Enzyme Engineering
ISSN: 2329-6674

Garg and Singh, Enz Eng 2015, 4:2
DOI: 10.4172/2329-6674.1000129

Review Article Open Access

Extreme Environments: Goldmine of Industrially Valuable Alkali-stable Proteases

Satyendra Kumar Garg* and Sanjay Kumar Singh

Center of Excellence, Department of Microbiology, Dr. Ram Manohar Lohia Avadh University, Faizabad, Uttar Pradesh, India

Abstract

Since the advent and release of Bio 40 (the first detergent with bacterial alkaline protease) in 1959, exploration of the microbial alkaline proteases has been exploited beyond expectations. The inventory of microbial alkaline proteases is difficult to draft as it is increasing daily. Most of these proteases are the result of studies in which one or a few isolates were targeted for an alkali-stable enzyme. Although, the worldwide research on microbial alkaline proteases has been widely performed, we still need an improved enzyme capable of catalyzing reactions under extreme conditions (also called as extremozymes), e.g., high alkalinity and salinity, anhydrous environment, cold and hot environment, etc. As the search for extremozymes is in progress, it is imperative to explore the extreme environments to get some novel microbial strains (extremophiles) for alkali-stable proteases. This review article is an effort to compile the scattered information on some extremophiles and their alkali-stable proteases, which may have better specific industrial applications. It includes: (i) various extreme environments relevant to industrial biocatalysts, (ii) strategies of extremophilic microbes and enzymes which make them able to tolerate such conditions, and (iii) an overview of some important work done so far to explore industrially relevant extremophilic enzymes from extremophiles.

Keywords: Alkali-stable protease; Extremophiles; Extremozymes

Introduction

Proteases are a complex group of enzymes which have immense physiological as well as commercial importance as they possess both degradative and synthetic properties. They differ substantially in their origin, catalytic mechanism, substrate specificity and active site. Proteases are broadly divided as exopeptidases and endopeptidases depending on their sites of action. If the enzyme cleaves the peptide bond proximal to the amino or carboxy terminus of the substrate, they are classified as exopeptidases. If the enzyme cleaves peptide bonds distant from the terminus of a substrate, they are classified as endopeptidases. Further, on the basis of functional group(s) present at the active site and enzymes' catalytic mechanism, proteases are categorized into four groups: serine-, cysteine-, aspartic- and metallo-proteases. Proteases are universally present in animals, plants and microorganisms. However, microbes are a goldmine of proteases and represent the preferred source of enzymes [1].

Although protease production is an inherent property of every microorganism, only those organisms that produce a significant amount of extracellular protease have been exploited commercially [2]. Proteases have several applications in food, dairy and detergent industries since time immemorial. Probably, the first application of cell-free protease was in cheese making, where rennin from calf stomach was used. The first commercial enzyme was reported in Germany in 1914 by Röhm, and the application of animal trypsin and chymotrypsin was shown to improve washing efficiency of detergent over traditional products [3]. Development of an improved quality detergent fortified with protease, triggered efforts towards the selection of proteases fit for detergent applications. Subsequently, a breakthrough in the commercial use of enzyme occurred with the introduction of microbial protease in detergents at an affordable cost. At present, microbial alkaline proteases rule the worldwide enzyme market, accounting for nearly two-third share of the detergent industry [4]. Alkaline proteases (EC 3.4.21-24, 99) are defined as those enzymes which are active at neutral to alkaline pH range. They either have a serine center (serine protease) or are of metallo-type (metalloprotease). These enzymes have broad substrate specificities, and function to some extent under the extreme conditions encountered in domestic washing conditions of 20-70°C, a pH up to 11 and at high concentration of detergents, polyphosphates, chelating agents [e.g., ethylene diamine tetra acetic acid (EDTA)] and oxidizing agents (e.g., sodium perborate) [5].

Although, more than 3000 different enzymes have been identified to date, and many of them have found their way into biotechnological/industrial applications, the present enzyme array is still insufficient to meet the global demands [6]. The extreme/harsh environment (to which several enzymes are subjected during process applications) has given thrust to screening of extremophiles (thermophiles, psychrophiles/psychrotrophs, halophiles, solvent tolerant microorganisms, etc.) for enzymes having necessary activity and stability. Extremophiles have been a challenging area of research since the time of their discovery. To survive under extreme environments, microorganisms possess enzymes that function under extreme conditions. Such extremophiles may be exploited for better and novel enzymes of enhanced industrial applications [6,7]. In addition, enzymes found in one type of extreme environment (e.g., high temperature) are usually tolerant to other extreme conditions also (e.g., organic solvents); thus, naturally exsisting array of robust enzymes may be employed in a variety of harsh environments [7]. This review presents a brief account of the isolation, characteristics and application of alkaline proteases from extremophiles.

***Corresponding author:** Satyendra Kumar Garg, Center of Excellence, Department of Microbiology, Dr. Ram Manohar Lohia Avadh University, Faizabad-224 001, Uttar Pradesh, India, Tel: +91-9454755166; +91-5278-245330; Fax: +91-5278-246330; E-mail: sk_garg001@yahoo.com

Received August 24, 2015; **Accepted** September 26, 2015; **Published** October 05, 2015

Citation: Garg SK, Singh SK (2015) Extreme Environments: Goldmine of Industrially Valuable Alkali-stable Proteases. Enz Eng 4: 129. doi:10.4172/2329-6674.1000129

Copyright: © 2015 Garg SK, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Alkaline proteases from thermophiles

Thermophiles inhabit different environmental conditions, including hot springs, geothermal sediments, marine solfatares or fermenting composts as well as in industrial environments, e.g., hot water pipelines, etc. [8]. On the basis of their optimum growth temperature range, thermophilic microorganisms can be further classified as (i) moderate thermophiles (35-70°C), (ii) extreme thermophiles (55-85°C) and (iii) hyperthermophiles (mainly comprising of archaea, 80-115°C) [9-11].

Thermophilic microbes are one of the most studied extremophiles, as their enzymes are usually thermostable; hence greatly suitable for industrial applications, especially in detergent formulations. It is reasonable to exploit thermostable enzymes, as the solubility of many reaction components, mainly polymeric substrates, is improved significantly at high temperatures. In addition, high temperature also reduces the risk of microbial contamination, which may cause undesired complications. Although, there is no firm evidence to suggest that thermostable enzymes are necessarily derived from thermophilic organisms, nevertheless there is a greater chance of exsistance of thermostable enzymes from thermophiles. Therefore, a wide range of microbial proteases from thermophilic microbial species has been extensively purified and characterized. These include Thermus sp., Desulfurococcus strain Tok12S1 and Bacillus sp. Among them, heat stable alkaline proteases derived from thermo-alkaliphilic bacilli are known to be active and stable in highly alkaline conditions [12].

In 1977, Salleh and Friends reported a thermoalkaline protease, produced by a thermophilic *Bacillus stearothermophilus* strain F1. The strain was capable of growing optimally at 60°C [13], and its thermoalkaline protease was optimally active in the pH range of 8-10 and was stable for 24 hr at 70°C [12]. Highly thermoalkaline proteases appear to have better washing properties, and if fortified in detergents, the washing can be conveniently performed at 50-60°C [14]. Bio 40, the first detergent with bacterial proteases, was produced by Gebruder Schnyder in Switzerland. The Danish company, Novo Nordisk introduced Alcalase, an alkaline protease produced by *Bacillus licheniformis*, followed by Maxatase marketed by Gist Brocades. Alcalase was formulated in detergent product known as Biotex that stimulated the development of many enzyme based detergents [15].

Hyperthermophiles are also known to produce heat stable enzymes, having activity in temperature range of 80-115°C. The majority of hyperthermophiles belong to the archaea group growing usually under anaerobic environments [9]. Under unconventional growth conditions of many hyperthermophiles, relatively low cell growth and enzyme yield, and sometimes generation of toxic/corrosive metabolites cause technical difficulties during industrial scale enzyme production [16,17]. Furthermore, the fermentation conditions (physical/nutritional), responsible for protease production in hyperthermophiles are still not sufficiently standardized.

Hence, industrial production of thermostable proteases is more imperative using thermophilic microbes rather than hyperthermophiles. A large number of thermophilic microbes belonging to bacteria, fungi, yeasts and actinomycetes are known to produce heat stable alkaline proteases [1,18-20], and bacteria represent the leading group of thermoalkaline protease producers. Although alkaline proteases are produced by several bacteria, the enzymes produced by Bacillus sp. are the best known ones [1,4,19], and are by far the most important source of many commercial microbial enzymes. At present, industrial production of proteases is carried out using thermophilic strains of genus Bacillus [13]. Thermolysin, a neutral metalloprotease isolated from B. stearothermophilus (half-life 1 hr at 80°C), is a good example of that group [1,12]. Thermoalkaline proteases can also withstand high concentration of chemical denaturing agents and non-aqueous environments [9,21]. Bacilli can be cultivated under extreme temperature and pH conditions to produce enzymes that are stable in a wide range of harsh environments. Table 1 summarizes few important alkaline proteases used in detergent formulations.

Generally, the optimum temperature of alkaline proteases ranges between 30-80°C [1,2,4,20]. However, an enzyme of alkaliphilic Bacillus P-2 showed higher optimum temperature of 90°C. The protease was optimally active at pH 9.6, retaining more than 80% of its activity in the pH range of 7–10. Its thermostability analysis revealed that it retained 100, 95 and 37% activities after 60 min incubation at 90, 99 and 121°C, respectively. The half-life of protease at 121°C (autoclaving temperature) was 47 min. Bacillus P-2 was the only mesophile reported until 2001, producing a protease stable even at autoclaving (121°C) and boiling temperatures [22]. B. stearothermophilus strain F1 reportedly produced high levels of extracellular alkaline serine protease at 60°C after 24 hr incubation [23]. An oxidant and sodium dodecyl sulphate (SDS)-stable alkaline protease secreted by a marine haloalkaliphilic Bacillus clausii produced maximum protease yield (15,000 U ml-1) under submerged fermentation conditions at 42°C (pH 9.6). The optimal pH and temperature of partially purified enzyme activity were

Trade name	Source organism	Enzyme's optimal Temperature (°C)	pH	Manufacturer
Alcalase	*Bacillus licheniformis*	60	8-9	Novo Nordisk
Savinase	*B. lentus*	55	9-11	Novo Nordisk
Esperase	*B. lentus*	60	9-11	Novo Nordisk
Maxacal	*B. alcalophilus*	60	11	Gist-Brocades
Maxatase	*B. licheniformis*	60	9.5-10	Gist-Brocades
Opticlean	*B. alcalophilus*	50-60	10-11	Solvay Enzymes
Optimase	*B. licheniformis*	60-65	9-10	Solvay Enzymes
Protosol	*Bacillus sp.*	50	10	Advanced Biochemicals
Wuxi	*Bacillus sp.*	40-50	10-11	Wuxi Synder Bioproducts

Table 1: List of some thermostable proteases used as detergent additive.

Organism	Enzyme's optimal pH	Enzyme's optimal (°C) Temperature	References
Bacillus sp. JB-99	11	70	[20]
Bacillus sp. P-2	9.6	90	[22]
Bacillus sp. No. AH101	12-13	80	[25]
Bacillus sp. B18'	13	85	[26]
Bacillus licheniformis	9.0	70	[27]
Bacillus brevis	10.5	60	[28]
Bacillus sp. SB5	10	70	[29]
Bacillus sp. NCDC 180	11-12	55	[30]
Bacillus sphaericus	10.5	55	[31]
Bacillus sp. RGR-14	11	60	[32]
Bacillus mojavensis	10.5	60	[33]
Bacillus pumilus UN-31-C-42	10	55	[34]
Bacillus clausii I-52	11	60	[35]
Bacillus subtilis megatherium	9.1	60	[36]
Bacillus licheniformis RP1	10-11	65-70	[37]
Bacillus mojavensis A21	8-10	60	[38]
Bacillus circulans	11	70	[39]
Bacillus sp. B001	10	60	[40]
Bacillus cereus SIU1	9.0	45-55	[41]

Table 2: Temperature and pH optima of some thermoalkaline proteases of Bacillus sp.

11.5 and 80°C, respectively [24]. Table 2 presents some thermoalkaline proteases of thermophilic Bacillus species.

Adaptation strategies of thermophilic microbes and their enzymes: To survive at above ambient temperature, microbes have developed several modifications at cellular and macro molecular level:

Modifications at cellular level: 1. The cell membrane of thermophiles contains saturated fatty acids, which creates a hydrophobic environment for the cell, and maintains the cell rigidity at elevated temperatures [42].

2. The hyperthermophilic archaea have lipids linked with ether on the cell wall, which is responsible for heat resistance [43].

3. Lately, tetraether membrane lipids were reported in a thermoacidophilic euryarchaeota Candidatus "*Aciduliprofundum boonei*" [44].

Modifications at macromolecular level: In addition to various structural adaptations of cell wall and cell membrane, thermophiles contain reverse DNA gyrase enzyme, which increases the melting point by producing positive super coils in the DNA [45].

Presence of small DNA binding protein increases the thermostability of DNA as well as promotes the annealing of complementary strands above the melting point. It is also responsible for ATPase-dependent rescue of the aggregated proteins (due to high temperature) [46,47].

Thermophiles are reported to have thermostable proteins which are generally resistant to denaturation and proteolysis [48]. The enzymes from some thermophilic bacteria, actinomycetes and archaea adapt to high temperatures by increased (a) hydrophobic bondings at the core of the molecules, (b) ion pairs, (c) number of non-polar amino acids, (d) conformational changes in the molecules, i.e., more globular structure, (e) hydrogen bonding due to large number of basic amino acids, (f) disulphide bridges and (g) hydrophobic interactions in their proteins [12,49-51].

Kabsch and Sander [52] concluded that with increasing temperature, moderate thermophilic proteins have a slight increase in helices (highly variable though), whereas hyperthermophilic proteins seem to have more increase in beta structures. A comparative structural study of a non-haem iron protein (rubredoxin) from *Pyrococcus furiosus* (archaeal hyperthermophile) indicated that its structure is very similar to that of mesophilic rubredoxins, and it was suggested that additional hydrogen bonds within the beta-sheet and a few extra electrostatic interactions at the protein surface contribute for the greater stability of the *Pyrococcus rubredoxin* [53,54]. An extensive, well packed hydrophobic core is a common characteristic of thermostable globular proteins. However, it is found more prevailing in the hyperthermophiles growing below 100°C than in those growing ≥ 100°C [55,56].

Besides the above mentioned mechanisms, thermophiles produce heat shock proteins, known as 'chaperons', which help to refold the proteins to native form and restore their functions under high temperature conditions [57,58].

Stability rendered by other molecules: Presence of certain metal ions (e.g., Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, etc.), salts (e.g., polyamines), substrate molecules (e.g., NADPH), as well as exchange of few amino acid residues and/or their different localization in molecule are known to impart thermostability to enzymes [59].

The presence of co-solvents also affects the thermostability of enzymes. The degree of co-solvent mediated protein stabilization under various denaturing conditions depends largely upon the nature of the co-solvent, its interaction with water molecules and nature of the protein itself [60-62]. Preferential interaction parameters provide the evidence that co-solvents stabilize protein molecules by their partial preferential exclusion from protein surfaces, but the exact detailed mechanism of stabilization is not clearly understood [63].

Evolution of acclimatized macromolecules: Arnold et al. [64] concluded that evolution produces the special subset of functional enzymes which can be generated from extant enzymes by mutation and selection, e.g., a thermophilic enzyme might share only 20-30% of its amino acid sequence with its counterparts that have adapted to cooler climates possibly leaving hundreds of differences among a typical set of sequences. The crystal structures of extremophilic enzymes unambiguously indicate a pattern in the molecular adaptations to temperature. There is certainly an apparent increase in the amount and strength of all known weak interactions/structural factors, responsible for protein stability from psychrophiles to mesophiles (living at intermediate temperatures close to 37°C) and to thermophiles [59,65].

Prevention of access of water to active sites: According to Peters and coworkers [66], the thermophilic enzymes are also more compact and generally lack regions such as exposed loops or extended ends. In fact, a thermostable protein is almost impenetrable to water, thus preventing water from competing for interaction sites that stabilize the fold.

Alkaline proteases from psychrophiles/psychrotrophs

Life under low temperature conditions was identified as early as 1840 by Hooker, who observed that algae were associated with sea ice. In nature, many bacteria can grow harmoniously in very hostile environments such as polar region, cold water, acidic warm springs, salterns, dry rock surfaces, deserts, or at depth in the sea. Such organisms are able to withstand harsh conditions, and they are often subjected to rapid variations of the environment. Cold environment on the earth represents about 80% of the biosphere, and 90% of the marine environment in which cold adapted microbes (psychrophiles and psychrotrophs) are present which produce psychro-tolerant enzymes [67]. Psychrotrophs are also credited to produce extracellular proteases, active in broad temperature range [68]. These enzymes become useful for washing purposes. Psychrophiles have optimal growth temperatures below 15°C with an upper limit of 20°C and a minimal temperature for growth at 0°C or below, while psychrotrophs (psychrotolerants), have the ability to multiply at low temperature but grow optimally at temperatures around 20-25°C [67]. Washing at low temperature is preferable as it protects the colours of fabrics, and reduces the energy consumption also. However, the enzymes that are added to detergents for removal of macromolecular stains, e.g., subtilisin, lipase and glycosidases, are poorly active at the tap water tempeature, and required to be substituted by cold active enzymes [69]. Psychrophiles and psychrotrophs, both the groups are known to produce enzymes with high level of activity at low temperatures [70,71]. When compared to their mesophilic counterpart, these enzymes display high catalytic efficiencies in order to maintain proper metabolic fluxes at low temperatures [68,71].

Generally, the industrially exploited proteases are adapted to work either under cold environments or at high temperatures. If an enzyme acclimatized only for cold environment is employed at elevated temperatures, it results in poor or no enzyme activity and vice-versa. Hence, an enzyme active in a broad temperature range is always a center of interest for the researchers worldwide. In their natural habitats, psychrotrophs are frequently submitted to large and rapid

temperature changes. This ability to cope with such temperature shifts must be accompanied by adaptive changes; hence, their enzymes also function in broad range of temperature up to 40°C [72]. This property of psychrotrophs is exploited by many workers to produce proteases, active in a broad temperature range of 10-40°C [70,73]. Henceforth, enzymes from psychrophiles/psychrotrophs have become attention-grabbing for industrial applications, to a certain extent for less energy expenditure. For example, there is an increasing effort to apply cold active enzymes in commercial detergents. As a result, it becomes possible to develop laundry applications that can be performed at lower temperatures efficiently. Some psychrophilic/psychrotrophic alkaline proteases are listed in Table 3.

Most of the investigations in the past have dealt with proteases produced by food-spoiling psychrotrophs; mainly from *Pseudomonas fluorescens* [88]. Therefore, substrate utilization, nutrient requirement and enzymatic studies of cold bacteria have been sparse [89-91]. Among various psychrophilic/psychrotrophic alkaline protease producers, Pseudomonas sp., Bacillus sp., Aeromonas sp., Serratia sp., Exiguobacterium sp., Burkholderia sp., etc., are the major genera of bacteria. Besides heat tolerant alkaline proteases from thermophilic microbes, production of thermostable enzymes from psychrophilic/psychrotrophic bacterial isolates has also been reported by several researchers. So far, few psychrotrophic bacterial isolates have been reported for thermostable alkaline protease production as compared to thermophiles. Jackman et al. [70] reported production of heat-stable proteases from psychrotrophic pseudomonads. Mitchell and Marshall [92] isolated four psychrotrophic *P. fluorescens* strains (P26, 32A, P1 and P27) capable of thermostable protease production. The enzymes produced by these strains were stable in the temperature range of 5.0-62.5°C for 30 min, with optimal activity at 37°C. Burkholderia cepacia produced an extracellular protease, with maximum activity at 45°C [93]. A psychrotrophic *P. fluorescens* 114 isolate was capable of growing at 0°C, with optimum growth at 20°C, and its protease was active in the temperature range of 0-45°C [75]. *P. pseudomallei* produced an extracellular alkaline metalloprotease, optimally active at 60°C [94]. *P. aeruginosa* strain PST-01 produced an organic solvent-stable protease with an optimum temperature of 55°C for maximum activity [95]. An extracellular enzyme, protease IV, is a serine protease of *P. aeruginosa* PA103-29, which exhibited maximum activity at optimum 45°C [96]. Heat stable protease production at 25°C from a psychrotrophic *P. fluorescens* RO98 was reported by Koka and Weimer [97]. Joshi et al. [86] reported maximum protease production at pH 9.0 and 25°C from *Bacillus cereus* MTCC 6840 isolate. Kasana and Yadav [85] reported thermoalkalistable protease from a psychrotrophic Exiguobacterium sp. SKPB5 (MTCC 7803) active optimally at 50°C. Singh et al. [98] reported a psychrotrophic *P. putida* SKG-1 isolate, capable to grow and produce thermoalkaline protease in temperature range of 10-40°C with maximum production at 25°C and pH 9.0. Its protease was active in the temperature range of 10-70°C with 100% stability at 10-40°C at optimum pH 9.5 [99].

Microorganisms	Enzyme's optimal Temperature (°C)	Enzyme's optimal pH	References
Pseudomonas fluorescens	25	7.5	[70]
Xanthomonas maltophila	50	8.0	[74]
Pseudomonas fluorescens 114	35	10	[75]
Bacillus coagulans	37	8.0	[76]
Aromonas hydrophila	37	8.5	[77]
Serratia liquefaciens	55	8.0	[78]
Pseudomonas fluorescens CY091	40-45	7.0-8.0	[79]
Bacillus polymyxa	50	7.5	[80]
Flavobacterium balustinum P104	40	8.0	[81]
Bacillus amyloliquefaciens S94	45	8.0 and 10	[82]
Pseudomnas strain DY-A	40	10.0	[83]
Bacillus cereus	60	9.0	[84]
Exiguobacterium sp. SKPB5	50	8.0	[85]
Bacillus cereus MTCC 6840	20	9.0	[86]
Pseudomonas putida SKG-1	40	9.5	[87]

Table 3: Temperature and pH optima of some alkaline proteases of psychrophilic/psychrotrophic microbes.

Adaptation mechanisms of psychrophilic/psychrotrophic microbes and their enzymes: Psychrophiles and psychrotrophs belong to extremely diverse genera, and psychrotrophic microorganisms are more ubiquitous than psychrophilic bacteria, which are numerous (both quantitatively and in terms of number of species) even in permanently cold environments. In their natural habitats, they repeatedly encounter large and rapid temperature changes, and therefore, they can develop over a wide temperature range (up to 40°C, whereas the temperature range that permits growth of most other bacteria does not exceed 30°C). This ability to deal with such wide temperature changes are accompanied by several adaptive changes in biophysical and biochemical parameters of cell including membrane fluidity, protein conformation, solubility, reaction kinetics, stability and alteration in gene expression [72]. The major possible mechanisms for low temperature adaptations are as under:

1. In order to withstand temperatures below freezing, synthesis of cryo-protectants (antifreeze proteins) take place to protect the cell from ice formation [100].

2. Maintenance of membrane fluidity at low temperature by increasing the ratio of unsaturated fatty-acyl residues and/or cis double bonds, chain shortening, and in some infrequent cases, methyl branching [72,101,102].

3. Synthesis of cold shock proteins (Csp) that assist the covalent folding or unfolding and the assembly or disassembly of other macromolecular structures. They regulate cellular protein synthesis (at the level of transcription as well as initiation of translation) and mRNA folding (acting as chaperone preventing the formation of an mRNA secondary structure). Cold shock proteins are inducible proteins [103].

4. Another aspect of adaptation to cold concerns is the synthesis of a specific set of proteins at low temperature that are not (or poorly) present at milder temperatures [72]. This particular class of proteins, referred to as cold acclimation proteins (Caps), is permanently synthesized during continuous growth at low temperature [72,104-107], and they are likely to play a fundamental role for life in the cold. In contrast to Csps, these proteins are continuously synthesized during prolonged growth at low temperature. These proteins may be involved in important metabolic function(s) at low temperature by maintaining membrane fluidity and/or by replacing cold-denatured peptides. Some 'Caps' could act as cold-specific proteases that eliminate denatured proteins whose accumulation would be deleterious for the cells [72].

5. Other adaptive strategies developed by psychrophilic organisms involve the regulation of ion channel permeability, seasonal dormancy and structural changes in alpha-tubulin protein [108].

In order to have increased catalytic efficiency at low temperature, cold-adapted enzymes have evolved toward a high conformational flexibility, but the enzyme stability has been compromised [109,110]. The correlation between conformational flexibility and enzyme activity has been studied by comparing few cold adapted enzymes and their mesophilic homologues: β-lactamase from the Antarctic psychrophile *Psychrobacter immobilis* A5 [109], α-amylase from Alteromonas haloplanctis [111] and subtilisin from the Antarctic Bacillus TA41 [112]. It was deduced that several factors play important role to gain protein flexibility, including reduction of electrostatic non-covalent weak interactions (salt bridges, polar interactions between aromatic side chains, hydrogen bonds) and decrease of hydrophobicity [113,114]. Also, molecular adaptation appears to have induced looser surface loops, more polar surfaces exposed to solvent, lower affinity of Ca^{2+} binding sites and less stabilized α-helices [71,111,115].

However, no general rule governs this adaptative strategy, and each enzyme can gain flexibility by one or a combination of the above modifications [113]. The need for more flexible molecules in order to gain functionality at low temperature is probably not limited to proteins. For example, high levels of dihydrouridine have been found in the tRNA of three psychrotrophic bacteria (ANT-300 and Vibrio sp. 5710 and 29-6), which are responsible for the maintenance of conformational flexibility and dynamic motion in RNA at low temperature [116]. This, together with protein modifications, may provide an advantage in organisms growing under conditions where thermal motion, enzymatic reaction rates and intermolecular interactions of biomolecules are compromised. The major adaptations are (a) structural adaptations at active site, (b) structural adaptations related to stability, and (c) kinetic adaptations.

The reactions that are catalysed by psychrophilic enzymes proceed with a large and unfavourable entropic contribution. This has been related to the occurrence of large changes in conformation at the active site [117]. This assumption has received strong experimental support using a transition-state analogue [65]. Accordingly, it is likely that a mobile and flexible active site binds its substrate weakly, and certainly most psychrophilic enzymes have higher Km values than their mesophilic counterparts [118]. It follows that numerous cold-active enzymes increase kcat at the expense of Km. Nevertheless, there are several examples from nature as well as protein engineering studies showing that the structural features involved in stability or/and activity can be very different, and can act independently [6]. Crystallographic studies of psychrophilic proteins reveal that they do not contain extraordinary conformations, but instead share a high resemblance with their mesophilic and thermophilic homologues [119]. To increase flexibility, many structural modifications that lead to decrease in strength and/or number of stabilizing factors (enthalpic or entropic) have been noticed. General modifications include: reduction of several ion pairs, hydrogen bonds and hydrophobic interactions; decreased inter subunit interactions; increased interaction with the solvent; reduced apolar fraction in the core; higher accessibility to the active site; increased exposure of apolar residues to the solvent; decreased cofactor binding; clustering of glycine residues; and a lower proline and arginine content [119].

Alkaline proteases from alkaliphiles

Enzymes from microorganisms that can grow luxuriantly under extreme pH could be predominantly useful for applications under highly acidic (e.g., cheese industry) or highly alkaline (e.g., detergent formulation) environments. Although, these microorganisms maintain a neutral pH internally, it never helps the extracellular proteins, which generally have to function under extreme environments, e.g., high pH environments in the case of alkaliphiles. Alkaline proteases, capable to function at high pH and in the presence of chelates/salts are highly desirable for modern detergent/laundry formulations. This desire provoked the screening of alkaliphilic bacteria and archaea for their ability to produce such enzymes. To date, several industrially valuable enzymes have already been identified. Generally, thermo-alkaliphilic bacilli that could grow at >pH 9.0, are employed for production of oxidation-resistant alkaline proteases [120]. Alkaliphilic bacilli can be found mostly in alkaline environments such as soda soils and soda lakes, neutral environments and deep-sea sediments. Animal manure, man-made alkaline environments such as effluents from food, textile, tannery, potato processing units, paper manufacturing units, calcium carbonate kilns and detergent industry, are also good sources [121]. Alkaline proteases of the genus Bacillus generally show an optimal activity and a good stability at high alkaline pH values. The optimum pH range of Bacillus alkaline proteases is generally between 9.0 and 11.0, with a few exceptions of higher pH optimum of 11.5 [122], 11.0-12.0 [30] and 12.0-13.0 [25,26,123,124]. The first report concerning an alkaline protease from alkaliphilic Bacillus strain 221 was published by Horikoshi in 1971. This strain isolated from soil, produced an alkaline protease that differed from the subtilisin group. The optimum pH of purified enzyme was found to be 11.5 and 75% residual activity was maintained at pH 13.0 [125]. Since then, various types of alkaline proteases were characterized, and their potential industrial applications have been explored. The major applications of these enzymes are in detergent formulations, food industry, leather processing, peptide synthesis and ptoteinaceous-waste treatment [2,126]. Table 2 provides a brief account of some alkaliphilic microbes.

High-alkaline proteases, the majority of which are extracellularly produced by alkaliphilic microorganisms, are one of the important members of subtilisin family. It is well known that the increase of protein function and stability is one of the major strategies for extremophiles to deal with extreme environments [127]. Therefore, enzymes from the alkaliphilic microorganisms have developed specific structural and catalyzing properties towards high-alkaline condition which can be used to acclimatize harsh industrial requirements, especially for the modern laundry/detergent formulations [127,128]. Since the first discovery of protease no. 221, a large array of highly alkaline proteases has been successfully characterized [1,2,25,129,130]. For example, M-protease, one of the intensively studied alkaline protease from alkaliphilic *B. clausii* KSM-K16, has been found stable in the pH range of 5.0-12.0 and used successfully as an additive in heavy duty laundry detergents [131].

Considerable number of reports appeared in the literature about the alkaline proteases produced by alkaliphilic bacilli. The optimal pH and temperature values of alkaline protease purified from *B. licheniformis* MIR29 were respectively 12.0 and 60°C [123]. The alkaline protease of facultative alkaliphilic *B. brevis* strain was found to be most active at pH 10.5 and 37°C [28]. An alkaliphilic hyper-producer of alkaline protease, Bacillus sp. NG 312, has been found to be fairly stable at a wide range of pH from 7.0 to 11.0 with maximum stability at pH 9.0-10.0 [132]. The thermostable alkaline proteases from various alkaliphilic and thermophilic Bacillus sp. having maximum activity at pH 11.0 and 70°C were reported by Takami et al. [25] and Johnvesly and Naik [21]. Kaur et al. [22] reported highly thermostable alkaline protease from an obligate alkaliphilic Bacillus sp. P-2 maximally active at pH 9.6 and 90°C. Oberoi et al. [32] reported SDS-stable alkaline protease from Bacillus sp. RGR14, optimally active at pH 11.0 and 60°C. Singh et al. [133] reported a serine alkaline protease produced by a Bacillus sp. SSR1 isolate having

optimal activity at pH 10.0 and 40°C. The protease of B. horikoshi exhibited highest activity at 45°C and pH 9.0 [134]. Beg and Gupta [33] reported the temperature and pH optima of commercial alkaline protease from *B. mojavensis* at 60°C and 10.5, respectively. Gessesse et al. [135] isolated two alkaliphilic bacterial strains, Nesternkonia sp. AL-20 and *B. pseudofirmus* AL-89, from alkaline soil sample collected from Lake Abjata, an alkaline soda lake with a pH of 10.0-10.5. Protease AL-20 was active in a broad pH range displaying over 90% of its maximum activity between pH 7.5 and 11.5 with a peak at pH 10.0. Protease AL-89, on the other hand, displayed a pH optimum of 11.0 and retained about 70% or more of its original activity between pH 6.5 and 11.0. Huang et al. [34] reported an extracellular alkaline serine protease of *B. pumilus* showing maximal activity at pH 10.0 and 55°C. An alkaliphilic *B. clausii* GMBAE 42 was capable of growing at pH 10.0, and produced extracellular alkaline protease with optimal temperature and pH at 60°C and 11.0, respectively [136]. An alkaliphilic Bacillus sp. B001 was able to grow at pH 8.0-11.0 with an optimal pH of 10.0 and at 15-40°C with an optimal temperature of 30°C. Maximum protease activity at pH and temperature optima, respectivey at 10.0 and 60°C suggested its potential application in various industrial processes [40].

Alkaline proteases of Pseudomonas sp. also hold an important share of enzymes, with major application in peptide synthesis, detergent formulations and biotechnological research [137-143]. Several Pseudomonas species from various stressed environments have been explored, and employed for alkaline protease production. However, efficient alkaline protease producing isolates are the strains of *Pseudomonas aeruginosa* and *P. fluorescens* [1,70,79,144,145]. The alkaline proteases of Pseudomonas sp. are generally Zn^{2+} dependent metallozymes with a consensus zinc binding sequence at their N-terminal proteolytic domain [115,146,147]. Another well described zinc dependent metalloprotease is a 56 kDa aminopeptidase of by *P. aeruginosa*. Catalytic function of this enzyme was zinc dependent and was inhibited by dithiothreitol and 1,10-phenanthroline, but not affected by serine protease inhibitors [148]. Singh et al. [99] reported a psychro-thermoalkalistable protease from psychrotrophic *Pseudomonas putida* isolate. The purified protease was maximally active at pH 9.5 and 40°C, with 100% stability in pH and temperature range of 6.0-11.0 and 10-40°C, respectively.

Commercially exploited Subtilisins, a clan of the subtilase superfamily, are one of the well studied alkaline proteases. Currently, based on the amino acid sequences, subtilisin family has been further alienated into six clans, e.g., true-subtilisins, high-alkaline proteases, intracellular proteases, oxidatively stable proteases, high-molecular-mass subtilisins and phylogenetically intermediate subtilisins [131]. High-alkaline proteases, generally produced extracellularly by alkaliphiles, are one of the important members of subtilisin family. To understand the mechanism of adaptation of thermophilic/psychrophilic condition, the sequences and structures of several members of the subtilase superfamily, such as M-protease, psychrophilic proteinase of Vibrio, thermitase, subtilisin BPN' and Carlsberg have been analyzed [128,129;149-151]. However, structural analysis of high-alkaline proteases is still scantly reported. Determination of enzyme 3D structures is also of great importance to get the insights of biophysical and structural forces, which affect the enzyme properties and its function under changing environments [152,153].

Mechanisms of alkali tolerance in microbes and their enzymes

There are two kinds of naturally occurring stable alkaline environments on the earth. First, high Ca^{2+} environments [ground waters bearing high $Ca(OH)_2$] and second, low Ca^{2+} environments (soda lakes and deserts, dominated by sodium carbonate) [154]. Soda lakes and soda deserts are most stable naturally occurring alkaline environments distributed worldwide. These environments are characterized by high concentrations of Na_2CO_3. Diverse industrial activities including food processing (KOH mediated removal of potato skins), cement manufacture (or casting), alkaline electroplating, leather tanning, paper and board manufacture, indigo fermentation, rayon and herbicide manufacture generate anthropogenic sources of alkaline type environments [126,154].

Adaptations at cellular level: The major mechanisms by which bacteria survive at high alkalinity include:

(i) increased metabolic acid production through amino acid deaminases and sugar fermentation,

(ii) increased ATP synthase that couples H^+ entry to ATP generation,

(iii) changes in cell surface properties, and

(iv) increased expression and activity of monovalent cation/proton antiporters [155].

Na^+/H^+ antiporter-dependent pH homeostasis is the major strategy for pH tolerance of alkaliphilic Bacillus species. Although such bacteria have multiple Na^+/H^+ antiporters, the unusual hetero-oligomeric Mrp antiporter has an indispensable role at high pH. A point mutation in the mrpA gene of alkaliphilic *Bacillus halodurans* C-125 resulted in a non-alkaliphilic mutant strain accompanied by loss of alkaline pH homeostasis [155,156].

A majority of alkaliphilic microorganisms require Na^+ ions for growth. For instance, *Bacillus firmus* and *Exiguobacterium auranticum* use Na^+/H^+ antiporter system in the region of pH 7.0-9.0, with usual respiration-coupled extrusion of Na^+ ions, being replaced by at least 2 antiporter proteins for the uptake of protons [157]. Bacteria extrude protons by primary transport systems to generate a proton motive force that can be used for ATP synthesis. Organisms growing at neutral pH usually maintain their internal pH slightly higher than the external pH. At very high environmental pH levels, however, alkaliphilic bacteria reverse their pH gradient (inside more acidic than the exterior) in order to maintain external pH near neutral. This reversed pH gradient contributes a negative component to the value of the proton-motive force, so the electrical membrane potential must be high to compensate and give a sufficiently energetic proton-motive force ATP synthesis [157,158].

It is currently unknown how alkaliphiles undertake processes that normally require inward translocation of proton-coupled ATP synthetase and pH homeostasis, which depend on an antiport of sodium and protons. It has been hypothesized that alkaliphilic organisms may have a sodium-motive force instead of a proton-motive force. Research into cell envelope analysis of the alkaliphiles indicated that there was some correlation between the density of high charge on the cell membrane and the degree of pH regulation exhibited by alkaliphilic species [157]. This is one of the reasons why a cell grown in alkaline pH is stable. This causes cellular adaptation of organisms for growth in alkaline pH. Study of B. firmus OF4 chromosomal DNA has indicated the presence of several open reading frames for Na^+/H^+ antiporters that may play roles in pH homeostasis [126].

Studies revealed that alkaliphiles have a lower internal than external pH (8.0-11.0) [126]. The internal pH is maintained at around 8.0, despite high external pH. Therefore, one of the key features in alkaliphily is

associated with the cell surface, which separates and maintains a neutral intracellular environment, in contrast to the extracellular alkaline environment [126]. Since the protoplasts of alkaliphilic Bacillus strains lose their stability in alkaline environments, it is clear that the cell wall plays a key role in protecting the cell from alkaline environments. Comparative studies of cell wall components of alkaliphilic and neutrophilic Bacillus sp. revealed that, besides peptidoglycan, cell wall of alkaliphiles also contains certain acidic polymers, such as galacturonic, gluconic, glutamic, aspartic and phosphoric acids [126,159].

The negative charges on the acidic non-peptidoglycan components enable the cell surface to adsorb cations (Na^+, H_3O^+) and repulse anions, and in turn, assist bacterial cells to grow under alkaline environments. Cell walls containing acidic polymers also function as a negatively charged matrix and possibly reduce the pH value at the cell surface. The surface of the plasma membrane should be kept below pH 9.0, as it is very unstable at alkaline pH values (pH 8.5-9.0), much below the pH required for optimum growth. Plasma membranes also employ Na^+/H^+ antiporter system to maintain pH homeostasis [126].

Adaptations at enzyme level: Changes in amino acids ratio also play an important role for alkaliphilic nature of enzymes. For alkaline proteases, a decrease in the number of Asp and Lys residues and an increase in Arg, His and Gln residues were noticed during the adaptation process under alkaline conditions [160]. Arginine and lysine are positively charged basic amino acids, and play significant roles in protein stability by forming ionic interactions and hydrogen bonds (intra-molecular as well as with water molecules). Although both amino acids are basic, arginine provides more stability to protein structure than lysine due to its geometric structure. Sokalingam et al. [161] concluded that replacement of lysine by arginine results in increased number of electrostatic interactions (salt bridges and hydrogen bonds), compared to lysine and plays an important role in enzyme stability under extreme conditions, such as high alkalinity. Zhao et al. [162] studied alkaline mannanases, and noticed an increase in the hydrophobic residues and Arg content and a decrease in polar residues of the primary protein structure.

Besides changes in the position and length of helices, strands and loops were also noticed, which might affect the pH-dependent properties [162]. As reported for other alkali-stable enzymes, negatively charged residues were increased on the surface of alkaline mannanases, at the cost of polar residues [162]. This fact may be correlated with the protein secondary structure also. Ratio of secondary structural elements affects the stability of enzymes under alkaline conditions. Yamagata et al. [163] studied the secondary structure of alkaline proteases subtilisin Sendai and subtilisin NAT by circular dichroism. They noticed that there was a lower β-content in the conformation of subtilisin Sendai than in subtilisin NAT. Subtilisin Sendai was more stable than NAT under the alkaline conditions at pH 12.0; the lesser amount of β-structure in the conformation of Sendai might be related to the tolerance for alkaline conditions.

Singh et al. [41] also studied the secondary structure of B. cereus SIU1 alkaline protease by circular dichroism. This protease was most active and stable at pH 9.0 and existed as α-, β- protein, with a large fraction of β-structure. Decrease in pH of environment resulted in loss of β-structure along with protease activity with an increase of unordered elements. Similar findings were reported by Singh et al. [99] for the alkaline protease of P. putida SKG-1. The protease secondary structure at pH 9.5 revealed it to be an α-, β- protein, with dominance of β-like content. The presence of acidic pH concomitantly decreased the β-structure along with protease activity, while the turns and unordered structure increased to maximum level. In fact, β-sheets align for nearly perfect hydrogen bond formation. The close internal packing of the backbone atoms in β-sheet structure serves to optimize Vandervaals interaction and minimizes energetically favorable hydrophobic interactions between non-polar protein groups and water molecules in the environment. These factors increase the enzyme stability relative to other structures [99].

Alkaline proteases from halophilesα

Halophiles are the group of extremophiles which grow at dual extremities of high salt and alkaline pH and therefore, their extracellular enzymes might be active and stable under these conditions. The first haloalkaliphilic bacterium was isolated by Tindall in 1984 [164]. Enzymes derived from such bacteria are potential biocatalysts due to their optimal activities under the conditions of high salt, surfactants, high temperature and alkaline pH [74], whereas the mesophilic counterparts fail to act under similar conditions [165].

Halophiles can survive in hypersaline habitats by means of their ability to preserve osmotic balance. They tend to accumulate salts such as NaCl or KCl up to concentrations that are in equilibrium with the environment. Consequently, proteins from halophiles have to survive under high salt concentrations (e.g., KCl ~4 M and NaCl >5 M) [166,167]. The enzymes maintain a relatively large number of negatively charged amino acid residues on their surface to survive this condition and avoid precipitation. Thus, under lower salt environment, the solubility of halophilic enzymes is often very poor and could limit their applicability [168]. Still, this property has been exploited by using halophilic enzymes in aqueous/organic and non-aqueous environments [169]. An extracellular protease from *Halobacterium halobium* has been exploited for efficient peptide synthesis in water/N-N-dimethylformamide [170]. Novel protease from haloalkaliphilic *Natronococcus occultus* was active in the presence of 1-2 M NaCl or KCl and lost activity below 0.5 M NaCl [171]. A protease from moderately halophilic bacterium Pseudoalteromonas and Nesterenkonia sp. was reported to be active between 0-4 M NaCl, the optimum being at 7.5%, a feature desirable for industrial applications [172]. An alkaline protease of haloalkaliphilic Bacillus sp. was found active in the range of pH 8.5-12.0 with the optima at 10.0-11.0. The requirement of salt for enzyme catalysis increased with increase in temperature, and a shift in temperature optima from 37°C to 55°C was evident in the presence of 2% salt [173]. The protease from haloalkaliphilic archaeon *Natrialba magadii* required high salt concentration to exhibit elevated stability in the presence of organic solvents [174].

A few proteases from extreme halophiles have been reported from archaeal halophiles [171,175,176], but some from moderately halophilic and halotolerant bacteria have also been purified and studied [27,172,177,178]. As moderate halophiles are adapted to grow over a wide salt range (optimal growth at 3-15% NaCl), they represent an interesting group of organisms with great industrial potential [177]. An alkaline protease producing haloalkaliphilic bacterium (isolate Vel) was isolated from west coast of India. The secreted protease was purified (10-fold) with 82% yield by a single step method on Phenyl Sepharose 6 Fast Flow column. The enzyme was active over the pH range of 8.5-12.0, the optimum being 10.0-11.0 [179]. A halotolerant *B. clausii* I-52 produced >50% of alkaline protease when incubated with 5% (w/v) NaCl. Even in the presence of 10 and 20% NaCl, it was capable of protease production. The protease retained 78% activity even in the presence of 20% NaCl [180]. An alkaline protease of halotolerant *B. cereus* MTCC 6840 exhibited 100% stability in the presence of 5% NaCl [86], and showed good activity in the range of 6-10% (w/v) NaCl concentrations. An

alkaline protease from moderately halophilic bacterium Salinivibrio sp. strain AF-2004 showed high stability in the presence of 20% water soluble organic solvents or alcohols such as ethanol and butanol [180,181]. An alkaliphilic and salt tolerant actinomycete, *Streptomyces clavuligerus* strain Mit-1 was isolated from Mithapur, the western coast of India. The strain could grow and produce protease with 0-10% NaCl (w/v), the optimum being 5%. Growth and protease production was optimum at initial pH 9.0, with maximum enzyme yield during early stationary phase [20].

Akolkar et al. [182] isolated halophilic archaeal species belonging to genera Halobacterium, Haloarcula and Haloferax from Kandla salt pans, India. The isolates had an optimum requirement of 25% NaCl for growth. The authors noted an increased organic solvent tolerance of isolates at higher salt concentrations. Among the three isolates, *Halobacterium sp. SP1(1)* was more solvent tolerant and its extracellular protease exhibited higher solvent tolerance compared to the organism itself. The enzyme was tolerant to toluene, xylene, n-decane, n-dodecane and n-undecane. Dodia et al. [165] isolated an alkaline protease secreting haloalkaliphilic bacterium (Gene bank accession number EU118361) from the Saurashtra Coast in Western India. The enzyme displayed catalysis and stability over pH 8.0-13.0, optimally at 9.0-11.0. It was stable in 0-4 M NaCl and required 150 mM NaCl for optimum catalysis at 37°C [165]. A haloalkaliphilic *Bacillus pseudofirmus* Mn6 isolate was found to produce an alkaline protease with a high potency (500 U/ml/min) at 60°C, pH 10.0 and salinity 0.5% [183].

An alkaline protease from salt tolerant alkaliphilic actinomycete *Nocardiopsis alba* strain OK-5 was purified to homogeneity by 27 and 13 fold with a yield of 35 and 13% using two- and one-step methods, respectively. The purification methods involved hydrophobic interaction on phenyl sapharose matrix, and the apparent molecular mass of protease was 20 kDa. The temperature optimum shifted from 70 to 80°C in 4 M NaCl and 30% Na-glutamate, with significant stability at 60-80oC in Na-glutamate [184]. A halotolerant *Bacillus cereus* SIU1 was able to grow in the presence of 10% NaCl and its alkaline protease exhibited ~100% stability at 0-2% (w/v) NaCl concentrations. Further increase in salt concentration resulted in decreased protease stability. The protease retained 98, 92, 87, 80, 67, 43 and 28% activity at 2.5, 3.0, 4.0, 5.0, 7.0, 9.0 and 10.0% NaCl concentrations, respectively. The stability in the presence of high salt concentrations revealed moderately to highly halotolerant nature of the protease [41].

The protease stability at high salt concentration is a desirable characteristic as NaCl is used as a core component in granulation of protease prior to addition in detergents [185]. Also, ground water available in different Indian geoclimatic regions is saline [186], which may be detrimental for the cleaning potential of detergents. Hence, the presence of halotolerant alkaline protease in detergents can make the washing efficient and effortless [187].

Adaptation mechanisms of halophilic microbes and their enzymes: Halophiles have developed two different adaptive strategies to cope with the osmotic pressure induced by high NaCl concentration of the natural environments they inhabit.

Adaptations at cellular level: The mechanisms responsible for salt tolerance and bacterial growth under hyper-saline conditions involve:

1. Modification of membrane composition and fluidity [188]

2. Use of Na^+/H^+ efflux pumps [189]

3. Increased production and/or uptake of compatible solutes [190]

4. Employment of general stress tolerance proteins [191].

The halobacteria and some extremely halophilic bacteria accumulate inorganic ions in the cytoplasm (K^+, Na^+, Cl^-) to balance the osmotic pressure of the medium, and their proteins are also salt stable. In contrast, moderate halophiles accumulate high amounts of specific organic osmolytes in their cytoplasm, which maintains the osmotic balance without altering the normal metabolism of cell [192]. To date, various genes responsible for providing salt tolerance, enabling the cells to grow at elevated salt concentration, involved in synthesis/uptake of various compatible solutes have also been identified from different bacteria [193,194].

Adaptations at macromolecular level: Water has an essential role in biological functions of proteins by binding to the surface and incorporating into interior of protein molecules. Water has a tendency to form ordered cages around hydrophobic groups on the protein surface [195]. It is well known that the presence of high salt concentration destabilizes the ion pairs, alters electrostatic interactions between charged amino acids, ultimately leading to enzyme denaturation [41,196]. Increased salt concentration removes water molecule from hydrophobic regions of protein surfaces, causing their inactivation [197,198]. Non-halophilic proteins are generally less able to compete with salts, and lose their structure and activity at relatively lower ionic concentration.

Contrary to that, halophilic proteins need presence of salt for their optimum activity, and they are enabled to compete successfully with salt ions for hydration and maintenance of functional conformation [199,200]. Such halophilic proteins have multilayered hydration shells that are of considerably greater size and order as compared to their non-halophilic counterparts [195]. An increased number of charged amino acids on protein surface, especially acidic residues, are a common feature of halophilic proteins [168,195,201]. Studies on Halobacterium sp. NRC-1 and other species have revealed that an increase in the number of acidic (glutamic acid, and to a lesser extent, aspartic acid) over basic amino acid residues is a general property of proteins predicted from the genomes of halophilic microorganisms [197,202]. The negatively charged amino acids bind hydrated cations, preserve a surface hydration layer which in turn reduce their surface hydrophobicity and maintain a mutual electrostatic repulsion [199].

Karan and Khare [203] studied the effect of NaCl on secondary structure contents of protease produced by haloalkaliphilic Geomicrobium sp. They reported that at high NaCl concentration (5 and 10%, w/v), the protease had predominantly α-helix content. As the salt concentration was shifted away from the optimal, loss of α-helix occurred with simultaneous increase in random coil. The effect of salt on protein stability can be understood as the balance between two contributions: i) the non-specific (weak) interactions between the co-solute and the protein backbone that favor extended conformations and ii) the solvation of the hydrophobic moiety of the biomolecule that stabilizes compact folding of the protein [204]. Similar behavior of a halophilic esterase was seen by Rao et al. [205] in the case of *Haloarcula marismortui*. The Far-UV CD spectra indicate that the enzyme adopted an inadequate secondary structural conformation at low and high salt concentrations as studied and could not perform its catalytic activity. The enzyme adopted its proper conformation in 3.4 M NaCl solution, as marked by dominance of the α-helix structure, which in turn activated its catalytic property [205].

It has also been observed that halophilic proteins bear a low level of bulky hydrophobic side chains on their surface, in contrast to non-

halophilic proteins. The number of bigger hydrophobic amino acid residues (phenylalanine, isoleucine and leucine) is reduced as compared to small (glycine and alanine) and borderline hydrophobic (serine and threonine) amino acid residues [168,197,201]. Such characteristics play an important role in increased flexibility, more surface hydration and reduced surface hydrophobicity of halophilic proteins.

Salts are also known to play a critical role in protein-DNA interactions. O'Brien et al. [206] studied the effect of salt on the thermodynamic-structural relationship of the binding of TATA box-binding protein (TBP) from *Pyrococcus woesei*, a moderately halophilic organism, to its DNA binding site. The authors hypothesized that uptake of cations and discharge of water accompanies protein-DNA complex formation.

Alkaline proteases from organic solvent tolerant microbes

In general, organic solvents are toxic for microbial population, and have been extensively employed as classical microbicidal agents. The first report on the isolation of a bacterial strain *P. putida*, growing in the presence of toluene, was published by Inoue and Horikoshi in 1989 [207]. Since then a new class of microbes "solvent tolerant microbes" has emerged, and extensive studies are underway for screening of microbes that can adapt and grow in the presence of organic solvents [99,208,209]. Such organisms have drawn considerable attention due to their unique capability to survive in the broad range of organic solvents [210,211].

Solvent-tolerant microbes serve as a rich source of novel alkaline proteases of industrial significance. It is now unfolding that such enzymes display new properties in the presence of organic solvents. Enzyme catalysis in non-aqueous or low-water containing media is finding increasing applications because one can use water insoluble/solvent soluble substrate and facilitate the product recovery and favor synthesis reaction such as peptide synthesis and trans-esterification, which are thermodynamically unfavorable in water [212,213]. Generally, enzymes from normal microbial sources give low rate of reactions under non-aqueous environment, and get inactivated [214]. In nearly dry organic solvents, enzymes are insoluble, and are used as suspended powder, which reduce their catalytic performance. The polar organic solvents inactivate the enzymes by stripping the water that is essential for activity and stability.

Several physical and chemical methods such as chemical modification [215], immobilization [216], protein engineering [217] and directed evolution [218] have been employed for obtaining enzymes stable in the presence of organic solvents [219,220]. However, if enzymes are naturally stable and exhibit high activities in the presence of organic solvents, the stabilization of enzymes will not be required. Studies on enzymes from solvent tolerant microbes appear promising in this regard [209]. Some of these microbes are reported to be rich sources of industrially important proteases [95,221]. These enzymes are logically attuned to work under solvent rich environment, hence generally stable in organic solvents [209].

Various organic solvent-tolerant proteases have been obtained from bacteria, especially from Pseudomonas sp. Most of these strains are reported to be organic solvent-tolerant. Ogino et al. [95] reported, for the first time, an organic solvent-tolerant bacterium *P. aeruginosa* PST-01, which grew well in the presence of organic solvents such as hexane, toluene, benzene, and secreted a protease stable to various organic solvents [95,137]. Geok and co-workers [221] isolated an organic solvent-tolerant protease producer *P. aeruginosa* strain K out of 11 isolates of benzene-toluene-xylene-ethyl benzene tolerant bacteria. This bacterium was a polycyclic aromatic hydrocarbon degrader. Its protease was also stable towards large variety of organic solvents. Karadzic et al. [222] isolated *P. aeruginosa* san-ai strain from water-soluble cutting oil used in industrial metal-working process. This strain, when grown in a mixture of surfactants and mineral oil, produced an extracellular organic solvent-stable proteolytic enzyme. Purified protease preparation from *P. aeruginosa* PseA strain was completely stable in the presence of 25% (v/v) benzene, toluene, cyclohexane and n-hexane, and exhibited ~60-80% residual activity in n-butanol, n-heptane and isooctane, after 72 h incubation [141]. An organic solvent tolerant *P. aeruginosa* PD100 strain produced solvent stable protease, which exhibited >50% residual activity in the presence of 10% (v/v) of all the organic solvents tested, except acetone. The enzyme showed 80-90% activity in the presence of 20% (v/v) methanol, ethylene glycol, xylene and toluene. Presence of acetone at any concentration reduced the protease activity completely. These findings indicate that the protease exhibited high stability against denaturation/unfolding in the presence of most of organic solvents [223].

Tang et al. [224] reported an alkaline protease from *P. aeruginosa* PT121, quite stable in the presence of hydrophilic organic solvents. The residual protease activities were 82, 95, 76, 84, 76, 68, 60, 70, 71 and 95% after 24 hr incubation at 30°C in the presence of chloroform, benzene, n-hexanol, isoamyl alcohol, n-butanol, isopropyl alcohol, acetone, ethanol, DMF and DMSO, respectively, as compared to the crude protease containing no organic solvent at 0 h. Singh et al. [99] reported a solvent stable thermoalkaline protease of P. putida SKG-1. The protease exhibited ≥ 100% stability (over two weeks) in the presence of all the solvents under study, except for ethanol and methanol, which slightly reduced the activity to 88 and 72%, respectively. The stability of protease was remarkable (92-174%) in the presence of hydrophobic organic solvents (with log P value ≥ 2.0) within 24 h incubation. While savinase and α-chymotrypsin were not so stable and exhibited residual activities ranging 44-95% and 16-118%, respectively. In the presence of hydrophilic organic solvents also, the SKG-1 protease was remarkably stable by retaining 82-191% activities during 24 h incubation. Under similar conditions, savinase also exhibited remarkable protease activity (108-136%); however, residual activity of α-chymotrypsin reduced in the range of 62-136%.

Solvent tolerant proteases have also been reported from Bacillus sp. [225-227]. Castro [228] studied the effect of 14 neat organic solvents on the enzymatic activity of subtilisin, and observed 500 fold higher activity in the presence of glycerol compared to ethylene glycol, N-methylformamide, 1,2- and 1,3- propanediol. The half-life of *B. cereus* BG1 protease in the absence of an organic solvent was about 40 days at 30°C, which increased in the presence of organic solvents. Further, the enzyme exhibited good stability in the presence of DMSO, DMF, methanol, ethanol and 2-propanol [225]. In the case of Bacillus sp. APR-4, the incubation time for solvent-stability of protease was much shorter than most of *P. aeruginosa* proteases [226].

Abusham et al. [229] also reported an alkaline protease of *B. subtilis* strain Rand with enhanced activity (30 min incubation) in the presence of organic solvents (25%, v/v). It is a general observation that the enzymes of halophiles are sufficiently active in the presence of organic solvents. Halophilic microorganisms generally produce salt requiring enzymes as a result of adaptation to high salt conditions. Since salt reduces water activity (a feature in common with organic solvent systems), halophilic enzymes are considered to be valuable tools as biocatalysts in aqueous-organic media [195,230,231]. A summary of organic solvent stability profile of some alkaline proteases is listed in Table 4.

Microorganisms	Incubation conditions of proteases	Stable in organic solvents	References
Pseudomonas putida SKG-1	30°C, 14 days	In presence of (25%, v/v) EtOH, ButOH, Ben, Tol, Xyl, CH, Hex, Hep, Oct, IOct, Dec and DD.	[99]
P. aeruginosa PST-01	30°C, 15 days	In presence of (25%, v/v) EG, BD, PD, EtOH, HexOH, MeOH, DMSO, ProOH, TEG, tButOH, HepOH, DMF, OctOH, ButOH, Ace, DecOH, DO and Tol.	[137]
P. aeruginosa PseA	30°C, 72 h	In presence of (25%, v/v) Ben, Tol, CH, Hep and Hex.	[141]
P. aeruginosa strain K	37°C, 14 days	In presence of (25%, v/v) DecOH, IOct, Dec, DD and HD.	[221]
P. aeruginosa san-ai	30°C, 10 days	In presence of (25%, v/v) DMF.	[222]
P. aeruginosa PD100	55°C, 20 min	In presence of (10% or 20%, v/v) MeOH, EtOH, Xyl, Tol and Ben.	[223]
P. aeruginosa PT121	30°C, 5 or 14 days	In presence of (50%, v/v) Dec, Oct, Hep, Hex, CH, Tol, Ben and DMSO.	[224]
B. cereus BG1	30°C, 1-55 days	In presence of (25%, v/v) DMSO, MetOH, EtOH and ProOH.	[225]
Bacillus sp. APR-4	4°C, 24 hours	In presence of (50%, v/v) MeOH, EtOH, ProOH, Ben and ButOH.	[226]
B. pumilus	37°C, 30 min	In presence of (25%, v/v) Hex, DecOH, IOct, DD and TD.	[227]
Natrialba magadii	30°C, 24 hours, 1.5M NaCl	In presence of (15%, v/v) GlyOH, DMSO and PPG and TD.	[174]
Halobacterium sp. SP1(1)	20°C, 30 min	In presence of (33%, v/v) Tol, Xyl, Dec, UDec and DD.	[182]
Gamma-Proteobacterium	30°C, 10 days	In presence of (33%, v/v) EG, EtOH, ButOH, Ace, DMSO, Xyl and PCE.	[231]

[Ace, acetone; AN, acetonitrile; Ben, benzene; ButOH, 1-butanol; BD, 1,4-butanediol; tButOH, tert-butanol; CHL, chloroform; CH, cyclohexane, DE, diethylether; DMF, N,N-dimethylformamide; DMSO, dimethylsulfoxide; Dec, n-decane; DecOH, 1-decanol, DD, n-dodecane; EtOH, ethanol; EG, ethylene glycol; GlyOH, glycerol; Hep, n-heptane; HepOH, heptanol; HD, n-hexadecane; Hex, n-hexane; HexOH, 1-hexanol; IOct, isooctane; MeOH, methanol; Oct, n-octane; OctOH, 1-octanol; PCE, perchloroethylene; PD, 1,5-pentanediol; PPG, polypylenglycol; ProOH, 2-propanol; TD, n-tetradecane; THF, tetrahydrofuron; Tol, toluene; TEG, triethylene glycol; Xyl, p-xylene; UDec, n-undecane].

Table 4: Some solvent stable alkaline proteases from various bacterial strains.

Solvent tolerance strategies of microbes and their enzymes: Generally, most of the organic solvents prove extremely lethal to microorganisms by affecting the rigidity and stability of plasma membrane, thus resulting in the leakage of intracellular contents and eventual death [232]. Toxicity of any organic solvent is measured in terms of its log P value [log P is the logarithmic value of the partition coefficient (P) of solvent between n-octanol and water]. It is a quantitative measure of the polarity of solvent [233], and increasing log P corresponds with greater hydrophobic nature of the solvent, which in turn results in reduced toxicity [87]. Solvents having log P value in the range of 1.5-4.0 are considered as toxic [232]. Generally, Gram negative bacteria are more tolerant to organic solvents than Gram positive bacteria due to the presence of outer membrane, which functions as a barrier. Microorganisms that are able to tolerate high concentration of organic solvents (10-50%) are considered as extremophiles.

Protective mechanisms of solvent tolerance at cellular level: To tackle the lethal effects of organic solvents, bacteria have developed various metabolic, biochemical and physiological protective mechanisms:

1. Biotransformation of solvents into nontoxic by-products [232],

2. Membrane modification [234],

3. Compartmentalized vesicles to remove solvents [235] and

4. Extrusion of organic solvents via energy dependant efflux pumps [236].

Among various bacterial strains, members of genus Pseudomonas (*P. putida* DOT-T1E or *P. putida* S12) are extremely tolerant to organic solvents, and have been studied well to understand the mechanism of solvent tolerance. Thrusting out of organic solvents, using dedicated efflux pumps, is considered as the major mechanism of solvent tolerance [236,237]. Membrane modification is another important adaptive mechanism to survive in the presence of organic solvents. Alterations in the overall degree of fatty acid saturation or in the cis-trans isomerization of unsaturated fatty acids directly depends on toxicity and concentration of organic solvent. Synthesis of trans-fatty acids comes about by direct isomerization of the respective cis-configuration of the double bond without shifting the position [234,238-241]. Addition of toluene caused a rapid increase in the ratio of trans-unsaturated fatty acids by ~10 folds in *P. putida* membrane, immediately within 5 min and resulted in increased membrane rigidity [240,241].

Adaptive strategies at enzyme level: Organic solvent stability of proteases is an important property concerning application in non-aqueous medium for peptide synthesis, as the presence of aqueous environment is unfavorable. Generally, the proteases are inactivated in the presence of organic solvents. In aqueous environments, enzyme possesses conformational mobility/flexibility necessary for optimum catalysis. In contrast, organic solvents lack water-like ability to engage in hydrogen bonding, and also have lower dielectric constant leading to stronger intra-protein electrostatic interactions. Consequently, enzyme molecules become more rigid leading to reduced activity/denaturation [169,242].

The major factor responsible for loss of enzyme activity in organic solvents is the removal of crucial water molecules [145]. The low water content restrains protein conformation mobility, and affects K_m and V_{max} values [243]. This rigidity increases the resistance to thermal vibrations and reduces the enzyme-substrate interactions, leading to a reduced catalytic rate [195,244]. Loss of water can limit hydrogen bond formation between protein subunits on the exterior surface and active site interactions in the interior of proteins may be weakened. The presence of organic solvents might cause disruption of the forces important for hydrophobic core due to increased hydrophobicity of the medium.

Enzymes in non-aqueous systems can be active if the surface and active site region are well hydrated [220], as low water activity may limit

diffusion of substrates and affects the enzyme conformation too. Hence, the proteases active in the presence of organic solvents suggest that they are able to resist conformational denaturation posed by the solvents, and can form multiple hydrogen bonds with water molecules available for structural flexibility and optimum catalysis. Sardessai and Bhosle [211] observed the pH as an important factor influencing the enzyme stability. The pH of the environment determines the ionization state of the enzyme. However, no protonation/deprotonation can occur in an organic environment. The pH of the environment from which the enzyme is taken before being placed in the water-poor environment of the organic solvent, therefore determines the ionization state of the enzyme. This pH is retained in the enzyme and the property is referred to as pH memory.

Ogino et al. [245] studied the mechanism of organic solvent tolerance in a *P. aeruginosa* PST-01 protease, employing site-directed and random mutagenesis. They reported that disulfide bonds and amino acid residues located on enzyme surface play important roles in organic solvent stability. The structural analysis of *P. aeruginosa* protease (after treatment with organic solvent) revealed the presence of two disulfide bonds and hydrophobic patches at the protein surface, and was thought to be a key factor for solvent-stable nature of the enzyme [246,247]. Gaur et al. [247] carried out bioinformatic analysis to find the structural basis for the solvent tolerant nature of *P. aeruginosa* PseA aminopeptidase, and found that its primary structure contained 52% hydrophobic residues (folded in a conformation that favored its stability in organic solvents). In this protein, out of 52.2% hydrophobic amino acids, 44% were found on the surface. This increased surface hydrophobicity imparted organic solvent stability to the enzyme. Karabec et al. [248] analyzed the crystal structure of alcohol dehydrogenase from *Rhodococcus ruber* DSM 44541, and suggested that salt-bridges play a significant role in the stability of enzyme in non-aqueous media. Although there are several reports regarding solvent stable proteases, the published studies on the mechanism of enzyme's adaptation to function in organic solvents are relatively scanty [195] and require more efforts to reveal the interactions playing behind.

Conclusions

Now-a-days, extremozymes have several indispensable industrial applications, which will grow exponentially in near future. The survey of extremophiles has clearly indicated them as useful sources of alkali-stable proteases, active under extreme conditions. Alkaline proteases are suitable detergent additives for efficient washing characteristics. Currently, the interest of an industrialist is focused on such an alkaline protease which is psychro-, thermo-, halo-tolerant. To achieve this goal, the researchers are now searching for psychrotrophic bacteria, which are expected to produce a protease with above novel properties. The proteases from psychrotrophic halophiles are likely to remain active and stable under the extremities of high salt, broad temperature and alkaline pH conditions. The stability of alkaline protease under high salt level is desirable because (i) sodium chloride is employed as an important ingredient for granulation of enzyme before fortification in the detergents, and (ii) the ground water available in most of the geoclimatic regions is saline, and the salinity is detrimental for washing property of the detergents. The use of broad temperature range halotolerant alkaline protease in detergents can significantly improve its washing efficiency.

The solvent tolerance is an additional characteristic of certain halotolerant-psychro-thermo-alkaline proteases, and their producing microbes can be rich sources of novel alkaline proteases of industrial significance. Such proteases are likely to exhibit novel characteristics, such as therapeutic peptide synthesis, under non-aqueous environments in the presence of organic solvents. Currently available commercial alkaline proteases are unable to act under such conditions for peptide synthesis due to their poor organic solvent tolerance. Although, to date, many alkali-stable proteases have been reported from extremophiles, unremitting hunt for more relevant industrial enzymes is imperative to overcome the limitations/drawback(s) of current enzyme array.

Acknowledgements

Sanjay Kumar Singh is thankful to University Grants Commission for providing research fellowship under the scheme "Research Fellowships in Science for Meritorious Students". The assistance provided by the Government of Uttar Pradesh and Department of Science and Technology, Government of India, respectively under the schemes of Center of Excellence and DST-FIST, is duly acknowledged.

Conflict of Interest

The authors declare that they have no conflicts of interest.

References

1. Rao MB, Tanksale AM, Ghatge MS, Deshpande VV (1998) Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62: 597-635.
2. Gupta R, Beg QK, Lorenz P (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 59: 15-32.
3. Nierstrasz VA, Warmoeskerken MMCG (2003) Process engineering and industrial enzyme applications. In: Cavaco-Paulo A, Gubitz GM (Eds.), Textile processing with enzymes. CRC Press, Boca Raton, USA, pp. 120-157.
4. Gupta R, Beg QK, Khan S, Chauhan B (2002) An overview on fermentation, downstream processing and properties of microbial alkaline proteases. Appl Microbiol Biotechnol 60: 381-395.
5. Cowan DA (1994) Industrial Enzymes. In: Moses V, Cape RE (Eds.), Biotechnology-The cience and the business. Harwood Academic Publishers, Switzerland, pp. 326-328.
6. van den Burg B (2003) Extremophiles as a source for novel enzymes. Curr Opin Microbiol 6: 213-218.
7. Liszka MJ, Clark ME, Schneider E, Clark DS (2012) Nature versus nurture: developing enzymes that function under extreme conditions. Annu Rev Chem Biomol Eng 3: 77-102.
8. Rothschild LJ, Mancinelli RL (2001) Life in extreme environments. Nature 409: 1092-1101.
9. Antranikian G, Rudiger A, Canganella F, Klingeberg M, Sunna A (1995) Biodegradation of polymers at temperatures up to 130°C. JMS Pure Appl Chem 32: 661-669.
10. Fujiwara S (2002) Extremophiles: developments of their special functions and potential resources. J Biosci Bioeng 94: 518-525.
11. Synowiecki J (2010) Some applications of thermophiles and their enzymes for protein processing. Afr J Biotechnol 9: 7020-7025.
12. Rahman RNZRA, Razak CN, Ampon K, Basri M, Yunus WMZW, et al. (1994) Purification and characterization of a heatstable alkaline protease from Bacillus stearothermophilus F1. Appl Microbiol Biotechnol 40: 822-827.
13. Haki GD, Rakshit SK (2003) Developments in industrially important thermostable enzymes: a review. Bioresour Technol 89: 17-34.
14. Garg SK, Johri BN (1999) Proteolytic Enzymes. In: Johri BN, Satyanarayana T, Olsen J (edn), Thermophilic Moulds in Biotechnology. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 191-218.
15. Poldermans B (1990) Proteolytic enzymes. In: Gerhartz W (edn) Proteolytic Enzymes in Industry: Production and Applications. VCH Publishers, Weinheim, SGermany, pp. 108-123.
16. Danson MJ, Hough DW, Lunt GG (1992) The archaebacteria: biochemistry and biotechnology. Portland Press, London.
17. Huber H, Stetter KO (1998) Hyperthermophiles and their possible potential in biotechnology. J Biotechnol 64: 39-52.

18. Li DC, Yang YJ, Shen CY (1997) Protease production by the thermophilic fungus Thermomyces lanuginosus. Mycol Res 1: 18-22.

19. Kumar CG, Takagi H (1999) Microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol Adv 17: 561-594.

20. Thumar J, Singh SP (2007) Secretion of an alkaline protease from a salt-tolerant and alkaliphilic, Streptomyces clavuligerus strain MIT-1. Braz J Microbiol 38: 766-772.

21. Johnvesly B, Naik GR (2001) Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic Bacillus sp. JB-99 in a chemically defined medium. Process Biochem 37: 139-144.

22. Kaur S, Vohra RM, Kapoor M, Beg QK, Hoondal GS (2001) Enhanced production and characterization of a highly thermostable alkaline protease from Bacillus sp. P-2. World J Microbiol Biotechnol 17: 125-129.

23. Rahman RNZRA, Basri M, Salleh AB (2003) Thermostable alkaline protease from Bacillus stearothermophilus F1; Nutritional factors affecting protease production. Ann Microbiol 53: 199-210.

24. Kumar CG, Joo HS, Koo YM, Paik SR, Chang CS (2004) Thermostable alkaline protease from a novel marine haloalkalophilic Bacillus clausii Isolate. World J Microbiol Biotechnol 20: 351-357.

25. Takami H, Akiba T, Horikoshi K (1989) Production of extremely thermostable alkaline protease from Bacillus sp. No. AH-101. Appl Microbiol Biotechnol 30: 120-124.

26. Fujiwara N, Yamamoto K, Masui A (1991) Utilization of a thermostable alkaline protease from an alkalophilic thermophile for the recovery of silver from used X-ray film. J Ferment Bioeng 72: 306-308.

27. Manachini PL, Fortina MG (1998) Production in sea-water of thermostable alkaline proteases by a halotolerant strain of Bacillus licheniformis. Biotechnol Lett 20: 565-568.

28. Banerjee CU, Sani RK, Azmi W, Soni R (1999) Thermostable alkaline protease from Bacillus brevis and its characterization as a laundry detergent additive. Process Biochem 35: 213-219.

29. Gupta R, Gupta K, Saxena RK, Khan S (1999) Bleach-stable, alkaline protease from Bacillus sp. Biotechnol Lett 21: 135-138.

30. Kumar CG, Tiwari MP, Jany KD (1999) Novel alkaline serine proteases from alkalophilic Bacillus spp.: purification and some properties. Process Biochem 34: 441-449.

31. Singh J, Vohra RM, Sahoo DK (1999) Alkaline protease from a new obligate alkalophilic isolate of Bacillus sphaericus. Biotechnol Lett 21: 921-924.

32. Oberoi R, Beg QK, Puri S, Saxena RK, Gupta R (2001) Characterization and wash performance analysis of an SDS-stable alkaline protease from a Bacillus sp. World J Microbiol Biotechnol 17: 493-497.

33. Beg QK, Gupta R (2003) Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from Bacillus mojavensis. Enz Microb Technol 32: 294-304.

34. Huang Q, Peng Y, Li X, Wang H, Zhang Y (2003) Purification and characterization of an extracellular alkaline serine protease with dehairing function from Bacillus pumilus. Curr Microbiol 46: 169-173.

35. Joo HS, Kumar CG, Park GC, Paik SR, Chang CS (2003) Oxidant and SDS-stable alkaline protease from Bacillus clausii I-52: production and some properties. J Appl Microbiol 95: 267-272.

36. Gerze A, Omay D, Guvenilir Y (2005) Partial purification and characterization of protease enzyme from Bacillus subtilis megatherium. Appl Biochem Biotechnol 121-124: 335-345.

37. Sellami-Kamoun A, Haddar A, El-Hadj Ali N, Ghorbel-Frikha B, Kanoun S, et al. (2008) Stability of thermostable alkaline protease from Bacillus licheniformis RP1 in commercial solid laundry detergent formulations. Microbiol Res 163: 299-306.

38. Haddar A, Agrebi R, Bougatef A, Hmidet N, Sellami-Kamoun A, et al. (2009) Two detergent stable alkaline serine-proteases from Bacillus mojavensis A21: Purification, characterization and potential application as a laundry detergent additive. Biores Technol 100: 3366-3373.

39. Subba Rao Ch, Satish T, Ravichandra P, Prakasham RS (2009) Characterization of thermo- and detergent stable serine protease from isolated Bacillus circulans and evaluation of eco-friendly applications. Process Biochem 44: 262-268.

40. Deng A, Wu J, Zhang Y, Zhang G, Wen T (2010) Purification and characterization of a surfactant-stable high-alkaline protease from Bacillus sp. B001. Bioresour Technol 101: 7111-7117.

41. Singh SK, Singh SK, Tripathi VR, Garg SK (2012) Purification, characterization and secondary structure elucidation of a detergent stable, halotolerant, thermoalkaline protease from Bacillus cereus SIU1. Process Biochem 47: 1479-1487.

42. Herbert R, Sharp R (1992) Molecular Biology and Biotechnology of Extremophiles. Chapman and Hall, NY.

43. De Rosa M, Morana A, Riccio A, Gambacorta A, Trincone A, et al. (1994) Lipids of the archaea: a new tool for bioelectronics. Biosens Bioelectr 9: 669-675.

44. Schouten S, Baas M, Hopmans EC, Reysenbach AL, Damste JSS (2008) Tetraether membrane lipids of Candidatus "Aciduliprofundum boonei", a cultivated obligate thermoacidophilic euryarchaeote from deep-sea hydrothermal vents. Extremophiles 12: 119-124.

45. López-García P (1999) DNA supercoiling and temperature adaptation: A clue to early diversification of life?. J Mol Evol 49: 439-452.

46. Guagliardi A, Cerchia L, Moracci M, Rossi M (2000) The chromosomal protein Sso7d of the crenarchaeon Sulfolobus solfataricus rescue aggregated proteins in an ATP hydrolysis dependent manner. J Biol Chem 275: 31813-31818.

47. Ciaramella M, Pisani FM, Rossi M (2002) Molecular biology of extremophiles: recent progress on the hyperthermophilic archaeon Sulfolobus. Antonie Van Leeuwenhoek 81: 85-97.

48. Kumar S, Nussinov R (2001) How do thermophilic proteins deal with heat? Cell Mol Life Sci 58: 1216-1233.

49. Fitter J (2003) A measure of conformational entropy change during thermal protein unfolding using neutron spectroscopy. Biophys J 84: 3924-3930.

50. Ladenstein R, Ren B (2006) Protein disulfides and protein disulfide oxidoreductases in hyperthermophiles. FEBS J 273: 4170-4185.

51. Pedone E, Limauro D, Bartolucci S (2008) The machinery for oxidative protein folding in thermophiles. Antioxid Redox Signal 10: 157-169.

52. Kabsch W, Sander C (1983) Dictionary of protein secondary structure: pattern recognition of hydrogen-bonded and geometrical features. Biopolymers 22: 2577-2637.

53. Blake PR, Park JB, Zhou ZH, Hare DR, Adams MW, et al. (1992) Solution-state structure by NMR of zinc substituted rubredoxin from the marine hyperthermophilic archaebacterium Pyococcus furiosus. Protein Sci 1: 1508-1521.

54. Day MW, Hsu BT, Joshua-Tor L, Park JB, Zhou ZH, et al. (1992) X-ray crystal structures of the oxidized and reduced forms of the rubredoxin from the marine hyperthermophilic archaebacterium Pyrococcus furiosus. Protein Sci 1: 1494-1507.

55. Russell RJ, Taylor GL (1995) Engineering thermostability: lessons from thermophilic proteins. Curr Opin Biotechnol 6: 370-374.

56. Pfeil W, Gesierich U, Kleemann GR, Sterner R (1997) Ferredoxin from the hyperthermophile Thermotoga maritima is stable beyond the boiling point of water. J Mol Biol 272: 591-596.

57. Laksanalamai P, Robb FT (2004) Small heat shock proteins from extremophiles: a review. Extremophiles 8: 1-11.

58. Singh SP, Purohit MK, Aoyagi C, Kitaoka M, Hayashi K (2010) Effect of growth temperature, induction and molecular chaperones on the solubilization of over-expressed cellobiose phosphorylase from Cellvibrio gilvus under in-vivo conditions. Biotechnol Bioprocess Eng 15: 273-276.

59. Vieille C, Zeikus GJ (2001) Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. Microbiol Mol Biol Rev 65: 1-43.

60. Kaushik JK, Bhat R (2003) Why is trehalose an exceptional protein stabilizer? An analysis of the thermal stability of proteins in the presence of the compatible osmolyte trehalose. J Biol Chem 278: 26458-26465.

61. Mukaiyama A, Koga Y, Takano K, Kanaya S (2008) Osmolyte effect on the stability and folding of a hyperthermophilic protein. Proteins 71: 110-118.

62. Yadav JK, Prakash V (2009) Thermal stability of alpha-amylase in aqueous cosolvent systems. J Biosci 34: 377-387.

63. Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003) Microbial a-amylases: a biotechnological perspective. Process Biochem 38: 1599-1616.

64. Arnold FH, Wintrode PL, Miyazaki K, Gershenson A (2001) How enzymes adapt: lessons from directed evolution. Trends Biochem Sci 26: 100-106.

65. D'Amico S, Marx JC, Gerday C, Feller G (2003) Activity-stability relationships in extremophilic enzymes. J Biol Chem 278: 7891-7896.

66. Peters J, Bothner B, Kelly S (2007) Unfolding the mystery of protein thermostability. Yellowstone Science 15: 5-14.

67. Morita RY (1975) Psychrophilic bacteria. Bacteriol Rev 39: 144-167.

68. Feller G, Narinx E, Arpigny JL, Aittaleb M, Baise E, et al. (1996) Enzymes from psychrophilic organisms. FEMS Microbiol Rev 18: 189-202.

69. Feller G, Gerday C (2003) Psychrophilic enzymes: hot topics in cold adaptation. Nat Rev Microbiol 1: 200-208.

70. Jackman DM, Bartlett FM, Patel TR (1983) Heat-stable proteases from psychrotrophic pseudomonads: comparison of immunological properties. Appl Environ Microbiol 46: 6-12.

71. Arpigny JL, Feller G, Davail S, Genicot E, Narinx E, et al. (1994) Molecular adaptations of enzymes from thermophilic and psychrophilic organisms. Adv Comp Environ Physiol 20: 269-295.

72. Hébraud M, Potier P (1999) Cold shock response and low temperature adaptation in psychrotrophic bacteria. J Mol Microbiol Biotechnol 1: 211-219.

73. Kuddus M, Ramteke PW (2008) A cold-active extracellular metalloprotease from Curtobacterium luteum (MTCC 7529): enzyme production and characterization. J Gen Appl Microbiol 54: 385-392.

74. Margesin R, Schinner F (2001) Potential of halotolerant and halophilic microorganisms for biotechnology. Extremophiles 5: 73-83.

75. Hamamoto T, Kaneda M, Horikoshi K, Kudo T (1994) Characterization of a Protease from a Psychrotroph, Pseudomonas fluorescens 114. Appl Environ Microbiol 60: 3878-3880.

76. Ram MS, Singh L, Alam SI, Aggarwal MK (1994) Extracellular protease from Bacillus coagulans, a psychrotrophic, Antarctic bacterium. World J Microbiol Biotechnol 10: 356-357.

77. Singh L, Sai Ram M, Agarwal MK, Alam SI (1994) Simultaneous production of amylase and protease by Aeromonas hydrophila PC5, a psychrotrophic bacterium. J Gen Appl Microbiol 40: 339-348.

78. Ramana KV, Lokendra Singh (2004) Microbial Degradation of Organic Wastes at Low Temperatures. 50: 371-382.

79. Liao CH, McCallus DE (1998) Biochemical and genetic characterization of an extracellular protease from Pseudomonas fluorescens CY091. Appl Environ Microbiol 64: 914-921.

80. Matta H, Punj V (1998) Isolation and partial characterization of a thermostable extracellular protease of Bacillus polymyxa B-17. Int J Food Microbiol 42: 139-145.

81. Morita Y, Hasan Q, Sakaguchi T, Murakami Y, Yokoyama K, et al. (1998) Properties of a cold-active protease from psychrotrophic Flavobacterium balustinum P104. Appl Microbiol Biotechnol 50: 669-675.

82. Son ES, Kim J II (2003) Multicatalytic alkaline serine protease from the psychrotrophic Bacillus amyloliquefaciens S94. J Microbiol 41: 58-62.

83. Zeng R, Zhang R, Zhao J, Lin N (2003) Cold-active serine alkaline protease from the psychrophilic bacterium Pseudomonas strain DY-A: enzyme purification and characterization. Extremophiles 7: 335-337.

84. Kim JI, Lee SM, Jung HJ (2005) Characterization of calcium-activated bifunctional peptidase of the psychrotrophic Bacillus cereus. J Microbiol 43: 237-243.

85. Kasana RC, Yadav SK (2007) Isolation of a psychrotrophic Exiguobacterium sp. SKPB5 (MTCC 7803) and characterization of its alkaline protease. Curr Microbiol 54: 224-229.

86. Joshi GK, Kumar S, Sharma V (2007) Production of moderately halotolerant, SDS stable alkaline protease from Bacillus cereus MTCC 6840 isolated from lake Nainital, Uttaranchal state, India. Braz J Microbiol 38: 773-779.

87. Singh SK, Singh SK, Tripathi VR, Khare SK, Garg SK (2011) A novel psychrotrophic, solvent tolerant Pseudomonas putida SKG-1 and solvent stability of its psychro-thermoalkalistable protease. Process Biochem 46: 1430-1435.

88. Patel TR, Jackman DM, Williams GJ, Bartlett FM (1986) Physico-chemical properties of heat stable protease from psychrotrophic Pseudomonas. J Food Prot 49: 183-188.

89. Juffs HS (1976) Effects of temperature and nutrients on proteinase production by Pseudomonas fluorescens and Ps. aeruginosa in broth and milk. J Appl Bacteriol 40: 23-32.

90. McKellar RC (1982) Factors influencing the production of extracellular proteinase by Pseudomonas fluorescens. J Appl Bacteriol 53: 305-316.

91. Fairbairn DJ, Law BA (1987) The effect of nitrogen and carbon sources on proteinase production by Pseudomonas fluorescens. J Appl Bacteriol 62: 105-113.

92. Mitchell SL, Marshall RT (1989) Properties of Heat-Stable Proteases of Pseudomonas fluorescens: Characterization and hydrolysis of milk proteins. J Dairy Sci 72: 864-874.

93. McKevitt AI, Bajaksouzian S, Klinger JD, Woods DE (1989) Purification and characterization of an extracellular protease from Pseudomonas cepacia. Infect Immun 57: 771-778.

94. Sexton MM, Jones AL, Chaowagul W, Woods DE (1994) Purification and characterization of a protease from Pseudomonas pseudomallei. J Microbiol 40: 903-910.

95. Ogino H, Yasui K, Shiotani T, Ishihara T, Ishikawa H (1995) Organic solvent-tolerant bacterium which secretes an organic solvent-stable proteolytic enzyme. Appl Environ Microbiol 61: 4258-4262.

96. Engel LS, Hill JM, Caballero AR, Green LC, O'Callaghan RJ (1998) Protease IV, a unique extracellular protease and virulence factor from Pseudomonas aeruginosa. J Biol Chem 273: 16792-16797.

97. Koka R, Weimer BC (2000) Isolation and characterization of a protease from Pseudomonas fluorescens RO98. J Appl Microbiol 89: 280-288.

98. Singh SK, Singh SK, Tripathi VR, Khare SK, Garg SK (2011) Comparative one-factor-at-a-time, response surface (statistical) and bench-scale bioreactor level optimization of thermoalkaline protease production from a psychrotrophic Pseudomonas putida SKG-1 isolate. Microb Cell Fact 10: 114.

99. Singh SK, Singh SK, Tripathi VR, Khare SK, Garg SK (2013) Downstream processing, characterization, and structure-function relationship of solvent-, detergent-, psychro-, thermo-, alkalistable metalloprotease from metal-, solvent-tolerant psychrotrophic Pseudomonas putida SKG-1 isolate. Biotech Prog 29: 99-108.

100. Ewart KV, Lin Q, Hew CL (1999) Structure, function and evolution of antifreeze proteins. Cell Mol Life Sci 55: 271-283.

101. Shaw MK, Ingraham JL (1967) Synthesis of macromolecules by Escherichia coli near the minimal temperature for growth. J Bacteriol 94: 157-164.

102. McElhaney RN (1982) The use of differential scanning calorimetry and differential thermal analysis in studies of model and biological membranes. Chem Phys Lipids 30: 229-259.

103. Ermolenko DN, Makhatadze GI (2002) Bacterial cold-shock proteins. Cell Mol Life Sci 59: 1902-1913.

104. Roberts ME, Inniss WE (1992) The synthesis of cold shock proteins and cold acclimation proteins in the psychrophilic bacterium Aquaspirillum arcticum. Curr Microbiol 25: 275-278.

105. Whyte LG, Inniss WE (1992) Cold shock proteins and cold acclimation proteins in a psychrotrophic bacterium. Can J Microbiol 38: 1281-1285.

106. Hebraud M, Dubois E, Potier P, Labadie J (1994) Effect of growth temperatures on the protein levels in a psychrotrophic bacterium, Pseudomonas fragi. J Bacteriol 176: 4017-4024.

107. Berger F, Morellet N, Menu F, Potier P (1996) Cold shock and cold acclimation proteins in the psychrotrophic bacterium Arthrobacter globiformis SI55. J Bacteriol 178: 2999-3007.

108. Willem S, Srahna M, Devos N, Gerday C, Loppes R, et al. (1999) Protein adaptation to low temperatures: a comparative study of alpha-tubulin sequences in mesophilic and psychrophilic algae. Extremophiles 3: 221-226.

109. Feller G, Zekhnini Z, Lamotte-Brasseur J, Gerday C (1997) Enzymes from cold-adapted microorganisms. The class C beta-lactamase from the antarctic psychrophile Psychrobacter immobilis A5. Eur J Biochem 244: 186-191.

110. Merlino A, Russo Krauss I, Castellano I, De Vendittis E, Rossi B, et al. (2010) Structure and flexibility in cold-adapted iron superoxide dismutases: the case

of the enzyme isolated from Pseudoalteromonas haloplanktis. J Struct Biol 172: 343-352.

111. Feller G, Lonhienne T, Deroanne C, Libioulle C, VanBeeumen J, et al. (1992) Purification, characterization and nucleotide sequence of the thermolabile alpha-amylase from the Antarctic psychrotroph Alteromonas haloplanctis A23. J Biol Chem 267: 5217-5221.

112. Davail S, Feller G, Narinx E, Gerday C (1994) Cold adaptation of proteins. Purification, characterization, and sequence of the heat-labile subtilisin from the antarctic psychrophile Bacillus TA41. J Biol Chem 269: 17448-17453.

113. Feller G, Gerday C (1997) Psychrophilic enzymes: molecular basis of cold adaptation. Cell Mol Life Sci 53: 830-841.

114. Michaux C, Massant J, Kerff F, Frère JM, Docquier JD, et al. (2008) Crystal structure of a cold-adapted class C beta-lactamase. FEBS J 275: 1687-1697.

115. Zhang L, Conway JF, Thibodeau PH (2012) Calcium-induced folding and stabilization of the Pseudomonas aeruginosa alkaline protease. J Biol Chem 287: 4311-4322.

116. Dalluge JJ, Hamamoto T, Horikoshi K, Morita RY, Stetter KO, et al. (1997) Posttranscriptional modification of tRNA in psychrophilic bacteria. J Bacteriol 179: 1918-1923.

117. Lonhienne T, Zoidakis J, Vorgias CE, Feller G, Gerday C, et al. (2001) Modular structure, local flexibility and cold-activity of a novel chitobiase from a psychrophilic Antarctic bacterium. J Mol Biol 310: 291-297.

118. Xu Y, Feller G, Gerday C, Glansdorff N (2003) Metabolic enzymes from psychrophilic bacteria: challenge of adaptation to low temperatures in ornithine carbamoyltransferase from Moritella abyssi. J Bacteriol 185: 2161-2168.

119. D'Amico S, Collins T, Marx JC, Feller G, Gerday C (2006) Psychrophilic microorganisms: challenges for life. EMBO Rep 7: 385-389.

120. Saeki K, Hitomi J, Okuda M, Hatada Y, Kageyama Y, et al. (2002) A novel species of alkaliphilic Bacillus that produces an oxidatively stable alkaline serine protease. Extremophiles 6: 65-72.

121. Akbalik G (2003) Screening for industrially important extracellular enzymes from alkalophilic Bacillus genus. Master's Thesis, Izmir Institute of Technology, Izmir, Turkey.

122. Fujiwara N, Yamamoto K (1987) Production of alkaline protease in a low-cost medium by alkalophilic Bacillus sp. and properties of the enzyme. J Ferment Technol 65: 345-348.

123. Ferrero MA, Castro GR, Abate CM, Biagori MD, Sineriz F (1996) Thermostable alkaline proteases of Bacillus licheniformis MIR 29: isolation, production and characterization. Appl Microbiol Biotechnol 45: 327-332.

124. Miyaji T, Otta Y, Nakagawa T, Watanabe T, Niimura Y, et al. (2006) Purification and molecular characterization of subtilisin-like alkaline protease BPP-A from Bacillus pumilus strain MS-1. Lett Appl Microbiol 42: 242-247.

125. Horikoshi K (1971) Production of alkaline enzymes by alkalophilic microorganisms Part I. Alkaline protease produced by Bacillus no. 221. Agric Biol Chem 35: 1407-1414.

126. Horikoshi K (1999) Alkaliphiles: some applications of their products for biotechnology. Microbiol Mol Biol Rev 63: 735-750, table of contents.

127. Fujinami S, Fujisawa M (2010) Industrial applications of alkaliphiles and their enzymes--past, present and future. Environ Technol 31: 845-856.

128. Arnórsdóttir J, Kristjánsson MM, Ficner R (2005) Crystal structure of a subtilisin-like serine proteinase from a psychrotrophic Vibrio species reveals structural aspects of cold adaptation. FEBS J 272: 832-845.

129. Kobayashi T, Hakamada Y, Adachi S, Hitomi J, Yoshimatsu T, et al. (1995) Purification and properties of an alkaline protease from alkalophilic Bacillus sp. KSM-K16. Appl Microbiol Biotechnol 43: 473-481.

130. Kobayashi T, Lu J, Li Z, Hung VS, Kurata A, et al. (2007) Extremely high alkaline protease from a deep-subsurface bacterium, Alkaliphilus transvaalensis. Appl Microbiol Biotechnol 75: 71-80.

131. Saeki K, Ozaki K, Kobayashi T, Ito S (2007) Detergent alkaline proteases: enzymatic properties, genes, and crystal structures. J Biosci Bioeng 103: 501-508.

132. Jasvir S, Gill N, Devasahayam G, Sahoo DK (1999) Studies on alkaline protease produced by Bacillus sp. NG312. Appl Biochem Biotechnol 76: 57-63.

133. Singh J, Batra N, Sobti RC (2001) Serine alkaline protease from a newly isolated Bacillus sp. SSR1. Process Biochem 36: 781-785.

134. Joo HS, Kumar CG, Park GC, Kim KT, Paik SR, et al. (2002) Optimization of the production of an extracellular alkaline protease from Bacillus horikoshii. Process Biochem 38: 155-159.

135. Gessesse A, Hatti-Kaul R, Gashe BA, Mattiasson B (2003) Novel alkaline proteases from alkaliphilic bacteria grown on chicken feather. Enz Microb Technol 32: 519-524.

136. Denizci AA, Kazan D, Abeln EC, Erarslan A (2004) Newly isolated Bacillus clausii GMBAE 42: an alkaline protease producer capable to grow under higly alkaline conditions. J Appl Microbiol 96: 320-327.

137. Ogino H, Watanabe F, Yamada M, Nakagawa S, Hirose T, et al. (1999) Purification and characterization of organic solvent-stable protease from organic solvent-tolerant Pseudomonas aeruginosa PST-01. J Biosci Bioeng 87: 61-68.

138. Ogino H, Yamada M, Watanabe F, Ichinose H, Yasuda M, et al. (1999) Peptide synthesis catalyzed by organic solvent-stable protease from Pseudomonas aeruginosa PST-01 in monophasic aqueous-organic solvent systems. J Biosci Bioeng 88: 513-518.

139. Bayoudh A, Gharsallah N, Chamkha M, Dhouib A, Ammar S, et al. (2000) Purification and characterization of an alkaline protease from Pseudomonas aeruginosa MN1. J Ind Microbiol Biotechnol 24: 291-295.

140. Beg QK, Sahai V, Gupta R (2003) Statistical media optimization and alkaline protease production from Bacillus mojavensis in a bioreactor. Process Biochem 39: 203-209.

141. Gupta A, Roy I, Khare SK, Gupta MN (2005) Purification and characterization of a solvent stable protease from Pseudomonas aeruginosa PseA. J Chromatogr A 1069: 155-161.

142. Gupta A, Khare SK (2006) A protease stable in organic solvents from solvent tolerant strain of Pseudomonas aeruginosa. Bioresour Technol 97: 1788-1793.

143. Ghorbel-Bellaaj O, Hayet BK, Bayoudh A, Younes I, Hmidet N, et al. (2012) Pseudomonas aeruginosa A2 elastase: purification, characterization and biotechnological applications. Int J Biol Macromol 50: 679-686.

144. Gupta A, Khare SK (2007) Enhanced production and characterization of a solvent stable protease from solvent tolerant Pseudomonas aeruginosa PseA. Enz Microb Technol 42: 11-16.

145. Doukyu N, Ogino H (2010) Organic solvent-tolerant enzymes. Biochem Eng J 48: 270-282.

146. Feltzer RE, Trent JO, Gray RD (2003) Alkaline proteinase inhibitor of Pseudomonas aeruginosa: a mutational and molecular dynamics study of the role of N-terminal residues in the inhibition of Pseudomonas alkaline proteinase. J Biol Chem 278: 25952-25957.

147. Rahman RNZRA, Geok LP, Wong CF, Basri M, Salleh AB (2010) Molecular investigation of a gene encoding organic solvent-tolerant alkaline protease from Pseudomonas aeruginosa strain K. J Basic Microbiol 50: 143-149.

148. Cahan R, Axelrad I, Safrin M, Ohman DE, Kessler E (2001) A secreted aminopeptidase of Pseudomonas aeruginosa. Identification, primary structure, and relationship to other aminopeptidases. J Biol Chem 276: 43645-43652.

149. Kuhn P, Knapp M, Soltis SM, Ganshaw G, Thoene M, et al. (1998) The 0.78 A structure of a serine protease: Bacillus lentus subtilisin. Biochemistry 37: 13446-13452.

150. Nonaka T, Fujihashi M, Kita A, Saeki K, Ito S, et al. (2004) The crystal structure of an oxidatively stable subtilisin-like alkaline serine protease, KP-43, with a C-terminal beta-barrel domain. J Biol Chem 279: 47344-47351.

151. Almog O, González A, Godin N, de Leeuw M, Mekel MJ, et al. (2009) The crystal structures of the psychrophilic subtilisin S41 and the mesophilic subtilisin Sph reveal the same calcium-loaded state. Proteins 74: 489-496.

152. de Souza N (2007) From structure to function. Nat Methods 4: 771.

153. Jaouadi B, Aghajari N, Haser R, Bejar S (2010) Enhancement of the thermostability and the catalytic efficiency of Bacillus pumilus CBS protease by site-directed mutagenesis. Biochimie 92: 360-369.

154. Ulukanli Z, Digrak M (2002) Alkaliphilic micro-organisms and habitats. Turk J Biol 26: 181-191.

155. Padan E, Bibi E, Ito M, Krulwich TA (2005) Alkaline pH homeostasis in bacteria: new insights. Biochim Biophys Acta 1717: 67-88.

156. Krulwich TA, Sachs G, Padan E (2011) Molecular aspects of bacterial pH sensing and homeostasis. Nat Rev Microbiol 9: 330-343.

157. Ivey DM, Ito M, Gilmour R, Zemsky J, Guffanti AA, et al. (1998) Alkaliphile Bioenergetics. In: Horikoshi K, Grant WD (edn) Extremophiles: Microbial Life in Extreme Environments. Wiley Liss, NY, pp. 181-200.

158. Ni S, Boone DR (1998) Extremophilic Methanogenic Archaea and Their Adaptation Mechanisms. In: Horikoshi K, Grant WD (edn) Extremophiles: Microbial Life in Extreme Environments. Wiley Liss, NY, pp. 211-232.

159. Aono R, Horikoshi K (1983) Chemical composition of cell walls of alkalophilic strains of Bacillus. J Gen Microbiol 129: 1083-1087.

160. Shirai T, Suzuki A, Yamane T, Ashida T, Kobayashi T, et al. (1997) High-resolution crystal structure of M-protease: Phylogeny aided analysis of the high-alkaline adaptation mechanism. Protein Eng 10: 627-634.

161. Sokalingam S, Raghunathan G, Soundrarajan N, Lee SG (2012) A study on the effect of surface lysine to arginine mutagenesis on protein stability and structure using green fluorescent protein. PLoS One 7: e40410.

162. Zhao Y, Zhang Y, Cao Y, Qi J, Mao L, et al. (2011) Structural analysis of alkaline Î²-mannanase from alkaliphilic Bacillus sp. N16-5: implications for adaptation to alkaline conditions. PLoS One 6: e14608.

163. Yamagata Y, Isshiki K, Ichishima E (1995) Subtilisin Sendai from alkalophilic Bacillus sp.: molecular and enzymatic properties of the enzyme and molecular cloning and characterization of the gene, aprS. Enzyme Microb Technol 17: 653-663.

164. Tindall BJ, Ross HNM, Grant WD (1984) Natronobacterium gen. nov. and Natronococcus gen. nov., two new genera of haloalkalophilic archaebacteria. Syst Appl Microbiol 5: 41-57.

165. Dodia MS, Rawal CM, Bhimani HG, Joshi RH, Khare SK, et al. (2008) Purification and stability characteristics of an alkaline serine protease from a newly isolated Haloalkaliphilic bacterium sp. AH-6. J Ind Microbiol Biotechnol 35: 121-131.

166. Danson MJ, Hough DW (1997) The structural basis of protein halophilicity. Comp Biochem Physiol 117: 307-312.

167. Demirjian DC, Morís-Varas F, Cassidy CS (2001) Enzymes from extremophiles. Curr Opin Chem Biol 5: 144-151.

168. Madern D, Ebel C, Zaccai G (2000) Halophilic adaptation of enzymes. Extremophiles 4: 91-98.

169. Klibanov AM (2001) Improving enzymes by using them in organic solvents. Nature 409: 241-246.

170. Kim J, Dordick JS (1997) Unusual salt and solvent dependence of a protease from an extreme halophile. Biotechnol Bioeng 55: 471-479.

171. Studdert CA, Seitz MKH, Gilv MIP, Sanchez JJ, De Castro RE (2001) Purification and biochemical characterization of the haloalkaliphilic archaeon Natronococcus occultus extracellular serine protease. J Basic Microbiol 6: 375-383.

172. Sánchez-Porro C, Mellado E, Bertoldo C, Antranikian G, Ventosa A (2003) Screening and characterization of the protease CP1 produced by the moderately halophilic bacterium Pseudoalteromonas sp. strain CP76. Extremophiles 7: 221-228.

173. Patel RK, Dodia MS, Joshi RH, Singh SP (2006) Purification and characterization of alkaline protease from a newly isolated haloalkaliphilic Bacillus sp. Process Biochem 41: 2002-2009.

174. Ruiz DM, De Castro RE (2007) Effect of organic solvents on the activity and stability of an extracellular protease secreted by the haloalkaliphilic archaeon Natrialba magadii. J Ind Microbiol Biotechnol 34: 111-115.

175. Kamekura M, Seno Y (1990) A halophilic extracellular protease from a halophilic archaebacterium strain 172 P1. Biochem Cell Biol 68: 352-359.

176. Ryu K, Kim J, Dordick JS (1994) Catalytic properties and potential of an extracellular protease from an extreme halophile. Enzyme Microb Technol 16: 266-275.

177. Ventosa A, Nieto JJ, Oren A (1998) Biology of moderately halophilic aerobic bacteria. Microbiol Mol Biol Rev 62: 504-544.

178. Giménez MI, Studdert CA, Sánchez JJ, De Castro RE (2000) Extracellular protease of Natrialba magadii: purification and biochemical characterization. Extremophiles 4: 181-188.

179. Gupta A, Roy I, Patel RK, Singh SP, Khare SK, et al. (2005) One-step purification and characterization of an alkaline protease from haloalkaliphilic Bacillus sp. J Chromatogr A 1075: 103-108.

180. Joo HS, Chang CS (2005) Oxidant and SDS-stable alkaline protease from a halo-tolerant Bacillus clausii I-52: enhanced production and simple purification. J Appl Microbiol 98: 491-497.

181. Karbalaei-Heidari HR, Ziaee AA, Amoozegar MA (2007) Purification and biochemical characterization of a protease secreted by the Salinivibrio sp. strain AF-2004 and its behavior in organic solvents. Extremophiles 11: 237-243.

182. Akolkar AV, Deshpande GM, Raval KN, Durai D, Nerurkar AS, et al. (2008) Organic solvent tolerance of Halobacterium sp. SP1(1) and its extracellular protease. J Basic Microbiol 48: 421-425.

183. Abdel-Fattah YR, El-Enshasy HA, Soliman NA, El-Gendi H (2009) Bioprocess development for production of alkaline protease by Bacillus pseudofirmus Mn6 through statistical experimental designs. J Microbiol Biotechnol 19: 378-386.

184. Gohel SD, Singh SP (2012) Purification strategies, characteristics and thermodynamic analysis of a highly thermostable alkaline protease from a salt-tolerant alkaliphilic actinomycete, Nocardiopsis alba OK-5. J Chromatography B 889-890: 61-68.

185. Jaouadi B, Ellouz-Chaabouni S, Rhimi M, Bejar S (2008) Biochemical and molecular characterization of a detergent-stable serine alkaline protease from Bacillus pumilus CBS with high catalytic efficiency. Biochimie 90: 1291-1305.

186. Tyagi NK, Sakthivadivel R, Sharma DK, Ambast SK, Agrawal A (2003) Farmers decision making in irrigation commands: The need and scope for improvement. CSSRI Synthesis Paper 1. Central Soil Salinity Research Institute, Karnal, India, pp. 1-11.

187. Singh SK, Tripathi VR, Jain RK, Vikram S, Garg SK (2010) An antibiotic, heavy metal resistant and halotolerant Bacillus cereus SIU1 and its thermoalkaline protease. Microb Cell Fact 9: 59.

188. Sakamoto T, Murata N (2002) Regulation of the desaturation of fatty acids and its role in tolerance to cold and salt stress. Curr Opin Microbiol 5: 208-210.

189. Waditee R, Hibino T, Nakamura T, Incharoensakdi A, Takabe T (2002) Overexpression of a Na+/H+ antiporter confers salt tolerance on a freshwater cyanobacterium, making it capable of growth in sea water. Proc Natl Acad Sci USA 99: 4109-4114.

190. Roberts MF (2005) Organic compatible solutes of halotolerant and halophilic microorganisms. Saline Systems 1: 5.

191. Hillmann F, Fischer RJ, Bahl H (2006) The rubrerythrin-like protein Hsp21 of Clostridium acetobutylicum is a general stress protein. Arch Microbiol 185: 270-276.

192. de Lourdes Moreno M, Pérez D, García MT, Mellado E (2013) Halophilic bacteria as a source of novel hydrolytic enzymes. Life (Basel) 3: 38-51.

193. Zuleta LF, Italiani VC, Marques MV (2003) Isolation and characterization of NaCl-sensitive mutants of Caulobacter crescentus. Appl Environ Microbiol 69: 3029-3035.

194. Naughton LM, Blumerman SL, Carlberg M, Boyd EF (2009) Osmoadaptation among Vibrio species and unique genomic features and physiological responses of Vibrio parahaemolyticus. Appl Environ Microbiol 75: 2802-2810.

195. Karan R, Capes MD, Dassarma S (2012) Function and biotechnology of extremophilic enzymes in low water activity. Aquat Biosyst 8: 4.

196. Dzubiella J (2008) Salt-specific stability and denaturation of a short salt-bridge-forming alpha-helix. J Am Chem Soc 130: 14000-14007.

197. Paul S, Bag SK, Das S, Harvill ET, Dutta C (2008) Molecular signature of hypersaline adaptation: insights from genome and proteome composition of halophilic prokaryotes. Genome Biol 9: R70.

198. Spitzer J (2011) From water and ions to crowded biomacromolecules: in vivo structuring of a prokaryotic cell. Microbiol Mol Biol Rev 75: 491-506.

199. Frolow F, Harel M, Sussman JL, Mevarech M, Shoham M (1996) Insights into protein adaptation to a saturated salt environment from the crystal structure of a halophilic 2Fe-2S ferredoxin. Nat Struct Biol 3: 452-458.

200. Britton KL, Baker PJ, Fisher M, Ruzheinikov S, Gilmour DJ, et al. (2006) Analysis of protein solvent interactions in glucose dehydrogenase from the

extreme halophile Haloferax mediterranei. Proc Natl Acad Sci U S A 103: 4846-4851.

201. Fukuchi S, Yoshimune K, Wakayama M, Moriguchi M, Nishikawa K (2003) Unique amino acid composition of proteins in halophilic bacteria. J Mol Biol 327: 347-357.

202. Kennedy SP, Ng WV, Salzberg SL, Hood L, DasSarma S (2001) Understanding the adaptation of Halobacterium species NRC-1 to its extreme environment through computational analysis of its genome sequence. Genome Res 11: 1641-1650.

203. Karan R, Khare SK (2011) Stability of haloalkaliphilic Geomicrobium sp. protease modulated by salt. Biochemistry (Mosc) 76: 686-693.

204. Ortega G, Laín A, Tadeo X, López-Méndez B, Castaño D, et al. (2011) Halophilic enzyme activation induced by salts. Sci Rep 1: 6.

205. Rao L, Zhao X, Pan F, Li Y, Xue Y, et al. (2009) Solution behavior and activity of a halophilic esterase under high salt concentration. PLoS One 4: e6980.

206. O'Brien R, DeDecker B, Fleming K, Sigler PB, Ladbury JE (1998) The effects of salt on the TATA binding protein-DNA interaction from a hyperthermophilic archaeon. J Mol Biol 279: 117-125.

207. Inoue A, Horikoshi K (1989) A Pseudomonas thrives in high concentrations of toluene. Nature 338: 264-266.

208. Aono R, Ito M, Inoue A, Horikoshi K (1992) Isolation of novel toluene-tolerant strain of Pseudomonas aeruginosa. Biosci Biotechnol Biochem 56: 145-146.

209. Ogino H, Ishikawa H (2001) Enzymes which are stable in the presence of organic solvents. J Biosci Bioeng 91: 109-116.

210. Isken S, de Bont JA (1998) Bacteria tolerant to organic solvents. Extremophiles 2: 229-238.

211. Sardessai YN, Bhosle S (2004) Industrial potential of organic solvent tolerant bacteria. Biotechnol Prog 20: 655-660.

212. Klibanov AM (1989) Enzymatic catalysis in anhydrous organic solvents. Trends Biochem Sci 14: 141-144.

213. Gupta MN (2000) Methods in Non-aqueous Enzymology. Birkhauser-Verlag, Basel, Switzerland.

214. Gupta MN (1992) Enzyme function in organic solvents. Eur J Biochem 203: 25-32.

215. Jiang Y, Dalton H (1994) Chemical modification of the hydroxylase of soluble methane monooxygenase gives one form of the protein with significantly increased thermostability and another that functions well in organic solvents. Biochim Biophys Acta 1201: 76-84.

216. Persson M, Wehtje E, Adlercreutz P (2002) Factors governing the activity of lyophilised and immobilised lipase preparations in organic solvents. Chembiochem 3: 566-571.

217. Martinez P, Arnold FH (1991) Surface change substitutions increase the stability of a-lytic protease in organic solvents. J Am Chem Soc 113: 6336-6337.

218. Arnold FH, Moore JC (1997) Optimizing industrial enzymes by directed evolution. Adv Biochem Eng Biotechnol 58: 1-14.

219. Khmelnitsky YL, Rich JO (1999) Biocatalysis in nonaqueous solvents. Curr Opin Chem Biol 3: 47-53.

220. Gupta MN, Roy I (2004) Enzymes in organic media. Forms, functions and applications. Eur J Biochem 271: 2575-2583.

221. Geok LP, Razak CNA, Rahman RNZRA, Basri M, Salleh AB (2003) Isolation and screening of an extracellular organic solvent-tolerant protease producer. Biochem Eng J 13: 73-77.

222. Karadzic I, Masui A, Fujiwara N (2004) Purification and characterization of a protease from Pseudomonas aeruginosa grown in cutting oil. J Biosci Bioeng 98: 145-152.

223. Najafi MF, Deobagkar D, Deobagkar D (2005) Potential application of protease isolated from Pseudomonas aeruginosa PD100. Elect J Biotechnol 8: 197-203.

224. Tang XY, Pan Y, Li S, He BF (2008) Screening and isolation of an organic solvent-tolerant bacterium for high-yield production of organic solvent-stable protease. Bioresour Technol 99: 7388-7392.

225. Ghorbel B, Kamoun AS, Nasri M (2003) Stability studies of protease from Bacillus cereus BG1. Enz Microb Technol 32: 513-518.

226. Kumar D, Bhalla TC (2005) Microbial proteases in peptide synthesis: approaches and applications. Appl Microbiol Biotechnol 68: 726-736.

227. Rahman RN, Mahamad S, Salleh AB, Basri M (2007) A new organic solvent tolerant protease from Bacillus pumilus 115b. J Ind Microbiol Biotechnol 34: 509-517.

228. Castro GR (1999) Enzymatic activities of proteases dissolved in organic solvents. Enz Microb Technol 25: 689-694.

229. Abusham RA, Rahman RN, Salleh AB, Basri M (2009) Optimization of physical factors affecting the production of thermo-stable organic solvent-tolerant protease from a newly isolated halo tolerant Bacillus subtilis strain Rand. Microb Cell Fact 8: 20.

230. Selleck GA, Chaudhuri JB (1999) Biocatalysis in organic media using enzymes from extremophiles. Enz Microb Technol 25: 471-482.

231. Sana B, Ghosh D, Saha M, Mukherjee J (2006) Purification and characterization of a salt, solvent, detergent and bleach tolerant protease from a new gamma-Proteobacterium isolated from the marine environment of the Sundarbans. Process Biochem 41: 208-215.

232. Ramos JL, Duque E, Gallegos MT, Godoy P, Ramos-Gonzalez MI, et al. (2002) Mechanisms of solvent tolerance in gram-negative bacteria. Annu Rev Microbiol 56: 743-768.

233. Laane C, Boeren S, Vos K, Veeger C (1987) Rules for optimization of biocatalysis in organic solvents. Biotechnol Bioeng 30: 81-87.

234. Heipieper HJ, Fischer J, Meinhardt F (2010) Cis-Trans Isomerase of Unsaturated Fatty Acids: An Immediate Bacterial Adaptive Mechanism to Cope with Emerging Membrane Perturbation Caused by Toxic Hydrocarbons. In: Timmis KN (edn) Handbook of Hydrocarbon and Lipid Microbiology. Springer-Verlag, Berlin, Heidelberg, pp. 1605-1614.

235. Kobayashi H, Uematsu K, Hirayama H, Horikoshi K (2000) Novel toluene elimination system in a toluene-tolerant microorganism. J Bacteriol 182: 6451-6455.

236. Rojas A, Duque E, Mosqueda G, Golden G, Hurtado A, et al. (2001) Three efflux pumps are required to provide efficient tolerance to toluene in Pseudomonas putida DOT-T1E. J Bacteriol 183: 3967-3973.

237. Kieboom J, Dennis JJ, de Bont JA, Zylstra GJ (1998) Identification and molecular characterization of an efflux pump involved in Pseudomonas putida S12 solvent tolerance. J Biol Chem 273: 85-91.

238. Weber FJ, de Bont JA (1996) Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. Biochim Biophys Acta 1286: 225-245.

239. Ramos JL, Duque E, Rodríguez-Herva JJ, Godoy P, Haïdour A, et al. (1997) Mechanisms for solvent tolerance in bacteria. J Biol Chem 272: 3887-3890.

240. Junker F, Ramos JL (1999) Involvement of the cis/trans isomerase Cti in solvent resistance of Pseudomonas putida DOT-T1E. J Bacteriol 181: 5693-5700.

241. Bernal P, Segura A, Ramos JL (2007) Compensatory role of the cis-trans-isomerase and cardiolipin synthase in the membrane fluidity of Pseudomonas putida DOT-T1E. Environ Microbiol 9: 1658-1664.

242. Rahman RNZRA, Geok LP, Basri M, Salleh AB (2006) An organic solvent-stable alkaline protease from Pseudomonas aeruginosa strain K: Enzyme purification and characterization. Enz Microb Technol 39: 1484-1491.

243. Zhu X, Zhou T, Wu X, Cai Y, Yao D, et al. (2011) Covalent immobilization of enzymes within micro-aqueous organic media. J Mol Catal B 72: 145-149.

244. Torres S, Castro GR (2004) Non-aqueous biocatalysis in homogeneous solvent systems. Food Technol Biotechnol 42: 271-277.

245. Ogino H, Uchiho T, Yokoo J, Kobayashi R, Ichise R, et al. (2001) Role of intermolecular disulfide bonds of the organic solvent-stable PST-01 protease in its organic solvent stability. Appl Environ Microbiol 67: 942-947.

246. Gupta A, Ray S, Kapoor S, Khare SK (2008) Solvent-stable Pseudomonas aeruginosa PseA protease gene: identification, molecular characterization, phylogenetic and bioinformatics analysis to study reasons for solvent stability. J Mol Microbiol Biotechnol 15: 234-243.

247. Gaur R, Grover T, Sharma R, Kapoor S, Khare SK (2010) Purification and characterization of a solvent stable aminopeptidase from Pseudomonas aeruginosa: Cloning and analysis of aminopeptidase gene conferring solvent stability. Process Biochem 45: 757-764.

248. Karabec M, Lyskowski A, Tauber KC, Steinkellner G, Kroutil W, et al. (2010) Structural insights into substrate specificity and solvent tolerance in alcohol dehydrogenase ADH-'A' from Rhodococcus rubber DSM 44541. Chem Commun (Camb) 34: 6314-6316.