for 10 min. Released reducing sugars were determined by DNS method described above.

**Effect of temperature and pH on the enzyme stability.** The thermostability of recombinant pullulanase was investigated at temperatures 60, 70 and 80°C after incubation of the enzyme solutions in absence of a substrate for 30, 60, 210, 270, 340 and 960 min. Residual activities were determined by DNS method as described above. An effect of $Ca^{2+}$ on thermostability was tested by the addition of the calcium ion at a final concentration of 1 and 10 mM to the enzyme solutions. The pH stability of recombinant pullulanase was investigated at pH 4–10 after incubation of the enzyme solution in absence of the substrate for 60 min at 60°C. The following buffers were used: 50 mM acetate (pH 4/60°C), 50 mM MES (pH 5–6/60°C), 50 mM Tris-HCl (pH 7–9/60°C) and 50 mM glycine-NaOH (pH 10/60°C).

**Analysis of hydrolysis products.** Thin-layer chromatography (TLC) of mono- and oligosaccharides was done on Silica gel 60 $F_{254}$ plates (Merck KGaA, Germany). The reactions mixtures were incubated as described above. 4 μL of each reaction mixture was applied to the plate and separated with the *n*-butanol–acetic acid–water (5 : 5 : 3, vol/vol/vol) solvent system. Sugars were detected after the plate was dried and sprayed with 20% sulfuric acid solution in ethanol and then incubated at 120°C for 10 min [19]. Mixture of glucose, maltose, and maltotriose (10 mg $mL^{-1}$ each) was used as the marker.

**Nucleotide sequence accession number.** Nucleotide sequence of AmyA1 was deposited in the GenBank under the no. KF192948.

## RESULTS

**LC/MS-based identification of thermostable pullulanase AmyA1.** According to LC/MS-based analysis, the nearest relatives of the tested enzyme were thermostable pullulanases type I of *G. thermodenitrificans* NG80-2 and *Geobacillus* sp. G11MC16 with the protein score 60 and sequence coverage 4%. Three peptides were identified: TPAFDDQFFYEGK, LIHPDSGDAR, and GFALGDASGR. The latter two correspond to the fragments of conserved domains cd02860 and cd11341 found in pullulanases and related enzymes. It should be noted that characteristics of pullulanases type I of *G. thermodenitrificans* NG80-2 and *Geobacillus* sp. G11MC16 are unknown. Gene sequences of these two pullulanases were only identified in the sequenced genomes.

**Cloning and sequence analysis of thermostable pullulanase AmyA1.** Pullulanase type I gene sequences of *G. thermodenitrificans* NG80-2 and *Geobacillus* sp.

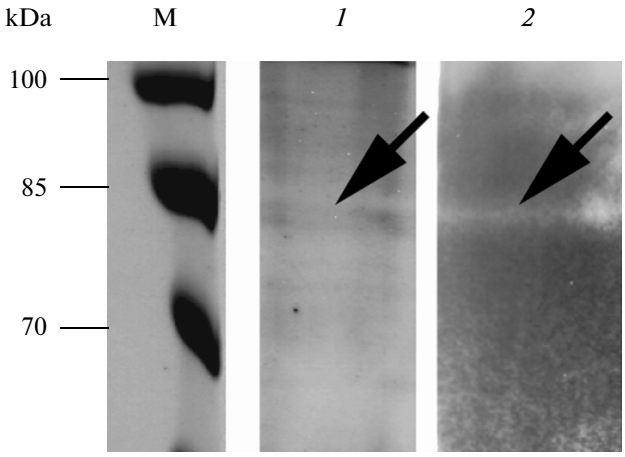


**Fig. 1.** SDS-PAAG and starch zymogram with samples of extracellular proteins of thermophilic starch enrichment culture AmyA1. The zone of activity on zymogram and corresponding band excised from the equivalent SDS/polyacrylamide gel without substrate are marked by arrows. Lanes: *1*—SDS/polyacrylamide gel without substrate; *2*—starch zymogram; M—PageRuler$^{TM}$ Unstained Protein Ladder (Thermo Fisher Scientific).

G11MC16 were aligned and compared. The primers AmyA1-F and AmyA1-R targeting the ends of the sequences were designed. It should be noted that our attempts to isolate discrete strains from the enrichment culture using starch agar plates were unsuccessful. Consequently, the enrichment culture was used for the further work. PCR product was sequenced and analysed. Sequence of the gene of pullulanase AmyA1 was 2181 bp in length coding protein of 726 amino acids in length. Molecular weight was calculated to be 81.7 kDa. The peptides identified through LC/MS analysis of the protein band excised from SDS/polyacrylamide gel (Fig. 1) were found in AmyA1 amino acid sequence. AmyA1 pullulanase gene was longer than the gene sequence of the previously published pullulanase type I (2157 nt) of *G. thermoleovorans* US105 [7]. Similarity between the gene of the latter pullulanase and that of AmyA1 was 78.3%. Amino acid sequence similarity between AmyA1 and the pullulanase of *G. thermoleovorans* US105 was 82.9%. Similarity with amino acid sequences of pullulanases of *G. thermodenitrificans* NG80-2 and *Geobacillus* sp. G11MC16 was higher—99.2 and 99.3%, respectively. AmyA1 protein sequence encoded conserved regions of pullulanases and other amylolytic enzymes belonging to glycoside hydrolase family 13: 293YNWGYNP, 334GLRVVMDAVCNHV, 406NGFRFDLMGV, 438YGEGWDL, and 517PLQSLNYVECHDNHTFWD [20].

**Expression and purification of recombinant pullulanase.** In order to characterize pullulanase AmyA1, it was subcloned into pET-28c(+) and expressed in *E. coli* BL21 (DE3). Recombinant enzyme was detected in the soluble fraction after disruption of cells. Recombinant pullulanase was purified 29 fold by two chromatographic separation steps. After purification, >95% pure pullulanase was obtained. The purification results are summarized in Table 1. Approximately 2.1 mg of purified pullulanase with a specific activity of 134.3 U mg$^{-1}$ was obtained.

**Characterization of recombinant pullulanase AmyA1.** In order to determine the characteristics of recombinant pullulanase AmyA1, its activity was assayed by measuring the released reducing sugars using DNS method. It should be noted that recombinant pullulanase AmyA1 carried additional amino acids at the N-terminal end. Therefore, the characteristics of the recombinant enzyme can differ from those of the native one.

The pullulanase was active in the temperature range of 4–95°C. Optimum temperature was determined to be 60°C with the 87.11 and 81.36% of activity at 50 and 70°C respectively (Fig. 2a). 13.94 and 9.45% of activity were detected at 4 and 95°C respectively. The highest activity of recombinant pullulanase was observed at pH 6 with 95.97 and 77.91% of activity at pH 7 and pH 5 respectively (Fig. 2b). ~26% of activity was retained at pH 4 and pH 10.

The effects of different metal ions and other reagents on the recombinant pullulanase are shown in Table 2. The presence of $Na^+$ and $K^+$ at a concentration of 50 mM did not affect activity of the recombinant pullulanase. Addition of $Li^+$, $Ba^{2+}$ and $Ca^{2+}$ had no effect either. But addition of divalent cations, such as $Co^{2+}$, $Cu^{2+}$, $Ni^{2+}$, $Sn^{2+}$, $Zn^{2+}$, as well as trivalent cation $Fe^{3+}$, reduced the pullulanase activity by more than 44%. Divalent cations $Mg^{2+}$ and $Mn^{2+}$ as well as dithiothreitol (DTT), Brij 35, Brij 58, and glycerol (at

**Table 1.** Purification of the recombinant pullulanase AmyA1 after expression in *E. coli* BL21(DE3)

| Purification step | Total activity, U | Total protein, mg | Specific activity, U mg$^{-1}$ | Yield, % | Purification fold |
|---|---|---|---|---|---|
| Crude extract | 714 | 156 | 4.6 | 100 | – |
| Affinity chromatography | 350 | 26.8 | 13.1 | 49 | 3 |
| Ion exchange chromatography | 282 | 2.1 | 134.3 | 39 | 29 |

One unit (U) of pullulanase catalyzes the formation of 1 μmol of reducing sugars per min under the defined conditions. Maltotriose was used as a standard.

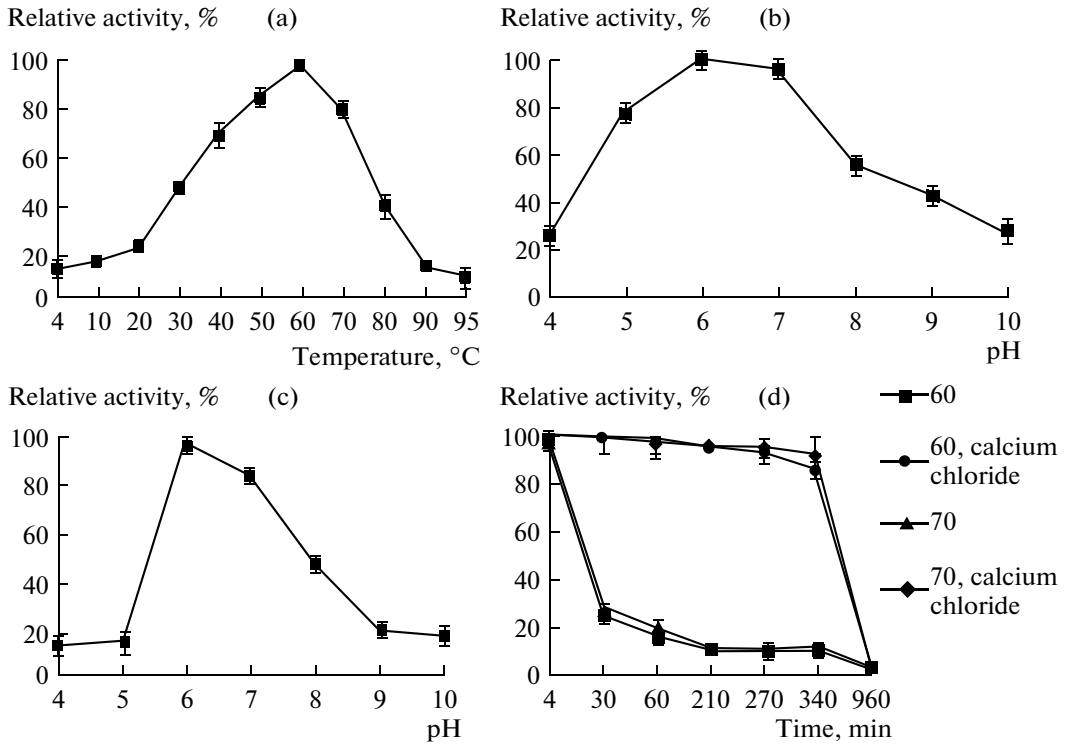


**Fig. 2.** Effects of temperature and pH on the recombinant pullulanase activity and stability: (a) the recombinant pullulanase was assayed at various temperatures ranging from 4 to 95°C for 10 min under pH 6 in order to determine the effects of temperature on activity; (b) effects of pH on the recombinant pullulanase activity were assayed using different buffers at 60°C for 10 min; (c) the pH stability of recombinant pullulanase was investigated at pH 4–10 after incubation of the enzyme solution in absence of the substrate for 60 min at 60°C; (d) the thermostability of recombinant pullulanase was investigated after incubation of the enzyme solutions in absence of a substrate for 30, 60, 210, 270, 340 and 960 min at pH 6. 60 and 70—the incubation temperature; calcium chloride—the addition of the calcium ion at a final concentration of 10 mM to the reaction mixture.

a concentration of 0.1%) had a stimulating effect on the enzymatic activity. Incubation in the presence of surfactants Tween-20, Tween-40, Tween-60 and Tween-80 remarkably reduced the activity of AmyA1. The enzyme was completely eliminated by SDS and 1% Triton-X305. Triton X-100 at a concentration of 1% inhibited the enzyme.

Pullulanase AmyA1 was active against pullulan, glycogen, and soluble starch (Table 3). All of them have α-1,6 glycosidic linkages in their structure. The highest activity was determined for pullulan, and the lowest activity was observed for starch. Removal of His-tag from the recombinant enzyme enhanced the activity against all substrates. Although $Ca^{2+}$ did not affect activity of His-tagged AmyA1, it stimulated activity of the enzyme after removal of His-tag against all substrates. TLC results showed the complete conversion of pullulan to maltotriose. Long chain oligosaccharides were detected after hydrolysis of glycogen and starch (data not shown).

Pullulanase AmyA1 was stable in the range of pH 6–8 for 60 min at 60°C. 12–19% of activity was retained at pH 4–5 and 9–10 (Fig. 2c). Recombinant pullulanase was thermostable at 60°C for 340 min showing 84.54% residual activity (Fig. 2d). At 70°C only 24.73% of activity was detected after incubation for 30 min. The enzyme was not stable at 80°C; complete inactivation was recorded after 15 min of incubation (data not shown). It should be noted that $Ca^{2+}$ had no effect on thermostability of pullulanase AmyA1.

## DISCUSSION

Pullulanase AmyA1 was identified by LC/MS analysis of extracellular proteins of the enrichment culture. Similarity of AmyA1 with previously uncharacterized pullulanases type I of *G. thermodenitrificans* NG80-2 and *Geobacillus* sp. G11MC16 allowed us to suggest AmyA1 to be pullulanase of *Geobacillus* as well as to design the primers for the successful amplification and cloning of this enzyme. The highly conserved seven-residue motif typical of all type I pullulanases, YNWGYNP [20], was identified in the sequence of AmyA1 confirming that this enzyme belongs to pullulanases type I. Analysis of substrate specificity and hydrolysis products confirmed this finding. AmyA1 hydrolysed pullulan to maltotriose and was also active against glycogen and soluble starch—activities well known for pullulanases type I [20]. Similarly to pullu-

**Table 2.** Influences of different reagents on pullulanase AmyA1 activity

| Reagent | Concentration | Relative activity, % |
|---|---|---|
| None | | 100 |
| KCl | 1 mM | 101.63 ± 2.41 |
| | 50 mM | 99.07 ± 1.79 |
| LiCl | 1 mM | 100.37 ± 0.46 |
| NaCl | 1 mM | 103.99 ± 1.04 |
| | 50 mM | 99.65 ± 1.93 |
| $BaCl_2$ | 1 mM | 101.26 ± 0.25 |
| $CaCl_2$ | 1 mM | 102.93 ± 0.19 |
| | 10 mM | 99.50 ± 2.02 |
| $CoCl_2$ | 1 mM | 55.13 ± 1.87 |
| $CuCl_2$ | 1 mM | 18.45 ± 2.31 |
| $MgCl_2$ | 1 mM | 113.82 ± 0.53 |
| $MnCl_2$ | 1 mM | 139.48 ± 1.97 |
| $NiCl_2$ | 1 mM | 41.95 ± 2.48 |
| $SnCl_2$ | 1 mM | 52.92 ± 3.77 |
| $ZnCl_2$ | 1 mM | 16.16 ± 1.63 |
| $FeCl_3$ | 1 mM | 22.43 ± 1.79 |
| Dithiothreitol | 1 mM | 116.36 ± 0.47 |
| Tris(2-carboxyethyl) phosphine | 1 mM | 99.62 ± 0.28 |
| 2-Mercaptoethanol | 1 mM | 97.52 ± 3.02 |
| Urea | 100 mM | 91.15 ± 0.56 |
| | 4000 mM | 88.85 ± 1.74 |
| Guanidine HCl | 1 mM | 91.50 ± 2.67 |
| SDS | 0.1% | 0 |
| DMSO | 0.1% | 94.63 ± 2.5 |
| | 1% | 81.23 ± 3.07 |
| Methanol | 1% | 91.51 ± 0.59 |
| | 5% | 87.57 ± 0.52 |
| Ethanol | 1% | 97.41 ± 0.43 |
| | 5% | 91.87 ± 0.9 |
| Ethylene glycol | 0.1% | 96.56 ± 1.05 |
| | 1% | 95.96 ± 0.92 |
| Glycerol | 0.1% | 109.39 ± 0.85 |
| | 1% | 98.74 ± 1.06 |
| Triton X-100 | 0.1% | 95.71 ± 2.91 |
| | 1% | 89.85 ± 2.78 |
| Triton X-305 | 0.1% | 41.74 ± 4.6 |
| | 1% | 0 |
| Tween 20 | 0.1% | 77.11 ± 1.99 |
| | 1% | 70.45 ± 2.04 |
| Tween 40 | 0.1% | 53.07 ± 2.38 |
| | 1% | 45.68 ± 2.82 |
| Tween 60 | 0.1% | 53.95 ± 1.67 |
| | 1% | 52.95 ± 2.6 |
| Tween 80 | 0.1% | 73.24 ± 3.85 |
| | 1% | 51.29 ± 2.42 |
| Brij 35 | 1 mM | 113.11 ± 0.74 |
| Brij 58 | 1 mM | 112.10 ± 0.49 |

lanase of *G. thermoleovorans* US105 [7], hydrolysis of soluble starch did not result in formation of oligosaccharides with short chain length.

The highest gene sequence similarity was obtained for the pullulanases type I of *Geobacillus* and *Thermus*. Molecular weight of AmyA1 was calculated to be 81.7 kDa. This size was comparable with that of pullulanases type I of *G. thermoleovorans* US105 (80 kDa; [7]), *G. stearothermophilus* TRS128 (83 kDa; [6]), *T. thermophilus* HB8 (80 kDa; [13]) and *T. aquaticus* YT-1 (83 kDa; [11]). Optimal pH of AmyA1 was 6.0, and this value is common for a number of bacterial pullulanases type I including those of geobacilli. pH range of activity of AmyA1 was 4–10, even ~26% of activity was retained at pH 4 and pH 10. To our knowledge, this is the widest pH range of activity among the thermostable pullulanases type I described so far. Although activity at pH of this range was also tested for pullulanases of *G. stearothermophilus* TRS128 [6], *A. gottschalkii* [1], *T. neapolitana* [10], *T. thermophilus* HB8 [13], only <10% of activity was detected if any.

Optimum temperature of AmyA1 was determined to be 60°C. This temperature is one of the lowest among thermostable pullulanases type I, such optimum temperature was previously reported only for *B. acidopullulyticus* [2]. But the temperature range of activity was very wide, from 4 to 95°C. None of the thermostable pullulanase type I described so far retained its activity below 20°C [5, 6], while AmyA1 showed 13.94% of activity at 4°C.

Thermostability of AmyA1 was quite low. At 70°C, only 24.73% of activity was detected after incubation for 30 min, while pullulanase of *G. thermoleovorans* US105 had half-life duration of 4 h at this temperature [8]. Pullulanase of *T. thermophilus* HB8 was also more thermostable than AmyA1 [13]. But AmyA1 was more thermostable than pullulanase of *G. stearothermophilus* TRS128, which lost its activity after incubation for <10 min at 70°C [6] as well as pullulanase of *B. acidopullulyticus* which completely lost its activity after incubation for 30 min at 70°C [2]. It is noteworthy, that our experiments carried out to reveal temperature effect on activity and stability clearly showed considerable stabilizing effect of substrate on enzyme thermostability.

$Ba^{2+}$ and $Ca^{2+}$ had no effect on activity of His-tagged AmyA1. After removal of His-tag, addition of $Ca^{2+}$ stimulated activity of the enzyme suggesting the activity of the native AmyA1 to be dependent on calcium ions. It should be noted that thermostability of AmyA1 was not enhanced by the addition of $Ca^{2+}$. This is in contrast with pullulanase of *G. thermoleovorans* US105 which required $Ca^{2+}$ for its stability but not activity [8]. $Ba^{2+}$ and $Ca^{2+}$ stimulated activity of other pullulanase of *Geobacillus*—native pullulanase of *G. stearothermophilus* TRS128 [6]. Stimulating effect of $Mn^{2+}$ on AmyA1 activity should be noted. Usually $Mn^{2+}$ has no effect or acts as inhibitor on pullulanase activity [1, 8, 10]. Stimulating effect of $Mn^{2+}$

**Table 3.** Substrate specificity of pullulanase AmyA1 and influence of $Ca^{2+}$ on enzymatic activity against different substrates

| | Substrate | Relative activity, % | | | | | |
|---|---|---|---|---|---|---|---|
| | | 10 min | 960 min | 10 min 1 mM $CaCl_2$ | 960 min 1 mM $CaCl_2$ | 10 min 10 mM $CaCl_2$ | 960 min 10 mM $CaCl_2$ |
| With His-tag | Pullulan | 100.00 | ND | 102.93 | ND | 99.50 | ND |
| | Glycogen | 34.51 | 43.59 | 32.55 | 43.36 | 31.92 | 43.13 |
| | Soluble starch | 0 | 21.60 | 0 | 19.73 | 0 | 19.04 |
| After removal of His-tag | Pullulan | 105.44 | ND | 113.14 | ND | 121.12 | ND |
| | Glycogen | 48.13 | 50.40 | 53.49 | 57.08 | 56.54 | 59.65 |
| | Soluble starch | 0 | 24.35 | 0 | 28.78 | 0 | 32.05 |

ND—not determined.

was previously reported only for pullulanases of *G. stearothermophilus* TRS128 [6] and *T. caldophilus* GK-24 [12]. Among reducing reagents, only DTT had positive effect on AmyA1 activity, while both DTT and 2-mercaptoethanol had stimulating effect on activity of other pullulanases type I [5, 10]. Pullulanase AmyA1 showed exceptional stability during incubation in the presence of 4 M urea as compared with other thermostable pullulanases type I [5, 10]. To our knowledge, stimulating effect of surfactants, namely Brij 35 and Brij 58, has never been previously reported for thermostable pullulanases type I.

In conclusion, using LC/MS analysis of the extracellular proteins of the thermophilic enrichment culture, novel thermostable pullulanase type I was identified. Some unique biochemical characteristics including the very wide pH and temperature range of activity as well as stimulating effect of some surfactants on its activity distinguished pullulanase AmyA1 from other thermostable pullulanases type I.

## ACKNOWLEDGMENT

This work was supported by the ThermozymOS project (no. SVE-08/2011) from the Research Council of Lithuania.

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