Review

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Recapitulation of stability diversity of microbial α -amylases

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Abstract: α -Amylases from a huge number of sources have been isolated and characterised but very few of them meet the demands of the industries. The industrial processes take place under conditions hostile to biocatalysts thus increasing the industrial demand for a highly stable enzyme in good titre level. Improved understanding of biomolecular aspects of α -amylases has led to the advanced understanding of their catalytic nature. Enzymes with high stability are obtained from extremophiles. Extensive studies have demonstrated the importance of regulating expression and catalytic efficiency of non-extremophiles through genetic engineering, directed evolution and chemical modifications. The inability to culture most microorganisms in the environment by standard methods has also led to the focus on the development of metagenomics for getting improved biocatalytic functions. The present review aims to compile the studies reported by researchers in manipulating non-extremophiles and improving stability through directed evolution, metagenomics and protein engineering.

Keywords: amylase; extremophiles; metagenomics; protein engineering; stability; directed evolution.

Abbreviations

GH, glycoside hydrolases; TAKA/TAA, BLA, BSUA, BAA, BStA, AHA, PWA, PFA – α -amylase from *Aspergillus oryzae*, *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus stearothermophilus*, *Alteromonas haloplanktis*, *Pyrococcus woesei*, *Pyrococcus furiosus*, respectively; TIM, triosephosphate isomerase.

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1 Introduction

α -Amylase (EC 3.2.1.1), one of the important industrial enzymes, is capable of hydrolysing the internal α -1,4-glycosidic linkages to glucose, maltose and dextrin and retains the α -anomeric configuration in the products [1]. This property makes amylases a prominent enzyme in different industries with wide spectrum of applications in fields such as starch saccharification, textile industry, detergent industry, agricultural, fermentation, pharmaceuticals, paper, food, baking, brewing and distilling industries [2,3]. Microbial α -amylases are the most popular enzymes used in industries in comparison to the α -amylases from animals and plants [4]. The ease of large scale production and genetic manipulation, wide range of stability and specificity, and high productivity facilitate their extensive industrial applications [5]. Despite the increase in the number of amylases isolated from various microbes such as hyperthermophilic bacteria and archaea, the naturally existing amylases are not optimal for the use in harsh conditions of the industries. The majority of industrial processes employing α -amylases are performed in extreme conditions of temperature and pH, and these conflicting surroundings of the enzyme in the industries result in non-optimal enzymatic action in most of the applications. The starch hydrolysis steps, which include liquefaction and saccharification, work best with thermostable and acid stable α -amylases, and most of the other starch-based industries carry out α -amylase-mediated reactions under extreme conditions of pH and temperature [6]. The detergent industry demands for enzymes with alkaline and oxidative stabilities, calcium ion independence and activity at a broader temperature range [7]. Thus stable α -amylases are on a continuous demand to fulfil the requirements of the various industries [8]. These variations have led to the high demand to enhance the stability of enzymes and meet the specifications set by specific industries and applications [8]. Thus, the exploration of novel α -amylases and engineered amylases with improved properties that can tolerate the harsh conditions of the industry has gained a great deal of research attention. This review focuses

on the characteristic features of α -amylase enzyme, the extremophilic sources and types, factors providing stability, and the molecular strategies and techniques used for enhancing their catalytic properties.

2 Structural features of α -amylases

α -Amylase (1,4- α -D-glucan glucanohydrolase) has been classified in the CAZy database (<http://www.cazy.org/>) [9,10]. It belongs to the family 13 of glycoside hydrolases (GH13), forming with families GH70 and GH77 the clan GH-H, but it is also found in families GH57, GH119 and GH126 (α -amylase activity not fully confirmed) [11,12]. Family GH13, considered as the main α -amylase family, contains more than 104,000 sequences and 42 CAZy curator-defined subfamilies [10], containing many activities, such as hydrolysis, transglycosylation, and isomerisation [11]. The characteristic features of the GH13 α -amylases are the retention of configuration hydrolysis mechanism, possession of 7 conserved sequence regions and a TIM-barrel domain fold with the GH13 catalytic machinery [11]. They are endo-hydrolases, which randomly cleave the α -1,4 linkages between adjacent glucose units of amylose and amylopectin chain, generating glucose, maltose, maltotriose and small dextrans as end products. The enzymatic and physicochemical properties of α -amylases vary as its source microorganism changes. The molecular weight of α -amylases generally lies between 10 to 210 kDa. The substrate specificity is highest towards starch and then followed by amylose, amylopectin, cyclodextrin, glycogen and maltotriose. α -Amylases are stable over a wide range of pH from 4 to 11 [1]. The X-ray crystallography studies of α -amylases isolated from *Aspergillus oryzae* (TAKA/TAA), *Bacillus licheniformis* (BLA), *Bacillus subtilis* (BSUA), *Bacillus amyloliquefaciens* (BAA), *Alteromonas haloplanktis* (AHA), *Pyrococcus woesei* (PWA), and *Pyrococcus furiosus* (PFA) revealed common structural features [13,14]. The tertiary structure of α -amylases is comprised of three distinct domains. The main catalytic domain, domain A [$(\beta/\alpha)_8$ -barrel or TIM-barrel] is highly conserved and forms the core of the molecule. The active site is located at the centre of the A domain and contains a triad of catalytic residues consisting of two aspartates and one glutamate residue. Domain B protrudes out of the TIM-barrel as a longer loop between the strand β 3 and helix α 3 and succeeded at the C-terminal end by domain C exhibiting the lowest degree of conserved sequence in the GH13 family and adopting an antiparallel β -sandwich fold [11-17].

Individual GH13 members with a given specificity may contain additional domains on either terminus of their

polypeptide chain. Although their functions have still not been completely understood, such domains are often involved in binding starch, glycogen, and other related saccharides [18-22]. Typical starch-binding domains (SBDs) have also been classified within the CAZy database as the CBM families [10] with CBM20 as a representative of the C-terminal SBD [15,23-28]. At present, fifteen CBM families are considered as SBD families [29]: CBM20, CBM21, CBM25, CBM26, CBM34, CBM41, CBM45, CBM48, CBM53, CBM58, CBM68, CBM69, CBM74, CBM82 and CBM83.

3 α -Amylase from extremophiles

3.1 α -Amylases from thermophiles

Stable microbial α -amylases can be obtained either from extremophiles (thermophiles, hyperthermophiles, halophiles, alkaliphiles, acidophiles, piezophiles, metallophiles and psychrophiles) and genetically manipulated non-extremophiles. Compared to other extremophiles, thermophiles and their thermostable enzymes are in great demand in different industrial sectors. Therefore, screening of thermozymes following conventional methods is still going on as an alternative over the tedious bioengineering procedures applied for enzymes of mesophilic origin [30]. Among these microbes, the superior producers of thermostable α -amylases that are stable at a temperature from 37 °C to 60 °C include *B. amyloliquefaciens* (BAA), *B. licheniformis* (BLA), *B. stearothermophilus* (BStA) and *P. furiosus* (PFA) [3]. Thermostable α -amylase from *B. licheniformis* shows extraordinary heat stability due to 469 amino acid residues and three domains, which lead to reduced surface area, increased ionic interactions, and increased packing interactions in the interior [31]. More ionic interactions, disulphide bridges and prolines, a greater extent of hydrophobic-surface burial, as well as improved core packing, shorter surface loops, increased number of hydrogen bonds, better hydrophobic interaction, metal binding, reduced entropy of unfolding, post translational modifications and higher states of oligomerization, etc., have been proposed to be responsible for increased thermostability of enzyme [8,14]. The isolation and storage of pure cultures of extremophiles capable of producing highly stable α -amylases are difficult. Hence, many studies have been done to clone the extremophilic genes to mesophilic hosts. The advantage of this method is that α -amylases, which have all properties of thermostable enzymes, can be produced at ambient temperatures

Table 1: Common expression systems of α -amylases.

Microorganism	Expression system ^b	pH _{opt}	T _{opt} (°C)	Ref.
<i>Bacillus amyloliquefaciens</i>	<i>Escherichia coli</i> BL21	5	50	[2]
<i>Pyrococcus woesei</i>	<i>Halomonas elongata</i>	5.5-6.0	90-100	[101]
<i>Thermococcus hydrothermalis</i>	<i>Escherichia coli</i>	5.0-5.5	75-85	[102]
Ba-Gt (chimeric) ^a	<i>Pichia pastoris</i>	4.0	60	[76]
<i>Thermotoga neapolitana</i>	<i>Escherichia coli</i>	6.5	75	[103]
<i>Bacillus subtilis</i> DR8806	<i>Escherichia coli</i>	5.0	70	[104]
<i>Bacillus subtilis</i> PY22	<i>Pichia pastoris</i>	7.0	60	[105]
<i>Thermobifida fusca</i>	<i>Yarrowia lipolytica</i>	7.0	60	[106]
<i>Rhizopus oryzae</i>	<i>Pichia pastoris</i>	4.0-6.0	60	[107]
<i>Geomyces pannorum</i>	<i>Aspergillus oryzae</i>	5	40	[81]
<i>Pseudoalteromonas</i> sp. M175	<i>Escherichia coli</i>	8	25	[82]
<i>Arthrobacter agilis</i>	<i>Escherichia coli</i> BL21	3	30	[85]
<i>Bacillus</i> sp. DR90	<i>Escherichia coli</i> BL21	4.5	70	[73]

^a Ba-Gt (chimeric), *Bacillus acidicola* & *Geobacillus thermoleovorans*.

^b Expression system and/or mesophilic producer.

using mesophilic organisms like *Escherichia coli*. Table 1 gives reported expression systems used for expression of thermostable α -amylases.

3.2 Cold-active α -amylases

The recently found microbial sources of cold active α -amylase enzyme include *Alteromonas* sp. from Antarctic sea water [32], *Arthrobacter psychrolactophilus* ATCC 700733 [33], *Aspergillus ochraceus* [34], *Lactobacillus plantarum* [35] and *Nocardiopsis egyptia* [36]. These microorganisms produce α -amylases, which function efficiently at cold temperatures with high degree of catalysis in comparison with the little or no activity shown by mesophiles or thermophiles at low temperatures [37]. The low activation energy makes it possible for the psychrophiles to work at the low temperatures [38]. Along with the integration of unsaturated fatty acids in cell membranes to sustain membrane fluidity, these organisms produce cold-adapted enzymes with high catalytic activity to thrive effectively at low temperature environments [39]. The reduced number of salt bridges and the less proline residues in loops provides flexibility to the enzyme, and this flexibility helps in easier accommodation of substrates at low temperatures [40]. Cold adaptation is also due to the reduced number of disulphide bridges, increased number

of acidic residues, increased hydrophobic residues on the protein surface, reduction in proline residues, lower Arg/Lys ratio, increased number of methionine residues, lower affinity for Ca²⁺ and decreased number of aromatic interactions [41]. Halophilic proteins when compared to non-halophilic proteins generally contain a large excess of acidic residues resulting in a higher negative surface potential that makes them more soluble and renders them more flexible at high salt concentrations [42].

3.3 Stabilizers of α -amylase

Apart from the intrinsic factors, such as amino acid sequence, structural properties, folding and unfolding processes, various extrinsic factors also account for the stability of the α -amylases. The presence of calcium and other stabilizers are regarded as the main factors, which contribute to the thermostability of α -amylase [43]. Most of the known α -amylases contain at least one calcium ion per protein molecule and they can significantly increase protein stability. Single conserved calcium ion is replaced by calcium-sodium-calcium metal triad in enzymes like BLA, BAA and BStA [44]. Besides a conserved calcium ion, the existence of auxiliary calcium ions bound to the thermostable enzymes substantially increases the free energy of the unfolding barrier [45] and calcium can

also be utilized to promote refolding of non-aggregated unfolded states [46]. Thermostabilization by Ca^{2+} can be justified by the increased rigidity of enzyme structure through salting out of hydrophobic residues by this metal ion [8]. In case of PFA, Ca^{2+} is required for thermostability only at elevated temperatures and it is also assumed that Zn^{2+} plays a significant role in the improvement of thermal stability of this enzyme [47].

4 Biotechnological approaches for the improvement of enzyme stability

The stability of enzymes is a crucial factor to determine whether its application will be commercially successful. Since last decade many researches have focused on the improvement of α -amylase behaviour in various extreme and harsh conditions. Most of such studies concentrated on improving the thermal stability of the enzyme. Few studies also focused on improving the acid/base stability, and the stability towards oxidative stress or salts. The common strategies adopted for these enhancements include immobilisation, chemical modification, use of additives, recombinant DNA technology and genetic engineering through cloning, site-directed mutagenesis and directed evolution. In recombinant DNA technology for heterologous α -amylase production, a highly efficient α -amylase gene is incorporated or cloned into a suitable vector system and transferred to an excellent expression system to produce a larger quantity of recombinant enzyme with or without the help of expression-vector promoter-inducing agents. Cloning the genes is a very useful technique for enhancing the stability and activity of the enzymes and it is mostly done for hyperproduction of various proteins and enzymes with high specific activity, which facilitates their easier purification during industrial production [48]. In 2010, Gangadharan et al. [2] amplified, cloned and overexpressed the α -amylase gene of *B. amyloliquefaciens* ATCC 23842 in *E. coli* BL21 (DE3) cells and found that the behaviour of the overexpressed enzyme at different pH and temperature was in accordance with the parent enzyme developed by them in 2009.

The most widely studied method for the stabilization of α -amylases is protein engineering, which includes random mutagenesis and site-directed mutagenesis. Random mutagenesis is performed using error prone PCR, DNA shuffling, UV irradiation, chemical mutagenesis, etc., which include introduction of mutations at random

along the entire length of a gene. But this process does not facilitate the prediction of structural information. In the process of site-directed mutagenesis, a mutation is generated at the demarcated site of the protein [49]. Alteration is made at a specific site in an amino acid, whose structure and mechanism of action is known, and the mutated protein is evaluated to determine whether it performs better than the wild or native protein. The structural information of the targeted proteins or enzymes is the primary requirement for this process [50]. In order to meet the industrial specifications, various site-directed mutations have been perfectly introduced in α -amylases to improve the thermostability, pH stability, and oxidative stability. Thus, through the process of site-directed mutagenesis, the activity, specificity, stability, solubility of the biocatalysts can be improved [51]. Reports on the stability improvement of α -amylase after UV irradiation and chemical mutagenesis by ethyl methane sulfonate or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine are scarce even though there are many studies reported [52]. In order to fulfil the industrial requirements, a number of well-known site-directed mutation concepts have been successfully applied to improve thermostability, pH stability, and oxidative stability of α -amylase.

It is also proven that directed evolution is a robust method for the synchronous improvement of thermostability and enzyme activity, especially when utilized in blend with rational or semi-rational engineering strategies [3]. Even though the directed protein evolution is an extremely advantageous process in recent day biotechnology, it has certain drawbacks due to its inability to produce mutant libraries of top quality containing more useful variants [53]. The variations in the enzyme activity and the stability of α -amylases by the application of these strategies are reviewed below.

Metagenomics is the study of genetic material recovered directly from environmental samples. Few reports are available on the discovery of α -amylases from metagenomic samples. In 2004, Yun et al. [54] characterized an amylolytic enzyme coded by a gene (*amyM*) obtained from the metagenomic library derived using pUC19 vector. The gene was overexpressed, purified and found that the enzyme was stable at pH 9.0 and 42 °C. From soil metagenomic libraries constructed for North-western Himalayas, Sharma et al. [55] in 2010 identified a gene (*pAMY*) of 909 bp encoding α -amylase. The enzyme was found to be Ca^{2+} independent and indicated that pAMY was closely related to uncultured bacteria [55]. Another example of α -amylase from faecal microbial metagenomes was given by Xu et al. [56] in 2014 where the authors identified the gene *amyPL*, which became the earliest

report on the α -amylase extracted from gastrointestinal metagenomic library.

4.1 Strategies to improve stability at high temperature

The increased interest in improving the stability of α -amylase at elevated temperature is because of the fact that α -amylases have to catalyse most of the reactions and processes at high temperatures in different industrial applications. Some examples of various techniques used for enhancing thermal stability are discussed.

There are many reports regarding the stability improvement of α -amylases by protein engineering methods, such as site-directed mutagenesis and directed evolution. The α -amylase from *B. licheniformis* (BLA) is the commonly used enzyme model for thermostability improvement studies. The thermostability of BLA is focused in the domain B and at its interface with the central A domain, and the mutations His133Ile and Ala209Val increased the half-life of BLA by 10 folds at 90 °C [57]. The alterations in the amino acids (Asn75, Ser76 and His77) at the calcium binding sites of α -amylase from *B. megaterium* WHO by the site-directed mutagenesis resulted in improved thermal stability (the recombinant enzyme was stable at 5°C higher than the optimum temperature) and half-life of the enzyme increased than the wild type [58]. The half-life of the Ca-independent α -amylase (BKA) from *Bacillus* sp. KR-8104 was improved by the substitution of deamidation residue asparagine with aspartic acid and serine [59]. The α -amylase from *Bacillus acidicola* was altered by fusing with highly thermostable α -amylase gene of *Geobacillus thermoleovorans* and the protein thus formed had a melting temperature of 73.8 °C, which is greater than the native form [60]. In the study by Kim et al. [61] in 2003, using the techniques like random mutagenesis and DNA shuffling, the thermal stability of α -amylase from *Thermus* sp. strain IM6501 was amplified [61]. Jones et al. [62] found that by employing the error-prone PCR and DNA shuffling at pH 4.5 the thermal stability of Novamyl from *Bacillus* sp. TS-25 could be improved [62]. Richardson et al. [63] increased the thermostability of α -amylases from uncultured organisms produced in *Pseudomonas fluorescens* at low pH in absence of added calcium using directed evolution technique. Apart from addition, substitution, and shuffling, deletion of certain residues in α -amylase can enhance its resistance to high temperatures. Thermostabilization observed by deletions or loop shortenings could be attributed to the formation of additional intramolecular interactions. The deletion

of a loop (Arg176-Gly177) in domain B and substituting with alanine for Lys269 and aspartic acid for Asn266 can increase the thermal stability of BAA up to great extent [64]. It was through the deletion of residues Arg178-Gly179 and two substitutions (Gly211Val and Asn192Phe) the T_m of α -amylase from *Bacillus* sp. 406 was amplified [65]. The thermostability was improved and calcium requirement of BStA was reduced by shortening the loop created by five residues (Arg212, Gly213, Ile214, Gly215 and Lys216) through deletion of Gly213-Ile214 or Ile214-Gly215. But the deletion of Gly213-Ile214-Gly215 caused a reduction in the optimum temperature by 17 °C [66,67]. A combination of two substitutions (Gly211Val and Asn192Phe) and deletion of residues Arg178-Gly179 resulted in a drastic enhancement in the T_m of α -amylase from *Bacillus* sp. 406 [63]. Introduction of prolines in loop regions is a common protein stabilizing strategy [68]. The substitution of Arg124 with proline increased the thermostability of α -amylases of *Bacillus* sp. NCIB 12512 [68] and *Bacillus* sp. KSM-1378 [69].

Manipulation of the disulphide bonds have also been proven successful for enzyme stabilization [70]. New disulphide bonds at A and C domains interface were introduced by mutating Ser336 and Ser437 to cysteines in *Saccharomycopsis fibuligera* R64 α -amylase [71]. The stability of the mutant α -amylase showed improved stability without any effect on its activity when overexpressed in the *Pichia pastoris* KM71H [71]. Introduction of disulphide bridge by replacing S450 and K415 with cysteines near a Zn^{2+} binding site in the C domain significantly improved the activity and thermostability of the α -amylase from *Flavobacteriaceae sinomicrobium* and the role of domain C has also been reported to play a significant role in maintaining the stability of α -amylases in extreme conditions [72].

In 2011, metagenomic library of Western Ghat soil was developed by Vidya et al. [73] using the fosmid vector (pCC1FOS) and characterized a novel α -amylase with optimum temperature at 60 °C and optimal pH of 5.0. The enzyme retained 30% of its activity after incubation at 80 °C for 60 min and retained 70% activity in presence of 1.5 M NaCl [73]. An α -amylase gene (*amyM*) was identified from the metagenomic library using pUC19 vector by Yun et al. [54] in 2004. The expressed enzyme had stability at 42 °C with ability to hydrolyse soluble starch and cyclodextrins resulting in production of maltose [54]. The thermostable α -amylase gene from *Thermococcus profundus* was expressed in *E. coli* by Lee et al. [74] and determined that the heterologous expression was 155-fold greater than the native strain [74]. The truncated α -amylase gene from *B. acidicola* expressed heterologously in *E. coli* had activity at 60 °C and pH 6.0 [75]. Compared to wild type,

the recombinant α -amylase produced extracellularly in *P. pastoris* showed 10.7-fold times improved activity and the expressed enzyme exhibited optimal activity at pH 4.0 and 60 °C. Since the enzyme could saccharify both soluble and raw starch and release maltose, it is used in baking and sugar syrup industries [76].

Directed evolution in combination with rational or semi-rational engineering strategies has proven to be a powerful method for the simultaneous improvement of thermostability as well as enzyme activity. Random mutagenesis and DNA shuffling methods have shown a significant improvement in thermostability of α -amylase from *Thermus* sp. strain IM6501 [61]. Error-prone PCR and gene shuffling studies were performed on the genes coding for the wild-type BAA and the mutants BAA Ser201Asn and BAA Asn297Asp [51,77]. Screening of the transformants yielded the mutants BAA 42 and BAA 29, in which the activity of BAA 42 was improved at pH 10 and an improvement in activities was noted in BAA 29 and BAA 42 [51,77]. The thermostability of *P. fluorescens* produced Biovar I α -amylase was increased at low pH in absence of added calcium using directed evolution [63]. Thermostability was increased at pH 4.5 by employing the error-prone PCR and DNA shuffling in Novamyl from *Bacillus* sp. TS-25 [62]. Although directed protein evolution is an invaluable process in the present-day biotechnology, it has some limitations due to its inability to generate high-quality mutant libraries containing more beneficial variants [53].

4.2 Strategies to improve stability during cold adaptation

The cold-active enzymes demand upgradation in terms of both quality as well as quantity due to shoot up in its use and applications. This can be accomplished by adopting the established recombinant DNA technology and protein engineering aspects. The strain improvement done by site-directed mutagenesis helps in quantitative enhancement of the enzyme characteristics. The possibility of these methods and the available examples are discussed in the following sections. Insights into the cold adaptation of proteins were gained on the basis of a psychrophilic protein's molecular structure. The structure of the recombinant form of a psychrophilic α -amylase from *Alteromonas haloplanctis* (AHA) at 2.4 Å resolution have been compared with the structure of the wild-type enzyme, solved at 2.0 Å resolution, and with available structures of their mesophilic counterparts [78]. The comparative studies have enabled to identify possible

determinants of cold adaptation and it was proposed that an increased resilience of the molecular surface and a less rigid protein core, with less interdomain interactions, are determining factors of the conformational flexibility that allows efficient enzyme catalysis in cold environments [78]. The crystal structures of the α -amylase (AHA) secreted by this bacterium, in its native state to 2.0 Å resolution as well as in complex with Tris to 1.85 Å resolution, were the first experimentally determined three-dimensional structures of a psychrophilic enzyme. It resembled those of other known α -amylases of various origins with a greatest similarity to mammalian α -amylases. It was found to contain a chloride ion, which activates the hydrolytic cleavage of substrate α -1,4-glycosidic bonds. The chloride-binding site situated ~ 5 Å from the active site was characterized by a triad of acid residues (Asp174, Glu200 and Asp264), which were involved in the firm binding of the Tris moiety [78]. A reaction mechanism for substrate hydrolysis was proposed on the basis of the Tris inhibitor binding and the chloride activation. A triad of residues (Ser303, His337, Glu19), which resembled the serine protease-like catalytic triad was found ~ 22 Å from the active site and suggested that it could be responsible for the autoproteolytic events observed in solution for this cold-adapted α -amylase [79]. Chloride has been reported as the allosteric effector of vertebrate pancreatic and salivary α -amylases and of the bacterial α -amylase from *Alteromonas haloplanctis*. Activation experiments of AHA by different monovalent anions showed that a negative charge, which was not restricted to that of Cl^- , is essential for the amylolytic reaction. Engineering of the chloride-binding site revealed that a basic residue is an essential component of the site. The Cl^- binding properties were altered by the mutation Lys337Arg, whereas the mutation Lys337Gln produces an active, chloride-independent enzyme. The dependence of the binding affinity on the chloride coordination mode by this basic residue was proven by the comparison of the K_d values for Cl^- in three homologous α -amylases. Analysis of substrate and chloride binding according to the allosteric kinetic model showed that the chloride effector is not involved in substrate binding. The chloride ions were proven to have an active role in the pK_a shift of catalytic groups that interacts with active-site carboxyl groups, the pH dependence of its activity [80].

Cold-active α -amylases could be used for different industrial applications once their properties are modified by protein engineering techniques. Microbial cold-active α -amylases with high catalytic activity at low temperatures can be successfully produced through site-directed mutagenesis and directed evolution. When the α -amylase

(AmyA1) from Antarctic psychrotolerant fungus *Geomyces pannorum* was cloned and expressed in *Aspergillus oryzae* system, the recombinant AmyA1 maintained about more than 20% of maximum activity at 0–20 °C with optimal activity at pH 5.0 and temperature 40 °C [81]. In 2018, a novel α -amylase-producing strain *Pseudoalteromonas* sp. M175 (KU726544) was identified from Antarctic ice cover by Wang et al. [82] and the α -amylase gene *amy175* isolated from this microbe was cloned and expressed in *E. coli*. By analysing the characteristics of the expressed enzyme, it was concluded that the α -amylase gene *amy175* could be used as a novel α -amylase source for industrial application with highest activity at 25 °C and pH 8.0, exhibiting the extreme salt-resistance [82]. The stain removal efficiency of various tested commercial detergents got improved with the incorporation of Amy175, and thus it can be considered as a novel α -amylase source for industrial application. When the wild-type α -amylase wtAmy175 from *Pseudoalteromonas* sp. M175 was expressed and studied, the optimum temperature and pH for enzyme activity were found as 30 °C and 7.5, respectively [82]. The enzyme also showed high activity and remarkable stability in 0–10 mM sodium dodecyl sulphate suggesting its capability as an outstanding candidate in detergent and textile industries [83]. The heterologous expression of α -amylase from Antarctic bacterium *Pseudoalteromonas* sp. 2-3 in *E. coli* BL21 (DE3) showed the optimum temperature at 20 °C with activation by Ca^{2+} , the enzyme being active on potato starch giving a K_m of 6.94 mg/mL and V_{max} of 0.27 mg/mL.min [84]. Kim et al. [85] in 2017 boosted the stability of psychrophilic α -amylase by cloning the gene encoding an α -amylase from a psychrophilic *Arthrobacter agilis* PAMC 27388 strain into a pET-28a (+) vector and this was heterologously expressed in *E. coli* BL21 (DE3). The recombinant α -amylase exhibited optimal activity at 30 °C with pH 3.0. In addition, the enzyme was highly stable at altering temperatures (30–60 °C) within the pH range of 4.0–8.0 [85].

Protein engineering studies on *Alteromonas haloplanktis* α -amylase reported five mutations improving significantly the stability of the protein [86]. The three mutations Lys300Arg, Asn150Asp and Val196Phe restored electrostatic interactions (H-bond, ion pair and aromatic interaction, respectively), while both mutations Gln164Ile and Thr232Val reconstructed hydrophobic core clusters. All mutations engineered in the psychrophilic AHA are located outside the catalytic cleft but both catalytic parameters k_{cat} and K_m of the mutants were largely shifted towards typical mesophilic values. Even if the reaction pathway of the α -amylase has not been modified, the ability of the active-site residues to perform catalysis

was altered, probably by modulating the plasticity of the whole catalytic cleft. This study confirmed the central role of weak interactions in regulating the balance between stability and activity of an enzyme in order to adapt to the environmental temperature [86].

In 2015, Vester et al. [87] isolated a cold-adapted α -amylase Amy13c6 that exhibited optimal activity at 10–15 °C from a metagenomic library of cold and alkaline environment of Greenland. The enzyme had the potential to be used in low temperature laundry process since it maintained about 70% activity at 1 °C and pH 8.0–9.0 [87].

4.3 Enhancement of acid/base stability by protein engineering strategies

The α -amylases used at commercial scale lack adequate acid/base stability, which limits their applications. For its extensive use, the pH should be adjusted to optimum range. Altering the pH stability is a tedious task and it lacks rational approaches. So, the acid/base stability of α -amylases can be tuned by employing the usual stability engineering techniques, like helix capping, cavity filling and removal of deamidating residues [88]. Most of the stable enzymes have an optimum pH, which is different from the pH of the environment where their action is required. In such circumstances, the fundamental strategy done to improve the enzyme stability is replacing the amino acid residues by site-directed mutagenesis. For example, Yang et al. [89] in 2013 suggested the replacement of histidine residues with aspartic acids (His275/293Asp, His275/310Asp, His293/310Asp and His275/293/310Asp) in *B. subtilis* α -amylase by site-directed mutagenesis to amplify the stability and catalytic activity under acidic conditions. Hashida et al. [49] through the process of site-directed mutagenesis (His156Tyr, Ala181Thr, Asn190Phe, Ala209Val and Gln264Ser) developed a calcium ion independent Termamyl LCTM from the α -amylase of *B. licheniformis*. The mutant was reconstructed further by region specific random mutagenesis, which introduces random mutations at seven regions and all interfaces between the domains A, B and C. The selected variants (Amy a, b, c) had much higher acid stability than TermamylTM and Termamyl LCTM at pH 4.5 [49]. Site-directed mutagenesis can also be applied to produce alkali-stable mutants for the detergent industry [90]. Priyadharshini et al. [91] randomly mutated a region comprising residues from the position 34–281 in BLA. They stated that, compared to the wild-type, the mutant α -amylase with substitution of two amino acids, Ile157Ser and Trp193Arg (located in the solvent accessible flexible loop region in

domain B), exhibited better activity at extreme acidic and alkali conditions [91].

Apart from creating mutations and substitutions, protein engineering and heterologous expression also seem to be ideal options for strengthening the pH stability of α -amylases. By cloning and expressing pH stable α -amylase encoding genes from acidophiles and alkalophiles in mesophilic hosts, the production of acid- or alkali-stable α -amylases can be made cost-effective. Asoodeh et al. [92] could express the acid-stable α -amylase gene of *Bacillus* sp. DR90 in *E. coli* BL21 as an intracellular active protein, which was stable over a range of pH from 3.5 to 7 with an optimum around 4.5, and optimum temperature at 70 °C. The truncated α -amylase gene of *B. acidicola* cloned into the pET28a (+) vector and expressed in *E. coli* BL21 (DE3) showed activity in the pH range 3.0-7.0 with optimum activity at pH 4.0 [60]. When the gene *amyZ2* of the marine bacterium *Zunongwangia profunda* (MCCC 1A01486), which codes for the alkali-stable α -amylase, was cloned and expressed in *E. coli*, it showed maximum activity at pH 7.0, and maintained 143% and 126% of initial activity at pH 10.0 and 11.0, respectively, after 5 days of incubation at 25 °C [93]. The metagenomic approach also helps in identifying different pH stable α -amylases. A classic example for this is represented by the cold adapted α -amylase, Amy13c6 from a metagenomic library of the cold and alkaline environment of Greenland. This enzyme had an optimal pH of 8.0-9.0, so that it can be used in laundry applications at very low temperatures [84]. The α -amylase gene (*amyM*) derived using the pUC19 vector from soil metagenomic library when overexpressed and purified was stable at pH 9.0 [54].

Substitution of Leu134 and Ser320 with arginine and alanine residues, respectively, in BLA [94], followed by expression in *B. subtilis* DB403 and in protease-deficient strain *B. subtilis* WB600 [95], exhibited improved stability of modified protein at pH below 6.0. Similarly, a double mutant of BLA, Leu134Arg/Ser320Ala, showed a strong stability at low pH compared to the wild-type [96]. Calcium ion independent Termamyl LC™ was obtained by site-directed mutagenesis (His156Tyr, Ala181Thr, Asn190Phe, Ala209Val and Gln264Ser) of α -amylase from *B. licheniformis*, Termamyl™ (Novozymes A/S). It was further modified by region-specific random mutagenesis introducing random mutations at seven regions, all interfaces between the domain A, B and C. The selected variants (Amy a, b, c) showed much higher acid stability than Termamyl™ and Termamyl LC™ at pH 4.5 [49]. Alkali-stable mutants for detergent industry have also been developed by site directed mutagenesis. The activity of BAA Ser201Asn at pH 10 and 11 was increased by 16% and

50%, respectively, compared to the wild-type, whereas the activity of BAA Asn297Asp at pH 11 was improved by 50% [75]. Mutant BLA Thr353Ile/His400Arg produced by error-prone PCR exhibited stronger tolerance towards a lower pH as compared to wild-type [97].

4.4 Improvement of stability towards oxidative stress

One of the important qualities required by α -amylase, majorly for the use in detergents industry with a strongly oxidizing environment, is the oxidative stability. Calcium-chelating agents and builders present in liquid detergent generally soften the water during washing, which can decrease the stability of calcium-dependent α -amylase. Hence, it is a prerequisite to have a Ca^{2+} independent or low Ca^{2+} requiring α -amylase for detergent industry. Methionine and cysteine amino acids in α -amylase are especially oxidation-sensitive residues. It was shown that the oxidation of these amino acid residues can result in reduced activity or complete inactivation of the α -amylase [98]. The oxidative stability can be raised by protein engineering with switching off methionine with oxidation-resistant residues like alanine or leucine. Simultaneous improvement in thermal and oxidative stabilities was observed in case of a thermostable variant derived from the truncated α -amylase of alkaliphilic *Bacillus* sp. strain due to Met231 replacement by leucine and site-directed mutagenesis of 483rd codon in the gene to stop codon (TAA) [7]. The thermostability and oxidative stability of α -amylase from the mutant of *B. stearothermophilus* got revamped along with a low Ca^{2+} ion requirement when Met197 was substituted with alanine [66]. The oxidative stability of alkaline α -amylase of *Alkalimonas amylolytica* also got polished up with the replacement of methionine by serine [99]. Thus, by introducing similar modifications, the oxidative stability of the α -amylase could be enhanced, and mould them as a potential candidate for detergent and allied industries. By mutating the methionine residues at positions 43, 44, 55 and 62 to alanine, the oxidative stability of α -amylase isolated from *Thermotoga maritima* was strengthened, since the mutants had 50% activity in comparison with the wild-type [100].

5 Conclusion

α -Amylase is probably the most studied starch-degrading enzyme and the use of stable and engineered α -amylases dominate the major starch-based industries. Strategies

including protein engineering, directed evolution and metagenomics combined with *in-silico* predictions have been extensively explored in improving the stability. Major developments in the field of protein engineering have led to the achievements in the improvement of properties of α -amylase to meet the industrial demands. The focus on developing highly stable α -amylases has been on improving pH tolerance and also adapting to high and low temperature besides the tolerance to acid/alkali, oxidative stress, stability, etc., and here we brought an overview of such attempts reported in the recent past. Directed evolution in combination with other approaches, like DNA shuffling has also shown promising results to improve the varying stability factors. The use of stable biocatalysts will have greater commercialization potential since they can support novel and unique applications.

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