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An Overview of Cold-active Microbial α -amylase: Adaptation Strategies and Biotechnological Potentials

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Abstract: Absolutely the largest proportion of the Earth's biosphere is comprised of organisms that thrive in cold environments are known as psychrophiles and psychrotrophs. Their ability to proliferate in the cold is predicated on a capacity to synthesize cold-adapted enzymes like amylases, proteases, lipases, pectinases, cellulases, etc. that could be used in low-energy processes. Amylases have most widely been reported to occur in microorganisms, although they are also found in plants and animals. Cold-active α -amylases confer low activation energies and high activities at low temperature which are favorable properties for the production of relatively insubstantial compounds. In addition, these enzymes have an advantage under extreme low temperature conditions due to their inherent greater membrane fluidity, production of cold-acclimation proteins and the mechanism of freeze tolerance. The low temperature stability of cold-active amylases has been regarded as the most important characteristics for use in the industry because of considerable progress towards energy savings but unfortunately these enzymes have largely been overlooked. Now this situation is changing which recently fascinated the scientific community to focus in many fields, such as clinical, medicinal and analytical chemistries, as well as their widespread biotechnological applications such as food processing, additive in detergents, waste-water treatment, biopulping, environmental bioremediation in cold climates and molecular biology applications. This review addresses the present status of knowledge on the source, structure, production and molecular characteristics of cold-active α -amylases and their biotechnological applications.

Key words: Psychrophiles, psychrotrophs, extremozymes, cold-active amylase, starch degrading enzymes

INTRODUCTION

Extremophiles are microorganisms that can grow and thrive in extreme environments, like high or low temperature, high or low pH, high salinity, very low water activity, high pressure, low oxygen etc. Extremophiles are structurally adapted at the molecular level to withstand these harsh conditions and among them biocatalysts play a major role which are called as extremozymes produced by these microorganisms. There are various extremozymes such as cellulases, amylases, xylanases, proteases, pectinases, keratinases, lipases, esterases, catalases, peroxidases and phytases which function under extreme conditions. The application of cold-active enzymes enables lowering of temperature without loss of efficiency which results in saving of energy consumption and have great potential for various biotechnological processes (Ramteke and Bhatt, 2007; Kuddus and Ramteke, 2008a).

Currently, only ~2% of the microorganisms on the earth have been commercially exploited and amongst these there are only a few examples of extremophiles (Gomes and Steiner, 2004). Around 85% of earth is occupied by cold ecosystems including the ocean depths, polar and alpine regions. Out of which ~70% is covered by oceans that have a constant temperature of 4-5°C, irrespective of the latitude. Remaining 15% included Polar regions to which the glacier and alpine regions must also be added. Extremophiles successfully colonized on these eternally cold environments which we can call as psychrophiles (Feller and Gerday, 2003). The classical definition of psychrophiles is given by Morita (1975) which is frequently used in the literature. This definition proposes that psychrophilic microorganisms have optimum growth temperatures of <15°C. But a psychrotrophic term is used for those cold-adapted organisms that have an optimum growth temperature of

~15-20°C but are able to grow upto 30°C. During the past decade it has been recognized that cold-adapted microorganisms and their enzymes provide a wide biotechnological potential and offering numerous economic and ecological advantages over the thermophilic enzymes/microbes (Kuddus and Ramteke, 2008b).

Alpha amylases (endo-1,4- α -D-glucan glucohydrolase, EC 3.2.1.1) belongs to the enzyme class of hydrolases which randomly cleaves the 1,4- α -D-glucosidic linkages between the adjacent glucose units in linear amylose chain of starch. Most of the α -amylases are metallo-enzymes which require calcium ions for their activity, stability and structural integrity. They belong to family 13 (GH-13) of the glycoside hydrolase group of enzymes (Bordbar *et al.*, 2005). The specificity of the bond attacked by α -amylases depends on the sources of the enzymes. Currently, two major classes of α -amylases are commercially produced through microbial fermentation. Based on the points of attack in the glucose polymer chain, they can be classified into two categories, liquefying and saccharifying. Bacterial α -amylase randomly attacks only the α -1,4 bonds, it belongs to the liquefying category. On the other hand, the fungal α -amylase belongs to the saccharifying category and attacks the second linkage from the non-reducing terminals (i.e. C4 end) of the straight segment, resulting in a disaccharide called maltose. The bond breakage is thus more extensive in saccharifying enzymes than in liquefying enzymes. Thus, amylases have emerged as one of the leading biocatalysts with proven potential to find usage in a wide array of industrial applications, such as additives in processed food industries, additives in detergents, waste-water treatment, biopulping, bioremediations and in molecular biology. These enzymes account for about 30% of the world's enzyme production (Maarel Van der *et al.*, 2002).

Cold active amylases: Cold-adapted amylolytic microorganisms produce cold-active amylases which function effectively at cold temperatures with high rates of catalysis in comparison to the amylases from mesophiles or thermophiles which shows little or no activity at low temperature. These amylases have evolved a range of structural features that confer a high level of flexibility, particularly around the active site are translated into low activation enthalpy, low-substrate affinity and high specific activity at low temperatures. Moreover, the maximum level of activity of these amylases is shifted towards lower temperatures with a concomitant decrease in thermal stability (Kuddus and Roohi, 2010).

Structural features of α -amylase

General α -amylase structure: The α -amylase family is the largest family of glycoside hydrolases, transferases and isomerases comprising nearly 30 different enzyme specificities (Henrissat, 1991). A large variety of enzymes are able to act on starch. These enzymes can be divided basically into four groups: endoamylases, exoamylases, debranching enzymes and transferases (Maarel van der *et al.*, 2002):

- **Endoamylases:** Cleave internal α -1,4 bonds resulting in α -anomeric products
- **Exoamylases:** Cleave α -1,4 or α -1,6 bonds of the external glucose residues resulting in α -or β anomeric products
- **Debranching enzymes:** Hydrolyze α -1,6 bonds exclusively leaving long linear polysaccharides and
- **Transferases:** Cleave α -1,4 glycosidic bond of the donor molecule and transfer part of the donor to a glycosidic acceptor forming a new glycosidic bond

Alpha amylase is classified as family 13 of the glycosyl hydrolases. The catalytic mechanism of the α -amylase family is that of the α -retaining double displacement. α -retaining mechanism is the characteristic feature of the enzymes from the α -amylase family. They vary widely in their reaction specificities. The attachments of different domains to the catalytic site or to extra sugar binding sub sites around the catalytic site is the prime reason for these differences (Maarel van der *et al.*, 2002). The catalytic domain-A is the most conserved domain in the α -amylase family. It consists of an amino terminal (β/α)₈-barrel structure as revealed in three dimensional structure of amylase from *Aspergillus* sp. (Matsuura *et al.*, 1984).

Structural modifications for cold adaptation: A reduction in temperature, one of the most important environmental factors for life as it influences most biochemical reactions, slows down most physiological processes, changes protein-protein interactions, reduces membrane fluidity and provokes an increased viscosity of water. Moreover, enzymes are also subject to cold denaturation, leading to the loss of enzyme activity at low temperatures (Somero, 1981; Yancey and Somero, 1978). Psychrophilic microorganisms producing cold-active amylases are structurally modified by an increasing flexibility of the polypeptide chain enabling an easier accommodation of substrates at low temperature. They must modify their lipid composition to maintain membrane fluidity at environmental temperatures. This can be done in many ways.

- Unlike cold-adapted proteins which improve their structural mobility, the thermal adaptation of membrane lipids does not involve the synthesis of fatty acyls that have increased degrees of freedom but rather the introduction of steric constraints that reduce the packing of acyl chains in the membrane. These steric constraints destabilize the membrane and reduce the lipid viscosity (Margesin *et al.*, 2002; Russell and Hamamoto, 1998; Russell, 1997)
- Presence of a *cis*-unsaturated double bond in the chain that induces a 30° bend. Such bending creates a cavity in the lipid layer and perturbs the packing density. Trans-unsaturated double bonds are also observed but are less efficient as they only produce a modest kink of the acyl chain
- The occurrence of branched lipids mainly methyl-branched fatty-acyl chains also perturbs the compactness of neighbouring chains owing to the steric hindrance that is caused by the side-chain group. The position of this branching along the chain also modulates the gel-phase transition temperature
- Finally, shorter fatty-acyl chains reduce the contacts between adjacent chains and increase fluidity (Fong *et al.*, 2001; Jagannadham *et al.*, 2000)

Besides the variations in membrane structure and its lipid composition, another important feature in these cold-adapted microorganisms is the presence of Cold-acclimation Proteins (CAPs). These are a set of ~20 proteins which is permanently synthesized during steady-state growth at low temperatures but not at milder temperatures (Hebraud and Potier, 2000; Berger *et al.*, 1996; Hebraud *et al.*, 1994). Interestingly, some of the CAPs that have been identified in cold-adapted bacteria actually acting as cold-shock proteins in mesophiles, such as the RNA chaperone CspA (Berger *et al.*, 1997). It has been proposed that these CAPs are essential for the maintenance of both growth and the cell cycle at low temperatures (Hebraud and Potier, 2000) but their function is still poorly understood. Cold-shock proteins, Csp, are an additional type of adaptation for psychrophilic organisms in cold regions. These proteins act mainly on the regulation of cellular protein synthesis, particularly at the level of transcription and the initiation of translation; and they also act as chaperone by preventing the formation of mRNA secondary structures. Advantage of these Csp's is that the synthesis of housekeeping gene products is not inhibited by cold-shock which is normally occurring in their mesophilic and thermophilic homologues (Cavicchioli *et al.*, 2000; Berger *et al.*, 1996; Mayr *et al.*, 1996).

Antifreeze proteins, AFPs, are more frequently occurring in fishes, insects, plants, fungi and some microorganisms which decrease the freezing point of cellular water by binding to ice crystals and prevent the destruction of cell membranes and the disruption of osmotic balance. Besides contributing to freeze resistance and freeze tolerance, AFPs also helped to increase species diversity in some of the harshest and most inhospitable environments (Barrett, 2001; Jia and Davies, 2002). Although antifreeze proteins have been reported in several eukaryotes, there is no supporting evidence for the occurrence of such glycopeptides in psychrophilic prokaryotes.

Structure of antarctic psychrophile *Alteromonas haloplanctis* α -amylase: The cold-active α -amylase from the Antarctic psychrophile, *Alteromonas haloplanctis* has been studied extensively (Feller *et al.*, 1992). The enzyme has a molecular mass of 49 kDa with few salt bridges, aromatic interactions, small hydrophobic cluster, few arginine residues and weak stabilisation of helix dipoles. It is the first cold-active α -amylase which has been successfully crystallized and the 3-D structure resolved at 1.85 Å (Aghajari *et al.*, 1996; Ramteke and Bhatt, 2007). Also, this α -amylase was successfully expressed in mesophilic host *E. coli* preserving genuine properties of a psychrophilic enzyme (Feller *et al.*, 1998).

The overall fold of *A. haloplanctis* α -amylase is very similar to those reported for mesophilic α -amylases (Aghajari *et al.*, 1998a). Three characteristic domains as well as ion-binding sites are found: domain A (residues 1-86 and 147-356); the central N-terminal domain with a $(\beta/\alpha)_8$ -barrel fold; a minor domain B (residues 87-146, an insertion between α_3 and β_3) that protrudes from domain A and comprises a loop structure, short β strands and a short α helix; and the C-terminal domain C consisting of eight β strands that form a Greek-key motif (the number of β strands in other α -amylases varies from five in barley to ten in human salivary). The largest variations in primary structures between these enzymes from different species have been found in domain C (Jespersen *et al.*, 1993; MacGregor, 1988) and domain B (Janecek *et al.*, 1997) but it should also be mentioned that, throughout the α -amylase family, only eight residues are invariant in the $(\beta/\alpha)_8$ barrel (Svensson, 1994). These include seven residues at the active site and a structurally important glycine. As in the mammalian α -amylases, binding sites for calcium and chloride ions have been located in the structure of *A. haloplanctis* α -amylase. The structure of psychrophilic *A. haloplanctis* α -amylase exposed that amino acid residues (Gln58 and Ala99) replace the cysteines involved in the disulfide bridge between domains A and B (Aghajari *et al.*, 1998b).

Production of cold active α -amylase

Sources of cold active amylases: Cold-active enzymes can be produced by prokaryotic as well as eukaryotic organisms. Till now, most of them originate from bacteria and fish living in polar regions, especially in Antarctic sea water which represent a permanently cold ($0\pm 2^\circ\text{C}$) and constant temperature habitat (Feller *et al.*, 1994). Psychrophilic (cold-loving) or psychrotolerant (cold-adapted) micro-organisms are found inhabiting the low temperature environments of the Earth, including polar regions, high mountains, glaciers, ocean deeps, shallow subterranean systems (i.e., caves), the upper atmosphere, refrigerated appliances and the surfaces of plants and animals living in cold environments, where temperatures never exceed 5°C . In fact, deep oceans which cover over 70% of the Earth's surface, represent the major ecosystem on the planet. Many psychrophiles live in biotopes having more than one stress factors, such as low temperature and high pressure in deep seas (piezo-psychrophiles) or high salt concentration and low temperature in sea ice (halo-psychrophiles). A diverse range of psychrophilic microorganisms, belonging to bacteria, archaea, yeast and fungi have been isolated from these cold environments. These psychrophiles are able to degrade a wide range of polymeric substances such as starch, cellulose, xylan, pectin, chitin, protein and lipid and produce enzymes like amylase, cellulase, xylanase,

pectinases, chitinase, protease and lipase, respectively (Georlette *et al.*, 2004; Feller and Gerday, 2003; Van den Burg, 2003; Margesin *et al.*, 2002; Deming, 2002; Demiorijan *et al.*, 2001; Eichler, 2001; Cavicchioli *et al.*, 2000). In addition, some recent examples of cold-active amylase producing bacteria are shown in the Table 1.

Fermentation conditions for cold active α -amylase production:

Cold active amylases are mostly extra cellular and are highly influenced by nutritional and physicochemical factors such as temperature, pH, agitation, nitrogen source, carbon source, inducers, inorganic sources and dissolved oxygen. To meet the demand of industries, low-cost medium is required for the production of α -amylase. Both Solid State Fermentation (SSF) and Submerged Fermentation (SmF) could be used for the production of α -amylases, although traditionally these have been obtained from submerged cultures because of ease of handling and greater control of environmental factors such as temperature and pH. SSF has been used for long to convert moist agricultural polymeric substrates such as wheat, rice, soy, cassava, etc. (Table 2) into fermented food products including industrial enzymes such as α -amylase (Pandey *et al.*, 1995). SSF is generally defined as the growth of microorganisms on moist solid substrates with negligible free water (Selvakumar *et al.*, 1998). The solid substrate

Table 1: Cold-active amylase producing microbes

Microorganisms	Source	Reference
Beta-proteobacteria	Roopkund Glacier, Himalayan range, India.	Suman <i>et al.</i> (2010)
<i>Nocardopsis aegyptia</i>	Marine sediment of Abu Qir Bay, Alexandria, Egypt	Abou-Elela <i>et al.</i> (2009)
<i>Streptomyces</i> 4Alga	Soil and vegetation (East Antarctica)	Mihaela <i>et al.</i> (2009)
<i>Micrococcus antarcticus</i>	Antarctica	Fan <i>et al.</i> (2009)
Bacterial strains	Sediment samples from Midtre Lovenbreen Arctic glacier	Reddy <i>et al.</i> (2009)
Culturable bacteria	Sediment and a soil from Kongsfjorden and Ny-Alesund, Svalbard, Arctic.	Srinivas <i>et al.</i> (2009)
<i>Nocardopsis</i> sp. 7326	Deep sea sediment of Prydz Bay, Antarctic.	Zhang and Zeng (2008)
<i>Lactobacillus plantarum</i> MTCC 1407	Central Tuber Crop Research Institute, Bhubaneswar, India	Smita <i>et al.</i> (2008)
Amy I and Amy II	Earthworm, Osaka, Japan.	Ueda <i>et al.</i> (2008)
<i>Arthrobacter psychrolactophilus</i> ATCC 700733	Pennsylvania soil	Smith and Zahnley (2005)
<i>Aspergillus oryzae</i>	Culture from University of Agriculture, Abeokuta, Nigeria	Akpan and Adelaja (2004)
Gamma-Proteobacteria	Permanently cold fjords of Spitzbergen, Arctic Ocean	Groudieva <i>et al.</i> (2004)
<i>Aspergillus ochraceus</i>	Soil	Nahas and Waldemarin (2002)
<i>Atheromonas</i> sp.	Antarctic sea water	Chessa <i>et al.</i> (1999)

Table 2: Agro substrates used for α -amylase production

Substrate	Organism	Activity (U g^{-1})	Reference
Wheat bran	<i>Bacillus</i> sp. PS-7	464000	Sodhi <i>et al.</i> (2005)
Rice bran	<i>Bacillus</i> sp. PS-7	145000	Sodhi <i>et al.</i> (2005)
Com bran	<i>Bacillus</i> sp. PS-7	97600	Sodhi <i>et al.</i> (2005)
Coconut oil cake	<i>A. oryzae</i>	3388	Ramachandran <i>et al.</i> (2004)
Spent brewing grain	<i>A. oryzae</i> NRRL 6270	6583	Francis <i>et al.</i> (2003)
Rice husk	<i>B. subtilis</i> 21	760	Baysal <i>et al.</i> (2003)
Amaranthus grains	<i>Aspergillus flavus</i>	1920	Viswanathan and Surlikar (2001)
Maize bran	<i>B. coagulans</i>	22956	Babu and Satyanarayana (1995)
Mustard oil cake	<i>B. coagulans</i>	5953	Babu and Satyanarayana (1995)
Gram bran	<i>B. coagulans</i>	8984	Babu and Satyanarayana (1995)

may provide only support or both support and nutrition. SSF is preferred to SmF because of simple technique, low capital investment, lower levels of catabolite repression and end-product inhibition, low waste water output, better product recovery and high quality production (Zadrazil and Puniya, 1995; Regulapati *et al.*, 2007). Although, emerging trends highlight that semi-solid and solid-state fermentation systems have real prospective for the sensible management of bacteria in industry (Joseph *et al.*, 2011).

Temperature and pH: The influence of temperature on amylase production is related to the growth of the organism. Thus, the optimum temperature for enzyme production depends on whether the culture is mesophilic, thermophilic or psychrophilic. Among the fungi, most amylase production studies have been done within temperature range of 25-37°C (Francis *et al.*, 2003; Ramachandran *et al.*, 2004). Bacterial α -amylases are produced at a much wider range of temperature. *Bacillus amyloliquefaciens*, *B. subtilis*, *B. licheniformis* and *B. stearothermophilus* are among the most commonly used *Bacillus* sp. reported to produce α -amylase at temperatures 37-60°C (Mishra *et al.*, 2005; Mendu *et al.*, 2005; Syu and Chen, 1997; Mielenz, 1983). A cold active α -amylase from Antarctic psychrophile *Alteromonas haloplanktis* was reported to exhibit maximum α -amylase production at 4°C (Feller *et al.*, 1998). pH is also one of the important factors that determine the growth and morphology of microorganisms as they are sensitive to hydrogen ions concentration present in the medium. pH

is known to affect the synthesis and secretion of α -amylase just like its stability. Earlier studies have revealed that fungi required slightly acidic pH and bacteria required neutral pH for optimum growth. Bacterial cultures such as *B. subtilis*, *B. licheniformis* and *B. amyloliquefaciens* required an initial pH of 7.0 (Saban-Tanyildizi *et al.*, 2005; Ikram-ul-Haq *et al.*, 2005; Syu and Chen, 1997). Hyperthermophilic archaee such as *Pyrococcus furiosus*, *P. woesei* and *T. profundus* yielded optimum α -amylase at pH 5.0 (Vieille and Zeikus, 2001). The amylase from *L. brunescens* is active from pH 5.0 to 7.5 (Tigerstrom and Stelmaschuk, 1987). A list of various cold active α -amylase producing psychrophilic and psychrotrophic bacteria and their production parameters are presented in Table 3.

Carbon and nitrogen sources: These are necessary for the growth and metabolism of organisms. Various carbon sources are tried to optimize the maximum production of cold-active α -amylase for different bacterial species (Table 4). For enhanced growth and metabolism of organisms, nitrogen also play very important role just like carbon. A large variety of nitrogen sources for bacterial species are available which give rise to maximum production of cold-active α -amylase (Table 5).

Other fermentative conditions: In the fermentation medium surfactants are known to increase the production of extracellular amylase enzymes by increasing cell membrane permeability. Some common surfactants are Tween 80, polyethylene glycols, Cholic acid etc. which are

Table 3: Production parameters for cold active amylase

Microbes	Incubation period (h)	Optimum temp. (°C)	Optimum pH	Reference
<i>Nocardiopsis caegyptia</i>	48	25	5.0	Abou-Elela <i>et al.</i> (2009)
<i>Streptomyces</i> sp.	60	20	NM	Mihaela <i>et al.</i> (2009)
<i>Micrococcus antarcticus</i>	64	12	8.0	Fan <i>et al.</i> (2009)
<i>A. niger</i>	120	30	5.0	Gupta <i>et al.</i> (2008)
<i>S. albidoflavus</i>	84	30	6.5	Narayana and Vijayalakshmi (2008)
<i>Lactobacillus plantarum</i>	36	35	7.0	Smita <i>et al.</i> (2008)
<i>Arthrobacter psychrolactophilus</i>	NM	22	NM	Smith and Zahnley (2005)
<i>Aspergillus oryzae</i>	NM	4	NM	Akpan and Adelaja (2004)
<i>Aspergillus ochraceus</i>	48	30	5.0	Nahas and Waldemarin (2002)
<i>Flavobacterium balustinum</i> A201	NM	30	NM	Morita <i>et al.</i> (1997)
<i>Bacillus</i> sp. A-001	NM	35	7.5	Lealem and Gashe (1994)

NM: Not mentioned

Table 4: Carbon sources used for maximum α -amylase production

Bacterial species	Best C-source	Reference
<i>Bacillus megaterium</i>	Maltose	Gurudeeban <i>et al.</i> (2011)
<i>B. subtilis</i> IMG22	Starch and Glycerol	Sodhi <i>et al.</i> (2005)
<i>Bacillus</i> sp. PS-7	Starch and Glycerol	Saban-Tanyildizi <i>et al.</i> (2005)
<i>Bacillus</i> sp. I-3	Starch and Glycerol	Goyal <i>et al.</i> (2005)
<i>Thermomyces lanuginosus</i>	Maltodextrin	Nguyen <i>et al.</i> (2000)
<i>Bacillus</i> sp.	Lactose	Hamilton <i>et al.</i> (1999)
<i>B. stearothermophilus</i>	Soluble starch	Srivastava and Baruah (1986)
<i>B. licheniformis</i>	Galactose, glycogen and inulin	Chandra <i>et al.</i> (1980)

Table 5: Nitrogen sources used for maximum α -amylase production

Bacterial species	Best N-source	Reference
<i>Actinomyces</i> AE-19	L-histidine	Poomima <i>et al.</i> (2008)
<i>Bacillus</i> sp. I-3	Soya bean meal	Sodhi <i>et al.</i> (2005), Francis <i>et al.</i> (2003)
<i>B. licheniformis</i> SPT 278	Peptone	Aiyer (2004)
<i>Thermomyces lanuginosus</i>	L-asparagine	Nguyen <i>et al.</i> (2000)
<i>A. oryzae</i> A1560	Casein hydrolysate	Pedersen and Nielson (2000)
<i>B. amylolyticus</i>	Peptone and yeast extract	Dettoni <i>et al.</i> (1992)

Table 6: Methods of one-step purification of α -amylases

Method	Adsorbent	Yield (%)	Purification fold	Reference
Affinity adsorption chromatography	Cyclodextrin-iminodiacetic acid	95	-	Liao and Syu (2005)
Substitute affinity method	Insoluble corn starch at 4°C	78	163	Najafi and Kumbhavi (2004)
Expanded bed chromatography	Alginate acid-cellulose cell beads	69	51	Amritkar <i>et al.</i> (2004)
Magnetic affinity adsorption	Magnetic alginate microparticles	88	9	Safarikova <i>et al.</i> (2003)

used in different concentrations. Supplementation of salts of certain metal ions provided good growth of microorganisms and thereby better enzyme production (as most α -amylases are known to be metalloenzymes). Some frequently used metal ions are CaCl_2 , NaCl , MgSO_4 , FeCl_3 , Mn^{2+} , Zn^{2+} etc. In SSF system some additional fermentative conditions plays very vital role viz. selection of a substrate and microorganism; pre-treatment of the substrate; particle size (inter-particle space and surface area) of the substrate; water content; relative humidity; type and size of the inoculum; removal of metabolic heat; period of cultivation; maintenance of uniformity in the environment of SSF system and the gaseous atmosphere, i.e. oxygen consumption rate and carbon dioxide evolution rate.

Purification and characterization of cold active α -amylases: Most of the purification schemes for amylases are based on multistep strategies. However, in these years new techniques have been developed that may yield high recovery. Based on the nature of amylase produced by the organism one has to design the protocol for purification and in the downstream processing the purification process depends on the market need, processing cost, final quality and available technology. There are also various methods are available in which α -amylases can be purified in one-step. The necessity for low cost, large-scale, effective purification of enzymes has resulted in progression of techniques that provide rapid, competent and economical protocols in fewer processing steps. Few purification techniques that produce homogeneous preparation of α -amylases in a single step are given in Table 6.

The purification efficiency is determined by total yield and purification factor (Kademi *et al.*, 2005). Pre-purification steps involve concentration of the protein containing amylases by ammonium sulphate precipitation and ultra filtration by dialysis. The characterization of cold active α -amylases can be studied in terms of optimum pH and pH stability, optimum temperature and

thermo-stability and effect of metal ions, chelating agents, inhibitors, nature of substrate, substrate concentration, enzyme concentration, solvents and stabilizing agents. One can think that whether cold-active enzymes do not follow the general principles of biochemistry viz., enzyme activity generally decreases approximately one-half with each decrease of 10°C but in actual fact they also do not disobey this concept rather they simply shift their peak activities to temperature ranges lower than those generally observed for enzymes from mesophilic organisms; just as enzymes from thermophiles often have optimal activity at temperatures higher than found for mesophilic enzymes (Feller and Gerday, 2003). The optimal activity temperature of *Nocardia* sp. 7326 amylase was 35°C and the enzyme was stable between pH 5 and 10 with maximal activity at pH 8 (Zhang and Zeng, 2008). The activity of α -amylase from *Streptococcus bovis* JB1 was optimal at pH 5 to 6. The enzyme was relatively stable at temperatures below 50°C (Freer, 1993). Ueda *et al.* (2008) purified and characterized the novel cold-adapted α -amylase (Amy I and Amy II) which are most active at pH 5.5 and stable at pH 7-9. Both Amy I and II exhibited activities at 10°C. The optimal temperature and pH for the purified amylase from *Micrococcus antarcticus* were 30°C and 6, respectively (Fan *et al.*, 2009). It still showed high activity at low temperature 10-15°C. It was sensitive to high temperature but was stable at pH 6.0-10.0 with at least 70% activity remained.

Biotechnological approaches in cold active α -amylase:

An emerging area of research in the field of enzymology is to develop radically different and novel biocatalysts through various molecular approaches including r-DNA technology, protein engineering, directed evolution and the metagenomic approach. As a whole, amylase biotechnology has just reached the end of lag phase and the beginning of the exponential phase: it demands extension in terms of both quality and quantity. Qualitative improvements in restructuring amylase gene and its protein can be achieved by employing already

established r-DNA technology and protein engineering. Quantitative enhancement needs strain improvement, especially through site-directed mutagenesis and standardizing the nutrient medium for the overproduction of cold active α -amylase.

Gene cloning: To date, a very few number of cold active α -amylase genes were isolated and the related studies have been carried out. Early successes in the production of heterologous proteins were achieved using *Escherichia coli* as host and various kinds of proteins were expressed in *E. coli*. However, expression of eukaryotic proteins in *E. coli* became very difficult due to formation of inclusion bodies, protein misfolding and safety issues. Other expression systems were developed among yeasts, fungi, plants and animals. Cloning and expression of the cold-active amylase gene from *Alteromonas haloplanctis* has been reported (Feller *et al.*, 1992, 1998). The α -amylase secreted by the Antarctic bacterium *Alteromonas haloplanctis* displays 66% amino acid sequence similarity with porcine pancreatic α -amylase. The psychrophilic α -amylase is however characterized by a seven fold higher k_{cat} and k_{cat}/K_m values at 4°C and a lower conformational stability with respect to the porcine enzyme.

Protein engineering: Psychrophilic organisms and their enzymes have, in recent years, increasingly attracted the attention of the scientific community due to their peculiar properties that render them particularly useful in investigating the possible relationship existing between stability, flexibility and specific activity and as valuable tools for biotechnological purposes. Although α -amylase carry significant commercial value, biotechnologically produced or engineered cold active α -amylases may represent the focus of industrial interest in future. Cold active α -amylases could generate avenues for industrial applications, once their specific properties are improved through enzyme engineering. Determination of three-dimensional structures of more cold active amylases would allow the detailed analysis of protein adaptation to temperatures at molecular level. This may include increased thermolabile nature and/or catalytic activity at low temperatures or the modification of pH profiles. Cold active α -amylases from microorganisms retaining high catalytic activity at low temperatures are successfully produced using site directed mutagenesis and directed evolution. α -amylase from the Antarctic psychrophile *Alteromonas haloplanctis* is synthesized at 0±2°C by the wild strain. This heat-labile α -amylase folds correctly when over expressed in *Escherichia coli* (Feller *et al.*, 1998). It was found that this heat-labile enzyme is the

largest known multi domain protein exhibiting a reversible two-state unfolding (Feller *et al.*, 1999).

Patents in cold active α -amylases: The number of companies involved in funding cold α -amylase research, nevertheless the high risk and cost concerned of this unexplored field. Even though some noteworthy discoveries based on Antarctic amylases and with potential commercial applications were made in collaboration with industrial partners. One important patent is concern to *B. licheniformis* amylase in which specific activity was increased at temperatures from 10 to 60°C (US Patent number 6673589) by Borchert and coworkers with industrial partner Novozymes. Most patents are process, rather than product based on an isolate from an organism. Though, it appears that none of these discoveries has led to commercialization yet.

Applications of cold active α -amylases

Desizing operation (removal of starch) in textile industry:

The desizing of grey fabric with crude amylase is very promising in textile industry. The strength of the textile is improved by warping the starch paste to textile weaving. After weaving the cloth, the starch is usually removed by application of α -amylase and goes to scouring and dyeing. By using cold-active α -amylase the required temperature may be decreases that will reduce energy consumption. It was observed that the desizing of fabric increased with increase in enzyme concentration up to certain limit. But as the concentration of the enzyme increased than the optimum level, it showed adverse effects on the desizing of the fabric. It might be due to the fact that appropriate enzyme to substrate ratio is essential to obtain optimal results (Allan *et al.*, 1997).

Application in detergent industry for cold washing:

Cold-active enzymes can be very useful for domestic processes. The activity at low temperature indicates the possibilities of these enzymes as a detergent additive for cold washing. Washing with detergents also use a lot of energy particularly when done at high temperature because peroxide-based bleaches need higher temperature (60°C) to work properly. Thus, lowering the wash temperature by using cold-active enzymes can save lots of energy and may be used to protect environment because it is biodegradable (Kuddus and Ramteke, 2009). Cold-active alkaline amylases can be used in detergents since washing clothes at low temperatures protect the colors of fabrics and reduce energy consumption.

Direct fermentation of starch to ethanol: For large-scale processing, the bioconversion of biomass resources,

especially starchy materials, to ethanol, is very useful because it can be used as a biofuel and as the starting material for various chemicals. However In the present scenario its cost of production is very high because of three main reasons; ethanol production from starchy materials via fermentation consists of two or three steps, large quantity of α -amylase is needed and starchy materials need to be cooked at a high temperature (140 to 180°C). However methods are developed in which cost of production can be minimized; by fermenting starch to ethanol in one step using co-cultures of two different strains and by using low-temperature-cooking fermentation systems (that succeeded in reducing energy consumption by approximately 50%) (Matsumoto *et al.*, 1985, 1982).

Manufacture of maltotetraose syrup: Maltotetraose syrup (G4 syrup) is produced by breaking of starch into maltotetraose by the action of amylase enzyme. The sweetness of the syrup is as low as 20% of sucrose. Therefore in foods, G4 syrup can be successfully used in place of sucrose which reduces the sweetness without altering their inherent taste and flavor. It has high moisture retention power which maintains integrity of starch particles and retains suitable moisture in foods. G4 syrup improves the food texture because of its high viscosity than sucrose. It further lowers down the freezing point of water than sucrose or high fructose syrup, so can be used to control the freezing points of frozen foods (Aiyer, 2005).

Manufacture of maltose: Maltose, a disaccharide, is made up of its monomeric units called glucose. It is the main component of maltosugar syrup (Sugimoto, 1997). Maltose is commonly used as sweetener and also as intravenous sugar supplement. It has a great value in food industries since it is non-hygroscopic and does not easily crystallize. For the manufacturing of maltose potato, sweet potato, corn and cassava starches are frequently used. Cold-active α -amylase from psychrophilic organisms may be used.

Manufacture of high molecular weight branched dextrins: High molecular weight branched dextrins are used as extender for production of powdery foods. These are produced by the action of α -amylase on corn starch. Degree of hydrolysis depends on the type of starch and the physical properties desired. Branched dextrins can be collected as powder after chromatography and spray drying.

Treatment of starch containing waste water: Food processing wastewater offers a unique challenge to any treatment system. Biotechnological treatment of food

processing starch waste water can produce valuable products such as microbial biomass protein and also purifies the effluent at low temperature (Aiyer, 2005).

Other applications: In food industry cold active α -amylase can be used for the reduction of haze formation in juices and retardation of staling in baking industry. Cold active α -amylase is also very useful for paper industry as it reduces the viscosity of starch for appropriate coating of paper. In pharmaceutical industry they can be used as a digestive aid. Psychrophilic microorganisms have also been proposed for the bioremediation of polluted soils and waste waters during the winter in temperate countries, when the degradative capacity of the endogenous microflora is impaired by low temperatures. Glycosidases are often used in the baking industry but can retain residual activity after cooking that alters the structure of the final product during storage; this can be avoided by the use of psychrophilic glycosidases. Lactose intolerance is a problem for approximately two-thirds of the world's population. The removal of lactose from milk by a psychrophilic β -galactosidase during cold storage has recently been patented. An important achievement in the field has been the construction of a host-vector system that allows the over expression of genes in psychrophilic bacteria (Tutino *et al.*, 2001): expression at low temperatures prevents the formation of inclusion bodies and protects heat-sensitive gene products. Using enzymes with high activity below 20°C in food processing to limit the growth of other contaminating microorganisms, shorten the process times and avoid designing expensive heating steps. Cold-active α -amylases could be used in the brewing industry to speed the mashing phase at low temperatures. Psychrophilic microorganisms and their enzymes are already crucial to nutrient cycling and biomass degradation and production. We can take advantage of the natural role of psychrophiles and use ones producing useful enzymes in waste-water treatment and biopulping in cold climates (Joseph *et al.*, 2008). Cold active enzymes, as a result of high catalytic efficiency and unique specificity at low and moderate temperatures and its producing microorganisms may also be ideal for bioremediation purposes (Ramteke *et al.*, 2005).

Conclusions and future prospects: Analysis of the literature reveals that cold-active enzymes offer several advantages over mesophilic/thermophilic enzymes. Cold active α -amylases are promising enzymes to replace the conventional enzyme processes of the biotechnological industries. Although, a more extensive exertion is required to overcome several bottlenecks such as high enzyme cost, low activity and/or stability under environmental conditions and the low biodiversity of

psychrophilic/psychrotrophic microbes explored so far. The comparatively latest introduction and progress of novel r-DNA technologies such as, metagenomics and site-directed mutagenesis have an intense positive effect on the expression and production of greater and greater amounts of recombinant proteins which means more competitive prices, by introducing new or tailored catalytic activities of these enzymes at low temperature. Therefore, efforts have to be made in order to achieve economical over production of cold active α -amylase in heterologous hosts and their alteration by chemical means or protein engineering to obtain more robust and active amylases. Genetically improved strains, appropriate for specific cold-active enzyme production, would play an important role in various industrial and biotechnological applications.

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