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Review

Molecular improvements in microbial α-amylases for enhanced stabi[illegible] and catalytic efficiency

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HIGHLIGHTS

- Stable and catalytically efficient amylases are required for industrial processes.
- Prospecting α-amylases from extremophiles and metagenome in heterologous e[illegible]ession syste[illegible]
- Discusses molecular strategies for enhancing the stability of α-amylases.

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ABSTRACT

α-Amylases is one of th[illegible]os[illegible]portant industrial enzyme which contributes to 25% of the industrial enzyme m[illegible] Though[illegible]produced by plant, animals and microbial source, those from microbial source[illegible]s t[illegible]ve pote[illegible] applications due to their stability and economic viability. However a large num[illegible]of α-am[illegible]ases from[illegible]ferent sources have been detailed in the literature, only few numbers of th[illegible]uld w[illegible]d the ha[illegible]sh industrial conditions. Thermo-stability, pH tolerance, calcium independ[illegible]ency[illegible]ant s[illegible]y and starch hydrolyzing efficiency are the crucial qualities for α-amylase in starch ba[illegible]industries. Microbes can be genetically modified and fine tuning can be done for the production of enz[illegible] with desired characteristics for specific applications. This review focuses on the native [illegible]nd recombin[illegible]α-amylases from microorganisms, their heterologous production and the recent molec[illegible] strategies which help to improve the properties of this industrial enzyme.

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1. Introduction

Enzymes are finding wide applications in industries for the productions of bioproducts. These biological catalysts are an indispensible component of biological reactions. The use of chemical catalysts is diminishing mainly because of the high costs, requirement of harsh reaction conditions and more importantly due to environmental concerns. These limitations can be overcome by the use of enzymes. Enzymes work at milder conditions and are highly specific and catalyze reactions faster than chemical catalysts (Prasad and Manjunath, 2011). Enzymes are now being used in various sectors of industry such as food, detergents, paper, textile etc. Enzymes have been in use since ancient times (Gupta et al., 2003) and they have been used in saccharification of starch, production of beverages like beer, treatment of digestive disorders and production of cheese from milk. Among the many enzymes that are widely used α-amylase has been in increasing demand due to its crucial role of starch hydrolysis and the applications of this hydrolytic action.

The major advantages of microbial α-amylases are their cost-effective industrial level production, easy genetic manipulation of the genes since no complex pathway is involved, well established tools for protein engineering for desired enzyme characte[ristics] and high productivity, and broad spectrum of stability and [specificity] (Gupta et al., 2003). In most of the industrial processes [the] reactions involving α-amylase are carried out under harsh co[ndi]tions at very low or high pH and temperature. The [commer]cial sta[rch] hydrolysis reaction involves two steps: liquefa[ction] and [sac]charifi[ca]tion, which involves thermostable and [acid] stable [α-]amylases (Jiang et al., 2015). However detergent [compa]tible amylase with alkaline and oxidative [stab]ilities, [calci]um ion independency and activity at broad ra[nge] of tempera[ture]. Therefore, there is a continuous demand of [novel] stable α-amy[lases] to meet the specific requirements of [various] industrial processes. This review addresses recent [develo]pments [in] molecular improvements in α-amylases for [enha]nced stability and [cata]lytic efficiency.

2. Structural and functional characteristics of α-amylase

Based [on] the m[ode] of acti[on] starch hydrolyzing enzymes may be endo- [or] exo- a[cting] type. α-amylases or α-(1-4)-glucan-4-glucanohydro[lase] (EC [3.2.1.1]) are categorized to glycosyl hydrolase family 13 (GH-13), which are endo-acting enzymes and together with glycoside hydrolase families 70 and 77 constitute the clan H of glycoside hydrolases (GH-H) which randomly cleave the α-1,4 linkages between adjacent glucose units in the linear amylose and amylopectin chain of starch and eventually generate glucose, maltose, maltotriose and small dextrins (Coutino and Henrissat, 1999). Based on the X-ray crystallographic analysis of α-amylase identified from different microbial sources such as *A. oryzae* (TAKA/TAA), *B. licheniformis* (BLA), *B. subtilis* (BSUA), *B. amyloliquefaciens* (BAA), *P. haloplanktis* (AHA), *P. woesei* (PWA) and *P. furiosus* (PFA) disclosed that these enzymes share common structural features such as: (i) they have 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 (Fitter, 2005). The three-dimensional structures of α-amylases mostly contain three domains namely A, B and C domain. The domain A acts as catalytic domain adopts a central $(\beta/\alpha)_8$ barrel structure consists of a highly symmetrical fold which includes eight parallel β-strands organized in a [barrel] with a border of eight α-helices. The N-terminal end of the [protein] contains domain A. This $(\beta/\alpha)_8$ barrel was first observed in chicken muscle triose phosphate isomerase (TIM), hence named [as] the structure as TIM barrel. This structure is present in a [large] number of alpha amylase family (Banner et al., 1975). The B domain is a protruding loop which is overhang between third β-strand and third α–helix of TIM barrel. It has irregular [β]-sheet structure and form large substrate binding cleft and varies among different α-amylases. This domain is supposed to play a role in substrate specificity differences observed in the α-amylases (Janecek et al., 1997). Most members of the family carry domain C, C-terminal anti-parallel β-sheet composed of [4]0 strands following the catalytic $(\beta/\alpha)_8$-barrel. It plays an important role in stability/folding of the protein and in substrate binding (Ali et al., 2011). The domain B and C are located at the opposite ends of TIM barrel. The substrate binding site (active site of the protein) is located in the long cleft located between at the carboxyl end of domain A and B and substrate binding sub sites are made from the side chains of residues located at the C-terminal ends of β-strands of barrel structure. [One] study showed that the domain C has also play a role in raw starch binding in barley α-amylase (Robert et al., 2003). In barley α-amylase, a "pair of sugar tongs" site in the domain C, formed by Ser378 and Tyr380, had been shown to function in starch recognition and binding (Bozonnet et al., 2007). In addition to the main three domains (ABC), the type and the number of extra domains such as domain D and domain E is located at the C-terminus shows wide variety within the α-amylase family (Janecek et al., 1997). The function of domain D is unknown but recent investigation by Tan et al. (2008) on the crystal structure of a thermo-stable α-amylase AmyB from *H. orenii* showed that this domain has additional domain in the N-terminal region called N domain which forms a large groove, the N—C groove. It was shown that the N domain helps in binding of enzymes to the raw starch. This N-domain is also found in maltogenic amylases, which is responsible for the thermo stabilization via oligomerization and substrate affinity modifications in maltogenic amylases of *G. thermoleovorans* and *Thermus* sp. (Mehta and Satyanarayana, 2013b). The domain E is referred to as carbohydrate-binding module (CBM) or starch-binding domain (SBD), which is a distinct sequence-structural module, which improves the efficiency of an amylolytic enzyme on raw starch (Motyan et al., 2011).

Most of α-amylases vary considerably with respect to their properties. In general, all known α-amylases contain a conserved calcium ion, which is found at the interface between domain A and B (Linden et al., 2003). This calcium ion is essential for its stability and activity of α-amylases. Calcium ion is positioned far away from the active site to contribute directly in catalysis and plays a role in stabilization of thermo stability of α-amylases.

Three steps are involved in catalytic mechanism of alpha amylase for retaining its glycosyl hydrolases. First step consist of glycosidic oxygen is protonated by the proton donor (Glu261). This is followed by a nucleophilic attack on the C1 of the sugar residue in subsite-1 by Asp231 (Nielsen et al., 1999). Once the glycon part of the substrate leaves, a water molecule is activated presumably

by the deprotonated Glu261. This water molecule hydrolyses the covalent bond between the nucleophilic oxygen and the Cl of the sugar residue in subsite-1, thereby completing the catalytic cycle (Nielsen et al., 1999).

3. Enzymatic stability of amylase and sources of stable α-amylases

The stability and catalytic efficiency of enzymes are determined by the non-covalent interactions, such as hydrogen bonds, ionic interactions, hydrophobic and van der Waals interactions and any factors negatively influencing these will result in unfolding or denaturation of the protein. According to Lumry-Eyring model, catalytic inactivation is a two-step process that includes a reversible unfolding step followed by an irreversible step (Fitter, 2005). Irreversibility is normally caused by aggregation of protein, misfolding, and modification of chemical moieties (Feller, 2010). Severable reports are available on the irreversible inactivation of the α-amylases, which is mainly due to high concentrations of solvents and chemical denaturants (Kumari et al., 2010; Nazmi et al., 2006). Strucksberg et al. (2007) found reversible unfolding of TAKA amylase in alkaline pH.

Highly stable microbial α-amylases can be isolated from three sources: (i) extremophiles (ii) Biotechnologically engineered extremophiles and non-extremophiles (iii) Metagenomic approaches for prospecting novel amylases from uncultivable microbes. Thermophilic microorganisms are the most sought after organisms among extremophiles for prospecting of highly stable α-amylases.

3.1. Prospecting of extremophiles for stable α-amylases

α-amylases from extremophiles are stable at extreme temperatures. As these are active under conditions that would obviously be regarded as incompatible, the extremozymes are gaining great attention recently.

3.1.1. Thermostable and cold active α-amylases

Thermophiles are the organisms which can grow above 55 °C. These are mainly classified into three groups: moderate thermophiles (grow above 65 °C), extreme thermophiles (grow above 75 °C) and hyperthermophiles (grow above 90 °C). Thermophilic microorganisms have been isolated from different habitats such as terrestrial, marine and extreme environments, hot spring, petroleum reservoirs and deep-sea hydrothermal vents (Vieille and Zeikus, 2001). Thermostability is a shared characteristic of most of the industrial enzymes. Generally thermophilic proteins contain higher concentration of charged residues on the surface. There is a significant increase in the proportion of Arg, Glu, Lys and Val whereas, a decrease proportion of Asn, Gln, Ser and Thr in thermophiles (Cambillau and Claverie, 2000). Residues at the surface of proteins tend to be flexible and show free intra protein interactions (surface salt bridges). These interactions enhance the thermotolerance of proteins from thermophilic organisms (Loladze et al., 1999). Thermostable α-amylase from *Bacillus licheniformis* shows extraordinary heat stability. It consists of 469 amino acid residues. The polypeptide chain of this α-amylases folds into three separate domains. The first domain (domain A) forms a (β/α) 8-barrel structure and this consisting of 291 residues. The second domain (domain B) with 104–206 residues is inserted between the third beta strand and the third α-helix of domain A. The third C-terminal domain (domain C) folds into an eight-stranded antiparallel beta barrel and consists 397–482 residues. The factors responsible for the extraordinary thermo-stability of this enzyme may be reduced surface area, increased ionic interactions, and increased packing interactions in the interior (Hwang et al., 1997). Calcium ions have been implicated in mechanisms involving thermal inactivation of *Bacillus* amylases. Almost all of the α-amylases require a certain quantity of calcium ions in the application, because their thermostability depends on the presence of structural calcium ions (Chi[illegible] et al., [illegible]).

Thermostable amylases isolated from thermophilic organisms have found a number of applications. With the availability of thermostable enzymes, a number of new ways for industrial processes have emerged. Thermostable amylases have extensively used in various industrial applications, detergent industries, brewing and baking industries, sugar industries, textile and paper industries. As enzymatic liquefaction and saccharification of starch are performed at high temperatures (100–110 °C), thermostable amylolytic enzymes have been currently analysed to improve industrial processes of starch degradation. Thermostable amylases from *Bacillus stearothermophilus* or *Bacillus licheniformis* are being currently used in starch processing industries. Thermostable amylases help to reduce in cooling costs, increase in solubility of substrate, reduce chance of microbial contamination and resistance to denaturing agents (Fincan and Enez, 2013).

Literatures are available on production of α-amylase from psychrophilic isolate. Psychrophilic organisms are those cold adapted organisms that grow at temperature ranging from 15 to 20 °C. To thrive effectively at low temperature environments, these microorganisms have evolved a multiple range of structural and functional adaptations. These are the production of cold active enzymes with high catalytic efficiency at low temperatures, the integration of unsaturated fatty acids in cell membranes to sustain membrane fluidity and the production of cold shock protein at low temperatures (Nam and Ahn, 2011). Cold active α-amylases are active at low temperatures and they have very low activation energies and lower melting temperatures, i.e. the temperatures at which half the protein is unfolded (Feller, 2010). These proteins can act at very low temperature because of their flexibility helps an easier accommodation of substrates at low temperatures. This flexibility of protein structure is because of reduce in the number of salt bridges, less proline residues in loops as the cyclic structure of proline provides rigidity to the proteins (Feller and Gerday, 1997). The low temperature stability of cold-active amylases is the most vital characteristic for use in the industries because of considerable progress towards energy savings. It also reduces undesirable chemical reactions occurring at high temperatures. Cold-active amylases are very useful in detergents since low temperature washing gives colour protection to fabrics.

Table 1
α-Amylase obtained from function based screening of metagenomic libraries.

Source	Vector	Properties	Reference
Soil	Cosmid	Cold-active	Sharma et al. (2010)
Soil	Fosmid	Saline and thermo-tolerant	Vidya et al. (2011)
Fecal sample	pEASY-E1 expression vector	–	Xu et al. (2014)
		Cold-active	Vester et al. (2015)
Soil	Lambda	pH and thermo-tolerance	Richardson et al. (2002)

4. Metagenomics strategy

Metagenomics refers to a culture independent strategy based on the total DNA that is isolated directly from communities of environmental samples. Metagenomics to study microbial diversity has been conducted in a variety of environments like soils, marine sediments, mangrove sediments, hot springs, hyper-saline habitats, acid mine drainage, polar ice caps, gut and skin microbiome. Diversity of biological species in metagenome can be evaluated either by sequence driven approach or functional metagenomics. In functional metagenomics the libraries were screened for various bioactives through heterologous expression (Schloss and Handelsman, 2003. Through functional metagenomics several enzymes, antimicrobial compounds, various pathway genes and antibiotics were identified. Recent molecular biology techniques revealed the potential of uncultured world as a reservoir for several potent biomolecules. Table 1 gives an overview of α- amylase reported based on functional screening of metagenomic libraries.

Several reports are available on discovery of α- amylase with improved properties from diverse habitats. Sharma et al., 2010 reported molecular cloning and characterization of amylase from soil metagenomic library constructed from Northwestern Himalayas. A gene (pAMY) of 909 bp was identified from metagenomic library encoding for amylase. Phylogenetic analysis as well as amino acid sequence comparison indicated that pAMY was closely related to uncultured bacteria. The enzyme was found to be ca^{2+} independent. This amylase works at low temperatures is unique for amylases reported so far reveal the potential of this enzyme for industrial applications.

Isolation and characterization of a novel α-amylase fr[illegible] a metagenomic library of Western Ghat soil was reported by V[illegible]a et al., 2011. Metagenomic library was constructed in fosmid ve[illegible] (pCC1FOS) and screened for α-amylase. The e[illegible] exhibit[illegible] optimum temperature at 60 °C and retained [illegible]0% ac[illegible]ty afte[illegible] incubation at 80 °C for 60 min. The enzym[illegible] hibited [illegible] optimal pH of 5.0 and retained 70% activity in pre[illegible]. Sequence analysis showed 95% simi[illegible]ty wit[illegible] *iguobacterium* sp. AFB 11 and AFB 18 with sequen[illegible] overage of [illegible]

Xu et al., 2014 cloned and cha[illegible]d a novel α-a[illegible]lase from fecal microbial metagenome. In this stud[illegible]ght amylolytic fosmid clones were observed ba[illegible]on activity s[illegible]ening. A novel α-amylase gene amyPL w[illegible]dentified. This is t[illegible]first report on α-amylase isolated fr[illegible]a gast[illegible]testinal metagenomic library. The study revealed t[illegible]gu[illegible]icroorganisms are a good source for novel hydrolase gen[illegible]

An excep[illegible] cold [illegible]ted [illegible] amylase – Amy$_{13c6}$ from a metagenom[illegible] libra[illegible] of cold [illegible]lkaline environment of Greenland w[illegible] reporte[illegible]by Vester et al., 2015. Sequence analysis revealed [illegible]ase was similar to α-amylase from class Clostri[illegible] The enzyme exhibited optimal activity at 10–15 °C and reta[illegible] more than 70% activity at 1 °C with optimal pH of 8.0–9.0. Th[illegible]esults indicate the potential of this amylase as a detergent enzyme in low temperature laundry processes.

Richardson et al. (2002) reported a novel high performance enzyme for starch liquefaction using sequence based screening approach. The enzyme was found to be stable at 95 °C and pH 4.5 without addition of endogenous Ca^{2+}. To combine the best aspects for improved starch liquefaction, gene reassembly was done and the chimeric sequences were screened using a process – specific, high-throughput activity assay and the mutants exhibited improved thermo-stability and optimal pH which leads to exceptional process compatibility and economic viability.

Yun et al. (2004) characterized a novel amylolytic enzyme encoded by a gene from a soil – derived metagenomic library using pUC19 vector. The putative amylase gene (*amyM*) was over-expressed and purified. The enzyme was stable at pH 9.0 and 42 °C. The enzyme hydrolyzes soluble starch and cyclodextrins and produced high levels of maltose and also exhibited high trans-glycosylation activity. The trans-glycosylation as well as enzymatic saccharification of AmyM suggests it has novel properties.

5. Heterologous expression systems and cell factory for improved amylase production

The recombinant techniques for he[illegible] amylase production involve the selection of amyla[illegible]ene, clo[illegible]in appropriate expression vector and transforma[illegible]into appro[illegible]e expression host. Several experimental att[illegible]mpts [illegible] been ma[illegible]on heterologous expression of amylas[illegible]ding ge[illegible]rom [illegible]erent bacteria and other microbes in *E*[illegible]. A hi[illegible]ly the[illegible]le amylase gene was isolated from *The*[illegible]*coccus*[illegible]*ofundus* (c[illegible]ding for 401-amino acid protein), and e[illegible]pre[illegible]*coli*. Th[illegible]eterologous production was found to be[illegible]5-fold [illegible]er tha[illegible]e wild strain (Lee et al., 1996). Anoth[illegible] amylase ge[illegible]thermophilic bacteria *Pyrococcus* sp.[illegible] mbinantly [illegible]ressed in *E. coli*. The enzyme showed on[illegible]y <40%[illegible]mology to other amylases which indicate the n[illegible]lty of the is[illegible]d amylase. The optimum temperature fo[illegible]hermostable e[illegible]me was 90 °C and pH 6.5 (Tachibana e[illegible]l., 1996). The hyperthermophilic archea bacteria *Pyrococcus*[illegible]*esei* produ[illegible]highly thermophilic α-amylase and this gene w[illegible]cloned in[illegible] halophile *Halomonas elongata* (Frillingos et al., 20[illegible] Anoth[illegible]maltogenic α-amylase gene from highly thermoph[illegible]*ilus* sp. WPD 616 was isolated, cloned and expressed [illegible]*E. coli*, which could withstand up to 50 °C and optimum pH at [illegible]mylase gene (Amy N) from *B. licheniformis* NH1 was recombinantly expressed in *E. coli* expression system. The thermostability of recombinant amylase was found to be 85 °C which is higher than the wild amylase (Hmidet et al., 2008). Karakas et al., 2010 expressed α-amylase from *B. subtilis* PY22 in *Pichia pastoris* expression system using the expression vector pPICZα, which is a tightly controlled methanol inducible expression system and allows extracellular secretion of the protein. The heterologous expression yielded high levels of extracellular amylase secretion (22 mg/L). An α-amylase which is highly thermostable and favourable enzyme for starch conversion process has been isolated from *Staphylothermus marinus* and over expressed in *E. coli*. The properties of the enzyme were interesting which is active in acidic conditions with an optimal pH of 5.0, and highly thermostable with a temperature maximum of 100 °C (Li et al., 2010). The N and C terminal truncated α-amylase gene from *B. acidicola* was cloned in pET28a (+) and heterologously expressed in *E. coli* (Sharma and Satyanarayana, 2012) and the enzyme was active 60 °C and pH 6.0. Another thermostable and acidic α-amylase from *G. thermoleovorans* including the signal peptide was expressed in *E. coli* gave an activity of 1723 U mg^{-1} protein and active at pH 5.0 and 80 °C (Mehta and Satyanarayana, 2013a).

Another efficient gene encoding α-amylase from *Exiguobacterium* sp. was isolated and expressed in *E. coli* with maximum enzyme activity at 30 °C and optimum pH 6.5. The enzyme was found to be halotolerant and retains its 50% of activity at 0 °C (Emampour et al., 2015).

A novel recombinant strain of *B. licheniformis* was constructed which over expresses homologous *Bacillus licheniformis* α-amylase carrying a recombinant plasmid pHY-amyL. The strain could produce around 26-fold increase in the amylase than the control. For the cost effective production of the protein, soybean meal and cotton seed meal has been used as carbon source and resulted in 17.6 mg/ml growth (Niu et al., 2009). Alkaline amylase

Table 2
Recombinant expression of α-amylases in heterologous hosts.

Source organism	Host	Temperature optimum	pH optimum	Reference
Thermococcus hydrothermalis	*E. coli*	75–85	5.0–5.5	Horvathova et al. (2006)
P. woesei	*Halomonas elongata*	90–100	5.5–6.0	Frillingos et al. (2000)
B. subtilis strain AS01a	*E. coli*	70	6.0	Roy et al. (2013)
Exiguobacterium sp. SH3	*E. coli*	30	6.5	Emampour et al. (2015)
Rhizopus oryzae	*Pichia pastoris* GS115	60	4.0–6.0	Li et al. (2011)
Thermococcus sp. CL1	*E. coli*	85	5	Jeon et al. (2014)
Thermotoga neapolitana	*E. coli*	75	6.5	Park et al. (2010)
B. subtilis DR8806	*E. coli*	70	5.0	Emtenani et al. (2015)
Thermobifida fusca	*Yarrowia lipolytica*	60	7.0	Yang et al., 2010
G. thermoleovorans	*E. coli*	60	7.0	Mehta a rayana (2014)
B. subtilis WB800	*E. coli*	60	6.0	Chen (20
Geomyces pannorum	*Aspergillus oryzae*	40	5.0	H (2017)
B. acidicola and *Geobacillus thermoleovorans* (Chimeric)	*Pichia pastoris*	60	4.0	and Satyan na (2017)
Arthrobacter agilis	*E. coli*	30	4.0	Kin (2016)
Staphylothermus marinus	*E. coli*	100	5.0	Li et a 0)
B. acidicola	*E. coli*	60	4.0	Sharma a yana (2012)
Pyrococcus sp. ST04	*E. coli*	95	5.0	et al. (2

has several potential applications in textile, paper and detergent industries. ARTP (atmospheric and room temperature plasma) has been identified as a novel technology for mutagenesis of microbes to improve the yield of industrial enzymes (Zhang et al., 2015). A mutant *Bacillus subtilis* was screened using this technology (Ma et al., 2015). In another study ARTP assisted mutagenesis screening method significantly increased the yield of recombinant pro in *Ba su* and the yield was 1.57 U/mg·h (1 higher tha ild type) (Ma et al., 2016).

Liu et al. (4a) mpted inve e metabolic engineering in *S. cerevisiae* to improve lase secretion. In this study they combined l dom mutag s and selection for growth on starch was formed to find out b st mutant strains producing recombinan amylase 5-fold above the level produced by the wild type

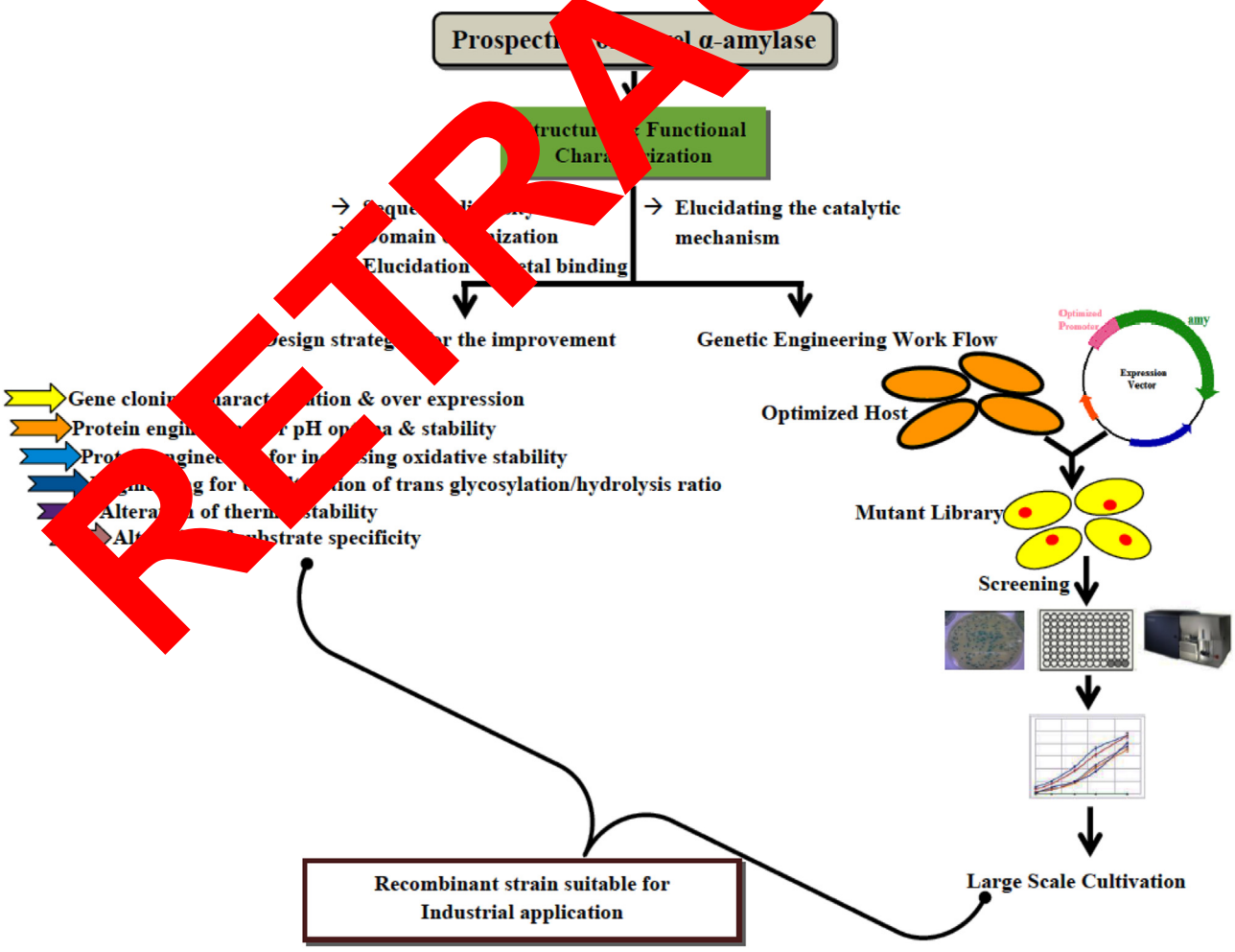

Fig. 1. Schematic representation of molecular improvement strategies for α amylase.

strain. Several genomic mutations were also observed in high amylase secretion strains identified through whole genome sequencing. Cyclodextrin degrading α-amylase was cloned and expressed from *G. thermoleovorans* with an optimum temperature of 80 °C and pH activity between 5.0 and 9.0 (Mehta and Satyanarayana, 2013b). Roy et al., 2013 over expressed raw starch hydrolysing in *E. coli* extracellularly and the recombinant enzyme showed optimum activity at 70 °C and pH 6.0.

A novel α-amylase (AmyA1) containing an open reading frame of 1482 bp was cloned from Antarctic psychrotolerant fungus *G. pannorum* and then expressed in the newly constructed *Aspergillus oryzae* system. The recombinant AmyA1 was optimally active at pH 5.0 and 40 °C, and retained over 20% of maximal activity at 0–20 °C. AmyA1 also exhibited broad substrate specificity (He et al., 2017). Recombinant chimeric α-amylase (Ba-Gt-amy) has been produced extracellularly in *Pichia pastoris* under strong AOX promoter resulted in 10.7-fold enhancement in α-amylase titre as compared to the wild type. The pure Ba-Gt-amy expressed in *P. pastoris* is a glycoprotein of 75 kDa, which is optimally active at pH 4.0 and 60 °C. The enzyme saccharifies soluble as well as raw starches efficiently and generates maltose and maltooligosaccharides, thus, useful in baking and sugar syrup industries (Parashar and Satyanarayana, 2017). Heterologously expressed α-amylases are listed in the Table 2.

6. Site directed mutagenesis and directed evolution as a tool for the improvement of α-amylase

Site-directed mutagenesis is one of the tools implemented to construct novel proteins that serve as efficient biocatalysts t[o] the industrial application. In site-directed mutagenesis an a[mi]no acid at specific site of a protein, whose structure and mecha[ni]sm of action is known, is edited and the mutated protein is evalua[ted] whether the modified protein performs better th[an] wild pr[o]tein. Site-directed mutagenesis enhances s[pe]cificity, stability, activity, solubility, and expression of th[e] biocatal[yst] (Bessler et al., 2003). The most important criteria for [de]sign[ing] mutagenesis is the selection of appr[o]priate a[mi]no acid residues and 3D structure analysis can provi[de] exact inform[ation] about this (Wind et al., 1998). Overview of [the] designing strategi[es] which can be applied to α-amylase is s[ch]emati[cally] represented in the Fig. 1. Recently the α-amy[lases] from *Ano[xy]bacillus* species were found to be a novel grou[p] the α-amylase fa[mily] GH13. Four residues from the amino [and] con[ser]ved regions were thus selected, and the mutants F1[83] (C[SR-I]), Y187F and L189I (CSR-II) and A161D (CSR-V) were [cha]racterized [a]mong the site directed mutants, onl[y] 161D [ha]d [spe]cific activity, kcat and kcat/km higher [1.]3, 1.[4] and 2.8[-fo]lds, respectively) than the corresponding [val]ues d[e]termined for the wild type (Ranjani et al., 2014).

6.1. Thermo-stability improvement

Thermo-stabi[lity] is the most important quality required for alkaline α-amylases for applications like desizing in the textile industry which decreases environmental hazards, reduces damage to textile fabric, and increases the efficiency of textile pretreatment .The protein engineering of disulfide bridges is the most widely accepted strategy for enhancing thermo-stability. Disulfide bridges stabilize enzymes undergoing reversible unfolding by decreasing the main-chain entropy of their unfolded states (Mansfeld et al., 1997). They also stabilize irreversibly unfolding proteins by decreasing the unfolding rate (Clarke and Fersht, 1993). In addition to disulphide bonds, hydrogen bonds and salt bridges also contribute to the thermo-stability of enzymes (Russell et al., 1997). These properties can be changed by site-directed mutagenesis or by directed evolution, for increasing its thermo-stability. Site-directed mutagenic changes in the amino acids Asn-75, Ser-76, and His-77 calcium binding sites of α-amylase from *B. megaterium* WHO increased the thermo-stability as well as enhancement in the half-life of the enzyme and recombinant enzyme showed 5 °C higher optimum temperature than the wild type (Ghollasi et al., 2013). The *Bacillus acidicola* α-amylase was modified by fusing the partial N- and C-terminal amino acids of highly thermostable α-amylase gene of *G. thermoleovorans*. The fusion protein was found to have a melting temperature of 73.8 °C which is higher than the native form (Parashar and Satyanarayana, 2016).

Wang et al., 2012 proposed a novel method, combinatorial coevolving-site saturation mutagenesi[s] which selects the crucial coevolving sites of proteins a[s] hotspot re[sidu]es to construct mutant libraries. They have used [the] technique [to] improve the thermo-stability of α-amylase from *[Ba]cillus subtili[s]* N7 (Amy7C). The results indicated that t[he] CCSM ca[n] identify [no]vel beneficial mutation sites, and enh[ance] the therma[l] ity of wild-type Amy7C by 8 °C, which [co]uld no[t] achieve [e] with the ordinarily rational introduction o[f] [a] a doubl[e] point mutation.

Suzuki et al., 1[9]9 repo[rted] that del[etion] of a loop (R176–G177) in domain B [of] *amyloliquef[aciens]* amylase (BAA), subsequent substitutio[n] [of] ine for Lys[176] and aspartic acid for Asn266 increased t[he]rmal [stab]ility of BAA. In another study *B. stearothermophilus* α-amylase ([BS]) thermo-stability was enhanced and calci[um] [requ]irement was r[edu]ced by shortening the loop composed [of] ve residues (R212, G213, I214, G215 and K216) and further [del]etion of G2[13] – I214 or I214–G215. However the deletion of G[213]–I214–G2[15] reduced the optimum temperature by 17 °C (K[uddus]khem[ov] al., 2009; Ali et al., 2006). Another thermo-stabi[lity] study reported the enhancement in the Tm of α-amylase [fr]om *Bacillus* sp. by substitution of amino acid residues (Gly211Val [and] 192Phe) and deletion of residues Arg178-Gly179 (Kachan and Evtushenkov, 2013)

The reversible nature of thermal denaturation and catalytic efficiency of *Bacillus licheniformis* α-amylase were improved by mutating Ser 187, Asn 188, Ala 269. Seven mutants carrying the mutations S187D, N188T, N188S, A269K, A269K/S187D, S187D/N188T, and A269K/S187D/N188T were generated and characterized. The highest thermo-stable mutant, A269K/S187D/N188T, exhibited a 9-fold enhancement in half-life at 95ÊC and pH 5.5 which is higher than the wild type (Li et al., 2017).

6.2. pH stability improvement

Site directed mutagenesis is an established technique employed for changing the optimum pH and increasing acid/basic stability of an enzyme. The stability of α-amylase at extreme pH can be manipulated by introducing stability enhancing engineering techniques like helix capping, removal of de-amidating residues and cavity filling. Substitution of Leu134 and Ser320 with Arg and Ala residues, respectively in BLA (Takazaki and Hirose, 1996), followed by heterologous expression in protease deficient *B. subtilis* WB600 (Liu et al., 2008a) resulted in improved stability of enzyme at pH below 6.0. In another study mutation of L134R/S320A, showed improved stability at acidic pH compared to wild type (Liu et al., 2008b). The optimum pH of the thermo-stable BLA is 6; and the enzyme is found to be unstable in acidic environment which is necessary for large scale industrial starch liquefaction process. Replacement of the basic residues with acidic amino acids by site directed mutagenesis is the fundamental step for the improvement of enzyme stability at low pH. The catalytic efficiency and stability of α-amylase from *B. subtilis* under acidic conditions has been increased by site directed mutagenesis of four basic histidine (His) residues His^{222}, His^{275}, His^{293}, and His^{310} in the catalytic domain which is crucial and single, double as well as triple mutants were constructed at these sites. The acidic stability of

enzyme was significantly enhanced after mutation, and 45–92% of initial activity of mutants was retained after incubation at pH 4.5 and 25 °C for 24 h as compared to the wild-type (39.5%) and catalytic efficiency was also found to be enhanced. Due to increase in the hydrogen bonds and salt bridges after mutation, an obvious shift of the basic limb toward acidity was observed. These changes around the catalytic domain contributed to the significantly improved protein stability and catalytic efficiency at low pH (Yang et al., 2013).

Calcium ion independent Termamyl LCTM was obtained by site-directed mutagenesis (H156Y, A181T, N190F, A209V and Q264S) of α-amylase from *B. licheniformis*, TermamylTM. The mutants were further modified by domain specific random mutagenesis by introducing mutations at seven regions, between the domain A, B and C. The selected mutants (Amy a, b, c) exhibited higher acid stability than Termamyl TM and Termamyl LCTM at pH 4.5 (Hashida and Bisgaard-Frantzen, 2000). Several studies reported the improvement of pH stability in α-amylase. For example the activity of BAA S201N at pH 10 and 11 was enhanced by 16% and 50%, respectively, compared to the wild type; however, the activity of BAA N297D at pH 11 was increased by 50% (Bessler et al., 2000). Priyadharshini et al. (2010) reported that amino acid residues from the position 34–281 was randomly mutated and further screening proved that mutant with I157S and W193R which is located in the loop region of the domain B resulted in the enhanced activity at extreme acidic and alkaline pH. Liu et al., 2008b observed that the mutations at two crucial positions Leu_{134} and Ser_{320} together affected the acid resistance of the α-amylase of *B. licheniformis* CICC 10181.

6.3. Oxidative stability improvement

Oxidative stability is one of the crucial qualities for the use alkaline amylase, especially in detergents industry where the reac tion environment is strongly oxidizing (Ito et al., 19 ethionine (Met) and cysteine amino acids in α-amyla are e cially oxidation sensitive (Vogt, 1995; Brosnan et a 992). T oxidation of methionines and cysteines increased th ze chain and resulted in the steric hindran the a site of the enzyme, leading to the significant red on or comp inactivation (Khemakhem et al., 2009; Hagi 1, 2001). Th dation of this amino acid residue has b n rep d to be involved in decrease in activity or com te inactiva of the amylase (Igarashi et al., 2003; Niel and Borchert, 2 To overcome the inactivation caused oxidat substitution of methionine residue by any oxidativ ist amino acid may work. The oxidation resistant amino ac dues in e serine (Ser), leucine (Leu), isoleuci th ine ar), and alanine (Ala) (Khemakhem al., 9). Rep ent of Met208 residue in a *Bacillus* s S-23 a ylase wi oxidation resistant leucine improved i bil n peroxide, and the enzyme activity was furthe eased from 35 to 84% with 500 mM H_2O_2 (Lin et al., 2003). M of α-amylase from *Geobacillus stearothermophilus* US110 wa bstituted by Ala, and 70% of the mutant activity was retained in the presence of 1.8 M H_2O_2 after 60 min of treatment (Khemakhem et al., 2009). The oxidative stability of α-amylase coding gene isolated from *Thermotoga maritima* was improved by mutating methionine residues at 43, 44, 55 and 62 to alanine. The mutants retained 50% activity in the presence of H_2O_2 compared to wild type (Ozturk et al., 2013). Substitution of methionine by serine also enhanced the oxidative stability of amylase of *Alkalimonas amylolytica* (Yang et al., 2012)

6.4. Directed evolution

Directed evolution is like natural evolution but at relatively faster speed. Past few decades witnessed tremendous impact of directed evolution. In directed evolution random genome mutations are introduced and the selection is based on the desired function. The major advantage of directed evolution is its tremendous pace compared to natural evolution which takes millions and millions of years (Tracewell and Arnold, 2009). Directed evolution comprises three main steps 1) Construction of mutant library 2) Screening/selection of mutants with improved function and 3) Isolation of improved genes.

Directed evolution and a high-throughput robotic screen were established to improve the properties of amylase (Novamyl) from *Bacillus* sp. *TS-25*. For improving low pH tolerance for bread industry applications such as sourdough and r r-prone based PCR libraries were generated, and r mbinant xpressed in *Bacillus subtilis* and screened for va with imp d thermal stability and activity under lo pH ditions. A umber of mutants exhibited more than C rise i rmal bility at pH 4.5 (Jones et al., 2008). Th tability of *Ba heniformis* α-amylase (BLA) under aci ndition as enha d through direct evolution using the erro polymera chain reaction. One beneficial mutation e, H28 as obt d in BLA. The specific activity of H281 161/352 U ch was 62.6/27.5% higher than that of t ype (WT) (6 U/mg) at pH 4.5/6.5 and 95 °C (Liu et , 2014

The thermo-stability *fluorescens* α-amylase was increased by d evolution at pH (Richardson et al., 2002). In an r study, thermo-stability of α-amylase from *Thermus* sp. str IM6501 w nhanced significantly with the help of random mu nesis and NA shuffling (Kim et al., 2003). The thermo-stabi f Nov yl from *Bacillus* sp. TS-25 was improved by employi prone PCR and DNA shuffling at pH 4.5 (Jones 2008). Liu et al., 2012 reported that BLA mutant with T 9R developed by error-prone PCR possesses stronger bility towards a lower pH compared to wild type.

7. Novel applications of α-amylases

Amylases found application in various industries including starch processing, textile and paper industry, as a detergent additive, food and pharmaceutical industries and in alcohol and other various fermentation processes (Monteiro and de Oliveira, 2010). Recent developments in pharmaceutical and chemical industries lead to extension of its applications to novel areas like therapeutic applications to cancer, wound healing etc. It also finds applications in the synthesis of drugs and agrochemicals (Samrot and Vijay, 2009). Oishi et al., 1991 reported prevention of obesity without any physical exercise by taking oral tablets containing α-amylases. This enzyme is an important component in several pharmaceutical enzyme preparations which is used for the treatment of pancreatic insufficiency. α-amylases finds applications in treatment of cystic fibrosis where the enzyme production was obstructed due to absence of trans-membrane regulator. Supplementation of α-amylases will prevent malnutrition (Li and Somerset, 2014). α-amylases also finds applications in clinical diagnosis. A molecular application of amylase as reporter gene assays serves as a tool for the study of gene regulatory elements and gene expression. Hence this serves as an additional strategy for selection of successful integration of a reporter construct and the insertion of foreign DNA into amylase gene can be identified by simple iodine staining (Aubel et al., 2001).

8. Conclusion and future perspectives

Recently research in α-amylase is being directed towards the development of highly thermo-stable and pH stable α-amylases for various industrial applications. Lot of improvement has been

achieved in engineering of α-amylase. Cloning and over expression has made tremendous improvement in enzyme production titres. In addition to that protein engineering studies of α-amylases have been carried out for the evolution of enzyme with several desirable characteristics. Wide scope still exists on the screening and selection of wild and novel organisms from extreme environmental conditions for the production of α-amylase and their further improvement with the aid of newer technologies.

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