AEM
Journals.ASM.org

Novel Maltotriose-Hydrolyzing Thermoacidophilic Type III Pullulan Hydrolase from *Thermococcus kodakarensis*

Nasir Ahmad,[a,b] Naeem Rashid,[a] Muhammad Saleem Haider,[b] Mehwish Akram,[a] Muhammad Akhtar[a,c]

School of Biological Sciences, University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan[a]; Institute of Agricultural Sciences, University of the Punjab, Quaid-e-Azam Campus, Lahore, Pakistan[b]; School of Biological Sciences, University of Southampton, Southampton, United Kingdom[c]

A novel thermoacidophilic pullulan-hydrolyzing enzyme (PUL) from hyperthermophilic archaeon *Thermococcus kodakarensis* (TK-PUL) that efficiently hydrolyzes starch under industrial conditions in the absence of any additional metal ions was cloned and characterized. TK-PUL possessed both pullulanase and α-amylase activities. The highest activities were observed at 95 to 100°C. Although the enzyme was active over a broad pH range (3.0 to 8.5), the pH optima for both activities were 3.5 in acetate buffer and 4.2 in citrate buffer. TK-PUL was stable for several hours at 90°C. Its half-life at 100°C was 45 min when incubated either at pH 6.5 or 8.5. The K_m value toward pullulan was 2 mg ml^{-1}, with a V_{max} of 109 U mg^{-1}. Metal ions were not required for the activity and stability of recombinant TK-PUL. The enzyme was able to hydrolyze both α-1,6 and α-1,4 glycosidic linkages in pullulan. The most preferred substrate, after pullulan, was γ-cyclodextrin, which is a novel feature for this type of enzyme. Additionally, the enzyme hydrolyzed a variety of polysaccharides, including starch, glycogen, dextrin, amylose, amylopectin, and cyclodextrins (α, β, and γ), mainly into maltose. A unique feature of TK-PUL was the ability to hydrolyze maltotriose into maltose and glucose.

Pullulan is a linear α-glucan that consists of repeating units of maltotriose linked by α-1,6 glycosidic linkages (1). Pullulanases (EC.3.2.1.41) are pullulan-hydrolyzing enzymes that are commonly known as debranching enzymes because of their hydrolytic action toward α-1,6 glycosidic linkages in starch and other branched polysaccharides (2, 3). This property makes pullulanase an enzyme of great interest for the hydrolysis of starch, because other starch-hydrolyzing enzymes, including α-amylase and glucoamylase, are unable to efficiently hydrolyze branch points containing α-1,6 glycosidic linkages (4). Complete hydrolysis of starch can only be achieved in the presence of a debranching enzyme (2). The process of starch hydrolysis requires highly thermostable amylolytic enzymes that are active at acidic pH (4, 5). In the last 3 decades, enormous efforts have been made to explore the amylolytic potential of thermophilic and hyperthermophilic microorganisms (4, 5), and a number of thermostable amylolytic enzymes have been cloned and characterized (3–5). However, more-efficient enzymes are still needed for starch processing.

In sequence-based classification, pullulanases, along with most of the starch-hydrolyzing enzymes, are grouped into glycosyl hydrolase family 13 (GH13) (6, 7), whereas on the basis of substrate specificity and reaction end products, pullulan-hydrolyzing enzymes are categorized either as pullulanases (types I and II) or pullulan hydrolases (types I, II, and III) (2). Pullulanases (types I and II) hydrolyze only α-1,6 linkages in pullulan, and they are unable to hydrolyze α-1,4 linkages in this substrate. Pullulanase type I specifically hydrolyzes α-1,6 glycosidic linkages in pullulan and yields maltotriose as an end product (8). However, pullulanase type II (amylopullulanase) has an additional ability to hydrolyze α-1,4 glycosidic linkages in starch and other polysaccharides (9). Pullulan hydrolases (type I and type II) can only hydrolyze α-1,4 linkages in pullulan and are unable to hydrolyze α-1,6 linkages of this glucan. The final products of hydrolysis may be panose (in the case of pullulan hydrolase type I) or isopanose (in the case of pullulan hydrolase type II) (2, 10). Pullulan hydrolase type III is a unique enzyme that is capable of hydrolyzing both α-1,4 and α-1,6 glycosidic linkages in pullulan, and its final reaction products include a mixture of maltose, panose, and maltotriose (2). Several pullulanases (types I and II) and pullulan hydrolases (types I and II) have previously been reported from all three domains of life (8, 11–13). However, the pullulanase from *Thermococcus aggregans* is the only pullulan hydrolase type III reported until now (10).

Thermococcus kodakarensis KOD1 is an anaerobic hyperthermophile that grows optimally at 85°C and pH 6.5 as an obligate heterotroph (14, 15). The complete genome of *T. kodakarensis* (GenBank accession no. AP006878) has been published, and it contains several genes coding for putative amylolytic enzymes (16). A few of them, including α-amylase, 4-α-glucanotransferase, cyclodextrin glucanotransferase, and amylopullulanase, have been cloned and characterized (17–20). In this study, we report a novel pullulan hydrolase of *T. kodakarensis* (TK-PUL) that was previously annotated (locus tag TK0977, GenBank accession no. BAD85166.1) and reported as a pullulanase type II (16, 21). We prove here with convincing experimental results that TK-PUL is a pullulan hydrolase type III, rather than a type II. Furthermore, to our knowledge, this is the only pullulan hydrolase that is capable of hydrolyzing saccharides as small as maltotriose.

MATERIALS AND METHODS

Reagents and chemicals. The reagents and chemicals used in this study were of high purity and were purchased either from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Leicestershire, United Kingdom). The

Received 18 September 2013 Accepted 18 November 2013
Published ahead of print 2 December 2013
Address correspondence to Naeem Rashid, naeemrashid37@hotmail.com.

doi:10.1128/AEM.03139-13

restriction endonucleases, InsTAclone PCR cloning kit, DNA extraction kit, T_4 DNA ligase, DNA and protein size markers, *Taq* DNA polymerase, RNase, and deoxynucleoside triphosphates (dNTPs) were from Thermo Scientific (Thermo Scientific Life Science Research, MD). Maltooligosaccharides (maltoheptaose to maltotriose), cyclodextrins (α and γ), and polysaccharides, including pullulan from *Aureobasidium pullulans*, starch from potato and corn, glycogen from oyster, and amylopectin derived from potato were purchased from Sigma-Aldrich, while β-cyclodextrin was from Acros Organics (Geel, Belgium).

Strains, plasmids, and media. *Escherichia coli* DH5α cells and plasmid pTZ57R/T (Thermo Scientific) were routinely used for cloning and general DNA manipulations. *E. coli* BL21-CodonPlus(DE3)-RIL (Stratagene, La Jolla, CA), and pET21a(+) (Novagen, Madison, WI) were used for gene expression. *E. coli* strains were grown normally in Luria-Bertani (LB) medium at 37°C. The growth medium contained ampicillin (100 μg ml^{-1}) when required for plasmid maintenance. Recombinant *E. coli* cells containing pET21a(+) were selected on LB agar containing ampicillin (100 μg ml^{-1}), whereas X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg ml^{-1}) and 1 mM IPTG (isopropyl-β-D-galactopyranoside) were added when blue/white screening of recombinant *E. coli* cells containing pTZ57R was required.

Cloning of TK-PUL gene. A 2,298-bp open reading frame (TK0977, accession no. BAD85166.1) annotated as a pullulanase type II of the GH13 family was identified in the genome of *T. kodakarensis* KOD1. A set of primers, Pul-N (5′-CATATGAGCGGATGTATCTCGGAGAGCAACG-3′, corresponding to the 5′ end of the gene) and Pul-C (5′-GAAGCGGG GGTCAACCCCGCTCAAG-3′, corresponding to the 3′ end of the gene), was synthesized commercially (Gene Link, Hawthorne, NY). An NdeI restriction site was introduced in the forward primer (underlined sequence). The TK0977 gene was amplified by PCR using this pair of primers as priming strands and genomic DNA of *T. kodakarensis* KOD1 as the template. The PCR-amplified product was cloned in pTZ57R/T, and the resulting plasmid was named pTZ-PUL. *E. coli* DH5α cells were transformed using pTZ-PUL. The recombinant plasmid pTZ-PUL was digested with NdeI and BamHI to liberate the pullulanase gene, which was purified and subsequently cloned in expression vector pET-21a (Novagen, Madison, WI) by utilizing NdeI and BamHI restriction sites, and the resulting plasmid was named pET-PUL.

Sequence analysis. The sequence of the pullulanase gene in pET-PUL was confirmed by DNA sequencing on an automated DNA sequencer (Beckman Coulter CEQ8000; Beckman Coulter, Inc., Fullerton, CA). Multiple sequence alignment was performed with ClustalW using BioEdit Sequence Alignment Editor (22).

Production in *E. coli* and purification of TK-PUL. All procedures of enzyme purification were performed at room temperature unless stated otherwise. *E. coli* BL21 CodonPlus (DE3)-RIL cells containing pET-PUL were grown in LB medium at 37°C until the optical density at 600 nm (OD_{600}) reached 0.4. Gene expression was induced with 0.1 mM IPTG. After 4.5 h of induction, cells were harvested by centrifugation at 12,000 × *g* for 10 min at 4°C. The cell pellet was resuspended in 50 mM Tris-Cl, pH 8.0, and disrupted by sonication using the Bandelin SonoPlus HD 2070 sonication system (Bandelin Electronic, GmbH, Berlin, Germany). Cell debris was removed by centrifugation at 20,000 × *g* for 10 min at 4°C. The supernatant thus obtained was heated at 80°C for 30 min to denature heat-labile proteins from host cells, which were removed by centrifugation. The recombinant TK-PUL obtained in the supernatant was precipitated by fractional ammonium sulfate precipitation. The precipitates obtained after 40% and 60% ammonium sulfate saturation were pooled, dialyzed, and applied on a Resource Q column using the AKTA purifier (GE Healthcare, Piscataway, NJ). Elution of the proteins bound to the column was done with a 0-to-1 M sodium chloride linear gradient solution prepared in 50 mM Tris-Cl, pH 8.0.

Protein estimation. The protein concentration was estimated by Coomassie dye-binding assay using the Quick Start Bradford protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). Bovine serum albumin was used as the standard for protein quantification.

Determination of molecular mass. The molecular mass of recombinant TK-PUL was determined by size exclusion chromatography using a Superdex 200 10/300 GL column on the ÄKTA Explorer fast protein liquid chromatography (FPLC) system according to the instructions of the manufacturer (GE Healthcare, Piscataway, NJ). The molecular mass of recombinant TK-PUL was also determined by matrix-assisted laser desorption ionization-time of flight mass spectroscopy (Autoflex III smartbeam system; Germany). The salt-free TK-PUL (6 μg/μl) was mixed with 2,5-dihydroxybenzoic acid (50 mg/ml in methanol) in a 1-to-19 ratio, and a 1-μl sample (containing about 3.5 pmol protein) was loaded on the sample plate. The sample was allowed to dry at room temperature for 10 to 15 min. The spectrum was obtained by striking 10,000 shots at 85% laser intensity in a detection range of 20,000 to 160,000 Da.

Enzyme activity assay. The pullulanase activity of recombinant TK-PUL was measured in terms of the amount of reducing sugars liberated upon incubation with pullulan. Maltose was used as the standard for reducing sugars. In a standard assay mixture, 125 μl of 0.5% (wt/vol) pullulan in 50 mM sodium citrate buffer (pH 4.2) was mixed with 125 μl of properly diluted (0.8 to 1.2 U/ml) TK-PUL and incubated at 90°C for 10 min. The reaction was stopped by quenching in ice water, and the reducing ends released were determined by the dinitrosalicylic acid (DNS) method (23). The reducing groups released by the nonenzymatic factors were subtracted from the experimental values. One unit of pullulanase activity was defined as the amount of enzyme that released 1 μmol of reducing sugars in 1 min under standard assay conditions. The α-amylase activity of the recombinant TK-PUL was measured by the same procedure but with pullulan replaced by 1% (wt/vol) starch as the substrate.

Effects of pH and temperature on enzyme activity. The effect of pH on the activity of recombinant TK-PUL was examined at 90°C using various buffers at 50 mM strength. The buffers used were sodium citrate (pH 2.5 to 4.5), sodium acetate (pH 3.2 to 6.5), and sodium phosphate (pH 6.5 to 8.5). The pH values were adjusted at room temperature.

To examine the effect of temperature on the enzymatic activity, assay mixtures were prepared either in 50 mM sodium citrate buffer, pH 4.2, or in 50 mM sodium acetate, pH 6.5, and incubated for 10 min at temperatures from 40 to 120°C. An oil bath was used for temperatures above 90°C, and incubations were performed in tightly screw-capped Hungate tubes to prevent boiling of the samples.

pH stability of recombinant TK-PUL. The pH stability of the recombinant TK-PUL was studied at 4°C in buffers of various pH values (50 mM sodium citrate, pH 4.2, adjusted with citric acid; 50 mM sodium acetate, pH 6.5, adjusted with acetic acid; and 50 mM Tris-Cl, pH 8.5, adjusted with HCl). The purified recombinant enzyme was diluted (40 μg/ml final concentration) in the respective buffer and incubated at 4°C for up to 56 h. Aliquots were withdrawn at regular intervals, and the pH stability was studied by measuring residual pullulanase activity.

Thermostability of recombinant TK-PUL. For thermostability analysis, the purified enzyme was diluted in 50 mM buffers of various pH values (sodium citrate, pH 4.2, sodium acetate, pH 6.5, and Tris-Cl, pH 8.5) to a final concentration of 40 μg/ml and incubated at 90°C and 100°C. All incubations were performed in tightly screw-capped Hungate tubes to prevent boiling of the samples. At various time intervals, samples (approximately 2 μg protein) were taken and examined for residual pullulanase activity at 90°C.

Effects of metal ions and other reagents on recombinant TK-PUL. The effects of various substances, including metal ions, inhibitors, detergents, and modifying agents, on the enzyme activity were studied by incubating purified recombinant TK-PUL (1.7 U/ml, final concentration) with various concentrations of these reagents at 60°C for 15 min. Samples were then withdrawn, and pullulanase activity was examined at 90°C.

Substrate specificity and characterization of the hydrolysis products. The substrate preference and relative hydrolysis rates of various polysaccharides, including pullulan, starch, glycogen, amylose, amylopec-

TABLE 1 Percent identity of TK-PUL with other amylolytic enzymes

Amylolytic enzyme and source	% identity with TK-PUL[a]									
	1	2	3	4	5	6	7	8	9	10
TK-PUL, Q5JID9[b] (*T. kodakarensis*)	100	62	38.3	21.7	21.3	21.3	21.3	20.2	20.2	19.5
Pullulan hydrolase type III, Q9P9A0 (*T. aggregans*)		100	41.3	21.8	20.7	22	21	21.2	20.2	19.8
Pullulanases, Q9HHB0 (*D. mucosus*)			100	24.5	23.7	25	24.1	22.2	22.8	21.6
Maltogenic α-amylase, P32818 (*Bacillus acidopullulyticus*)				100	43.8	41.3	57.4	55.7	58.7	55.7
Cyclomaltodextrin hydrolase, P29964 (*T. ethanolicus*)					100	47.7	47.7	44.8	46.7	45.7
Neopullulanase, Q08751 (*T. vulgaris*)						100	45.9	42.1	45.1	43.4
Neopullulanase, P38940 (*B. stearothermophilus*)							100	57.7	69.6	59.5
Neopullulanase, Q57482 (*Bacillus* sp.)								100	60.5	58.6
Maltogenic amylase, Q45490 (*G. stearothermophilus*)									100	64
Neopullulanase, Q819G8 (*B. cereus*)										100

[a] BioEdit Sequence Alignment Editor was used to determine the sequence identity matrix after multiple alignment of sequences with ClustalW.
[b] UniProt accession number.

tin, dextrin, and cyclodextrins, were determined by incubating each of them at a final concentration of 0.25% (wt/vol) with recombinant TK-PUL. Substrate solutions were prepared in 50 mM sodium citrate buffer (pH 4.2), and after the addition of purified enzyme (0.15 U), incubated at 90°C for 2 to 30 min. The hydrolysis rates (μmol reducing sugar min^{-1} ml^{-1}) of these substrates were measured every 2 min by the DNS method (23). To characterize the oligosaccharides obtained as hydrolysis products, incubations were done under similar conditions for various time intervals. The products were then analyzed by high-performance liquid chromatography (HPLC) on an Aminex HPX-42A column (300 by 78 mm; Bio-Rad Laboratories, Inc., Hercules, CA). Signals were detected with a differential refractive index detector (S3580; Sykam GmbH, Germany). Double-distilled deionized water was used as the mobile phase/solvent. During separation, the column temperature was maintained at 85°C and the detector temperature was maintained at 45°C.

For identification of di- (maltose or isomaltose) and tri- (maltotriose or panose) saccharides in the pullulan hydrolysates, the reaction products obtained after 16 h of incubation with TK-PUL were further incubated with α-glucosidase from *Saccharomyces cerevisiae* at 37°C for 2 h in 50 mM sodium phosphate buffer, pH 6.0. A control experiment containing maltotriose (at equal concentration and under similar conditions), instead of pullulan hydrolysates, was incubated with α-glucosidase.

RESULTS

Gene cloning and sequence analysis of TK-PUL. The genome sequence of *T. kodakarensis* was searched, and an open reading frame (TK0977, TK-PUL) coding for a putative pullulanase type II of the GH13 family was identified. The gene consisted of 2,298 nucleotides, encoding a polypeptide of 765 amino acid residues. A signal peptide of 17 amino acids was predicted using SignalP 3.0 software (24). TK0977 (accession no. YP_183390.1) was located at positions 851740 to 854037 on the *T. kodakarensis* chromosome and was flanked by TK0976 (accession no.YP_183389.1), encoding a small nuclear ribonucleoprotein, and TK0978 (accession no. YP_183391.1), encoding a glycyl-tRNA synthetase.

Among the characterized enzymes, TK-PUL displayed the highest homology (62%) with pullulan hydrolase from *T. aggregans*. Less than 38% homology was found with the sequences of cyclomaltodextrin hydrolases, pullulanases, neopullulanases, and maltogenic amylase (Table 1). Among the uncharacterized enzymes from the family *Thermococcaceae*, TK-PUL showed 73%, 67%, and 66% identities with pullulanase type II from *Thermococcus* sp. strain CL1, pullulan hydrolase type III from *T. gammatolerans*, and maltodextrin glucosidase/pullulanases from *Thermococcus* sp. strain AM4, respectively. TK-PUL did not show a significant homology with pullulanases from *Thermococcus hydrothermalis* (25), *Pyrococcus furiosus* (26), and *Pyrococcus abyssi* (UniProt accession no. Q9V294). Four highly conserved amino acid sequence regions typical of almost all amylolytic enzymes (27) were also identified in TK-PUL (Fig. 1). These regions were not found in the pullulanase sequences of *T. hydrothermalis*, *P. furiosus*, and *P. abyssi* (5). Three acidic residues crucial for catalytic activity were also conserved, at positions 503 (Asp^{503}), 601 (Asp^{601}), and 534 (Glu^{534}).

Gene expression in *E. coli* and purification of recombinant TK-PUL. The expression of the gene in *E. coli* resulted in the production of recombinant TK-PUL in soluble form which remained in solution even after heat treatment at 80°C for 30 min, while most of the host proteins were denatured and precipitated. The precipitated proteins were separated by centrifugation. TK-PUL in the supernatant, after centrifugation, was further purified by fractional precipitation with ammonium sulfate. Several contaminating proteins were precipitated at 20 to 30% ammonium sulfate saturation. TK-PUL was precipitated at 40 and 60% ammonium sulfate saturation. Further purification by anion-exchange chromatography resulted in an apparent homogeneity of TK-PUL on SDS-PAGE (see Fig. S1 at http://pu.edu.pk/images/publication/AEM-%2003139-13.pdf) and an 11.19-fold higher specific activity (70.5 U/mg) than in the crude extract (6.3 U/mg). The overall yield was 89% (see Table S1 at http://pu.edu.pk/images/publication/AEM-%2003139-13.pdf).

Molecular mass determination. The molecular mass of the recombinant TK-PUL appeared to be nearly 80 kDa on SDS-PAGE, whereas the theoretically calculated mass of TK-PUL without the signal peptide, determined by the ProtParam tool (http://www.expasy.ch/tools/protparam.html), was 84,399.9 Da. In order to know the exact molecular mass, the recombinant TK-PUL was analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry, and the results revealed that the mass of purified recombinant TK-PUL was 84,402.053 Da (data not shown), which was in good agreement with the theoretically calculated mass of the mature protein. The N-terminal sequence of the first five amino acid residues of the purified recombinant TK-PUL was determined commercially. The sequence matched with the N-terminal amino acid sequence of TK-PUL except for the starting methionine, which probably was truncated by the methionine aminopeptidase of the host *E. coli*. When the recombinant TK-PUL was analyzed by size exclusion chromatography for

Enzyme source	Region I	Region II	Region III	Region IV
	Pos. 412-423	Pos. 498-507	Pos. 526-538	Pos. 598-607
		#	#	#
Q5JID9	GMRVIFDFVPNH	DGIRVDVPNE	PDAYLVGEIWTL	DSHDTSRVLT
Q9P9A0	GIRIIFDFVPNH	DGIRIDAPQE	PDAYIVGEIWEL	SSHDTSRVLT
Q9HHB0	GIKVIFDFVPDH	DGLRIDTPLD	PDAYIVGEIWDY	GSHDTSRVLT
P32818	GIKVMLDAVFNH	DGWRLDVANE	PDLYILGEIWHD	GSHDTPRILT
P29964	GIKVIFDAVFNH	DGWRLDVANE	PEAIIVGEVWHD	GSHDTERFLT
Q08751	GIKIILDAVFNH	DGWRLDVANE	PDALIVGEIWHD	DSHDTERFLT
P38940	GIRVMLDAVFNH	DGWRLDVANE	PDVYILGEIWHD	GSHDTSRILT
Q57482	GIRVMLDAVFNH	DGWRLDVANE	PDVYILGEVWHD	GSHDTPRILT
Q45490	AIRVMLDAVFNH	DGWRLDVANE	PDAYILGEIWHD	GSHDTPRLLT
Q819G8	GIKVMLDAVFNH	DGWRLDVANE	PEVYILGEIWHD	DSHDTPRILT

FIG 1 Regions conserved among pullulanases and other amylolytic enzymes. Three amino acid residues essential for catalytic activity are marked by a hash symbol (#), identical residues are shown in white with a black background, and similar residues are shown in black with a gray background. The sequences, identified by their UniProt accession numbers, are as follows: Q5JID9, TK-PUL; Q9P9A0, pullulan hydrolase type III from *T. aggregans*; Q9HHB0, pullulanases from *D. mucosus*; P32818, maltogenic α-amylase from *B. cidopullulyticus*; P29964, cyclomaltodextrin hydrolase from *Thermoanaerobacter ethanolicus*; Q08751, neopullulanase from *Thermoactinomyces vulgaris*; P38940, neopullulanase from *B. stearothermophilus*; Q57482, neopullulanase from *Bacillus* species; Q45490, maltogenic amylase from *G. stearothermophilus*; and Q819G8, neopullulanase from *Bacillus cereus*.

molecular mass determination, it eluted at a retention volume of 13.7 ml, corresponding to a molecular mass of 84 kDa, indicating that TK-PUL existed in a monomeric form.

Biochemical characteristics of TK-PUL. Examination of the enzyme activity of TK-PUL at various pH values revealed that it was active over a broad pH range (3.0 to 8.5). The highest pullulanase and α-amylase activities were observed at pH 3.5 in acetate buffer and 4.2 in citrate buffer (Fig. 2). Despite showing the highest activity in acidic pH, TK-PUL was more stable at alkaline pH, and it displayed 84%, 77%, and 57% of the maximal activity after 56 h of incubation at 4°C and pH values of 8.5, 6.5, and 4.2, respectively (data not shown).

When the enzyme activity was examined at various temperatures with the pH kept constant either at 4.2 (in citrate buffer) or 6.5 (in acetate buffer), TK-PUL exhibited the highest activities at 95°C at pH 4.2 and 100°C at pH 6.5. More than 50% activity was observed even at 120°C (Fig. 3). Prolonged incubation at elevated temperatures demonstrated that TK-PUL was extremely thermo-

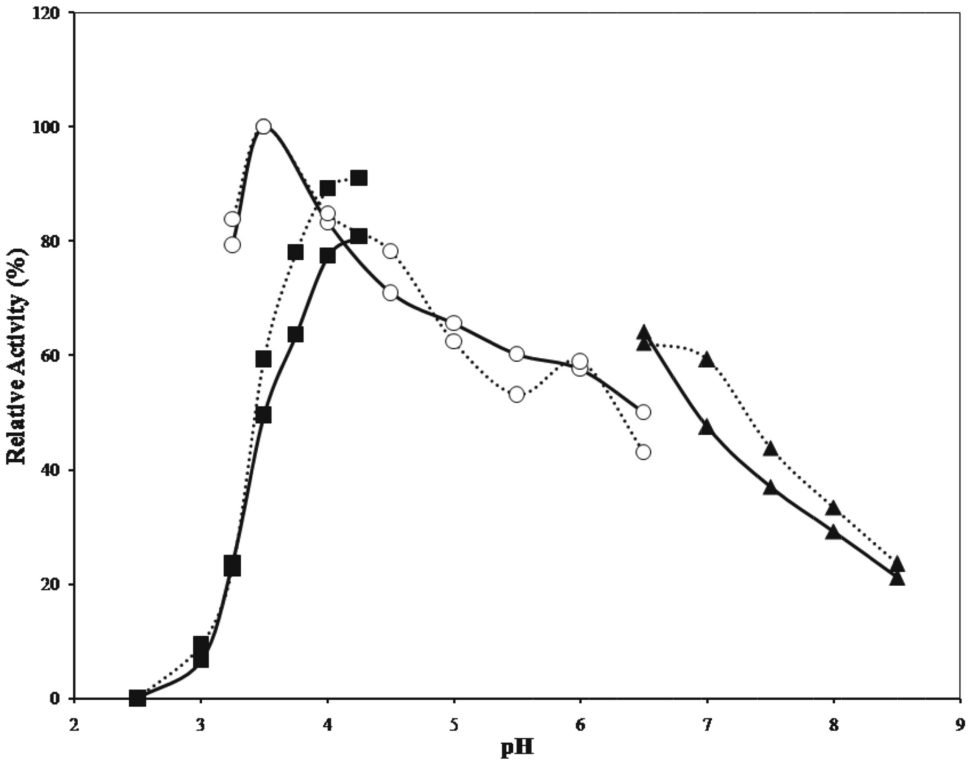

FIG 2 Comparison of pullulanase and α-amylase activities of recombinant TK-PUL at various pH values in sodium citrate (■), sodium acetate (○), and sodium phosphate (▲) buffers. Each buffer was used at a final concentration of 50 mM. Pullulanase activity is shown by solid lines, while dotted lines represent amylase activity.

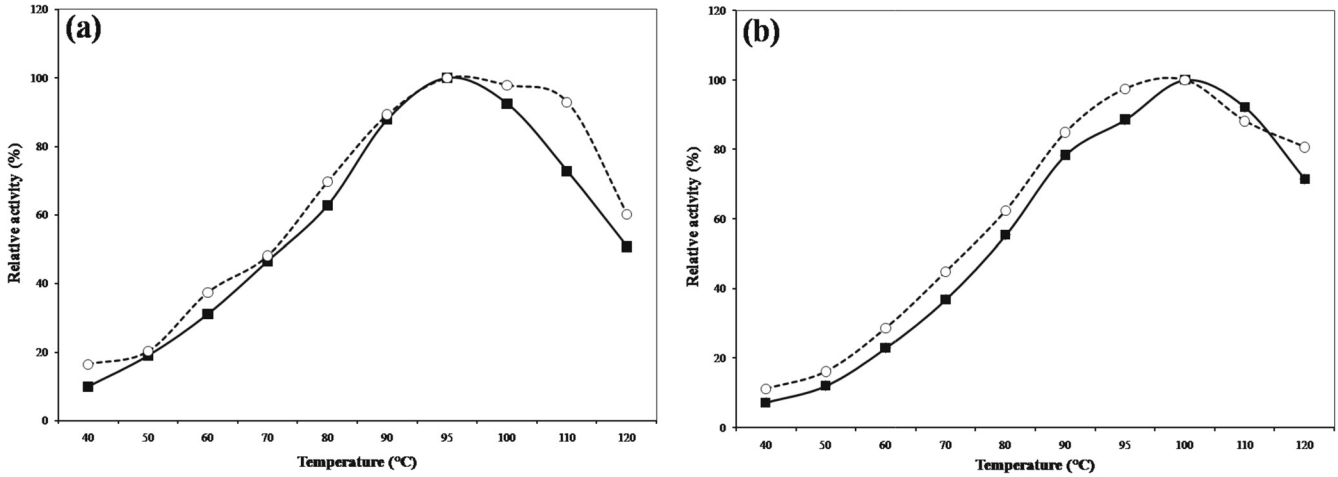

FIG 3 Comparison of pullulanase and α-amylase activities of recombinant TK-PUL at various temperatures. (a) Activity in sodium citrate buffer, pH 4.2. (b) Activity in sodium acetate buffer, pH 6.5. Pullulanase activity is shown by solid lines, while dotted lines represent amylase activity.

stable, displaying more than 90% activity even after 10 h of incubation at 90°C in sodium acetate buffer of pH 6.5. The half-life of TK-PUL was 45 min at 100°C when incubated either at pH 6.5 or 8.5. Under optimal conditions, TK-PUL displayed a specific activity of 70.5 μmol min^{-1} mg^{-1}. The K_m value toward pullulan was calculated to be 2 mg ml^{-1}, and the V_{max} was 109 U mg^{-1}.

Effects of metal ions and other reagents on TK-PUL activity. There was no significant difference in the enzyme activity of TK-PUL in the presence or absence of Ca^{+2}, which indicated that, unlike other amylolytic enzymes, recombinant TK-PUL does not depend on calcium for its activity. The activity of TK-PUL was also not affected by the presence of Mg^{+2}, Mn^{+2}, Co^{+2}, or Zn^{+2} whereas Ni^{+2}, Cu^{+2}, and Fe^{+2} had inhibitory effects at 5 mM concentration but no effect at 0.05 mM concentration (see Table S2 at http://pu.edu.pk/images/publication/AEM-%2003139-13.pdf). The activity of TK-PUL was slightly enhanced by the addition of nonionic detergents like Triton X-100 and Tween 20, whereas the ionic detergent SDS strongly inhibited the activity. Iodoacetamide had no significant effect when added to the reaction mixture at a final concentration of 20 mM. Ammonium sulfate and guanidine HCl inhibited the pullulanase activity of the recombinant TK-PUL when present at a concentration of 0.5 M or above (see Table S2 at http://pu.edu.pk/images/publication/AEM-%2003139-13.pdf).

The enzyme activity was strongly inhibited in the presence of *N*-bromosuccinimide even at a final concentration of 0.01%. β-Cyclodextrin and *p*-chloromercuribenzoic acid slightly inhibited TK-PUL activity when added to the standard assay mixture at final concentrations of 0.1% and 0.01%, respectively (see Table S3 http://pu.edu.pk/images/publication/AEM-%2003139-13.pdf). The strong inhibition with *N*-bromosuccinimide indicates the possibility that tryptophan residues are critically involved at the active site, whereas the insignificant inhibition by *p*-chloromercuribenzoic acid suggested the possibility of noninvolvement of sulfhydryl groups for TK-PUL activity. The above-described effects were identical for both the pullulanase and α-amylase activities of TK-PUL.

Substrate preference and analysis of hydrolysis end products. In order to determine the substrate preference, various α-glucans, each at a final concentration of 0.25% in 50 mM sodium citrate buffer, pH 4.2, were used as substrates. Pullulan was the most preferred substrate of TK-PUL. Surprisingly, γ-cyclodextrin was hydrolyzed at the second highest velocity, after pullulan, with a relative activity of 76%. This property of TK-PUL is unique, because cyclodextrins are well-known competitive inhibitors of pullulanases (11). Other carbohydrates were hydrolyzed in the following order of preference: pullulan (100%) > γ-cyclodextrin (76%) > potato starch (60%) > amylose (46%) > dextrin (43%) > corn starch (41%) > amylopectin (37%) > glycogen (26%) > β-cyclodextrin (5%). Recombinant TK-PUL was able to hydrolyze glycogen with a 26% relative activity, in contrast to the pullulanases from *T. aggregans* and *Desulfurococcus mucosus* (10, 11), which were unable to hydrolyze glycogen. Dextran, although it contains mainly α-1,6 linkages, was not hydrolyzed by TK-PUL.

Investigation of the hydrolysis end products was first performed by thin-layer chromatography (see Fig. S2 at http://pu.edu.pk/images/publication/AEM-%2003139-13.pdf) and then validated by HPLC analysis. The pullulan hydrolysis catalyzed by TK-PUL was so efficient that, after even 10 min of incubation at 90°C, at a final concentration of 0.25% in 50 mM sodium citrate buffer, pH 4.2, and 2.6 U, or approximately 40 μg, of purified TK-PUL, pullulan was completely converted to maltotriose (98.3%) and maltotetraose (1.7%). Subsequent hydrolysis of maltotriose (DP3 [DP, degree of polymerization; DP3 indicates a trisaccharide]) resulted in the formation of maltose and glucose. Peaks corresponding to maltose (DP2) and glucose (DP1) were detected after 1 h of incubation under similar conditions, and after 16 h of incubation, most of the maltotriose (60%) was hydrolyzed to maltose (40%) and glucose (20%) (Fig. 4). These results indicate that, in addition to hydrolyzing α-1,6 bonds in pullulan, TK-PUL also possesses the ability to hydrolyze α-1,4 linkages in this substrate. To our knowledge, none of the previously reported enzymes could hydrolyze pullulan so efficiently or was able to hydrolyze a trisaccharide, such as maltotriose. The smallest oligosaccharide hydrolyzed by previously reported pullulanases is maltotetraose (10, 11). In order to find the nature of the di- and trisaccharides, the pullulan hydrolysates obtained after 16 h of incubation at 90°C with TK-PUL were further incubated with α-glucosidase from *S. cerevisiae*. In a control experiment, maltotriose was incubated instead of pullulan hydrolysates, at equal concentration and under

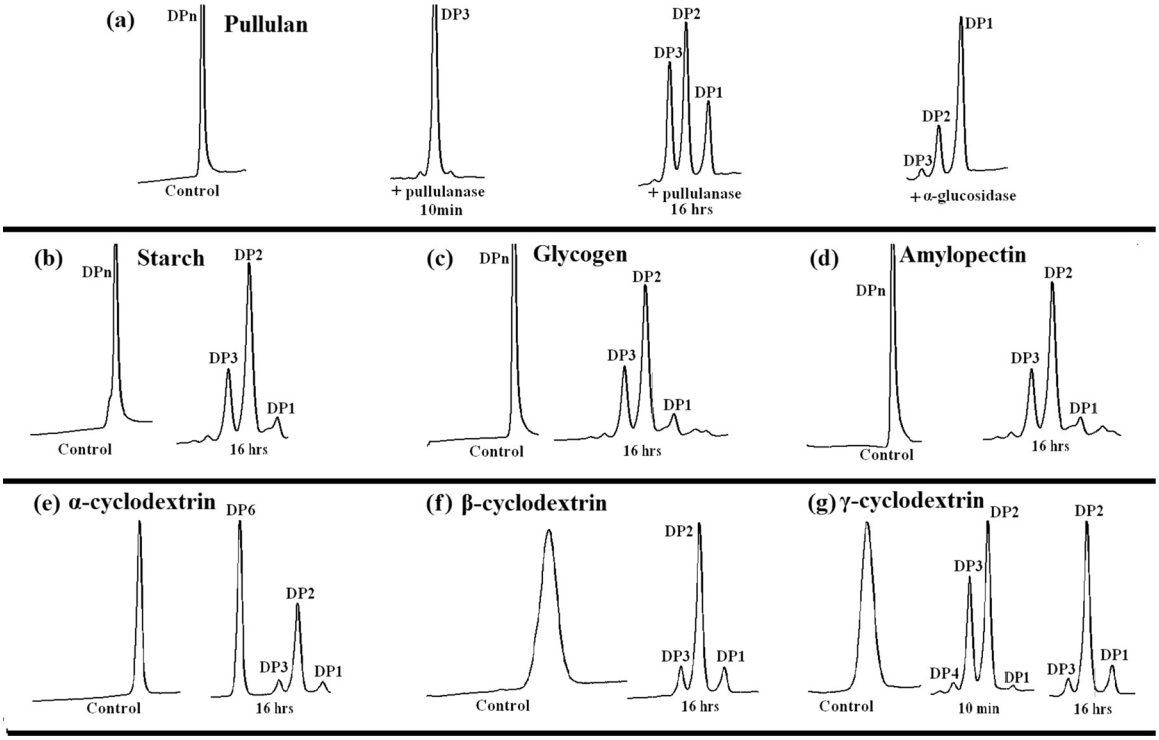

FIG 4 HPLC peaks showing hydrolysis products of various polysaccharides by the action of TK-PUL. Purified TK-PUL (2.6 U, or approximately 40 μg) was mixed in a total volume of 250 μl with each indicated substrate at 0.25% final concentration in 50 mM sodium citrate buffer, pH 4.2, and incubated at 90°C. Aliquots were withdrawn at various intervals, and after the reaction was quenched on ice, 20-μl amounts were analyzed on an Aminex HPX-42A column. Pullulan hydrolysates obtained after 16 h of incubation at 90°C were further incubated with 10 U of α-glucosidase from *S. cerevisiae* in 50 mM sodium phosphate buffer, pH 6.0, for 2 h at 37°C. DP, degree of polymerization; DPn, a polysaccharide of *n* number of glucose residues.

similar conditions, with the same amount of α-glucosidase. Surprisingly, in contrast to the control, which was completely converted to glucose, the di- and trisaccharides in pullulan hydrolysates were only partially hydrolyzed to glucose (Fig. 4a). These results indicated that the pullulan hydrolysates possibly contained two types of di- and trisaccharides, maltose and maltotriose, which were hydrolyzed to glucose by α-glucosidase, and isomaltose and panose, which could not be hydrolyzed because α-glucosidase from yeast was unable to cleave their α-1,6 linkages. This interesting feature of isomaltose production from the hydrolysis of pullulan has not been previously reported; however, the production of panose in addition to maltotriose has been reported in the case of pullulanase from *T. aggregans* (10).

The recombinant TK-PUL hydrolyzed a wide variety of high-molecular-weight polysaccharides, including starch, glycogen, amylose, amylopectin, and dextrin, as well as cyclodextrins, to a mixture containing maltotriose, maltose, and glucose with predominant concentrations of maltose (Fig. 4). More than 95% of the substrates (other than cyclodextrins) were hydrolyzed within 20 min of incubation at 90°C, while complete conversion (to maltotriose, maltose, and glucose) was attained after 16 h. Smaller linear oligosaccharides, from maltoheptaose to maltotriose, were also hydrolyzed but at lower rates. The most interesting feature of the recombinant TK-PUL was its ability to hydrolyze maltotriose (see Fig. S3 at http://pu.edu.pk/images/publication/AEM-%2003139-13.pdf) and cyclodextrins (α, β, and γ). Under similar conditions, the hydrolysis rate of γ-cyclodextrin was almost similar to that of pullulan (i.e., complete hydrolysis within 10 min), while α- and β-cyclodextrins were hydrolyzed at much lower rates (Fig. 4).

DISCUSSION

Industrial starch hydrolysis is a two-step process, and pullulanase is normally added during the second step (saccharification) to hydrolyze α-1,6 linkages at branch points that were not attacked by α-amylase in the first step (liquefaction) (4). Complete solubilization of starch in water can only be achieved at a temperature above 100°C, and the natural pH of this solution is 4.5. Therefore, the use of highly thermostable amylolytic enzymes capable of working efficiently in this acidic environment would directly benefit the starch industry (3). We describe here a novel thermoacidophilic pullulan-hydrolyzing enzyme from *T. kodakarensis*. The amino acid sequence of TK-PUL displayed homology with type II pullulanases, and it was annotated accordingly during the analysis of the genome sequence of *T. kodakarensis* (16).

The archaeal pullulanases reported to date show maximal activity in the pH range 5.0 to 6.5, with the exception of pullulanase type II from *Pyrodictium abyssi*, which exhibits its highest activity at pH 9.0 (5). In accordance with the acidic catalysis mechanism proposed for amylolytic enzymes (28), the recombinant TK-PUL has an optimum pH of 3.5 in sodium acetate and 4.2 in sodium citrate buffer and is active over a broad pH range (3.0 to 8.5).

A major problem for the starch industry is that Ca^{2+} has to be added to starch slurry to enhance the thermostability and activity of amylases. This added Ca^{2+} inhibits the activity of glucose isomerase, which is used in later steps for isomerization of glucose to fructose during high-fructose syrup production (29). Calcium oxalate is also produced as a waste product and deposits in the pipes and heat exchangers. This deposition chokes the pipes and

in turn increases the production cost. With the development of Ca^{2+}-independent thermostable enzymes, this problem can be solved. TK-PUL does not need Ca^{2+} for activity and thermostability. Its Ca^{2+} independence, along with its high thermostability and pH optima of 3.5 in acetate buffer and 4.2 in citrate buffer, make TK-PUL an attractive candidate for application in the starch industry.

As both the α-1,4- and α-1,6-hydrolyzing activities of recombinant TK-PUL were inhibited at the same rate in the presence of 0.1% β-cyclodextrin, 0.01% *p*-chloromercuribenzoic acid, and 0.01% *N*-bromosuccinimide (see Table S3 at http://pu.edu.pk/images/publication/AEM-%2003139-13.pdf), it can be speculated that recombinant TK-PUL possesses a single active site for the hydrolysis of both α-1,4 and α-1,6 glucosidic linkages. However, detailed studies are required to reach such a conclusion. Amylopullulanases from *D. mucosus* (11), *Thermoanaerobium* strain Tok6-B1 (30), and *C. thermohydrosulfuricum* (31) have also been shown to possess only one active site, whereas amylopullulanases from *Bacillus circulans* F-2 (32), *Bacillus* sp. strain KSM-1378 (33), and *P. woesei* (34) have been reported to possess two different active sites responsible for dual catalytic activities. TK-PUL was able to hydrolyze a variety of substrates, with the highest preference toward pullulan and then γ-cyclodextrin. The pullulanase activity was slightly inhibited in the presence of β-cyclodextrin, possibly due to substrate competition, as the enzyme was able to hydrolyze all types of cyclodextrins (α, β, and γ). The slight inhibition may be attributed to a possible single catalytic site being involved in both types of reactions. Among previously reported pullulanases, only two enzymes, from *T. aggregans* (10) and *D. mucosus* (7), were able to hydrolyze cyclodextrins, while others were competitively inhibited.

Recombinant TK-PUL was able to hydrolyze maltotriose into maltose and glucose. This is a unique feature of TK-PUL that was not previously reported for any of the pullulanases. The smallest maltooligosaccharide hydrolyzed by pullulanases from *T. aggregans* (10) and *D. mucosus* (11) was maltotetraose. The incubation of pullulan hydrolysates obtained by the action of TK-PUL with α-glucosidase from yeast resulted in incomplete conversion of the tri- and disaccharides into monosaccharides. This indicated the probability of the presence of panose and isomaltose, in addition to maltotriose and maltose. On the basis of these strong evidences, recombinant TK-PUL should be regarded as a pullulan hydrolase type III, instead of type II as annotated in the genome sequence of *T. kodakarensis*. These annotations were based on the amino acid sequence comparisons. Sequence-based classification of putative enzymes may not always be correct, as observed in the case of the well-characterized branching enzyme TK1436 from *T. kodakarensis*. TK1436 was annotated as a "probable α-amylase" in the genome sequence, but the biochemical characterization was in contrast with the sequence-based annotation and showed that it was a branching enzyme (35). Similarly, heteromeric amino acid transporter proteins do not possess any amylolytic activity but are classified as members of clan glycoside hydrolases-H (GH-H) because of similarity in amino acid sequence (36). A very recent report described the pullulan-hydrolyzing enzyme from *T. kodakarensis* (TK0977, TK-PUL) as a pullulanase type II on the basis of sequence similarity and initial data for enzyme characterization (22). Detailed analysis proves that TK-PUL is actually a pullulan hydrolase type III. To our knowledge, the pullulanase reported from *T. aggregans* is the only enzyme belonging to pullulan hydrolases type III that has been characterized (10). TK-PUL displayed a 62% homology with *T. aggregans* pullulanase. Comparison of both enzymes shows that their K_m values toward pullulan are quite similar (TK-PUL, 2.0 mg ml^{-1}, and *T. aggregans*, 2.38 mg ml^{-1}), whereas TK-PUL exhibits a very high V_{max} value of 109 U mg^{-1}, compared to 16.6 U mg^{-1} for the pullulanase from *T. aggregans* (Table 2) (10). The optimum temperatures for activity of these two enzymes are quite similar, whereas the optimum pH values are entirely different.

TABLE 2 Comparison of the properties of type III pullulan hydrolases

Property	Result for pullulan hydrolase type III from:	
	T. kodakarensis KOD1	*T. aggregans*
Molecular mass (kDa)	84.4	80
Catalytic subunits	Monomer	ND
Optimum temp (°C)	95–100	95
Activity at 120°C (%)	>60	>35
pH range	3.0–8.5	3.5–8.5
Activity at pH 3.5 (%)	100	50
Optimum pH for activity	3.5	6.5
Ca^{2+} requirement	No	No
Half-life at 100°C (min)	45	90
K_m (mg/ml) for pullulan	2.0	2.38
V_{max} (U/mg) for pullulan	109.17	16.6
Complete hydrolysis of pullulan	10 min	16 h
End products of pullulan hydrolysis	Maltotriose, panose, maltose, isomaltase, and glucose	Maltotriose, panose, maltose, and glucose
Hydrolysis of glycogen	Yes	No
Hydrolysis of cyclodextrins	All types	Only β- and γ-cyclodextrins
Smallest oligosaccharide hydrolyzed	Maltotriose	Maltotetraose

In conclusion, TK-PUL is a novel thermoacidophilic pullulan hydrolase type III, and its calcium independence, extraordinary stability over a broad pH range, highest activity at pH 3.5 or 4.2, and high thermostability make it an ideal enzyme for the starch industry.

REFERENCES

1. **Leathers TD.** 2003. Biotechnological production and applications of pullulan. Appl. Microbiol. Biotech. **62:**468–473. http://dx.doi.org/10.1007/s00253-003-1386-4.
2. **Hii SL, Tan JS, Ling TC, Ariff AB.** 2012. Pullulanase: role in starch hydrolysis and potential industrial applications. Enzyme Res. **2012:** 921362. http://dx.doi.org/10.1155/2012/921362.
3. **van der Maarel MJ, van der Veen B, Uitdehaag JC, Leemhuis H, Dijkhuizen L.** 2002. Properties and applications of starch-converting enzymes of the alpha-amylase family. J. Biotechnol. **94:**137–155. http://dx.doi.org/10.1016/S0168-1656(01)00407-2.
4. **Lévêque E, Janeček Š, Haye B, Belarbi A.** 2000. Thermophilic archaeal amylolytic enzymes. Enzyme Microb. Tech. **26:**3–14. http://dx.doi.org/10.1016/S0141-0229(99)00142-8.
5. **Bertoldo C, Antranikian G.** 2002. Starch-hydrolyzing enzymes from

thermophilic archaea and bacteria. Curr. Opin. Chem. Biol. **6:**151–160. http://dx.doi.org/10.1016/S1367-5931(02)00311-3.
6. **Janeček Š, Svensson B, MacGregor EA.** 27 June 2013. α-Amylase: an enzyme specificity found in various families of glycoside hydrolases. Cell. Mol. Life Sci. http://dx.doi.org/10.1007/s00018-013-1388-z.
7. **MacGregor EA, Janecek S, Svensson B.** 2001. Relationship of sequence and structure to specificity in the alpha-amylase family of enzymes. Biochim. Biophys. Acta **1546:**1–20. http://dx.doi.org/10.1016/S0167-4838(00)00302-2.
8. **Wasko A, Polak-Berecka M, Targonski Z.** 2011. Purification and characterization of pullulanase from *Lactococcus lactis*. Prep. Biochem. Biotechnol. **41:**252–261. http://dx.doi.org/10.1080/10826068.2011.575316.
9. **Nisha M, Satyanarayana T.** 2013. Recombinant bacterial amylopullulanases: developments and perspectives. Bioengineered **4**(6)**:**300–312. http://dx.doi.org/10.4161/bioe.24629.
10. **Niehaus F, Peters A, Groudieva T, Antranikian.** 2000. Cloning, expression and biochemical characterisation of a unique thermostable pullulan-hydrolysing enzyme from the hyperthermophilic archaeon *Thermococcus aggregans*. FEMS Microbiol. Lett. **190:**223–229. http://dx.doi.org/10.1111/j.1574-6968.2000.tb09290.x.
11. **Duffner F, Bertoldo C, Andersen JT, Wagner K, Antranikian G.** 2000. A new thermoactive pullulanase from *Desulfurococcus mucosus*: cloning, sequencing, purification, and characterization of the recombinant enzyme after expression in *Bacillus subtilis*. J. Bacteriol. **182:**6331–6338. http://dx.doi.org/10.1128/JB.182.22.6331-6338.2000.
12. **Fujita N, Toyosawa Y, Utsumi Y, Higuchi T, Hanashiro I, Ikegami A, Akuzawa S, Yoshida M, Mori A, Inomata K, Itoh R, Miyao A, Hirochika H, Satoh H, Nakamura Y.** 2009. Characterization of pullulanase (PUL)-deficient mutants of rice (*Oryza sativa* L.) and the function of PUL on starch biosynthesis in the developing rice endosperm. J. Exp. Bot. **60:**1009–1023. http://dx.doi.org/10.1093/jxb/ern349.
13. **Li Y, Zhang L, Niu D, Wang Z, Shi G.** 2012. Cloning, expression, characterization, and biocatalytic investigation of a novel bacilli thermostable type I pullulanase from *Bacillus* sp. CICIM 263. J. Agric. Food Chem. **60:**11164–11172. http://dx.doi.org/10.1021/jf303109u.
14. **Atomi H, Fukui T, Kanai T, Morikawa M, Imanaka T.** 2004. Description of *Thermococcus kodakaraensis* sp. nov., a well studied hyperthermophilic archaeon previously reported as *Pyrococcus* sp. KOD1. Archaea **1:**263–267. http://dx.doi.org/10.1155/2004/204953.
15. **Morikawa M, Izawa Y, Rashid N, Hoaki T, Imanaka T.** 1994. Purification and characterization of a thermostable thiol protease from a newly isolated hyperthermophilic *Pyrococcus* sp. Appl. Environ. Microbiol. **60:**4559–4566.
16. **Fukui T, Atomi H, Kanai T, Matsumi R, Fujiwara S, Imanaka T.** 2005. Complete genome sequence of the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 and comparison with *Pyrococcus* genomes. Genome Res. **15:**352–363. http://dx.doi.org/10.1101/gr.3003105.
17. **Guan Q, Guo X, Han T, Wei M, Jin M, Zeng F, Liu L, Li Z, Wang Y, Cheong G-W.** 2013. Cloning, purification and biochemical characterization of an organic solvent-, detergent-, and thermo-stable amylopullulanase from *Thermococcus kodakarensis* KOD1. Process Biochem. **48:**878–884. http://dx.doi.org/10.1016/j.procbio.2013.04.007.
18. **Rashid N, Cornista J, Ezaki S, Fukui T, Atomi H, Imanaka T.** 2002. Characterization of an archaeal cyclodextrin glucanotransferase with a novel C-terminal domain. J. Bacteriol. **184:**777–784. http://dx.doi.org/10.1128/JB.184.3.777-784.2002.
19. **Tachibana Y, Fujiwara S, Takagi M, Imanaka T.** 1997. Cloning and expression of the 4-α-glucanotransferase gene from the hyperthermophilic archaeon *Pyrococcus* sp. KOD1, and characterization of the enzyme. J. Ferment. Bioeng. **83:**540–548. http://dx.doi.org/10.1016/S0922-338X(97)81134-8.
20. **Tachibana Y, Leclere MM, Fujiwara S, Takagi M, Imanaka T.** 1996. Cloning and expression of the α-amylase gene from the hyperthermophilic archaeon *Pyrococcus* sp. KOD1, and characterization of the enzyme. J. Ferment. Bioeng. **82:**224–232. http://dx.doi.org/10.1016/0922-338X(96)88812-X.
21. **Han T, Zeng F, Li Z, Liu L, Wei M, Guan Q, Liang X, Peng Z, Liu M, Qin J, Zhang S, Jia B.** 2013. Biochemical characterization of a recombinant pullulanase from *Thermococcus kodakarensis* KOD1. Lett. Appl. Microbiol. **57:**336–343. http://dx.doi.org/10.1111/lam.12118.
22. **Hall TA.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. **41:**95–98.
23. **Bernfeld P.** 1955. Amylases, alpha and beta. Methods Enzymol. **1:**149–158. doi.10.1016/0076-6879(55)01021-5. http://dx.doi.org/10.1016/0076-6879(55)01021-5.
24. **Bendtsen JD, Nielsen H, von Heijne G, Brunak S.** 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. **340:**783–795. http://dx.doi.org/10.1016/j.jmb.2004.05.028.
25. **Erra-Pujada M, Debeire P, Duchiron F, O'Donohue MJ.** 1999. The type II pullulanase of *Thermococcus hydrothermalis*: molecular characterization of the gene and expression of the catalytic domain. J. Bacteriol. **181:**3284–3287.
26. **Dong G, Vieille C, Zeikus JG.** 1997. Cloning, sequencing, and expression of the gene encoding amylopullulanase from *Pyrococcus furiosus* and biochemical characterization of the recombinant enzyme. Appl. Environ. Microbiol. **63:**3577–3584.
27. **Nakajima R, Imanaka T, Aiba S.** 1986. Comparison of amino acid sequences of eleven different α-amylases. Appl. Microbiol. Biotechnol. **23:**355–360.
28. **Kim T-J, Kim M-J, Kim B-C, Kim J-C, Cheong T-K, Kim J-W, Park K-H.** 1999. Modes of action of acarbose hydrolysis and transglycosylation catalyzed by a thermostable maltogenic amylase, the gene for which was cloned from a *Thermus* strain. Appl. Environ. Microbiol. **65:**1644–1651.
29. **Wang L, Zhou Q, Chen H, Chu Z, Lu J, Zhang Y, Yang S.** 2007. Efficient solubilization, purification of recombinant extracellular alpha-amylase from *Pyrococcus furiosus* expressed as inclusion bodies in *Escherichia coli*. J. Ind. Microbiol. Biotechnol. **34:**187–192. http://dx.doi.org/10.1007/s10295-006-0185-1.
30. **Plant AR, Clemens RM, Morgan HW, Daniel RM.** 1987. Active-site- and substrate-specificity of *Thermoanaerobium* Tok6-B1 pullulanase. Biochem. J. **246:**537–541.
31. **Mathupala S, Saha BC, Zeikus JG.** 1990. Substrate competition and specificity at the active site of amylopullulanase from *Clostridium thermohydrosulfuricum*. Biochem. Biophys. Res. Commun. **166:**126–132. http://dx.doi.org/10.1016/0006-291X(90)91920-N.
32. **Kim CH, Kim YS.** 1995. Substrate specificity and detailed characterization of a bifunctional amylase-pullulanase enzyme from *Bacillus circulans* F-2 having two different active sites on one polypeptide. Eur. J. Biochem. **227:**687–693. http://dx.doi.org/10.1111/j.1432-1033.1995.tb20189.x.
33. **Hatada Y, Igarashi K, Ozaki K, Ara K, Hitomi J, Kobayashi T, Kawai S, Watabe T, Ito S.** 1996. Amino acid sequence and molecular structure of an alkaline amylopullulanase from *Bacillus* that hydrolyzes α-1, 4 and α-1, 6 linkages in polysaccharides at different active sites. J. Biol. Chem. **271:**24075–24083. http://dx.doi.org/10.1074/jbc.271.39.24075.
34. **Rudiger A, Jorgensen PL, Antranikian G.** 1995. Isolation and characterization of a heat-stable pullulanase from the hyperthermophilic archaeon *Pyrococcus woesei* after cloning and expression of its gene in *Escherichia coli*. Appl. Environ. Microbiol. **61:**567–575.
35. **Murakami T, Kanai T, Takata H, Kuriki T, Imanaka T.** 2006. A novel branching enzyme of the GH-57 family in the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. J. Bacteriol. **188:**5915–5924. http://dx.doi.org/10.1128/JB.00390-06.
36. **Janeček Š Svensson B, Henrissat B.** 1997. Domain evolution in the α-amylase family. J. Mol. Evol. **45:**322–331. http://dx.doi.org/10.1007/PL00006236.