Review Article

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Archaeal and bacterial thermostable amylopullulanases: characteristic features and biotechnological applications

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Abstract: Amylopullulanases are endoacting bifunctional enzymes capable of hydrolyzing α -1,4- and α -1,6-glycosidic linkages in starch, amylose, pullulan, amylopectin and related oligosaccharides. These enzymes possess single or dual active site(s) for cleaving α -1,4- and α -1,6-glycosidic bonds; the former are called amylopullulanases, and the latter, α -amylase-pullulanases. These are grouped into GH13 and GH57 families based on the architecture of the catalytic domain and the number of conserved sequence regions. The amylopullulanases/ α -amylase-pullulanases are produced by bacteria as well as archaea, and among them, thermophilic and hyperthermophilic species are the major producers. The thermostable amylopullulanases find application in one-step starch liquefaction-saccharification to form various sugar syrups and maltooligosaccharides. The starch saccharification process catalysed by amylopullulanases minimizes the use of other amylolytic enzymes, like α -amylase and glucoamylase, thereby reducing the cost of sugar syrups. The enzymes also find applications in bread making as an anti-stale and as a detergent additive.

Keywords: amylopullulanase; α -amylase-pullulanase; starch saccharification; anti-stale; maltooligosaccharides

Abbreviations: CSR, conserved sequence region; DP, degree of polymerization; FnIII, fibronectin type III domain; GH, glycoside hydrolase; SBD, starch binding domain.

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1 Introduction

Amylopullulanases or α -amylase-pullulanases form a class of endo-acting debranching enzymes of the glycoside hydrolases (GHs) included in the sequence-based classification of Carbohydrate-Active enZymes, the CAZy database [1]. The amylopullulanase is a bifunctional enzyme, which cleaves both α -1,4- and α -1,6-glycosidic linkages in starch, amylose, amylopectin, glycogen, pullulan and related oligosaccharides [2-5]. They hydrolyze α -1,4-glycosidic linkages in amylose to form products with degree of polymerization (DP) 2-4, while pullulan is generally cleaved at α -1,6- linkages with release of maltotriose. The enzymes are multi-domain proteins having two or three catalytic residues depending of the respective GH family [3,4]. They are grouped into family GH13 or GH57 based on the architecture of the catalytic domain and the number of conserved sequence regions (CSRs) [5-8]. The amylopullulanases of GH13 family possess six or seven CSRs in the catalytic domain, which is folded into a $(\beta/\alpha)_8$ or TIM barrel [named after triose phosphate isomerase, the first enzyme discovered to have a $(\beta/\alpha)_8$ fold] and the catalytically important amino acid residues, i.e. aspartate (catalytic nucleophile), glutamate (proton donor) and aspartate (transition-state stabilizer) present on the strands β 4, β 5 and β 7, respectively [2,3]. While the GH57 family enzymes possessing five CSRs fold into a $(\beta/\alpha)_7$ (i.e. an incomplete TIM barrel), with the catalytic machinery formed by glutamate (catalytic nucleophile) on strand β 4 and aspartate (proton donor) on β 7 [9,10]. The amylopullulanases from mesophiles belong to GH13 family, while the thermostable amylopullulanases belong to either GH57 or GH13 family [10]. The enzymes of both families employ the retaining mechanism of enzyme action.

Based on the number of active sites within the protein, the enzymes are categorized into two subgroups: the enzymes which possess two active sites occupying separate catalytic domains for cleaving α -1,4- and α -1,6-glycosidic bonds are termed α -amylase-pullulanases,

while amylopullulanases possess a single active site for hydrolyzing both α -1,4- and α -1,6-glycosidic linkages. Amylopullulanases from thermophilic anaerobes possess single active site [12-14], while those from aerobic microbes have either one [15,16] or two active sites for cleaving the α -1,4- and α -1,6-glycosidic linkages [17,18].

Amylopullulanases find potential applications in starch saccharification for making sugar syrups, as anti-stale in bread and other bakery products, and as an additive in detergents [19]. In the conventional process of starch saccharification, ~30-35% of dry starch slurry is gelatinized (at ~60 to 90 °C) to disrupt the structure of the starch granules followed by subsequent liquefaction at 95-105 °C (pH 6.5) by bacterial α -amylase to dextrins. The dextrins are subsequently saccharified by amylolytic enzymes, like pullulanase, β -amylase and glucoamylase leading to release of glucose [20]. The α -amylases currently employed in the starch conversion process are active at 95 °C and pH 6.8, and are Ca^{2+} -dependent. The process, therefore, requires the addition of Ca^{2+} that inhibits glucose isomerase used in the isomerization of glucose to fructose and can also lead to the formation of calcium oxalate, which clogs heat exchangers and pipes over time [21]. The removal of Ca^{2+} from the product streams by ion exchangers adds to the cost of the process and the final product. Moreover, the pH of the starch slurry (pH 4.5) [22] needs to be raised to 6.8, and the reversion products, such as isomaltose and maltose formed by glucoamylase at the cost of glucose needs to be minimized. The thermostable and Ca^{2+} -independent amylopullulanases would prove advantageous in the starch conversion process, as it allows increasing substrate concentration, reduces the reaction time, increases maltose and limits the use of glucoamylase, thereby making the process economical [4]. Owing to high thermostability, the amylopullulanases from thermophilic microorganisms of the genus *Geobacillus* act as efficient biocatalysts for one-step starch liquefaction-saccharification. The advances in molecular biology and protein engineering have led to improvement in enzyme titers, thermostability as well as catalytic activity enabling their efficient use in various industrial processes [23]. This review focuses on the developments in production, molecular aspects, characteristics, protein engineering and applications of amylopullulanases.

2 Bacterial and archaeal amylopullulanases

Amylopullulanase is produced by both aerobic as well as anaerobic bacteria, and archaea (Fig. 1), the anaerobic

bacteria being the highest producers [12]. Among aerobes, amylopullulanases are reproduced by certain species of *Bacillus* and *Geobacillus*, most of which are thermophilic. *Bacillus* sp. KSM-1378 [24], *Bacillus* sp. XAL 601 [25], *Bacillus* sp. TS-23 [26], *Bacillus* sp. 3183 [27], *Bacillus* sp. US149 [28], *Bacillus subtilis* [29], *Bacillus* sp. DSM 405 [30], *Bacillus circulans* F-2 [31-33], *Geobacillus thermoleovorans* NP33 [34], *Geobacillus stearothermophilus* L14 [35] and *Cohnella* sp. [36] have been reported to produce amylopullulanases (Table 1). Among thermophilic anaerobes, *Thermoanaerobium brockii* [12], *Clostridium* sp. strain EM1 (now *Thermoanaerobacterium thermosulfurogenes*) [37], *Thermoanaerobacter ethanolicus* 39E [38], *Thermoanaerobium* Tok6-B1 [39,40], *Thermoanaerobacterium thermosaccharolyticum* [41], *Thermoanaerobacter finni* [42], *Thermobacteroides acetoehtylicus* [42, 43] and *Thermotoga maritima* [44] are reported as producers of amylopullulanases. Hyperthermophilic archaea *Pyrococcus furiosus* [45,46], *Pyrococcus woesei* [47], *Thermococcus litoralis* [46], *Thermococcus celer* [48], *Thermococcus hydrothermalis* [49,50] and *Thermococcus profundus* [51] produce highly thermostable amylopullulanases (Table 1) belonging to the family GH57 [5,9].

3 Native amylopullulanases of the genus *Geobacillus*: production and characteristics

Among *Geobacillus* species, amylopullulanases have been produced using *G. thermoleovorans* NP33 [34] and *G. stearothermophilus* L14 [35]. Thermophiles, in general, have been reported to produce low biomass and enzyme titres, thus demand optimization of various cultural parameters [19]. Various physical and chemical parameters (temperature, pH, dissolved oxygen, agitation, carbon and nitrogen sources) have been optimized for increasing the biomass and enzyme production. These parameters have been shown to influence the growth as well as enzyme production.

G. thermoleovorans NP33 [34] and *G. stearothermophilus* L14 [35] produce amylopullulanases in the temperature range between 45 and 80 °C. The impact of temperature on the enzyme production is related to the growth of the organism. Amylopullulanase is maximally produced by *G. thermoleovorans* NP33 in the late exponential growth phase, at 70 °C, pH 7.0, and 200 rpm [34]. Starch and ammonium sulphate supported maximum enzyme production. The enzyme synthesis by the strain is partially inducible. A low basal level of constitutive enzyme

Figure 1. Neighbour-joining tree showing the phylogenetic relationship of the amylopullulanases from bacteria and archaea based on their amino acid sequences. GenBank gi: *Geobacillus* sp. MAS1, 559464335; *Geobacillus* sp. A8, 1408737936; *Geobacillus* sp. CAMR5420, 635250714; *Geobacillus thermoleovorans* NP33, 386276905; *Geobacillus stearothermophilus* ATCC12980, 88193077; *Geobacillus* sp. LC300, 909996913; *Geobacillus* sp. JF8, 752598410; *Geobacillus kaustophilus* HTA426, 56381557; *Geobacillus stearothermophilus* TS-23, 12006232; *Anoxybacillus amylolyticus*, 1024279633; *Thermoanaerobacter thermohydrosulfuricus*, 144720; *Thermoanaerobacterium saccharolyticum*, 237688402; *Clostridium perfringens* F262, 380306416; *Clostridium butyricum*, 758313218; *Thermococcus parvalvinellae*, 573025504; *Pyrococcus furiosus*, 2435438; *Thermococcus* sp. AM4, 345650580; *Pyrococcus abyssi*, 499169028; *Pyrococcus* sp. NCB100, 1001992508; *Thermococcus litoralis* DSM 5473, 374743225; *Yersinia rohdei* ATCC 43380, 238709313; *Lactobacillus plantarum*, 161085685; *Thermoanaerobacter kivui*, 694167291; *Bacillus* sp. NFR08, 1434604948; *Bacillus halodurans* C-125, 10176321. Amylopullulanases from *Geobacillus thermoleovorans* NP33 [49], *Geobacillus stearothermophilus* TS-23 [50], *Thermoanaerobacterium saccharolyticum* [4], *Pyrococcus furiosus* DSM 3638 [40,42], *Thermococcus litoralis* DSM 5473 [42], *Lactobacillus plantarum* [27] and *Bacillus* sp. [13] have been experimentally characterized.

degrades exogenous substrate resulting in low-molecular weight products, which enter the cells and induce enzyme synthesis. There is no requirement for additional trace elements [34]. Batch fermentation in the laboratory fermenter led to two-fold increase in amylopullulanase production by *G. thermoleovorans* NP33 as compared to that attained in shake flasks [34]. The strain has been

shown to possess multiple amylopullulanases [46]. The amylopullulanase of *G. thermoleovorans* NP33 (apu105) had a molecular mass of 105 kDa, while the molecular mass of *G. stearothermophilus* L14 amylopullulanase was 100 kDa. The amylopullulanase of *G. thermoleovorans* NP33 (apu105) [52] is highly thermostable with optimum activity at 80 °C and pH 7.0, while 65 °C and pH 5.5 are

Table 1. Characteristics of bacterial and archaeal amylopullulanases.^a

Source	Mw	pH	Temp.	Ref.
Mesophilic bacteria				
<i>Bacillus</i> sp. KSM-1378	210	10	50	24
<i>Bacillus circulans</i> F-2	220	7-8.5	50	31-33
<i>Bacillus subtilis</i>	450	7.0	30	37
Thermophilic bacteria				
<i>Bacillus</i> sp. US149	200	5.0	60	28
<i>Bacillus</i> sp. 3183	-	5.0	62	27
<i>Bacillus</i> sp. TS-23	140	9.0	55	26
<i>Bacillus</i> sp. DSM 405	126	6.0-6.5	70	30
<i>Bacillus</i> sp. XAL 601	224	9.0	70	25
<i>Clostridium</i> sp. strain EM1	-	5.9	60	37
<i>Cohnella</i> sp. A10	70	6.0	60	36
<i>Desulfococcus mucosus</i>	74	5.8	85	48
<i>Geobacillus thermoleovorans</i> NP33	-	7.0	80	34
<i>Geobacillus stearothermophilus</i> L14	100	5.5	65	35
<i>Thermoanaerobium brockii</i>	105	-	-	12
<i>Thermoanaerobium</i> Tok6-B1	120	5.6	70	39,40
<i>Thermoanaerobacterium thermosaccharolyticum</i>	150	5.0-5.5	65	41
<i>Thermoanaerobacter</i> strain B6A	450	5.0	75	101
<i>Thermoanaerobacter ethanolicus</i> 39E	133	5.5		38
<i>Thermobacteroides acetoethylicus</i>	-	5.0-6.0	75	42,43
<i>Thermoanaerobacter finni</i>	-	5.0-6.0	75	42
<i>Thermotoga maritima</i>	96	-	-	44
Hyperthermophilic archaea				
<i>Desulfurococcus amylolyticus</i> JCM9188	71	5.0	95	63
<i>Pyrococcus furiosus</i>	89	5.5	110	45,46
<i>Pyrococcus woesei</i>	90	6.0	100	47
<i>Thermococcus litoralis</i>	119	5.5	110	46
<i>Thermococcus hydrothermalis</i>	128	5.5	95	49,50
<i>Thermococcus profundus</i>	43	5.5	80-90	51
<i>Thermococcus celer</i>	-	5.5-6.0	90	48
<i>Thermofilum pendens</i>	65	3.5	95-100	102

^a Mw, molecular weight (kDa); pH, pH optimum; Temp., optimal temperature for activity (°C); Ref, reference(s).

optima for that of *G. stearothermophilus* L14 [35]. The enzyme possesses single active site for hydrolyzing both α -1,4- and α -1,6-glycosidic linkages in soluble and raw starches, pullulan, and malto-oligosaccharides, and it does not hydrolyse panose and cyclodextrins. The action of amylopullulanase from *G. thermoleovorans* NP33 [46] and *G. stearothermophilus* L14 [35] on different

substrates released glucose, maltose, and maltotriose as major end products. Similar findings have been recorded in the amylopullulanase of *Anoxybacillus* sp. SK3-4 [53]. Amylopullulanases, in general, cleave pullulan to form maltotriose as the only product [3,35,49,55]. The enzyme exhibits Ca^{2+} -independency, stability over acidic pH and the ability to degrade raw starches, the characteristics

which are highly valued in the starch saccharification process [21].

4 Cloning, expression and characteristics of recombinant amylopullulanases

As the levels of production and the specific activity of the amylopullulanases achieved with the wild bacterial and archaeal strains are very low, cloning of the enzyme encoding genes and their expression in heterologous hosts have opened up gateways for improving the protein yield. The genes encoding amylopullulanase are found in *Geobacillus* sp. MAS1 (GenBank Acc. No.: ESU72863.1), *Geobacillus* sp. A8 (EQB96130.1), *Geobacillus* sp. CAMR5420 (KDE46452.1), *Geobacillus* sp. LC300 (WP050367846.1), *G. kaustophilus* HTA426 (BAD77465.1), *Geobacillus* sp. JF8 (WP_041267797.1), *G. stearothermophilus* ATCC12980 (KOR93301.1), *G. stearothermophilus* TS-23 (WP_061567453.1) and *G. thermoleovorans* NP33 (AFI70750.1). Molecular biology and recombinant DNA technology are of considerable importance for not only enhancing enzyme production, but also improving the efficiency in industrial starch saccharification process [4]. The thermostable proteins cloned and expressed in mesophilic hosts have been shown to display proper secondary structure and sustain their stability at high temperatures, resistance to host proteolysis and simplify the purification by thermal precipitation.

The amylopullulanases from *G. thermoleovorans* NP33 (Gt-apu) [3] and *G. stearothermophilus* TS-23 [56] with a molecular mass of 182 kDa and 220 kDa, respectively, have been cloned and expressed in *E. coli*. In *E. coli*, the recombinant amylopullulanase of *G. thermoleovorans* NP33 was located in the intracellular fraction, while in the case of *G. stearothermophilus* TS-23, the intracellular, extracellular and periplasmic fractions contained recombinant amylopullulanase, with major portion being in the extracellular pool. The recombinant amylopullulanase of *G. thermoleovorans* NP33 is optimally active at 60 °C and pH 7.0 [3]. The enzyme has a single active site for both α -amylase and pullulanase activities, based on the kinetics with competing substrates (starch and pullulan) and response to inhibitors. The enzyme forms products with DP 2-4 upon hydrolysis of soluble and raw starches, amylose, amylopectin and related oligosaccharides, with maltopentaose being the smallest substrate to be acted upon by the enzyme. The amylopullulanases of *Anoxybacillus* sp. SK3-4 [53] and

G. stearothermophilus L14 [56] hydrolyze pullulan to glucose. The amylopullulanase of *G. thermoleovorans* NP33 hydrolyses pullulan to maltotriose, but does not hydrolyze panose and cyclodextrins. The high-molecular weight amylopullulanase of *G. thermoleovorans* NP33 exhibits high thermostability, Ca^{2+} -independency and raw starch degrading activity, as recorded for the native amylopullulanase (apu105). The α -amylase and pullulanase activities have been stimulated by Zn^{2+} , while Hg^{2+} and Cu^{2+} , *N*-bromosuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide inhibit the activity.

The protein engineering efforts to truncate the N- and C-terminal portions (Gt-apu Δ N1 and Gt-apu Δ C) of the enzyme [3,57] resulted in improved thermostability and specific enzyme activity in comparison with the native enzyme (Gt-apu). A relative decrease in the starch adsorption and saccharification rates of Gt-apu Δ N1 suggested the role of the N-terminus (N1 domain of Gt-apu that comprises two copies of the X25 module) in raw starch binding. The N1 truncation has also resulted in significant changes with respect to the substrate specificity of the enzyme. The Gt-apu Δ N1 displayed high hydrolytic action on pullulan over starch and hydrolysed maltotetraose as the smallest substrate as opposed to maltopentaose for Gt-apu and Gt-apu Δ C. The thermodynamic parameters for enzyme deactivation and the structure-function study using far-UV circular dichroism spectroscopy, intrinsic fluorescence emission and aniline-naphthalene-sulphonic-acid fluorescence spectroscopy revealed enhanced conformational and thermal stability of the truncated variants of Gt-apu (Gt-apu Δ N1 and Gt-apu Δ C). The thermostability of the enzymes is correlated with low number of cysteine residues [54,58] and disulphide bridges [59]. The heavily glycosylated Gt-apu Δ C secreted from the recombinant *Pichia pastoris* displayed higher thermostability and starch saccharification efficiency than that expressed in *E. coli*. Moreover, the post-translational modification of the enzyme secreted by *P. pastoris* resulted in altered substrate specificity that hydrolysed maltotriose and maltotetraose unlike that produced in *E. coli* [60].

The recombinant amylopullulanase encoding gene from *G. stearothermophilus* TS-23 [56] expressed in *E. coli* was found to be degenerated, as suggested by zymogram analysis with more than six specific bands of the α -amylase activity on polyacrylamide agarose gel electrophoresis. The molecular mass of the largest polypeptide was 220 kDa, while the smallest was of 105 kDa. The degeneracy may have been the result of degradation of the newly synthesized polypeptides and/or from incomplete translation.

5 Structure of amylopullulanases and role of different domains in catalysis

The amylopullulanases have been classified in families GH13 and GH57 on the basis of the architecture of catalytic domain and the number of CSRs [8,61]. The GH13 family enzymes fold into a catalytic $(\beta/\alpha)_8$ -barrel with catalytic machinery consisting of β 4-aspartate (catalytic nucleophile), β 5-glutamate (proton donor) and β 7-aspartate (transition-state stabilizer) and have four to seven CSRs [5-7]. These possess [3] the N-domain classified as the family carbohydrate binding module (CBM) 34, the catalytic $(\beta/\alpha)_8$ -barrel (i.e. TIM-barrel) fold succeeding by the α -amylase-type C-terminal domain (AmyC), fibronectin type III domain (FnIII), CBM20 domain, and in some cases, also the S-layer homology domain (Fig. 2a).

The GH57 family enzymes adopt a $(\beta/\alpha)_7$ TIM barrel (i.e., an incomplete TIM barrel) and the catalytic machinery encompasses the strand β 4-glutamate (catalytic nucleophile) and β 7-aspartate (proton donor) and five conserved sequences [5,61]. The GH57 amylopullulanases, in general, possess [5,9,45,49,62] a signal peptide preceding the catalytic domain, N-terminal glycoside hydrolase family 57 catalytic domain and, two surface layer homology (SLH) like domains and a threonine-rich region in the C-terminal region (Fig. 2b). The GH13 includes all the amylopullulanases from mesophiles, while the thermostable amylopullulanases are grouped into either GH57 or GH13 family. Both families GH57 and GH13, however, are similar in having the same retaining reaction mechanism [63-65]. Many members of the GH13 family have been assigned to subfamilies in 2006 [11]. The characteristics of the amylopullulanases of GH13 subfamilies are listed in Table 2.

Figure 2. (a) Schematic representation of the conserved domain structure in GH13 amylopullulanases of the thermophilic *Geobacillus* species, *G. thermoleovorans* NP33 (UniProt Acc. No.: I1WWV6), *G. stearothermophilus* TS-23 (Q9EZ24) and *G. kaustophilus* HTA426 (Q5KV21). Symbols are as follows: horizontal lines, X25 domain; white dotted box, CBM34; diagonal lines, catalytic domain; starred box, AmyC domain; grey box, fibronectin type III domain; vertical lines, CBM20 domain; boxes with triangles, S-layer homology domain. (b) Putative multidomain structure of GH57 amylopullulanases, in general. GH57, catalytic domain; SLH1 and SLH2, two repeats of surface layer homology motif bearing domain; TR, a threonine rich region.

Table 2. Characteristics of amylopullulanases belonging to different GH13 subfamilies.^a

GH13	Source	GenBank	Mw	pH	Temp.	CBM family	Activity	Ref.
12	<i>Streptococcus suis</i> P1/7	CAR47543.1	230	-	-	CBM41	dual	103
	Uncultured bacterium	AGL50935.1	168	9.0	40	CBM41, CBM48	dual	104
14	<i>Anoxybacillus</i> sp.	ARM71174.1	225	7.5	60	CBM20	single	53
	<i>Bifidobacterium breve</i> UCC2003	AAY89038.1	182	-	-	CBM48, CBM25	dual	105
20	<i>Lactobacillus amylophilus</i> DSM 20533	APT18553.1	90	6.5	37	CBM48	single	33
28	Uncultured bacterium	AGL50935.1	168	9.0	40	CBM41, CBM48	dual	104
39	<i>Geobacillus thermoleovorans</i> NP33	AFI70750.1	182	7.0	60	CBM20	single	3
	<i>Geobacillus stearothermophilus</i> TS-23	AAG44799.1	223	-	-	CBM20	single	56
	<i>Lactobacillus paracasei</i> B41	ADK95117.1	67	5.0	45	-	single	106
	<i>Thermoanaerobacterium saccharolyticum</i> NTOU1	ACR15165.1	~184	5.0-6.0	70	CBM20	single	107
41	<i>Streptococcus suis</i> P1/7	CAR47543.1	230	-	-	CBM41	dual	103

^a GH13, GH13 subfamily; GenBank, GenBank accession No.; Mw, molecular weight (kDa); pH, pH optimum; Temp., optimal temperature for activity (°C); Activity, number of catalytic domain for α -amylase and pullulanase activities; Ref, reference(s).

5.1 N-domain

The amylopullulanases of the genus *Geobacillus* are known to possess an additional region of around 350 amino acids in the N-terminus relative to α -amylases. The N-terminus of the *G. thermoleovorans* NP33 amylopullulanase has N1 and N2 domains, the latter has been designated as the CBM34. The N1 domain (57-149 and 161-251 amino acids) is composed of two copies of X25 domain, which has been shown to play a role in the raw starch adsorption and hydrolysis [57]. The important role of these auxiliary domains is to bind polysaccharides and bring the enzyme into close vicinity with its substrate for carbohydrate hydrolysis [66]. The region is homologous to the X25 domain of unknown function, present in the X45 domain of *Bacillus acidopullulyticus* pullulanase [67]. The N-terminal domain of amylopullulanases does not form a part of the active site in contrast to that reported for isoamylase [68]. The sequence alignment of the N1 domain of Gt-apu with CBM20 of gt-apu, CBM48 of the α -amylase-pullulanase of *Bacillus* sp. XAL601 and the CBM69 of AmyP [69-71] has helped in identifying the amino acid residues of the starch-binding sites 1 and 2. The homology model of *G. thermoleovorans* NP33 amylopullulanase and its N1 domain built using *Thermoactinomyces vulgaris* α -amylase II (PDB code: 1BVZA) and *B. acidopullulyticus* pullulanase (PDB code: 2WANA) as templates suggested that the N1 domain (residues 1-257) of Gt-apu is positioned as a flexible region away from the rest of the structure (Fig. 3a). The residues 23-257 of the N1 domain adopt an immunoglobulin-like fold (β -sandwich) of FnIII (Fig. 3b).

5.2 Catalytic domain

The catalytic domain of the *Geobacillus* amylopullulanases, as those of other enzymes of the α -amylase family GH13, has domain A, i.e. the $(\beta/\alpha)_8$ -barrel, and domain B. The parallel β -strands are surrounded by α -helices [72].

The α -amylase family GH13 members are characterized by the presence of 6-7 CSRs (CSR I to VII) [72,73] located on the strands β_3 , β_4 , β_5 , β_7 , β_2 , β_8 and on loop 3, near the C-terminus of domain B (Fig. 4). The loop 3 protruding from the catalytic $(\beta/\alpha)_8$ -barrel domain interconnects the strand β_3 with the third α -helix and, in fact, represents the domain B. It contains the CSR-V, which is differentiated by the calcium binding aspartate residue. The catalytically important amino acids – aspartate (catalytic nucleophile), glutamate (proton donor) and aspartate (transition-state stabilizer) – are located on the strands β_4 , β_5 and β_7 , respectively, of the catalytic $(\beta/\alpha)_8$ -barrel. The CSR-VI on strand β_2 is characterized by the presence of the conserved glycine residue separated from proline by seven amino acid residues. A conserved glycine residue and a proline residue are present at the start of the CSR-VII on β_8 strand. The *G. thermoleovorans* NP33 amylopullulanase has six conserved regions of the GH13 family around the strands β_2 , β_3 , β_4 , β_5 , β_7 and β_8 , as follows: CSR-VI, ⁴⁷⁷GVNTIYLN⁴⁸⁵; CSR-I, ⁵²⁹LDGVFNH⁵³⁵; CSR-II, ⁶⁶⁵GWRLDVANE⁶⁷³; CSR-III, ⁷⁰⁸EIWD⁷¹¹; CSR-IV, ⁷⁶⁸LIGSHD⁷⁷³; and CSR-VII, ⁸¹⁸GAPTIYYGDE⁸²⁷. On the other hand, the amylopullulanases from the family GH57 possess the N-terminal catalytic domain that folds into an incomplete TIM-barrel, i.e. a seven-stranded $(\beta/\alpha)_7$ -barrel [5,7,63],

Figure 3. (a) The *G. thermoleovorans* NP33 amylopullulanase model generated by Swiss-Model (Automated Mode) using *Thermoactinomyces vulgaris* α -amylase II as template. The model is composed of amino acids from V263 to G953 (labelled in red). The domain N2 (CBM34 domain; residues 270-384) and the α -amylase domain C (AamyC domain; residues 878-953) are shown in brown and blue colour, respectively. The catalytically important amino acids in the α -amylase catalytic domain (residues 397-87), Asp669-Glu708-Asp773, are indicated by spheres. (b) Model of the N1 domain of *G. thermoleovorans* NP33 amylopullulanase created by Swiss-Model using the pullulanase of *B. acidopullulyticus* as template. The model is composed of amino acids from G123 to P255 (labelled in red). The N1 domain composed of two X25 modules is indicated in different colours – blue depicts a part (residues 123-149) of the first X25 module (residues 57-149), whereas the second X25 module (residues 161-251) is specified by brown.

share five CSRs and their catalytic machinery consists of two residues, glutamic acid (catalytic nucleophile) and aspartic acid (proton donor) at the strands β_4 and β_7 , respectively [5,7].

5.3 C-domain

The C-terminus of *G. thermoleovorans* NP33 has the domains AamyC, the FnIII domain and CBM20. The AamyC domain is composed of β -sheets and facilitates proper orientation of the substrate onto the active site [74]; this plays a role in substrate binding site of the catalytic domain in separating α -glucan chains of starch and the disruption of the starch granule. The FnIII domain has similar β -sandwich fold like immunoglobulin domain, and has been reported to play a role in substrate binding [75-78]. Highly diverse CBM20s are present in families GH13, GH14, GH15 and GH77 [79]. Starch binding domains (SBDs) constitute a CBM subgroup and assist in starch binding [80]. Currently, SBDs have been found in CBM families 20, 21, 25, 26, 34, 41, 45, 48, 53, 58, 68, 69, and 74, [69,80]. SBDs are usually 100 to 130 amino acids long [69,81] and mainly present in GH13 α -amylases, GH14 β -amylases, GH15 glucoamylases and GH77 amylomaltases [81-83].

The C-terminal region of *G. stearothermophilus* TS-23 possesses nine repeats of the six-amino-acid sequence (Pro-Gly-Ser-Gly-Thr-Thr). The region shares the highest degree of similarity with the *Bacillus* sp. XAL601

amylopullulanase and is similar to the exoprotein glycine-rich repeats (GGXGXD)₄₋₃₆ of Gram-negative bacteria [84] involved in the secretion of polypeptides [85].

The C-terminal region of GH57 amylopullulanases contains two SLH motif bearing domains and a threonine-rich region [49,86] (Fig. 2b).

6 Applications of amylopullulanases

6.1 Starch liquefaction and saccharification

The global enzyme market was dominated by the food and beverage industry in 2010, with the major market share occupied by the baking enzymes. Starch industry approximately accounts for 15-20% of the global sales of industrial enzymes. The industrial starch hydrolysis requires action of different endo- and exo-acting enzymes including α -amylase, pullulanase, glucoamylase, glucose isomerase, and others. The acid stable, Ca^{2+} -independent and thermostable amylopullulanases from thermophilic microbes, such as *Geobacillus* spp., have potential application in the starch processing industry as these are thermostable and Ca^{2+} independent with raw starch degrading ability. Because of the debranching ability and debranching action of the enzymes, thermostable amylopullulanases can be employed as biocatalysts in one-step starch liquefaction-saccharification for producing

<i>B. breve</i> UC2003	RTSVGAPGDVIA--IAFQTNWNSVAKECTEAYGPEGVGYVQVSPPMESIQGTEWWTSYQP	58
<i>G. thermoleovorans</i> NP33	KGTSGYDGDGEWSNDFFGGDIAGIE-QKLDYQLSLGVNTIYLNPIAN----APSNHKYDASN	53
<i>G. stearothermophilus</i> TS-23	KATPGYDGDGIWSNDFFGGDIAGIE-QKLDYQLSLGVNTIYLNPIAH----APSNHKYDAHD	53
<i>B. breve</i> UC2003	-VSY-KLDSKLGTEAEFKTMIATCNAAGV [★] DIADSVINH [★] TTGADQGE [★] GTGVAGSKYDGE [★] GN	117
<i>G. thermoleovorans</i> NP33	-YKELDPMFGSP [★] EEFQS [★] FVQALANRGMH [★] ILDGVFNH [★] VSDDSI-----YFDRYHR-----	106
<i>G. stearothermophilus</i> TS-23	--YKAIDPMFGTPEEFESFVQAVASRGMH [★] ILDGVFNH [★] VSDDSI-----YFDRYGK-----	106
<i>B. breve</i> UC2003	FPAIPYT [★] KENFHDCTKNIGDYTNADEVQNCRLTSLQDLDT [★] SQEYVQDKLADYMNRLDLG	177
<i>G. thermoleovorans</i> NP33	YPTVGAYE--YWEA--VYDL [★] MNEKGLSE-----EEARKQVEEK [★] F--KQEGQTFS-----	149
<i>G. stearothermophilus</i> TS-23	YPTVGAYE--YWSA--VYDL [★] MNDKGLSE-----EEARVQVEQK [★] F--KDEGQQFS-----	149
<i>B. breve</i> UC2003	---VY [★] GFRVDAVKH [★] LA [★] TADVAAIKAKLA [★] EKSGRNAD [★] DIFFE [★] QEVIG [★] IA-SE-AAEIQPSNYVA	235
<i>G. thermoleovorans</i> NP33	-----GWRLDVANE [★] VDPAFWREFRQELLQGS-----EQPLILCEIWD [★] DASKYFLGDQYDSVM	284
<i>G. stearothermophilus</i> TS-23	-----GWRLDVANE [★] VDTEFWREFRQELLQGD-----QQPLILCEIWD [★] DASKYFLGDQYDSVM	284
<i>B. breve</i> UC2003	NGKVSEFNFTNHL [★] SVAFAGDITDASKGLAKVGGD [★] GWVSSDKAAV [★] WVTNWD [★] TERGSS---	291
<i>G. thermoleovorans</i> NP33	NYR---FRGAVLDFLKN [★] GNAAEADKRLTAIR---ED---YPSEAFYAL [★] MNLIGSHD [★] TARAVFL--	339
<i>G. stearothermophilus</i> TS-23	NYR---FRGAVLDFLRNGKAAEIDARLTAIR---ED---YPSEAFYAL [★] MNLIGSHD [★] TARAVFL--	339
<i>B. breve</i> UC2003	IT-----YKKGSKYLLANAFMLAYDYGQPHIYSGYYFDNSDDGAPGATETSV [★] PDMECPT	345
<i>G. thermoleovorans</i> NP33	LGNGTDSSERAELDPNYNEELGKKRLKLA [★] VILQMGYPGAPTIIY [★] YGDEAGVTGSRDPNRR [★] T	393
<i>G. stearothermophilus</i> TS-23	LGNGTDSYERAELDPNYNEQLGKKRLKLA [★] AILQMGYPGAPTIIY [★] YGDEAGVTGSKDPDDR [★] RT	393
<i>B. breve</i> UC2003	---DGSMTSGTWQCAERWTAIR	364
<i>G. thermoleovorans</i> NP33	YPWGKEDQNL LSHYQKVGHIR	420
<i>G. stearothermophilus</i> TS-23	YPWGSEDTTEL IAHYQKVGTVR	420

Figure 4. Multiple amino acid sequence alignment of α -amylase-type catalytic domains of GH13_14 and GH13_39 amylopullulanases showing the difference in their conserved sequence regions. The amylopullulanase from *Bifidobacterium breve* UC2003 (UniProt Acc. No.: F9Y041) belongs to the subfamily GH13_14, whereas both *Geobacillus thermoleovorans* NP33 (I1WWV6) and *Geobacillus stearothermophilus* TS-23 (Q9EZZ4) amylopullulanases are grouped into the subfamily GH13_39. Four conserved sequence regions are highlighted in boxes and the catalytic triads (Asp-Glu-Asp) are marked with stars. The *B. breve* UC2003 amylopullulanase possesses two separate catalytic domains specific for α -amylase (subfamily GH13_32) and pullulanase (GH13_14) located in the N-terminal and C-terminal parts, respectively, while amylopullulanases of *G. thermoleovorans* NP33 and *G. stearothermophilus* TS-23 have a single catalytic domain for both α -amylase and pullulanase activities.

different sugar syrups by replacing amylolytic enzymes, like glucoamylase and α -amylases, thus minimizing the use of glucoamylase by 50%, thereby reducing the total reaction time and the cost of sugar production.

The *G. thermoleovorans* NP33 amylopullulanase pre-treated raw starches have been saccharified to a greater extent by the glucoamylase in comparison to the starch saccharification achieved with the α -amylase pre-treated starches and glucoamylase alone [52]. An improved thermostability and catalytic efficiency achieved with the protein engineering effort via C-terminal truncation enabled efficient use of the enzyme in starch saccharification [3].

6.2 Preparation of maltooligosaccharides

Maltooligosaccharides are composed of glucopyranosyl units linked by an α -1,4-linkages, while the ones containing both α -1,4- and α -1,6-glucosidic linkages are called branched oligosaccharides or isomaltooligosaccharides [87]. According to the International Union of Pure and Applied Chemistry, linear maltooligosaccharides are defined as polymers of monosaccharides with DP between 3 and 10. The maltooligosaccharides are widely used in beverage, food and pharmaceutical industries [88]. Maltooligosaccharides are characterized by high

solubility, less sweetness, low calories, less water activity, good moisture level, acid resistance and heat stability. These can be made into various healthcare and nutritional foods including baby foods, confectionary items, healthcare candies, beverages, healthcare beers and ice creams. Maltotriose, maltotetraose and maltopentaose prevent migration of moisture from starch granules, reduce retrogradation by inhibiting realigning of amylose and amylopectin chains and interfere with starch-gluten interaction; these properties make them useful as anti-staling agent in the bread industry [89-91].

Maltooligosaccharide syrups serve as the feed of high nutrition to infants and the aged [92]. Maltooligosaccharides come under soluble non-digestible carbohydrates [93], which stimulate the intestinal microflora, especially the Bifidobacteria and form fermentation products in the colon [30,94]. These non-digestible oligosaccharides are called prebiotics [25], and are categorized as functional food ingredients, which can escape from low-pH stomach fluids and digestive enzymes, and are metabolized in large bowels. Maltooligosaccharides can be gradually absorbed in human body, and thus, provide energy continuously for a long period. Furthermore, maltose and maltooligosaccharides have low calories, thus regulating blood glucose levels, and decreasing post-prandial blood glucose levels [95]. Therefore, it is beneficial for the cardiovascular, diabetes and obese patients. In addition, maltooligosaccharides provide increased body endurance, immunity and induce good therapeutic effect to persons with hypoglycemia and brain disorder associated with overconsumption of energy [96]. Maltooligosaccharides are commercially available as Fuji Oligo syrups, a product of Nihon Shokuhin Kako Kogyo Kabushiki Kasha (Tokyo, Japan).

6.3 Anti-staling agent in making bread and other bakery products

The amylopullulanases can be used in the bread industry as an anti-stale. Staling refers to all the undesirable changes, such as loss of the moisture content in the bread crumb, reduced crispness of the crust and diminished bread flavour that occurs upon storage of bread. The *G. thermoleovorans* NP33 amylopullulanase [52] has been reported to act as an anti-stale, which in turn improves the shelf life of bread and other bakery products.

Staling occurs because of retrogradation of the amylopectin of starch [97,98]. An anti-staling agent acts by shortening the amylopectin chain. The α -amylase presently used in making bread results in sticky bread when added in excess because of the production of

branched maltodextrins [99]. The debranching action of amylopullulanase enables the hydrolysis of the branched maltodextrins, thereby eliminating the stickiness of the α -amylase-treated bread and other bakery products [100]. The supplementation of whole wheat dough with the *G. thermoleovorans* NP33 amylopullulanase markedly improves the quality of the bread by enhancing the shelf-life, texture of bread and the loaf volume [52]. Moreover, maltooligosaccharides generated in the bread by the enzyme action will act as prebiotics in ameliorating human health.

6.4 Detergent applications

The alkali-tolerant amylopullulanase of *G. thermoleovorans* NP33 finds application as a detergent additive [52]. The enzyme exhibits significant stability in different commercially available detergents and efficiently removes the starch stain as revealed from the wash performance analysis of the stained cotton fabric. An increase in reflectance when washed with the combination of enzyme and the detergent as compared to the detergent alone suggested the potential use of the enzyme as detergent additive.

7 Conclusions

The amylopullulanases have received considerable attention in the past few decades because of their action in cleaving both α -1,4- and α -1,6-glycosidic linkages in different complex polysaccharides. The thermostable amylopullulanases from thermophilic bacteria and archaea possess high thermostability, raw starch hydrolysing efficiency and Ca^{2+} independence. They, therefore, find potential application in the starch processing industry for making maltose and maltooligosaccharide syrups. Because of their action on multiple substrates and debranching action, they can be used for one-step starch liquefaction-saccharification. The multiple amino acid sequence alignment and the domain organization revealed six to seven CSRs of the α -amylase family. The protein engineering efforts via truncation enabled to attribute functions to some of the domains, whose functions have not yet been understood, besides improving catalytic activity and thermostability. The limited information available on the three-dimensional structures of amylopullulanases, however, limits our understanding of the structure-function relationship of the enzymes, which demands further research efforts.

Conflict of interest: The authors declare no conflict of interests.

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