Chapter 1
Review of Literature
1.1. Introduction

In the search of technologies that would expand the usage of renewable resources, scientists and engineers have evaluated replacement of chemical processes with biological ones. The pool of microorganisms used in bio-processing, however, is limited to a few well characterized microbial species. Extremophiles - the organisms living in extremes of temperature, pressure, pH, salinity or some combination of otherwise abiotic conditions offer interesting conversion potential. The ability of these organisms to withstand harsh conditions highlight their importance in industrial biotechnology (reviewed in Podar & Reysenbach, 2006, Turner et al., 2007). Extremophilic microorganisms are of particular interest for environmental biotechnologists as they can serve to degrade toxic components present in the environment under extreme conditions (Maloney et al., 1997; Kalin et al., 2005; Aislabie et al., 2006; Brakstad & Bonaunet, 2006; Takeuchi & Sugio, 2006). A large number of industries including - beverage, pulp and paper and cement industries release wastewater of highly alkaline nature. The potential of extremophilic bacteria growing at such high pH could be exploited for treatment of highly alkaline of wastewater. In addition, these extremophilic organisms also provide experimental opportunities for examination of physiological processes under conditions in which the stress of the extreme environment brings issues of general biological importance into special focus (Krulwich, 1995). The aim of this study was to characterize a novel alkaliphilic bacterium at molecular level in order to gain insight into the basic physiology of alkaliphiles with a goal to develop more robust bioremediation applications using alkaliphilic bacteria.

What are Alkaliphiles?

The term "Alkaliphile" (alcali from Arabic, soda ash, phile, loving) is used for microorganisms that grow optimally or very well at pH values above 9 but cannot grow or grow only slowly at the near-neutral pH value of 6.5 (Horikoshi, 2011). Alkaliphiles, which include prokaryotes, eukaryotes and archaea, can be further classified on the basis of their requirement of high pH for growth or survival (Krulwich & Guffanti, 1989) (Fig 1.1).
a) **Obligate alkaliphiles**

Organisms which are able to grow only in alkaline conditions but fail to grow at neutrality are termed as obligate alkaliphiles.

b) **Facultative alkaliphiles**

Organisms which have optimal growth at pH 10 or above and are also able to grow well at neutrality are termed as facultative alkaliphiles.

c) **Alkalitolerants**

Organisms capable of growing at pH 9 or 10, but with optimum growth rates at around neutrality or less, are referred to as alkalitolerants (Krulwich, 1986).

In the environment, alkaliphiles are often subjected to other stresses in addition to alkaline stress. These include temperature and salinity stress. Accordingly alkaliphiles have been classified as thermophilic (high temperature loving), psychrophilic (low temperature loving), and halophilic (salt loving) alkaliphiles (Pikuta *et al.*, 2007) (Fig. 1.2).

### 1.2. Distribution and diversity of alkaliphiles

Alkaliphilic microorganisms coexist with neutralophilic microorganisms, as well as occupy specific extreme environments in nature. They are distributed in a wide range of...
both man-made and natural alkaline environments. Alkaline environments can be placed into several broad categories depending on the nature of the process generating alkalinity (Fig. 1.3). Generation of alkaline conditions depends on a continuous process, either microbial or chemical, to maintain an alkaline pH and counter the buffering effect of CO₂ which tends to maintain more a neutral or acidic pH (Jones et al., 1998).

Fig 1.2: Intersection of pH stress with (a) Temperature and (b) Salinity stress. (Adapted from Pikuta et al., 2007).
(a) Naturally occurring alkaline environments

(i) Transient alkaline environments

Soil microbial processes such as ammonification and sulfate reduction generate transient and localized alkaline environments. However, these rarely lead to stable pHs in excess of pH 10. Natural geochemical processes such as the weathering of silicate minerals can also lead to alkaline water of pH close to 11 because of Ca(OH)$_2$ (Grant, 1992). These silicate waters have an extremely low buffering capacity, the pH stability being rather limited by the low solubility of Ca(OH)$_2$ and exposure to atmospheric CO$_2$. Carbonate ions are rapidly removed from solution as insoluble calcium or magnesium minerals. Similarly, in volcanic areas, alkaline hot springs have been reported with pH upto 9.5 (Hensel et al., 1997) where alkalinity is probably also generated by decomposition of silicates. Here too, the maintenance of a stable alkaline pH is limited because of exposure to O$_2$ and outgassing of CO$_2$. 

Fig 1.3: Classification of alkaline habitats. The horizontal axis indicates natural or artificial environments and the vertical axis indicates the scale of the habitat. The third axis indicates time of exposure to alkaline conditions. P: permanent alkaline condition, O: occasionally alkaline condition. Adapted from Yumoto et al., 2011.
(ii) Stable alkaline environments

There are three kinds of naturally occurring stable alkaline environments in the world.

1. Soda lakes and soda deserts

These represent the most stable high-pH environments on Earth. Although widely distributed, they are often located in inaccessible continental interiors. Conditions suitable for the formation of soda lakes are found in closed hydric basins with low calcium, high sodium geological composition located in arid, semiarid, tropical or semitropical areas where evaporative concentration rates exceed the water inflow rates such that salts accumulate. The alkalinity is due to a shift in the \( \text{CO}_2 / \text{HCO}_3^- / \text{CO}_3^{2-} \) equilibrium towards a predominance of \( \text{CO}_3^{2-} \) that in the absence of \( \text{Ca}^{+2} \) or \( \text{Mg}^{+2} \) can remain in solution (in the presence of these ions, carbonate will precipitate as \( \text{CaCO}_3 \) or \( \text{MgCO}_3 \)). Therefore, these lakes are characterized by high concentrations of sodium carbonate and also represent saline and sometimes hyper-saline environments due to the concomitant increase in the Cl\(^-\) concentration (Jones et al., 1998).

2. Non saline alkaline environments

Non saline environments are much rarer. Their genesis is dependent on a single geochemical process known as serpentinization. Briefly, this process includes weathering of silicate minerals present in mafic and/or ultramafic rocks by \( \text{CO}_2 \) charged waters. This process is important for the development of the characteristics of a variety of environments such as groundwater associated with kimberlite formation and groundwater and surface water associated with some ophiolites. Serpentinization has been implicated in the formation of a new class of seafloor hydrothermal system composed of variable mixtures of calcite, aragonite and brucite, known as the lost city field, which is considered a potential environment for the emergence of life on the Earth’s ocean floor (Kelley et al., 2001). Remarkably, water associated with ophiolites and serpentinization activity has also been considered to be a habitat analog of Mars (Schulte & Blake, 2003).

3. Gut of Insects

Termites harbor abundant microorganisms in their gut. The first proctodeal segment of the gut in higher termites has a high pH (i.e., pH 10-12) and is rich in K\(^+\). The lifestyle
and food strongly affects the bacterial community in termite gut (Thongaram et al., 2003) with the majority of species belonging to genus *Bacillus* and some to genus *Paenibacillus*. Similarly, the bacterial community in the midgut of gypsy moth which typically has a pH of 8-10 has also been investigated in which several new bacterial phylotypes were identified (Broderick et al., 2004).

(b) Man-made alkaline environments

Human industrial activity by processes such as cement manufacture (Ca(OH)$_2$), mining operations, paper and pulp production (NaOH), alkaline electroplating, leather tanning, indigo fermentation, rayon manufacture, soft drink beverage and herbicide manufacture and food-processing effluents (KOH mediated removal of potato skins), all generate highly alkaline environments, often in excess of pH 11. The first indigo-reducing bacterium had been isolated from indigo fermentation liquor by Takahara and Tanabe (1960). Similarly, alkaliphiles have been isolated from effluent from potato processing plant (Collins et al., 1983), fishery processing plant (Yumoto et al., 2004b) and effluent derived from the preparation of edible olives (Ntougias & Russel, 2000).

1.3. Basic physiological properties of alkaliphiles

1.3.1. Internal pH

Most alkaliphiles have an optimal growth pH at around 10, which is the most significant difference from well-investigated non-alkaliphilic microorganisms (Fig. 1.4). More interesting is the fact that alkaliphiles are able to maintain their internal pH much lower even in highly alkaline medium conditions. This capacity of pH homeostasis has been investigated in several alkaliphilic bacteria using the distribution of weak acid or base across the cell membrane or using fluorescent probes. For example, Guffanti et al. (1978) used $^{14}$C-methylamine to determine intracellular pH values in *B. alcalophilus* by flow dialysis, and found that the cytoplasmic pH remained at 9.0-9.5 over a range of external pH values from 9.0 to 11.5. Similarly, other alkaliphilic organisms such as facultative alkaliphilic *B. halodurans* C-125 and *Bacillus* strain YN-2000 are also able to maintain pH homeostasis as studied using fluorescent probe BCECF (Aono et al., 1997; Horikoshi, 2011). Intracellular pH values have also been estimated from the protein synthesizing systems of alkaliphilic bacteria. Phenylalanyl tRNA synthetase activity at different pH
values indicated no remarkable differences between alkaliphilic *Bacillus halodurans* C-125 and *B. subtilis*. Thus, the pH optima of protein synthesizing machinery also strongly suggests that the internal pH value may be 8-8.5 but not 10 (Horikoshi, 2011).

### 1.3.2. Cytoplasmic buffering

The buffering capacity of the cytoplasm to sequester or release protons upon pH flux is used by alkaliphiles as a pH homeostasis mechanism (Booth, 1985). The buffering capacity of the cytoplasm is due to the presence of titratable groups which include small organic molecules such as amino acids, as well as ionizable groups on proteins and inorganic polymers such as polyphosphate; typical buffering capacities for different species range from 50 to 200 mM protons per pH unit shift (Leone *et al*., 2007, Slonczewski *et al*., 2009). Other buffering molecules present within the cell include phosphoric acid (H₃PO₄) which has a pKa of 7.2, and at near-neutral pH the addition or removal of protons has a negligible effect on the pH of this molecule. On the other hand, proteins, which have a wide range of pKₐ values, can provide buffering in a broad range. Cytoplasmic buffering is also known to be important in pH homeostasis under acidic conditions (Baker-Austin & Dopson, 2007).

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**Fig 1.4: Alkaline pH homeostasis by representative alkalophilic and neutralophilic bacteria.** Reported cytoplasmic pH data at the indicated external pH values are shown for two model neutralophilic bacteria, *B. subtilis* (red square) (Shioi *et al*., 1980) and *Escherichia coli* (green circle) (Padan *et al*., 1981) and for the following alkalophilic bacteria: *B. pseudofirmus* OF4 (■) (Sturr *et al*., 1994); *B. halodurans* C-125 (Navy blue circle) (Ito and Aono, 2002); *B. cohnii* YN-2000 (brown diamond) (Suigyama *et al*., 1986); *B. pseudofirmus* RAB (magenta triangle) (Kitada *et al*., 1982), *B. alcalophilus* ATCC 27647 (sky blue circle) (Hoffmann & Dimroth, 1991). Adapted from Krulwich *et al*., 2011.
1.3.3. Cell shape and size

High pH also affects cell shape and size. A relation between the cell surface aspects, size and growth pH has been found in case of alkaliphilic strain YN-2000 (Yumoto *et al.*, 2000). The cell surface was found to be thicker at pH 10 versus pH 7 which might aid in proton trapping for producing a lower pH region in the vicinity of cell membrane. This phenomenon has also been observed in alkaliphilic strain *Bacillus halodurans* C-125 (Aono *et al.*, 1995). In addition, there are also reports that the cells become longer in alkali medium (Yumoto *et al.*, 2000; Sturr *et al.*, 1994; Aono, 1995). On the contrary, some bacteria are known to reduce their surface area: volume ratio in response to stress (Neumann *et al.*, 2005). This relative reduction of the cell surface–volume ratio has been proposed to be an effective way for the cells to lower the toxic effects of environmental stress factors just by decreasing the attachable/exposed surface in relation to the whole cell volume.

1.3.4. Cell wall characteristics

The cell surface in alkaliophiles is exposed directly to adverse external pH. However, they maintain their internal pH much less than the external alkaline pH (Krulwich, 1995). Moreover, the protoplast of alkaliphilic *Bacillus* species is known to lose stability in alkaline conditions (Aono *et al.*, 1992). It follows that the cell surface might possess some distinct features which help alkaliphiles to survive in alkaline conditions. Certain alkaliphiles, for example, *Bacillus pseudofirmus* OF4 possesses specialized surface layers which are over-expressed at alkaline pH values (Gilmour *et al.*, 2000). Cell wall composition of alkaliphiles is also found to be different from their neutralophilic counterparts. The cell wall in alkaliophiles appears to be rich in acidic polymers such as glucuronic acid, galacturonic acid, glutamic acid, aspartic acid and phosphoric acid (Aono & Horikoshi, 1983). The negative charges on the acidic non-peptidoglycan components may give the cell surface its ability to adsorb sodium and hydronium ions and repulse hydroxide ions and, as a consequence, may assist cells to grow in alkaline environments (Horikoshi, 1999a) (Fig 1.5). The peptidoglycan of alkaliphilic *Bacillus* spp. appears to be similar to that of *B. subtilis*. However, its
composition was characterized by an excess of hexosamines and amino acids in the cell walls compared to that of the neutralophilic *B. subtilis* (Horikoshi, 1999a). Several alkaliphilic bacteria have been found to contain secondary cell wall polymers. *Bacillus halodurans* C-125 contains teichuronic acid and teichuronopeptides (Aono et al., 1999) whereas *Bacillus pseudofirmus* OF4 contains S layer proteins (Gilmour et al., 2000). The S layer is found to be more acidic than the homologues in neutralophilic bacteria.

![Proton trapping nature of the alkaliphile cell wall](image)

**Fig 1.5:** Proton trapping nature of the alkaliphile cell wall.

### 1.3.5. Cell membrane

The alkaliphilic bacteria contain high concentration of squalene and anionic phospholipids, especially of cardiolipin (Clejan *et al*., 1986), as compared to bacteria inhabiting neutral and low saline niches. Cardiolipin acts as proton trap and is known to have essential function in proper alignment of oxidative phosphorylation components (Haines & Dencher, 2002). In addition, the hydrophilic part of the membrane spanning proteins which faces alkaline exterior, is found to be rich in acidic residues in case of alkaliphiles (Krulwich *et al*., 2011) e.g. respiratory cytochrome c complexes (Fig 1.6).
Fig 1.6: An alignment of a region of subunit II of Bacillus cytochrome c(2) complexes that shows that this region in alkaliphiles (species in blue) is significantly more acidic than the same stretches found in neutralophiles. Acidic residues are shown in red and basic residues in blue. The acidic and basic residue composition of each species is shown on the right, along with the predicted isoelectric point (pI) of that region. The numbering refers to the mature form of the B. pseudofirmus OF4 subunit II (Adapted from Krulwich et al., 2011).

1.3.6. Interacting sodium and proton cycles

The cell envelope and cytoplasmic buffering only provides marginal protection against the external alkaline pH. The major mechanism of pH homeostasis is the active uptake of H(+) mediated by Na(+)/H(+) antiporters. Na(+)/H(+) antiporters are membrane transport systems that are energized by the substantial trans-membrane electrical potential across the cytoplasmic membrane. The electric potential is generated by active ion extrusion by primary ion pumps such as the respiratory chain components, light-driven cation pumps, ATPases that extrude cations (as in C. paradoxum), or membrane-embedded exergonic enzymes whose activity is coupled to cation extrusion (von Ballmoos et al., 2009). The bacterial Na(+)/H(+) antiporters that play a role in cytoplasmic H(+) accumulation, relative to the outside milieu, specifically extrude cytoplasmic Na(+) in exchange for H(+) and a greater number of H(+) are taken up than Na(+) extruded, so that the overall exchange is electrogentic, with net positive charge moving inward during each turnover of the antiporter. This makes it possible for the antiporter-mediated H(+) uptake that is coupled to Na(+) efflux to be energized by the negative-inside trans-membrane potential. In neutralophilic bacteria such as E. coli and B. subtilis, both Na(+) (Li(+) + H(+) and K(+)/H(+) antiporters participate significantly in alkaline pH homeostasis, whereas in the alkaliphilic bacteria studied to date, Na(+) (Li(+) + H(+) antiporters have an essential, dominant, and perhaps exclusive role in this central physiological function (Hanhe et al., 2009; Padan et al., 2005; Slonczewski et al., 2009). Na(+) is therefore required for alkaliphile growth and pH homeostasis. The sodium ions moving out of the cell are coupled to the solute symport and rotation of flagellar motor. This completes the sodium cycle. The
protons which move into the cell can again be used for the generation of ATP via the \( F_0 - F_1 \) ATP synthase. So, the interaction of the sodium and proton cycles (Fig 1.7) is centrally important for the pH homeostasis and energy generation in alkaliphiles.

![Diagram](image)

**Fig 1.7: Interacting Na\(^+\) and H\(^+\) cycles in alkaliphiles.**

1.3.7. **Energetic challenge in alkaliphiles**

All aerobically respiring microorganisms generate energy via the movement of protons out of the cell via the respiratory chain to generate an electrochemical gradient (\( \Delta p \)) (Mitchell, 1961). This electrochemical gradient or the proton motive force is made up of two components the electrical gradient (\( \Delta \psi \)), which is + outside, and the concentration gradient or the pH gradient (\( \Delta pH \)), which is acid outside. However, the alkaliphiles are thought to be energy limited because the pH gradient in this case is reversed owing to the external high pH (Fig 1.8 a). But when we compare the growth of alkaliphile growing at high pH to that of neutrophile growing at neutral pH, the rate of growth is comparable. This means that the alkaliphiles have to have an efficient energy generation mechanism to counteract the low pmf.

Several hypotheses have been put forward for explaining the energy coupling (Krulwich, 1995). One theory anticipates that the oxidative phosphorylation by extreme alkaliphiles could be energized using Na\(^+\) as a coupling ion to bypass the low \( \Delta p \). However, both extreme and moderate alkaliphiles studied to date use H\(^+\)-coupled rather than Na\(^+\)-coupled ATP synthases (Krulwich et al., 2007; von Ballmoos et al., 2008). Although Na\(^+\)-coupled \( F_1F_0 \)-ATPases are found in alkaliphilic anaerobes, they do not synthesize ATP with the enzyme under physiological conditions. They use it in the hydrolytic direction that generates an SMF (Ferguson et al., 2006).
Fig 1.8: Energetics in alkaliphiles. (a) Comparison of membrane potentials in alkaliphiles and neutralophiles and (b) Hypotheses for explaining energy coupling in alkaliphiles.

Other proposals that explain the discrepancy between the bulk pmf and ATP synthesis have focused on different ways in which the H⁺ pumped by the respiratory chain may be sequestered from equilibration with the bulk medium (Fig 1.8 b). For example, it has been hypothesized that rapid transfers of pumped H⁺ along the membrane surface allow the H⁺ to reach the ATP synthase before they are equilibrated with the bulk phase outside the cell. The alkaliphile OXPHOS capacity may rely upon close proximity of
critical respiratory chain pumps, especially the terminal oxidases, to the ATP synthase; these partners in H\(^+\) transfers during alkaliphile OXPHOS might be found in clusters (Goto et al., 2005; Liu et al., 2007). The high concentration of membrane cardiolipin has been suggested as a possible mediator of proximity and/or H\(^+\) transfer itself (Haines and Dencher, 2002). There could be lateral movement of protons along the surface of membrane which could be fast enough so that the electron is captured by the ATP synthase before it could be lost to the bulk medium. Or there could be intra-membrane transfer of protons from the respiratory chain to the ATP synthase so that all the protons moving out via the respiratory chain are efficiently used for ATP synthesis. These theories are based on the study of interacting residues in the ATP synthase and various respiratory chain complexes.

Special adaptations of OXPHOS machinery in response to specific environmental challenges are widespread (von Ballmoos et al., 2008). Alkaliphile-specific sequence motifs have been identified in both the F\(_1\)F\(_0\)-ATP synthase (Fig 1.9) and the Cta caa3-type cytochrome oxidase (Ivey & Krulwich, 1992; Quirk et al., 1993). In addition, unusual redox features of alkaliphile respiratory chain components have been described (Goto et al., 2005; Hicks & Krulwich, 1995; Muntyan & Bloch, 2008). ATP synthase motifs have been shown to play important roles in OXPHOS at high pH and low pmf. For example, the “alkaliphile-specific” a-subunit Lys180 plays a critical role in synthesis at high pH and is proposed to be required for H\(^+\) capture from the outside surface of the synthase and passage to the interface between the a-subunit and the c-ring rotor (McMillan et al., 2007; Wang et al., 2004). There are also critical motifs on both helices of the hairpin-like c-subunits that constitute the oligomeric rotor; their mutational change to the consensus sequence for non-alkaliphiles severely compromises ATP synthesis at high pH (Liu et al., 2009; Wang et al., 2004). Another proposed adaptation is the number of c-subunits that comprise the c-ring rotor. The stoichiometry of the c-ring varies among different organisms in which it has been determined from 10–15 (von Ballmoos et al., 2008; von Ballmoos et al., 2009). An especially high c-subunit stoichiometry would help address the problem of OXPHOS at low PMF (Meier et al., 2007; von Ballmoos et al., 2008).
Fig 1.9: Amino acid sequence alignment of ATP synthase c subunits of alkaliphilic (blue) and non-alkaliphilic bacteria. (Adapted from Ivey & Krulwich, 1992).

The cytochrome c which transfers the protons from respiratory chain complex III (bc1) to the complex IV(cytochrome oxidase) is found to have a much lower midpoint redox potential than the neutralophilic bacteria (Goto et al., 2005) (Fig 1.10). Since the midpoint redox potential of cytochrome oxidase remains the same in the two cases, there could be a relatively high capture of energy at this critical step of the terminal part of the respiratory chain.

Fig 1.10: Midpoint redox potential of cytochrome c-553 of alkaliphilic Bacillus cohnii YN-2000. (Adapted from Goto et al., 2005).

1.4. Economic importance of alkaliphiles

Organisms which thrive in extreme alkaline environments have gathered unique adaptations of fundamental biological processes. They not only constitute unique
models for investigations on how biomolecules are stabilized when subjected to extreme conditions, but also, offer a multitude of actual or potential applications in various fields of biotechnology. Some of the applications of products from alkaliphilic bacteria are listed in Table 1.1.

**Table 1.1: Applications of alkaliphilic bacteria.**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Application</th>
<th>Product</th>
<th>Advantage</th>
<th>Organism</th>
<th>Reference</th>
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<tbody>
<tr>
<td>1</td>
<td>Detergent additives</td>
<td>Alkaline protease</td>
<td>Effective at alkaline pH of detergents, higher hydrolyzing activity against insoluble fibrous natural proteins such as elastin and keratin</td>
<td><em>Bacillus</em> spp., AH-101, AB42 and PB12, <em>Bacillus pumilus</em>, <em>Bacillus</em> sp. strain KSM-K16</td>
<td><em>Kobayashi et al.</em>, 1995</td>
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<td><strong>Cellulase</strong></td>
<td>Effective at alkaline pH of detergents. Acts on cellulose in interfibre spaces and helps detergent to remove oily substances like sebum from cotton fabrics</td>
<td><em>Bacillus</em> sp. strains N4 and 1139, KSM-635</td>
<td><em>Fukumori et al.</em>, 1985; <em>Horikoshi et al.</em>, 1984; <em>Ito et al.</em>, 1989</td>
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<td></td>
<td><strong>Lipase</strong></td>
<td>Effective at alkaline pH of detergents. Removes oily substances like sebum from fabric</td>
<td><em>Bacillus</em> sp. strain A30-1</td>
<td><em>Gupta et al.</em>, 2004; Reviewed in <em>Fuciones et al.</em>, 2012</td>
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<td></td>
<td><strong>Amylase</strong></td>
<td>Effective at alkaline pH of detergents</td>
<td><em>Bacillus</em> spp., <em>Halobacterium salinarium</em>, <em>Streptomyces gulbargensis</em>, <em>Rhizobium</em> sp.</td>
<td>Reviewed in <em>Sarethy et al.</em>, 2011</td>
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<td><strong>Pullulanase</strong></td>
<td>De-branching enzyme active at alkaline pH</td>
<td><em>Bacillus</em> sp. S-1 and a <em>Micrococcus</em> sp. Y-1</td>
<td><em>Kim et al.</em>, 1993</td>
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<td>2</td>
<td>Dehairing</td>
<td>Keratinolytic enzymes</td>
<td>Effective at operational pH during dehairing (8.0-10.0). Results in more rapid water absorption and lessened soaking time, reduces environmental pollution by limiting use of sodium sulphide</td>
<td><em>Bacillus licheniformis</em> strain, PWD-1</td>
<td><em>Cheng et al.</em>, 1995; <em>Zaghloul et al.</em>, 1998</td>
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<td>4</td>
<td>Biostoning, of fabrics</td>
<td>Alkaline cellulases</td>
<td>Diminish indigo back staining under higher pH</td>
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<td>Bhat, 2000</td>
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<td>5</td>
<td>Biopolishing of fabrics</td>
<td>Alkaline cellulases</td>
<td>Eliminate the rough cellulose lumps formed on cloth thereby providing and even finish to the fabric as well as a brighter color</td>
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<td>Anish et al., 2007; Pazarlioglu et al., 2005</td>
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<td>6</td>
<td>Deinking of paper</td>
<td>Alkaline Cellulases</td>
<td>Cellulases act on the paper fiber thereby dislodge the ink and facilitate its removal by washing or flotation under alkaline conditions.</td>
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<td>Oksanen et al., 2000</td>
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<td>7</td>
<td>Biobleaching of wood pulp, removal of color from pulp and paper mill effluents</td>
<td>Alkaline xylanases</td>
<td>Thermostable and alkali stable</td>
<td>B. stearothermophilus T-6, Bacillus sp. strain BP-23, Streptomyces thermoviolaceus</td>
<td>Khasin et al., 1993, Blanco et al., 1995, Garg et al., 1996</td>
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<td>8</td>
<td>Plant fiber processing for textile and paper making</td>
<td>Pectinases</td>
<td>Effective at operational pH during fiber processing</td>
<td>Bacillus sp., Strains NT-2, NT-6, NT-33, and NT-82</td>
<td>Yoshihara &amp; Kobayashi, 1982, Cao et al., 1992</td>
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<td>9</td>
<td>To form cyclodextrins from starch</td>
<td>Cyclomaltodextrin Glucanotransferases (CGTases)</td>
<td>Used for degradation, deodorization, masking of taste and solubilization of water insoluble materials in the field of foods, pharmaceuticals, supplements, cosmetics and toiletries</td>
<td>Bacillus sp. strain 38-2, B. macerans</td>
<td>Nakamura &amp; Horikoshi, 1976; Nomoto et al., 1986</td>
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<td>10</td>
<td>Biocontrol agents of agricultural pests</td>
<td>Chitinases</td>
<td>Effective for alkaline soils</td>
<td>Bacillus sp. strain BG-11, Nocardiosis albus subsp. prasina OPC-131</td>
<td>Tsujibo et al., 1992; Bhushan &amp; Hoondal, 1998; Bansode &amp; Bajekal, 2006</td>
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<td>11</td>
<td>Organic synthesis</td>
<td>Lipase</td>
<td>Lipolytic enzymes functional in water-organic solvents mixtures broaden choices for industry since these enzymes can act on even those substrates that are only moderately soluble in water.</td>
<td><em>Bacillus</em> sp. strain A30-1</td>
<td>Gupta <em>et al.</em>, 2004; Reviewed in Fuciños <em>et al.</em>, 2012</td>
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<td></td>
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<td>Alkaline proteases Cause degradation of prion protein (PrPSc)</td>
<td></td>
<td>Hirata <em>et al.</em>, 2010</td>
</tr>
<tr>
<td>13</td>
<td>Microbially enhanced oil recovery</td>
<td>Surfactants , Acid production</td>
<td>Controlled induction of gelation</td>
<td>Strain SL-1A, SL-2A</td>
<td>Bailey <em>et al.</em>, 2000</td>
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<td>14</td>
<td>Indigo Reduction</td>
<td>Whole cell (fermentation)</td>
<td>High operational pH</td>
<td><em>Bacillus</em> sp. No. S-8, <em>Alkalibacterium psychrotolerans</em></td>
<td>Takahara &amp; Tanabe, 1962; Yumoto <em>et al.</em>, 2004a</td>
</tr>
<tr>
<td>15</td>
<td>Metabolites</td>
<td>2- phenyl amine</td>
<td>Used as the initial starting material for synthesis of medicinal products</td>
<td><em>Bacillus</em> sp. strain YN-2000, <em>B. cohnii</em></td>
<td>Hamasaki <em>et al.</em>, 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Siderophore</td>
<td>Isolates 15,37,E1, H, O, W2</td>
<td>Gascoyne <em>et al.</em>, 1991a, b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cholic acid derivatives</td>
<td><em>Bacillus</em> strains</td>
<td>Kimura <em>et al.</em>, 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Food supplement</td>
<td><em>Spirulina platensis</em></td>
<td>Grant &amp; Tindall, 1986</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Organic acid</td>
<td>isobutyric, a-oxoisocaproic and phenylacetic acid</td>
<td>Paavilainen <em>et al.</em>, 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Polyamines: Spermine, Putrescinein, spermidine</td>
<td><em>B. firmus</em> OF-4, <em>B. firmus</em> RAB RA-1, and <em>B. alkalophilus</em></td>
<td>Hamasaki <em>et al.</em>, 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Carotenoids</td>
<td><em>Bacillus</em> sp. strains A-40-2, 2B-2, 8-1, and 57-1</td>
<td>Aono &amp; Horikoshi, 1991</td>
</tr>
</tbody>
</table>
1.5. Application of alkaliphiles in bioremediation of alkaline wastewater

Anthropogenic extreme environments are among the most interesting sites for the bio-
prospection of extremophiles since the selection pressures may favor the presence of
microorganisms of great interest for taxonomical and astrobiological research as well as
for bioremediation technologies and industrial applications (Brito et al., 2012). A large
number of industries including - beverage, pulp and paper and cement industry, release
highly alkaline wastewater. Highly alkaline waters are a multifarious source of
pollution. High pH (>9) itself can be directly toxic to higher aquatic fauna such as
salmonids (Wilkie & Wood, 1996), while rapid rates of calcium carbonate precipitation
smother benthic habitats and reduce light penetration to primary producers (e.g. Effler,
1996; Koryak et al., 2002). In addition, elevated sulphate concentrations and the release
of some anionic/amphoteric metals or metalloids such as As, Cr, Ni and V can be at
levels of potential impact to aquatic biota and compliance with regulatory discharge and
surface water standards. Management options for highly alkaline waters generally fall
within three categories: 1) direct chemical neutralization (e.g. Forbes et al., 1981), 2)
aeration/CO₂ sparging to encourage calcium carbonate precipitation (which consumes
constituents of sample alkalinity: e.g. Schramke, 1992; Roadcap et al., 2005) and 3)
dilute and disperse (e.g. Føllesdal, 2005). The latter is the most commonly adopted
course of action at abandoned industrial sites where legal liabilities for clean-up are
either absent or unclear. The ability of alkaliphiles to tolerate high pH (Kruilwich et al.,
2001) and Na⁺ (Kruilwich et al., 1997) combined with the observation that they can alter
the pH of even a well buffered medium (Makela et al., 1990) makes them an attractive
choice for neutralization of alkaline wastewater. Although alkaliphiles have been
utilized as a source of large number of enzymes (Ito et al., 1998; Horikoshi, 1999b)
used in industries such as detergent, hide dehairing, pulp milling and food processing
and for gelatin decomposition from spent X-ray films (Fujiwara et al., 1987), they have
not been fully exploited for the treatment of industrial wastewaters. Therefore, it will be
worthwhile to look for novel autochthonous alkaliphilic bacteria from the same
ecological niche as the polluted area and to apply them for bioremediation of the
polluted site.
1.6. Global approaches for studying alkaliphily

The 'age of omics' has revolutionized our way of studying microbial physiology by introducing global analysis tools such as comparative genomics and global expression techniques including transcriptomics and two-dimensional protein gel electrophoresis (proteomics). Global approaches provide a valuable integrated picture of responses of genes and proteins to various growth conditions and stresses. Genome mining can not only be used to explore the hidden biosynthetic potential in sequenced bacterial chromosomes, but also to search for novel niche specific markers. Moreover, studies investigating changes in expression patterns in response to various environmental conditions at the level of both the transcriptome and proteome will help us gain a deeper understanding of the physiology of extremophiles, bacterial resistance mechanisms as well as stress response networks.

1.6.1. Genomics

The science of genomics has largely been driven by the desire to understand the organization and function of genomes. The growing body of microbial genome sequences, accumulating rapidly in public databases, has advanced the field of comparative genomics and is leading to new insights into how genomes change over time, how microbes survive in extreme environments and contribute to natural, environmental and biological processes (Fraser-Liggett, 2005). Unraveling these details could potentially lead to the development of novel conversion strategies, the creation of useful antimicrobial compounds, and the design of innovative strategies to modify bacteria for applications such as bioremediation (Binnewies et al., 2006; Sakharkar & Chow, 2008). More recently, there has been a growing interest in microorganisms isolated from various environments. The objective is to exploit available methodologies to identify specific properties that allow these organisms to grow in diverse environments, including extreme ecological niches; to understand the fundamental mechanisms of adaptation to specific environments and to develop practical applications (Podar & Reysenbach, 2006). These studies should also lead to a better understanding of ecosystems themselves. Moreover, they should lead to the identification of novel functions that may be exploitable for biotechnological applications, in particular the bioremediation of contaminated environments.
Alkaliphiles have clearly gained large amounts of genetic information by evolutionary processes and exhibit an ability in their genes to cope with particular environments; therefore their genes are a potentially valuable source of information waiting to be explored and exploited by the biotechnologists. They have been studied primarily to understand biological mechanisms of adaptation to extreme pH conditions and their possible applications in biotechnology. Microbial physiologists have long been interested in alkaliphiles as they employ mechanisms allowing them to maintain life processes at pH where rates of reactions and molecular properties present challenges. However, no single consistent answer has emerged to account for adaptation to high pH extremes.

Recently the complete genomes of a few alkaliphilic species have been sequenced to obtain large scale information for the industrial applications and microbial studies of alkali characteristics. The first complete genomic sequence of alkaliphilic *Bacillus halodurans* C-125 was determined by Takami and colleagues (2000). The genome was compared with *B. subtilis* which is taxonomically related to *B. halodurans* strain C-125, except for the alkaliphilic phenotype. Apart from the gene *tupA*, encoding teichuronopeptide, which contributes to alkaliphily, 10 unique σ factors which belong to the extra-cytoplasmic function family were found suggesting that they may have a role in the special mechanism of adaptation to an alkaline environment (Takami *et al.*, 2000). The genome of *B. halodurans* C-125 has also been analyzed from an industrial point of view (Takami & Horikoshi, 2000), highlighting the hidden potential in genomics of alkaliphilic bacteria.

Genome sequence of *Oceanobacillus iheyensis* HTE831 which is an alkaliphilic and extremely halotolerant *Bacillus* –related species isolated from deep sea sediment was determined next and the candidate genes involved in alkaliphily were determined based on comparative analysis with three *Bacillus* species and two other Gram positive species (Takami *et al.*, 2002). The orthologs identified between only the two alkaliphiles (*O. iheyensis* and *B. halodurans*) included various ABC transporters, transporters associated with C₄-dicarboxylate, organic osmotic solute transport and Na⁺ uptake. ABC transporters coding for branched chain amino acids have been speculated to be responsible for maintaining the cytoplasmic pH around 8-8.5. The branched chain
amino acids such as leucine, isoleucine and valine are believed to be converted to L-glutamate in the presence of 2-oxoglutarate and pyridoxal 5-phosphate by a branched chain amino acid amino-transferase (Schadewaldt et al., 1995; Kagamiyama & Hayashi, 2000). Since L-glutamate should be negatively charged at pH values higher than its $pK_a$ (3.9 or 4.3), the converted L-glutamate and its accompanying proton could contribute to the acidification of the cytoplasm. Similarly 6 genes coding for oligopeptide transporters showed orthologous relationship only with *B. halodurans*. They can contribute to the acidification of the cytoplasm if the acidic amino acids, such as glutamate and aspartate are released by digestion of the incorporated oligo-peptide by a peptidase or are converted to free amino acids from other digest by and an amino-transferase. These acidic amino acids are an important source of protons and a resource for acidic polymers in the cell wall (Takami et al., 2002). *tupA* gene product involved in TUP biosynthesis in *B. halodurans* was also found to be common between the two alkaliphiles (Takami et al., 2000). *Oceanobacillus iheyensis* also possesses two sets of C$_4$-dicarboxylate carriers, and one permease large protein, which are shared only between the alkaliphiles. In aerobic bacteria, dicarboxylate transport carriers catalyze uptake of C$_4$-dicarboxylates by a H$^+$ or Na$^+$ symport (Janausch et al., 2002). This transport system may play a role in regulation of pH homeostasis and the sodium cycle of alkaliphilic bacterial cytoplasm. A putative protein showing significant similarity to voltage gated sodium channel of *B. halodurans* (Ren et al., 2001) was also identified in *O. iheyensis* genome. This channel, which was not detected in any other prokaryotic genome except for the two alkaliphiles, likely provides a transient supply of Na$^+$ for the sodium cycle under both alkaline and neutral pH conditions. Later a voltage gated sodium channel Na$_V$BP was also identified in *Bacillus pseudofirmus* OF4 (Fujinami et al., 2007) which is also an alkaliphile.

Recently the genome of *Bacillus pseudofirmus* OF4 was analyzed (Janto et al., 2011). The genome reveals a more acidic pl profile for proteins exposed on the outer surface than found in neutralophiles. The modest indication of a more acidic character to cytoplasmic proteins from some alkaliphilic vs. neutralophilic *Bacillus* species may be part of a broader set of global adaptations of cytoplasmic proteins that underpins retention of function at cytoplasmic pH > 9. A large array of transporters and regulatory
genes are predicted to protect the alkaliphile from its overlapping stresses. In addition, unanticipated metabolic versatility was observed, which could ensure requisite energy for alkaliphily under diverse conditions. However, certain genes found to be crucial for pH homeostasis in *Bacillus halodurans* C-125 were not observed in *Bacillus pseudofirmus* OF4. These include genes for synthesis of acidic cell wall associated teichuronic acids- tuaG and tuaA gene.

Availability of complete genome sequences provides the opportunity to search all of the proteins of the organisms for similarities and differences that might have bearing on the ability of the organism to grow at high pH. However, no single consistent mechanism has emerged to account for adaptation to high pH extremes. Moreover, the alkaliphilic genome sequences currently available are dominated by alkaliphilic *Bacillus* or *Bacillus* related species. Thus, our genomic understanding of alkaliphily relies mainly on the studies of these few alkaliphilic members of genus *Bacillus*. It will be interesting to figure out whether the principles of alkaliphily deduced in these species are extendable to other genera or not. This could be answered by taking up whole genome sequencing projects of non-*Bacillus* alkaliphilic species.

### 1.6.2. Transcriptomics

With the progress in sequencing technologies, the number of microbial genomes deposited in databanks has grown in an accelerated manner. As more genomes are added, it becomes easier to elucidate relevant biological processes. Since sequencing on its own does not give us information about these processes, the next step is investigation based on post-genomics processes, such as transcriptomics. A transcriptome is a collection of all the transcripts (RNAs) present in a given cell, evaluated qualitatively and quantitatively at a particular moment of cell development or during a specific physiological condition (Wang *et al.*, 2009).

During the last decades, the techniques for evaluating gene expression have advanced greatly in the volume of data obtained, which has progressed from one or a few genes, analyzed for example, through Northern blotting, quantitative real-time polymerase chain reaction (RT-PCR), and nuclease protection assay, to analyzing a large number of genes, through subtractive hybridization, differential display, serial analysis of gene
expression (SAGE), and microarray (Moody, 2001). However, we are approaching the technical limits of microarray technology; it is being substituted by transcriptomics, using new generation sequencing (NGS) of RNA on an “ultra-large-scale”.

The differential analysis in various growth conditions of the whole protein content (‘proteome’) or transcripts (‘transcriptome’) allows the simultaneous quantification of gene expression or the accumulation of the corresponding products in an organism (Kahn, 1995). The mechanisms of stress tolerance in various extreme conditions such as high temperature and low pH have been studied. However the so called “alkaline stress response” is still not fully understood. Response to alkaline pH in mesophilic organisms has been studied via genome wide approaches. For example, in *E.coli*, microarray studies show that among the genes repressed at the alkaline pH are genes encoding two respiratory chain complexes that pump protons outward during electron transport, cyo and nuo, as well as flagellar and chemotaxis genes (Maurer et al., 2005). Induced genes include those encoding the F$_1$F$_0$-ATP synthase that imports protons during ATP synthesis, and the alternate cyd-encoded terminal oxidase, that generates a PMF without outward proton pumping. These observations indicate that alkali challenge, under growth conditions, changes the gene expression profile: robust oxidative phosphorylation together with the ATP synthase maximize proton retention and proton capture by the cell. This elegant study also shows that pH differentially regulates a large number of periplasmic and envelope proteins.

Microarray studies have also been conducted on mesophilic *Bacillus subtilis* following a shift to pH 8.9 (Weigert et al., 2001). A group of the upregulated genes is under the control of σ$^w$, one of the extracytoplasmic function family of σ factors (ECF) that commonly regulate groups of genes in response to extracytoplasmic stimuli (Helmann, 2002). It has been proposed that the induction of the σ$^w$ regulon reflects an intersection of alkali-stress with cell wall stress and Na$^+$-stress (Cao et al., 2002). Additional genes that are up-regulated after the alkaline shock include *Bsmrp* (Weigert et al., 2001) genes that encode the Mrp Na$^+$/(K$^+$)/H$^+$ antiporter (Ito et al., 1999; Ito et al., 2000; Kosono et al., 1999). Another transporter gene locus that is upregulated in the alkaline shock experiment is the YhaSTU locus that catalyzes K$^+$ efflux in *B. subtilis* and is regulated in a complex way by alkaline pH and Na$^+$ stress (Fujisawa et al., 2004). YhaSTU may
extrude NH$_4^+$ (in addition to K$^+$), as does in its alkaliphile homologue AmhT (Wei et al., 2003). This could contribute to acidification of the medium.

Alkali tolerance response in *Listeria monocytogenes* has been studied by both transcriptomics and proteomics approach (Giotis et al., 2008). Adaptive intracellular gene expression involved genes that are associated with virulence, the general stress response, cell division, and changes in cell wall structure and included many genes with unknown functions. Interestingly, several alkali induced genes/proteins can provide a cross protective overlap to other types of stresses. Transcriptome patterns of alkali and non-alkali-stressed *L. monocytogenes* 10403S cells has also been studied to elucidate the mechanisms by which *Listeria* adapts and/or grows during short- or long-term alkali stress (Giotis et al., 2010). Rapid (15 min) changes in expression included upregulation of genes encoding for multiple metabolic pathways (including those associated with Na$^+$/H$^+$ antiporters), ATP-binding cassette transporters of functional compatible solutes, motility, and virulence-associated genes as well as the σ(B) controlled stress resistance network. Slower (30 min and more) responses to alkali shock and adaptation during growth in alkaline conditions involved a different pattern of changes in mRNA concentrations, and genes involved in proton export.

The molecular response of *Shewanella oneidensis* MR-1 to variations in extracellular pH was investigated based on genome wide gene expression profiling (Leaphart et al., 2006). Microarray analysis revealed that cells elicited both general and specific transcriptome responses when challenged with environmental acid (pH 4) or base (pH 10) conditions over a 60-min period. Global responses included the differential expression of genes functionally linked to amino acid metabolism, transcriptional regulation and signal transduction, transport, cell membrane structure, and oxidative stress protection, whereas the molecular response to alkaline pH was characterized by upregulation of *nhaA* and *nhaR*, which are predicted to encode an Na$^+$/H$^+$ antiporter and transcriptional activator, respectively, as well as sulfate transport and sulfur metabolism genes. Collectively, these results suggest that *S. oneidensis* modulates multiple transporters, cell envelope components, and pathways of amino acid consumption and central intermediary metabolism as part of its transcriptome response to changing external pH conditions.
Thus alkaline shock response and alkali tolerance response, as studied via transcriptomics in model neutralophilic organisms, includes both global and specific responses. Some of these responses are overlapping stress responses but majority of modulated genes are different in different organisms. Moreover, it is not clear whether the alkaliphilic bacteria behave in a similar manner as their neutralophilic counterparts in terms of their transcriptome profile in alkaline vs non-alkaline conditions or they have specialized pathways for coping with alkaline stress. The availability of differential transcriptome data for facultative alkaliphilic species which are able to withstand both alkaline and non-alkaline conditions will shed light on the response of these alkaliphilic microorganisms to high pH and their adaptive strategies.

1.6.3. Proteomics

Proteomics provides direct information of the dynamic protein expression in whole cells, giving us a global picture. Complementing proteomic technologies enable an unbiased view of cellular adaptation and thus may provide a new understanding of cellular physiology, particularly for microorganisms because a major fraction of their proteome is accessible to currently available technology. One important aspect of proteomics is to characterize proteins differentially expressed by cells imposed to different environmental conditions. Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) in combination with mass spectrometry is currently the most widely used technology for comparative bacterial proteomics analysis (Gygi et al., 2000).

Two-dimensional gel electrophoresis of the proteome of bacteria grown at various pH values identifies changes in the level of individual proteins in response to a change in external pH. The proteins are identified by mass spectrometry. Findings from such screens are well represented by the study of Stancik et al. (Weigert et al., 2001) in E. coli. Under aerobic conditions (in LB medium, which contains tryptone, yeast extract and NaCl), the abundance of a variety of proteins is increased by high pH, including the periplasmic protein i.e. YceI, MalE and OmpA porins and the virulence-associated OmpX porin (Weigert et al., 2001). These changes presumably lead to metabolic patterns that are adaptive. For example, MalE would increase availability of an acid-generating carbohydrate. Several enzymes of amino acid catabolism are also increased.
in abundance at alkaline pH, including TnaA (tryptophan deaminase) and TnaB (the tryptophan transporter) as well as serine deaminase (SdaA). Deaminases provide an acid-generating mechanism that is adaptive to alkaline challenge just as decarboxylases promote alkalinization that is adaptive for an acid challenge (Blankenhorn et al., 1999; Richard et al., 2004), as predicted by early work by Gale and colleagues (1942). Increased production of TnaA has also been implicated in a specific and remarkable defense against alkali when anaerobic *E. coli* uses TMAO as its terminal electron acceptor. Use of TMAO generates an alkaline reduction product and in apparent “anticipation” of that alkaline challenge, the Tor phospho-relay system that detects TMAO increases expression of tnaA. This results in a functional TnaA- and tryptophan-dependent defense against alkali (Bordi et al., 2003). It has not yet been shown whether deaminase activities lower cytoplasmic pH, external pH or both. Overall, the proteomic data are consistent with a model in which *E. coli* modulates multiple metabolic pathways of amino acid consumption so as to minimize the effect of an increase in the external pH (Blankenhorn et al., 1999). A mechanism based on a change of external pH itself can only be realistic when the culture volume is small and/or the fluid layer surrounding the cells is unstirred so that its composition is not in equilibrium with that of the bulk external fluid. In general, the internal volume of cells is much smaller than the external volume. Hence, modest changes in intracellular acid or base production and/or in the protons pumped out of or taken up by the cytoplasm can have a significant impact on maintenance of a constant intracellular pH.

The proteome of a *B. subtilis* mutant disrupted in Bs-mrpA (a Na⁺(K⁺)/H⁺ antiporter) and grown at high pH shows elevated levels of salt stress proteins as well as of the ATP synthase (Kosono et al., 2004). The latter is consistent with the alkali adaptive value of ATP synthase inferred in *E. coli* (Maurer et al., 2005). By contrast, ATP synthase genes are downregulated during acid-adaptation of *H. pylori* (Wen et al., 2003) and in fermentative bacteria, the H⁺-pumping, hydrolytic mode of the ATPase is a critical piece of acid-adaptation (Belli et al., 1991; Kobayashi et al., 1986; Kuhnert et al., 2004).

Response of cytoplasmic and membrane proteome of *Corynebacterium glutamicum* to pH changes has been studied by classical 2D electrophoresis and by anion exchange
chromatography followed by SDS PAGE (Barriuso-Iglesias et al., 2008). The only cytoplasmic protein that increases clearly in response to alkaline pH was identified as a catalase (KatA) whereas the membrane proteins found to be upregulated at alkaline pH included the three subunits of succinate dehydrogenase complex, subunits b, δ and α of the F$_0$F$_1$-ATP synthase complex, a hypothetical secreted/membrane protein and the α subunit of the nitrate reductase II. In addition, 3 membrane protein spots with poorly significant abundance changes were identified as β subunit of F$_0$F$_1$ ATP synthase, glutamate binding protein and a polyketide synthase involved in secondary metabolism.

The majority of proteins in alkaliphilic Bacillus pseudofirmus OF4 grown at pH 7.5 and 10.5, as studied by two-dimensional gel electrophoresis analyses, did not exhibit significant pH-dependent variation (Gilmour et al., 2000). A new surface layer protein (SlpA) was identified in these studies. SlpA was similar in size to homologues from non alkaliphiles but contained fewer Arg and Lys residues. An slpA mutant strain (RG21) lacked an exterior S-layer showed a diminished capacity for Na$^+$-dependent pH homeostasis upon a sudden upward shift of external pH from 8.5 to 10.5. The energy cost of retaining the SlpA layer at near-neutral pH is apparently adverse, but the constitutive presence of SlpA enhances the capacity of the extremophile to adjust to high pH.

Thus, the proteomic studies of neutralophilic or moderately alkaliphilic species has revealed up-regulation of proteins involved in metabolism and energy generation. However, the most widely studied alkaliphilic species i.e. Bacillus pseudofirmus OF4 did not show any significant differences in the proteomic profile in alkaline vs non-alkaline conditions except for surface layer protein having a role in sudden upward shift in pH. This might be due to experimental/technology limitations at that time. Therefore, there is a need for more extensive proteomic studies in alkaliphilic bacteria for deciphering the global alkali adaptation strategies in these bacteria.

The aim of this study was to characterize a novel alkaliphilic bacterium at molecular level in order to gain insight into the basic physiology of alkaliphiles with a goal to
develop more robust bioremediation applications using alkaliphilic bacteria. The availability of the whole genome sequence as well as the global transcriptome and proteomic response to high vs neutral pH is expected to provide an integrated picture of the alkali adaptation in this organism and will increase the overall understanding of alkaliphily in bacteria. This will also aid the identification of novel functions that may be exploitable for biotechnological applications, in particular the bioremediation of contaminated environments.