MICROBIAL AMYLASES

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INTRODUCTION

Amylases of microbial origin are the basis for many commercial applications of hydrolytic enzymes. Large quantities of these enzymes are used in textile industry for the desizing of fabrics, in the paper industry for the preparation of starch slurries for coating paper, and in the food industry for the preparation of syrups for sweetening agents (Pazur, 1965).

These enzymes are usually extracellular, inducible and remain in the culture fluids after removal of the microorganisms (Alexander, 1977 & 1981). But the ability of microorganisms to form amylolytic enzymes depends on the type of starch.

The optimum pH and the isoelectric points of these enzymes are also quite similar. The amino acid composition and its values for some of the microbial α-amylase are recorded in Table 1. Significant differences are apparent in amino acid composition among the amylases from the different organisms. Investigations thus far reported on the determination of N-terminal and carboxyl-terminal amino acids indicate that a bacterial amylase is composed of two polypeptide chains presumably held together by disulfide bonds, whereas a fungal amylase may consist of a branched structure (Pazur, 1965 and Kulp, 1975).

2. Microorganisms as a source of amylases and certain properties of these enzymes

Amylases are commonly secreted by microorganisms. These enzymes have been isolated and purified (Ammar *et al* 1988; Shah *et al.*, 1991; Ramesh & Lonsane, 1991; Selim *et al.*, 1992; El-Saady, 1993, and Rabie *et al.*, 1995).

Bacteria and actinomycetes have the capacity to hydrolyze starch. Among the

Table. 1. Amino acid composition of some amylases*.

Amino acid	*A. oryzae* alpha-amylase	*B. subtilis* alpha-amylase	*B. stearothermophilus* alpha-amylase	Human salivary amylase	Surine pancreatic amylase	*A. niger* gluco-amylase
Aspartic acid	15.91	14.49	9.30	19.3	14.4	12.7
Threonine	8.38	5.59	6.20	4.5	3.9	11.5
Serine	6.04	5.21	4.2	7.8	4.1	11.8
Glutamic acid	8.09	12.94	20.36	9.6	10.5	10.6
Proline	4.22	3.37	16.27	3.6	3.6	3.7
Glycine	5.68	6.01	4.24	6.8	6.7	4.7
Alanine	5.92	5.29	4.77	4.4	6.9	7.6
Valine	6.03	6.14	8.53	6.9	7.8	6.1
Methionine	2.14	1.47	2.66	2.4	2.1	0.6
Isoleucine	6.34	4.55	6.05	5.8	11.5	4.7
Leucine	7.64	6.16	7.22	5.8	---	7.5
Tyrosine	10.88	9.05	2.96	5.5	5.3	5.3
Phenylalanine	3.99	6.01	6.40	7.2	10.1	4.3
Histidine	1.82	3.90	4.33	3.2	3.9	0.9
Lysine	4.77	7.42	5.22	6.3	4.9	2.8
Arginine	2.97	6.09	3.36	8.7	5.8	4.1
Tryptophan	3.78	6.22	---	7.2	6.7	---
Cystine/2	2.09	---	3.42	4.4	2.3	0.9

* Grams of amino acid per 100 g. of protein (Pazur, 1965).

bacteria are gram positive and gram negative, spore formers and nonspore formers, aerobes and obligate anaerobes. In addition to many physiologically different groups (Alexander, 1977 & 1981 and El-Saady, 1993). Amylases are produced by various bacteria including *Bacillus, Lactobacillus, Clostridium, Micrococcus, Pseudomonas* and *Streptomyces.* Actinomycetes also produced amylases which are used for industrial purposes (Shimizu *et al.*, 1978; Nakamura & Crowell, 1979; Hidaka & Adachi, 1980; Kassim, 1983; Srivastava & Mathur, 1983; Priest & Thirunavukkarasu, 1985; Meki *et al.*, 1988; Yoo *et al.*, 1988; Bajpal & Bajpal, 1989; Ariga *et al.*, El-Hawary & El-Refai, 1991; Mizukami *et al.*, 1992; Haroun *et al.*, 1993a & b; Swamy *et al.*, 1994; Ray *et al.*, 1995; Abdel-Aty, 1996; Rabie *et al.*, 1995; and Rabie, 1997).

Many filamentous fungi are also capable of excreting amylase, e.g., *Aspergillus niger* (Mahmoud *et al.*, 1975; Tang *et al.*, 1977; El-Fadly & El-Hawary, 1988; and Mohamed, 1996), Aspergillus foetidus (Michelena and Castillo, 1984), and *Aspergillus* oryzae (El-Sahy, 1990; Selim *et al.*, 1992; and Terebiznik et al., 1996). Amylolytic yeasts are potentially valuable in the utilization of starchy substances and these are known to produce at least two types of amylases α-and glucoamylase (Glymph & Stutzenberger, 1977; Moulin & Galzy, 1979; Clementi *et al.*, 1980; Estrela *et al.*, 1982; Wilson & Ingledew, 1982; Simoes-Mendes, 1984; DeMot *et al.*, 1984 & 1985; DeMot & Verachtert, 1986; and Gogoi *et al.*, 1987). Amylolytic enzymes are used in the conversion of starch into oligosaccharides.

pH and temperature optimal for amylases are summarized in Table 2. Amylases are the diversity in their properties, possibly due to their different sources. These characters illustrate the great potential of microbial enzymes in industry. From the diversity between these enzymes, the enzyme which is the most appropriate for each industrial use can be selected and screened without much difficulty from a wide spectrum of microorganisms.

3. Starch As Substrate

Starch is the major source of carbohydrate in human diet, but apart from it being an important food in its own right, it can be readily hydrolysed to produce syrups or solids containing dextrose, maltose and other oligosaccharides. Starch occurs in various sites in the plants: in seeds as in cereal grains, in the root and tuber as in tapioca and potato, and (more rarely) in the stem pith as in the sago plant. The counterpart of starch in the animal kingdom is glycogen, a compound starch princi-

Table. 2. Optimum pH and temperature of certain microbial amylases.

α-amylase character Enzyme source	Temp. °C	pH	Reference
Bacillus subtilis	65	---	Taha *et al.*, (1970)
Bacillus subtilis	60	5.0 - 8.0	Rabie *et al.*, (1995)
Bacillus subtilis	50	7.0	Hauka, 1997
Bacillus polymyxa	45	7.5	Murao *et al.*, (1979)
Bacillus coagulans	70	7.5 - 8.5	Medda & Chandra, (1980)
Bacillus stearothermophilus	55 - 70	7.0 - 9.0	Morgan & Priest, 1981
Bacillus stearothermophilus	---	4.5 - 6.5	Selim *et al.*, (1985)
Bacillus licheniformis	70 -90	7.0	Morgan & Priest, 1981
Lactobacillus acidophilus	40	6.4	Champ *et al.*, (1983)
Lactobacillus bulgaricus	70	5 and 8	Rabie, 1997
Thermomonospira curvata	65	5.0 - 7.0	Glymph & Stutzenberger, 1977
Aspergillus oryzae	40 - 70	6.0	Selim *et al.*, (1992)
Soccharomycopsis fibuligero	60	4.8 - 6.0	Gogoi *et al.*, (1987)

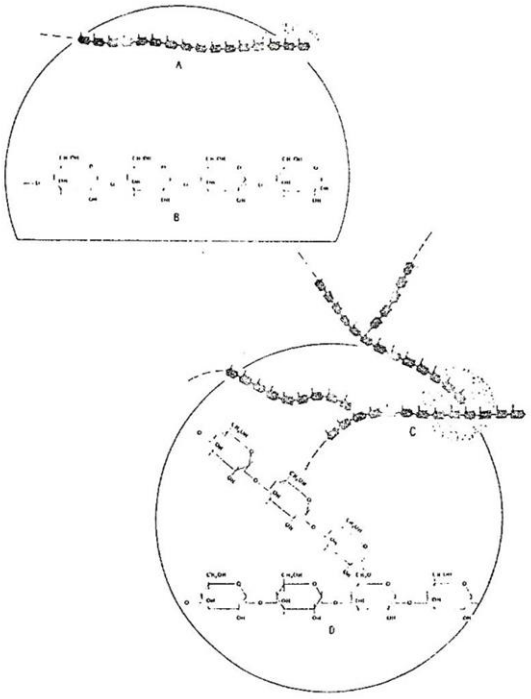

Fig. 1. Structure of the amylose and amylopectin components of starch. A: diagram a portion of an amylose molecule; B: enlarged view of the shaded section showing chemical formula; C: diagram of a portion of an amylopectin molecule; D: enlarged view of shaded area showing chemical formula (Pazur, 1965).

pally present in the liver and resembling amylopectin in many respects (Pazur, 1965; Kulp, 1975; Alexander, 1977 & 1981; Norman, 1981; and El-Saady, 1993).

Starch granules contain two polymers of glucose, amylose and amylopectin, which are evently distributed throughout the granules and are most probably associated with other by hydrogen bonds. A representation of these structural features of the amylase and amylopectin molecules are shown in Fig. 1. Starches commonly contain 70 to 90 percent amylopectin and 10 to 30 percent amylose, but exceptions are not uncommon. The enzymatic susceptibility of the granules also depends on the type and condition of the granules.

Amylose is a flexible, linear chain molecule of 500 or more glucose units (Fig. 2). The glucose residues are joined by α-1, 4-glucosidic linkages. There has been some doubt about the complete linearity of this polymer, since it cannot be completely converted by the action of β-amylase to maltose. The complex of amylose and iodine, which produce a blue color, is the basis for the quantitative determination of amylose.

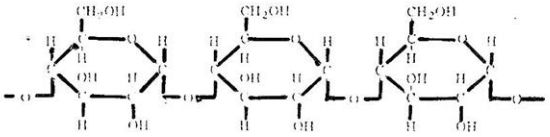

Fig. 2. Amylose struture (Alexander, 1977)

Amylopectin is a glucose polymer having a highly branched, treelike configuration composed of linear chains similar to those of amylose, but connected at the branch points by α-1, 6-likages. These branch points are belived to occur at intervals of about 20-30 glucose units, and some evidence indicates that there may also be some branching at C (2) and C (3). In contrast to amylose, amlopectin does not form complexes; a red color is produced with iodine (Radley, 1968; Greenwood, 1970; Kulp, 1975; Norman, 1981; and El-Saady, 1993).

4. The Mode of Action of Amylases

Amylases act on the starch components in an essentially random (endo) manner, (α-amylase) or an exo-attack mechanism (β-amylase and Amyloglucosidase). Among these are the amylases, which act on starch, glycogen and derived polysaccharides hydrolyze the α-1, 4-glycosidic linkages. Amylases may be divided or classified into three main groups, as follows: (Norman, 1981).

1- Endo-amylases:

1-a Thermostable:
B. licheniformis amylase.
B. amyloliquefaciens amylase.
1-b Thermostable:
Fungal-α-amylase (A.oryzae)
2- Exo-amylases:
2-a Glucogenic:
Glucoamylase (A.niger)
2-b Maltogenic:
β-amylases (cereal, microbial)
3- Debranching enzymes:
3-a Direct:
Isoamylase, Pullulanase
3-b Indirect:
Amylo-1, 6-glucosidase

Endoamylases (α-amylases) split the bonds in the interior of the substrate. The β-amylases hydrolyze units from the non reducing end of the substrate (exoamylases). The glucoamylases, which split off glucose units from the non reducing terminal of the substrate molecules. Diagramatically, the action of α-, β – amylases and other enzymes may be visualized as shown in Fig. 3 (Kulp, 1975; Alexander, 1977; Norman, 1981; and El-Saady, 1993).

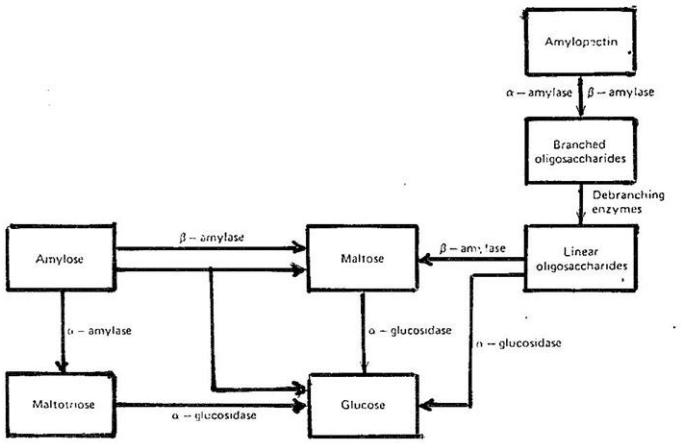

Fig. 3: Enzymatic conversion of starch to glucose initiated by α- and β–amylases (Alexander, 1977).

The activity of amylases are usually determined by measurements of the degradation of the substrate. The activity is estimated by measuring the reduction in iodine-staining ability of the substrate or by the decrease in the viscosity of the substrate. In many cases the activity is expressed in arbitrary units or measured by the colorimitric determination of reducing sugars (Fischer & Stein, 1961; Ogasahara *et al.*, 1970; Hernandez and Pirt, 1975; Gogoi *et al.*, 1987; Kichakova, 1991; Selim *et al.*, 1992; El-Saady, 1993; and Rabie *et al.*, 1995).

4.a. Endo-amylases or α-amylases:

Endo-amylases are generally α-amylases α-1, 4-D-glucanohydrolase, EC. 3.2.1.1, endo-amylase), which affect a rapid fragmentation of the whole starch molecule by hydrolysis or cleaving 1, 4-α-glucosidic bonds in amylase, amylopectin and related polysaccharides. The products of hydrolysis, which are oligosaccharides of varying chain length, have the α-configuration at the C1 of the reducing glucose unit (Robyt & Whelan, 1968; Norman, 1981; and Fogarty, 1983).

As the name suggests, endo-amylases hydrolyze the bonds located in the inner regions of the substrate, and therefore, causing a rapid decrease in the viscosty of starch solutions and a decrease in the iodino-staining power of amylose. These enzymes are also able to by-pass 1, 6-α-branch points in amylopectin (Robyt & Whelan, 1968; Norman, 1981; and El-Saady, 1993).

Recently, Marked differences were found in the mode of action of various α-amylases, i.e., *Bacillus subtilis* produces two types of α-amylases, saccharifying and liquefying amylase when acting on starch. α-amylases. were divided into two groups according to the chain length of saccharide, with a new reducing end formed by the first attack: 1) Liquefying *Bacillus subtilis, Bacillus stearothermophilus* and malt α-amylases which hydrolyze by maltohexaasyl or maltopentaasyl units, and 2) Saccharifying bacillus subtilis and Endomycopsic, several fungal and animal α-amylases which attack by maltotrisoyl units (Norman, 1981).

The action of α-amylase on amylose fraction of starch proceeds in two stages. Initially, a complete, rapid degradation of amylose into maltose and maltotriose takes place. This step is a random attack on the substrate by the enzyme and is accompained by a rapid loss of viscossity and or the iodine-staining power of the amylose. The second stage is a slow hydrolysis of the oligosaccharides, with the formation of glucose and maltose as final products (Kulp, 1975; Fogarty, 1983; and El-Saady, 1993).

The α-amylolysis of amylopectin yields glucose, maltose and a series of α-limit dextrins, oligosaccharides of four or more glucose residues, all containing α-1, 6-glucosidic bonds. The hydrolysis of these products are slowly, effecting a break-down of certain linkages in the regions of the branch points of the molecules (Kulp, 1975 and Fogarty, 1983).

Norman, (1979 & 1981) divided the endo-amylases of industrial importance into two groups, thermotable α-amylases which are used mainly for high temperature liquefaction and thermolabile α-amylases which are used for saccharification. α-Amylase produced by *Bacillus licheniformis* is a more robust enzyme than that produced by *Bacillus amyloliqefaciens*. It can tolerate higher operating temperature, it is less dependent of calcium ions for stability and it is active over a wider pH range.

4.b. Exo-Amylases:

Exo-Amylases (β-amylases and glucoamylase) will hydrolyze 1, 4-α-glucosidic bonds in amylose, amylopectin and related polysaccharides. The glucogenic exo-amylases are able to hydrolyse 1, 6-α-glucosidic bonds in isomaltose, panose of branched oligosaccharides, though at a slower rate. On the other hand, maltogenic exo-amylases such as cereal β-amylases are not able to by-pass 1,6-α-aglucosidic branch points (Banks & Green Wood, 1975 and Norman, 1981). Action of β-amylases on amylopection results in 50-60% conversion to maltose and formation of a β-limit dextrin which is the parent polymer with the outer chains trimmed down close to the outer most branch points (Robyt and Whelan, 1968; Murao *et al.*, 1979; and Norman, 1981).

Exo-amylases catalyse the hydroysis of α-glucosidic bonds by successively removing low-molecular-weight products such as glucose or maltose from the non-reducing chain-end in a step wise manner. But, high-molecular weight limit dextrin remains. In contrast of the action of endo-amylases, this results in a slow decrease in the viscosity and iodine-staining power of starch solutions. The products of hydrolysis generally have the β-configuration at the C1 of the reducing glucose unit (Robyt & Whelan, 1968).

4.b.1. β-Amylases:

β–Amylases (α–1-4-glucan maltohydrolase, EC 3.2.1.2) were also called saccharogenic amylases. The action pattern of these enzymes were found. β–Amylase of

Bacillus megaterium was studied by Ray *et al.*, (1994 & 1995). They observed that the enzyme acted on substares from the non-reducing end in an exo fashion and released maltose. *Bacillus cereus* var mycoides β–Amylases produced maltose alone from amylose, amylopectin, starch and glycogen. The maltose produced had the β–configuration and α–1, 6-linkages in pullulan and β–limit dextrin were not hydrolysed by the systems (Takasaki and Yamanobe, 1981).

4.b.2. Amyloglucosidase:

Other names employed for enzymes of this type include amyloglucosidase, glucoamylase and gamma-amylase. Glucoamylase (α–1, 4-glucohydrolase, EC 3.2.1.3) is an exo-splitting enzyme that removes the glucose units consecutively from the non reducing ends of the starch polymers. The end product of the reaction is glucose, which clearly differentiates this enzyme from the α and β–amylase (Kulp,. 1975 and Alexander, 1977). It also hydrolyza α–1,6 and α–1,3 linkages althouhg at a much slower rate than α–1,4 linkages (Fleming, 1968 and Fogarty, 1983).

Amyloglucosidases are classified into two groups (Fleming, 1968), One yielding total hydrolysis of starch and β–limit dextrins, and the second yielding 80% hydrolysis of starch and 40% conversion of β–limit dextrins to glucose. Panose and limit dextrins are fully hydrolysed by both groups (Fleming, 1968 and Fogarty, 1983).

Amyloglucosidases occur almost exclusively in fungi, e.g., *Aspergillus oryzae* (El-Sahy, 1990) and *Aspergillus niger* (Mohamed, 1996). The enzyme from *Cephalosporium charticola* hydrolysed starch at five times the rate, amylose three times and maltotriose twice, as rapidly as maltose. The two forms of amyloglucosidase from *Penicillium oxalicum* hydrolysed amylose and maltotriose five times and three times faster than maltose. The enzyme from *Aspergillus niger* had relative affinities for starch, maltotriose and maltose of 100, 68 and 31, respectively (Fogarty, 1983).

4.c. Debranching Enzmes:

Debranching enzymes hydrolyze 1,6-α branch points in amylopectin, glycogen and certain branched maltodextrins and oligosaccharides. Two classes of debranching enzymes exist (Lee & Whelan, 1971; Norman, 1981; and Fogarty, 1983), namely "indirect" and "direct" debranching enzymes. With indirect enzymes (Amylo-1,6-glucosidase-EC 3.2.1.33) the substrate has first to be modified by the action of oth-

er enzymes (phosphorylase and transferease) before they can act. Thus, these are not of industrial significane (Fogarty, 1983).

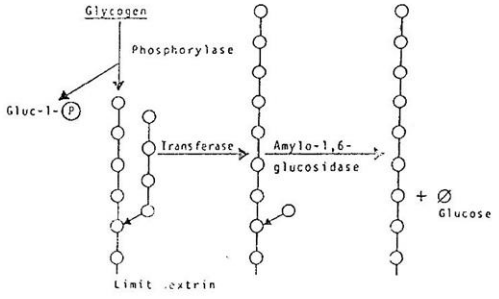

Fig. 4. Action of amylo-1,6-glucosidase. (Norman, 1981).

The direct enzymes can act on unmodified glycogen and amylopectin (Norman, 1981), and release side chains of varying length. These enzymes may be classified into two groups (Pullulanases and Isoamylases) on the basis of substrate specificity. Pullulanses are a1, 6-α glucosidase which degrade pullulan (poly-1,6 maltotriose). Whereas isoamylase do not (Lee & Whelan, 1971). The structure of pullulan, which is produced by the fungus *Aureobasidium pullulans*, is shown in Fig. (5). It is a linear polymer consisting essentially of maltotrisoyl units connected by 1,6-bonds (Norman, 1981). These enzymes are produced by a number of microorganisms but only the pullulanases produced by *Aerobacter aerogenes, Klebsiella pneumoniae, Bacillus cereus* var. mycoides, *Pseudomonas stuttzeri*. Enzymes of *Bacillus macerans* and *Bacillus polymyxa* are of inductrial importance (El-Saady, 1993).

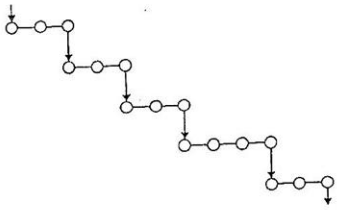

Fig.5. Generalised structure of pullulan. (Norman, 1981).

On the other hand, glucoamylase are able to hydrolyse 1,6-α-glucosidic links, but the reaction proceeds relatively slowly. In these cases the use of a debranching enzymes would have obvious advantages.

4.c.1. Pullulanase-EC 3.2.1.41

Bacterial and plant pullulanases attack the 1, 6-α links in a random endomanner, releasing maltotriose and a series of branched maltotriosyl oligosaccharides and maltotriose Fig. (6).

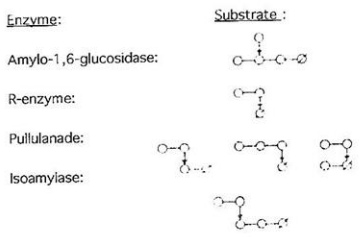

Fig.6. Minimum substrates for debranching enzymes (Norman, 1981).

Other pullulan-hydrolysing enzymes which specifically attack the 1, 4 α-glucosidic linkages have been described. Isopullulanases (Pullulan-4-glucanohydrolase) EC 3.2.1.54, isolated from *Aspergillus niger* and *Arthrobacter globiformis,* hydrolyse the 1,4-α-glucosidic links adjacent to the 1,6 μ-glucosidic links in a random endo-manner to produce isopanose and a series of isopanose-containing oligosaccharides initially and isopanose finally (Norman, 1981).

An α-amylase isolated from *Thermoactinomyces vulgaris* has also been shown to hydrolyse pullulan (Shimizu *et al.*, 1978). this enzyme attacks the other 1,4-α-glucosidic bond in a similar manner, producing panose.

From the pH-activity curve in Fig. (7), it can be seen that the optimum pH for pullulanase activity is 5.5-6.0 under the conditions of the analysis, below pH 5 the activity drops dramatically. The data were obtained by incubating a 2% pullulan solution in a 0.08 M acetate buffer at the appropriate pH for 30 min. at 50°C. The enzyme is stable between pH 5.5-7.5.

The effect of temperature on enzyme activity is shown in Fig. (8). The optimum temperature for activity under the conditions of the analysis is 50-55°C.

Above 55°C the activity declines sharply. The data were obtained by incubating, at various temperature, a 2% pullulan solution in 0.1 M acetate buffer at pH 5 (Norman, 1981).

Calcium ions have been shown to have an activating effect on pullulanase, but inhibition is observed at levels above 10^{-2} M. No other metal ions appear to have this dual effect, copper, iron, aluminium and mercury being strongly inhibitory (Norman, 1981).

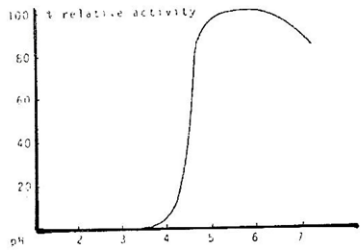

Fig.7. The influence of pH on *K. pneumoniae* pullulanase activity at 50°C. Substrate: 2% pullulan, 0.08 M acetate buffer. Reaction time: 30 Min. (Norman, 1981).

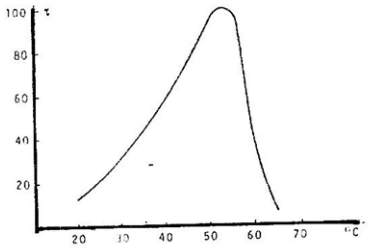

Fig.8. The influence of temperature on *K. pneumoniae* pullulanase activity. Substrate: 2% pullulan, pH 5, 0.1 M acetate buffer. Reaction time: 30 min. (Norman, 1981).

4.c.2. Isoamylase-Ec 3.2.1.68

Isoamylase is a debranching enzyme that hydrolyses 1,6-α-linkages in amylopectin, glycogen and certain branched maltodextrins and oligosaccharides, unlike pullulanase it has a high activity twowards amylopectin and gylcogen (Which are

completely debranched) and has a very low activity towards pullulan (Yokobayashi *et al.*, 1970). A comparison of the action *K. pneumoniae* pullulanase and *Ps. amyloderamosa* isoamylase on various substrates is given in Table (3).

Table 3. Action of pullulanase and isoamylases on various substrates (Norman, 1981).

Substrate	Relative rate of hydrolysis	
	Isoamylase	Pullulanase
Pullulan	v. low	100
Amylopectin	100	15
Glycogen (oyster)	124	1
6^3-O-α-Maltosyl-maltotriose	2.8	22
6^3-O-α-Maltotriosyl-maltotriose	9.7	162
6^3-O-α-Maltotriosyl-maltotetraose	33	146

The effect of pH on enzyme activity is shown in Fig. (10). The pH optimum of isoamylase is 4.0-4.5 at 50°C. The data were obtained by incubating a 1% waxy maize starch substrate in 0.07 M acetate buffer at the appropriate pH for 30 minutes. The enzyme is stable at 40°C within the pH range 3.0-3.5 (Yokobayashi *et al.*, 1970) and this contrasts markedly with the pH-activitystability properties of *K. pneumoniae* pullulanase, which is unstable and inactive below pH 5.

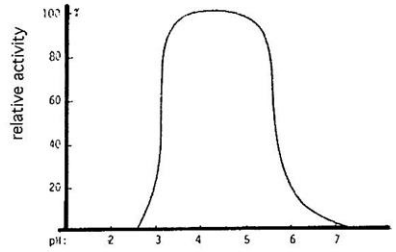

Fig.9. The influence of pH on *Ps. amyloderamosa* isoamylase activity at 50°C. Substrate: 1% waxy-maize starch, 0.07 M acetate buffer. Reaction time: 30 min. (Yorkobayashi *et al.*, 1970)

The effect of temperature on enzyme activity is shown in Fig. (11). A 1% waxy maize starch substrate in 0.17 M acetate buffer at pH 4 was used. The optimum temperature for activity is about 52.5°C under the conditions of the analysis. The enzyme is therefore similar to *K. pneumoniae* pullulanase in that it is healt-labile (Yokobayashi *et al.*, 1970).

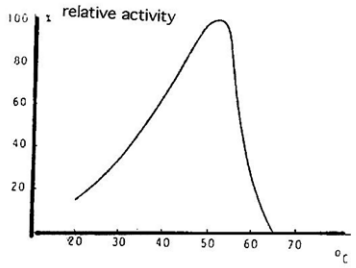

Fig.10. The influence of temperature on *Ps. amyloderamosa* activity. Substrate: 1% waxy-maize starch, pH 4, 0.17 M acetate buffer. Reaction time: 30 min. (Yorkobayashi *et al.*, 1970).

5. Thermostable Endo-Amylases

5.1. Thermostable endo-amylases application.

The most important application of thermostable endo-amylases in the starch-processing industry is in the so-called liquification process. Liquification is the term used to describe the dispersion of starch molecules in aqueous solution, followed by partial hydrolysis. If an aqueous starch suspension is heated to above 60°C, the starch granules swell and eventually disrupt, dispersing the starch molecules into solution. The adhering protein separates and coagulates during this process. The temperature required for complete dispersion or gelatinization depends on the source of starch, but 105-110°C is sufficient for most starches (Norman, 1981).

The traditional thinning agent used in the starch industry was acid. In the so-called acid-liquefication process. The pH is adjusted to about 2 with a strong acid. Hydrochloric acid is normally used. Oxalic acid is also used. However, the non-specific catalytic action of the acid can result in the formation of undesirable by-products such as 5-hydroxymethyl-2-furfuraldehyde and anhydro-glucose compounds (Birch & Shallenberger, 1973). In addition, the colour and ash contents are high, with the result that purification costs become significant.

If a thermostable endo-amylase is used as a catalyst, the processing conditions are milder, by-product formation is not a problem and refining costs are lower.

Until 1973, the only thermostable endo-amylases which were commercially avaiable were derived from *Bacillus amyloliquefaciens* (*B. subtilis* var. *amyloliquefaciens*). But now, a more heat-stable α-amylase is isolated form *Bacillus licheniformis* (Fig. 11) was introduced commercially (Madsen *et al.*, 1973). The optimum tem-

perature of *B. licheniformis* amylase is 92°C, whereas for the *B. amyloliquefaciens*, it is only 70°C. This has a considerable effect on enzyme stability and enables the *B. licheniformis* amylase to be used at temperatures up to 110°C for short reaction periods, while, ***B. amyloliquefaciens*** is only suitable for 85-90°C.

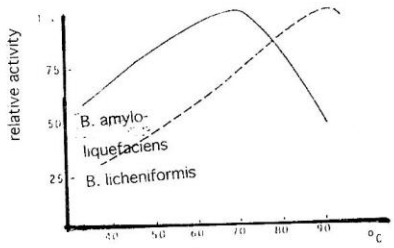

Fig.11. The influence of temperature on α-amylase activity. Substrate: 0.46% soluble starch, 0.05 M phosphate buffer, pH 5.7, 4.3 mM calcium. Reaction time: 7-20 minutes (Madsen *et al.*, 1973).

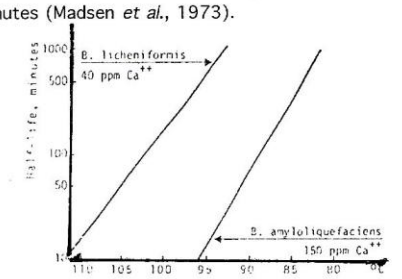

Fig.12. Effect of temperature on half-life in minutes for the two enzymes under typical industrial conditions (Madsen *et al.*, 1973).

The effect of pH on enzyme activity at 60°C is illustrated in Fig. (14). It is clearly seen that the *B. licheniformis* amylase is active over a much wide pH range under the conditions of anaysis.

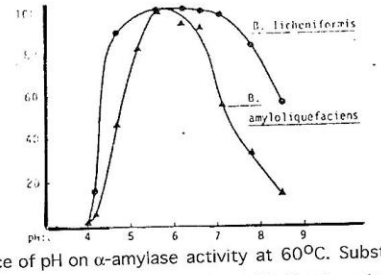

Fig. 13. The influence of pH on α-amylase activity at 60°C. Substrate: 0.46% soluble starch, 0.05 M acetate buffer (pH 4.0-5.6), 0.05 M tris-maleate buffer (pH 6.0-8.5), 4.3 mM calcium. Reaction time: 3-10 minutes (Norman, 1981).

The effect of calcium ions on enzyme stability at 70°C in the absence of substrate is illustrated in Fig (14). An addition of 3.4 ppm calcium stabilised the *B. licheniformis* amylase completely under these conditions. The enzyme preparation itself contributed approximately 1 ppm calcium, therefore, 5 ppm is sufficient for stabiliazation at 70°C.

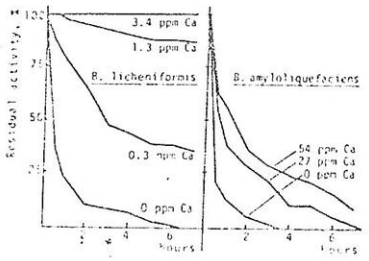

Fig. 14. The influence of added calcium on α-amylase stability in the absence of substrate at 70°C, pH 6.5-7 (Norman, 1981).

5.2. Action on starch:

Thermostable α-amylases are used for liquefication and the reaction is normally terminated before significant starch hydrolysis has taken place. The *B. licheniformis* amylase produces mainly maltose, maltotriose and maltopentaose. The maltohexaose formed initially is almost completely hydrolysed. The *B. amylosliquefaciens* amylase produces mainly maltohexaose and, unlike the *B. licheniformis* amylases, it does not appear to be able to hydrolyse this further.

Two liquefication processes based on the use of *B. licheniformis* amylase are shown in Fig. (15) and the layout of the pilot plant in which the process has been evaluated is shown in Fig. (16).

The process is as follows: A starch slurry containing 30-40% dry solids is prepared in the feed tank. The pH is adjusted to 6.0-6.5 with sodium hydroxide, and calcium salts may be added if the level of free calcium ions is below about 50 ppm. In a small-scale, the liquifying enzyme well be added to the feed tank, but in a large-scale industrial process the enzyme would be metered directly into the steam emerging from the feed tank (Norman, 1981).

The slurry is then pumped continuously through a jet-cooker where the temperature is raised to 105°C by the direct injection of live stem. The viscosity-

reducing action of the enzyme takes place and then some mechanical thinning also takes. Peak viscosities are therefore avoided.

The slurry is maintained at this high temperature in the pressurised holding cells for a period of minutes, after which it is flash-cooled to atmospheric pressure and pumped through a multi-stage reaction vessel where enzyme action is allowed to continue for about 2hours at 95°C. After this treatment the liquefied starch will have a dextrose equivalent (DE) of 8-12, depending on the amount of enzyme used.

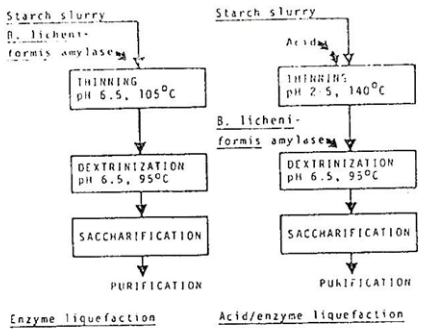

Fig. 15. Starch liquefaction processes. (Norman, 1981).

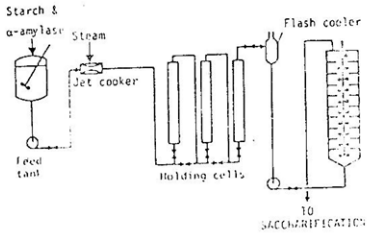

Fig.16. Diagram of starch liquefaction pilot-plant. (Norman, 1981).

The most important advantage of this process, a part from its simplicity, is that the energy consumption is relatively low, because the maximum operating temperature is only 105°C compared with 140-150°C normally used. However, careful control of temperature is required. If it is too low the starch will not be completely gelatinised and filtration problems will occur. If the temperature runs too high, a significant amount of enzyme activity will be destroyed.

6. Mechanism of Starch Hydrolysis

The hydrolysis of starch and oligosaccharides by enzymes involves the addition of the elements of water to a D-glucosidic bond. In the enzymic reactions, the catalysis occurs on the surface of the enzyme. Initially, some of the functional group at the active site of the enzyme molecule must interact with the functional groups of the substrate molecules to form the enzyme-substrate complex. Some possible arrangments of the functional group of the substrate and enzyme in the complex are shown in Fig. (17). In the complexes, the D-glucosidic-bond oxygen has been protonated by hydrogen ions from amino or imidazole groups of the enzyme and the electron deficient center at C-1 of the bond attracts from donor groups such as hydroxyl groups. The resulting strained structure is cleaved on the C-1 carbon side of the bond forming a carbonium ion intermediate and a neutral D-glucosyl fragment. The final step involves the addition of a hydroxyl ion (or a water molecule) to the carbonium ion intermediate. With some enzyme, the last step occurs with inversion of configuration and with others by retention of the original configuration at C-1. (Pazur, 1965).

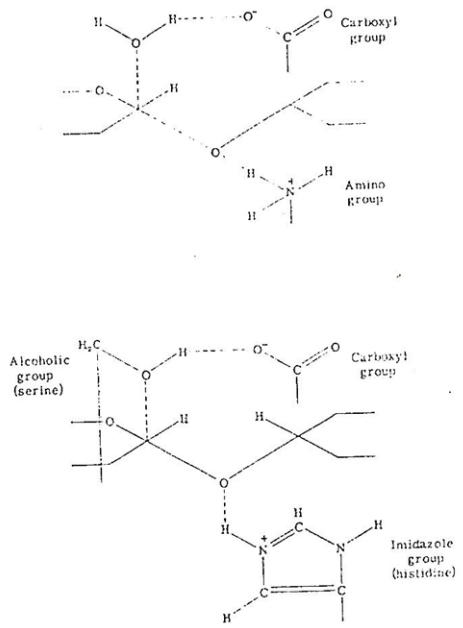

Fig. 17. Possible arrangements of functional groups at the active center of the enzyme and a D-glucosidic bond of starch (Pazur, 1965).

The alpha-amylases of microbial origin have been obtained in crystalline form from bacterial sources, *Bacillus polymyxa* (Robyt and French, 1964), Bacillus *subtilis* and *Bacillus Stearothermophilus* (Manning & Campbell, 1961; Ogasahare *et al.*, 1970; and Davis *et al.*, 1980), and from fungal sources. *Aspergillus oryzae* (Michelena & Castillo, 1984; and Shah *et al.*, 1991). The amino acid values for several of the microbial alpha-amylases are recorded in Table 1. Significant differences are apperent in amino acid composition among the amylases from the different organisms. Investigations thus far reported on the determination of N-terminal and carboxyl-terminal amino acids indicate that bacterial amylase is composed of two polypeptide chains presumably held together by disulfide bonds, whereas a fungal amylase may consist of a branched structure (Pazur, 1965).

Beta-amylase hydrolyses starch and begins at the nonreducing ends of the outer chains and proceeds by stepwise removal of maltose units. Since the maltose liberated in the reaction is of beta configuration, the term beta-amylase was adopted for this enzyme. Amylose and amylopectin are hydrolysed beginning at the nonreducing ends of the outer chains. However, since beta-amylase cannot hydrolyze or bypass an D-(1--> 6) bond, a high-molecular-weight limit dextrin containing all the original α-D-(1--> 6) linkages is produced. A large portion of the amylopectin molecule is, therefore, not hydrolyzed by beta-amylase. Several possible structures for the outer chains of the beta-amylase limit dextrin have been suggested by and are shown in Fig. 18. The mode of attack of the enzyme in the linear glucose polymers is also shown in this Figure (Pazur, 1965; Norman, 1981; Fogarty, 1983; and El-Saady, 1993).

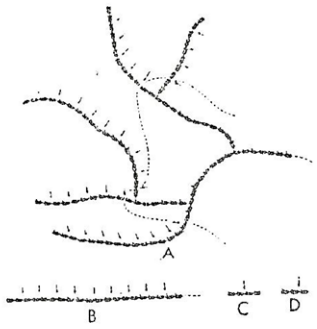

Fig. 18. Action pattern of beta-amylase on amylopectin (A), Amylose (B), maltotetraose (C), and maltotriose (D). Dotted arrow indicates slow rate of hydrolysis, solid arrows indicate rapid rate of hydrolysis, and dotted line indicates the limit of hydrolysis of amylopectin (Pazur, 1965).

Glucoamylase is composed of most of the common amino acids but is particularly low in the sulfur-containing acids and relatively high in the hydroxyl and the dicarboxylic amino acids. Of interest is the recent finding that glucoamylase from *A. niger* is a glycoprotein and contains a significant amount of D-mannose and smaller amounts of D-glucose and D-galactose (Pazur 1965).

Pure glucoamylase hydrolyzes amylopectin, amylose and the maltooligosaccharides completely to D-glucose. Other alpha-glucosides are also slowly hydrolyzed by glucoamylase as the enzyme apparently has a relatively broad substrate specificity. Radioactive oligosaccharide labeled at the reducing end have been used to show conclusively that the enzyme acts predominately by a multi-chain mechanism (Pazur, 1965).

7. Amylases use in the baking industries

The use of amylases in the milling and baking industries are very important in providing machining and other functional properties in doughs. The enzymes traditionally used are amylases from malted wheat and barley and amylases & proteases from fungal sources. More recently, various enzymes from bacterial sources have played an important role in the production of soda crackers, snack foods, and pizza (Barrett, 1975; Fogarty & Kelly, 1980; and Price & Stevens, 1989).

Flour has only small amounts of fermentable sugars. The concentration of such mono-and disaccharieds is about 0.5%. This level of sugars is not sufficient to sustain vigorous yeast fermentation, which is needed to produce lively doughs and large loaf volumes. The addition of extra sucrose or dextrose to doughs does not solve this problem, since the gas production rate must coincide with the ability of the dough to trap the gas and to expand into a controlled structure. Added free sugars are fermented too fast with the resulting loss of nutrients and gas. Consequently, the production of quality bread depends on the addition of α-amylase for the sustained formation of maltose during the fermentation period. The β-amylase naturally present in the flour completes the breakdown of the starch to maltose, which is used by the yeast to form carbon dioxide and ethanol (Barret, 1975; and Price & Stevens, 1989).

7.1. Significance of damaged starch

A certain number of starch granules are damaged (ruptured or torn open) when wheat is milled into flour. Found a range from 6.7 to 10.5% damaged starch in

a wide selection of domestic white bread flours. The level of damaged starch normally present in bread flour is ample to support proper and vigorous fermentation if the starch can be converted to maltase. The β-amylase naturally present in the flour does not attack damaged starch granules and cannot convert enough starch to maltose. α-Amylase does attack damaged starch to produce sugars (dixtrins), which are then hydrolyzed by β-amylase to maltose. Therefore, it is desirable and even essential to supplement a bread flour at the mill or to add α-amylase in the bakery when preparing a dough (Barrett, 1975).

7.2. Effect of amylase supplementation on bread quality

Modern bread production methods require fermentation at a rapid and uniform rate. Timing of sugar production is, therefore, important. The yeast must be supplied with fermentable sugars for its own metabolism, which produces carbon dioxide, alchol and other fermentation intermediates. Sucrose and/or dextrose can, of course, be added (and they usually are added), but this does not necessarily meet the demands of a uniform fermentation rate for the production of quality products. α-Amylase supplementation of flour does not only sustain the fermentation rate but produces additional sugar in the finished bread. This is important, as the trend over the past 20 years has been to increase sugar levels in bread. This increased sugar level improves the taste, crust color, and the toasting qualities of the bread. Besides, the cost of the enzyme and the limited amount of damaged starch available for sugar production have to be considered (Barrett, 1975) Table 3 shows the level of residual sugars in bread when anylases from different sources are used.

Table 3. Effect of amylases from different sources on the level of sugar in bread (α-amylase units/700 gm of flour).

Type of amylase	Amount of amylase	Sugar in bread crumb mg/gm	
		Glucose	Maltose
Control	None	4	1
Cereal	140	6	4
Cereal	560	8	6.5
Cereal	1120	11.2	6.9
Fungal	140	6	2
Fungal	560	8	2
Fungal	1120	10.5	2
Bacterial	35	6	--
Bacterial	140	11.2	4.8

α-Amylase in conjunction with glucoamylase increased loaf volumes at low levels of added sugar. However, with high levels of added sugars, use of the enzymes was not effective (Barrett, 1975).

7.3. Bread quality

The hydrolysis of starch by amylases produces not only loaves with better volume, grain and texture but it also yields bread with a softer, more compressible crumb. With respect to crump softeness. Bacterial α-amylase is much more effective than either the fungal or amylase malted wheat (Barrett, 1975; and Price & Stevens, 1989).

7.4. Sources of amylases

7.4.1. Fungi

Aspergillus oryzae has been approved by the U.S. Food and Drug Administration for general food use. Fungal enzyme concentrates are generally available with activities of 25,000 to 50, 000 α-amylase units per gram. Diluted powders are available at any desired activity level and fungal enzyme tablets are generally standardized at 5000 α-amylase units per tablet (Barrett, 1975; and Price & Stevens, 1989).

7.4.2. Bacteria:

The organism commonly used for production of bacterial α-amylase is *Bacillus subtilis* (Barrett, 1975).

8. Conclusion

Several important new developments have taken place during recent years in enzyme technology which have been of benefit to the starch syrups industry. The production of sucurose from fructose and starch might also become a techincal possibilty. Also, these enzymes are used in textile industry for the desizing of fabrics, in the paper industry for the preparation of starch slurries for coating paper, and in the baking industry for providing machining and other functional properties in doughs.

Amylases are commonly secreted by microorganism. Bacteria, actinomycetes as well as filamentous fungi and yeast have the capacity to hydrolyze starch and these are known to produce amylases. These enzymes are usually extracellular, in-

ducible and remain in the culture fluids after removal of the microorganisms. Amylases act on the starch components in essentially random (endo) manner, (α-amylase, α-1, 4-D-glucanohydrolase, EC 3.2.1.1) or an exo-attack mechanism (β-amylase, α-1-4-glucan maltohydrolase, EC 3.2.1.2 and amylogucosidase, α-1-4-glucohydrolase, EC 3.2.1.3.

Microbial amylases, especially thermostable one has many benefits in the starch processing industry is in the so-called liquification process. A more heat-stable α-amylase was isolated from *Bacillus licheniformis* and this enzyme was introduced commercially. The optimum temperature of *Bacillus licheniformis* amylase is 92oC and its optimum pH around 6.0. This enzyme produces mainly maltose, maltotriose and maltopentaose (from starch). *Aspergillus oryzae* and *Bacillus subtilis* are commonly used for amylase production.

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