

Review

Structural adaptation and biocatalytic prospective of microbial cold-active α -amylase

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Amylases have most widely been reported to occur in microorganisms, although they are also found in plants and animals. Microbial cold-active α -amylases confer high activities at low temperature that favours production of comparatively insubstantial compounds. Cold-active α -amylases have an advantage under extreme low temperature conditions due to their freeze tolerance mechanism, intrinsic greater membrane fluidity and production of cold-acclimation proteins. Due to considerable progress towards energy savings process in industries, the low temperature stability has been regarded as the most important characteristics of cold-active amylases. Cold active enzymes attract more and more attention nowadays. Comparatively little information is available on cold-active microbial α -amylases and its catalytic properties. Now the situation is changing which recently enchanted the scientific community to focus in various fields, such as analytical, medicinal and clinical chemistry, as well as their extensive industrial applications such as starch industry, food processing, additive in detergents, waste-water treatment, biopulping, textile industry, environmental bioremediation in cold climates and other molecular biology applications.

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

INTRODUCTION

Extremophiles are structurally adapted at the molecular level to withstand with extreme environments such as high or low temperature, high or low pH, high pressure, high salinity, low oxygen etc., and among them biocatalysts play a major role which is called as extremozymes produced by the microorganisms. There are various extremozymes including amylases which have great potentials in various industrial and biotechnological processes. Approximately 85% of the Earth is occupied by cold ecosystems out of which ~70% is covered by oceans and remaining ~15% included Polar Regions. Extremophiles effectively colonized on these cold environments and called as psychrophiles (Feller

and Gerday, 2003). However, according to classical definition of Morita psychrophilic microorganisms have optimum growth temperature of $<15^{\circ}\text{C}$ and a psychrotropic term is used for those cold-adapted organisms that have an optimum growth temperature of $\sim 15\text{-}20^{\circ}\text{C}$ but are able to grow up to 30°C (Morita, 1975).

Alpha amylases (EC 3.2.1.1) belong to the enzyme class of hydrolases which randomly cleaves the 1, 4- α -D-glucosidic linkages between the adjacent glucose units in linear amylase chain of starch. The specificity of the bond attacked by α -amylases depends on the source of the enzyme. 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 α -1, 4 bonds and belongs to the liquefying category. Alternatively, the fungal α -amylase belongs to the saccharifying category and attacks the second linkage from the non-reducing (C4) end of the

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straight chain, resulting in a disaccharide called maltose. Cold-adapted amylolytic microorganisms produce cold-active amylases, which function effectively at low temperatures with high rates of catalysis in comparison to the amylases from mesophiles or thermophiles, which shows little or no activity at low temperature. Therefore, cold-active α -amylases have emerged as one of the prime biocatalyst having broad potential in industrial applications, such as additives in processed food, in detergent for cold washing, waste-water treatment and bioremediations in cold climates and in molecular biology applications.

COMPARISON BETWEEN GENERAL AND COLD-ACTIVE α -AMYLASE

Structure of general α -amylase

Alpha amylase is classified as family 13 of the glycosyl hydrolases which is the largest family of glycoside hydrolases, transferases and isomerases comprising nearly 30 different enzyme specificities (Henrissat, 1991). The first reported structure in the α -amylase family is Taka Amylase A (Matsuura et al., 1979, 1984). The molecular structure of Taka-amylase A from *Aspergillus oryzae*, has been studied at 6 Å resolution by X-ray diffraction analysis. The molecule was an ellipsoid and contains a hollow which may correspond to the active centre. It consists of 478 amino acid residues which contain glutamic acid (Glu230) and aspartic acid (Asp297) as catalytic residues (Matsuura et al., 1984). The general structure of α -amylase is shown in Figure 1a. The catalytic mechanism of the α -amylase family is that of the α -retaining double displacement. They vary widely in their reaction specificities. The attachment of different domains to the catalytic site is the prime reason for these differences (van der Maarel et al., 2002).

Structural modifications in microbes for cold adaptation

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. This can be done in many ways; (a) 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 it is 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 et al., 1998; Russell and Hamamoto,

1997); (b) 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; (c).

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; (d) finally, shorter fatty-acyl chains reduce the contacts between adjacent chains and increase fluidity (Fong et al., 2001; Jagannadham, 2000).

In addition to variations in membrane structure and its lipid composition, another significant 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. 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. 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 cold-active 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

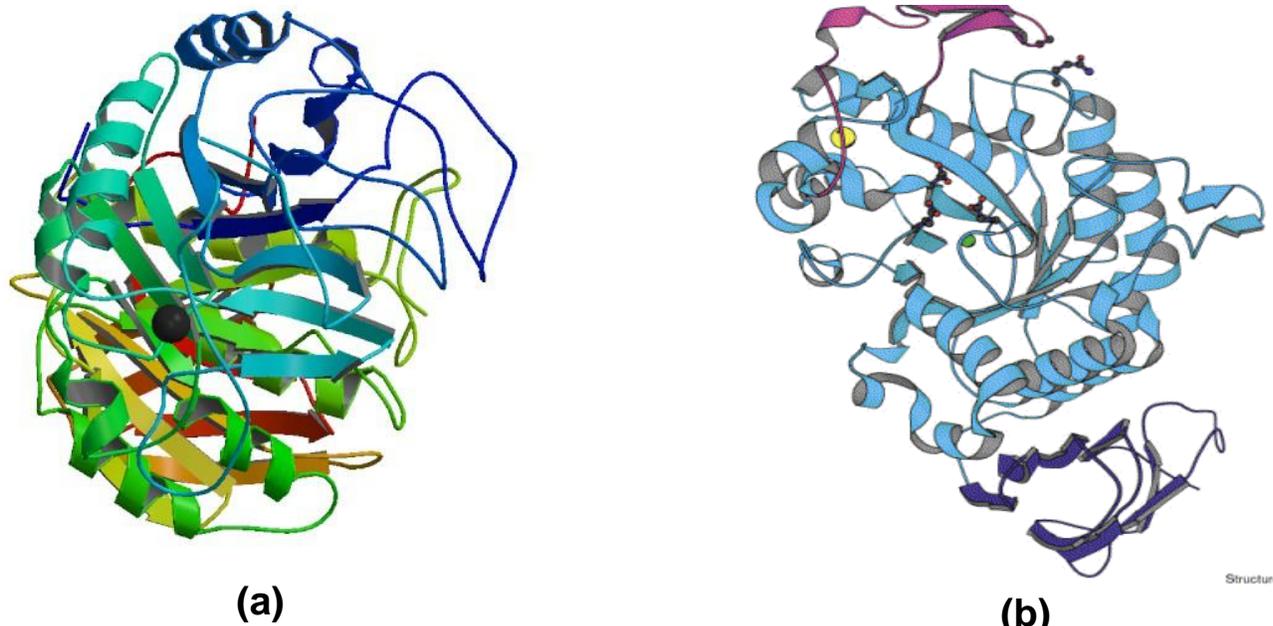


Figure 1. (a) The three dimensional structures of α -amylase from *Aspergillus* sp. (Matsuura et al., 1984); (b) Overall structure of psychrophilic AHA with residues (Gln58 and Ala99) that replace the cysteines involved in the disulfide bridge between domains A and B in MAA highlighted (in the upper right part of the figure). Domain A is colored in cyan, domain B in pink and domain C in blue. The active site with the three catalytic amino acids is shown, as are the calcium ion (yellow sphere) and chloride ion (green sphere) (Aghajari et al., 1998a).

crystallized and the 3-D structure resolved at 1.85 Å (Aghajari et al., 1996). 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., 1998). 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 (Figure 1b) (Aghajari et al., 1998a).

SOURCES AND FERMENTATION CONDITIONS FOR COLD ACTIVE α -AMYLASE PRODUCTION

Cold active α -amylase can be produced by prokaryotic as well as eukaryotic organisms. Most of the amylases produced from bacteria and fish living in Polar Regions, especially in Antarctic sea water (Feller, 1994). Psychrophilic/ psychrotolerant micro-organisms are found inhabiting the low temperature environments of the Earth, where temperatures never exceed 5°C such as polar regions, glaciers, ocean deeps, high mountains, refrigerated appliances and the surfaces of plants and animals living in cold environments. In fact, deep oceans, which cover over 70% of the Earth's surface, represent the major ecosystem on the planet. A diverse range of cold adapted microorganisms have been isolated from these cold environments which are able to degrade wide range of polymers 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; Burg, 2003; Margesin et al., 2002; Deming, 2002; Demirjian et al., 2001; Eichler, 2001). Some latest examples of cold-active amylase producing microbes are shown in the Table 1.

Most of the cold-active amylases are extracellular and the production are extremely influenced by physicochemical and nutritional factors such as pH,

Table 1. Source and nutritional requirements of cold-active amylase producing microorganisms.

Microbes	Microbial source	Best C-source	Best N-source	Reference
<i>Microbacterium foliorum</i> GA2	Gangotri glacier, India	Lactose	Yeast extract	Roohi et al. (2011)
<i>Wangia</i> sp. C52	Southern Okinawa	NM	NM	Liu et al. (2011)
β -proteobacteria	Roopkund Glacier, Himalayan range, India.	NM	NM	Suman et al. (2010)
<i>P. arctica</i> GS230	Seawater from Gaogong island, China	Soluble starch	Beef extract	Lu et al. (2010)
<i>Nocardioopsis aegyptia</i>	Abu Qir Bay, Alexandria, Egypt	NM	NM	Abou-Elela et al. (2009)
<i>Streptomyces</i> 4Alga	Soil and vegetation (East Antarctica)	NM	NM	Mihaela et al. (2009)
<i>Micrococcus antarcticus</i> Bacterial strains	Antarctica Midtre Lovenbreen Arctic glacier	NM NM	NM NM	Fan et al. (2009) Reddy et al. (2009)
Culturable bacteria	Sediment and soil from Svalbard, Arctic.	NM	NM	Srinivas et al. (2009)
<i>Nocardioopsis</i> sp. 7326	Deep sea sediment of Prydz Bay, Antarctic.	NM	NM	Zhang and Zeng (2008)
<i>L. plantarum</i> MTCC 1407	CTCRI, Bhubaneswar, India	NM	NM	Smita et al. (2008)
<i>Eisenia foetida</i>	Earthworm, Osaka, Japan.	NM	NM	Ueda et al. (2008)
<i>Arthrobacter psychrolactophilus</i>	Pennsylvania soil	NM	NM	Smith and Zahnley (2005)
<i>Bacillus</i> sp. PS-7	NM	Starch and Glycerol	Peptone	Tanyildizi et al. (2005)
<i>Bacillus</i> sp. I-3	NM	Starch and Glycerol	NM	Goyal et al. (2005)
Gamma-Proteobacteria	Spitzbergen, Arctic Ocean.	NM	NM	Tatiana et al. (2004)
<i>B. licheniformis</i> SPT 278	NM	NM	Peptone	Aiyer (2004)
<i>Aspergillus ochraceus</i>	Soil	NM	NM	Nahas and Mirela (2002)
<i>Thermomyces lanuginosus</i>	NM	Maltodextrin	Asparagine	Nguyen et al. (2000)
<i>A. oryzae</i> A1560	NM	NM	Casein hydrolysate	Pederson and Nielson (2000)

NM = Not mentioned

temperature, agitation, carbon source, nitrogen source, inducers, inorganic sources and dissolved oxygen. However, to meet the demand of industries, low-cost medium is required for the production of α -amylase by using solid state and submerged fermentation. Usually

submerged fermentation has been used due to easy handling and greater control on environmental factors. Solid state fermentation has been used for long to convert moist agricultural polymeric substrates into industrial enzymes such as α -amylase (Pandey et al.,

Table 2. Recent literatures showing parameters for cold-active amylase production.

Microbes	Incubation period (h)	Optimum temp. (°C)	Optimum pH	Activity (unit)	Reference
<i>M. foliorum</i> GA2	120	20	9	5870	Roohi et al. (2011)
<i>P. arctica</i> GS230	24	20	8	780.4	Lu et al. (2010)
<i>N. aegyptia</i>	48	25	5	2255	Abou-Elela et al. (2009)
<i>M. antarcticus</i>	64	12	8	2.6	Fan et al. (2009)
<i>L. plantarum</i>	36	35	7	4022	Smita et al. (2008)
<i>A. psychrolactophilus</i>	-	22	-	-	Smith and Zahnley (2005)
<i>A. ochraceus</i>	48	30	5	56.7	Nahas and Mirela (2002)

1995). The optimum temperature for production of amylase depends on whether the culture is mesophilic, thermophilic or psychrophilic because temperature affects growth of the organisms. In comparison to fungi bacterial α -amylases are produced at a much wider range of temperature (Mishra et al., 2005; Mendu et al., 2005; Syu and Chen, 1997). A cold-active α -amylase from Antarctic psychrophile *A. haloplanktis* was reported to exhibit maximum α -amylase production at 4°C (Feller et al., 1998). pH is also known to affect the synthesis and secretion of α -amylase as the growth of microorganisms is sensitive to hydrogen ions concentration present in the medium. The literature revealed that fungi required slightly acidic pH and bacteria required neutral pH for optimum growth (Kuddus et al., 2011). Various parameters for cold active α -amylase production are given in Table 2.

Carbon and nitrogen source are essential for the growth and metabolism of microorganisms. There are different carbon and nitrogen source which give rise to maximum production of cold-active α -amylase (Table 1). In addition, surfactants are also known to increase the production of extracellular cold-active amylase by increasing permeability of cell membrane. Some common surfactants are Tween 80, polyethylene glycols, Cholic acid, etc. Supplementation of salts of certain metal ions also provided good microbial growth and thereby better amylase production. Some frequently used metal ions are CaCl_2 , NaCl , MgSO_4 , FeCl_3 , Mn^{2+} , Zn^{2+} , etc. In solid state fermentation some additional conditions plays very crucial role viz. pre-treatment of the substrate; particle size of the substrate; relative humidity; type and size of the inoculum; period of cultivation, and the gaseous atmosphere, that is, oxygen consumption and carbon dioxide evolution rate.

PURIFICATION OF COLD ACTIVE α -AMYLASES

Based on the nature of amylase produced by the organism one has to design the protocol for purification. Most of the purification processes are based on multistep strategies. However, in these years new techniques have been developed that may yield high recovery. Few

purification techniques that produce homogeneous preparation of α -amylases in a single step are reviewed (Kuddus et al., 2011). Pre-purification steps involve concentration of the protein containing amylases by salt precipitation followed by dialysis. The efficiency of purification is determined by total yield and purification factor (Kademi et al., 2005). Characterization of cold active α -amylases can be studied in terms of optimum pH and pH stability, optimum temperature and thermostability 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).

MOLECULAR APPROACHES IN COLD ACTIVE α -AMYLASE

In these years an emerging area of enzyme research is to develop radically different and novel biocatalysts through various molecular approaches including r-DNA technology, protein engineering, directed evolution and the metagenomic approach. 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. So far very few number of cold-active α -amylase genes is isolated and the related studies have been carried out. Early successes in heterologous proteins production were achieved using *Escherichia coli* as host and different kinds of proteins are expressed.

Cloning and expression of the cold-active amylase gene from Antarctic bacterium *A. haloplanctis* has been reported (Feller et al., 1992, 1998). The secreted psychrophilic α -amylase displays 66% amino acid sequence similarity, seven fold higher k_{cat} and k_{cat}/K_m values and lower conformational stability with respect to porcine pancreatic α -amylase. Although α -amylase carry significant commercial value in recent years, 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/protein engineering. This may include analysis of protein adaptation to temperatures, 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 *A. haloplanctis* is synthesized at $0\pm 2^\circ\text{C}$ by the wild strain (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. 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 co-workers 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

Detergent additive for cold washing

The activity of α -amylases at low temperature indicates the potential of these enzymes as a detergent additive for cold washing that may be useful for domestic processes. Washing with common 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 (Kuddus et al., 2011). Therefore, lowering the wash temperature by using cold-active amylase can reduce energy consumption, protect the colours of fabrics and may be used to protect environment because it is biodegradable (Kuddus and Ramteke, 2009).

Starch removal in textile industry

In textile industry, crude α -amylase has promising potential for desizing of grey fabric. The strength of the textile is improved by warping the starch paste to textile weaving which is usually removed after weaving by using α -amylase and goes to scouring and dyeing. The required temperature may be decreases by using cold-active α -amylase that will reduce energy consumption (Allan and Henrik, 1997).

Bioremediation in cold environment

Industrial and household waste water offers a unique challenge to any treatment system. Treatment of food processing starch waste water by using cold-active α -amylase may produce valuable products such as microbial biomass protein and also purifies the effluent at low temperature (Aiyer, 2005). In fiber recycling mills starches, used for sizings, can accumulate in the waste water and obstruct the drainage. Reducing the viscosity of the backwater by using cold-active α -amylase can improve drainage system. 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.

Production of maltose and maltotetraose syrup

Maltose, a disaccharide made from glucose units, is the main component of maltosugar syrup (Sugimoto, 1977). It is commonly used as sweetener and has a great value in food industries because it is non-hygroscopic and does not crystallize easily. For the production of maltose from potato, sweet potato, corn and cassava starches cold-active α -amylase may be used. Maltotetraose syrup may be produced by the action of cold-active amylase that breaks starch into maltotetraose. It can be used successfully as a sweetener in place of 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).

Other applications

In pharmaceutical and food industry cold active α -amylases can be used as a digestive aid and for the reduction of haze formation in juices, respectively. Cold-active α -amylases could be used in the brewing industry to speed the mashing phase at low temperatures. 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. These enzymes are also very useful for paper industry as it reduces the viscosity of starch for appropriate coating of paper. 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, biopulping and bioremediation in cold climates (Joseph et al., 2008).

FUTURE PROSPECTS

The review of literatures concluded that cold active α -amylases are promising enzyme to replace the conventional enzyme processes of the biotechnological industries as they offer several advantages over mesophilic/thermophilic enzymes. However, more extensive efforts are 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. Comparatively, introduction of novel recombinant DNA technologies such as, metagenomics and site-directed mutagenesis have a tremendous positive effect on the expression and production of more recombinant proteins, having specific catalytic activities at low temperature. Accordingly, efforts have to be made in order to achieve inexpensive over production of cold active α -amylases in heterologous hosts and their alteration by protein engineering to obtain more specific and active amylases. Genetically improved strains, appropriate for specific cold active α -amylase production, would play a significant role in various biotechnological and industrial applications.

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