

Gene Cloning and Characterization of a Type II Pullulanase Hydrolase from a Hyperthermophilic Archaeon, *Pyrobaculum calidifontis*

Masood Ahmed Siddiqui,^{1*} Habib-ur-Rehman¹ and Naeem Rashid²

¹Department of Chemistry, Biotechnology Research Laboratory, University of Balochistan, Quetta 87300, Pakistan

²School of Biological Sciences, University of the Punjab, Quaid-e-Azam Campus, Lahore 54590, Pakistan

Abstract.- The genome search of the hyperthermophilic archaeon *Pyrobaculum calidifontis* revealed the presence of an open reading frame, Pcal_1616, encoding for a type II pullulanase hydrolase. Pcal_1616 composed of 1006 amino acid residues with a molecular mass of 111 kDa including a 17-residue signal peptide. The amino acid sequence analysis revealed the presence of five conserved regions that are characteristic of GH57 family hydrolases. Pcal_1616 gene was cloned and expressed in *Escherichia coli*. The recombinant enzyme exhibited the highest activity at 95°C. The optimum pH of the enzyme activity was 5.5. However, Pcal-1616 exhibited more than 80% activity over a broad pH range (4.0-8.0). The metal ions including Ca²⁺ did not show a significant effect on the enzyme activity.

Key words: Archaea, hyperthermophile, *Pyrobaculum calidifontis*, type II pullulanase hydrolase.

INTRODUCTION

Starch is one of the most abundant polysaccharide present in nature and is a polymer of glucose residues which are linked together by α -1,4 and α -1,6 glycosidic bonds. For the industrial production of glucose enzymes are used especially in liquefaction and saccharification processes. For liquefaction process pH 6 is needed with high temperature around 105°C and for the next saccharification process pH 4.5 and a temperature of 60°C is required as described previously (Leveque *et al.*, 2000; Richardson *et al.*, 2002; Vieille and Zeikus, 2001). Such variations of parameters and specificity of enzymes for two steps cause time consuming and waste of cost in the industry (Linko and Wu, 1993). To date use of pullulanases for starch hydrolyzing industry are becoming interesting due to their increasing hydrolysis efficiency one step starch catalysis ability for liquefaction and saccharification processes and decreasing commercial cost.

Pullulan is a polymer of maltotriose linked by α -1,6 glycosidic bonds and a small number of α -1,4 glycosidic linked maltotriose units (Domań-Pytka

and Bardowski, 2004). Pullulanases (E.C. 3.2.1.41) are enzymes which degrade pullulan (a linear α -glucan that consists of repeating subunits of maltotriose linked by α -1,6 glycosidic linkages) in to different types of saccharides. Up till now five types of pullulanase enzymes have been reported according to Hii *et al.* (2012). First type of pullulanases belonging to type I, hydrolyses α -1,6 glycosidic bonds in pullulan and branched polysaccharides (Rudiger *et al.*, 1995; Kim *et al.*, 1996; Koch *et al.*, 1997; Bertoldo *et al.*, 1999; Ben-Messaoud *et al.*, 2002). Second type of pullulanases belonging to type II, also known as amylopullulanases (APUs) hydrolyses α -1,6 glycosidic bonds in pullulan and starch and also α -1,4 glycosidic linkage of starch, amylose and amylopectin (Mathupala *et al.*, 1993; Melasniemi, 1988; Ramesh *et al.*, 1994; Saha *et al.*, 1988; Spreinat and Antranikian, 1990). The third and fourth types are pullulan hydrolase I also called as neopullulanase and pullulan hydrolase II also known as isopullulanase hydrolyze α -1,6 glycosidic bonds in pullulan only (Duffner *et al.*, 2000; Niehaus *et al.*, 1999; Matzke *et al.*, 2000), while fifth type is pullulan hydrolase III has the ability to degrade α -1,4 and α -1,6 glycosidic bonds in pullulan (Niehaus *et al.*, 2000).

The enzymes involved in hydrolysis of glycosidic bonds in carbohydrates are called glycoside hydrolases (GH). Nearly 132 GH families

* Corresponding author: masoodbioeng@hotmail.com
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have been reported in carbohydrate active enzymes (CAZy) database, (Cantarel *et al.*, 2009). All the mesophilic APUs so far reported belong to GH13 family however, hyperthermophilic APUs may belong to GH13 or GH57 family such as APUs from *Pyrococcus furiosus* (Dong *et al.*, 1997) and from *Thermococcus hydrothermalis* (Erra-Pujada *et al.*, 1999, 2001), belong to GH57 family.

APUs enzymes discovered from hyperthermophiles are replacing the use of other amylolytic enzymes in starch industry. However, these enzymes are Ca²⁺ dependent for their activity and there is need to search for Ca²⁺ independent APUs. *Pyrobaculum calidifontis* strain VA1 by Amo *et al.* (2002) is a hyperthermophilic archaeon that was isolated from a terrestrial hot spring in the Philippines. Cells of the strain VA1 grow optimally at 90 to 95°C and pH 7.0 in atmospheric air as an obligate heterotroph. The complete genome sequence of *P. calidifontis* (Gen bank accession No. CP000561) has been reported. A number of *Pyrobaculum* species have been reported but neither a native nor a recombinant APU has been characterized from *Pyrobaculum* species. We report here cloning and characterization of Pcal_1616, a type II pullulanase hydrolase.

MATERIALS AND METHODS

Chemicals, restriction enzymes and reagents

All the chemicals used in this study were molecular biology or analytical grade and were purchased from Sigma (St. Louis, Mo., USA). Restriction endonucleases, *Taq* DNA polymerase, PCR cloning kit, deoxyribonucleic acid (DNA) extraction and ligation kits, protein markers and DNA markers were obtained from Fermentas Life Sciences (Hanover, MD, USA).

Growth condition of Pyrobaculum calidifontis

The hyperthermophilic archaeon strain *P. calidifontis* was cultured in 05-liter medium as previously described by Amo *et al.* (2002) containing 0.1 % yeast extract, 1.0 % tryptone and 0.5 % NaCl. The pH of the medium was adjusted to 7.0 with NaOH before autoclaving.

Host bacterial strains, plasmids and media

The *Escherichia coli* strains for DNA manipulations was DH5 α (*FsupE44* Φ 80 δ lacZ Δ M15 Δ (lacZYA-argF) U169 endA1recA1 hsdR17 (*rk,mk*⁺) *deoRthi-1* λ ⁻ *gyrA96 relA1*). Plasmid pTZ57R/T (Fermentas) was used as a cloning vector. pET-28a (Novagen, Madison, WI, USA) vector and *E. coli* BL21(DE3) *hsdS gal* (λ *clts857 ind1 Sam7 nin5 lacUV5-T7 gene1*) strain were used for gene expression. All *E. coli* strains were cultivated at 37°C.

Sequence analyses

GENETYX software (Genetyx Corporation, Tokyo, Japan) was used for amino acid sequence analyses. Homology search was obtained by using the Basic Local Alignment Search Tool (BLAST) program (Altschul *et al.*, 1990). Multiple sequence alignment was performed by using CLUSTALW program (Thompson *et al.*, 1994) by using the information of DNA Data Bank of Japan (<http://clustalw.ddbj.nig.ac.jp/top-e.html>).

Cloning of Pcal-apu gene

Based on the open reading frame sequence of *P. calidifontis* Pcal_1616 available by the complete genome sequence of *P. calidifontis* (GenBank accession No. CP000561). Forward primer Pcal_1616F:5'CCATGGATGATATTGCACAACCAC CAGC-3', and a reverse Pcal_1616R:5'-CTTGTAGC CATGGAGATCCTCGCCCTCCT-3' primer were designed for the amplification of Pcal_1616 gene. The amplification of genes was performed by polymerase chain reaction (PCR) using *Taq* DNA polymerase as follows: 3 min at 98°C; 30 s at 94°C, 30 s at 55°C and 1 min at 74°C (30 cycles) in a thermal cycler (Gene Amp PCR System 24000, Perkin Elmer, Foster, Calif). The resulting amplified PCR product for Pcal_1616 gene was ligated into pTZ57R/T vector to obtain pTZ- Pcal_1616 plasmid. *E. coli* DH5 α cells were transformed using pTZ- Pcal_1616 plasmid.

DNA sequencing

Specific restriction fragment cloned into pTZ57R/T and pET-28a expression vectors were sequenced on an automated DNA sequencer (Beckman Coulter 121 CEQ8000; Beckman Coulter Inc., Fullerton, CA).

Gene expression

For expression of Pcal_1616 gene, the cloned restriction fragment into pTZ57R/T plasmid was cleaved utilizing the *Nco*I and *Bam*HI restriction enzymes, gene cleaned and ligated in pET-28a expression vector using the same restriction enzymes *i.e.* *Nco*I and *Bam*HI. The resulting plasmid was designated as pET- Pcal_1616. The *E. coli* BL21 (DE3) cells were transformed with pET-Pcal_1616. Cells harboring recombinant vector were grown overnight in LB medium at 37°C ampicillin (100 µg/mL). The preculture was inoculated (1%) into fresh LB medium containing ampicillin. In the exponential phase of growth gene expression was induced by the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside and incubation was continued for further 16 h at 37°C. Cells were harvested by centrifugation at 5,000×g, 4°C for 10 min and washed with 50 mM Tris-HCl buffer (pH 8.0). The cell pellet was resuspended in the same buffer, and the cells were disrupted by sonication. Soluble and insoluble fractions were separated by centrifugation at 14,000 × g, 4°C for 15 min. The recombinant protein appeared in the soluble fraction. The expression was monitored by measuring optical density (OD) using spectrophotometer and by SDS-PAGE.

Enzyme assay

Pullulanase activity was determined by measuring the amount of reducing sugar released during enzymatic hydrolysis of 1% pullulan or soluble starch in 125 µL 50mM acetate buffer (pH 5.6) containing 0.5 mM Ca²⁺ with further addition of 125 µL diluted recombinant enzyme and incubation at 90°C for 15 min. A control without addition of enzyme was used. The amount of reducing sugar released was determined by the dinitrosalicylic acid method (Bernfeld, 1955). One unit of enzyme activity was defined as the amount of enzyme that released 1µmole of reducing sugars in one minute under standard assay conditions.

RESULTS AND DISCUSSION

Pcal-apu gene

During the genome sequence search of *P. calidifontis* an open reading frame (Pcal_1616)

coding for a putative pullulanase from GH57 family was identified. The gene consisted of 3018 nucleotides encoding a polypeptide of 1006 amino acid residues. Pcal_1616 (accession No. YP_001056498) was located on *P. calidifontis* chromosome at position 1505971-1508991 and was flanked by Pcal_1615 (accession No. YP_001056498) that code for molybdenum cofactor guanylyltransferase and Pcal_1617 (accession No. YP_001056500) coding for maltooligosaccharide binding protein.

Among the APU enzymes characterized from family *Thermococcaceae*, Pcal-APU exhibited highest homology (50%) with amylopullulanase from *P. furiosus* (Dong *et al.*, 1997) and *Pyrococcus yayanosii*, while 49% with APU enzyme from *Thermococcus kodakaraensis*, *Thermococcus barophilus* and 48% with pullulanase from *Thermococcus hydrothermalis* (Erra-Pujada *et al.*, 1999). Among the APU enzymes from family *Thermoproteaceae* to whom *P. calidifontis* belongs, displayed homology 85% with APU enzyme from *Pyrobaculum oguniense* and *Pyrobaculum arsenaticum*, 84% with *Pyrobaculum aerophilum* and 83% homology was found with APU from *Pyrobaculum islandicum*. All these enzymes from family *Thermoproteaceae* are uncharacterized.

The amino acids sequences of amylopullulanases belonging to GH57 family have been aligned in Figure 1. Pcal_1616 had four conserved motifs that were typically reported in almost all amylolytic enzymes of GH57 family (Zona *et al.*, 2004). Three conserved acidic residues crucial for catalytic activity of amylopullulanases were also present at positions 277 (Asp²⁷⁷), 376 (Asp³⁷⁶) and 374 (Glu³⁷⁴). Based on the APU enzymes amino acids sequences multiple alignment and presence of conserved Asp-Glu-Asp reported sequence, the single catalytic active site of Pcal_1616 has been postulated. However, some APU enzymes have been reported to have two different catalytic sites such as *Pyrococcus woesei* (Rudiger *et al.*, 1995), *Bacillus circulans* F-2 (Kim and Kim, 1995) and *Bacillus* sp. KSM-1378 (Hatada *et al.*, 1996) therefore further work such as mutational studies are required for the presence or absence of second catalytic active site of *P. calidifontis* APU enzyme.

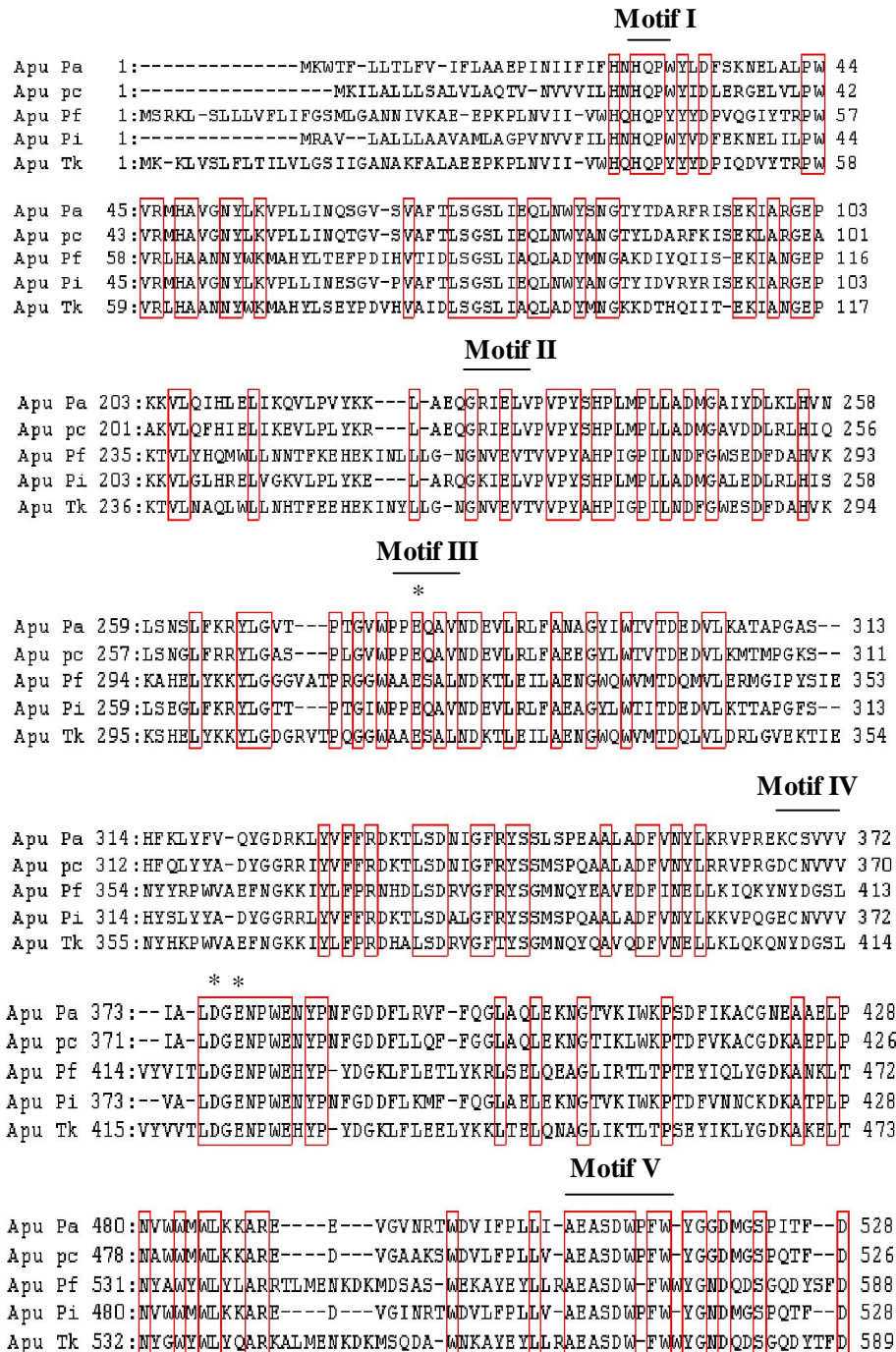


Fig. 1. Multiple sequence alignment of Pca1_1616 with other amylopullulanases of GH57 family members. Amino acid residues conserved in all the aligned sequences are boxed. The five conserved motifs I, II, III, IV and five of GH57 family amylopullulanases were lined. The catalytic amino acid residues are indicated by asterisks. Apu Pa, amylopullulanase *Pyrobaculum aerophilum* (accession no AE009441 [GenBank]); Apu Pc, amylopullulanase *Pyrobaculum calidifontis* (accession no NC_009073 [GenBank]); Apu Pf, amylopullulanase *Pyrococcus furiosus* (accession no NC_003413 [GenBank]); Apu Pi, amylopullulanase *Pyrobaculum islandicum* (accession no CP000504 [GenBank]); Apu Tk, amylopullulanase *Thermococcus kodakaraensis* (accession no NC_006624 [GenBank]).

A signal peptide of 17 amino acids was predicted using Signal P 3.0 software (Bendtsen *et al.*, 2004). The residues Gln¹⁵-Thr¹⁶-Val¹⁷ are also in accordance with the rule summarized from the statistical data as the signal-sequence cleavage site of exported proteins in prokaryotes (Simonen and Palva, 1993).

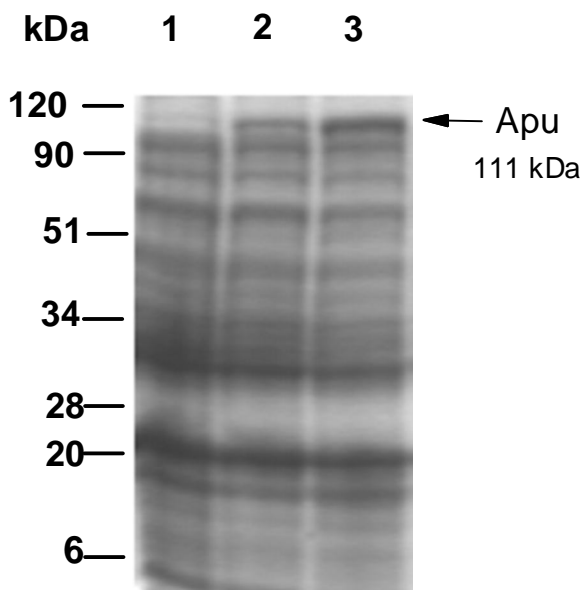


Fig. 2. CBB stained 12% SDS- PAGE demonstrating the production of recombinant Pcal₁₆₁₆ (APU) in *E. coli* BL21 (DE3) cells. Lane 1, APU-pET-8c in *E. coli* BL21 after 16 h with out induction; lane 2, APU-pET-8c in *E. coli* BL21 after 6 h with 1mM IPTG induction; APU-pET-8c in *E. coli* BL21 after 16 h with 1mM IPTG induction.

Heterologous expression of Pcal-apu gene

In order to know whether the Pcal₁₆₁₆ gene product exhibits the amylopullulanase activity, the encoding gene was expressed in to pET-28a vector utilizing the T7 promoter expression system. The resulting plasmid was introduced into mesophilic *E. coli* BL21 (DE3) cells for expression. Heterologous gene expression was achieved with 0.1 mM isopropyl- β -D-galactopyranoside in 16 h. According to SDS-PAGE analysis approximately 111kDa Pcal₁₆₁₆ protein was produced (Fig. 2) whereas the theoretically calculated average molecular mass of Pcal₁₆₁₆ determined by GENETYX software (Genetyx Corporation, Tokyo, Japan) without signal

peptide was 109830.38 Da. The molecular mass of Pcal-APU on SDS-PAGE was in good agreement with the theoretically calculated mass of the mature protein. Beside our continuous efforts, the amylopullulanase could not be purified from the *E. coli* culture the apparent reason might be due to low expression level of Pcal₁₆₁₆ gene. There are number of APU enzymes reported that could not be purified due to low gene expression or proteolytic effect (Duffner *et al.*, 2000; Chen *et al.*, 2001). The Pcal₁₆₁₆ exhibited the specific activity (7.3 U/mg) utilizing the crude extract.

Biochemical characterization of Pcal-apu gene

The pH dependent activity of Pcal₁₆₁₆ was measured in 50 mM sodium acetate; Tris-HCl and potassium phosphate buffers. Recombinant Pcal₁₆₁₆ enzyme exhibited maximum activity at pH 5.5 whereas the enzyme was highly active between the pH ranges 4.0-8.0 (Fig.3A). The enzyme showed more than 80% activity at pHs 4.0-8.0.

The recombinant APU enzyme from *P. calidifontis* did not show detectable activity at room temperature. Its activity was found directly proportional with temperature, up to the highest level at 100°C (Fig. 3B). Most of the enzymes belonging to the α -amylase family, including APUs, are Ca²⁺ dependent, but in the case of Pcal₁₆₁₆ the addition of 0.5 mM Ca²⁺ in the reaction mixture did not show a significant effect on the enzyme activity of recombinant Pcal₁₆₁₆.

Analysis of the end product

Pullulanases type II or amylopullulanases hydrolyse α -1,6 glycosidic bonds in pullulan to produce maltotriose as main product. APU acts on starch and hydrolyses α -1,6 glycosidic bonds of amylopectin and also hydrolyses α -1,4 glycosidic linkage of, amylose and amylopectin to produce maltohexaose, maltopentaose, maltotetraose, maltotriose, maltose, and glucose. In order to confirm the enzyme specificity as amylopullulanase 5 mg/mL pullulan, starch or amylopectin were mixed with 10 μ L Pcal₁₆₁₆ enzyme and incubated at 90°C and pH 5.5 for various time. The hydrolysis products were analyzed using thin layer chromatography (data not shown). The end products

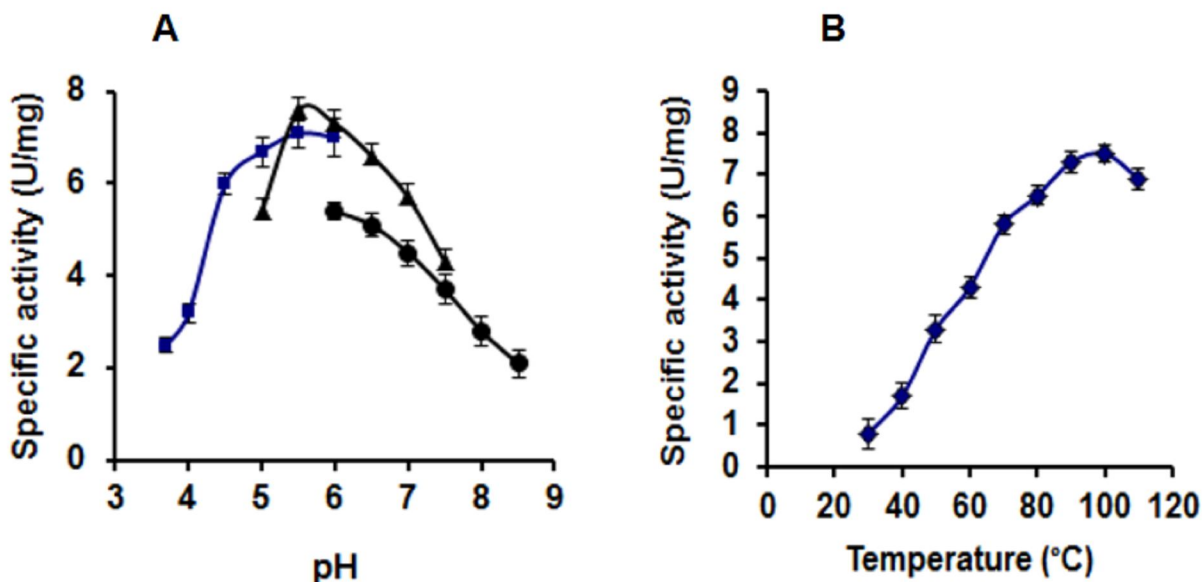


Fig. 3. Effects of pH and temperature on recombinant Pcal_1616 enzyme activity. A) Enzyme activity was assayed at 90°C in 50 mM sodium acetate (■), 50 mM Tris-HCl (▲) and 50 mM potassium phosphate (●). B) Enzyme activity was assayed in 50 mM sodium acetate (pH 5.6) at various temperatures. The parameters reported here are the means of three determinations.

maltotriose and maltose were found when pullulan was used as substrate while using starch or amylopectin as substrate main products were maltohexaose, maltopentaose, maltotetraose, maltotriose, maltose, and glucose.

In conclusion, we have characterized a pullulanase hydrolase type II from a hyperthermophilic archaeon, *P. caldifontis*. The primary structure of the protein displayed low similarity with those of previously characterized starch-degrading enzymes. As Pcal_1616 seems to be Ca²⁺-independent thermostable enzyme, therefore, it is a good candidate to replace Ca²⁺-dependent enzymes being used in starch industry.

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