Microbial cold-active α-amylases: From fundamentals to recent developments

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Alpha-amylases randomly cleave the 1,4-α-D-glycosidic linkages between the adjacent glucose units in linear amylose chain of starch. Cold-active α-amylases isolated from living and metabolically active psychro-tolerant microorganisms confers low activation energies and high activities at low temperature which are favorable properties for the production of relatively insubstantial compounds and therefore enzymatically driven reactions can be carried out at low temperature. The low temperature stability of cold-active amylases has regarded as the most important characteristics for use in industry because of considerable progress towards energy savings but unfortunately these enzymes have lately attracted attention and their diversity, physiology and potential have largely been overlooked. 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. Cold-adapted microorganisms are potential source of cold-active amylases and they have been isolated from cold regions including Antarctic continent and studied. Amylase isolated from different sources has a wide range of properties depending on their sources with respect to positional specificity, thermo-stability, pH optimum etc. Cold-active amylases cover a broad spectrum of biotechnological applications like additives in processed food industries, additives in detergents, waste-water treatment, biopulping, bioremediations in cold climates and molecular biology applications. Thus, Cold-active amylases are becoming promising enzymes to replace successfully the conventional enzyme for biotechnological industries running at low temperature and serve as a world-wide choice for biotechnologists, microbiologists, biochemists, pharmacists, biochemical and process engineers.

Keywords: extremozymes; cold-active amylase; starch degrading enzymes; applications

1. 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 tension, 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 that function under extreme conditions. There are various extremozymes like cellulases, amylases, xylanases, proteases, pectinases, keratinases, lipases, esterases, catalases, peroxidases and phytases, which have great potential for application in various biotechnological processes. Currently, only 1-2 % of the microorganisms on the Earth have been commercially exploited and amongst these there are only a few examples of extremophiles [1]. 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 below a depth of 1,000 m, 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 (which literally means cold-loving) [2]. The classical definition of psychrophiles is given by Morita [3] 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.
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 metalloenzymes, which require calcium ions (Ca^{2+}) for their activity, structural integrity and stability. They belong to family 13 (GH-13) of the glycoside hydrolase group of enzymes [4]. 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. The hydrolysis reaction catalyzed by this class of enzymes is usually carried out only to the extent that, for example, the starch is rendered soluble enough to allow easy removal from starch-sized fabrics in the textile industry. The paper industry also uses liquefying amylases on the starch used in paper coating where breakage into the smallest glucose subunits is actually undesirable. 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 biocatalyst 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 molecular biology. These enzymes account for about 30 % of the world's enzyme production [5].

2. Cold active amylases

Cold-adapted amylolytic microorganisms produce amylases, which function effectively at cold temperatures with high rates of catalysis in comparison to the amylases from mesophiles or thermopiles, 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. The knowledge of cold active amylolytic enzymes is increasing at a rapid and exciting rate.

3. Structural features of α-amylase

3.1. General α-amylase structure

The α-amylase family is the largest family of glycoside hydrolases, transferases and isomerases comprising nearly 30 different enzyme specificities [6]. 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 [5]:

a) endoamylases: cleave internal α-1,4 bonds resulting in α-anomeric products,
b) exoamylases: cleave α-1,4 or α-1,6 bonds of the external glucose residues resulting in α- or β anomeric products,
c) debranching enzymes: hydrolyze α-1,6 bonds exclusively leaving long linear polysaccharides, and
d) 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 [5]**.** The catalytic domain-A is the most conserved domain in the α-amylase family. It consists of an amino terminal $(\beta/\alpha)_8$-barrel structure. The structure of amylase is shown in Figure 1.

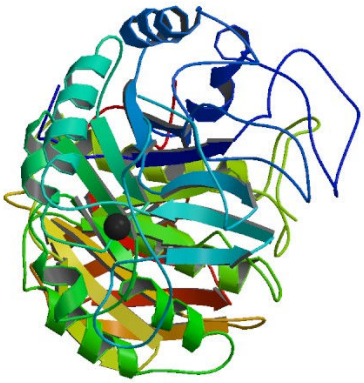

Fig. 1 The three dimensional structures of amylase from *Aspergillus* sp. [7]

3.2. Structural modifications for cold adaptation

Temperature is one of the most important environmental factors for life, as it influences most biochemical reactions. A reduction in temperature 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 [8-9]. 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;

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 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 [10-12].
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 [13-14].

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 [15-17]. Interestingly, some of the few CAPs that have been identified in cold-adapted bacteria actually acting as cold-shock proteins in mesophiles, such as the RNA chaperone CspA [18]. It has been proposed that these CAPs are essential for the maintenance of both growth and the cell cycle at low temperatures [15] 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 [19, 17, 20].

Antifreeze proteins, AFPs, (are peptides and glycopeptides of various sizes) 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 [21-22]. Although antifreeze proteins have been reported in several eukaryotes, there is no supporting evidence for the occurrence of such glycopeptides in psychrophilic prokaryotes.

3.3. Structure of Antarctic psychrophile *Alteromonas haloplanctis* α-amylase

The cold-active α-amylase from the Antarctic psychrophile, *Alteromonas haloplanctis* has been studied extensively [23]. 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 [24]. Also, this α-amylase was successfully expressed in mesophilic host *E. coli* preserving genuine properties of a psychrophilic enzyme [25].

The overall fold of *A. haloplanctis* α-amylase is very similar to those reported for mesophilic α-amylases [26]. 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 (residues 357-453, with the last five residues, 449-453, not visible in the electron density in AHA_{wt} as well as in AHA_r) 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 [27-28] and domain B [29], but it should also be mentioned that, throughout the α-amylase family, only eight residues are invariant in the $(\beta/\alpha)_8$ barrel [30]. 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 2).

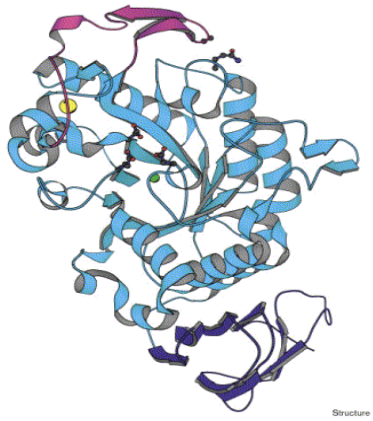

Fig. 2 Overall structure of psychrophilic *A. haloplanctis* α-amylase. The active site with the three catalytic amino acids is shown, as are the calcium ion (yellow sphere) and chloride ion (green sphere) [32].

4. Production of cold active α-amylase

4.1. Sources of cold active amylases

Enzyme adapted to cold can be produced by prokaryotic as well as by eukaryotic organisms. Up to now, most of them originate from bacteria and fish living in polar regions and especially in Antarctic sea water which represent a permanently cold (0±2 °C) and constant temperature habitat [33]. 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. The potentials of psychrophiles and psychrophilic enzymes have been reviewed. 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 [2, 10, 19, 34]. 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 [2, 10, 19, 34-38]. In addition, some recent examples of cold-active amylase producing bacteria are shown in the Table 1.

Table 1 Cold-active amylase producing microbes

Microorganisms	**Source**	**Reference**
Alteromonas sp.	Antarctic sea water	[39]
Amy I and Amy II	Earthworm, Osaka, Japan.	[40]
Arthrobacter psychrolactophilus ATCC 700733	Pennsylvania soil	[41]
Aspergillus oryzae	University Culture Collection Center, University of Agriculture, Abeokuta, Nigeria	[42]
Aspergillus ochraceus	Soil	[43]
Bacillus sp. A-001	Fermenting tef	[44]
Lactobacillus plantarum MTCC 1407	Central Tuber Crop Research Institute, Bhubaneswar, India	[45]
Nocardiopsis aegyptia	Marine sediment of Abu Qir Bay, Alexandria, Egypt	[46]
Strain A201	Snow-covered soil of Ishikawa, Japan.	[47]
Streptomyces 4Alga	Antarctic vegetation (East Antarctica)	[48]

4.2. 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, agitation, pH, 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 [49].

Solid state fermentation is generally defined as the growth of microorganisms on moist solid substrates with negligible free water [50]. The solid substrate may provide only support or both support and nutrition. SSF constitutes an interesting alternative since the metabolites so produced are concentrated and purification procedures are less costly [49, 51-52]. 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 [53]. Among the different substrates used for SSF, wheat bran has been reported to produce promising results [54-55]. A list of various cold active α-amylase producing psychrophillic and psychrotrophic bacteria and their production parameters are presented in Table 3.

Table 2 Various agro substrates used for α-amylase production

Substrate	Organism	Activity (U/g)	Reference
Wheat bran	*Bacillus* sp. PS-7	464 000	[56]
Spent brewing grain	*A. oryzae* NRRL 6270	6583	[57]
Maize bran	*B. coagulans*	22 956	[58]
Rice bran	*Bacillus* sp. PS-7	145 000	[56]
Rice husk	*B. subtilis* 21	760	[59]
Coconut oil cake	*A. oryzae*	3 388	[60]
Mustard oil cake	*B. coagulans*	5 953	[58]
Corn bran	*Bacillus* sp. PS-7	97 600	[56]
Amaranthus grains	*Aspergillus flavus*	1 920	[61]
Gram bran	*B. coagulans*	8 984	[58]

Table 3 Overview of production parameters for cold active amylase

Microbes	Incubation period	Optimum temp.	Optimum pH	Reference
Nocardiopsis aegyptia	2 days	25 °C	5	[46]
Arthrobacter psychrolactophilus	NM	22 °C	NM	[41]
Bacillus sp. A-001	NM	35 °C	7.5	[44]
Flavobacterium balustinum A201	NM	30 °C	NM	[47]
Aspergillus ochraceus	2 days	30 °C	5.0	[43]
Aspergillus oryzae	NM	4 °C	NM	[42]
Lactobacillus plantarum MTCC 1407	36h	35 °C	7	[45]
Eisenia foetida (Amy I and Amy II)	NM	50 °C	5.5	[40]
Streptomyces 4Alga	20-60 min	α-amylase at 30°C	NM	[48]
	50 min	β-amylase at 30°C	NM	
	60 min	β-amylase at 20°C	NM	

NM = not mentioned

4.2.1. Temperature

The influence of temperature on amylase production is related to the growth of the organism. Hence, the optimum temperature depends on whether the culture is mesophilic, thermophilic or psycrophilic. Among the fungi, most amylase production studies have been done with mesophilic fungi within the temperature range of 25–37 °C [57, 60]. 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 [62-65]. A cold active α-amylase from Antarctic psychrophile *Alteromonas haloplanktis* was reported to exhibit maximum α-amylase production at 4 °C [25].

4.2.2. pH

pH is also one of the important factors that determine the growth and morphology of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium. Earlier studies have revealed that fungi required slightly acidic pH and bacteria required neutral pH for optimum growth. pH is known to affect the synthesis and secretion of α-amylase just like its stability [66]. Bacterial cultures such as *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* required an initial pH of 7.0 [62, 67-68]. *Rhodothermus marinus* was reported to yield good enzyme levels at initial pH range 7.5 to 8 [69]. Hyperthermophilic archae such as *Pyrococcus furiosus, P. woesei* and *Thermococcus profundus* yielded optimum α-amylase at pH 5.0 [70]. Thermophilic anaerobic bacteria *Clostridium thermosulfurogenes* gave maximum titres of α-amylase at pH 7 [71].

4.2.3. Carbon 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).

Table 4 Various carbon sources used for maximum α-amylase production

Bacterial species	Best C-source	Reference
B. licheniformis	Galactose, glycogen and inulin	[72]
B. subtilis IMG22 *Bacillus* sp. PS-7 *Bacillus* sp. I-3	Starch and Glycerol	[56, 67, 73]
B. stearothermophilus	Soluble starch	[74]
Bacillus sp.	lactose	[75]
Thermomyces lanuginosus	maltodextrin	[76]

4.2.4. Nitrogen sources

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).

Table 5 Various nitrogen sources used for maximum α-amylase production

Bacterial species	Best N-source	Reference
Bacillus sp. I-3	Soya bean meal	[56-57, 73]
B. stearothermophilus *B. amylolyticus*	1 % Peptone, 0.5% Yeast extract and 0.5 % maltose	[77]
B. licheniformis SPT 278	Peptone	[78]
Thermomyces lanuginosus.	L-asparagine	[76]
A. oryzae A1560	Casein hydrolysate	[79]

4.2.5. Other fermentative conditions:

Surfactants in the fermentation medium 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 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, $LiSO_4$, $MgSO_4$, $FeCl_3$, Mn^{2+}, Zn^{2+} etc. In SSF system some additional fermentative conditions plays very vital role viz. selection of a suitable 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. O_2 consumption rate and carbon dioxide evolution rate.

4.3. Purification and characterization of cold active α-amylases

Most 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.

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

Method	Adsorbent	Yield (%)	Purification fold	Reference
Affinity adsorption chromatography	b-cyclodextrin-iminodiacetic acid-Cu^{2+}	95	-	[80]
Expanded bed chromatography	Alginic acid-cellulose cell beads	69	51	[81]
High speed counter current chromatography	PEG4000-aqueous two-phase system	73	-	[82]
Magnetic affinity adsorption	Magnetic alginate microparticles	88	9	[83]
Substitute affinity method	Insoluble corn starch at 4 °C	78	163	[84]

The purification efficiency is determined by total yield and purification factor [85]. Pre-purification steps involve concentration of the protein containing amylases by ammonium sulphate precipitation, ultra filtration by dialysis. The characterization of cold active α-amylases can be studied in terms of optimum pH and stability, optimum temperature, 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 [2].

5. 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 recombinant 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 recombinant 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-amylases.

5.1. 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 [23, 25].

5.2. 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*, providing the culture temperature is sufficiently low to avoid irreversible denaturation [25]. The thermal stability of the cold-active α-amylase (AHA) secreted by the Antarctic bacterium *Alteromonas haloplanctis* has been investigated by intrinsic fluorescence, circular dichroism, and differential scanning calorimetry. It was found that this heat-labile enzyme is the largest known multi domain protein exhibiting a reversible two-state unfolding [86].

6. Applications of cold active α-amylases

6.1. Manufacturing of maltose

Maltose is a disaccharide made up of glucose units. It is the main component of maltosugar syrup [87]. 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. For production of medical grade the concentration of starch slurry is adjusted to be 10-20 % maltose and for food grade 20-40 % maltose is used.

6.2. Manufacture of high fructose containing syrups

High fructose containing syrups 42 F (Fructose content = 42 %) is prepared by enzymatic isomerization of glucose with glucose isomerase. The starch is first converted to glucose by enzymic liquefaction and saccharification.

6.3. 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. G4 syrup imparts gloss and can be used in industry such as a paper sizer.

6.4. Manufacturing of high molecular weight branched dextrins

High molecular weight branched dextrins are used as extender for production of powdery foods and a glozing agent for rice cakes. 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.

6.5. Removal of starch sizer from textile (desizing)

In textile industry, strength of the textile is improved by warping the starch paste to textile weaving. It also prevents the loss of string by friction, cutting and generation of static electricity on the string by giving softness to the surface of string due to laid down warp. After weaving the cloth, the starch is removed and the cloth goes to scouring and dyeing. The starch on cloth is usually removed by application of α-amylase.

6.6. 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 %) [88-89], but it is still necessary to add large amounts of amylolytic enzymes to hydrolyze the starchy materials to glucose.

6.7. Treatment of starch containing waste water

Food processing wastewater offers a unique challenge to any treatment system. Often containing multiple types of contaminants that pose serious threats to the ability of a standard sewage treatment facility, causes pollution problem

also. 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 [90].

6.8. Other applications

Psychrophilic enzymes can be very useful for domestic processes as amylases; especially cold-active alkaline amylases can be used in detergents since washing clothes at low temperatures protect the colors of fabrics and reduce energy consumption. 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 [91]: 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, biopulping and bioremediation in cold climates [92]. The expansion of experimental models to include plants, nematodes, some cold-blooded animals such as fish and frogs, and other microorganisms may create the need for enzymes with higher activities at lower temperatures. In addition, reporter genes making cold-active enzymes would be valuable additions to the arsenal of molecular tools.

6.9. Patents in cold active α-amylases

It is not surprising that 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.

7. 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 recombinant 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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