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Structural and Catalytical Features of Different Amylases and their Potential Applications

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Abstract

Amylases can hydrolyze the O-glycosyl linkage of starch and related polymers. They are ubiquitously present in all living systems. However, microbial amylases meet the demands of industrial applications. Amylase and related enzymes are classified in different glycosyl hydrolases (GH) families. The GH13 is the largest family in the carbohydrate-active enzyme (CAZy) database, which comprises α-amylase, α-glucosidase, maltogenic amylase, debranching enzymes, CGTase, pullulanase, neopullulanase, and others. Despite GH13, some other families also contain α-amylase and related enzymes. Most of the starch-degrading enzymes have a common $(\beta/\alpha)_8$ barrel structure and four or five conserved sequences containing catalytic residues. α-Amylase and related enzymes follow the α-retaining double displacement mechanism during catalysis. The enzymes of the α-amylase family are potentially applied in food, pharmaceutical, textile, paper, detergent, biofuel, and animal feed producing industries. α-Amylase-mediated liquefaction and saccharification of starch is the essential step for the production of glucose, maltose, high fructose-containing syrups, maltooligosaccharides, cyclodextrins. These products are potentially used for the preparation of geriatric and infant foods. In conclusion, starch degrading enzymes bear a common structural arrangement and catalytic activity, and are broadly exploited in different sectors ranging from food, pharmaceutical industries to wastewater treatment.

Keywords: Amylases, starch, glycosyl hydrolases, catalytic properties, maltooligosaccharides

1. Introduction

Starch is the major storage polysaccharide in plant products and is an important source of energy for human and other monogastric animals. Starch is also widely used in different industries. About 75 million tons of starch was utilized in the year 2012; the expected annual growth rate will be continued by 2-3%. Amylolytic enzymes are essential in starch-based industries for the preparation of commercial products. The physicochemical properties of natural starch do not meet the requirements of industrial use (Park *et al.,* 2018). Previously, various physical methods (heat-moisture treatments, freezing, and ultrahigh-pressure treatments) and chemical modification were applied for starch processing (Chung *et al.,* 2010; Park *et al.,* 2018). To overcome the hazards of these treatments, enzymatic modification is the best as the process is safe, healthier, and eco-friendly. The enzymatic processing is mostly mediated by a group of amylolytic enzymes.

Varieties of starch degrading enzymes such as α-amylase, isoamylase, α-glucosidase, CGTase (cyclodextrin glycosyltransferase), branching enzymes, pullulanase, amylopullulanase, and neopullulanase commonly belong to the α-amylase family. Earlier, Kuriki and Imanaka (1999) had classified the amylases into α-amylase (EC 3.2.1.1), debranching enzymes such as pullulanase (EC 3.2.1.41), isoamylase (EC 3.2.1.68), CGTase (EC 2.4.1.19), and branching enzyme (EC 2.4.1.18). The carbohydrate-active enzyme (CAZy) database is the tool for the classification of carbohydrases. This database carries 168 glycosyl hydrolase (GH) families (GH1–GH168) having 18 clans (A–R). CAZy database was constructed for the distribution of all carbohydrate-splitting enzymes (Lombard *et al.,* 2014; https://www.cazy.org/). GH13, GH31, GH57, and GH119 are the most important family concerning amylolytic enzymes. Almost all the common amylolytic enzymes (family GH13, GH31, GH57, GH77, and GH119) have multiple domains and similar internal structures containing $(\beta/\alpha)_8$ barrel topology. The molecular structure of amylases, mechanism of catalysis, and mutational effects can improve overall strategies of applications of amylases.

The progression of biotechnology tremendously increases the applications of amylases and other industrial enzymes like cellulase, xylanase, pectinase, and proteases. The global market of industrial enzymes is growing exponentially from the last decade and is projected to be continued in the future. According to the global industrial enzymes market report, the annual growth had started with a value of 5.8% from the year 2017; the estimated values had touched the level of US$ 5.9 billion in the previous year (2020). The expected estimated values will reach up to US$ 8.7 billion in 2026 (Industrial Enzymes Market 2016; https://www.businesswire.com/news/home/20161219005619/en/Global-Industrial-Enzymes-Market-Analysis-2016--

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). The enzymes of the amylase family are potentially used in the brewing process, food and pharmaceutical industry, animal feed preparation, paper recycling, biofuel production, desizing process, detergent preparation, and waste management (Yan and Wu, 2016). Amylases themselves have a 25% share in the world's enzyme market (Rajagopalan and Krishnan, 2008; John, 2017) and more specifically used in different purposes (detergents-37%, textiles-12%, starch processing-11%, baking-8%, and animal feed production-6%) (Deb *et al.,* 2013).

The commercial production of amylase was started in 1894 when Jōkichi Takamine isolated "Takadiastase" from a wheat bran culture of *Aspergillus oryzae.* Numerous bacteria (e.g. *Bacillus licheniformis, B. amyloliquefaciens, B. subtilis*, *B. stearothermophilus*) and fungi (e.g. *Aspergillus oryzae*, *A. niger*, *A. awamori, Rhizopus* sp.) were exploited for the large scale production of amylases (Gupta *et al.,* 2003; Mehta and Satyanarayana, 2016; Samanta, 2020a). In the commercial sectors, different multinational companies (Novozymes, DuPont Danisco, AB Enzymes, Dyadic, BASF, DSM, and others) are involved in the production of amylases; they cover more than 70% of the total enzyme market. Indian biotech sector has achieved a vital position in manufacturing and research services in the enzyme sector (Li *et al.,* 2012; Chandel *et al.,* 2007; Kumar *et al.,* 2014; Industrial Enzymes Market, 2016). The enzyme-based preparation of infant and geriatric foods, confectionery products, digestive aids, animal feed, detergents, biofuel, and many other products depend on the supply of amylases and other industrial enzymes from these multinational companies. The present review has focused on the amylases of GH13 and associated families, their sources, production, catalytic properties, primary structure. Special emphasis has been given to the applications of α-amylase, α-glucosidase, maltogenic amylase, debranching enzymes, CGTase, pullulanase, and neopullulanase.

2. Methodology

The CAZy database (Lombard *et al.,* 2014) was thoroughly reviewed for the study of the classification of amylases. Then the literature survey had been done for the collection of information related to molecular structure, catalytic strategies, mechanism of action, and applications of amylolytic enzymes in different sectors. For this purpose, several databases such as NCBI website database PubMed, Springer Nature, Science Direct (Elsevier), Google Scholar, ResearchGate, and others were viewed for searching the relevant articles. Different keywords like classification of amylases, glycosyl hydrolase families, the molecular structure of amylases, mechanism of catalysis by amylases, and applications of amylases were used during the literature survey. Then, this review has been prepared on the basis of the searching content.

3. Starch and some important saccharides

Starch is the major component of the human diet and comprises half of the ingested carbohydrates. Several starch-based products like glucose syrups, high fructose-containing syrup, malto-oligosaccharides, cyclodextrins, anomalously linked oligosaccharides mixture (Alo mixture), branched dextrin are produced through enzymatic treatment for the formulation of geriatric and infants' foods. Moreover, starch has other industrial applications such as thickener, colloidal stabilizer, gelling, bulking, and water holding agent due to its molecular properties (Singh *et al.,* 2007). Amylases are used for the removal of starch during cloth making, production of finished paper, biofuel production, and wastewater treatment of the sugar and paper industries. Another important use of amylases is in the brewing industry where starchy products are the initial components for making wine. Several digestive medicines are formulated by fungal α-amylase. Recently amylase is used in the diagnostic kit.

Starch is regarded as a biopolymer of glucose. It is usually present inside the plant cells as compact insoluble granules, which may be spherical, lens-shaped, or ovoid. The major sources of starch are maize, rice, tapioca, potato, wheat, and cassava. Natural starches consist of an unbranched amylose chain (15–20%) and branched amylopectin (80–85%). Amylose is a polymer of D-glucopyranosyl units, consists of 300-3000 glucose residues joined by α-1→4 glycosidic linkages. Amylose can form inclusion complexes with iodine (Zobel, 1988) and generate a colored complex. A minimum of 18 glucose units containing an oligosaccharide chain is required to form a starch iodine color complex (Bailey and Whelan, 1961). However, the intensity of color increases linearly with chain length to about 70 glucose units. The structure of amylopectin is more complex. It is the largest polymer in nature with a DP (degree of polymerization) ranging from 3×10^5 to 3×10^6 D-glucopyranosyl units; however, the individual chains vary between 10 and 100 glucose units (Bijttebier *et al.,* 2008). The backbone of amylopectin is much longer than that of amylose. It contains up to 10^5 glucose residues joined by α-1,4 glycosidic linkages with glucan sidechains having 20-25 residues linked by α-1,4 linkages. Sidechains are attached to the backbone by α-1,6 glycosidic bonds (Fig. 1A). The detailed structure of amylopectin is still speculative; it is composed of 3 types of chains, A, B, and C with the degree of polymerization (DP) in the range of 12–75 glucose residues. A-chains (DP 12-16) are linked to B-chains. These B-chains are further linked to other B-chains and to the C-chain, which carries the only reducing glucose residue. The B-chains can be further divided into B1- (DP 20-24), B2- (DP 42-48), B3- (DP 69-75) and B4-chains (Hizukuri, 1986, 1996) (Fig. 1B). In consideration of general properties, starch appears as polymeric glucan, osmotically inactive, stable, white soft amorphous powder, lacks sweetness, and is insoluble in water, alcohol, and ether at room temperature. It is highly hydrated since it contains many exposed hydroxyl groups.

Pullulan is a fungal polysaccharide (extracellular) that was initially isolated from *Pullularia pullulans.* It comprises maltotriose units linked by α-1,6-glucosidic bonds (Fig. 1C). The other important molecules are isopanose and panose. They are trisaccharides, contain α-1,4- and α-1,6-glycosidic bonds. (Fig. 1D). Another component is isomaltose, which contains only α-1, 6-glycosidic linkage. Although the α-1, 6-glycosidic linkages are similar to amylopectin, starch-debranching enzymes can cleave the pullulan polysaccharide. Pullulan is a water-soluble polysaccharide, produces a colorless

visco-adhesive solution. The food, pharmaceutical, and biomedical industries are the major user of pullulan.

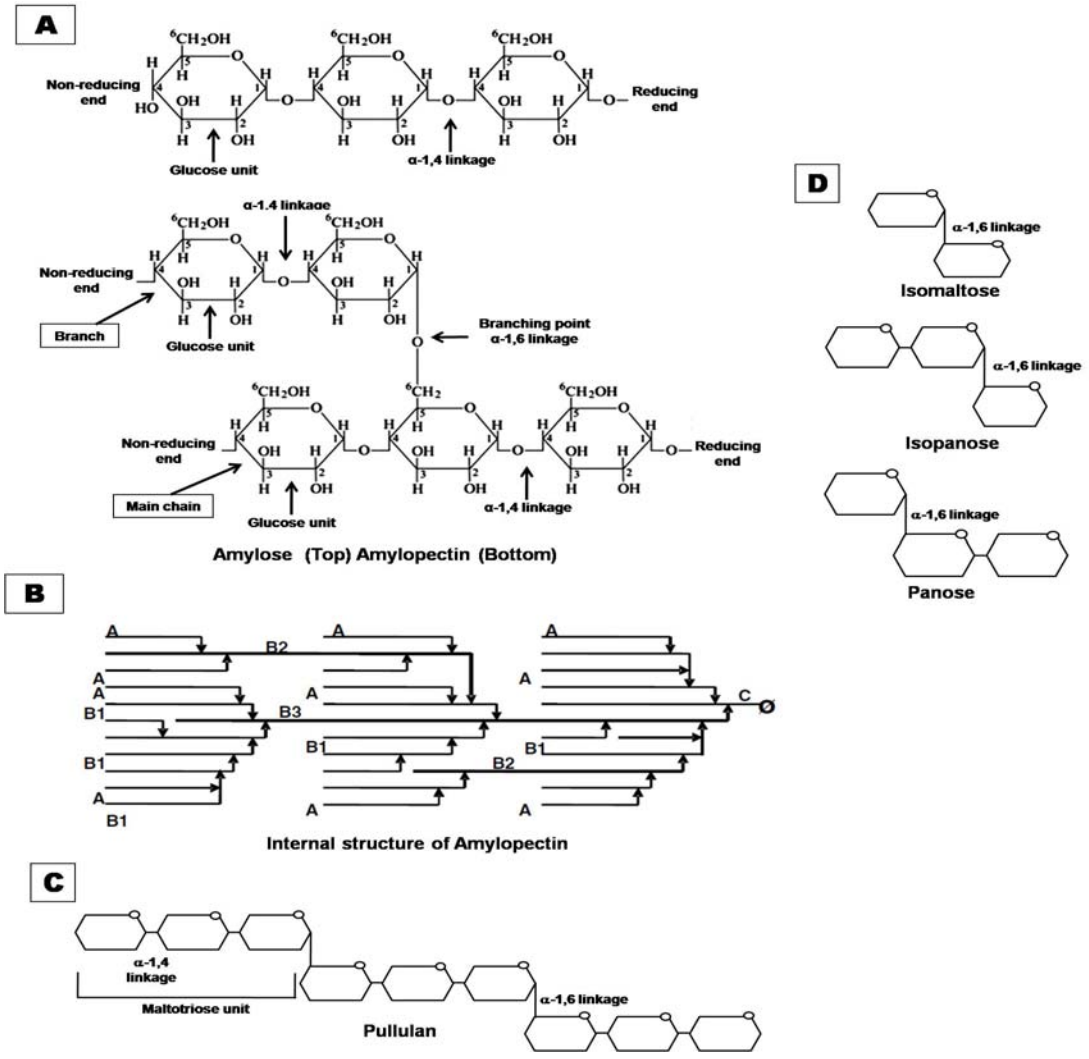

Figure 1. A: Schematic representation of amylose and amylopectin. Amylose molecule has repeating α-1, 4 linkages. Amylopectin molecule showing the α-1, 4, and α-1,6 chain linkages. B: Molecular arrangement of amylopectin, composed by A, B, and C chains. Solid line: α-1,4 bound glucose units; arrow: α-1,6 linkage; ø: reducing glucose residue. (details are given in the text). (Adapted from Hizukuri 1986, 1996). C: Schematic representation of the structure of pullulan. D: Diagramatic representation of isomaltose, isopanose, and panose.

4. An overview on glycosyl hydrolase family and classification of amylolytic enzymes

Different starch degrading enzymes are most abundant in nature and responsible for metabolism as well as industrial applications. Earlier, R. Kuhn (1925) and later E. Ohlsson (1930) observed the contrastive differences of the enzymatic-hydrolytic products of starch and then classified the starch degrading enzymes in α-and β-amylases according to the anomeric type of sugar products. The classification of amylases had not completed properly at the initial stage of categorization because several starch-degrading enzymes had shown the activity of transfer or condensation reaction in addition to the hydrolytic reaction. Initially, these enzymes had not classified as amylases. The molecular study of these enzymes (cyclodextrin glycosyltransferase, pullulanase isopullulanase, etc.) had shown that they had four conserved regions of sequence in their primary structure for substrate binding and catalytic activity, which is similar to Taka amylase. These four conserved sequences were not present in β-amylase (EC 3.2.1.2) and glucoamylase (EC 3.2.1.3).

Based on the action pattern, K. Myrback and G. Neumuller (1950) classified amylases into endo-amylases and exo-amylases. Endoamylases cleave internal α-1,4 bonds that are present in the inner part (endo) of the amylose or amylopectin chain. The hydrolytic reaction occurs randomly in the interior of the starch molecule. The end products are α-anomeric oligosaccharides with varying chain lengths and α-limit dextrins, which constitute branched oligosaccharides. The term "alpha" relates to the initial anomeric configuration of the reducing groups of the liberated product and is not related to the configuration of the hydrolyzable linkage. Exoamylases start hydrolysis from the non-reducing end; the subsequent result is the production of short end-products. These

enzymes cleave α-1,4 or α-1,6 bonds of the external glucose residues of amylose or amylopectin resulting, in the formation of α- or β-anomeric products. There are several types of exoamylases. Amylogucosidase (glucoamylase/γ-amylase, EC 3.2.1.3) and α-glucosidase (EC 3.2.1.20) produce glucose as the sole end product from starch and related polymers. β-amylase produces maltose from amylose, maltose, and β-limit dextrin from amylopectin and glycogen. Maltogenic -amylase (EC 3.2.1.133), maltotetraose forming amylase (EC 3.2.1.60), maltohexaose forming amylase (EC 3.2.1.98) are also the example of exoamylases. The cyclodextrin producing enzyme cyclodextrin glycosyltransferase (CGTase, EC 2.4.1.19) and branching enzyme (EC 2.4.1.18) have an additional activity of transglycosylation. They belong to the transferase group. CGTase hydrolyzes starch to a homologous series of cyclic, non-reducing D-glucosyl polymers, called cyclodextrins. Besides these, debranching enzymes hydrolyze only α-1,6 bonds exclusively, producing long linear polysaccharides. Enzyme transferases cleave α-1,4 glycosidic bond of the donor molecule, transfer the part of the donor molecule to a glycosidic acceptor, forming a new glycosidic bond. Thus, the classifications of amylolytic enzymes of microbial origin are based on action pattern and end-product formation.

From the year 1991, the α-amylase family belonged to the glycosyl hydrolases family 13 after the establishment of sequence-based classification of all glycoside hydrolases, transferases (Henrissat, 1991). Finally, the concept of the α-amylase family was proposed by Takata (1992). Currently, carbohydrate-splitting all the enzymes are distributed in different glycosyl hydrolase families (GH family). According to the carbohydrate-active enzymes (CAZy) database, there are 168 glycosyl hydrolases families (GH1–GH168) with 18 clans (A–R). The starch degrading or modifying enzymes are distributed in GH families 3, 13, 14, 15, 31, 57, 63, 77, 97, 119, 122, 126, and 133 (Table 1). Mostly useful amylolytic enzymes are commonly found in GH13, GH57, GH119, and GH126 family (Lombard *et al.,* 2014; Janeček *et al.,* 2014; Kerényiová and Janeček, 2020a). The family GH57 was established more than twenty years ago when the three-dimensional structure of some α-amylases and related enzymes were elucidated that contained a $(\beta/\alpha)_7$-barrel (an irregular TIM-barrel domain) in their configuration (Sarian *et al.,* 2017). Later, family GH119 also was established (Janeček and Kuchtová, 2012; Blesák and Janeček, 2013). Initially, there were some disputes about the members of GH126 that were resolved after the crucial contribution of Koseoglu *et al.* (2015), Wu *et al.* (2019), and Janecek *et al.* (2019). The family GH126 was created by Ficko-Blean in the year 2011 (Ficko-Blean *et al.,* 2011). Up to July 2020, this family counts more than 1000 sequences exclusively from the bacteria under the phylum Firmicutes (Kerényiová and Janeček, 2020a). The *in silico* study had revealed that this family has relationships with other GH families. The members of this family bear seven conserved sequence regions; they comprise catalytic $(\alpha/\alpha)_6$-barrel structure in their three-dimensional configuration (Kerenyiova and Janecek, 2020b). Moreover, α-glucosidase (EC 3.2.1.20) belongs to the families GH31, GH63, GH97, and GH122 along with GH13 and glucoamylase (EC 3.2.1.3) is present in the separate family GH15 and GH97.

Table 1. Selected glycosyl hydrolases family (GH family) and their fundamental characteristics (Lombard *et al.,* 2014).

Family	Enzyme name and EC No.	Structure	Mechanism of
GH13	α-Amylase (3.2.1.1); oligo-1,6-glucosidase (3.2.1.10); α-glucosidase (3.2.1.20); pullulanase (3.2.1.41); cyclomaltodextrinase (3.2.1.54); maltotetraose-forming α-amylase (3.2.1.60); isoamylase (3.2.1.68); dextran glucosidase (3.2.1.70); trehalose-6-phosphate hydrolase (3.2.1.93); maltohexaose-forming α-amylase (3.2.1.98); maltotriose-forming α-amylase (3.2.1.116); maltogenic amylase (3.2.1.133); neopullulanase (3.2.1.135); malto-oligosyltrehalose trehalohydrolase (3.2.1.141); limit dextrinase (3.2.1.142); maltopentaose-forming α-amylase (3.2.1.-); amylosucrase (2.4.1.4); sucrose phosphorylase (2.4.1.7); branching enzyme (2.4.1.18); cyclomaltodextrin glucanotransferase (CGTase) (2.4.1.19); 4-α-glucanotransferase (2.4.1.25); isomaltulose synthase (5.4.99.11); trehalose synthase (5.4.99.16).	Number of domains : 3 (A, B, C).	Retaining
GH Clans: GH-H		Types of fold: $(\beta/\alpha)_8$-barrel.	Some enzymes have transglycosylation activity.
		Catalytic site: $(\beta/\alpha)_8$-barrel in domain A.	Proton donor: Glu
			Nucleophile: Asp
GH14	β-Amylase (3.2.1.2)	Single domain $(\beta/\alpha)_8$	Inverting
			Proton donor: Glu
			Nucleophile: Glu
GH15	Glucoamylase (3.2.1.3); glucodextranase (3.2.1.70); α,α-trehalase (3.2.1.28); dextran dextrinase (2.4.1.2)	Types of fold: $(\alpha/\alpha)_6$ barrel	Inverting
GH Clans: GH-L		Some have super-β-sandwich.	Proton donor: Glu Nucleophile: Glu
GH31	α-Glucosidase (3.2.1.20); α-galactosidase (3.2.1.22); α-mannosidase (3.2.1.24); α-1,3-glucosidase (3.2.1.84); sucrase-isomaltase (3.2.1.48) (3.2.1.10); α-xylosidase (3.2.1.177); α-glucan lyase (4.2.2.13); isomaltosyltransferase (2.4.1.-); oligosaccharide α-1,4-glucosyltransferase (2.4.1.161); sulfoquinovosidase (3.2.1.-).	Types of the fold: $(\beta/\alpha)_8$-barrel.	Retaining
GH Clans: GH-D		N-terminal β-sandwich also present.	
		β-folded proximal and distal C-terminal domain is present in the structure.	Proton donor: Asp Nucleophile: Asp.
GH57	α-Amylase (3.2.1.1); α-G\galactosidase (3.2.1.22); amylopullulanase (3.2.1.41); cyclomaltodextrinase (3.2.1.54); branching enzyme (2.4.1.18); 4-α-glucanotransferase (2.4.1.25).	Number of domains: 3 (A, B, C).	Retaining
		Types of fold: $(\beta/\alpha)_8$-barrel.	
		Catalytic site: $(\beta/\alpha)_8$-barrel in domain A.	Proton donor: Asp Nucleophile: Glu.
		Some cases $(\beta/\alpha)_7$ irregular $(\beta/\alpha)_8$-barrel / pseudo TIM-barre may be present.	
GH77	Amylomaltase or 4-α-glucanotransferase (2.4.1.25)	4-7 conserved sequences are present, but domain C is absent.	Trans-glycosylation
GH Clans: GH-H		The TIM-barrel structure is disrupted in the sub-domains B1, B2 and B3 by insertions. Subdomain B1 consists of a highly twisted four-stranded antiparallel β-sheet with two α-helices.	Proton donor: Glu Nucleophile: Asp.
GH97	Glucoamylase (EC 3.2.1.3); α-glucosidase (EC 3.2.1.20); α-galactosidase (EC 3.2.1.22)	Number of domain: 3	Inverting and retaining
		N-terminal β-super-sandwich domain, followed by $(\alpha/\beta)_8$ barrel containing central domain, carries catalytic site and a C-terminal β-sheet domain.	Proton donor: Glu
			Nucleophile: Glu for inverting; Asp for retaining.
GH119	α-Amylase (EC 3.2.1.1)	Distantly related to family GH57;	Retaining
	Created after Watanabe et al. (2006).		
GH122	α-glucosidase (EC 3.2.1.20)		
	Created after Confort et al. (2008).	-	-

Among the glycosyl hydrolase families, GH13 is the largest family in the CAZy database having more than 30 types of amylolytic enzymes with different characteristics (Lombard *et al.,* 2014). At present, the GH13 family counts more than 80,000 sequences (Janeček and Zámocká, 2020). The GH13 has 42 subfamilies (Janeček and Gabriško, 2016; Valk *et al.,* 2016; Janeček and Zámocká, 2020). However, the number of subfamilies is still emerging. Currently, another novel GH13 subfamily is proposed after the bioinformatics study of the α-amylase from the halophilic archaeon *Haloarcula hispanica* (Janeček and Zámocká, 2020). Considering the different subfamilies, only eleven subfamilies contain α-amylases activity: GH13_1 (fungi), GH13_5 (bacterial liquefying enzymes that produce short-chain dextrin from starch and reduce the viscosity of starch suspension), GH13_6 (plants), GH13_7 (archaea), GH13_15 (insects), GH13_24 (animals), GH13_27 (proteobacteria), GH13_28 (bacterial saccharifying enzymes, mostly exoamylases and produce glucose, maltose, maltotetraose, etc.), GH13_32 (bacteria), GH13_36 (intermediary α-amylase), and GH13_37

(marine bacteria) (Janeček and Gabriško, 2016; Møller and Svensson, 2016). Neopullulanase, cyclomaltodextrinase, and maltogenic amylases are classified in the subfamily GH13_20 (Lombard *et al.,* 2014; Kuchtová and Janeček, 2016). These enzymes have the N-terminal starch-binding domain (SBD) that specifically belongs to the carbohydrate-binding modules (CBMs) family 34 (CBM34) (Machovic and Janeček 2006; Kuchtová and Janeček 2016). Additionally, pullulan hydrolase type III enzymes had been included in the subfamily GH13_20 (Lombard *et al.,* 2014). Valk *et al.* (2016) reported that about 10% of enzymes of the GH13 family carried SBDs. Fundamentally, SBDs are the subgroup of CBMs. Several authors observed that raw starch degrading α-amylases had additional CBM at their C-terminal ends (Boraston *et al.,* 2006; Machovič and Janeček, 2006; Lombard *et al.,* 2014). The SBDs are more important for the breakdown of thermally-untreated granular starch. SBD is the independent protein module within the raw starch digesting enzymes. SBD does not exhibit catalytic activity but holding the substrate in a proper position and represents it to the active site. The SBDs along with other CBMs are an integral part of the CAZy database. Currently, there are 85 CBM families; among them CBM20, 21, 25, 26, 34, 41, 45, 48, 53, 58, 68, 69, 74, 82 and 83 exhibit SBD functional characteristics. However, CBM74 is recognized as an extra-long module having a β-sandwich with 100 residues and carrying at least one substrate binding site (Janeček *et al.,* 2019).

The classification of the GH13 family is based on the amino acid sequence homology. Three main groups of enzymes like glycosyl hydrolases (endoamylases, exoamylases), transferases, and isomerases are the member of this family (Møller and Svensson, 2016). GH13 family was further classified based on a larger unit called clan, which is the three-dimensional structure of the catalytic region. A clan consists of two or more families having the same three-dimensional structure of the catalytic domain but with limited sequence similarities. Among the eighteen clans (A–R) of glycosidases and transglycosidases, GH-13 belongs to the clan eighth (GH-H) (MacGregor *et al.,* 2001; MacGregor 2005). Initially, Takata *et al.* (1992) gave the common features of the enzymes of this family: (i) they cleave α-glycosidic bonds and hydrolytic products are α-anomeric mono- or oligosaccharides, transglycosylation activity forms α-1-4 or 1-6 glycosidic linkages or a combination of both; (ii) TIM (triose phosphate isomerase) barrel or $(\beta/\alpha)_8$ barrel structure is the common features, which bears the amino acid residues at the catalytic site; (iii) four highly conserved sequence regions are present in their primary sequence, these hold catalytic residues and stabilize TIM barrel topology; (iv) Asp, Glu, and Asp residues form a triad at the catalytic site to exert hydrolytic cleavage by acid/base retaining mechanism. These three amino acid residues are also present in the catalytic sites of α-amylase, pullulanase, cyclodextrin glycosyltransferase (CGTases), isoamylase, and the branching enzymes (Kuriki and Imanaka, 1999). However, some additional features are also included in the α-amylase family, which cover hydrolases, transferases, and isomerases (Svensson *et al.,* 2002). The enzymes of this family can cleave not only α-1,4- and α-1,6-bonds but also α-1,1-, α-1,2-, α-1,3- and α-1,5-glucosidic linkages (Mac Gregor *et al.,* 2001). Later, three additional conserved sequence regions were also established along with the previous four conserved regions (Janeček, 2002).

5. Production and purification of amylases

The application of biocatalysts in different industrial sectors is a great success towards the clean and green world. Several biotech companies produce varieties of useful enzymes like amylases, cellulase, pectinases invertase, pullulanase, protease, lipase, lysozyme, and others, which are mostly obtained from microbial sources. Among the industrially important enzymes, amylases constitute a class of enzymes having approximately a quarter shares in the world's enzyme market (John, 2017). Amylases are ubiquitously present in all living organisms. However, in the commercial sectors, different bacteria, fungi, and recombinant microbes are used for amylase production.

Several studies had revealed that various bacteria under the genus *Bacillus* (*B. licheniformis*, *B. stearothermophilus*, B. *amyloliquefaciens*, *B. subtilis*, and others), non-Bacillus (*Pseudomonas stutzeri*, S*treptococcus brevis*, *Rhodothermus marinus*, *Corynebacterium gigantea*, *Clostridium acetobutylicum*, *Caldimonas taiwanensis*), and halophilic organisms (*Haloarcula hispanica*, *Halobacillus* sp., *Chromohalobacter* sp., *Bacillus dipsosauri*, *Halomonas meridian*) are the good sources of extracellular amylase production. Not only bacteria, but fungi have also shown good results in this purpose. Different species of *Aspergillus* (*A. oryzae*, *A. niger*, *A. awamori*, *A. fumigates* and others), *Penicillium* (*P. brunneum*, *P. fellutanum*, *P. expansum*, *P. chrysogenum*, etc.), and others (*Streptomyces rimosus*, *Thermomyces lanuginosus*, *Pycnoporus sanguineus*, *Thermomonospora curvata*) are regarded as efficient amylase producer (Samanta, 2020b). Additionally, the genus *Aspergillu*s and *Bacillus* are the major contributors of amylases in the industrial sectors, and they are constantly exploited for enzyme preparation. A short-list of amylase producers and their optimized conditions for enzyme production has been furnished in Table 2. To improve the characteristics (pH profile, thermostability, oxidant resistance, metal ion independency, and others) of amylase, recombinant organisms (Table 3) were developed for better purposes.

Production of microbial enzymes mostly depends upon the environmental and nutritional conditions of the culture medium (Table 2). The growth of microorganisms is determined by the organism's ability to utilize the essential nutrients from its surroundings as well as its physiological activities. Several physicochemical parameters, including nutrient supplementation (carbon, nitrogen, and phosphate), pH of the medium, water activity, oxygen supply, temperature, and level of contamination are the crucial factors for optimum growth of the microorganism as well as enzyme production. Fermentation is the process for the production of useful products through the mass culture of certain microorganisms. Production of amylases has been carried out in both solid-state fermentation (SSF) and submerged fermentation (SmF). Traditionally, SmF was used for enzyme production; later SSF is being more popular for large-scale production of amylases due to its cost-benefit and ease of handling. Biotechnological progress makes the SSF more fashionable to the industrial

sectors for the production of enzymes, food and pharmaceutical components, bio-bleaching agents, and others (Soccol *et al.,* 2017). To achieve the maximum yield, several parameters like surface area, porosity, moisture content, particle size, and nutrient supplementation are considered during culture (Farinas, 2015; Singhania *et al.,* 2009). The potential use of moist agricultural polymeric substrates such as wheat bran, rice bran, rice husk, cassava, sunflower meal, cottonseed meal, soybean meal, pearl millet, and others make the process more convenient and eco-friendly. Agricultural residues provide solid support and nutrients as they contain cellulose, hemicellulose, lignin, starch, pectin, protein, minerals, and others (Farinas, 2015). Supplementation of macro (protein) and micro-nutrients (vitamins and minerals) enhance many folds of enzyme synthesis.

The extracellular enzyme is present with other proteins and cell debris in the culture medium; so, it is essential to purify the enzyme up to its homogeneity. Most of the enzymes are purified from other proteins and non-protein contaminants based on their inherent properties like shape, size, charge, hydrophobicity, solubility, and biological activity. Traditionally, a multi-step purification system was the common process of purification. In this technique, centrifugation, or filtration of the culture medium, salting out, ion-exchange chromatography followed by gel filtration were performed in the purification process. High performance liquid chromatography (HPLC) is applied to increase the homogeneity of protein.

The conventional multi-step purification process is cost expensive, time-consuming, and always has a chance of loss of the desired product with low yield (Arauza *et al.,* 2009). The large scale cost-effective purification of the bulk enzyme for commercial purposes had been developed after the evolution of purification techniques, which are fast, efficient, and economically viable with fewer processing steps (Amritkar *et al.,* 2004). Forced affinity chromatography, expanded bed / fluidized bed chromatography, high-speed counter-current chromatography (HSCCC), and magnetic affinity adsorption chromatography are the single-step purification process. Forced affinity chromatography provides effective results for the purification of amylase on an industrial scale. Soluble starch, raw potato starch, carboxymethyl-starch, and guar gum are used for the preparation of the gel matrix by cross-linking the polysaccharides with epichlorohydrin. Pectin gel may also be prepared by using carbodiimide (EDC, l-ethyl-3-(3-dimethyl aminopropyl). Ammonium sulfate acts as a stimulator (forced affinity) for the binding of the enzyme to the gel. The features of single-step purification techniques are given in Table 4.

Molecular weights of α-amylases vary from about 10-148 kD (Nguyen *et al*., 2000; Valk *et al.,* 2016). The optimum pH value of α-amylase from various sources differs from 2-11 (Sivaramakrishnan *et al.,* 2006). Temperature optima for α-amylase activity vary from 25 °C to 100 °C (Mehta and Satyanarayana, 2016). Most of the α-amylases are calcium-dependent; however, calcium-independent amylase is equally important (Mehta and Satyanarayana, 2014; Samanta *et al.,* 2014; Xian *et al.,* 2015). The major characteristics of microbial α-amylases are presented in Table 5. Variations in the characteristics make them useful for their industrial applications. Despite α-amylase, del Moral *et al.* (2018) reported that fungal α-glucosidases were optimally active in 50 to 65 °C temperatures and pH 4.5–6. The molecular mass of the enzymes was observed in between 50 to 145 kD. Generally, they showed metal ion-independent activity. The molecular weight of isoamylase varied in the range of 60 to 120 kD. The pH and temperature optima were observed between 5–8 and 30 to 80 °C, respectively. The catalytic activity did not depend on metal ions (Ray, 2011).

Table 2: Sources and production of microbial α-amylase.

Source	Fermentation	Substrate	Enzyme production	pH for optimum enzyme production	Tempt. (°C) for optimum enzyme synthesis	Incuba-tion period (hours)	Ref.
Fungi							
Aspergillus oryzae (IFO 30103)	SSF	Spent-brewing grains	11296 U/ gds		30	48	Patel *et al.,* 2005
Aspergillus oryzae	SSF	Wheat bran	15095 U/gds	5.0	30	72	Sivaramakrishnan *et al.,* 2007
Aspergillus oryzae IFO 30103	SSF	Wheat bran		6.0	32	48	Dey and Banerjee, 2012
Aspergillus oryzae	SmF	Synthetic media	2.685 U/ml	7.0	45	72	Shah *et al*., 2014
Aspergillus niger	SmF	Synthetic medium		5.0	30	120	Gupta *et al.*, 2008
Aspergillus niger BAN 3E	SSF	Black gram bran	8 U/mg	5.5	37	120-144	Suganthi *et al.,* 2011
Aspergillus niger WLB42	SmF	Synthetic medium	2189 U/ml	7.0	30	48	Wang *et al.,*· 2016
A. fumigatus NTCC1222	SSF	Wheat bran	341.7 U/mL	6.0	35	144	Singh *et al*., 2014
Aspergillus terreus NCFT4269.10	SSF and liquid static surface (LSSF)	Pearl millet		7.0	30	96	Sethi *et al.*, 2016a and 2016b
Penicillium janthinellum NCIM)	SSF	Wheat bran	275 U/gds	5.0	35	96	Sindhu *et al.*, 2011
Bacteria							
Bacillus subtilis	SmF	Soluble starch	3790 U/ml		37	24	Özdemin *et al.,* 2011
B. amyloliquifaciens TSWK1-1	SmF	Synthetic medium	250 U	7.0	50	48	Kikani and Singh, 2011
B. amyloliquifaciens ATCC23842	SSF	Ground nut oil cake and wheat bran	1671 U/gm	5.0	37	72	Gangadharan *et al.,* 2011
B. amyloliquefaciens P-001	SmF	Synthetic medium with soluble starch	35.0 4 U/ml	9.0	42	48	Deb *et al*., 2013
B. methylotrophicus P11-2	SmF	Soluble starch	144 U/ml	7.2	37	70	Xie *et al*., 2014
B. licheniformis AI20	SmF	Soluble starch	166.5 U/ml	7.0	45	40	Abdel-Fattah *et al.,* 2013
B. licheniformis SKB4	SmF	Soluble starch	3.8 U/ml	6.5	42	24	Samanta *et al*., 2014
B. acidicola TSAS1	SmF	Starch	8300 IU/l	4.5	33	36	Sharma and Satyanarayana, 2011
B. cereus	SmF	Potato starch		6.5	35	48	Madhavi *et al*., 2010
Bacillus sp. GHA 1	SmF	Potato starch Soyameal		6.5-8.0	37	72	Ahmadi *et al*., 2010
Geobacillus sp. IIPTN	SmF	Starch containing medium with soybean meal	135 U/ ml	6.5	60	14	Dheeran *et al*., 2010
Geobacillus stearothermophilus HP 3	SmF	Maltose supplement	80 U/ml	9.0	55	24	Selim, 2012
Cronobacter sakazakii Jor 52	SmF	Soluble starch	2.2 U/ml	7.0	37	24	Samanta *et al*., 2013
Recombinant *B. licheniformis* CBBD302 (PHY-amyL)	SmF	Soybeam meal, cotton seed meal, corn step liquor, lactose	17.6 mg/ml	6.0	42	120	Niu *et al*., 2009

Tempt.: Temperature; Ref.: References

Table 3. Sources of recombinant organisms as α-amylase producer and some fundamental features of the enzyme

Organism	Host	Key features of α-amylase from recombinant organism	References
Bacillus licheniformis NH1	*Escherichia coli* BL21 Vector: pDEST17	MW: 58 kDa pH optima: 6.5 (pH range 5.0 to 10.0) Temperature optima: 90 °C Others: Show better therostability and maximum stability towards surfactants (SDS, Tween 20 and Triton X-100)	Hmidet *et al.*, 2008
B. subtilis WB800	*E. coli* DH5α Vector: pMD18-T	ORF sequence: 1545 bp encoding 514 amino acid residues Rate of production: 1.48-fold higher than wild type MW: 58.4 kDa (predicted) pH optima: 6.0 Temperature optima: 60 °C Others: sensitive to detergents/surfactants.	Chen *et al*., 2015
B. acidicola	*E. colii* BL21 (DE3) Vector: pET28a(+)	Rate of production: 15-fold higher than wild type MW: 62 kDa pH optima: 4.0 (pH range 3.0 to 7.0) Tempt. optima: 60 °C (tempt. range 30 to 100 °C) Others: Ca2+-independent, acidstable, and high maltose forming	Sharma and Satyanarayana, 2012
B. subtilis DR8806	*E. coli* BL21 (DE3) Vector: pET28a(+)	MW: 76 kDa pH optima: 5.0 (pH range 4.0 to 9.0) Tempt. optima: 70 °C (tempt. range 45 to 75 °C) Others: Show high stability towards ioninc detergents SDS, CTAB, and maltoriose and maltose forming	Emtenani *et al*., 2015
Geobacillus thermoleovorans	*E. coli*	ORF sequence: 1650 bp encoding 515 amino acid residues MW: 59 kDa pH optima: 5.0 Tempt. optima: 80 °C Others: Ca2+-independent, highly thermostable and raw starch digesting	Mehta and Satyanarayana, 2013a
Halomonas meridiana DSM 5425	*E. coli*	α-Amylase gene *AmyH* is the first extracellular-amylase-encoding gene isolated from halophiles and cloned into *E. coli*	Coronado *et al*., 2000b
Pseudoalteromonas sp.MY-1	*E. coli*	ORF sequence: 2007 bp encoding 669 amino acid residues MW: 73.77 kDa (predicted) pH optima: 7.0 Tempt. optima: 40 °C Others: oligosaccharides, and maltose forming	Tao *et al*., 2008
Staphylothermus marinus	*E. coli*	Amino acid residues: 696 MW: 82.5 kDa (predicted) pH optima: 5.0 (pH range 3.5 to 5.0) Tempt. optima: 100 °C Others: extremely thermostable; active against linear malto-oligosaccharides, starch, cyclodextrins, and cycloamylose; hydrolyze acarbose and pullulan to acarviosine-glucose and panose, respectively	Li *et al*., 2010
Thermobifida fusca NTU22	*Pichia pastoris* X-33 Vector: pGAPZalphaA	Rate of production: very high MW: 65 kDa pH optima: 7.0 Tempt. optima: 60 °C Others: highly thermostable; raw sago starch digesting	Yang *et al*., 2010
Thermoplasma volcanicum GSS1	*E. coli*	pH optima: 7.0 Tempt. optima: 75 °C and 80 °C Others: highly thermostable; longer subsite structure, highly maltogenic	Kim *et al*., 2007

Tempt.: temperature; MW: molecular weight; ORF: open reading frame; SDS: Sodium dodecyl sulphate; CTAB: cetyl trimethylammonium bromide

Table 4: Methods of one-step purification process of α-amylases.

Method	Chromatographic substances	Basis	Advantages
Affinity adsorption chromatography	Cross-linked starch, Epichlorohydrin or bifunctional epoxides activated agarose gel, α-, β-, and γ Cyclodextrin (CD) coupled with sepharose, divinyl sulphone.	Reversible interaction occurs between enzymes and specific ligand attached to chromatographic matrix.	High selectivity, high resolution, high capacity
	Immobilized metal ion affinity chromatography (IMAC): Cyclodextrin is treated by iminodiacetic acid (IDA) or nitrilotriacetic acid (NTA) and then the material is being further charged with cations (Ag^{+}, Cu^{2+}, Mg^{2+}, Ni^{2+}, Hg^{2+}, Fe^{2+} and Zn^{2+}).	Enzyme binds with ligand by non-covalent interaction	
	Desorption agent -elution buffer with different pH, salt solution, imidazole, N-protected histidines and tryptophan	The amino acid residues, such as aspartic acid (carboxyl group), glutamic acid (carboxyl group), histidine (imidazole group), cysteine (thiol group), tryptophan (indoyl group) or lysine (amino group) on the protein surface can specifically interact with different metal ions.	
Expanded bed chromatography / Fluidized bed chromatography	Alginic acid-cellulose cell beads Cross-linking cellulose with other OH containing polymers like starch, dextrins, sodium alginate, alginic acid, cellulose, DEAE cellulose, carboxymethyl cellulose and microcrystalline cellulose (MCC).	Sample feeding flow and elution flow regulate the entire process	Simple process, low downstream processing cost, reduced number of purification steps, high downstream efficiencies, industrially applicable; enzyme is eluted in increased concentration.
High speed counter current chromatography (HSCCC)	The combinations of aqueous polymer with high water content and low interfacial tension (two phase system) are applicable.	It is a continuous liquid–liquid partition chromatography without solid support matrix	No interaction between samples with solid support, little chance of denaturation of target products, high recovery, high efficiency, easy to scale-up, industrially compatible, reduces initial downstream steps.
	Aqueous two phase polymer is made by PEG4000-phosphate aqueous polymer or PEG4000-citrate aqueous polymer.		
Magnetic affinity adsorption	Magnetic alginate beads (microparticles) is prepared by using sodium alginate and ferromagnetic material (magnetic fluid - citrate-based ferrofluid).	Magnetic component carries magnetic biopolymer particles which have affinity to the enzyme	Applicable for separation of alpha amylase and other starch degrading enzymes, simple process, very few steps are necessary, all the steps of purification are done in a single vessel, less expensive, no needs of major equipments, time saving, high efficacy value for large scale separation
	Other substances are used for magnetic biopolymers synthesis are agarose, chitosan, and kappa carrageenan.	.	

Table 5: Physico-chemical characteristics of *α*-amylases.

Source	Purification levels	MW (kD)	pH optima for enzyme activity	Tempt. (°C) optima for enzyme activity	Kinetic properties	Effects of metal ions	Ref.
Fungi							
Aspergillus oryzae IFO 30103	Sp. Act. (U/mg)- 627 Pur. Fd.- 7.1 Yld. (%)- 40	51.3	5.5	50	K_m - 0.5% V_{max} - 1000 U/mg	↑ Co^{2+}, Ca^{2+}, Mg^{2+} ↓ Zn^{2+}, Cu^{2+}	Dey and Banerjee, 2015
A. oryzae (IFO 30103)	Sp. Act. (U/mg)- 51.08 Pur. Fd.- 7.14 (Up to $(NH_4)_2SO_4$ ppt.)	66	5.0	50		↑ Mn^{2+}, Fe^{2+} ↓ Hg^{2+}, Cu^{2+}	Patel *et al*., 2005
A. niger WLB42		50	7.0	45		↑ Co^{2+}, ↓ Zn^{2+}, Cu^{2+}, Mg^{2+}	Wang *et al*.,· 2016 ·
A. niger	Sp. Act. (U/mg)- 0.982 Pur. Fd.- 81.83		4.5	45		↑ K^{+}, Ca^{2+}, Mg^{2+} ↓ Hg^{2+}, Pb^{2+},	Aisien and Igbinosa, 2019

	Yld. (%)- 60.227						
A. flavus F_2Mbb	Sp. Act. (U/mg)-4348 Pur. Fd.- 161 Yld. (%)- 15.74	52.5	6.4	30	K_m - 0.5 mg/ml V_{max} – 17.78 mg/ml/min		Sidkey *et al*., 2011
A. flavus NSH9	Sp. Act. (U/mg)-48.1 Pur. Fd.- 2.55 Yld. (%)- 11.73	54	5.0	50	K_m – 4.22 mg/ml V_{max} – 65.52 U/mg	↑ Ca^{2+}, ↓ Zn^{2+}, Cu^{2+}, Mg^2	Karim *et al.*, 2018
Penicillium camemberti PL21	Sp. Act. (U/mg)-154.2 Pur. Fd.- 38.5 Yld. (%)- 23	60.5	5-6.0	30	K_m – 0.92 mg/ml V_{max} - 38.5 µmole/min	↑ Ca^{2+} ↓ Hg^{2+}, Ag^{+}	Nouadri *et al.*, 2010
P. citrinum HBF62	Sp. Act. (U/mg)-1451 Pur. Fd.- 18 Yld. (%)- 82	65	5.5	55	K_m – 0.2 mg/ml V_{max} - 5000 U/mg	↑ Mn^{2+}, Ca^{2+}, Co^{2+}, Fe^{3+}, Ba^{2+} ↓ K^{+}, Zn^{2+}. Hg^{2+}	Metin *et al.*, 2010
P. janthinellum NCIM)	Sp. Act. (U/mg)-696.66 Pur. Fd.- 44.06 Yld. (%)-30.73	42.7	5.0	50		↓ Hg^{2+}, Zn^{2+}, Cu^{2+}, Ag^{+}, Pb^{2+}, Fe^{2+}, Fe^{3+}	Sindhu *et al.*, 2011
P. olsonii	Sp. Act. (U/mg)-23.45 Pur. Fd.- 25.14 Yld. (%)- 36.5		5.6	30	K_m – 0.556 mg/ml	↑ Mn^{2+}, Mg^{2+}	Afifi *et al.*, 2008
Bacteria							
Bacillus cereus	Sp. Act. (U/mg)-50.0	56	6.0	50	K_m – 1.43 mg/ml V_{max} - 0.27µmol/min	↓ Zn^{2+}	Mahdavi *et al.*, 2010
B. subtilis ITBCCB148	Sp. Act. (U/mg)-40000 Pur. Fd.- 148 Yld. (%)- 8.8	67	6.5	60	K_m – 2.5 mg/ml V_{max} - 192.3 µmol/ml/min		Yandri *et al.*, 2010
B. amyloliquifaciens TSWK1-1	Sp. Act. (U/mg)-8000 Pur. Fd.- 13.3 Yld. (%)-45.71	43	7.0	70	K_m – 0.6 mg/ml V_{max} - 2632 µmol/ml/min	Metal ion independent	Kikani and Singh, 2011
B. methylotrophicus P11-2	Sp. Act. (U/mg)-330.7 Pur. Fd.- 13.1 Yld. (%)- 7.0	44	7.0	70		↑ Mg^{2+}, Ba^{2+}, Al^{3+} ↓ Hg^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}	Xie *et al.*, 2014
B. megaterium VUMB109.	Sp. Act. (U/mg)-240.19 Pur. Fd.- 27.39 Yld. (%)-38.43	150	7.75	93	K_m – 1.5 µM V_{max} – 0.56 µmol/mg/min	↑ Ag^{2+}, Cu^{2+}, Sn^{2+}, K^{+}, Mg^{2+} ↓ Hg^{2+}, Pb^{2+}, Zn^{2+}	Jana *et al.*, 2013
B. licheniformis AI20	Sp. Act. (U/mg)-748.9 Pur. Fd.- 59.34 Yld. (%)- 12.6	55	6-7.5	60-80	K_m – 0.709 mg/ml V_{max} - 454	↑ Ca^{2+}, Co^{2+} ↓ Hg^{2+}	Abdel-Fattah *et al.*, 2013

					mU/mg		
B. licheniformis	Sp. Act. (U/mg)-339.5 Pur. Fd.- 203.29 Yld. (%)- 23.62		7.5	90	K_m – 1.097% V_{max} - 44.54 U/min	↑ Ca^{2+}, Mg^{2+}, Co^{2+}, Fe^{2+} ↓ Zn^{2+}, Ba^{2+}	Adeyanju *et al*., 2007
B. licheniformis SKB4	Sp. Act. (U/mg)-827 Pur. Fd.- 214 Yld. (%)- 64.8	60	6.5	90	K_m – 6.2 mg/ml V_{max} – 1.04 µmol/mg/min	↑ K^+, Mg^{2+}	Samanta *et al*., 2014; Samanta *et al*., 2017
Bacillus sp. GHA1	Sp. Act. (U/mg)-250 Pur. Fd.- 131.6 Yld. (%)- 28	66	5.5-8			↑ Ca^{2+}	Ahmadi *et al*., 2010
Bacillus sp. SI-136	Sp. Act. (U/mg)-1193.07 Pur. Fd.- 2.93 Yld. (%)-84.3 (Up to $(NH_4)_2SO_4$ ppt.)	26	10	70-80		↑ Mn^{2+} ↓ Fe^{2+}, Mg^{2+}, Hg^{2+}, Zn^{2+}	Sarethy *et al.*, 2012
Geobacillus stearothermophilus HP 3	Sp. Act. (U/mg)-1.45 Pur. Fd.- 0.35 Yld. (%)- 13.9	64	9.0			↑ K^+, Mg^{2+}, Fe^{2+} ↓ Hg^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}	Selim, 2012
Geobacillus sp. IIPTN	Sp. Act. (U/mg)-1200 Pur. Fd.- 82 Yld. (%)- 31	97	5.0	80	K_m – 36 mg/ml V_{max} – 222 µmol/mg/min	↑ Mn^{2+}, Co^{2+}, Ca^{2+}, Ba^{2+}, Na^+, Fe^{3+} ↓ Cu^{2+}, Zn^{2+}, Mg^{2+}	Dheeran *et al*., 2010
Anoxybacillus beppuensis TSSC-1	Sp. Act. (U/mg)-10,000.00 Pur. Fd.- 19.51 Yld. (%)-58.53	43	7.0	80	K_m – 0.5 mg/ml V_{max} – 3571.42 µmol/ml/min	Metal ion independent	Kikani and singh, 2012

MW: Molecular weight; Tempt.: Temperature; Ref.: References; Sp. Act.: Specific activity; Pur. Fd.: Purification fold; Yld.: Yield; ppt.: Precipitation

6. The pattern of the domain structure of different amylases of GH13 and related families

The crystallographic structure of amylases revealed that they consist of a single polypeptide chain folded in three distinct domains A, B, and C (Fig. 2). The A domain is the most conserved structure in the amylase family. The N-terminal part of the A-domain consists of eight parallel β-strands, arranged in a barrel configuration, surrounded by eight α-helices ─ $(β/α)_8$ barrel also called TIM barrel (Prakash and Jaiswal, 2010). This $(β/α)_8$ barrel structure was first observed in the triosephosphate isomerase (TIM) of chicken muscle. Domain B and C are located on the opposite side of the TIM-barrel structure. Domain B is a β-strand rich structure. It is a projection part between β-strand 3 and α-helix 3 of the TIM-barrel (Janecek *et al.,* 1997). Both the domains A and B jointly form the substrate-binding cleft. There are seven other domains (C to I) in this family (Table 6) that are distributed either in front or behind the A domain. Domain C (β-sandwich domain) is present at the C terminal end of the enzyme (Nielsen *et al.,* 2004). The activity of CGTase is influenced by C-domain. E-domain has a role in the raw starch binding activity. N-terminal F-, H-, or G-domains are involved in the hydrolysis of α-1,6 glycosidic linkages (van der Maarel *et al.,* 2002). Most of the time, calcium ion is associated with amylase structure. It is present at the interface between domains A and B. Calcium ion increases the stability and activity of the enzyme. However, several reports support the calcium independence of amylase activity (Asoodeh *et al.,* 2010; Mehta and Satyanarayana, 2014; Samanta *et al.,* 2014; Xian *et al.,* 2015).

There are slight differences in the domain structure in different amylases. α-Amylase (EC 3.2.1.1) and its related enzymes have A, B, and C domains. Features of these domains are already described. The $(β/α)_8$ barrel topology is also observed in the structure of α-glucosidase (EC 3.2.1.20); although, the $(β/α)_8$ structure of GH31 is quite different from GH13 (Mohanan and Satyanarayana, 2018). The catalytic domain of all debranching enzymes (oligo-1,6-glucosidase, isoamylase, and pullulanase) is present in $(β/α)_8$ barrel structure. CTGase (EC 3.2.1.19) consists of five domains (A–E). The A domain is present at the N-terminal site, comprised $(β/α)_8$ barrel, and appeared as the major functional site. The B and C domains are involved in substrate binding and stability of the catalytic site (Knegtel *et al.,* 1996). Additionally, the C domain helps in

maltose-binding (Strokopytov *et al.,* 1995). The D domain is specifically present in CGTases, but its function is still unknown. E domain is also present in CGTase and other amylases (Table 6). E domain is associated with maltose-binding as well as raw starch binding (Qi and Zimmermann, 2005). Maltotetraose forming amylase (EC 3.2.1.60) has $(\beta/\alpha)_8$ barrel containing A domain; domain B has five standard anti-parallel β-sheets. At the molecular level, branching enzyme (EC 2.4.1.18) bears $(\beta/\alpha)_8$ barrel structure in their internal topology.

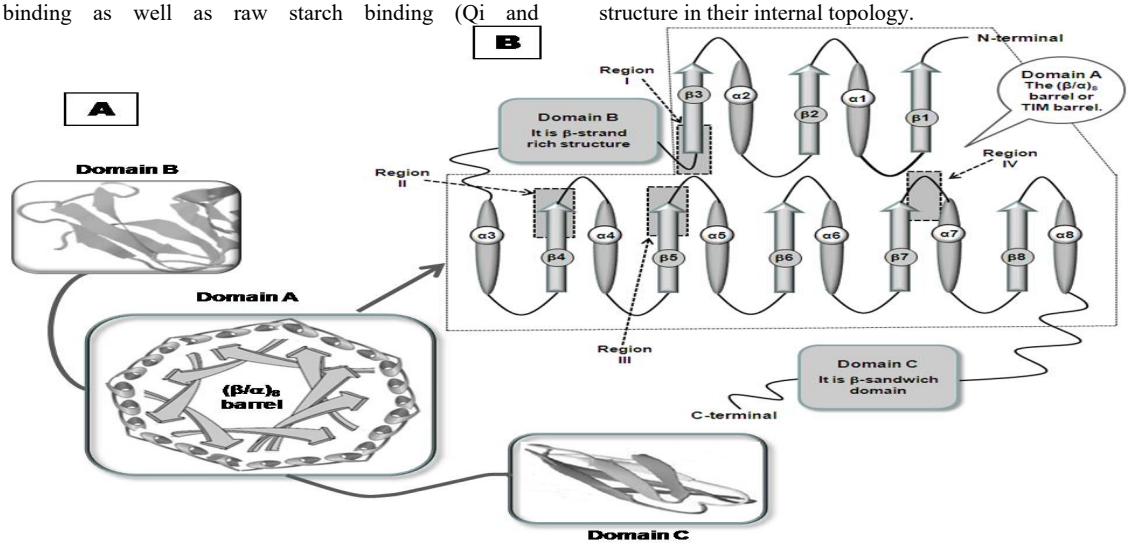

Figure 2. A: Schematic representation of the molecular structure of amylase. Three distinct domains are shown with a different configuration. $(\beta/\alpha)_8$ barrel structure is present in domain A. B: Topology of $(\beta/\alpha)_8$ barrel. The positions of four conserved sequence (I–IV) are indicated with shaded boxes.

Table 6: Different domains present in the enzymes of α-amylase family (van der Maarel *et al.,* 2002).

Common name	EC. No.	Domain	Features of domain
α- amylase	3.2.1.1	A, B, C	The A domain contains $(\alpha/\beta)_8$ barrel structure and catalytic site.
			The B domain (eight-stranded antiparallel β-sandwich structure) is a short loop structure closely associated with $(\alpha/\beta)_8$ barrel. It forms the wall of the catalytic cleft and few residues of substrate fit within the B domain during catalysis. Some times, Ca^{2+} can bind with B domain.
			The C domain is made by antiparallel β sandwich structure, folded in a Greek-key motif.
Oligo-1,6-glucosidase	3.2.1.10	A, B	Features are already given.
Isoamylase	3.2.1.68	A, B, F, 7	The domain F (α/β-barrel structure), located at the N-terminal part of $(\alpha/\beta)_8$ barrel
Pullulanase	3.2.1.41	A, B, H, G, 1	The domain G and H (both α/β-barrel structure) located at the N-terminal part of $(\alpha/\beta)_8$ barrel
Neopullulanase	3.2.1.135	A, B, G	Features already are given.
Cyclodextrin glycosyltransferase	2.4.1.19	A, B, C, D, E	The β strand of D domain forms immunoglobulin-like fold and E domain has separate β strand motif and also has starch binding capacity.
Branching enzyme	2.4.1.18	A, B, F	Features already are given.
Maltogenic -amylase.	3.2.1.133	A, B, C, D, E	Features already are given.
Maltotetraose forming amylase	3.2.1.60	A, B, C, E	Features already are given.

Isoamylase (EC 3.2.1.68) from *Pseudomonas amyloderamosa* contains 750 amino acid residues. Like other GH13 enzymes, it also contains $(\beta/\alpha)_8$ barrel fold, which is not fully completed. An extra domain is present at the N-terminal part of the enzyme. A long region between the third β-strand and third α-helix of $(\beta/\alpha)_8$ barrel form a globular cluster in association with the loop between the fourth β-strand and fourth α-helix of $(\beta/\alpha)_8$ structure. Ca^{2+} is also attached to the internal structure of the enzyme; however, the position of calcium is different from the α-amylase (EC 3.2.1.1) (Ray, 2011).

Pullulanases are the important debranching enzyme. They have been classified into Type I pullulanase and Type II pullulanase, also known as amylopullulanases. Type I pullulanase exclusively hydrolyzes α-1,6-glycosidic bonds in pullulan and other branched oligosaccharides resulting in the formation of maltotriose and linear oligosaccharides. Amylopullulanases (Type II pullulanase) can cleave both α-1,6-glycosidic and α-1,4-glycosidic linkages in branched and linear oligosaccharides (Bertoldo and Antranikian, 2002). Type I pullulanase belongs to GH13 while Type II pullulanase is distributed in GH13 and GH57 based on their structure and

catalytic residues (Nisha and Satyanarayana, 2013). All the pullulanases carry $(\beta/\alpha)_8$ barrel structure, the catalytic triad is composed of aspartate, glutamate, and aspartate residues at the β4, β5, and β7 strands, respectively. However, the amylopullulanases of the GH57 family contain $(\beta/\alpha)_7$ barrel structure where catalytic residues glutamate and aspartate are situated in β4 and β7 strands, respectively. Moreover, it has five conserved sequences (Nisha and Satyanarayana, 2013; Xu *et al.,* 2013). Previously, Zona *et al.* (2004) first reported that the families GH57 had five conserved sequence regions. GH119 also had five conserved sequence regions (Sarian *et al.,* 2017). Mikami *et al.* (2006) reported that the crystal structures of the pullulanase of *Klebsiella pneumoniae* had five domains (N1, N2, N3, A, and C). N1 domain could bind with maltotriose, maltotetraose and one calcium ion also. The N1 and N2 domains were the exclusive features of pullulanases, while the other three domains (N3, A, and C) had some similarities with *Pseudomonas* isoamylase. Moreover, isoamylase and pullulanase are the debranching enzymes, but the substrate specificities are not similar due to their separate organization of domains and active site. Later, it was revealed that type I pullulanase from A*noxybacillus* sp. LM18-11 consists of four domains (N1, N2, A, and C). The catalytic amino acids Asp413, Glu442, and Asp526 are arranged in domain A. The N1 domain is recognized as a carbohydrate-binding motif. Interestingly, four molecules of oligosaccharides were associated with the active configuration of pullulanase. In this concern, two molecules of oligosaccharides were present in domain A; another two molecules were located in the N1 domain and the loop between the third β-strand and the third α-helix of domain A (Xu *et al.,* 2013).

Neopullulanase has a close similarity with maltogenic amylase (EC 3.2.1.133) and cyclomaltodextrinase (EC 3.2.1.54) (Hondoh *et al.,* 2003). Neopullulanase, cyclomaltodextrinase, and maltogenic amylase exclusively exhibit the fifth conserved sequence region (CSR V) (Janeček, 2002). Neopullulanases are closely associated with Type I pullulanase (Nisha and Satyanarayana, 2016). The Crystal structures of neopullulanase from *Bacillus stearothermophilus* TRS40 had revealed that the active enzyme forms a dimmer. The monomeric configuration possesses four domains (N, A, B, and C). Like other amylases, $(\beta/\alpha)_8$ barrel is present in domain A. However, Hondoh *et al.* (2003) reported that the α-helix 5 and β-strand of the $(\beta/\alpha)_8$ barrel of A domain were incomplete in comparison to the ideal A domain structure. The active site of the neopullulanase lies between the domain of two monomers, located in between domain A and domain N of the other monomer. This type of arrangement makes active-site cleft narrower in comparison to α-amylase and helps to exert hydrolytic activity for both α-1,4 and α-1,6-glucosidic bonds (Hondoh *et al.,* 2003).

β-amylase (EC 3.2.1.2) is organized in a single domain structure that contains a large $(\beta/\alpha)_8$ barrel core, which is similar to α-amylase. A small lobe made by three loops from the β-strand adjacent to the β barrel with a long C-terminal loop is also observed. Glucoamylase (EC 3.2.1.3) has three functional domains. The catalytic domain consists of $(\alpha/\alpha)_6$ barrel structure; O-glycosylated linked domain comprises multiple O-glycosylated sites and two N-glycosylated sites; another domain is the starch binding domain. Besides these, other features of some amylases are presented in Table 7.

Table 7: Structural and functional comparison of different amylases

Parameters	α-amylase	CTGase	Maltotetraose forming amylase	β-amylase	Glucoamylase (γ-amylase)
E.C. number	3.2.1.1	2.4.1.19	3.2.1.60	3.2.1.2	3.2.1.3
	Endo-acting	Transglycosylation	Exo-acting	Exo-acting	Exo-acting
Sources	*Bacillus licheniformis* (BLA) *Aspergillus oryzae* (TAKA amylase),	*Bacillus stearothermophilus*	*Pseudomonas stutzeri*	*Bacillus cereus*	*Aspergillus awamori*
Amino acids residues	512 (BLA)	680	429	516	471
	549 (TAKA amylase)				
Active site	In domain A at conserved sequence region II, III and IV.	In domain A, another substrate binding site is present in domain D	In domain A	C-terminal end of β-barrel	N-treminal end of α-helix bundle
Catalytic residues	BLA: Asp231, Glu261,	Asp225, Glu253, and Asp324	Asp193, Glu219, and Asp294	Glu172 and Glu367	Glu179 and Glu400
	TAKA amylase Asp206, Glu230				
Calcium ion	2/3	02	02	01	-
Disulfide bond	01	01	02 Cys140:150, 216:251	Cys91:99	03 Cys210:213, 266:270, 222:449
	Cys30:38	Cys40:47			

7. Conserved region and distribution pattern of catalytic residues in the amylases of GH13 and related families

The enzymes of the α-amylase family have four (I–IV) highly conserved sequence regions in their TIM barrel structure. Despite these four conserved regions, there are three additional conserved sequences in the enzymes of the α-amylase family. The seven conserved sequence regions of different enzymes of the α-amylase family are given in Table 8. The four primary conserved sequence regions are present in the terminal end of β-strand 3, β-strands 4, 5, and in the loop connecting β-strand 7 to α-helix 7 (MacGregor *et al.,* 2001; Janeček, 2002) (Fig. 2). These regions form the substrate-binding site and catalytic center (Kuriki and Imanaka, 1999). Asp residue of the second region and Glu residue of the third region acts as nucleophile and proton donors, respectively in α-amylase and related enzymes. His residue of the first region and His and Asp residue of the fourth region helps to hold the substrate in the proper position (Prakash and Jaiswal, 2010). The amino acids of the catalytic triad are always present in the conserved sequence region II, III, and IV, but their position number is different among the amylases. The conserved residues exhibit similar functional activity, also contribute to the substrate and calcium-binding capacity (Kumari *et al.,* 2012). The additional three conserved sequence regions are present in the different enzymes (glycoside hydrolases, transferases, isomerases, and others) of clan H. Among the three regions, two regions (VI and VII) are located in the area of β-strand 2 and β-strand 8 of the $(\beta/\alpha)_8$ scaffold. The third region (V) is present near the C-terminus of domain B at the connecting region of β-strand 3 and α-helix 3 in the vicinity of calcium-binding aspartate (Janeček, 2002). Qi and Zimmermann (2005) reported that CGTase had 51 conserved amino acid residues that were distributed in the conserved sequence region of the α-amylase family.

Table 8: Four highly conserved sequence regions and amino acid residues of enzymes of amylase family.

Enzymes	Origin	CSRI	CSR II *	CSRIII *	CSR IV *
α-Amylase	*Aspergillus oryzae*	117DVVANH	202GLRIDTVKH	230EVLD	292FVENHD
Cyclodextrin Glucosyl transferase (CGTase)	*Bacillus macerans; Bacillus circulans* 251	135DFAPNH	225GIRFDAVKH	258EWFL	324FIDNHD
Isoamylase	*Pseudomonas amyloderamosa*	292DVVYNH	371GFRFDLASV	435EPWA	505FIDVHD
Branching enzyme	*Escherichia coli; Bacillus stearothermophilus*	335DWVPGH	401ALRVDAVAS	458EEST	521LPLSHD
Pullulanase	*Bacillus flaocaldarius KP 1228*	600DGVFNH	671GWRLDVPNE	704EIWH	827LLGSHD
Neopullulanase	*Bacillus stearothermophilus*	242DAVFNH	324GWRLDVANE	357EIWH	419LLGSHD
α -Glucosidase	*Saccharomyces carlsbergenesis*	106DLVINH	210GFRIDTAGL	276EVAH	344YIENHD
Oligo-1,6-glucosidase	*Bacillus cereus*	98DLVVNH	195GFRMDVINF	255EMPG	324YWNNHD

Numbering of the amino acid sequences starts from N-terminal. Highlighted parts are the conserved amino acids. * marked indicates the catalytic amino acids.

The additional three conserved regions are not furnished in this table. Here, only the major regions involved in substrate binding and catalysis are presented. Actually, the region VI is located before the region I, region V is placed in between region I and II, and VII is present after region IV (for details see review Janeček, 2002).

All the members of the GH13 family bear Glu as a proton donor and Asp as a nucleophile in their catalytic site (Table 1), but the respective position of the Glu and Asp differs according to their total number of amino acid residues (Table 7). Sarian *et al.* (2017) reported that an atypical α-amylase (BmaN1) from *Bacillus megaterium* NL3 contained only two invariant catalytic residues in place of three residues. The third residue of BmaN1 was histidine, which acts as the transition-state stabilizer instead of aspartate. Typically, this amylase produces glucose and maltose from soluble starch. However, most of the α-amylase gives maltooligomers and branched dextrins as enzyme-catalyzed end products. This specific α-amylase belongs to the group of α-amylases containing aspartate, glutamate, and histidine in their catalytic triad. After phylogenetic analysis, this group of α-amylases is branched into a separate subfamily under GH13 (Sarian *et al.,* 2017). Alternatively, in family GH57, Asp acts as a proton donor, and Glu is a nucleophile. The three-dimensional structure of 4-α-glucanotransferase of *Thermococcus litoralis* was determined first in the family GH57. Unlike the catalytic triad of the enzymes of the GH13 family, the members of GH57 carried two catalytic residues Glu and Asp in their active site as the nucleophile and the proton donor, respectively (Imamura *et al.,* 2003). The *In Silico* study of Janeček and Kuchtová (2012) had revealed that α-amylase from family GH119 showed the same catalytic residues Glu231 and Asp373 with GH57 family as nucleophile and proton donor, respectively (Table 1). However, in family GH31, both proton donor and nucleophile is Asp (Table 1). Oligo-1,6-glucosidase (EC 3.2.1.10) bears $(\beta/\alpha)_8$ barrel fold in domain A. β-strands 4, 5, and 7 of $(\beta/\alpha)_8$ barrel contain nucleophile (Asp198), proton donor (Glu240), and transition state stabilizer (Asp316), respectively. Domain B involves in substrate recognition. Domain C consists of antiparallel β-sheet, forms a β-sandwich structure. Ca^{2+} forms an octahedral coordination shell, linked with 3 Asp, Asn, and

Ile residues of domain A (Møller *et al.,* 2012). However, Ca^{2+} independent structure was also reported by Watanabe *et al.* (1997).

8. Strategies of catalysis of glycosidic bonds

α-Amylase randomly hydrolyzes α-1,4 glycosidic linkages in polysaccharides containing three or more α-1,4 linked D-glucose units. It cannot hydrolyze α-1,6 linkages and always produce α-anomeric products. The initial products are high molecular weight dextrins, as the reaction proceeds large amounts of maltose, maltotriose, and oligosaccharides (α-limit dextrin) have appeared (Fig. 3). The reaction shows a rapid loss of viscosity and blue loss property (loss of intensity of the blue color of the starch-iodine complex).

α-Glucosidase (EC 3.2.1.20) is an exo-acting enzyme, hydrolyzes terminal non-reducing α-1,4 glucosidic linkage. The hydrolytic product is a single glucose molecule in α-configuration. However, glucoamylase gives the same type of reaction, but the product is in β-configuration (Fig. 3). A recent report indicated that α-glucosidase can cleave not only the α-1,4 linkage but also the α-1,6-, α-1,2-, and α-1,3-glucosidic linkages. Besides these, this enzyme has a transglycosylation activity (Mohanan and Satyanarayana 2018). At the catalytic site, both proton donor and nucleophile is Asp.

Debranching enzymes like oligo-1,6-glucosidase (EC 3.2.1.10), isoamylase (EC 3.2.1.68), and pullulanase (EC 3.2.1.41) attack only α-1,6 glucosidic linkages in an endo-acting fashion. They produce a variety of products like maltooligosaccharides (G3, G4, G6) and maltose (Fig. 3). Another debranching enzyme, amylo-1,6-glucosidase (EC 3.2.1.33) mainly found in the animal system; however, few bacterial species produce this enzyme. It is a bifunctional enzyme. It shows 4-α-glucanotransferase (EC 2.4.1.25) activity when the substrate is digested with phosphorylase (EC 2.4.1.1). Isoamylase acts on amylopectin and glycogen but bypasses pullulan. However, pullulanase can break amylopectin, pullulan, and a lesser extent of glycogen. In the industrial sector, pullulanase, in combination with β-amylase is applied for the production of maltose.

Maltohexaose forming amylase (EC 3.2.1.98), maltotetraose forming amylase (EC 3.2.1.60), maltotriose forming amylase (EC 3.2.1.116), and maltogenic amylase (EC 3.2.1.133) specifically produce different maltooligosaccharides and maltose (Fig. 3). All these enzymes are exo-acting, generating the products in α-configuration. Except maltogenic amylase (EC 3.2.1.133), the other three enzymes attack definite α-1,4 glucosidic linkages from the non-reducing end, produce maltohexaose, maltotetraose, and maltotriose, respectively. The maltogenic amylase (EC 3.2.1.133) cleaves α-1,4 glucosidic linkages of the substrates containing only two glucose units at the non-reducing end; the end product appears as maltose.

Cyclodextrin glycosyltransferase (CGTase) catalyzes different reactions: cyclization, coupling, and hydrolysis. These enzymes generate different types of cyclodextrins [(CDs) cyclomaltohexaose (α-CD), cyclomaltoheptaose (β-CD), and cyclomaltooctaose (γ-CD)] (Feng *et al.,* 2011), and few maltooligomers. The cyclization reaction produces CDs. In this reaction, the enzyme incises the α-1,4 glycosidic bond of the glucan chain at a distance of 6, 7, or 8 oligosaccharide units from the non-reducing end. Later, the C-4 hydroxyl group of non-reducing glucose end of the existing glucan chain reacts with newly formed reducing end glucose to complete the cyclization reaction. A coupling reaction occurs in the presence of glucose and CDs, resulting in the formation of maltooligosaccharides. CGTase hydrolyzes α-1,4 glycosidic bond and forms α-1,4 glycosidic bond in their reaction system.

Neopullulanases (EC 3.2.1.135) is a specific class of enzyme, which hydrolyzes α-1,4-glycosidic linkages of pullulan and releases a particular class of trisaccharides called panose, isopanose as well as isomaltose (Fig. 3). Neopullulanases can cleave α-1,4- and α-1,6-bonds of starch and related polysaccharides with poor efficacy. Several authors indicated that this enzyme is polyspecific, exhibits transglycosylation reaction, can hydrolyze cyclomalto-dextrins, starch, and pullulan (Park *et al.,* 2000).

The branching enzyme (2.4.1.18) is a different class of enzymes responsible for starch synthesis and is abundantly present in plants. The branching enzyme forms α-1,6 glycosidic bonds by chopping and transferring the growing α-glucan chains to the C-6 hydroxyl group of another glucan chain. Microbial sources of this enzyme are also available. *Bacillus* Sp., *B. stearothermophilus*, and *Pseudomonas* sp. can produce branching enzymes (Mohanan and Satyanarayana, 2018).

Both plant and microbial β-amylases are placed in the GH14 family. It is an exo-acting enzyme, cleaves α-1-4 glucosidic bond from the non-reducing end and successively releases maltose in β-configuration as well as β-limit dextrin (Fig. 3). It cannot cleave α-1,6 linkages. In comparison to the mechanism of action, β-amylase exhibits inverting instead of retaining, in which the hydrolytic product is converted to β-configuration from an α-anomeric structure (Fig. 4). β-Amylase is organized in a single domain structure; both proton donor and nucleophile activity are mediated by glutamate (Glu).

Glucoamylase / γ-amylase / amyloglucosidase (EC 3.2.1.3) was initially isolated from fungi after the identification of α-amylase and β-amylase in Japan. It is an exo-acting enzyme, hydrolyzes terminal α-1-4 glucosidic linkages from the non-reducing end and also enables to hydrolyze α-1-6 bonds at a slower rate than cleavage of α-1-4 glucosidic linkages. The final product is exclusively β-D-glucose (Fig 3). Thus this enzyme completely hydrolyzes starch to glucose. Unlike $(\beta/\alpha)_8$ barrel structure of α-amylase and β-amylase, glucoamylase has an $(\alpha/\alpha)_6$ structure. Both the proton donor and the nucleophile are Glu.

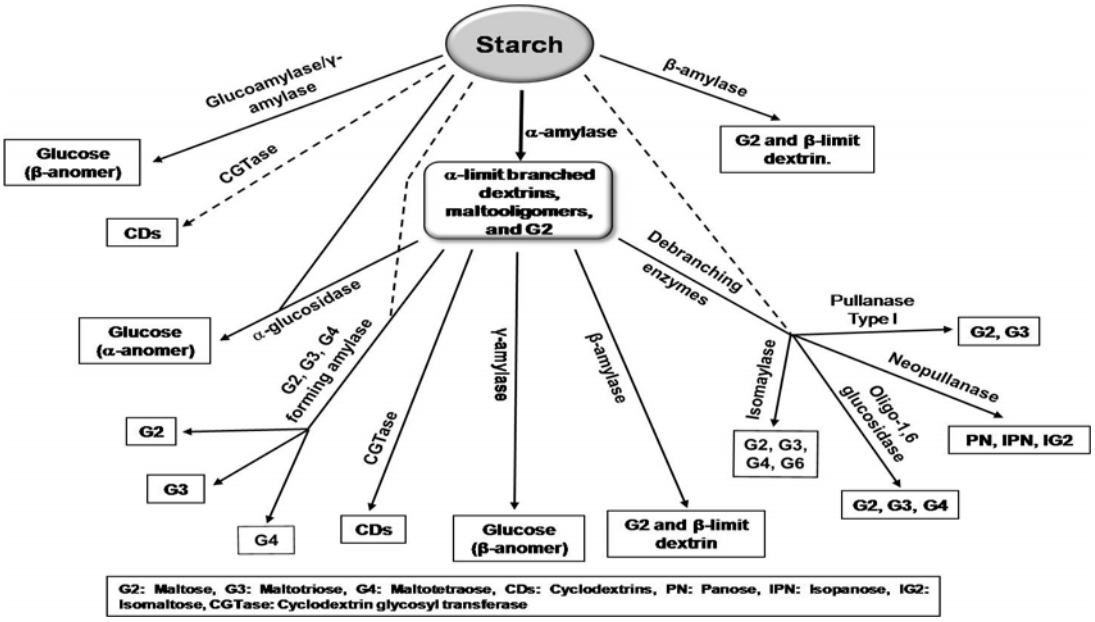

Figure 3. The pattern of product formation from starch and partially hydrolyzed substrate by different types of amylases.

9. Mechanism of catalytic activity

The hydrolytic activity of amylases is mediated through the α-retaining double displacement mechanism (Fig 4) (van der Maarel *et al.,* 2002). Four to ten glucose units of starch are fitted in the substrate-binding cleft. A particular subsite nomenclature is accepted for amylase activity. The nonreducing end of the substrate binds at the minus subsites; while, plus subsites hold the reducing end. The catalysis occurs in between +1 and –1 subsites (Fig 4a) (Davies *et al.,* 1997). Two aspartic acids (Asp) and one glutamic acid (Glu) involve in catalytic activity. Glutamic acid acts as a proton donor and aspartic acid acts nucleophile. During the reaction, Glu donates the proton to the glucosidic oxygen of the starch substrate, while aspartate starts nucleophilic attack to the C1 of glucose at subsite -1. The proton donation and nucleophilic attack finally cleave the glycosidic bond (Fig 4b). Later, water involves in the reaction system, re-protonates the glutamate and hydroxyl group of water attached to the C1 of the remaining substrate (Fig. 4b,c). The second aspartate residue plays an indirect role in catalysis by holding the substrate in the proper position (Uitdehaag *et al.,* 1999). Agirre *et al.* (2019) reported that α-amylase from *Alicyelobacillus* sp. can give the space to accommodate the branching point of starch. In the inverting reaction mechanism, the α-configuration products are formed after the reaction, which is converted to β-configuration (Fig 4c).

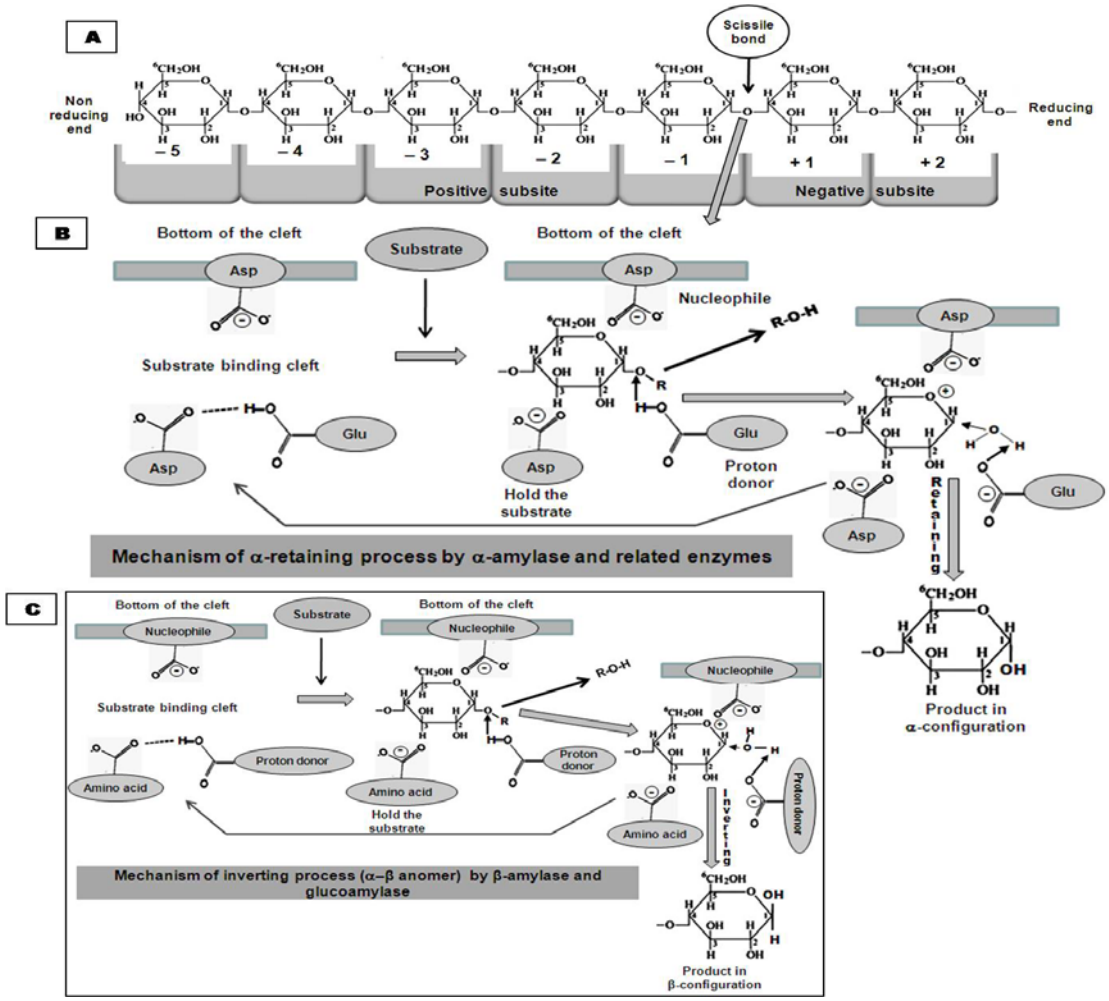

Figure 4: A: The pattern of subsite position, nomenclature, and presentation of a scissible bond. B: The α-retaining mechanism of the catalytic reaction. C: The inverting reaction and formation of β-anomeric product.

10. Application of α-amylase

The journey of applications of amylases was started from the early days of the nineteenth century when the Russian chemist Kirchhoff first discovered a starch degrading enzyme in wheat in the year 1811. Later, Payen and Persoz (1833) isolated a catalytic substance 'diastase', which converted gelatinized starch into sugars in experimental conditions. Magendie (1846) showed the diastatic properties of blood and proposed that blood was able to split starch into dextrin and glucose. The commercialization and industrial use of amylase were started after the isolation of fungal α-amylase 'Takadiastase' from *Aspergillus oryzae* by the Japanese scientist Jōkichi Takamine in 1894. In 1917, the French scientists, Boidin and Effront initiated the application of bacterial amylase (isolated from *Bacillus subtilis*) as a desizing agent.

10.1. Starch liquefaction, saccharification, and applications of hydrolyzed products

Starch is abundantly present in our environment. Different cereals like barley (50-60%), wheat (65-70%), sorghum (65-75%), rice (75-87%), corn (75-80%) contain large amount of starch. These ingredients are used in the starch processing industry for the production of different food ingredients, animal feed, biofuel, and therapeutic agents. Enzymatic hydrolysis of starch is a crucial part of the production of various commercially important products like glucose, fructose, high-fructose containing syrup (HFCS), maltose, maltooligosaccharides, dextrin, and cyclodextrins (Fig. 3). For this purpose, about 15%–20% of industrial enzymes are used in the starch industry. The endo- and exo- acting amylolytic enzymes (α-amylase, pullulanase, glucoamylase, glucose isomerase, and others) are potentially used for hydrolysis of starch at the industrial scale. Corn starch is mostly used for hydrolysis due to its huge availability.

Thermostable microbial α-amylase is added in the starch slurry for the production of low-molecular-weight dextrins after hydrolysis. The dextrins (DP3-25) are used for further applications in the preparation of commercial products. Debranching enzymes [oligo-1,6-glucosidase (EC 3.2.1.10), isoamylase (EC 3.2.1.68), and pullulanase (EC 3.2.1.41)] cleave the dextrin to DP2-5 products. The saccharification process is a vital step for starch processing. For this purpose, different enzymes such as α-glucosidase, maltogenic enzyme, maltooligosaccharide forming enzymes, β-amylase (microbial/plant), and γ-amylase are applied for the production of glucose,

fructose, HFCS, maltose, maltotriose, maltotetraose, and others. Pullulanases are widely used in the starch industries for the process of saccharification and preparation of maltose, maltotriose, and other oligosaccharides. This enzyme decreases the application of glucoamylase up to 50% and also reduces the reaction time. The mixture of glucoamylase and pullulanase is commercially available in the brand name of OPTIMAX from Genencor. Application of acid-stable, calcium-independent, thermostable amylopullulanase in the saccharification process makes reaction more convenient to produce different sugar syrups; this step is time-saving, and cost-effective (Nisha and Satyanarayana, 2013a, 2014). The neopullulanase breaks starch, CDs, and maltooligosaccharides, synthesizes large amounts of maltose and little amounts of glucose (α-anomer) during the reaction.

10.1.1. Production of maltose and maltooligosaccharides and their potential applications

Starch slurry is initially treated with the thermostable α-amylase for liquefaction; then saccharification is done with isoamylase, pullulanase, and β-amylase. The final product is prepared after downstream processing. Food grade maltose has several applications in bakery and confectionery products, preparation of infant and geriatric foods. Maltose is also used as a sweetener, quality improver and the preparative of foods. Moreover, highly purified medical-grade maltose is provided in intravenous infusion as it prevents a sudden increase in glucose levels (Samanta, 2020a).

Maltooligosaccharides have different gradations like maltotriose rich maltooligosaccharides, maltotetraose syrup (G4 syrup), and anomalously linked oligosaccharides mixture (Alo-mixture). Specifically, the maltotriose-rich maltooligosaccharides mixture contains glucose (2%), maltose (37.5%), maltotriose (46.5%), and other oligosaccharides (14.0%). The initial step of production is similar to maltose. β-amylase and pullulanase are used for the synthesis of the maltotriose-rich maltooligosaccharides mixture (Maltooligomer Mix). Maltotetraose syrup (G4 Syrup) is formed when saccharification is mediated by G4 amylase and isoamylase. Application β-amylase and fungal α-glucosidase at the saccharification level produces anomalously linked oligosaccharides mixture (Alo Mixture).

Linear maltooligosaccharides contain 3-10 monosaccharide units linked by α-1,4 linkages. Maltooligosaccharides have several beneficial properties and are widely used in the food industry. They have several unique characteristics, including high solubility, less sweet than sucrose, low calorigenic, less hygroscopic, high moisture retention capacity, and anti-retrogradation activity (Mohanan and Satyanarayana, 2018; Samanta, 2020a). Maltooligosaccharides are potentially used for the preparation of infant and geriatric foods, candies, ice creams, and beverages. In the bakery industry, maltooligosaccharides act as an antistaling agent as they interfere with starch–gluten interaction (Nagarajan *et al.,* 2006; Plácido Moore *et al.,* 2005).

Maltotriose-rich maltooligosaccharides mixture exhibits only 30% sweetness as compared to sucrose, low viscosity, low freezing point, less color formation than corn syrup, better hygroscopic properties. It is used as a sucrose substitution and maintains the hardness and texture of the frozen foods. At the commercial levels, this mixture is essential for the preparation of confectionery products (jams, jelly, cake, chewing gum, butter cream, custard cream), bakery products, frozen foods, canned coffee, cocoa, fruit drinks, and alcoholic beverages (Jana *et al.,* 2013; Samanta, 2014, 2020a).

Enzymatic preparation of maltooligosaccharides is enormously increasing in the last few decades. Two government agencies [Foods for Specified Health Use (FOSHU) and Foods with Nutrient Function Claims (FNFC)] of Japan enlisted 223 items as functional components of foods; among them, 50% are oligosaccharides (Jana *et al.,* 2013). Maltooligosaccharides are commercially available as Fuji Oligosyrups, a product of Nihon Shokuhin Kako Kogyo Kabushiki Kasha (Tokyo, Japan) (US9730464B2, US20130210764A1). G4 syrup contains about 50% maltotetraose, whereas Alo-mixture contains more glucose (40%) and isomaltose (17%). Properties of these two ingredients have some similarities with the maltotriose-rich maltooligosaccharides mixture, and the application fields are also similar. Maltooligosaccharides have health-beneficial effects. They are treated as a prebiotic agent and influence the growth of bifidobacteria in the colon and prevent dysbiosis. This property increases its value as bio preservatives and functional foods (Barreteau *et al.,* 2006). Maltooligosaccharides are poorly absorbed; they are low calorigenic agents. These properties are beneficial to prevent diabetogenic effects, cardiovascular diseases, and obesity (Kayode *et al.,* 2009).

10.1.2. High fructose containing syrup (HFCS) preparation and uses

Treatment of starch by thermostable α-amylase followed by saccharification with the fungal glucoamylase and debranching enzyme produces glucose. The conversion of glucose to fructose is mediated by the action of glucose isomerase (xylose isomerase; EC 5.3.1.5), which converts glucose to fructose as a common product. This conversion is essential for HCFS production, but the enzyme does not belong to the amylase family (Bhosale *et al.,* 1996). Recently, Mohanan and Satyanarayana (2018) had reported that every year, Japan produced 3 million tons of starch from which more than 60% are utilized for glucose and high fructose-containing syrup (HFCS) preparation. Annually, Japan and the United States produce one million tons and 8 million tons of HCFS, respectively. HFCS is the useful ingredients for the preparation of beverages, dairy products, bakery and confectionery products, canned foods, and frozen candies.

10.1.3. Applications amylases in the bakery industry

Amylopectin in wheat flour acts as a staling agent due to its retrogradation activity. Applications of plant β-amylase and maltogenic amylase (EC 3.2.1.133) along with α-amylase degrade the starch into dextrin as well as maltose, which improves the quality of bread by increasing loaf volume. Additionally, the application of amylopullulanase in dough hydrolyzes branched maltodextrins that decrease the stickyness of bread and

bakery foods, improves shelf-life, texture, crispiness, and loaf volume of the products. Moreover, the presence of maltooligosaccharides in bread acts as prebiotics for health benefits (Nisha and Satyanarayana, 2014).

10.1.4. Production of cyclodextrin and its applications

About 2000 tons of cyclodextrins are produced per year for industrial purposes. Cyclodextrins are prepared from gelatinized starch, which is made through boiling of starch in hydrated conditions. This treatment irreversibly changes the intermolecular bonding structure of starch. The production of cyclodextrins is done by the use of cyclodextrin glycosyltransferase (CGTase) enzyme for the treatment of gelatinized starch. Cyclodextrins (CDs) are cyclic-oligosaccharides having 6-8 glucose units linked through the α-1,4-glucosidic bond. Six, seven, and eight glucose units containing cyclodextrins are named as α, β, and γ cyclodextrin, respectively. The types of cyclodextrin production depend on the nature of the enzyme from which it is isolated. Branched cyclodextrin contains maltose or glucose through α-1,6-glucosidic linkage. In the industrial sectors, these types of cyclodextrins are made through a reverse reaction by using a thermostable pullulanase in the presence of high concentration of maltose and cyclodextrins. CDs and branched-cyclodextrin are mostly used in food, pharmaceutical, cosmetic, and plastic industries as emulsifiers. CDs have a hydrophobic cavity for their unique structure, which makes them unable to form inclusion complexes with different hydrophobic agents related to favor and taste enhancement. The hydrophobic volatile compounds from herbs and spices were packed with CDs. The formation of these inclusion complexes increases the solubility, stability, and bioavailability of hydrophobic components. Thus, CDs are applied as food additives. This conjugation retains the flavor of the foods and also acts as an antioxidant to preserve the foods and other commercial products (Qi and Zimmermann, 2005; Ray, 2011; Mohanan and Satyanarayana, 2018). Volatile flavoring agents were trapped within the hydrophobic cavity of CDs and mixed with different cooking spices. Administration of these conjugate spices in delicious preparation enhances the flavor and taste of served foods.

10.1.5. Production of isomaltooligosaccharides, resistant starch, and their health benefits

Break down of starch by α-amylase followed by treatment of various enzymes like α-glucosidase, oligo-1,6-glucosidase (EC 3.2.1.10), isoamylase (EC 3.2.1.68), pullulanase (EC 3.2.1.41), and neopullulanase (EC 3.2.1.135) produces maltooligosaccharides, isomaltooligosaccharides (IMO), panose, isopanose as well as resistant starch (RS) (Zhang *et al.,* 2012; Møller *et al.,* 2012; Li *et al.,* 2017; del Moral *et al.,* 2018). Recently, these ingredients are used as functional foods as well as dietary supplements for the preparation of therapeutic and healthy diet. IMO, panose and RS have prebiotic activity (Chen *et al.,* 2010; Møller *et al.,* 2012; Li *et al.,* 2017). IMO is poorly absorbed in the human gut and reaches the colon. It influences the growth of bifidobacteria and lactobacillus (Goffin *et al.,* 2011; Ketabi *et al.,* 2011). IMO is metabolized by the human colonic microbiota due to the presence of oligo 1,6-α-glucosidase (EC 3.2.1.10) and α-glucosidase (EC 3.2.120). Additionally, panose and isopanose also have bifidogenic activity. Thus, IMO and panose selectively stimulate the growth of beneficial bacteria; alternatively, they inhibit the growth of other harmful microbes that can synthesize amines and other toxicants. Thus, they maintain the health of the colon and prevent carcinogenic activity.

Another important component is RS. Enzymatic treatment (name already is given) produces RS that is used as dietary supplements. The application of RS as a functional food ingredient improves several properties of foods like color, flavor, gel formation, viscosity texture, and moisture-retaining capacity (Sajilat *et al.,* 2006; Simsek *et al.,* 2012). RS is called high-amylose starches, which is being resistant to hydrolysis by intestinal α-amylase. This effect is beneficial to regulate blood glucose concentrations. Though RS is not digested in the small intestine, the colonic microbiota starts fermentation of RS, resulting in the production of high amounts of short-chain fatty acid (butyrates, acetate). Decrease of blood glucose levels and production of short-chain fatty acids are beneficial to control diabetes and obesity and consequently lowers the risk of cardiovascular diseases (Fuentes-Zaragoza *et al.,* 2010; Zhang *et al.,* 2012). Moreover, short-chain fatty acids preserve colon health (Hii *et al.,* 2012).

10.1.6. Applications of maltitol, trehalose

Isoamylase is used for the preparation of glucose syrup, maltose, maltitol, trehalose, CDs, and RS from the starch substrate. Glucose and maltose are commonly used in the food and pharmaceutical industry. Maltitol is utilized as a sugar substitute in the production of non-calorigenic candies, chewing gum, and other confectionery products (Ray, 2011). Supplementation of trehalose in food enhances stability, controls humidity, and maintains the texture of the food (Olempska-Beer, 2007).

10.2. Preparation of animal feed

Animal feed is produced by using food grains like wheat, barley, maize, cassava, sorghum, rice hush, etc. Starch is the reserve food material in all types of food grains. In the cereals, starch has arranged in concentric layers. Application of un-boiled animal feed lowers the digestibility of the foods and decreases nutrition level. α-Amylases are applied for the preparation of animal feed, which partially cleaves the starch of the grains resulting in enhancement of quality improvement of feed as well as digestive capacity (de Souza and Magalhaes, 2010). Thus, pre-treatment of starchy material with amylases enhances the nutritional value of the feed. Isoamylase is also exploited for the preparation of animal feed. Resistant starch is present in cereal meal-based animal feed, which is partially digested in the gut of monogastric animals. Puspita *et al.* (2019) reported that isoamylase-treated cassava root meal starch can be used for poultry feed.

10.3. Application in desizing (Removal of starch sizer from textile)

Starch paste is applied as a sizer agent in the textile industry during weaving. The starch sizer increases the softness of the strings and protects them from friction, cutting, and generation of static electricity. After making the cloth, starch has removed (desizing) from the cloth. There are different processes of desizing, including enzymatic treatment, oxidative breakdown, acid wash, and fermentative removal. Among these, enzymatic treatment is the best process concerning the quality and safety of the cloth as well as the prevention of the use of harmful chemicals. Application of thermostable α-amylase in the desizing process eliminates the starch paste without affecting the fabric quality.

10.4. Application in papermaking

The application of starch paste during paper making protects the paper against mechanical damage and reduces the tendency of liquid absorption during drying. Removal of starch paste by α-amylase and pullulanase enhances the quality, texture, strength, smoothness, writing, and erasability capacity of the finished paper (Sundarram and Murthy, 2014). Pullulanase-treated high-amylose containing starches are blended in adhesive products and used in the manufacturing of papermaking agents and corrugated boards (Jobling, 2004).

10.5. Production of biofuel

Biofuel is the best choice to control pollution levels. Starch is the initial ingredient of biofuel production due to its low cost and abundant availability. There are three main stages for the production of ethanol from starch. Stage 1: thermostable α-amylase mediated starch liquefaction (Pervez *et al.,* 2014). This step reduces the viscosity of the starch slurry and increases dextrins and oligosaccharide contents. Stage 2: saccharification of liquefied starch by α-glucosidase, isoamylase, pullulanase, β-amylase, and glucoamylase that produce simple sugars and smaller oligosaccharides for alcoholic fermentation. Stage 3: fermentable sugars are then fermented by yeast to produce ethanol (Mohiuddin *et al.,* 2016; Saini *et al.,* 2017).

10.6. Application in detergent preparation

Enzyme-based liquid detergent is environmentally safe. α-Amylase along with protease, lipase, and cellulase are used in the preparation of enzyme-based high-quality detergent. Starch holds the dust particle on the surface of the cloth. Similarly, the residues of starchy food ingredients and some oily substances are present as a remnant over the used food-dishes. α-Amylase and other enzymes degrade the starchy and oily substances to clean the content properly. Alkalophilic amylases having a broad range of temperature profile, chelator insensitivity, and oxidant resistant capacity are potentially used in detergent formulation (Kumari *et al.,* 2012; Sundarram and Murthy, 2014).

10.7. Degradation of extracellular polymeric substances and inhibition of biofilm formation

Biofilm is a syntrophic association that makes the adherence of microbial cells on the biotic and abiotic surfaces. About 80% of microbial pathogenesis is directly associated with the formation of biofilm (Donlan and Costerton, 2002; Lahiri *et al.,* 2021b). Extracellular polymeric substances (EPS) are most important in the formation of biofilm. The biofilm contains proteins and nucleic acids; it stabilizes the microbial association and contributes nutrients to the sessile microbial communities (Lahiri *et al.* 2021c). EPS contains various types of cationic and anionic molecules, such as glycoproteins, glycolipids, and proteins that provide protection from invasion of the motile cells into the biofilm interior. (Nadell *et al*., 2015). Another important component of EPS is polysaccharides. EPS acts as a protective shield to prevent the penetration of antibiotics into the growing microbial cells. Collectively, biofilm contributes to generating resistance capacity against antimicrobial drugs that creates a crisis in the healthcare system (Jana *et al*., 2017). Enzymes, particularly amylases (α-amylase, β-amylase, glucoamylase, and α-glucosidase) play important roles in the degradation of biofilm. The breakdown of carbohydrate residues in the biofilm weakens the association between microbes and the host surface. Amylases can be used as antimicrobial components to denature the biofilm and restriction of pathogenesis (Lahiri *et al.* 2021c).

Several microbes like *Candida. albicans*, *C. glabrata*, *Enterococcus faecalis*, *Streptococcus mutans*, *Veillonella dispar*, and *Fusobacterium nucleatum* (Berger *et al*., 2018) are present in the oral cavity. Another important pathogen is *S. aureus,* causal agent of Skin infections, cellulitis, folliculitis, and others. α-Amylase from *Bacillus subtilis* degrades the EPS and is effective against biofilm formation of *S. aureus, P. aeruginosa*, and *V. cholerae* (Kalpana *et al*., 2012). In vitro studies had revealed that α-amylase blocks the biofilm formation of S. *aureus* and *P. aeruginosa* (Lahiri *et al*., 2021a). Bradford (2011) critically studied the role of amylase on biofilm formation by *S. aureus* and had reported that amylase reduced biofilm formation capacity up to 90%. Moreover, Watters *et al*. (2016) indicated that α-amylase is the potent inhibitor of biofilm formation in drug resistance *S. aureus*. From these observations, it has been stated that amylases can potentially be used in the ointment, mouth gel, and digestive medicine. Application of amylases in these medicines may block the pathogenesis by exerting anti-biofilm activity which will be helpful for the treatment of dermal infection, oral infection, and intestinal pathogenesis.

10.8. Clinical applications

α-Amylase from *Aspergillus oryzae* (diastase) is used for the preparation of digestive medicines, which are formulated in liquid (common composition α-amylase 100 mg; papain 50 mg/5ml) and capsule form. Many companies produce digestive medicines that are available in the market. Digestive medicines are commonly used for the treatment of indigestion, acute pancreatitis, and related diseases. A high level of serum α-amylase has been

observed in pancreatic fistula, acute stress, gastric aspiration, etc. (Yan and Wu, 2016). Ultrasensitive amylase biosensor has been developed for the detection of hyperamylasemia (Gibbs *et al.,* 2015; Wang *et al.,* 2015). This biosensor-mediated amylase detection tool can be used in the diagnostic field.

10.9. Other applications

α-Amylase, α-glucosidase, isoamylase, and pullulanase make the brewing process easier and cost-effective. These enzymes are applied for the production of low-calorie beer. These enzymes in association with pectinase and cellulase are used for making fruit juice and beverages. Commercially available amylase mixture is used for the treatment of starchy wastes containing effluent of starch-based industry. This process is eco-friendly and economically viable as the technique is sometimes associated with microbial biomass or bio-fuel production.

11. Conclusion

All the carbohydrate-splitting enzymes have been classified in different glycosyl hydrolase families based on their sequences, molecular structure, catalytic activity, and product formation. Among the carbohydrate splitting enzymes, amylases (hydrolases and transglycosylases) have multipurpose applications in the industrial sectors ranging from the food industry to waste management. Applications of amylases for the production of food ingredients, garments, paper, detergent, biofuel, and others are the eco-friendly process. To achieve this process, several novel characteristics, including thermostability, catalytic efficacy, pH profile, oxidation resistance capacity, chelator insensitivity are the essential factors in the point of industrial uses. Elucidation of molecular structure, amino acid sequences, and catalytic strategies have made the new arena to improve these profiles through protein engineering techniques. Multinational companies (Novozymes, DuPont Danisco, AB enzymes, Genencor, Calzyme, and others) are also involved in the production of advanced quality amylases by establishing their Research and Development wings. Thus, the multidimensional research in protein structure and protein engineering, and biotechnological progression will open many avenues to achieve the high quality of amylases that will change the scenario of their applications in the near future and will be a forward step towards a clean world. Finally, in conclusion, applications of amylases in various industries (food, pharmaceutical, brewing, textile, detergent) and the clinical system will be beneficial in all aspects, including our health, socio-economic development, and environmental protection in the upcoming days.

Conflict of interest

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