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THERMOALKALIPHILIC MICROBES

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1. Introduction

The interest in studying extremophilic microorganisms has increased immensely over the last two decades. Extremophiles are organisms that are adapted to grow at or near to the extreme ranges of environmental variables. Most extremophiles are microorganisms that thrive optimally at environmental and physicochemical parameters unsuitable for the typical and widely studied mesophilic microorganisms such as *Escherichia coli*, *Bacillus subtilis*, and *Neurospora crassa* that cluster around temperature 37 °C, pH 7.4, 0.9–3 % salinity, and 1 atm pressure, the conditions that are congenial for human beings. R. D. MacElroy was the first who coined the term "extremophile" in a 1974 paper entitled "Some Comments on the Evolution of Extremophiles." The definitions of extremophile and extreme are of course anthropocentric, from the point of view of the organism per se and the environment to which it is adapted to. A highly diverse group of organisms is known that can tolerate extreme conditions and grow, but not necessarily optimally in extreme habitats. These organisms have been called extremotrophs (Mueller et al., 2005). The distinction between extremophily and extremotrophy is not merely a semantic one but also highlights a number of fundamental issues relating to the experimental studies like:

- Methods which have been used to isolate putative extremophiles may be inappropriate.
- Putative extremophily may get compromised by subsequent serial cultivation under laboratory conditions.
- Claims of extremophily that may not have been tested rigorously.
- No further investigation whether organisms are adaptable to only small differences in environmental variables or really diverted from original conditions.

It also may be noted that many species can survive extreme conditions in a dormant state but are not capable of growing or reproducing indefinitely under those conditions. Particularly over the last century, exploration of other environments has shown that a large number of organisms live under, or actually require, more "extreme" conditions (conditions hostile to humans and most of their microbial commensals) (Grant et al., 1990; Aguilar, 1996; Aguilar et al., 1998; Antranikian et al., 2005). Isolation of many more extremophilic microorganisms has become possible due to improved or more avid culture techniques, which has made possible to look into environments formerly considered uninhabitable. There is no environment which is not inhabited by living organisms; one just has to know how to recognize their presence. One of the most useful example of this fact is the Dead Sea, thought to be lifeless but actually containing large variety of exciting prokaryotic (Arahal et al., 1999) and even eukaryotic (Buchalo et al., 1998) life forms. The growth profiles could be the best criteria for characterizing extremophiles using marginal data, under certain culture or environmental conditions such as pH (pH_{opt}, pH_{min}, pH_{max}) and temperature (T_{opt}, T_{min}, T_{max}) ranges.

In general extremophiles are defined by one extreme of life, but in many cases natural environments pose two or more extremes, such as alkaline hot springs, alkaline hypersaline lakes, acidic hot springs, and dry sandy deserts. These environments harbor thermoalkaliphiles, halophilic alkaliphiles, thermoacidophiles, and UV radiation-resistant oligotrophs. Extremophiles adapted to more than two extremes, called polyextremophiles, are much less common than organisms with single extreme. While dealing with multiple extremophiles, it is problematic to give descriptions of their optimal and marginal growth data because the optimal growth parameters are interdependent as the value of one of the extreme growth conditions could be affected by the other.

For example, pH measurement of medium depends on temperature because of changing pKa values of different medium components at different temperatures (Wiegel, 1998). Thus, the pH of the medium when measured at room temperature will be different from its pH when it is measured at the elevated growth temperature using temperature-calibrated electrodes and pH meters. The difference in pH is small for neutral pH (less than 0.3 pH units), which becomes larger (more than 1 pH unit) at acidic or alkaline pH. To maintain authenticity and comparability among published data, it is important to know the conditions under which the pH was determined. Wiegel (1998) has proposed that authors indicate the temperature at which the pH is measured and the pH meter was calibrated with a superscript (e.g., $pH^{37\,°C}$).

The wide distribution of thermoacidophiles is due to frequent occurrence of hot springs with acidic pH values (≤3.0). Acidic hot springs arise because of the presence of sulfuric acid formed by microbial and chemical oxidation of sulfur compounds. The scarcity of thermoalkaliphiles (with pH_{opt} ≥9.5, $temperature_{opt}$ ≥65 °C) has been attributed to physiological causes. Growth under both of these extreme conditions requires specific adaptations of the cell wall and membrane composition to minimize permeability to protons and cations. Thermoalkaliphiles are also faced with the burden of acidifying cytoplasmic pH while growing in a scarcity of protons and various other bioenergetic problems, such as suboptimal proton-motive force and phosphorylation potential. However, the existence of such isolates cannot be ruled out, as isolates like the obligately aerobic archaeal *Picrophilus* species have been reported at such temperatures on the acidic side of the pH scale from solfataric areas in Japan (Futterer et al., 2004). The genome of *Picrophilus torridus* contained genes that allow them to cope with cytoplasmic acidification and degradation of organic acids. Interestingly, all these adaptations have been acquired by horizontal gene transfer (Futterer et al., 2004). The acquisition of the genes required for surviving extremely acidic conditions by horizontal gene transfer is intriguing as this implies that hyperalkalithermophiles could have evolved by obtaining the necessary genes from extreme alkaliphiles and extreme thermophiles.

The study of extremophiles also stems from their possible utility in industrial processes and will provide possible clues as to how and where to look for extraterrestrial life (Stetter, 1996; Shock, 1997; Litchfield, 1998; Wiegel and Adams, 1998; Javaux, 2006; Lentzen and Schwarz, 2006; Villar and Edwards, 2006).

2. Life in Hot and Alkaline Conditions: General Considerations

Study of alkalithermophiles has been vindicated earlier by Wiegel and Adams (1998) as ancestor of life and model organism for the possible terrestrial life. The authors believe that life probably originated on mineral surfaces in moderate thermobiotic (e.g., 60–85 °C range), relatively shallow pools at the edges of the early Earth's oceans instead of hyperthermobiotic environments. The necessary dynamic conditions for frequent association and dissociation of prebiotic and biotic structures, which lead to superior surviving combinations, were supplied by drastic changes in physicochemical parameters over space and time in such a thermoalkaline environment (Shock et al., 1998; Baross, 1998; Miller and Lazcano, 1998). These assumed selection conditions propose to a "bush-like origin" of life as suggested by Kandler (1998) and thus is different from the frequently assumed quasi monophylogenetic progenote. As such, some form of thermoalkaliphile can be considered as a logical descendant of hypothetical early life forms. In some cases, it is assumed that extremophiles are phylogenetically older ones (e.g., thermophilic *Clostridia*), whereas in other instances, the extremophiles are assumed to be secondary adaptations (Wiegel and Adams, 1998).

3. Definitions and Taxonomical Importance

3.1. ALKALIPHILES

Organisms that are able to grow at a high pH (pH ≥9) have two categories: The first group is categorized as alkali-tolerant species; those can grow at pH 9, but their optimum growth pH is around 7. In addition, they cannot grow at a pH higher than 10, for example, several *Virgibacillus* spp. (e.g., *Virgibacillus chiguensis*

(Wang et al., 2008) and *Anoxybacillus* spp. (e.g., *Anoxybacillus flavithermus* (Pikuta et al., 2000)). They are able to grow at pH 9 but not at pH 10, and their optimum growth pH is around 7. The second group of bacteria is categorized as alkaliphilic organisms. Alkaliphilic species can be defined as the organisms that can grow at above or at pH 10 and/or grow equally well or better in terms of growth intensity or velocity above or at pH 9 compared with those grown at a pH lower than 9. Alkaliphilic species can be further divided into obligate alkaliphiles, which cannot grow well below or at pH 8, and facultative alkaliphiles, which can grow well below or at pH 8. Some genera of bacteria include all three neutrophilic, alkali tolerant, and alkaliphilic (e.g., *Bacillus*). There are so many cases reported where in same species of bacteria, some of them are alkaliphilic and some are alkali-tolerant bacteria (e.g., *Bacillus horikoshii* (Nielsen et al., 1995)). Even among the same species of bacteria, there are possibilities that some are obligate and others facultative alkaliphiles like *Bacillus pseudofirmus* (Nielsen et al., 1995). It is considered that the above-described differences in categorized bacteria are due to the differences in the physiological function for adaptation at high pH and/or neutral pH.

3.2. THERMOPHILES

Thermophilic prokaryotes have been known as those with optimum temperature for growth between 45 and 80 °C (Cavicchioli and Thomas, 2000). Due to the development of better isolation techniques, Stetter (1996) was able to isolate *Pyrolobus fumarii* from black “smoker” chimneys in deep-ocean thermal vent environments which can grow up to 113 °C and above under hydrostatic pressure. This investigation led to change in the definition, and Stetter categorized thermophiles as hyperthermophiles (T_{opt} 80 °C or above), extreme thermophiles (T_{opt} 65–80 °C), and moderate thermophiles (T_{opt} 45–65 °C); these are the generally adapted definitions (Mesbah and Wiegel, 2008; Wagner and Wiegel, 2008).

The discovery of deep-sea hydrothermal vents in 1977 led to the first study of an ecosystem based on primary production of chemosynthetic extreme and hyperthermophilic bacteria (Prieur et al., 1995). Mostly the hyperthermophilic organisms are archaea, and most of them perform common metabolic processes such as methanogenesis; anaerobic respiration via sulfate reduction, sulfur reduction, nitrate reduction, iron reduction, etc.; aerobic respiration; or even fermentation. For long time *P. fumarii* was considered as most thermophilic organism known with the record for highest T_{max} (113 °C), a T_{opt} of 106 °C, being unable to grow below 90 °C. However, the new record is held by *Methanopyrus kandleri* strain having a T_{max} of 122 °C under high atmospheric pressure isolated from the deep ocean near Japan (Takai et al., 2008). Representative genera include *Archaeoglobus*, *Thermodiscus*, *Thermoproteus*, *Acidianus*, *Pyrococcus*, *Thermococcus*, *Desulfurococcus*, and *Sulfolobus*, which can oxidize H_2S or elemental sulfur; the methanogens *Methanothermus*, *Methanococcus*, and *Methanopyrus*; and the nitrate reducers *Pyrobaculum* and *Pyrolobus*. These organisms are mostly chemosynthetic or have metabolic process that no doubt plays a major role in the local environment and biogeochemical cycles. Hyperthermophilic bacteria are also included in the genera *Thermotoga* and *Aquifex*.

Most of the extremely thermophilic bacteria are anaerobic Firmicutes, which include cellulolytic *Caldicellulosiruptor saccharolyticus* (Rainey et al., 1994), the ethanol-producing *Thermoanaerobacterium* (Wiegel, 1992; Wiegel and Ljungdahl, 1996), as well as the acetogenic facultative chemolithoautotrophic *Thermoanaerobacterium kivui* (Leigh and Wolfe, 1983) and denitrifying *Ammonifex degensii* (Huber et al., 1996).

Some important and interesting for both basic and applied research are aerobic extreme thermophiles that include the well-known *Bacillus stearothermophilus* (Firmicutes) and some species within the Gram-negative genus *Thermus*, inhabitants of hot water boilers. Recently described novel thermophilic species include the citrate-fermenting *Sporolituus thermophilus* (Ogg and Patel, 2009); the novel microaerophilic, nitrate- and nitrite-reducing thermophilic bacterium *Microaerobacter geothermalis* (Khelifi et al., 2010); the deep-sea bacterium *Nautilia abyssi* (Alain et al., 2009); the thermal mud-inhabiting *Anoxybacillus thermarum* (Poli et al., 2009); and finally the novel bacterial phylum *Caldiseria* (Mori et al., 2009).

Some important moderate thermophiles and thermotolerant organisms are the cellulolytic *Clostridium thermocellum*, the acetogenic *Moorella thermoacetica/thermoautotrophica*, and *Thermoanaerobacterium* (former *Clostridium*) *thermosaccharolyticum*, the "can-swelling" organism capable of growing in vacuum-packed foods (Kristjansson, 1992; Wiegel and Canganella, 2000; Prevost et al., 2010). The obligate mixotrophic *Thiomonas bhubaneswarensis*, the marine *Lutaonella thermophila*, the cellulolytic bacteria *Clostridium clariflavum* and *Clostridium caenicola*, the facultative microaerophilic *Caldinitratiruptor microaerophilus*, and a novel hydrogen-producing bacterium from buffalo dung have been described (Arun et al., 2009; Panda et al., 2009; Shiratori et al., 2009; Fardeau et al., 2010; Romano et al., 2010). Some important aerobic ones isolated in our laboratory are neutral amylase producer *Geobacillus thermoleovorans* NP54 (Malhotra et al., 2000), xylanolytic *Geobacillus thermoleovorans* (Sharma et al., 2007), and novel polyextremophilic endoxylanase-producing *Bacillus halodurans* TSEV1 (Kumar and Satyanarayana, 2011).

3.3. THERMOALKALIPHILIC MICROBES

Prokaryotes having ability to proliferate in environmental conditions having high pH extreme under elevated temperature are known as alkalithermophiles. Since they are able to sustain more than one extreme condition, these are considered polyextremophiles. Many definitions have been proposed that classify microorganisms based on their requirement or tolerance for salt, alkaline pH, and temperature (Kevbrin et al., 2004; Bowers et al., 2009). Since the minimum, optimum,

Table 1. Marginal data for defining alkaliphiles and thermophiles.

Alkali tolerant[a]	$pH_{min} \geq 6.0$	$pH_{opt} < 8.5$	$pH_{max} > 9.5$
Alkaliphiles[a]	$pH_{opt} \geq 7.5$	$pH_{opt} > 8.5$	$pH_{max} > 9.5$
Thermotolerant	T_{min} –	$T_{opt} < 50$ °C	$T_{max} < 60$ °C
Thermophiles	T_{min} –	$T_{opt} > 50$ °C	$T_{max} > 60$ °C
Extreme thermophiles	T_{min} usually ≥ 60 °C	$T_{opt} > 75$ °C	$T_{max} > 85$ °C
Hyperthermophiles	T_{min} usually > 65 °C	$T_{opt} > 80$ °C	$T_{max} \leq 122$ °C

[a]The temperature at which pH values were measured with a calibrated pH meter (Wiegel, 1998).

and maximum salt concentration, pH, and temperature for growth are dependent upon each other and can vary with changes in the growth medium composition, it is very difficult to draw sharp boundaries for what a thermoalkaliphile is. The definition described by Kevbrin et al. (2004) is accepted: microorganisms that grow optimally at or above pH 8.5 and temperatures greater than or equal to 50 °C are deemed as thermoalkaliphiles. More simplified definition is given in Table 1.

Alkalithermophiles are found within both *Bacteria* and *Archaea*. Of the thermoalkaliphilic bacteria, the anaerobic thermoalkaliphiles fall into the class Clostridia, phylum Firmicutes. *Clostridium paradoxum* is considered as the most thermoalkaliphilic anaerobe, having a pH_{opt} for growth of 10.3 and a maximum of 11.3, with T_{opt} around 56 °C, whereas *Thermococcus alkaliphilus* and *Thermococcus acidaminovorans* are the most thermophilic ones (Keller et al., 1995; Dirmeier et al., 1998), growing optimally around 85 °C, with pH_{opt} of only 9.0. Among the aerobes, *Bacillus alcalophilus* B-M20 represents the most alkaliphilic thermoalkaliphile, with a pH_{opt} around 10.5 and T_{opt} of 60–65 °C. It has been observed that with increasing T_{opt}, the pH_{opt} becomes less alkaline and vice versa: at a T_{opt} of 72 °C, the pH_{opt} is around 9.2 (*Bacillus* sp. TA2.A1), and at a T_{opt} of 80 °C, the pH_{opt} is only around pH 8.0 (*Bacillus caldotenax* YTG).

Two moderately thermophilic novel species of *Anoxybacillus*, *Anoxybacillus tengchongensis* and *Anoxybacillus eryuanensis* (T_{opt} 50 °C and T_{opt} 55 °C), have been isolated from hot springs in Tengchong and Eryuan counties in Yunnan Province (Zhang et al., 2011). A novel strict aerobic genus and species of Firmicutes, *Caldalkalibacillus thermarum*, had been reported, which is a moderate thermophile (T_{opt} 60 °C) and alkaliphile (pH_{opt} 8.5) (Xue et al., 2006).

Recently three moderately thermophilic alkaliphilic novel strains of *Bacillus halodurans* have been isolated from different environmental samples in our laboratory; all three are having T_{opt} 45–52 °C and pH_{opt} 9–10.5 (unpublished work). All of them are good producers of thermoalkalistable hydrolases. One of them is a good source of thermoalkalistable endoxylanase having potential applicability in various biotechnological processes (Kumar and Satyanarayana, 2011).

Prokaryotes can adapt to a wide range of environments compared with other organisms due to their tremendous genetic diversities. Particularly bacteria have gone through generations of changes over a long period on earth. In addition, bacteria are very well-known global material managers. Therefore, it can be said that the maintenance of environmental conditions on earth depends on the bacterial activity in ecological niches. On the basis of the above facts, it is important to know the environmental and taxonomic distributions of bacteria to understand their functions and communities in the environment. It is also beneficial to understanding the relationship between taxonomic diversity, geographical distribution, and the variation in adaptation strategy in an environment, along with the concomitant evolutional process of bacteria. Knowledge of all these parameters will contribute to the understanding of the contribution of microorganisms to environmental sustainability on earth. However, it is not easy to understand the genetic diversity of bacteria in a short term, even with limited environment and limitations of bacterial categorization. Furthermore, it is not easy to consider the ecological function of such species of bacteria. In case we consider only small groups of polyextremophiles like alkaliphilic and thermophilic bacteria, it would be much easier to think about the above problems because bacteria can be isolated selectively from an environmental sample. In addition, alkaliphilic and thermophilic bacteria can be isolated from a wide range of environmental samples, and therefore, we will be able to consider a wide range of environments for a limited category of bacteria in comparison with other polyextremophilic bacteria. This approach will reduce the difficult problem in microbial ecology and will bring us a simplified model of bacterial taxonomic diversity and environmental distribution as compared to an exhaustive approach.

Based on the size and natural or artificial environments, Yumoto et al. (2011) have adopted four environmental bacterial habitats for alkaliphiles: large natural and artificial environments and small natural and artificial environments. If we apply these categories to the habitats of alkaliphiles, we can categorize the bacterial habitats as follows: large natural environments such as warm alkaline soda lakes, large artificial environments such as alkaline wastewater treatment systems (paper and pulp industries, textile industries, indigo dye processing units, and others), small natural environments such as the gut of termites, and small artificial environments such as laboratory enrichment culture for alkaliphilic thermophiles. To a greater or lesser extent, these four environmental categories can classify most of the microorganisms. The global material circulation and the human society are very much dependent upon the actual status and dynamics of bacterial flora between natural and artificial environments, and their symbiotic associations. However, it is not very easy to understand these differences by exhaustively considering all bacteria. If we consider a more solid proposal of a study of the above-mentioned problem on all four categories of bacterial habitats, the differences in physiological function between the same species of bacterial strains isolated from different habitats may also clarify the relationship between ecological niches and their physiological function. It is also an interesting question why distribution of alkaliphilic and thermophilic bacteria is not limited to only thermoalkaline environments but also can be isolated from conventional garden soil. To resolve such a problem, it is important to accumulate data on the characteristics of corresponding species from different habitats. Targeting polyextremophiles will be useful from the physiological as well as microbial ecological points of view.

4. Habitats: Environmental Conditions Where They Thrive Well

Extreme habitats like hot springs, deep-sea hydrothermal vents, cold and hot deserts, and polar ice continue to provide a rich resource of novel microorganisms, appearing uniquely adapted for these extreme niches (Junge et al., 2002; Nagy et al., 2005; McCliment et al., 2006). Extreme environments that have received little attention are high-temperature soil systems such as those found on geothermally heated volcanic grounds.

It is generally assumed that the major environments from which thermoalkaliphiles can be isolated would be alkaline and thermobiotic such as naturally hot environments on earth ranging from terrestrial volcanic sites (including solfatara fields) to alkaline hot springs and the new alkaline hydrothermal vents of the "Lost City" or alkaline lakes like Lake Bogoria (Africa) and Rehai Geothermal Field, located in Tengchong County (China). There are also anthropogenic hot environments such as compost piles (usually around 60–70 °C but as high as 100 °C), slag heaps, industrial processes, and water heaters (Oshima and Moriya, 2008). As described in Sect. 1, the probability of isolation of alkaliphilic thermophiles is not restricted to environments having extreme conditions only; many thermoalkaliphiles have also been isolated from mesobiotic, slightly acidic to neutral habitats (Wiegel, 1998).

Thermoalkaliphiles have been generally categorized on the basis of their niches from which they have been isolated, their geographical distribution, and the geochemistry. The following cases can be distinguished based on spatial relationships: (1) narrow biogeography but with a relaxed biogeochemistry (organisms found are only in one very restricted location but various environmental niches like the nonspore-forming *Anaerobranca horikoshii* isolated from a specific location in Yellowstone National Park containing both acidic and alkaline springs next to each other (Engle et al., 1995)), (2) narrow and restricted biogeochemistry but relaxed biogeography (organisms can be isolated only from one type of environment but from different continents such as sporulating *C. paradoxum* found only in sewage sludge but in all tested sewage samples from various continents (Li and Poole, 1999)), and (3) relaxed biogeography and biogeochemistry (those thermoalkaliphiles which are ubiquitously distributed such as nonsporulating *Thermobrachium celere* in thermobiotic, mesobiotic, alkaline, and slightly acidic sediments from various continents (Engle et al., 1996)). An example of an aerobic thermoalkaliphile from a non-alkaline sample is *Bacillus* sp. Strain JB-99 isolated from slightly acidic sugarcane molasses (Johnvesly and Naik, 2001). Another example is *Thermalkalibacillus uzonensis* which was isolated from neutral green mat samples from hot springs of Kamchatka (Zhao et al., 2006).

In short, alkalithermophiles have been isolated from mesobiotic as well as thermobiotic environments of natural (sediments, soil, manure piles) and anthropogenic origin. Furthermore, most of the aerobes and anaerobes are sporeformers, which make this group of the thermoalkaliphiles ubiquitous with respect to their geographical distribution.

5. Physiological Properties: Adaptation or Natural Selection?

The thermoalkaliphiles isolated so far are neither the most alkaliphilic nor the most thermophilic of the extremophiles. From the presently characterized thermoalkaliphiles, it appears that the larger the temperature optimum, the lower the pH optimum, and vice versa. This is not only true for species among different genera but also for different strains of the same species (Kevbrin et al., 2004).

Microorganisms utilize a number of adaptive mechanisms in order to enable them to proliferate in extreme environments, and this is true to an even greater extent with polyextremophiles. Life at alkaline pH values and high temperatures undoubtedly requires special adaptive physiological mechanisms. Each extreme growth condition, whether it is high salt concentration, alkaline pH, or high temperature, poses a number of physiological and bioenergetics problems. The physiological changes adopted by polyextremophiles may be a combination of changes adopted by microbes when they are exposed to individual extreme of life. Even within a given group, a very wide range of environmental limits may be tolerated. Some well-recognized adaptive mechanisms are discussed below.

5.1. ADAPTIVE MECHANISMS FOR ALKALINE CONDITIONS

The bioactive proteins (enzymes, hormones, and others) play a crucial role in organisms' survival and have distinct ranges of pH within which they can function, which propel living cells to maintain pH homeostasis. In addition, cellular bioenergetics is intricately dependent on the proton concentration. The central energy currency of bacterial cell is proton-motive force (PMF), and the pH gradient (ΔpH) across the bacterial cell membrane is one of the two PMF components (transmembrane pH gradient (ΔpH) and a transmembrane electrical potential ($\Delta\psi$)). Higher eukaryotes (including humans) follow strict pH homeostasis (internal pH 7.3) with external pH 7.4 (Casey et al., 2010). Neutrophiles can grow between pH 5.5 and 9.0, but they maintain their internal pH $\approx 7.5 \sim 7.7$ (Padan et al., 2005; Slonczewski et al., 2009). In extremophiles, the strategies remain almost same as observed in neutrophiles, with some adaptations for responding to more extreme challenges. A large number of adaptive mechanisms are involved in maintaining pH homeostasis in alkaline environments, and some important ones include:

- Changes in cell surface properties
- Increased expression and activity of Na^+ $(K^+)/H^+$ antiporters
- Increased ATP synthase activity that couples H^+ entry to ATP generation
- Increased metabolic acid production through amino acid deaminases and sugar fermentation

The most important among the above are Na^+ $(K^+)/H^+$ antiporters because there is a well-established correlation between Na^+ dependence and alkaliphily for mesophilic alkaliphiles and thermoalkaliphiles. There is a major difference between the aerobic and anaerobic alkaliphiles. Aerobic alkaliphiles are well known for pH homeostasis and are even able to raise or lower the external media pH to obtain optimal growth conditions (Horikoshi, 1991), where as the anaerobic alkaliphiles change their intracellular pH with the extracellular pH and thus do not maintain a pH homeostasis; some possible reasons are discussed below.

The requirement of cytoplasmic Na^+ to support high levels of alkaliphile antiport activity is fulfilled by numerous Na^+/solute symporters and two Na^+ channels: a voltage-gated Na^+ channel (Na_VBP) and another flagella-associated (MotPS) channel (Krulwich et al., 1985; Ito et al., 2004a, b; Fujinami et al., 2007; Krulwich et al., 2009).

Less is known about the antiporters which have major roles in anaerobic alkaliphiles or Gram-negative alkaliphiles, and these are present in very small numbers than aerobes (Mesbah and Wiegel, 2008). Polyextremophilic anaerobe *Natranaerobius thermophilus*, a halophilic and thermophilic alkaliphile, has been shown to possess a large complement of both Na^+/H^+ and K^+/H^+ antiporters (Mesbah et al., 2009; Krulwich et al., 2009). From this it can be concluded that cytoplasmic pH regulation and homeostasis are not controlled only by different antiporters. The properties of an antiporter that control its impact on cytoplasmic pH acidification include the stoichiometry and kinetics of the exchange.

In aerobic alkaliphilic *Bacillus* spp., proton uptake that accompanies the ATP synthesis mediated by F_1F_0-ATP synthase contributes to pH homeostasis. While anaerobic and thermophilic alkaliphiles such as *N. thermophilus* and *Clostridium paradoxum* use their F_1F_0-ATPases in the hydrolytic direction to generate a $\Delta\psi$, they have to avoid proton loss, and hence, they do so by using Na^+-coupled F_1F_0-ATPases instead of H^+-coupled F_1F_0-ATPases (Ferguson et al., 2006; Mesbah et al., 2009).

In aerobic alkaliphilic *Bacillus* spp., ATP synthases work in the synthetic direction. Their proton-translocating subunits *a* and *c* have specific sequence motifs which support function at high pH and protects cytoplasmic proton loss during ATP synthesis (Ivey and Krulwich, 1992; Wang et al., 2004; Liu et al., 2009; Fujisawa et al., 2010). Wang and his colleagues (2004) reported that mutations in these motifs of non-alkaliphile consensus sequence lead to the reduced ATP synthase activity, usually with shift in pH from 7.5 to pH 10.5. It has been very well established that magnitude of the defect in ATP synthase activity correlates with a loss of the capacity for pH homeostasis during a sudden alkaline shift in external pH (Fujisawa et al., 2010; Wang et al., 2009). These mutations in motifs of ATP synthase also lead to proton leakiness (Liu et al., 2009; Fujisawa et al., 2010; Wang et al., 2009). Thus, it is possible that it was ATP synthase that adapted to promote both functions in pH homeostasis and ATP synthesis at high pH and elevated temperatures. Recently Preiss et al. (2010) had revealed atomic structure of the rotor of ATP synthase from the *B. pseudofirmus* OF4, a homo-oligomeric ring composed of 13 hairpin-like *c*-subunits, by three-dimensional X-ray crystallography. It was also evident that two major alkaliphile-specific motifs – AxAxAxA in the amino-terminal helix and PxxExxP in the carboxy-terminal helix – seem to influence the properties of the ion-binding site, including the presence of a water molecule, and are functionally important for high pH bioenergetics (Liu et al., 2009; Wang et al., 2009). Together these features of this *c*-ring have been proposed to support the high affinity of the binding sites for protons (Preiss et al., 2010), and some of these features decrease the growth capacity of *B. pseudofirmus* OF4 at a near-neutral external pH (Hicks et al., 2010). Similar types of adaptations are expected with other aerobic alkaliphiles growing at high temperatures.

Apart from Na^+-based mechanisms, other strategies are also in play for survival in alkaline and thermally heated environment. Organisms growing at high temperatures are faced with the challenge of controlling cytoplasmic membrane permeability to protons, due to increased intramolecular motion of lipids at elevated temperatures. Because of this motion, water molecules are trapped in the lipid core, allowing protons to hop from one molecule to the other. Diffusion is a temperature-dependent process; thus, membrane permeability to ions will increase as well. Other ions, unlike protons, can diffuse through the membrane (Konings et al., 2002). The presence of specific secondary cell wall polymers associated with peptidoglycan has been reported that plays a crucial role for survival at pH 10.5 and high temperature, but these are not required at 7.5 in mesophilic conditions (Padan et al., 2005). Large proportions of cardiolipin and squalene are also reported in alkaliphilic *Bacillus* and is hypothesized that both these membrane components are involved in trapping protons at the membrane surface. The phospholipid fatty acid (PLFA) profile of *N. thermophilus*, when grown at its optimal growth conditions of $pH^{55\,°C}$ 9.5, 1.5 M NaCl, and 53 °C, revealed a unique pattern of branched chain dimethyl acetals, which are expected to play a critical role in cell permeation mechanism (Mesbah et al., 2007). It was also found that the presence of acidic amino acids in the cell wall of *Bacillus halodurans* supports growth at alkaline pH (Horikoshi, 2011). The peptidoglycan hydrolysate of polyextremophilic *Nv. wadinatrunensis* was of the type α-4-β and does not contain diaminopimelic acid isomers. It primarily consists of amino acids like aspartic acid, glutamic acid, ornithine, alanine, and glycine (Mesbah and Wiegel, 2012). The PLFA profiles of the three halophilic thermoalkaliphiles did not show any fatty acids longer than 18 carbons, the hallmarks of thermophilic bacteria, and there are only small amounts of some branched and unsaturated fatty acids

(Mesbah and Wiegel, 2012). These kinds of structural changes are being considered as reasons of less T_{opt} for polyextremophiles.

Another challenge for organisms growing at high temperatures is that they must have mechanisms to preserve protein structure at elevated temperatures. In general the preferred adaptations for extreme conditions are change of amino acid sequence of a protein by mutations, optimization of weak interactions within the protein at the protein and solvent boundary, and the influence of extrinsic factors such as metabolites and cofactors. Thermal adaptations are consequence of a number of subtle interactions, often characteristic for each protein species. Comparison of protein sequences of thermophilic and mesophilic bacterial sp. revealed amino acid substitutions involved in adapting proteins to higher temperatures. The main changes were an increase in hydrophobic amino acid residues (Haney et al., 1999). This adaptation minimizes surface energy and the hydration of nonpolar surface groups while burying hydrophobic residues and maximizing packing of the core (Yip et al., 1995). Another group of compounds, low-molecular-weight aliphatic polycations, polyamines are involved in numerous processes and are reported to play critical roles in the stabilization of nucleic acids and proteins during exposure to extremes of temperatures, either hot or cold (Tabor and Tabor, 1985). Polyamines stabilize DNA and RNA in cells of thermophiles; the presence of the polyamine tetrakis(3-aminopropyl)ammonium and spermidine is critical for protein biosynthesis in *Thermus thermophilus* near the optimal growth temperature of 65 °C (Uzawa et al., 1993; Terui et al., 2005).

6. Use of Thermoalkaliphiles in Biotechnology

The thermoalkaliphiles and halothermoalkaliphiles are promising in terms of production of biomolecules suited for a variety of applications. Enzymes from these microorganisms have found major commercial applications such as in laundry detergents, for efficient food processing, in finishing of fabrics, and in pulp and paper industries. Among various extremophiles, the immense potential of alkaliphiles has been realized since the 1960s, primarily due to the pioneering work of Horikoshi (1999). Products of industrial importance from alkaliphiles have been commercialized, the most successful of which are alkaline proteases and amylases for detergent and food industries. In a recent industrial survey, it has been shown that the enzyme industry worldwide is valued \$5.1 billion and is predicted to show an annual increase in demand by 6.3 %. Enzymes with process-specific characteristics and those used for animal feed processing, food processing, detergent, and ethanol production are envisaged to have increased demand (http://www.freedoniagroup.com/World-Enzymes.html). It is very important to notice that the production of extremozymes having requisite properties for respective industrial application is very low and uneconomical. Since most of the industrial process uses high temperature and alkaline pH, thermoalkaliphiles are well known to produce biomolecules for such applications. Some important

Table 2. Industrially important enzymes produced by thermoalkaliphilic bacteria.

		Optimum production conditions		
Enzyme	**Organism**	T_{opt} **(°C)**	**pH**$_{opt}$	**References**
Proteases	*Bacillus* sp. GUS1	70	8.0–12.0	Seifzadeh et al. (2008)
	Nocardiopsis prasina HA-4	50	7.0 and 10.0	Ningthoujam et al. (2009)
	Bacillus circulans	70–80	Alkaline pH	Rao et al. (2009)
	Paenibacillus tezpurensis AS-S24-II	45–50	9.5	Rai et al. (2010)
Amylase	*Bacillus halodurans* LBK 34	60	10.5–11.5	Hashim et al. (2005)
	Bacillus halodurans 38C-2-1	50–60	10.0–11.0	Murakami et al. (2007)
	Bacillus sp. PN5	90	10.0	Saxena et al. (2007)
	Bacillus sp. A3-15	70	11.0	Arikan (2008)
	Bacillus flexus XJU-3	40	10.0	Zhao et al. (2008)
	Halobacterium salinarum MMD047	40	8.0	Shanmughapriya et al. (2009)
	Streptomyces gulbargensis	45	8.5–11.0	Dastager et al. (2009)
Xylanase	*Rhodothermus marinus* ITI376	61	7.5	Dahlberg et al. (1993)
	Dictyoglomus sp. B1	70	8.0	Adamsen et al. (1995)
	Bacillus sp. SPS-0	60	8.2	Bataillon et al. (1998)
	Clostridium absonum CFR-702	75	8.5	Rani and Nand (2000)
	Bacillus pumilus	45	10	Duarte et al. (2000)
	Bacillus sp. NCIM59	50	10	Nath and Rao (2000)
	Bacillus circulans b AB16	55	8.0	Dhillon et al. (2000)
	Bacillus sp. JB-99	50	10	Virupakshi et al. (2005)
	Paecilomyces thermophila J18	50	7.0	Yang et al. (2006)
	Geobacillus thermoleovorans AP07	70	7.5	Sharma et al. (2007)
	Streptomyces sp. 7b	50	8.0	Bajaj and Singh (2010)
	Bacillus halodurans TSEV1	45	9.0	Kumar and Satyanarayana (2011)
	Bacillus halodurans TSPV1	50	10.0	(our unpublished work)

extremozymes obtained from moderate thermoalkaliphilic bacteria are presented in Table 2.

6.1. PROTEASES

Commercially, proteases have major uses as components of detergent formulations and contact lens solutions, in cheese production, in processing of meat products, and for the recovery of silver from photographic films (earlier it was recovered by burning of films) (Ito et al., 1998; Gupta et al., 2002). To be components of detergent formulations, it is advantageous for proteases to have broad substrate specificity as well as function effectively at alkaline pH and high temperature. Proteases including thermoalkaline proteases are already reviewed earlier by many authors (Anwar and Saleemuddin, 1998; Gupta et al., 2002). Some recently reported thermoalkaline proteases produced mostly by aerobic thermoalkaliphilic bacteria are mentioned in Table 2. In the leather industry, routine hide-processing techniques entail the usage of harmful chemicals like sodium sulfide. Application of alkaline thermophilic keratinolytic proteases for the de-hairing step can result in improvement of leather quality as also less production of toxic waste (Gupta et al., 2002). There are several reports on the production methods and their functional utility in diverse fields such as agriculture and production of pharmaceuticals, cosmetics, and in protease preparations that cause cleavage of prion proteins (PrPSc) (Brandelli, 2008; Brandelli et al., 2010; Hirata et al., 2010).

6.2. AMYLASES

Thermostable α-amylases have been found to be beneficial for starch-processing and brewing industries, since these industrial processes utilize elevated temperatures to facilitate quicker and enhanced activity of reactants and to reduce process viscosity (Leveque et al., 2000; Pandey et al., 2000; van der Maarel et al., 2002). The enzyme preparations that contain alkaline debranching enzymes (pullulanases, amylopullulanase, neopullulanase, or isoamylase) in combination with amylases at high pH and temperature can remove stains more effectively (Ito et al., 1998). Ballschmiter and coworkers (2005) had reported, for the first time, a thermostable amylase having broad pH range from a thermoalkaliphilic *Anaerobranca gottschalkii* to exhibit transglycosylation on maltooligosaccharides as well as β-cyclodextrin glycosyltransferase (CGTase). CDs are cyclic molecules; a hydrophobic cavity is formed by them which facilitates inclusion of compounds of suitable size and polarity, which, in turn, changes the properties of these compounds and increases their industrial applications (Schmid, 1989). After the establishment of efficient industrial production of CDs using crude cyclomaltodextrin glucanotransferases (CGTase, EC 2.4.1.19), there are many reports on the production of thermostable and alkali-stable CGTases of *Bacillus* spp. and other bacterial strains (Matsuzawa et al., 1975; Martins and Hatti-Kaul, 2002; Thiemann et al., 2004; Yim et al., 1997; Atanasova et al., 2008). In spite of the apparent widespread occurrence of amylase-producing bacteria and efficient screening methods, a wide scope still exists for applications of amylases in other processes such as degradation of raw starch. Starch processing is widely used in the food industry for the production of maltodextrin and glucose syrups. It is performed in a two-step process consisting of liquefaction followed by saccharification. Thermostable α-amylases (EC 3.1.1.1) belonging mostly to family GH13 are added before the heat treatment, usually performed at 105–110 °C, to catalyze the dextrinization of starch. Commercial preparations of α-amylases from hyperthermophilic archaea, namely, *Pyrococcus furiosus*, *Pyrococcus woesei*, and *Thermococcus litoralis*, having the ability to resist for several hours at the operational conditions (75–100 °C), have been exploited (Turner et al., 2007). However, ideally thermostable enzymes active and stable at low pH (about 4.5) and calcium independent for stability and activity would be even more suitable for this process (Satyanarayana et al., 2012).

Another important application of thermoalkalistable amylases is in textile industries: before dyeing and bleaching, textiles have to be treated to remove the size in a process named desizing; this is usually performed enzymatically by using α-amylases. Desizing is performed by prewashing and an impregnation of the textiles at 75–80 °C with a desizing liquor containing surfactants to wet starch and to make it available for hydrolysis. These lacunae can be addressed by bioprospecting for high yielding strains as well as those producing amylases with better process performance characteristics.

6.3. CELLULASES

Extensive studies are going on globally for facilitating usage of cellulose-based substrates as renewable sources of bioenergy. Renewable agricultural residues and municipal wastes, cellulose and hemicellulose are not effectively utilized due to considerable costs associated with the conversion processes. Since lignocellulosic materials are more soluble at high-temperature and alkaline conditions, there is a need of lignocellulolytic enzymes which are active at high temperature and high pH. Several cellulases have been reported from thermophilic bacteria as recently reviewed by Klippel and Antranikian (2011); very few of them are having both alkalistability and thermostability simultaneously. In this panorama, cellulases from thermoalkaliphilic bacteria and archaea would be worthwhile to investigate. First, due to having intrinsic remarkable resistance to heat and alkali (Kevbrin et al., 2004) and other protein denaturants, their enzymes and proteins suit application in industrial processes. In addition, due to low complexity of the prokaryotic genomes, their genes are easily accessible and can be expressed and manipulated in heterologous hosts. Although cellulases from alkaline origin are being successfully utilized as detergent additives (Fukumori et al., 1985), thermoalkalistable cellulases find major applications in finishing of fabrics and clothes like biopolishing and biostoning of denim jeans (Pazarlioglu et al., 2005). Other major applications are deinking of wastepaper to improve the characteristics of recycled pulp (Bajpai and Bajpai, 1998). Recently Klippel and Antranikian (2011) have discussed in detail about thermostable cellulases and cellobioases produced by hyperthermophilic bacteria and archaea.

6.4. XYLANASES

Xylan is the major component of hemicellulose that contributes to 15–30 % of the total dry weight in angiosperms and 7–12 % in gymnosperms (Singh et al., 2003; Izydorczyk and Dexter, 2008). Owing to the heterogeneity and complex chemical nature of plant hemicelluloses, its complete breakdown requires the action of several hydrolytic enzymes with diverse substrate specificity, namely, β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid (ferulic and *p*-coumaric acid) esterase (Singh et al., 2003; Collins et al., 2005). Among them, β-1,4-endoxylanase has a major role to play in industries like paper and pulp industry, biofuel industry, food and feed industries, and other biotechnological applications as reviewed recently by Satyanarayana et al. (2012). It has been established by so many workers in the past that xylanases from thermoalkaliphilic origin will be beneficial for such applications. Many *Bacillus* and *Geobacillus* spp. have recently been reported for the production of thermoalkalistable xylanases using lignocellulosic agricultural residues as well as commercial xylan sources in submerged fermentation (Anuradha et al., 2007; Sharma et al., 2007; Sanghi et al., 2009; Ko et al., 2010; Nagar et al., 2010; Kumar and Satyanarayana, 2011; Satyanarayana et al., 2012). Some recent xylanases produced by thermoalkaliphilic bacteria are cited in Table 2.

Other important enzymes produced by thermoalkaliphilic bacteria and archaea are pectinases used in degumming of ramie fibers (a novel thermophilic and alkaliphilic *Geobacillus thermoglucosidasius* growing optimally at 60 °C and pH 8.5 was isolated for production of pectinase (Valladares Juárez et al., 2009)); thermoalkalistable catalases and peroxidases can be used to remove residual hydrogen peroxide from effluent streams of the textile processing industry. Due to their versatile uses, thermoalkaliphilic enzyme-producing bacteria and archaea have received undue attention in the recent years (Wiegel and Kevbrin, 2004).

7. Conclusions and Future Prospects

Thermoalkaliphiles are an exciting group of extremophilic microorganisms that comprises representatives from both bacteria and archaea. Their adaptations to high pH and elevated temperature draw attention not only as a model for studying adaptive mechanisms to extreme environmental parameters but also as sources of industrially valuable enzymes. Some of the chemolithotrophic thermoalkaliphiles (e.g., CO-oxidizing iron reducers) have been considered as one of the earliest microbial life forms on Earth. In short, many opportunities exist for studying them to answer many questions regarding their adaptations and biotechnological applications. Future research directions including the refinement of culture media and enrichment isolation techniques, culture-independent metagenomic approaches for understanding their diversity and gene mining, strategies based on cell-cell communication, high-throughput innovations, and a combination of these approaches with recombinant DNA technology will lead to novel insights into the world that once was thought to be hostile for any form of life.

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