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Review Article

BACILLUS SPP. AMYLASE: PRODUCTION, ISOLATION, CHARACTERISATION AND ITS APPLICATION

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Abstract

Amylase is one of the leading enzymes used in industry from decades. The preliminary function of this enzyme is the hydrolysis of the starch molecule into glucose units and oligosaccharides. Amylases have spectacular application in broad spectrum of industries such as food, detergent, pharmaceutical and fermentation industries. Among different type of amylases α- amylase is in utmost demand because of its striking features. This particular enzyme is a good substitute over the chemicals catalyst used in industries. α- amylases can be acquired from different sources such as microorganism, animals and plants. Microorganisms are the major source of production of amylase because of the ease of availability, manipulation and operation. The starch converting enzymes is basically generated using submerged fermentation. Some of the prominent characteristics of amylase are its mode of action, substrate specificity and operating condition (temperature and pH). Amylases from different bacterial sources contribute differently to the particular trait of the enzyme. Bacillus amylases have been studied and applied so far because of their robustness in nature and easy accessible pure form of it. Thus this makes it more specific and fit for distinct application in the industry. The purpose of this manuscript was the comparative analysis of the physical and chemical features of α amylases from Bacillus species. It also focuses on the unique characteristics of this enzyme and their industrial applications.

Keywords: Amylase; Bacillus; starch; fermentation; industrial application

Introduction

Starch which is a white granular polysaccharide manufactured by plants as a reserve food supply greatly influences the nutritional requirement of plant, animal and microorganism. The primary store for starch intake worldwide is cereals (wheat, maize and rice) and vegetables (potato and cassava). Besides this starch is the principal constituent present in domestic and commercial waste (Bozic *et al*., 2011). It is a hetero polysaccharide composed of amylose and amylopectin where the hydrolysis of this hetero polysaccharide results in various oligosaccharide that has cutting edge industrial application (Wang *et al*., 2010). It serves as an important source for the production of glucose, fructose and maltose syrup as a partial substitute of expensive malt in the brewing industry (Roy *et al.*, 2012). Branched oligosaccharide resulting from the hydrolysis of starch is effectively used as anticarcinogenic oligoisaccharides (Nakakuki *et al*., 2002). Moreover the hydrolysis of starch give rise to dextrin that has extensive biotechnological application in bread and baking industry (Windish and Mhatre, *et al*., 1965). Thus it is apparent that starch saccharification has wide application in baking, brewing, detergent, textile, paper and distilling industry (Souza and Magalhaes, *et al*., 2010). Starch hydrolysis has a significant broad range of application in modern biotechnological industries that was initially achieved by chemicals. The extensive consumption of chemicals for starch hydrolysis requires extreme condition such as high temperature, henceforth this escalates the overall costs of production. Nowadays this chemical method of hydrolysis is chiefly replaced by enzymatic hydrolysis of starch (Pandey *et al*., 2000).

Amylase is an enzyme also known as glycoside hydrolases that catalyzes the breakdown of starch into sugars. It plays a central role in the metabolism of carbohydrate in plant, animal, fungi and microorganism (Gomaa, *et al.*, 2013). Two prime forms of amylases are alpha and beta amylase. Alpha amylase (1,4-α-D-glucan-4-glucanohydrolase; EC 3.2.1.1) are endoglycosidases that acts at random locations cleaving the internal α-1,4-D-glycosidic linkages between adjacent glucose units in a linear amylose chain thereby producing smaller dextrins and oligosaccharides with a C1-OH group in the α-anomeric configuration (Najafi *et al.*, 2005). Beta amylase (1,4-α-D-glucan maltohydrolase; EC3.2.1.2) that catalyzes the hydrolysis of second α-1,4-D-

glycosidic linkage generating two glucose units (maltose) at a time. In the carbohydrate active enzyme (CAZy) database, α-amylases are restricted to the families of different glycoside hydrolase called as GHF based on its amino acid sequence. The major amount of α-amylases belong to GHF 13 and they are acknowledged to share a common supersecondary structure, the (β/α) 8-barrel. A minute number of α-amylase belongs to GHF 57, a family much smaller than GHF 13, though to date, only one α-amylase belongs to GHF 119 (Labes *et al.,* 2008).

Amylase approximately covers 25% of the enzyme market (Asgher *et al*, 2005, Burhan *et al*., 2003, Rao *et al*., 1998). It is extensively expressed in nature and can be derived from different type of sources such as plant, animal and microorganism. Microorganism is preferred for aplenty generation of amylase over plant and animal as it's a cost effective method for the bulk production and the ease of manipulation necessary for the proficient production of enzymes of desired characteristics (Pandey *et al*., 2000 and Fossi *et al.*, 2009). Most of the studies in developing countries target *Bacillus sp*. for the enzyme extraction probably because of its simple nature and nutritional requirement (Oyeleke and Oduwole, 2009). The production of amylase is generally carried out using submerged fermentation (Gomaa, *et al.*, 2013), although solid state fermentation appear more promising due to the natural potential and advantages offered by the same (Bogar *et al.*, 2002).

Amylase at present has an assortment of application in the sugar, animal nourishment, leather, paper and pulp, textile, detergents, baking, brewing, and distilling industries, production of cakes and starch syrups, preparation of digestive aids, and in pharmaceutical industries (Bano *et al.*, 2011). Recently, the use of bioethanol as a second transportation fuel has grabbed a lot of attention (Louime and Uckelmann, 2008). In this method of biofuel production, glucose is converted into ethanol by means of fermentation with the help of yeasts (Gomaa, 2013). Renewable biomasses are used as a substrate for the production of ethanol. Because *Saccharomyces cervisiae* lacks alpha amylase, the conversion of starch to glucose is accomplished by the supplementation of amylase from outsource (Fatoni and Zusfahair, 2012). In addition to this it can also be used for the exclusion of microbial biofilms from its root by degrading its major structural component particularly, Extracellular polymeric matrix (EPM). Thus all this industrial application demands for an enzyme that has unique desirable properties with respect to the specificity, stability, temperature, pH spectra and metal ion dependence (Wang *et al.*, 2010). Therefore, screening of the microorganism for enzyme production possibly will facilitate the discovery of novel amylase (Goswami and Chauhan, 2003).

Lately it has been revealed that in most of the cases enzymatic reaction is inhibited by higher concentration of substrate and product. In addition to that the efficiency of amylase is further reduced under repetitive and prolonged use (Konsoula *et al*., 2006). Thus, immobilization is an important tool for the stability and frequent use of enzymes in industrial application and for its rapid separation from the reaction mixture. Immobilization of the enzyme can be achieved in one of the following ways like entrapement, coplymerization, microencapsulation, cross-linking, covalent binding, chemical attachment and physical adsorption (Hasicri, Aksoy, and Tumturk, 2006; Rajagopalan and Krishnan, 2008; Reshmi, Sanjay and Sugnan, 2006). As a result, the above features are important for the development of economically feasible bioreactor.

Elucidating the parameters governing the properties of amylase enzyme:

Media Composition and cultivation conditions:-

Media composition and culture condition is the most crucial factor for the growth of the microorganism for the efficient production of amylase. Different microorganisms have special nutritional requirement as illustrated in Table 1.

Growth of the microorganism is followed up by taking the optical density at 600nm. Thus the yield of the enzyme (Table 2) corresponding to the maximum amylase production is then calculated from the growth curve (Bozic *et al.*, 2010). With reference to the table 2 the maximum yield is observed for *Bacillus subtilis*-AS-SO1a at stationary phase. To meet the ever increasing needs of new age, it is necessary to improve the yield and effectiveness of the system with no further enhancement in the cost of production.

Table 1: Summary of the media compositions and parameters governing the growth of different microorganisms**.**

S. No	Microorganisms	Ingredients	Quantity	Reference
1.	*Bacillus licheniformis* ATCC 9945a, pH6.5/37ºC/72hr	Soluble starch Tryptone $MgSO_4 \cdot 7H_2O$ K_2HPO_4 Na_2HPO_4 NaCl $(NH_4)_2SO_4$ $FeCl_3$ $CaCl_2 \cdot 2H_2O$ Incoulum culture	10 g/l 5 g/l 0.5 g/l 2 g/l 5 g/l 2 g/l 4 g/l 0.05 g/l 0.05 g/l 5ml	Bozic *et al.,* 2010

Table 1(Contd.): Summary of the media compositions and parameters governing the growth of different microorganisms**.**

2.	*B. subitils* NCTC10400 *and B. cereus* ATCC14579 35 ºC/72 hr	Substrate- Wheat bran-pretreated with 1% NaOH and autoclaved at 121 ºC for 20 minutes. KH_2PO_4 NaCl $MgSO_4$ $CaCl_2$ Inoculum culture	5g 0.1g/l 0.25g/l 0.01g/l 0.01g/l 1ml	Gomaa, 2013
3.	*Chryseobacterium taeanense* TKU001 30ºC/1-5days/pH9	Bacteria isolated from soil using red-koji rice as the sole carbon source. K_2HPO_4 $MgSO_4{\cdot}7H_2O$ Mung bean	1% 0.1% 0.5% 0.5-2.5%	Wang *et al.*, 2010
4.	*Bacillus subtilis* AX20 37 ºC/60 hr/ pH 6	Peptone Yeast extract Starch NaCl Inoculum culture	1% 0.2% 1% 1% 1%	Najafi *et al.*, 2005
5.	*Bacillus subtilis* JS2004 40ºC/144hr/ pH 7	KH_2PO_4 Na_2HPO_4 NaCl $(NH_4)_2SO_4$ $MgSO_4.\ 7H_2O$ $CaCl_2$ Tryptone Waste starch powder Inoculum culture	0.1% 0.25% 0.1% 0.2% 0.005% 0.005% 0.2% 1% 1ml	Asgher *et al*,. 2006
6.	*Bacillus subtilis* AS-SO1a 55ºC/pH 6	M9 Media (for inoculum) Starch Beef extract	 1.421% 0.131%	Roy *et al.*, 2011
7.	*Bacillus sp*. YX-1 37ºC/48hr/pH 4.5	Soluble starch Peptone Corn syrup $MgSO_4.\ 7H_2O$ KH_2PO_4 $CaCl_2.\ 6H_2O$ $FeSO_4.\ 7H_2O$ Inoculum- cultivation medium	5 g/l 5 g/l 20 g/l 0.5 g/l 3 g/l 0.5 g/l 0.1 g/l 5% (v/v)	Liu *et al.*, 2007
8.	*Bacillus megaterium* VUMB109 40ºC/24hr/pH 8.2	Starch $(NH_4)_2.HPO_4$ $MgSO_4{\cdot}7H_2O$ KCl Enriched Culture media	0.2% 0.4% 0.05% 0.1%	Jana *et al.*, 2012
9.	*Bacillus subtilis* KBIGE HAS 35ºC/24 hr/pH 7	Starch Bacto-peptone Yeast extract NaCl $MgSO_4{\cdot}7H_2O$ $CaCl_2{\cdot}2H_2O$ Inoculum	20g/l 10 g/l 4 g/l 0.5 g/l 0.5 g/l 0.2 g/l 10%	Bano *et al.*, 2011
10.	*Bacillus* strain HUTBS71 50 ºC/50 hr	Yeast extract Soluble starch Casein NaCl $CaCl_2$ $MgSO_4$	0.5% 1% 4% 2.5% 0.02 (g/l) 0.01 (g/l)	Quadan *et al.*, 2009
11.	*Marinobacter sp.* EMB8 35 ºC/54hr/pH 7	Starch Caesin enzyme hydrolysate K_2HPO_4 $MgSO_4.7H_2O$ NaCl Inoculum	50g/l 10g/l 0.87g/l 6.2g/l 50g/l 1% (v/v)	Khare *et al.*, 2011

Table 1(Contd.): Summary of the media compositions and parameters governing the growth of different microorganisms				
12.	*Aspergillus oryzae*	Coconut oil cake KH_2PO_4 NH_4NO_3 NaCl $MgSO_4.7H_2O$	5g/l 2g/l 5g/l 1g/l 1g/l	Ramachandran *et al.*, 2003

Table 2: Comparative analysis of the yield of Amylase from different microorganisms

S. N.	Microorganism	Yield	Maximum Growth	References
1.	*Bacillus licheniformis* ATCC 9945a	5.2 IU/mL	72hr (stationary phase)	Bozic *et al.*, 2010
2.	*B. subitils* NCTC10400 *and B. cereus* ATCC14579	74.60 and 56.66 U/g	72hr (exponential phase)	Gomaa, 2013
3.	*Chryseobacterium taeanense* TKU001	0.135 units per ml	5^{th} day (exponential phase)	Wang *et al.*. 2010
4.	*Bacillus subtilis* AX20	38U/ml	34-46 hr (stationary phase)	Najafi *et al.*. 2005
5.	*Bacillus subtilis* JS2004	44.84 U/ml	48hr (exponential phase)	Asgher *et al.*. 2006
6.	*Bacillus subtilis* AS-SO1a	440U/ml	60hr (stationary phase)	Roy *et al.*, 2011
7.	*Bacillus sp.* YX-1	53 U/ml	44hr (stationary phase)	Liu *et al.*, 2007
8.	*Bacillus subtilis* KBIGE HAS	13011U/mg	24hr	Bano *et al.*. 2011
9.	*Marinobacter sp.* EMB8	48.0 IU/mL	54hr (exponential phase)	Khare *et al.*, 2011
10.	*Aspergillus oryzae*	1752 U/gds	72 hr (exponential phase)	Ramachandran *et al.*, 2003

Enzyme production can be either substrate dependent or independent. A number of the microbes have shown highest production of amylase at the exponential stage. This clearly indicates the nutritional requirement of the enzyme for its maximal production. The depletion of nutrient at this time point results in the accumulation of toxic byproducts at the stationary phase, resulting in the fall of the productivity of the enzyme (Asgher *et al.*, 2006). The main reason behind this might be the proteolysis of amylase at stationary phase. Whereas the other microbes have shown maximum production of amylase at stationary phase even though there is a nutrient deprivation and this tends to decline even further when it enters the death phase (Liu *et al*., 2008).

Characterization of α- Amylase activity

Alpha-amylase activity is evaluated by measuring the amount of reducing sugars released during the hydrolysis of starch. The reaction mixture is mainly composed of the soluble starch that acts as a substrate to which the diluted enzyme is added along with the buffer at specific pH. The reaction mixture which is then incubated for 30 minutes in water bath is examined for the amount of reducing sugar liberated during this course. This is determined by the addition of Dinitrosalicylic acid (DNS) which was used to stop the reaction held in boiling water bath for 5 minutes. The absorbance was measured at 540 nm (Miller *et al.*, 1959) and specific amylase activity is determined as specified in table 3. Glucose can be used as a standard curve to discover the activity of unknown enzyme. One unit of amylase activity is define as the amount of enzyme that can release one 1 µmol of reducing end group per minute. As compared to the other bacillus species mentioned in the table 3 *Bacillus licheniformis* ATCC 9945a has revealed the maximum amylase activity when tested from the crude supernatant.

Determination of the reduction in blue value

The liquefying activity of the amylase is determined by the reduction in the blue value of the Gram's iodine solution. In this method of blue value determination, the reaction mixture comprising of substrate and amylase is taken out at different time points to which 0.1 N HCl was added to halt the reaction. Gram's iodine solution is added afterward to this. The function of iodine in this case is to bind the starch in the solution but when starch is hydrolyzed to oligosaccharides by amylase then this can no more bind to iodine and hence there is a diminution in the blue value of Iodine. Thus it can be inferred that the blue value of iodine is inversely proportional to the activity of amylase (Teodoro and Martins *et al.*, 2000).

Absolute purification of α-Amylase

The partial purification of enzyme is done with ammonium sulfate which is added to the crude cell free supernatant to 70% saturation at 4ºC for overnight. The precipitate obtained was recovered by centrifugation (9000g, 4ºC and 20 minutes) which is then dissolved in small amount of Tris buffer followed by dialysis. The enzyme obtained from the above step can be further purified and loaded onto Sephadex G-50 column (Bozic *et al*., 2010). The fraction that shows utmost amylase activity is collected and concentrated by ammonium sulphate precipitation. The enzyme obtained from Sephadex G-50 column can be loaded onto DEAE-Cellulose column for advance purification. The fractions with specific activity has to be pooled off and if required then it can be passed through Starch affinity chromatography (Najafi *et al.,* 2005). Accordingly this purified enzyme can be further analyzed for its activity.

Comparative analysis of the molecular mass determination from SDS and Native PAGE

The molecular weight of alpha amylase subunit after purification was determined by Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in comparison to the existing standards. The sample post purification is mixed with reducing sample buffer (62.5mM Tris pH 6.8, 2% SDS, 5% beta-mercapthoethanol, 10% glycerol and 0.002% bromophenol blue) and heated for 3 minutes in water bath (Laemmli *et al.,* 1970). Electrophoresis can be carried out in a 10% acrylamide gel. The standard proteins that are basically used for calibration are myosin (MW: 220,000), phosphorylase b (MW: 97,000), bovine serum albumin (MW: 66,000), ovalbumin (MW: 45,000), carbonic anhydrase (MW: 30,000), trypsin inhibitor (MW: 20,100), and lysozyme (MW: 14,300). The proteins were formerly visualized by silver staining. Consequently the molecular weight of the desired amylase subunit was deduced from the standard proteins (marker) in gel electrophoresis (Wang *et al.,* 2010).

The molecular weight of the native alpha-amylase is examined using gel filtration chromatography or Native PAGE (Liu *et al*., 2008) where the standard proteins mentioned above can be used for calibration. In gel filtration chromatography the molecules with larger molecular weight will elute first followed up by those that comes out through intermolecular spaces and then the smallest one which is trapped in the gel bead. As shown in Table 3, if the molecular weight of amylase from the SDS-PAGE (denatured form) is equivalent to that from the Native PAGE or Gel filtration chromatography then the enzyme is entitled as homogeneous and monomer. The Table 3 illustrates the comparative analysis of the molecular mass of amylase from different bacillus species where *Bacillus megaterium* VUMB109 has found to produce an amylase (monomer) of 150KDa. Of the entire Bacillus species listed in the table 3 *Bacillus subtilis* AX20 was found to be a homodimer with molecular mass 139440KDa.

Effect of pH on the activity of amylase

For the determination of optimum pH for the maximum enzyme activity the reaction mixture is incubated with different buffers from pH 4 to 10. For pH of 4.0 and 5.0, sodium-acetate buffer can be used, for pH of 6.0 and 7.0, sodium phosphate buffer can be used, for pH of 8.0 and 9.0, Tris-HCl can be used, whereas for pH of 10, borax NaOH buffer can be used. Alpha amylase production is greatly influenced by the extracellular pH as it alters the cell membrane permeability of the microorganism hence interfering with the transport of different components across the cell membrane (Gomaa *et al.,* 2013). Not only had this but the change in pH has distorted the amino side chain present at the active site of the enzyme as well. Hence at its optimum pH the enzyme is at its favorable confirmation. Thus as reported in Table 3 most of the enzymes show maximum activity at neutral pH and slightly acidic pH (Pandey *et al.*,2000). With increase in pH the activity of enzyme increases to a certain point and beyond this a sharp decline is speculated as the metabolic activity of microbes is very sensitive to higher pH. The optimum pH for amylase from different species was listed down in table 3 where *Bacillus sp*. YX-1 shows the maximum stability at an extreme range of pH from 8 to 12. Whereas the amylase from other species were found to be most stable at an optimum pH of 6 to 7. A very small part of industrial enzyme amylase has been noted down that remains stable at higher pH which is of great importance to the detergent and textile industries.

Effect of temperature on amylase activity

For the determination of optimum temperature for the maximum amylase activity the reaction mixture was incubated at different temperature from 30 to 100º C. Temperature plays a great role in shifting the confirmation of the protein to enzyme inhibition (Gomaa *et al.,* 2013). Thus as shown in Table 3, with increase in the temperature the kinetic energy increases which corresponds to the increase in the collisions between the substrate and the enzyme resulting in enzyme-substrate complex (Lehninger *et al.,* 2005 and Mohammadi *et al.*, 2010). Therefore with increase in temperature initially the enzyme activity increases and it may remain stable at a broad range of its optimal temperature (Prakash *et al.,* 2010 and Kindle *et al.,* 1983). If the temperature is increased beyond this then it ruptures the secondary, tertiary and quaternary bonds leading to denaturation of the enzyme (Ciobanu *et al.,* 1976 and Schokker *et al.,* 1999). As specified from the table 3, *Bacillus* strain HUTBS71 shows the maximum amylase activity at an optimum temperature of 100ºC which is industrially very significance.

Table 3: Comparison of different microorganisms based on the specific activity, molecular mass and the effect of temperature, pH , metal ions, and inhibitors on the ativity of purified alpha-amylase

S. N.	Microorganism	Specific Activity (Crude supernatant)	Molecular Mass	Optimum pH	Optimum Temperature (ºC)	Cations and Inhibitors	References
1.	*Bacillus licheniformis* ATCC 9945a	591.5 U/mg	32KDa (monomer)	6.5	90	Activated by Ca2+ & Inhibited by Hg 2+, EDTA.	Bozic *et al.*, 2010
2.	*B. subitils* NCTC10400 *and B. cereus* ATCC14579	296.31 and 218.67 U/g	NA	7.0	50	Activated by Ca2+ & Inhibited by EDTA and Cu2+.	Gomaa, 2013
3.	*Chryseobacterium taeanense* TKU001	0.0008IU/mg	46KDa (monomer)	9.0	50	Activated by Ca2+ & Inhibited by EDTA	Wang *et al.*, 2010
4.	*Bacillus subtilis* AX20	110.7 U/mg	139440KDa (homodimer)	6.0	60	Metal independent	Najafi *et al.*, 2005
5.	*Bacillus subtilis* AS-SO1a	201U/mg	21KDa (monomer)	8.0	70	Activation by Ca2+ & Inhibited by Co2+, Cu2+, Hg2+.	Roy *et al.*, 2011
6.	*Bacillus sp*. YX-1	17.9 U/mg	56KDa (monomer)	(8-12)	55	Strongly affected by PMSF and 4-bromophenacylchloride.	Liu *et al.*, 2007
7.	*Bacillus megaterium* VUMB109	8.77U/mg	150KDa (monomer)	5.0	40-50	Metal independent, Strongly inhibited by Hg2+,Cu2+, Fe3+, Mn2+and Zn2+.	Jana *et al.*, 2012
8.	*Bacillus subtilis* KBIGE HAS	135.0U/mg	56KDa (monomer)	7.5	50	NA	Bano *et al.*, 2011
9.	Bacillus strain HUTBS71	357 U/mg	58.8KDa (monomer)	7.8	100	Activated by CoCl2, MnCl2, MgSO4, ZnSO4 and MnSO4	Quadan *et al.*, 2009
10.	*Marinobacter sp.* EMB8	3.0IU/mg	72KDa (monomer)	7.0	80	Inhibited by PCMB and Hg2+	Khare *et al.*, 2011

Effect of metal ions and inhibitors on amylase activity

For determining the effect of metal ions, the reaction mixture was incubated with different concentration of metals where the untreated samples were taken as control. Ca^{2+}, Cu^{2+}, Fe^{2+}, K^{+}, Mg^{2+}, Mn^{+}, Na^{+} and Zn^{2+} can be used as cations to find its possible effect on amylase activity.

Similarly, it can be checked for inhibitors incubated at different concentrations. The inhibitors that are basically used to keep a check on the amylase activity is ethylenediamide tetraacetic acid (EDTA), ethyleneglycol-bis (b-aminoethylether) N,N,N0,N0 tetraaceticacid (EGTA), triethylenetetramine hexactic acid (TTHA), diethyldithiocarbamate (DTC), phenanthrolien and phenylmethylsulphonyl fluroide (PMSF) (Delkash-Roudsari *et al*., 2013). It is reported that on the addition of Ca^{2+} to the reaction mixture there is increase in the activity of amylase. It is suspected that may be calcium ion is present at the active site of the enzyme (Machius *et al*., 1995 and Strobl *et al.*, 1998). Ca^{2+} has stabilizing effect on the thermostability of the enzyme as a result of which salting out of the hydrophobic residues take place hence resulting in the compact structure of the protein. This correlates with the higher activity of the enzyme. On the addition of EDTA which chelates the calcium ion resulting in the decrease in the activity of the enzyme. This confirms the presence of calcium ion at the active site of amylase. Whereas according to Delkash-Roudsari 2013, DTC chelates Cu^{2+} and TTHA chelates Mg^{2+}. On other hand when an enzyme is activated by Cu^{2+} then it is deduced that might be Cu^{2+} is interacting with some amino acid residue present at the active site of the enzyme. Thus this might alter the confirmation of the enzyme that corresponds to higher activity of amylase. The inhibition of enzyme by Hg^{2+} indicates the presence of indole amino acid residues which is required for the proper functioning of the enzyme (Gupta *et al*., 2003). The inhibition by other metal ions could be due to the competition between the ions that are added exogenously and the cations associated with proteins resulting in decreased metalloenzyme activity. Some of the enzymes are strongly influenced by the presence of PMSF and 4-bromophenacylchloride which marks the role of serine and histidine residue in the enzyme catalysis (Roy *et al.*, 2004). Hence most of the enzymes as reported in Table 3 are calcium dependent that has taken the cost of its production to a very next level as calcium is required to stimulate the activity of amylase (Malhotra *et al.*, 2010 and Ghollasi *et al.*, 2010). The industries demands the metal independent enzyme that are extremely useful as it cut downs the cost of production making the process economically feasible because the addition of calcium ions insert an extra step of its subsequent exclusion by ion exchange chromatography (Najafi *et al.*, 2005). It is known that a very small source of amylase are metal independent derived from *Bacillus subtilis* AX20 and *Bacillus megaterium* VUMB109 as depicted in Table 3.

Degree of starch hydrolysis

Enzymes which are capable of hydrolyzing raw starch granule are economically very attractive. Several amylases are reported that can hydrolyze raw starch in single step and at the temperature below the gelatanization temperature of the starch (Goyal *et al.,* 2005) Disaccharides and oligosaccharides such as maltose and maltotriose obtained from the hydrolysis of the starch are widely accepted by the food industries (Ivanova *et al.*, 1991). The end product of hydrolysis can be determined by analyzing the paper chromatogram for the digestion of disaccharide via solvent system butanol, pyridine and water (6:3:4). Thin layer chromatography following the principle of HPTLC can also be used to analyze the end product of hydrolysis (Jana *et al.*, 2012). For the identification of the hydrolyzed product the maltooligosaccharide markers can be used, visible by spraying silica plates with dipheyl amine in acetonitrile.

According to Liu *et al*., 2008, the extent of hydrolysis of the raw starch is calculated as:

$$Rh\ (\%) = (A1/A0) \times 100,$$

Where A1 is the amount of reducing sugar released after the hydrolysis reaction and A0 is the amount of sugar released before the reaction.

The optical rotation of the hydrolyzed product can also be measured periodically using polarimeter accompanied by sodium light. Thus the mutarotation of the hydrolysate is determined by adding 5mg of solid sodium carbonate per ml of mixture after the optical rotation is almost constant (Konsula and Liakopoulou-Kyriakides, 2004). Depending on whether the optical rotation has shifted downward or upward it can be deduced that if the hydrolyzed products have alpha or beta anomeric configuration respectively. This study verifies the family of the enzyme to which it fits in (Anindyawati *et al.*, 1998).

Industrial Application of amylase

Amylases have prospective application in a wide spectrum of industries such as food, fermentation and pharmaceutical as cited in Table 4. The application of amylase has widened to an unusual field of research as well. Some of them are discussed below:

Production of Bioethanol

The most common used method for the production of bioethanol is possible via the fermentation of biomass (starch) by commercial yeast. The starch is the cheapest source and is easily available in most regions of the world; which involves liquefaction and saccharification step for its conversion to ethanol (Trovati *et al*., 2009). The initial step is the hydrolysis of starch to glucose that cannot be attained by yeast as this lack amylase. So amylase is added extracellularly to the reaction mixture for the efficient and

rapid conversion of starch to glucose and then finally to bioethanol. To the glucose solution obtained post amylase hydrolysis, yeast is added and incubated in an anaerobic condition at 37ºC for 7 days (Suhaimi *et al.*, 2012). To determine the amount of bioethanol produced the solution can be assayed using gas chromatography equipped with flame ionization detector. It is known from the literature that *B.subitils* NCTC10400 *and B. cereus* ATCC14579 play a chief role in the production of bioethanol.

Biofilm removal efficacy

It has been reported that enzymes are useful for the degradation of the multistructural extracellular polymeric matrix (EPS), a base on which the biofilms are developed (Lequette *et al.*, 2010). EPS is mainly made of polysaccharides, proteins, lipids and nucleic acid. The mechanism by which enzymes attack EPS is by weakening the integrity of the proteins, carbohydrate and lipid (Xavier *et al.*, 2005). Thus to determine the efficacy of amylase for removal of biofilm can be achieved by adding microorganisms to the polystyrene microtiter plate and it is incubated for 48hr until the microorganisms is completely attached to the surface of plate (Pitts et al., 2003). To this amylase is added and kept for some time. Crystal violet is used for staining and absorbance is measured at 600 nm.

According to Gomaa 2013, the percentage reduction in the biofilm is used for the evaluation of efficacy of the enzymes:

Percentage Reduction = [(C-B)-(T-B)/ (C-B)] × 100%

Where B denotes, the average absorbance per well for blank (no biofilm, no treatment); C denotes the average absorbance per well for control wells (biofilm, no treatment) and T denotes the average absorbance per well for treated wells (biofilm and treatment).As evident from the Table 4, *B. subitils* NCTC10400 *and B. cereus* ATCC14579 are constructive for the degradation of EPS.

Table 4: Summary of the Industrial Application and distinctive feature of different microorganisms.

S. N.	Microorganism	Industrial Application	Distinct feature	References
1.	*Bacillus licheniformis* ATCC 9945a	Produce highly efficient alpha amylase for the digestion of raw starch below gelatinization temperature- less energy expenditure.	Degradation of starch using very low dose of enzyme (0.07 U/mg of starch)	Bozic *et al.*, 2010
2.	*B. subitils* NCTC10400 *and B. cereus* ATCC14579	Production of Bioethanol and removal of Biofilms.	Active at higher temperature and over a wide range of pH. Stable at high range of pH, moderate thermostability.	Gomaa, 2013
3.	*Chryseobacterium taeanense* TKU001	Strong antioxidant activity and ability of enhancing the growth of probiotic bacterium	Metal independent Enzyme	Wang *et al.*, 2010
4.	*Bacillus subtilis* AX20	Digestion of maltose to produce glucose at higher levels.	Active at a wide range of temperature greater than 70ºC	Najafi *et al.*, 2005
5.	*Bacillus subtilis* JS2004	Thermostable alpha-amylase	Alkaline alpha amylase shows highest activity at pH 9.	Asgher *et al.*, 2006
6.	*Bacillus subtilis* AS-SO1a	Compatible in presence of oxidant, surfactants, EDTA and commercial laundry detergent.	Stable at a very low pH.	Roy *et al.*, 2011
7.	*Bacillus sp.* YX-1	Raw starch digesting novel enzyme	Higher affinity for starch as compared to other substrates (amylase, amylopectin and glycogen)	Liu *et al.*, 2007
8.	*Bacillus megaterium*VUMB109	End product of hydrolysis: Maltotetraose and Maltopentose – used as a food supplement for baby and aged people.	Optimum pH and temperature is 7.8 and 100ºC, stable over a broad range of pH and temperature, 5.2-10pH and 80-115ºC.	Jana *et al.*, 2012
9.	Bacillus strain HUTBS71	Highly thermostable, extremely hyperthermophilic and alkalitolerant	End product of hydrolysis : maltooligosaccharides,	Quadan *et al.*, 2009
10.	*Marinobacter sp.* EMB8	Application in bread making and other food industries secretes solvent and heat stable enzyme.	Optimum temperature is 80ºC and stable at a high concentration of solvents.	Khare *et al.*, 2011
11.	*Aspergillus oryzae*	Coconut oil cake (byproduct obtained after oil extraction from copra) used as a substrate for the production of alpha-amylase is commercially very important.	Enhanced enzyme titre- 1827 U/gds	Ramachandran *et al.*, 2003

Immobilization of amylase

Immobilization is required for repetitive and prolonged use of enzyme. It is basically the technique in which enzyme is enclosed in an aqueous solution within a semi permeable membrane. Encapsulation in calcium-alignate gels requires a very gentle condition that facilitates the ease of operation and suppresses the cost (Dey *et al.,* 2003). Other characters of the capsule can be controlled by modifying the conditions of the gelatin such as thickness or permeability of different substrates to the gel membrane (Bradley *et al.*, 2004). The optimum conditions required for the encapsulation of alpha-amylase is 2% (w/v) sodium alignate, 5% $CaCl_2$ (Konsula *et al.*, 2004). The encapsulation efficiency is enhanced on the further addition of 2% (w/v) silica gel. Glyoxyl and glutatraldehyde supports can also be used for the stabilization of the enzyme by multipoint attachment (Tavano *et al.*, 2013). Thus the most important aspect that has to be considered when immobilizing the amylase is the huge size of the substrate that cannot penetrate through the pores of the gel resulting in an inadequate accessibility of the enzyme to the substrate.

Conclusion

Amylase is one of the most vital enzymes with incomparable application in different industries. The marked application of alpha amylase as described has widely entrapped modern biotechnology. As visible the food, detergent, textile and pharmaceutical industries demands finer enzyme with respect to the conditions required for the optimization of the efficient production of amylase. Food and starch based industries are the key consumer of the amylase and covers a hefty market in the biotechnology fields. The global market of amylase is rapidly increasing and expected to rise at an annual growth rate of 3.3%. Commercially the production of amylase is carried out in submerged fermentation, but solid state fermentation seems to be a potential tool for its production, particularly when agroindustrial residues are used as a substrate.

A great number of microbial sources are used for the efficient production of amylase. Only few of the strains are highlighted commercial that meets the ever increasing demand of the industries. Microbes are gold mine, a favored source for the production of amylase in context to rapid growth, limited space and its accessibility to genetic exploitation. Thus the search for a novel microorganism for the production of amylase is an unremitting process. Moreover, recently many authors have presented a better approach towards the proficient production of amylase. Also, the chemical modification of enzyme also called protein engineering enhances the activity and the affinity of the enzyme for the substrate. Thus the use of overproducing mutant, genetically engineered strains, reasonable raw material and novel enzyme with respect to its dependency on temperature, pH and metal ions would be the real achiever of the coming era.

Amylase is an eco-friendly and biodegradable enzyme as it can be produced from various biological sources. The invincible properties of amylases mainly constancy, robustness and reusability is crucial for industrial application. It has wide range of application in diverse industries from food to the clinical sectors. Moreover it is an alternative source of energy and a new ray of hope for the society where fuel is depleting at an elevated rate.

Different approaches are proposed for the rapid, high production and prolonged use of enzymes. Immobilization of the enzyme is a worthier approach towards the economical production of enzyme. It has considerable effects on the kinetics of the enzyme. Thus this influences the partition of material between the product stage and the enzyme stage and enforces restrictions on the rate of diffusion of materials. Consequently, enzyme immobilization has both its promises and pitfalls.

The current scenario craves for the screening of the microbes for the novel enzyme that is profitable to the man and society. With enhanced function spectra of the industrial application of enzyme, the demand is for the enzyme with absolute specificity. It is obvious that amylase will provide great and novel opportunities to the biotechnological sectors as a biocatalyst.

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