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NOTE

Purification and characterization of a cold-adapted pullulanase from a psychrophilic bacterial isolate

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Abstract There is a considerable potential of cold-active biocatalysts for versatile industrial applications. A psychrophilic bacterial strain, *Shewanella arctica* 40-3, has been isolated from arctic sea ice and was shown to exhibit pullulan-degrading activity. Purification of a monomeric, 150-kDa pullulanase was achieved using a five-step purification approach. The native enzyme was purified 50.0-fold to a final specific activity of 3.0 U/mg. The enzyme was active at a broad range of temperature (10–50 °C) and pH (5–9). Optimal activity was determined at 45 °C and pH 7. The presence of various metal ions is tolerated by the pullulanase, while detergents resulted in decreased activity. Complete conversion of pullulan to maltotriose as the sole product and N-terminal amino acid sequence indicated that the enzyme is a type-I pullulanase and belongs to rarely characterized pullulan-degrading enzymes from psychrophiles.

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Introduction

Tailor-made enzymes for specific, often harsh, process conditions in industrial applications are highly demanded. These candidates can be either developed by protein engineering techniques or isolated from environments that are extreme from a human point of view, such as hydrothermal vents, solfataric fields, alkaline and salt lakes or ice samples (Antranikian and Egorova 2007; Davids et al. 2013). In this context, enzymes from psychrophilic microorganisms are promising candidates for versatile biotechnological applications especially food industry due to the reduced risk of microbial contamination, minimized energy consumption and the fact that reacting compounds are often instable at increasing temperatures (Trincone 2011). Among the most industrially relevant biocatalysts are starch-hydrolyzing enzymes, such as amylase, pullulanase, glucoamylase or α-glucosidase, that are widely used in food, feed, textile, pharmaceutical and detergent industries.

Starch is composed of the linear polymer amylose and the branched component amylopectin. Both contain α-linked glucose molecules, that are either α-1,4-linked in both polymers or α-1,6-linked at branch sites in amylopectin (Bertoldo and Antranikian 2002; Elleuche and Antranikian 2013). Pullulanase (EC 3.2.1.41) is a diverse group of enzymes that catalyzes hydrolysis of glucosidic linkages and can be classified into five groups based on substrate specificity and products formation: (a) pullulanase type-I, (b) pullulanase type-II, (c) pullulan hydrolase type-I, (d) pullulan hydrolase type-II, and (e) pullulan hydrolase type-III (Bertoldo and Antranikian 2002). Pullulanase type-I specifically hydrolyzes

α-1,6 linkages in pullulan, amylopectin or limit dextrins with high specificity. Pullulan is completely degraded in a random fashion to maltotriose. These enzymes require at least two α-1,4 linked glucose units in the vicinity of the α-1,6 linkages (Bertoldo and Antranikian 2002). Unlike pullulanase type-I, type-II enzymes (amylopullulanases) show dual specificity by hydrolyzing α-1,4 and α-1,6 glucosidic linkages in starch. Pullulan hydrolase type-I (neopullulanase) and pullulan hydrolase type-II (isopullulanase) hydrolyze the α-1,4 linkages in pullulan, liberating panose and isopanose, respectively. Both enzymes are unable to hydrolyze α-1,6 linkages in branched substrates or pullulan. Pullulan hydrolase type-III attacks α-1,4 as well as α-1,6 linkages in pullulan, producing maltotriose, maltose, panose and glucose (Niehaus et al. 2000).

True pullulanases have been found and isolated from a variety of bacteria. Pullulanase type-I has predominantly been identified in mesophilic and thermophilic microorganisms, such as *Anaerobranca gottschalkii*, *Bacillus acidopullulyticus*, *Bacillus flavocaldarius*, *Caldicellulosiruptor saccharolyticus*, *Fervidobacterium pennivorans, Geobacillus thermoleovorans, Klebsiella pneumoniae*, *Thermotoga maritima* and *Thermotoga neapolitana* (Kornacker and Pugsley 1990; Suzuki et al. 1991; Kelly et al. 1994; Albertson et al. 1997; Bertoldo et al. 1999; Kriegshauser and Liebl 2000; Bertoldo et al. 2004; Zouari Ayadi et al. 2008; Kang et al. 2011). Pullulanase type-II is widely distributed in microbial strains such as *B. acidocaldarius*, *Desulfurococcus mucosus*, *G. thermoleovorans* and others (Koivula et al. 1993; Duffner et al. 2000; Nisha and Satyanarayana 2013a, b). Based on their substrate specificities and sequence composition, true pullulanases can be grouped into glycoside hydrolase families 13 and 57 (www.cazy.org). To the best of our knowledge, only a single cold-adapted amylopullulanase (pullulanase type-II) has been described from *Exiguobacterium* sp. SH3, while no type-I pullulanases were reported from psychrophiles (Rajaei et al. 2013).

In our previous study, we have isolated psychrophilic bacterial strain *Shewanella arctica* 40-3 from arctic sea ice samples in Spitsbergen. In a first plate-assay screening approach, this bacterial strain was shown to exhibit multiple biotechnological-relevant enzymatic activities including the capability to degrade pullulan (Qoura et al. 2014). In this work, we report in the purification of type-I pullulanase from cultured wild-type cells and present detailed biochemical properties of the 150-kDa enzyme.

Materials and methods

Bacterial strain, medium and growth condition

Shewanella arctica 40-3 (DSMZ 16509, JCM 14208) was grown in a complex marine medium consisting of basal medium supplemented with a solution of different carbon sources (Qoura et al. 2014). The basal medium contained (volume g l^{-1}) NaCl, 28.13 g; KCl, 0.77 g; $CaCl_2 \times 2$-H_2O, 0.02 g; $MgSO_4 \times 7H_2O$, 0.5 g; NH_4Cl, 1.0 g; iron-ammonium-citrate, 0.02 g; yeast extract, 0.5 g; tenfold concentration trace element solution (DSM 141), 1 mL; tenfold concentration vitamin solution (DSM 141), 1 mL; KH_2PO_4, 2.3 g and $Na_2HPO_4 \times 2H_2O$, 2.9 g. The carbon source mixture solution (volume g l^{-1}) contained pullulan, 10 g; Na-acetate, 0.5 g; Na_2-succinate, 0.5 g; Na-pyruvate, 0.5 g; DL-malate, 0.5 g; D-mannitol, 0.5 g and glucose, 2 g. The final pH of the complex medium was adjusted to 7. Incubation was carried out at 15 °C for 4 days.

Bacterial strain cultivation and native crude enzyme extraction

Cells of *S. arctica* 40-3 were harvested from 5-l cell culture sample by centrifugation (producing 80 g wet weight cell mass), washed 3 times with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH of 7.4), and resuspended in buffer A (20 mM Tris–HCl, 100 mM NaCl, 1 % (v/v), Triton X-100, pH 8) in a constant ratio of 1:5 wet weight cell mass (g) to buffer volume (mL). The resuspended cells were disrupted with a French press (LM-AMINCO, Spectronic instruments), 3 times at 2500 psi, and the suspension was centrifuged at 10,000*g* for 30 min at 4 °C. The supernatant produced was used as the native enzyme crude extract.

Purification of native pullulanase

All purification steps were carried out at room temperature. The native pullulanase crude extracts were dialyzed against 100 volumes of 50 mM Tris–HCl, pH 8. The crude extract was then loaded onto a Q-sepharose column (2.5 by 20 cm) equilibrated with 50 mM Tris–HCl, pH 8, washed with the same buffer until no absorbance at 280 nm was detectable. The column was washed with the same buffer, and proteins were eluted with a linear 0–1 M NaCl gradient with the same buffer. Active fractions were pooled and concentrated by ultrafiltration. The enzyme solution was adjusted with ammonium sulfate to a final concentration of 1 M and applied at a flow rate of 20 mL/h to a phenyl-Sepharose column (2.5 by 20 cm) that was equilibrated with 50 mM Tris–HCl, pH 8, containing 1 M ammonium sulfate. After the column was washed with 50 mL of the same buffer, the pullulanase was eluted with a linear 1–0 M ammonium sulfate gradient in the same buffer. Active fractions were pooled and dialyzed against 20 mM phosphate buffer (pH 7). The enzyme solution was then loaded onto a Hydroxylapatite column (GHT Ceramic) (2.5 by 20 cm) equilibrated with 5 mM phosphate buffer (pH 7). Pullulanase

was eluted with a linear 5–200 mM phosphate gradient in the same buffer. Active fractions were pooled and dialyzed against 50 mM Tris–HCl containing 0.15 M NaCl (pH 8). The enzyme solution was then applied to a Superdex S-200 column (2.6 by 96 cm; Amersham Biotech Inc., Germany) equilibrated with 50 mM Tris–HCl, 0.15 M NaCl buffer (pH 8). Elution was performed with the same buffer and 2 mL fractions were collected at a flow rate of 2 mL/min. Active fractions were pooled, concentrated by ultrafiltration (10-kDa cut off) and dialyzed against 25 mM Tris–HCl (pH 8). The enzyme solution was then applied to a Mono Q HR 5/5 column (Pharmacia LKB, Germany), which was equilibrated with 50 mM Tris–HCl (pH 8). Pullulanase was eluted with a linear 0–0.5 M NaCl gradient in the same buffer.

N-terminal sequencing of purified pullulanase

N-terminal protein sequencing was carried out by automated Edman degradation with electroblotted samples on PDVF membrane analyzed on pulsed liquid protein (model 492, PE-Biosystem, USA) connected online to an HPLC apparatus (HP 1100 Hewleh Packard) for phenylthiohydantoin derivative identification.

Gel electrophoretic analysis

SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) as described by Laemmli (1970) was routinely performed under reducing conditions with 8 % polyacrylamide gels by using a Bio-Rad mini-SUB CELL GT II electrophoresis unit (Bio-Rad, Germany). SDS high- and low-molecular-weight markers (Amersham Biosciences, Germany) were used to determine the apparent molecular weight of isolated proteins. Samples were mixed with loading buffer, without heat treatment, before loading. After electrophoresis, the gels were rinsed in 0.5 % (v/v) Triton X-100 at 4 °C for 30 min to remove SDS. Zymogram staining for pullulanase activity was performed by staining the rinsed gel with 5 % (w/v) red-pullulan (in water) (Megazyme, Ireland) for 6 h at 4 °C. The active band appeared (as clearing zone corresponding to red-pullulan hydrolysis) after incubating the stained gel for 30–60 min under optimal assay conditions. Gels that were not used for activity staining were stained using coomassie blue solution.

Enzyme assays

Pullulanase activity was determined by measuring the amount of reducing sugar released during incubation with pullulan (molecular weight 200,000; ICN, Merkenheim, Germany). To 50 µl of 1 % (w/v) pullulan dissolved in 50 mM Tris–HCl buffer (pH 7), 50 or 100 µl of enzyme solution was added, and the samples were incubated at different temperatures for 30–60 min. The reaction was stopped by cooling on ice, and the amount of reducing sugar released was determined by the dinitrosalicylic acid method (Bernfeld 1955). Protein concentration was determined according to Bradford method (Bradford 1976). Sample blanks were used to correct for non-enzymatic release of reducing sugar. One unit of pullulanase activity is defined as the amount of enzyme that releases 1 µmol of reducing sugars (with maltose as standard) per min. The following polysaccharide substrates were tested: 0.5 % (w/v) pullulan, 0.5 % (w/v) soluble starch, 0.5 % (w/v) amylopectin, and 0.5 % (w/v) amylose.

In order to study the influence of temperature and pH, experiments were carried out with the purified native enzyme (3 U/mg). To determine the influence of temperature on the enzymatic activity, samples were incubated at temperatures from 10 to 60 °C for 60 min (pH 7). The influence of pH on the enzymatic activity was determined at 45 °C for 60 min by using the standard assay protocol. 50 mM Tris–HCl buffer was used to obtain pH range from 6 to 9 and sodium acetate buffer to obtain pH range from 2 to 5. Thermostability studies were carried out at high temperatures (40, 50 and 65 °C) for 0–6 h. After various incubation time intervals, samples were withdrawn and clarified by centrifugation, and the enzymatic activity was determined using standard conditions (45 °C, pH 7).

The effect of metal ions and several reagents on the pullulanase activity was investigated on the purified native enzyme (3 U/mg). Various substances were pre-incubated with enzyme at room temperature for 60 min. Samples were withdrawn and tested for pullulanase activity using standard conditions. The following metal ions were tested with a concentration of 1 mM: $MnCl_2$, $CoCl_2$, $CuCl_2$, $NiCl_2$, $MgCl_2$ and $ZnCl_2$. Other reagents were: 1 % (w/v) SDS, 0.4 and 0.8 M guanidine, 1 mM EDTA (ethylenediaminetetraacetic acid), 0.025 % (w/v) α, β, and γ-cyclodextrin, 0.01 % (w/v) *N*-bromosuccinimide, 10 mM DTT (dithiothreitol) and 10 and 20 mM idoacetamide.

HPLC analyses

The hydrolysis products arising from the action of pullulanase on linear and branched polysaccharides were analyzed by high-performance liquid chromatography (HPLC) with an Aminex HPX-42 a column (300 by 78 mm) (Bio-Rad, Hercules, Calif). Double-distilled water was used as the mobile phase at a flow rate of 0.3 mL/min (Rüdiger et al. 1995). Enzyme was incubated with 0.5 % (w/v) pullulan at 45 °C for 60 min. In order to distinguish maltotriose (only α-1,4 bonds) from panose or isopanose (α-1,4 and α-1,6 bonds), enzymatic assays using yeast α-glucosidase (10 U/mg final concentration) in a 50 mM potassium phosphate buffer (pH 7) were performed. Co-incubation of α-glucosidase and pullulanase was done in a two-step

Table 1 Purification of the native pullulanase

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude extract	1225	68.2	0.06	100	1
Q-sepharose	235	58.8	0.3	86.2	5.0
Phenyl-sepharose	17.1	10.0	0.6	14.7	10.0
Hydrohylapatite	1.0	2.1	2.0	3.0	33.3
Superdex S-200	0.5	1.3	2.6	1.9	43.3
Mono Q	0.3	0.7	3.0	1.0	50.0

After the growth of *S. arctica* 40-3 at 10 °C, a 5 L culture was centrifuged (80 g of wet weight cells), cells were washed 3 times with PBS buffer, and resuspended with Tris-NaCl-Triton X-100 buffer (pH 8). Cells were disrupted with French press, centrifuged and the supernatant was used for purification

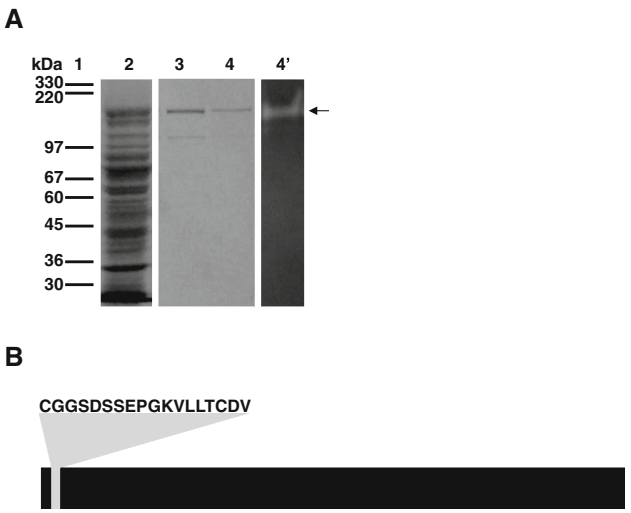

Fig. 1 SDS–PAGE analysis, zymogram and schematic protein illustration of pullulanase. **a** Purification and zymogram of native pullulanase. *Lane 1* molecular weight marker, *lane 2* crude extract, *lane 3* mono Q pool (13 µg) and *lane 4* mono-Q pool (25 µg). *Lane 4'* mono-Q pool (25 µg). *Arrow* indicates the position of pullulanase. **b** Schematic representation of putative pullulanase from *Shewanella* sp. strain HN-41 and location of conserved sequence range (CGGSDSSEPGKVLLTCDV) identified by Edman degradation using purified pullulanase from *S. arctica* 40-3

reaction at 45 °C for 60 min followed by incubation at 37 °C (T_{opt} of α-glucosidase) for 60 min. DP3 (maltotriose) was also incubated as a control with α-glucosidase at 37 °C for 60 min. Samples were withdrawn and the reaction was stopped by incubation of the mixtures on ice. DP1 (glucose), DP2 (maltose), DP3 (maltotriose) and DP4 (maltotetraose) were used as standards.

Results

Purification of native pullulanase

Shewanella arctica 40-3 culture supernatant showed low pullulanase activity that was only detectable by incubation on red-pullulan agar plates. Crude cell extracts exhibited a specific pullulanase activity of 0.06 U/mg indicating that pullulanase is probably an intracellular enzyme. Five-step purification procedure resulted in a 50-fold increase in specific activity and a yield of 1 % (Table 1). Proteins from the purification steps were separated by 8 % SDS–PAGE (Fig. 1a). The enzyme migrates as a single band with an apparent molecular mass of 150 kDa. This matched the molecular mass of the single active band detected on the zymogram pattern (8 % SDS–PAGE activity gel of the purified native enzyme).

N-terminal sequencing and BLAST analysis

Edman degradation analysis of the purified native pullulanase was carried out to reveal the N-terminal sequence. BLASTP-analysis using 18 amino acids found in the N-terminal sequence (CGGSDSSEPGKVLLTCDV) of the purified native pullulanase revealed 100 % identity to amino acid residues 23–40 from an uncharacterized, putative pullulanase from *Shewanella* sp. strain HN-41 (Accession no. WP_007649744) (Fig. 1b). This annotated protein is composed of 1,440 amino acid residues with a predicted molecular mass of 155.4 kDa, which is in good agreement with purified protein band on SDS–PAGE and zymogram (Fig. 1a).

Biochemical properties and substrate specificity

Catalytic activity was measured in a temperature range between 10 and 60 °C and the optimal temperature was 45 °C. The enzyme retained more than 50 % of activity between 20 and 45 °C. Up to 27 % of activity is retained at 10 °C, while the functionality dramatically decreased above 45 °C (Fig 2a). However, the decline of catalytic activity above 45 °C is also a result of the low thermostability of the enzyme at high temperatures. The influence of temperature on the stability of pullulanase was examined by measuring the enzymatic activity after pre-incubation at

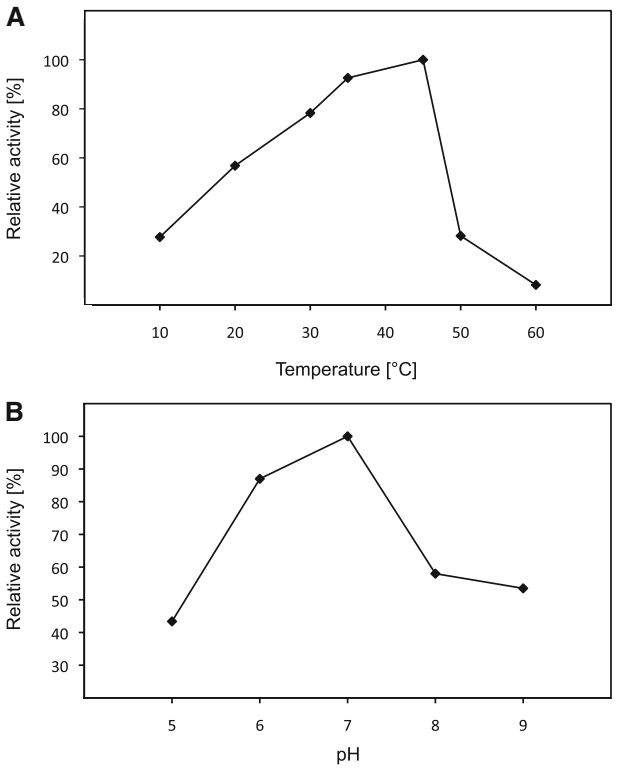

Fig. 2 Influence of temperature and pH on the activity of pullulanase from *Shewanella arctica* 40-3. **a** 3 U/mg native pullulanase was incubated in Tris–HCl buffer (pH 7) for 30 min at different temperatures. **b** Pullulanase was incubated at 45 °C for 30 min at different pH to determine the pH optimum

different temperatures. Pullulanase retained about 80 % residual activity at 40 °C for 3 h and exhibited a half-life of 44 min at 50 °C (data not shown). Purified native pullulanase showed activity over a broad pH range (pH 5–9), with an optimum at pH 7 (Fig. 2b). A number of different substrates were incubated with the native pullulanase. As shown in Table 2, pullulanase preferentially hydrolyzed pullulan. Amylopectin (65.8 ± 1.7 %) and starch (71.7 ± 14.7 %) and amylose (5.9 ± 1.7 %) were also hydrolyzed. The apparent kinetic parameters for Michaelis–Menten were determined at 45 °C and pH 7 with different concentrations of pullulan. An affinity for pullulan of $K_m = 0.18$ % and a maximal reaction rate of 6.8 µmol/min was obtained. In order to analyze products after pullulan hydrolysis, monosaccharides and small oligosaccharides were identified by HPLC analysis. The hydrolysis pattern after the pullulanase action on pullulan revealed the conversion of the substrate to give maltotriose (data not shown). In order to confirm that the hydrolysis product from pullulan was maltotriose (possessing two α-1,4 glycosidic linkages) and not panose or isopanose (possessing α-1,4 and α-1,6 glycosidic linkages), the product of pullulan hydrolysis was incubated with α-glucosidase from yeast (data not shown). The formation of glucose as the main product confirmed the exclusive formation of maltotriose from pullulan.

Table 2 Substrate specificity of pullulanase

Substrate (0.5 %)	Relative activity (%)
Pullulan	100 ± 0.5
Soluble starch	71.7 ± 14.7
Amylopectin	65.8 ± 1.7
Amylose	5.9 ± 1.7

Experiments were done at 45 °C and pH 7. Enzymatic activity on pullulan was defined as 100 %. Each experiment was performed in triplicate

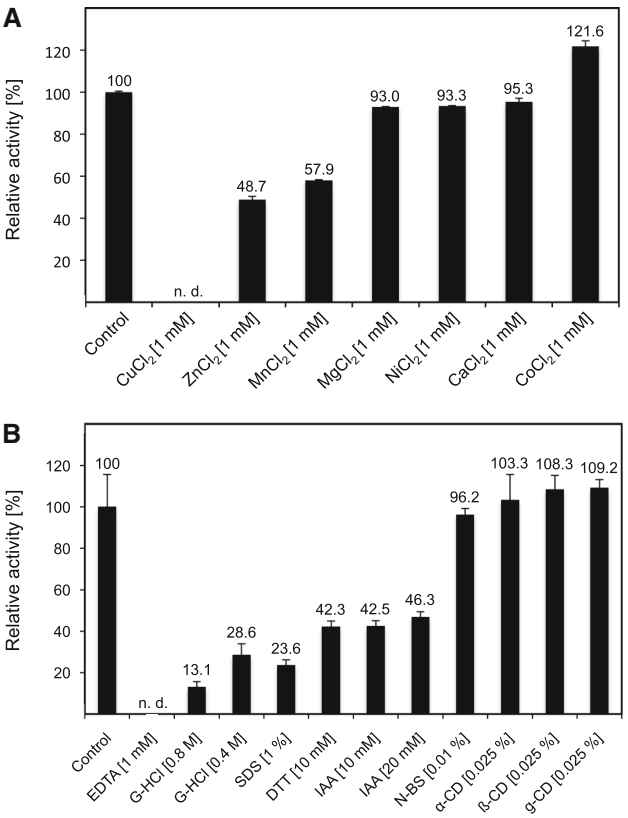

Fig. 3 Influence of metal ions and other compounds on the catalytic activity of pullulanase. **a** Inhibitory effect of simple metal ions was tested after pre-incubation at room temperature for 60 min followed by standard activity assays (incubation at 45 °C, pH 7 for 60 min). **b** The effect of various substances on the enzyme activity was examined after pre-incubation at room temperature for 60 min. *G-HCl* guanidine-HCl, *IAA* iodacetamide, *N-BS* *N*-bromosuccinimide, α,- β-, γ-*CD* cyclodextrins

Effect of metal ions and other reagents on pullulanase activity

The effect of divalent cations on pullulanase was tested after pre-incubation for 60 min at room temperature.

Table 3 Comparison of type-I pullulanases from different sources

Source	(U/mg)	T_{opt} (°C)	pH_{opt}	(kDa)	Reference
Bacillus subtilis	24.1	40	6.0	81.0	(Malle et al. 2006)
Shewanella arctica 40-3	3.0	45	7.0	~150	This study
Bacillus macerans	n. d.[a]	50–55	7.0	n. d.[a]	(Adams and Priest 1977)
Bacillus thermoleovorans	3.7[b]	75	5.6–6.0	~80	(Messoud et al. 2002)
Geobacillus thermoleovorans	12.0	70	6.0	80	(Zouari Ayadi et al. 2008)
Thermotoga maritima	28.7	80	6.5	93	(Kang et al. 2011)
Anaerobranca gottschalkii	56.0	65–70	8.0–8.5	96	(Bertoldo et al. 2004)
Thermoactinomyces thalpophilus	1,352.0	70	7.0	79	(Odibo and Obi 1988)

[a] Not determined

[b] Crude extract was used for activity measurements

Incubation in the presence of $CuCl_2$ resulted in complete inhibition, while $MnCl_2$ (57.9 ± 0.4 %), and $ZnCl_2$ (48.7 ± 1.6 %) partly inhibited pullulytic activity (Fig. 3a). Due to the stimulating effect of $CoCl_2$, the purified enzyme was incubated in the presence of increasing concentrations of divalent Co^{2+} ions. A slight activation of pullulytic activity was measured in the low molar range (0.5–10 mM), while higher concentrations resulted in an opposite effect (Figure S1). Other tested metal ions neither interfered with catalytic activity nor stimulated performance of the enzyme (Fig. 3a). Chelating agent EDTA (1 mM) completely inhibited pullulanase indicating the relevance of metal ions for catalytic activity. Moreover, the activity of the enzyme was not affected by α-, β-, and γ-cyclodextrins, which are generally known as possible competitive inhibitors of archaeal pullulanases. The activity was also not affected by *N*-bromosuccinimide, which oxidizes tryptophan residues, while SDS, DTT, guanidine-HCl and iodocetamide had inhibitory effects on pullulanase (Fig. 3b).

Discussion

Several locations on nordic island Spitsbergen were identified to be inhabited by diverse prokaryotic species containing versatile hydrolases including proteases, xylanases, esterases and starch-degrading enzymes (Groudieva et al. 2003; Groudieva et al. 2004; Kim et al. 2008; Srinivas et al. 2009; Al Khudary et al. 2010; Elleuche et al. 2011; Konneke et al. 2013). These microorganisms and their biocatalysts are of special interest for academia and industry due to their high catalytic efficiency and capability to work at low temperatures (Leiros et al. 2000).

In this study, a novel pullulanase was purified from crude extracts of psychrophilic isolate *S. arctica* 40-3. The putative monomeric quaternary structure resembles that of other debranching enzymes described so far (Michaelis et al. 1985; Suzuki et al. 1991; Albertson et al. 1997; Bibel et al. 1998; Bertoldo et al. 1999; Messoud et al. 2002; Bertoldo et al. 2004). N-terminal sequencing indicated that homologues of this enzyme are well conserved among bacterial species, but have not yet been characterized in detail. Conserved domain search in a putative pullulanase from related *Shewanella* sp. strain HN-41 (Accession no. WP_007649744) indicated the presence of protein regions typical for type-I pullulanase (data not shown). Homologous pullulanases have neither been identified in screening approaches nor have they been cloned yet from completed genome sequences, which might probably be due to the large size of their deduced open reading frames.

A complex purification approach, composed of five steps, was necessary to isolate the pullulanase from wild-type cells. Catalytic activity of 3 U/mg is rather low compared to type-I pullulanases from other (mainly thermophilic) microorganisms (Table 3). However, partial purification of native pullulanase from *A. gottschalkii* resulted in a catalytic activity of 0.33 U/mg, while a recombinant enzyme displayed 56.0 U/mg indicating the potential of heterologous production of type-I pullulanases (Bertoldo et al. 2004). The optimal pH of the native pullulanase (pH 7) is slightly higher than most pullulanases, where pH optima usually range between 5.5 and 6.0, such as *Bacillus thermoleovorans*, *Thermus caldophilus* and *T. maritima* (Kim et al. 1996; Bibel et al. 1998; Messoud et al. 2002). Most pullulanases show weak activity in alkaline pH range (pH 8–10), which is optimal pH range of pullulanase from alkaliphilic *A. gottschalkii* (Bertoldo et al. 2004). Optimal temperature of 45 °C is in agreement with a pullulanase from mesophilic *Bacillus subtilis*, but different from most reported type-I pullulanases, which have been mainly isolated from thermophilic species (Rüdiger et al. 1995; Bertoldo et al. 1999; Duffner et al. 2000; Malle et al. 2006; Zouari Ayadi et al. 2008).

S. arctica 40-3 pullulanase was inhibited by metal ions Mn^{2+} and Zn^{2+}, while Ni^{2+}, Mg^{2+} and Ca^{2+} had no inhibitory effect. The latter divalent cation was shown to be essential for catalytic activity of different pullulanases and amylases (Elleuche and Antranikian 2013). Pullulanase from *G. thermoleovorans* was influenced by Ca^{2+} in a

temperature-dependent manner, while *B. acidopullulyticus* was completely unaffected by this metal ion (Stefanova et al. 1999; Zouari Ayadi et al. 2008). Moreover, complete inhibition of *S. arctica* 40-3 pullulanase was observed with EDTA, which is capable of binding metal ions that are relevant for catalytic activity. However, activity of heat-stable type-I pullulanase from *T. maritima* was even enhanced in the presence of EDTA (Kang et al. 2011). Other tested compounds, such as α-, β-, and γ-cyclodextrins and *N*-bromosuccinimide did not affect the activity of *S. arctica* 40-3 pullulanase, which is in contrast to other pullulanases described so far (Rüdiger et al. 1995; Duffner et al. 2000; Bertoldo et al. 2004). The hydrolysis pattern of the characterized pullulanase on pullulan revealed the complete conversion of the substrate to give maltotriose. Similar results were presented with other type-I pullulanases (Rüdiger et al. 1995; Duffner et al. 2000; Messoud et al. 2002; Bertoldo et al. 2004).

Together with homologies to completely sequenced related enzymes, these results indicate that this pullulanase is most probably the first characterized type-I pullulanase from a psychrophilic bacterium. However, the production efficiency and the purification approach need to be improved to further elucidate its biochemical properties, structure–function relationships and the potential of application in industrial processes. Using different strategies including a genome library in combination with activity-based screening approaches and/or degenerated primers deduced from related putative pullulanases will be necessary for the production of recombinant enzyme at high concentrations.

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