Microbial Life in Cold Regions of the Deep Sea

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Abstract

Deep sea ecosystem is not only the largest but also the most remote biome of the biosphere. Exploration of sea depths has resulted in the discovery of several new microbial habitats with unique nutritional composition and high microbial diversity. Microorganisms present in deep sea play a fundamental role in global biogeochemical cycles, and with their functional activities, they allow the existence of life. To survive and multiply in cold regions of deep sea, microorganisms should be able to adapt to a variety of changing conditions and stresses. Adaptations to fluctuations in temperature and pressure are possibly the most common; thus, psychropiezophilic microbes dominate in cold regions of the deep sea. These microorganisms make numerous adjustments to cope up with temperatures and pressure lower or higher than optimum. Benthic microbes exhibit both autotrophic and heterotrophic modes of nutrition in obligate oligotrophic environments of the deep sea. The rearrangement of simple metabolic strategies might help these microbes to metabolize in nutrient-poor environments. Further understanding of the genetic switches regulating the metabolism versatility at the deep sea could help us use and manipulate deep sea microbial strains for improved bioprocesses.

Keywords

Adaptions · Deep sea · Microbial diversity · Metabolism · Piezopsychrophiles

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3.1 Introduction

Ecological biodiversity is always a subject of interest for researchers, and a vast amount of information about the distribution of microorganisms around the world has also been collected. Several researchers have focused in recent years on the great potential of marine microbial treasures as a prolific producer of bioactive substances as well as a potential source of drug and antimicrobial compounds (Gimmler et al. 2016). Furthermore, the use of deep sea microbes in biogeochemical processes, biotechnology, pollution, and health has become increasingly interesting. The microbes that inhabit these unusual habitats are usually extremophiles. Extremophiles are the microbes that are capable of surviving in extreme environments. These microbes can survive in conditions like elevated (thermophilic) or low-temperature (psychrophilic), heavy ionic strength (halophilic), acid or alkaline conditions (acidophilic, alkalophilic), anaerobic environment, higher pressure (piezophilic), UV rays and polyextremophilic conditions, such as thermoacidophilic and thermohalophilic values. In cold regions of the deep sea, temperatures would be cold (2–3 °C), and the pressure can be more than 10 MPa, and thus microbes living there are called psychropiezophiles, and if the temperature is high like 400 °C (near hydrothermal vents), then microbes living there are called thermo-piezophiles, respectively (Fang et al. 2010). Extremophiles thrive in hot, cold, and high-pressure environments owing to their lipids, enzymes, and other biopolymers having specific properties/features to function in extreme conditions.

The Earth's biosphere is dominated by low-temperature ecosystems which are effectively colonized by a wide number of cold-adapted organisms. Although microorganisms, particularly, bacteria, yeasts, archaea, and protists, predominate in these cold habitats, microorganisms such as algae and microalgae have also been reported in these ecosystems. The ability of psychrophilic microorganisms to prevail in these conditions reflects their adaptability to the cold deep sea environment (Margesin and Collins 2019). This is accomplished through a set of morphological and physiological adaptations of all cellular elements, from a molecule level to whole cells and even complete ecosystems. In the deep sea, life is encountered with low temperature and high pressure. In addition to reduced thermal energy, low temperatures often contribute to more physicochemical constraints such as higher viscosity of solvents and solubility of the gases (such as oxygen and reactive oxygen species). Low temperature also lead to decreased solubility of solutes and nutrients, reduced diffusion, increased osmotic tension, desiccation, and ice formation. Various cold habitats are also marked with other extreme conditions including high salinity, oxidative stress, low nutrient levels, low water activity, and freeze-thaw cycles. Microorganisms in extreme sea interior and subglacial conditions are often subjected to the additional stress of high pressure. Thus, a multitude of synergistic adaptations are required for life in the cold biosphere, to react to not only the low-temperature threat but also the multitude of other interactive stresses imposed by particular environmental conditions. Importantly, many of these methods have multiple uses and can be used to address a variety of problems or combinations of problems. Unraveling the various interacting parameters and deciphering the precise

role of a specific trait, whether it is a specific response to low temperatures or another (or other) environmental stressor (s) common to a particular habitat, is a common problem with the classification of cold-adapted microorganisms. Moreover, microorganisms do not necessarily use all resources in their "cold adaptation" toolbox. In reality, each organism will use its strategy or combination of strategies, depending on its specific requirements and the environmental parameters, and the microbial community structure.

High hydrostatic pressure (HHP) is an important parameter in the deep oceans as the average hydrostatic pressure is estimated at 38 MPa. Piezophiles are the species that survive at a pressure higher than ambient pressure (0.1 MPa) with an optimum growth rate. The effects of HHP on the physiological functioning of microbes have been studied in piezosensitive mesophilic (e.g., *Escherichia coli*) and psychrophilic bacteria (e.g., *Photobacterium profundum* SS9). In piezo-sensitive bacteria, due to HHP, compaction of lipid constituents of the cytoplasmic membrane occurs, and it turns to a rigid structure, whereas piezophiles counteract this constraint by altering the composition of the membrane lipids, particularly the ratio of monounsaturated fatty acids. For example, *P. profundum* SS9, a piezophilic bacterium, has a high ratio of unsaturated/saturated membrane lipids in the membrane which increases the membrane fluidity under HHP. The effect of HHP on piezophiles could arise through multiple pathways directly related to the composition of the membrane-like altered functioning of cellular transporters, motility, and respiratory chain components. Therefore, understanding the regulation of genes and enzymes involved in the respiratory chain of piezophiles is important to have an insight into differential mechanisms involved to counteract the effect of HHP. In this chapter, we will discuss the various concepts about psychrophiles and piezophiles, living in cold regions of the deep sea.

3.2 Deep Sea as a Microbial Habitat

3.2.1 With Low Temperature

For the past three million years, the relationship between microbial diversity and temperature is one of the most fascinating ecological phenomena (Tittensor et al. 2010). The mechanisms involved in this relationship have been explained through various hypothetical theories based on ecology and evolution. However, due to the inability to reach the deep sea region, accessible microbial diversity is still restricted to a few taxa. Also, the numerous theories suggested so far are still controversial (Brown and Thatje 2014), posing the requirement for more detailed studies with comparative analysis under natural conditions. Since current Intergovernmental Panel on Climate Change (IPCC) scenarios indicate that temperatures in most ocean regions will change rapidly in the coming decades, one of the main objectives of current ecological research is to gain a better understanding of potential responses to these changes. During the glacial/interglacial cycles of the Late Quaternary, deep sea temperatures in glacials were ~4 °C cooler than in interglacials.

Palaeoceanographic data showed 1–2 °C temperature variations in deep sea both on millennial and centennial time scales. Deep-water temperatures in the Labrador sea have demonstrated complex decadal variation at rates of change of up to 0.5 °C per decade over the last 60 years. Abrupt changes in temperature of deep-water temperature can impact physical and biological processes occurring down in the sea (Canals et al. 2006). There are significant differences in deep sea temperature, especially between oceans. There are exceptionally high deep sea temperatures in certain marginal seas, such as the Mediterranean, Red, and Sulu seas (from around 13 °C for the Mediterranean to >20 °C for the Red sea at a depth of 2000 m). Some deep waters are very cold at high latitudes, with temperatures close to −2 °C (e.g., Antarctic bottom water). Sometimes deep sea organisms are sensitive to even minor temperature changes because they encounter less seasonal variation in temperature compared to surface-sea organisms. The microorganisms that travel from shallow-sea environments to the deep sea are thought to be more temperature tolerant compared to those that originated at deep sea.

3.2.2 With High Pressure

The relationship between the rate of change in pressure and ocean depth is linear. In the shallower areas, the relative rate of pressure change is much higher with depth. For example, a microbe descending from 500 to 1000 m will experience a 101.2% pressure change, whereas an organism going from 9500 to 10,000 m will experience a pressure change of just 5.3%, while the absolute change is still ~500 dbar (decibar) per 500 m. Therefore, vertical migration does not generally account for any shifts in the regular pressure faced by benthic fauna. Likewise, the pressure difference is also negligible if an organism traverses a smooth and vast abyssal plain. If, however, an entity travels in any direction inside the trench perpendicular to the trench axis, then compression (if heading toward the axis) or decompression is encountered (if moving away from the axis). The pressure increases by 30–60 dbar per km across the abyssal plains (4000–6000 m). As the sea surface rises and falls, the atmospheric hydrostatic pressure is subject to tidal cycles regardless of changes in depth or distance traveled overground. Pressure data from the Kermadec Trench from 4329 to 8547 m, taken both in 2007 and 2009, indicate a cumulative mean tidal duration of 12.42 h 0.64 S.D. (semidiurnal), implying the presence of an internal tidal period of M2 (*lunar* semidiurnal tide). The M2 tidal cycle is one of the region's dominant semidiurnal tides and rotates around New Zealand anti-clockwise (Chiswell and Moore 1999). It is also common in conditions that are bathyal and abyssal. It was found that the mean amplitude (peak to trough) of these cycles of pressure was 1.26 dbar 0.19 S.D., approximating a swell of 1 m. In the Kermadec Trench, as well as in all other stations examined during the HADEEP project, these tidal cycles have been found as deep as 9900 m, regardless of depth (Kermadec, Tonga, Izu-Bonin, Japan, and Peru-Chile trenches). The same signature of the cycle is seen as deep as 7700 m in the North Pacific trenches. Hadal species would therefore likely be able to detect minor tidal variations in pressure.

3.3 Microbial Diversity in Deep Sea

Microbes are found everywhere on Earth. Microbial activities (nitrogen fixation, phosphate solubilization, etc.) are affected by various environmental factors and climatic changes (Kaur et al. 2014; Kaur and Gosal 2015, 2017). The deep seafloor comprises the largest ecological realm of the world. In deep sea sediments, bacteria and archaea (mostly in deep sea hyperthermal vents) and some fungi comprise the largest fraction of taxonomic richness and biomass at deep sea, playing a major role in remineralizing the organic matter as well as in nutrient cycling (Jørgensen and Boetius 2007; Wei et al. 2010). The highest sea depth reported for microbial occurrence in the deep sea is 10,898 m for bacteria *Dermacoccus abyssi* MT1.1 T (Pathom-aree et al. 2006) and *Shewanella benthica* DB21MT-2 (Kato et al. 1998; Nogi and Kato 1999). Understanding the spatial patterns of microbial diversity could pave the way toward better insight into mechanisms of diversification in the deep sea (Varliero et al. 2019) (Table 1.1).

3.4 Microbial Adaptations at Deep Sea

Microorganisms are novel living agents which can tolerate extreme environmental conditions existing on earth. Many environmental conditions on earth may be unideal for the survival of living agents. These conditions may exist normally or due to some external forces. Among them, physical extreme conditions, temperature, and pressure are important. Below are the details of various adaptation mechanisms adopted by microorganisms to thrive under deep sea extreme environmental conditions like low temperature and high pressure.

3.4.1 Low-Temperature Adaptations

Permanently cold environments existing on earth (like the deep sea and polar regions) have been successfully colonized by microbial species. Microbes surviving in deep sea or polar regions are referred to as psychrotolerants or psychrotrophs, based on their ability to grow at different temperature ranges (Morita 1975). Depending on the temperature, the abundance and composition of the microbial community vary. Variation in temperature only changes the types of microbes but not their ability to grow under such an environment. This novel property has made psychrophiles able to tolerate the effects of lower temperature like high viscosity (increases by drop-down of 2 °C) and negative effects on biochemical reactions. These effects of low temperature are successfully overcome by some of the psychrophiles (*Moritella profunda*). *Moritella profunda* has the ability to survive under a temperature range of 2–12 °C (Xu et al. 2003a, b). Other microorganisms also show adaptations to the cold environment by evolving various mechanisms which are discussed below.

Table 1.1 Microorganisms isolated from deep sea cold regions

Microbial type	Most resembles with	Isolation			Optimum growth		Reference
		Site	Source	Depth	Temp.	Pressure	
Bacteria	*Psychromonas kaikoae* JT7304T	Japan trench	Cold-seep sediment	7434 m	10 °C	50 MPa	Yayanos and Dietz (1979) and Yayanos (1986)
	Colwellia sp. strain MT41	Mariana trench	Decaying amphipod	10,476 m	8 °C	103 MPa	Yayanos et al. (1981) and Yayanos (1986)
	Colwellia hadaliensis BNL-1 T	Puerto Rico trench	Seawater	7410 m	10 °C	90 MPa	Deming et al. (1988)
	Shewanella violacea DSS12	Ryukyu trench	Sediment	5110 m	10 °C	30 MPa	Kato et al. (1995)
	Shewanella benthica DB6101	Ryukyu trench	Sediment	5110 m	10 °C	50 MPa	
	Shewanella benthica DB5501	Suruga bay	Sediment	2485 m	15 °C	60 MPa	Kato et al. (1995)
	Shewanella benthica DB6705	Japan trench	Sediment	6356 m	5 °C	60 MPa	
	Shewanella benthica DB6906	Japan trench	Sediment	6269 m	15 °C	60 MPa	
	Shewanella benthica F1A	Atlantic Ocean	Water column	4900 m	8 °C	30 MPa	Jannasch and Taylor (1984), Kato et al. (1996)
	Shewanella benthica DB172R	Izu-Bonin trench	Sediment	6499 m	10 °C	60 MPa	Kato et al. (1996)
	Shewanella benthica DB172F	Izu-Bonin trench	Sediment	6499 m	10 °C	70 MPa	
	Desulfovibrio profundus 500-1 T	Japan Sea	Sediment core 518 mbsfb	900 m	25 °C	15 MPa	Bale et al. (1997)
	Shewanella benthica DB21MT-2	Mariana trench	Sediment	10,898 m	10 °C	70 MPa	Kato et al. (1998) and Nogi and Kato (1999)
	Psychromonas profunda 2825 T	Atlantic Ocean	Sediment	2770 m	10 °C	25 MPa	Xu et al. (2003a, b)
	Colwellia piezophila Y223GT	Japan trench	Sediment	6278 m	10 °C	60 MPa	Nogi et al. (2004)
	Dermacoccus abyssi MT1.1 T	Mariana trench	Sediment	10,898 m	28 °C	40 MPa	Pathom-aree et al. (2006)

	Psychromonas sp. strain CNPT3	Central North Pacific	Decaying amphipod	5800 m	12 °C	52 MPa	Nogi et al. (2007)
	Psychromonas hadalis K41GT	Japan trench	Sediment	7542 m	6 °C	60 MPa	
	Shewanella sp. strain KT99	Kermadec trench	Amphipod homogenate	9856 m	2 °C	~98 MPa	Lauro et al. (2007)
	Carnobacterium sp. strain AT7	Aleutian trench	Water column	2500 m	20 °C	20 MPa	
	Rhodobacterales bacterium PRT1	Puerto Rico trench	Water	8350 m	2 °C	–	Eloe et al. (2011)
	Gammaproteobacteria Alphaproteobacteria; *Bacteroides Actinobacteria* and *Fermicutes*	Eastern Mediterranean deep sea	Sediment; water	2800–4400 m; 500 m–4000 m	14 °C	52 MPa	Gärtner et al. (2011)
	Rhodobacterales bacterium PRT1	Puerto Rico trench	Seawater	8350 m	10 °C	80 MPa	Zeng et al. (2009)
Yeast	*Rhodotorula rubra* and *Rhodosporidium sphaerocarpum*		Marine water	4000 m	–	40 MPa	Lorenz (1997)

3.4.1.1 Maintenance of Membrane Structure by the Generation of Unsaturated Fatty Acids

Enzymes are responsible for various conversions (Kaur et al. 2020). Due to a decrease in temperature, certain enzyme-mediated changes occur in the microbial cell membrane fatty acid profile. One such conversion is a change of saturated fatty acids to unsaturated fatty acids. This conversion is carried out by desaturase enzyme. These changes may occur to maintain optimum fluidity. Desaturase is also known to preferentially synthesize various types of fatty acids which may include short-chain fatty acids, branched-chain fatty acids, and anteiso fatty acids (Suutari and Laakso 1994). Some of the microbes involved in carrying out these functions are *Micrococcus roseus*, *Sphingobacterium antarcticus*, and *Pseudomonas syringae* (Chattopadhyay and Jagannadham 2001). Anteiso saturated fatty acid (a-C15:0) plays a major role in the survivability of psychrophiles (Annous et al. 1997). Kumar et al. (2002) described the role of hydroxy fatty acids in homeoviscous adaptation (an adaptation of lipid composition in the cell membrane) of outer membrane fluidity. It was demonstrated using *P. syringae* that when bacteria were incubated at a low temperature, there was an increased concentration of hydroxy fatty acids in lipopolysaccharides. When *Bacillus subtilis*, a mesophilic bacterium, was incubated in a psychrophilic condition, there was a transcriptional upregulation of some of the genes which were involved in coding those enzymes that degrade amino acid with branched chains (Kaan et al. 2002). Compounds like isobutyryl-CoA and α -methylbutyryl-CoA which are the intermediate product of valine and isoleucine degradation are utilized as a part of the cellular mechanism (synthesis of branched chain fatty acids) to maintain fluidity at low temperature. This indicates that not only anabolic pathways but catabolic pathways are also involved in maintaining membrane fluidity.

To thrive under cold conditions, many bacteria have evolved various other different mechanisms like the synthesis of unsaturated fatty acids in the case of *B. subtilis* under low temperature. In order to regulate glycerophospholipid, *B. subtilis* harbors a sensory system called DesK (dimeric histidine kinase). It has two domains that include five transmembrane helical domains and cytosolic kinase/phosphate domains. Under cold conditions, DesK changes from phosphatase active site to kinase active site leading to autophosphorylation. This phenomenon activates the DesR which in turn activates 5-lipid desaturase, which transforms saturated lipid acyl chains to unsaturated. Once the conditions become normal, DesK returns to the phosphatase active site, and dephosphorylation occurs, inactivates the DesR, and stalls the production of desaturase.

3.4.1.2 Cold-Shock Proteins (CSP)

A sudden downshift in temperature leads to harmful effects on the cells. Such damages are counteracted by proteins called cold-shock proteins (Phadtare 2004). These proteins are activated only under cold shock. Immediately after its synthesis, other proteins are recruited for growth synthesizes leading to the growth under cold conditions but at a slower pace (Ermolenko and Makhatadze 2002). Recent studies

have revealed the role of cold-shock proteins in bacterial stress tolerance (Schmid et al. 2009).

CSPs are "small nucleic acid-binding proteins" whose length ranges between 65 and 75 amino acids (Czapski and Trun 2014). These proteins occur in psychrophiles as well as mesophiles (Jin et al. 2014). There is a total of nine CSPs (CspA to CspI) which are homologous to each other and share 46–91% similarity (Yamanaka et al. 2001). Among all, CspA plays an important role during cold shock, and its role has been described in *E. coli* (Goldstein et al. 1990).

Even though all CSPs share structural similarity, still their thermostability varies (Jin et al. 2014). CspA protein can even tolerate the mesophilic temperature of 40 °C (Lee et al. 2013). This nature of the protein helps them survive in varying temperatures (Jin et al. 2014). It was first identified in *Listeria monocytogenes* (Jin et al. 2014). At mesophilic temperature, mRNA of *csp*A is highly unstable. Usually under mesophilic range, half-life will be very less (12 s) but increases up to 20 min under cold conditions (Mitta et al. 1997). Under cold conditions, it is essential to transiently stabilize *cspA* mRNA as it plays a greater role in inducing CspA (Phadtare and Severinov 2005).

Functions of Cold-Shock Proteins

The highly conserved nucleic acid-binding domain of CSPs is called cold-shock domain (CSD) (Graumann and Marahiel 1996). Ribonucleoproteins 1 and 2 are two important nucleic acid-binding motifs of CSD (Lee et al. 2013). These help the protein to bind to its target RNA or DNA. Jiang et al. (1997) stated that the binding ability of CspA to RNA is weak and is responsible for minimal specificity for RNA. CSPs are known as molecular chaperones because they disrupt the secondary structure of RNA thereby helping transcription and translation to occur smoothly. This process is highly dependent on the mode of attachment of CSP to RNA. If CSP binds strongly to RNA, then, their role as molecular chaperon will be interrupted. CSPs are also known as anti-terminators as they terminate the formation of hairpin structures, which halt the transcription (Phadtare et al. 2002). Usually, during cold shock, CspA, CspB, CspE, CspG, and CspI are induced, but during the first temperature downshift, only CspA and CspB are synthesized (Jung et al. 2010).

3.4.1.3 Viable but Non-Culturable Cell (VBNC)

Viable but non-culturable cells (VBNC) are live bacteria that neither grow nor divide but are alive and capable of performing necessary metabolic operations for their survival. Generally, VBNC has greater physical and chemical resistance compared to culturable cells because of reduced metabolic activity and high content of peptidoglycan (Signoretto et al. 2000). Bacteria do not directly enter into VBNC state; before it, they enter into a persister cell phase (Bigger 1944). Persister cells refer to phenotypic variants in the population. Till today, persister cells are considered to be a nongrowing state of the cell. These are also known to tolerate antibiotics. Ayrapetyan et al. (2015) stated that "VBNC and persister cells are closely related states of a shared dormancy continuum." It suggests that logarithmic phase cells may enter into the persister state before entering the VBNC state.

Mechanism of VBNC Formation

The mechanism by which bacteria enter to VBNC state is not thoroughly understood. Various hypotheses have been put forward to explain the mechanism lying behind it. Among them, three important include the following: firstly, the severe conditions may lead to cells with poor quality which may result in null activity, and such cells cannot be cultured (Nystrom 2003). Secondly, it is described as a strategy of survivability in order to overcome harsh environmental conditions (Oliver 2005). Thirdly, it has been stated that genes are involved in the formation of VBNC (Ayrapetyan and Oliver 2016). The third hypothesis is widely accepted by scientists. Although the molecular mechanism of VBNC formation is not understood completely, several genes involved in its formation have been identified. One among them is the rpoS gene, which codes for the stress regulator protein RpoS which is known to enhance the efficiency of bacterial survivability under extreme conditions. If bacteria cannot produce this protein, then bacteria may enter VBNC. Research over a longer period revealed the role of ppGpp in VBNC formation. ppGpp is considered as a regulatory signaling molecule that regulates RpoS. The higher the ppGpp concentration, the greater the synthesis of RpoS, which contributes to resistance and persistence of cells under stress. Thus, ppGpp is considered as an inducer of the VBNC state.

3.4.1.4 Antifreeze Proteins

Antifreeze proteins (AFPs) refer to a class of polypeptides produced by certain animals, plants, fungi, and bacteria that enable their survivability in freezing temperature. These proteins are also known as ice structuring proteins. The main functions of AFPs are to bind to ice crystals and prevent growth and recrystallization (Collins and Margesin 2019). Unlike ethylene glycol (automotive antifreeze agent), they will not reduce freezing point but instead work in a non-colligative manner. This phenomenon enables them to be better antifreeze agents. These are known to act as an antifreeze agent at a concentration of 1/300th to 1/500th. As it is highly effective at lower concentrations, it doesn't have any side effects on the organism. A unique property of AFP is to bind to the particular ice crystals and immediately prevent their formation. AFPs mechanism is completely based on thermal hysteresis (TH) (Zhang et al. 2008). Thermal hysterisis refers to "a difference between the melting and freezing point" (busting temperature of AFP bound ice crystal). Thermodynamically favored growth of ice crystals can be inhibited by the addition of AFP between the solid ice and liquid water. Kinetically, ice growth can be inhibited by AFP that covers the water-accessible surfaces of ice.

Mechanism of AFP

The mechanism adopted by AFP is not frozen avoidance but freeze tolerance. Crystallization involves two major steps: nucleation (formation of a stable crystal nucleus) and extending the synthesis of crystals by nucleus growth. Based on the occurrence, nucleation is classified into two groups, i.e., nucleation taking place around the foreign molecule called heterogeneous nucleation and spontaneous formation of nucleus due to natural fluctuations called homogeneous nucleation.

Homogeneous nucleation occurs in the case of absolutely pure water. Crystallization is a cyclic process that may occur again and again; this is due to fluctuation in temperature within the subzero range. This fluctuation is the result of the dissolving of small crystals and the formation of larger crystals. This phenomenon may cause more damage to the cells and tissues (Hassas-Roudsari and Goff 2012).

As mentioned earlier, AFP mechanism is based on TH, which prevents the death of cells by various mechanisms like modification of ice crystal morphology (Kontogiorgos et al. 2007), recrystallization inhibition (Zhang et al. 2008), and intensifying integrity of the cell. All these properties are the result of interactions occurring between AFP, water, and ice. Freezing point depression occurs through a non-colligative mechanism (occurrence of protein between water ice interface to modify the growth of ice crystals). On to the outer world, the mechanism seems to be the adsorption-inhibition process (antifreeze agents bind to the surface of growing crystals). According to this, crystals of ice grow between adjacent antifreeze molecules with high surface curvature. High energy is required for the addition of water molecules to the convex surface. The whole process is nothing but maintenance of freezing point keeping the melting point at constant. This phenomenon is called as Kelvin effect. There are two models (mattress model and step pinning model) that justify the Kelvin effect. In the mattress model, the growth of ice crystals perpendicular to the ice surface is prevented by adsorbed molecules, whereas in the case of the step pinning model, molecules are blocked by ice growth (Bouvet and Ben 2003).

3.4.1.5 Adaptation Mechanism of Psychrophilic Enzymes

A higher degree of structural flexibility, lower thermostability, and specific activity are some of the characteristics of psychrophilic enzymes. Increased structural flexibility of psychrophilic enzymes may be restricted to a catalytic site which helps them exist in a disordered state. Increased flexibility in turn intensifies the degree of compatibility between catalytic site and substrate. This leads to an increase in substrate turnover rate and a decrease in activation energy. Multiple mechanisms have been evolved by psychrophilic enzymes to enhance their flexibility and activity and decrease thermostability. Among them, one mechanism involves reducing amino acids like arginine and proline. These amino acids are known to reduce conformational flexibility by the formation of a large number of hydrogen bonds and salt bridges. This mechanism has been observed in many psychrophilic enzymes. Some of the psychrophilic bacteria (*Shewanella* sp.) were known to have less alanine content, while others (*Psychrobacter arcticus*) lack proline/arginine content (particularly in those proteins involved in reproduction and cell division) (Zhao et al. 2010). Some other compositional variations found in the psychrophilic enzymes are increased content of methionine, asparagine, and glycine. These amino acids are found especially in the catalytic site which is known to contribute to local mobility. Increased lysine/arginine ratio is known to lower the hydrogen bond and salt bridge formation. Psychrophilic proteins with a longer external loop and reduced proline content result in less compact and highly stable proteins and also a catalytic site with more flexibility and mobility. Electron

microscopic study of cold-adapted enzymes revealed that they contain a greater number of cavities with a larger size compared to that of mesophiles (Paredes et al. 2011). Larger cavities can hold a maximum number of hydrophilic groups thereby binding the large number of water molecules which enhance the flexibility by increasing internal salvation. For example, a region present near the helical lid of the psychrophilic enzyme lipase M37 found in *Photobacterium lipolyticum* consists of the surface cavity (Jung et al. 2008). The destabilizing effects of these surface cavities may provide flexibility to the helical lid thereby enhancing the lateral movement when substrate binds to it.

3.4.1.6 Piezophiles/Barophiles

Piezophiles or barophiles are organisms with the ability to survive under high pressure (depth of sea/ocean). Piezophiles are primarily found in ocean depths, with an average pressure of 10 MPa (megapascals). Some of the microbes are also found in the deepest point in the ocean (Mariana trench) where the pressure is around 110 MPa (Abe and Horikoshi 2001). *Pyrococcus yayanosii*, an extremophile, could survive in pressures ranging up to 150 MPa (Zeng et al. 2009). To counteract the effects of the elevated pressure, these organisms have evolved various mechanisms. As of yet, very little information is gathered regarding the piezophiles. Preliminary research on piezophiles indicated their potential applications in the industrial and biotechnological field (Abe and Horikoshi 2001).

3.4.2 Adaptation Mechanism of Piezophiles (High-Pressure Adaptations)

3.4.2.1 Membrane Lipid Adaptation

The effect of high pressure is similar to that of low temperature as both are involved in decreasing fluidity by increasing packing of the fatty acyl chains of phospholipids. Piezophiles found in the depths of the ocean need to acclimatize not only to high pressure but also low temperature. Intense hydrostatic pressure reduces membrane fluidity which results in the formation of the gel-like membrane that may interfere with the uptake of nutrients and cell signaling. These problems in piezophiles can be avoided by increasing the number of mono- and polyunsaturated fatty acids in their membranes. These fatty acids are difficult to be packed tightly. This nature of fatty acids makes the movement of the membrane easier (Bartlett 1999). An example is mentioned below.

Synechocystis, a phototrophic bacterium, has a two-component regulatory system to control the expression of desaturase which is involved in regulating membrane viscosity. A key regulatory element involved in inducing the expression of desB gene (codes for desaturase) is histidine kinase 33 (Hik33) (Suzuki et al. 2001). Hik33 are the key regulatory element in homeoviscous adaptation also known to regulate more than two dozens of the gene. There are several highly conserved domains in Hik33 which include HAMP domain (histidine kinases, adenyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases), a leucine zipper domain which

transfers signals to the 2-helix bundle in DesK, and a PAS domain (Per, Arnt, Sim sensor proteins) acting as a light-sensitive module in Hik33. Two helical regions of HAMP domain present adjacent to each other are involved in converting cold stress signal by structural modification. HAMP domain signal transmission is mediated by homo-dimeric, four-helical, parallel coiled coils. Hik33 gets activated by the enhanced molecular lipid packing (Los and Murata 2004), but the underlying sensor mechanism remains to be unknown.

3.4.2.2 Outer Membrane Porins

In response to high pressure, the membrane becomes highly rigid which has a greater influence on the movement and nutrient uptake. Many different proteins are involved in order to get acclimatized to these situations. The best example is *Photobacterium profundum* SS9, which regulates the outer membrane protein under high pressure. These bacteria consist of a specialized protein called OmpH. Its concentration increases with increasing pressure. It is usually expressed at a pressure of 28 MPa which is the minimum pressure required by the *P. profundum* SS9 for its growth (Chi and Bartlett 1993). Another protein involved is ompL which acts simultaneously along with ompH, but ompL is encoded by pressure-regulated genes that express at 0.1 MPa (decreases with increasing pressure); therefore, ompH contributes more to the pressure regulation when compared to ompL (Le Bihan et al. 2013). These both are the fourth most expressed proteins at high pressure. At elevated pressure, *P. profundum* lacking the ompH is not greatly affected. OmpH is also known to play a greater role under nutrient-deprived low-pressure conditions. There are a series of nine genes that are known to be present on the outer membrane, but the functions of these genes are not analyzed yet. Among these genes, ompC and two others, hypothetical maltoporins, are studied to some extent. These two maltoporins are known to express usually at constant pressure, while other genes, pbpra2139, express at elevated pressure. Porin-encoding genes that express at high pressure are a counter-intuitive example to show how difficult it would be to survive at high pressure. Increased porin produces many different compounds to boost survivability in the nutrient-scare ecosystem (Bartlett et al. 1993).

3.4.2.3 Membrane Transport

High hydrostatic pressure (HHP) has a greater influence on the transportation system in the bacterial cell membrane (Vezzi et al. 2005). Due to high hydrostatic pressure, fluidity is affected which may interfere with the transportation of nutrients across the membrane. HHP leads to an increase in volume, inhibits certain reactions, and makes amino acid (histidine, lysine, leucine, and tryptophan) movement difficult (Abe and Horikoshi 2001). Some of the bacteria in the sea/ocean use a higher amount of acetate and glutamate at elevated pressure. In *P. profundum* SS9, amino acid synthesis and ion transport were upregulated at 0.1 MPa. This is the best example that represents the adaptation of *P. profundum* SS9 transporters to high pressure. A variety of transporters such as ion, sugar, and phosphate transporters have isoforms known to function at varying pressure. It is essential to regulate the transporters in order to survive in the case of marine bacteria. Some hypothetical models have been

explained to reveal the mechanism of transportation under high pressure, but a much detailed study has not been done yet.

3.4.2.4 Respiratory Chain

The respiration mechanism in piezophiles is quite different from other organisms to survive under extreme conditions. These consist of two kinds of electron transport systems in the inner membrane. A model organism used to study the respiratory chain in the deep sea is *Shewanella benthica* (Kato et al. 1999). A series of steps are involved in the respiratory chain of this organism: Initially, $NADH_2$ is oxidized to NAD by transferring two electrons to quinone(Q), that quinone get reduces to quinol (QH2), and it is carried out by NADH-dehydrogenase (ionic complex I). Within complex III (cytochrome c-551), electrons are exchanged between quinol and cytochrome c-551. These electrons are then passed to the active complex which covalently binds to the terminal cytochrome oxidase (a soluble protein). Later, oxygen is broken down to water by periplasmic oxidase and pumps proton to the cell. Not only periplasmic oxidase but also BC1 complex pumps proton to the cell, and this leads to the synthesis of ATP in the cytoplasm which is catalyzed by an enzyme called ATP synthase.

Under high pressure (60 MPa), the respiratory chain becomes more compact. During this situation, electrons are donated to quinol oxidase reducing the supply of oxygen to cytochrome c-551. This leads to the pumping of protons to the periplasmic vacuum. At elevated pressure, cytochrome c-552 will not be produced. The ability of a piezophile *Shewanella violacea* DSS12, to survive under high pressure depends 40% on its strain and 60% on cytochrome bc-1 complex. *Streptococcus* existing under high pressure contain two forms of soluble cytochrome. Under high hydrostatic pressure, cytochrome cA (belongs to c5 group) is constitutively expressed, whereas cytochrome cB is repressed. Three terminal oxidases exist under HHP, i.e., one terminal cytochrome c-oxidase, two bo, and bd-type quinol oxidases. At low oxygen high pressure, bd-type quinol oxidase increases, while all other terminal oxidase genes decrease. Bd-type quinol oxidase plays a greater role at high pressure and also makes a significant contribution to respiration. These kinds of variation do not occur in all types of microbes but only in piezophiles.

3.4.2.5 Motility Under High Pressure

Motility is a critical process for the survivability of bacteria as it enables bacteria to escape unfavorable conditions and helps to move toward the nutrient-rich environment. Motility occurs due to flagella. Flagella are found attached to cell envelopes and extended to extracellular space (Schuhmacher et al. 2015). The basic structure of flagella includes basal body, hook, and filament. The basal body has a C-shaped ring with a rod attached to it. All three parts of the flagellum are assembled by a type III secretion system. Approximately 25 different types of proteins are assembled in flagella. An important component of flagella is flagellin protein; 20,000–30,000 flagellin subunits are found at the distal end of the flagella. The synthesis of flagella is a highly complex process as there is the involvement of many genes. These genes

are classified as early, middle, and late genes based on their involvement in the synthesis (Merino et al. 2006).

Flagellar structure and its role under high pressure are studied using two piezophiles, i.e., *Shewanella piezotolerans* WP3 and *Photobacterium profundum* SS9; these will have either polar or lateral flagella (Campanaro et al. 2005). Lateral flagella (LF) have a complex structure, encoded by 40 genes, and have higher GC content. There are two different kinds of motors for the motion of flagella, i.e., sodium-driven motors (*Shewanella piezotolerans* WP3) and proton-driven motors (*Photobacterium profundum* SS9) (Wang et al. 2008). Two genes identified to be away from the flagellar cluster were responsible for the movement of both types of flagella. Two flagellin genes (*fla*A, *fla*C) are known to regulate flagella in *S. piezotolerans* WP3 whereas three (*fla*A, *fla*C, and *fla*b) in case of *P. profundum* SS9. Recent studies have confirmed that high pressure inhibits the *fla*A and *fla*C and prevents motility. Under high physical tension and viscosity, lateral flagella enable the bacteria to swarm rather them swim. Under nutrient-deprived status, *mot*A and *fla*B are not expressed leading to non-motility. Mutants of any of the genes in the flagella inhibit either swimming or swarming. It is revealed that the developmental process is responsible for the proper functioning of flagella. Destruction of polar flagella either genetically or physically leads to activation of lateral flagella and vice versa.

3.4.2.6 Enzymes Adaptations Under High Pressure

Microbes are the richest source of enzymes; they help microbes to carry out biochemical reactions (Kaur et al. 2020). These proteins under high pressure may undergo physical damage; hence, microbes have developed a certain mechanism for their protection. Piezophilic microbes contain proteins that are homologs mesophilic proteins. When a comparison is made between them, some mesophilic proteins are found to be pressure adapted but not all. Microbes are found in the deepest region of the ocean, i.e., Mariana trench (temp. 1–4 °C, pressure 1.1 kbar) and in the hydrothermal vent (temp. $\geq$100 °C, pressure ~0.5 kbar), describing microbes' ability to survive by acclimatizing to varying temperature and pressure (Prieur et al. 2009). Therefore, it is confirmed that microbes' survival is based on their adapting mechanism; among them, protein protection mechanisms are explained below.

Low Stability

One of the common enzymes found in the piezophiles is DHFR (dihydrofolate reductase) which is used as a model to explain piezotolerant enzymes. Usually, enzymes found in high-pressure areas will have low stability because low stability is associated with high flexibility. So far, DFHR is less stable which is indicated by ΔG_u. Unlike fatty acid conversion in psychrophiles, low stability is not a driving force for survivability. It just prevents enzymes from being ruptured under high pressure. It is not essential to adapt a feature of low stability in the piezophilic microbe. Low stability may also exist in microbes under normal pressure. Overall, it can be inferred that low stability and greater flexibility appear to be favorable for the survival of enzymes under cold and high-pressure conditions.

High Compressibility

A common feature found in all piezophilic enzymes is high compressibility. The presence of a larger internal cavity makes the protein more susceptible to pressure unfolding. Maintaining normal protein structures under high pressure without leading them to get distorted keeps the microbes highly active (Kato et al. 1998). A study on crystal structures of piezophilic enzymes revealed that enzymes are loosely packed and highly hydrated with a large internal cavity. Piezophilic enzyme will have a greater number of small cavities instead of a single larger cavity (Fig. 3.1).

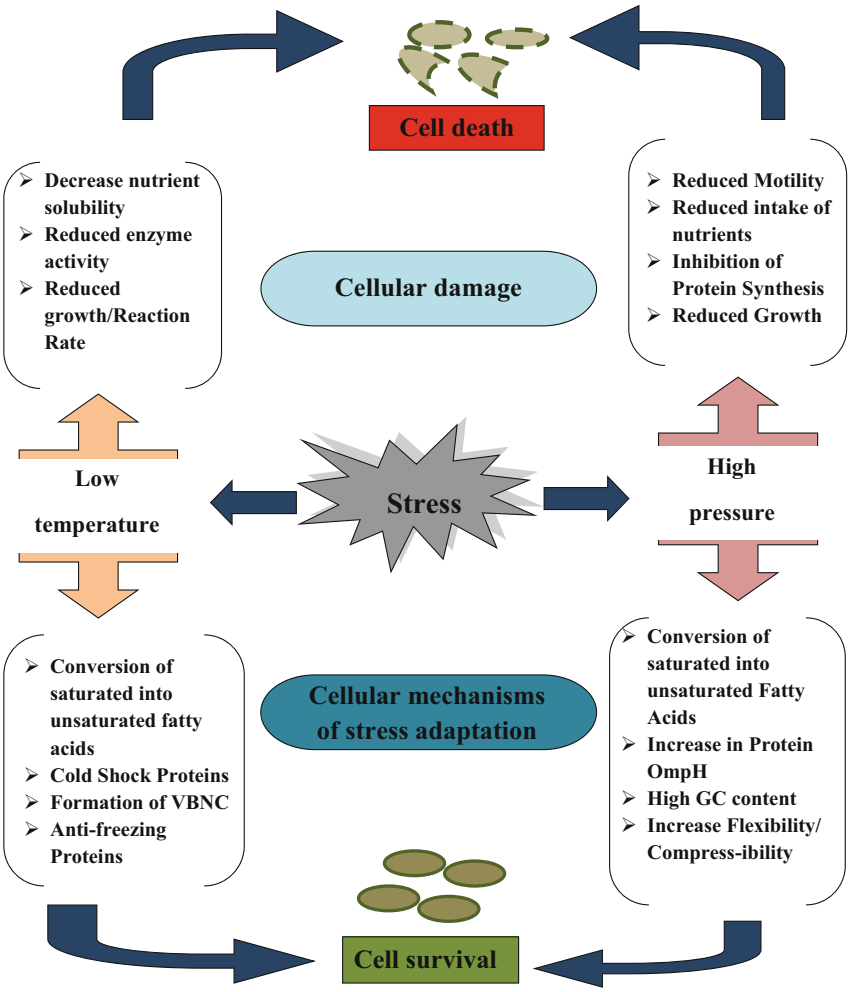

Fig. 3.1 Schematic diagram representing effects and adaptation mechanism of microbes at low temperature and high pressure

High Absolute Activity

Regardless of temperature and pressure, one mechanism adapted by microbes to retain their catalytic activity is maintaining high absolute activity. The turnover number (k_{cat}) of most of the piezophilic microbes is usually four or five times greater than other enzymes. It indicates that even activity of microbes is reduced at low temperature and high pressure, and still they can survive at their GTP. This is due to greater versatility, as they have lower stability and novel modifications to enhance the catalytic activity. But, some of the enzymes isolated from *Shewanella* normally have high catalytic activity. They do not possess any absolute activity to retain catalytic activity. Recent studies have confirmed that the catalytic activity of enzymes greatly depends on microbes rather than the condition they exist.

High Relative Activity at High Pressures

Increased relative activity helps the piezophilic microbes to maintain catalytic activity under high-pressure conditions. Many piezophilic enzymes maintain high relative activity to retain catalytic activity with available GTP. In some enzymes, increased pressure enhances both relative and absolute activity. An example is D27E, a mutant of DFHR which shows increased relative activity with a 50% increase in k_{cat} and a slight 2 kJ/mol increase in ΔG_u (Ohmae et al. 2013). An amino acid residue Asp27 in DFHR plays a central role in hydride transfer (Schnell et al. 2004). This indicates that DFHR has a slightly opened substrate-binding cleft which is the explanation for the increased activity of DFHR under high pressure. Low stability of DFHR leads them to decrease their activity above 500 bar.

3.5 Microbial Nutrition and Metabolism in Deep Sea

3.5.1 Chemistry of Deep Sea

Seawater is an open ecosystem serving as a sink of nutrients from various reservoirs. The knowledge of chemical features of seawater can provide an insight into the net functioning as well as the inflow and outflow of energy from the system. Sea surfaces have complex nutritional composition and are rich in ionic forms of nutrients especially nitrogen and sulfur. Urban storm, water runoff, irrigation drainage, agricultural runoff, creeks, rivers, and estuaries are the general portals of entry of nutrients into the marine environment (Akinde and Obire 2011). Unlike the organisms of the terrestrial ecosystem, marine organisms are dependent on dissolved forms of nutrients. Carbon, the major building block of life forms, can enter the sea interior from the atmosphere through a network of processes, where it can be stored or sequestered for millennia. Of the stored carbon on Earth, deep ocean zones constitute the largest reservoir (3150 Pmol, 1 Petamole = 1015 moles). It also corresponds to more than 50 times the amount of carbon present in the atmosphere (currently estimated as 62.5 Pmol) and more than one order of magnitude greater than all the carbon present in microbes, terrestrial vegetation, and soils combined.

Table 3.2 Major nutrient forms at deep sea levels and their source of origin [Source: Jørgensen et al. (2019), Thompson and Johnston (2017), Voss et al. (2013), Jasińska et al. (2012)]

Nutrient form	Source
Carbon	
Dissolved organic carbon (DOC)	Living plants and marine organisms (mainly phytoplankton), organic-rich detritus, or as dissolved organic carbon
Inorganic (carbonic acid, bicarbonate, and carbonate)	Atmospheric carbon dioxide
Sulfur	
Sulfate (So_4^{2-})	Sediments, weathering and leaching of rocks, biological or chemical oxidation of sulfides, sulfur partitioning, and riverine inflow
Sulfide	Sulfate (So_4^{2-}) reduction by marine microbes
Iron sulfide (FeS); pyrite (FeS_2)	Product of sulfide oxidation in the presence of C_{org} and Fe^{3+}; Pyritization of H_2S and FeS
Nitrogen	
Dissolved organic nitrogen (DON)	Rivers, atmospheric processes, wind, Ekman upwelling, biomass of surface autotrophs
No_2^-	Regeneration of particles, microbial nitrification
No_3^-	Wind, convective overturning, and Ekman upwelling, eddy activity/gyres, rivers, atmospheric deposition, shelf processes, regeneration of particles, microbial nitrification
NH_4^+	Diffusion, atmospheric deposition, regeneration of particles, decomposition of debris
N_2O	Bacterial and archaeal nitrification, intermediate of denitrification

Aside from atmospheric carbon, there are many other sources of carbon entry into the marine system (Table 3.2).

3.5.2 Microbial Metabolism in Deep Sea

Nutrient availability is a key factor for microbial existence and activity in any environment. The deep sea is an extreme oligotrophic environment that is often thought to set limits for microbial activity. However, this challenging environment is a habitat for great microbial diversity (Molari et al. 2013). The ability to survive in such contrasting extremes of temperature and pressure is assumed to have arisen from the adaptive route they followed to reprogramme their metabolism, scavenge the limiting nutrients, and bypass starvation.

The dark conditions of the deep sea enable microbes to develop photopigments; therefore, the major source of energy for benthic microbes is the downward flux of organic matter from primary producers on the sea surface (Danovaro et al. 2014). In marine environments, phytoplanktons are the primary producers (Azam and Malfatti 2007) and thereby the continuous source of organic matter for other life forms. A

large fraction of their primary production is released as dissolved organic matter (DOM) into the system, either by the producers or by the degradative action of other organisms (Ducklow and Carlson 1992). Almost half of the DOM is consumed by higher-trophic-level organisms, while the remaining (1–2%) reach the benthic microbes (chemoorganoheterotrophs), which they assimilate into their biomass and re-mineralize the excess into inorganic nutrients that re-enter the nutrient cycle. Therefore, microbes play a critical role in the marine food web by organic matter turnover and establishing a balance between net energy flux (Mason et al. 2009). It is estimated that about one-third of the CO_2 produced in oceans originates from the microbial transformations at sea bottom (Arístegui et al. 2005). Despite the heterotrophic C metabolism, there is evidence for the existence of microbes with an expression of enzymes involved in autotrophic nutrition, especially the Calvin cycle and 3-hydroxypropionate pathway subsidizing autotrophic nutrition.

Benthic microbes can also metabolize several reduced inorganic compounds to accomplish heterotrophic nutrition. These compounds usually serve as an additional source of energy for support. The most prevalent of these is the oxidation of sulfur compounds via chemolithoheterotrophy (Ghosh and Dam 2009). On an average, 10% of microbes from marine environments are found with genes (*sox*) for sulfur oxidation (Venter et al. 2004). Microbes at sea sediments are also found with the ability of nitrification, the oxidation of ammonia to nitrite and nitrate. These microbes hold 21–50% of the total oxygen demand of the deep sea and mainly belong to the group of *Gammaproteobacteria* and *Archaea* (Könneke et al. 2005; Swan et al. 2011). The energy derived from nitrification is usually associated with carbon-dioxide fixation (chemolithoautotrophs) in dark regions of the deep sea. The process of carbon-dioxide fixation in these heterotrophs is not to derive biomass carbon like autotrophs but for the transformation of organic compounds into precursors of central metabolism using assimilatory carboxylases (Wuchter et al. 2006; Middelburg 2011). This is often termed as "mixotrophic nutrition," since the fixation of inorganic CO_2 without photoactivity often costs much energy to an organism, while, here, the energy is supplied from heterophony.

Despite the evidence of diverse metabolic activity at deep sea levels, it is important to understand the stress-derived metabolic alterations in microbes in their natural environment. Studies are based on quantifying the metabolic fluxes of marine microbes under conditions of varying temperature and pressure. The metabolism of model psychrophilic bacterium *Colwellia psychrerythraea* 34H at 4 °C (the temperature at natural environment) and under heat-stressed conditions was compared with cold-stressed and mesophilic *E.coli*, respectively (Jeffrey et al. 2018). Genetic analysis revealed that both bacteria had a similar metabolic network, but 34H had certain metabolic alterations that allow it to survive as an obligate psychrophile. These include the ability to suppress catabolic repression under a complex medium, activation of anaplerotic reactions to supplement TCA intermediates via CO_2 fixation, and therefore the maintenance of high cell biomass and metabolic flux. In contrast to *E. coli*, 34H favored ED pathway as the primary glycolytic route under glucose-rich medium. The potential driving force behind is the thermodynamic advantage of the ED pathway ($\Delta G = -36$ kJ/mol) to the

bacterium as compared to EMP pathway ($\Delta G = -8$ kJ/mol) at low temperature. Therefore, it can be said till further evidence that marine heterotrophs also use simplified metabolic strategies but are rearranged to overcome the thermodynamic constraints imposed by the environment.

3.6 Conclusion

The microorganisms thriving the extreme environmental conditions indeed have uniquely adapted enzymatic and metabolic systems as discussed in the chapter. These unique metabolic and enzymatic mechanisms are the most promising resources for the isolation of cold-adapted and pressure-tolerant enzyme systems. This potential appears even larger with psychrophiles than for peizophiles in terms of their diversity and potential uses in industries. Presently, huge data is available on biochemical/physicochemical reaction and protein and enzyme structure of peizizophiles and psychrophiles which open two major research avenues: (1) detailed insights in survival mechanism of these extremophiles and (2) application of these mechanisms for biotechnological applications. These extremophiles provide an immense genetic resource for manipulating industrial strain to work under extreme environmental conditions, thus delimiting the various stress factors during industrial production. Finally, combining the basic knowledge of extreme pressure and temperature effects on biochemical/physical reaction and advanced molecular biology techniques opens greater possibilities toward generating clean, efficient, and energy-saving industrial applications.

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