Pullulanases are endo-acting enzymes capable of hydrolyzing α-1,6-glycosidic linkages in starch, pullulan, amylopectin, and related oligosaccharides, while amylopullulanases are bifunctional enzymes with an active site capable of cleaving both α-1,4 and α-1,6 linkages in starch, amylose and other oligosaccharides, and α-1,6 linkages in pullulan. The amylopullulanases are classified in GH13 and GH57 family enzymes based on the architecture of catalytic domain and number of conserved sequences. The enzymes with two active sites, one for the hydrolysis of α-1,4-glycosidic bond and the other for α-1,6-glycosidic bond, are called α-amylose-pullulanases, while amylopullulanases have only one active site for cleaving both α-1,4- and α-1,6-glycosidic bonds. The amylopullulanases produced by bacteria find applications in the starch and baking industries as a catalyst for one step starch liquefaction-saccharification for making various sugar syrups, as antistaling agent and in bread and as a detergent additive.

**Introduction**

Amylopullulanases (E.C. 3.2.1.1/41) form a class of debranching enzymes belonging to the family of glycoside hydrolases (GHs) organized in the sequence-based classification of carbohydrate-enzymes belonging to the family of glycoside hydrolases (GHs) of the GH-13, GH70, and GH77; these account for a small portion of enzyme specificities and forms a clan GH-H along with α-amylase family comprising more than 30 different enzymes.1 Among glycoside hydrolases, GH13 represents a large number of conserved sequences. The enzymes with two active sites, one for hydrolyzing α-1,4-glycosidic bond and the other for α-1,6-glycosidic bond, are called α-amylose-pullulanases, while amylopullulanases have only one active site for cleaving both α-1,4- and α-1,6-glycosidic bonds. The amylopullulanases produced by bacteria find applications in the starch and baking industries as a catalyst for one step starch liquefaction-saccharification for making various sugar syrups, as antistaling agent in bread and as a detergent additive.

**Keywords:** amylopullulanase, amylose-pullulanase, pullulan, starch, thermostability, sugar syrups, site directed mutagenesis

**Microorganisms Producing Amylopullulanases**

Amylopullulanase is produced by both aerobic and anaerobic bacteria, the latter being the highest producers.20 Among aerobes, certain species of *Bacillus* and *Geobacillus* are known to produce amylopullulanase, most of which are thermophilic. *Bacillus* sp. 3L83,23 *Bacillus* sp. TS-23,23 *Bacillus subtilis*,24 *Bacillus* sp. XAL 601,25 *Bacillus circulans* F-2,26 *Bacillus* sp. KSM-1378,27 *Bacillus* sp. DSM 405,28 *Geobacillus thermoleovorans* NP33,29 *Bacillus* sp. US149,30 *Geobacillus stearothermophilus* L434,31 *Thermotoga maritima*,32 *Clostridium thermohydrodsulfurosum*,33 *C. thermohydrodsulfurosum* Z 21–109,34 *Clostridium* sp. strain EM1 (now *Thermanaeroanaerobacter thermosulfurosum*),33,35 *Thermoaerobacter brockii*,36 *Thermanaeroanaerobacter T0k6-BI*,37 *Thermnaeroanaerobacter brockii* 39E,38 *Thermnaeroanaerobacter fumarii*,39,40 *Thermohalophilus aestuariivorus*,40,41 *T. ethanolicus*,40,42 *Thermotoga maritima*,40 and *Thermococcus profundus*40 are identified as producers of amylopullulanase. The GH57 family amylopullulanases have been produced from archaea *Pyrococcus furiosus*,40,43 *P. woesei*,43 *Thermococcus litoralis*,43 *T. oceanis*,43 *T. hydrothermalis*,43 and *T. isidii*.43

**Cloning and Expression of Amylopullulanases**

As the production levels and often the specific activity of the starch hydrolyzing enzymes achieved with the native hosts is
inadequate, the molecular cloning of the corresponding genes and their expression in a homologous and heterologous hosts, which are genetically modified, have opened up gates for improving the protein yield. Thus the higher yields would permit economic utilization of enzymes for biotechnological applications. A large number of amylopullulanase encoding genes from the bacterial genomes have been cloned, and their expression aspects have been investigated (Table 1). Dating back to 1987, Coleman et al. cloned the amylopullulanase gene from a thermophilic anaerobe, T. brockii, into Escherichia coli and Bacillus subtilis. Amylopullulanase genes have also been cloned from other thermophilic anaerobes including C. thermosaccharolyticum, T. ethanolicus, and expressed in E. coli. Among aerobes, the gene was cloned from Bacillus sp KSM-1378, Bacillus sp. strain XAL601, Bacillus stearothermophilus TS-23, and G. thermoleovorans NP39 and the expression was checked in E. coli. The amylopullulanase gene from Bacillus sp. KSM-1378 had also been expressed in B. subtilis. The amylopullulanase encoding gene from lactic acid bacterium Lactobacillus plantarum L137 had been cloned and expressed in E. coli and Bacillus subtilis. Coleman et al.13

Table 1. Cloning of amylopullulanase genes from various bacteria

| Organism                  | Molecular mass (kDa) | Host                  | Remarks                                                                 | Reference |
|---------------------------|----------------------|-----------------------|-------------------------------------------------------------------------|-----------|
| Bacillus sp. KSM-1378     | 211                  | E. coli and Bacillus subtilis | The N-terminal and carboxyl-terminal half of the enzyme constitutes the amylase and pullulanase domains, and are separated by a 35 amino acid sequence tandem repeat. | Hatada et al.15 |
| Bacillus sp. strain XAL601 | 220                  | E. coli               | The α-amylase-pullulanase has been overexpressed in E. coli and was found to be alkaline in nature. The enzyme has been found to adsorb strongly to crystalline cellulose (Avicel) and raw corn starch. | Lee et al.16 |
| Bacillus stearothermophilus | 223                  | E. coli               | The expressed gene products obtained were found degenerate with the largest active polypeptide of 220 kDa and the smallest one of about 105 kDa. | Chen et al.17 |
| Clostridium thermosaccharolyticum DSM 5783 | 165, 130, 100       | E. coli               | Immunoblotting has revealed more than ten α-amylase-pullulanase specific polypeptides. The largest polypeptide was found to have a molecular weight of about 165 kDa, while the smallest enzymatically active polypeptide was about 100 kDa. | Melenioni and Palkoheimo 18 |
| Geobacillus thermoleovorans NP39 | 182                | E. coli               | The 350 amino acid truncation from the C-terminus enhanced the production, specific enzyme activity, thermostability and starch saccharification efficiency. | Nishia and Satyanarayana20 |
| Lactobacillus plantarum L137 | 200                   | L. plantarum NCL127 | The N-terminal and C-terminal regions of the enzyme was found to possess amino acid sequence repeats. The truncation of the 100 amino acid repeat region of the C-terminus enhanced the production and specific activity of the enzyme. | Kim et al.21, Kim et al.22 |
| Thermoaerobacter ethanolicus 39E | 162                  | E. coli               | The 300 amino acid truncation from the C-terminus enhanced the production, specific enzyme activity, thermostability and starch saccharification efficiency. | Mathupala et al.23, Lin and Leu24 |
| Thermoaerobacter saccharolyticum B6A-R1 | 140                | E. coli               | Highly conserved amino acid residues of the protein have been identified by hydrophobic cluster analysis and multiple sequence alignment. | Ramesh et al.25 |
| Thermoaerobacter brockii | 70–100               | E. coli and Bacillus subtilis | Secretion of enzyme increased from 0.23 U/ml (in T. brockii) to 0.80 to 1.0 U/ml, when B. subtilis was used as an expression host. | Coleman et al.26 |

Table 2. Four-fold higher enzyme...
| Organism                  | Mol mass (kDa) | Purification strategy                                                                 | Opt. Tem (°C) | Opt. pH | Specific activity (U/mg) | Fold purification/ yield (%) | Inhibitors                          | Stabilizers | Additional properties                                                                 | Reference |
|--------------------------|----------------|----------------------------------------------------------------------------------------|---------------|--------|--------------------------|-------------------------------|-----------------------------------|-------------|---------------------------------------------------------------------------------------|-----------|
| Bacillus sp. KSM-1378    | 210            | Ultrafiltration, DEAE-cellulose (pH 8.0), α-cyclodextrin coupled with Sepharose 6B (pH 8.0), Sephacryl S-200 | 50            | 8.5    | 42.6                     | 203/11                       | Hg²⁺, Cd²⁺, Pb²⁺, and Mn²⁺ (pullulanase) | Co²⁺       | Km: pullulan 0.72 (mg/ml), amylose 0.27 V<sub>max</sub> pullulan 5.7 | Ara et al. |
| Bacillus circulans F-2   | 220            | (NH₄)₂SO₄ (80% saturation), starch adsorption, DIAION-type anion exchange chromatography (pH 8.0), hydrophilic interaction chromatography using TSK Gel Phenyl-SAX (pH 7.5) | 70            | 7.0    | 81.7                     | 167/4                        | Hg²⁺, Cd²⁺, Pb²⁺, and Mn²⁺ (α-amylase) | Co²⁺       | Km: pullulan 0.77 (mg/ml), soluble starch 0.53 V<sub>max</sub> amylase 0.56 | Sata et al.|
| Bacillus sp. DSS-405     | 126            | Corn starch adsorption (pH 6.0), hydrophilic interaction chromatography using phenyl-Sepharose Q-48 (pH 7.0), ultrafiltration and gel filtration | 70            | 6.5    | 11000                    | 1400                         | Hg²⁺, Cd²⁺, Pb²⁺, and Mn²⁺ (α-amylase) | Co²⁺       | Km: pullulan 0.81 (mg/ml), soluble starch 0.64 V<sub>max</sub> amylase 1.39 | Brunswick et al.|
| Lactobacillus amylophilus G6 | 90             | (NH₄)₂SO₄ (pH 6.0) and sepharose 5-200 column chromatography | 37            | 6.5    | 15.238                   | 1114                         | Cu²⁺, Ni²⁺, Ca²⁺, Fe³⁺, Ba²⁺, Zn²⁺, EDTA, SOD, and Tween 80 | Co²⁺       | Km: pullulan 0.48 (g/l), soluble starch 44 V<sub>max</sub> pullulan 44 (μmol/ml) soluble starch 80 min | Vishnu et al.|
| Geobacillus stearothermophilus L14 | 100          | (NH₄)₂SO₄, DEAE-Sephacore column (pH 9.0), DEAE-Sepharose column (pH 8.0), ultrafiltration and sephadex G-100 gel filtration column | 65            | 5.5    | 947                      | 100/10                        | Cu²⁺, Ni²⁺, Ca²⁺, Fe³⁺, Ba²⁺, Zn²⁺, EDTA, SOD, and Tween 80 | Co²⁺       | Km: pullulan 0.48 (g/l), soluble starch 44 V<sub>max</sub> pullulan 44 (μmol/ml) soluble starch 80 min | Zareian et al.|

Table 2. Characteristics of the native GH13 amylopullulanases.
production was achieved with the recombinant *L. plantarum* NCL21 harboring the *L. plantarum* L137 amylopullulanase gene than that of the native strain. In the recombinant *B. subtilis* containing *T. brockii* amylopullulanase gene, the enzyme production was 3.4–4.3-fold higher than that in the native host.

**Molecular Mass of the Recombinant Amylopullulanases**

The amylopullulanases of both GH-13 and GH-57 family vary in gene sequence and length. The protein is usually of a high molecular weight among other glycosyl hydrolases. The molecular mass of the protein ranges between 80 and 250 kDa, and furthermore, some are glycoproteins.

**Domain Architecture**

The amylopullulanases are multidomain proteins. GH13 amylopullulanases possess the cyclodextrin and pullulan degrading enzyme N-terminus domain, the (α/β)8 barrel core and the C-terminal region containing one α-amylase C-terminal all-β domain (AamyC), one or two fibronectin type III (FnIII) domains, and one putative carbohydrate-binding module 20 domain (CBM20) (Fig. 1).

An α-amylase catalytic domain present in the form of a barrel of eight parallel β-strands surrounded by eight α-helices is common to all members of the α-amylase family. These β-strands are linked to the adjacent α-helices by irregular structures in the form of loops. These loops carry the catalytic amino acid residues of the active site (for details see ref. 55). A distinct N-terminal domain is present preceding the (α/β)8 catalytic domain and the amino acid residues of the N-terminal domain may not form part of the active sites unlike that reported for isoamylase.

The AamyC is an all-β-domain of α-amylase present in the C-terminus. The possible functions of AamyC is in disrupting the starch granule and separating the α-glucan chains together with the other substrate binding site in the α-amylase catalytic domain. The domain is considered to secure proper orientation of the active site of the enzyme on the substrate chains. It has also been suggested that the AamyC domain stabilizes the (α/β)8 catalytic domain by shielding the hydrophobic residues of the domain from the solvent.

The fibronectin type III (FnIII) domain is a small folding unit of about 100 amino acid residues and possesses a seven-stranded β-sandwich structure. The β-sandwich structure of FnIII is similar to that of immunoglobulin domains. No specific role has been assigned to the FnIII domain. It is possibly involved in the binding of the enzyme and the polysaccharide substrates.

The carbohydrate-binding domain is composed of 40 to 200 amino acids and is characterized by a discrete fold that possesses carbohydrate binding property. The CBM facilitates the interaction between the insoluble substrate and the enzyme by bringing the substrate to the catalytic domain, and thereby improving the substrate hydrolysis. Currently CBMs are divided into 39 families based on the amino acid sequence similarity. The family 20 carbohydrate-binding module (CBM20) is also known as the
starch-binding domain and is found in a large number of starch hydrolyzing enzymes including α-amylase, β-amylase, glucoamylase, amylopullulanases, and CGTase (cyclodextrin glucanotransferase). CBM20 adopts an antiparallel β-barrel structure with two starch binding sites (SBS1 and SBS2) (for a review, see ref. 63). The two tryptophans and a lysine forms the evolutionary conserved SBS1, while SBS2 possesses a tryptophan residue (for a review, see refs. 61 and 62). These two sites are thought to differ functionally with SBS1 involved in the initial starch recognition site and SBS2 participate in the specific recognition of appropriate regions of starch.

Conserved Sequences

The short stretches of sequences are well conserved within the amylopullulanases of GH13 family. According to the definition proposed by Takata et al. the members of the α-amylase family contain four highly conserved sequences (I to IV). The conserved sequences I, II, III, and IV are located on strands β-3, β-4, β-5 and β-7, respectively. The catalytically important amino acid residues corresponding to two aspartic acids and one glutamate have been reported in α-amylase family enzymes (for a review, see ref. 5). The fifth conserved sequence region of the amylopullulanases is characterized by the presence of calcium binding aspartate and are located on loop 3 that protrudes from the (α/β)8 catalytic domain and connects the β-3 strand and the third α-helix. The cyclomaltooligosaccharases, maltogenic amylases, and neopullulanases, are unable to bind to the calcium ion as Asp is substituted by Lys in this region. The sixth conserved sequence region present on β-2 strand has been identified by the evolutionary conserved glycine and proline residues linked by seven or eight amino acid residues.

Amylopullulanase and other α-amylase family members have seven amino acid residues, while CGTases have eight residues separating glycine and proline residues. The seventh conserved sequence region is differentiated by a well conserved glycine residue at the start of the region followed by a proline residue. A single active site is responsible for the bifunctionality of the enzyme in case of T. ethanolicus 39E, T. thermohydrosulfuricum, C. thermohydrosulfuricum, Bacillus sp. strain XAL 601, and G. thermoleovorans NP33. Kinetic experiments on competitive inhibition with mixed substrates and chemical modification with different inhibitors were used by Brunovwick et al. to determine the number of active sites in the amylopullulanase of Bacillus sp. DSM 405. Both these approaches suggested the presence of a single active site for the dual hydrolytic activities.

Active Site and Catalytic Mechanism

The bacterial amylopullulanases from both GH13 and GH57 families possess one or two active sites for hydrolyzing α-1,4 and α-1,6-glycosidic linkages. A single active site is responsible for the hydrolytic activity. Dual hydrolytic activities associated with different active sites have been reported in α-amylase-pullulanase from Bacillus sp. KSM 1378. The partial hydrolysis of the enzyme with papain has revealed the presence of two functional domains for α-1,4- and α-1,6-hydrolytic activity. The amylose and pullulan-hydrolyzing polypeptides were visualized as a mixture of differently sized globular molecules joined by a thin short linker region under transmission electron microscope. The role of acidic amino acids at the active site has been shown by Mathupala and Zeikus for T. ethanolicus 39E amylopullulanase. The two conserved aspartate residues on strands β4 and β7 act as catalytic nucleophile and proton donor, respectively, and one β5-glutamate residue involved in the transition state stabilizer have been found to play a catalytic role in GH13 amylopulullanases. The catalytic residues were identified at the C-terminal
In *Bacillus* sp. KSM-1378 amylopullulanase, the putative catalytic triads were identified as Asp550-Glu579-Asp645 for the amylase activity and Asp1464-Glu1493-Asp1581 for the pullulanase activity. The amylopullulanases of *T*. *ethanolicus* 39E12 and *T*. *saccharolyticum* B6A-RI have Asp597-Glu626-Asp703 and Asp594-Asp700-Glu623 as catalytic triads, respectively for amylase and pullulanase activities.

Site directed mutagenesis of the three catalytic amino acid residues to amides has led to complete loss of both α-amylase and pullulanase activities.

Hydrophobic cluster analysis (HCA) is a 2-D illustration of the amino acid sequences of the protein and is used for comparing the shapes and relative location of hydrophobic clusters in proteins. The shape of hydrophobic clusters predicts the secondary structure of the protein and the similarity of the clusters suggests the similarity in the polypeptide folding of the proteins.

Figure 2. The sequence alignment of the α-amylase catalytic domain, (α/β)8 barrel of amylopullulanases of selected GH13 family members. The multiple sequence alignment of the amino acid sequence of protein was generated using the software, ClustalW2 of the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The seven conserved regions around the strands β-2, β-3, β-4, β-5, β-7, β-8, and on loop 3 are highlighted in boxes with different colors. Numbers on the left side of the amino acid sequence indicate amino acid positions of the sequences. The catalytic triad residues, 2Asp and 1Glu, are marked with a red star.

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| Protein                  | Accession | Sequence Length |
|-------------------------|-----------|-----------------|
| G. thermoleovorans NP33 | 443       |                 |
| G. kaustophilus MTHA246 | 453       |                 |
| G. stearothermophilus   | 525       |                 |
| Bacillus sp. XAL061     | 521       |                 |
| G. thermoleovorans NP33 | 502       |                 |
| G. kaustophilus MTHA246 | 512       |                 |
| G. stearothermophilus   | 584       |                 |
| Bacillus sp. XAL061     | 580       |                 |
| G. thermoleovorans NP33 | 562       |                 |
| G. kaustophilus MTHA246 | 571       |                 |
| G. stearothermophilus   | 643       |                 |
| Bacillus sp. XAL061     | 639       |                 |
| G. thermoleovorans NP33 | 620       |                 |
| G. kaustophilus MTHA246 | 630       |                 |
| G. stearothermophilus   | 702       |                 |
| Bacillus sp. XAL061     | 698       |                 |
| G. thermoleovorans NP33 | 679       |                 |
| G. kaustophilus MTHA246 | 689       |                 |
| G. stearothermophilus   | 761       |                 |
| Bacillus sp. XAL061     | 757       |                 |
| G. thermoleovorans NP33 | 738       |                 |
| G. kaustophilus MTHA246 | 748       |                 |
| G. stearothermophilus   | 820       |                 |
| Bacillus sp. XAL061     | 816       |                 |
| G. thermoleovorans NP33 | 797       |                 |
| G. kaustophilus MTHA246 | 879       |                 |
| G. stearothermophilus   | 875       |                 |
| Bacillus sp. XAL061     | 856       |                 |
| G. thermoleovorans NP33 | 866       |                 |
| G. kaustophilus MTHA246 | 938       |                 |
| G. stearothermophilus   | 934       |                 |
and pullulanase activities, suggesting a single active site for the dual catalytic activity in T. ethanolicus 39E. The three residues are located in close proximity with each other, forming a single active site for the dual activities in both T. ethanolicus 39E and T. saccharolyticum B6A-R1, in contrast to the dual active sites for the α-amylase-pullulanase of alkaline amylopullulanase from Bacillus sp. KSM-1378. The GH3 amylopullulanases have also been found to contain two histidine residues that are critical for the transition state stabilization.

It was also noted that the active site of an enzyme is made up of subites, each capable of binding to a monosaccharide residue. The subites are the amino acid side chains present in the loops of the enzyme structure that links the C-terminal ends of β-strands to the N-terminal ends of the adjacent α-helices of the catalytic domain. The catalytic activity of an enzyme requires the interaction of a glucose residue of the substrate to the -1 subsite. The different enzyme specificities vary with the nature of the substrate portion binding onto the subites +1 and +2. The three catalytic triads, 2 Asp and 1 Glu as well as the 2His residues involved in the transition state stabilization have been reported to occupy the subite -1, while the amino acid residues of the conserved sequence regions III and IV on strands β-4 and β-5, respectively, constitute subites +1 and +2. The flexible nature of the subite +1 at the active site of the amylopullulanases or in an α-amylase-pullulanase might be responsible for the action of the enzyme on more than one type of linkage. It has also been shown that the enzymes having amino acid sequence VANE at the C-terminal end of the amylopullulanase activity was attained in the amylopullulanase from T. saccharolyticum B6A-R1 on incubation at 65 °C for 1 h. The α-amylase-pullulanase from C. thermohydrosulphuricum DSM 3793 retained 60% of the enzyme activity, even after 2 h at 85 °C.

The L. plantarum L137 amylopullulanase was found stable at pH 2.5–6.5 and was reported to be less stable above neutral pH in comparison with its C-terminal truncated variant. The truncated amylopullulanase of G. thermoleovorans NP33 had been shown to have high pH stability than the full-length variant. The effect of metal ions. The amylopullulanases from different bacterial sources have shown diverse behavior toward metal ions for their activity. Some enzymes require metal ions, some are inhibited by metal ions and some are unaffected by their presence. The α-amylase and pullulanase activities of the T. ethanolicus 39E were found to be stimulated by Ca2+, Mn2+, Ba2+, while Hg2+, Ni2+, Zn2+, and Fe2+ were strong enzyme inhibitors. Both α-amylase and pullulanase activities of the L. plantarum L137 amylopullulanase were strongly inhibited by EDTA, N-bromosuccinimide and γ-cyclodextrin; the γ-cyclodextrin and Cu2+ ions inhibited both α-amylase and pullulanase activities of the L. plantarum L137 amylopullulanase, while Co2+ ions stimulated the activity. Zn2+ stimulated the enzyme activities of G. thermoleovorans NP33 amylopullulanase, while the enzyme activity was almost completely lost in the presence of Hg2+ and Cu2+ ions. A moderate loss of the enzyme activity was observed by Fe2+ and Mg2+, while Co2+, Mn2+, and Ca2+ ions did not affect the enzyme activity.

Effect of inhibitors. EDTA, N-bromosuccinimide and α-cyclodextrin strongly inhibited the amylopullulanase activity in T. ethanolicus 39E. The inhibition by EDTA suggested that either the enzyme is metal dependent or the EDTA is changing the conformation of the protein instead of acting as a chelating agent. Enzyme inhibition by N-bromosuccinimide suggests the involvement of tryptophan residues in the catalytic activity. Cyclodextrins act by forming inclusion complexes between aromatic amino acid residues of amylopullulanase and cyclodextrin. The L. plantarum L137 amylopullulanase was strongly inhibited by N-bromosuccinimide, guaianide-HCl, and urea and moderately by β-cyclodextrin and γ-cyclodextrin; the enzyme activity was unaffected by EDTA and β-cyclodextrin. Both α-amylase and pullulanase activities of G. thermoleovorans NP33 have been significantly affected by EDTA, EGTA,
| Organism | Molecular mass (kDa) | Purification strategy | Opt. Tam (°C) | Opt. pH | Specific activity (U/mg) | Fold purification/yield (%) | Inhibitors | Stabilizers | Additional properties | Reference |
|----------|----------------------|-----------------------|----------------|---------|-------------------------|-----------------------------|------------|-------------|------------------------|-----------|
| Lactobacillus plantarum L137 | 215.6 | Ultrafiltration, DEAE-Sepharose CL-4B and superose 6 | 40–45 | 4.0–4.5 | 431 | 1.5/25 | Hg²⁺, Cu²⁺, N-bromosuccinimide, guanidine-HCl, urea, moderately by α-cyclodextrin, γ-cyclodextrin | Co²⁺ | K₅ pullulan 6.9 (g/l) soluble starch 7.7 amylose 2.5 V₅₀ pullulan 3.79 (U/mg) soluble starch 53.4 amylose 32.3 | Kim et al. 35,54 |
| Lactobacillus plantarum L137 (C-terminal truncated) | 150–250 | Ultrafiltration, DEAE-Sepharose CL-4B and superose 6 | 40–45 | 4.0–4.5 | 596 | 1.5/29 | Hg²⁺, Cu²⁺, N-bromosuccinimide, guanidine-HCl, and urea, moderately by α-cyclodextrin and γ-cyclodextrin | Co²⁺ | K₅ pullulan 2.6 (g/l) soluble starch 5.0 amylose 3.3 | Kim et al. 54 |
| Thermoanaerobacterium saccharolyticum B6A-RI | 142 ± 2 | Heat treatment of the recombinant E. coli cells, Q-sepharose, and β-cyclodextrin-coupled sepharose affinity chromatography | 65 | 6.0 | 498 | 17/15.2 | Nd | Nd | K₅ pullulan 0.40 (mg/ml) soluble starch 0.43 | Ramswish et al. |
| Thermoanaerobacter ethanolicus 39E | 169 (thermostable region) | Ni-nitrotoxycarbonyl affinity purification using His-bond resin | 90 | 6.0 | Nd | Nd | EDTA, N-bromosuccinimide, and α-cyclodextrin | Cu²⁺, Mn²⁺, Ba²⁺ | K₅ pullulan 3.79 (mg/ml) soluble starch 1.38 V₅₀ pullulan 90 μmol/min/mg | Mathupala et al. 11 |
| Bacillus sp. KSM-1278 | 210 | DEAE-cellulose, affinity chromatography on Sepharose 6B-α-cyclodextrin and gel filtration on sephacryl S-200 | 50 | 9.5 | 47 (for soluble starch) 84 (for pullulan) | Nd | Nd | Nd | Nd | Hatada et al. 16 |
| Bacillus sp strain XAL601 | 225 | Ammonium sulfate precipitation, mono S HRP/5, superose 20HR 10/30 | 70 | 90 | 56.7 U/ml (for soluble starch) 67.3 U/ml (for pullulan) | 6.6 | Nd | Nd | Nd | Lee et al. 13 |
| Geobacillus thermodenitrificans NFP33 | 182 | Nickel NTA affinity chromatography | 60 | 70 | 851 (for soluble starch) 975 (for pullulan) | Nd | Hg²⁺, Cu²⁺, EDTA, EDTA, N-bromosuccinimide, guanidine-HCl, and EDAC | Zn²⁺ | K₅ pullulan 3.3 mg/ml soluble starch 0.833 V₅₀ pullulan 640 soluble starch 666.4 | Nisha and Satyanarayana 66 |
| Geobacillus thermodenitrificans NFP33 (C-terminal truncated) | 190 | Nickel NTA affinity chromatography | 60 | 70 | 1200 (for soluble starch) and 1169 (for pullulan) | Nd | Hg²⁺, Cu²⁺, EDTA, EDTA, N-bromosuccinimide, guanidine-HCl, and EDAC | Zn²⁺ | K₅ pullulan 2.8 mg/ml soluble starch 0.598 V₅₀ pullulan 1192 soluble starch 133.2 | Nisha and Satyanarayana 66 |
The action of amylopullulanases of _T. ethanolicus_ 39E, _Bazillia sp._ strain XAL601, and _G. thermoleovorans_ NP33 on starch, amylase, amylopectin and glycogen liberated maltose, maltotriose, and maltotetraose as the end products. The amylopullulanase of _T. plantarum_ L137 hydrolyzed amyllose to maltotriose, maltotetraose, and maltopentaose. Maltose or glucose was not detected in amyllose hydrolysate. _T. saccharolyticum B6A-R1_ amylopullulanase efficiently degraded starch, amylase, amylopectin, glycogen, and pullulan. The action of most of the bacterial amylopullulanases on pullulan forms maltotriose as the only hydrolysis product unlike that reported for the native amylopullulanase from _G. stearothermophila_ L14 that produced glucose from pullulan hydrolysate.

### Circular Dichroism and Fluorescence Spectrometry

The effect of truncation and the comparative analysis of the secondary structures of some amylopullulanases have been studied by using circular dichroism spectroscopy and fluorescence spectroscopy studies. The circular dichroism spectroscopy is based on the proteins' unequal absorption of right- and left-handed circularly polarized light, while fluorescence spectroscopy measures the intrinsic fluorescence generated from aromatic amino acids such as tryptophan, phenylalanine, and tyrosine. The secondary structural analysis of the amylopullulanase from _T. ethanolicus_ 39E has been made and compared with that of its C-terminal truncated mutant (with deletion of 100 amino acids from C-terminus) using fluorescence emission and CD spectroscopy.1 The enzymes have been found to exhibit similar fluorescence spectra upon denaturation with urea and renaturation. The comparative analysis of the secondary structures of full-length amylopullulanase and its C-terminal truncated mutant using far-UV CD spectroscopy has also revealed the CD spectra of equal intensity. Both the enzymes exhibited identical secondary structure. The fluorescence and circular dichroism spectrometric methods have also revealed highly indistinguishable structure for the full-length amylopullulanase from _T. saccharolyticum_ and its C-terminal truncated mutant. An identical active conformation was attained for both enzymes on fluorescence spectra. A similar thermal unfolding and a one-step melting curve were observed upon far-UV CD measurements. The truncation experiments on amylopullulanase from _T. ethanolicus_ 39E suggested that a large part of the C-terminal carbohydrate-binding module family 20, a portion of the first fibronectin III motifs and the second fibronectin type III could be deleted without causing a significant change in the structure and action of the enzyme on soluble starch and pullulan,2 and a similar experiment on _T. saccharolyticum_ NTU01 amylopullulanase revealed non-essentiality of the C-terminal fibronectin domains of calcium-dependent GH3 amylases.18

### Applications of Amylopullulanases

Amylopullulanases are one of the emerging enzymes for use in the industrial starch processing industry. Because of the enzyme's debranching ability, bifunctionality as well as the calcium independence in some will make it extremely useful for the current starch conversion process (Fig. 3). The enzyme can be used as a catalyst for one step starch liquefaction-saccharification process, and therefore, can replace other amylosytic enzymes like α-amylases and β-amylases. Besides its application in the starch industry for the production of various sugar syrups including maltose, maltotriose, and maltotetraose and as anti-stale in baking, the amylopullulanase and α-amylase-pullulanase find application in the detergent industry. The addition of the enzyme in starch liquefaction process has been found to increase the yield of maltose, thereby reducing the amount of branched oligosaccharides. The α-amylases employed in the current starch conversion process are calcium dependent and does not act at a pH below 5.9. Therefore, the process requires the addition of calcium and the pH adjustment to that of the starch slurry (pH 4.5). The reverse production such as maltose and isomaltose formed by glucoamylase at the expense of glucose need to be minimized. The amylopullulanases would, therefore, prove advantageous in the starch conversion process as it increases the production of maltose, reduces the reaction time, allows an increase in substrate concentration, and limits the use of glucoamylase, thereby making the process economical. Maltose and maltoligosaccharide syrups are employed in food, beverage, pharmaceutical, and chemical industries. Maltose-containing syrups are used in the baking, soft drink, canning, confectionery, and other food industries. Maltotriose syrup has been reported to have low freezing point depression and solution viscosity, good heat stability, mild sweetness, keeps in moisture, prevents the starch retrogradation in foodstuffs, and forms less color as compared with maltose, glucose, and sucrose syrups. These properties are very important in food and pharmaceutical industries. High maltotriose syrup also finds application in the food industry in making desserts and in baking and brewing. The maltose and maltotriose syrups are being used in pharmaceutical industry as a substitute for glucose in intravenous feeding. Transgenic rice seeds containing a thermostable and bifunctional amylopullulanase from _T. ethanolicus_ 39E enzyme have also been generated, which would facilitate the industrial production of sweeteners and fermentation products. The granule-bound amylopullulanase activity has also been found to be associated with the reduction of amylase in developing transgenic rice
The enzyme has also been used in the production of slowly digestible starch. Slowly digestible starch has an impact on human health, as it is associated with a low glycemic index for the treatment and prevention of various diseases, such as cardiovascular diseases, non-insulin diabetes, obesity, and provides sustained and stable energy for athletes. Amylopullulanase has also been found to increase the resistant starch content. During starch hydrolysis process, the gelatinized starch may revert to a form that is highly resistant to α-amylase hydrolysis and is called resistant starch. The resistant starch has potential application in the food industry and has attracted much attention of the nutritionists as it causes reduced levels of plasma glucose and insulin, increased fecal bulk, and short-chain fatty acid (SCFA) production through fermentation in the large intestine. RS has been produced by a heating-cooling process and chemical modification. However, the chemical modification may not be safe, and the heating-cooling process alone may lower the RS content due to the structure of starch.

The use of amylopullulanase in the alcohol and brewing industries along with glucoamylase can increase the amount of fermentable sugars and may facilitate filtration steps. Amylopullulanases also find use in the production of low carbohydrate (low calorie) “lite beer.” The enzyme can be added with fungal α-amylase or glucoamylase to the wort during fermentation instead of pullulanase.

The enzyme has also been used in the production of slowly digestible starch. Slowly digestible starch has an impact on human health, as it is associated with a low glycemic index for the treatment and prevention of various diseases, such as cardiovascular diseases, non-insulin diabetes, obesity, and provides sustained and stable energy for athletes. Amylopullulanase has also been found to increase the resistant starch content. During starch hydrolysis process, the gelatinized starch may revert to a form that is highly resistant to α-amylase hydrolysis and is called resistant starch. The resistant starch has potential application in the food industry and has attracted much attention of the nutritionists as it causes reduced levels of plasma glucose and insulin, increased fecal bulk, and short-chain fatty acid (SCFA) production through fermentation in the large intestine. RS has been produced by a heating-cooling process and chemical modification. However, the chemical modification may not be safe, and the heating-cooling process alone may lower the RS content due to the structure of starch.

The enzyme has also been used for the production of branched cyclodextrins. Branched cyclodextrins possess one or more saccharide chains such as glucose, maltose, and other saccharides linked to cyclodextrins by an α-1,6 bond. The branched cyclodextrins are more soluble in water and organic solvents than cyclodextrins which have no branches and thus are likely to form more soluble inclusion complexes with various chemicals.
Cyclodextrins are used in the food, cosmetic, pharmaceutical, and plastic industries as emulsifiers, antioxidants, and stabilizing agents. Ara et al. reported the applicability of an alkaline pullulanase from alkaliphilic Bacillus sp. KSM-1876 in dishwashing and laundry detergents.

Conclusions

Amylopullulanases are receiving considerable attention as bifunctional and debranching enzymes for the industrial starch saccharification process. Attempts have been made in cloning amylopullulanase encoding genes and overexpressing them for attaining high enzyme yield and to characterize the proteins. The truncation experiments on many amylopullulanases enabled to enhance specific enzyme activity and thermostability.

The primary structures of bacterial amylopullulanases revealed the domain architectures of most of the amylopullulanases. The tertiary structure of the enzyme needs to be studied for understanding the catalytic mechanism and possible role of different domains and demarcation from other amyloolytic enzymes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Castedo RL, Guimaraes PM, Caramelli C, Bernard T, Leendert V, Hanstein B. The Carbohydrate-Active Enzymes database (CAzDB): an expert resource for Glycogenomics. Nucleic Acids Res 2009; 37(Database issue):D228-36. PMID:19383991. http://dx.doi.org/10.1093/nar/gkp960.

2. Janowska S. Amylobranch families of glycocalyx hydrolases from the family 8-57. Biologia (Bratisl) 2005; 60:491-100. PMID:16297919.

3. Menendez V, Romanos M, Handoa W, Kakolate M. Structure and possible catalytic residues of Taka-amylo-1,4 pullulanase from Bacillus sp. KSM-1378. Biochim Biophys Acta 1995; 1243:315-22. PMID:8798645.

4. Noorwali F, Fogarty WM. The amylopullulanase of Bacillus sp. DSM 405. Appl Microbiol Biotechnol 1999; 51:176-9. PMID:10019322. http://dx.doi.org/10.1007/s002530051378.

5. Sato H, Umeda M, Kim C, Hirotsu J, Kobayashi T, et al. Proteinase and molecular structure of an alkaline amylopullulanase from Bacillus sp. B876. J Protein Chem 1989; 8:183-94. http://dx.doi.org/10.1007/BF00164533.

6. Hada Y, Igarashi K, Onishi K, Hitomi J. Structural and possible catalytic residues of Taka-amylo-1,4-pullulanase from Bacillus sp. KSM-1378. Biochim Biophys Acta 1995; 1243:315-22. PMID:8798645.

7. Kim CH, Kim YS. Substrate specificity and detailed characterization of an alkaline amylo-1,4 pullulanase enzyme from Bacillus circulans F-2 having two different active sites on one polypeptide. Eur J Biochem 1995; 227:687-93. PMID:7532585. http://dx.doi.org/10.1111/j.1432-1033.1995.tb20189.x.

8. Janecek S. The α-amylase family: structural similarity and common catalytic mechanism. Wei Sheng Wu Xue Bao 2011; 51:21-8; PMID:21867052. http://dx.doi.org/10.1007/s11239-010-9135-8.

9. Hatada Y, Saeki K, Ito S. An alkaline amylo-1,4 pullulanase with both α-1,4 and α-1,6 transglycosylation catalyzed by a thermostable and Ca2+ independent amylopullulanase by an extreme thermophile. J Biochem (Tokyo) 1994; 116:1633-42. PMID:7645492.

10. Imaoka T, Takekoshi S, Kusunoki M, Harada W, Kakudo M. Sequence analysis of the aapT gene and characterization of its product, alpha-amylase family. Biologia (Bratisl) 2005; 60:1644-51; PMID:16566734. http://dx.doi.org/10.1271/bbb1961.51.9.

11. Reddy GV, Viskhetti P, Mallik V, Vedula S. Amylopullulanase -a novel enzyme of Bacillus sp. AMD33. Trends Biotechnol 1989; 7:23-6. http://dx.doi.org/10.1016/0167-7799(89)90013-9.

12. Mathupala SP, Lo KM, Chong VW, Bhattacharya R, Maitreyee Y. Purification of α-amylase-pullulanase bifunctional enzyme by high-performance ion-exchange and hydrophobic-interaction chromatography. J Chromatogr A 2003; 980:293-300. http://dx.doi.org/10.1016/S0021-9673(01)89479-6.

13. Noorwali F, Kusumawardana D, Reddy G. Amylopullulanase -a novel enzyme of Bacillus sp. GV6 in direct fermentation of starch to L (+)-arabinose. Enzyme Microb Technol 2010; 46:57-63. http://dx.doi.org/10.1016/j.enzmictec.2009.09.012.

14. Chen JT, Chen MC, Chen LL, Chu WS. Subtilisin-like α-amylase-pullulanase bifunctional α-amylase-pullulanase from an α-amylase. Biotechnol Lett 2004; 26:419-24. PMID:15023681. http://dx.doi.org/10.1023/B:BIOTE.0000024313.85089.b0.

15. Lee SP, Morikawa M, Lee KY, Lee HK. Purification and characterization of thermostable pullulanase and glucoamylase entrapped into immobilized starch. J Ferment Bioeng 1996; 82:240-5. PMID:8798645. http://dx.doi.org/10.1016/0021-9797(89)90013-9.
42. Rodiguez A, Ingenzael J, Anturian, G, Isolation and characterization of a thermophilic amylopulullanase from Thermotoga maritima. Starch 1999; 51:135-42; PMID:10218582.

41. Takata K, Kikuchi T, Okada S, Takeda Y, Iienka M, Miyazaki N, et al. Action of neopolysaccharase. Neopolysaccharase catalyze both hydrolysis and transglycosylating. FEBS Lett 1992; 300:25-30; PMID:1264784.

40. Southall SM, Simpson PJ, Gillett HJ, Wilkinson G, Wilkinson MP. The crystal-binding domain from glycoconjugate Xyloglucan the trans-4 xylolation. FEBS Lett 1999; 446:6-10; PMID:10218582.

43. Naito M, Tsuchiya H, Kaizumi T. Characterization of two truncated amylases from Thermomonospora fusca. J Bacteriol 1997; 179:290-8; PMID:9052429.

39. McMillen RI. The starch-binding domain from gels. Carbohydr Polym 1999; 39:589-95; PMID:10322035.
80. Shiur SL, Kim HJ, Ha HJ, Lee SH, Moon TW. Effect of hydrothermal treatment on formation and structural characteristics of slowly digestible waxy maize sweet potato starch. Starch 2005; 57:421-30. http://dx.doi.org/10.1002/star.200400577.
81. Wold EW, Baez LL, Faley GC Jr. Effects of chemical modification on in vitro and in vivo digestibility of food starches: an attempt to discern a slowly digested starch. J Agric Food Chem 1999; 47:6178-83. PMID:10523787. http://dx.doi.org/10.1021/jf980316q.
82. Illman GC. Starch in food: structure, function and applications. Woodhead Publishing Limited. Cambridge, 2006: 477-905.
83. Zhang H, Jia Z. Preparation of starch starch by hydrolysis of maize starch with pullulanase. Carbohydr Polym 2011; 83:865-7. http://dx.doi.org/10.1016/j.carbpol.2010.08.066.
84. Annison G, Tipping DL. Nutritional role of resistant starch. Chemical structure vs. physiological function. Anna. Rev. Nutr 1996; 14:247-320. PMID:7946522. http://dx.doi.org/10.1146/annurev.nu.14.070194.001501.
85. Mac SH, Shi M. Mild hydrolysis of resistant starch from maize. Food Chem 2006; 96:115-21. http://dx.doi.org/10.1016/j.foodchem.2005.02.015.
86. Weszka N, Yasumura K, Tanaka W, Ohba T, Kobayashi S. A Novel Method to Produce Branched α-cyclodextrins: pullulanase-α-glucosidase-mixed method. J Ferment Bioeng 1997; 83:43-47. http://dx.doi.org/10.1016/S0922-338X(97)87325-4.
87. Bruch D, Pilley M, El-Effahbghi D, Reiglje MA, Abdill A. Studies on the Schardinger dextrins. XII. The molecular size and structure of the δ, ε, ζ, and η dextrins. Arch Biochem Biophys 1965; 111:155-60. PMID:6394862. http://dx.doi.org/10.1016/0003-9861(65)90134-6.
88. Ara K, Iizuki K, Saki K, Kamii S, Ito S. Purification and some properties of an alkaline pullulanase from alkalophilic Bacillus sp. KSM-1976. Bior Technol Biotechnol Biochem 1992; 56:46-5. http://dx.doi.org/10.1271/bbb.56.62.