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Cloning, Expression, Characterization, and Biocatalytic Investigation of a Novel Bacilli Thermostable Type I Pullulanase from *Bacillus* sp. CICIM 263

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Ⓢ *Supporting Information*

ABSTRACT: The *pulA1* gene, encoding a novel thermostable type I pullulanase PulA1 from *Bacillus* sp. CICIM 263, was identified from genomic DNA. The open reading frame of the *pulA1* gene was 2655 base pairs long and encoded a polypeptide (PulA1) of 885 amino acids with a calculated molecular mass of 100,887 Da. The *pulA1* gene was expressed in *Escherichia coli* and *Bacillus subtilis*. Recombinant PuLA1 showed optimal activity at pH 6.5 and 70 °C. The enzyme demonstrated moderate thermostability as PuLA1 maintained more than 88% of its acitivity when incubated at 70 °C for 1 h. The enzyme could completely hydrolyze pullulan to maltotriose, and hydrolytic activity was also detected with glycogen, starch and amylopection, but not with amylose, which is consistent with the property of type I pullulanase. PulA1 may be suitable for industrial applications to improve the yields of fermentable sugars for bioethanol production.

KEYWORDS: *Bacillus sp., thermostable type I pullulanase, gene cloning and expression*

■ INTRODUCTION

Complete starch hydrolysis requires a consortium of enzymes including *endo*-amylases, glucoamylases, and α-glucosidases as well as α-1,6- cleaving enzymes (known as debranching enzymes or pullulanase and isoamylase). Pullulanase, α-dextrin 6-glucanohydrolase (EC 3.2.1.41), is one of the most important enzymes in starch-bioprocessing industry for it specifically hydrolyzes α-1,6-glycosidic linkages at branching points in amylopectin and pullulan.[1]

Pullulanase belongs to the glycoside hydrolase (GH) super-family,on the basis of amino acid sequence similarity.[2] The enzymes exhibiting pullulanase activities are currently classified within GH family 13 (see http://www.cazypedia.org). Based on substrate specificity and released products, pullulanase can be classified into type I enzyme and type II enzyme. The type I ones cannot attack α-1,4-glycosidic linkages compared with type II ones, and mainly forms maltotriose and linear oligomers.[3−6] Although most of well-investigated pullulanases to date are type II enzymes, type I enzymes with thermo tolerance (activating beyond temperature of 60 °C) are more preferable by industrial application for the reason that they would release long polymers of α-1,4 linked glucan units which would allow more efficient and more rapid conversion reactions in starch processing industry.[7] Among thermostable type I pullulanase ever characterized, those from *Bacillus acidopullulyticus*,[8−10] *Bacillus cereus*,[11] *Fervidobacterium pennavorans*,[7] *Anaerobranca gottschalkii*,[12] and *Thermotoga neapolitana*[13] have been studied at the genetic level. Sequence information reveals a low level of overall conservation among type I enzymes.

For obtaining a new pullulanase with its properties more suitable for starch processing, in the previous research, a strain, *Bacillus* sp. CICIM 263, was screened and isolated from soil of volcano in hot spring from Yunnan, China. It produced a remarkable extracellular thermostable pullulanase. In this report we cloned and sequenced the gene encoding the novel pullulanase from *Bacillus* sp. CICIM 263, followed by the expression of the enzyme in *Escherichia coli* and bacilli hosts. The properties of the recombinant enzyme in *E. coli* were also determined.

■ MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions. The bacterial strains and recombinant DNA constructs are listed in Supporting Information (SI) Table 1. *Bacillus* sp. CICIM 263 was grown in a medium containing (g L^{-1}) dextrin 8; beef extract 12; NaCl; and $MgSO_4·7H_2O$ 0.5 in 40 mM Na_2HPO_4-KH_2PO_4 buffer, pH 6.0.While necessary, tetracycline was supplemented with the final concentration of 20 μg mL^{-1}. *E. coli* and *B. subtilis* were was cultivated in Luria−Bertani (LB) broth medium complemented with 20 μg mL^{-1} tetracycline or 25 μg mL^{-1} kanamycin when necessary. All bacteria were cultured at 37 °C with aeration.

Cloning of DNA from *Bacillus* sp. In previous studies, purified PulA from *Bacillus* sp. CICIM 263 was digested by trypsin and the released peptides were analyzed by nanoflow liquid chromatography coupled to electrospray ionization quadrupole time-of flight tandem mass spectrometry (nanoLC-ESI-Q-TOF/MS/MS). Based on the internal amino acid sequences detected, gene encoding PulA was characterized and cloned. Degenerate primer DNAs of BPD1 (AAARTTGGRGRTAAGTGKACYGAAGC) and BPD2 (TKCATSGTTTCATAAYCATRARTACCYAT) were designed based on the identified internal peptides sequences (KIGGEWTE and MGIHDTETM), to amplify part of the gene. They were prepared with a DNA synthesizer (Applied Biosystems) and were purified with a DNA Refining System by Sangon Biotech. Genomic DNA from *Bacillus* sp.

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CICIM 263 was prepared by using the DNeasy Kit (Qiagen). PCR was performed in a DNA thermal cycler (TPerformance standard gradient thermalcycler, Biometra), using each primer plus genomic DNA (1.0 μg). The reaction conditions were as follows: 96 °C for 30 s, 52 °C for 30 s, and 72 °C for 2 min for 30 cycles. The reaction mixture contained 200 μM dNTPs, 2.5 mM $MgSO_4$, 25 mM KCl, 5 mM $(NH_4)_2SO_4$, 1.25 units of *Ex Taq* DNA polymerase (TAKARA), and 10 mM Tris-HCl buffer (pH 8.85) in a reaction volume of 50 μL. In order to determine the integral sequence of the gene, inverse PCRs were applied to characterize the flanking sequences of the known fragment. First, genomic DNA of *Bacillus* sp. CICIM 263 was digested with *Kpn*I, *Bam*HI, and *Hind*III, respectively, followed by self-ligation under conditions that favored the formation of monomeric circles[19] by T4 DNA ligase. The first round inverse PCRs were conducted with primers Inv-11 and Inv-12. Then, a new round inverse PCRs were conducted using templates generated by *Sal*I, *Xba*I, and *Eco*RI, respectively, and suitably synthesized primers Inv-21 and Inv-22. Finally, determined sequences by the two round inverse PCRs, as well as the sequence of the product with primers BPD1 and BPD2, were analyzed with DNAMAN V7, to assemble the whole encoding gene, *pulA1*.

Construction of Expression Plasmid. The coding region of *pulA1* was amplified by PCR using sense primers B-1 to B-6 and antisense primer B-end (SI Table 1), respectively. PCRs were performed with *Ex Taq* DNA polymerase under the program of 96 °C for 30 s, 54 °C for 30 s, and 72 °C for 4 min for 30 cycles, followed by directional cloning into pET-28a (+) at *Nco*I/*Bam*HI sites, to yield pETP1-pETP6. The plasmids were analyzed by digestion with restriction enzymes and DNA sequencing, and then transferred to *E. coli* BL21 (DE3) for protein expression assays.

Expression of *pulA1* Gene in *E. coli*. *E. coli* BL21 (DE3) was transformed with pETP1−6 constructed. The enzyme was expressed as a fusion protein that consisted of PulA1 plus a carboxyl-terminal tag with six histidines. *E. col*i BL21 (DE3) harboring plasmids pETP1−6 were cultivated in 50 mL of LB medium that contained kanamycin (0.05 mg/mL) at 37 °C, with shaking, to an optical density of 0.6 at 600 nm. After addition of isopropyl-1-thio-β-D-galactoside (IPTG) to a final concentration of 1 mM, the culture was incubated at 25 °C for 8 h with agitation. The cells were harvested and lysed, with the lysate, pullulanase activity was assayed and sodiun dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the system of Laemmli (1970).

Expression of *pulA1* Gene in *Bacillus subtilis*. *E.coli* clone plasmid pMDQG was digested by *Bam*HI and *Nco*I, successively, the released fragment of 1132 bp containing the artificial promoter PQ and gentamycin resistant gene was recovered. The fragment was ligated with *E. coli* expression plasmid pETP digested by *Bgl*II and *Nco*I, so as to replace the T7 promoter, to form pETPQ. Expression of *pulA1* gene under the two promoters was compared with fermentation and IPTG induction, respectively. Subsequently, for deletion of the *Bam*HI site on the shuttle vector pHY300-PLK (the site also exists in *pulA1*), the vector was linearized at the *Bam*HI site, and blunting was carried out by Quick Blunting Kit (NEB). The vector with blunt end was recircularized with T4 DNA ligase (TAKARA), resulting in plasmid pHY-*Bam*HI$^-$. After digestion of *Xho*I and *Sph*I on pETPQ, a 4330-bp fragment containing *pulA*, PQ and gentamycin resistant gene was recovered and blunted with Quick Blunting Kit (NEB). The obtained blunt fragment was ligated with pHY-*Bam*HI$^-$ opened by *Sma*I. The ligation product was transformed to *B. subtilis* 1A717 using chemical transformation method described by Spisizen,[14] to yield a recombinant strain harboring plasmid pHY-WZX-*pulA1*. Fermentation of the recombinant *B. subtilis* was performed according to the method of Aiba.[15]

Purification of Recombinant PulA1. Recombinant PulA1 produced by *B. subtilis* was expressed as His-tag fusion protein, and it was purified to homogeneity in two steps as reported previously.[16] For this, the crude enzyme solution was precipitated with 30% saturation of ammonium sulfate to remove impurities and loaded on a Histrap chelting column (GE healthcare). The bound enzyme was eluted with 200 nM imidazole in 20 mM phosphate buffer (pH 7.0). A single peak of pullulanase was detected and corresponding fractions were pooled and concentrated with Vivaspin (Sartorius stedium biotech) to 1.0 mL. The concentrated solution was loaded on a HiTrap desalting column (GE healthcare) in order to remove the imidazole.

Pullulanase Assay. Pullulanase activity was determined by measuring the amount of reducing sugars released during incubation with pullulan. To 50 mL of 1% (wt/vol) pullulan dissolved in a 50 mM sodium acetate buffer (pH 6.0), 25 or 50 mL of the enzyme solution was added, and the samples were incubated at different temperatures for 10−60 min. The reaction was stopped by cooling the mixture on ice, and the amounts of reducing sugars released were determined by the dinitrosalicylic acid method. Sample blanks were used to correct for the nonenzymatic release of reducing sugars. One unit of pullulanase is defined as the amount of enzyme that releases 1 μmol of reducing sugars (with maltose as the standard) per min under the assay conditions specified. Pullulanase activity was routinely determined in a 50 mM sodium acetate buffer (pH 6.5) at 70 °C with 0.5% (wt/vol) pullulan.

SDS-PAGE Analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 3% stacking and 10% separating gels containing 0.1% SDS, as described previously.[17] The protein standards (New England BioLabs, Ipswich, MA) used for estimation of subunit molecular masses were MBP-β-galactosidase (175 kDa), MBP-truncated-β-galactosidase (80 kDa), MBP-CBD (58 kDa), CBD-*Mxe* intein-2CBD (46 kDa), CBD-*Mxe* intein (30 kDa), and CBD-*E. coli* Par (23 kDa).

Kinetic Measurements. All kenetic measurements were performed at 70 °C in 0.05 M sodium acetate buffer (pH 6.5). The kinetic parameters for PulA1 were determined by using glycogen as substrate with concentrations ranging from 100 μM to 10 mM. All concentrations were measured in triplicate, and data were calculated according to the Hanes-Woolf equation.[18] The inhibition kinetics of Fe^{3+} on PulA1 was also investigated with three concentrations of 5, 10, and 50 mM.

Determination of Protein Concentration. The protein concentration was determined using the Bio-Rad protein assay kit, with bovine serum albumin as the standard.

Influence of pH and Temperature. To determine the influence of temperature on the enzymatic activity, standard assay reaction mixtures were incubated at temperatures ranging from 30 to 80 °C for 1h in 0.05 M sodium phosphate (pH 6.5) buffer.The pH of the buffers was generally adjusted (0.05 M HAc- NaAc for pH 4.0−6.0, 0.05 M sodium phosphate for pH 6.5−7.5 and 0.05 M Tris-HCl for pH 8.0−9.0) at the temperature used for the experiment. Experiments were carried out with the purified recombinant enzyme. The influence of pH on pullulanase activity was determined at 60 °C by using the assay protocol described above.

Biocatalysis Investigation. Sunbstrate specificity of recombinant pullulanase was investigated including: pullulan, amylopectin, sticky rice starch, maize starch, soluble starch, malto-dextrin, and glycogen. Enzyme activity was assayed as described above.

Accession Number. The nucleotide sequence of the amplified gene of pullulanase from *Bacillus* sp. CICIM 263 has been deposited under Genbank accession number JX018171.

RESULTS

Cloning of Gene for Pullulanase and Genome Walking by Inverse PCR. Degenerate primers were designed based on the peptide sequences obtained from nanoLC-ESI-Q-TOF/MS/MS analysis to amplify a partial sequence of the PulA1 gene from *Bacillus* sp. CICIM 263. A 2.1 kb amplicon was obtained by PCR using the degenerate pair, BPD1 and BPD2. To amplify the full-length of the *pulA*1 gene, 2 round inverse PCRs were applied. Primers (SI Table 1) were designed based on the determined 2.1-kb fragment. *Bam*HI digestion-self-ligated genomic DNA coupled with primer Inv-11 and Inv-12 resulted in a 1.7 kb fragment. The sequence of the fragment included a sequence that encoded the deduced aminoacid sequence TFNQI-TLETNFPFQWRD, which was identical to the sequence of the internal Family 41 Carbohydrate-Binding Module from pullulanase-like enzymes (cd10315, http://www.ncbi.nlm.nih.gov/Structure/cdd). This indicated that the gene sequences

encoded the N-terminal of the pullulanase had been identified. *Eco*RI digestion-self-ligated genomic DNA coupled with primer Inv-21 and Inv-22 resulted in a 1.4 kb fragment. Assembly of sequences from 2.1 kb and 1.4 kb fragments suggested that an intact alpha amylase catalytic domain (found in pullulanase, cd11341, http://www.ncbi.nlm.nih.gov/Structure/cdd) was characterized. Furthermore, a potential TAA stop codon was observed in the sequence of 1.4-kb fragment. At the end of this experiment, a 3.3 kb-sequence containing the open reading frame (ORF) of pullulanase encoding gene was identified.

Construction and Expression of the Entire pulA1 Gene. After analysis of the sequence, six possible initiation codons and one termination codon were predicted. Consequently, six sense primers (B1−B6) and one antisense primer (B-end) were designed to amplify fragments with lengths of 3.0, 2.8, 2.7, 2., 2.5, and 2.4 kb, respectively. An expression system pET/BL21 was used to testify the function of the six amplified fragments. The six amplified fragments were subcloned into the pMD18-T vector, after verified by sequencing; they were inserted into pET-28a (+) at *Nco*I/*Bam*HI sites. The plasmids were named pETP1−6 and transformed into *E. coli* cell, followed by sequencing to confirm correct sequences. After induction of IPTG, enzyme assay of the lysate suggested that only the 2.8 kb fragment functionally expressed. The specific activity of the pullulanase expressed in *E. coli* was 0.63 U/mg. The molecular mass of recombinant protein was estimated to be approximately 100 kDa by SDS-PAGE (Figure 1). Finally, a 2655 bp-full length ORF encoding *Bacillus* sp. CICIM 263 pullulanase was identified, cloned, and expressed.

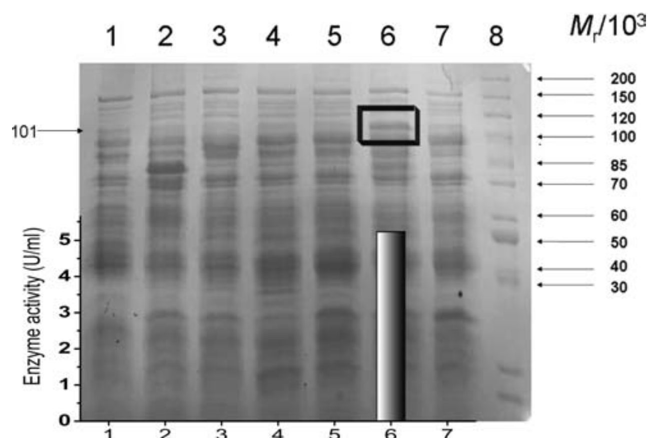

Figure 1. SDS-PAGE analysis of IPTG induced protein in *E. coli* BL21 (DE3) harboring pETP1−6. Lane 1 *E. coli* BL21 harboring pET-28a (+), Lane 2−7 *E. coli* BL21 harboring pETP1−6, Lane 8 Protein ladder. Except the fragment of 3.0 kb, each of the other five amplified fragments could result a recombinant protein after induction of IPTG ranging from 88 to 101 kDa. While enzyme assay suggested only the fragment of 2.8 kb functionally expressed (with an enzyme activity of 5.22 U/mL lysate). The other four proteins without function may translate from the initiation codon of the vector pET-28a (+).

As for *Bacillus subtilis* host, expression and secretion of pullulanase was under the control of an expression cassette consists of *P* promoter and *S* signal peptide region of *Bacillus licheniformis* amyl.[20] The recombinant enzyme displays activity, as determined by halo formation on red dyed pullulan plates and measurement of reducing sugars produced from pullulan as described above. Comparison of the pullulanase production per cell suggested that the expression level of *Bacillus subtilis* 1A717 was 3 times higher than that of *E. coli*.

Analysis of the pulA1 Gene Sequence. The recombinant plasmid from the positive clone, pETP, had 2760 bp of foreign DNA in the *Nco*I and *Bgl*II site of pET28-a (+). Sequencing of the pETP DNA insert revealed one major ORF which was 2655 bp in length, started from an ATG codon and ended with a TAA stop codon (Figure 2). A putative ribosome-binding site (Shine Delgarno sequence, AAGAAGGAGA) is observed at 6−15 bp upstream from the ATG codon. The sequence would have a free energy (ΔG) of −813.17 kcal/mol (3404.65 kJ/mol), as calculated by the method of Gruber,[21] when bound to the 3′ end of the 16S rRNA from *E. coli*. Analysis of the sequence upstream of the initiation codon showed that no presumable promoter region could be identified.

Analysis of Amino Acid Sequence of pulA1 Product. *PulA1* was predicted to encode a polypeptide (designated PulA1) of 886 amino acids which, as shown under the nucleotide sequence in Figure 2. Analysis of the amino acid sequence with Conserved Domain Database (CDD; http://www.ncbi.nlm.nih.gov/cdd/[22] revealed that five carbohydrate binding sites, 15 active sites, three calcium binding sites and three catalytic sites are distributed at N-terminal and C-terminal ends, repectively (Figure 3). Intervals between them contain an E_set_Pullulanase domain (Early set domain associated with the catalytic domain of pullulanase), may be related to the immunoglobulin and/or fibronectin type III superfamilies. These domains are associated with different types of catalytic domains at either the N-terminal or C-terminal end and may be involved in homodimeric/tetrameric/dodecameric interactions. As predicted previously, only moderate homology between the *pulA1* deduced amino acid sequence and known pullulanase was revealed. The enzyme appears to be a novel enzyme displaying less than 65% sequence identity with the pullulanase known. The best homologues are bacillus pullulanases, type I of *Bacillus megaterium* QM B1551 (64%), type I of *Bacillus cereus* Rock1-3(61%), and α-dextran endo-1,6-α-glucosidase of *Bacillus cereus* G9241 (60%). According to the system proposed by Hengrissat, the amino acid sequence of PulA1 reveals it to be a member of glycoside hydrolase family 13 (2). Further analysis of the conserved domains suggested that PulA1 has a carbohydrate-binding module family 48 (CBM48) domain, which is often found in a range of enzymes that act on branched substrates, that is, isoamylase, pullulanase, and branching enzyme. CBM48 specifically binds glycogen.[23−26]

Purufication of PulA1 Protein from Recombinant *B. subtilis*. The His_6-tagged recombinant pullulanase expressed was purified in two steps. First, 8.8 g ammonium sulfate was added to 50 mL lysate of *E. coli* BL21 (DE3) harboring pETP to reach a saturation of 30%. The sediment was abandoned as impurity. Second, the supernatant of ammonium sulfate precipitation was adjusted to pH 7.3 with phosphate buffer and loaded into Histrap. After affinity adsorption process, a linear gradient of 0−500 mM imidazole was performed to elute the bound protein. Finally, a specific pullulanase was purified 52.1-fold with a specific activity of 73 U/mg and a yield of 63.3% (Table 1). It clearly showed a single band, and its molecular mass was estimated to be 101 kDa by SDS-PAGE, which accorded with that predicted by gene sequence (Figure 4).

Biochemical Characterization of Recombinant PulA1. The maximum activity was measured at 70 °C, while at a temperature above 80 °C the activity is very low (see Figure 5A). The enzyme was quite tolerant to high temperature that after incubation at 70 °C for 1 h, it could retain 85% initial activity. However, at 80 °C, the thermostability declined sharply (see

Figure 2. Nucleotide sequence of *Bacillus* sp. CICIM 263 pullulanase gene and predicted amino acid sequence. The gene is 2655 bp, coding for an 886-aa protein with predicted molecular mass of 100 887 Da. The initial ATG codon, the stop codon TAA and terminal sequence are shaded in gray. Shine-Dalgarno sequence underlined is a putitive ribosome-binding site. An asterisk indicates a stop codon. Peptide sequences obtained by LC-MS/MS are boxed.

Figure 5A). Interestingly, in presence of 5 mM Ca^{2+}, Mg^{2+} and Mn^{2+}, thermostability of the enzyme displayed significant change. In details, Ca^{2+} not only protected PulA1 from inactivation at high temperatures but its activation effect enhanced sharply as the temperature increased. With the protection and activation of Ca^{2+}, PulA1 exhibited 254% and

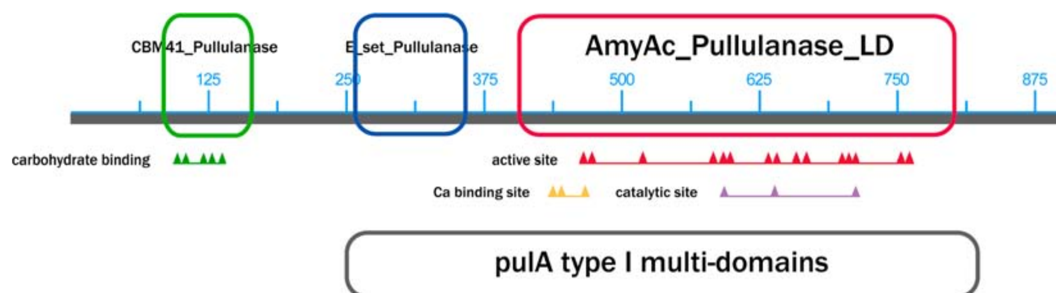

Figure 3. Structure of the amino acid sequence of *Bacillus* sp. CICIM 263 pullulanase. The amino acid sequence was deduced from *pulA1*. DNA sequence that determined completely for both strands. Three main conserved domains are characterized: CBM_Pullulanase, Family 41 carbohydrate-binding module from pullulanase-like enzymes; E_set_Pullulanase, Early set domain associated with the catalytic domain of pullulanase; and AmyAC_Pullulanase_LD-like, α-amylase catalytic domain found in pullulanase, limit dextrinase, and related proteins. Five carbohydrate binding sites, 15 active sites, 3 calcium binding sites, and3 catalytic sites are indicated with triangles, respectively, and underlined. A part of the product from Glu263 to Lys865 consists of several pullulanase type I conserved domains (pula type I multidomains), which may involved in multiple typical functions of type I pullulanase.

Table 1. Purification of Recombinant Pullulanase

purification step	total activity (U)	total protein (mg)	specific activity (U/mg)	purification fold	yield (%)
crude cell extract[a]	76.4 ± 2.3	53.1 ± 3.3	1.4 ± 0.05	1	100
ammonium sulfate precipitation	64.3 ± 1.6	7.1 ± 0.5	9.1 ± 0.3	6.5	84.2
Histrap	40.7 ± 1.9	0.6 ± 0.03	73.5 ± 4.1	52.1	63.3

[a]Obtained from 2 g (wet weight) of *E. coli* BL21 pETP.

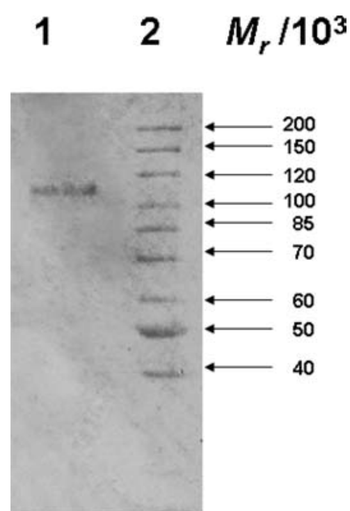

Figure 4. SDS-PAGE of purified recombinant PulA1. Lane 1, affinity purified PulA1-His tag; lane 2, molecular weight marker. After two steps of purification, recombinant PulA1 appeared a single band on SDS-PAGE with a molecular weight of about 100 kDa.

112% of original activity at 80 and 70 °C, respectively, much higher than 45% residual activity in absence of the bivalent cation. Mn^{2+} had limited protection and activation effect on PulA1 at high temperatures compared to Ca^{2+}. On the other hand, even Mg^{2+} notably activated PulA1 at 40 °C, it could not avoid denaturing of it at 80 °C. The optimum pH was measured at pH 6.5 in phosphate buffer. Recombinant PulA1 preferred alkaline and neutral conditions. It was suggested that the enzyme was most stable at pH 7.0, which less than 5% of initial activity was lost after 1 h incubation (see Figure 5B).

Hydrolytic and Kinetic Properties of Recombinant PulA1. A series of carbohydrate compounds were investigated to explore the substrate specificity and hydrolysis pattern of recombinant PulA1. As shown in Table 2, substrate specificity may relate to the branching degree of the substrate. After reaction, pullulan (contain only α-1,6 linkages) was completely converted to maltotriose. While other branched oligosaccharides containing α-1,6- as well as α-1,4-linkages such as glycogen, starch, and amylopectin, were partially hydrolyzed by the enzyme, generating maltose and maltotriose as the main products. Substrates containing only α-1,4-linkages, such as maltoheptaose, maltohexaose, maltopentaose and maltotetraose, could not be hydrolyzed by the enzyme, which is also consistent with the results that small oligosaccharides could not be hydrolyzed by type I pullulanase.[7,12] Accordingly, the enzyme specifically attack only α-1,6-linkages and can be classified as pullulanase of type I.

PulA1 followed a typical Michaelis–Menten kinetics. Using the Lineweaver–Burk plot, a K_m value of 4.03 ± 0.18 μM/mL and a V_{max} value of 0.18 ± 0.01 mM/(min·mL) were established for pullulan hydrolysis. The inhibition kinetics suggested that the three curves intersected at the same point on the horizontal axis when the enzyme acting on substrates with different concentrations, that is, they had the invariable K_m value. Meanwhile, V_{max} changed following concentration of the inhibitor and the reaction velocity was decreased with the increase of inhibitor concentration. From the above, Fe^{3+} concentration could change reaction velocity but not Michaelis constant which indicated the inhibition of Fe^{3+} on PulA1 was noncompetitive, probably because the substrate and inhibitor combined in different sites of the enzyme.

DISCUSSION

Thermoactive and thermostable pullulanase is valuable in starch processing industry,[27] as high efficient enzymatic liquefaction and saccharification are performed at elevated temperature (up to 117 °C). Moreover, the current starch conversion techniques need a cooling step to 60 °C or below before saccharification,[4] because the commercially available saccharification enzymes are unstable above 60 °C. The application of thermostable pullulanase could dramatically improve the energy- and time-

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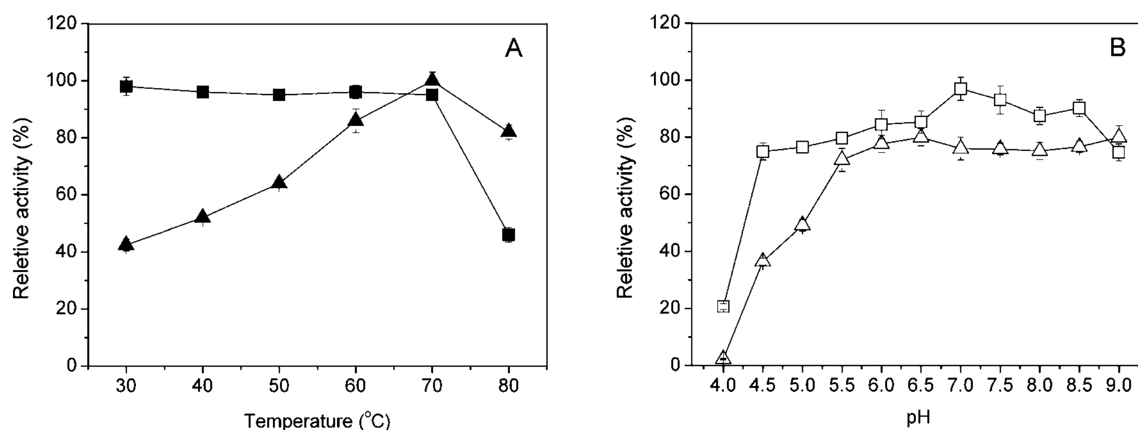

Figure 5. (A and B) Influence of pH on the activity and influence of temperature on the activity and stability of the recombinant pullulanase. (A) Influence of temperature on activity of purified recombinant pullulanase (■) was investigated by assaying the enzyme at temperatures ranging from 30 to 80 °C in 0.05 M sodium phosphate (pH 6.5); thermostability of the enzyme (▲) was studied by incubating at different temperatures for 1 h under pH 6.5 and assaying the residue activities. (B) Searching for optimal catalytic pH (△) for recombinant pullulanase was done by assaying the purified enzyme in buffers of different pH (0.05 M HAc-NaAc for pH 4.0−6.0, 0.05 M sodium phosphate for pH 6.5−7.5 and 0.05 M Tris-HCl for pH 8.0−9.0) at a same temperature of 60 °C; influence of pH on stability of the enzyme (□) was investigated by incubating in different pH conditions at 60 °C for 1 h and assaying the residue activities.

Table 2. Relative Activity and Amylolytic Products of Purified PulA1 on Different Carbohydrate Compounds[a]

		products (%)			
substrate	relative activity (%)	DP_1[b]	DP_2	DP_3	DP_4
pullulan	100			99	
glycogen	37		68	30	
starch	49		65	27	2
amylopectin	52		67	29	
amylose					
maltoheptaose					
maltohexaose					
maltopentaose					
maltotetraose					
isomaltose					

[a]Relative activity was expressed as a percentage of the activity against pullulan, which was given a value of 100%. HPLC analysis of hydrolysis products formed after incubation of the purified pullulanase (10 U/mL) with 1% polysaccharides. After 12 h-incubation at 50 °C, samples were withdrawn and analyzed on an Agilent Zorbax NH_2 column for oligosaccharides. DP_1, glucose; DP_2, maltose; etc.

consuming of the process. Until very recently, several pullulanases have been characterized from aerobic and anaerobic strains with high pH value and elevated optimal temperatures. Most of them are not convenient for use in starch processing industry due to their extremely low yield. A thermostable pullulanase (up to 70 °C) worked at acid condition from *Bacillus acidopullulyticus* has been developed for commercial use in preparing high glucose and maltose syrup.[28] Here a novel type I thermostable pullulanase from a newly isolated strain *Bacillus* sp. CICIM 263 was cloned and expressed in *E. coli*, which works well at 70 °C and prefers alkaline and neutral conditions. The enzyme can completely hydrolyze pullulan efficiently as well as part of starch. When pullulan was used as a substrate, the main product was maltotriose. One might predict that the enzyme will be very helpful in starch debranching process.

Amylolytic enzymes are widely distributed among microorganisms and all of its members have different catalytic properties and structures, however, as they share similar substrates and modes of action, strong similarities exist in their amino acid sequences between substrate binding sites and catalytic sites.[29] Compared of PulA1 amino acid sequence with that of other starch degrading enzyme (Table 3) revealed that four regions (region I, II, III, and IV) have been characterized conserved in amylolytic enzymes catalyzing the hydrolysis of α-1,4-glucosidic bonds of amylase, amylopectin and related oligosaccharides.[30,31] The consensus sequence proposed for each region is aligned in Table 3. Besides the four typical amylolytic conserved regions, PulA1 pullulanase shares a TNWGYDP motif with other type I pullulanases originated from *Bacillus acidopullulyticus*, *Bacillus cereus*, *Lactobacillus iners LactinV*, and *Fervidobacterium pennivorans*. All the five regions are involved in the active site architecture within the catalytic domain of glycoside hydrolase family 13 enzymes, and like majority enzymes of that family, *B.* sp CICIM 263 pullulanase has an active site cleft found between domains A and B where a triad of catalytic residues (Asp509, Glu609, and Asp699) performs catalysis.[32−34] The third Trp of *Bacillus* sp. neopullulanase and *Thermoanaerobacter thermohydrosulfuricus* amylopullulanase (α-1,4 and α-1,6 activity) in region II were conserved in contrast to a common Phe of type I pullulanase and isoamylase (only α-1,6 activity) at the same position, which may be involved in the substrate binding catalysis.[35,36] The pattern of action against substrate for pullulanase is similar with that of isoamylase, which is supported by the fact that the Trp611 residue corresponds to the Trp479 of *Flavobacterium* sp. isoamylase and the Trp481 of *Pseudomonas* sp. isoamylase in region III, and both enzymes specifically hydrolyze α-1,6 glycosidic linkages.[23,37]

It has been proposed that, enhanced thermostability of *Bacillus* oligo-1,6-glucosidases could be gained by increasing the frequency of proline occurrence at β-turns and the total hydrophobic residues, which was referred to as the proline theory.[38] For pullulanase from *Bacillus* sp. CICIM 263, proline, phenylalanine, and leucine account for 13.65% (mol/mol) of the total amino acids, which may contribute to improving the potential of hydrophobic interaction and thus to enhancing thermostability. It was worthy noting that, addition of calcium strengthens the pullulanase both in catalysis and thermostability. Three calcium binding sites are found in catalytic domain, resembles three found in the A domain of pullulanase from

Table 3. Regions Conserved among Type I Pullulanases and Other Amylolytic Enzymes[a]

enzyme	YNWGYDP		region I		region II		region III		region IV	
	position	sequence	position	sequence	position	sequence	position	sequence	position	sequence
B. sp. pullulanase	461	YNWGYDP	503	QLRVVMDVVYNHV	575	DGFRFDLMGIHD	607	IGEGWDL	688	PEQAVTYAEAHDNHTLWDK
B. ac. type I pullulanase	504	YNWGYDP	545	RIGVNMDVVYNHT	617	DGFRFDLMALLG	649	YGEPWTG	725	PSETINYVTSHDNMTLWDK
B. ce. type I pullulanase	425	YNWGYDP	466	NLRVVMDVVYNHM	538	DGFRFDLMGIHD	570	HGEGWDL	651	PEQVLTYVEAHDNHTLWDK
L. la. type I pullulanase	139	YNWGYDP	180	GLRVSMDVVYNHV	251	DGFRFDLMGILD	283	YGEGWDM	369	PMQTINYVECHDNYTLFDK
Eu. re. type I pullulanase	187	YNWGYDP	228	GLGVIMDVVYNHT	300	DGFRFDLMGVLD	332	YGEGWTG	407	PMQSINYLSCHDNYTLWDR
F. pe. type I pullulanase	424	YNWGYDP	465	GIRVILDMVFPHT	538	DGFRFDQMGLMD	570	YGEPWGG	648	PQETINYVEVHDNHTLWDK
B. sp. neopullulanase			235	GIRVMLDAVFNHS	322	DGWRLDVANEVD	354	LGEVWHD	417	LLGSHD
Th. th. amylo-pullulanase			513	GIKVILDGVFNHT	623	DGWRLDVANEIA	655	IAELWGD	729	LLGSHD
F. sp. isoamylase			320	GIKVYIDVVYNHT	405	DGFRFDLASVLG	476	WAEWNGl	528	FVVAHD
Ps. sp. isoamylase			312	GIKVYMDVVYNHT	396	DGFRFDLASVLG	478	WSEWNGL	531	FIDVHD
B. am. amylase			131	GIKVIVDAVINHT	211	DGFRFDAAKHIE			304	WVESHD
B. lich amylase			122	DINVYGDVVINHK	254	DGFRLDAVKHIK			351	FVDNHD
B. lich maltogenic-amylase			232	GMKIMLDAVFNHI					410	LLDSHE

[a]Underlined amino acid residues are residues identified as being highly conserverd between amylolytic enzymes. Abbreviations and accession numbers of the sequences: *B.* sp. pullulanase, *Bacillus* sp. (this study); *B. ac.*, *Bacillus acidopullulyticus* (accession number 2WAN_A); *B. ce.*, *Bacillus cereus* (accession number YP_084052); *L. la.*, *Lactobacillus iners LactinV* (accession number ZP_07700625); *Eu. re.*, *Eubacterium rectale* (accession number CBK94658.1); *F. pe.*, *Fervidobacterium pennivorans* (accession number ZP_09162612.1); *B.* sp. neopullulanase, *Bacillus* sp. (accession number BAA02521); *Th. th.*, *Thermoanaerobacter thermohydrosulfuricus* (accession number AAA23201.1); *F.* sp., *Flavobacterium* sp. (accession number AB62256.1); *Ps.* sp., *Pseudomonas* sp. (accession number CAA00929.1); *B. am.*, *Bacillus amyloliquefaciens* (accession number ADH93703.1); *B. lich* amylase, *Bacillus licheniformis* (accession number CAA01355.1); *B. lich* maltogenic-amylase, *Bacillus licheniformis* (accession number CAA47612.1).

Klebsiella pnumoniae.[39] It is thought that these calcium sites are involved in constitution of an octahedral geometry interacting with water molecules or other amino acid residues nearby, which may contribute to the increase in the thermolstability of the enzyme.

The size of recombinant *Bacillus* sp. pullulanase (101 kDa) resembles typical type I pullulanase, and its monomeric quaternary structure is common among debranching enzymes described so far,[38,40−45] except for that of pullulanase from *Fervidobacterium pennivorans*[7] and *Geobacillus thermoleovorans*,[46] which have dimeric structures. The temperature and pH profiles of PulA1 and its excellent tolerance to thermal inactivation at temperatures up to 70 °C (after incubation at 70 °C for 1 h, 85% initial activity remained) are in agreement with the suitable growth conditions of *Bacillus* sp. CICIM 263.[47] To our knowledge, this enzyme is one of the few enzymes that are active at an alkaline pH (8.0−9.0) and a relatively high temperature (60−70 °C).

■ ASSOCIATED CONTENT

Supporting Information

Two additional figures and Table 1 are available. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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