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Review article

STRUCTURE-FUNCTION RELATIONSHIP AND THEIR APPLICATION IN INDUSTRY OF α, β AND GLUCOAMYLASE

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ABSTRACT
Amylolytic enzymes are one of the oldest industrial enzymes and hold the maximum market share of enzyme sales. With the advent of new frontiers in biotechnology the spectrum of the application of the enzymes have expanded to major area of industry. Among different amylolytic enzymes, α amylase, β amylase and glucoamylase are the most useful and best known enzymes. This review focuses on the sources, structure-function and some important properties of the three amylolytic enzymes. Research is focused on improving enzymes, modifying them to achieve desired properties of the three enzymes.α-amylase uses double displacement mechanism whereas β-amylase and glucoamylase use single displacement mechanism.The active site of α-amylase contains a trio of acidic groups, β-amylase contains thiol groups, glucoamylase contains tryptophan.

KEYWORDS: amylolytic enzyme, Substrate binding affinity, Starch binding domain, Metalloenzyme, Thermostability.

1. INTRODUCTION

Starch consist of two polysaccharides namely, amylose and amylopectin linked up by the α-glucosidic bonds and are large molecules. It is used in the food and confectionary industry as a thickener, stabilizer and as a gelling agent; in the pharmaceutical industry to prepare syrups and in the brewery industry to prepare alcohol. This would require the hydrolysis (degradation) of starch as a primary source for sugar (Weinheim *et al.* 1994). The use of amylolytic enzymes are the most preferred method for starch degradation as it offers a number of advantages including improved yields and favorable economics (Satyanarayana *et al.* 2004). Although 30 different amylolytic and related enzymes have been identified till date (Janeček, 1997), α-amylase (1, 4, α- D-glucanglucanohydrolase, E.C.3.2.1.1), β-amylase (1,4-α-D-glucanmaltohydrolase, E.C.3.2.1.2) and glucoamylase (1,4-α-D-glucanglucohydrolase, EC 3.2.1.3) are three most well-known amylolytic enzymes. These enzymes play a key role in the starch degradation (hydrolysis) process. α-amylase is an endohydrolase that catalyzes the hydrolysis of α-1,4-O-glycosidic linkage in polysaccharides with the retention of α-anomeric configuration (Sivaramakrishnan *et al.* 2006). β-amylase is an exohydrolase which releases β maltose from the non-reducing ends of α-1,4 linked poly and oligoglucans until the first α-1,6 branching point is encountered (Ziegler, 1999) . Glucoamylase (also known as γ-amylase) is an exohydrolase that catalyses hydrolysis of α-1, 3, α-1, 4 and α-1,6 glycosidic linkages to release β-D glucose from the non-reducing ends of starch (Sauer *et al*. 2000). Amylase constitutes approximately 25% market of the enzyme production (Sindhu *et al.* 1997). It is interesting to note that, although amylase has been used since 9thcentury for the conversion of malt starch to sweetener, a significant increase in amylases production and utilization of amylase occurred in 1960s (Aqeel and Umar, 2010). The enzymesare found in a wide variety of microorganisms. They are also produced by certain animals and plants such as human α-amylase,cereals β-amylas e (Pandey, 1995).The present review focuses on three amylolytic enymes, namely α-amylase, β-amylase and glucoamylasein terms of their structure, functions, applications and their sources of production. Several industrial problems like low catalytic activity, accumulation of by-product and caused due to these enzymes and the solutions to overcome these problems are also discussed.

2. Sources

Fungal sources like *Aspergillus oryzae* and *Aspergillus niger* produce large amount of α-amylase which are extensively used in the industry and as such fungal α-amylases are more suitable due to the more accepted Generally Recognized as Sate (GRAS) status. For the production of dextrose from starch, α-amylase and glucoamylase are used from *Bacillus subtilis* and *Aspergillus niger* respectively. Mesophilic fungus like *Aspergillus awamori*, *Aspergillus oryzae*, *Aspergillus niger*and thermophillic fungus like *Thermomyces lanuginosus*are a good producer of α-amylase. Bacterial strains also producethermostable α-amylase byboth submerged fermentation (SmF) as well as solid state fermentation (SSF) on an industrial scale, although the latter has been found to be more advantageous than the former (Sivaramakrishnan *et al.* 2006). Using SSF, α-amylase is produced from *B.subtilis, B.licheniformis, B. polymyxia, B.mesentericus* and *B.vulgarus.* In harsh

industrial conditions, halophilic microorganisms are used to produce α-amylase at a high salinity. Most halobacterial enzymes are thermotolerant and stable at room temperature for long periods. Halophilic bacteria such as *Chromohalobacter sp.*, *Halobacillussp.*, *Haloarculahispanica*, *Halomonasmeridiana*, *B. dipsosauri* are used for industrial α-amylase production and β-amylase have found mostly in higher plants and in some microorganisms(Adachi *et al.*1998, Mikami *et al.*1999). β-amylase plays an important role during germination and malting of cereal grains in the degradation of starch to fermentable or metabolizable sugars (Okamoto *et al.*1980). It is present in yeasts, molds, bacteria, seeds of many plants and some archaebacteria (Sunna *et al.* 1997). Glucoamylase is produced by different microorganism like *A. niger, A. foetidus, A. candidus*, *B. subtilis and B. amyloliquefaciens*. It also occurs in methanogenicarchaeon *Methanococous jannaschii* (Bult *et al.* 1996). There are several methods to identify α-, β- and glucoamylase enzymes. Physical properties for example, stability in high temperature and extreme pH are used to differentiate these enzymes. An useful method to identify these proteins is through by-product characterization α-amylase releases high proportion of oligosaccharides along with α-D-maltose, β-amylase releases only β-D-maltose and glucoamylase releases single terminal glucose unit. These products can be identified by paper chromatography, HPLC and by optical rotation. These three proteins can also be separated by SDS PAGE based on the average molecular weight. α-amylase is small with average molecular weight of 20-55 KDa while the molecular weight of β-amylase is 53-64 KDa (Ziegler, 1999)and that of glucoamylase is 48 - 112 KDa (Minami *et al.*1999).

3. STRUCTURE

α-amylase is a metalloenzyme which requires calcium (Ca^{+2})ions for their activity, structural integrity and stability. It has a single polypeptide chain ((Nakajima *et al.* 1986)and four highly conserved regions consisting of around 405 amino acid residues. α-amylase consists of three distinct domains. Domain *A* is interconnected with domain *B* by disulphide bond and by a simple polypeptide chain with domain*C*. Domain *A* has a typical barrel-shaped (α/β) 8superstructure of triosephosphateisomerase. Domain *B* is inserted between the third β sheets. Domain *C*-carries a carbohydrate chain while Ca^{+2} ion is between domains *A* and *B*. Mostly β-amylase does not require any co-enzyme metal ion or prosthetic group for its activity (Thoma *et al.*1971; Shinke, 1988). But sometimes it requires Ca^{+2} for its activity (Rani and Jemamoni 2012). It is also composed of a core (α/β)8 barrel domain that has three long loops surrounding the barrel helices (Mikami *et al.* 1993).It was observed that the two side chains of Glu-residues (Glu186 and Glu380) act as an acid and base pair in the catalytic process (Mikami *et al.* 1999). The amino acid sequences how a degree of homology to plant β-amylase. The C-terminal sequence of soybean β-amylase is 30 residues shorter than barely β-amylase and has Gly-rich repeats (Mikami *et al.* 1999). Glucoamylase is composed of two different domains, a catalytic domain of 471 residues and a non-essential starch binding domain of 108 residues (Ford, 1999). The catalytic domain is (α/α) 6 barrel-shaped composed of 13 α helices linked by β turn regions. The enzymes from different species contain the same amount of α helix, but differ in their β sheet amount. The glucoamylase from various sources are different in their aromatic amino acid and cystine content. It must be noted that α-and β-amylases are structurally different. Besides their structural difference, there are some differences in their functions as well. In this regard, the degradation abilities of α- and β-amylase from *Bacillus sp.* were tested on raw starch granules from various sources like potato, sweet potato, wheat, rice and corn. It was observed that the enzymatic pattern of action were different on the starch granules. The β-amylase was found to be less efficient than α-amylase. The latter used both centrifugal and centripetal hydrolysis pattern on wheat, rice and corn granules, but used only centrifugal hydrolysis on potato granules. It was also reported that bacterial β-amylase was 60% more active than soybean β-amylase at the same temperature. β-amylase is more acid stable and less heat stable than α-amylase(Ziegler,1999).

3.1Active site

The widely-accepted mechanism of hydrolysis involves proton transfer to the glycosidic oxygen of the scissile bond formation of an oxo-carbenium ion and a nucleophilic attack of water(McCarter and Withers 1994).The active site of α-amylase contains a trio of acidic groups that do most of the work.Ca^{+2}ion stabilizes the structure of the enzyme and a Cl^- ion is bound underneath the active site in many α-amylase to assist the reaction.Inactivation of β-amylase in the presence of thiol inhibitors (p-chloro-mercuribenzoate and N-ethylmaleimide) proved that the presence of thiol at the active site of the enzyme(Ray and Chakraborty, 1998). Cereal β-amylase contains a highly conserved glutamine residue (Wang *et al.* 1997). In increased thermo-stabilization of β-amylase in the presence of a reducing agent and accelerated thermo-inactivation in the presence of an oxidizing agent (Cu^{+2}), it is proposed that thermo-inactivation of β-amylase probably involve oxidation of thiols.β-amylase cannot directly attack starch granules; they can attackafter degrading solubilizedintermediates released from the granules by α-amylase (Maeda *et al.*1978). β-amylase contains a sulphydryl group which is not a part of active site, but is needed for enzyme activity although sorghum β-amylase do not contain this part (Robbin and Egan, 1992). β-amylase shows potential of glycosylation sites, but change of carbohydrate would not be expected for cytosolic proteins (Ziegler,1999). Tryptophan is observed at the active site of glucoamylase. Replacement of tryptophan residues with photo-oxidation (Jolleyand Gray 1976) or by N-bromosuccinimide (NBS) (Inokuchi *et al.* 1982) resulted in loss of activity. Only one active site presents in glucoamylase to break the glycosidic linkage α-1,4, α-1,6 and α-1,3. Glucoamylase can hydrolyzes maltose (α-1,4 linkage), isomaltose (α-1,6 linkage)andnigerose (α-1,3 linkage) at the same time.

3.2 Subsite binding of amylase

A part of active site of amylase which can interact with glucose molecule is called subsite of amylase. The affinity to glucose, which is measured by the unitary free energy decrease due to interaction is called subsite affinity (A_i), where *i* is the number of subsite counting from the terminal point where non reducing end is situated. The model of amylase subsite is developed by (Robyt and

French, 1970).The subsite is complementary to the substrate monomer unit. Exoenzymes like β-amylase and glucoamylase have single binding mode for each substrate. Therefore subsite mapping is used specifically for exoenzymes. On the other hand, endoacting enzyme such as α-amylase has many binding mode and form a complex product. The subsite binding energy is measured by bond cleavage frequency of the enzyme.

3.3 Mechanism of action

α-amylase degrades starch by retaining mechanism, while β-amylase and glucoamylase uses inverting mechanism for starch hydrolysis. α-amylase uses double displacement mechanism whereas β-amylase and glucoamylase use single displacement mechanism (Fig 1,2). In the case of β- and glucoamylase, two active sites of carboxylic acid are oriented accordingly. One acts as a base to attack water, another acts as an acid to break glycosidiclinkage. By the formation of a covalent glucosyl-enzyme intermediate,α-amylase can act.The action pattern of the carboxylic acid residues is different from inverting mechanism. One of the active site of carboxylic acid residues acts as a nucleophile and attacks at the anomeric carbon atomof the sugar to form glycosyle-enzyme intermediate. Other active site of carboxylic acid residues acts as an acid base catalyst.In the first step, it protonated the glycosidic oxygen and in the second it deprotonated the water. Both reactions are carried on by an oxocarbonium ion (Horvathova *et al.* 2000).

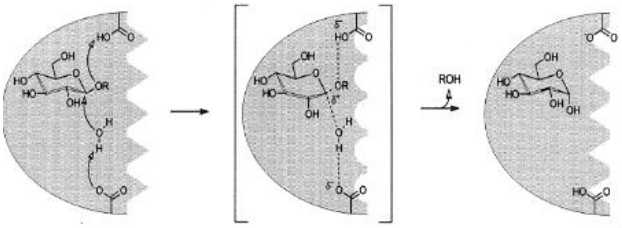

FIGURE 1: Mode of action of β and glucoamylase
Source: Horvathova *et al.*, 2000

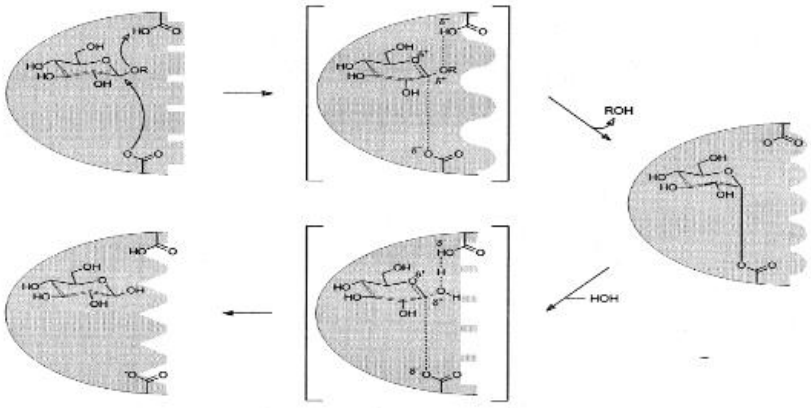

FIGURE 2: Mode of action of α amylase
Source: Horvathova et al., 2000

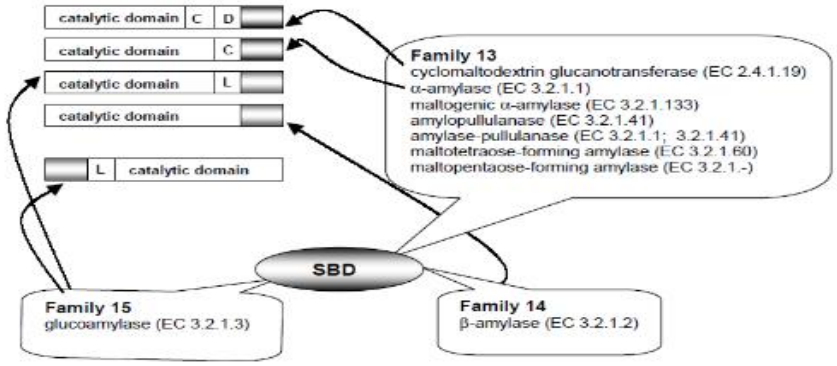

FIGURE 3: starch binding domain of α, β and glucoamylase
Sources: Juge *et al.*, 2002

3.4 Starch Binding Domain

For digesting granular starch, the starch binding domain (SBD) plays a function to the catalytic site of an enzyme (Fig 3). The SBD is present some amylolytic enzymes of glycoside hydrolase family (Svensson *et al*, 1983). It is found in α-amylase (in family 13), β-amylase (in

family14), glucoamylase (in family 15) etc. The SBD always occurs at the C-terminal end of α-amylase.At β-amylase the SBD can be connected directly to the catalytic domain and in glucoamylase it plays as a linker of O-glycosylatedregion (Juge *et al.* 2002).

4. PROPERTIES OF AMYLOLYTIC ENZYMES

Amylolytic enzymes have some properties which are important key role in the time of fermentation**.** Temperature and pH are the physical factors which are greatly influenced on amylolytic enzymes**.**

4.1Role of Phosphate

Phosphate acts as a regulatory component in the synthesis of primary and secondary metabolites in microorganism (Dean, 1972). It affects the growth of the organism and production of α- amylase. It is observed that a 0.2 M phosphate level is the optimum concentration of α-amylase production in *A. oryzae* (Gupta *et al.* 2003). On the other hand high phosphate concentration were inhibitory to enzyme production by *B.amyloliquefaciens* (Zhang *et al.* 1983).

4.2 Role of pH

pH plays an important role by inducing morphological change in the organism and helps enzyme secretion. pH is also responsible for the product stability of the medium. Phosphate buffer have been used traditionally to control pH, but participation of Ca^{+2} ions create calcium phosphate, it is a problem for conservation. Acetate buffer may be used instead of phosphate buffer. The major advantage of acetate buffer is that it does not interact with the solubility of any metallic ions (Fridrich *et al.* 1989). The pH value plays as a indicator of the initiation and end of enzyme synthesis. pH 3.2 – 4.2 in the case of *A.oryzae* DAE1679, 7.0-8.0 in *A.oryzae* EI 212 and 6.8 for *B. amyloliquefaciens* MIR – 41is reported as optimum. *A. niger* glucoamylase was stable at pH 2, where both the *A. candidus* and *Rhizopus* glucoamylase has lost their activity. At pH 7 glucoamylase from *A. niger* changed its conformation.

4.3 Role of temperature

The production of amylases varies greatly with temperature; α-amylase has been produced in a wide range of temperature among bacteria and fungi. Optimum yield of α-amylase were observed at 30-37°C for *A. oryzae* and 36°C for *B. amyloliquefaciens.* Thethermophilic fungi *Thermomonosporafusca* produced α-amylase at 55°C. The hyperthermophile *Thermococcusprofundus* produced amylase at 80°C with increasing temperature, glucoamylase is unfolded.In this unfolded condition, inter or intra molecular crosslinking of disulfide bond is occurred. The disulfide bonds play an important role in structural stability. At very high temperature glucoamylase of *A. candidus* and *Rhizopus* species aggregate.

5. INDUSTRIAL ASPECTS OF AMYLOLYTIC ENZYMES

Amylolytic enzymes are very useful for industrial purposes. Thermostability, metal ion effect, inhibitors, substrates are the major industrial aspects for amylolytic enzyme.

5.1Thermostability

Due to the improvement of the industrial degradation process of starch there has been a great interest in thermo-stable amylolytic enzymes. One of the most widely used thermostable enzymes is the α-amylase in the starch industry (Poonam and Dalel, 1995, Crab and Mitchinson 1997, Sarikaya *et al.*2000). α-amylase should be active at the high temperatures for gelatinization process (100-110ºC) and liquefaction process (80-90º C) for industrial purpose. So there has been needed for more thermophilic and thermostable α-amylase (Sindhu *et al.* 1997). Several *Bacillus* sp. like *B.subtilis*, *B. stearothermophilus* and *B. Amyloliquefaciens* and thermostable *Actinomycetes* including *Thermomonspora* and *Thermoactinomyces* are mostly used strain for the production of the α-amylase. A extremely thermostable α-amylase is available from the mesophile *B. licheniformis* (Morgan and Priest, 1981). Various additives like metal ions, surfactants, exogenous thiols, polyols,bile salt and detergents (SDS) were increased thermostability of β-amylase (Ray and Chakraborty, 1998). Archea were found to be a good source of hyperthermostable enzymes (Woese, 1987). For glucoamylase and β-amylase there have been comparatively less reports on their thermostability (Horvathova *et al.* 2000). One of the most thermostable β-amylase has been isolated from thermophilic bacterium *Clostridium thermosulfurogenes* (Kitamoto *et al.* 1998) with 75ºC as the optimal temperature. Thermophilic fungus *Thermomyceslanuginosus* have been identified for thermostable glucoamylase (Mishra and Maheshwari 1996). A few thermostable wild-type glucoamylase have been identified such as *H. grisea var. Thermoidea*, *A. fumigates* and the gene sequence of thermostable glucoamylase from *clostnidium* sp. (strain *G0005*). The gene sequence of this microorganism is approximately 40% sequence identity with *Aspergillus* (Sauer *et al.* 2000).

5.2 Effect of Metal ion

Many metal ions such as Ca^{+2}, Ba^{+2}, Co^{+2}, Mg^{+2}, Zn^{+2}, Cu^{+2}, Al3+, Fe^{+2} and H^{+2} at 5mM concentration were tested for ragi amylase (Muralikrishna and Nirmala 2005).Ca^{+2} and Ba^{+2} were found activating and stabilizing effectwhere Co^{+2} and Mg^{+2} are having negligible effect on activity. On the other hands, Al^{3+}, Fe^{+2} and Hg^{+2} completely inactivated the α-amylase (Nirmala and Muralikrishna 2003).

5.2.1 Effect of Ca^{+2} ions on α-amylase

Ca^{+2} ions play an important role in α-amylase activity such as:

1. In presence of Ca^{+2}, thermal stability of α-amylase is increased.
2. Ca^{+2} play an important role in the induction and secretion of α-amylase from aleurone or scutellar tissues.
3. Ca^{+2} helps in maintaining the three dimensional structure of this enzyme (Bush *et al.* 1989).
4. $Cacl_2$ (5-7.5mM) is enhanced the thermal stability of α-amylase.
5. It may act as allosteric activator.

Mn^{+2} ion increases the thermostability of β-amylase. Addition of Mn^{+2} (4 mM) bring about a fourfold increase the half-life of enzyme. In the presence of heavy metals (Cu^{+2}, Ag^{+2}, Hg^{+2}) can be partially overcome by Mn^{+2} ion. If a combination of β marcaptoethanol (0.5 mM) and Mn^{+2} (2mM) are added in an inactivated β-amylase, it is

reactivated with an increase of about 43±5 % residual activity. It was reported that in the presence of Mg^{+2} ion and phosphate ion glucoamylase activity was increased. But in the presence of K^{+1}, $Fe^{+2}$and Zn^{+2} glucoamylase activity was decreased (Jambhulkar, 2012). It has been shown that successive addition of Cu^{+2} decreased glucoamylase activity by 59%. By Mn^{+2} and Fe^{+2}, in solid substrate fermentation by *A.niger* glucoamylase activity was stimulated (Selvakumar *et al.* 1996). Supplemented with Zn^{+2} and Ca^{+2} ions in the medium showed high glucoamylase activity in *Rhizopus* Sp.A-11 (Yusaku and Hiroshi, 1996). But in the case of thermophillus fungi *Thermomyces lanuginous* had been shown that Zn^{+2} strongly inhibit enzyme activity whereas Mn^{+2} and Fe^{+2} ions are act as a activator (Quang *et al.* 2002). Glucoamylase produced by *Aspergillus flavus* was activated by Mn^{+2}, Co^{+2}, Ba^{+2} and was inhibited by Hg^{+2}, Fe^{+2}, Zn^{+2} and Cu^{+2}(Oznur and Kubilay, 2010).

5.3 Synthetic substrate

Many substrates can enhance the activity of the enzyme. In the presence of maltose generally induced the action of α-amylase (Tonomura *et al.* 1961). There had been shown when maltose and starch were used as substrate (inducers) enzyme activity increased 20 fold in *A.oryzae* (NRC 401013) (Eratt *et al.* 1984). The carbon sources like lactose, trehalose, α-methyl-D-glucoside also act as inducers of amylase (Yabuki *et al.* 1977). α-amylase production is catabolically repressed by glucose (Morkeberg *et al.* 1995). For α-amylase production many non-conventional sources are used. Lactose (Kelly *et al.* 1977), casitone (Emanuilova and Toda, 1984), fructose (Welker and Campbell, 1963) oilseed cake (Krishnan and Chandra 1982) has been reported for good substrate of α-amylase. The chemically modified artificial oligosaccharide contains more suitable physical and biological properties than their natural forms. A synthetic substrate P-nitrophenylmaltopentaoside (PNPGS) was used with sweet potato for the production of β-amylase. Himaltosin acts as a stabilizer for β-amylase production. α-methyl-D-glycoside (synthetic analogue of maltose) used as a substrate for α-amylase production from *A. fumigates* have also been reported (Goto *et al.*1998). For glucoamylase synthetic substrate p-nitrophenyl-α-D glucoside showed in slight changes in secondary structure without any change in near-UV CD spectrum. If the substrate binds to the tyrosine-tryptophan residues, there are certain changes in the conformation of glucoamylase (Shenoy *et al.* 1985).

5.4 Inhibitors

Abscissic acid inhibits the α-amylase synthesis at the stage of transcription and translation **(**Chrispeels and Varner, 1966**).** β-amylase is inactivated in the presence of some thiol group such as p-chloro-mercuribenzoate.*A. candidus*, *Rhizopus* and *A. niger* glucoamylase are inactivated with denaturants such as urea (8M). Maltose protects β-amylase against inactivation (Ohnishi and Hiromi 1976). The pseudotetradaccharideacarbose and pseudodisaccharide acarviosine binds with higher affinity to Glucoamylase (Sigurskjold *et al.*1994).These are important inactivators of glucoamylase. A transferred NOE NMR experiment indicates that inhibiton is occured in bound conformation which is the crystal structure of the D- gluco-dihydroacrarbose complex (Weimar *et al.* 2000). Aacarbose and 1-deoxynojirimycin are bound to a number of glucoamylase mutants with single amino acid substitutions in catalytic domain have been reported. All of the glucoamylase mutants had almost wild type affinity for 1-deoxynohirimycin (Berland *et al.* 1995). Thioglucoside disaccharide is an inhibitor of Glucoamylase (Andrews *et al.* 1995). Transferred NOE NMR measurements also show that methy 5'-thio-4-N-α maltoside, P-nimophenyl-α-D-glucoryranoside are bound with glucoamylase and also inhibit its action (Mirgorodskaya *et al.* 1999). It is said that a ring sulphur and nitrogen in the interglycosidic linkage is efficient for glucoamylase inhibition (Randell *et al.*1999). The molecular mechanism, synthesis and regulation of the three enzymes are the most important area of research in this inhibition studies. Gibberellic acid was found to enhance the synthesisof mRNA specific for α-amylase.

6. PROTEIN ENGINEERING OF AMYLOLITIC ENZYME ENHANCING INDUSTRIAL APPLICABILITY

Some molecular mechanism is used for the improvement of enzyme for the industrial purpose. The synthesis of a series of 5 – thio – D – glucopyranosylamines by reaction of 5 - thio – D – glucopyranose pentaacetate with the corresponding arylamine and mercuric chloride catalyst is reported (Nikolov *et al.* 1909). The products were obtained as anomeric mixtures of the tetracetates. The tetracetate were deprotected to give α/β mixtures of the parent compounds which were identified as inhibitors of the hydrolysis of maltose by glucoamylase. Kinetic modeling is also useful technique for the improvement of starch hydrolysis (Veille and Zeikus, 2001). Sequence alignments, amino acid content comparisons, crystal structure comparisons and mutagenesis experiments referred that hyperthermophillic enzymes are very similar to their mesophillic homologues (Chen *et al.* 1995). The method i.e. ion pairs, hydrogen bonds, hydrophobic interactions, disulfide bridges, packing decrease of the entropy of unfolding and intersubunit interaction involved in protein thermostabilization. The potential operating temperature of Aspergillus *awamori* glucoamylase has been increased by several mutations that reduce irreversible thermo-inactivation (Table1).

TABLE 1: some site directed mutation to enhanced enzyme activity

Mutation	Effect on free energy of thermoinactivation	Effect on melting temperature	Effect on activity
Asn182→Ala	Decreased thermoinactivation rates 2.5-fold at 60 and 65°C	NR	NR
Gly137→Ala	Increased ΔΔG 0.8 kJ/mol at 65°C	Increased 1.2°C	No reduction in specific activity
Asn20→Cys/ Ala27→Cys	Increased ΔΔG 1.2 kJ/mol at 65°C	Increased 1.4°C	No negative effects on activity
Ser30→Pro	Increased ΔΔG 1.6 kJ/mol at 65°C	Increased 1.2°C	No reduction in specific activity
Gly137→Ala, Ser 30→Pro and Asn20→Cys/ Ala27→Cys	Increased ΔΔG 4.4 kJ/mol at 65°C	Increased 3.9°C	Specific activity 10% higher than wild type (24.5 IU/mg)
Ala246→Cys	NR	Increased 4°C	Roughly half as active as wild type at 45°C, but twice as active as wild type at 66°C
Gln400→Cys	NR	NR	Reduced catalytic rate to 0.2% of wild type – chemical modification to cysteinesulfonic acid increased activity to 160% of wild type
Ser411→Ala	Increased ΔΔG 0.9 kJ/mol at 45°C	NR	Specific activity 32% of wild-type; twofold decrease in the ratio of α-1,6-linked isomaltose to α-1,4-linked maltose formation; increased glucose yield from saccharification of 2%

Source: Ford, 1999

Other mutations have been isolated that increase selectivity of α-1, 4 over α-1, 6 glycosidic bonds, resulting in fewer α-1, 6 linked reversion products thus increasing glucose yield. Some mutations also increase selectivity and yield, suggesting that enzyme flexibility plays a role in accommodating unwanted α-1, 6 bonds in the active site (Chen *et al.* 1994). Glucoamylase I from *Aspergillus awamori var. kawachi* composed of three functional domains: the amino terminal catalytic GA I domain, the threonine and serine rich o-glycosylaed GP-I domain, the carboxy-terminal raw starch-biniding CP domain. In order to investigate the role of GP-I domain, an additional repeat of GP-I and internal deletion of the entire GP-I sequence or parts of the GP-I sequence were introduced within GP-I (Devi *et al.* 2012).These biotechnological approaches are implemented for increased acceptance of starch hydrolyzing enzyme in industrial application.

To improve the potential productivity of amylases, the organism genome may be modified in two ways:

- Classical strain improvement by mutation and selection
- The uses of recombination of amylases are produced by microorganisms using submerged and solid state fermentation (Alia *et al*, 2003).

This productivity is also achieved by immobilization of enzyme (Sadhukhan *et al.* 1993, Bickerstaff 1997, Strumeyer *et al.* 1974, Linko *et al*. 1975). Site directed mutagenesis is a technique that alters the properties of an enzyme based on its structural information. Information from 3D structures, chemical modifications and other studies aids in understanding the functions of various enzyme domains and offers the key amino acids for catalytic activity and to develop stability under strained conditions (Linko *et al.* 1975). Rotational protein engineering and site directed mutagenesis studies on α-amylase have been carried out to tailor glycosyl hydrolases in terms of increasing their thermostability profile (Dumitnui and Popa, 1985), altering both pH activity (Emne'us and Gorton, 1990) and product specificity (Za). α- amylase, being acid stable and Ca^{+2} independent is preferred over the currently used enzymes in starch processing α- amylase plays a active role to prevent the staling of baked goods like bread and also improves texture and shelf-life. In order to improve the volume, texture, flavor, shelf life and reduce the firmness of bread during storage, the bacterial maltogenic and acidic α amylase with intermediate thermostability have been reported to act as antistaling agents functioning by reducing the shortening of amylopectin chain length due to the formation of malto-oligosaccharides. A brewing process for rice grape wine in which rice powder and grapes are concurrently fermented was developed. Rice powder was mixed with starch degrading enzyme (Cong *et al*, 1995, Kurakake *et al*. 1997, Aksoy *et al*. 1998,).

7. LIMITATION OF AMYLOLYTIC ENZYME

There is some limitation of amylolytic enzyme like glucoamylase for their industrial uses.Mutation can partially overcome the performance of enzyme.

7.1 Limitations of glucoamylase

The main limitation is the low catalytic activity of glucoamylase. The first step in starch processing is the liquefaction of α-amylase to produce dextrin. The process takes five minutes at 105ºC or an hour at 95ºC. The dextrin is cooled at 55-60ºC because glucoamylase cannot active at the high temperature like α-amylase. Glucoamylase takes few days to complete hydrolysis of dextrin at this temperature (Ford, 1999). The other limitation is that due to accumulation of disaccharide and trisaccharide, glucoamylase is loss of yield at a high glucose concentration (to about 96% glucose) (Linko *et al.*1975). At high concentration of glucose, the enzyme hydrolyzes dextrin to glucose, and glucose is used as a substrate in a reverse reaction to synthesize α–1,6 linked isomaltose and isomaltotriose.

7.2 Overcoming the limitations

Mutation can enhance the operating performance of glucoamylase. The two main goals in mutagenizing glucoamylase for industrial purposes are:

1. Increasing thermostability allows higher temperatures to be used for dextrin hydrolysis, increasing the rate of enzyme activity.
2. Decreasing α-1,6 activity (synthesis and hydrolysis)while retaining nominal α-1,4 activity to allow the hydrolysis the main bond in dextrin (Ford, 1999)

There is a continuous demand to improve stability in α-amylase. Microbial strains such as *Pyrococcuswoesei* are collected from extreme environment conditions such as hydrothermal vents, salt, soda lakes and brine pools (Dumitnui and Popa, 1985). It has better thermostability than the other enzyme. Genetic engineering has been successfully utilized for screening novel thermostable isoamylase from *Sulfolobus* species and *Rhodothermusmarinus* species. Some amino acid regions are identified in *B. licheniformis* and *B. amyloliquefaciens* which are responsible for thermostability. Researches also identified some site which are responsible for pH stability (8-10.5) improved Ca^{+2} ion stability and increased specific activity at 30-40°C. Replacement of proline with arginine at position 124 of α-amylase in *Bacillus* species increases the stability of the enzyme. It is also observed that introduction of disulfide bonds in the enzyme can also improve stability. Glucoamylase is one of the most industrially important enzymes. It is preferable that glucoamylase should be active at high temperature for saccharification in starch industry. Site directed mutagenesis is applied on the enzyme to get appropriate dimension for industrial purpose, such as glycine being replaced in α-helices (Emne'us and Gorton 1990), the fragile Asp-X bonds is eliminated (Zanin *et al.* 1984) substituted the asparagine in Asn-Gly sequence (Zanin *et al.* 1984).

7.3 Recombinant glucoamylase production

A report (Sauer *et al.* 2000) has indicated that glucoamylase can be produced by genetic engineering. The cDNA of *A. awamoni* which encodes glucoamylase is genetically transferred into the methylotrophic yeast *P. Pastoris*. Glucoamylase from *A. niger*and *A. awamoti* are identical in conformation. The three microorganisms are used as host for recombinant enzyme production. The produced enzyme is essentially identical in their catalytic properties but differs in thermostability.

8. IMMOBILIZATION

The use of enzymes in soluble or free form is considered as a wasteful treatment because they cannot be recovered at the end of the reaction. Immobilization involves separation of enzyme from starch hydrolysis products which decreases labor and overhead costs (Weinheim *et al.* 1994). It increases process yield per unit of enzyme and protects enzymes from their inactivation by various physical and chemical denaturing agents. Besides protection and stabilization, immobilization enhances enzyme properties and their repetitive utilization (Cong *et al.* 1995). Enzymes can be immobilized to a multitude of different methods like entrapment, adsorption, ionic binding and covalent binding. Biocompatibility, easily controlled diffusion properties, higher fermentative growth, non-toxic nature tolerance to harsh environmental conditions and reusability system are various advantages of an immobilization system (Kurakake *et al.* 1997). Entrapment is a common method applied for immobilization which causes little damage to the enzyme.

8.1. Immobilization technique

Some methods have been developed to immobilize α-amylase including entrapment within cross linked polyacrylamide gel, covalent binding to the surface of sepharose gel beads induced by cyanogens bromide (CNBr) and entrapment within calcium alginate beads (Aksoy *et al.* 1998).Among these methods, physical entrapment in calcium alginate beads has been shown to be easy, rapid and safe (Tanyolac *et al.* 1998).But there are a wide varitely of carriers have been proposedfor immobilization of amylase (Sardar and Gupta 1998, Chitra and Baradarajan 1989, Mislouicova *et al*,1998, Nigam and Singh1995, Tischer *et al.*1999, Tischer and Wedekind 1999). Aliginate gel has also been used for purification of amylase from a mixture of proteins encountered in downstream processing. Their results shows α- amylase has an affinity for alginate. The β-amylase from *Vignaradiata* is immobilized onto nitrated and chlorinated woven Bombyxmori silk fabric through covelent coupling with glutararaldehyde (Rani and Jemamoni 2012).Whole cell immobilization method for β-amylaseiseffective in both industrial and academic field. Immobilization of β-amylase by gel entrapment and covalent crosslinking increased thermo-tolerance with 14 fold increased in catalytic half-life. Glucoamylase has been immobilized using different methods. Alginate immobilization is widely used for microorganisms. Glucoamylase and *Saccharomyces cercvisiae* were co-immobilized within alginate matrix. Glucoamylase was covalently linked through cyanuric chloride method to prevent the leakage of the enzyme out of alginate beads. Three different types of glucoamylase binding on cellulose supports were proposed. The biospecific binding is the most successful immobilization technique in industrial purpose.In this process the activity of glucoamylase is not decrease and leakage is minimum during storage and operation.

9. CONCLUSION

The present review deals with α-, β- and glucoamylase which are considered as the most widely distributed enzyme in nature (Table 2). But only a few selected strains of fungi and bacteria fulfill the criteria for commercial production. Research for new organisms for amylase production is a continuous process. These enzymes are closely related in their structural and functional properties, but they do not share common structure and reaction mechanism. Research is focused on improving thermotolerant enzymes from microbes, modifying them genetically or applying site directed mutagenesis to achieve desired properties of the three enzymes. During the last three decades, these enzymes have been used by the vegetables starch processing industry as a replacement of acid hydrolysis. However the future will see a change in the focus of enzyme engineering. The focus of α-,β- and glucoamylase engineering will cover the dimension of stability enhancement.

TABLE 2: Comparative study between α, β, glucoamylase

Properties	α amylase	β amylase	Glucoamylase
1.Pattern of action	Endohydrolase	Exohydrolase	Exohydrolase
2.Release	α- D- maltose	β-maltose	β-D- glucose
3.Avarage molecular weight	20-55Kda	53-64Kda	48-112Kda
4.Composed of core barrel structure	$(\alpha/\beta)_8$	$(\alpha/\beta)_8$	$(\alpha/\beta)_6$
5.Domain	3 Domains,Domains A, Domains- B,Domain- C	Single domains with 3 long loops	2 domains,Catalytic domains, starch binding domains
6.Varying from different Species	Contains same amount of α-helix,but differ in their β-sheet	Differ in their C- terminal sequence	Differ in their aromatic amino acid and cystine content
7.Active site	Contains trio acidic groups	Contains thiol groups	Contains tryptophan
8.Subsite binding	Multiple binding mode	Single binding mode	Single binding mode
9.Starch binding domain	Connected with C- terminal domain	Connected with catalytic domain	Connected with O-glycosylated region
11.Mechanism of action	Retaining Mechanism (Double displacement)	Inverting Mechanism (Single displacement)	Inverting Mechanism (Single displacement)
12.Thermostability	Active at 80-100°C	Comparatively less thermostable	Comparatively less thermostable
13.Common Synthetic substrate	α- methyl- D- glycoside	p- nitrophenylmaltopentaoside	p-nitrophenyl-α-D-glucoside
14.Common Inhibitors	Abscissic acid	p- chloromercuribenzoate	Urea
15. glycoside hydrolyses family	Family 13	Family 14	Family 15

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