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Starch and Microbial α -Amylases: From Concepts to Biotechnological Applications

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1. Introduction

Starch is a polymer of glucose linked to one another through the C1 oxygen, known as the glycosidic bond. Amylases are capable of digesting these glycosidic linkages found in starch. Amylases have been isolated from diversified sources including plants, animals, and microbes, where they play a dominant role in carbohydrate metabolism. In spite of the wide distribution of α -amylase, microbial sources are used for the industrial production. This is due to their advantages such as cost effectiveness, consistency, less time and space required for production as well as ease of process modification and optimization.

In the present day scenario, α -amylases have applications in all the industrial processes such as in food, detergents, textiles and paper industry, for the hydrolysis of starch. They can also be of potential use in the pharmaceutical and fine chemical industries. In this light, microbial α -amylases have completely replaced chemical hydrolysis in the starch processing industry. Despite this, interest in new and improved α -amylase is growing and consequently, the research is intensified as well to meet requirements set by specific applications.

2. Starch

Starch and starch-containing substrates are wide spread in nature and also in industrial praxis. They can predominantly find their application in many industrial processes.

2.1. Sources and utilization

Starch occurs mainly in the seeds, roots and tubers of higher plants. Some algae produce a similar reserve polysaccharide called phytyglycogen. Plants synthesize starch as a result of

photosynthesis. It is synthesized in plastids as a storage compound for respiration during dark periods. It is also synthesized in amyloplasts found in tubers, seeds, and roots as a long-term storage compound. In these latter organelles, large amounts of starch accumulate as water-insoluble granules. The shape and diameter of these granules depend on the botanical origin. Regarding to commercial starch sources, the granule sizes range from 2–30 μm (maize starch) to 5–100 μm (potato starch) (Robyt, 1998). A variety of different enzymes are involved in the synthesis of starch. Sucrose is the starting point of starch synthesis. It is converted into the nucleotide sugar ADP-glucose that forms the actual starter molecule for starch formation. Subsequently, enzymes such as soluble starch synthase and branching enzyme synthesize the amylopectin and amylose molecules (Smith, 1999).

Starch-containing crops form an important constituent of the human diet. Besides the direct use of starch-containing plant parts as a food source, starch is harvested and chemically or enzymatically processed into a variety of different products such as starch hydrolysates, glucose syrups, fructose, starch or maltodextrin derivatives, or cyclodextrins. In spite of the large number of plants able to produce starch, only a few plants are important for industrial starch processing. The major industrial sources are maize, tapioca, potato, and wheat.

2.2. Structure and properties

Starch is a polymer of glucose linked to one another through the C1 oxygen by a glycosidic bond. This glycosidic bond is stable at high pH but hydrolyzes at low pH. At the end of the polymeric chain, a latent aldehyde group is present. This group is known as the reducing end. Two types of glucose polymers are present in starch: (i) amylose and (ii) amylopectin. While amylopectin is soluble in water, amylose and the starch granule itself are insoluble in cold water.

Amylose is a linear polymer consisting of up to 6000 glucose units with α , 1-4 glycosidic bonds (Fig. 1a.). The number of glucose residues, also indicated with the term DP (degree of polymerization), varies with the origin. The relative content of amylose and amylopectin varies with the source of starch. The average amylose content in most common starches, e.g. in barley, corn and potato, is 20-30% (Marc et al., 2002).

Amylopectin consists of short α , 1-4 linked linear chains of 10–60 glucose units and α ,1-6 linked side chains with 15–45 glucose units (Fig. 1b.). The average number of branching points in amylopectin is 5% (Thompson, 2000), but varies with the botanical origin. The complete amylopectin molecule contains about 2 000,000 glucose units, thereby being one of the largest molecules in nature (Marc et al., 2002). The most commonly accepted model of the structure of amylopectin is the cluster model, in which the side chains are ordered in clusters on the longer backbone chains (Bertoft, 2007; Thompson, 2000). In general, Zhu et al. (2011) suggested that the internal part of amylopectin is critical to the physical behavior of granular starch.

The diameter of starch granules ranges from 2 to 100 μm (Whistler & Daniel, 1985) depending on its source. The orientation of the starch chains is thought to be perpendicular

to the granule surface (French, 1984). Native starch is partly crystalline. The crystallinity of native starch varies between 15 and 45% depending on the origin and pretreatment (French, 1984). According to the currently accepted concept, amylopectin forms the crystalline component whereas amylose exists mainly in the amorphous form (Hanashiro et al., 1996; Marc et al., 2002; Zobel, 1992). Structural studies have shown that native starch has crystalline polymorphism. In x-ray diffraction, cereal starch typically gives A-type patterns of monoclinic symmetry, and tuber starch gives B-type patterns of hexagonal symmetry (Gerard et al., 2000; Imberty et al., 1991). The crystal lattice of B-type starch contains more water molecules than the A- structures, which is proposed to be the reason for higher stability of the A- structure. Both structures' molecular conformations are practically identical. They have left-handed double helices with parallel strands. Double helices contain six glucose units per turn in each chain and the glucose units are in a chair conformation. Within the double helix, there are inter-chain but no intra-chain hydrogen bonds. In addition, parallel packed double helices are connected through a hydrogen bonding network.

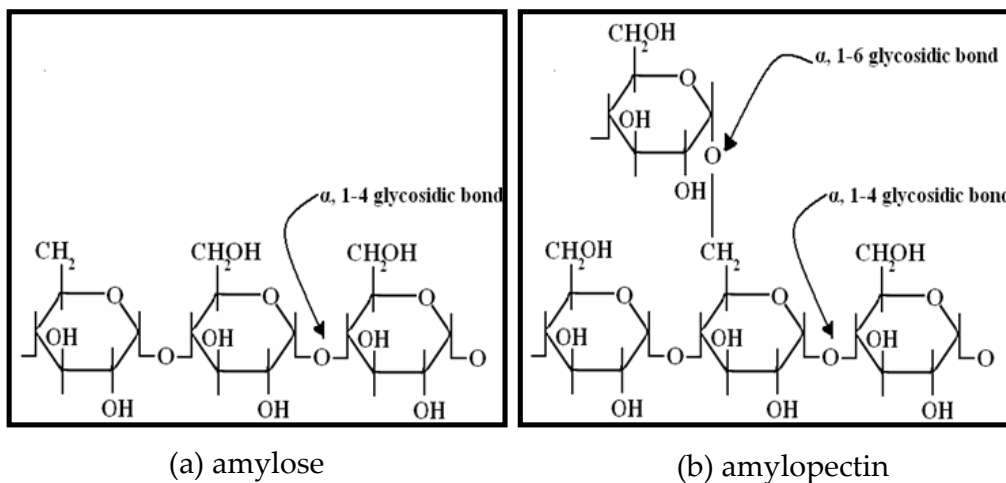


Figure 1. Amylose and amylopectin chain structure

3. Amylases

Amylases are a class of enzymes that are capable of digesting these glycosidic linkages found in starches. Amylases can be derived from a variety of sources. They are present in all living organisms, but the enzymes vary in activity, specificity and requirements from species to species and even from tissue to tissue in the same organism. Raw-starch digesting amylases are produced by a variety of living organisms, ranging from microorganisms including fungi, yeast, and bacteria to plants and humans.

3.1. Microbial sources of amylases

Several amylase-producing bacteria, fungi and other microorganisms have been isolated and characterized over many decades. Bacteria and fungi secrete amylases outside their cells to carry out extra-cellular digestion.

Among mold species producing high levels of amylase, those of *Aspergillus niger*, *Aspergillus oryzae* (Aunstrup, 1979), *Thermomyces lanuginosus* (Arnesen et al., 1998) and *Penicillium expansum* (Doyle et al., 1989) in addition to many species of the genus *Mucor* (Domsch et al., 1995; Petruccioli & Federici, 1992; Zare-Maivan & Shearer, 1988). It was reported that four species of *Ganoderma* mushrooms could produce relatively weak amylase in sawdust medium (Y.W. Wang & Y. Wang, 1990). Amylolytic yeasts differ strongly with regard to amylase secretion and the extent of starch hydrolysis (De Mot et al., 1984a, 1984b). Strains of *Filobasidiium capsuligenum* are capable of extensive starch hydrolysis (De Mot et al., 1984c; McCann & Barnett, 1984).

Regarding to bacteria, *Bacillus* spp and the related genera produce a large variety of extracellular enzymes, of which amylases are of particular significance to the industry e.g., *B. cereus* (Rhodes et al., 1987), *B. circulans* (Siggins, 1987), *B. subtilis* (El-Banna et al., 2007), *B. licheniformis* (El-Banna et al., 2008) and *Clostridium thermosulfurogenes* (Hyun & Zeikus, 1985a). Bacteria belonging mainly to the genus *Bacillus* have been widely used for the commercial production of thermostable α -amylase (Tonkova, 2006). However, most of the *Bacillus* liquefying amylases, such as the enzymes from *B. amyloliquefaciens* and *B. stearothermophilus* have pH optima of between 5 and 7.5 (Yamamoto, 1988). Many alkaline amylases have been found in cultures of *Bacillus* sp. (Hayashi et al., 1988; Kim et al., 1995). This alkaline amylases are all of the saccharifying type, except for the enzymes from *Bacillus* sp. strain 707 (Kimura et al., 1988) and *B. licheniformis* TCRDC-B13 (P. Bajpai and P.K. Bajpai, 1989). Thermostable β -amylases have been isolated from *Bacillus* species (Shinke et al., 1974; Takasaki, 1976). Also, *Lactobacillus plantarum* strain A6 was selected for its ability to synthesize large amounts of extracellular α -amylase (Giraud et al., 1991). Furthermore, a variety of ruminal bacteria exhibit the ability to utilize starch as a growth substrate and are present in the rumen in sufficient numbers to be of quantitative significance in the fermentation of this substrate. These species include *Bacteroides ruminicola*, *Ruminobacter amylophilus*, *Butyrivibrio fibrisolvens*, *Selenomonas ruminantium*, and *Streptococcus bovis* (Russell, 1984).

Genes encoding intracellular α -amylases have been reported for *Escherichia coli* and *Streptococcus bovis* (Satoh et al., 1997; Whitehead & Cotta, 1995). Although there has been some characterization of these activities, no clear physiological role for intracellular α -amylase has been established for either *E. coli* or *Streptococcus bovis*. However, it is postulated that it plays an important role in rapid cell growth in *Streptococcus bovis* (Brooker & McCarthy, 1997).

Many hyperthermophilic microorganisms possess starch-hydrolyzing enzymes in their genomes even though they live in environments where starch is rare (Sambrook et al., 1989). Among the polysaccharide-degrading enzymes of *Thermotoga maritime* described so far are two α -amylases, one is an extracellular putative lipoprotein (AmyA) (Liebl et al., 1997) and one is located in the cytoplasm (AmyB) (Lim et al., 2003). *Geobacillus thermoleovorans* has been found to produce hyperthermostable, high maltose-forming and Ca^{2+} independent α -amylase (Malhotra et al. 2000; Narang & Satyanarayana 2001). Numerous

hyperthermophilic Archaea, especially deep-sea *Thermococcale* and *Sulfolobus* species have been reported to produce α -amylases (Leuschner and Antranikian, 1995; Sunna et al., 1997).

The industrial potential of high-maltose forming α -amylases from *Thermomonospora curvata* (Collins et al., 1993) is limited by their moderate thermostability and Ca^{2+} requirement.

α -Amylases are secreted by several species of *Streptomyces*, for example *S. albus* (Andrews & Ward, 1987), *S. griseus* IMRU3570 (Vigal et al., 1991), *S. thermocyaneoviolaceus* (Hang et al., 1996). Gene encoding extracellular α -amylase has been cloned from many *Streptomyces* species (Bahri & Ward, 1990; Virolle et al., 1988). In addition, α -amylase activity of *Thermoactinomyces* species was first reported by Kuo & Hartman (1966). After that, several α -amylases with different characters were found in other studies (Obi & Odibo, 1984; Omar et al., 2011; Shimizu et al., 1978; Uguru et al., 1997). Within actinomycetes, available reports on β -amylase production are scanty and refer mainly to nonthermostable enzyme (Shinke et al., 1974).

3.2. Amylases types

Enzymes belonging to amylases, endoamylases and exoamylases, are able to hydrolyse starch. These enzymes are classified according to the manner in which the glycosidic bond is attacked. The starch degrading enzymes are found in the numerous glycoside hydrolase (GH) families (13, 14 and 15), mainly in GH family 13 (Coutinho & Henrissat, 1999; Henrissat, 1991).

Endoamylases are able to cleave α ,1-4 glycosidic bonds present in the inner part (endo-) of the amylose or amylopectin chain. α -amylase (EC 3.2.1.1) is a well-known endoamylase. It is found in a wide variety of microorganisms, belonging to the Archaea as well as the Bacteria (Pandey et al., 2000). The end products of α -amylase action are oligosaccharides with varying length with α -configuration and α -limit dextrins, which constitute branched oligosaccharides. α -amylases are often divided into two categories according to the degree of hydrolysis of the substrate (Fukumoto & Okada, 1963). Saccharifying α -amylases hydrolyze 50 to 60% and liquefying α -amylases cleave about 30 to 40% of the glycosidic linkages of starch.

Enzymes belonging to the second group, the exoamylases, either exclusively cleave α ,1-4 glycosidic bonds such as β -amylase (EC 3.2.1.2) or cleave both α ,1-4 and α ,1-6 glycosidic bonds like amyloglucosidase or glucoamylase (EC 3.2.1.3) and α -glucosidase (EC 3.2.1.20). Exoamylases act on the external glucose residues of amylose or amylopectin and thus produce only glucose (glucoamylase and α -glucosidase), or maltose and β -limit dextrin. β -amylase and glucoamylase also convert the anomeric configuration of the liberated maltose from α to β . Glucoamylase and α -glucosidase differ in their substrate preference: α -glucosidase acts best on short maltooligosaccharides and liberates glucose with α -configuration while glucoamylase hydrolyzes long-chain polysaccharides best. β -amylases and glucoamylases have also been found in a large variety of microorganisms (Pandey et al., 2000).

3.3. α -amylases actions and structure

3.3.1. Mode of action

In general, it is believed that α -amylases are endo-acting amylases which hydrolyze α -(1-4) glycosidic bonds of the starch polymers internally. Several models for amylase action pattern have been proposed, such as the random action and the multiple attack action. Random action has also been referred to as a single attack or multi-chain attack action (Azhari & Lotan, 1991). In the former, the polymer molecule is successively hydrolysed completely before dissociation of the enzyme-substrate complex. While, in the latter, only one bond is hydrolysed per effective encounter. The multiple attack action is an intermediate between the single-chain and the multi-chain action (Bijttebier et al., 2008) where the enzyme cleaves several glycosidic bonds successively after the first (random) hydrolytic attack before dissociating from the substrate.

In short, it can clearly be seen that the multiple attack action is generally an accepted concept to explain the differences in action pattern of amylases (Kramhøft et al. 2005; Svensson et al. 2002). However, most of the endoamylases have a low to very low level of multiple attack action (Bijttebier et al., 2008). Although only few reports deal with the influence of pH and temperature on the action pattern of amylases, this influence was confirmed. Bijttebier et al. (2007) showed that the level of multiple attack of several endoamylases increased with temperature to a degree depending on the amylase itself.

3.3.2. Molecular weight

Despite wide difference of microbial α -amylases characters, their molecular weights are usually in the same range 40-70 kDa (Gupta et al., 2003). Ratanakhanokchai et al. (1992) reported the highest molecular weight of α -amylases, 210 kDa, for *Chloroflexus aurantiacus*. Whereas, 10 kDa of *Bacillus caldolyticus* α -amylase was reported to be the lowest value (Gupta et al., 2003).

This molecular weight may be raised due to glycosylation as in the case of *T. vulgaris* α -amylase that reach 140 kDa (Omar et al., 2011). In contrast, proteolysis may lead to decrease in the molecular weight. For example, α -amylase of *T. vulgaris* 94-2A (AmyTV1) is a protein of 53 kDa and smaller peptides of 33 and 18 kDa that have been shown to be products of limited AmyTV1 proteolysis (Hofemeister et al., 1994).

3.3.3. Modular structure

α -amylases from different organisms share about 30% amino acid sequence identity and all belong to the same glycosyl hydrolase family 13 (Henrissat & Bairoch, 1993). The three dimensional (3D) structures of α -amylases have revealed monomeric, calcium-containing enzymes, with a single polypeptide chain folded into three domains (A-C).

The most conserved domain in α -amylase family enzymes, the A-domain, consists of a highly symmetrical fold of eight parallel β -strands arranged in a barrel encircled by eight α -helices.

The highly conserved amino acid residues of the α -amylase family involved in catalysis and substrate binding are located in loops at the C-termini of β -strands in this domain. This is typical to all enzymes belonging to the α/β -barrel protein family (Farber & Petsko, 1990).

α -amylases have a B-domain that protrudes between β -sheet no 3 and α -helix no. 3. It ranges from 44 to 133 amino acid residues and plays a role in substrate or Ca^{2+} binding (Marc et al., 2002). The sequence of this domain varies most; in *Bacillus* α -amylases it is relatively long and folds into a more complex structure of β -strands (Machius et al., 1995), whereas in barley α -amylase there is an irregularly structured domain of 64 residues (Kadziola et al., 1994).

All known α -amylases, with a few exceptions, contain a conserved Ca^{2+} binding site which is located at the interface between domains A and B (Linden et al., 2003; Prakash & Jaiswal, 2010). In addition, α -amylase produced by *Bacillus thermooleovorans* was found to contain a chloride ion binding site in their active site (Malhotra et al., 2000), which has been shown to enhance the catalytic efficiency of the enzyme, presumably by elevating the pKa of the hydrogen-donating residue in the active site (Prakash & Jaiswal, 2010).

α -amylases have a domain C which is relatively conserved and folds into an antiparallel β -barrel. The orientation of domain C relative to domain A varies depending on the type and source of amylase (Bayer et al., 1995). The function of this domain is unknown.

Structural studies have confirmed that the active sites of glycosyl hydrolases are composed of multiple binding sites, or subsites, for the sugar units of polymeric substrates. The open active site cleft is formed between domains A and B, so that residues from domain B participate in substrate binding. The substrate binding sites are commonly lined with aromatic residues which make hydrophobic stacking interactions with the sugar rings. In addition, the active sites contain many residues which form hydrogen bonds to the substrate either directly or via water molecules (Aleshin et al., 1994; Svensson & Sogaard, 1993).

In Taka-amylase A, the first examined protein α -amylase by X-ray crystallography, three acidic residues, i.e., one glutamic and two aspartic acids were found at the centre of the active site (Matsuura et al., 1984), and subsequent mutational studies have shown that these residues are essential for catalysis (Janecek, 1997; Svensson, 1994). The glutamic acid residue is now believed to be the proton donor, while the first of the two conserved aspartic acids appearing in the amino acid sequence of an α -amylase family member is thought to act as the nucleophile. The role of the second aspartic acid is less certain, but it has been suggested to be involved in stabilising the oxocarbenium ion-like transition state and also in maintaining the glutamic acid in the correct state of protonation for activity (Uitdehaag et al., 1999). These residues occur near the ends of strands 3, 4, 5 and 7 of the α/β -barrel and are found in four short sequences, long-recognised as being conserved in α -amylase family enzymes.

3.3.4. Glycosylation

Glycosylation is one of the major post-translation modifications that affect a variety of enzyme functions including secretion, stability, and folding (Barros et al., 2009; Shental-

Bechor & Levy, 2009). Oligosaccharides are usually linked to asparagine side chains (N-linked glycosylation) or to serine and threonine hydroxyl side chains (O-linked glycosylation) (Shental-Bechor & Levy, 2009).

Glycoproteins have been detected in α -amylases of *A. oryzae* (Eriksen et al., 1998), *B. stearothermophilus* (Srivastava, 1984) and *B. subtilis* strains (Matsuzaki et al., 1974; Yamane et al., 1973). Generally, this is about 10 % for most α -amylases (Vihinen & Mantsala, 1989). These carbohydrate moieties are thought to be responsible for high molecular weight of some α -amylases. A carbohydrate content as high as 56 % has been reported in *S. castelii* (Sills et al., 1984). Also, the high molecular weight α -amylase of 140 kDa produced by *T. vulgaris* (Abou Dohara et al., 2011) is a good example of highly glycosylated α -amylase (Omar et al., 2011). Using SDS-PAGE, glycoproteins can be detected by initial oxidation of carbohydrates by periodic acid and subsequent staining with cationic dyes such as alcian blue (Wardi & Michos, 1972).

3.4. Production of microbial α -amylases

The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and easy manipulation of microbes to obtain enzymes of desired characteristics (Lonsane & Ramesh, 1990). Screening for the α -amylase producers is a key step for production. Starch hydrolysis is usually detected directly on plates as clear zones surrounding the colonies. The diameter of the area of hydrolysis, within limits, was always related to the potency of the amylase (Dhawale et al., 1982).

3.4.1. Factors affecting production

The production and stability of α -amylase in the medium is affected by a variety of physicochemical factors. In spite of expression's possibility under a wide range of culturing conditions, α -amylase could be denatured under some conditions. Many proteins easily aggregate into so-called inclusion bodies during expression in bacterial systems (Espargaro et al., 2008). Inhibition of protein aggregation during fermentation/expression can be achieved by adjusting the production conditions (Bahrami et al., 2009; Hao et al., 2007).

Regarding to the incubation period, many investigators have found that extracellular α -amylase production is growth associated (Abou Dohara et al., 2011; Asoodeh et al., 2010; Murthy et al., 2009). The changes in productivity of extracellular enzymes can be attributed to the differences in the timing of induction of separate components of the enzyme system, the inhibition by products of substrate hydrolysis and differential inactivation by proteases and/or variation in the pH during cultivation conditions (Tuohy & Coughlan, 1992; J.P. Wang et al., 1993). The accumulation of sugars over a critical concentration in the medium is well documented to inhibit the enzyme production (Dona et al., 2010; J.P. Wang et al., 2006).

Among the physical parameters, the temperature and pH of the medium play an important role in α -amylase production and stability. Generally, the influence of temperature on amylase production is related to the growth of the organism. Hence, the optimum

temperature depends on whether the culture is mesophilic, thermophilic or psychrophilic. Among the fungi and actinomycetes, most amylase production studies achieved the optimum yields within the range 25°C- 40°C (Gupta et al., 2003). However, thermophilic fungi, such as *Thermomyces lanuginosus* (Mishra & Maheshwari, 1996), and actinomycetes, namely; *Thermomonospora fusca* (Busch & Stutzenberger, 1997) and *Thermoactinomyces vulgaris* (Abou Dohara et al., 2011) have been reported to produce α -amylase optimally at 50 °C, 55 °C and 55 °C, respectively. On the other hand, it has been produced at a wider range of optimal temperature by bacteria reaching to 90 °C in *Thermococcale* and *Sulfolobus* species (Leuschner & Antranikian, 1995; Sunna et al., 1997). Also, the pH values were reported to serve as an indicator of the initiation and end of enzyme synthesis (Friedrich et al., 1989) because the change in pH affects α -amylase stability in the medium (Calamai et al., 2005). It is worth noting that the α -amylase active site consists of a large number of charged groups (Lawson et al., 1994; Strokopytov et al., 1996; Uitdehaag et al., 1999) which explain the fact that most α -amylases had optimum pH in the acidic to neutral range (Bozic et al., 2011; Pandey et al., 2000; Sun et al., 2010).

In general, amylase activity is connected with the substrate utilization. The inducibility nature of α -amylase has been assured in different microorganisms (Abou Dohara et al., 2011; Aiyer, 2004; Asodeh et al., 2010; Ryan et al., 2006). α -amylase production is also appeared to be subjected to catabolite repression by maltose and glucose, like most other inducible enzymes that are affected by substrate hydrolytic products (Bhella & Altosaar, 1988; Morkeberg et al., 1995). However, α -amylase synthesis by *Bacillus* strains was reported to not subject to catabolite repression by monosaccharides (Kalishwaralal et al., 2010). Gupta et al. (2003) have classified xylose and fructose as strongly repressive to α -amylase synthesis. Addition of starch to the medium has normally been employed for the production of α -amylase from various microorganisms as reported in the literature.

Nitrogen source as a basal component of the medium is a major factor affecting α -amylase production. Its effect was not only as a nitrogen source but also as a metal ion source and a pH controller as well. Many investigators had recorded that organic nitrogen sources supported maximum α -amylase production by various bacteria (Abou Dohara et al., 2011; Aqeel & Umar, 2010; Mrudula & Kokila, 2010; Saxena et al., 2007). The increased α -amylase production by organic nitrogen sources could be attributed to the high nutritional amino acids and vitamins content. However, various inorganic salts have been reported to support better production in fungi (Gupta et al., 2003). As a metal ion source, ammonium chloride was found to enhance the production of the α -amylase by *T. vulgaris*, where chloride is a stabilizer, over that of other ammonium salts (Abou Dohara et al., 2011). In addition, the same authors also reported different productivity of α -amylase by using sodium nitrate from potassium nitrate.

3.4.2. Activity measurement of enzyme

The diversity and heterogeneity of natural substrates coupled with the mixed specificities of individual enzymes presents a problem in the characterization of amylases. Furthermore,

the enzymatic degradation of native insoluble substrates involves steps and mechanisms which are not yet understood at the molecular level. Therefore biochemical studies always use starch in some modified form to simplify analyses. There are basically four different types of substrates used for activity measurements: purified insoluble substrates approximated to a native substrate, modified insoluble substrates, soluble modified polysaccharides and soluble oligosaccharides. Catalytic activity is usually measured by quantifying formed soluble saccharides or chromophoric aglycon. The action of enzyme on insoluble substrates can also be assayed by other means. For example, a viscosimetric method has been used to measure α -amylase activity on starch pastes (Marciniak & Kula, 1982).

The measurement of soluble products from insoluble or soluble polymeric substrates often means assaying the formed reducing sugars. One of the simplest and most widely used is the 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959). However, the colour development in the reaction is not strictly proportional to the number of reducing sugars present, but also to the length of the oligosaccharides, leading to higher apparent reducing values with longer sugars (Robyt & Whelan, 1972). DNS itself also breaks down the substrate. Several other reducing sugar determination methods have also been developed. In some cases dye groups have been attached to the polymeric substrate, e.g. dyed amylose and amylopectin (Klein et al., 1970) and dyed and cross-linked starch (Cesk et al., 1969). The enzymatic assay is based on colour released from the substrate.

Starch forms a deep blue complex with iodine and with progressive hydrolysis of the starch, it changes to red brown. Several procedures have been described for the quantitative determination of amylase based on the reduction in blue colour intensity resulting from enzyme hydrolysis of starch (Swain et al., 2006). This method determines the dextrinising activity of α -amylase in terms of decrease in the iodine colour reaction. Also, the coupled assay methods have been used for amylases, in which the concentration of released glucose is determined either by glucose oxidase/peroxidase (Kunst et al., 1984) or by hexokinase/glucose-6-phosphate dehydrogenase method (Rauscher, 1984).

Generally, various available methods for the determination of α -amylase activity are based on decrease in starch-iodine colour intensity, increase in reducing sugars, degradation of colour-complexed substrate and decrease in viscosity of the starch suspension.

3.4.3. Purification of enzyme

Purification is a key step in the enzymes production where residual cell proteins and other contaminants are removed. Different techniques have been developed for purification of enzymes based on their properties, prior to their characterization or use in biotechnological and industrial processes. The commercial use of α -amylase generally does not require purification of the enzyme but enzyme applications in food industries, pharmaceutical and clinical sectors require high purity amylases. The enzyme in purified form is also a prerequisite in studies of structure-function relationships and biochemical properties.

The used methods to purify amylases can vary considerably, but most purification protocols involve a series of steps (Sun et al., 2010). The choice of purification protocol naturally depends on the intended use, the highest purity usually being required for basic purposes in which even separation of isozymes may be important. The purity and the yield attained depend on the number of steps and separation techniques employed.

The purification of α -amylases from microbial sources in most cases has involved classical purification methods. These methods involve separation of the culture from the fermentation broth, selective concentration by precipitation using ammonium sulphate or organic solvents. The crude enzyme is then subjected to chromatography. The most commonly used techniques are usually affinity chromatography, ion exchange, and/or gel filtration. Cross-linked starch or starch derivatives are useful affinity adsorbents for the isolation of bacterial α -amylases (Somers et al., 1995). Primarini & Ohta (2000) isolated and separated two pure α -amylases from *Streptomyces* sp. using starch adsorption, α -CD Sepharose 6B and DEAE-Toyopearl 650M. Adsorption of α -amylase, from *Streptomyces* sp. E-2248; on starch followed by separation on DEAE-Toyopearl and Toyopearl-HW55S gave the highest purification (2130-fold) (Kaneko et al., 2005). Also, α -amylase from *Bacillus licheniformis* has been purified 6-fold with a yield of 38% using by two gel filtration chromatography steps on Sephadex G-100 and Superose 12 column (Bozic et al., 2011).

In addition to the classical chromatographic techniques, immunoaffinity chromatography has been applied for the preparation of highly purified amylases (Jang et al., 1994). Recent advances in the understanding of the physical and functional properties of amylases, and of the selectivity and capacity of the adsorbents, have led to greater rationality in the design of separation methods. However, the potential of the methods for the separation of amylases has not been fully exploited.

3.4.4. Industrial desirable aspects

The stability of biocatalysts is often a limiting factor in the selection of enzymes for industrial applications due to the elevated temperature or extreme pH of many biotechnological processes. Therefore, there is a continuing demand to improve the stability of the enzymes and thus meet the requirements set by specific applications.

As an example, the problem with traditional detergent enzymes is that they have to function in a washing machine under conditions that are very unfavorable for the stability of the enzyme. The pH is highly alkaline in washing conditions. The high temperature (55–60°C) in a dishwasher requires thermostable enzymes. In addition, it is preferred to be resistant to various detergent ingredients, such as surfactants, chelating and oxidative agents (bleach).

In general, temperature has a complex effect on protein either directly or indirectly for both physical and chemical induced aggregation processes (Y.W. Wang et al., 2010). Therefore, it is the most critical environmental factor for consideration when proteins are handled during the entire development and commercialization processes. The advantages for using thermostable α -amylases in industrial processes include the decreased risk of

contamination, the increased diffusion rate and the decreased cost of external cooling. In short, almost all industries need thermostable enzymes. Besides thermostability and other factors such as activity with high concentrations of starch, i.e. more than 30% dry solids, or the protein yields of the industrial fermentation are important criteria for commercialization (Schäfer et al., 2000). Also, α -amylases with wide pH range is desired to satisfy all applications either acidic as glucose syrup production or alkali as detergents industries.

However, there is a recent trend to use intermediate temperature stable (ITS) α -amylases (Ahuja et al., 1998, as cited in Gupta et al., 2003). Olesen (1991) found that this feature render the enzyme to be useful for baking industry through avoiding stickiness in bread. Also, a modern trend among consumers is to use colder temperatures for doing the laundry or dishwashing. At these lower temperatures, detergents with α -amylases optimally working at moderate temperatures and alkaline pH would be favourable (Marc et al., 2002). Although a wide variety of microbial α -amylases is known, α -amylase with 'ITS' property has been reported from only a few microorganisms (Gigras et al., 2002).

Another important desirable feature is calcium independency. Most known α -amylases, with a few exceptions, contain a conserved Ca^{2+} binding site (Linden et al., 2003; Prakash & Jaiswal, 2010) which make calcium be important to the enzyme activity. In manufacture of fructose syrup, the Ca^{2+} ions inhibit the glucose isomerase enzyme used in the final step of the process (Tonkova, 2006) and may lead to the formation of inorganic precipitates which have deleterious effects on fermentation and downstream processing (Kelly et al., 2009). Because the removal of these metal ions is both cost and time consuming to the overall industrial process (Kelly et al., 2009), the use of stable and functional α -amylases in the absence of Ca^{2+} ions at high temperatures would be highly favored.

4. Biodegradation of starch

The degradation of starch occurs mainly through the action of microorganisms in plant litter and soil. Since the native substrate is water-insoluble and cannot penetrate into cells, the biodegradation of starch occurs extracellularly. Amylases are mainly secreted into the medium or are found membrane-bound. Some microbial strains are known to produce intracellular amylases; the reason for this is unknown (Vihinen & Mantsala, 1989).

4.1. Enzymatic degradation of starch

The effective hydrolysis of starch demands the action of many enzymes due to its complexity, although a prolonged incubation with one particular enzyme can lead to (almost) complete hydrolysis. Few microorganisms produce a complete set of enzymes capable of degrading starch efficiently. There are basically four groups of starch-converting enzymes: (i) endoamylases; (ii) exoamylases; (iii) debranching enzymes; and (iv) transferases.

Endoamylases are able to cleave α ,1-4 glycosidic bonds present in the inner part (endo-) of the amylose or amylopectin chain. Exoamylases act on the external glucose residues of

amylose or amylopectin and thus produce only glucose (glucoamylase and α -glucosidase), or maltose and β -limit dextrin (β -amylase).

The third group of starch-converting enzymes is the debranching enzymes that exclusively hydrolyze α ,1-6 glycosidic bonds: isoamylase (EC 3.2.1.68) and pullulanase type I (EC 3.2.1.41). These enzymes exclusively degrade amylopectin, thus leaving long linear polysaccharides. There are also a number of pullulanase type enzymes that hydrolyze both α , 1-4 and α ,1-6 glycosidic bonds. These belong to the group II pullulanase and are referred to as α -amylase–pullulanase or amylopullulanase. The main degradation products are maltose and maltotriose.

The fourth group of starch-converting enzymes are transferases that cleave an α ,1-4 glycosidic bond of the donor molecule and transfer part of the donor to a glycosidic acceptor with the formation of a new glycosidic bond. Enzymes such as amyломaltase (EC 2.4.1.25) and cyclodextrin glycosyltransferase (EC 2.4.1.19) form a new α , 1-4 glycosidic bond while branching enzyme (EC 2.4.1.18) forms a new α ,1-6 glycosidic bond. Cyclodextrin glycosyltransferases have a very low hydrolytic activity and make cyclic oligosaccharides with 6, 7, or 8 glucose residues and highly branched high molecular weight dextrans, the cyclodextrin glycosyltransferase limit dextrans. Amyломaltases are very similar to cyclodextrin glycosyltransferases with respect to the type of enzymatic reaction. The major difference is that amyломaltase performs a transglycosylation reaction resulting in a linear product while cyclodextrin glycosyltransferase gives a cyclic product.

Depending on the relative location of the bond under attack as counted from the end of the chain, the products of this digestive process are dextrin, maltotriose, maltose, and glucose, etc. Dextrans are shorter, broken starch segments that form as the result of the random hydrolysis of internal glucosidic bonds. A molecule of maltotriose is formed if the third bond from the end of a starch molecule is cleaved; a molecule of maltose is formed if the point of attack is the second bond; a molecule of glucose results if the bond being cleaved is the terminal one; and so on.

Most of the enzymes that convert starch belong to one family based on the amino acid sequence homology: the α -amylase family or family 13 glycosyl hydrolases according to the classification of Henrissat (1991). Other little enzymes that convert starch don't belong to family 13 glycosyl hydrolases like β -amylases that belong to family 14 glycosyl hydrolases (Henrissat & Bairoch, 1993); and glucoamylases which belong to family 15 glycosyl hydrolases (Aleshin et al., 1992).

4.2. Catalytic mechanism and substrate binding

The α -glycosidic bond is very stable having a spontaneous rate of hydrolysis of approximately $2 \times 10^{-15} \text{ s}^{-1}$ at room temperature (Wolfenden et al., 1998). Members of the α -amylase family enhance this rate so enormously that they can be considered to belong to the most efficient enzymes known.

The α -amylase family enzymes always carry strictly conserved three essential catalytic residues (Matsuura et al., 1984). Of these three residues, the roles of Glu230 and Asp206 have been generally accepted as working for acid (proton donor) and base (nucleophile) catalyst, respectively (Janecek, 1997; Svensson, 1994). The catalytic mechanism has been discussed mostly on the basis of these two residues. However, the critical role of the third residue Asp297 seems to be still undefined and under dispute, except the facts that it plays an important role in the distortion of the substrate (Uitdehaag et al., 1999).

The generally accepted catalytic mechanism of the α -amylase family is that of the α -retaining double displacement (Koshland, 1953). The mechanism involves two catalytic residues in the active site; a glutamic acid as acid/base catalyst and an aspartate as the nucleophile. It involves five steps: (i) after the substrate has bound in the active site, the glutamic acid in the acid form donates a proton to the glycosidic bond oxygen, i.e. the oxygen between two glucose molecules at the subsites -1 and +1 and the nucleophilic aspartate attacks the C1 of glucose at subsite -1; (ii) an oxocarbenium ion-like transition state is formed followed by the formation of a covalent intermediate; (iii) the protonated glucose molecule at subsite +1 leaves the active site while a water molecule or a new glucose molecule moves into the active site and attacks the covalent bond between the glucose molecule at subsite -1 and the aspartate; (iv) an oxocarbenium ion-like transition state is formed again; (v) the base catalyst glutamate accepts a hydrogen from an incoming water or the newly entered glucose molecule at subsite +1, the oxygen of the incoming water or the newly entered glucose molecule at subsite +1 replaces the oxocarbenium bond between the glucose molecule at subsite -1 and the aspartate forming a new hydroxyl group at the C1 position of the glucose at subsite -1 (hydrolysis) or a new glycosidic bond between the glucose at subsite -1 and +1 (transglycosylation). Studies with cyclodextrin glycosyltransferase have shown that the intermediate indeed has a covalently linked bond with the enzyme (Uitdehaag et al., 1999).

Other conserved amino acid residues e.g. histidine, arginine, and tyrosine play a role in positioning the substrate into the correct orientation into the active site, proper orientation of the nucleophile, transition state stabilization, and polarization of the electronic structure of the substrate (Lawson et al., 1994; Strokopytov et al., 1996; Uitdehaag et al., 1999).

5. Biotechnological application

Nowadays, α -amylases represent one of the most important enzyme groups within the field of biotechnology. These enzymes are present in numerous biotechnological and industrial applications such as in food, detergents and textiles as well as in paper industry, for the hydrolysis of starch. They can also be of potential use in the pharmaceutical and fine chemical industries.

5.1. Industrial production of glucose and fructose from starch

The acid hydrolysis method for glucose production has been replaced recently by enzymatic treatment, with three or four different enzymes, in which α -amylase is the first (Crabb & Shetty, 1999).

For the complete conversion into high glucose syrup, the first step is the liquefaction into soluble, short-chain dextrans. Dry solids starch slurry (30–35%) of pH 6 is mixed with α -amylase and passed through a jet cooker after which the temperature is kept at 95–105 °C for 90 min to assure the removal of lipid–starch complexes. The dextrose equivalent value of starch hydrolysate syrup depends on the time of incubation and the amount of added enzyme. The drawback of the currently used α -amylases is that they are not active at a pH below 5.9 at the high used temperatures. Therefore, the pH has to be adjusted from the natural pH 4.5 of the starch slurry to pH 6. Also Ca^{2+} needs to be added because of the Ca^{2+} -dependency of these enzymes (Tonkova, 2006). The next step is the saccharification of the starch hydrolysate syrup to high concentration glucose syrup, with more than 95% glucose. This is done by using an exo-acting glucoamylase. The final step is the conversion of high glucose syrup into high fructose syrup by using glucose isomerase (Bhosale et al., 1996).

5.2. Bakery and anti-staling

The baking industry is a large consumer of starch and starch-modifying enzymes. Amylases can be added to degrade the damaged starch in the flour into smaller dextrans, which are subsequently fermented by the yeast. Upon storage, all undesirable changes together are called staling. Retrogradation of the starch fraction in bread is considered very important in staling (Kulp & Ponte, 1981). Staling is of considerable economic importance for the baking industry since it limits the shelf life of baked products. Several additives may be used in bread baking (Spendler & Jørgensen, 1997) to delay staling and improve texture, volume and flavor of bakery products. Enzymes active on starch have been suggested to act as anti-staling agents, especially α -amylases (Sahlstrom & Brathen, 1997).

α -amylase supplementation in flour not only enhances the rate of fermentation and reduces the viscosity of dough resulting in improvements in the volume and texture of the product (De Stefanis & Turner, 1981) but also it generates additional sugar in the dough, which improves the taste, crust colour and toasting qualities of the bread (Van Dam & Hille, 1992). A recent trend is to use intermediate temperature stable (ITS) α -amylases (Ahuja et al., 1998, as cited in Gupta et al., 2003) since they become inactive much before the completion of the baking process which avoid sickliness in bread.

5.3. Cyclodextrin formation

Cyclodextrins are cyclic α ,1-4 linked oligosaccharides mainly consisting of 6, 7, or 8 glucose residues. The glucose residues in the rings are arranged in such a manner that the inside is hydrophobic while the outside is hydrophilic. This enables cyclodextrins to form inclusion complexes with a variety of hydrophobic guest molecules. The formation of inclusion complexes leads to changes in the chemical and physical properties of the guest molecules. These altered characteristics of the encapsulated compounds have led to various applications of cyclodextrins in analytical chemistry, agriculture, pharmacy, food and cosmetics. For the industrial production of cyclodextrins, starch is first liquefied by a heat-

stable α -amylase and then the cyclization occurs with a cyclodextrin glycosyltransferase (Riisgaard, 1990).

5.4. Detergent industries

A growing area of application of α -amylases is in the fields of laundry, dish-washing detergents and spot removers (Borchet et al., 1995; Kennedy et al., 1988). Amylases have the function of facilitating the removal of starchy stains by means of catalytic hydrolysis of the starch polysaccharide, and have been used for this purpose for a fairly long time in dishwashing detergents and textile laundering (Speckmann et al., 2001).

Traditional detergent enzymes are functional under alkaline conditions, thermostable and resistant to various detergent ingredients, such as surfactants, chelating and oxidative agents. On the other hand, a modern trend among consumers is to use colder temperatures for doing the laundry or dishwashing. At these lower temperatures, the removal of starch from cloth and porcelain becomes more problematic. Detergents with α -amylases optimally working at moderate temperatures and alkaline pH can help solve this problem (Marc et al., 2002).

5.5. Ethanol production

For large-scale processing, the bioconversion of starchy materials to ethanol is very useful because it can be used as a biofuel and as the starting material for various chemicals. The production of ethanol from starchy biomass commonly involves three-step processes: liquefaction of starch by an endoamylase such as α -amylase to reduce the viscosity of the gelatinized starch produced after the cooking of the grains, enzymatic saccharification of the low-molecular-weight liquefaction products to produce glucose, and fermentation of glucose.

However, the present process for ethanol production from starchy materials via fermentation requires improvement of cost production. Although noncooking and low-temperature-cooking fermentation systems (Matsumoto et al., 1982) have succeeded in reducing energy consumption by approximately 50% (Matsumoto et al., 1982), it is still necessary to add large amounts of amylolytic enzymes to hydrolyze the starchy materials. Many researchers have reported attempts to resolve this problem by using recombinant glucoamylase-expressing yeasts with the ability to ferment starch to ethanol directly (Kondo et al., 2002). Also, a noncooking fermentation system using a cell surface-engineered yeast strain promises to be very effective in reducing the production costs of ethanol (Shigechi et al., 2004). On the other hand, fermentation of starch to ethanol in one step using co-cultures of two different strains has been suggested and has potential application for the direct bioconversion of starch into ethanol (Zeikus, 1979).

5.6. Miscellaneous applications

Besides amylases' use in the saccharification or liquefaction of starch, these are also used for the clarification of formed haze in fruit juices, the pretreatment of animal feed to improve

the digestibility (Marc et al., 2002). α -amylase is used for the production of low viscosity, high molecular weight starch for coating of paper (Bruinenberg et al., 2004). Starch is a good sizing agent for the finishing of paper. It is added to the paper in the size press and paper picks up the starch by passing through two rollers that transfer the starch slurry. The temperature of this process lies in the range of 45–60 °C. A constant viscosity of the starch is required for reproducible results at this stage. The mill also has the flexibility of varying the starch viscosity for different paper grades. The viscosity of the natural starch is too high for paper sizing and is adjusted by partially degrading the polymer with α -amylases in a batch or continuous processes. Also, good desizing of starch sized textiles is achieved by the application of α -amylases, which selectively remove the size and do not attack the fibers. It also randomly cleaves the starch into dextrans that are water soluble and can be removed by washing.

Furthermore, high molecular weights amylases were found in culture supernatants of an environmentally derived microbial mixed culture selected for its ability to utilize starch-containing plastic films as sole carbon sources (Burgess-Cassler et al., 1991). This suggests a new application for amylases in biodegradation. With the advent of new frontiers in biotechnology, the spectrum of amylase applications has expanded into many other fields, such as clinical, medicinal and analytical chemistry (Becks et al., 1995).

A modern trend is to use starch for production of a more efficient and specific degradation products through a particular combination of activities. Amylase from *Aspergillus niger*, a saccharifying enzyme which produces maltose, maltotriose and some glucose, is capable of alcoholysis for the synthesis of methyl-glucosides from starch in the presence of methanol. As these products are a series of methyloligosaccharides, from methyl-glucoside to methyl-hexamaltoside, the biotechnological applications of using starch as substrate for the production of alkyl-glucosides is analyzed (Santamaria et al., 1999).

Moreover, it becomes possible to produce lactic acid directly from starch by an efficient simultaneous saccharification and fermentation from soluble starch by recombinant *Lactobacillus* strains (Okano et al., 2009). Finally, α -amylase is suggested as an enzyme that contributes to the reduction of AuCl_4^- to gold nanoparticles (Au-NPs) which makes it ideal for the production of Au-NPs (Kalishwaralal et al., 2010).

6. Conclusion

Despite the fact that several different α -amylase preparations are available with various enzyme manufacturers for specific use in varied industries, amylase biotechnology demands extension in terms of both quality and quantity. Qualitative improvements in amylase gene and its protein can be achieved by recombinant DNA technology and protein engineering. Quantitative enhancement needs strain improvement through site directed mutagenesis and/or standardizing the nutrient medium for the overproduction of active α -amylases. Another approach is to screen for novel microbial strains from extreme environments.

All the above-mentioned approaches are aimed to increase stability, improve product specificity, alter pH optimum, improve thermostability, achieve free Ca⁺² requirement; by using the currently available insights into the structure–function relationships of the amylase family enzymes.

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