

Chapter 4

Alkaliphilic Bacteria: Diversity, Physiology and Industrial Applications

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4.1 Introduction

Microorganisms are ubiquitous in nature occupying not only different moderate habitats on earth but are also found to adapt and colonize certain harsh environments, once assumed to be sterile by man. Such organisms thriving in extreme environments are called extremophiles and alkaliphiles are a diverse group of organisms that thrive in highly alkaline environments with pH optima for growth of 9.0 or above. They are represented in a large number of bacterial genera and physiological types, but share common challenges that include cytoplasmic pH homeostasis, associated problems of bioenergetic work and function of surface-associated and secreted proteins that must function at very high pH (Krulwich and Guffanti 1989). Alkaliphilic microorganisms have made a large impact with their application and manufacture of mass-market consumer products in industries, especially with enzymes active at high pH. The immense potential of alkaliphiles and a remarkable versatility regarding the production of their primary and secondary metabolites has resulted in their exploitation for use in industries.

4.2 Ecological Niches of Prevalence of Alkaliphiles

Alkaliphilic microorganisms, particularly prokaryotes are widely distributed extremophiles some of which grow in alkaline niches where the pH is above 12. These niches include alkaline soda lakes, which are found throughout the world, providing natural enrichments for an impressively diverse array of alkaliphiles. They

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can be found not only in alkaline environments but also in nonalkaline environments such as neutral and acidic soils, although counts of alkaliphiles are higher in alkaline environments (Grant 2006). In addition, alkaliphiles can be isolated from more specialized alkaline niches such as alkaline ground water (Roadcap et al. 2006), termite gut (Thongaram et al. 2003, 2005), the guts of insect larvae (Aizawa et al. 2010), man-made alkaline environments such as indigo dye balls (Aino et al. 2008; Ghauri et al. 2006) and alkaline effluents of various industries (Borkar et al. 2003; Blanco et al. 2012) as presented in Table 4.1.

4.3 Genesis of Natural Alkaline Environments

The most stable naturally occurring alkaline environments such as eutrophic soda (Na_2CO_3) lakes, soda deserts and alkaline ground waters are caused by a combination of geological, geographical and climatic condition (Grant et al. 1990). Lake Magadi in Kenya, Lake Wadi Natrum in Egypt of the East African rift valley and the western soda lakes of the USA are probably the most stable and highly alkaline environments on the earth with a consistent pH of 10.5–12.0. These environments are characterised by the presence of large amounts of sodium carbonate and sodium chloride, formed by the evaporative concentration, giving rise to environments that are both alkaline and saline. In India, such alkaline and saline lakes are Lake of Lonar situated in Maharashtra having an average pH 10.5 (Joshi et al. 2008; Kanekar et al. 1999) and Deodani Kyars salt lake in Rajasthan (Upsani and Desai 1990).

The genesis of alkaline lakes is due to volcanism, contributing to a source of sodium carbonate to the surface environment or biological activity and weathering, which produce CO_3^{2-} charged surface waters, thus forming a bicarbonate–carbonate solution that leaches surrounding minerals. In most environments, such ground waters rapidly become saturated with Ca^{2+} and Mg^{2+} , resulting in the precipitation of calcite (CaCO_3), magnesite (MgCO_3) and dolomite ($\text{MgCa}(\text{CO}_3)_2$). Carbonate is removed from the solution and the genesis of alkaline brine is inhibited, as it is the case for most ground waters. However, when the CO_3^{2-} concentration exceeds that of Ca^{2+} and Mg^{2+} , alkalinity develops, usually with Na^+ as the dominant cation (Fig. 4.1).

Bacterial sulphate reduction in surrounding swamps has also been proposed to contribute towards the alkalinity of Wadi Natrum depression and Hungarian soda lakes (Grant et al. 1990). Transient localised alkaline conditions can also be generated due to animal excreta and biological activities such as proteolysis, ammonification, sulphate reduction or photosynthesis (Horikoshi 1991).

Table 4.1 Ecological niches of prevalence of alkaliphiles

Source	Reference	Source	Reference
Natural environments		High-organic-content ecosystems	
Alkaline springs	–	Biogas plants	Horikoshi (1991)
Aqua de Ney, Siskiyou country, California	Nimura et al. (1987)	Bioterriated casein-containing building material	Karlisson et al. (1988)
Black bird valley, Stanislaus country, California	Nimura et al. (1987)	Chicken manure digester	Lowe et al. (1993)
Ground water calcium springs in Oman	Grant et al. (1990)	Compost	Horikoshi (1991)
Hyperthermal springs in Bulgaria	Dimitrov et al. (1997)	Alkaline soil	Gupta et al. (2000)
Yellow Stone National Park	Wang et al. (1995), Li et al. (1994)	Japanese forest soil	Nakamura et al. (1993)
Hvergerdi Island	Hansen and Ahring (1997)	Decomposed manure	Horikoshi (1991)
Hot water spring in Bombay–Thane India	Chauthaiwale and Rao (1994)	Dung	Gordon and Hyde (1982)
Alkaline ground water	Roadcap et al. (2006)	Faeces of man and animals	Horikoshi (1991)
Soda lakes	–	Horn meal	Gordan and Hyde (1982)
Alkaline Soda Lake, Ethiopia	Gessesse (1998)	Indigo dye ball	Aino et al. (2008)
Big Soda Lake, Nevada	Grant et al. (1990)	Lime-treated garden soil	Guffanti et al. (1980)
Hungarian Soda Lakes	Borsodi et al. (2008)	Horn meal	Gordon and Hyde (1982)
Lake Ashanti, Bosumatwi, Ghana, Africa	Grant et al. (1979)	Neutral garden soil	Ghosh et al. (2007)
Lake Bogoria, Kenya	Grant et al. (1979)	Sewage	Boyer and Ingle (1972)
Lake Hannington, Kenya	Grant et al. (1979)	Termite-infested mound soil	Rajaram and Varma (1990)
Lake Magadi, Kenya	Florenzano et al. (1985)	Deep-sea mud of Mariana trench	Georganta et al. (1993), Takami et al. (1997)
Lake WadiNatrun, Kenya	Florenzano et al. (1985)	Sandy soil	Lee et al. (2008)

Table 4.1 (continued)

Source	Reference	Source	Reference
Lake Canyon Diable, Arizona, USA	Horikoshi (1991)	Mangolian soda soil	Sorokin et al. (2008)
Lake Nakuru (Pine Lake)	Grant et al. (1990)	Industrial effluents	–
Lake Quebec, Labrador, Canada, North America	Horikoshi (1991)	Battery-cell-manufacturing units	Horikoshi (1991)
Lonar Lake, Maharashtra, India	Kanekar et al. (1999)	Cement manufacturers and casting	Grant et al. (1990)
Owenn's Lake	Horikoshi (1991)	Detergent factories	Horikoshi (1991)
Soda Lake of Kulunda steppe	Horikoshi (1991)	Electro plating	Grant et al. (1990)
Soda Lake Baikal region	Zhilina et al. (2005)	Lye treatment of animal hide	Grant et al. (1990)
Soda Lake in Taiwan	Nomoto et al. (1988)	Paper-processing factories	Grant et al. (1990)
Tibetan soda lake	Xu et al. (1999)	Potato-/food-processing plants	Gee et al. (1980)
Soda Lake of Tuva	Khmelenina et al. (1997)	Textile mills	Horikoshi (1991)
Southwestern Siberian soda lake	Banciu et al. (2008)	Cassava starch waste water	Blanco et al. (2012)
Alkaline salterns	–	Agrochemical factory	Borkar et al. (2003)
–	–	Other environments	–
Great Salt Lake, Utah	Lowe et al. (1993)	Air contaminants	Gordon and Hyde (1982)
Sambhar Salt Lake, Rajasthan, India	Upsani and Desai (1990)	Acidic soil	Horikoshi (1991)
–	–	Deep-sea water	Gordon and Hyde (1982)
–	–	Gut of higher termites	Thongaram et al. (2005)
–	–	Guts of Japanese horned beetle larvae	Aizawa et al. (2010)

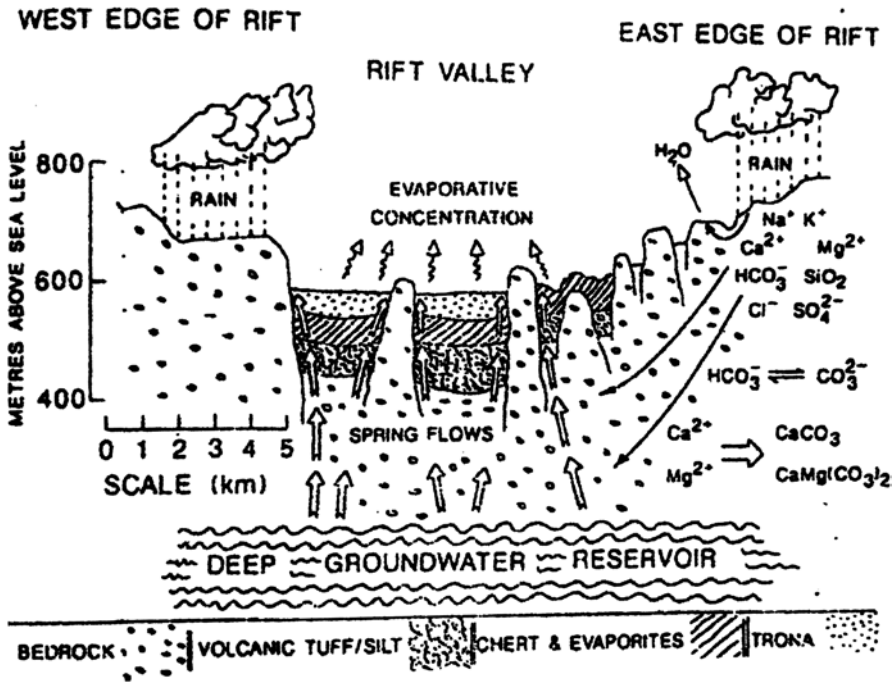


Fig. 4.1 Schematic representation of the possible mechanisms involved in the function of alkaline soda lakes. (Source: Grant et al. 1990)

4.4 Biodiversity and Physiological Groups of Alkaliphiles

4.4.1 Biodiversity

The alkaliphilic microorganisms are known as a diverse group consisting of a heterogeneous collection of eubacteria and archaeobacteria (Yumoto 2007) often adapted to multiple environmental challenges such as high/low temperature, high pressure or high sodium concentrations in addition to high pH. Koki Horikoshi, 'The Explorer of Extremophiles', has isolated a large number of alkaliphiles since 1969 and purified many alkaline enzymes (Horikoshi 1991; Horikoshi and Bull 2011). Extreme alkaliphilic eubacteria are known to be members of Gram-positive group belonging to genera such as *Bacillus* (Aizawa et al. 2010; Blanco et al. 2012; Georganta et al. 1993; Ghosh et al. 2007; Lee et al. 2008; Nogi et al. 2005; Sorokin et al. 2008; Takimura et al. 2007; Yumoto et al. 2003), *Micrococcus* (Kimura and Horikoshi 1990), *Staphylococcus* (Gupta et al. 2000), *Vagococcus* (Joshi and Kanekar 2011), *Arthrobacter* (Horikoshi 1991), *Corynebacterium* (Bahm et al. 1998), *Streptomyces* (Garg et al. 1998), *Actinomyces* (Borgave et al. 2012; Tsujibo et al. 1988),

Clostridium (Zhang et al. 2005; Zhilina et al. 2005), *Exiguobacterium* (Collins et al. 1983), Spirochaetes (Zhilina et al. 1996), magnetotactic bacteria (Lefevre et al. 2011), including cyanobacteria (Florenzano et al. 1985) to Archaeobacteria (Jangir et al. 2012; Khmelenina et al. 1997) with reports also on eukaryotes growing at high pH (Bhushan et al. 1994).

Reports are also available on Gram-negative alkaliphiles belonging to the genera like *Aeromonas* (Krulwich and Gufantii 1989), *Pseudomonas* (Horikoshi 1991; Kanekar et al. 1999), *Vibrio* (Krulwich and Gufantii 1989), *Alkaliflexus* (Zhilina et al. 2004), *Nitrobacter* (Sorokin et al. 1998); *Nitritalea halalkaliphila* (Jangir et al. 2012), photosynthetic *Ectothiorhodospira* (Grant et al. 1979); sulfur-oxidizing bacteria (Banciu et al. 2008), methanotrophic bacteria (Khmelenina et al. 1997) and *Flavobacterium* (Horikoshi 1991).

4.4.2 Physiological Groups

Alkaliphiles consist of two main physiological groups:

4.4.2.1 Alkaliphiles

These organisms require an alkaline pH of 9.0 or more for growth with optimal growth pH of 10.0. Based on pH preference, such alkaliphiles are grouped into two broad categories: Alkali-tolerant organisms that show optimal growth in the pH range of 7.0–9.0 but cannot grow above pH 9.5 and alkaliphilic organisms that show optimal growth between pH 10.0 and 12.0. The extreme alkaliphiles are further subdivided into facultative alkaliphiles, which show optimal growth at pH 10.0 or above but can grow well in neutral pH range and obligate alkaliphiles which show optimal growth above pH 10.0 but do not grow below pH 9.0. They can be isolated on alkaline media with alkalinity generated using sodium carbonate, sodium bicarbonate, sodium hydroxide, trisodium phosphate or sometimes sodium borate. These salts are added in concentration of about 0.5–2.0% depending on the microorganism used, giving a pH of 8.5–11.0 (Gee et al. 1980; Krulwich and Gufantii 1989; Krulwich and Ito 2013; Horikoshi 1991). Although most of the alkaliphiles are aerobic or facultatively anaerobic, there are reports of alkaline tolerant strictly anaerobic strains such as *Clostridium* and *Methanobacterium* (Ferguson et al. 2006; Karlsson et al. 1988). *Clostridium proteolyticum* was isolated from a chicken manure digester and *Clostridium collagenovorans* from a sewage sludge digester (Lowe et al. 1993). *C. bifermentans* and *C. sporogenes* have been isolated from biodeteriorated casein containing building materials having maximal pH tolerance of 12.2 and 11.7, respectively, (Karlsson et al. 1988). Kevbrin et al. (1998) isolated alkaliphilic obligately anaerobic *Tindallia magadi* from deposits of lake Magadi Kenya.

There are alkaliphiles reported to grow at high temperature under aerobic (Kitada et al. 1987) and anaerobic (Hansen and Ahring 1997) conditions. Enzymes from these thermophilic alkaliphiles are both alkalostable and thermostable, used for protein engineering and production of thermostable enzymes.

Anaerobic alkalithermophiles have been isolated from alkaline hot spring in Yellowstone National Park in North America (Li et al. 1994). Interestingly, Yumoto et al. (2004) have isolated several strains of bacteria which can grow at high pH and temperatures as low as 0°C. Alkaliphilic and barophilic bacteria have also been reported from deep-sea sediments collected from depths up to 10,898 m of Mariana trench (Georganta et al. 1993; Horikoshi 1999; Takami et al. 1997).

4.4.2.2 Haloalkaliphiles

These organisms isolated from alkaline and highly saline environments such as alkaline soda lakes of Wadi Natrum in Egypt (Mesbah et al. 2007, 2009), lake Magadi in Kenya (Rees et al. 2004; Vargas et al. 2005), Mongolian soda soil (Sorokin et al. 2008), Hungarian soda lake (Borsodi et al. 2008), Lonar Lake, India (Joshi et al. 2007, 2008; Joshi and Kanekar 2011) and Sambhar lake, India (Upsani and Desai 1990) also require high salinity achieved by addition of 20% NaCl to the isolation medium. *Natronobacterium* and *Natronococcus* are two well-recognized genera of haloalkaliphilic bacteria growing only at pH values above 8.0–9.0 and extremely high salt concentration (Tindall et al. 1980). Xu et al. (1999) isolated two haloalkaliphilic archaea from the soda lake in Tibet belonging to a new genus *Natronorubrum*. Complete genome sequence of the anaerobic, halophilic alkalithermophile *Natranaerobius thermophilus* has been studied by Zhao et al. (2011). A number of anaerobic halophilic eubacteria have been isolated from hypersaline alkaline environments such as Big Soda Lake, Aqua de Ney spring (Nimura et al. 1987). Haloalkaliphilic sulfur-oxidizing bacteria have been reported by Sorokin and Kuenen (2005) and Sorokin et al. (2011).

4.5 Molecular and Biochemical Adaptations in Alkaliphiles

Organisms that grow at extreme pH values are faced with a central problem of pH homeostasis. The mechanisms by which these organisms solve and develop survival strategies have been extensively studied and investigated during the past decades. Yet, complete understanding of any of the specific adaptation in all extreme alkaliphiles needs more attention. Some adaptative and regulatory mechanisms have been elucidated in aerobic, alkaliphilic *Bacillus* sp. and have been characterised as passive and active mechanisms.

4.5.1 *Passive Mechanisms*

Alkaliphilic bacteria are known to change external pH values to a pH suitable for growth, thereby creating their own environment. From an ecological point of view, this phenomenon explains the survival strategies of alkaliphiles in nonalkaline and acidic soils by creating a microcosm favourable for their proliferation.

Alkaliphilic bacteria also show significantly higher external (Bo) and internal cytoplasmic (Bi) buffering capacities under alkaline conditions in comparison to the other nonalkaliphiles (Krulwich et al. 1985, 2011a, 2011b; Ruis and Loren 1998). It is postulated that this property is conferred by high levels of basic proteins or polyamines in alkaliphilic cells (Gilmour et al. 2000). The cell wall components of alkaliphiles also play a key role in protecting the cell from alkaline environments since the protoplasts of alkaliphilic *Bacillus* strains were found to lose their stability in alkaline environments (Ito and Aono 2002). In addition to the peptidoglycan, alkaliphilic *Bacillus* sp. grown at pH 10.0 contain acidic polymers such as galacturonic acid, glucuronic acid, glutamic acid, aspartic acid, phosphoric acid and teichuronopeptides which are more negatively charged than in cells grown at pH 8.0. The negative charges on the acidic nonpeptidoglycan components adsorb sodium and hydronium ions and repulse hydroxide ions assisting cells to grow in alkaline conditions/environments/econiches.

The cytoplasmic membranes of alkaliphiles have a higher concentration of membrane lipid: membrane protein ratio with high cardiolipin content and fatty acid composition consistent with a very fluid membrane (Padan et al. 2005). *Bis* monoacyl-glycero phosphate (BMP), a novel lipid, absent in neutrophiles, is found in alkaliphiles (Horikoshi 1991). Like other *Bacillus* spp., the alkaliphilic bacilli possess a diverse group of branched chain fatty acids in their membrane lipids and also contain variable amounts of saturated and unsaturated straight chain fatty acids. Further, all alkaliphilic bacilli have squalene and dehydro or tetrahydro squalene with substantial quantities of C₄₀ and smaller quantities of C₅₀ isoprenoids in the neutral lipid fraction of cell membrane along with phosphatidyl ethanolamine and phosphatidyl glycerol (Krulwich et al. 1998).

Facultative alkaliphiles, however, are found to contain much lower content of unsaturated fatty acids and branched chain fatty acids than obligate alkaliphiles. Studies indicate that fatty acid composition of obligate alkaliphile membrane is a factor precluding growth at low pH values (Yumoto et al. 2000). Facultative alkaliphiles lack desaturase activity and lose their ability to grow at near neutral pH when supplemented with an unsaturated fatty acid (Dunkley et al. 1991). The proteins secreted into the external environment by alkaliphilic bacteria consist mainly of acidic amino acid moieties (Krulwich 1995). A salient feature of alkaliphiles is that their cytoplasmic membranes contain a high concentration of respiratory chain components such as cytochromes including a-, b- and c-type and are red in colour (Krulwich et al. 1988), believed to be crucial to life at high pH. This high concentration of respiratory chain components may provide the mechanism by which the alkaliphiles maximise productive proton transferring collisions between respiratory chain components and the ATPase (Fujisawa et al. 2010; Hicks et al. 2010; Krulwich et al. 1998, 2011a; Yumoto et al. 2003).

4.5.2 Active Mechanisms

A typical alkaliphile, which grows at an alkaline pH range of 9.0 to 11.0, maintains an internal pH 1–2 units lower than the external pH (Horikoshi 1999; Ito and Aono 2002). This mechanism is found to be present in obligate as well as facultative alkaliphiles but is absent in alkali-tolerant bacteria which cannot grow at pH values above 9.0 (Guffanti et al. 1980). As a result of this, the pH gradient is reversed (acid in) with respect to a chemiosmotic driving force. Yet, the cells of alkaliphiles like other aerobic bacteria and mitochondria, extrude protons during respiration acidifying a narrow region near the membrane. In alkaliphiles, however, primary proton extrusion is followed by a Na^+ dependent proton accumulation resulting in a proton gradient (pH) H^+ in $>$ H^+ out and a Na^+ gradient Na^+ in $<$ Na^+ out. This function is performed by Na^+/H^+ antiporter, which exchanges internal Na^+ for external H^+ , both the ions, transported against their concentration gradients at high pH (Janto et al. 2011; Krulwich 1995; Krulwich et al. 2009, 2011a, 2011b; Krulwich and Ito 2013). Most alkaliphilic bacteria therefore require the presence of Na^+ not only for growth (Pogoryelov et al. 2003) but also for motility, (Fujinami et al. 2009; Ito et al. 2005) and sporulation (Kosono et al. 2000). When alkaliphilic bacilli are either transferred to Na^+ free buffers or subjected to a shift in external pH from near neutral to highly alkaline conditions in the absence of Na^+ , cytoplasmic pH rapidly equilibrates to the alkaline external pH, thereby resulting in a complete failure of pH homeostasis. This alkalinisation of the cytoplasm is immediate in spite of the observation that, the cytoplasmic buffering capacity of the bacilli is relatively high in alkaline range (Krulwich et al. 1985). All alkaliphilic bacilli growing at alkaline pH possess F_1F_0 ATPase localised within the cytoplasmic membrane which helps in the translocation of protons (Ferguson et al. 2006; Hicks et al. 2010; Krulwich et al. 1998, 2011b; Mesbah and Wiegel 2011; McMillan et al. 2007).

A diagrammatic summary of properties of alkaliphilic bacilli relating to their bioenergetics is as represented in Fig. 4.2.

4.6 Industrial Applications

Alkaliphilic microorganisms have made a large impact with their application and manufacture of mass-market consumer products in industries (Nogi et al. 2005; Sarethy et al. 2011). Indigo fermentation is the first industrial application of alkaliphilic bacteria in the world where reduction of indigo from indigo leaves is brought about by alkaliphilic *Bacillus* sp. (Aino et al. 2008; Grant et al. 1990; Horikoshi 1999; Krulwich and Guffanti 1989). The so-called biological detergents contain enzymes obtained from alkaliphilic and alkali-tolerant bacteria. The traditional craft industry of leather tanning uses a series of highly alkaline and hostile processes where the application of alkaline enzymes has brought significant process improvements. Paper and textile processing industries also require enzymes for enzymatic pulping process under alkaline conditions, which help the plant tissues

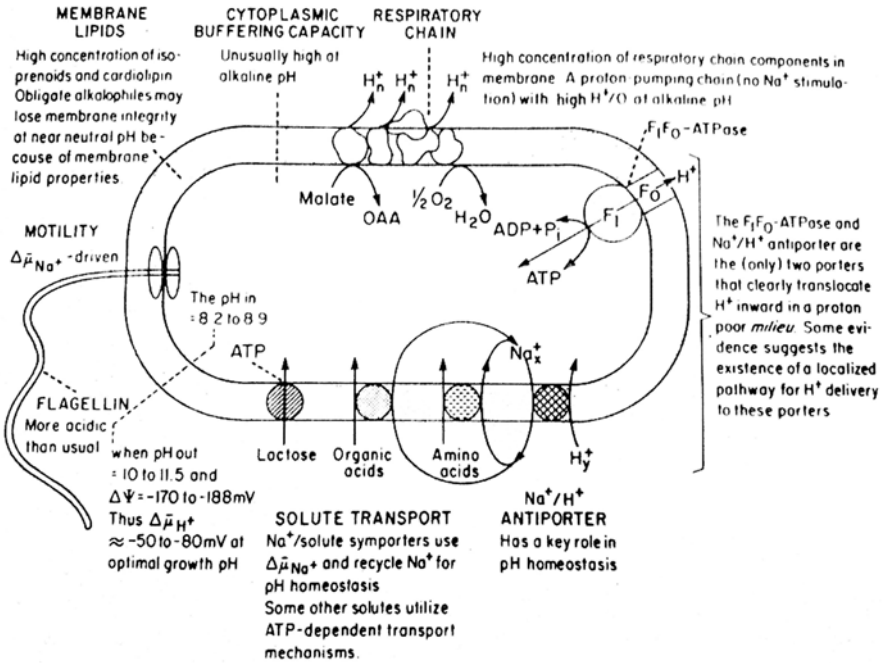


Fig. 4.2 Diagrammatic summary of properties of alkaliphilic bacilli relating to their bioenergetics. (Source: Krulwich et al. 1988)

to swell and facilitate the degradation of pectic substances. Alkaliphiles have also been used for the production of cyclodextrins from starch by the action of cyclomaltodextrin gluconotransferases. These cyclodextrins are used as emulsifying, foaming, stabilizing agents and as molecular capsules to wrap up fragrances or drugs for slow release and hence find wide applications in food, fine chemicals and pharmaceutical industries. Alkaline enzymes have significantly been used in waste treatment (De Graaff et al. 2011), retting process (Yoshihara and Kobayashi 1982), degradation of aromatic compounds (Yumoto et al. 2003) and appear as useful candidates for bioremediation of sites contaminated with toxic chlorinated compounds (Kanekar et al. 1999), since increase in pH can increase the bioavailability of these compounds with the resultant decrease in the toxicity.

The use of microbial enzymes in laundry processes is not a new idea, e.g. Bio-tex[®], a prewash laundry detergent containing an alkaline protease called Alcalase[®] was launched in the early 1960s (Grant et al. 1990). During the 1980s, there was a dramatic revival of detergent enzymes due to remarkable technical innovations and improvements such as the exclusion of sodium tripolyphosphates (STPP) from formulations, resulting from the environmental concerns of phosphate pollution.

Since detergent enzymes account for 30% of the total worldwide enzyme production, the continuous screening of improved alkaline enzymes from alkaliphilic

microorganisms is a good example of a successful commercial exploitation of biology by industry.

The most commonly used enzymes in detergent formulations are alkaline amylases and proteinases with the recent introduction of cellulases and lipases, as discussed below:

4.6.1 *Amylases*

Amylases are one of the most widely exploited enzymes to date in the laboratory and in industry. These enzymes hydrolyse starch, including raw starch and have tremendous potential in different industries like food, detergent, textile, paper, adhesives, fine chemicals, pharmaceutical and fermentation. Although amylases produced by members of the plant and animal kingdom have been traditionally used for various commercial purposes, microbial intracellular or extracellular amylases are preferred for commercial exploitation due to their multifold properties, easy extraction procedure, lower production cost and unlimited supply (Ray and Nanda 1996).

Amylases as detergent additives at high pH remove starch-based stains often working synergistically with proteinases to dissolve protein–starch combinations in food stains. Products include Termanyl[®] (Novo) and Maxamyl[®] (IBIS), which are effective up to 100 °C and exhibit activity up to pH 10.0. Rapidase[®] is used in desizing and softening of denim (Horikoshi 1999). The first alkaline amylase was produced in Horikoshi II medium by cultivating alkaliphilic *Bacillus* sp. A-40-2 (Horikoshi 1971). Many alkaliphilic strains have now been reported to produce alkaline amylases (Shirai et al. 2007).

4.6.2 *Proteases*

These are the most commonly used enzymes in detergent formulations and have the largest market segment obtained from alkaliphilic microorganisms (Grant et al. 1990; Krulwich and Guffanti 1989; Saeki et al. 2007). Serine proteinases produced from alkaliphilic *Bacillus* spp. are endopeptidases with a reactive serine moiety at the active site. Commercial products include Alcalase[®] and Esperase[®] (Novo industries) and Maxatase[®] and Maxacal[®] (International Biosynthetics—IBIS). Maxatase[®] is an alkaline protease active between pH 7.0 and 11.0 with an optimum activity at pH 9.5–10.0, the pH of many detergents. In their encapsulated form, they are added as 0.4–0.6% to detergents for the hydrolysis of proteins and removal of proteinaceous stains such as blood, egg, grass and body secretions. Besides lifting proteinaceous soil, proteinases ensure that coagulated protein is not redeposited on the fabric during the wash, which gives a grey unclear appearance to whites. (Horikoshi 1991).

Alkaline proteases have also been used in the hide dehairing process, where dehairing is carried out at pH values between 8.0 and 10.0 (Horikoshi 1999). An interesting application of alkaline protease was developed by Fujiwara et al. (1991) for the removal of the gelatinous coating of X-ray films for the recovery of silver.

4.6.3 Cellulases

Alkaline cellulases with an optimum pH of 10.0 for activity were first reported by Horikoshi et al. (1984) who found two *Bacillus* sp. strains N4 and 1139 producing extracellular alkaline carboxy methyl cellulases. This discovery led to an industrial application of cellulases as laundry detergent additives. Cellulase 103, introduced by Genecor International (Rochester, NV) and launched as 'Attack[®]' detergent with added cellulases, isolated from alkaliphilic bacterium is the first large-scale commercial application of an extreme molecule.

Cellulases exhibit fabric-softening and colour-brightening properties, besides removing soil (Horikoshi 1991) by removing or opening up the microfibrils that appear on the surface of the cotton fabrics due to wear and repeated washing, thereby restoring the original appearance and smooth fibre structure. Cellulases can also be used in waste treatment, since the undigested material is mainly cellulose (Shikata et al. 1990). Ito (1997) has extensively studied the use of alkaline cellulases in detergents.

4.6.4 Lipases

These enzymes degrade fats into more hydrophilic fatty acids. This property makes them desirable as detergent additives, since hydrophobic stains caused by cosmetics, body fats and oil-based foods are more difficult to remove. Introduction of alkaline lipases as detergent additives is only recent, although their market value is comparable to proteases. Wang et al. (1995) reported purification and characterisation of thermophilic and alkaliphilic tributyrin esterase from *Bacillus* A-30-1, isolated from a hot spring area of Yellow Stone National Park. Lesuisse et al. (1993) have characterised an extremely pH-tolerant lipase while Bhushan et al. (1994) found a lipase produced from an alkaliphilic *Candida* sp. by solid state fermentation.

In addition to these enzymes, there are reports on alkaline xylanases (Aizawa et al. 2010; Gessesse 1998; Gupta et al. 2000), pectinases (Cao et al. 1992), phosphatases (Nomoto et al. 1988), chitinases (Bhushan 2000), cyclomaltoglucanotransferases (Salva et al. 1997) and urease (Singh 1995).

Alkaliphiles also exhibit remarkable versatility in regards to the production of their secondary metabolites such as 2-phenylamine (Hamasaki et al. 1993), carotenes (Aono and Horikoshi 1991), siderophores (McMillan et al. 2010), antibiotics (Tsujibo et al. 1988) and exopolymers (EPs) (Joshi and Kanekar 2011; Kumar et al. 2004).

4.7 Bioprospecting Alkaliphilic Bacteria from Varied Econiches of Goa

4.7.1 Isolation

For the isolation of alkaliphilic bacteria, samples representing neutral, acidic and alkaline econiches were serially diluted in carbonate-bicarbonate buffer (pH 10.0) and plated on alkaline media: (1) polypeptone yeast extract glucose agar (PPYG) (Gee et al. 1980), (2) Horikoshi I and (3) Horikoshi II (Horikoshi 1991). The plates were incubated for 24–48 h at room temperature and colonies were counted to estimate cfu/ml or g.

All the samples were found to yield the presence of alkaliphilic bacteria and the counts were higher on PPYG agar as compared to Horikoshi I and Horikoshi II media (Table 4.2). Interestingly, sample with acidic pH also showed the existence of alkaliphiles with higher counts of 2.06×10^5 cfu/g on PPYG, 1.2×10^3 cfu/g on Horikoshi I and 1.5×10^1 cfu/g on Horikoshi II media.

Predominant isolates (126) from all the samples were purified and characterised. The isolates had varied cultural and morphological features varying from sporulating rods to cocci and pleomorphic forms. A large number of organisms were found to be filamentous with varying degrees of branching. All organisms were found to be Gram-positive except one. The isolates further varied in their tolerance to high pH and temperature. Equal distribution of obligate alkaliphiles (61) and facultative alkaliphiles (65) was observed. It was interesting to note that 39.3% of obligate alkaliphiles and 29.23% of facultative alkaliphiles were also tolerant to high temperature (55 °C). A wide variety of organisms were pigmented with the colour ranging from buff-yellow-orange-pink-red-green.

Table 4.2 Total viable cell counts of Alkaliphiles from acidic, neutral and alkaline econiches

Samples analysed	pH	c. f. u./ml/g		
		PPYG	Horikoshi I	Horikoshi II
Acidic soil	6.00	2.06×10^5	1.2×10^3	1.5×10^1
Garden soil	7.00	7.00×10^8	6.20×10^8	6.50×10^8
Termite mound soil	7.00	2.0×10^4	1.1×10^4	1.56×10^5
Marine water	7.354	1.20×10^7	4.0×10^6	2.0×10^7
Mangrove sediments	8.00	4.50×10^3	3.25×10^2	2.1×10^2
Mangrove water	7.80	1.00×10^2	2.50×10^1	1.0×10^1
Salt pans	8.00	2.57×10^6	1.55×10^4	1.2×10^4
Alkaline soil	8.50	2.50×10^4	1.2×10^2	–
Mining effluent	8.00	1.60×10^8	1.8×10^5	2.0×10^4
Agro chemical factory effluent	10.00	5.30×10^9	1.50×10^5	1.3×10^4

4.7.2 Enzyme Profiles of the Isolates

The enzyme activities of the isolates from PPYG agar (pH10.5) were further determined qualitatively by spot inoculating on media with specific substrate: example, Horikoshi II agar for amylase, skimmed milk agar for protease, carboxy-methylcellulose (CMC) agar for cellulase and tributyrin/tween 80 agar for lipase, all at pH 10.5.

The studies on enzyme production showed the presence of extracellular enzymes by obligate and facultative alkaliphiles at high pH. Presence of cellulase was found in 79.35 % of the total isolates. Amylase production was shown by only 58.72 % and protease by 47.61 %. However, only 29.35 % of the total isolates exhibited lipase activity. Further, 16 % of the isolates showed the production of multiple enzymes as has also been reported by Nihalani and Satyanarayan (1992). The two isolates, a mesophilic obligate alkaliphile (SB-D) and, a thermophilic, facultative alkaliphile (SB-W) produced maximum amylase activity at alkaline pH in comparison with other isolates. Hence, these isolates were selected for further studies.

Enzymes from both the isolates were found to belong to the group of α -amylases. The temperature for optimum enzyme activity was found to be 25 °C for amylase from isolate SB-D, whereas amylase from SB-W showed maximum activity at 55 °C, irrespective of the pH being either 7 or 10.3. Interestingly, the enzyme from isolate SB-W was found to be stable between pH 7.3 and 10.3 and temperature up to 65 °C, while isolate SB-D had more thermolabile enzyme being stable only up to 35 °C and pH between 9.3 and 10.3.

The activity of the enzymes in the presence of organic solvents was also monitored. It was interesting to note that the purified enzyme from isolate SB-W retained almost 80 % activity in butanol, isopropanol and diethyl ether. However, α -amylase from isolate SB-D showed no activity in the presence of organic solvents. Interestingly, both the enzymes obtained from isolates SB-D and SB-W were found to be stable in the presence of detergent additives.

4.7.3 Characterisation of Potential Isolates

Isolates SB-D and SB-W were found to be spore forming, catalase positive, motile and aerobic rods. Scanning electron micrographs of the cells (Figs. 4.3 and 4.4) also revealed a rod shaped morphology. Both the strains showed acid production from glucose, arabinose, xylose and mannitol, hydrolysed starch and casein and were oxidase positive. Based on Bergey's Manual of Systematic Bacteriology (Sneath et al. 1986) and 16 S rRNA sequencing, the organisms were found to belong to genus *Bacillus* with isolate SB-D identified as *Bacillus lehensis* and isolate SB-W as *Bacillus halodurans*.

The effect of sodium ions was determined as per the procedure followed by Koyama and Nosoh (1995). Both the isolates demonstrated the necessity of sodium ions for growth at pH 10.5. Interestingly, the presence of sodium was

Fig. 4.3 SEM of *Bacillus lehnensis* SB-D

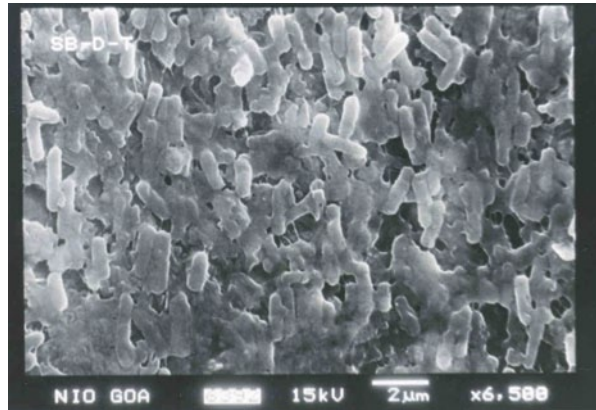
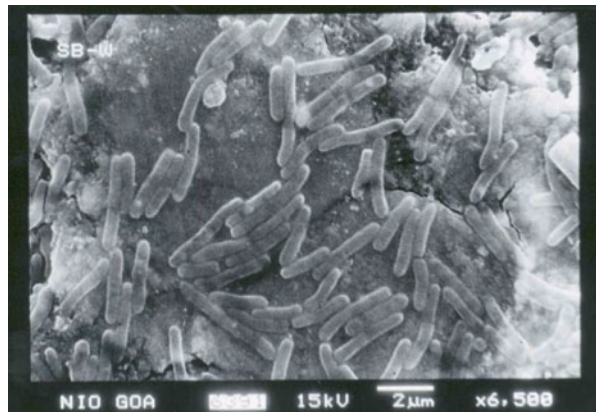


Fig. 4.4 SEM of *Bacillus halodurans* SB-W



also needed for growth of facultative alkaliphile at not only pH 10.5 but also at pH 7.4. The absence of sodium failed to support the growth of both the isolates at alkaline pH.

Determination of intracellular pH as performed by the method of Cook et al. (1996) showed that the intracellular pH of *B. halodurans* SB-W was only 7.7 as compared to the obligate alkaliphile *B. lehnensis* SB-D which exhibited a pH of 8.02 when grown at pH 10.5.

In the present study, the buffering capacities of *B. lehnensis* SB-D and *B. halodurans* SB-W and two neutrophilic isolates were determined (Borkar and Bhosle 2003; Krulwich et al. 1985). It was interesting to note that the alkaliphiles, SB-D and SB-W required 920 and 490 μl of 0.05 M KOH respectively for change of 1 pH unit. In comparison, the neutrophiles *S. aureus* and *B. subtilis*, however, needed only 40 and 85 μl , respectively, for 1 pH unit change to occur. Alkaliphilic cultures, however, were found to lose this property on treatment with Triton X-100 as a drastic reduction in the KOH requirement (30–350 μl) was observed.

Fig. 4.5 Viscosity of culture broth of *Bacillus lehensis* SB-D in Horikoshi II medium



4.7.4 Exopolymer Production by *B. Lehensis* Strain SB-D

During the studies on purification of amylase from *B. lehensis* SB-D, it was found that the viscosity of Horikoshi II medium increased when the culture was grown under shaking conditions for 16 h and kept stationary for 4 h (Fig. 4.5) indicating the production of EP. It was, therefore, envisaged to isolate and characterise the EP using analytical methods.

Preliminary screening for cell-free/cell-bound EP was carried out using alcian blue adsorption assay (Bar-or and Shilo 1987). For isolation, purification and characterisation of EP from viscous culture supernatant, isopropanol was added in the ratio of 1:1 and the precipitate obtained after spooling on a bent glass rod was purified by dialysis and then lyophilised. Emulsification property of the fermentation broth and polymer, metal adsorption and adhesive properties of EP were also studied.

It was interesting to note that the alcian blue was adsorbed on the cells obtained from the medium and viscous supernatant indicating the presence of cell-associated and extracellular EP. Physicochemical analysis of dialysed and lyophilised EP revealed an organic content of 76% with 212 $\mu\text{g}/\text{mg}$ of carbohydrates and 130 $\mu\text{g}/\text{mg}$ of lipids. The compound also contained uronic acids, pentoses, phosphates and pyruvate. The product appears to be a glycolipid complex as the major components are carbohydrates and lipids.

Prior to the addition of isopropanol, 24-h culture broth supernatant (Fig. 4.6) as well as the pelleted cells demonstrated a strong water-in-oil emulsification activity. This property was not evident in the EP removed by the glass rod after the addition of isopropanol as well as the precipitated polymer. The low activity of the polymer but the high activity of the supernatant appears to indicate the requirement of water soluble component for optimum activity. The EP possessed strong metal binding affinity towards copper, as reported for EP (Mc Lean et al. 1990). The most significant feature of EP was that this polymer possessed strong adhesive property, which

Fig. 4.6 Emulsification activity of culture broth of *Bacillus lehensis* SB-D

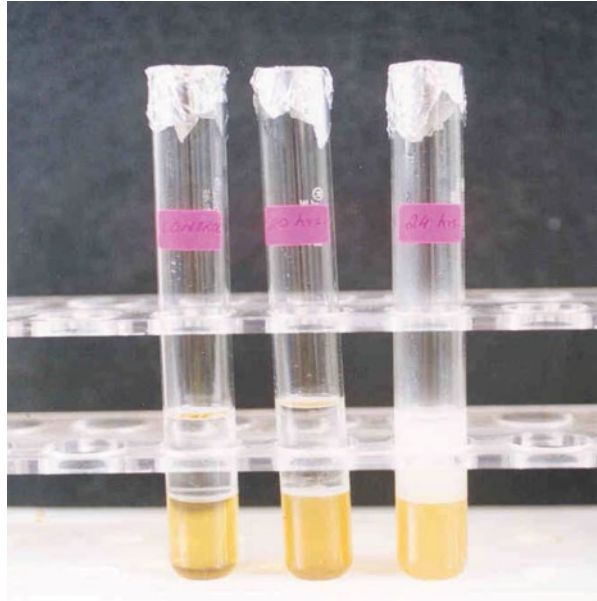


Fig. 4.7 Adhesive property of viscous EP



could be demonstrated by its ability to adhere glass-on-glass as well as glass-on-paper as seen in Fig. 4.7.

4.8 Conclusions and Future Prospects

From the results of the present study, it can be conclusively inferred that the alkaliphiles are a diverse group of organisms present in varied niches. They exhibit an interesting phenomenon of maintaining their internal pH more than two units

less than the external pH and possess high cytoplasmic buffering capacity. In addition, they are a potential source of several enzymes not only active at pH 10.3 but also other harsh conditions such as high temperature, presence of organic solvents and detergent additives, therefore having wide industrial applications. They are also a versatile source of other new metabolites/bioactive compounds. Hence, future research on this group of organisms may be directed towards the role of membrane proteins/components in the mechanism of pH tolerance. Studies on hydrolytic extracellular enzymes such as cellulase, amylase, protease, lipase, xylanase, etc., of industrial application for potential use in organic matter degradation and bioremediation of toxic effluents can be investigated. The production of biopolymer obtained from *B. lehensis* SB-D strain including adhesive and metal scavenging property for industrial exploitations can be undertaken.

Thus, alkaliphiles, by virtue of several unique characteristics, hold immense potential for the production of the enzymes active at alkaline pH with wide applications in various industries. Since alkaliphilic bacterial strains are more tolerant to toxic compounds and have better bioavailability, efficiency of biodegradation and removal of pollutants from waste treatment systems can be undertaken. The five major extreme groups of microorganisms, i.e. *Thermophiles*, *Halophiles*, *Acidophiles*, *Barophiles* and *Alkaliphiles* have been rightly termed by Horikoshi in 1991, as *Super Bugs* on account of their potentiality. Basic research, directed towards isolation of extremophiles producing unique extremozymes and bioactive products need to be continued, strengthened, and pursued with renewed scientific vigour so that their potentials are revealed and exploited.

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