Improvement of microbial α-amylase stability: strategic approaches

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Graphical Abstract

**Advancements in α-amylase stability**
1. Discovery of novel α-amylases from extremophiles
2. Genetic manipulation of mesophilic α-amylases
3. Immobilization, chemical modification and use of additives
4. Protein engineering

**Main obstacles:**
1. Thermal and pH tolerance
2. Ca$^{2+}$ independency
3. Oxidant stability

**Need of stable α-amylase that suits industrial processing conditions**
**Highlights**

- Stable microbial α-amylases are required for industrial applications in robust conditions
- Thermo and pH tolerance, Ca$^{2+}$ independency and oxidant stability are major concerns
- Stable α-amylases are obtained from extremophiles and genetically manipulated non extremophiles
- Stability of α-amylases can also be improved by different methods
- This review summarized various strategies used for the improvement of α-amylase stability.
Abstract:

Microbial α-amylase being a vital enzyme in industrial biotechnology, has also received enormous attention in academic field. Although a huge number of α-amylases from different sources have been mentioned in the literature, only few of them are able to meet the industrial demands. Thermostability, pH tolerance, calcium independency and oxidant stability and very high starch hydrolyzing efficiency are the important criteria for the diverse applications of α-amylase in starch based industries. Owing to biotechnological importance, its stability is a major concern for its economic viability. Stable microbial α-amylase can be obtained: (i) from extremophiles and genetically manipulated non extremopliles, and (ii) through improving stability by immobilization, chemical modification, protein engineering and augmenting with different additives. The present review is an attempt to compile the different strategies employed till date to obtain stable α-amylase.

Key words: α-amylase; industrial biotechnology; stability; thermostable; starch; protein engineering; directed evolution

Abbreviations used: TAKA/TAA: α-amylase from Aspergillus oryzae, BLA: α-amylase from Bacillus licheniformis, BSUA: α-amylase from Bacillus subtilis, BAA: α-amylase from Bacillus amyloliquefaciens. AHA: α-amylase from Pseudoalteromonas haloplanctis, PWA: α-amylases from Pyrococcus woesei, PFA: α-amylase from Pyrococcus furiosus, BStA: α-amylase from Bacillus stearothermophilus, TIM: Triosephosphate isomerase, ILs: ionic liquids
1. Introduction

α-Amylase (EC 3.2.1.1) is one of the oldest industrial enzymes of great significance for industrial biotechnology. Despite of their wide distribution across animals, plants and microbes, α-amylases of microbial origin have proved to be of great value in all starch based industries such as food, agricultural, fermentation, textiles, pharmaceuticals, detergent, brewing, baking and paper [1]. Other potential applications include treatment of starch processing waste water, bio-ethanol production, and the manufacturing of oligosaccharides mixtures and high molecular weight branched dextrins for production of powdery foods, rice cakes, etc. [2]. The major advantages of microbial α-amylases are their cost-effective bulk production, an easy manipulation of the microorganisms for desired enzyme characteristics and high productivity, less time and space requirement, and broad spectrum of stability and specificity [3]. In most of the industrial sectors, α-amylase mediated reactions are carried out under extreme conditions at very low or high pH and temperature. The conventional starch hydrolysis bioprocessing involves two steps: liquefaction and saccharification, where thermostable and acid stable α-amylases are required [4]. On the other hand alkaline and oxidative stabilities, calcium ion independency and activity at a broader temperature range are important aspects need to be possesses by α-amylase for their use in detergent industry [5]. Therefore, there is a continuous demand of stable α-amylases to meet requirements of such specific applications in various industrial sectors [6]. In this context, the discovery of new and robust α-amylases, with respect to mainly their pH and temperature tolerance, has become a trend in biotechnology. Such enzymes can be obtained from extremophiles or can be produced by the genetic manipulated of non extremophiles. Moreover, the stability of α-amylases can be improved by different processes such as immobilization, chemical modification, protein engineering and by different additives. Modification of α-amylase
properties by means of protein engineering is a relatively popular and routine practice for both academic and industrial sectors. In the present review, an emphasis has been given on compiling different strategies employed for improving the stability of α-amylase.

2. Major amylases and their common properties

α-Amylase (1,4-α-D-glucan glucanohydrolase) is one of the extensively studied amylolytic enzymes and most frequently utilized in all starch based industries. Most α-amylases belong to the family 13 of glycoside hydrolases (GH13) [7] which randomly cleave the α-1,4 linkages between adjacent glucose units in the linear amylose and amylopectin chain and ultimately generate glucose, maltose, maltotriose and small dextrins.

The X-ray crystallography elucidating the structures of α-amylases isolated from A. oryzae (TAKA/TAA), B. licheniformis (BLA), B. subtilis (BSUA), B. amyloliquefaciens (BAA), P. haloplanktis (AHM), P. woesei (PWA), and P. furiosus (PFA) revealed that these enzymes share common structural features such as: (i) they possess a (β/α)$_8$ or TIM barrel structure containing the catalytic site residues, (ii) they have four highly conserved regions in their primary sequence which contains all the catalytic and most of the important substrate-binding sites, and (iii) contain Asp, Glu and Asp residues as catalytic sites [8,9]. However, a truncated mutant of B. stearothermophilus US100 α-amylase (AmyTM) is the only exception which contains the βαβα instead of (β/α)$_8$ unit as the minimal subdomain associated to an enzymatic function. Moreover, it is devoid of the above mentioned three catalytic residues but still retains catalytic activity with the regeneration of a catalytic site [10].

As shown in Fig. 1, α-amylase consists of two large domains, namely N-terminal and C-terminal domains. N-terminal domain is further divided into two domains, viz., A and B. The catalytic domain A contains (β/α)$_8$ or TIM barrel structure and domain B consists of three-
stranded antiparallel β-sheet structures and protrudes between β3 and α3 of domain A. Domain B is involved in functional diversity and stability. Domain C with a β-sheet structure is located in the C-terminal part of the polypeptide chain [8,11]. Similar to all TIM-barrel enzymes, the α-amylase family carries the catalytic and substrate binding residues at the C-terminal of β-strands and in loops that extend from these strands [12].

3. Stability of α-amylases

The stability and functionality of native proteins are maintained by a subtle balance among the non-covalent forces or interactions, such as H bonds, ion pairs, hydrophobic and van der Waals interactions and weakening of these will result in unfolding or denaturation of the protein. According to the commonly accepted notion (Lumry-Eyring model), enzyme inactivation is a two-stage process that involves an early reversible unfolding step followed by an irreversible step [9] (Eq. 1):

\[ N \text{ (folded)} \leftrightharpoons U \text{ (unfolded)} \rightarrow D \text{ (final denatured state)} \]  (Eq.1)

Irreversibility is generally caused by aggregation, misfolding, and chemical modification or in the absence of chaperones [13]. Reports are available on the stability and irreversible inactivation of the α-amylases mostly on TAKA, BLA, BAA, BStA and PFA. In some cases, thermal unfolding transitions of α-amylases have shown a partial reversibility due to the use of high concentrations of co-solvents and chemical denaturants [14–17]. Strucksberg et al. [18] found reversible unfolding of TAKA in alkaline pH and GndHCl, whereas, for BLA it was only under high concentrations of protecting osmolytes (glycerol). This phenomenon supports that aggregation of the unfolded state works as the main obstacle for a proper refolding.

3.1. Sources of stable α-amylases
Stable microbial α-amylases can be obtained from two sources: (i) extremophiles (thermophiles, hyperthermophiles, halophiles, alkaliphiles, acidophiles, piezophiles, metallophiles and psychrophiles), and (ii) genetically manipulated non extremophiles. Compared to other extremophiles, thermophiles and their thermostable enzymes are in great demand in different industrial sectors. Therefore, screening of thermostymes following conventional methods is still going on as an alternative over the tedious bioengineering procedures applied for enzymes of mesophilic origin [19]. Some important and recently isolated thermophiles with efficient thermostable (optimum temperature ≥60°C) α-amylase production capacity are listed in Table 1. Among them B. amyloliquefaciens (BAA), B. licheniformis (BLA), B. stearothermophilus (BSTA) and Pyrococcus furiosus (PFA) are the most potent producers of thermostable α-amylase at 37°C to 60°C. The isolation and maintenance of pure cultures of extremophiles are difficult tasks and most of them do not possess significant starch hydrolyzing efficiency. Hence, a more convenient approach has been explored for a long time to clone extremophiles genes into suitable mesophilic hosts. This gives the advantages of high productivity and high stability [20]. Some of the examples of genetic manipulations of mesophiles for the production of thermostable α-amylases are mentioned in Table 2. In this process, α-amylases were produced at ambient temperature by genetically manipulated mesophilic hosts like E. coli or Pichia pastoris retaining all the properties of the thermostable enzyme.. The developments of genetically modified thermophilic hosts could help in overexpression of the thermostable enzymes which could not be produced in active form using conventional mesophilic host.

3.2. Different factors responsible for α-amylase stability

Besides intrinsic factors (e.g., amino acid sequence) that confer resistance to the higher temperature to α-amylases from thermophiles, some extrinsic factors such as pH, presence of
calcium ion, etc. also play significant roles in structural stability of α-amylases [21]. In spite of strong sequence similarities, BLA is more thermostable compared to BStA and BAA, primarily due to the presence of additional salt-bridge involving three lysine residues (K88, K253 and K385). The extra thermostability of this enzyme is achieved by lowering the rate constant of monomolecular conformational scrambling, which serves as the cause of irreversible thermoinactivation for the other two α-amylases [22]. Various determinants and mechanisms have been identified for the structural stability of enzyme under extreme environmental conditions. For example, 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 as compared to non-halophilic proteins [23]. 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 [6, 9]. Whereas, the cold adaptation in AHA and other cold adapted enzymes may result from reduced number of disulfide 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 Ca$^{2+}$ and decreased number of aromatic interactions [24].

The primary kinetic adaptation in a psychrophilic enzyme (AHA) is that the enzyme activity is less temperature dependent compared to the mesophilic homologues, as the enzyme possesses a more flexible structure, and consequently is more thermolabile. This low conformational stability and high flexibility of AHA probably arise from a reduced number of
weak ionic interactions in the protein that may facilitate cooperativity and reversible unfolding [25]. It has been suggested that thermophilic or hyperthermophilic enzymes have lower catalytic activity at a given temperature than the corresponding mesophilic enzymes [26]. “Activity-stability trade-off hypothesis” is a widely assumed view, which postulates that the decrease in the structural flexibility of thermophilic enzymes is responsible for their low catalytic activity, i.e., flexibility and catalysis are directly correlated [27].

Calcium ion has been shown to regulate the structural stability and catalytic activity of most α-amylases [28, 29]. Thermostabilization by Ca$^{2+}$ can be justified by the increased rigidity of enzyme structure through salting out of hydrophobic residues by this metal ion [6]. Most of the α-amylases are known to contain at least one Ca$^{2+}$ per molecule and it is located at the interface between the domains A and B [28], however BLA, BAA and BSTA contain calcium-sodium-calcium metal triad instead of single conserved calcium ion [30]. High concentrations of Ca$^{2+}$, some α-amylases have been reported to be inhibited due to secondary binding at the catalytic site of the enzyme other than calcium binding sites [28, 31]; however, the thermodynamic stability is enhanced because of the subtle changes in the tertiary structure with a higher rigidity and compactness [32]. 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 [33].

3.3. Improvement of α-amylase stability

3.3.1. Immobilization

Enzyme immobilization offers huge advantages over free enzymes such as possibility of reuse, continuous operation and better control of reactions, improved stability and adaptability to various engineering designs [34]. A variety of methods have been developed for immobilizing
enzymes for different purposes like adsorption, covalent attachment, entrapment, crosslinking, and encapsulation. It is very difficult to predict completely how stability would increase after immobilization. It could be due to mutual spatial fixation of enzyme molecules against aggregation or autolysis of proteases, increase in their rigidity, and their protection from possible inactivators [35]. Among all the immobilization techniques, multipoint covalent attachment is the most effective thermal stabilization process due to strong rigidification of the enzyme structure, mainly if a very intense multipoint covalent attachment is achieved on highly activated pre-existing supports via short spacer arms and involving many residues present on the enzyme surface [36, 37]. In that case, all the residues involved in the immobilization process can maintain their relative positions under any drastic conditions (e.g., at higher temperature or in presence of organic solvents, urea, guanidine, etc.), and native structure/activity of the immobilized enzyme is retained [36]. Some of the recent studies on the stability improvement of α-amylase by immobilization procedures are compiled in Table 3. Immobilization onto spore surfaces by cross linking and covalent attachment is a promising method of α-amylase stabilization and durability [38]. The resistant power of the microbial spores in hostile environmental conditions and the spore surface characteristics of being anion rich, hydrophobicity and the presence of functional groups facilitate immobilization of enzymes, moreover, this process is simple and cost effective [38, 39]. Cross-linked enzyme aggregates (CLEAs) also serve as a simple, rapid and economical immobilization strategy with advantages of high volumetric activity, substantial space time yield, operational and storage stability, easy recyclability, amenability of easy scale-up and, most importantly, possibility of using crude enzymes without purification [40]. Very recently, Nadar et al. [41] have found macromolecular cross-linked enzyme aggregates (M-CLEAs) of α-amylase to be more thermostable as compared
to the traditionally prepared glutaraldehyde CLEAs. They have used non toxic, biodegradable, biocompatible, renewable polysaccharide based macromolecular cross-linkers such as agar, chitosan, dextran, and gum arabic.

3.3.2 Chemical modification

The chemical modification of proteins helps identifying specific residues at active sites, which are involved in substrate binding or chemical catalysis. This process is also used for tailoring the specificities and stability of enzymes [42]. Some of the previous reports proved that chemical modification is a powerful technique for the improvement of α-amylase stability. Acylation and acetylation are common chemical modification processes applied for the improvement of enzyme stability. B. subtilis α-amylase was stabilized by acylation of free amino groups with esters based on N-hydroxysuccinimide and 1-hydroxybenzotriazole [43] and D-glucono-δ-lactone [44]. Thermostability of the same enzyme was also increased at temperatures above 70 °C, but, was decreased at temperatures below 67 °C by acetylation with p-nitrophenyl acetate [45]. At very high temperatures, BLA and BAA are inactivated primarily due to deamidation of asparagine and glutamine residues. One of the five flexible regions in BAA contains a -Lys-Asn-sequence and it was suggested that modification of this particular lysine might decrease deamidation of asparagine at 80 °C. Chemical modification of lysine residues by citraconic anhydride brought about dramatic enhancement of thermal stability of BAA and catalytic activity of BLA [42,46]. Citraconic anhydride is a specific blocking agent for lysine residues (Fig. 2A), which alters a positive charge (pKₐ ~10) to a negatively charged carboxylic group with a much lower pKₐ. At the same time, the bulky group attached to the modified lysine residues enhances the flexibility with greater ease of the expansion of protein structure. Instead of high flexibility in modified BAA, the enhanced thermostability was proposed to be the result of a high resistance
toward irreversible thermoinactivation as deamidation was diminished [42]. Sorbitol was also reported to be very effective in the protection of native as well as chemically modified BAA against irreversible thermoinactivation [46]. Hence, combination of medium and chemical modification may provide an effective strategy for enhancement of protein activity and stability. Compared to the method described in an earlier study [46], a greater enhancement of thermostability was attained upon treatment of BAA with ethylene glycol bis (succinic acid N-hydroxy succinimide ester) [47] due to intra-molecular cross-links between lysine residues (Fig. 2B). The result of CD spectra and fluorescence quenching by acrylamide showed modification of positive ε-amine charges of lysine residues to neutral charges, which bestowed higher rigidity or compactness and lower hydrophobicity to the protein structure. Moreover, a more distinct stabilization was observed in presence of 10 mM CaCl$_2$ [47]. Arginine residues have shorter hydrophobic arms, larger hydrophilic groups, higher pI values and, thus, may be less motile than lysine. These residues are expected to impart more enzyme thermostability than lysine residues by facilitating more ionic (two salt bridges and five H bond) interactions through their guanidino group [24]. Psychrophilic amylases like AHA have been predicted to be structurally flexible and conformationally unstable due to a high Lys-to-Arg ratio. Modification of lysine to homo-arginine (hR) by guanidination (K106hR) resulted in a conformationally more stable and less active AHA. In this process, an intra-Domain B salt bridge was formed stabilizing the active site and decreasing the cooperativity of unfolding. This modification also altered Ca$^{2+}$ and Cl$^-$ binding in the active site [24].

The carboxyl groups of TAA were chemically modified by L-arginine methyl ester dihydrochloride (AME 2HCl) [48]. The modified TAA exhibited 200% improvement in starch-hydrolyzing efficiency at 60 °C. Moreover, the modified TAA formed a thermostable molten
globule (MG) state and this thermostabilization was not enthalpically driven, it was associated with a decrease in the entropy of the transition state arose from a higher helical content and/or better organization of sol vent water molecules on newly exposed hydrophobic surfaces of transition state [48]. Chemical modification of Saccharomycopsis fibuligera R64 α-amylase by acid anhydrides (nonpolar groups), dimethyl adipimidate (DMA) (cross-linking) and polyethylene glycol (PEG) (hydrophilization) increased enzyme stability against thermal, chelator (calcium ion) and proteolytic inactivation (tryptic digestion), respectively [49]. 3.3.3.3.3.3.

**Protein engineering approaches**

Protein engineering is the mostly studied method for the stabilization of α-amylases. This method can be classified as random mutagenesis and site-directed mutagenesis. Random mutagenesis includes introduction of mutations at random along the entire length of a gene using error prone PCR, DNA shuffling, UV irradiation, chemical mutagenesis, etc. In this process mutation sites can’t be predicted by structural information. In site-directed mutagenesis process a mutation is created at a defined site in a DNA molecule, thus structural information of the targeted enzyme is essential [50]. Although several studies have been carried out for the production of hyper-active α-amylase after UV irradiation and chemical mutagenesis by ethyl methanesulfonate (EMS) or N-methyl-N′-nitro-N-nitrosoguanidine (NTG), eventhough reports on the stability improvement of α-amylase are in scarce by these processes [51]. In order to fulfill 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.

**3.3.3.1 Thermostability improvement by protein engineering**
BLA is an interesting model for protein engineering in order to investigate enzyme thermostability. The overall thermostability of BLA concentrates in domain B and at its interface with the central A domain [52]. A series of substitutions has already been reported in BLA to stabilize the enzyme by many research groups [29]. For example, H133I and A209V mutations increased half-life of BLA by 10 folds at 90 °C [53–55]; whereas, accumulation of 5 substitutions namely H133I-N190F-A209V-Q264S-N265Y or to each single mutation enhanced thermostability of this enzyme [56]. The substitution of deamidation residue asparagine with aspartic acid and serine increased the half-life of a Ca-independent Bacillus sp. KR-8104 α-amylase (BKA) [57].

In addition to site-directed mutagenesis, some workers showed that deletion of some residues in α-amylase enhances the resistance to high temperatures. Suzuki et al. [58] reported that deletion of a loop (R176–G177) in domain B and substitutions of alanine for Lys269 and aspartic acid for Asn266 increased thermal stability of BAA up to large extent. In a similar vein, thermostability was improved and calcium requirement was reduced in BStA by shortening the loop created by five residues (R212, G213, I214, G215 and K216) through deletion of G213–I214 or I214–G215. However, the deletion of G213–I214–G215 reduced the optimum temperature by 17°C [59,60]. A drastic enhancement in the Tm of α-amylase from Bacillus sp. 406 was noted on a combination of two substitutions (Gly211Val and Asn192Phe) and deletion of residues Arg178-Gly179 [61]. Thermostabilization observed by shortening of the loop was possibly due to the formation of additional intramolecular interactions.

Introduction of prolines in loop regions is a common protein stabilizing strategy and it is only possible if Pro can be accommodated in the structure [62]. For paradigm, replacement of
Arg124 with proline increased the thermostability of α-amylases derived from an alkalophilic Bacillus species (NCIB 12512) [62] and Bacillus species KSM-1378 [63].

Disulphide bond engineering is also a successful technique for enzyme stabilization, yet the reasons behind the stabilizing effect of this bridge are not well characterized [64]. In some cases, domain C plays an important role for stability of α-amylases in extreme conditions. Introduction of disulphide bridge by replacing S450 and K415 with cysteines near a Zn$^{2+}$ binding site in the C domain resulted in a significant improvement of activity and thermostability of Flavobacteriaceae sinomicrobium α-amylase [65]. Whereas, Ser336 and Ser437 were mutated to cysteines in order to introduce a new disulphide bond at A and C domains interface of Saccharomycopsis fibuligera R64 α-amylase. The mutant α-amylase was overexpressed in the Pichia pastoris KM71H, and showed improved stability without any affect on its activity [66].

### 3.3.3.2 pH stability improvement

The stability at extreme pH can be engineered by employing normal protein stability engineering techniques including helix capping, removal of deamidating residues and cavity filling [29]. The optimum pH of the thermostable BLA is 6; and the enzyme is unstable in acidic environment which is necessary for industrial-scale starch liquefaction process. Replacement of basic residues with acidic amino acids by site directed mutagenesis is the fundamental strategy for the improvement of enzyme stability at low pH. For example, replacement of histidine residues of B. subtilis α-amylase by site directed mutagenesis with aspartic acid residues (H275/293D, H275/310D, H293/310D and H275/293/310D) showed enhanced stability and catalytic activity under acidic condition [67]. Substitution of Leu134 and Ser320 with Arg and Ala residues, respectively in BLA [68], followed by expression in B. subtilis DB403 and in protease-deficient
strain *B. subtilis* WB600 [69] exhibited improved stability of modified protein at pH below 6.0. Similarly, a double mutant of BLA, L134R/S320A, showed strong stability at low pH compared to wild type [70].

Calcium ion independent Termamyl LC™ was obtained by site-directed mutagenesis (H156Y, A181T, N190F, A209V and Q264S) 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 [50]. Alkali stable mutants for detergent industry have also been developed by site directed mutagenesis. The activity of BAA S201N at pH 10 and 11 was increased by 16% and 50%, respectively, compared to the wild type; whereas, the activity of BAA N297D at pH 11 was improved by 50% [71]. A region comprising residues from the position 34-281 was randomly mutated in BLA by Priyadharshini et al. [72]. According to their report, an amylase mutant with substitution of two amino acids, I157S and W193R (located in the solvent accessible flexible loop region in domain B), showed improved activity at extreme acidic and alkali pH values compared to the wild type.

### 3.3.3.3 Oxidative stability enhancement

Oxidative stability of α-amylase is one of the most important properties for its usage in detergents. 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. Oxidative stability can be improved by protein engineering through replacement of methionine to oxidation-resistant residues like alanine or leucine. Substitution of Met197 by
alanine increased thermostability and oxidative stability in a mutant of *B. stearothermophilus* α-amylase along with a low Ca$^{2+}$ ion requirement [59]. 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 483$^{\text{th}}$ codon in the gene to stop codon (TAA) [5]. Replacement of methionine by serine also significantly increased the oxidative stability of alkaline amylase of *Alkalimonas amylolytica* [73]. Therefore, these engineered enzymes can potentially be used in detergent industry.

### 3.3.3.4 Directed evolution

Directed evolution has been proved to be a powerful molecular tool to improve enzyme properties like substrate specificity, thermostability, pH stability, and organic solvent tolerance for specific applications [74]. In comparison to the rational design of site-directed mutagenesis, this technique modifies enzyme properties without requiring much knowledge of their structure [75]. This process relies on Darwinian principles of mutation and selection [76] but it is performed under a controlled selection pressure with repeated rounds of three steps: (i) generation of a random gene library by different mutagenesis strategies like random mutagenesis by error-prone PCR, DNA shuffling, etc., (ii) gene expression in a suitable host and (iii) screening or selection of high-performing mutants [75]. A number of studies have proved that directed evolution is a powerful method for the simultaneous improvement of thermostability as well as enzyme activity, especially when used in combination with rational or semi-rational engineering strategies. Using directed evolution, the thermostability of *P. fluorescens* Biovar I α-amylase was increased at low pH in absence of added calcium [77]. Similarly, thermostability of α-amylase from *Thermus* sp. strain IM6501 was improved significantly by random mutagenesis
and DNA shuffling [78]. The genes coding for the wild-type BAA and the mutants BAA S201N and BAA N297D [71] were subjected to error-prone PCR and gene shuffling. Screening of 960 transformants and their subsequent rescreening yielded the mutants BAA 42 and BAA 29 in which activity of BAA 42 was improved at pH 10 and an overall improvement in activities was noted in BAA 29 and BAA 42 [79]. Thermostability was increased at pH 4.5 by employing error-prone PCR and DNA shuffling in Novamyl from Bacillus sp. TS-25 [80]. Mutant BLA T353I/H400R produced by error-prone PCR exhibited stronger tolerance towards a lower pH as compared to wild type [81].

Although directed protein evolution is an invaluable process in present day biotechnology but it has some limitations due to its inability to generate high quality mutant libraries containing more beneficial variants [82]. The construction of focused mutant libraries at defined hotspot residues is a potential way of improving this process. A new method, combinatorial coevolving-site saturation mutagenesis (CCSM), which chooses the coevolving sites of proteins as hotspot residues to construct focused mutant libraries, has been applied for the improvement of thermostability of an α-amylase from B. subtilis CN7 [82].

3.4 Stabilization by different additives

Stability of α-amylase can be improved by numerous stabilizing agents following different principles. Water molecules act as plasticizer, which allow the enzyme molecules to unfold or denature, resulting in the loss of activity. α-Amylase stability can be improved by supplementing some stabilizing agents (e.g. sugars or polyols) in the reaction mixture, which modify the water structure or strengthen hydrophobic interactions inside the protein molecules [83]. Polyols, dimethyl formamide and dimethyl sulfoxide increased the half life of BStA by almost two fold, which suggested that the enzyme's structure is stabilized against thermal denaturation through
Among different sugars (sucrose and trehalose) and polyols (mannitol, sorbitol, lactitol and glycerol), sucrose showed maximum protective effect on thermostability of A. oryzae α-amylase [85]. On the contrary, Yadav and Prakash [86] found trehalose as a more effective stabilizer in comparison to sorbitol, sucrose and glycerol. Usually trehalose occupies a volume that is at least 2.5 times larger than sucrose in aqueous solution and it has less dynamic conformations compared to any of the other sugars due to its anisotropic hydration adjacent to glycosidic oxygen [86] [87]. Therefore, it may substitute more water molecules in solution and act as a better stabilizer compared to other sugars [86]. Sorbitol was found to be very effective against irreversible thermoinactivation of BAA and BLA [83]. Yoon and Robyt [88] evaluated activation and stabilization effect of Triton X-100, polyethylene glycols (PEG) and polyvinyl alcohols on 10 starch-degrading enzymes. They proposed the mechanism that binding of additives to enzyme gives a single tertiary structure resulting in the maximum enzyme activity, whereas without additives the enzymes have several tertiary structures in equilibrium, each with a different specific activity resulting in an average lower activity.

In a dry condition, many sugar molecules (e.g., maltodextrin, trehalose) are able to form a “glassy state” which causes reduction of mobility of the enzyme molecules decreasing rate of enzyme inactivation or denaturation. For example, freeze-dried α-amylase of A. oryzae NRRL showed higher thermostability compared to its aqueous form. Moreover, an extraordinary stabilization was achieved when the enzyme was dehydrated in presence of trehalose [89]. Similarly, A. oryzae α-amylase was far more thermostable in maltodextrin systems at reduced moisture content than in aqueous solutions [90].

An enhancement in the α-amylase thermostability in presence of starch is a well known phenomenon [91,92]. Polysacharides provide rigidity and hydration to enzyme; hence, covalent
binding of dextran increased both thermal and pH stability to α-amylase of B. licheniformis [93,94].

Nowadays, ionic liquids (ILs) have gained a considerable attention as potential environmental friendly solvents. Enzyme activity, enantioselectivity, thermal stability and reusability can all be improved in ILs [95]. However, the activity and stability of both the enzymes, BAA and BLA, were reduced in presence of two ILs namely 1-butyl-3-methylimidazolium chloride [BMIm][Cl] and 1-Hexyl-3-methylimidazolium chloride [HMIm][Cl] [96]. Similarly, activity of recombinant α-amylase from B. subtilis DR8806 was inhibited in 1-ethyl-3-methylimidazolium bromide ([EMIm][Br]), 1-butyl-3-methylimidazolium bromide ([BMIm][Br]), 1-hexyl- 3-methylimidazolium bromide ([HMIm][Br]) and [BMIm][Cl] [32]. Plenty of reports are available on thermostabilization of α-amylase in presence of different metal ions, mainly calcium [3]. Recently, two heavy atoms, gadolinium (Gd) and samarium (Sm) ions, have been shown to enhance the thermostability of A. oryzae α-amylase [97].

4. Conclusion

Currently research is being focused towards the discovery of highly stable α-amylases with respect to mostly their pH and temperature. Developments in protein engineering confer great achievements in the α-amylase properties to meet various specific application demands. Now engineered α-amylases have become predominant enzymes for most of the starch based industries. A huge number of protein engineering processes along with other strategies have already been explored in order to improve stability of α-amylases. Some patents have also been filed on different stabilization methodologies for α-amylases. Among different approaches, ‘directed evolution’ is a promising one for the stability improvement of various enzymes, especially when it is being used in combination with other approaches, but few reports are
available on α-amylase on this aspect. Each of the strategies employed for the improvement of α-amylase stability has its own limitations. According to a very latest approach, a fusion protein composed of oligopeptide (AEAEAKAKAEAEAKAK) attached to the N-terminus of alkaline α-amylase from Alkalimonas amylolytica was expressed in E. coli exhibiting improved specific activity, catalytic efficiency, alkaline stability, thermal stability and oxidative stability [67]. According to the achievements obtained so far, it may be possible to discover few more strategies to enhance the α-amylase stability to many folds in the near future.

Acknowledgement

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References


Captions of the Figures:

**Fig. 1. Domains of α-amylase (PDB ID: 6TAA) of *Aspergillus oryzae*.** (a) Amino acid sequence of α-amylase showing domain A (green: residues 1-121 and 177-380 AA) which fold into \((\beta/\alpha)_8\) barrel, domain B (blue: residues 122-176) which lies within the domain A and domain C (orange colour: residue 384-478) which fold into eight-strand β-sandwich ; (b) Rasmol generated 3D image (strand model) of 6TAA depicting domain A (green), B (blue) and C (orange).

**Fig. 2 Chemical modification of Lysine residue of α-amylase** by (A) citraconic anhydride and (B) ethylene glycol bis (succinic acid N-hydroxy succinimide ester)
Fig. 2

[A]

Citraconic anhydride

[B]

Ethylene glycol bis (succinic acid N-hydroxy succinimide ester)
Table 1: Some important α-amylase producing thermophiles.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Optimal temperature (°C)</th>
<th>Optimal pH</th>
<th>Kinetic parameters</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. stearothermophilus</td>
<td>70-80</td>
<td>5.0-6.0</td>
<td>Km=14 mg/ml</td>
<td>[98]</td>
</tr>
<tr>
<td>Pyrococcus woesei</td>
<td>100</td>
<td>6.5 – 7.5</td>
<td>NM</td>
<td>[99]</td>
</tr>
<tr>
<td>Pyrococcus furiosus</td>
<td>100</td>
<td>5.5</td>
<td>T1/2 =&gt;12 h , Km=3.69 mg/ml, Tm= &gt;110 °C</td>
<td>[17,100]</td>
</tr>
<tr>
<td>B. stearothermophilus US100</td>
<td>80–82</td>
<td>5.6</td>
<td>T1/2 =&gt;90 min at 90 °C</td>
<td>[101]</td>
</tr>
<tr>
<td>Thermococcus (HJ21)</td>
<td>95</td>
<td>5</td>
<td>T1/2 =5 h, Km= 45 mg/ml</td>
<td>[102]</td>
</tr>
<tr>
<td>Bacillus sp. Ferdowsicus</td>
<td>70</td>
<td>4.5</td>
<td>T1/2 = 48 min at 80 °C</td>
<td>[103]</td>
</tr>
<tr>
<td>Geobacillus sp. IIPTN</td>
<td>40-120</td>
<td>5.0 -10.0</td>
<td>Km = 36 mg/ml</td>
<td>[104]</td>
</tr>
<tr>
<td>Paecilomyces varioti</td>
<td>60</td>
<td>4</td>
<td>T1/2 = 53 min, Km= 4.3 mg/ml</td>
<td>[105]</td>
</tr>
<tr>
<td>Geobacillus sp. LH8</td>
<td>80</td>
<td>5.0 - 7.0</td>
<td>Km= 3 mg/ml</td>
<td>[106]</td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td>110</td>
<td>8</td>
<td>NM</td>
<td>[107]</td>
</tr>
<tr>
<td>B. amyloliquifaciens TSWK1-1</td>
<td>70</td>
<td>7</td>
<td>T1/2 =12h; Km= 0.6 mg/ml; Tm=82 °C</td>
<td>[108]</td>
</tr>
<tr>
<td>B. licheniformis ATCC 9945a</td>
<td>90</td>
<td>6.5</td>
<td>T1/2 = 250 min, Tm=102 °C</td>
<td>[12,109]</td>
</tr>
<tr>
<td>Bacillus strain HUTBS62</td>
<td>90</td>
<td>4.4</td>
<td>T1/2 =2h</td>
<td>[110]</td>
</tr>
<tr>
<td>Anoxybacillus beppuensis TSSC-1</td>
<td>80</td>
<td>7</td>
<td>T1/2 =6h (at 90 °C), Km=0.5 mg/ml</td>
<td>[108]</td>
</tr>
<tr>
<td>B. laterosporus</td>
<td>60</td>
<td>7</td>
<td>NM</td>
<td>[111]</td>
</tr>
<tr>
<td>B. megaterium VUMB109</td>
<td>93</td>
<td>7.7</td>
<td>Km = 1.5 M; Vmax/Km = 0.38 and Kcat/Km = 2.5 × 106</td>
<td>[112]</td>
</tr>
<tr>
<td>B. methylotrophicus strain P11-2</td>
<td>70</td>
<td>7</td>
<td>NM</td>
<td>[113]</td>
</tr>
<tr>
<td>A. penicillioides TISTR3639</td>
<td>80</td>
<td>9</td>
<td>Vmax = 1.05 μmoles/min/mg , Km = 5.41 mg/ml</td>
<td>[114]</td>
</tr>
<tr>
<td>B. licheniformis TSI-14</td>
<td>70</td>
<td>7</td>
<td>NM</td>
<td>[115]</td>
</tr>
<tr>
<td>Laceyella sacchari TSI-2</td>
<td>70</td>
<td>7</td>
<td>Km = 2.71 mg/ml, Vmax = 7.589 μmoles/min/ mg Kcat=4.31×10⁻² s⁻¹</td>
<td>[116]</td>
</tr>
</tbody>
</table>
Tables 2: Some recent examples of genetically manipulated thermostable α-amylases

<table>
<thead>
<tr>
<th>Thermophilic producer</th>
<th>Expression system/mesophilic producer</th>
<th>Temperature optimum (°C)</th>
<th>pH optimum</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. subtilis</em> PY22</td>
<td><em>Pichia pastoris</em></td>
<td>60</td>
<td>7.0</td>
<td>[117]</td>
</tr>
<tr>
<td><em>Thermotoga neapolitana</em></td>
<td><em>E. coli</em></td>
<td>75</td>
<td>6.5</td>
<td>[118]</td>
</tr>
<tr>
<td><em>Thermobifida fusca</em> NTU22</td>
<td><em>Yarrowia lipolytica</em> P01g</td>
<td>60</td>
<td>7.0</td>
<td>[119]</td>
</tr>
<tr>
<td><em>Rhizopus oryzae</em></td>
<td><em>Pichia pastoris</em> GS115</td>
<td>60</td>
<td>4.0-6.0</td>
<td>[120]</td>
</tr>
<tr>
<td><em>Geobacillus thermoleovorans</em></td>
<td><em>E. coli</em> BL21(DE3)</td>
<td>80</td>
<td>5.0</td>
<td>[13, 121]</td>
</tr>
<tr>
<td><em>Pyrococcus</em> sp. ST04</td>
<td><em>E. coli</em></td>
<td>95</td>
<td>5.0</td>
<td>[122]</td>
</tr>
<tr>
<td><em>Thermococcus</em> sp. CL1</td>
<td><em>E. coli</em></td>
<td>85 (α-1,4-glycosidic linkage) &amp; 98 (α-1,6-glycosidic linkage)</td>
<td>5.0 (α-1,4-glycosidic linkage) &amp; 6 (α-1,6-glycosidic linkage)</td>
<td>[123]</td>
</tr>
<tr>
<td><em>B. subtilis</em> DR8806</td>
<td><em>E. coli</em> BL21 (DE3)</td>
<td>70</td>
<td>5.0</td>
<td>[32]</td>
</tr>
<tr>
<td><em>Geobacillus</em> sp. 4j</td>
<td><em>E. coli</em> BL21</td>
<td>60-65</td>
<td>5.5</td>
<td>[4]</td>
</tr>
</tbody>
</table>

NM, not mentioned
**Table 3:** Some recent studies on α-amylase stabilization by immobilization process

<table>
<thead>
<tr>
<th>Source</th>
<th>Process</th>
<th>Properties</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. subtilis</em></td>
<td>Immobilized on chitosan bead via <strong>adsorption</strong> and exposed in 0.15 T static magnetic field</td>
<td>Activity was increased by 19.53%, pH and storage stability increased</td>
<td>[124]</td>
</tr>
<tr>
<td>α-amylase from Sigma-Aldrich</td>
<td>Natural halloysite nanotubes through <strong>physical adsorption</strong></td>
<td>Exhibited thermal stability, good storage stability and reusability</td>
<td>[125]</td>
</tr>
<tr>
<td>Diastase α-amylase (Hi Media Lab. Pvt. Ltd., Mumbai)</td>
<td><strong>Physical adsorption and covalent binding</strong> onto polyanilines</td>
<td>Enhanced storage stability</td>
<td>[126]</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>Glutaraldehyde modified polyaniline</td>
<td>Thermal stability increased in presence of CaCl₂</td>
<td>[127]</td>
</tr>
<tr>
<td>Analytical grades of α-amylase (Lobachem)</td>
<td>Tetramethoxysilane produced polysaccharide nanohybrids</td>
<td>Improved the overall stability, affinity and catalytic property</td>
<td>[128]</td>
</tr>
<tr>
<td>Thermoalkalophilic amylase from environmental bacterial isolate</td>
<td>Magnetic poly glycidyl methacrylate [m-poly (GMA)] beads</td>
<td>Increased pH and thermostability</td>
<td>[129]</td>
</tr>
<tr>
<td>A109181, Aladdin company</td>
<td>Cu-Metal ceramic powder (MCP) as <strong>carrier</strong></td>
<td>Improved thermal stability</td>
<td>[130]</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td><strong>Covalently immobilized</strong> on the surface of silica coated modified magnetite nanoparticles</td>
<td>Increased thermal, pH and storage stability</td>
<td>[131]</td>
</tr>
<tr>
<td><em>B. licheniformis</em></td>
<td><strong>Cross-linked</strong> enzyme aggregates with BSA and calcium and sodium ions</td>
<td>Half-life increased from 43.31 to 115 min (about 3-folds) at 110°C</td>
<td>[132]</td>
</tr>
<tr>
<td><em>Bacillus</em> sp.</td>
<td>Aminated poly(glycidyl methacrylate-co-ethylendimethacrylate)</td>
<td>Improved acid resistance (the optimal pH from 7 to 5), presented better thermal stability at 90°C</td>
<td>[133]</td>
</tr>
<tr>
<td>α-amylase of <em>B. licheniformis</em> expressed in <em>E.coli</em> BL21</td>
<td>Immobilized on the spore surface by the <strong>covalent</strong> (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and Nhydroxysulfosuccinimide (NHS)) and <strong>adsorption</strong> methods</td>
<td>Shifting of pH from 5 to 8 and temperature from 50°C to 60°C</td>
<td>[38]</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td><strong>Ionic exchange and hydrophobic interactions</strong> on gold nanorods (GNRs) synthesized via sequential seed mediated growth method</td>
<td>Increased temperature, pH and storage stability</td>
<td>[134]</td>
</tr>
<tr>
<td>Organism</td>
<td>Description</td>
<td>Result</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-----------------------------------------------------------------------------</td>
<td>-------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Glutaraldehyde-containing amino group functionalized calix[4]arene was used to immobilize α-amylase covalently</td>
<td>Increased thermostability and durability</td>
<td>[135]</td>
</tr>
<tr>
<td><em>Laceyella sacchari</em> TSI-2</td>
<td>DEAE cellulose with Glutaraldehyde cross linking</td>
<td>Increased temperature, pH, solvents and surfactants stabilities</td>
<td>[136]</td>
</tr>
</tbody>
</table>