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Psychrophiles: A source of cold-adapted enzymes for energy efficient biotechnological industrial processes

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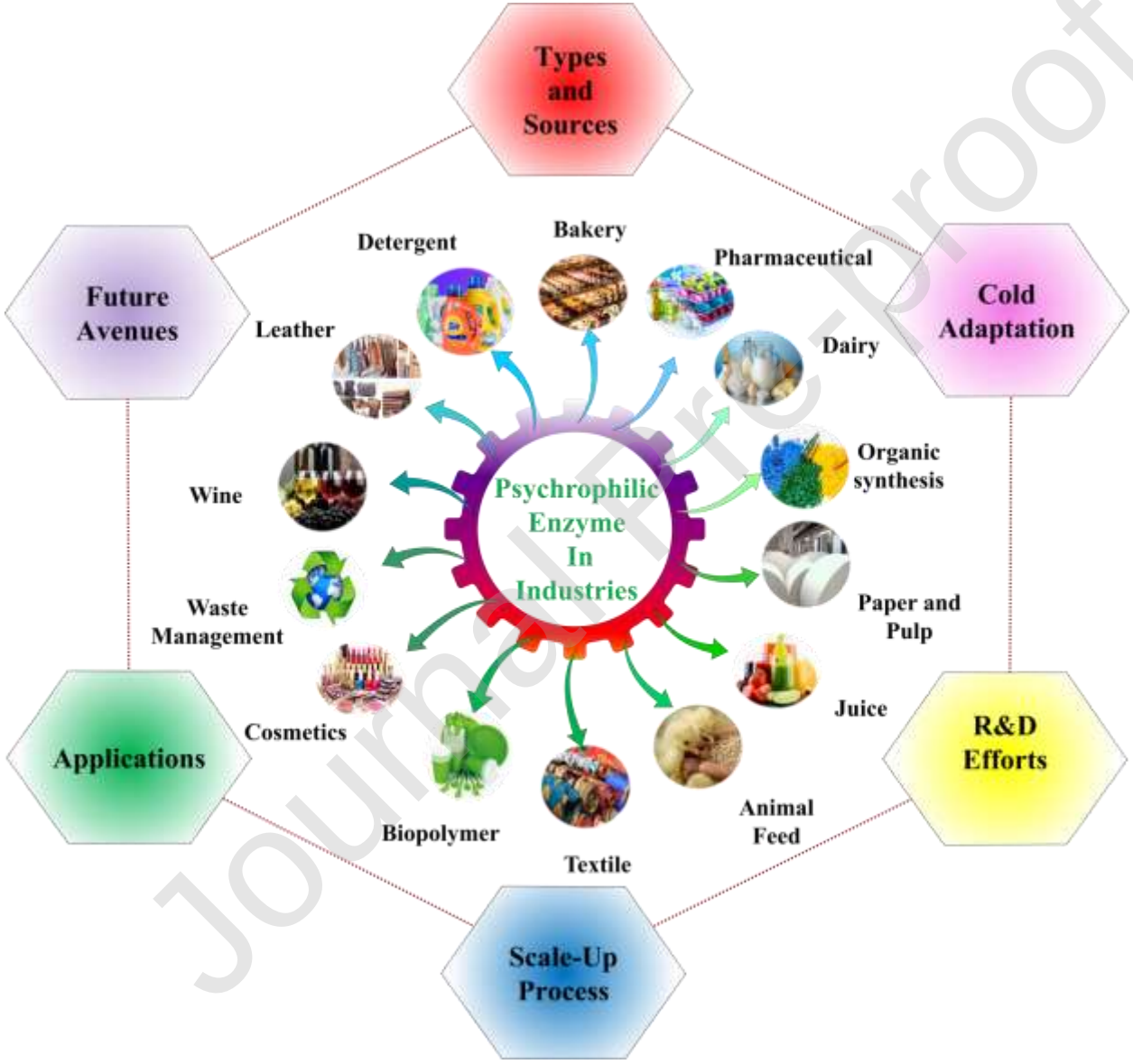
Graphical abstract



Highlights

- New insights and update in the research of psychrophilic enzymes
- Adaptation strategies and specific features of psychrophilic organism
- Exploitation of adaptive features of psychrophiles for commercial applications
- Role of psychrophilic enzymes in industrial processes
- In depth analysis of various applications of psychrophilic enzymes

Abstract

Biocatalysts are the backbone of bioprocessing industries that are going through a phase of transition with reference to the requirement of extraordinary enzymes for various biochemical processes. This transition is well reported and documented by various researchers through elucidation of different features and applications of mesophilic and thermophilic enzymes. However, there is little information available about psychrophilic enzymes and their involvement in industrial processes. Therefore, understanding the features and functions of psychrophilic enzymes could suggest some of their novel applications in various industries such as food, agriculture, chemicals, pharmaceuticals, and waste management etc. Currently, different industries are looking for such novel psychrophilic enzymes to develop efficient biochemical processes that will help to reduce the reaction time, lower the energy inputs and as well as be eco-friendly. These bioprocesses will help to increase the profit margin by reducing the overall cost of the final products. This review article will provide new insights in technical and scientific analysis of psychrophilic microbes, their enzymes and low energy biochemical processes that are useful in various industries for the production of valuable products. It will also further strengthen the understanding of academia and industry about these ubiquitous biocatalysts.

Keywords: Psychrophiles, adaptation, R&D, scale-up, energy efficient, industry, applications.

1. Introduction

Psychrophiles (cold-adapted organisms) have adjusted themselves to survive in the frozen ecosystems of biosphere including glaciers, deep sea, and ice lakes [1]. These microorganisms have developed essential mechanisms to overcome the limitations of low temperature by necessary changes in their cellular structures and functional organization. These adaptive features resulted into production of extracellular polymeric substances, antifreeze and ice-nucleating proteins, membrane fatty acids and pigments, lipopolysaccharide, compatible solutes, bio surfactants, chaperones, storage constituents like polyhydroxyalkanoates and cyanophycins etc. [2-4]. These diverse array of bioactive metabolites and bio-products have properties such as antimicrobials, anticancer, anti-inflammatory and antioxidants and well reported for various biotechnological applications [5-6]. However, these all moieties also make these organisms more interesting in scientific community to explore them for various commercial and industrial applications.

Considering all types of enzymes required by various industries, the global market of enzymes was approximately US $5.5 billion in 2018 and projected to achieve US$ 7.0 billion by 2023 [7]. Currently, scientists have focused to search for novel enzymes for their significant role to replace industrial chemical catalysis by enzymatic processes; since, contrary to chemical processes, enzymes are more sustainable and ecofriendly [8-10]. Industrial production of commodities at commercial scale often involves processes that are carried out in harsh conditions of very high and low pH, temperature, and salinity at which standard enzymes are denatured [10]. Therefore, there is always a need to search for novel biocatalysts that can withstand these extreme conditions [11-14].

Most of the industrial enzymes have been identified, screened and functionally characterized by using numerous “omics” tools such as metagenomics, genome mining, bioinformatics, and in-silico analysis to predict the specific enzyme function from cultured or uncultured psychrophilic microorganisms [15-16]. Such techniques are very crucial in the present time for cold-adapted enzymes modification to make them better for various industrial applications [17-18]. In the current article, we will discuss different types of psychrophilic microorganisms, their morphological and molecular characteristics, as well as action mechanism of their enzymes. Along with this global market value and significant applications of psychrophilic enzymes in different industries also presented.

2. Sources of psychrophilic enzymes

Psychrophiles, also known as cryophiles, are organisms capable of growth and reproduction in temperatures ranging from −20 to +10 °C. These cold-loving organisms are generally found in places that continuously remain frozen for two or more years and are located on land or under the ocean [19]. Approximately three-quarters of the earth's surface are dominated by the ice-cold environment ranging from the Arctic to the Antarctic and from high-mountain regions to the deep ocean. Most of the psychrophiles thriving in low-temperature environments include the deep sea, snow-covered land, permanent snow-covered areas, ice in the sea and glaciers of the land surface [4, 20]. Other cold environments also include cold-water lakes, cold soils, cold deserts, and caves. These cold zones are occupied by psychrophilic bacteria, yeast, archaea, algae, insects, and fish in addition to viruses. They are able to thrive and even maintain metabolic activity in these ubiquitous regions [14]. Various cold adapted microorganisms, their sources and typical feature are described in Table 1. These microorganisms are of great interest in different industries for commercial production of various commodities as well as potential environmental applications and wide range of other underexplored applications. Various emerging

technologies like metagenomics hold great promise for increasing our knowledge and understanding of psychrophiles diversity and adaptation to these stressful areas as well as reveal novel proteins and enzymes of biotechnological interest [1].

3. Adaptations in psychrophiles

To thrive in extreme environments, organisms require various adaptation features at all levels starting from cell structure to enzymes to perform various metabolic activities efficiently [44-45]. It has been found that various structure stabilizing factors interact among them to provide stability to the structural protein and help in their compactness, kinetic stability, thermodynamic stability and make them resistant towards chemical denaturation and harsh environment [2, 15, 46]. These factors have been analysed through molecular changing aspects. To achieve the desired enzyme functions and activity, these factors must be refrained from to cope with low temperature and perform the biochemical reactions efficiently to facilitate life in these harsh conditions. The cold-adapted enzyme must be modified structurally without affecting their cold-induced functions. Being a cold adaptive enzyme, their active site domain is always sensitive to heat whereas the rest of the neighbouring protein may be stable like mesophilic proteins [38]. Therefore, various molecular and evolutionary changes not only affect the catalytic site residues but also the surrounding protein residues. Strong evolutionary pressure may change the amino acid residues in a polypeptide chain as a consequence of substitutions, insertions or deletions in the nucleotide sequence [15, 47]. In this way, psychrophiles have developed unique adaptive strategies at gene and protein level to maintain their metabolic activity so that they can survive in the harsh cold conditions. These adaptive strategies include alterations in transcription and translational processes, membrane fluidity, expression of the cold-shock protein (CSP), production of

antifreeze/ice-nucleating proteins, compatible solutes, exo-polysaccharides and biocatalysts capable of catalysing biochemical reactions at low temperature [15, 48].

Flavobacterium bomensis sp. nov. isolated and studied by Liu et al. [26] showed that besides producing various useful enzymes, including trypsin, the genes of this strain also developed various adaptive strategies such as biosynthesis of carotenoid, polyhdroxyalkanoates, regulation of osmotic and oxidative stress, cold-shock protein, and ice-binding protein etc. Cold stress can also induce changes in DNA methylation patterns as an adaptive strategy as was shown by Turchetti et al. in psychrophilic and psychrotolerant *Naganishia* yeast species [49]. The necessary cold adaptation of microorganisms produces a wide range of biomolecules including various proteins and enzymes that may find application in existing and future biotechnological processes. To make use of cold-adapted enzymes we must explore the dynamics of specific adaptations, structural features, metabolism, enzymology, and genetics of psychrophiles besides their biodiversity from the cold regions.

3.1 Synthesis of cryoprotectants

Cryoinjuries leads to osmatic shrinkage and dehydration of the cell interior which deactivate enzymes causing negative effects on cell function and survival. But, psychrophiles protect their cell content by producing a variety of novel cryoprotectants including, compatible solutes, ice-nucleating proteins, extracellular polymeric substances (EPS) and bio-surfactants [50]. Organic osmolytes such as betaine, glycine, glycerol, mannitol, sarcosine, sorbitol, sucrose and trehalose are examples of various types of low molecular weight and non-toxic cryoprotectants that have been synthesised as shown in Table 2. These organic osmolytes accumulate inside the

cell to prevent the shrinkage of cell and suppress the freezing of solution inside the cell membrane to protect it from cryoinjuries during freezing conditions [51].

Arctic isolate *Mesorhizobium* sp. Strain N33 revealed an increment in the accumulation of sarcosine, threonine and valine when this bacterium was grown at 4 °C, probably acting as cryoprotectants [52]. Qin et al. produced exo-polysaccharide (EPS) under laboratory conditions at 10 °C, and reported a yield of 5.25 g/L (dry weight) under optimal growth conditions [53]. Besides providing protection against the freezing conditions, these cryoprotectants are also involved in scavenging free radicals and promotes folding and stabilising of proteins at low temperatures [54].

3.2 Production of anti-freezing proteins

In response to the cold temperature, these are unique proteins synthesised by psychrophiles and can bind to ice to prevent their further growth and recrystallization. All the cell components are designed to operate at low temperatures, and the antifreeze protein helps prevent the growth of ice crystals [55]. This long-term adaptation also results in the genetic modifications in these microbes to produce enzymes active in low temperature [38]. Various Anti-freez proteins (AFPs) of diverse structure and nature have been reported in various bacteria, fungi, diatoms, plants, insects, and crustaceans. These proteins are frequently glycosylated and/or lipidiated for rapid reaction and stability [50, 56]. Bacteria in which the presence of antifreeze activity was demonstrated are *Rhodococcus erythropolis, Micrococcus cryophilus*, *Sphingomonas* sp., *Halomonas* sp., *Pseudoalteromonas* sp., *Stenotrophomonas maltophilia*, *Bacillus aquamarinus*, *Psychrobacter* sp., *Enterobacter agglomerans, Pseudomonas fluorescens* and *Marinomonas protea* [11].

AFPs bind irreversibly to ice crystals, block nucleation events, and prevent further growth of ice and thermal hysteresis (TH). The activities of TH ranging from the temperature ~0.1 °C to ~13 °C and further may be enhanced by the addition of other AFPs and ionic solutes [57]. Besides TH, AFPs have other important functions such as stabilising of the cell membranes and protection of cell structural integrity [58-59]. Based upon their important functions in psychrophiles, AFPs are used in the food industry to preserve yogurt and save the texture of ice creams [55].

3.3 Synthesis of Ice-nucleating proteins

Most of the psychrophilic organisms synthesise ice-nucleating proteins (INPs) attached to the outer membrane, they promote ice growth at high sub-zero temperatures to protect the organisms [69]. These proteins start assorted crystallisation of ice at sub-zero temperatures and act as a basic pattern for stabilisation of water molecules to form the ice-like structure [2, 59]. It has been reported that a number of cold loving microorganisms produced INPs as repetitive multimeric aggregates, with larger extracellular complexes. INPs are capable of lowering the activation energy, and creating favourable conditions to nucleate ice and its growth at about -2 °C and prevent the formation of ice crystals inside the cell on further decrease in temperature [59].

Besides playing important roles in psychrophiles, INPs are also employed for a number of other applications including artificial seeding of clouds, freezing various products in the food industry and in freeze-thaw valves of microfluidic devices [69]. Qiu et al. also found that aggregation increased the ability of ice-binding proteins to induce ice nucleation that is in consistent with the findings of Cascajo-Castresana et al. that aggregates are responsible for the ice nucleation activity at higher temperatures [70, 71]. All the proteins that have been screened so far could induce freezing up to at least −8 °C. This suggests that the ice nucleation-active

species is oligomers or aggregates of those proteins. Indeed, casein, known to form micelles has consistently induced high-temperature freezing (from −8 °C to −13 °C) [72]. In contrast, the hydrophobins (HPA and HPB), which form monolayer coatings on surfaces rather than aggregates, exhibit only a small fraction of sites that are active between −6 and −8 °C. The ice-binding protein LeIBP with only a few nucleation events above −10 °C is known to dimerize in solution, but might have a low tendency to form larger aggregates [73]. Folding and the structural basis for interactions between water and the ice-nucleating protein InaZ from the INA bacterium *Pseudomonas syringae* strain R10.79 also has been studied by Roeters et al. [74].

3.4 Salt bridges among amino acids and H-bonding

Amino acids form salt bridges among themselves which result in weak electrostatic interactions that help to stabilise the protein conformation. Several psychrophilic enzymes lack surface salt bridges but arginine preferably helps to form salt bridges which play a significant role in cold temperature adaptation [55]. Besides forming the salt bridges, the presence of H-bonds in protein also contributes towards the stability of protein structures and makes them a predominant factor [2, 58]. Arginine mediated networks of ion pairs appear to be responsible for the unusual stability of glutamate dehydrogenase from the hyper-thermophilic archaeon *Pyrococcus furiosus* [60, 68] studied the structure of exo-β-1,3-glucanases and found that the hydrogen bonds and salt bridges are reduced in the active site of this enzyme which results in better active site flexibility and catalytic activity in cold environment. Molecular dynamics simulations (MDS) of Antarctic salmon elastases showed that among the various amino acids predicted, valine and isoleucine were critical residues at its catalytic site. The simulations carried out at five different temperatures proved that the salmon enzyme retains a higher catalytic rate as the temperature is lowered, as would be expected for a cold-adapted enzyme [27].

3.5 Alpha-helices interactions and metal ion binding

Alpha-helix secondary structure is stabilised by the electrostatic interactions of N- or C-terminal and play a significant role in the conformation of a psychrophilic triosephosphate isomerase which is composed of eight α and β barrel polypeptide chains [56]. These interactions are further stabilised and strengthened by binding of various metal ions. Ca^{2+} ions binding to psychrophilic proteins has been found to form eight or nine possible coordination bonds that are able to bridge between secondary structures of proteins to provide extra stability [59].

3.6 Cold-shock responses

Exposure of organisms to low temperature results in upward or downward regulation of some specific genes to produce cold-shock proteins (Csps). These proteins further play an important role in transcription, translation, protein folding and even in the regulation of membrane fluidity and permeability. However, the studies of these special proteins are still in their infancy due to limited availability of these extremophiles [15, 75]. Fluidity of cell membrane is crucial to maintain its structural integrity and function. The membrane of psychrophilic organisms have higher ratio of polyunsaturated to saturated fatty acid for better adaptability to cold conditions (Figure 1) and have been extensively reviewed [61,76]. Various other proteins such as CspA, chaperones, GroEL and DnaK that bind to the nucleic acid at molecular level also show an increased level of expression. Many of the Csps observed in mesophiles act as cold-acclimation proteins (Caps) in psychrophiles and are expressed constitutively at low temperatures [52, 77]. Furthermore, this regulation of psychrophilic genes at low temperature indicates that a temperature sensory system exists in psychrophiles at the membrane level that can sense the changes in outside environment.

3.7 Hydrophobic proteins and solvent interactions

Hydrophobic interactions in the side chains of the protein core helps to get folded conformation and provide stability to the active site as well as to the rest of the protein. Different researchers have evaluated the hydrophobic index (HI) of individual amino acids present in the core of the protein using hydrophobic cluster analysis [78, 79]. Various cold adapted enzymes display amino acid substitutions in the hydrophobic core which results in a sharp decrease of the HI as compared to their mesophilic and thermophilic counterpart [47]. Most of the psychrophilic organisms have different fluidics environment inside and outside of their cells and this variation in solvents help in unfolding of the proteins due to competing of water molecules for the internal H-bonds of the protein. These persistent interactions of various hydrophobic polypeptides on the surface and inside of the psychrophiles provide them protection and stability against the freezing conditions [80]. MDS of exo-β-1,3-glucanases (GaExg55) performed by Mohammadi et al. [68] using GROMACS software revealed an increased exposure of the hydrophobic side chains in the active site of this enzyme which proved its stability at varying low temperatures.

3.8 Structure of catalytic site

The catalytic centre of cold adaptive enzymes are highly conserved which is well demonstrated by the study of their crystal structures. Study of a protease from a psychrophilic *Pseudomonas* sp. revealed that additional bounded ion present in the backbone of the catalytic site pulls its entrance and increases its accessibility for the substrate at lower energy which decreases the activation energy required for the formation of the enzyme-substrate complex [81]. These conformational changes in the active site may also facilitate the release of products more easily and alleviate the effect of a rate-limiting step on the overall reaction carried out by

psychrophilic enzymes [82]. As shown in Table 1, various cold adaptive enzymes like citrate synthase, malate dehydrogenase, uracil-DNA glycosylase, elastase, and trypsin etc. are well characterized and show differences in the electrostatic potentials near the active site region which make them robust candidates to work under low temperature [8, 76, 83]. The structural analysis of GaExg55 with large catalytic cleft and wide active site pocket confirmed the high activity of GaExg55 to hydrolyze polysaccharide substrates [68].

3.9 Broader substrate specificity

Various psychrophilic enzymes show broader substrate specificity due to active site dynamics. A cold active α-amylase showed that its active site is more flexible due to which it can easily accommodate various polysaccharides with variable size and can result in better product formation at low temperature as compared to the mesophilic amylases [76, 84]. Furthermore, on studying the inhibition pattern of the psychrophilic α-amylase, it is clearly seen that due to its larger catalytic site it can form enzyme, substrate, and inhibitor complex. In contrast, the more rigid catalytic site present in the mesophilic homologue can only form enzyme and substrate or inhibitor complex [81, 85]. Several simulated crystal structures of psychrophilic enzymes also proved this point that increased flexibility of the catalytic site is due to molecular dynamics of the active site [59, 86].

3.10 Up and down regulation of proteins

It has been observed that during the exposure of psychrophilic organisms to cold conditions there is rapid up-regulation of genes involved in membrane biogenesis, such as fatty acid and lipopolysachharides, peptidoglycan, carotenoids, exopolymeric and other protein biosynthesis [3, 87]. Transcriptomic analysis has shown that membrane transport proteins and peptide transporter genes are up regulated under cold conditions to facilitate the uptake of nutrients and compatible solutes for peptidoglycan biosynthesis.

Carotenoid pigments also act as modulators in membrane fluidity [48, 52, 79]. In contrast, some of the genes, such as those of outer membrane proteins and structures, flagella, chemotaxis proteins and iron uptake receptors are down regulated at cold temperatures (Figure 1). In *Psychrobacter urativorans*, they may account for up to 14% of the cell lipid content, and in *P. arcticus,* the wax ester synthase is constitutively expressed [56].

4. Uniqueness of cold-adapted enzymes

Classically, cold adapted enzymes have low optimum temperature for catalytic activity and tend to lose their activity at higher temperatures. However, many reports from different research groups indicate that there are some cold-adapted enzymes which show unusual characteristics.

Florczak et al. [88] reported a cold-adapted lipase, LipG7, from the Antarctic filamentous fungus *Geomyces* sp. P7 which retained 15% of its maximum activity at 0 °C. Its uniqueness was that the enzyme retained 100% of its catalytic activity after 1h incubation at 100 °C. Three superoxide dismutases (SOD), belonging to two families, identified in the Antarctic psychrophilic ciliate *Euplotes focardii* demonstrate tolerance to high temperatures (Tm in the range of 50-70 °C) in combination to cold adaptation [40]. An aerobic gram-positive bacterium (*Planococcus halocryophilus* strain Or1) was isolated from an arctic permafrost layer in Canada that can survive at -15 °C [54]. Another study reported a psychrophilic laccase linked with copper oxide nanoparticles entangled in single walled nanotube. The enzyme was not only active at low temperature (4 °C) but also showed enhanced activity when switched to higher temperature (80 °C) [89]. A recent study revealed that a flexible active site yet an overall rigid structure and large amounts of stable valine and lysine residues and strong electrostatic interactions at both N- and C- terminals were responsible for the

thermostability of a cold active transglutaminase [90]. Few other cold active enzymes with thermostable properties isolated from various microorganisms are given in Table 1. Organisms living in the deep-sea environments are exposed to low temperatures as well as high hydrostatic pressure [29, 91]. Therefore, it is imperative that their metabolic enzymes adapt to such conditions for survival of the organism. Hence, it can be assumed that enzymes from these organisms may function at low temperatures and under high pressure. *Shewanella piezotolerans* WP3 is a psychrotolerant and piezotolerant bacterium, isolated from western Pacific Ocean sediment sample from a depth of 1,914 m, which grow optimally at 15-20 °C and can tolerate up to 20 MPa pressure [92]. This bacteria expresses two nitrate reductases (NAP-α and NAP-β) in its peri-plasm. NAP-α was shown to be more tolerant of elevated pressures (highest activity at 40 MPa) than Nap-β [93]. Several other microorganisms have been isolated which have adapted to life at cold temperature and high pressures of the deep sea for example *Shewanella benthica* DB21MT-2 [94], *Colwellia marinimaniae* sp. *nov.*, [41]. A β-glucosidase from the bacterium *Martelella mediterranea* is cold active and also highly alkali stable. It was found to retain more than 50% of its maximum activity at 4 °C and 80% of the maximum activity after 24h pre-incubation with pH 11 buffers [95]. Certain cold adapted enzymes already have commercial applications while others are potential candidates [78]. Enzymes that also function at high hydrostatic pressures, are thermostable or work at extreme pH in addition to being cold active may also be potential candidates for biotechnological applications or industrial processes such as sterilization and food preservation.

4.1 Chaperons

Chaperons are ubiquitous proteins which are widely distributed in prokaryotes as well as in eukaryotes. These specific proteins help the newly synthesized polypeptide chains and immature proteins fold to their native conformation to become stable and active [96, 97]. The genes encoding for various varieties and families of chaperones are induced under stressful conditions and depends upon the stressed environment and organism.

A chaperon from *Psychrobacter frigidicola* (TFPf) cloned and expressed in *E. coli* showed that these cold adapted chaperons assist in the folding of proteins. Cold-active chaperones Cpn60 and Cpn10, named as Arctic Express, are cloned, and expressed in *E. coli* and commercialized by Agilent Technologies [98]. Kim et al. reported a cold active chaperonin (PsyGroELS) from the psychrophilic bacterium *Psychrobacter* sp.PAMC21119 co-expressed with a cold active esterase in an *E. coli* and suggested that this chaperonin is suitable for co-expression of other stable psychrophilic proteins using GroES/GroEL chaperones reported another example of chaperon eco-expression [53, 65].

4.2 Cold-active promoters

A gene cloned in *E. Coli* with cold-shock expression vectors (pColdI-IV) harboring the csp A promoter from CspA showed high expression of proteins when induced by a cold sock protein [75]. Only few expression vectors have been reported so far from psychrophilic organisms, including pColdI vector that is used to express cold-active β- galactosidase (rBglAp) in *E. coli* [99]. A cold-active lipase gene, Lip-948, from the Antarctic psychrotrophic bacterium *Psychrobacter* sp. *G* has been cloned into the plasmid pColdI of expression vector *E.coli* BL 21. This resulted in 39% higher expression of the lipase-948. Half of all the cold-adapted genes are cloned in plasmids from the pET system for their expression [42].

4.3 Expression hosts

It is well documented and reported by various researchers that a number of psychrophilic genes have been identified, cloned and expressed in suitable hosts and have resulted into highly active enzymes which are quite stable at various temperatures [22, 68, 93, 100]. However, it has been found that mesophilic expression systems such as *E. coli* misread the psychrophilic genes and results in altered expression of psychrophilic proteins and enzymes. Such problems clearly shows that there is a need of new expression hosts to enhance the yield of protein and overall productivity in psychrophiles to make them most suitable for industrial applications [22, 100].

Kishishita et al. used homologous expression system of glucoamylase promoter to express cellulases from *T. cellulolyticus* in *E. coli* and the yield of the recombinant enzymes were estimated to be over 100 mg/L [21[. Parra et al. over expressed cold adapted lipase in *E. coli* by using an expression vector obtained from Antarctic bacteria [101]. Low-temperature expression systems were developed using psychrophilic native plasmids including the Gram-negative Antarctic bacteria, *Psychrobacter* sp. and *S. livestonensis* by Miyake et al., [102]. Tutino et al. also expressed heat-labile α- amylase in *Pseudoalteromonas haloplanktis* in cold-adapted Gram negative bacteria [103]. Low temperature controlled promoting regions of *Shewanella livingstonensis* was fused to the *Desulfotalea psychrophila* β-lactamase reporter gene and cloned to the broad plasmid host range pJRD215 [102]. *E. Coli* expression system was also developed using groEL from the Antarctic bacterium *Oleispira antarctica* in order to allow *E. coli* to grow at low temperatures and for overexpression of cold adapted proteins [104]. Such developments in low temperature expression systems are advantageous for expressing at low temperature, even for mesophilic and thermophilic proteins.

4.4 Protein engineering

Stability and activity of enzymes can be increased by protein engineering methods such as through mutations, rational designing, error-prone PCR (epPCR) and DNA shuffling to alter protein sequence under *in vitro* conditions [47]. Thermal stability of cold adapted enzymes improved via protein engineering are well-documented.

A single-point mutation introduced by rational designing in the mesophilic *Bacillus subtilis* lipase LipJ, resulted in downshifting the lipase activity to low temperature, i.e. from 30 °C to 17 °C [105]. Similarly, an aromatic amino acid replaced by flexible amino acids in the active site of psychrophilic alkaline phosphatase led to increased stability in the psychrophilic character of the wild enzyme. A single mutation incorporated using epPCR in a metagenomically-isolated mesophilic *Bacillus* lipase led to a 7-fold enhancement in lipase activity with better optimal activity at 10 °C [106]. It has been found that when directed evolution combined with DNA shuffling was done for a cold adapted glycine oxidase from *Bacillus licheniformis*, it resulted in an increased catalytic activity against the herbicide glyphosate. The engineered cold adapted glycine oxidase can be used to confer glyphosate resistance on genetically modified crops [107]. A cold-adapted protease, subtilisin, has been isolated through evolutionary engineering and improved through *in vitro* mutagenesis to generate the mutant, m-63; which exhibited 100% higher catalytic efficiency at 10 °C compared to the wild type [23].

4.5 Kinetic improvement

Kinetic analysis of an enzyme is an important factor to find its utility at commercial level but unfortunately most of the psychrophilic enzymes reported so far lack detailed kinetic analyses. Evidently, most of the cold-active enzymes can retain their activity between 0-10 °C [15]. Many scientists have investigated the effect of low temperature on binding of substrate to the catalytic

site of psychrophilic enzymes to elucidate their kinetic features. Cold adaptive enzymes such as alkaline phosphatase from *Vibrio* sp. showed turnover number (K_{cat}) of 24000S^{-1} and Michaelis constant (k_m) of 0.3 mM at 4 °C; whereas another cold adapted pectin methyl esterase showed K_{cat} of 15.78 mmol/min/mg and k_m of 0.55 mg/ml at 10 °C in pH 5 [11, 97]. Various characterized cold adaptive enzymes with their optimal temperature, pH and kinetic parameters like k_{cat} and K_m are shown in Table 1. Various optimized cold-adapted enzymes showed that if there is an improvement of k_{cat} at the expense of K_m then both k_{cat} and K_m increases; whereas on improving k_{cat}/K_m ratio, increase of k_{cat} and decrease of K_m has been observed as found in some cold-adapted enzymes such as a phosphoglycerate kinase from *Pseudomonas* sp. TACII18 and glycine oxidase from *Bacillus licheniformis* respectively [107, 108].

4.6 Immobilization

Efforts have been made by researchers to find and characterize the psychrophilic enzymes as well as to develop novel strategies to enhance their activity. However, very little efforts have been made on immobilization of cold-adapted enzymes on a solid matrix to make them efficient and suitable for continuous use for various industrial applications [109-111]. Immobilization of cold adapted enzymes have resulted in increased stability, reusability, and efficient handling of the biocatalyst [112]. A cold active enzyme lipase, CalB, from *C. antarctica* immobilized on epoxy-activated macroporous acrylic resin is commercially available for industrial applications [10]. Recently, cold adapted enzymes have been immobilized using magnetic nanoparticles, single-walled carbon nanotubes (SWCNTs) and graphene oxide besides conventional immobilization matrices.

Graphene oxide, a two-dimensional carbon nanosheet with oxygen-containing functional groups (alcohols, epoxides, and carboxylic acids), has recently been used for enzyme immobilization [89, 110]. It is noteworthy that, most of the attempts for unleashing the full biotechnological potential of cold-adapted enzymes as biocatalysts rely on protein engineering strategies and immobilization for successfully improving the stability of cold-adapted enzymes for industrial processes.

5. R&D Efforts in psychrophiles

Cold adapted enzymes have been subjected to intensive investigations by the scientific community to explicate the origins of these ubiquitous enzymes. Attempts have been made to find out the possible features responsible for high cold activity and weak stability besides understanding the mechanism of action of these enzymes under cold conditions [46].

Conventionally, psychrophiles with potential cold active biocatalysts are isolated from extreme environments by cultivation of the psychrophiles in laboratory using organic-rich medium. It has been found that only <1% of microorganisms are culturable by traditional cultivation techniques from a given soil/water sample [15]. Beyond the conventional culturing techniques, some emerging biotechnological tools such as metagenomics, enables the direct isolation of novel genes and encoding enzymes and other biomolecules from microbial communities. Advances in computational analysis are enabling the mining of environmental metagenomic DNA as well as prediction of their translational products in a very short span of time and also facilitates the identification of gene products with potential industrial applications [75].

Amalgamation of basic knowledge with functional genomics using proteomics and microarray technologies provide new tools and techniques to understand the mechanisms of cold adaptation at both molecular and cellular levels to enhance

their performance and properties [113]. Several strategies suggested for modifying the cold-active enzymes to improve their activity, stability and yield are summarized in Figure 2. These strategies will further advance our understanding of psychrophilic enzymes adaptation and production processes to make them useful in different energy efficient industrial processes.

6. Scale up of psychrophilic proteins and enzymes

With an increasing growth in green industrial processes, the scientific community has traced out, characterized, and exploited a variety of psychrophilic enzymes for more efficient and ecofriendly industrial processes. Psychrophilic enzymes have the added benefit of enhanced selectivity and stereo-specificity at low and moderate temperatures [32, 114]. Psychrophilic enzymes proved a milestone for the food industry especially for preserving product quality along with carrying out reactions. Similarly, these enzymes are the gold standard for molecular biology where low temperature is the prime requirement for process or product [11, 115]. The use of psychrophilic enzymes in market represents 30-40% of all enzymes at industrial scale worldwide.

Two essential types of alkaline proteases, subtilisin Carlsberg and subtilisin novo are obtained from *Bacillus* sp., and can be used as industrial enzymes to produce zein hydrolysates [23]. Likewise, the best-known psychrophilic enzyme is a lipase commercially called Novozym 435 (sold by Novozymes, Denmark) that is obtained from the polar yeast *C. Antarctica* and used in food/feed, cosmetics and pharmaceutical industries [29]. This enzyme has also been used in detergent industry, especially for washing of clothes at low temperatures to protect the colours of fabrics. Similarly, cold-active lipase from Antarctic *Bacillus* sp. became the best enzyme to be used in washing detergents for optimal washing results in tap water temperatures and proved as the best example for the potentials of psychrophilic enzyme to reduce energy consumption [10]. Psychrophilic Xylanase, one of highest produced enzyme

at large scale, extracted from the Antarctic bacterium *P. haloplanktis* and commercialised by Puratos (Belgium) is used as dough conditioners at industrial level to improve quality of bread along with a positive effect on loaf volume [87, 97]. A cold-active lactase was extracted from an Antarctic bacterium and produced in large quantities by Nutrilab NV (Belgium) to hydrolyze lactose to produce high value sweetener D-tagatose during the storage of milk at low temperatures [53]. Unilever (The Netherlands–England) has been using antifreeze proteins cloned and expressed in the baker's yeast *Saccharomyces cerevisiae* at commercial level to preserve the taste and texture of ice-creams under the name of ice-structuring protein [13]. Cold-active polygalacturonase (PGase) from a marine psychrophile, *Thalassospira frigidphilosprofundus* S3BA12, resulted in a 4-fold increase of cell mass from 0.84 dcw g/L in submerged culture to 3.22 g/L, which commensurately increased the PGase titre from 21.0 U/ml to 85.25 U/ml [110]. Daskaya et al. produced three different enzymes pectinase, amylase and protease from *Rhodosporidiobolus, Cystofilobasidium* and *Yamadazyma* fermented in different media at 15 °C and their activities were determined in the range of 0.76–1.73, 0.5–1.57 and 2.11–10.53 U/mL respectively [76].

There are approximately forty-three companies which are involved in research, development and commercialisation of various cold adaptive enzymes isolated from arctic area [14]. The production of psychrophilic enzymes by these companies fall into broad categories as shown in the graphical abstract. The data shows that a number of patents have been filed for the use of psychrophilic enzymes in medicine, cosmetics, nutraceuticals, dietary supplements and other health products, animal health products including aquaculture and food technology; all of which can be transformed into successful industrial process in the upcoming days [7, 32]. Cold adapted esterase(s) and lipases from *C. psychrerythraea* are used to produce polyhydroxyalkanoate (PHA) polyesters that are of

industrial interest for their thermoplastic and elastomeric properties and as sources for fine chemical synthesis [20]. Despite the immense biotechnological potentials psychrophilic enzymes remain under-used, due to the production cost.

7. Significance of psychrophilic enzymes in industrial processes

Since the first commercial application in detergents, psychrophilic enzymes have been extensively used in diverse industrial sectors. In this regard, the quest for enzymes has turned towards extremophilic organisms adapted to extreme environmental conditions [6, 31]. Among such enzymes of extremophilic origin, psychrophilic or cold-adapted enzymes are particularly interesting as they solve many of the issues in an industrial setting and many of these enzymes are already used or have potential industrial uses (Table 3).

The psychrophilic enzymes have high catalytic activity at low temperature, and this is one of the main characteristics that allow their extensive use in various industries [8, 10]. High activity means that, at optimal conditions, lower amounts of psychrophilic enzyme will be required to achieve the same results as compared to its mesophilic or thermophilic counterpart. Enzymes in general, have considerable techno-economic advantages in various industries by providing savings on raw materials and energy compared to conventional processes; and this has been documented well in scientific literature [111, 116-118]. Therefore, in addition to being more productive, psychrophilic enzymes may also hold the potential to exclude the need for energy-requiring heating steps in the production process, which could translate to energy savings and cost reduction [62, 119].

Cold-adapted enzymes have potential application for organic synthesis in mixed aqueous-organic or non-aqueous solvents, especially chiral drugs, due to their inherent versatility, which counteracts the stabilising effects of low water activity in organic

solvents. In addition, as chiral drugs are twice as potent as a racemic combination, cold-adapted enzyme sterospecificity can be useful for the synthesis of chiral drug enzymes [19]. Cold-adapted esterases and lipases during fine chemical synthesis have been found to have a high degree of stereospecificity. Furthermore, reactions can be continued during the winter seasons and permanently cold regions when psychrophilic enzymes are employed in the manufacturing process [19, 120]. Performing reactions at low temperatures curtail unwanted side reactions and generation of by-products, which could be produced at high temperature for that particular reaction [10]. This property is significant in pharmaceutical, food processing and fine-chemical organic synthesis industries where substrates and products may be prone to heat alteration. Additionally, catalysis at low temperatures can also minimize bacterial contaminations [8].

Thermolabile properties of psychrophilic enzymes offer the advantage of selectively deactivating them by minimally raising the temperature in a reaction mixture when required in a production process without using chemicals [19]. Heat inactivation is also vital in the field of molecular biology where enzymes need to be inactivated in sequential reactions after it has served its function. Hence, cold-active enzymes allow the heat inactivation to be performed at lower temperatures at which the structural integrity of other molecules such as double stranded DNA are not compromised [2, 59]. An example of psychrophilic enzymes application would be proteinase K, a serine protease that is used to degrade undesired enzymes in crude extracts or contaminating enzymes from earlier steps in an experiment procedure. Removal of the enzyme from an assay by phenol-chloroform extraction/ethanol precipitation or by heating at 95 °C leads to sample loss or denatured target product respectively [76]. A psychrophilic version of the enzyme would

eliminate the need for chemical or high temperature treatments for deactivation, which is done for all currently available commercial proteinase K [10].

The impacts of cold-active enzymes are not limited to industrial processes alone; end consumers also experience advantages of such enzymes. For example, addition of cold-active lipases and amylases in commercial detergents allows the possibility of low temperature washing which retains fabric color and prolongs garment lifetime [13, 29]. It has been reported that energy consumption can be reduced by decreasing wash cycle temperature. Setting wash cycle at 30 °C instead of 40 °C reduces electricity usage by 30%, which also means reduction of 150-300 g of CO_2 emission [121]. An additional 10 °C decrease can cut CO_2 emissions by half. Therefore, low temperature wash along with 50% reduction in surfactant usage without compromising wash performance is possible when cold-active enzymes are added in detergent formulations. In the food industry, use of psychrophilic enzymes during food processing helps to retain volatile molecules, preserves flavor, taste and nutritional value which could otherwise be lost at high temperatures [19].

8. Other significant applications of psychrophilic enzymes

Most of the enzymes and bio-products used in various applications have been obtained from mesophilic organisms [44, 147]. The abundance of novel bioactive molecules and recent growing interest in psychrophiles led to the exploration of numerous new products using modern high throughput techniques such as metagenomics to find out novel bioactive compounds [91]. Psychrophiles are believed to have potential as probiotics for use as dietary supplements for aquatic livestock's health and nutrition [148, 149].

Besides exploration of bioactive compounds, cold-adapted microorganisms have also shown potential for use in bioremediation to degrade a wide range of organic compounds including mineral oil hydrocarbons, phenolic compounds, poly-aromatic hydrocarbons, pesticides and persistent pollutants [1, 150, 151]. Using psychrophiles in bioremediation of sewage proves a game changer especially in the cold regions. Anaerobic membrane bioreactor (AnMBR) treatment of simulated domestic wastewater was carried out at 3 ℃ by Samith et al. [145] and resulted in >95% removal of COD using psychrophilic consortia along with methane recovery. Similarly, an anaerobic submerged membrane bioreactor (AnSMBR) used for treating municipal wastewater at 18 ± 2 °C resulted in 90% removal of COD and also produced 19.1 ± 0.84 mg CH_4/L [125]. However, only a few of the characterized enzymes were studied for a real industrial application and most of them in the food industry. It will be interesting to see more original articles covering other examples of a concrete use of these remarkable enzymes in the future, which are known to be very relevant for various industrial processes and whose applications will be potentially widespread in the following years.

9. Challenges to use psychrophilic enzymes in commercial applications Despite the enormous advantages of psychrophilic enzymes in different industrial applications, the actual number of usable cold sensitive biocatalysts and bio-catalytic tools is still in their infancy. Working with psychrophiles includes implementing and developing new methods, methodologies, assays, and techniques in addition to existing ones [10]. Many of the methods commonly used in the experiments with classical microbiology and biochemistry cannot be extended to psychrolphilic research because they do not possess the chemical and/or mechanical properties to withstand extreme conditions. At low temperatures, cold-active enzymes are able to grasp available water molecules more tightly because they have a low inherent surface hydrophobicity so, cold-active enzymes preserve their catalytic activity in

organic solvents, additives, such as metal ions, EDTA, DTT, β-mercaptoethanol, and protease inhibitors as these additives help to maintain a tight hydration shell and use of these additives add to the cost of overall bioprocess [152]. Similarly, techniques for researching common microorganisms need to be further adjusted to fit the requirements of psychrophiles. The role is not only related to the development of media composition or assay, but also requires the production of tools and instruments which can withstand and work optimally under extreme conditions. Bioreactors working under extreme conditions, such as high and low pH, elevated temperatures or high salt concentrations, shorten the life of sensors and seals in the bioreactors [153]. The discovery of new genetic sequence-based enzymes also does not always provide accurate information, especially for less studied organisms such as psychrophils. Furthermore, the great technological distance between generating an enzyme under laboratory conditions and achieving a final marketable product remains a problem for the production of new biocatalysts [118]. To get industry-quality results for new enzymes, these approaches require further processing through directed evolution and protein engineering. Also, in the case of functional metagenomic screenings, there are several technical and scientific challenges need to be addressed to fully realize the potential of psychrophilic enzymes for industrial applications.

10. Future avenues in psychrophilic enzymes research

Cold-active enzymes have been characterized for their physiochemical features at low temperatures where mesophilic counterparts do not work well so that they can be used for specific commercial applications [13]. Due to the emerging importance of cold adapted enzymes in catalysing diverse biochemical reactions at low temperatures, psychrophilic enzymes need to be improved in

terms of structural flexibility, amplitude and molecular motions of amino acids present in the active site to increase the activity and stability as the relationships between these factors are still poorly understood [56].

As a result of their biophysical peculiarities, psychrophilic enzymes are interesting and useful models in folding and dynamic studies of proteins. Better exploration of fundamental properties of cold adaptive enzymes with advanced biotechnological tools will stimulate further advances in the field of psychrophilic enzymes. Although significant progresses have already been made by number of researchers in understanding of cold adaptive enzymes and proteins, still many questions remain to be answered (Figure 3). Extensive investigations are needed for these queries which are with regards to i) molecular adaptations to carry out transcription and translations at low temperature, ii) adaptation of amino acid sequence and how polypeptide chains are synthesized and folded at low temperature, iii) physiological requirements for substrate binding to the active site for efficient enzyme activity at low temperature, iv) analysis of local or global flexibility for better activity and stability, v) selective pressures to maintain a balance between stable and flexible cold adaptive proteins, vi) analysis of psychrophilic enzymes with more sophisticated techniques such as NMR, neutron scattering, and H/D exchanges, vii) refinements in the activity-flexibility-stability relationships (AFSR) models of psychrophilic enzymes, viii) psychrophilic proteins adaptive mechanisms to low temperatures etc. High-throughput screening (HTS) such as metagenomic screening and genome mining technologies are currently being applied by pharmaceutical companies to classify new drugs and chemicals, but the need for sufficient hosts to heterologically express recovered genes from metagenomics data remains a challenge. In addition, the development of new culture techniques for uncultivated bacteria, such as iChip, may help to recognise and produce new industrial enzymes [154, 155].

Apart from these future avenues, more efforts are also required to overcome various hurdles such as high enzyme cost, low stability and limited biodiversity in cold adapted regions to make cold-active enzymes more useful in the field of biotechnology to shorten process times and save energy costs of the industrial processes.

11. Conclusions

Cold adapted enzymes from psychrophiles are needed for bioprocesses as they would minimize the expensive heating of reactors and provide potential economic benefits. These enzymes have gained considerable attention to increase their activity and stability for industrial applications. However, there are still number of technical glitches to be overcome before the cold adaptive enzyme technology can be transferred from laboratory to energy efficient industrial process for getting a final commercialized product. No doubt we have addressed in-depth studies of various aspects of cold-active enzymes by giving examples and discussing of various studies to give more evidences of the advantages of cold adaptive enzymes and their sources but still needed more experimental based information to fulfill the demand of energy efficient industrial processes. Current biotechnological methods must be used to explore and advance our understanding of the adaptive traits and novel metabolic peculiarities of psychrophilic enzymes for use as innovative tools for various industrial applications.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure legend

Fig. 1 Psychrophilic microbial cell showing various adaptation features to cold stress *1) Biosynthesis of trehalose 2) Ice-Nucleating Proteins 3) Anti-freezing Proteins 4) Compatible osmatic solutes 5) Extracellular polymeric substances 6) Lipo-polysaccharide biosynthesis 7) Up-regulation of outer membrane proteins 8) Adaptation of phospholipid membrane 9) Up-regulation of membrane transporters 10) Up-regulation of peptidoglycan layer 11) Biosynthesis of carotenoids 12) Down regulated flagella movement.*

Fig. 2 Novel strategies for improving the cold active enzymes.

Fig. 3 Future possibilities in the research of psychrophiles and their enzymes.

Fig. 1

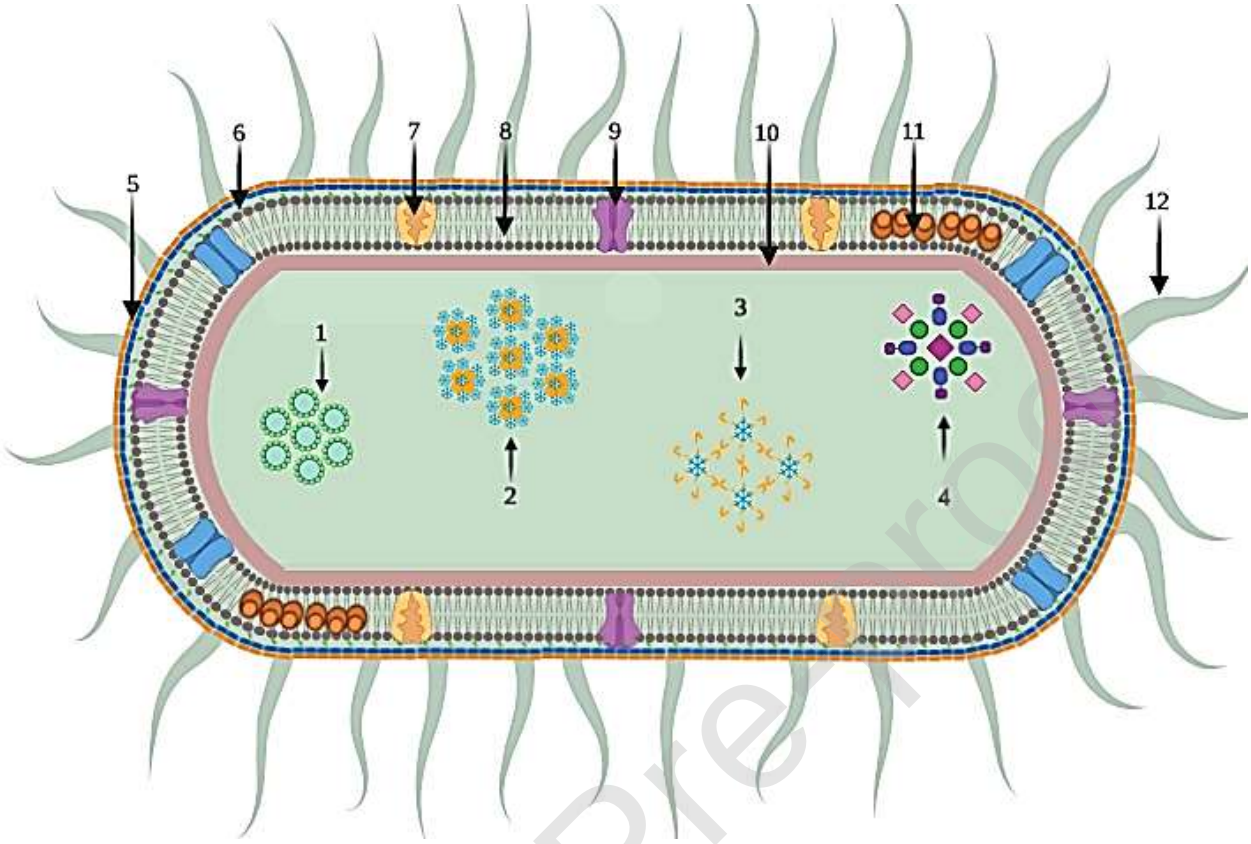

Fig. 2

Fig. 3

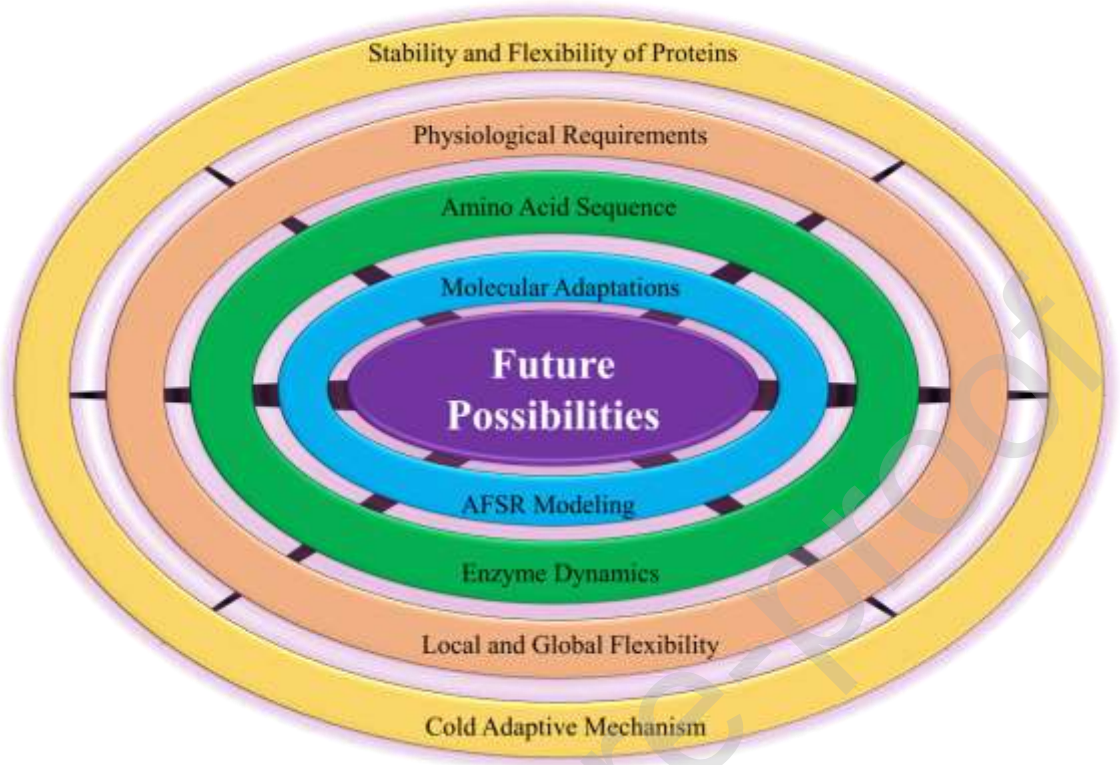

Table 1: Different psychrophilic enzymes, sources, physicochemical properties, and their cold adaptation features.

Source	Organism	Enzyme	Physicochemical properties (Temp, pH, k_{cat}, K_m)	Psychrophilic adaptation product	Possible role in cold adaptation	References
Antarctic	*Pyrococcus* sp.	Cellulase	5°C, ND, ND	*Membrane fatty acids:*	Maintain Membrane	[21]
Antarctic	*Geomyces pannorum*	α-amylase	4°C,5,233 s^{-1},820 mM	Unsaturated fatty acids,	fluidity	[22]
Antarctic	*Bacillus* sp.	Subtilisin	5°C, ND, 32 s^{-1}, 26 mM	Long-chain poly-unsaturated fatty acids		[23]
Deep Sea	*Flammeovirga Pacifica* WPAGA1	Xylanase	4°C, 8, 15 s^{-1}, 2.5 g:l	(LC-PUFA)	Cryo-protactants to prevent cryo-injury	[24]
Antarctic	*P. immobilis*	b-Lactamase	7°C, 984 s^{-1}, 226 mM	*Membrane pigments:*	Osmo-protectants	[25]
Tibetan glacier	*Flavobacterium bomense* sp.	Trypsin	5°C, 29 min^{-1}, 121 mM	Carotenoids Compatible solutes	Against osmotic stress	[26]
Atlantic salmon	*Gadus morhua*	Elastase	10°C, 25 s^{-1}, 0.8 mM			[27]
Atlantic cod	*Gadus morhua*	Chymotrypsin	10°C, 117 s^{-1} , 0.12 mM	*Anti-freezing proteins*	Ice growth inhibition (thermal hysteresis)	[26]
Antarctic soil	*Sporosarcina aquimarina* and *Algoriphagus antarcticus*	Protease	0°C, 8, 5.186, 0.175% (w/v)		Membrane stabilisation	[28]
Antarctic	*Pseudomonas fluorescence*	β-lactamase	ND	*Salt bridge and metal ions*	To prevent folding of proteins under cold stress	[25]
Antarctic	*Arthrobacter* sp.	Citrate synthase	ND		Strong binding to substrate	[27]
Antarctic	*Arthrobacter* sp. *Paracoccus* sp.	β-galactosidase	5°C, 7.5, 140s^{-1}, 4.28 mM			[8]
Antarctic	*Bacillus* sp.	Subtilisine	5°C, 8.5, ND, ND	*Hydrophobic amino acids*	Increased hydrophobic surface	[29]
Marine	*Nesterenkonia* sp.	Aliphatic amidase	ND		residues	[30]
Arctic	*Colwellia psychrerythraea*	Aminopeptidase	ND		Reduced core hydrophobic residues	[8]
Arctic	*Colwellia psychrerythraea*	Phenylalanine hydroxylase	ND			
Arctic	*Aquaspirillum arcticum*	Malate dehydrogenase	ND	*Secondary metabolic pathways*	Depressed oxidative metabolism	[30]
Arctic	*Desulfotalea psychrophila*	Isocitrate dehydrogenase	ND	Glyoxylate and Methyglyoxal production		[3]
Deep-sea	*Bacillus globisporus* and *Marinibacillus marinus*	Adenylate kinase	ND			[31]
Marine	*Vibrio marinus*	Triose-phosphate isomerase	ND	*Ice-nucleating proteins*	Extracellular ice crystal nucleation	[8]
Arctic ice	*Pseudoalteromonas arctic* and *Psychrobacter*	Esterase	10°C, 9, 3.31s^{-1}, 0.162 mM		Prevention of damaging intracellular ice formation	[11]

	cryohalolentis K5^T			*Cell wall modification*	Structural adjustment of	
Marine	*Shewanella* sp.	Tyrosine phosphatase	ND	*Lipopolysachhride(LPS), EPS, Metal ions bonded*	cell wall to protect from cryo-injury	[32]
Marine habitat	*Aliivibrio salmonicida*	Superoxide dismutase	ND	*Choline*		[33]
Antarctic	*Vibrio* sp. and *Shewanella* sp.	Alkaline phosphatase	4°C, 9.5, 24,000s^{-1}, 0.3 mM	*Extracellular polymeric substances*	Ice growth inhibition, Osmo-protection	[34]
Antarctic fish	*Champsocephalus gunnari*	Lactate dehydrogenase	ND		Reduction of osmotic stress,	[8]
Atlantic salmon	*Salmo salar*	Trypsin, Elastase	3°C, 9, 6 s^{-1}, ND		Protection from	[31]
Marine	*Aliivibrio salmonicida*	Catalase Endonuclease I	1°C, ND		desiccation	[35]
Marine sediment	*Photobacterium lipolytica*	Lipase	4°C, 8, 199s^{-1}, 0.27 mM	*Active catalytic site*	Broader substrate capability	[36]
Antarctic marine	*Pseudoalteromonas* sp. SY39	Chitosanase	ND		High catalytic efficiency	[37]
Waste water of Food processing	*Penicillium chrysogenum* F46	*Pectin methylesterase*	10°C, 5, 15.78 mmol/min/mg 0.55 mg/ml		Adaptation to work at low temperature Effective conversion under stressed conditions	[38]
Soil sample	*Paenibacillus polymyxa* Nws-pp2	Pullulanase	10°C, 8.5, 37s^{-1}, 2.8 mg/ml			
					Protection from freezing	[39]
phosphate rock stacking site	*Sphingomonas* sp. JB13	β-mannanase	10◦C, 6.5, 211.9s^{-1}, 5 mg/ml		desiccation	[27]
	Slagofa -			*Bio-surfactants*	Maintenance of adequate	
Soil sample from the cold deserts of Himachal Pradesh	*Paenibacillus* sp. IHB B 3084 Lake sediment	Endoglucanase	5◦C, 5, 0.692s^{-1}, 40.5 mg/ml		metabolic flux	[38]
	C. psychrerythraea			*Cold-adapted enzymes*		
Antarctic	*Euplotes focardii*	SOD	ND		Promotion of protein folding and stability,	[40]
Mariana Trench	*Colwellia marinimaniae sp. nov.*	NA	ND		Destabilisation of	[41]
				Chaperones	RNA/DNA secondary	
Antarctic	*Psychrobacter cryohalolentis*	NA	ND		structures	[42]
Marine	*Alcanivorax dieselolei* B- 5(T)	Leucine dehydrogenase	0-30◦C, 6.5-8.5, ND		Cryoprotectants, Acts as carbon and nitrogen source	[43]
				Storage compounds		
				Polyhydroxyalkanoates, Cyanophycins		

Table 2: Various types of cold-adaptive compounds produced by different psychrophiles and their possible function.

Name of Organism	*Type of cold adaptive compounds*	*Function*	*Reference*
Pseudomonas fluorescens KUIN-1	Cryo-protective protein COR26	Cryoprotection of enzymes	[60]
Pantoea agglomerans NBRC12686	Cryo-protective protein Ribose-1-phosphate	Cryoprotection of enzymes	[61]
Pantoea ananatis KUIN-3	Cold acclimation protein HSC25 Glucose	Depression of freezing points	[62]
Pseudomonas putida GR12-2	Glycolipoprotein	*Anti-freezing protein*	[60]
Moraxella sp.	Lipoprotein	*Anti-freezing protein*	[63]
Acinetobacter baumannii ATCC19606	Lipopolysachharide	*Antifreeze lipids*	[64]
Psychrobacter sp.	*Chaperonin*	*Anti-freezing protein*	[65]
Colwellia psychrerythraea strain 34H	*Extracellular polymeric substances (EPS)*	Cryoprotectant	[62]
Pseudoalteromonas sp.	Sulfated hetero-polysaccharide, high levels of uronic acids with acetyl groups	Cryoprotection	[66]
Pseudoalteromonas strain SM9913	Linear arrangement of α-(1→6) linkage of glucose with a high degree of acetylation	Cryoprotection	[51]
Planococcus halocryophilus	Calcium carbonate and choline rich exo-polysaccharide	Cryoprotection	[38]
Pseudoalteromonas sp. MB-16	Highly complex Mannose rich EPS	Cold adaptation	[67]
Glaciozyma Antarctica PI12	Reduced hydrogen bonds and salt bridges in amino acids	Cold Adaptation	[68]
Colwellia marinimaniae	Rich in cellular fatty acid docosahexaenoic acid	Freeze and high pressure adaptation	[41]

Table 3: Applications of some cold-active enzymes in various industries.

Cold-active Enzyme	**Industry**	**Applications**	**Reference**
Pectinase	Food/Beverage	• Clarification processes of fermented beverage • Decreasing bitter taste and cloudiness of fruit juice. • Degrading other pectic substances	[100] [99]

Protease	Food, detergent, leather, feed	• Food processing/preparations (e.g. tenderizing meat at low temperatures swith cold active collagenase) • Detergent formulations • Dehairing and bating of hides • As supplements in poultry and swine feed: to degrade stored proteins in plant material	[76] [32]
Ribonuclease	Biotechnology, pharmaceutical	• RNase R can be a potential candidate as antimicrobial drug target • Molecular biology applications (oligoribonucleases can be used for complete digestion of small RNA molecules)	[122] [123]
Cellulase	Food, textile	• Pretreatment of garment with cellulose can increase the softness and lifetime of the fabric • Cellulose ethanol fermentation	[112] [14]
Ectoine synthase	Cosmetics, Biomedial	• Used in production of ectoine which can stabilize protein, membrane and cell to be used in cosmetics and other medical applications	[85]
Serine hydroxymethyl transferase (SHMT)	Pharmaceutical	• Potential use in stereoselective synthesis of β-hydroxy-α-amino acids, bioactive compounds that are important pharmaceutical ingredients • SHMT itself is a potential target for anticancer therapy	[17] [31]
Fuculose aldolase	Pharmaceutical	• Potential use in the synthesis of rare sugars in a stereoselective manner • Can be used to make iminocyclitols (inhibitors of glycosidases and glycosyltransferases)	[86] [19]
Penicillin V acylase	Pharmaceutical	• Commonly used in the synthesis of β-lactam antibiotics	[11]
Aminotransferase	Chemical, pharmace	• Transamination reactions used to synthesize natural and non-natural amino acids	[84]

	utical		
Pullulanase	Food, beverage, biofuel, detergent, textile	• Saccharification in the production process of glucose, maltose, fructose etc. • Preparation of low carbohydrate beer • Bioethanol production	[39]
Peroxiredoxin	Food, pharmaceutical	• Potential applications in the development of drugs (due to antioxidant, anti-cancer properties)	[13]
5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)	Agriculture, biotechnology	• Key enzyme in the shikimate pathway of aromatic amino acid synthesis • Target of the herbicide glyphosate • Development of herbicide tolerant transgene plants	[124]
Deacetoxycephalosporin C Synthase (DAOCS)	Pharmaceutical	• Manufacture of cephalosporin antibiotics via the 7-aminodeacetoxycephalosporanic acid (7-ADCA). DAOCS converts penicillin substrates into 7-ADCA which is converted to cephalosporins	[16]
Alkane hydroxylase	Bioremediation	• Degradation of anthropogenic alkanes	[125] [109]
Nitroreductase	Pharmaceutical, bioremediation, biotechnology	• Potential target for development of new antibiotics • Applications in cancer therapy: as activating enzymes in gene-directed enzyme prodrug therapy (GDEPT) and other prodrug therapy approaches • Nitroreductase probes for bioluminescent imaging • Developing transgenic zebrafish for tissue-specific cell ablation	[126] [127]
haloalkane dehalogenase	Bioremediation, biotechnol	• Degrading halogenated aliphatic pollutants into less toxic compounds • Biosensors	[59]

	ogy		
Chitosanase	Pharmaceutical, food, biomedical	• Production of chitooligosaccharide (COS) from the hydrolysis of chitosan. (COS were shown to have antimicrobial, antioxidant, hypoglycemic, anti-tumor properties) • Other potential applications in industries where COS are required.	[128] [53]
Alanine racemase	Pharmaceutical, biotechnology	• Used in the manufacture of D-amino acids • This enzyme is a potential target for antibacterial agents	[129] [130]
α-amylase	Food, detergent	• Laundry/dishwashing detergent formulations to degrade starchy food residues	[81]
Lactate dehydrogenase	Pharmaceutical, Biotechnology	• Diagnostics, nanotoxicology, manufacture of chirally pure pharmaceutical agents	[113] [19]
Exonuclease	Biotechnology	• Molecular biology applications (cleavage of nucleotides from 3'-5' single stranded DNA)	[131]
Superoxide dismutase	Pharmaceutical, cosmetics	• Anti-inflammatory agent • As an anti-aging additive in cosmetics and other personal care products	[33]
Catalase	Food, Dairy	• Used as food preservative	[13]
Myrosinase	Food, feed	• Hydrolysis products of glucosinolates by myrosinase produces various bioactive compounds such as anticarcinogens • Detoxification of feed	[19]
Xylanase	Food, textile, paper	• Fruit and vegetable processing, brewing • Retting of flax, jute	[24]

Glycine oxidase	Biotechnology	• Biobleaching • Development of biosensors to assay for glycine and sarcosine as biomarkers in different biological fluid samples • Development of transgenic crops	[107]
Alkaline phosphatase	Biotechnology, dairy	• Molecular biology tool, use in clinical assays, biosensors, recombinant DNA technology • Indicator of successful pasteurization	[132] [97]
Sedoheptulose 7-phosphate isomerase	Pharmaceutical	• Potential target for antibiotic development	[133]
Carboxyl esterase	Food, bioremediation/ environmental monitoring	• Commonly used in synthesis of fragrances and flavor compounds • Carboxyl esterases can be used to breakdown agrochemicals such as pyrethroids • Bioassays based on carboxyl esterase activity to monitor agrochemical contamination of the ecosystem	[120] [109]
DNA gyrase	Pharmaceutical	• Various antibiotics work by targeting bacterial DNA gyrase	[134]
Epoxide hydrolase	Pharmaceutical	• Potential therapeutic agent against inflammation, pain and neurodegenerative disorders	[135]
β-lactamase	Pharmaceutical	• Degradation of antibiotics	[25]
N-acetylneuraminic acid synthase	Pharmaceutical, food	• Potential use in the synthesis of sialic acid (*N*-Acetylneuraminic acid, Neu5Ac) [Neu5Ac has commercial applications in drug manufacture, food supplements, stabilization of drug delivery carrier molecule]	[119]

Lysozyme	Food, pharmaceutical	• Preservative for fish, meat and vegetable products due to its antimicrobial activity • In pharmaceutical industries it is widely used against viral, bacterial and inflammatory diseases	[114]
Desulfinase	Petroleum	• Removal of sulfur from petroleum during refining	[136]
N-acetylneuraminate lyase (NAL)	Food, pharmaceutical	• NALs catalyze reversible cleavage and synthesis of sialic acids. Therefore by using favorable reaction conditions NALs can be used to synthesize Neu5Ac from pyruvate and *N*-acetylmannosamine, ManNAc	[137]
Lipase	Bioremediation, leather, food, biofuel, detergent, pharmaceutical, biotechnology	• Biodegradation of petroleum contaminants or other greasy effluents in cold environments • Removal of natural fats from animal hide • Manufacture of flavor compounds, dairy products • Modification of waste fats and oils to produce biodiesel • Detergent additive for removal of fatty food stains under low temperature wash conditions • Drugs for treatment of obesity, inflammation, cardiovascular disease • Applications in biosensors and bioassays	[36] [29] [138]
DNA ligase	Biotechnology	• Molecular biology tool	[119]
Glucanase	Biofuel	• Has potential to be developed into a cost effective biocatalyst for biofuel industry: conversion of cellulosic material to bioethanol.	[68]
Glycogen branching enzyme	Food, paper	• Improvement of food (cookies, bread, cakes) quality • Starch modification for coating step in paper manufacture	[11]
Endonuclease	Biotechnology	• Molecular biology applications (digesting all types of DNA and RNA) *(Cryonase, a cold active nuclease, supplied by Takara-*	[139]

		Clontech)	
Nudix hydrolase	Pharmaceutical	• Nudix hydrolase (MutT) inhibitors as potential alternatives to antibiotics for treatment of pathogenic bacterial infections	[35]
Inulinase	Food, biofuel	• Potential use in the low temperature conversion of inulin to fructose • Conversion of inulin derived fructose feedstock to ethanol	[140] [115]
β-Mannanase	Detergent, food, paper	• Bioconversion of lignocellulosic materials in various industries • Reducing viscosity of food items during processing	[97]
Feruloyl esterase	Food, pharmaceutical	• Potential use in isolation of ferulic acids which can be converted to vanillin as a food precursor and • Ferulic acid and its derivatives used in medicines	[5]
Glutathione S-transferase	Pharmaceutical, biotechnology, food	• Scavenging organic hydroperoxides and reactive oxygen species (ROS) • Indicator of diseases such as hepatic infection, biomarkers of toxicity • Potential use in low temperature health food as an antioxidant additive	[141]
Arabinose isomerase	Food, biotechnology	• Industrial scale bioconversion of D-galactose into D-tagatose (a natural sweetener) • Production of L-ribulose from L-arabinose within recombinant GRAS bacterial cells.	[13]
Laccase	Paper & pulp, food/beverage, textile	• Delignification of wood pulp • Wine stabilization and quality improvement of food products • Used for bleaching of cotton for a whiter appearance and indigo-dyed denim	[89] [142]
Uracil-DNA	Biotechno	• Molecular biology tool (eliminate carry-over	[11]

N-glycosylases (UNGs)	logy	contamination during PCR, RT-PCR, site directed mutagenesis etc.)	[134]
Leucine dehydrogenase	Pharmaceutical	• Bioconversion of nonproteinogenic L-*tert*-leucine from its α-keto acid trimethylpyruvate (TMP) [L-*tert*-leucine is an important intermediate in the synthesis of chiral drugs] • The cold adapted enzyme maybe be suitable to convert α-keto acids that are unstable in lengthy incubation at moderate temperatures	[43] [143]
Invertase	Food/beverage	• Potential use in the manufacture of non-crystallizable sugar syrup from sucrose, alcoholic beverages made by fermentation of sucrose containing substrates in cold conditions	[121]
Phytase	Feed, agriculture	• As supplements in the diet of monogastric animals: to enhance bioavailability of phosphorus and other nutrients bound to phytate • Potential use in improving bioavailability of phosphorus in cultivation soil: release inorganic phosphate from organic phytate	[144] [32]
Glutathione synthetase	Biotechnology	• Enzyme in the second step of glutathione biosynthesis pathway. Kits based on the enzyme for biochemical assays at low temperature maybe developed to be used for research purposes	[30]
Chitinase	Bioremediation, biofuel, biotechnology	• Chitinase degrade chitin from krill and other shellfish. • Conversion of chitin to ethanol • Chitinase as a potential target for bio pesticides	[27] [145]
Luciferase	Biomedical,	• Luciferase reporter kit, Diagnostics, *in vivo* bioluminescence imaging	[146]

	biotechnology	• Potential use in bioluminescent plants as alternative to traditional light sources