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Alkaliphiles in Biotechnology

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Alkaliphiles in Biotechnology

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Preface

The notion that on earth there is life where there is liquid water seems holding true. The biosphere has many extreme environments which were once thought free of life. However, studies indicated that even these extreme sites that are bizarre to humans are found inhabited by richly diverse life forms known as extremophiles. Alkaliphiles are a class of extremophiles that thrive in alkaline habitats. To thrive in the extremely alkaline habitats, alkaliphiles evolved unique structural and functional adaptive features. These features are of great importance to biotechnology. High pH conditions are part of several industrial and environmental processes. Thus, alkaliphiles and their products have got several applications such as in detergent, paper and pulp, textile, and leather industries. These applications and human curiosity of understanding nature led to isolation and studies of a wide range of alkaliphiles from various alkaline environments. In recent years, these studies are contributing to expand the application horizon of alkaliphiles to new territories and technologies. This volume on "Alkaliphiles in Biotechnology" was thus intended to bring attention to these fascinating extremophiles and highlight their immense biotechnological potential.

Alkaline habitats are widely distributed in the biosphere. However, the genesis and actual chemical composition of these environments are quite different, a factor which influences the diversity of the community inhabiting the alkaline environments. This variation also affects the success of isolating alkaliphiles from different habitats. Moreover, like other extremophiles, isolation of alkaliphiles is not efficient and only a very tiny fraction of alkaliphiles community are culturable. However, there is some progress made on the isolation and cultivation of this group of organisms. These advances in isolation and cultivation of alkaliphiles are included in this volume.

The emergence of molecular techniques such as cloning and heterologous expression, metagenomics, system biology, metabolic engineering, and systems metabolic engineering which integrates genetic engineering, synthetic biology, and systems biology approaches has started to contribute to the advancement of biotechnology.

To utilize alkaliphiles efficiently, it is necessary to implement these molecular techniques and hence, it is desirable to understand their genetic makeup. Thus, there have been studies on the genomes of alkaliphiles for which a chapter is dedicated in this volume.

It is not only the applications of alkaliphiles that are enthralling, but the mechanism of how these organisms adapted to the extreme pH habitats which are often lethal to other life forms is also intriguing. There are some progresses that depict part of the alkaline adaptations. In this volume, some of the most important adaptation mechanisms are highlighted and new insights which might stimulate further studies are included.

At least some of the unique products and physiologies of alkaliphiles which are of great importance to biotechnology emanate from the high pH adaptation mechanism. For instance, alkaliphiles produce organic acids that alleviate the high pH stress, an ability that can be tapped for commercial production of organic acids. Similarly, alkaliphiles adaptation resulted in novel siderophores, exopolysaccharides, carotenoids, biosurfactants, and many other metabolites with immense application potential. One of the most important and well-studied alkaliphiles adaptations, which attracted a great deal of interest, is their alkaline-active and -stable enzymes. These unique enzymes are suitable for high pH biocatalysis and are implemented in several processes. Among alkaline active enzymes, those enzymes which modify starch, hemicellulose, and protein are relatively the most studied and account for the greater portion of the current alkaline active enzymes' applications. The real and potential applications of these extraordinary extracellular enzymes of alkaliphiles are covered in the volume.

In addition to their products, alkaliphiles whole cells have got applications such as in food and feed supplementation, waste treatment, biofuel, and other chemical production. Another newly emerging application of alkaliphiles is in the construction industry where alkaliphiles are winning growing attention to treat concrete. Alkaliphiles have been tried to make concrete surface coatings, repair concrete cracks, and engineer self-healing concretes. Moreover, it can also serve in many other related applications such as in bioclogging, bioaggregation, and as concrete admixture. This book highlights the major advances of alkaliphiles applications in the construction industry.

Although alkaliphiles are one of the oldest organisms, it is among the newcomers into the circle of biotechnology. Hence, an active research is ongoing and some of these studies trickle in new fascinating applications and discoveries. The emergence of new techniques, the desire for new products, the growing demand for organic products and biotechnological processes, and the proven versatility of alkaliphiles in serving different sectors of biotechnology may indicate the possible expansion of alkaliphiles biotechnological utilization in years to come.

As editors of this volume, we have acquired a lot from the chapters written by scientists who are specialists in the field and hence, we would like to extend our heartfelt gratitude to all the authors for their outstanding contribution and efforts for

making this book an interesting, important source of information on biotechnology of alkaliphiles. In the meantime, we would also like to take the opportunity to thank the series editor of *Advances in Biochemical Engineering/Biotechnology* for believing in the significance of alkaliphiles in biotechnology and the inspiration to edit this volume.

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Alkaliphiles: The Versatile Tools in Biotechnology

Gashaw Mamo and Bo Mattiasson

Contents

Abstract The extreme environments within the biosphere are inhabited by organisms known as extremophiles. Lately, these organisms are attracting a great deal of interest from researchers and industrialists. The motive behind this attraction is mainly related to the desire for new and efficient products of biotechnological

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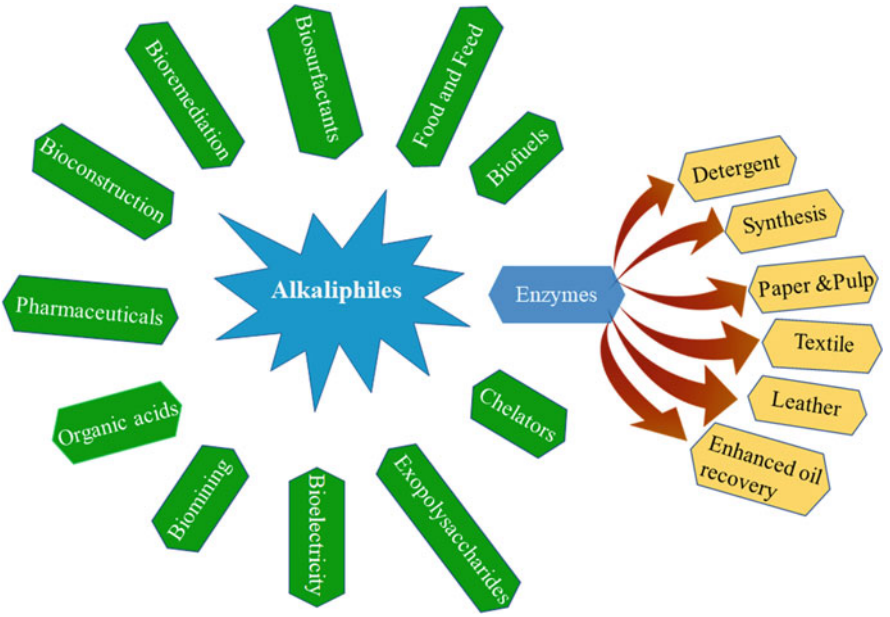
importance and human curiosity of understanding nature. Organisms living in common "human-friendly" environments have served humanity for a very long time, and this has led to exhaustion of the low-hanging "fruits," a phenomenon witnessed by the diminishing rate of new discoveries. For example, acquiring novel products such as drugs from the traditional sources has become difficult and expensive. Such challenges together with the basic research interest have brought the exploration of previously neglected or unknown groups of organisms. Extremophiles are among these groups which have been brought to focus and garnering a growing importance in biotechnology. In the last few decades, numerous extremophiles and their products have got their ways into industrial, agricultural, environmental, pharmaceutical, and other biotechnological applications.

Alkaliphiles, organisms which thrive optimally at or above pH 9, are one of the most important classes of extremophiles. To flourish in their extreme habitats, alkaliphiles evolved impressive structural and functional adaptations. The high pH adaptation gave unique biocatalysts that are operationally stable at elevated pH and several other novel products with immense biotechnological application potential. Advances in the cultivation techniques, success in gene cloning and expression, metabolic engineering, metagenomics, and other related techniques are significantly contributing to expand the application horizon of these remarkable organisms of the 'bizarre' world. Studies have shown the enormous potential of alkaliphiles in numerous biotechnological applications. Although it seems just the beginning, some fantastic strides are already made in tapping this potential. This work tries to review some of the prominent applications of alkaliphiles by focusing such as on their enzymes, metabolites, exopolysaccharides, and biosurfactants. Moreover, the chapter strives to assesses the whole-cell applications of alkaliphiles including in biomining, food and feed supplementation, bioconstruction, microbial fuel cell, biofuel production, and bioremediation.

Graphical Abstract



Keywords Antibiotics, Biocatalysis, Bioconstruction, Biofuel, Biomining, Bioremediation, Biosurfactants, Carotenoids, Cyanobacteria, Enzymes, Extremolytes, Extremophiles, Extremozymes, High pH adaptation, Secondary metabolites, Siderophores

Abbreviations

AFPs	Antifreeze proteins
ATP	Adenosine triphosphate
CD	Cyclodextrins
CGTase	Cyclo-maltodextrin glucanotransferase
CMC	Critical micelle concentration
DP	Degree of polymerization
ECF	Elemental chlorine free
EDTA	Ethylenediaminetetraacetic acid
EPS	Exopolysaccharide
GWh	Gigawatt hours
IFT	Interfacial tension
MEOR	Microbial enhanced oil recovery
MFC	Microbial fuel cell
MPa	Megapascal
mW	Milliwatt
NADH	Reduced nicotinamide adenine dinucleotide

NADPH	Reduced nicotinamide adenine dinucleotide phosphate
PCR	Polymerase chain reaction
PGP	Plant growth promoting
PGPR	Plant growth-promoting rhizobacteria
TCF	Total chlorine free
Wa	Water activity
Wh	Watt-hour

1 Introduction

Biotechnology is one of the most important disciplines of the modern era which is advancing at breathtaking pace. Being an important field of science, it is revolutionizing human life in unprecedented ways and magnitude and plays numerous roles in our daily lives. It is involved in different processes ranging from the food we eat to cloths we wear and drugs we use to treat ailments. This technology is expanding and integrating to many other disciplines which resulted in modification or alteration of existing chemical processes and engraving its own new applications. The growing environmental concern, the consumer inclination to organic products, and emergence of new techniques partly contributed to its expansion. Indeed, agricultural, medicinal, industrial, and environmental fields have become increasingly intertwined in one or another way to biotechnology. This trend is clearly marked by the pleasant assimilation of biotechnology to the global economy which, in recent years, is growing by leaps and bounds. The global market value of biotechnology was estimated to be just around USD 216 billion in the year 2011, which grew to about USD 369 billion in 2016 [1] and projected to reach around USD 730 billion by the year 2025 [2]. Considering the amount of fund flowing to biotechnology research, products which are in pipeline, and the growing demand for organic products and biotechnology processes, one expects a huge market growth in the years to come and beyond.

Microorganisms are the formidable pillars which uphold the vital tasks of biotechnology and ensure its success. These organisms are the crucial sources for several products including enzymes, pharmaceutical compounds, industrial chemicals, agricultural inputs, materials, biofuels, etc. In addition to their products, whole microbial cells have been used to process food and feed, to manage residential and industrial waste, to fertilize soil, to promote health (probiotics), etc. The great majority of the microbes used in these applications have been those that thrive under "normal" conditions, normal from anthropocentric perspective. These organisms have been in focus for decades. But, the need for better and novel products, emergence of new applications, and human curiosity have extended the search for biotechnologically important organisms in previously unexplored habitats. One of these habitats that attracted a great deal of attention is "extreme" environments.

In the last few decades, exploration studies made on extreme environments such as those with high or low temperature or pH emerged with big surprises [3–7]. These

environments which once have been considered too extreme to host life are found inhabited by richly diverse group of organisms. The discovery of these organisms dwelling in extreme environments not only heralds a new dawn in biotechnology but also brought a substantial change in human perception regarding the limits of habitable environments. Although the limits of extreme conditions at which organisms can survive and thrive are not exactly known, the results of these studies support the notion that no matter what the condition may be, there could be life at least where there is liquid water. Indeed, this contributed to the dramatic expansion of human knowledge on adaptability of life, which flourishes from environments of frozen water of around −20°C [8] to hydrothermal vents which are above 120°C [9], and in extreme pH values as low as pH 0 [6] and as high as pH 13.5 [4], and in many other extreme conditions such as high salinity, high level of radiation, and high pressure [10]. The organisms adapted to these extreme environments are often referred to as extremophiles and are known to have immense biotechnological application potentials.

2 Extremophiles

Extremophiles are organisms evolved to exist in a variety of extreme environments which could be natural, manmade, temporary, or permanent. Extreme environments are ubiquitous and differ according to their characterizing condition(s) such as acidic, alkaline, cold, or saline. The organisms adapted to these habitats also vary as the nature of the extreme condition. Thus, the categorization of these organisms in one group, extremophiles, is just for simplicity but does not reflect any biological commonality. As shown in Table 1, extremophiles are named according to the nature of their habitats or conditions that they are adapted to and fall into several classes.

Taxonomical studies on organisms dwelling in extreme habitats revealed that extremophiles are diverse, and the great majority belongs to the domains of Archaea and Eubacteria [11–14]. However, a variety of unicellular and multicellular eukaryotic organisms have also been reported [15–17].

Extremophiles evolved ingenious structural and functional adaptations which allow them not only to survive but also thrive in their specific extreme environments. These adaptations are of great importance in biotechnology and can be harnessed for various applications (Fig. 1).

The use of enzymes in industrial processes has many advantages including shorter processing time, low energy consumption, process cost minimization, safe to use, high selectivity, and environmentally friendly [18]. However, most industrial processes involve harsh conditions which are tough for most enzymes to cope with. Thus, there has always been the interest for robust enzymes that are amenable to industrial conditions. Extremophiles are proven sources of fascinating enzymes (which are also known as extremozymes) that function under extreme conditions in which biocatalysts from their mesophilic counterparts could neither be active nor stable. Due to their desirable properties, several extremozymes have made their ways into various industrial processes (Table 2).

Table 1 Classes of extremophiles with their distinguishing characters and examples

Class	Optimal growth	Examples
Alkaliphile	At or above pH 9	*Alkalibacterium psychrotolerans, Bacillus halodurans, Alkaliphilus transvaalensis*
Acidophile	At or below pH 3	*Picrophilus oshimae, Acidianus brierleyi, Ferroplasma acidarmanus*
Thermophile	60–80°C	*Caldicellulosiruptor saccharolyticus, Thermoanaerobacter italicus, Thermotoga neapolitana, Methanogenium frigidum, Colwellia psychrerythraea*
Hyperthermophile	Above 80°C	*Pyrolobus fumarii, Pyrococcus furiosus, Geogemma barossii*
Psychrophile	At or below 15°C	*Desulfotalea psychrophile*
Halophile	At or above 0.5 M NaCl	*Dunaliella salina, Wallemia ichthyophaga, Halobacterium salinarum*
Xerophile	Capable of growing below water activity (Wa) of 0.8	*Xeromyces bisporus, Trichosporonoides nigrescens, Artemia salina, Methanosarcina barkeri*
Piezophile (barophiles)	At or above 50 MPa (obligate piezophiles)	*Thermococcus piezophilus, Shewanella benthica*
Radiophile	Tolerate high levels of radiation	*Deinococcus radiodurans, Thermococcus gammatolerans*
Endolith	Rock-dwelling	*Ostracoblabe implexis, Lithopythium gangliiforme, Desulfovibrio cavernae*
Metallophiles	Tolerate high concentration of heavy metals	*Ralstonia metallidurans*

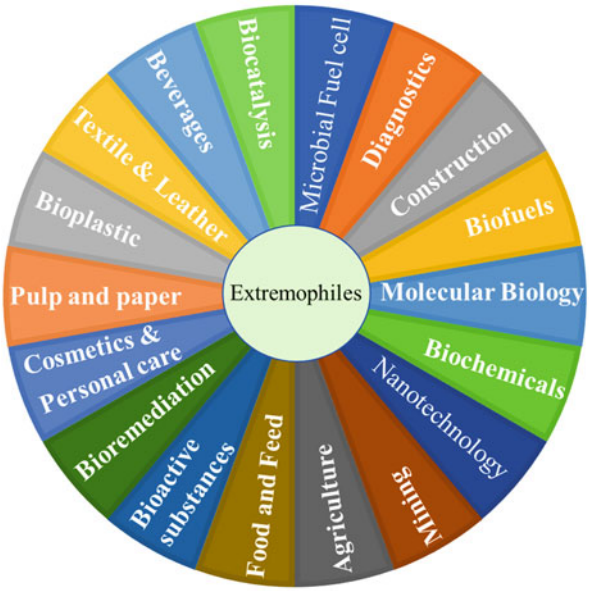


Fig. 1 Some of the application areas of extremophiles

Table 2 Examples of extremozymes and their biotechnological applications

Extremophile	Enzymes	Industrial applications
Acidophiles	Oxidases	Desulfurization of coal
	Proteases, cellulases	Feed component
	Amylases, glucoamylases	Starch processing
Alkaliphiles	Pectinases	Textile, degumming of ramie
	Xylanases	Pulp and paper, xylooligosacchrides
	Proteases, cellulases, lipases	Detergent
	Lipases and proteases	Leather tanning
Halophiles	Protease and lipase	Synthesis reaction
Thermophiles	Amylases	Starch hydrolysis
	Lipases	Detergents, food processing
	Xylanases	Pulp bleaching
	DNA polymerases	Molecular biology (PCR)
	Alkaline proteases	Detergents
	Acid proteases	Food processing
	Neutral proteases	Baking, brewing
	Pullulanases	High glucose syrups
	Cellulases	Cellulose hydrolysis
Psychrophiles	Pectinases	Clarification of fruit juices
	Lipases	Food and cosmetics
	Proteases, amylases, lipases	Low-temperature detergents
	Proteases	Cheese making
	ß-Galactosidase	Lactose hydrolysis

Extremophiles evolved various mechanisms to deal with the stress stemming from the effect of their extreme habitat. One of these adaptive strategies is to produce and accumulate low molecular substances, extremolytes, which are capable of protecting macromolecules against denaturing stress conditions [19, 20]. Due to their protective nature, extremolytes have been used in formulation of skin care products and sunscreens, and stabilization of proteins and cells, and have also been considered as antioxidants, anticancer drugs, cell-cycle-blocking agents, etc. [21–27]. A myriad of other bioactive substances of pharmaceutical and nutraceutical importance have been discovered from extremophiles, and a handful of these metabolites entered clinical trials [28]. In addition to these bioactive substances, extremophiles are sources of industrial chemicals such as organic acids, lipids, carotenoids, exopolysaccharides, etc. [29–31] which are of great importance in several applications. An issue worth to mention here in relation to chemical production is the impressive ability of production and tolerance of extremophiles to alcohols which serve as industrial chemicals or biofuels.

Extremophiles are known to play prominent role in biofuel production [32]. Moreover, their robust enzymes often used in the hydrolysis of lignocellulosic materials to generate fermentable sugars, extremophiles have been successfully used as fermentative organisms to produce bioethanol and butanol [33, 34], the two important industrial and biofuel alcohols. Another biofuel that requires the touch of extremophiles is biodiesel. In biodiesel production, the lipases of extremophiles are used in processing lipid to biodiesel [35]. Moreover, certain

extremophiles such as *Cyanidium caldarium* [36] and *Galdieria sulphuraria* [37] are highly lipogenic and are attractive for biodiesel production. Extremophiles are also considered for production of fuel hydrogen. In fact, large-scale processes have been tried using the thermophilic strains of *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii* [38]. Studies on *Thermoanaerobacterium* [39], *Pyrococcus* [40], and *Aeropyrum* [41] indicated the promising potentials of these organisms in fuel hydrogen production. The extraordinary potential of extremophiles has motivated the engineering of hyperthermophiles for production of biohydrogen [42].

As shown in Fig. 1, extremophiles are leaving their outstanding marks on many applications including agriculture and mining. Agriculture is known to be one of the beneficiaries of biotechnological innovations. Microorganisms and their products have been used not only to boost productivity but also to improve the quality of the yield. Extremophiles, in addition to their use in food and feed processing, compost making, etc. are found with enormous potential that can significantly improve agricultural productivity in cold [43], dry [44–46], and saline [47] environments. It seems, in the time ahead, extremophiles together with techniques of molecular biology will play a pivotal role in shaping the future of agriculture.

In mining, among the many possibilities that extremophiles can be used or applied, leaching is one of the most important processes. Leaching is a prominent mining activity that extracts valuable minerals from their respective ores. The traditional leaching process requires the use of chemicals including toxic substances such as cyanide and mercury [48], which makes mining one of the most polluting human activities. The alternative technology, bioleaching, uses microorganisms instead of chemicals to leach out metals from the ores in environmentally benign way. This process has been used to mine copper, gold, silver, nickel, zinc, uranium, etc. and, hence, becoming increasingly important in the mining sector. Since the leaching process involves extreme conditions such as low pH, high metal concentration, and high temperature, organisms considered for biomining should tolerate the extreme conditions which makes extremophiles the primary choice. The acidophilic strains that belong to the genera *Acidithiobacillus*, *Leptospirillum*, *Ferroplasma*, *Sulfolobus*, and *Metallosphaera* [49, 50] can be mentioned as examples that are widely used in bioleaching processes.

As biotechnology becomes increasingly important in our daily lives, extremophiles are emerging as one of the most important contributors revolutionizing the field of biotechnology. Several industrial, agricultural, medicinal, nutraceutical, energy, personal care and hygiene, environmental, and other astonishingly diverse applications are involving extremophiles directly or indirectly. Alkaliphiles are among these extraordinary extremophiles of great biotechnological importance, and this chapter focuses on this class of extremophiles and tries to depict the immense biotechnological potential.

3 Alkaliphiles

Alkaliphiles are one of the most important groups of extremophiles which are adapted to thrive in high pH environments that are often lethal to other forms of life. These organisms grow optimally at or above pH 9, and the most extreme strains can grow lavishly as high as at pH 13.5 [4]. Alkaliphiles are believed to be the earliest life forms on earth which originated billions of years ago in deep-ocean alkaline hydrothermal vent systems [51, 52]. Today, alkaliphiles thrive in many geographical locations across the planet, both in natural and manmade alkaline environments. Alkaline soda lakes, soda deserts, and saline soda soils are the most important ecological niches from which the largest number of alkaliphiles is isolated. Other natural habitats in which alkaliphiles flourish include the alkaline serpentine lakes, oceanic bodies, ikaite tufa columns, and alkaline hydrothermal vents. There are also a range of highly alkaline anthropogenic driven environments such as drainages, lakes, and ponds associated, among others, with steel, soda, and bauxite processing sites. Moreover, alkaliphiles have also been isolated from neutral environments [53]. The isolation of alkaliphiles from a range of alkaline and non-alkaline habitats indicates that these organisms are quite common and are ecologically important. The isolation and cultivation of alkaliphiles are discussed in detail in this volume (see [54]).

These fascinating organisms evolved structural and functional adaptive features to thrive in the extreme alkaline environments, in which other organisms may die instantly. Understanding the genetic makeups that are responsible for these adaptions is an interest to both applied and basic research (see [55]). In line with this, the genome of several alkaliphiles has been sequenced and analyzed [56–58]. The knowledge generated from genome and gene sequencing not only helps to unravel the mechanism of high pH adaptations but also helps to tap the potential of these organisms in biotechnological applications. Like other extremophiles, the great majority of alkaliphiles are unculturable [59], and hence, direct isolation and screening of these microbes is limited. However, advances in molecular and computational biology such as bioinformatics, genomics, data mining, sequencing, metagenomics, cloning, and heterologous expression gave the possibility of utilizing these unculturable alkaliphiles in various biotechnological applications. Moreover, the breakthroughs in these disciplines together with the integrative approach may lead to the discovery of alkaliphiles' genetic blueprints for high pH adaptations and capabilities in producing novel biotechnologically important substances.

Genetic, physiological, and biochemical studies revealed that alkaliphiles deploy several intriguing strategies to thrive in their extreme habitats. Some of the most important adaptations are shown in Fig. 2. These adaptive features not only allow these organisms to flourish in the high pH environments but also make alkaliphiles the most efficient ATP producers as well as fastest and denser growers compared to their non-alkaliphile counterparts (see [60]). Most of the adaptive features of alkaliphiles are of great importance in biotechnology. Acid production, efficient siderophores, highly anionic cell surface, alkaline active enzymes, etc. are high pH adaptations of alkaliphiles which are useful in various applications.

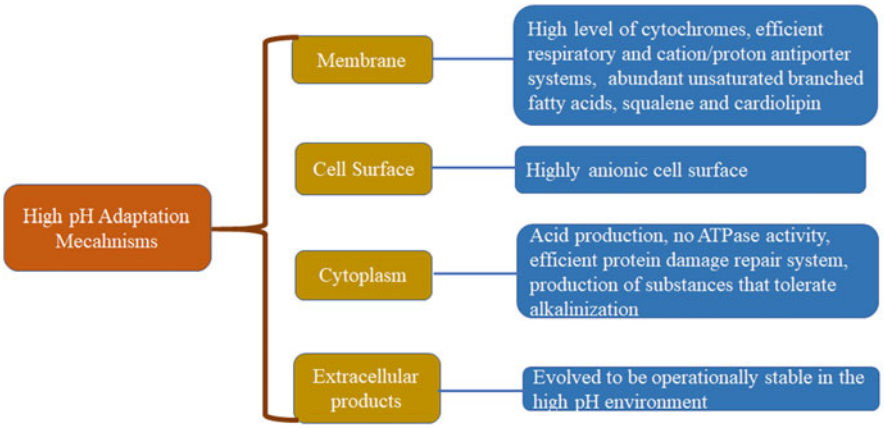


Fig. 2 Some of the high pH adaptations that alkaliphiles deploy to thrive in their extreme habitat

4 Alkaliphiles in Biotechnology

Alkaliphiles are one of the most applied extremophile classes which is used in a growing number of applications. The prominent existing applications of these organisms are related to their alkaline active enzymes which are used in detergent formulation, textile and leather processes, and pulp and paper production. Alkaliphiles are also known in producing a variety of biochemicals which are important for various applications. Among others, alkaliphiles can be promising potential sources for novel carotenoids, bioactive substances, siderophores, biosurfactants, etc. Moreover, the use of alkaliphiles in production of organic acids and biofuels seems to have, at least in some respects, a better edge than the conventional organisms. Some of the most important cell-free and whole-cell associated applications of these remarkable extremophiles are discussed below.

4.1 Alkaline Active Enzymes

Enzymes which are operationally stable at high pH have got several applications. Although some alkaline active enzymes have been reported from non-alkaliphiles, alkaliphiles are the major and reliable sources of these enzymes, and, hence, wherever there is application of alkaline active enzymes, alkaliphiles are the primary choices. Among enzymes of alkaliphiles, the enzymes which are secreted out to the environment are truly evolved to work optimally in alkaline condition, and, hence, the interest on alkaline active enzymes hovers around these extracellular biocatalysts of alkaliphiles. Some of the most common applications of these extracellular enzymes of alkaliphiles are discussed below.

4.1.1 Detergent Formulation

Over years of studies and evaluations, the chemical composition of detergents reached at its peak, and it had been difficult to further improve their washing performance. Detergents that are available in the market were formulated with related ingredients which clean dirt based on similar mechanisms. Later, studies on enzymes as detergent additives revealed their great potential in boosting the washing performance of detergents [61]. This has brought the emergence of enzyme applications in detergent formulation, a new game that improves not only the washing performance but also reduces the wash cycles, wash duration, and energy consumption. Trypsin was the first enzyme introduced to the detergent world. Although the washing performance of detergents supplemented with trypsin was better than the cleaning power of the enzyme-free detergent, the enzyme was unstable due to the detergent alkalinity. This triggered the search for enzymes that are more compatible with detergents and the washing process. Since alkaline active enzymes are more operationally stable in alkaline conditions, the search has been focused on this type of enzymes. This led to an extensive screening of alkaliphiles for production of alkaline active enzymes which are amenable for detergent applications. Today, heavy-duty powder and automatic dishwasher detergents are often formulated with alkaline active enzyme(s). Alkaline active proteases, amylases, cellulases, mannanases, lipases, etc. are used in the formulation of heavy-duty laundry and dishwashing detergents, and these applications absorb the lion share of the world industrial enzymes market.

In addition to improving the total washing performance, enzymes effectively remove stains and allow effective low-temperature (30–40°C) washing. The use of alkaline active enzymes of psychrophiles which are optimally active at low temperature may even allow to achieve efficient washing performance at room temperature or below. Reduction in washing temperature and cycles is expected to have positive impact on the environment by reducing the energy and water consumption [62, 63]. Moreover, low water consumption infers to low discharge volume from washing process. The reduction of the washing effluent volume can substantially cut down the amount of detergent containing effluent discharged to the aquatic bodies. Studies made by [63] indicated that a 3% reduction in CO_2 emissions and energy consumption can be achieved for every 25% increase in consumer cold-water washing in the United States. The authors draw a conclusion that a shift to low-temperature washing, use of energy saving-washing machines, and elimination of mechanical drying can reduce the release of 105 million metric tons of carbon dioxide and save 142,000 GWh electricity which is almost equal to removing about 16 million passenger cars or 23 coal power plants. Moreover, it can reduce up to 60% of the country water consumption.

Most detergent enzymes degrade the component of dirt and facilitate its removal. Alkaline active proteases degrade proteinaceous stains like those of blood, egg, gravy, milk, sweat, etc. into small readily soluble fragments which can be easily removed from the surface of the object subjected to cleaning. However, not all

proteases of alkaliphiles are desirable in detergent formulation. The alkaline active proteases used in detergent formulation are those which are non-specific serine proteases capable of cleaving most peptide bonds. Unlike serine proteases, cysteine (thiol) proteases and metalloproteases cannot be used in detergent formulation. These proteases can be inactivated by the detergent bleaching agents which oxidize cysteine proteases and chelators that strip the metal cofactor of metalloproteases. Thus, the focus has always been on serine proteases. Since Novo Nordisk developed Alcalase (from *Bacillus licheniformis*), the first alkaline active detergent protease in 1958, several serine proteases have been developed and implemented in detergent formulation. The alkaline active Esperase and Maxtase (from alkaliphilic strains of *Bacillus licheniformis*) and Savinase (from alkaliphilic *B. amyloliquefaciens*) are among the well-known examples of commercial detergent proteases. In recent years, alkaline active proteases from *Bacillus cereus*, *Bacillus pumilus* strain CBS, *Streptomyces* sp. strain AB1, *Bacillus licheniformis*, *Aspergillus flavus*, *Bacillus brevis*, and *Bacillus subtilis* AG-1 have exhibited excellent detergent compatibility [64].

Like protein-based dirt, lipid-containing dirt is an important target in cleaning. Alkaline active lipases which are compatible to detergent ingredients such as chelators, surfactants, and oxidants have become vital detergent enzymes. Nearly 5% of the world phosphate produced globally had been used in detergent formulation which in addition to reducing water hardness helps to remove fatty and greasy dirt. However, due to environmental concern, there is a need to limit or avoid phosphate in detergent formulations [65]. This led to an increase in the need for lipases that degrade and remove fat-containing dirt in non-phosphate detergents. This demand has brought screening of several alkaliphiles from which a range of interesting lipases have been reported [66–68].

It is not only dirt-degrading enzymes that are considered to used in detergent formulation, enzymes which modify the surface of textiles have also been considered. An interesting approach in this regard could be the introduction of alkaline active cellulases as detergent enzymes for washing cotton fabrics [69]. Unlike other detergent enzymes, cellulases in detergents do not directly hydrolyze the dirt, but it modifies the fabric surface and facilitates the dirt removal. Garments made from cellulose fabric over time tend to have small fibers on its surface which in addition to reducing the color intensity can easily attach to dirt. These small fibers are mostly amorphous in structure and hence are susceptible to enzymatic hydrolysis. Thus, cellulases intended for this application should remain operationally stable in the detergent/washing condition and degrade the fine cellulose fibers but are inert toward the main crystalline cellulose fibers of the garment. The hydrolysis of the fine fibers from the surface of the garment removes the dirt attached to these fibers and enhances the brightness to its "new" condition [70].

In its journey of over 100 years, detergent enzymes have astonishingly progressed and revolutionized the entire detergent industry. However, there are still some issues that deserve a proper look. Although detergent enzymes have been in the market for a long time, it is not yet readily available in most developing countries. So far, the application seems restricted in the developed world. The main limiting factors that contribute to low usage of detergent enzymes in many third-world countries seem to

be due to high price, poor availability, and awareness. However, like any other technological innovations, this will also slowly trickle in and become available to the rest of the world. In recent years, the market started to gather momentum in some Asia-Pacific countries such as China and India. That means, potentially, the market will expand until it covers all geographical locations, and hence, it is not farfetched to expect a huge market growth for detergent enzymes in the years to come. Another issue worth to mention here is the limited portfolio of detergent enzymes. Since the days of its infancy, enzymatic detergent formulations are confined to proteases, lipases, cellulases, and amylases. Perhaps there is still a room for expansion. Mannanases and xylanases have shown remarkable potential [71, 72]; however, the market integration is still lagging. Another area of expansion could be the use of enzymes that in situ produce bleaching agents such as hydrogen peroxide. Thus, enzymes such as lipoxygenases, glucose oxidases, glycerol oxidases, etc. can be considered in the formulation of detergents with bleaching efficacy. The introduction of such new enzymes in detergents is expected to expand the market.

4.1.2 Textile and Fiber Processing

Alkaline active enzymes have got several notable applications in textile and fiber processing. Cotton scoring and bast fiber degumming are two examples that show the technical, economic, and environmental benefits of using alkaline active enzymes.

Bioscouring of Cotton

Among the natural fibers used in textile manufacturing, cotton is undeniably the prominent one. Processing of cotton to textile yarn involves several treatment steps. Removal of the fiber protecting pectin from the raw cotton is one of these steps used in making yarn. This treatment is known as scouring. Conventionally, the cotton scouring is done at elevated temperature using sodium hydroxide solution which solubilizes the pectin, hemicellulose, and lignin fractions of the cotton. As this process depends on high concentration of sodium hydroxide and high temperature, it is unfavorable from economic point of view. Moreover, to remove the sodium hydroxide, an extensive washing is a necessary step. The high energy and water consumption leave a negative impact on the environment. Thus, there has been an interest for an alternative process that reduces the amount of alkali and the energy consumption.

An enzyme-based scouring which is often referred to as bioscouring has emerged as an attractive alternative. In this process, enzymes which selectively degrade the pectin at ambient temperature have been used. Since high pH facilitates the solubilization of pectin and inhibits the growth of cellulose-degrading fungal strains, the use of diluted alkali is beneficial. Thus, the enzymes planned for this application are desired to be alkaline active. The use of alkaline active pectinases that are operationally stable in diluted sodium hydroxide dramatically reduced the processing cost

and render the process environmentally benign [73]. Moreover, the reduction in the alkali concentration improves the quality of the cotton fiber [74] and subsequently enhances the textile quality.

Bast Fiber Degumming

Bast fibers which are known for their high tensile strength have been used for thousands of years in making ropes, cloths, yarn, bags, and sails, while in recent times, bast fibers are used to manufacture different items ranging from building materials to plastic composites [75, 76]. For instance, blended hemp, flax and kenaf fibers have been used as a natural insulating material that competes with the traditional fiberglass insulation [77]. However, the bast fiber should be recovered from the biomass to be used in these applications. Like cotton scouring, the bast fiber recovery process involves the removal of cementing substances that cover and bundle the cellulosic fibers. This process of removing the gummy substance is known as degumming. The traditional degumming process uses up to 20% (w/v) sodium hydroxide solution containing some wetting and reducing agents [78]. The plant biomass will be soaked for 1 day in this alkaline solution and then boiled for about 4 h to dissolve the gummy substance and release the fibers. After boiling, the fibers will be extensively washed and neutralized. As in the case of the cotton scouring process described above, the high concentration of sodium hydroxide, the boiling of the suspension, and the accompanied extensive washing and neutralization make the process not only expensive but also time-consuming and polluting. The use of enzymes that allow to recover the fibers at reduced concentration of sodium hydroxide and energy consumption can make the process more attractive from environmental as well as economic stands.

Alkaline active pectinases have been successfully tried to degum flax, ramie, jute, and hemp fiber [79, 80]. The use of enzymes allows to reduce the sodium hydroxide concentration, shorten the soaking time, and lower the cooking temperature used to release the fibers. Diluted alkali is often implemented in the degumming process which, in addition to preventing the growth of cellulose-degrading contaminants, helps to remove effectively the lignin components from the fibers, and this minimizes the consumption of bleaching chemicals in the subsequent steps. Thus, the enzymes used to degum the fibers should be operationally stable at high pH. Such enzymes are often obtained from alkaliphiles [80–82].

Biostoning

Textile fiber-modifying enzymes have been used in the finishing of fabrics and clothes in a process known as biopolishing. A prominent application in this area is biostoning of denim. Denim jeans dyed with synthetic indigo seems to be one of the most popular garments. Due to the fashion desire, denim garments are often subject to wash cycles that give the garments a slightly worn look. In early conventional

process, denim used to be washed with pumice stones. However, this abrasive process was cost incurring due to machine repair and high energy consumption. Moreover, the abrasion can damage the fabric and hence lower the quality of the fabric. Thus, it was necessary to develop an alternative method that gives the same effect. The use of enzymes, specifically cellulases, emerged as replacement for the stone washing, and the process is named biostoning [83, 84]. The action of the cellulase on the cellulosic fibers results in certain indigo dye loss which gives the garment a uniform aged look under mild conditions. Neutral and acid active cellulases have been used in biostoning process. However, the low pH biostoning suffers from the undesirable effect of the indigo dye back staining. On the other hand, denim biostoning at alkaline conditions diminishes the indigo back staining effect, and hence, the use of alkaline active cellulases in biostoning process has been recommended [85]. Alkaline active cellulases which are potentially suitable for this application have been reported from different alkaliphiles [69, 85–87].

4.1.3 Paper and Pulp Processing

Pulp and paper production has been a multibillion-dollar business, and its market value for 2019 was estimated to be about 63 billion USD [88]. In the year 2017, the world has produced about 184 million metric tons of pulp [89] and close to 420 million metric tons of paper and cardboard [90]. To process such large volume of products, the input it takes and the effluent it discharges are expected to be high. Thus, if the process involves the use of hazardous chemicals, its pollution impact can be significant and poses a serious threat. Pulp and paper production had been using different potentially hazardous chemicals, and bleaching was one of the major pollution sources.

Unbleached pulp has brownish color primarily due to the residual lignin and lignin derivatives which are covalently attached to the hemicellulose fraction. Removal of this lignin involves a multistage bleaching process, which used to involve elemental chlorine. Although chlorine-based bleaching of pulp is effective, it is also associated to formation of chlorinated organic by-products which are known to have highly persistent toxic and mutagenic effects. Because of the growing public concern about environment and strict legislations regarding pollution, the search for alternative ways to reduce or avoid the release of chlorogenic compounds with pulp mill bleaching effluent has been promoted. Different environmentally friendly bleaching chemicals like H_2O_2, O_2, and O_3 have been substituting the toxic chlorine-based bleaching chemicals [91]. Currently, the oxidizing agent, chlorine dioxide, is the major bleaching chemical and being used widely in what is known as elemental chlorine-free (ECF) bleaching process [92]. On the other hand, the total chlorine-free (TCF) bleaching, which is the most environmental benign process, accounts only for a tiny fraction of pulp bleaching.

ECF substituted the elemental chlorine-based bleaching and is accepted by the United Nations Environment Programme, the International Joint Commission, the

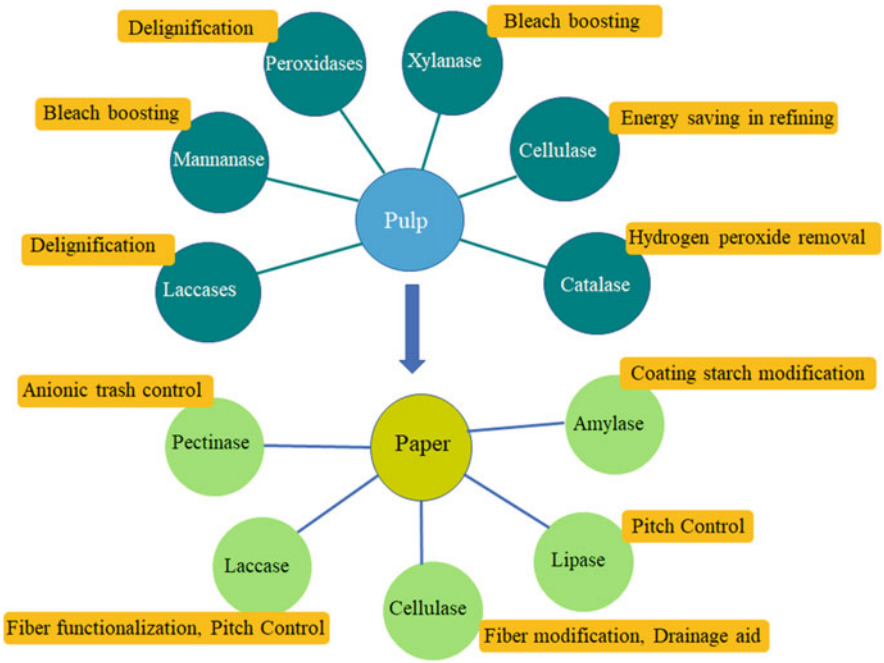


Fig. 3 The most important applications of different alkaline active enzymes in processing of pulp and paper

European Commission, and other organizations as the best available technology for pulp bleaching. Thus, due to its economic and environmental benefits, more than 90% of the current global pulp bleaching depend on it. Although chlorine dioxide is not making the chlorogenic compounds, dioxins and furans, it is not risk-free. It is highly unstable, extremely toxic, and explosive. Thus, any green approach that helps in bleaching or any of the processes that allow to minimize the chemical and energy consumption of the bleaching process can be considered as environmentally benign. One of the success stories in this line is the use of enzymes as pre-bleaching aids which avoids or reduces the use of bleaching chemicals. Xylanases, mannanases, cellulases, lipases, laccases, and peroxidases have shown promising results in processing of pulp and paper and reduce the chemical load [93–98], and some of these enzymes are already applied by different mills [99]. The most important roles of enzymes in processing pulp and paper is shown in Fig. 3. Often, cellulases and lipases are used in papermaking, while xylanases and laccases are primarily used in pulp production processes. The main benefit of using cellulases is in fiber modification, while lipases are used to control the pitch. Lipases hydrolyze the resin triglycerides and prevent pitch deposit on paper machines. The free fatty acids released by the lipase are easily removed by washing with sodium hydroxide solution.

Laccases, mannanases, peroxidases, and xylanases are applied in pulping process to delignify the pulp and boost the bleaching. Although cellulases are primarily applied in papermaking, there has been a growing interest of using these enzymes in the pulping process as well. The refining and grinding of wood in conventional mechanical pulping result in pulp rich in fines and bulk and higher stiffness; hence, in the subsequent steps, the energy consumption is high. On the other hand, the use of cellulases in a mechanical pulping process known as biomechanical pulping substantially reduces the energy consumption (up to 40%) during the subsequent refining process and enhances the strength properties [100–102].

In paper mills, there are some fines, peel off fibrils, and other dissolved and colloidal substances which often cause drainage problems. Alleviating this problem can improve the process productivity. The use of cellulases and hemicellulases, in addition to enhancing pulp beatability, helps to solubilize drain plugging constituents and considerably improve the drainage [103]. These effects of enzymatic treatment are accompanied by an overall improvement in the mill performance [101]. Moreover, cellulase treatment is known to decrease defibrillation which concomitantly reduces fiber coarseness [104]. Manufacturing of easily degradable cardboard and soft paper such as sanitary paper and paper towels utilizes such enzymes.

Application of laccases in pulp and paper industry is versatile. Application of laccases, as aforementioned, delignifies and brightens pulp. Like lipases, laccases are also capable of removing lipophilic substances which are responsible for pitch deposition on papermaking machines [105]. Since laccases generate reactive radicals with lignin and functionalize lignocellulosic fibers, in addition to delignification, it can also improve the chemical and physical properties of the pulp [106, 107]. Moreover, laccases which mediate polymerization and depolymerization reactions can remove colored and toxic compounds from mill effluent and, hence, render bioremediation purpose.

Kraft pulping is the major pulping process which involves alkaline treatment. Therefore, the enzymes intended for pulp and paper application preferably should be alkaline active to avoid or reduce the time-consuming and cost-incurring pH adjustments. Hence, alkaliphiles which are major sources of alkaline active enzymes are vital in these multibillion-dollar processes.

4.1.4 Paper Recycling

Paper recycling is a flourishing business with great economic and environmental impact. It has become mandatory for most pulp and paper industries and believed playing a remarkable role in the fight against deforestation. In addition to saving trees, paper recycling significantly cuts down the water and energy consumption and hence contributes toward greener environment. However, regeneration of high-quality secondary fibers from old papers is not a simple task. Deinking and removal of adhering materials are among the most important challenges in paper recycling.

Ink removal is one of the major technical obstacles, and over the years, several chemical and enzymatic processes have been tried in alleviating problems related to deinking process. Compared to the traditional chemical-based deinking, the use of the enzymatic alternative has some advantages including better brightness, greater freeness, and superior strength of fibers [108]. Moreover, the enzymatic process is more efficient and can reduce the use of deinking chemicals, and hence it is considered as environmentally benign [108]. Enzymatic deinking, in addition to higher pulp cleanliness, allows to improve strength properties, reduce alkali usage, render fiber brightness, increase pulp freeness, and minimize the presence of fine particles in the pulp [109].

Cellulases, lipases, amylases, laccases, pectinases, and xylanases have been used in recycled paper deinking and bleaching processes [110–113]. The mechanism of these enzymes in achieving the desired deinking is quite different. For instance, the lipase hydrolyzes the oil-based binders/resins in the inks, whereas cellulases and xylanases detach the ink from the fiber surface through hydrolysis of the fine fibers from toner surfaces [114]. Amylases degrade the starch used in the papermaking and thereby facilitate the ink release and its removal by the subsequent flotation step. However, it is essential to note that the enzymes, specially cellulases, should not be used at higher doses as it can affect the quality of the pulp. Significant loss of fines may result in undesirable poor fiber bonding [115].

Paper recycling involves swelling of the cellulose fibers, a phenomenon which facilitates the removal of ink and other additives from the cellulose fibers [116]. Since high pH renders better swelling of fibers, paper recycling is often accomplished at higher pH. Moreover, the alkali can directly interact with the dye, weaken its association to the fibers, and facilitate its removal [117]. Thus, enzymes considered for paper recycling applications should be operationally stable at alkaline conditions, and this offers economic, technical, as well as environmental benefits.

The other challenge in paper recycling is the complete removal of adhesive contaminants which are often referred to as stickies [118–121]. The presence of stickies can pose serious problems in recycling process and affect the quality of the recycled paper properties. It can adhere to the recycling and papermaking machines and lead to fouling and equipment plugging. Macro-stickies which are in the range of 100 to 1,000 μm in size are the most difficult adhesive contaminants to remove during the recycling process [122]. Efficient removal of these stickies has been one of the major issues in paper recycling. An enzymatic method that uses alkaline active esterases and lipases which act on adhesives resulted in an encouraging removal efficiency of stickies and concomitantly improve the pulp quality [122, 123].

4.1.5 Synthesis

With the emergence of green chemistry, the synthesis of substances using enzymes has become increasingly important. These synthetic reactions often use enzymes which are cell-free or cell-bound in processes respectively known as biocatalysis and biotransformation. These processes are widely considered as safe, mild, efficient,

specific, and environmentally sound. In some cases, it serves as simple replacement for existing toxic, hazardous, complex, or uneconomical chemical- or chemo-physical-based processes [124, 125]. While in other cases such as when the substrate/product is complex and hardly possible to synthesize it using the conventional chemical methods, biotransformation or biocatalysis processes may be the only way forward. Here, a good example could be PCR, a reaction that is the heartbeat of the modern-day biotechnology. The polymerases used in this reaction duplicate the template (DNA strands) of largely unknown sequence (often except that of primers) which is impossible to achieve with the conventional chemistry approach.

Synthesis reactions may require harsh conditions such as extreme pH, low water activity, high temperature, vigorous mixing, etc. Hence, the biocatalysts used for synthesis reactions should be robust enough, and extremophiles and extremozymes have been known more suitable to catalyze such kind of synthesis reactions. Synthetic reactions that require high pH conditions can deploy alkaliphiles and their enzymes. Thus, alkaliphiles, through simple whole-cell biotransformation or enzymatic biocatalysis, can potentially be used to produce an array of valuable organic compounds.

Alkaliphiles and their enzymes have been tried in various synthesis reactions, and peptide synthesis is one of the interesting mediations achieved by these biocatalysts. Unlike the conventional method of chemical synthesis, enzymatic peptide synthesis is stereoselective, clean, and mild and avoids the time-consuming protection-deprotection procedures [126]. Moreover, it may offer other technical advantages which are difficult to achieve using the chemical method. For instance, production of peptides which are longer than dipeptides has been a challenge for the chemical-based synthetic route. However, it is possible to synthesize such peptides through enzymatic synthesis route [127, 128]. Efficiency of peptide synthesis is better in alkaline condition, which is considered as a natural process that happens around hydrothermal vents and believed to be part of the prebiotic chemical evolution of biological molecules. Recently, this reaction is mimicked and successfully used in vitro to synthesize peptides on rock surfaces at pH 10–11 [129]. Thus, by combining the effect of alkaline condition and catalytic efficiency of enzymes, one expects better synthesis of longer peptides. Indeed, this has been demonstrated by [130] who reported higher degree of peptide polymerization (DP) by enzymatic reactions at alkaline medium than at neutral condition. This shows the potential of alkaline active proteases in the synthesis of peptides for a variety of applications. However, not all alkaline active proteases are equally applicable. Since the peptide synthesis reactions are more efficient in nonaqueous medium, proteases which are alkaline active and operationally stable in low water activity are more suitable.

There has been an effort in obtaining alkaline active proteases which are active at low water activity. Alkaline active proteases from *Aspergillus flavus*, *Bacillus pseudofirmus* SVB1, *Pseudomonas aeruginosa* PseA, *Bacillus pumilus* strain CBS, *Streptomyces* sp. strain AB1, and Alcalase (the most well-known alkaline active proteases) are known to be highly active and stable in the presence of organic solvents, and their promising potential in peptide synthesis has been verified [131–135]. Although it is possible that alkaline active proteases can be obtained from

neutralophiles, alkaliphiles are the most promising sources of alkaline active proteases. Moreover, alkaliphiles often exhibit halophilicity, and their enzymes tend to show relatively better activity in low water activity than their non-alkaliphilic counterparts. Thus, proteases from alkaliphiles are expected to be more effective in nonaqueous medium and are more interesting for peptide synthesis.

Alkaline active proteases are also used in synthesis of other compounds. Synthesis of 2H-1-benzopyran-2-one and polymerizable vinyl guaifenesin ester has been demonstrated using the alkaline active proteases from *Bacillus licheniformis* [136] and *Bacillus subtilis* [137], respectively. Other alkaline active enzymes used in synthesis of valuable substances are cyclo-maltodextrin glucanotransferases (CGTases) which are capable of producing cyclodextrins (CDs), substances widely applied in pharmaceutical, food, and chemical industries [138, 139]. In 2019, several tons of cyclodextrins with an estimated global sell of US $180 million have been produced by enzymatic conversion using alkaline active CGTases of alkaliphiles [140]. Alkaline active CGTases are covered in this volume [141].

Lipases and esterases have also been considered in catalyzing synthesis reactions. For instance, the alkaline active lipase from haloalkalophilic *Bacillus atrophaeus* FSHM2 is used to synthesize ethyl valerate and methyl valerate [142], and the alkaline active esterase is applied to make racemic resolution of *O*-benzyl lactic acid ethyl ester, an intermediate in the production of the third-generation wide-spectrum antibiotic, levofloxacin [143]. In a similar way, an alkaline active epoxide hydrolase of *Maritimibacter alkaliphilus* was used to obtain enantiopure (*R*)-glycidyl phenyl ether by selectively hydrolyzing the (*S*)-glycidyl phenyl ether in the racemic mixture to diol. Thus, this alkaliphilic enzyme can be used in production of enantiopure epoxides and diols [144]. An alkaline active esterase from *Pelagibacterium halotolerans* converts dimethyl 3-(4-fluorophenyl)glutarate (3-DFG) into methyl (*R*)-3-(4-fluorophenyl)glutarate ((*R*)-3MFG), precursor for the synthesis of the antidepressant paroxetine hydrochloride [145]. Some of these reactions mediated by alkaline active enzymes are shown in Fig. 4.

It is not only isolated enzymes which have been considered for synthesis, whole alkaliphile cells have been used to make a wide range of chemicals. Here are some examples. The alkaliphilic strains of *Alkalibacterium iburiense*, *Alkalibacterium psychrotolerans*, and *Bacillus* spp. [146–149], which play pivotal role in the production of indigo dye, can be mentioned as examples. In the dye making vat, a number of oxidation and reduction reactions occur in sequential steps, and the alkaliphilic microbes play the crucial reduction role [150]. Another example could be the biotransformation of ferulic acid to vanillin using an alkaliphilic *Bacillus* [151].

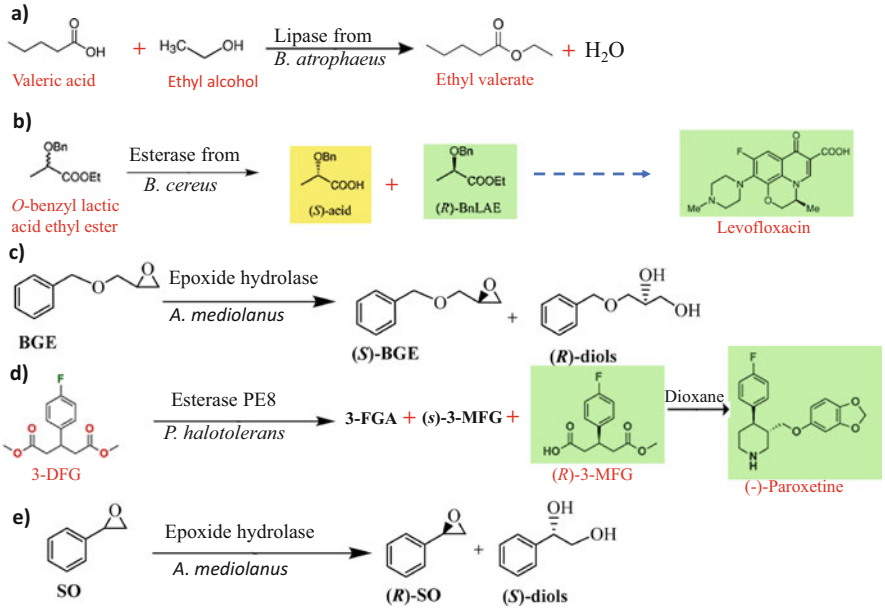


Fig. 4 Examples of biocatalysis reactions accomplished by alkaline active enzymes. (**a**) Synthesis of ethyl valerate; (**b**) racemic resolution of *O*-benzyl lactic acid ethyl ester (BnLAE) by *Bacillus cereus* esterase, a precursor for synthesis of levofloxacin; (**c**) kinetic resolution of racemic benzyl glycidyl ether (BGE) by *Agromyces mediolanus* epoxide hydrolase; (**d**) racemic resolution of (R)-3MFG used for synthesis of (−)-paroxetine, and (**e**) resolution of racemic styrene oxide (SO)

4.2 *Pharmaceutically Important Metabolites*

The rate of new bioactive substance discovery from conventional sources has been declining, and, hence, there has been interest in expanding the search for pharmaceutically important compounds to non-conventional sources such as extremophiles. Alkaliphiles, being one of the prominent extremophiles, have been subject to such explorations, and several interesting bioactive compounds are already reported. Some of these compounds are shown in Table 3.

In addition to metabolites, alkaline active enzymes have also been considered in therapeutic applications. Bioactive peptides which have health-promoting effects are often obtained from proteins through enzymatic hydrolysis. These peptides are known to have, among others, antimicrobial, antihypertensive, and antitype 2 diabetes mellitus activities [169]. The protein amino acid sequence and the specificity of the protease used to hydrolyze the protein are the two most important parameters in the production of peptides with desired effects. In this line, alkaline active proteases which are optimally active in the pH range of 9–10 such as those from *Cryptococcus aureus*, *Issatchenkia orientalis*, *Aureobasidium pullulans*, and *Yarrowia lipolytica* have been reported for their promising bioactive peptide production properties from different protein sources [170]. These organisms are not alkaliphiles, but their

Table 3 Examples of potent bioactive substances produced by alkaliphiles

Alkaliphile	Bioactive substance	Active against	References
Bacillus halodurans	Haloduricin (antimicrobial)	*Staphylococcus aureus, Enterococcus faecium, Enterococcus faecalis, Streptococcus* sp., *Pediococcus* sp.	Danesh et al. [152], Lawton et al. [153]
Bacillus alkalophilshaggy JY-827	Aminoglycoside antibiotic	*Streptococcus mutans*	Chun et al. [154]
Streptomyces sannanensis	Antimicrobial	Gram-positive bacteria such as *Bacillus cereus, B. megaterium, Staphylococcus aureus*	Vasavada et al. [155]
Streptomyces tanashiensis	Antifungal	*Candida albicans, Fusarium moniliforme*	Singh et al. [156]
Streptomyces sp.	Antimicrobial	Active against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli, Proteus vulgaris, Salmonella typhi*	Kharat et al. [157]
	Anticancer	Active against human lung carcinoma	Kharat et al. [157]
Streptomyces aburaviensis	Antimicrobial	Active against *Bacillus subtilis*, *Staphylococcus aureus*, *B. megaterium Bacillus cereus*	Thumar et al. [158]
Nocardiopsis sp.	Naphthospironone A Antimicrobial	*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli, Aspergillus niger*	Ding et al. [159]
	Anticancer	HeLa, L929, AGZY cells	Ding et al. [159]
Synechocystis aquatilis	Antimicrobial	*Escherichia coli, Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa*	Deshmukh and Puranik [160]
Piscibacillus sp.	Anticancer	Breast cancer MDA-MB-231 cells	Neelam et al. [161]
Streptomyces sp. AK 409	Pyrocoll	Bacteria, filamentous fungi, pathogenic protozoa, and some human tumor cell lines	Dieter et al. [162]
Streptomyces sanglieri AK 623	Lactonamycin Z	Gastric adenocarcinoma cells	Höltzel et al. [163]
Nocardiopsis sp.	Griseusin D	Human leukemia cells (HL60), human lung adenocarcinoma cell lines (AGZY), and fungi	Li et al. [164]
Streptomyces violascens	Belongs to polyene antifungal class	Fungal	Bisht et al. [165]
Nocardiopsis alkaliphila YIM-80379	Nocardiopyrones A and B	*Pseudomonas aeruginosa*, *Enterobacter aerogenes, Staphylococcus aureus*, and *Candida albicans*	Wang et al. [166]

(continued)

Table 3 (continued)

Alkaliphile	Bioactive substance	Active against	References
Streptomyces strain Acta 2,930	Warkmycin	Gram-positives, mouse fibroblast cell line NIH-3T3, and human cancer cell lines HepG2 and HT29	Helaly et al. [167]
Streptomyces castaneoglobisporus AJ9	2-Hydroxy-4-methoxy-6-methyl-, methyl ester	Bacterial, fungal, and viral	Adlin et al. [168]

enzymes are alkaline active; hence alkaliphiles which are producing alkaline active enzymes by far and large are expected to demonstrate at least equally excellent bioactive peptide production properties; however, this needs to be experimentally proven. In addition to bioactive peptides, alkaline active proteases can be used directly in treatments. Studies have indicated the potential of alkaline active proteases with fibrinolytic activities in the treatment of thrombosis and cancer [171–173]. Moreover, an alkaline active serine protease with elastolytic property has been used to make elastoterase [174], a formulation used to treat burns, abscesses, carbuncles, and other wounds [175].

4.3 Carotenoids of Alkaliphiles

Different plants, algae, and microorganisms are known producing carotenoids, pigment molecules with polyene backbone which consist of a series of conjugated C=C bonds. The modification of the carbon chain due to oxidation, hydroxylation, and other reactions led to the presence of a large variety of carotenoids, with more than 700 known structures so far. These substances are yellow, orange, or red in color and among other things are known to offer photoprotection and antioxidant activity, properties that led to applications of carotenoids as food colorants, source of vitamin A, feed supplements, and nutraceuticals for cosmetic and pharmaceutical applications [176]. Due to increasing eye problems and cancer cases, and the shift of consumers to organic and natural products, the carotenoid market is expected to grow from about 2.3 billion USD in 2017 to USD 3.6 billion by 2025 as reported by [177]. This indicates that the carotenoid market is expanding. This has led to look for new products as well as better alternatives to the traditional synthetic production method. Due to the low cost, high yield, and safety to consumers and the environment, microbial-based production of carotenoids has become a trend [178, 179].

A wide variety of alkaliphiles are known producing carotenoids, and it is believed that these substances are possibly involved in the high pH adaptation mechanisms of alkaliphiles (see [60]). For instance, astaxanthin, the most important commercial carotenoid which is known for its anticancer [180, 181], antioxidant, and immunomodulatory activity [182], is known to be produced by a range of alkaliphiles.

A strain of *Paracoccus bogoriensis* secretes 0.4 mg astaxanthin per gram of wet cells [183]. A variety of other carotenoids such as spirilloxanthin, spheroidene, demethylspheroidene, demethylspheroidenone, canthaxanthin, and lycopene type of carotenoid pigments have been reported from different genera of alkaliphiles including *Dietzia*, *Microbacterium*, *Thiorhodospira*, *Ectothiorhodospira*, *Roseibacula*, etc. (see [184]). Novel carotenoids containing glucoside esters are also reported from the alkaliphilic strains of *Heliobacteria*, *Heliorestis acidaminivorans*, and *Candidatus Heliomonas lunata* [185, 186]. Thus, it seems that alkaliphiles have a great potential to serve as rich sources of carotenoids.

4.4 Siderophores of Alkaliphiles

Chelators have got various applications in food, pharmaceutical, cosmetic, and chemical industries. In addition, these substances are being used in agriculture, in water treatment, and in dental and other medical applications. Due to the consumer inclination to organic natural products and environmental concerns, there has been an effort to look for efficient organic chelators. Like many other biotechnological important products, microorganisms have been the primary targets of the search. Studies have indicated that microorganisms produce low molecular weight chelating compounds known as siderophores, and so far, more than 500 siderophores have been reported [176]. Most of these chelators, at least partly, seem evolved to acquire iron, one of the most vital substances in cellular processes. Iron is involved in the electron transport chain system that generates ATP and serves as a cofactor for enzymes that mediate an array of biochemical reactions.

Production of siderophores is known to be affected by iron availability. The lower the iron, the higher the siderophore production and vice versa. In alkaline environments, the solubility of inorganic iron and other metal ions is extremely low [187]. In some alkaline habitats, there is another factor that further dwindles iron and other metal availability, high level of oxygen. Alkaline environments such as soda lakes are known to be the most productive lakes [188]. The impressive primary productivity is associated with abundant oxygen release mostly by the photosynthetic cyanobacteria. The oxygen oxidizes the metal ions including iron and precipitates it out. This precipitation further shrinks the availability of the metal ions far below than what is optimally required for normal growth of most organisms. Thus, alkaliphiles which are thriving in these environments, to ensure acquiring enough iron and other important metal ions, are expected to produce siderophores which are very efficient. Thus, it is possible that alkaliphiles may be sources of novel siderophores. Indeed, structural analysis of some siderophores of alkaliphiles supports this expectation. For instance, novel siderophores have been reported from the alkaliphilic strains of *Caldalkalibacillus thermarum* [189] and *Halomonas* [190]. Thus, as siderophores are potentially applicable in areas where chemical chelators are in use and alkaliphiles seem capable of producing efficient and novel

siderophores, it may be rewarding to further study alkaliphiles as sources of novel siderophores that are amenable for different applications.

4.5 Biosurfactant Production by Alkaliphiles

Biosurfactants are surface-active amphiphilic biomolecules produced by plants and microorganisms. These surface-active agents are more efficient, and their critical micelle concentration (CMC) is 10–40 times lower than that of chemical surfactants CMC [191]. The low CMC values indicate that it is possible to reduce the surface tension effectively using very low amount of biosurfactants. In general, their unique properties such as low toxicity, high efficiency, better environmental compatibility, higher stability, etc. have brought interest on biosurfactants. Some of which have got their ways into a range of applications such as in chemical, petroleum, mining, agrochemical, fertilizer, food, beverage, cosmetic and pharmaceutical industries. Biosurfactants are used as foaming agents, emulsifiers, demulsifiers, wetting agents, spreading agents, functional food ingredients, and detergents [192]. The global biosurfactant market is expanding at a reasonable pace. For the year 2018, the market was estimated to be about USD 4.2 billion, and in year 2026 it is forecasted to reach USD 7.25 billion [193]. The demand for green products in personal and home care industries seems to be the major impetus for the market growth.

Several microorganisms have been isolated and studied for surfactant production. Among alkaliphiles, strains of *Cronobacter sakazakii* [194], *Achromobacter xylosoxidans* [195], *Exiguobacterium* [196], *Natronolimnobius innermongolicus* [197], *Ochrobactrum intermedium* [198] are proven surfactant producers. As biosurfactants produced by alkaliphiles are believed to have evolved to work at high pH conditions, biosurfactants of alkaliphilic origin may be of special interest to applications that involve high pH conditions such as detergents and oil extraction. Characterization studies made on surfactants from alkaliphiles revealed interesting results. The biosurfactants from a haloalkaliphilic archaea is stable in pH range of 5–12 and high salinity (up to 35% (w/v)) [197]. The surfactant produced by *Klebsiella* sp. RJ-03 is found to be compatible to detergents and effective for enhanced oil recovery [194]. The *Ochrobactrum intermedium* biosurfactants have shown remarkable stability at pH 10–13 and up to 90°C [198]. These interesting properties such as operational stability at high pH, temperature, and salinity indicate the potential of alkaliphiles to become sources of excellent biosurfactants.

4.6 Exopolysaccharides of Alkaliphiles

Microbial exopolysaccharides (EPSs) are high molecular weight sugar-containing substances secreted by microorganisms. These substances contain distinct monosaccharide units as well as non-carbohydrate substituents and are often species specific.

EPSs are very heterogeneous which greatly vary in composition, structure, functional properties, etc. and have got numerous applications in the food, pharmaceutical, cosmetic, nutraceutical, agricultural, and other industries [199]. The market demand and the scientific interest for these products led to the search for novel EPSs. Screening studies made so far resulted in the discovery of a vast array of EPS producing extremophiles including halophiles, psychrophiles, acidophiles, and alkaliphiles [30].

Microorganisms produce EPSs for various reasons [200]. In alkaliphiles, EPSs play a vital role in their high pH adaptations that allow them to thrive in their alkaline habitats. A variety of alkaliphiles such as *Cyanospira rippkae* and *Cyanospira capsulata* are known producing thick EPS capsule [201]. Thus, with further studies, such alkaliphiles can potentially serve as important sources of EPSs. Probably, the EPSs of alkaliphiles differ from other groups of microbial EPSs due to their largely anionic nature. This may indicate the possibility that some alkaliphiles produce unique EPSs. Indeed, structural studies made so far revealed the presence of novel EPSs from alkaliphiles [202, 203]. Due to their high density of anions, EPSs from alkaliphiles have high metal sorption capacity and, hence, can be ideal to trap metal ions and other cationic pollutants. Thus, the biomass of exopolysaccharide depositing alkaliphilic strains can be used to remove cationic pollutants from industrial effluents. In this regard, EPS-producing alkaliphilic cyanobacteria have been widely studied in the removal of heavy metals, and the results indicate their promising potential [204–206]. EPSs of alkaliphiles have also shown bioflocculant, antioxidant, emulsifying, antivirus, and antitumor properties [207–209] which indicates its potential in various applications.

4.7 Alkaliphiles in Biofuel Production

Due to environmental concerns and the dwindling of petroleum reserves, the world has resorted to utilize renewable resources (biomass) to produce fuels. In the conventional processes of biofuel production, the polymeric biomass is converted to fermentable sugars which are used by the microbes to make the biofuel. Food and feed crops such as wheat, sugar beet, sorghum, sugarcane and maize are relatively easy to hydrolyze to fermentable sugars, and the biofuel production from these resources can be considered simple, straightforward, and efficient. The biofuels produced from these kinds of substrates are known as first-generation biofuel. From economic and environmental point of views, production of first-generation biofuels is not favorable primarily due to direct competition with food and feed [210]. Thus, the use of lignocellulosic biomass for production of biofuels has been promoted. The biofuel produced from agricultural residues or another lignocellulosic biomass is known as the second-generation biofuel. However, the production of biofuels from lignocellulosic biomass is not simple. The main challenges in the second-generation biofuel production process include biomass hydrolysis efficiency, formation of fermentation inhibitor substances during biomass hydrolysis, risk of

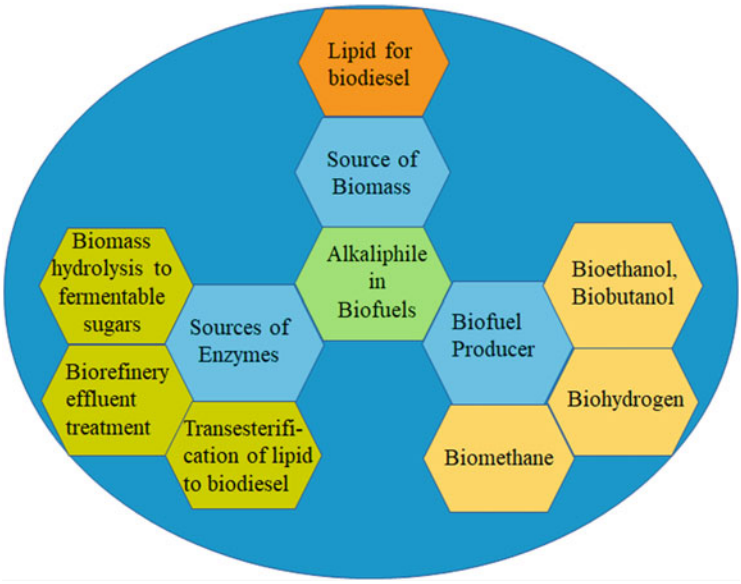


Fig. 5 The potential of alkaliphiles in production of biofuels

contamination, and lack of efficient pentose and disaccharides (e.g., cellobiose) fermenting commercial strains. To alleviate these problems, extremophiles such as acidophiles [211, 212] and thermophiles [213] have been assessed. However, the effort made so far on utilizing alkaliphiles is insignificant despite their great potential.

As shown in Fig. 5, alkaliphile can be used in biofuel production in three different ways. First, the hydrolytic enzymes of alkaliphiles can be used to hydrolyze lignocellulosic biomass to fermentable sugars and facilitate biofuel production. Several enzymes of alkaliphiles exhibit remarkable activity around neutral pH, and these hydrolases can be used to process pretreated biomass like the conventional enzymes from non-alkaliphiles. On the other hand, alkaline active enzymes can be used to hydrolyze biomass at high pH, and this potentially offers many advantages (see [214]). For an efficient conversion of lignocellulosic materials to biofuel, the biomass must be pretreated properly. This pretreatment is the main operation cost. Thus, a cost-effective and efficient pretreatment process is essential to cut down the operation cost and enhance process productivity. Alkaline pretreatment is cheaper, easier, and safer. However, it requires extensive washing and neutralization before enzymatic hydrolysis which is undesirable. If direct hydrolysis of the alkali-treated biomass is done with alkaline active enzymes, the washing and neutralization can be avoided. Even this hydrolysate can be beneficially used directly without neutralization. This is because biofuel fermentation is accompanied by pH drop, and hence the alkaline hydrolysate in addition to its use as carbon source can also be used to regulate the pH drop during the fermentation. Thus, by adjusting the feed rate of the

hydrolysate, it is possible to control the fermentation without pumping base. Moreover, in mixed culture system, better ethanol production has been reported at higher pH [215, 216].

The second possibility of alkaliphiles in biofuel production is their use as biofuel producers. In fact, this approach allows direct utilization of alkaline hydrolysate, and it is attractive from economic as well as technical stand. *Bacillus marmarensis*, an obligate alkaliphile that grows optimally at pH 10, was used as model organism in an effort of evaluating the true potential of alkaliphiles in biorefineries. An engineered *B. marmarensis* is reportedly producing 38 g/l ethanol at yield of 65% using glucose as carbon source in unsterilized media at very high pH [217]. These authors also indicated that the alkaliphile can produce ethanol from xylose and cellobiose at a titer of 12 g/l and 50% yield in media prepared with unsterilized seawater. This approach of successfully running unsterilized fermentation indicates the possibility of contamination-resistant biorefining which utilizes pentoses and disaccharides using alkaliphiles. The approach has a great potential in minimizing the biofuel production cost. In addition to bioethanol production, alkaliphiles have been also used successfully to produce biohydrogen [218] and biogas [219].

The third possibility of using alkaliphiles in biofuel production is related to biodiesel. Certain alkaliphilic organisms accumulate lipid which can be of interest for biodiesel production. Lipogenic alkaliphiles such as *Neochloris oleoabundans* [220] and *Chlorella vulgaris* BA050 [221] accumulate high amount of lipids. Studies have indicated that biodiesel production using these kinds of alkaliphilic lipogenic organisms could be energy efficient [222]. Although outdoor cultivation can significantly reduce the production cost, cultivating of non-alkaliphiles is often challenged by poor economic feasibility due to the high cost of CO_2 supply and contamination problems [223]. On the other hand, unlike non-alkaliphiles, the outdoor cultivation of alkaliphilic algae is very attractive for production of biofuels. This is due to the high pH growth condition which promotes CO_2 solubility and avoids the need for CO_2 sparging. Moreover, the high pH heavily minimizes the possibility of contamination and further promotes the economic viability [221].

4.8 Organic Acids

Organic acids are versatile substances with a wide range of applications in several industries such as in food, beverage, feed, chemicals, personal care and pharmaceuticals. Commercially, these valuable compounds are produced through multi-step chemical synthesis from petroleum-based resources or by microbial fermentation. Compared to the chemical synthesis route, the microbial production approach has several advantages including high purity, enantioselectivity, cost-efficiency and its environmental benign nature.

Organic acid production is among the adaptive strategies deployed by alkaliphiles to thrive in high pH habitats. This ability of alkaliphiles can be potentially attractive for commercial production of organic acids. So far, the studies in this regard are

Table 4 Lactic acid production by alkaliphiles using glucose as carbon source

Alkaliphile	Production (g/l)	Yield (g/g)	Optical purity (%)	References
Exiguobacterium sp.	125	0.98	L, 100	Jiang et al. [224]
Bacillus sp. WL-S20	225	0.99	–	Meng et al. [225]
Bacillus sp. N16-5	144	0.96	L, 99.85	Assavasirijinda et al. [226]
Halolactibacillus halophilus	66	0.83	L, 98.8	Calabia et al. [227]
Enterococcus casseliflavus	103	0.8	L. 99.5	Yokaryo and Tokiwa [228]
Enterococcus sp. AY 103	153	0.99	L, 100	Yoshimune et al. [229]
Enterococcus sp. L-120	149	0.97	L, 100	Yoshimune et al. [229]
Enterococcus hirae BoM 1–2	181	0.96	–	Abdel-Rahman et al. [230]
Psychrobacter maritimus	141	0.94	–	Abdel-Rahman et al. [231]

focused on lactic acid, and a number of high-level and optically pure lactic acid-producing organisms have been reported (Table 4). In addition to high productivity, production of organic acids by alkaliphiles has another advantage. In the conventional filamentous fungi or lactic acid bacteria, the organic acid is removed during the fermentation process through $CaCO_3$-induced precipitation. Although this avoids excessive pH drop and promotes the acid production, it makes mixing difficult. However, in alkaliphile organic acid production process, NaOH can be used to maintain the alkaline condition of the culture, and this renders easy mixing. Moreover, the high pH minimizes the risk of culture contamination. Thus, alkaliphiles can be potentially considered as new promising organic acid producers.

4.9 Alkaliphile Biomass as Food and Feed Supplement

In addition to its carotenoids, organic acids, enzymes, and exopolysaccharides which can be potentially applied in food and feed processing, alkaliphile biomass has been used directly as food and feed supplement. One of the most common and long-established business is the use of the alkaliphilic cyanobacteria (*Arthrospira platensis*, which is previously known as *Spirulina*) as food and feed. For centuries, these alkaliphiles have been part of human diet and are still marketed as food supplements. *Arthrospira* is among the richest sources of proteins which accounts about 60–70% weight of its biomass [232] and high levels of beta-carotene, thiamine, riboflavin, and vitamin [233, 234]. It is believed that *Arthrospira* is one of the

richest sources of vitamin B12. Its nutritional efficiency as a dietary supplement has been the subject of many studies with positive results [232, 235]. Although toxicity studies indicate that *Arthrospira* consumption did not exhibit any toxicity [236], in recent years, in some countries there have been concerns regarding the potential toxicity and long-term effects on human health. Thus, there is strict legislation for microcystin and other toxins of *Arthrospira* biomass destined to be dietary supplement, and this restricts the marketing of the whole algal biomass [237]. However, its global sell value has continued to expand. The global market size which was estimated to be around USD 348 million in 2018 is projected to reach USD 779 million by 2026 [238].

Arthrospira biomass has served as highly nutritious feed for many agriculturally important animals. Studies made using *Arthrospira* biomass as chicken, pig, cattle, rabbit, and sheep feed resulted in encouraging results that show better animal growth, nutritional product quality, fertility, and aesthetic values [239].

Arthrospira is also known for producing an array of bioactive compounds with antioxidant, anticancer, antibacterial, antifungal, and antiviral activities [240]. This makes it one of the favored nutraceuticals and cosmetic ingredient which in turn significantly contributed to the market expansion. Further purification and characterization of the bioactive substances are necessary to determine if there is any novel substance of interest for drug development.

Alkaliphiles are known for their fast and dense growth, and these alkaliphilic cyanobacteria make the East African soda lakes the most productive ecosystems [188]. In addition to its productivity, *Arthrospira* can be easily and cheaply cultivated in open pond systems. In non-alkaline cultivations, cultures crash due to microbial contamination and predators [241, 242]. On the other hand, the alkaline growth condition mitigates culture crashes by preventing the growth of contaminants and predators as mentioned above in Sect. 4.8. Thus, considering that alkaliphilic cyanobacteria are fast growers, nutritious, produce a range of useful bioactive substances, and easily grow outdoor, it seems rewarding to deeply study and evaluate their potential in not only food and feed applications but also as sources of bioactive substances. One interesting study area could be to isolate toxin-free strain or metabolically engineer it to be toxin-free to ease the restrictions in some countries and raise consumers' confidence, which may be a step for further expansion of the market.

4.10 Alkaliphiles in Bioelectricity: Microbial Fuel Cell

Generation of electrical energy from renewable organic matters contained in biomass has begun attracting attention. One means of generating bioelectricity is through microbial fuel cells (MFCs). In MFCs, microorganisms oxidize organic or inorganic substrates and generate current which can be tapped as sustainable energy. The electron generated during the oxidation of substances will be attracted to the terminal (anode) of the microbial fuel cell which subsequently move to the positive terminal

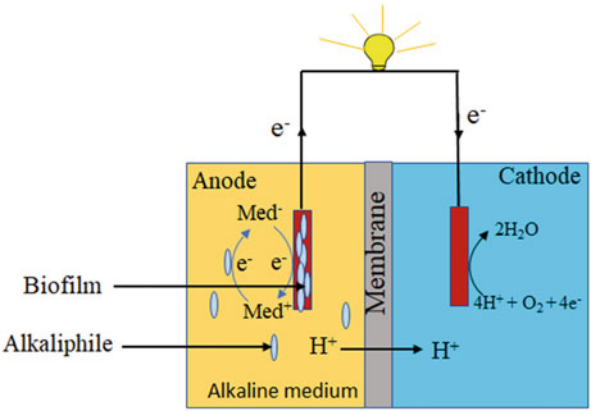


Fig. 6 Schematic presentation depicting the electron (e^-) generation and extraction in alkaliphilic microbial fuel cell system in the presence or absence of mediator (Med). In the absence of the mediator, the biofilm on the anode directly transfer the e^- to anode

(cathode) through conducting material (Fig. 6). A cell voltage increases with increasing anode pH [243, 244], which can be substantiated by the steeper pH gradient created by the elevated pH. This shows the possibility of getting more energy from MFC systems running at higher pH values. Thus, alkaliphiles are exceptionally interesting for developing MFC systems. In line with this, strains of alkaliphiles such as *Pseudomonas alcaliphila* MBR and *Corynebacterium* sp. strain MFC03 have been used to make MFC systems [245, 246]. An interesting co-production of methane and electricity in continuous stirred tank reactor linked to MFC system using the alkaliphilic *Arthrospira maxima* has also been demonstrated [247].

MFC system is relatively a new technology and needs improvements to be competitive. For instance, although the potential is high, the energy efficiency of MFC systems is still low compared to that of anaerobic fermentation processes [248]. This is mainly due to the poor efficiency of the microbes at the anode in utilizing the substrate and the competition by fast-growing contaminants. However, the use of alkaliphiles allows to run the system at high pH where there is better electricity generation and inhibition of contaminant growth. Thus, by selecting very efficient strains that effectively utilize substrates, alkaliphiles can play a very important role in the future MFCs and probably contribute to the realization of large-scale processes. When a glucose molecule undergoes complete oxidation, it produces 24 electrons, and, hence, with complete (100%) coulombic efficiency, a kg of glucose potentially can generate about 4,430 Wh [249]. Therefore, considering the waste biomass the world generates each year, one expects an interesting amount of power that can be generated with MFC system. Encouraging results on alkaliphile-based MFCs started to appear in the literature, which probably ushers the beginning of the long journey. An example could be the generation of electricity at 63% coulombic efficiency from food waste digest leachate supplemented with 100 mM NaCl at pH 9 MFC [250]. The report on utilization of wastewater from seafood processing plant that generates up to 105 mW/m^2 by an MFC system working at pH 9 [251] could be another good example.

4.11 Alkaliphiles in Bioconstruction

Microorganisms contribute to the construction industries in various ways through a discipline known as construction biotechnology. One of the growing trends is the consideration of calcite precipitating alkaliphiles in repairing existing concrete cracks, surface biodeposition (biocoating) and making self-healing concrete (see [252]). Since concrete is very alkaline in nature, the use of alkaliphiles is ideal, and several of them have been studied for these applications. The same principle of microbial-induced carbonate precipitation has been used in the construction industries for several years as a mineral-plugging material, a technique suitable for enhanced oil recovery, cementing unconsolidated ground, restricting the flow of contaminants to aquifer, and plugging pores and channels in soil or fractured rock to limit hydraulic conductivity [253–255]. Moreover, it is used in increasing soil particle cohesion to reduce erosion and dust emission [256, 257] as well as to bind sand grains together to mitigate desert expansion. One of the most important parameters for carbonate precipitation is pH. The higher the pH, the better the precipitation and the more stable are the crystals. Thus, alkaliphiles with calcite precipitation properties could be attractive alternatives for these applications.

In addition to biomineralization processes, microbes can be used in construction activities through their organic products. An important product in this line could be the exopolysaccharides such as xanthan, curdlan, succinoglucan, and welan which are used in preparations including wall plasters, concrete and dry-mix mortars, injection grouts, and self-leveling underlayers [258]. These admixtures are used to improve the water retention property, viscosity, set retarding, and flowability of the preparations [259]. As aforementioned, some alkaliphilic strains are known to be producing high amount of exopolysaccharides, and, hence, alkaliphiles potentially can be interesting sources of exopolysaccharides for the construction industry. Alkaliphiles can be applied in construction sector in various ways, and some of these applications are shown in Fig. 7.

4.12 Alkaliphiles in Mining and Oil Drilling

There is not much published work in the use of alkaliphiles in mining and oil drilling. However, the limited studies done so far show the potential of some alkaliphiles in oil extraction and mining. For instance, the alkaliphilic strain *Alkaliphilus metalliredigens* is known to grow at extremely high pH using chelator-bound metal ions such as Co(III)-EDTA, Fe(III)-citrate, Fe(III)-EDTA, and Cr(VI) as electron acceptors. Such kind of metal-reducing alkaliphilic bacterium may be useful for bioleaching of metals at alkaline and anaerobic conditions [260]. Indeed, *A. metalliredigens*-driven metal reduction and mineral formation has been demonstrated by [261]. Another alkaliphile that reduces Cr(VI) at alkaline condition is a strain of *Amphibacillus* sp. [262].

Fig. 7 Potential applications of alkaliphiles in construction biotechnology

Alkaliphiles, at least potentially, are also interesting in gas and oil recovery. Desulfurization of natural gas and disposal of H_2S are the most common problems in the natural gas industry. Microorganisms which are known as lithoautotrophs oxidize inorganic substances such as H_2S as source of energy. The H_2S-oxidizing lithoautotrophs can be used for natural gas desulfurization applications. Since the transfer velocity of H_2S between water and air at pH 10 is about 200-fold higher than at pH 5 [263] and the use of alkaline solutions such as (bi)carbonate solution selectively absorbs H_2S [264], desulfurization is conventionally accomplished at elevated pH. Studies made on an alkaliphilic lithoautotrophic strain of *Thioalkalivibrio* revealed that it can effectively remove H_2S in haloalkaline medium, which is a condition required for desulfurization of natural gas [265].

Alkaliphiles have also been considered in the recovery of oil. Oil drilling companies use different methods to enhance oil recovery from the ground. One among such recent techniques is known as microbial enhanced oil recovery (MEOR). Microorganisms used in the oil recovery facilitate the flow of oil from bedrocks to reservoirs and from the reservoirs to the surface [266]. The microbes improve the flow such as through (1) production of low molecular weight acids which can cause rock dissolution, (2) production of biosurfactants which lower the interfacial tension (IFT) and enhance oil in water micelle formation, (3) reduction of the oil's viscosity due to enzymatic conversion of large hydrocarbons into smaller molecules, and (4) increasing the formation pressure by generating gases such as carbon dioxide and nitrogen. Since alkaline flooding and alkali-polymer and alkali-surfactant-polymer injection are common procedures in enhanced oil recovery, alkaliphiles are the

choice if MEOR is considered in the extraction process. For instance, guar gum polymer is used to improve oil recovery by fracturing the bedrock. However, after the fracturing, it is necessary to reduce the polymer viscosity to ease the oil flow. Reduction in viscosity of the guar gum can be achieved by using mannanases which degrade the polymer. Since the polymer flooding is alkaline, the wells are alkaline, and, hence, it is imperative to use mannanases that are operationally stable at alkaline condition, and such enzymes have been engineered specifically for this application [267].

4.13 Alkaliphiles in Environmental Biotechnology

Microbial cells and their enzymes have been playing crucial roles in waste management. Hazardous substances in effluents, often from industries, are degraded or biotransformed into harmless products by microorganisms. Moreover, microbes decrease the biological oxygen demand (BOD) and chemical oxygen demand (COD) of effluents which in turn allows easy disposal after treatment. For such applications, the pH of the effluent (waste) dictates which kind of microbes or enzymes to be considered for treatment. If the effluent is alkaline, the use of alkaliphiles or alkaline active enzymes is a primary choice. The other option is neutralization of the effluent and treatment with neutralophiles or their enzymes which are often optimally active around neutrality. However, the later choice is time-consuming and cost-incurring and may also increase the salinity which is an environmental concern. On the other hand, direct use of alkaliphiles or their enzymes is attractive to treat alkaline effluents due to its time-saving, technical, economic, and environmental benefits.

There are some that show cases where alkaliphiles or alkaline active enzymes are implemented in treating waste. Azo dyes which are widely used in many industries are hazardous and need to be removed from effluents. One of such dyes is Reactive Orange 16. This dye has been removed in a cost-effective way using the alkaliphilic microbe *Bacillus flexus* VITSP6 at pH 11, which is able to biotransform the toxic dye to harmless low molecular products [268]. Similarly, the degradation of toxic azo dyes by the alkaliphilic bacteria *Nocardiopsis alba* [269], *Bacillus cohnii* MTCC 3616 [270], and consortium [271] has been reported.

Alkaline active enzymes are considered to treat nitroaromatic compounds. These substances are among the most common industrial chemicals that are currently in use and have become one of the major classes of pollutants. Nitroaromatics are highly toxic and mutagenic [272, 273], and, hence, waste containing these pollutants should be treated effectively. Although microbial degradation has been tried widely [274, 275], little is done on the use of isolated enzymes on detoxification of nitroaromatics. However, some enzymes of alkaliphilic origin have been tried successfully. The alkaliphile *Bacillus badius* flavin-free NADH azoreductase is used for biotransformation and detoxification of nitroaromatic compounds such as 1-chloro-2-nitrobenzene, 3-nitrobenzoic acid, 3-nitrotoluene, and 4-nitrotoluene

using [276]. Similarly, the degradation of azo dyes and nitroaromatics by NDP(H)-dependent azoreductase from an alkaliphilic strain *Aquiflexum* sp. DL6 is reported [277].

Petroleum industries and oil refineries often contaminate aquatic and terrestrial environments with a range of hydrocarbons. Removal of these pollutants has always been important, and it seems that alkaliphiles have a great potential in remediation of environments polluted with hydrocarbons and its derivatives. An array of alkaliphiles belonging to the genera *Dietzia*, *Micrococcus*, *Bacillus*, *Oceanobacillus*, *Citricoccus*, *Cronobacter*, *Marinobacter*, *Psychralcaliphila*, *Halomonas*, *Alteromonas*, etc. are capable of efficiently degrading and metabolizing these pollutants [194, 278–282]. Moreover, benzoates and pyrenes which are widely used in several industries and appear in many environments as pollutants can be degraded and removed by alkaliphilic microbes such as *Bacillus krulwichiae*, *Mycobacterium* sp., *Halomonas campisalis*, and *Bacillus badius* [283–285], a phenomenon which indicates the alkaliphiles' bioremediation potential.

Activities such as jewelry-making, gold mining, steel and aluminum processing, electroplating, and production of nitrile pesticides generate highly toxic cyanide-containing wastewater which need to be treated. Although cyanides are known to be highly toxic and lethal, a handful of organisms are known to resist this effect. One of such organisms is the alkaliphilic strain of *Pseudomonas pseudoalcaligenes* which utilizes cyanide as sole nitrogen source and has been used effectively to treat wastewater containing cyanide [286, 287].

Heavy metal pollution is a serious concern not only due to its toxicity but also due to its recalcitrant nature. Unlike organic pollutants, heavy metals are not biodegradable, and removing these contaminants from polluted environments has been a challenge. Microbes which are capable of precipitating metal ions are very attractive in handling heavy metal pollution. In this regard, alkaliphiles which are known for their effective siderophores, organic acids, and innate abilities of reducing metals can be of great importance. For example, the alkaliphilic bacteria *Alkaliphilus metalliredigens* QYMF cells are known to effectively reduce iron in anaerobic alkaline condition and precipitate it [260, 261]; this shows the possibility of using such alkaliphiles in removing metal contaminants from alkaline effluents. The alkaliphile-induced metal precipitation is expected to reduce the metal mobility which subsequently prevents the spreading of the pollutant.

Another area where alkaliphiles are useful in managing waste is in the textile and leather industry. The roles of alkaliphiles in the leather industry are discussed in this volume(see [288]) and hence, will not be discussed here. In the textile industry, bleaching is performed using alkaline hydrogen peroxide which is accompanied by washing. However, if the copious amount of water used in the extensive washing of the bleaching agent must be recycled, the hydrogen peroxide should be removed. In this regard, the use of alkaline active catalases or alkaliphiles is the most attractive approach which provides economic, environmental, and technical rewards [289–292].

As a way of counteracting the high pH effect of their habitats, alkaliphiles often produce organic acids. This ability of alkaliphiles can be tapped to neutralize

alkaline effluents from different industries and avoids the use of mineral acids. This has been demonstrated by the alkaliphilic strain of *Exiguobacterium* which reduces the pH of an industrial waste from 12 to 7.5 [293] and an alkaliphilic strain of *Enterococcus faecium* which reduced a chlor-alkali industrial effluent pH from 12 to 7 within 3 h [294].

Alkaliphiles are also useful in managing poultry wastes, which are rich in keratin. Due to their tough to degrade nature, keratin wastes are often disposed by chemical and mechanical hydrolysis or by incineration to avoid their accumulation. But these approaches are not benign to the environment. On the other hand, the use of microbes or enzymes that degrade these wastes is desirable because of its efficiency in solubilizing keratin at milder conditions and its ability of valorizing the keratin waste to value-added products without any negative impact on the environment. Keratinolytic alkaliphiles can be used effectively in managing poultry wastes and an impressive range of publications that demonstrate this potential are available as reviewed by [295].

5 Concluding Remarks

Alkaliphiles have come a long way in the history of biotechnology, a journey depicted with several success stories. Indeed, today, alkaliphiles are well-established commercial sources of alkaline active enzymes used such as in detergent, paper and pulp, and leather and textile industries. With further studies, this application range of alkaline active enzymes can dramatically expand and play a crucial role in the enzyme market. To tap the great biocatalytic potential, it is imperative to expand the exploration to the nontraditional enzymes and look for new applications. However, as it stands now, most of the studies on enzymes of alkaliphiles focus on few hydrolases which are related to the existing established market. Moreover, the story of alkaliphiles goes well beyond alkaline active enzymes. Several other studies have shown the promising potential of alkaliphiles in the production of novel as well as known biochemicals of great biotechnological importance. On the other hand, the industrial application of all these interesting substances is still lagging behind. This may be partly due to the unavailability of the products in the market which discourage industrialists to try and apply it. There could be several reasons why these products are not available in the market. One of the bottlenecks that limit the production of the valuable substances may be the cultivation of alkaliphiles is not well-established unlike the cultivation of the common microbes such as yeast, *Aspergillus*, and *Escherichia coli*. Even most of the alkaline active enzymes available in the market are produced heterologously in non-alkaliphilic expression hosts. Thus, in order to efficiently utilize the full potentials of alkaliphiles, it is necessary to develop the cultivation system and develop few selected alkaliphiles as hosts for heterologous expression, metagenome library construction, metabolic engineering, and other related uses. It is delighting to see that some laboratories are moving in this direction and interesting results from expression of proteins as well as engineering of

alkaliphiles are trickling in. With such moves, one expects the rise in the interest among industrialists to realize the enormous potential of alkaliphiles.

References

1. Transparency (2016) Global Biotechnology market recovering from post-recession crunch, expected to reach US$414.5 bn by 2017. https://www.transparencymarketresearch.com/pressrelease/global-biotechnology-market.htm. Accessed 18 Feb 2020
2. Global Market Insights (2019) Biotechnology market size to exceed $729bn by 2025. https://www.gminsights.com/pressrelease/biotechnology-market. Accessed 18 Feb 2020
3. Fiala G, Stetter KO (1986) *Pyrococcus furiosus* sp. nov., represents a novel genus of marine heterotrophic archaebacteria growing optimally at 100°C. Arch Microbiol 145:56–61
4. Kisková J, Stramová Z, Javorský P, Sedláková-Kaduková J, Pristaš P (2019) Analysis of the bacterial community from high alkaline (pH > 13) drainage water at a brown mud disposal site near Žiar nad Hronom (Banská Bystrica region, Slovakia) using 454 pyrosequencing. Folia Microbiol 64:83–90
5. Mei N, Postec A, Erauso G, Joseph M, Pelletier B, Payri C et al (2016) *Serpentinicella alkaliphila* gen. nov., sp. nov., a novel alkaliphilic anaerobic bacterium isolated from the serpentinite-hosted Prony hydrothermal field, New Caledonia. Int J Syst Evol Microbiol 66:4464–4470
6. Schleper C, Pühler G, Kühlmorgen B, Zillig W (1995) Life at extremely low pH. Nature 375:741–742
7. Xu Y, Nogi Y, Kato C, Liang Z, Rüger HJ, De Kegel D, Glansdorff N (2003) *Moritella profunda* sp. nov. and *Moritella abyssi* sp. nov., two psychropiezophilic organisms isolated from deep Atlantic sediments. Int J Syst Evol Microbiol 53:533–538
8. Margesin R, Collins T (2019) Microbial ecology of the cryosphere (glacial and permafrost habitats): current knowledge. Appl Microbiol Biotechnol 103:2537–2549
9. Kashefi K, Lovley DR (2003) Extending the upper temperature limit for life. Science 301:934
10. Rothschild L, Mancinelli R (2001) Life in extreme environments. Nature 409:1092–1101
11. Grant S, Grant WD, Jones BE, Kato C, Lina L (1999) Novel archaeal phylotypes from an East Aftrican alkaline saltern. Extremophiles 3:139–145
12. Rampelotto PH (2013) Extremophiles and extreme environments. Life (Basel) 3:482–485
13. Satyanarayana T, Raghukumar C, Sisinthy S (2005) Extremophilic microbes: diversity and perspectives. Curr Sci 89:78–90
14. Ventosa A, de la Haba RR, Sánchez-Porro C, Papke RT (2015) Microbial diversity of hypersaline environments: a metagenomic approach. Curr Opin Microbiol 25:80–87
15. Aguilera A (2013) Eukaryotic organisms in extreme acidic environments, the Río Tinto case. Life (Basel) 3:363–374
16. Kavembe GD, Meyer A, Wood CM (2016) Fish populations in East African saline lakes in *Soda Lakes of East Africa*. Springer, Cham, pp 227–257
17. Lanzén A, Simachew A, Gessesse A, Chmolowska D, Jonassen I, Øvreås L (2013) Surprising prokaryotic and eukaryotic diversity, community structure and biogeography of Ethiopian soda lakes. PLoS One 8(8):e72577
18. Singh R, Kumar M, Mittal A, Mehta PK (2016) Microbial enzymes: industrial progress in 21st century. 3 Biotech 6:174. https://doi.org/10.1007/s13205-016-0485-8
19. Lentzen G, Schwarz T (2006) Extremolytes: natural compounds from extremophiles for versatile applications. Appl Microbiol Biotechnol 72:623–634
20. Van-Doan T, Hashim S, Hatti-Kaul R, Mamo G (2012) Ectoine mediated protection of enzyme from the effect of pH and temperature stress: a study using *Bacillus halodurans* xylanase as a model. Appl Microbiol Biotechnol 97:6271–6278

21. Aguilera JA, Bischof KB, Karsten UK, Hanelt DH, Wiencke CW (2002) Seasonal variation in ecophysiological patterns in macroalgae from an Arctic fjord. II. Pigment accumulation and biochemical defence systems against high light stress. Mar Biol 140:1087–1095
22. Bünger J (1999) Ectoine added protection and care for the skin. Eur Secur 7:22–24
23. de la Coba F, Aguilera J, de Galvez MV, Alvarez M, Gallego E, Figueroa FL, Herrera E (2009) Prevention of the ultraviolet effects on clinical and histopathological changes, as well as the heat shock protein-70 expression in mouse skin by topical application of algal UV-absorbing compounds. J Dermatol Sci 55:161–169
24. Gabani P, Singh OV (2013) Radiation-resistant extremophiles and their potential in biotechnology and therapeutics. Appl Microbiol Biotechnol 97:993–1004
25. Heinrich U, Garbe B, Tronnier H (2007) *In vivo* assessment of ectoin: a randomized, vehicle-controlled clinical trial. Skin Pharmacol Physiol 20:211–218
26. Llewellyn CA, Airs RL (2010) Distribution and abundance of MAAs in 33 species of microalgae across 13 classes. Mar Drugs 8:1273–1291
27. Roenneke B, Rosenfeldt N, Derya SM, Novak JF, Marin K, Krämer R, Seibold GM (2018) Production of the compatible solute α-d-glucosylglycerol by metabolically engineered *Corynebacterium glutamicum*. Microb Cell Fact 17:94
28. Giddings LA, Newman DJ (2015) Bioactive compounds from terrestrial extremophiles. Springer, Berlin
29. Bosma E, Oost J, De Vos W, van Kranenburg R (2013) Sustainable production of bio-based chemicals by extremophiles. Cur Biotechnol 2:360–379
30. Nicolaus B, Kambourova M, Oner ET (2010) Exopolysaccharides from extremophiles: from fundamentals to biotechnology. Environ Technol 31:1145–1158
31. Rodrigo-Baños M, Garbayo I, Vílchez C, Bonete MJ, Martínez-Espinosa RM (2015) Carotenoids from Haloarchaea and their potential in biotechnology. Mar Drugs 13:5508–5532
32. Barnard D, Casanueva A, Tuffin M, Cowan D (2010) Extremophiles in biofuel synthesis. Environ Technol 31:871–888
33. Demain AL, Newcomb M, Wu JH (2005) Cellulase, clostridia, and ethanol. Microbiol Mol Biol Rev 69:124–154
34. Jiang Y, Guo D, Lu J, Dürre P, Dong W, Yan W et al (2018) Consolidated bioprocessing of butanol production from xylan by a thermophilic and butanologenic *Thermoanaerobacterium* sp. M5. Biotechnol Biofuels 11:89. https://doi.org/10.1186/s13068-018-1092-1
35. Salameh M, Wiegel J (2007) Lipases from extremophiles and potential for industrial applications. Adv Appl Microbiol 61:253–283
36. Luca PD, Musacchio A, Taddei R (1981) Acidophilic algae from the fumaroles of Mount Lawu (Java), locus classicus of *Cyanidium caldarium* Geitler. Gionr Bot Ital 115:1–9
37. Pulz O, Gross W (2004) Valuable products from biotechnology of microalgae. Appl Microbiol Biotechnol 65:635–648
38. de Vrije T, de Haas GG, Tan GB, Keijsers ERP, Claassen PAM (2002) Pretreatment of *Miscanthus* for hydrogen production by *Thermotoga elfii*. Int J Hydrogen Energy 27:1381–1390
39. Ren N, Cao G, Wang A, Lee DJ, Guo W, Zhu Y (2008) Dark fermentation of xylose and glucose mix using isolated *Thermoanaerobacterium thermosaccharolyticum* W16. Int J Hydrogen Energy 33:6124–6132
40. Baker SE, Hopkins RC, Blanchette CD, Walsworth VL, Sumbad R, Fischer NO et al (2009) Hydrogen production by a hyperthermophilic membrane-bound hydrogenase in water-soluble nanolipoprotein particles. J Am Chem Soc 131:7508–7509
41. Nishimura H, Sako Y (2009) Purification and characterization of the oxygen-thermostable hydrogenase from the aerobic hyperthermophilic archaeon *Aeropyrum camini*. J Biosci Bioeng 108:299–303
42. Lipscomb GL, Schut GJ, Thorgersen MP, Nixon WJ, Kelly RM, Adams MW (2006) Engineering hydrogen gas production from formate in a hyperthermophile by heterologous production of an 18-subunit membrane-bound complex. J Biol Chem 289:2873–2879

43. Sun X, Griffith M, Pasternak J, Glick B (1995) Low temperature growth, freezing survival, and production of antifreeze protein by the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. Can J Microbiol 41:776–784
44. Rolli E, Marasco R, Vigani G, Ettoumi B, Mapelli F, Deangelis ML et al (2015) Improved plant resistance to drought is promoted by the root-associated microbiome as a water stress-dependent trait. Environ Microbiol 17:316–331
45. Saikia J, Sarma RK, Dhandia R, Yadav A, Bharali R, Gupta VK, Saikia R (2018) Alleviation of drought stress in pulse crops with ACC deaminase producing rhizobacteria isolated from acidic soil of Northeast India. Sci Rep 8:3560. https://doi.org/10.1038/s41598-018-21921-w
46. Ullah A, Nisar M, Ali H, Hazrat A, Hayat K, Keerio AA et al (2019) Drought tolerance improvement in plants: an endophytic bacterial approach. Appl Microbiol Biotechnol 103:7385–7397
47. Kearl J, McNary C, Lowman JS, Mei C, Aanderud ZT, Smith ST et al (1997) Salt-tolerant halophyte rhizosphere bacteria stimulate growth of alfalfa in salty soil. Front Microbiol 10:1849. https://doi.org/10.3389/fmicb.2019.01849
48. Hidayati N, Juhaeti T, Fauzia S (2009) Mercury and cyanide contaminations in gold mine environment and possible solution of cleaning up by using phytoextraction. HAYATI J Biosci 16:88–94
49. Podar M, Reysenbach AL (2006) New opportunities revealed by biotechnological explorations of extremophiles. Curr Opin Biotechnol 17:250–255
50. Vera M, Schippers A, Sand W (2013) Progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation – part A. Appl Microbiol Biotechnol 97:7529–7541
51. Herschy B, Whicher A, Camprubí C, Eloi WC, Dartnell L et al (2014) An origin-of-life reactor to simulate alkaline hydrothermal vents. J Mol Evol 79:213–227
52. Sojo V, Herschy B, Whicher A, Camprubí E, Lane N (2016) The origin of life in alkaline hydrothermal vents. Astrobiology 16:181–197
53. Horikoshi K (1991) Microorganisms in alkaline environments. Kodansha-VCH, Tokyo
54. Kevbrin VV (2019) Isolation and cultivation of alkaliphiles. Adv Biochem Eng Biotechnol. https://doi.org/10.1007/10_2018_84
55. Lebre PH, Cowan DA (2019) Genomics of alkaliphiles. Adv Biochem Eng Biotechnol. https://doi.org/10.1007/10_2018_83
56. Cheevadhanarak S, Paithoonrangsarid K, Prommeenate P, Kaewngam W, Musigkain A, Tragoonrung S et al (2012) Draft genome sequence of *Arthrospira platensis* C1 (PCC9438). Stand Genomic Sci 6:43–53
57. Takami H, Nakasone K, Takaki Y, Maeno G, Sasaki R, Masui N et al (2000) Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with Bacillus subtilis. Nucleic Acids Res 28:4317–4331
58. Wernick DG, Choi KY, Tat CA, Lafontaine Rivera JG, Liao JC (2013) Genome sequence of the extreme obligate alkaliphile *Bacillus marmarensis* strain DSM 21297. Genome Announc 1 (6):e00967-e00913. Doi:https://doi.org/10.1128/genomeA.00967-13
59. Grant WD, Heaphy S (2010) Metagenomics and recovery of enzyme genes from alkaline saline environments. Environ Technol 31:1135–1143
60. Mamo G (2019) Challenges and adaptations of life in alkaline habitats. Adv Biochem Eng Biotechnol. https://doi.org/10.1007/10_2019_97
61. Röhm O (1913) German Patent DE 283923
62. Cameron BA (2007) Laundering in cold water: detergent considerations for consumers. Fam Consum Sci Res J 36:151–162
63. Golden JS, Subramanian V, Irizarri GMAU, White P, Meier F (2010) Energy and carbon impact from residential laundry in the United States. J Integr Environ Sci 7:53–73
64. Sundus H, Mukhtar H, Nawaz A (2016) Industrial applications and production sources of serine alkaline proteases: a review. J Bacteriol Mycol Open Acces 3:191–194

65. Kirschner EM (1997) Soaps and detergents: despite price pressures, ingredients suppliers aim to provide better quality and service, address environmental concerns, and introduced new products. Chem Eng News 75:30–46
66. Chen SJ, Cheng CY, Chen TL (1998) Production of an alkaline lipase by A*cinetobacter radioresistens*. J Ferment Bioeng 86:308–312
67. Cherif S, Sami M, Hadrich F, Abdelkafi S, Sayadi S (2011) A newly high alkaline lipase: an ideal choice for application in detergent formulations. Lipids Health Dis 10:221
68. Wang YX, Srivastava KC, Shen GJ, Wang HY (1995) Thermostable alkaline lipase from a newly isolated thermophilic *Bacillus*, strain A30-1 (ATCC 53841). J Ferment Bioeng 79:433–438
69. Ito S, Shikata S, Ozaki K, Kawai S, Okamoto K, Inoue S et al (1989) Alkaline cellulase for laundry detergents: production by *Bacillus* sp. KSM-635 and enzymatic properties. Agric Biol Chem 53:1275–1281
70. Karmakar M, Ray RR (2011) Current trends in research and application of microbial cellulases. Res J Microbiol 6:41–53
71. Bettiol JP, Showell MS (2002) Detergent compositions comprising a mannanase and a protease, US Patent No. 6 376 445
72. Kumar BK, Balakrishnan H, Rele MV (2004) Compatibility of alkaline xylanases from an alkaliphilic *Bacillus* NCL (87-6-10) with commercial detergents and proteases. J Ind Microbiol Biotechnol 31:83–87
73. Bruhlmann F, Kim KS, Zimmerman W, Fletcher A (1994) Pectinolytic enzymes from *Actinomycetes* for degumming of ramie bast fibers. Appl Environ Microbiol 60:2107–2112
74. Hoondal GS, Tiwari RP, Tewari R, Dahiya N, Beg QK (2002) Microbial alkaline pectinases and their industrial applications: a review. Appl Microbiol Biotechnol 59:409–418
75. Dalmay P, Smith A, Chotard T, Turner PS, Gloaguen V, Krausz P (2010) Properties of cellulosic fibre reinforced plaster: influence of hemp or flax fibres on the properties of set gypsum. J Mater Sci 45:793–803
76. Shah DU, Schubel PJ, Clifford MJ (2013) Can flax replace E-glass in structural composites? A small wind turbine blade case study. Compos Part B 52:172–181
77. Takagi H (2019) Review of functional properties of natural fiber-reinforced polymer composites: thermal insulation, biodegradation and vibration damping properties. Adv Compos Mater 28:525–543
78. Cao J, Zheng L, Chen S (1992) Screening of pectinase producer from alkalophilic bacteria and study on its potential application in degumming of ramie. Enzyme Microb Technol 14:1013–1016
79. Kapoor M, Beg QK, Bhushan B, Singh K, Dadhich KS, Hoondal GS (2001) Application of an alkaline and thermostable polygalacturonase from *Bacillus* sp. MG-cp-2 in degumming of ramie (*Boehmeria nivea*) and sunn hemp (*Crotalaria juncea*) bast fibers. Process Biochem 36:803–807
80. Kobayashi T, Higaki N, Suzumatsu A, Sawada K, Hagihar H, Kawai S, Ito S (2001) Purification and properties of a high-molecular-weight, alkaline exopolygalacturonase from a strain of *Bacillus*. Enzyme Microb Technol 29:70–75
81. Kobayashi T, Koike K, Yoshimatsu T, Higaki N, Suzumatsu A, Ozawa T et al (1999) Purification and properties of a low-molecular-weight, high-alkaline pectate lyase from an alkaliphilic strain of *Bacillus*. Biosci Biotechnol Biochem 63:65–72
82. Sorokin D, Panteleeva A, Tourova T, Kaparullina E, Muyzer G (2011) *Natronoflexus pectinivorans* gen. nov. sp. nov., an obligately anaerobic and alkaliphilic fermentative member of bacteroidetes from soda lakes. Extremophiles 15:691–696
83. Doshi R, Shelke V (2001) Enzymes in the textile industry-an environment-friendly approach. Indian J Fibre Text Res 26:202–206
84. Shelke V (2001) Enzymatic decolourization of denims: a novel approach. Colourage 48(1):25
85. Anish R, Rahman MS, Rao M (2007) Application of cellulases from an alkalothermophilic *Thermomonospora* sp. in biopolishing of denims. Biotechnol Bioeng 96:48–56

86. Fukumori F, Kudo NT, Horikoshi K (1985) Purification and properties of a cellulase from alkaliphilic *Bacillus* no 1139. Gen Microbiol 131:3339–3345
87. Kim J, Hur S, Hong J (2005) Purification and characterization of an alkaline cellulase from a newly isolated alkalophilic *Bacillus* sp. HSH-810. Biotechnol Lett 27:313–316
88. Global Paper and Pulp Market (2019) Paper and pulp market 2019 industry size, trends, global growth, insights and forecast research report 2024. https://www.360marketupdates.com/global-paper-and-pulp-market-13684507. Accessed 12 Feb 2020
89. Statista (2019) Distribution of pulp production worldwide in 2017 by region. https://www.statista.com/statistics/596035/pulp-production-distribution-worldwide-by-region/. Accessed 23 Jan 2020
90. Statista (2019) Paper industry – statistics & facts. https://www.statista.com/topics/1701/paper-industry/. Accessed 23 Jan 2020
91. Abad S, Santos V, Parajó JC (2001) Totally chlorine-free bleaching of Acetosolv pulps: a clean approach to dissolving pulp manufacture. J Chem Technol Biotechnol 76:1117–1123
92. AET (Alliance for Environmental Technology) (2006) Trends in world bleached chemical pulp production: 1990-2005. http://www.aet.org/science_of_ecf/eco_risk/2005_pulp.html. Accessed 9 Feb 2020
93. Arnand A, Sharma N, Mishra S, Bajpai P, Lachenal D (2006) Enzymes improve ECF bleaching of pulp. Bioresources 1:34–44
94. Gangwar A, Prakash R, Nagaraja Tejo P (2014) Applicability of microbial xylanases in paper pulp bleaching: a review. Bioresources 9:3733–3754
95. Lin XQ, Han SY, Zhang N, Hu H, Zheng SP, Ye YR, Lin Y (2013) Bleach boosting effect of xylanase a from *Bacillus halodurans* C-125 in ECF bleaching of wheat straw pulp. Enzyme Microb Technol 52:91–98
96. Singh A, Kuhad ARC, Ward OP (2007) Industrial application of microbial cellulases. In: Kuhad RC, Singh A (eds) Lignocellulose biotechnology: future prospects. I.K. International Publishing House, New Delhi, pp 345–358
97. Suominen P, Reinikainen T (1993) Foundation for biotechnical and industrial fermentation research, in proceedings of the 2nd symposium on *Trichoderma reesei* cellulases and other hydrolases (TRICEL '93), vol 8, Espoo, Finland
98. Thakur VV, Jain RK, Mathur RM (2012) Studies on xylanase and laccase enzymatic prebleaching to reduce chlorine-based chemicals during CEH and ECF bleaching. Bioresources 7:2220–2235
99. Viikari L, Suurnäkki A, Grönqvist S, Raaska L, Ragauskas A (2009) Forest products: biotechnology in pulp and paper processing. In: Encyclopedia of microbiology. Academic Press, New York, pp 80–94
100. Akhtar M (1994) Biochemical pulping of aspen wood chips with three strains of *Ceriporiopsis subvermispora*. Holzforschung 48:199–202
101. Bhat MK (2000) Cellulases and related enzymes in biotechnology. Biotechnol Adv 18:355–383
102. Pere J, Puolakka A, Nousiainen P, Buchert J (2001) Action of purified *Trichoderma reesei* cellulases on cotton fibers and yarn. J Biotechnol 89:247–255
103. Dienesm D, Egyházi A, Réczey K (2004) Treatment of recycled fiber with *Trichoderma* cellulases. Ind Crop Prod 20:11–21
104. Mansfield SD, Wong KKY, De Jong E, Saddler JN (1996) Modification of Douglas-fir mechanical and kraft pulps by enzyme treatment. Tappi J 79:125–132
105. Virk A, Sharma P, Capalash N (2012) Use of laccase in pulp and paper industry. Biotechnol Prog 28:21–32
106. Chen Y, Wan J, Ma Y, Tang B, Han W, Ragauskas AR (2012) Modification of old corrugated container pulp with laccase and laccase - mediator system. Bioresour Technol 110:297–301
107. Mocchiutti P, Zanuttini M, Kruus K, Suurnäkki A (2008) Improvement of the fiber-bonding capacity of unbleached recycled pulp by the Laccase/Mediator treatment. Tappi J 7:17–22

108. Saxena A, Chauhan PS (2017) Role of various enzymes for deinking paper: a review. Crit Rev Biotechnol 37:598–612
109. Pathak P, Bhardwaj NK, Singh AK (2011) Optimization of chemical and enzymatic deinking of photocopier waste paper. BioRes 6:447–463
110. Knutson K, Ragauskas A (2004) Laccase-mediator biobleaching applied to a direct yellow dyed paper. Biotechnol Prog 20:1893–1896
111. Leduc C, Lanteigne-Roch LM, Daneault C (2011) Use of enzymes in deinked pulp bleaching. Cell Chem Technol 45:657–663
112. Mohandass C, Raghukumar C (2005) Biological deinking of inkjet-printed paper using *Vibrio alginolyticus* and its enzymes. J Ind Microbiol Biotechnol 32:424–429
113. Singh G, Arya SK (2019) Utility of laccase in pulp and paper industry: a progressive step towards the green technology. Int J Biol Macromol 134:1070–1084
114. Kuhad RC, Mehta G, Gupta R, Sharma KK (2010) Fed batch enzymatic saccharification of newspaper cellulosics improves the sugar content in the hydrolysates and eventually the ethanol fermentation by *Saccharomyces cerevisiae*. Biomass Bioenergy 34:1189–1194
115. Salonen SM (1990) Method for manufacturing paper or cardboard and product containing cellulase. US patent 4980023
116. Wielen LCV, Panek JC, Pfromm PH (1999) Fracture of toner due to paper swelling. Tappi J 82:115–121
117. Shrinath A, Szewczak JT, Bowen IJ (1991) A review of ink removal techniques in current deinking technology. Tappi J 74:85–93
118. Chakrabarti S, Verma P, Tripathi S, Barnie S, Varadhan R (2011) Stickies: management and control. IPPTA J 23:101–107
119. Friberg T (1996) Cost impact of stickies. Prog Pap Recycl 6:70–72
120. Oldack RC, Gustafson FJ (2005) Initiative to promote environmentally benign adhesives (IPEBA): solving a sticky issue. Prog Pap Recycl 14:6–8
121. Venditti RA, Lucas BE, Huo X, Jameel H, Chang HM (2007) Paper recycling factors affecting the screening of pressure sensitive adhesives. Prog Pap Recycl 16:18–31
122. Fabry B, Delagoutte T, Serieys L, Schelcher M (2014) Enzyme in paper recycling: effect of enzyme on stickies. ATIP Association Technique de L'Industrie Papetiere 68:10–18
123. Zhang E, Zhai W, Luo Y, Scott K, Wang X, Diao G (2016) Acclimatization of microbial consortia to alkaline conditions and enhanced electricity generation. Bioresour Technol 211:736–742
124. Savile CK, Janey JM, Mundorff EC, Moore JC, Tam S, Jarvis WR et al (2010) Biocatalytic asymmetric synthesis of chiral amines from ketones applied to sitagliptin manufacture. Science 329:305–309
125. Xie X, Watanabe K, Wojcicki WA, Wang CC, Tang Y (2006) Biosynthesis of lovastatin analogs with a broadly specific acyltransferase. Chem Biol 13:1161–1169
126. Yazawa K, Numata K (2014) Recent advances in chemoenzymatic peptide syntheses. Molecules 19:13755–13774
127. Narai-Kanayama A, Hanaishi T, Aso K (2012) α-Chymotrypsin-catalyzed synthesis of poly-L-cysteine in a frozen aqueous solution. J Biotechnol 157:428–436
128. Uyama H, Fukuoka T, Komatsu I, Watanabe T, Kobayashi S (2002) Protease-catalyzed regioselective polymerization and copolymerization of glutamic acid diethyl ester. Biomacromolecules 3:318–323
129. Takahagi W, Seo K, Shibuya T, Takano Y, Fujishima K, Saitoh M et al (2019) Peptide synthesis under the alkaline hydrothermal conditions on Enceladus. ACS Earth Space Chem 3:2559–2568
130. Baker PJ, Numata K (2012) Chemoenzymatic synthesis of poly(L-alanine) in aqueous environment. Biomacromolecules 13:947–951
131. Chen ST, Chen SY, Hsiao SC, Wang KT (1991) Application of industrial protease "Alcalase" in peptide synthesis. Biomed Biochim Acta 50:S181–S186

132. Gupta A, Khare SK (2007) Enhanced production and characterization of a solvent stable protease from solvent tolerant *Pseudomonas aeruginosa* PseA. Enzyme Microb Technol 42:11–16
133. Jaouadi B, Badis A, Fodil D, Ferradji F, Rekik H, Zaraî N, Bejar S (2010) Purification and characterization of a thermostable keratinolytic serine alkaline proteinase from *Streptomyces* sp strain AB1 with high stability in organic solvents. Bioresour Technol 101:8361–8369
134. Sen S, Dasu VV, Dutt K, Mandal B (2011) Characterization of a novel surfactant and organic solvent stable high-alkaline protease from new *Bacillus pseudofirmus* SVB1. Res J Microbiol 6:769–783
135. Yadav SK, Bisht D, Shikha DNS (2011) Oxidant and solvent stable alkaline protease from *Aspergillus flavus* and its characterization. Afr J Biotechnol 10:8630–8640
136. Wang CH, Guan Z, He YH (2011) Biocatalytic domino reaction: synthesis of 2H-1-benzopyran-2-one derivatives using alkaline protease from *Bacillus licheniformis*. Green Chem 13:2048–2054
137. Wang N, Wu Q, Liu BK, Cai Y, Lin XF (2004) Enzyme catalyzed regioselective synthesis of lipophilic guaifenesin ester derivatives. J MolCatal B: Enzym 27:97–102
138. Fujinami S, Fujisawa M (2010) Industrial applications of alkaliphiles and their enzymes – past, present and future. Environ Technol 32:845–856
139. Qi Q, Zimmermann W (2005) Cyclodextrin glucanotransferase: from gene to applications. Appl Microbiol Biotechnol 66:475–485
140. Fior Markets (2019) Global cyclodextrin market growth 2019–2024. https://www.fiormarkets.com/report/global-cyclodextrin-market-growth-2019-2024-373093.html. Accessed 10 Feb 2020
141. Hashim SO (2019) Starch-modifying enzymes. Adv Biochem Eng Biotechnol. https://doi.org/10.1007/10_2019_91
142. Ameri A, Shakibaie M, Faramarzi MA, Ameri A, Amirpour-Rostami S, Rahimi HR, Forootanfar H (2017) Thermoalkalophilic lipase from an extremely halophilic bacterial strain *Bacillus atrophaeus* FSHM2: purification, biochemical characterization and application. Biocatal Biotransformation 35:151–160
143. Grosse S, Bergeron H, Imura A, Boyd J, Wang S, Kubota K et al (2010) Nature versus nurture in two highly enantioselective esterases from *Bacillus cereus* and *Thermoanaerobacter tengcongensis*. J Microbial Biotechnol 3:65–73
144. Woo JH, Kang JH, Hwang YO, Cho JC, Kim SJ, Kang SG (2010) Biocatalytic resolution of glycidyl phenyl ether using a novel epoxide hydrolase from a marine bacterium, *Rhodobacterales bacterium* HTCC2654. J Biosci Bioeng 110:509–509
145. Wei XL, Jiang XW, Ye LD, Yuan SF, Chen ZR, Wu M et al (2013) Cloning, expression and characterization of a new enantioselective esterase from a marine bacterium *Pelagibacterium halotolerans* B2T. J Mol Catal B: Enzym 97:270–277
146. Aino K, Hirota K, Okamoto T, Tu Z, Matsuyama H, Yumoto I (2018) Microbial communities associated with indigo fermentation that thrive in anaerobic alkaline environments. Front Microbiol 9:2196
147. Nakajima K, Hirota K, Nodasaka Y, Yumoto I (2005) *Alkalibacterium iburiensesp*. nov., an obligate alkaliphile that reduces an indigo dye. Int J Syst Evol Microbiol 55:1525–1530
148. Padden AN, Dillon VM, Edmonds J, Collins MD, Alvarez N, John P (1999) An indigo-reducing moderate thermophile from a woad vat, *Clostridium isatidis* sp. nov. Int J Syst Bacteriol 49:1025–1031
149. Takahara Y, Tanabe O (1960) Studies on the reduction of indigo in industrial fermentation vat (VII). J Ferment Technol 38:329–331
150. Park S, Ryu JY, Seo J, Hur HG (2012) Isolation and characterization of alkaliphilic and thermotolerant bacteria that reduce insoluble indigo to soluble leuco-indigo from indigo dye vat. J Korean Soc Appl Biol Chem 55:83–88

151. Yan L, Chen P, Zhang S, Li S, Yan X, Wang N, Liang N, Li H (2016) Biotransformation of ferulic acid to vanillin in the packed bed-stirred fermenters. Sci Rep 6:34644. https://doi.org/10.1038/srep34644
152. Danesh A, Mamo G, Mattiasson B (2011) Production of haloduracin by *Bacillus halodurans* using solid-state fermentation. Biotechnol Lett 33:1339–1344
153. Lawton EM, Cotter PD, Hill C, Ross RP (2007) Identification of a novel two-peptide lantibiotic, Haloduracin, produced by the alkaliphile *Bacillus halodurans* C-125. FEMS Microbiol Lett 267:64–71
154. Chun JY, Ryu IH, Park JS, Lee KS (2002) Anticaries activity of antimicrobial material from *Bacillus alkalophilshaggy* JY-827. J Microbial Biotechnol 12:18–24
155. Vasavada SH, Thumar J, Singh SP (2006) Secretion of a potent antibiotic by salt-tolerant and alkaliphilic actinomycete *Streptomyces sannanensis* strain RJT-1. Curr Sci 91:1393–1397
156. Singh LS, Mazumder S, Bora TC (2009) Optimisation of process parameters for growth and bioactive metabolite produced by a salt-tolerant and alkaliphilic actinomycete, *Streptomyces tanashiensis* strain A2D. J Mycol Med 19:225–233
157. Kharat KR, Kharat A, Hardikar BP (2009) Antimicrobial and cytotoxic activity of *Streptomyces* sp. from Lonar Lake. Afr J Biotechnol 8:6645–6648
158. Thumar JT, Dhulia K, Singh SP (2010) Isolation and partial purification of an antimicrobial agent from halotolerant alkaliphilic *Streptomyces aburaviensis* strain Kut-8. World J Microbiol Biotechnol 26:2081–2087
159. Ding ZG, Li MG, Zhao JY, Ren J, Huang R, Xie MJ et al (2010) Naphthospironone a: an unprecedented and highly functionalized polycyclic metabolite from an alkaline mine waste extremophile. Chemistry 16:3902–3905
160. Deshmukh D, Puranik P (2010) Application of Plackett-Burman design to evaluate media components affecting antibacterial activity of alkaliphilic cyanobacteria isolated from Lonar Lake. Turk J Biochem 35:114–120
161. Neelam DK, Agrawal A, Tomer AK, Bandyopadhayaya S, Sharma A, Jagannadham MV et al (2019) A *Piscibacillus* sp. isolated from a soda lake exhibits anticancer activity against breast cancer MDA-MB-231 cells. Microorganisms 7:34. https://doi.org/10.3390/microorganisms7020034
162. Dieter A, Hamm A, Fiedler HP, Goodfellow M, Müller WEG, Brun R, Beil W, Bringmann G (2003) Pyrocoll, an antibiotic, antiparasitic and antitumor compound produced by a novel alkaliphilic *Streptomyces* strain. J Antibiot 56:639–646
163. Höltzel A, Dieter A, Schmid DG, Brown R, Goodfellow M, Beil W et al (2003) Lactonamycin Z, an antibiotic and antitumor compound produced by *Streptomyces sanglieri* strain AK 623. J Antibiot 56:1058–1061
164. Li YQ, Li MG, Li W, Zhao JY, Ding ZG et al (2007) Griseusin D, a new pyranonaphthoquinone derivative from a alkaphilic *Nocardiopsis* sp. J Antibiot 60:757–761
165. Bisht G, Bharti A, Kumar V, Gusain O (2012) Isolation, purification and partial, characterization of an antifungal agent produced by salt-tolerant alkaliphilic *Streptomyces violascens* IN2-10. Proc Natl Acad Sci India Sect B Biol Sci 83:109–117
166. Wang Z, Fu P, Liu P, Wang P, Hou J, Li WJ, Zhu W (2013) New pyran-2-ones from alkalophilic actinomycete, *Nocardiopsis alkaliphila* sp. Nov. YIM-80379. Chem Biodivers 10:281–287
167. Helaly SE, Goodfellow M, Zinecker H, Imhoff JF, Süssmuth RD, Fiedler HP (2013) Warkmycin, a novel angucycline antibiotic produced by *Streptomyces* sp. Acta 2930. J Antibiot 66:669–674
168. Adlin J, Remya R, Velmurugan S, Babu M, Citarasu T (2018) *Streptomyces castaneoglobisporus* AJ9, a haloalkaliphilic actinomycetes isolated from solar salt works in southern India and its pharmacological properties. Indian J Mar Sci 47:475–488
169. Daliri EB, Oh DH, Lee BH (2017) Bioactive peptides. Foods 6(5):32. https://doi.org/10.3390/foods6050032

170. Li J, Chi Z, Wang X, Peng Y, Chi Z (2009) The selection of alkaline protease-producing yeasts from marine environments and evaluation of their bioactive peptide production. Chinese J Oceanol Limnol 27:753–761
171. Kim W, Choi K, Kim Y, Park H, Choi J, Lee Y, Oh H, Kwon I, Lee S (1996) Purification and characterization of a fibrinolytic enzyme produced from *Bacillus* sp. strain CK 11-4 screened from Chungkook-Jang. Appl Environ Microbiol 62:2482–2488
172. Mukherjee AK, Rai SK (2011) A statistical approach for the enhanced production of alkaline protease showing fibrinolytic activity from a newly isolated gram-negative *Bacillus* sp. strain AS-S20-I. N Biotechnol 28:182–189
173. Simkhada JR, Mander P, Cho SS, You JC (2010) A novel fibrinolytic protease from *Streptomyces* sp. CS684. Process Biochem 45:88–93
174. Kudrya VA, Simonenko IA (1994) Alkaline serine proteinase and lectin isolation from the culture fluid of *Bacillus subtilis*. Appl Microbiol Biotechnol 41:505–509
175. Davidenko T, Chuenko AV, Kovalenko VN (1997) Immobilized elastoterase. Pharm Chem J 31:396–398
176. Mamo G, Mattiasson B (2016) Alkaliphilic microorganisms in biotechnology. In: Pabulo HR (ed) Biotechnology of extremophiles. Springer, Cham, pp 243–272
177. Fior Markets (2019) Global carotenoids market is expected to reach USD 3.59 billion by 2025. https://www.globenewswire.com/news-release/2019/10/15/1929461/0/en/Global-Carotenoids-Market-is-expected-to-reach-USD-3-59-billion-by-2025-Fior-Markets.html. Accessed 12 Feb 2020
178. Lee PC, Schmidt-Dannert C (2002) Metabolic engineering towards biotechnological production of carotenoids in microorganisms. Appl Microbiol Biotechnol 60:1–11
179. Mata-Gómez LC, Montañez JC, Méndez-Zavala A, Aguilar CN (2014) Biotechnological production of carotenoids by yeasts: an overview. Microb Cell Fact 13:12
180. Miyashita K (2009) Function of marine carotenoids. Forum Nutr 61:136–146
181. Palozza P, Torelli C, Boninsegna A, Simone R, Catalano A, Mele MC, Picci N (2009) Growth-inhibitory effects of the astaxanthin-rich alga *Haematococcus pluvialis* in human colon cancer cells. Cancer Lett 283:108–117
182. Jyonouchi H, Sun S, Gross M (1995) Effect of carotenoids on in vitro immunoglobulin production by human peripheral blood mononuclear cells: Astaxanthin, a carotenoid without vitamin A activity, enhances in vitro immunoglobulin production in response to a T-dependent stimulant and antigen. Nutr Cancer 23:171–183
183. Osanjo GO, Muthike EW, Tsuma L, Okoth MW, Bulimo WD, Lünsdorf H et al (2009) A salt Lake extremophile, *Paracoccus bogoriensis* sp. nov., efficiently produces xanthophyll carotenoids. Afr J Microbiol Res 3:426–433
184. Khalikova E, Somersalo S, Korpela T (2019) Metabolites produced by alkaliphiles with potential biotechnological applications. Adv Biochem Eng Biotechnol. https://doi.org/10.1007/10_2019_96
185. Sarethy IP, Saxena Y, Kapoor A, Sharma M, Sharma SK, Gupta V, Gupta S (2011) Alkaliphilic bacteria: applications in industrial biotechnology. J Ind Microbiol Biotechnol 38:769–790
186. Asao M, Takaichi S, Madigan MT (2012) Amino acid-assimilating phototrophic heliobacteria from soda lake environments: *Heliorestis acidaminivorans* sp. nov. and 'Candidatus Heliomonas lunata'. Extremophiles 16:585–595
187. Drechsel H, Jung G (1998) Peptide siderophores. J Pept Sci 4:147–181
188. Melack JM, Kilham P (1974) Photosynthetic rates of phytoplankton in East African alkaline, saline lakes. Limnol Oceanogr 19:743–755
189. McMillan DGG, Velasquez I, Nunn BL, Goodlett DR, Hunter KA, Lamont I, Sander SG, Cook GM (2010) Acquisition of iron by alkaliphilic Bacillus species. Appl Environ Microbiol 76:6955–6961

190. Figueroa LS, Schwarz B, Richards AM (2015) Structural characterization of amphiphilic siderophores produced by a soda lake isolate, *Halomonas* sp. SL01, reveals cysteine, phenylalanine and proline containing head groups. Extremophiles 19:1183–1192
191. Anjum F, Gautam G, Edgard G, Negi S (2016) Biosurfactant production through Bacillus sp. MTCC 5877 and its multifarious applications in food industry. Bioresour Technol 213:262–269
192. Karlapudi AP, Venkateswarulu TC, Tammineedi J, Kanumuri L, Ravuru BK, Dirisala VR, Kodali VP (2018) Role of biosurfactants in bioremediation of oil pollution-a review. Petroleum 4:241–249
193. Maximize Market Research (2019) Biosurfactant market – global industry analysis and forecast (2019–2026) by type, by application and by geography. https://www.maximizemarketresearch.com/market-report/global-biosurfactants-market/433/. Accessed 10 Feb 2020
194. Jain R, Mody K, Mishra A, Jha B (2012) Isolation and structural characterization of biosurfactant produced by an alkaliphilic bacterium *Cronobacter sakazakii* isolated from oil contaminated wastewater. Carbohydr Polym 87:2320–2326
195. Tambekar DH, Dose PN, Gunjakar SR, Gadakh PV (2012) Studies on biosurfactant production from Lonar Lake's *Achromobacter xylosoxidans* bacterium. Int J Adv Pharm Biol Chem 1:415–419
196. Shende AM (2013) Studies on biosurfactant from *Exiguobacterium* sp. Sci Res Repor 3:193–199
197. Selim S, El-Alfy S, Hagagy N, Hassanin A, Khattab R, Syaed E (2012) Oil-biodegradation and biosurfactant production by haloalkaliphilic archaea isolated from soda lakes of the Wadi an Natrun, Egypt. J Pure Appl Microbiol 6:1011–1020
198. Zarinviarsagh M, Ebrahimipour G, Sadeghi H (2017) Lipase and biosurfactant from *Ochrobactrum intermedium* strain MZV101 isolated by washing powder for detergent application. Lipids Health Dis 16:177. https://doi.org/10.1186/s12944-017-0565-8
199. Andhare P, Chauhan K, Dave M, Pathak H (2014) Microbial exopolysaccharides: advances in applications and future prospects. In: Biotechnology volume 3: microbial biotechnology. Studium Press LLC, Houston, pp 1–25
200. Costa OYA, Raaijmakers JM, Kuramae EE (2018) Microbial extracellular polymeric substances: ecological function and impact on soil aggregation. Front Microbiol 9:1636
201. Florenzano G, Sili C, Pelosi E, Vincenzini M (1985) *Cyanospira rippkae* and *Cyanospira capsulata* (gen. nov. and spp. nov.): new filamentous heterocystous cyanobacteria from Magadi lake (Kenya). Arch Microbiol 140:301–306
202. Corsaro MM, Grant WD, Grant S, Marciano CE, Parrilli M (1999) Structure determination of an exopolysaccharide from an alkaliphilic bacterium closely related to *Bacillus spp*. Eur J Biochem 264:554–561
203. Joshi AA, Kanekar PP (2011) Production of exopolysaccharide by *Vagococcus carniphilus* MCM B-1018 isolated from alkaline Lonar Lake, India. Ann Microbiol 61:733–740
204. De Philippis R, Micheletti E (2009) Heavy metal removal with exopolysaccharide-producing cyanobacteria. In: Wang LK, Chen JP, Hung YT, Shammas NK (eds) Heavy metals in the environment. CRC Press, Boca Raton, pp 89–122
205. De Philippis R, Paperi R, Sili C (2007) Heavy metal sorption by released polysaccharides and whole cultures of two exopolysaccharide-producing cyanobacteria. Biodegradation 18:181–187
206. Zinicovscaia I, Yushin N, Shvetsova M, Frontasyeva M (2018) Zinc removal from model solution and wastewater by *Arthrospira* (Spirulina) *Platensis* biomass. Int J Phytoremediation 20:901–908
207. Han PP, Sun Y, Wu XY, Yuan YJ, Dai YJ, Jia SR (2014) Emulsifying, flocculating, and physicochemical properties of exopolysaccharide produced by cyanobacterium *Nostoc flagelliforme*. Appl Biochem Biotechnol 172:36–49

208. Liu C, Wang K, Jiang JH, Liu WJ, Wang JY (2015) A novel bioflocculant produced by a salt-tolerant, alkaliphilic and biofilm-forming strain *Bacillus agaradhaerens* C9 and its application in harvesting *Chlorella minutissima* UTEX2341. Biochem Eng J 93:166–172
209. Xu L, Yong H, Tu X, Wang Q, Fan J (2019) Physiological and proteomic analysis of *Nostoc flagelliforme* in response to alkaline pH shift for polysaccharide accumulation. Algal Res 39:101444. https://doi.org/10.1016/j.algal.2019.101444
210. Tenenbaum DJ (2008) Food vs. fuel: diversion of crops could cause more hunger. Environ Health Perspect 116:A254–A257
211. Blomqvist J, Eberhard T, Schnürer J, Passoth V (2010) Fermentation characteristics of *Dekkera bruxellensis* strains. Appl Microbiol Biotechnol 87:1487–1497
212. Sharma A, Kawarabayasi Y, Satyanarayana T (2012) Acidophilic bacteria and archaea: acid stable biocatalysts and their potential applications. Extremophiles 16:1–19
213. Jiang Y, Xin F, Lu J, Dong W, Zhang W, Zhang M et al (2017) State of the art review of biofuels production from lignocellulose by thermophilic bacteria. Bioresour Technol 245 (Pt B):1498–1506
214. Mamo G (2019) Alkaline active hemicellulases. Adv Biochem Eng Biotechnol. https://doi.org/10.1007/10_2019_101
215. Temudo MF, Kleerebezem R, van Loosdrecht MCM (2007) Influence of the pH on (open) mixed culture fermentation of glucose: a chemostat study. Biotechnol Bioeng 98:69–79
216. Temudo MF, Muyzer G, Kleerebezem R, van Loosdrecht MC (2008) Diversity of microbial communities in open mixed culture fermentations: impacts of the pH and carbon source. Appl Microbiol Biotechnol 80:1121–1130
217. Wernick DG, Pontrelli SP, Pollock AW, Liao JC (2016) Sustainable biorefining in wastewater by engineered extreme alkaliphile *Bacillus marmarensis*. Sci Rep 6:20224. https://doi.org/10.1038/srep20224
218. Ananyev G, Carrieri D, Dismukes GC (2008) Optimization of metabolic capacity and flux through environmental cues to maximize hydrogen production by the cyanobacterium "*Arthrospira* (*Spirulina*) *maxima*". Appl Environ Microbiol 74:6102–6113
219. Mussgnug JH, Klassen V, Schlüter A, Kruse O (2010) Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. J Biotechnol 150:51–56
220. Santos AM, Janssen M, Lamers PP, Evers WAC, Wijffels RH (2012) Growth of oil accumulating microalga *Neochloris oleoabundans* under alkaline saline conditions. Bioresour Technol 104:593–599
221. Bell TA, Prithiviraj B, Wahlen BD, Fields MW, Peyton BM (2016) A lipid-accumulating alga maintains growth in outdoor, alkaliphilic raceway pond with mixed microbial communities. Front Microbiol 6:1480. https://doi.org/10.3389/fmicb.2015.01480
222. Chowdhury R, Keen PL, Tao W (2019) Fatty acid profile and energy efficiency of biodiesel production from an alkaliphilic algae grown in the photobioreactor. Bioresour Technol Rep 6:229–236
223. Vadlamani A, Viamajala S, Pendyala B, Varanasi S (2017) Cultivation of microalgae at extreme alkaline pH conditions: a novel approach for biofuel production. ACS Sustain Chem Eng 5:7284–7294
224. Jiang X, Xue Y, Wang A, Wang L, Zhang G, Zeng Q, Yu B, Ma Y (2013) Efficient production of polymer-grade l-lactate by an alkaliphilic *Exiguobacterium* sp. strain under nonsterile open fermentation conditions. Bioresour Technol 143:665–668
225. Meng Y, Xue Y, Yu B, Gao C, Ma Y (2012) Efficient production of l-lactic acid with high optical purity by alkaliphilic *Bacillus* sp. WL-S20. Bioresour Technol 116:334–339
226. Assavasirijinda N, Ge D, Yu B, Xue Y, Ma Y (2016) Efficient fermentative production of polymer-grade d-lactate by an engineered alkaliphilic *Bacillus* sp. strain under non-sterile conditions. Microb Cell Fact 15:3. https://doi.org/10.1186/s12934-015-0408-0
227. Calabia BP, Tokiwa Y, Aiba S (2011) Fermentative production of l-(+)-lactic acid by an alkaliphilic marine microorganism. Biotechnol Lett 33:1429–1433

228. Yokaryo H, Tokiwa (2014) Isolation of alkaliphilic bacteria for production of high optically pure L-(+)-lactic acid. J Gen Appl Microbiol 60: 270–275
229. Yoshimune K, Yamamoto M, Aoyagi T, Yumoto I (2017) High and rapid L-lactic acid production by alkaliphilic *Enterococcus* sp. by adding wheat bran hydrolysate. Ferment Technol 6:1. https://doi.org/10.4172/2167-7972.1000138
230. Abdel-Rahman MA, Hassan SE, Azab MS, Mahin AA, Gaber MA (2019) High improvement in lactic acid productivity by new alkaliphilic bacterium using repeated batch fermentation integrated with increased substrate concentration. Biomed Res Int 2019:7212870. https://doi.org/10.1155/2019/7212870
231. Abdel-Rahman MA, Hassan SED, Azab MS, Gaber MA (2016) Effective production of lactic acid by a newly isolated alkaliphilic *Psychrobacter maritimus* BoMAir 5 strain. J Appl Biotechnol Bioeng 1:68–76
232. Olvera-Novoa MA, Dominguez-Cen LJ, Olivera-Castillo L, Martinez-Palacios CA (1998) Effect of the use of the microalga *Spirulina maxima* as fish meal replacement in diets for tilapia. Aquacult Res 29:709–715
233. Plavsic M, Terzic S, Ahel M, Van Den Berg CMG (2004) Folic acid in coastal waters of the Adriatic Sea. Mar Freshw Res 53:1245–1252
234. Prasanna R, Sood A, Jaiswal P, Nayak S, Gupta V, Chaudhary V et al (2010) Rediscovering cyanobacteria as valuable sources of bioactive compounds (review). Appl Biochem Microbiol 46:119–134
235. Rabelo SF, Lemes AC, Takeuchi KP, Frata MT, Monteiro de Carvalho JC, Danesi EDG (2013) Development of cassava doughnuts enriched with *Spirulina platensis* biomass. Braz J Food Technol 16:42–51
236. Chamorro G, Salazar M, Favila L, Bourges H (1996) Pharmacology and toxicology of the alga *Spirulina*. Rev Invest Clin 48:389–399
237. Gantar M, Svirčev Z (2008) Microalgae and cyanobacteria: food for thought. J Phycol 44:260–268
238. Allied Market Research (2019) Spirulina market outlook – 2026. https://www.alliedmarketresearch.com/spirulina-market. Accessed 25 Feb 2020
239. Holman BWB, Malau-Aduli AEO (2012) Spirulina as a livestock supplement and animal feed. J Anim Physiol Anim Nutr 97:615–623
240. Nuhu AA (2013) *Spirulina* (*Arthrospira*): An important source of nutritional and medicinal compounds. J Mar Biol 2013:325636
241. Ganuza E, Sellers CE, Bennett BW, Lyons EM, Carney LT (2016) A novel treatment protects *Chlorella* at commercial scale from the predatory bacterium *Vampirovibrio chlorellavorus*. Front Microbiol 7:848. https://doi.org/10.3389/fmicb.2016.00848
242. Rego D, Redondo LM, Geraldes V, Costa L, Navalho J, Pereira M (2014) Control of predators in industrial scale microalgae cultures with pulsed electric fields. Bioelectrochemistry 103:60–64
243. Fan Y, Hu H, Liu H (2007) Sustainable power generation in microbial fuel cells using bicarbonate buffer and proton transfer mechanisms. Environ Sci Technol 41:8154–8158
244. Zhang Z, Lan D, Zhou P, Li J, Yang B, Wang Y (2016) Control of sticky deposits in wastepaper recycling with thermophilic esterase. Cellul 24:1–11
245. Yumoto I, Nakamura A, Iwata H, Kojima K, Kusumoto K, Nodasaka Y, Matsuyama H (2002) *Dietzia psychralcaliphila* sp. nov., a novel, facultatively psychrophilic alkaliphile that grows on hydrocarbons. Int J Syst Evol Microbiol 52:85–90
246. Zhang T, Zhang L, Su W, Gao P, Li D, He X et al (2011) The direct electrocatalysis of phenazine-1-carboxylic acid excreted by *Pseudomonas alcaliphila* under alkaline condition in microbial fuel cells. Bioresour Technol 102:7099–7102
247. Inglesby AE, Fisher AC (2012) Enhanced methane yields from anaerobic digestion of *Arthrospira maxima* biomass in an advanced flow-through reactor with an integrated recirculation loop microbial fuel cell. Energ Environ Sci 5:7996–8006

248. Foley J, Rozendal R, Hertle C, Lant P, Rabaey K (2010) Life cycle assessment of high-rate anaerobic treatment, microbial fuel cells, and microbial electrolysis cells. Environ Sci Technol 44:3629–3637
249. Azuma M, Ojima Y (2018) Catalyst development of microbial fuel cells for renewable-energy production. In: Shiomi N (ed) Current topics in biochemical engineering. IntechOpen, London. https://doi.org/10.5772/intechopen.81442
250. Li XM, Cheng KY, Wong JW (2013) Bioelectricity production from food waste leachate using microbial fuel cells: effect of NaCl and pH. Bioresour Technol 149:452–458
251. Jayashree C, Tamilarasan K, Rajkumar M, Arulazhagan P, Yogalakshmi KN, Srikanth M et al (2016) Treatment of seafood processing wastewater using upflow microbial fuel cell for power generation and identification of bacterial community in anodic biofilm. J Environ Manage 180:351–358
252. Mamo G, Mattiasson B (2019) Alkaliphiles: the emerging biological tools enhancing concrete durability. Adv Biochem Eng Biotechnol. https://doi.org/10.1007/10_2019_94
253. Ferris F, Stehmeier L, Kantzas A, Mourits FM (1997) Bacteriogenic mineral plugging. J Can Petrol Technol 36:56–61
254. Gollapudi UK, Knutson CL, Bang SS, Islam MR (1995) A new method for controlling leaching through permeable channels. Chemosphere 30:695–705
255. Ivanov V, Chu J (2008) Applications of microorganisms to geotechnical engineering for bioclogging and biocementation of soil in situ. Rev Environ Sci Biotechnol 7:139–153
256. Dejong JT, Fritzges MB, Nüsslein K (2006) Microbially induced cementation to control sand response to undrained shear. J Geotech Geoenviron Eng 132:1381–1392
257. Canakci H, Sidik W, HalilKili I (2015) Effect of bacterial calcium carbonate precipitation on compressibility and shear strength of organic soil. Soils Found 55:1211–1221
258. Stabnikov V, Ivanov V, Chu J (2015) Construction biotechnology: a new area of biotechnological research and applications. World J Microbiol Biotechnol 31:1303–1314
259. Plank J (2004) Application of biopolymers and other biotechnological products in building material. Appl Microbiol Biotechnol 66:1–9
260. Ye Q, Roh Y, Carroll SL, Blair B, Zhou JZ, Zhang CL et al (2004) Alkaline anaerobic respiration: isolation and characterization of a novel alkaliphilic and metal-reducing bacterium. Appl Environ Microbiol 70:5595–5602
261. Roh Y, Chon CH, Moon JW (2007) Metal reduction and biomineralization by an alkaliphilic metal-reducing bacterium, *Alkaliphilus metalliredigens* (QYMF). Geosci J 11:415–423
262. Ibrahim A, Eltayeb M, Elbadawi Y, Alsalamah A (2011) Isolation and characterization of novel potent Cr(VI) reducing alkaliphilic *Amphibacillus* sp. KSUCR3 from hypersaline soda lakes. Electron J Biotechnol 14:4–4. https://doi.org/10.2225/vol14-issue4-fulltext-4
263. Balls PW, Liss PS (1983) Exchange of H_2S between water and air. Atmos Environ 187:735–742
264. de Rink R, Klok JBM, van Heeringen GJ, Keesman KJ, Janssen AJH, Ter Heijne A, Buisman CJN (2020) Biologically enhanced hydrogen sulfide absorption from sour gas under haloalkaline conditions. J Hazard Mater 383:121104. https://doi.org/10.1016/j.jhazmat.2019.121104
265. Sorokin DY, Van Den Bosch PLF, Abbas B, Janssen AJH, Muyzer G (2008) Microbiological analysis of the population of extremely haloalkaliphilic sulfur-oxidizing bacteria dominating in lab-scale sulfide-removing bioreactors. Appl Microbiol Biotechnol 80:965–975
266. Nikolova C, Gutierrez T (2020) Use of microorganisms in the recovery of oil from recalcitrant oil reservoirs: current state of knowledge, technological advances and future perspectives. Front Microbiol 10:2996. https://doi.org/10.3389/fmicb.2019.02996
267. Zhang B, Huston A, Whipple L, Barrett H, Wall M, Hutchins R, Mirakyan A (2013) A superior, high-performance enzyme for breaking borate crosslinked fracturing fluids under extreme well conditions. SPE Prod Oper 28:210–216

268. Saha P, Rao B (2019) Biotransformation of reactive Orange 16 by alkaliphilic bacterium *Bacillus flexus* VITSP6 and toxicity assessment of biotransformed metabolites. Int J Environ Sci Technol 17:99–114
269. Shobana S, Thangam B (2012) Biodegradation and decolorization of reactive Orange 16 by *Nocardiopsis alba* soil isolate. J Bioremed Biodegr 3:6. https://doi.org/10.4172/2155-6199.1000155
270. Prasad ASA, Rao KVB (2013) Aerobic biodegradation of azo dye by *Bacillus cohnii* MTCC 3616; an obligately alkaliphilic bacterium and toxicity evaluation of metabolites by different bioassay systems. Appl Microbiol Biotechnol 97:7469–7481
271. Lalnunhlimi S, Krishnaswamy V (2016) Decolorization of azo dyes (Direct Blue 151 and Direct Red 31) by moderately alkaliphilic bacterial consortium. Braz J Microbiol 47:39–46
272. Kovacic P, Somanathan R (2014) Nitroaromatic compounds: environmental toxicity, carcinogenicity, mutagenicity, therapy and mechanism. J Appl Toxicol 34:810–824
273. Purohit V, Basu AK (2000) Mutagenicity of nitroaromatic compounds. Chem Res Toxicol 13:673–692
274. Ju KS, Parales RE (2010) Nitroaromatic compounds, from synthesis to biodegradation. Microbiol Mol Biol Rev 74:250–272
275. Spain JC (1995) Biodegradation of nitroaromatic compounds. Annu Rev Microbiol 49:523–555
276. Misal S, Humne VI, Lokhande PD, Gawai KR (2015) Biotransformation of nitro aromatic compounds by flavin-free NADHAzoreductase. J Bioremed Biodegr 6:2
277. Misal SA, Lingojwar DP, Gawai KR (2013) Properties of NAD(P)H azoreductase from alkaliphilic red bacteria *Aquiflexum* sp. DL6. Protein J 32:601–608
278. Al-Awadhi H, Sulaiman RH, Mahmoud HM, Radwan SS (2007) Alkaliphilic and halophilic hydrocarbon-utilizing bacteria from Kuwaiti coasts of the Arabian gulf. Appl Microbiol Biotechnol 77:183–186
279. Mcgenity T, Whitby C, Fahy A (2010) Alkaliphilic hydrocarbon degraders. In: Timmis KN (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin, pp 1931–1937. https://doi.org/10.1007/978-3-540-77587-4_141
280. Sorkhoh NA, Al-Awadhi H, Al-Mailem DM, Kansour M, Khanafer M, Radwan SS (2010) Agarolytic bacteria with hydrocarbon-utilization potential in fouling material from the Arabian Gulf coast. Int Biodeter Biodegr 64:554–559
281. Sugimori D, Dake T, Nakamura S (2000) Microbial degradation of disodium terephthalate by alkaliphilic *Dietzia* sp. strain GS-1. Biosci Biotechnol Biochem 64:2709–2711
282. Yumoto I, Yamaga S, Sogabe Y, Nodasaka Y, Matsuyama H, Nakajima K, Suemori A (2003) *Bacillus krulwichiae* sp. nov., a halotolerant obligate alkaliphile that utilizes benzoate and m-hydroxybenzoate. Int J Syst Evol Microbiol 53:1531–1536
283. Ahmed A, Othman M, Sarwade VD, Gawai KR (2012) Degradation of anthracene by alkaliphilic bacteria *Bacillus badius*. Environ Pollut 1:97–104
284. Habe H, Kanemitsu M, Nomura M, Takemura T, Iwata K, Nojiri H et al (2004) Isolation and characterization of an alkaliphilic bacterium utilizing pyrene as a carbon source. J Biosci Bioeng 98:306–308
285. Oie CS, Albaugh CE, Peyton BM (2007) Benzoate and salicylate degradation by *Halomonas campisalis*, an alkaliphilic and moderately halophilic microorganism. Water Res 41:1235–1242
286. Huertas MJ, Sáez LB, Roldán MD, Luque-Almagro VM, Martínez-Luque M et al (2010) Alkaline cyanide degradation by *Pseudomonas pseudoalcaligenes* CECT5344 in a batch reactor. Influence of pH. J Hazard Mater 179:72–78
287. Luque-Almagro VM, Blasco R, Huertas MJ, Martínez-Luque M, Moreno-Vivian C, Castillo F, Roldan MD (2005) Alkaline cyanide biodegradation by *Pseudomonas pseudoalcaligenes* CECT5344. Biochem Soc Trans 33:168–169

288. Wanyonyi WC, Mulaa FJ (2019) Alkaliphilic enzymes and their application in novel leather processing technology for next-generation tanneries. Adv Biochem Eng Biotechnol. https://doi.org/10.1007/10_2019_95
289. Costa SA, Tzanov T, Carneiro F, Gübitz GM, Cavaco-Paulo A (2002) Recycling of textile bleaching effluents for dyeing using immobilized catalase. Biotechnol Lett 24:173–176
290. Oluoch KR, Welander U, Andersson MM, Mulaa FJ, Mattiasson B, Hatti-Kaul R (2006) Hydrogen peroxide degradation by immobilized cells of alkaliphilic *Bacillus halodurans*. Biocatal Biotransformation 24:215–222
291. Paar A, Costa S, Tzanov T, Gudelj M, Robra K-H, Cavacao-Paulo A, Gübitz GM (2001) Thermo-alkali-stable catalases from newly isolated *Bacillus* sp. for treatment and recycling of textile bleaching effluents. J Biotechnol 89:147–153
292. Paar A, Raninger A, Desousa F, Beurer I, Cavaco-Paulo A, Gübitz GM (2003) Production of catalase-peroxidase and continuous degradation of hydrogen peroxide by an immobilized alkalothermophilic *Bacillus* sp. Food Technol Biotechnol 41:101–104
293. Kulshreshtha N, Kruthiventi A, Bisht G, Pasha S, Kumar R (2012) Usefulness of organic acid produced by *Exiguobacterium* sp. 12/1 on neutralization of alkaline wastewater. Sci World J 2012:345101. https://doi.org/10.1100/2012/345101
294. Jain RM, Mody KH, Keshri J, Jha B (2011) Biological neutralization of chlor-alkali industry wastewater. Mar Pollut Bull 62:2377–2383
295. Li Q (2019) Progress in microbial degradation of feather waste. Front Microbiol 10:2717. https://doi.org/10.3389/fmicb.2019.02717

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Isolation and Cultivation of Alkaliphiles

Vadim V. Kevbrin

Contents

Abstract Alkaliphilic microorganisms are ubiquitous and inhabit various econiches on Earth. Alkaline environments suitable for alkaliphilic microbial communities may be created by certain geological processes or human activities. Moreover, a significant contribution to the emergence of alkaline conditions may be due to the activity of neutralophilic microorganisms through certain reactions, which explains the widespread distribution of alkaliphiles. Alkaliphilic microorganisms are part of extremophiles and become interesting and useful in environmental and industrial microbiology. With increasing knowledge of alkaliphiles, we greatly increase their biotechnological and industrial application potential. New microorganisms from

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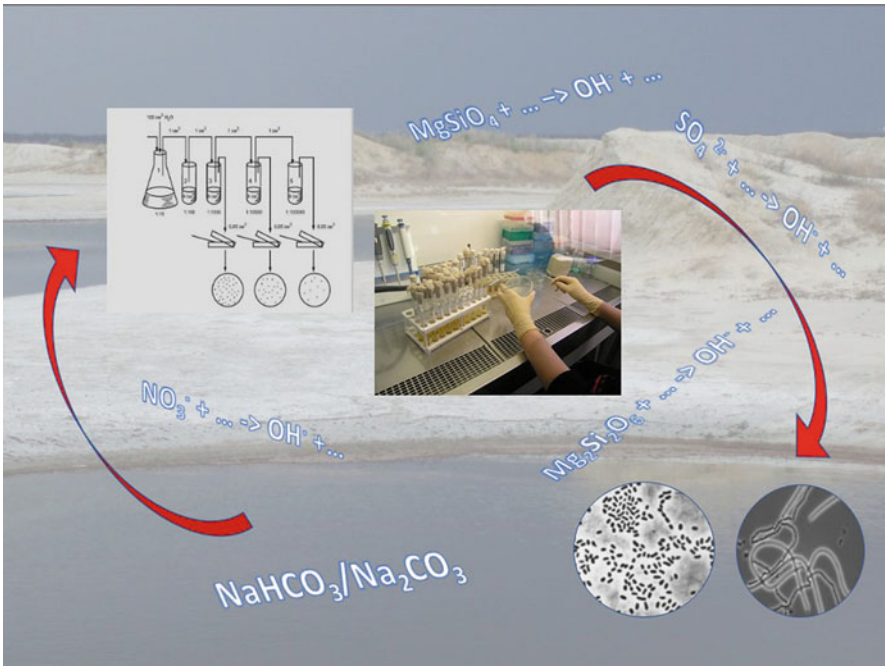
natural habitats serve as a practically endless source of new enzymes. This chapter summarizes the scattered data on alkaliphiles isolated from habitats other than soda lakes. The conditions for occurrence of alkaline habitats are also considered. Moreover, the chapter reviews some important features on preparation of media for the isolation and cultivation of alkaliphiles. The chapter also includes relevant information on chromatographic analysis of alkaliphiles culture.

Graphical Abstract



Keywords Alkaline environments, Alkaliphiles, Alkaliphilic eukaryotes, Chromatography, Extremophiles, Isolation of alkaliphiles, Microbial communities

Abbreviations

FID	Flame ionization detector
GLC	Gas-liquid chromatography
HPLC	High-performance liquid chromatography
OTU	Operational taxonomic unit

1 Definitions

Alkaliphiles are referred to microorganisms growing at alkaline pH range, i.e., at a pH of the medium higher than 7. A few definitions of alkaliphiles do exist. Despite the fact that they are known since the end of the nineteenth century, some uncertainty in their definition still persists, and, at present, Prof. Koki Horikoshi, a pioneer of the study of alkaliphilic microorganisms, defines alkaliphiles as "bacteria with pH optima for growth in excess of pH 8, usually pH 9–12" [1]. According to his classification which is now well established, alkaliphiles can be divided into two distinct groups based on their pH profiles for growth. Particularly, those that grow starting from pH 7 to 12 have been classified as obligate alkaliphiles and those where the lower limit for growth is slightly less than 7 (not less than 6.0) as facultative alkaliphiles. Another division classifies alkaliphiles as *obligate alkaliphiles* (synonymously to true) which do not grow at pH 7 or lower, and their optimum pH value for growth is always greater than 7, and the other group is classified as alkalitolerant, whose growth optimal pH can be at 7 and slightly lower (not less than 6.0) but the upper limit for growth extends over 8.0–8.5. In a strict sense, alkalitolerant microorganisms are not alkaliphiles, they are neutralophiles, the upper limit of which extends over 8, whereas in common neutralophiles, it usually does not exceed 8.0–8.5. An *Extremophiles Handbook* offers even more strict definition: "Alkaliphile: an organism with optimal growth at pH values above 10" [2].

Alkaline environments in nature, except for some cases, mostly caused by carbonates ($NaHCO_3/Na_2CO_3$) with varying buffering capacity. The evaporation process can significantly raise the carbonate concentration up to saturation as it is, for example, in some African or Asian soda lakes. Accordingly, alkaliphiles isolated out there; besides a requirement for alkaline conditions (high pH), frequently possess a requirement for high sodium content, usually >1 M of Na^+. Such alkaliphiles are referred to haloalkaliphiles; they are virtually double extremophiles and growth ranges both for sodium and pH are equally important for their characterization. In the dependency of anion associated with sodium, haloalkaliphiles, in turn, can be subdivided on haloalkaliphiles *sensu stricto* which require both carbonate and chloride and natronophiles (in Arabic natrono – pertaining to soda, sodium carbonate) which require only carbonates, while sodium chloride can enhance growth or do not affect it. At the moment, the molecular backgrounds for the discrepancy between haloalkaliphiles and natronophiles are not clear. Other group of double extremophiles includes the less numerous thermoalkaliphilic (or alkalithermophilic) microorganisms that are introduced at the end of the twentieth century by Wiegel [3]. Based on sodium requirement, alkalithermophiles can also be halophilic, and these organisms are triple extremophiles or polyextremophiles. Such microorganisms exist in nature, and the reader can learn more about them in the chapter "Anaerobic alkaliphiles and alkaliphilic poly-extremophiles" written by Wiegel in the *Extremophiles Handbook* [4].

2 Habitats

Historically, the first well-documented isolate of alkaliphilic bacterium, *Bacillus alcalophilus*, is dated by 1934 [5], and short history of alkaliphiles before 1968 had been surveyed by Horikoshi [6]. Yet, there was an earlier but less documented isolate dated by 1889: *Urobacillus pasteurii* → *Bacillus pasteurii* and now *Sporosarcina pasteurii* [4]. All of the early alkaliphiles were aerobic, and it took decades before the first alkaliphilic anaerobes, facultative anaerobic *Amphibacillus xylanus* [7], and obligate anaerobic *Methanosalsum zhilinae* (former *Methanohalophilus zhilinae*) [8] were isolated. Since then, the growing interest in the unusual, for that time, group of microorganisms has led to the isolation and identification of a large number of new species and genera of alkaliphiles. The continuing interest of microbiologists in this group of extremophiles can be illustrated by a long list of aerobic and anaerobic alkaliphiles isolated merely from soda lakes around the world, with a total number of nearly a 150 species [9].

How to find a place where alkaliphiles live? The notion that "Alkaliphiles should be looked for in alkaline locations" as we will see it further below may not be evident. This statement is correct, but only in part. The geochemical pathways led to creation of natural alkaline environments which are known and briefly considered below. Most prominent example includes soda lakes which are widely distributed around tropical, subtropical, and intracontinental cryoarid zones of Earth. In such lakes, alkalinity is created by chemical weathering of igneous or metamorphic volcanic carbonatite-containing rocks by CO_2-bearing waters (runoff and groundwater). Various dissolved carbonate species are released into water making it alkaline. The common feature of all soda lakes in hydrologically closed basins is a progressive evaporative concentration of inorganic salts, where sodium carbonates are prevailing [10]. Depending on local climatic features, soda lakes mineral saturation can be both low and high. It is important to emphasize that the composition of the brine will be substantially different (depending on the particulate waterbody) compared to the evaporation of seawater where sodium chloride dominates and the ratio chloride/sulfate will near constant up to the beginning of the solid precipitation. The highest pH value achieved in the soda lake is determined by the pH of the sodium carbonate solution and usually does not exceed 11. Numerous examples of soda lakes can be found in the East African Rift Valley (Ethiopia, Kenya, Tanzania), in the eastern Turkey (Lake Van, the largest alkaline lake on Earth), alkaline lakes of the Great Basin in the western USA, and numerous Asian alkaline shallow lakes of Mongolia, China, India, and the southern Siberia in Russia. In addition to their biotechnological potential as sources of new extremophilic microorganisms, soda lakes have attracted the attention of paleomicrobiologists as possible counterparts of Precambrian water bodies, whose microorganisms probably gave the entire modern variety of prokaryotes. The idea of a high biodiversity of the microbial population of soda lakes, put forward by the Russian microbiologist George Zavarzin in the 1990s [11], was confirmed in the following years, when the number of publications on the isolation and description of new alkaliphiles began to increase rapidly. The microbial communities inhabiting soda lakes are mostly

phototrophic and autochthonous and characterized by high productivity, which is due to (1) unlimited source of substrate (inorganic carbon) for phototrophs, (2) high sunlight incidence, and (3) the variety of substances circulating inside the community and deriving from the decay of the primary producers' biomass after they die off. Considering the duration of existence of phototrophic microbial communities on Earth and provided similarity of geological features of formation of alkaline water bodies, it will not be surprising to find similar communities on other planets [12]. To assess the biodiversity of the soda lakes microbiomes and to find particular microorganisms out there, the reader is referred to the recently published excellent Grant and Jones' survey [9], and therefore alkaliphiles from soda lakes will not be mentioned more in this chapter. It can only be noted that despite most of the known taxa of alkaliphiles have been isolated from soda waterbodies, their potential as a source of valuable microorganisms has not yet been exhausted, and ongoing publications on the description of new alkaliphiles from soda lakes can easily be found in the literature.

2.1 The Sites of Serpentinization

Another geochemical alkaline system is presented by the serpentinization process which is a metamorphic transformation of olivine- and pyroxene-rich rocks by water into minerals of the serpentine group. Serpentinization is a rather complex cascade of reactions [13], and the first step is characterized by dissolution of silicates that is accompanied by a concomitant increase in the ambient pH:

$$\text{For forsterite (the species of olivine)} : \mathrm{Mg_2SiO_4} + 2\mathrm{H_2O} = 2\mathrm{Mg^{2+}} + \mathrm{SiO_{2(aq)}} + 4\mathrm{OH^-}$$

$$\text{For enstatite (the species of pyroxene)} : \mathrm{Mg_2Si_2O_6} + 2\mathrm{H_2O} = 2\mathrm{Mg^{2+}} + 2\mathrm{SiO_{2(aq)}} + 4\mathrm{OH^-}$$

The serpentinizing systems can be both terrestrial and submarine. Unlike soda lakes, alkalinity is generated by hydroxyl anions, and for terrestrial systems where the presence of carbonates is minimal, the pH can reach 12. The sites of serpentinization are less abundant and more diverse in ionic composition comparatively to carbonate systems. The process of serpentinization as a geochemical phenomenon has been known since the twentieth century [14], but only with the onset of the era of molecular techniques, a large variety of microorganisms inhabiting these extreme places has been discovered. Terrestrial sites of modern serpentinization with outflowing alkaline waters are found in Jordan [15], Portugal [16], Newfoundland, Canada [17], the Philippines [18], northern Italy [19], Oman [20], Turkey [21], Costa Rica [22], and the Cedars springs in California [23]. How this interesting geochemical system works can be seen on the example of the Cedars springs (Fig. 1).

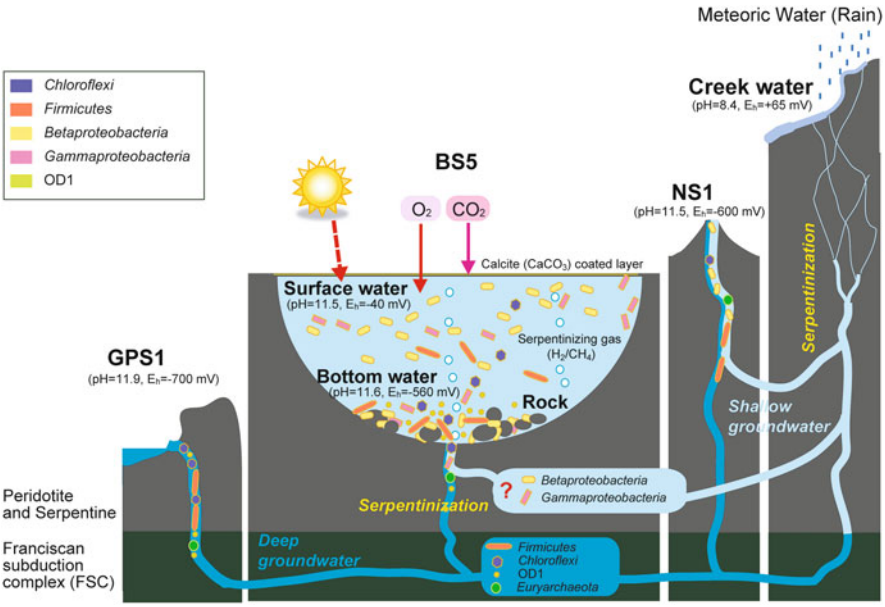


Fig. 1 A schematic diagram showing the geological setting of The Cedars springs studied and their microbial communities. Dark and light blue colors indicate the deep or shallow groundwaters, respectively. Rods and circles in various colors indicate microbes. Taken from Suzuki et al. [23]

In the year 2000, there was a significant discovery of the submarine serpentinite-hosted Lost City hydrothermal field near the Mid-Atlantic Ridge [24–26]. One of the characteristic features of serpentinization is an abiogenic generation of dissolved hydrogen and methane [27]. It is not surprising that microbes could not "pass by" such energetically beneficial substances. Taking into account that carbonate and sulfur species always present in submarine hydrothermal vents, places of submarine serpentinization were densely populated by chemoorganotrophic microbial communities. Metagenomic investigation of the Lost City hydrothermal field revealed high bacterial (*Firmicutes*, *Chloroflexi*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Epsilonproteobacteria*, *Planctomycetes*) and low archeal (*Methanosarcinales*, *Euryarchaeota*, *Crenarchaeota*, and ANME) diversity [24]. Besides the Lost City, another good example of the developed ecosystem existing in the submarine serpentinizing site is a Prony Hydrothermal Field in New Caledonia [28, 29]. Unlike the Lost City field, Prony is more accessible for sampling because it is located in shallow area. The archaeal part of community comprised of *Euryarchaeota* and *Thaumarchaea*, the bacterial part mainly consisted of *Firmicutes*, *Chloroflexi*, *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Deltaproteobacteria* and of the candidate division NPL-UPA2. The surprisingly high diversity of the Prony's biocoenosis makes it possible to consider it as a source of not only new alkaliphiles but potentially other extremophiles, particularly thermophiles. Although, to date, not many valid names of alkaliphiles from this site have been published, *Acetoanaerobium pronyense* [30], *Alkaliphilus hydrothermalis* [31], and

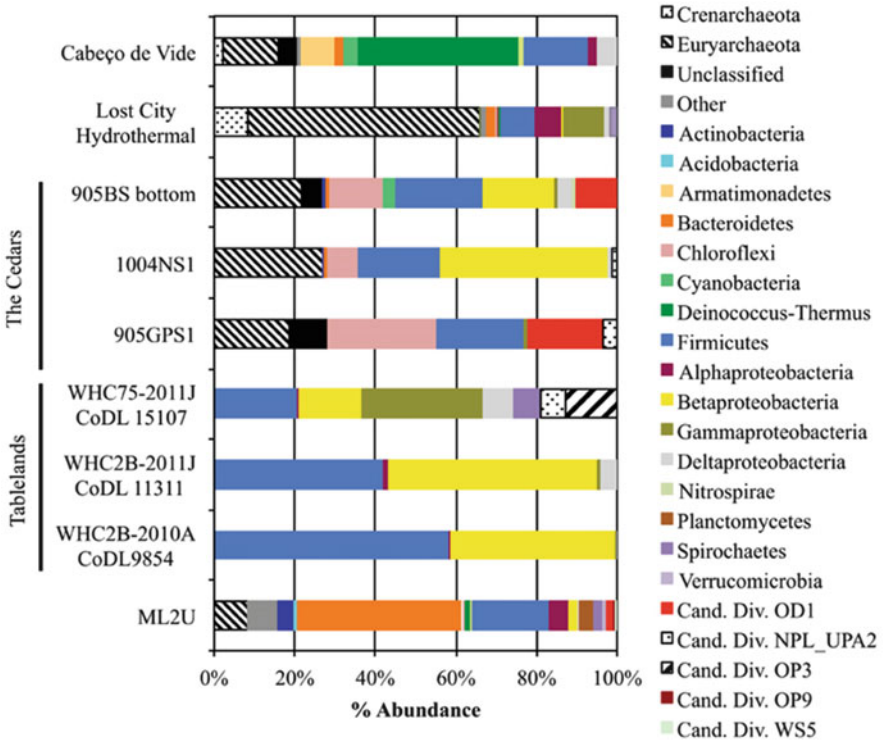


Fig. 2 A comparison between phylotypes from the different sources of serpentinizing systems separated by phylum (and class, in the phylum *Proteobacteria*). Examples include subterranean serpentinizing aquifer, Cabeço de Vide, in Portugal [16], submarine Lost City Hydrothermal Field in Atlantic Ocean [25], spring waters in the Cedars in the Coast Range Ophiolites, USA [23]; terrestrial serpentinizing seeps from Tablelands Ophiolite Complex in Newfoundland, Canada [17]; and terrestrial serpentinizing seep from Manleluag, Philippines (ML2U) [18]. Taken from Woycheese et al. [18]

Serpentinicella alkaliphila [32] can be noted. All three are anaerobic; the latter two uses a rather limited set of substrates including fructose, lactate, pyruvate, crotonate, and proteinaceous substances. Three strains of obligate alkaliphiles with an optimum for growth at pH 11 and are capable of autotrophic growth using hydrogen, calcium carbonate, and oxygen have been isolated from highly alkaline (pH 11.6) serpentinizing springs at the Cedars, California [33]. It is proposed to place them in a new genus "*Serpentinomonas*"; however, it has not been validated yet. An earlier publication describes strictly aerobic, heterotrophic *Algoriphagus alkaliphilus* (former *Chimaereicella alkaliphila*) isolated from a highly alkaline (pH 11.4) groundwater environment at Cabeço de Vide, in Southern Portugal [34]. All isolated cultures were natronophilic, and the lack of NaCl requirements for organisms isolated from terrestrial serpentinization sites may be a consequence of their adaptation to a habitat that is characterized by low or zero NaCl content. A comparison between phylotypes found in several serpentinizing systems is shown on Fig. 2. As one can see, the

diversity of the sites of serpentinization, at least some of them, is not inferior to the phototrophic communities of soda lakes. For "alkalimicrobiologists," these habitats are less studied compared to soda lakes, and, obviously, one can expect isolation of new microbes in the future. Moreover, interest to sites of serpentinization is warmed up by the observation that their physicochemical conditions resemble to those that are proposed to exist in the early Earth. Previously, the possible participation of serpentinization process in the origin of life has been proposed [35–37].

2.2 Ocean

Another geochemical system that has a pH on the alkaline side, although not so high, is the sea. In public consciousness, the sea is usually not regarded as an alkaline environment; however, seawater has a pH value of near 8.3, and, formally, sea is a slightly alkaline environment and, hence, can also be the source of alkaliphiles. Although the alterations of the pH of seawater in course of the Earth's evolution is still a matter of debates, nowadays, the World Ocean secures a slightly alkaline but rather stable environment, comparable with those in low-mineralized soda lakes. The first evidence of the presence of alkaliphiles in deep-sea mud samples had been collected by Horikoshi in the late 1990s of the twentieth century in course of search for extremophiles living on the ocean floor [38, 39]. Since then, microbiologists have been isolating various strictly and facultatively aerobic alkaliphiles including the obligate aerobes *Oceanobacillus*, *O. profundus* [40], *O. pacificus* [41], and an extreme halotolerant (0–21% (v/w) NaCl) *O. iheyensis* [42] and species of aerotolerant *Alkalibacterium*, *A. thalassium*, *A. pelagium*, *A. putridalgicola*, *A. kapii* [43], and *A. subtropicum* [44]. *Alkalibacterium* species are often referred to as marine lactic acid bacteria (lactate is a main product in anaerobic growth) and can be isolated from decaying marine algae, seagrass, and raw and salted fish. By their alkaliphilicity, they are distinctly different from common terrestrial lactic acid bacteria, which are acid tolerant or neutralophilic. To them, one can add psychrotolerant, collagenolytic *Alkalimonas collagenimarina* [45]; alkalitolerant, urea-lytic *Bacillus nanhaiisediminis* [46]; alkalitolerant species of *Idiomarina* (all are former *Pseudidiomarina*), *I. donghaiensis*, *I. maritima* [47], and *I. sediminum* [48]; and haloalkaliphilic species of *Aliidiomarina*, *A. taiwanensis* [49], non-valid "*A. haloalkalitolerans*" [50], *A. iranensis* [51], *A. sedimenti* [52], and *Salinispirillum marinum* [53]. The latter three species were isolated from a Gomishan wetland located along the eastern shore of the Caspian Sea in Iran. This area, although of marine origin, is characterized by pH 8.5–9.3. Other examples include facultatively anaerobic *Alkalimarinus sediminis* [54] and strictly aerobic, alkalitolerant *Alkalimicrobium pacificum* [55]. Strictly anaerobic alkaliphiles of marine origin except for those who inhabit submarine serpentinization sites has not been isolated yet. Considering the global scale of the world oceans, we should expect more descriptions of new marine alkaliphiles.

2.3 Soils

Another natural alkaline system where alkaliphiles live is soil, especially alkaline soils. Soil is a highly heterogeneous mix of organic and mineral residues. Unlike soda lakes or seawater, soils have complex chemical compositions which highly depend on local geological background, inhabiting biota, and climatic features. Soils of all types are rich reservoirs of different microorganisms, and no wonder that the first alkaliphiles were isolated from soil, e.g., alkaliphilic strain of *Bacillus circulans* by Horikoshi in the 1950s [2]. Surface layers of the soil are usually well-aerated and have a low-water content which contributes to the survival of sporeformers. Because of this, traditionally, soil samples serve as good source for the representatives of genus *Bacillus* and bacilli-like organisms. Among various soil types classified in the World Reference Base for soil resources, solonchak soils ("soils with limitations to root growth and with high concentration of soluble salts" [56]) are the most likely habitat for soil alkaliphiles. This soil has a high content of both sodium chloride and sodium carbonates and, hence, an alkaline pH that supports the growth of alkaliphiles. Solonchaks (or saline soda soils, or saline-alkaline soils, or Natracualf in Argentina) are located in the arid zones and are found in southern Siberia (dry steppe), north-eastern Mongolia, Northern China, Egypt, India, Pakistan, Hungary, Argentina, and North American prairie. Interestingly, despite the fact that a lot of soil alkaliphilic bacteria are known, not so many alkaliphiles have been isolated from solonchak soils located far from the shallow soda lakes. The latter often accompany solonchaks on the Asian continent, and, theoretically, alkaliphiles can migrate from alkaline water to alkaline soil and back. Known alkaliphilic representatives include *Actinobacteria* isolated from a solonchak soil sample of Xinjiang province in China: *Lipingzhangella halophila* [57], *Phytoactinopolyspora alkaliphila* [58], haloalkaliphilic *Egibacter rhizosphaerae* [59], and *Bacillus tamaricis* [60]. The latter two were isolated from the soil of the rhizosphere of a tree *Tamarix hispida* Willd which tolerate saline-alkaline conditions. Other rhizosphere-associated soil alkaliphiles include *Bacillus patagoniensis* from a perennial shrub *Atriplex lampa* in Argentina [61] and *Bacillus kiskunsagensis* from the bayonet grass *Bolboschoenus maritimus* in Hungary [62]. The rhizosphere supplies nutrients to soil bacteria, as is well known for nodule bacteria. Saline and alkaline soils have their own vegetation, so it was not surprising to expect the isolation of alkaliphilic bacteria from the rhizosphere of such plants.

Metagenomic diversity in two physicochemically different soils from the coastal region of Gujarat, India, were investigated using 16S rRNA gene clone libraries [63]. The phylogenetic diversity of bacteria in a haloalkaline soil (pH 9.5) was compared with a normal soil (pH 7.2). Clones representing phyla *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Actinobacteria*, *Acidobacteria*, and *Planctomycetes* were found in both soils. *Cyanobacteria*, *Verrucomicrobia*, OP10, and *Bacteria incertae sedis* were detected in normal soil, whereas *Nitrospira* was found only in haloalkaline soil. The dominant phylum in the haloalkaline soil was *Bacteroidetes* followed by *Proteobacteria*, whereas normal soil was dominated by *Proteobacteria* and *Actinobacteria*. One third of the total sequences from both soil

samples showed low BLAST identities (<95%) suggesting that these soils may harbor unique, novel taxa.

Two diazotrophic haloalkaliphiles, *Natronobacillus azotifigens* from solonchak soil in the Kulunda Steppe in Russia [64] and *Anaerobacillus alkalidiazotrophicus* (former *Bacillus alkalidiazotrophicus*) from northeastern Mongolia solonchak samples [65] have been isolated by Sorokin. The authors concluded that both nitrogen-fixing organisms, together with *B. arseniciselenatis* and *Paenibacillus* spp., form a separate cluster on the tree of the *nif*H gene, and, thus, there is more to the ability of the Gram-positive bacteria (other than clostridia) to fix nitrogen that is currently recognized. Later, Sorokin and coworkers have been conducting more detailed study on the solonchak samples [66]. Although it concerned only aerobic cultivated hydrolytic species, the work performed made possible to assess the rich potential of solonchak soils as a source of new extremophilic microorganisms. Using samples of solonchak soils with pH > 9 brought from eight regions of the Earth, it was shown that these soils are inhabited mainly by the two largest groups of bacteria belonging to the phyla *Actinobacteria* and *Firmicutes*. The *Actinobacteria* were mostly represented by two genera *Nocardiopsis* and *Streptomyces*, and the *Firmicutes* was expectedly represented by genus *Bacillus*. Of course, this study was not exhaustive, but the trend is obvious, and other genera belonging to these two phyla will eventually be isolated from saline soda soils.

Transitional formations between saline-alkaline soils and soda lakes (so-called salt pans) have an alkaline pH and can also serve as a source of alkaliphilic microorganisms. Examples are *Jeotgalibacillus alkaliphilus* (tolerates up to 24% (w/v) NaCl) isolated from the salt pan near seashore in India [67], *Alkalibacillus almallahensis* from an inland solar saltern in Spain [68], *Nesterenkonia pannonica* [69], *Nitrincola alkalilacustris*, and *Nitrincola schmidtii* [70] from the salt pan located in Kiskunság National Park in Hungary.

2.4 A Near-Bottom Alkaline Sites

A small-scale natural alkaline environment has been found in ikaite ($CaCO_3 \cdot 6H_2O$) tufa columns which harbor the bottom of the Ikka Fjord in southwest Greenland. Ikaite columns are formed when alkaline freshwater springs rich in sodium carbonates meet the cold, calcium-rich seawater, which leads to an immediate oversaturation of calcium and carbonate ions and a subsequent growth of the column. The interior is filled with a cold (4°C), alkaline (pH 10.4), and low salinity (0.9%) spring water floating through the column, and this peculiar environment harbors a large diversity of bacterial species [71, 72]. The 16S rRNA gene sequences from 67 cultured isolates and 109 clone library phylotypes showed similarity to phylotypes of 6 bacterial phyla where *Alphaproteobacteria* and *Gammaproteobacteria* dominated, and 22 of the 67 ikaite isolates showed less than 97% sequence similarity to sequences from known species. The place seems promising in terms of the search and isolation of new alkaliphiles as producers of a diversity of enzymes, and researchers succeeded in

isolating 322 strains of aerobic alkaliphiles [73], 3 of which formed new genera: red-pigmented, psychrophilic *Rhodonellum psychrophilum* [74], *Arsukibacterium ikkense* [75], and natronophilic *Alkalilactibacillus ikkensis* [76]. Interestingly, the similar but opposite mechanism (spring water with high calcium content meets carbonate containing lacustrine water) was found in alkaline and hypersaline Mono Lake in California (a tufa formation) [77] and in Lake Van in the eastern Turkey [78] resulting in the same hydrochemical phenomenon as underwater carbonate columns, but their prokaryotic diversity descriptions are beyond the scope of this chapter.

2.5 Man-Made Alkaline Sites

Besides natural reasons, human activities can create alkaline environments. A striking example of an extreme environment hosting microbial community is found in the Lake Calumet area (Illinois, USA) [79]. Over decades of industrial waste dumping, large-scale infilling of the wetlands with steel slag has created an aquifer with pH values as high as 12.8. Compared to other alkaline environments, the overall microbial diversity is poor. However, representatives of *Alphaproteobacteria*, *Betaproteobacteria*, and *Firmicutes* were found. Another example of artificial alkaline environment is the huge (200 ha) repository of saline soda lime in Janikowo, Poland [80]. The repository was established in 1957 as a waste storage site from a local chemical plant producing soda by the Solway method. Molecular probing has revealed a diverse microbial community in this highly saline-, alkaline- (pH 10.8), and nutrient-poor environment. The dominated bacterial phyla were *Proteobacteria* (representing 52.8% of the total bacterial community) and *Firmicutes* (16.6%). It is interesting that a significant portion of the sequences (27%) constituted chloroplasts of algae instead of cyanobacteria, as one might expect, and most of the organic matter present in this artificial environment originates from allochthonous airborne material and atmospheric deposition on the lime surface. Other industrial facilities with high pH and potentially regarded as habitats for alkaliphiles may include aluminum production (*Bacillus vedderi* from a bauxite-processing red mud tailing pond in Canada [81]) and mining (strictly anaerobic *Alkaliphilus transvaalensis* from a containment dam at 3.2 km below land surface in an ultra-deep gold mine in South Africa [82]). One more artificial alkaline habitat is an alkaline silica sol which is an important fine chemical product and has been widely used in the chemical, textile, and material industries. The strains of facultative alkaliphiles with protease and amylase activities belonging to six different genera have been isolated from a putrid alkaline silica sol [83]. Although such a habitat is fully artificial and in no way connected with the biosphere, isolation of alkaliphilic microorganisms from there can be interesting, if the task is to find microorganisms of possible producers of valuable enzymes. One more alkaline artificial habitat for alkaliphiles is a process of indigo fermentation. The dye has been produced from the leaves of plants specific for certain regions of the Earth, e.g., *Polygonum tinctorium* Lour. in China, Korea, and Japan; *Strobilanthes cusia* in the Ryukyu Islands, Japan; Indigofera (*Indigofera tinctoria* L. and *Indigofera suffruticosa* Mill. in India; and woad (*Isatis tinctoria* L.)

in Europe, in two-step processing. Both steps are carried out by microorganisms, and the second step (conversion of the insoluble dye to soluble one) is carried out anaerobically at a pH > 10. Although the process has been known for several centuries, microbiologists drew attention to it only in the 1960s of the twentieth century, but the first indigo-reducing alkaliphile, "*Bacillus alkaliphiles*," was lost [84]. The second one, *Alkalibacterium psychrotolerans*, was isolated only by 2004 [85]. The fermentation broth at the different steps and ages in the production of indigo proved to be a fertile material for the isolation of alkaliphiles, many of which represented new genera and species. Hydrolysates of wheat bran and composted indigo leaves (sukumo) have been proposed to use as ingredients in the fermentation fluid in the selective medium for isolation of new indigo-reducing bacteria [86]. Readers can find detailed information on indigo-reducing alkaliphiles in papers published by Yumoto's group and coworkers [84, 87, 88].

Besides indigo production, there are other technological processes which use alkaline chemicals and accompanied by alkaline wastewater from factories and can potentially be habitat for alkaliphiles. A good example is an isolation of the facultatively anaerobic *Exiguobacterium aurantiacum* from potato-processing alkaline effluent which is formed in course of potato peeling by lye [89]. Other alkaliphilic species of *Exiguobacterium*, *E. alkaliphilum*, had been isolated from alkaline wastewater drainage sludge of a soft drink beverage factory located near New Delhi, India [90]. Species of genus *Exiguobacterium* were found to be ubiquitous, and at the time of writing this chapter, 16 species from various natural habitats, including Antarctica, are known and have been described. The olive oil extraction process also has an alkaline step. The olive mill wastewater (alpeorujo) is treated by calcium hydroxide (pH varies from 10.5 to 13.5) to absorb undesirable odor. The first species of the genus *Alkalibacterium*, *A. olivoapovliticus*, was isolated from such a habitat [91]. Currently, the genus comprises ten species. A later work on the molecular identification of strains of microorganisms isolated from the alkaline alpeorujo revealed the presence of *Alphaproteobacteria*, *Gammaproteobacteria*, *Firmicutes*, and *Actinobacteria* representatives, some of which form novel phylogenetic lineages [92]. *Halomonas alkalicola* has recently been isolated from a soapmaking tank (pH 9.0–10.5) of a household product plant in China [93]. The organism is noteworthy due to its high upper pH, reaching 12.5.

2.6 Microbially Mediated Alkalinization

All the above examples are related to conditions where the alkalinity of the medium was specified either naturally or artificially. However, there is also another way of alkalinization, the microbiological or transient alkalinization in microniches. Some experimental approaches were demonstrated by Horikoshi [1] where growing bacteria (*Bacillus* sp.) spontaneously increased the pH of unbuffered broth. Similar observation has been done on aerobic spirillum *Alkalispirillum mobile* [94]. However, more detailed studies on this interesting direction are required, and the

Table 1 Microbially mediated reactions leading to external alkalinization

Photosynthesis	$HCO_3^- + H_2O = (CH_2O)^a + O_2 + OH^-$ (oxygenic) $HCO_3^- + H_2O + 2HS^- = (CH_2O)^a + 2S^o + 3OH^-$ (anoxygenic)
Sulfate and thiosulfate reduction[b]	$8[H] + SO_4^{2-} = HS^- + 3H_2O + OH^-$ $6[H] + SO_4^{2-} = S^o + 2H_2O + 2OH^-$ $4[H] + S_2O_3^{2-} = 2S^o + H_2O + 2OH^-$
Nitrate and nitrite reduction	$8[H] + 2NO_3^- = N_2O + 3H_2O + 2OH^-$ $10[H] + 2NO_3^- = N_2 + 4H_2O + 2OH^-$ $8[H] + NO_3^- = NH_4^+ + H_2O + 2OH^-$ $4[H] + 2NO_2^- = N_2O + H_2O + 2OH^-$ $6[H] + 2NO_2^- = N_2 + 2H_2O + 2OH^-$ $6[H] + NO_2^- = NH_4^+ + 2OH^-$
Methanogenesis[c]	$4HCOO^- + H_2O = CH_4 + 2HCO_3^- + CO_3^{2-}$
Sulfide oxidation	$2HS^- + O_2 = 2S^o + 2OH^-$
Fermentation of proteins and nucleic acids	Proteins, DNA, RNA $\rightarrow NH_4^+$

[a]Means biomass
[b]Thiosulfate reduction to sulfide do not lead to alkalinization
[c]Out of possible methanogenic substrates, only formate degradation leads to alkalinization

following theoretical prerequisites are possible. There are several microbiological reactions carried out by "ordinary", neutralophilic bacteria with a concomitant alkalinization of the milieu (Table 1). The most notable example is photosynthesis of both types, oxygenic and anoxygenic. In a recent publication, ten strains of cyanobacteria belonging to two novel genera *Pantanalinema* and *Alkalinema*, after 14 days of growth, regardless of the starting pH, have shifted the final pH of unbuffered BG-11 culture medium to 8.4–9.9, which convincingly demonstrates the ability of these strains to induce alkalinization of the culture medium [95]. Besides to the ability to shift the pH, phototrophic bacteria are the prime producers of organic carbon and serve as suppliers of organic substances for organotrophic bacteria, living at the expense of phototrophs (so-called dissipotrophs). Mutualism in such associations can be strongly pronounced, and thus it is not always possible to isolate the phototroph into a pure culture as prescribed by the microbiological standard. This phenomenon is well known to microbiologists working with cyanobacteria. Most laboratory strains of cyanobacteria are axenic, i.e., free of other cyanobacteria but have some non-phototrophic contaminants (satellites). Satellitic bacterium can be an interesting object in itself, and, besides, it may well be alkaliphile, since its existence will be supported both by alkalinization of the medium due to photosynthesis and by supplementation of organic substances excreted by the phototroph. Probably, in this way, it was possible to isolate aerobic spirillum *Alkalispirillum mobile*, a satellite at anoxygenic phototroph *Halorhodospira halophila* [94], *Silanimonas algicola* from laboratory culture of *Microcystis* sp. cyanobacterium [96], and

Aliidiomarina sanyensis from the cultivation pool for *Spirulina platensis* cyanobacterium [97].

Other alkalinizing reactions include anaerobic processes, e.g., sulfate and nitrate reduction and fermentation of the nitrogenous biopolymers such as nucleic acids and proteins. The latter were released into surrounding medium after dying off prime producers' cells. Hydrolytic microflora or fermenters in case of anaerobes have hydrolyzed biopolymers with concomitant release of ammonium ions that increase a pH value in external medium. Although the alkalinization proceeds on a microscale, unlike a geochemical process, alkaliphiles from the outside, once getting this favorable environment can stay in it and can, possibly, evolve inside such microbial community provided the community is stable for enough time. Certainly, evident alkaline localities such as alkaline lakes, soils, springs, etc. will probably contain more alkaliphiles; however, in view of a microscale alkalinization, it is not a rule. That is why alkaliphiles can be isolated from many neutral environments provided microbial life is possible there. The reader can find numerous examples of obligate alkaliphiles isolated from apparently neutral, unobvious in the sense of the search for alkaliphiles, habitats. They include *Halomonas desiderata* [98], *Clostridium paradoxum* [99], and *Clostridium thermoalcaliphilum* [100] isolated from municipal wastewaters, anaerobic sulfate reducer *Desulfotomaculum alkaliphilum* from cow/pig manure [101], CO-oxidizing anaerobic acetogen *Alkalibaculum bacchi* from livestock-impacted soil [102], moderately halophilic and alkalitolerant *Natribacillus halophilus* from the garden soil [103], haloalkaliphilic *Alkalibacillus silvisoli* from non-saline forest soil [104], *Algoriphagus trabzonensis* from freshwater river [105], *Bacillus marmarensis* from mushroom compost [106], hydrocarbon-utilizing *Dietzia psychralcaliphila* [107], and *Exiguobacterium oxidotolerans* with an extremely high catalase activity [108], the latter two from a drain pool of a fish processing plant. Microbiological alkalinization also seems to be responsible for the existence of alkaliphiles in the intestine of insects, e.g., *Alkalispirochaeta odontotermitis* (former *Spirochaeta odontotermitis*) from a termite [109], *Alkalispirochaeta cellulosivorans* from a wood-eating cockroach [110], *Bacillus trypoxylicola* from the larvae of the Japanese horned beetle [111]. The latter is interesting in that it shows a preference for K^+ over Na^+. Three isolated strains, SU1^T, 36AC4, and 36AC6, showed good growth up to 12% (w/v) KCl. And finally, alkaliphiles can be isolated even from cheese, as in the case of *Alkalibacterium gilvum* [112].

2.7 Alkaliphilic Eukaryotes

Although this chapter is primarily devoted to alkaliphilic prokaryotes, it also mentions the alkaliphilic eukaryotic organisms. Since it is assumed that alkaline habitats existed throughout the entire geological history of the Earth [113], eukaryotes had to develop certain mechanisms of adaptation to life at alkaline ambient pH. And, indeed, they are (e.g., an altered content of membrane lipids and cytoprotectant

molecules in fungus *Sodiomyces tronii* [114]), although eukaryotic biodiversity at the alkaline pH is noticeably less than at near-neutral pH. Historically, alkaliphilic eukaryotes from soda lakes have been studied in the most detail. Comprehensive reviews of particular eukaryotes inhabiting African soda lakes (diatoms, green algae, protozoa, invertebrates, fish, etc.) the reader can find in a valuable book edited by Michael Schagerl [115], while eukaryotes from the other soda or alkaline lakes were also the matter of exploring, e.g., picophytoplankton diversity in the Transylvanian Basin (Romania) [116], fungi in the Mono Lake (USA) area [117], diatoms in Lake Van [118], and assessment of the overall eukaryotic diversity in the three US alkaline lakes plus a biofilm from the chimney at the Lost City hydrothermal vent with a pH 11 [119]. The latter contemporary study has been fulfilled by the method of massively parallel pyrotag sequencing targeting the V9 hypervariable region of the 18S rRNA gene and aimed to compare eukaryotic communities living at acidic ($pH < 3$) and alkaline ($pH > 9$) environments. It has been found that the number of OTUs for alkaline lakes exceeds that for acid habitats, although the DNA derived from allochthonous sources or environmental DNA (inactive or dead cells) may contribute. It has been found that the most cosmopolitan OTU was a diatom (*Fragillariophyceae*) that occurred both acidic and alkaline samples. Among the shared alkaline OTUs were *Frontonia* and *Lacrymaria* (ciliates), *Protaspa* (a cercozoan), a maxillopod metazoan OTU, a tracheophyte OTU and various opisthokont OTUs including fungi. A unicellular chlorophyte *Picocystis* was expectedly found in the Mono Lake sample because this alga accounts for up to 50% of the primary production there as it has been shown earlier [120].

In the 1980–1990s of the twentieth century, an interest of microbiologists to alkaliphiles sparked the researchers to search for alkaliphilic fungi. Although the ability of fungi to grow at pH 10 has been known for a long time [121], only a targeted search for alkaliphilic fungi gave the first results in the beginning of the 1990s [122, 123]. The definition of alkaliphilicity at mycologists is slightly different from that adopted by microbiologists. An earlier definition designated fungus which could grow at pH up to 10, but not below pH 5–6, as alkaliphilic and those grew at pH 10 and below pH 5–6 are regarded as alkalitolerant [122]. In a later definition, it was proposed that fungal species be considered alkaliphilic provided their optimum growth lies at pH above 8 and the acidic pH limit constraint 4–5 [124]. Among fungi there are no species that would not grow at pH 7, so there are no fungal obligate alkaliphiles in the "bacteriological sense." The importance of using an alkaline medium for the search for alkaliphilic fungi was already emphasized in the first papers [122, 125]. However, the past decades of research have shown that the diversity of, namely, alkaliphilic fungi by now, is not so large compared to alkaliphilic bacteria, and the majority of species/genera of fungi/yeasts isolated at pH 10 are often to be alkalitolerant [125–128].

This trend was recently confirmed in an extensive study, including morphological and metagenomic analysis, on the search for alkaliphilic fungi from alkaline soils of varying degrees of salinity sampled from Russia, Mongolia, Kazakhstan, Kenya, Tanzania, and Armenia [124]. Among over 100 isolated strains assigning to various taxons, only strains belonging to two genera, *Sodiomyces* and *Thielavia*, were turned

to be alkaliphilic. The former composes three species, *S. tronii*, *S. magadii*, and *S. alkalinus*, and all of them are alkaliphilic. Interestingly, all the isolated strains could only be recovered if the soil clumps put directly on the medium instead of the common suspending in the medium. In summary, by now, the list of known alkaliphilic fungi (noteworthy, all are within the *Ascomycota* division) includes *Acremonium alcalophilum* (isolated from pig feces compost in Japan) [123]; *Gliocladium cibotii*, *Phialophora geniculata*, *Stachylidium bicolor*, and *Stilbella annulata* (from slightly alkaline grassland in Indonesia) [122]; *Acremonium* sp. and *Chrysosporium* sp. (from limestone cave in Japan) [129]; *Emericellopsis minima*, *Neosartorya stramenia*, and *Melanospora zamiae* (from different soil types including Natracualf, in Argentina) [126]; *Sodiomyces* (from alkaline soils including solonchak type of soils of Russia, Mongolia, Kenya, Tanzania); and *Thielavia* sp. (from alkaline soils of Russia and Armenia) [124]. There is no doubt that the search for alkaliphilic fungi will be continued, since the fungi in general are valued producers of many biologically active substances and are widely used in the bioindustry.

Of course, in one review, it is impossible to embrace all alkaliphiles in all habitats and a world experience based on published papers has convincingly shown that alkaliphiles habit many various biocoenoses. In this sense, alkaliphiles, similar to neutralophiles, follow the known Beijerinck's principle, but not all taxa or physiological groups are existing in any habitats. Now and before, choosing the "right" sample for isolation is mostly a matter of an intuition and experience of a particulate microbiologist.

3 Isolation, Media, and Cultivation

How to isolate an alkaliphile? The techniques of isolation for alkaliphiles are mostly the same as for neutralophilic microorganisms, and it includes getting enrichments on an appropriate substrate, physical separation on solid media (getting colonies), serial dilutions, selective inhibition by antibiotics or inhibitors, etc., and any peculiarities in relation to alkaliphiles are mainly related to the preparation of media. Before to start isolation, it is very important to understand which media will contribute to isolating a particular strain and to what physiological group the target isolate will belong to, because the current diversity of alkaliphiles is too high to isolate "simply" alkaliphile. In this connection, a matter of importance is a choice for the natural or man-made source where putative alkaliphile can live.

How to prepare a medium for the isolation and subsequent maintenance of an alkaliphile? Looking through a large number of published papers relating to alkaliphilic microorganisms, the reader will discover the same great variety of media used. Apparently, there are no universal formulations, although an attempt was made by Grant to standardize the medium formulations for the isolation of aerobic heterotrophic alkaliphiles [130]. Nevertheless, one point is obvious: to be successful in isolation of alkaliphiles, it is necessary that the pH of the medium for

isolation be in the alkaline range. To fulfill this condition, a mixture of two sodium salts, carbonate, and/or bicarbonate in various ratios is added to the medium. This is the main difference between an alkaline medium and the "usual" one that is used for neutralophiles. The mixture of both salts (sometimes is referred to as a sesquicarbonate solution) forms an alkaline buffer, and, depending on their ratios, it is possible to get different pH values in the range of 8.3–11.5. While sodium ions will be present in the carbonate buffer, it is still recommended to include sodium chloride in the formulation at least at the initial step of isolation. Chloride ions are inherent in many habitats of alkaliphiles and are absolutely necessary for the isolation of haloalkaliphiles and can significantly enhance the growth for natronophiles.

When selecting substrates for an alkaline medium, it should always be remembered that when heated at an elevated temperature and high pH condition, very few substances remain unchanged. These are mainly low molecular organic acids, alcohols, most of amino acids (but not all), sugar alcohols, and already hydrolyzed substrates like peptone or casamino acids. All complex organic substances such as polymers (pectin, casein, starch, etc., except for cellulose) and sugars will irreversibly decompose by thermoalkaline hydrolysis during autoclaving, and, thus, the convenient "all-in-one" principle does not always fit. There are two possible solutions for this problem. The first one is the well-known approach, labile substance prepared as concentrated solution which is sterilized separately either in the same autoclave but as neutral solution or by filtrating through membrane filter with a pore size of 0.2 μm. Then, separately sterilized component is poured into the bulk of the medium. However, not all substances can be prepared as concentrated solutions (e.g., most polymers, poorly soluble or insoluble substances), so another approach called the "double concentration technique" is used. In this case, sodium carbonate and sodium bicarbonate (sometimes sodium chloride is also included) are dissolved in a separate screw or crimp cap closed bottle with concentration double of that required (an alkaline solution I), and all the other components, including the labile one, are also dissolved with a double concentration in the flask with a cotton plug (a neutral solution II). After separate autoclaving, both solutions are mixed together (solution I is poured into solution II), and the final solution will have the given concentrations of all components. When mixing, the volumes of both solutions must be the same. A closed bottle is necessary, since sodium bicarbonate reversibly decomposes on heating. In the case of extreme haloalkaliphiles ($Na^+ > 2$ M), it may be necessary to divide solution I into two parts: a mixture of carbonates and a separate NaCl solution. In case an anaerobic medium is prepared, solution II should also be prepared in a closed bottle, and both bottles must be previously purged with nitrogen prior to autoclaving. A reducing agent that is needed for strict anaerobes can be added either in solution I or solution II, depending on its stability toward pH. We found that the redox indicator resazurin used in working with strict anaerobes proved to be stable to pH 10 and destroyed above this value. When several labile substances are used, as in the case of testing of the spectrum of substrates used, individual solutions II can be prepared in test tubes (or in Hungate tubes for anaerobes), and solution I is used as the stock solution. In case of doubt, whether the particular substance is resistant to thermoalkaline hydrolysis, the common advice will be either to search for its

properties in the reference books or to assay its content by an appropriate analytical technique (e.g., chromatographically or colorimetrically) before and after autoclaving. In particular, by measuring the content of total free amino acids that released into the alkaline medium with albumin or caseinate after autoclaving, we found that half of the amounts of these proteins added was destroyed and, hence, should be added separately [131]. When isolating proteolytic alkaliphiles in our lab, sodium caseinate turned out to be more convenient to use than egg albumin, since the aqueous solution of the former could be safely autoclaved staying soluble. No free amino acids were revealed. Sodium caseinate is a special processed casein to give the latter a good solubility, but with the preservation of the polypeptide chain.

Agar is also destroyed during autoclaving, so the preparation of solid media for alkaliphiles is possible only by the double concentration technique. It has been proposed to use carrageenan as an agar substitute. This sulfated polysaccharide withstands thermoalkaline hydrolysis and forms a strong gel even at a pH of 13.5 [132]. However, the use of labile substrates negates the benefits of carrageenan, since it still requires separate preparation of the constituents.

In addition to the destruction of organic molecules, heating of aqueous alkaline solutions causes a gradual erosion of glassware used for the cultivation of alkaliphiles, and, over time, the test tubes become turbid (etched) and have to be discarded. Unfortunately, manufacturers do not make glassware especially for "alkalimicrobiologists," yet alkali-resistant varieties of glass do exist, e.g., Schott Glass 8436 has the highest an alkali resistance class A1. Although borosilicate glassware (e.g., Duran or Pyrex brands) is more resistant to alkali than soda lime glass, it is thermo- rather than alkali-resistant and has an alkali resistance class A2.

Divalent cations of alkaline-earth metals (Mg and Ca) precipitate out in alkaline medium as insoluble carbonates; therefore, their content should be minimized, but not completely eliminated as both relate to biogenic elements. The precipitation threshold for each of them strongly depends on both the pH and the ionic strength of the solution, and it is significantly lower for calcium than for magnesium. Unfortunately, because of too great a variety of combinations of sodium carbonate, bicarbonate, and chloride, it is impossible to calculate in advance the thresholds for precipitation for each formulation, so, if needed, their selection is possible only empirically. In our experience, the critical concentration for magnesium was 3 mM and for calcium was 0.2 mM, and in practice, we use 0.5 mM for Mg^{2+} and 0.1 mM for Ca^{2+}.

As noted by many microbiologists, alkaliphiles, regardless of the carbon source used, often require some additives like yeast extract (usually up to 0.5 g/L) and/or the mixture of vitamins different in content, and only some of them can grow in a defined (minimal) medium. In this, alkaliphiles resemble halophiles where both additives serve for anabolic purposes. Yeast extract is a source of B vitamins, trehalose [133], and peptides of uncertain composition, and it can significantly enhance the growth or be essential; therefore, at the step of isolation, it is better to include it in the medium at a concentration of at least 0.1 g/L. An artificial mixture of vitamins does not always replace yeast extract, and, in case of revealing of the stimulating effect of yeast extract, it is desirable to check whether such a substitution

is possible. Most vitamins are complex in structure and should be added as a separate filter-sterilized solution (the only exceptions are nicotinic and p-aminobenzoic acids, resistant to thermoalkaline hydrolysis) yet the parts of vitamin's molecule can still possess a stimulating effect [134].

4 Getting the Right pH and Na^+ Concentration Range for Alkaliphiles

Finding the pH and Na^+ concentration range/optimum is an important part of physiological characterization of newly isolated strains of alkaliphiles. At present, it is mandatory for description of new taxon to include the data of pH and Na range/optimum for growth. A routine task to determine a pH range for growth of a given alkaliphilic strain sometimes may not be unequivocal. For example, for alkaliphiles which are facultative aerobes, a search for the growth pH range should carry out on fermentable rather than nonfermentable substrate as these alkaliphiles have different pH ranges depending on the type of the used substrate: growth on nonfermentable carbon sources (succinate) occurs in more narrow pH range than on fermentable substrate (sucrose) [135]. For anaerobic alkaliphiles, pH optimum can shift depending on the substrate used, if non-dissociated (methanol) or dissociated (methylamine) as it has been shown earlier for *Methanosalsum zhilinae* [136]. A generally accepted technique to get a pH range is to prepare the growth medium with two or more buffer substances (one buffer is insufficient) which will embrace a full possible growth pH range. For this purpose, Good's buffers at a concentration of near 0.1 M or less are commonly used [137]. In addition to buffer substances, it is necessary to leave in a medium a small amount of carbonates for anabolic purposes since many alkaliphiles cannot grow in the complete absence of carbonates in the medium. Some of the Good's buffers are costly, but they have been specially designed to be biological compatible, so the use of simple, inexpensive buffers such as organic acids or amino acids should be used with caution, after making sure that the test strain grows in the medium with 0.1 M or more of the test buffer. Phosphate salts which are commonly used for cultivation of neutralophiles are ineffective in the alkaline growth range. Although the isolation and cultivation of alkaliphiles are carried out in carbonate buffer, its use to obtain the pH range is possible but less convenient because of a significant shift in the pH of the medium after autoclaving especially at the neutral region. In any case, when preparing a series of tubes with different pHs, it is necessary to prepare one extra tube for each point that will be used for the pH measurement after autoclaving. It is the pH value verified after autoclaving that should be taken for further plotting. For maintenance and further cultivation, after finding the optimal pH, the ratio of carbonate and bicarbonate is chosen in such a way as to provide this optimum pH. Addition of NaCl may be

necessary if the test strain requires. It should be noted that although low NaCl practically do not affect pH, high concentrations can lower the pH value by suppressing the dissociation of bicarbonate/carbonate. The effect of NaCl on the dissociation constant of the carbonate buffer is gradual; therefore, an extra check of the pH may be required. A carbonate-free buffered medium with the optimal pH and supplemented with an optimal concentration of NaCl can be used to determine the strain's requirement for carbonates. To be sure that the strain does not need carbonates, up to five successive transfers on this medium are usually enough. Growth in the fifth transfer means that given culture does not require carbonates.

The amount of substrate when determining the pH range should be minimal. This is especially true for anaerobic alkaliphiles, fermenting sugars to organic acids. The molar concentration of sugar should not exceed (less is better) the molar concentration of the buffer; otherwise, the initial pH will last only for a short time of the growth curve, depending on the buffer capacity of the medium. This applies to any fermentable substrate, when ionic products are excreted to the medium. Readers can find in Sorokin's letter [138] other precautions that should be taken into account when determining the pH range for growth of alkaliphiles. Features of pH measurement at elevated temperatures, which are required when working with alkalithermophiles, can be found in the publication of Mesbah and Wiegel [139]. Pouring anaerobic media with various pHs is conveniently carried out in a specially designed conical flask (Fig. 3). The anaerobic medium with the initial pH, freed from oxygen (by vacuum or boiling), is pumped under a slight nitrogen pressure into a flask with attached accessories. The flask should be previously purged by nitrogen. Depending on the direction of the pH change, the plastic syringe is filled with concentrated HCl or NaOH and attached to a thin flexible tubing to accurately supply the titrant to the bulk of the medium. After stabilization of reading, the medium with the set pH is poured over the tubes in a nitrogen flow.

Determining the sodium concentration range for growth of alkaliphiles can possibly be achieved by two methods. The first one is the same as that used for halophiles, i.e., the calculated weights of dry NaCl are put into the culture tubes and brought to the mark by a 0.1–0.2 M buffered (by carbonate for alkaliphiles) medium with optimal pH. Accordingly, sodium, introduced with carbonates, should be taken into further account. In the case of natronophiles with a high demand for carbonates, it is preferable to use the second method. It implies the mixing of solutions I and II in various proportions. Solution I is a saturated solution of carbonate buffer with the optimal pH for a given strain plus all other constituents. Solution II is an aqueous solution of all other constituents except for carbonates. The disadvantage of this method is the strong dependence of the solubility of the saturated sesquicarbonate solution on pH and, consequently, the inability to reach high concentrations of dissolved sodium. The solubility of sodium carbonate is worse (sodium bicarbonate is even much worse) than that of NaCl, and its addition to the saturated solution of the former leads to salting out of bicarbonate or carbonate, depending on the pH. After finding the optimum concentration for sodium, regardless of the method

Fig. 3 Experimental setup for pouring anaerobic medium with simultaneous measurement and setting the pH value. Arrows show: (**a**) A 1 L conical flask with a welded glass port (for mounting the pH electrode; made in glass-blowing workshop) placed on a magnetic stirrer. (**b**) A rubber stopper with three holes for three tubings: two for supplying nitrogen and titrants, the third for pouring the medium into a Hungate tube. (**c**) A plastic medical syringe with a connected narrow polypropylene tubing (inner diameter is ca. 1 mm; used in liquid chromatography) to accurately dispense titrants (concentrated HCl or NaOH) to the bulk of the medium

used, the ratio of carbonate and bicarbonate is chosen in such a way as to provide both the optimal sodium and pH. A chloride-free carbonate buffered with an optimal concentration of Na^+ medium can be used to determine the strain's requirement for chloride. Similar to a carbonate-free medium, up to five successive transfers on this medium are usually enough to discern whether the strain requires chloride or not.

5 Chromatographic Assays Employed in the Study of Alkaliphiles

Phenotypic characterization, especially for anaerobic alkaliphiles, often requires assays of some fermentative or anaerobic respiration products. Gaseous substances (e.g., H_2, N_2, CH_4) can be analyzed by gas chromatography which is widely used for their quantification. However, for quantification of soluble substances (sugars, organic acids, inorganic anions, etc.), precautions should be taken into account, if chromatographic methods planned to be used. The main source of problems in this case is the high sodium content (carbonates and chloride) of the medium. For some haloalkaliphiles, upper limit for growth may reach up to 4 M of sodium. Out of variety of chromatographic methods, the only insensitive one, working regardless of the salt content, is a gas-liquid chromatography (GLC). In fact, in order to maintain the column in good condition, it is sufficient to simply change a glass wool tampon in a pre-column (sometimes called a liner) equipped with any gas chromatograph. When an injected sample is evaporated there, salts will stay inside the pre-column and will not interfere with the column. Although manufacturers recommend change the tampon time to time, it has become especially topical in case of frequent injections of the samples with the high salt content. Otherwise, bad resolution and residual retention of the analyte are possible. With regard to specific protocols of separation, the manufacturers have their own columns for the separation of acidic, neutral, or basic substances, and the reader is referred to their catalogs or websites.

The sample preparation for the GLC determination generally includes centrifugation or filtration through membrane filter with a pore size of 0.45 μm. When using GLC, clarifying the sample is needed not only for the instrument but also for protecting the needle of the microliter syringe, which is easily clogged in the presence of insoluble impurities in the sample. After clarification of the sample, next actions depend on if acidic (e.g., organic acids) or basic (e.g., methylamines) volatile products are planned to be assayed. To organic acids, a clarified culture medium should be acidified by formic acid to pH 2–3 to convert nonvolatile organic anions into volatile free acids. Formic acid is the strongest volatile organic acid and compatible with all types of the columns designed for organic acid separation. Moreover, it does not detect by the FID detector and thus is "invisible" for the system. Injection of 1% formic acid into the column before and after a series of analyses is a good practice for maintaining the column in good condition. If the sample has high buffer capacity, acidity of formic acid may not be sufficient to shift a pH value into acidic range. In this case, first, the sample should be acidified by concentrated phosphoric acid to neutrality and then by formic acid to pH 2–3. Neither hydrochloric nor sulfuric acid should be used for neutralization as its vapors are highly aggressive toward the stationary phase of the column. To assay for volatile amines, ammonia solution should be added to the sample to facilitate amines' evaporation. Similar to formic acid, ammonia is also invisible for the FID detector. Interestingly, despite the fact that the capillary columns almost displaced the packed ones, our experience had shown that the Supelco packing (0.3% Carbowax 20M + 0.1% H_3PO_4, matrix 60/80

Carbopack C) provides better separation for all structurally possible C2–C5 acids than well-known FFAP capillary columns from different manufacturers. Nonvolatile substances (sugars, organic acids like succinic) can also be analyzed by the GLC method, but derivatization is required to convert them into volatile form [137] that complicates the assay.

The HPLC method developed for analyses of both volatile and nonvolatile substances are based on direct injection of the sodium containing sample into the eluent. Analysis of brines is a heavy test for any chromatographic system, and, until now, the "universal" column or common solution capable to assay any product or metabolite in the whole range of the sodium solubility still does not exist. Although pre-columns, cartridges, and centrifugal mini-columns (mini-inserts) for desalting the sample are available, they do not absorb sodium entirely at its high content, and an excess sodium can enter the column leading to unsatisfactory separation. Our experience with the widely used Aminex HPX-87H (BioRad) column (used to separate neutral and acidic fermentation products) has shown that the column can operate effectively up to 0.5–1.0 M of sodium, but above this concentration, the efficiency significantly deteriorates. Some success can be achieved if the peak of the target analyte is far enough from the "water (or salt) peak" (retention time for the void volume of the column). Significant progress has been made by Thermo Scientific Dionex in their IonPac AS5 column designed for the separation of strongly retained species such as thiocyanate and thiosulfate in 7% brine. Unfortunately, this approach will not work for every desired species, and, again, there is no common solution for every microbiologically interesting substance.

The sample preparation for the HPLC determination requires more attention, especially when an acid eluent is used. As applied to the Aminex HPX-87H column, after removing the cells, an alkaline culture fluid should be acidified by sulfuric acid to pH 2–3. Using hydrochloric acid is not recommended by most manufacturers as it has a corrosive effect even for the stainless steel. If a highly concentrated carbonate medium was used, a vigorous foaming is observed so this operation should be carried out in vials of larger volume to avoid spilling out the sample. The evolved carbon dioxide remains partly dissolved in the sample, and if the acidified sample is immediately injected into the column, a negative peak of CO_2 can be seen in the field of analytes' peaks when using a refractometric detector. Particularly for the Aminex HPX-87H column, a CO_2 peak is close to the propionate peak and interferes quantitatively in the estimation of the latter. To avoid this, the acidified samples are best left closed before injection for several hours or overnight. Another precaution is associated with the presence of sulfide in the anaerobic media. As pH of the eluent is acidic, sulfide is converted to hydrogen sulfide which, in turn, is easily oxidized to insoluble sulfur. Depending on its content, numerous injections of the sulfide-containing samples can lead to clogging of capillaries and the column. Out of our experience, if the sample contains no more than 0.5 g/L of $Na_2S{\cdot}9H_2O$ (a widely used concentration of sulfide as a reductant for anaerobic media), there is no need to take special efforts, and the best solution will be to leave the acidified sample for overnight for spontaneous oxidation of the sulfide inside the vial. The amount of elemental sulfur formed is negligible in this case; the sample remains clear and can

be injected into the column. However, if the sulfide content is high, there will be a precipitate that must be separated from the rest of the solution. For instance, this should be done when analyzing organic products in the course of anaerobic respiration with sulfate or sulfur when sulfide content in the sample can reach 1 g/L and even higher. In this case, after preliminary acidification, a small amount of 30% H_2O_2 is added. Precipitation of sulfur takes several hours (depending on the initial sulfide content), and after its completion, a little dry MnO_2 is put into the reaction mixture. The latter will decompose the unreacted H_2O_2, since in case of entering the column, hydrogen peroxide can disrupt covalent linkages in the ion-exchange resin. The reaction mixture must then be clarified by centrifugation or filtration, and the sample can be safely injected. Since hydrogen peroxide is a strong oxidizer, drawback of the method is the possible oxidation of unstable substrates/products, if they are present in the sample. If it is known a priori, another technique involving the use of heavy metal salts (Cu^{2+}, Sn^{2+}) can be applied. These cations bind the soluble sulfide and precipitate it in an insoluble form stable at acidic conditions. The reaction proceeds quickly, but this technique should be applied with caution, if used in excess the free metal ions in the reaction mixture can bind to the resin too and, subsequently, are difficult to wash off the column. Thiosulfate (often used as an electron acceptor) is also unstable in the acidic eluent and, if present, is degraded to sulfur. So, the abovementioned notes about sulfide removal are also valid for this sulfur species.

Another risk associated with the analysis of brines is the possibility of salt precipitation (the salting out effect) if the eluent contains some organic modifier (e.g., methanol or acetonitrile) that may result in clogging of the capillaries. To avoid, in advance, it is necessary to mix the test sample with the eluent in various proportions, in order to make sure that there is no salting out.

6 Concluding Remarks

The increasing number of publications on alkaliphilic microorganisms convincingly demonstrates their importance for both fundamental and applied research in microbiology and biotechnology. It is now generally accepted that alkaliphiles, along with thermophiles, are the oldest organisms on Earth, and their study from the standpoint of paleomicrobiology will provide more knowledge about Earth's history when the biosphere was exclusively prokaryotic. This knowledge can be especially valuable in the case of discovery of extraterrestrial life. At the same time, despite the large number of already known alkaliphiles and the commonness of the basic biochemical processes, nature gives practically "inexhaustible" combinations of biomolecules, including industrially important enzymes. Therefore, the search for this group of microorganisms will continue to isolate alkaliphiles which are still waiting for their discoverers.

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References

1. Horikoshi K (2011) General physiology of alkaliphiles. In: Horikoshi K (ed) Extremophiles handbook. Springer, Tokyo, pp 99–118
2. Horikoshi K, Bull AT (2011) Prologue: definition, categories, distribution, origin and evolution, pioneering studies, and emerging fields of extremophiles. In: Horikoshi K (ed) Extremophiles handbook. Springer, Tokyo, pp 3–15
3. Wiegel J (1998) Anaerobic alkalithermophiles, a novel group of extremophiles. Extremophiles 2:257–267
4. Wiegel J (2011) Anaerobic alkaliphiles and alkaliphilic poly-extremophiles. In: Horikoshi K (ed) Extremophiles handbook. Springer, Tokyo, pp 81–97
5. Vedder A (1934) *Bacillus alcalophilus* n. sp.; benevens enkele ervaringen met sterk alcalische voedingsbodems. Ant van Leeuwenhoek. J Microbiol Serol 1:143–147
6. Horikoshi K (2011) Introduction and history of alkaliphiles. In: Horikoshi K (ed) Extremophiles handbook. Springer, Tokyo, pp 19–26
7. Niimura Y, Koh E, Yanagida F, Suzuki K-I, Komagata K, Kozaki M (1990) *Amphibacillus xylanus* gen. nov., sp. nov., a facultatively anaerobic sporeforming xylan-digesting bacterium which lacks cytochrome, quinone, and catalase. Int J Syst Bacteriol 40:297–301
8. Mathrani IM, Boone DR, Mah RA, Fox GE, Lau PP (1988) *Methanohalophilus zhilinae* sp. nov., an alkaliphilic, halophilic, methylotrophic methanogen. Int J Syst Bacteriol 38:139–142
9. Grant WD, Jones BE (2016) Bacteria, archaea and viruses of soda lakes. In: Schagerl M (ed) Soda lakes of East Africa. Springer, Cham, pp 97–147
10. Deocampo DM, Renaut RW (2016) Geochemistry of African soda lakes. In: Schagerl M (ed) Soda lakes of East Africa. Springer, Cahm, pp 77–93
11. Zavarzin GA (1993) Epicontinental soda lake are probable relict biotopes of terrestrial biota formation. Microbiology RU 62:473–479
12. Zavarzin GA (2005) Recent microbiology and Precambrian paleontology. In: Hoover RB, Rosanov AY, Paepe R (eds) Perspectives in astrobiology. NATO science series. I: life and behavioural sciences, vol 366. IOS Press, Amsterdam, pp 201–216
13. Chavagnac V, Monnin C, Ceuleneer G, Boulart C, Hoareau G (2013) Characterization of hyperalkaline fluids produced by low-temperature serpentinization of mantle peridotites in the Oman and Ligurian ophiolites. Geochem Geophys Geosyst 14:2496–2522
14. Barnes I, Lamarche VC, Himmelberg G (1967) Geochemical evidence of present-day serpentinization. Science 156:830–832
15. Pedersen K, Nilsson E, Arlinger J, Hallbeck L, O'Neill A (2004) Distribution, diversity and activity of microorganisms in the hyper-alkaline spring waters of Maqarin in Jordan. Extremophiles 8:151–164
16. Tiago I, Veríssimo A (2013) Microbial and functional diversity of a subterrestrial high pH groundwater associated to serpentinization. Environ Microbiol 15:1687–1706
17. Brazelton WJ, Morrill PL, Szponar N, Schrenk MO (2013) Bacterial communities associated with subsurface geochemical processes in continental serpentinite springs. Appl Environ Microbiol 79:3906–3916

18. Woycheese KM, Meyer-Dombard DR, Cardace D, Argayosa AM, Arcilla CA (2015) Out of the dark: transitional subsurface-to-surface microbial diversity in a terrestrial serpentinizing seep (Manleluag, Pangasinan, the Philippines). Front Microbiol 6:44
19. Quéméneur M, Palvadeau A, Postec A, Monnin C, Chavagnac V, Ollivier B, Erauso G (2015) Endolithic microbial communities in carbonate precipitates from serpentinite-hosted hyperalkaline springs of the Voltri Massif (Ligurian Alps, Northern Italy). Environ Sci Pollut Res 22:13613–13624
20. Rempfert KR, Miller HM, Bompard N, Nothaft D, Matter JM, Kelemen P, Fierer N, Templeton AS (2017) Geological and geochemical controls on subsurface microbial life in the Samail Ophiolite, Oman. Front Microbiol 8:56
21. Neubeck A, Sun L, Müller B, Ivarsson M, Hosgörmez H, Özcan D, Broman C, Schnürer A (2017) Microbial community structure of a serpentine-hosted abiotic gas seepage at the Chimaera ophiolite, Turkey. Appl Environ Microbiol 83:e03430–e03416
22. Crespo-Medina M, Twing KI, Sánchez-Murillo R, Brazelton WJ, McCollom TM, Schrenk MO (2017) Methane dynamics in a tropical serpentinizing environment: the Santa Elena Ophiolite, Costa Rica. Front Microbiol 8:916
23. Suzuki S, Ishii S, Wu A, Cheung A, Tenney A, Wanger G, Wanger G, Gijs Kuenen J, Nealson KH (2013) Microbial diversity in The Cedars, an ultrabasic, ultrareducing, and low salinity serpentinizing ecosystem. Proc Natl Acad Sci U S A 110:15336–15341
24. Kelley DS et al (2005) A serpentinite-hosted ecosystem: the Lost City hydrothermal field. Science 307:1428–1434
25. Brazelton WJ, Schrenk MO, Kelley DS, Baross JA (2006) Methane- and sulfur-metabolizing microbial communities dominate the Lost City hydrothermal field ecosystem. Appl Environ Microbiol 72:6257–6270
26. Brazelton WJ, Ludwig KA, Sogin ML, Andreishcheva EN, Kelley DS, Shen C-C, Edwards RL, Baross JA (2010) Archaea and bacteria with surprising microdiversity show shifts in dominance over 1,000-year time scales in hydrothermal chimneys. Proc Natl Acad Sci U S A 107:1612–1617
27. Proskurowski G et al (2008) Abiogenic hydrocarbon production at Lost City hydrothermal field. Science 319:604–607
28. Postec A, Quéméneur M, Bes M, Mei N, Benaïssa F, Payri C, Pelletier B, Monnin C, Guentas-Dombrowsky L, Ollivier B, Gérard E, Pisapia C, Gérard M, Ménez B, Erauso G (2015) Microbial diversity in a submarine carbonate edifice from the serpentinizing hydrothermal system of the Prony Bay (New Caledonia) over a 6-year period. Front Microbiol 6:857
29. Frouin E, Bes M, Ollivier B, Quéméneur M, Postec A, Debroas D, Armougom F, Erauso G (2017) Diversity of rare and abundant prokaryotic phylotypes in the Prony Hydrothermal Field and comparison with other serpentinite-hosted ecosystems. Front Microbiol 9:102
30. Bes M, Merrouch M, Joseph M, Quéméneur M, Payri C, Pelletier B, Ollivier B, Fardeau M-L, Erauso G, Postec A (2015) *Acetoanaerobium pronyense* sp. nov., an anaerobic alkaliphilic bacterium isolated from a carbonate chimney of the Prony Hydrothermal Field (New Caledonia). Int J Syst Evol Microbiol 65:2574–2580
31. Ben Aissa F, Postec A, Erauso G, Payri C, Pelletier B, Hamdi M, Fardeau M-L, Ollivier B (2015) Characterization of *Alkaliphilus hydrothermalis* sp. nov., a novel alkaliphilic anaerobic bacterium, isolated from a carbonaceous chimney of the Prony Hydrothermal Field, New Caledonia. Extremophiles 19:183–188
32. Mei N, Postec A, Erauso G, Joseph M, Pelletier B, Payri C, Ollivier B, Quéméneur M (2016) *Serpentinicella alkaliphila* gen. nov., sp. nov., a novel alkaliphilic anaerobic bacterium isolated from the serpentinite-hosted Prony Hydrothermal Field, New Caledonia. Int J Syst Evol Microbiol 66:4464–4470
33. Suzuki S, Kuenen JG, Schipper K, van der Velde S, Ishii S, Wu A, Sorokin DY, Tenney A, Meng XY, Morrill PL, Kamagata Y, Muyzer G, Nealson KH (2014) Physiological and genomic features of highly alkaliphilic hydrogen-utilizing *Betaproteobacteria* from a continental serpentinizing site. Nat Commun 5:3900

34. Tiago I, Mendes V, Pires C, Morais PV, Veríssimo A (2006) *Chimaereicella alkaliphila* gen. nov., sp. nov., a gram-negative alkaliphilic bacterium isolated from a nonsaline alkaline groundwater. Syst Appl Microbiol 29:100–108
35. Schulte M, Blake D, Hoehler T, McCollom T (2006) Serpentinization and its implications for life on the early Earth and Mars. Astrobiology 6:364–376
36. Russell MJ, Hall AJ, Martin W (2010) Serpentinization as a source of energy at the origin of life. Geobiology 8:355–371
37. Sleep NH, Bird DK, Pope EC (2011) Serpentinite and the dawn of life. Philos Trans R Soc Lond Ser B Biol Sci 366:2857–2869
38. Takami H, Inoue A, Fuji F, Horikoshi K (1997) Microbial flora in the deepest sea mud of the Mariana Trench. FEMS Microbiol Lett 152:279–285
39. Takami H, Kobata K, Nagahama T, Kobayashi H, Inoue A, Horikoshi K (1999) Biodiversity in deep-sea sites located near the south part of Japan. Extremophiles 3:97–102
40. Kim Y-G, Choi DH, Hyun S, Cho BC (2007) *Oceanobacillus profundus* sp. nov., isolated from a deep-sea sediment core. Int J Syst Evol Microbiol 57:409–413
41. Yu C, Yu S, Zhang Z, Li Z, Zhang X-H (2014) *Oceanobacillus pacificus* sp. nov., isolated from a deep-sea sediment. Int J Syst Evol Microbiol 64:1278–1283
42. Lu J, Nogi Y, Takami H (2001) *Oceanobacillus iheyensis* gen. nov., sp. nov., a deep-sea extremely halotolerant and alkaliphilic species isolated from a depth of 1050 m on the Iheya Ridge. FEMS Microbiol Lett 205:291–297
43. Ishikawa M, Tanasupawat S, Nakajima K, Kanamori H, Ishizaki S, Kodama K, Okamoto-Kainuma A, Koizumi Y, Yamamoto Y, Yamasato K (2009) *Alkalibacterium thalassium* sp. nov., *Alkalibacterium pelagium* sp. nov., *Alkalibacterium putridalgicola* sp. nov. and *Alkalibacterium kapii* sp. nov., slightly halophilic and alkaliphilic marine lactic acid bacteria isolated from marine organisms and salted foods collected in Japan and Thailand. Int J Syst Evol Microbiol 59:1215–1226
44. Ishikawa M, Nakajima K, Ishizaki S, Kodama K, Okamoto-Kainuma A, Koizumi Y, Yamamoto Y, Yamasato K (2011) *Alkalibacterium subtropicum* sp. nov., a slightly halophilic and alkaliphilic marine lactic acid bacterium isolated from decaying marine algae. Int J Syst Evol Microbiol 61:2996–3002
45. Kurata A, Miyazaki M, Kobayashi T, Nogi Y, Horikoshi K (2007) *Alkalimonas collagenimarina* sp. nov., a psychrotolerant, obligate alkaliphile isolated from deep-sea sediment. Int J Syst Evol Microbiol 57:1549–1553
46. Zhang J, Wang J, Song F, Fang C, Xin Y, Zhang Y (2011) *Bacillus nanhaiisediminis* sp. nov., an alkalitolerant member of Bacillus rRNA group 6. Int J Syst Evol Microbiol 61:1078–1083
47. Wu Y-H, Shen Y-Q, Xu X-W, Wang C-S, Oren A, Wu M (2009) *Pseudidiomarina donghaiensis* sp. nov. and *Pseudidiomarina maritima* sp. nov., isolated from the East China Sea. Int J Syst Evol Microbiol 59:1321–1325
48. Hu ZY, Li Y (2007) *Pseudidiomarina sediminum* sp. nov., a marine bacterium isolated from coastal sediments of Luoyuan Bay in China. Int J Syst Evol Microbiol 57:2572–2577
49. Huang SP, Chang HY, Chen JS, Jean WD, Shieh WY (2012) *Aliidiomarina taiwanensis* gen. nov., sp. nov., isolated from shallow coastal water. Int J Syst Evol Microbiol 62:155–161
50. Srinivas TNR, Nupur, Anil Kumar P (2012) *Aliidiomarina haloalkalitolerans* sp. nov., a marine bacterium isolated from coastal surface seawater. Antonie Van Leeuwenhoek 101:761–768
51. Amoozegar MA, Shahinpei A, Abolhassan S, Fazeli S, Schumann P, Spröer C, Ventosa A (2016) *Aliidiomarina iranensis* sp. nov., a haloalkaliphilic bacterium from a coastal-marine wetland. Int J Syst Evol Microbiol 66:2099–2105
52. Shahinpei A, Amoozegar MA, Abolhassan S, Fazeli S, Schumann P, Spröer C, Ventosa A (2017) *Aliidiomarina sedimenti* sp. nov., a haloalkaliphilic bacterium in the family *Idiomarinaceae*. Int J Syst Evol Microbiol 67:2087–2092
53. Shahinpei A, Amoozegar MA, Fazeli SAS, Schumann P, Ventosa A (2014) *Salinispirillum marinum* gen. nov., sp. nov., a haloalkaliphilic bacterium in the family '*Saccharospirillaceae*'. Int J Syst Evol Microbiol 64:3610–3615

54. Zhao J-X, Liu Q-Q, Zhou Y-X, Chen G-J, Du Z-J (2015) *Alkalimarinus sediminis* gen. nov., sp. nov., isolated from marine sediment. Int J Syst Evol Microbiol 65:3511–3516
55. Zhang G, Yang Y, Wang S, Sun Z, Jiao K (2015) *Alkalimicrobium pacificum* gen. nov., sp. nov., a marine bacterium in the family *Rhodobacteraceae*. Int J Syst Evol Microbiol 65:2453–2458
56. IUSS Working Group WRB (2015) World Reference Base for Soil Resources 2014, update 2015 international soil classification system for naming soils and creating legends for soil maps. World Soil Resources Reports No. 106. FAO, Rome
57. Zhang Y-G, Lu X-H, Ding Y-B, Wang S-J, Zhou X-K, Wang H-F, Guo J-W, Liu Y-H, Duan Y-Q, Li W-J (2016) *Lipingzhangella halophila* gen. nov., sp. nov., a new member of the family *Nocardiopsaceae*. Int J Syst Evol Microbiol 66:4071–4076
58. Zhang Y-G, Lu X-H, Ding Y-B, Zhou X-K, Li L, Guo J-W, Wang H-F, Duan Y-Q, Li W-J (2016) *Phytoactinopolyspora alkaliphila* sp. nov., an alkaliphilic actinomycete isolated from a saline-alkaline soil. Int J Syst Evol Microbiol 66:2058–2063
59. Zhang Y-G, Wang H-F, Yang L-L, Zhou X-K, Zhi X-Y, Duan Y-Q, Xiao M, Zhang Y-M, Li W-J (2016) *Egibacter rhizosphaerae* gen. nov., sp. nov., an obligately halophilic, facultatively alkaliphilic actinobacterium and proposal of *Egibaceraceae* fam. nov. and *Egibacterales* ord. nov. Int J Syst Evol Microbiol 66:283–289
60. Zhang Y-G, Zhou X-K, Guo J-W, Xiao M, Wang H-F, Wang Y, Bobodzhanova K, Li W-J (2018) *Bacillus tamaricis* sp. nov., an alkaliphilic bacterium isolated from a *Tamarix* cone soil. Int J Syst Evol Microbiol 68:558–563
61. Olivera N, Siñeriz F, Breccia JD (2005) *Bacillus patagoniensis* sp. nov., a novel alkalitolerant bacterium from *Atriplex lampa* rhizosphere, Patagonia, Argentina. Int J Syst Evol Microbiol 55:443–447
62. Borsodi AK, Tóth E, Aszalós JM, Bárány A, Schumann P, Spröer C, Kovács AL, Márialigeti K, Szili-Kovács T (2017) *Bacillus kiskunsagensis* sp. nov., a novel alkaliphilic and moderately halophilic bacterium isolated from soda soil. Int J Syst Evol Microbiol 67:3490–3495
63. Keshri J, Mody K, Jha B (2013) Bacterial community structure in a semi-arid haloalkaline soil using culture independent method. Geomicrobiol J 30:517–529
64. Sorokin ID, Zadorina EV, Kravchenko IK, Boulygina ES, Tourova TP, Sorokin DY (2008) *Natronobacillus azotifigens* gen. nov., sp. nov., an anaerobic diazotrophic haloalkaliphile from soda-rich habitats. Extremophiles 12:819–827
65. Sorokin ID, Kravchenko IK, Tourova TP, Kolganova TV, Boulygina ES, Sorokin DY (2008) *Bacillus alkalidiazotrophicus* sp. nov., a diazotrophic, low salt-tolerant alkaliphile isolated from Mongolian soda soil. Int J Syst Evol Microbiol 58:2459–2464
66. Sorokin DY, Kolganova TV, Khijniak TV, Jones BE, Kublanov IV (2017) Diversity of cultivated aerobic poly-hydrolytic bacteria in saline alkaline soils. PeerJ 5:e3796
67. Srinivas A, Divyasree B, Sasikala C, Tushar L, Bharti D, Ramana CV (2016) Description of *Jeotgalibacillus alkaliphilus* sp. nov., isolated from a solar salt pan, and *Jeotgalibacillus terrae* sp. nov., a name to replace '*Jeotgalibacillus soli*' Chen et al. 2010. Int J Syst Evol Microbiol 66:5167–5172
68. Pérez-Davó A, Aguilera M, Ramos-Cormenzana A, Monteoliva-Sánchez M (2014) *Alkalibacillus almallahensis* sp. nov., a halophilic bacterium isolated from an inland solar saltern. Int J Syst Evol Microbiol 64:2066–2071
69. Borsodi AK, Szili-Kovács T, Schumann P, Spröer C, Márialigeti K, Tóth E (2017) *Nesterenkonia pannonica* sp. nov., a novel alkaliphilic and moderately halophilic actinobacterium. Int J Syst Evol Microbiol 67:4116–4120
70. Borsodi AK, Korponai K, Schumann P, Spröer C, Felföldi T, Márialigeti K, Szili-Kovács T, Tóth E (2017) *Nitrincola alkalilacustris* sp. nov. and *Nitrincola schmidtii* sp. nov., alkaliphilic bacteria isolated from soda pans, and emended description of the genus *Nitrincola*. Int J Syst Evol Microbiol 67:5159–5164

71. Schmidt M, Priemé A, Stougaard P (2006) High microbial diversity in permanently cold and alkaline ikaite columns from Greenland. Extremophiles 10:551–562
72. Trampe E, Castenholz RW, Larsen JE, Kühl M (2017) Phototrophic microbes form endolithic biofilms in ikaite tufa columns (SW Greenland). Environ Microbiol 19:4754–4770
73. Vester JK, Glaring MA, Stougaard P (2014) Discovery of novel enzymes with industrial potential from a cold and alkaline environment by a combination of functional metagenomics and culturing. Microb Cell Factories 13:72
74. Schmidt M, Prieme A, Stougaard P (2006) *Rhodonellum psychrophilum* gen. nov., sp. nov., a novel psychrophilic and alkaliphilic bacterium of the phylum *Bacteroidetes* isolated from Greenland. Int J Syst Evol Microbiol 56:2887–2892
75. Schmidt M, Priemé A, Stougaard P (2007) *Arsukibacterium ikkense* gen. nov., sp. nov, a novel alkaliphilic, enzyme-producing γ-*Proteobacterium* isolated from a cold and alkaline environment in Greenland. Syst Appl Microbiol 30:197–201
76. Schmidt M, Priemé A, Johansen A, Stougaard P (2012) *Alkalilactibacillus ikkensis*, gen. nov., sp. nov., a novel enzyme-producing bacterium from a cold and alkaline environment in Greenland. Extremophiles 16:297–305
77. Mono Basin Ecosystem Study Committee, Board on Environmental Studies and Toxicology, Commission on Physical Sciences, Mathematics, and Resources, National Research Council (1987) The Mono Basin ecosystem: effects of changing lake level. National Academy Press, Washington
78. Reimer A, Landmann G, Kempe S (2009) Lake Van, Eastern Anatolia, hydrochemistry and history. Aquat Geochem 15:195–222
79. Roadcap GS, Sanford RA, Jin Q, Pardinas JR, Bethke CM (2006) Extremely alkaline (pH > 12) ground water hosts diverse microbial community. Ground Water 44:511–517
80. Kalwasińska A, Felföldi T, Szabó A, Deja-Sikora E, Kosobucki P, Walczak M (2017) Microbial communities associated with the anthropogenic, highly alkaline environment of a saline soda lime, Poland. Antonie Van Leeuwenhoek 110:945–962
81. Agnew MD, Koval SF, Jarrell KF (1995) Isolation and characterization of novel alkaliphiles from bauxite-processing waste and description of *Bacillus vedderi* sp. nov., a new obligate alkaliphile. Syst Appl Microbiol 18:221–230
82. Takai K, Moser DP, Onstott TC, Spoelstra N, Pfiffner SM, Dohnalkova A, Fredrikson JK (2001) *Alkaliphilus transvaalensis* gen. nov., sp. nov., an extremely alkaliphilic bacterium isolated from a deep South Africa gold mine. Int J Syst Evol Microbiol 51:1245–1256
83. Ren L, Han Y, Yang S, Tan X, Wang J, Zhao X, Fan J, Dong T, Zhou Z (2014) Isolation, identification and primary application of bacteria from putrid alkaline silica sol. Front Chem Sci Eng 8:330–339
84. Aino K, Hirota K, Okamoto T, Tu Z, Matsuyama H, Yumoto I (2018) Microbial communities associated with indigo fermentation that thrive in anaerobic alkaline environments. Front Microbiol 9:2196
85. Yumoto I, Hirota K, Nodasak Y, Yokota Y, Hoshino T, Nakajima K (2004) *Alkalibacterium psychrotolerans* sp. nov., a psychrotolerant obligate alkaliphile that reduces an indigo dye. Int J Syst Evol Microbiol 54:2379–2383
86. Nishita M, Hirota K, Matsuyama H, Yumoto I (2017) Development of media to accelerate the isolation of indigo-reducing bacteria, which are difficult to isolate using conventional media. World J Microbiol Biotechnol 33:133
87. Aino K, Narihiro T, Minamida K, Kamagata Y, Yoshimune K, Yumoto I (2010) Bacterial community characterization and dynamics of indigo fermentation. FEMS Microbiol Ecol 74:174–183
88. Okamoto T, Aino K, Narihiro T, Matsuyama H, Yumoto I (2017) Analysis of microbiota involved in the aged natural fermentation of indigo. World J Microbiol Biotechnol 33:70
89. Collins MD, Lund BM, Farrow JAE, Schleifer KH (1983) Chemotaxonomic study of an alkalophilic bacterium, *Exiguobacterium aurantiacum* gen. nov., sp. nov. J Gen Microbiol 129:2037–2042

90. Kulshreshtha NM, Kumar R, Begum Z, Shivaji S, Kumar A (2013) *Exiguobacterium alkaliphilum* sp. nov. isolated from alkaline wastewater drained sludge of a beverage factory. Int J Syst Evol Microbiol 63:4374–4379
91. Ntougias S, Russell NJ (2001) *Alkalibacterium olivoapovliticus* gen. nov., sp. nov., a new obligately alkaliphilic bacterium isolated from edible-olive wash waters. Int J Syst Evol Microbiol 51:1161–1170
92. Ntougias S, Zervakis GI, Ehaliotis C, Kavroulakis N, Papadopoulou KK (2006) Ecophysiology and molecular phylogeny of bacteria isolated from alkaline two-phase olive mill wastes. Res Microbiol 157:376–385
93. Tang X, Zhai L, Lin Y, Yao S, Wang L, Ge Y, Liu Y, Zhang X, Zhang T, Zhang L, Liu J, Cheng C (2017) *Halomonas alkalicola* sp. nov., isolated from a household product plant. Int J Syst Evol Microbiol 67:1546–1550
94. Rijkenberg MJA, Kort R, Hellingwerf KJ (2001) *Alkalispirillum mobile* gen. nov., spec. nov., an alkaliphilic non-phototrophic member of the *Ectothiorhodospiraceae*. Arch Microbiol 175:369–375
95. Vaz MGMV, Genuário DB, Andreote APD, Malone CFS, Sant'Anna CL, Barbiero L, Fiore MF (2015) *Pantanalinema* gen. nov. and *Alkalinema* gen. nov.: novel pseudanabaenacean genera (Cyanobacteria) isolated from saline–alkaline lakes. Int J Syst Evol Microbiol 65:298–308
96. Chun S-J, Cui Y, Ko S-R, Lee H-G, Oh H-M, Ahn C-Y (2017) *Silanimonas algicola* sp. nov., isolated from laboratory culture of a bloom-forming cyanobacterium, *Microcystis*. Int J Syst Evol Microbiol 67:3274–3278
97. Wang G, Wu H, Zhang X, Zhang H, Yang X, Tian X, Li J, Xiang W, Li X (2013) *Aliidiomarina sanyensis* sp. nov., a hexabromocyclododecane assimilating bacterium from the pool of *Spirulina platensis* cultivation, Sanya, China. Antonie Van Leeuwenhoek 104:309–314
98. Berendes F, Gottschalk G, Heine-Dobbernack E, Moore ERB, Tindall BJ (1996) *Halomonas desiderata* sp. nov., a new alkaliphilic, halotolerant and denitrifying bacterium isolated from a municipal sewage works. Syst Appl Microbiol 19:158–167
99. Li Y, Mandelco L, Wiegel J (1993) Isolation and characterization of a moderately thermophilic anaerobic alkaliphile, *Clostridium paradoxum*, sp. nov. Int J Syst Bacteriol 43:450–460
100. Li Y, Engle M, Mandelco L, Wiegel J (1994) *Clostridium thermoalcaliphilum* sp. nov., an anaerobic and thermotolerant facultative alkaliphile. Int J Syst Bacteriol 44:111–118
101. Pikuta E, Lysenko A, Suzina N, Osipov G, Kuznetsov B, Tourova T, Akimenko V, Laurinavichius K (2000) *Desulfotomaculum alkaliphilum* sp. nov., a new alkaliphilic, moderately thermophilic, sulfate-reducing bacterium. Int J Syst Evol Microbiol 50:25–33
102. Allen TD, Caldwell ME, Lawson PA, Huhnke RL, Tanner RS (2010) *Alkalibaculum bacchi* gen. nov., sp. nov., a CO-oxidizing, ethanol-producing acetogen isolated from livestock-impacted soil. Int J Syst Evol Microbiol 60:2483–2489
103. Echigo A, Minegishi H, Shimane Y, Kamekura M, Usami R (2012) *Natribacillus halophilus* gen. nov., sp. nov., a moderately halophilic and alkalitolerant bacterium isolated from soil. Int J Syst Evol Microbiol 62:289–294
104. Usami R, Echigo A, Fukushima T, Mizuki T, Yoshida Y, Kamekura M (2007) *Alkalibacillus silvisoli* sp. nov., an alkaliphilic moderate halophile isolated from non-saline forest soil in Japan. Int J Syst Evol Microbiol 57:770–774
105. Inan K, Kacagan M, Ozer A, Belduz AO, Canakci S (2015) *Algoriphagus trabzonensis* sp. nov., isolated from freshwater, and emended description of *Algoriphagus alkaliphilus*. Int J Syst Evol Microbiol 65:2234–2240
106. Denizci AA, Kazan D, Erarslan A (2010) *Bacillus marmarensis* sp. nov., an alkaliphilic, protease- producing bacterium isolated from mushroom compost. Int J Syst Evol Microbiol 60:1590–1594
107. Yumoto I, Nakamura A, Iwata H, Kojima K, Kusumoto K, Nodasaka Y, Matsuyama H (2002) *Dietzia psychralcaliphila* sp. nov., a novel, facultatively psychrophilic alkaliphile that grows on hydrocarbons. Int J Syst Evol Microbiol 52:85–90

108. Yumoto M, Hishinuma-Narisawa M, Hirota K, Shingyo T, Takebe F, Nodasaka Y, Matsuyama H, Hara I (2004) *Exiguobacterium oxidotolerans* sp. nov., a novel alkaliphile exhibiting high catalase activity. Int J Syst Evol Microbiol 54:2013–2017
109. Sravanthi T, Tushar L, Sasikala C, Ramana CV (2015) *Spirochaeta odontotermitis* sp. nov., an obligately anaerobic, cellulolytic, halotolerant, alkaliphilic spirochaete isolated from the termite *Odontotermes obesus* (Rambur) gut. Int J Syst Evol Microbiol 65:4589–4594
110. Sravanthi T, Tushar L, Sasikala C, Ramana CV (2016) *Alkalispirochaeta cellulosivorans* gen. nov., sp. nov., a cellulose-hydrolysing, alkaliphilic, halotolerant bacterium isolated from the gut of a wood-eating cockroach (*Cryptocercus punctulatus*), and reclassification of four species of *Spirochaeta* as new combinations within *Alkalispirochaeta* gen. nov. Int J Syst Evol Microbiol 66:1612–1619
111. Aizawa T, Urai M, Iwabuchi N, Nakajima M, Sunairi M (2010) *Bacillus trypoxylicola* sp. nov., xylanase-producing, alkaliphilic bacteria isolated from the guts of Japanese horned beetle larvae (*Trypoxylus dichotomus septentrionalis*). Int J Syst Evol Microbiol 60:61–66
112. Ishikawa M, Kodama K, Yasuda H, Okamoto-Kainuma A, Koizumi K, Yamasato K (2007) Presence of halophilic and alkaliphilic lactic acid bacteria in various cheeses. Lett Appl Microbiol 44:308–313
113. Kempe S, Kaźmierczak J (2003) Modern soda lakes: model environments for an early alkaline ocean. In: Müller T, Müller H (eds) Modelling in natural sciences; design, validation and case studies. Springer, Berlin, pp 309–322
114. Bondarenko SA, Ianutsevich EA, Danilova OA, Grum-Grzhimaylo AA, Kotlova ER, Kamzolkina OV, Bilanenko EN, Tereshina VM (2017) Membrane lipids and soluble sugars dynamics of the alkaliphilic fungus *Sodiomyces tronii* in response to ambient pH. Extremophiles 21:743–754
115. Schagerl M (ed) (2016) Soda lakes of East Africa. Springer, Cham
116. Keresztes ZG, Felföldi T, Somogyi B, Székely G, Dragoş N, Márialigeti K, Bartha C, Vörös L (2012) First record of picophytoplankton diversity in Central European hypersaline lakes. Extremophiles 16:759–769
117. Steiman R, Ford L, Ducros V, Lafond JL, Guiraud P (2004) First survey of fungi in hypersaline soil and water of Mono Lake area (California). Antonie Van Leeuwenhoek 85:69–83
118. Golubic S, Buch B (1978) Diatoms in Lake Van Sediments. In: Degens ET, Kurtman F (eds) Geology of Lake Van. Miner Res Explor Inst Turkey, vol 169. Ankara, pp 111–114
119. Amaral-Zettler LA (2013) Eukaryotic diversity at pH extremes. Front Microbiol 3:441
120. Roesler CS, Culbertson CW, Etheridge SM, Goericke R, Kiene RP, Miller LG, Oremland RS (2002) Distribution, production, and ecophysiology of *Picocystis* strain ML in Mono Lake, California. Limnol Oceanogr 47:440–452
121. Di Menna ME (1959) Some physiological characters of yeasts from soils and allied habitats. J Gen Microbiol 20:13–23
122. Nagai K, Sakai T, Rantiatmodjo RM, Suzuki K, Gams W, Okada G (1995) Studies on the distribution of alkalophilic and alkali-tolerant soil fungi I. Mycoscience 36:247–256
123. Okada G, Niimura Y, Sakata T, Uchimura T, Ohara N, Suzuki H, Kozaki M (1993) *Acremonium alcalophilum*, a new alkalophilic cellulolytic hyphomycete. Trans Mycol Soc Jpn 34:171–185
124. Grum-Grzhimaylo AA, Georgieva ML, Bondarenko SA, Debets AJM, Bilanenko EN (2016) On the diversity of fungi from soda soils. Fungal Divers 76:27–74
125. Aono R (1990) Taxonomic distribution of alkali-tolerant yeasts. Syst Appl Microbiol 13:394–397
126. Lisichkina GA, Bab'eva IP, Sorokin DY (1993) Alkalitolerant yeasts from natural biotopes. Microbiology RU 72:618–620
127. Kladwang W, Bhumirattana A, Hywel-Jones N (2003) Alkaline-tolerant fungi from Thailand. Fungal Divers 13:69–83
128. Eliades LA, Cabello MN, Voget CE (2006) Contribution to the study of alkalophilic and alkali-tolerant *Ascomycota* from Argentina. Darwiniana 44:64–73

129. Nagai K, Suzuki K, Okada G (1998) Studies on the distribution of alkaliphilic and alkali-tolerant soil fungi II: fungal flora in two limestone caves in Japan. Mycoscience 39:293–298
130. Grant WD (2006) Cultivation of aerobic alkaliphiles. In: Rainey FA, Oren A (eds) Methods in microbiology. Extremophiles, vol 35. Elsevier, New York, pp 439–449
131. Kevbrin V, Boltyanskaya Y, Zhilina T, Kolganova T, Lavrentjeva E, Kuznetsov B (2013) *Proteinivorax tanatarense* gen. nov., sp. nov., an anaerobic, haloalkaliphilic, proteolytic bacterium isolated from a decaying algal bloom, and proposal of *Proteinivoraceae* fam. nov. Extremophiles 17:747–756
132. Datta S, Mody K, Gopalsamy G, Jha B (2011) Novel application of k-carrageenan: as a gelling agent in microbiological media to study biodiversity of extreme alkaliphiles. Carbohydr Polym 85:465–468
133. Kevbrin VV, Boltyanskaya Y, Garnova E, Wiegel J (2008) *Anaerobranca zavarzinii* sp. nov., an anaerobic, alkalithermophilic bacterium isolated from Kamchatka thermal fields. Int J Syst Evol Microbiol 58:1486–1491
134. Boltyanskaya Y, Detkova E, Pimenov N, Kevbrin V (2018) *Proteinivorax hydrogeniformans* sp. nov., an anaerobic, haloalkaliphilic bacterium fermenting proteinaceous compounds with high hydrogen production. Antonie Van Leeuwenhoek 111:275–284
135. McMillan DGG, Keis S, Berney M, Cook GM (2009) Nonfermentative thermoalkaliphilic growth is restricted to alkaline environments. Appl Environ Microbiol 75:7649–7654
136. Kevbrin V, Lysenko AM, Zhilina TN (1997) Physiology of the alkaliphilic methanogen Z-7936, a new strain of *Methanosalsus zhilinae* isolated from lake Magadi. Microbiology RU 66:261–266
137. Krieg NR, Padgett PJ (2011) Phenotypic and physiological characterization methods. In: Rainey F, Oren A (eds) Methods in microbiology. Taxonomy of prokaryotes, vol 38. Academic, Cambridge, pp 15–60
138. Sorokin DY (2005) Is there a limit for high-pH life? Int J Syst Evol Microbiol 55:1405–1406
139. Mesbah NM, Wiegel J (2006) Isolation, cultivation and characterization of alkalithermophiles. In: Rainey FA, Oren A (eds) Methods in microbiology. Extremophiles, vol 35. Elsevier, New York, pp 451–468

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Challenges and Adaptations of Life in Alkaline Habitats

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Contents

Abstract A vast array of organisms is known thriving in high pH environments. The biotechnological, medical, and environmental importance of this remarkable group of organisms has attracted a great deal of interest among researchers and industrialists. One of the most intriguing phenomena of alkaliphiles that engrossed researchers' attention is their adaptation to high pH and ability to thrive in the "extreme" condition which is often lethal to other organisms. Studies made in this line revealed that alkaliphiles deployed a range of adaptive strategies to overcome the various challenges of life in high pH environments. This chapter highlights some of the challenges and the most important structural and functional adaptations that alkaliphiles evolved to circumvent the hurdles and flourish in alkaline habitats. The fascinating alkaliphiles' pH homeostasis that effectively maintains a lower

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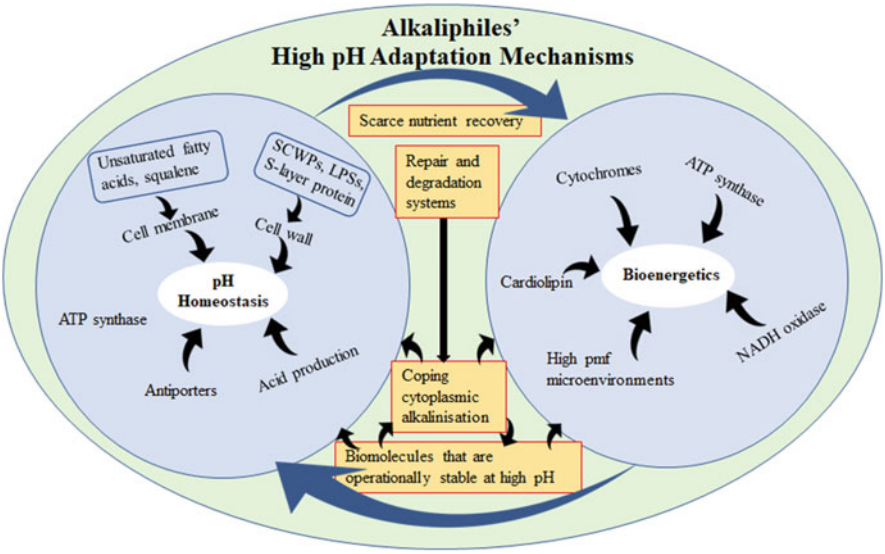
cytoplasmic pH than its extracellular environment and the remarkable bioenergetics that produce ATP much faster than non-alkaliphiles systems are reviewed in detail. Moreover, the adaptive mechanisms that alkaliphiles employ to keep the structural and functional integrity of their biomolecules at elevated pH are assessed.

It is undeniable that our understanding of alkaliphiles adaptation mechanisms to high pH is expanding with time. However, considering that little is known so far about the adaptation of life in alkaline milieu, it seems that this is just the beginning. Probably, there is a lot more waiting for discovery, and some of these issues are raised in the chapter, which not only summarizes the relevant literature but also forwards new insights regarding high pH adaptation. Moreover, an effort is made to include the largely neglected eukaryotic organisms' adaptation to high pH habitats.

Graphical Abstract



Keywords Alkaliphiles, Alkaliphiles adaptation, Antiporter, ATP synthase, Bioenergetic, Cardiolipin, Cytochrome, Eukaryotes, Extremophiles, pH homeostasis, Secondary cell wall, S-layer, Squalene, Unsaturated fatty acids

Abbreviations

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BMB	Bis(monoacylglycero) phosphate
CPA	Cation/proton antiporters

GH	Glycoside hydrolase
LPS	Lipopolysaccharide
Mrp	Multiple resistance and pH
MS	Mass spectrometry
NMDA	*N*-methyl-D-aspartate
NMR	Nuclear magnetic resonance
OXPHOS	Oxidative phosphorylation
PCM	L-isoaspartyl protein carboxyl methyltransferase
PG	Peptidoglycan
P_i	Inorganic phosphate
pI	Isoelectric points
Pmf	Proton motive force
SCWP	Secondary cell wall polymers
S-layer	Cell surface layer
SLH	S-layer homology
SlpA	Surface-layer protein A
Smf	Sodium motive force
TCDB	Transporter classification database
TMH4	Trans-membrane helix-4
TMH5	Trans-membrane helix-5
TUA	Teichuronic acid
TUP	Teichuronopeptide
UT-A	Urea transporter A

1 Introduction

Organisms interact with their environment to ensure survival. They acquire resources such as nutrients from their environment and discharge waste and products out of their bodies. These fascinating complex processes are accomplished at astonishingly high rate of fidelity through numerous metabolic activities which give the identity of life. To sustain life, organisms should adjust to their environment. Thus, organisms evolve various mechanisms including structural and physiological features that allow them not only to survive but also thrive in their respective habitats. This process of evolving structural and functional mechanisms to thrive in an environment is known as adaptation.

The incredible evolution of organisms in response to environmental conditions gave rise to an impressive diversity of adaptive solutions to a range of habitats. This stretches life almost to every corner of the planet. Even places that once have been considered too hostile for life are found to be inhabited by various life forms. One of these "unusual" places is the different natural and man-made alkaline environments which are found inhabited by diverse groups of organisms known as alkaliphiles. Soda lakes, which are stable alkaline environments, are important habitats for

alkaliphiles from which many alkaliphiles have been isolated [1, 2]. Similarly, soda deserts, soda pans, solonchak soils, salt pans, oceans, etc. are known supporting this remarkable group of organisms [3–7]. Alkaliphiles have also been isolated from the steady-state alkaline environments that exist in the body of other organisms such as insect guts [8–10]. Serpentinization, low-temperature weathering of silicate containing calcium and magnesium minerals like olivine ($MgFeSiO_4$) and pyroxene ($MgCaFeSiO_3$), forms a highly alkaline Ca^{2+}-rich environment [11]. Alkaliphiles such as *Alkaliphilus hydrothermalis* [12] and *Serpentinicella alkaliphila* [13] have been isolated from such serpentine environments. Anthropogenic activities such as indigo dye production, potato peeling using KOH, cement/concrete production, electroplating, leather tanning, paper and board manufacture, mining, and herbicide manufacturing create alkaline environments [14–18]. Regardless of the difference in the genesis, chemistry, and stability, all known alkaline environments are inhabited by alkaliphiles, and this is discussed in detail by Kevbrin [19].

High pH environments are not easy to live in without special adaptations. For instance, maintaining structural integrity, bioenergetics, and intracellular pH homeostasis are barriers for non-alkaliphiles to survive and thrive in this extreme environment. On the other hand, alkaliphiles evolved adaptive solutions to circumvent these barriers and thrive lavishly in environments with pH values of up to over 13 [16]. However, adaptation always comes with price. Indeed, there is no single organism that flourishes in a pH range of 1–13. At least in this case, the rule of nature seems clear, when an organism evolves adaptations to thrive in specific pH condition (acidic, neutral, or alkaline), its fitness to live in a habitat of different pH condition is often compromised. Thus, the adaptation range of alkaliphiles determines their ability to survive in neutral conditions. As the optimum pH for growth varies among alkaliphiles, the ability to grow in neutral zone is also different. The growth of obligate alkaliphiles is compromised around neutral condition. On the other hand, facultative alkaliphiles can grow at neutral pH but not as lavishly as neutralophiles [20–22]. Similarly, at or above pH 10, the growth yield of facultative alkaliphiles is often lower than that of obligate alkaliphiles. This may indicate that alkaliphiles evolved to colonize high pH environments at a cost of losing growth potency around neutrality.

This chapter presents the grand challenges of life in high pH environments and tries to summarize the adaptive mechanisms deployed by alkaliphiles to circumvent the challenges and successfully colonize high pH habitats. Most of the studies made on high pH adaptations of organisms are related to alkaliphilic bacteria, and hence, the discussion in this chapter largely revolves around this group of organisms. On the other hand, there are several groups of unicellular and multicellular eukaryotes that are adapted to alkaline habitats. Studies on high pH adaptations of these eukaryotic organisms still remain scarce. Here, an effort is made to include the available information on adaptive mechanisms of multicellular organisms to high pH environment, fish.

2 The Grand Challenges to Thrive in Alkaline Habitats from a Neutralophilic Standpoint

A wide range of "bizarre" environments exist in the biosphere. Hot and frozen environments, sulfurous springs, solfataras, the deep-sea black smoker vents and cold seeps, acidic environments of anthropogenic and natural origin, and salt lakes are some among the many that fall in this category. These environments have their own challenges for life to thrive in. However, such sites are often found inhabited by organisms, which have specific adaptive solutions to the challenges of the respective extreme habitats. Likewise, alkaline environments have their own challenges, and some of the most important ones are discussed below.

The biochemical reactions of life are not spontaneous or self-driven; rather they are highly regulated and are mediated by specific enzymes which are operationally stable within a range of pH. The cytoplasmic pH of cells from various organisms is known to be within the neutral range [22], and the enzymes that catalyze the myriad biochemical reactions occurring inside cells are evolved to work optimally around this pH, neutrality. As the pH drifts away from the neutral range, the catalytic efficiency of the enzymes dwindles, and the cellular functional integrity drops. Like the functional integrity, the structural integrity of intracellular biomolecules is tuned to the cytoplasmic pH. The integrity of at least some of the important macromolecules such as proteins, lipids, and genetic materials can be labile at elevated pH, and the molecules become prone to precipitation or breakdown [23–25]. This structural and functional integrity impairment can be fatal and bring cellular demise. Thus, for biochemical reactions to proceed without a flaw and ensure survival, the intracellular pH should be maintained in the neutral range. However, when organisms are exposed to high pH condition, maintaining their cytoplasmic pH within the neutral range becomes difficult, and an upward drift in the cytoplasmic pH can occur. If this cytoplasmic pH rise remains unchecked, it ultimately kills the cell/organism. Thus, thriving in high pH environment requires an effective way of maintaining the intracellular pH close to neutrality and ability to withstand some degree of alkalinization. This process of maintaining pH within physiologically favorable range regardless of the extracellular environment is known as pH homeostasis.

In addition to pH homeostasis, at least non-photosynthetic aerobic prokaryotic life forms face another daunting task in high pH environment, bioenergetics. Living organisms require energy to perform the phenomena of life such as growth, reproduction, structure maintenance, movement, etc. Moreover, cells maintain order against chaos/randomness with expenditure of energy. If there is no energy that a cell uses to maintain order, chaos reign, and it loses viability. Thus, life-sustaining metabolic processes enable organisms to generate and store energy. In this regard, ATP is the most vital molecule which lays at the center of cellular bioenergetics. It is known as the energy currency of life which can store and shuttle chemical energy within cells. This energy-rich molecule can be produced by various cellular processes, most typically by F_1F_0-ATP synthase-mediated oxidative phosphorylation

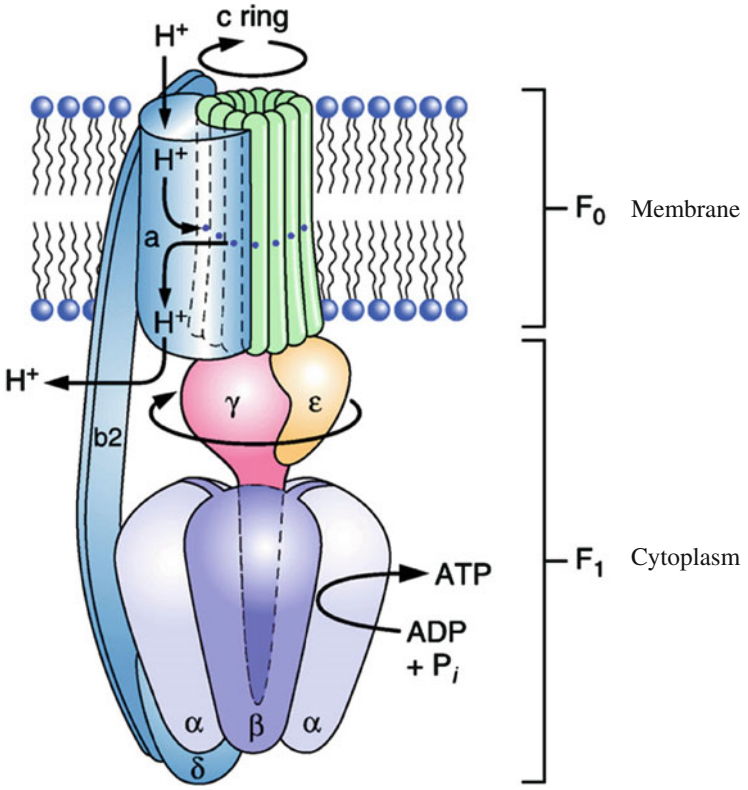


Fig. 1 A diagram representation of F_0F_1-ATP synthase with its subunits. Adopted from Hicks et al. [27] and reprinted with kind permission from Elsevier

(OXPHOS) or via photophosphorylation. However, some organisms in anoxic condition [26] and few aerobic cells such as matured red blood cells synthesize ATP through substrate level phosphorylation.

The F_1F_0-ATP synthase is a multi-subunit two-domain membrane-bound enzyme. The intracellular domain which is known as F_1 domain is hydrophilic, and the F_0 domain is hydrophobic, and most of it is embedded in the membrane. The F_0 domain has a functional center which captures protons from the bulk (extracellular) environment and channels them down to the cytoplasm (Fig. 1). This proton (H^+) translocation induces conformational changes in F_1 domain which in turn drives the synthesis of ATP from inorganic phosphate (P_i) and ADP. Neutralophiles and acidophiles have lower concentration of protons in their cytoplasm than their extracellular environment. Thus, the downhill movement of the ions across the membrane drives the ATP synthesis as explained by the chemiosmotic theory [28]. However, the efficiency of this proton motive force (pmf) diminishes in alkaline environments due to reversed proton gradient, which is higher in the cytoplasm than the extracellular environment. Thus, to flourish in alkaline

environments, it is necessary to evolve mechanisms that effectively generate energy carriers under this thermodynamically challenging condition.

High pH is known to degrade biological entities by disrupting molecular bonds between atoms in biomolecules. Thus, one of the challenges to thrive in alkaline environments is the maintenance of the cell structural integrity at high pH. Proteins, lipids, carbohydrates, and aromatic structures such as lignin which are the important structural components of different organisms are susceptible to alkaline conditions. For instance, keratin, a tough structural protein of hair, feather, horn, and nails decomposes at high pH [29]. This type of alkaline-mediated proteolysis of cellular proteins and peptides is sometimes referred to as liquefactive denaturation [23]. Studies on the degradation of plant biomass in aquatic environments have also revealed that the decomposition rate is linearly related to pH [30]. At high pH the plant cell wall cementing substance, lignin, decomposes, and the solubility of the hemicellulose fraction increases which results in degradation of plant biomass. Such susceptibility of structural biomolecules to high pH has led to the emergence of applications that use alkaline treatments to break down biological materials in various processes. Alkaline treatments are used in pulp and paper industry to break down the lignin and hemicellulose fractions of the plant biomass (Kraft pulping process), in molecular biology to digest bacterial cell wall during DNA (e.g., plasmid) extraction, in leather tanning to dehair skin and hides, in waste management to decompose keratin (e.g., feather) waste, etc. Lipids, which are important structural components of cells such as membranes, are also labile to high pH. The degradation process of lipids is known as saponification [23]. Degradation of structural components such as the cell membrane is lethal as it compromises the integrity of cells. In fact, alkaline solutions have long known for their disinfectant properties and are used as antimicrobial agents [31–34]. Alkaline solutions are also widely used as cleaning agents due to their ability of removing (by degrading and solubilizing) organic matter such as protein, lipid, and nucleic acids [32]. Thus, organisms that colonize alkaline habitats must evolve mechanisms which protect the cell integrity from the adverse effect of the extreme pH.

The challenges of life at high pH habitats are not restricted only to cell associated structures, but it also involves the structural and functional integrity of extracellular products. Cells produce and secrete various biomolecules to the extracellular environment to perform different tasks such as exopolysaccharides for protection, adhesion and biofilm formation, chemical signal molecules for cell-to-cell communication, enzymes for nutrient acquiring and recycling, bioactive compounds for defense and competition, etc. [35–40]. The efficiency of these biomolecules influences the success of the organism in colonizing a habitat. To fulfill the desired tasks, these products should be operationally stable in the habitat condition. Thus, the success of colonizing high pH habitats, at least partly, depends on the operational stability of the extracellular products. For instance, extracellular enzymes are very important to acquire nutrients by breaking down polymeric substrates to smaller pieces that can be transported to the cytoplasm across the cell envelope. But enzymes are optimally active and stable within a certain range of pH and can be denatured and cease to function outside this range. Since the extracellular biomolecules of

neutralophiles are evolved to function often around neutrality, the high pH of alkaline habitats can disrupt their activity and stability, which potentially starve the cell to death. Thus, one of the challenges in colonizing high pH habitats is to have extracellular products that are efficient and operationally stable at elevated pH.

Another important high pH environment challenge is related to nutrient availability. The bioavailability of some nutrients can be affected by the pH of the environment. Some nutrients become less available at high pH. However, since the genesis and chemistry of alkaline environments are different, the scarcity of nutrients could also vary from habitat to habitat. The scarcity problem in some soda lakes is mentioned here as an example. Nutrients such as P, Ca, Mg, Fe, etc. are less available in soda lakes [41, 42]. These substances either due to geological reasons or reactions with other constituents of the lakes form insoluble precipitates which severely diminish the bioavailability. However, these nutrients are important for normal metabolic process. For instance, several enzymes require metal ions such as Ca, Mg, and Fe for activity and/or stability [43]. Thus, the scarcity of these metals can severely impair the function of the enzymes and adversely affect the metabolic processes. The malfunctioning of metabolic processes can compromise cellular activities which can lead to survival deterioration. Thus, colonization of high pH habitats requires adaptive solutions to evade challenges related to poor nutrient availability.

What has been described in the above are some of the important challenges of life in alkaline environments, challenges that neutralophiles can face in high pH habitats. Probably, solving these challenges was the key adaptive evolution of alkaliphiles which allowed them to flourish in high pH environments. Below, the adaptive strategies deployed by alkaliphiles to circumvent these grand challenges of alkaline environments are discussed.

3 Adaptation of Alkaliphiles: Circumventing the Challenges of Alkalinity

Often, organisms colonizing extreme habitats have unique features that allow them not only to survive but also thrive in it. As discussed above, alkaline environments are bundled with tough challenges, and yet it is home for richly diverse group of organisms. The ability of lavishly growing at punishingly high pH in which neutralophiles cannot even survive for a while indicates the unique adaptive strategies deployed by alkaliphiles. Unraveling the secret of high pH adaptation of alkaliphiles has been the subject of several studies for decades. These studies have contributed to our knowledge of high pH adaptations of organisms. An effort is made here to summarize the important findings that depict the remarkable high pH adaptation mechanisms of alkaliphiles.

3.1 pH Homeostasis

The intracellular pH homeostasis is a vital adaptation shared by all alkaliphiles. Since Garland indicated that alkaliphiles maintain lower cytoplasmic pH than its external environment [44], the difference between the intracellular and extracellular pH values of alkaliphiles has been studied (Table 1). The results of these studies confirmed the established notion that alkaliphiles have an impressive capacity of maintaining low cytoplasmic pH while thriving in high pH environment. This pH homeostasis can uphold a difference of more than 2 pH units between the cytoplasm and extracellular environment [46–48, 50, 51]. Alkaliphiles use a variety of adaptive strategies to achieve this remarkable pH homeostasis (Fig. 2). Some of the most important adaptive mechanisms that play significant role in pH homeostasis such as acquiring H^+ from extracellular environment, reducing H^+ leakage from the cytoplasm, production of organic acid, and deterring the diffusion of OH^- from the extracellular environment are discussed below.

Table 1 The intracellular and extracellular pH values of some alkaliphiles

Organism	Extracellular pH	Intracellular pH	References
Exiguobacterium aurantiacum	9.4	8.4	McLaggan et al. [45]
Clostridium paradoxum	9.8	8.5	Cook et al. [46]
B. pseudofirmus OF4	10.5	8.3	Guffanti and Hicks [47]
B. pseudofirmus OF4	10.8	8.3	Sturr et al. [48]
B. pseudofirmus OF4	11.2	8.9	Sturr et al. [48]
B. pseudofirmus OF4	11.4	9.6	Sturr et al. [48]
B. halodurans C-125	9	7.9	Aono et al. [49]
B. halodurans C-125	9.5	8.1	Aono et al. [49]
B. halodurans C-125	10	8.2	Aono et al. [49]
B. halodurans C-125	10.5	8.4	Aono et al. [49]
B. alcalophilus	9	7.6	Guffanti and Hicks [47]
B. alcalophilus	10	8.6	Guffanti and Hicks [47]
B. alcalophilus	11	9.2	Guffanti and Hicks [47]

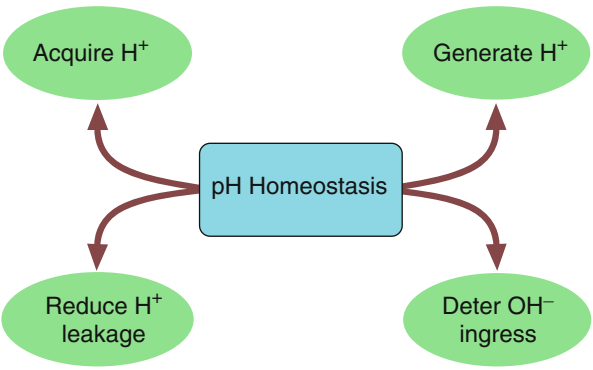


Fig. 2 The strategies alkaliphiles use to maintain low intracellular pH

3.1.1 High Level of Monovalent Cation/Proton Antiporters

Alkaliphiles tend to keep their cytoplasmic pH close to neutral range. To do this alkaliphilic cells maintain relatively high concentration of H^+ in their cytoplasm. One way of achieving this is by translocating H^+ from the extracellular environment into the cell and tightly controlling it. But there are two challenges to do this: the scarcity of H^+ in the extracellular environment and that the translocation and control are against concentration gradient. Alkaliphiles evolved mechanisms that solve these challenges. The monovalent cation/proton antiporters which exchange the intracellular cations such as Na^+ and Li^+ for the extracellular H^+ are believed to be the most important mechanism that alkaliphiles depend on for intracellular pH homeostasis [21, 52–59]. Based on the Transporter Classification Database (TCDB; http://www.tcdb.org), these antiporters are diverse and belong to two superfamilies. The cation/proton antiporters (CPA) superfamily which consists of five families including family CPA1 and CPA2 and the Na^+ transporting Mrp superfamily that comprises three families including family CPA3 which is among the most vital H^+ translocating antiporters of alkaliphiles [22, 60]. In addition to the families that belong to the two superfamilies, the Nha families, NhaA, NhaB, NhaC, and NhaD [61] are also involved in the homeostasis process [62].

Among the monovalent cation/proton antiporters, Na^+/H^+ antiporters which exchange cytoplasmic Na^+ for extracellular H^+ seem to be very crucial for pH homeostasis in alkaliphiles [21, 22, 54, 55]. Moreover, these antiporters are also used for Na^+ and volume homeostasis as well, like what it does in eukaryotic cells and their organelles [58, 63–66]. These antiporters avoid the accumulation of Na^+ to toxic level, while it maintains relatively higher H^+ concentration in the cytoplasm [21, 67]. The Na^+/H^+ antiporters are secondary active transporters which use the transmembrane electrical potential ($\Delta\psi$) generated by primary ion pumps such as the respiratory complexes [27] to efflux intracellular Na^+ [21, 54, 55, 68, 69]. In alkaliphiles, the monovalent cation/proton antiporter-mediated pH homeostasis is primarily specific for Na^+ but also accommodates Li^+ efflux. On the other hand, unlike alkaliphiles, neutralophiles use not only $Na^+(Li^+)/H^+$ antiporters but also K^+/H^+ antiporters [21, 22]. The specificity of the alkaliphiles monovalent cation/proton antiporters system to Na^+ is believed to avoid severe depletion of cytoplasmic K^+ that can potentially compromise some cytoplasmic processes [21] and enhances the cytotoxicity of Na^+ [21, 70, 71]. The other possibility might be that most of the studied alkaliphiles are adapted to habitats such as soda lakes with high level of Na^+; hence, it is ideal for such organisms to evolve a system that relies on the ample resource (Na^+).

Comparative analysis of genes encoding CPAs in genomes of alkaliphiles and neutralophiles revealed that there is no significant difference in the number of the genes between alkaliphiles and neutralophiles [54, 55]. However, the aggregate level of the Na^+/H^+ antiporter is much higher in alkaliphiles than in neutralophiles [21, 52, 53, 72]. This may be due to the greater burden of pH homeostasis at higher

extracellular pH values and the sole dependence of alkaliphiles on Na^+/H^+ antiporters unlike neutralophiles which also involve K^+/H^+ antiporters [21, 52].

Several studies have shown the vital importance of Na^+/H^+ antiporters in adapting high pH environments [73, 74]. This is clearly shown in the growth profile of neutralophiles with and without Na^+/H^+ antiporters. The growth of neutralophiles that lack functioning Na^+/H^+ antiporters is limited around neutrality, pH 6.3–7.7 [75, 76]. In these organisms, the rise in the environmental pH to alkaline range is accompanied by rapid alkalinization due to inefficient intracellular pH homeostasis, which hampers growth. But neutralophiles equipped with functional Na^+/H^+ antiporters can maintain their cytoplasmic pH around 7.5 and grow in environments with pH values of up to 8.5. Often, when the external pH value exceeds 8.5, neutralophiles start to grow slowly, and when the pH is over 9, their growth becomes severely impaired [21, 22]. At higher alkalinity, the neutralophiles fail to maintain their cytoplasmic pH below 8. Moreover, the physiology of these organisms is not adapted to function at high pH, and this results in a dramatic drop in growth rate as the external pH values increase. However, alkaliphiles which are endowed with high level of Na^+/H^+ antiporters can maintain their cytoplasmic pH at 7.5 even when growing in an environment of pH 9.5. Some of these alkaliphiles can grow at much higher pH, and in those conditions, the intracellular pH is expected to become way above the pH values at which neutralophiles can survive and/or grow.

Monovalent cation/proton antiporters can be products of a single gene or hetero-oligomers assembled from multiple gene products. The hetero-oligomer monovalent cation antiporters are known as Mrp [77]. Mrps are widely distributed among bacteria and archaea [77, 78] and involved in several physiological processes. In archaea, Mrps are used in the conversion of energy involved in metabolism and hydrogen production, while in bacteria, it is involved in nitrogen fixation, bile salt tolerance, arsenic oxidation, and pathogenesis [78]. In alkaliphiles it is believed that it plays a dominant role in pH homeostasis and sodium tolerance [21, 22, 60, 77, 79, 80]. The Mrp operon has six or seven genes which encode hydrophobic proteins required for optimal activity [79]. Structural analysis predicted that these antiporters have large surface which can facilitate the capturing of proton and funneling it into the antiporter [21, 22, 80].

The Na^+/H^+ antiporter systems exchange cytoplasmic Na^+ for extracellular H^+. However, the Na^+ leaving the cytoplasm must be replenished so that the antiporter-dependent pH homeostasis system works effectively; this is especially important when the extracellular Na^+ concentration is low [21, 22, 81–85]. Alkaliphiles use Na^+ solute symporters and Na^+-coupled motility channels known as MotPS for reentry of Na^+ to the cytoplasm [21, 78, 86, 87, 88]. Moreover, Na^+ uptake by alkaliphiles is accomplished through voltage-gated Na^+ channels known as NaChBac and NaVBP [86, 89–93]. The major Na^+ and H^+ entry and exit pathways are shown in Fig. 3.

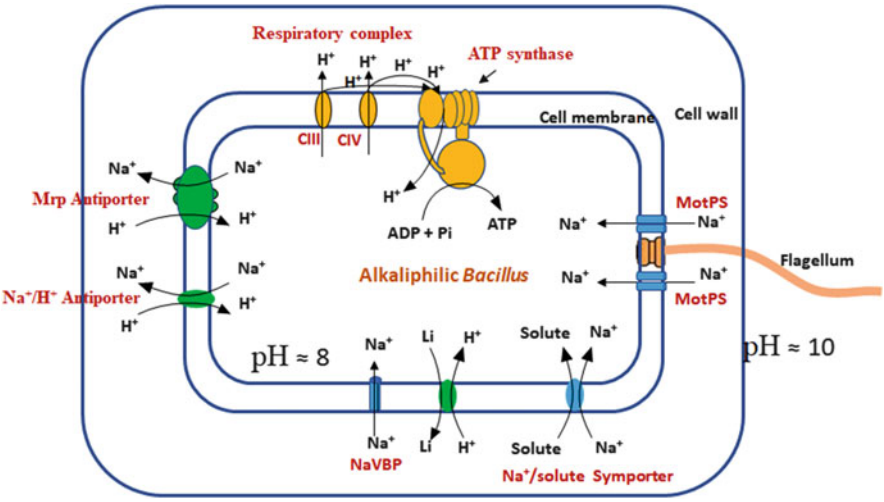


Fig. 3 A diagram of alkaliphilic *Bacillus* cell depicting some of the most important cellular entry and exit paths of H^+ and Na^+

3.1.2 Effective Proton Capturing by ATP Synthase and Inhibition of ATPase Activity

The capture and translocation of H^+ by F_1F_0-ATP synthases of cells adapted to neutral or acidic habitats are energetically favored as the H^+ concentration outside the cell is higher than that of the cytoplasm. On the other hand, alkaliphiles do not have the luxury of high H^+ concentration in their environment. H^+ is scarce in alkaline habitats, and hence, organisms adapted in such environments require an efficient system for capturing and translocating H^+. The analysis of the *atp* operon, the cluster of genes coding for F_1F_0-ATP synthase, of alkaliphilic *Bacillus* bacteria revealed a conserved lysine residue at position 180 (based on that of *B. pseudofirmus* OF4 numbering) of *a*-subunit [94] which exists only in alkaliphilic *Bacillus* gene sequences [95]. This conserved lysine in a thermoalkaliphilic strain, *Bacillus* sp. TA2.A1, was mutated to His, Arg, and Gly. Analyses of the ATP synthases carrying these mutations have shown that L180 is a specific adaptation of alkaliphiles that facilitate H^+ capture at high pH [94]. A broader mutational study on the ATP synthase of *a*-subunit of *B. pseudofirmus* OF4 indicated that the ATP synthase of alkaliphiles evolved to efficiently capture, translocate to the synthase core, and retain H^+ in the cytoplasm [95]. Therefore, ATP synthase is believed contributing to alkaliphiles pH homeostasis.

The ATP synthases of alkaliphiles have another remarkable contribution to the pH homeostasis. The F_1 domain of non-alkaliphiles ATP synthases is known not only synthesizing ATP but also hydrolyzing (ATPase activity) it. As shown in Fig. 4a, the hydrolysis of ATP drives the ATP synthase *c*-ring reverse rotation which pumps out proton to the extracellular environment. However, the ATP

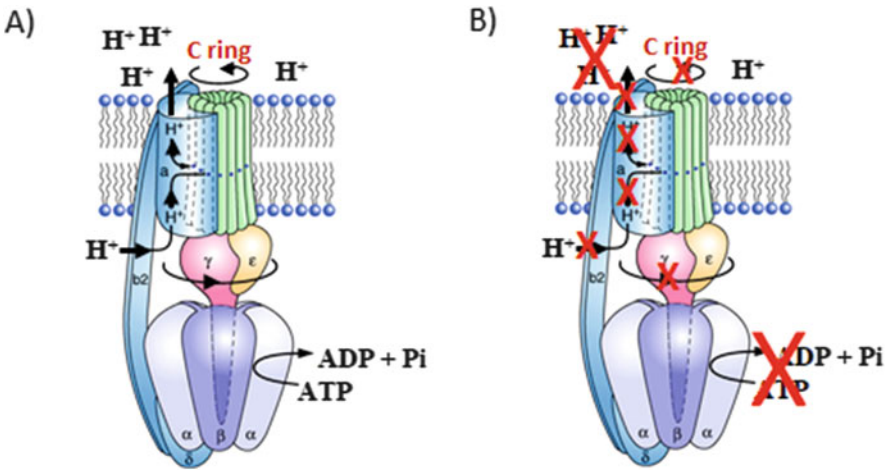


Fig. 4 The ATPase activity that pumps out H^+ in non-alkaliphiles (**a**) is absent in alkaliphiles, and cells retain H^+ (**b**)

synthase of aerobic alkaliphiles does not have the ATPase activity (Fig. 4b) and cannot translocate H^+ out of the cell [96–99], and this helps to retain the H^+ required for lowering the cytoplasmic pH. The importance of this adaptation is reflected on the *B. pseudofirmus* OF4 mutants K180H and K180G. These mutants exhibit high level of ATPase activity which compromised non-fermentative growth at pH 10.5 [95]. The adaptation features of the ATP synthase and the respiratory system that contributes to pH homeostasis in addition to ATP generation are discussed below in Sects. 3.2.1 and 3.2.2.

3.1.3 Acid Production

Extracellular pH is known to affect metabolic processes, and cells produce acids or alkali to offset the change in medium pH [100, 101]. For example, when *Escherichia coli* grows in high pH medium, it shifts its metabolism toward acid production [102]. The acid production process is facilitated by upregulating the deaminase, ATP synthase, and cytochrome *d* oxidoreductase activities. Like *E. coli*, many other organisms swing to acid production upon rise in the pH of the medium. Similarly, numerous alkaliphiles are known to produce acid that decreases the pH of the culture significantly [56, 103–105]. Alkaliphiles produce metabolic acid through sugar fermentation and amino acid deaminases. The acid production contributes to the pH homeostasis primarily by increasing the cytoplasmic H^+ concentration. Moreover, the acid production, in addition to preventing cytoplasmic alkalinization, can increase the availability of H^+ in the vicinity of the cell, and this can potentially contribute to alleviate the burden of capturing and translocating H^+ to cytoplasm.

3.1.4 Anion (OH^-) Deterring Cell Surface

The cell envelope, which comprises the cell membrane, cell wall, and associated cell surface depositions, is a barrier that prevents the cytoplasm from the direct effect of its environment. If cells must maintain a near-neutral cytoplasmic pH, the OH^- of the alkaline environment should not freely ingress into the cell. The cell surface of alkaliphiles contains acidic residues [106] that potentially repel the anions and prevent the rise in the cytoplasmic pH. Moreover, the anionic cell surface can capture H^+, especially H^+ pumped out of the cell such as by the respiratory complex. This ability of trapping H^+ might form a kind of H^+ reserve close to the membrane surface which hypothetically reduces the difficulty of capturing H^+ from the bulk alkaline environment. The H^+ trapped in the cell envelope can be retrieved such as by the Na^+/H^+ antiporters and ATP synthases and translocated to the cytoplasm, which contributes to the pH homeostasis. These trapped protons may also neutralize OH^- migrating to the cell membrane. Since the contribution of the cell envelope to high pH adaptation is significant, it is relevant to discuss it in detail.

3.2 Protective Cell Envelope

The cell envelope, which consists of cell membrane and cell wall (plus the outer membrane in Gram-negative bacteria), delineates the cell from its environment. This structure plays a vital role in the cell survival primarily by maintaining its content, while it allows controlled exchange of materials between the cell and its environment. Cells such as bacteria interact with their extracellular environment through their cell envelope. Thus, adaptations of such kind of organisms to their habitats often involve their cell envelope. There have been evidences that the cell envelopes of alkaliphiles are part of the high pH adaptation assemblies. Some of the most important studies that substantiate how cell envelope contributes to high pH adaptation of alkaliphiles are discussed below under the different components of the cell envelope.

3.2.1 Cell Wall: Secondary Cell Wall Polymers

Among alkaliphiles, members of the genus *Bacillus* are the most studied. Like other Gram-positive bacteria, the cell wall of *Bacillus* cells contains different polysaccharides. Most of these polysaccharides are covalently bonded to the peptidoglycan (PG), which is the prominent cell wall scaffolding structure. Based on structural properties, the cell wall polysaccharides of these organisms are categorized into three groups: (1) teichuronic acids [107, 108], (2) teichoic acids [109, 110], and (3) other polysaccharides which cannot be characteristically assigned to the other two groups [111, 112]. These polysaccharides have been considered to play

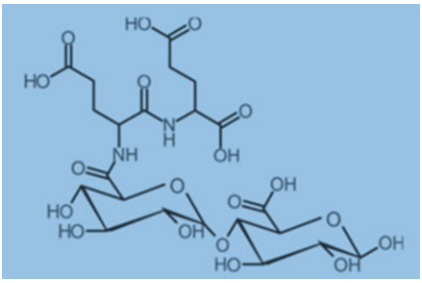


Fig. 5 A teichuronopeptide unit that forms the major polymeric cell wall component of alkaliphilic *Bacillus* strains

secondary role in cell wall function and hence referred to as "secondary" cell wall polymers (SCWPs). SCWP analysis of one of the most studied alkaliphiles, *B. halodurans* C-125, revealed that it is rich in negatively charged residues such as aspartic acid, galacturonic acid, glutamic acid, and phosphoric acid [113, 114]. The negatively charged residues glutamic and glucuronic acids form the major cell wall component of this alkaliphilic *Bacillus* strain, teichuronopeptide (TUP) (Fig. 5). This highly negatively charged cell wall structure interacts with cations such as H^+ [115]. The H^+ trapping by the alkaliphilic bacterial cell wall can delay the rapid loss of H^+ from the cell surface by equilibration effect of the alkaline bulk phase (the environment), and this significantly contributes to the pH homeostasis and bioenergetics of alkaliphiles. Moreover, the cell walls of alkaliphiles shield the cells from the detrimental effect of the high pH environment.

The negatively charged residues of SCWPs such as teichuronopeptide (TUP), teichuronic acid (TUA), and acidic amino acid chains in the cell envelope together with the trapped cations around the cell surface serve as barrier to OH^- [81, 83]. The anions of the SCWPs repel OH^-, while the trapped H^+ neutralizes OH^- escaping into the cell wall. Hence, SCWPs are expected to play a prominent cell protection role in high pH adaptation. Indeed, several studies confirmed its remarkable contribution to high pH adaptation. This includes (1) quantification of SCWPs revealed that cells produce more TUA and TUP when grown at alkaline than at neutral condition [106, 116], (2) removal of the cell wall of *B. halodurans* C-125 cells resulted in protoplasts that are not stable in alkaline medium [81, 83], and (3) *B. halodurans* C-125 mutants with disrupted TUP and TUA production poorly grow at pH 10.5 [81–84] and lose their alkaliphilicity [82, 84, 106, 117]. Moreover, electron microscopy analysis of an alkaliphilic *Bacillus* cell wall has shown that the thickness increases with increasing alkalinity of the growth medium [106]. The increase in the peptidoglycan and SCWPs was proportional [106], and hence, it can be speculated that at higher pH, the cells need a denser negative layer that ensures protection from the effect of high pH, and this is partly achieved by increasing the thickness of the cell wall.

3.2.2 Lipopolysaccharides

The nature of the Gram-negative and Gram-positive bacteria cell surfaces is different. Gram-negative cells lack SCWPs and do not seem to benefit from the high pH adaptation role of these structures. However, it seems that the outer membrane of Gram-negative bacteria plays more of the protection role. This membrane of Gram-negative bacteria contains lipopolysaccharides (LPS) which are exposed to the outer surface of the cells. Although, little is done on its involvement in high pH adaptation, it seems that it may function the same way as SCWPs of Gram-positive alkaliphiles. Indeed, structural analysis of the haloalkaliphilic strain, *Halomonas pantelleriensis* lipopolysaccharide *O*-chain revealed that it has a unique repeating unit, 4-*O*-((*S*)-1-carboxyethyl)-D-GlcA residue [118]. This repeating unit contains carboxyl groups which make the polymer highly negatively charged. Further, chemical, NMR, and MS study results show that the LPS of this haloalkaliphilic strain are very rich in carboxylate groups [118]. A similar observation of highly carboxylated LPS is reported from another Gram-negative bacteria, *H. magadiensis*. A protective buffering effect of this negatively charged LPS has been suggested [119], which is expected to be similar to that of SCWPs, repelling the OH^- by the anions of the LPS and neutralization by the trapped cations.

3.2.3 S-Layer Proteins

The cell envelopes of Gram-positive alkaliphiles are also known to have a special proteinaceous layer known as cell surface layer (S-layer) [120, 121]. S-layers are composed of identical (glyco) protein structures that form lattices on the bacterial cell surfaces. This layer is sometimes referred to as "nonclassical" SCWPs; however, based on compositional and structural analysis, Schäffer and Messner [122] suggested that it belongs to the third cell wall group of Araki and Ito [111]. Alkaliphilic cells express a range of S-layer proteins. For instance, 17 S-layer homology (SLH) domain-containing proteins, including S-layer protein A (SlpA), are identified in the genome sequence of *B. pseudofirmus* OF4 [123]. The contribution of S-layer to high pH adaptation has been assessed through mutational studies using *B. pseudofirmus* OF4 cells which produce SlpA both at neutral and alkaline conditions. Mutants that lack SlpA grow more slowly at pH 11 than the wild-type cells, especially when the Na^+ concentration was low [85]. On the other hand, the wild-type cells expressing SlpA grow slower at neutral condition than at high pH [85]. Although it seems that the expression of SlpA at neutral pH reduces growth efficiency, those facultative organisms expressing SlpA in the neutral range could benefit if sudden alkalinization happens. The results of the mutational studies indicate that the presence of SlpA on the cell surface has a high pH adaptive advantage. Like other cell wall proteins from alkaliphiles, SlpA has low (4.36) isoelectric point (pI) which is mainly due to its fewer arginine and lysine content. Like TUA and TUP, the relatively abundant negatively charged residues of SlpA favor H^+ accumulation and deter OH^- penetration [54, 55, 85, 124, 125].

Despite the fact that S-layer proteins exist in Gram-positive and Gram-negative bacteria [126], studies made so far on high pH adaptation role of S-layer proteins have been restricted to Gram-positive bacteria. Hence, relatively, little is known about the contribution of Gram-negative S-layer proteins to high pH adaptations. Scanning electron microscopy analysis of the surface of a Gram-negative bacterium, *Pseudomonas alcaliphila*, revealed that cells grown at pH 10 have rougher surface than those grown at pH 7 [127]. This might show the possibility that Gram-negative strains also make some surface depositions (S-layer proteins) to thrive in high pH environments. However, this needs specific experimental evidences. Further studies on other S-layer proteins of Gram-positive bacteria and probably other cell surface deposited proteins of alkaliphilic Gram-negative and Gram-positive bacteria may improve our understanding of these interesting proteins contribution to high pH adaptation.

3.2.4 Cell Membrane

The other component of the cell envelope that contributes to high pH adaptation is the cell membrane. The contribution of the outer membrane of Gram-negative bacteria is briefly discussed above in Sect. 3.2.1. In addition to serving as an anchor to negatively charged polymers, the cell membrane of alkaliphiles has shown a stunning difference in composition when compared to that of non-alkaliphiles. Even a difference is noted between the membrane of obligate and facultative alkaliphiles. For instance, a comparative analysis of the membrane fatty acid composition revealed that the unsaturated fatty acids account for 20% and up to 3% of the total phospholipid fatty acids of the obligate and facultative alkaliphilic *Bacillus* strains, respectively, when grown in alkaline condition [128]. Similarly, the membrane composition of an organism can vary with the pH of the growth medium. The membrane of facultative alkaliphiles grown in pH 7.5 medium was almost free of unsaturated fatty acids, while the unsaturated fatty acid content rises to about 3% when these cells were grown in pH 10.5 medium [128]. In another study, *Yersinia enterocolitica* cells were grown at pH 9 and pH 5, and the analysis of the fatty acid content of the cells revealed that the unsaturated fatty acid content was higher when it was grown at pH 9 and significantly decreased for cells grown at pH 5 [129]. A similar observation of high percentage of unsaturated membrane lipid has been reported for different alkaliphiles [130].

The rise in the content of unsaturated fatty acid seems correlated to the fatty acid desaturase (an enzyme that forms carbon double bonds in fatty acids) activity (Fig. 6). The membrane of obligate alkaliphiles has very high fatty acid desaturase activity, while the membranes of facultative strains do not have detectable activity [131]. As shown in Fig. 6, desaturase mediated reaction consumes oxygen. Aono et al. [132] have shown that the oxygen uptake rate of membrane vesicles of *Bacillus lentus* C-125 (which is later named *B. halodurans* C-125) grown at pH 9.9 is more than double than that of neutral grown. Part of this oxygen consumption may be related to the formation of unsaturated fatty acid bonds. However, this is yet to be experimentally proven. In general, very little is known about the role of desaturase in

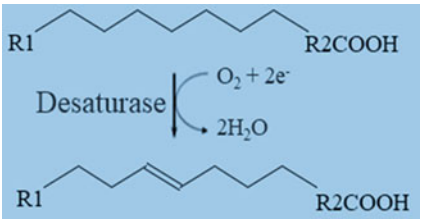


Fig. 6 Conversion of saturated fatty acid to unsaturated form by desaturase

high pH adaptation. Similarly, there are some substances that are known to be correlated to alkaliphiles or to growth at alkaline condition, but their high pH adaptation role is not clear. Bis(monoacylglycero) phosphate (BMB) could be an example. BMB exists in most alkaliphiles and known to be absent at least in many nuteralophiles [128, 133, 134]. But it is not clear if it contributes to high pH adaptation.

A difference is also observed in the membrane content of branched fatty acids. About 90% and 66–76% of the fatty acids in the phospholipids of obligate and facultative alkaliphiles, respectively, were found to be branched [128]. This branching may help in pH homeostasis by reducing H^+ leakage. It has been known that branched fatty acids are common in membranes maintaining H^+ gradient [135]. Moreover, studies revealed that the fatty acid chain length is tending to be shorter in facultative alkaliphiles cell membrane than those from obligate alkaliphiles. This may be related to inhibition of H^+ leakage. In the model proposed by Haines [136], it is indicated that branched fatty acids at the center of the bilayer are involved in preventing H^+ leakage. Thus, the longer the fatty acid chains, the better the chance it reaches at the center of the membrane bilayer. This probably suggests that the alkaliphilic membrane fatty acids which tend to be branched and longer plays a role in the pH homeostasis of these fascinating group of organisms.

Another interesting observation was made on the Gram-negative bacterium *Pseudomonas alcaliphila* fatty acid content which shows a difference in the amount of *cis*- and *trans*-unsaturated fatty acid with varying growth pH. When the bacterium is grown at high pH, the concentration of the *trans*-unsaturated fatty acid increases, while the amount of the *cis*-unsaturated fatty acid decreases proportionally [137]. But how this can contribute to the high pH adaptation is not clear. On the other hand, this phenomenon of high concentration of *trans*-unsaturated membrane fatty acids has been detected in bacteria exposed to environmental stresses including acidity [138–141]. Thus, one can speculate that its contribution to coping stress (including high pH) might be due to the better stability of the *trans* than the *cis* form.

Analysis of the content of alkaliphiles' membranes has also shown that it is rich in squalene and cardiolipin [128, 130, 142], which contain unsaturated bonds. In fact, one of the unique features of alkaliphiles membrane is the presence of high amount of isoprenes (including squalene) which accounts up to 40 mol% of the membrane lipids [128, 135]. The squalene and its derivatives account for about 10–11 mol% of the alkaliphilic *Bacillus* spp. total membrane lipid [128, 142]. Being apolar, this substance may occur inside the lipid bilayer and hence can serve as barrier and

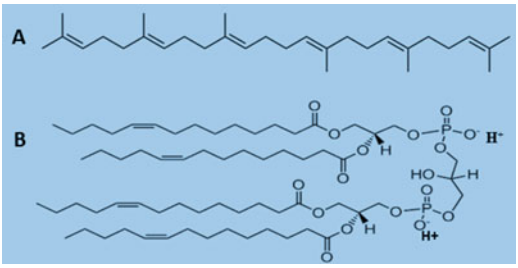


Fig. 7 Squalene (**a**) and cardiolipin (**b**), the two lipids abundant in the membrane of alkaliphiles

decrease membrane permeability for ions [128, 143]. It is interesting that these hydrocarbons are predominantly oriented parallel to the membrane plane [135], which probably enhances the barrier effect and minimizes H^+ leakage [136] and OH^- ingress. Thus, squalene seems be involved in the pH homeostasis of alkaliphiles. The other substance that exists at high concentration in alkaliphilic bacteria is cardiolipin [128], which is unsaturated anionic phospholipid. Cardiolipin has four unsaturated fatty acid chains an anionic structure which can trap H^+ [144]. Thus, like the other negatively charged residues of the cell envelope components such as SCWPs, it can trap cations and repel anions and hence play an important role in high pH adaptation. The structures of squalene and cardiolipin are shown in Fig. 7.

The composition, including its high content of unsaturated fatty acids, branched fatty acids, *trans*-unsaturated fatty acid, cardiolipin, and squalene, makes the membranes of alkaliphiles to function optimally at or above pH 9 [130, 131, 142, 143]. However, the membrane integrity of these alkaliphiles (especially that of obligate alkaliphiles) is compromised around neutral condition; it maintains low electrochemical ion gradient [145], becomes leaky, and tends to lyse [146]. This compromise can be one of the reasons why obligate alkaliphiles fail to grow at near-neutral pH while facultative alkaliphiles are able to grow well [147]. Thus, it is obvious that the cell membrane of alkaliphiles evolved adaptations for high pH environment. Among the membrane adaptations, the tendency of having more unsaturated fatty acid seems to be the most widely reported. However, so far, there is neither experimental nor theoretical explanation on how the unsaturated fatty acids contribute to high pH adaptation. Here, an attempt is made to propose an explanation how the unsaturated membrane lipid is involved in high pH adaptation.

The membranes of alkaliphiles are known to contain many proteins. Although there is no available information, at the time of writing, regarding the protein content difference among cells grown at neutral and alkaline conditions, one can speculate that there are more proteins bound to the membrane at high pH than at neutral condition. This is because the level of expression of proteins such as ATP synthase, cytochromes, antiporters, and other membrane proteins such as enzymes, etc. is high when alkaliphiles grow at elevated pH [21, 131, 148]. Moreover, the rate of denaturation due to the extreme pH condition is expected to be higher; hence, to compensate this, the synthesis of membrane-bound proteins could be enhanced at higher pH. The rise in the amount of proteins together with the enhanced level of

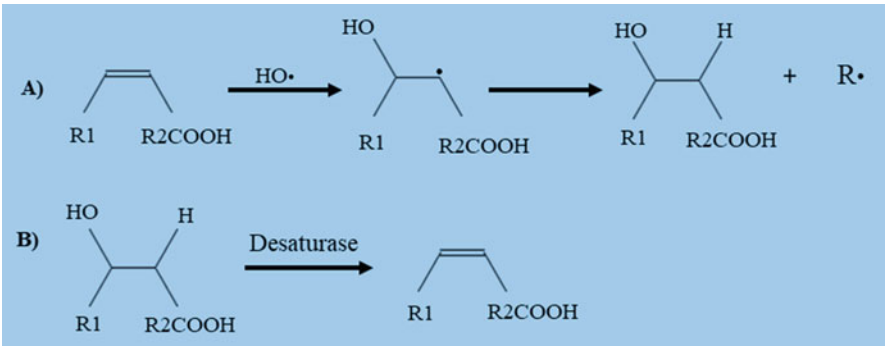


Fig. 8 The reaction of the hydroxy radical with C=C bond of the unsaturated fatty acid that results in saturation of the bond (**a**) and restoration of C=C bond by desaturase (**b**)

fatty acids and hydrocarbons can decrease the "free" volume within the alkaliphiles membrane. This molecular crowding can favor lipid-lipid interaction that may result in rigidity. Thus, it may be important to increase the membrane fluidity by increasing the unsaturated fatty acid content, and this is expected to alleviate the potential problems emanating due to membrane rigidity.

The other explanation for high amount of unsaturated fatty acids may be related to scavenging OH^-. The free radical OH^- is known to react with fatty acids or other hydrocarbon chains such as squalene in two alternative reaction routes. In one of the routes, H^+ is abstracted by OH^- from unsaturated bonds of lipids/hydrocarbons which are accompanied by the release of water. In the alternative route, the OH^- is added to the unsaturated bonds (Fig. 8). However, the addition of OH^- to the C=C is not only the dominant but also the fastest reaction route [149]. Thus, OH^- which somehow escapes through the outer barriers such as the cell wall and traversing the membrane will be captured by the double bonds of the unsaturated fatty acids (including cardiolipin's), squalene, and its derivatives in the same way antioxidants scavenge radicals. Thus, the presence of more unsaturated fatty acids in alkaliphiles cell membrane helps to capture efficiently the OH^- that traverses the membrane. The C=C readily reacts with OH^- and becomes saturated. However, one can speculate that the desaturase may act on the saturated fatty acid to unsaturated form, and the cycle continues (Fig. 8). However, this should be supported experimentally.

Thus, the double bonds between carbon atoms of unsaturated fatty acid can be involved in high pH adaptation through:

1. Improving membrane fluidity and facilitating material exchange.
2. The C=C bonds neutralize the OH^- traversing the membrane before it reaches the cytoplasm.

The phospholipid cardiolipin seems to have another important contribution to high pH adaptation, organization of membrane proteins, and facilitating ATP synthesis. Cardiolipin in mitochondria is known to facilitate the function of membrane-associated proteins, especially the formation of "supercomplex" proteins such as

those involved in shuttling of substances across the membrane and electron transport complexes [150–153]. As aforementioned, there is high presence of proteins in the membranes of alkaliphiles, and hence, one expects more cardiolipin at elevated pH to make these protein assortments assemble and function properly. Cardiolipin has a unique role in membranes involved in OXPHOS, aggregating the proteins involved in OXPHOS into a patch, and its headgroup serves as H^+ trap [144]. Since cardiolipin restricts pumped H^+ close to its headgroup domain, it possibly supplies H^+ to the ATP synthase [144]. Its close association to ATP synthase and respiratory complexes makes cardiolipin to play a unique role in the bioenergetics of alkaliphiles. As discussed below in Sect. 3.3.2, alkaliphiles pump out H^+ faster than non-alkaliphiles, and these protons need to be channeled to ATP synthase before it dissipates into the bulk phase. To this end, a microcircuit that facilitates the transfer of H^+ to ATP synthase has been proposed [27]. Based on its close association to cytochrome *c* oxidase and ATP synthase, and its unique role in patching these systems together, trapping H^+ and feeding it to ATP synthase, it seems that the microcircuit role is, at least partly, played by cardiolipin. Thus, it is not surprising that alkaliphiles have more cardiolipin in their membrane.

3.3 Bioenergetics

The pH homeostasis which effectively maintains a lower intracellular pH than that of the extracellular environment comes with bioenergetics challenge, difficulty of chemiosmotically driven ATP synthesis. Based on the chemiosmotic theory, cells generate ATP using pmf which is the sum of the transmembrane potential ($\Delta\Psi$) and the H^+ concentration gradient (ΔpH). In non-alkaliphiles, the relatively high concentration of H^+ in extracellular than in intracellular environment results in diffusion of H^+ to the cell, which is coupled to ATP synthesis by the ATP synthase. However, in alkaliphiles, this gradient is reversed, the intracellular H^+ concentration exceeds that of the extracellular, and hence, H^+ cannot diffuse to the cytoplasm. Although $\Delta\Psi$ increases at higher pH, it is not high enough to offset the chemiosmotically counterproductive pH gradient [20, 48, 50, 154]. Thus, it is obvious that the successful pH homeostasis raises problems concerning H^+-coupled OXPHOS-based ATP synthesis by prokaryotic alkaliphiles.

In photosynthetic alkaliphiles such as cyanobacteria, the ATP synthase is embedded in thylakoids which are suspended in the cytoplasm and hence not affected by the extracellular low H^+ concentration [155–157]. The pmf across the thylakoid membrane is higher than the pmf across the cytoplasmic membrane [158, 159]; thus, ATP can be produced chemiosmotically regardless of the high pH of their habitat. Probably, the same holds true for eukaryotic cells (organisms) that are adapted to high pH habitats and produce ATP using ATP synthase which is partly embedded in the inner membrane of mitochondria. However, there is no available information on how eukaryotic organisms thriving in alkaline environments generate ATP through OXPHOS.

Contrary to the thermodynamically unfavored condition, ATP synthesis by prokaryotic alkaliphiles is known to be more efficient at alkaline condition than in the near neutral range [47, 50, 51, 160]. Moreover, often aerobic alkaliphiles have higher growth rate and yield than neutralophiles [48, 161], which specifies that alkaliphiles are efficient in producing ATP. Studies have also shown that alkaliphiles, with few exceptions of anaerobes, depend on H^+-coupled ATP synthase to produce ATP and satisfy their energy requirement [20]. Thus, the fact that alkaliphiles do not exhibit energy shortage despite the thermodynamic hurdle of generating ATP in alkaline habitats marks that alkaliphiles have devised unique adaptive strategies to efficiently generate energy carriers at elevated pH. A number of experimentally supported and speculative adaptations have been forwarded to substantiate how prokaryotic alkaliphiles accomplish H^+-coupled ATP synthesis under the unfavorable low pmf. These adaptations which allow alkaliphiles to effectively generate ATP during high pH growth are discussed below.

3.3.1 ATP Synthase

Two types of ATP synthases are known in bacteria, those that are coupled to H^+ and those coupled to Na^+ [162]. Although it seems disadvantageous for alkaliphiles which are thriving in alkaline (low proton) environment to couple their energy carrier generating system to H^+, surprisingly non-fermentative aerobic alkaliphiles are entirely dependent on H^+-coupled ATP synthase [20, 98, 133, 163, 164]. Several studies have tried to decipher the reason behind why aerobic alkaliphiles couple their OXPHO-based ATP synthesis to H^+. Some of these studies have been focused on adaptation of alkaliphiles ATP synthase and able to identify certain unique features of the enzyme that seem to be correlated to high pH adaptation.

ATP synthase in non-alkaliphilic organisms is known to mediate both the synthesis and degradation of ATP. The ATPase activity that breaks down ATP to ADP and P_i is linked to pumping out H^+ from the cytoplasm. As discussed in Sect. 3.1.2, one of the phenomenal adaptations of this enzyme is inactivation of its ATPase activity. This inactivation, in addition to pH homeostasis, may contribute to energy saving. However, as revealed by several studies, ATP synthase mainly contributes to high pH adaptation through enhanced level of expression and specific adaptations of its subunits.

High Level Expression of ATP Synthase

One of the ATP synthase contributions to high pH adaptations seems to be related to the level of activity. Transcriptome and mutagenic studies revealed an increased expression and activity of ATP synthase at high pH. As pH increases, the energy demand to fuel cellular activities is also expected to rise [27], and hence an increase in the level of expression and activity of the synthase will compensate the high energy demand and contribute to minimize the low pmf effect on H^+-coupled ATP

synthesis. Moreover, it has been observed that ATP synthase expression increases when microbes such as *B. subtilis*, *Corynebacterium glutamicum*, *E. coli*, *Desulfovibrio vulgaris*, etc. are subjected to alkaline treatment [102, 148, 165–167]. ATP synthase synthesizes ATP when H^+ flows from cell surface to cytoplasm through it, and this contributes to the intracellular H^+ concentration. Hence, it is expected contributing to the pH homeostasis process. In fact, results of a mutational study reflect the pH homeostasis role played by ATP synthase. As aforementioned, Mrp antiporter is important in translocating H^+ from the extracellular environment to the cytoplasm and known to play a vital role in pH homeostasis. An Mrp antiporter deletion mutant of *B. subtilis* exhibited a rise in ATP synthase expression [168]. The rise in the level of synthase may compensate the loss in H^+ translocation due to the Mrp deletion. This also indicates that the bioenergetics and pH homeostasis processes are wired tightly.

Although high level ATP synthase expression is widely accepted and experimentally supported as means of high pH adaptation, it seems that the case is not universal. The transcriptome analysis of alkaline-stressed *Enterococcus faecalis* revealed that the ATP synthase was significantly downregulated when the cells were grown in pH 10 media [169]. The authors' findings also include a significant drop in the expression of the *nhaC* gene which encodes Na^+/H^+ antiporter. This also contradicts to the main stream notion that recognizes enhanced expression of the antiporter at high pH. However, the authors did not mention how these downregulations help the organism to survive the alkaline condition. However, it is tempting to speculate that *E. faecalis* is a lactic acid bacterium and in the presence of glucose, which the authors added in the medium they used, can produce acid, and this can possibly maintain low intracellular pH when grown in alkaline media. Thus, it is possible that in order to survive in the alkaline condition, the cells shift their metabolism to produce more acid and which may reduce the need for OXPHOS-based ATP synthase. However, this needs further studies. For instance, what will the transcriptome trend show if a non-fermentable medium is used?

Adaptations of the *a*-Subunit

ATP synthase is a multicomponent enzyme. One of these components believed to be involved in high pH adaptation is the *a*-subunit. Alignment studies on the *a*-subunit amino acid sequences of alkaliphilic and non-alkaliphilic *Bacillus* species revealed that the transmembrane helix-4 (TMH4) and transmembrane helix-5 (TMH5) are somehow distinct between these two groups of bacteria. The TMH4 of alkaliphiles has a conserved motif of 171MRxxxxVxxKxxxM, while TMH5 has two conserved residues, L205 and G212 [95, 170]. The fair conservation of these residues only among sequences of alkaliphiles suggests their possible role in high pH adaptation. Mutational studies on the conserved residues were done to elucidate their adaptive roles. Based on the analysis of the mutants, it seems that residues V177 and K180 are involved in H^+ uptake pathway [171]. Similarly, M171, M184, I185, and L205 are also believed to be relevant to the *a*-subunit proton pathway [95]. These authors also

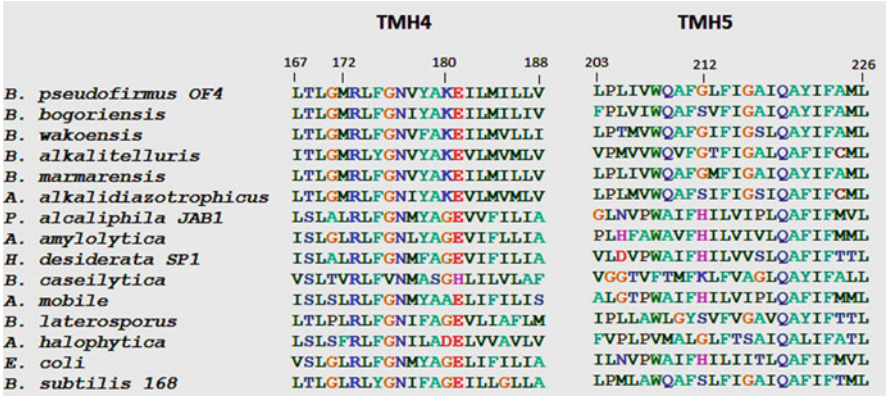


Fig. 9 Sequence alignment of the *a*-subunit of TMH4 and TMH5. The sequence GenBank accession numbers are given in parentheses. The alignment includes five Gram-positive alkaliphilic *Bacillus* strains – *B. pseudofirmus* OF4 (AAG48358), *B. bogoriensis* (WP_026672803.1), *B. wakoensis* (WP_034748151.1), *B. alkalitelluris* (WP_088077058.1), and *B. marmarensis* DSM 21297 (ERN51921.1). A sequence from a Gram-positive alkaliphilic anaerobe *Anaerobacillus alkalidiazotrophicus* (OIJ21792.1) is also included. Six sequences are from Gram-negative alkaliphiles – *Pseudomonas alcaliphila* JAB1 (APU32731.1), *Alkalimonas amylolytica* (WP_091342750.1), *Halomonas desiderata* SP1 (OUE41273.1), *Bogoriella caseilytica* (WP_123305435.1), *Alkalispirillum mobile* (WP_121443140.1), and *Brevibacillus laterosporus* (PCN45399.1). In addition, the alignment includes a sequence from alkaliphilic cyanobacteria *Aphanothece halophytica* (BAK19941.1) and two sequences of non-alkaliphilic strains, *B. subtilis* 168 (NP_391568.1) and *E. coli* (WP_078180285.1)

studied G212 mutant which exhibited H^+ leakiness. However, new sequence analysis (Fig. 9) shows that the conserved residues are not universal among alkaliphiles. These conserved residues are important only to alkaliphilic *Bacillus* strains and probably to related genera. If these residues are important as they are claimed to be, it would have been universal among alkaliphilic bacteria. But that does not seem to be the case. Even among alkaliphilic *Bacillus a*-subunit, some sequences lack conserved residues such as V177 or G212 [170]. Moreover, most of the mutational studies made so far did not pinpoint the exact high pH adaptive role of the conserved residues. However, the growing evidence suggests that the alkaliphilic *Bacillus* spp. ATP synthase *a*-subunit could be involved in preventing H^+ leakage. In addition, the results of the studies indicate a possibility that ATP synthase of alkaliphiles evolved an efficient system of capture and translocation of H^+ to cytoplasm. However, it needs further mutational and structural studies to establish the real high pH adaptation of these structures.

Adaptations of the *c*-Subunit

Amino acid sequence alignment studies on *c*-subunit of ATP synthase revealed the presence of two conserved motifs, 16AxAxAVA and 51PxxExxP, in alkaliphilic

Bacillus strains [170, 172, 173]. Neutralophilic *Bacillus* spp. have GxGxGNG motif in the TMH1. But, in alkaliphiles, this motif is substituted with 16AxAxAVA, which possibly indicates its potential importance in high pH adaptation. In the second conserved motif, the residue 51P is specific to alkaliphiles, and it is positioned close to the ion-binding residue E54. Mutational, structural, and sequence analysis of the *c*-subunit has shown features that may be recognized as high pH adaptations. Mutation of all the alanine of the 16AxAxAVA motif to glycine led to ATP synthesis activity loss by more than 80% [173]. P51 has been mutated to alanine and glycine. The mutant P51A exhibited a dramatic loss of ATP synthesis and non-fermentative growth at pH 10.5, whereas the P51G mutation did not affect the ATP synthesis capacity although it had growth problem at high pH and exhibits H^+ leakage [173]. Studies made on the mutant E54G revealed that it leaks H^+ and a 90% drop on non-fermentative growth. Although it needs further studies to expound how these identified motifs contribute to the high pH adaptation exactly, the results generated so far are valuable and indicate the involvement of the *c*-subunit in the adaptation of ATP synthesis at high pH. However, it is imperative to expand the study to other alkaliphilic genera as well to see the whole picture of the *c*-subunit high pH adaptation role.

3.3.2 Cytochrome

The adaptation of alkaliphiles respiratory system is believed compensating the pmf lost by the reversed transmembrane pH gradient [51]. To unravel this adaptive mechanism, different components of the respiratory system have been isolated and characterized. Analysis of the cytochrome content has shown that it increases with the cultivation pH [51, 161, 174]. This correlation possibly shows that the high pH adaptation of alkaliphiles involves cytochromes. In fact, characterization of isolated cytochrome *c* from alkaliphiles and neutralophiles revealed that the midpoint redox potential of alkaliphiles cytochrome *c* is much lower (<+100 mV) than that of neutralophiles (+220 mV) [161, 175]. However, the redox potential of cytochrome *c* oxidase, the terminal oxidase that accepts electron from cytochrome *c*, is similar between those of alkaliphiles and neutralophiles, +250 mV (cytochrome *a*) [161, 176]. The high midpoint redox potential difference between the terminal oxidase (cytochrome *a*) and cytochrome *c* drives the flow of H^+ and e^- faster across the membrane of alkaliphiles. This can create a H^+ gradient close to the membrane surface, especially the membrane part embedded with the respiratory system.

The H^+ gradient created by the respiratory complex activity is further enhanced and maintained by another unique feature of alkaliphiles cytochrome *c*, high electron retention capacity [177]. Studies made on soluble cytochrome *c*-552 of the Gram-negative facultative alkaliphilic *Pseudomonas alcaliphila* strain revealed that at alkaline pH, it has high electron retention ability and serves as an electron reservoir in the periplasmic space [174, 178]. A similar observation of electron retention is made for cytochrome *c*-550 of *Bacillus clarkii*, an obligate alkaliphile. The retention of electrons attracts H^+, and this contributes to the formation of high membrane

electrical potential ($\Delta\Psi$) for attracting H^+ from the outer surface membrane. This creates for each H^+ an enhanced ATP synthase driving force.

Faster pumping of H^+ by the respiratory complex may require an increased oxygen uptake and high level of electron donor (NADH). The respiratory and NADH activities of an alkaliphilic *Bacillus* have been studied [132]. The results indicate that the oxygen uptake and NADH oxidation activities increase with the rise in cultivation pH. The oxygen consumption studies revealed that the *Bacillus* grown at pH 7.2 and 9.8 has consumed 1.17 and 2.43 μmol oxygen atom/min/mg cell protein, respectively [132]. In addition to viable cells, the authors have also studied the activities of membrane vesicles prepared from the cell envelope of the alkaliphilic *Bacillus* cells. The trend was the same. When the cells were grown at pH 7–9 and 9.9, the oxygen uptake was 1.1–1.4 and 2.5 pmol oxygen atom/min/mg of the cell envelope protein used to make the membrane vesicles, respectively. Similarly, vesicles prepared from the *Bacillus* cells cultivated at pH 7–8.5 and 9.9 were able to oxidize 1.4–1.7 and 6.3 pmol NADH/min/mg cell envelope protein, respectively. On the other hand, an approximately 2.5 times lower oxygen consumption rate was reported for *B. clarkii* DSM 8720(T) cells at pH 10 than that of *B. subtilis* IAM 1026 cells at pH 7. This is despite the alkaliphilic *B. clarkii* 7.5 times higher rate of ATP synthesis than the neutralophilic *B. subtilis* [160]. Such a discrepancy regarding oxygen consumption is also reflected among different alkaliphiles [132], which suggests the high rate of oxygen uptake by NADH oxidation activities is not universal. In fact, it seems that at low level of aeration, the electron retention capacity of cytochrome *c* is more important in maintaining the transmembrane potential that drives the synthesis of ATP [177].

As it has been suggested, alkaliphiles generate pmf during the respiratory electron transport events [179]. At least theoretically, the rapid pumping of H^+ forms the high pmf before it gets equilibrated with bulk phase of the extracellular environment. However, the potential equilibration with the bulk phase should be minimized to tap the pmf for ATP synthesis. The high level of cytochrome *c* and cardiolipin that retains the H^+ close to the surface of the membrane seems to play a crucial role in preventing the dissipation of the pmf created by the respiratory complexes. Moreover, the retention of H^+ by the cytochromes and cardiolipin forms H^+ pool. If the cells have an effective means to shuttle the H^+ from the pool to the ATP synthase, it can generate ATP efficiently. This is expected to be more effective if the ATP synthases are located near to the respiratory complex, a task believed to be accomplished by cardiolipin. To this end, the presence of microcircuits that facilitate the transfer of H^+ to ATP synthase by connecting the surface of the H^+ pumping respiratory complexes and ATP synthases has been speculated [27]. The presence of specific interaction between cytochrome *c* oxidase and ATP synthase, which has been demonstrated in a reconstituted system [180], supports the speculation to some extent. The physical interaction between the respiratory and the ATP synthase complexes can efficiently sequester H^+ transfers during OXPHOS at high pH.

It seems that several factors contribute to enhance the efficiency of alkaliphiles' OXPHOS based ATP production. Among these factors, probably, the most important features include the presence of high amount of:

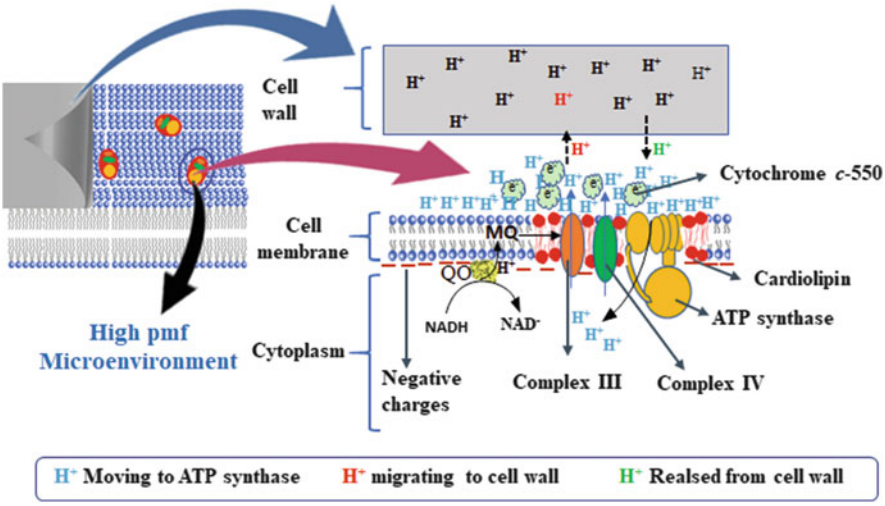


Fig. 10 An illustration of the high pmf microenvironment created by cardiolipin-induced aggregation of the respiratory complexes (complex III and IV). Quinone oxidoreductase (QO, NDH-2) transfers electrons to menaquinone (MQ) pool from which the electrons move to the menaquinol: cytochrome *c* (Complex III) and cytochrome *c* oxidase (complex IV). The rapid pumping of H^+ by the respiratory complexes and the retention of electrons by the cytochrome *c*-550 contribute to high pmf with more negative charges on the membrane facing the cytoplasm than the outer membrane

1. cardiolipin which aggregates the respiratory system together with the ATP synthase. It also restricts the H^+ coming from the respiratory complex close to ATP synthase and facilitate the H^+ transfer to the synthase [144],
2. cytochromes that pump out H^+ much faster than the normal pace of non-alkaliphilic organisms [161, 175] and cytochromes like cytochrome *c*-550 with high electron retention capacity [174, 177], and
3. ATP synthase which is efficient in translocating H^+ to its catalytic core (i.e. inhibition of H^+ leakage) [95, 170, 173].

The rapid pumping of H^+ by the respiratory complex and the restriction of these H^+ close to the surface of the aggregate create a microenvironment with high pmf which promotes the synthesis of ATP (Fig. 10). The H^+ translocated by the ATP synthase during ATP synthesis replenishes the H^+ pumped out by the respiratory complex, which contributes to maintain the low cytoplasmic pH. This microcircuit in the microenvironment produces ATP approximately seven times faster than that of neutralophiles [160, 177], and this may be one of the reasons why alkaliphiles grow faster and denser than neutralophiles.

3.4 Coping Intracellular Alkalinization

Although alkaliphiles are known to have an efficient pH homeostasis system that keeps the cytoplasmic pH well below that of the habitat, the intracellular pH can go above the neutral range. For instance, the intracellular pH of the facultative alkaliphile *B. psuedofirmus* has been reported reaching 9.6 when the cells were cultivated at pH 11.4 [48]. The maximum reported difference between the intracellular and extracellular pH of alkaliphiles is around 2.5 pH units [48]. As shown in Table 1, it seems that the cytoplasmic pH rises even higher as the cultivation pH for the organism reaches to its upper edge. Thus, although there is no available data, the cytoplasmic pH of alkaliphiles such as those growing at pH 13.5 [16] may exceed pH 10. In non-alkaliphiles, this high cytoplasmic pH can potentially impair cellular activities and integrities and ultimately kill the cell. But, as witnessed from their growth in extreme pH conditions, alkaliphiles evolved their intracellular system to remain active and stable at elevated pH. The possible strategies that alkaliphiles deploy to withstand cytoplasmic alkalinization may include (1) altering the expression/production profile of biomolecules, (2) evolving efficient intracellular repair system, and (3) production of biomolecules that are operationally stable at high pH. The production of biomolecules that are stable and functional at high pH is not restricted to intracellular products; it is an absolute necessity to extracellular products.

3.4.1 Altering the Expression/Production Profile of Biomolecules

Organisms endure stress by changing their gene expression profile and metabolic programming. Studies have shown that alkalinization is accompanied by up- and downregulation of several genes [102, 148, 169, 181]. Such changes bring the desired tolerance to the rising pH by (1) switching to alkali-tolerant variants, (2) increasing the level of biomolecules that mitigate the pH drift, (3) compensating the loss due to denaturation, and (4) activating the protein repair and degradation systems.

Switching Expression to Alkali-Tolerant Variants Some organisms have genes which encode variants of a product. These organisms, up on cytoplasmic pH rise, may switch to the expression of the alternative variant that encodes the protein which is operationally stable at alkaline condition [182]. Thus, inactivation of the alkali-sensitive proteins will not hamper the cellular process as the sensitive products are replaced by resistant variants. Alkaliphiles are known to produce biomolecules (such extracellular enzymes) that are active and stable at high pH. It is possible that these organisms use the same adaptation strategy to make alkali-resistant intracellular products. The adaptation mechanism for high pH operational stability of biomolecules is discussed in Sect. 3.5.

Producing More Biomolecules That Mitigate the pH Drift With rising intracellular pH, the level of some biomolecules such as ATP synthase, Na^+/H^+ antiporter, squalene, SCWPs, etc. increases [21, 102, 106, 128, 130, 148]. As aforementioned, these biomolecules play a significant role in the pH homeostasis of alkaliphiles, and hence, the upregulations of these biomolecules contribute to mitigate further increase in cytoplasmic pH. For instance, an increased level of ATP synthase expression results in pumping more H^+ to the cytoplasm which eases the cytoplasmic pH rise, while accumulation of squalene effectively limits H^+ leakage and OH^- ingress. In some organisms, cytoplasmic alkalinization is accompanied by metabolic acid production [102] which alleviates the cytoplasmic pH rise and protects the cell from the subsequent demise. In fact, many alkaliphiles are known producing organic acids and even reduce the culture pH significantly [56, 105].

Compensating Loss Due to Denaturation With the rise in cytoplasmic pH, the activity and integrity of intracellular biomolecules deteriorate. To compensate this loss, cells increase the production level of pH labile biomolecules. For example, as translation slows down and mRNAs are not stable at elevated pH, cells increase the level of mRNA to maintain the necessary level of protein synthesis [183, 184].

3.4.2 Activating Protein Damage Repair and Degradation Systems

The cells also use another strategy to maintain the necessary level of functional biomolecules, repairing the damage incurred by cytoplasmic alkalinization. Thus, it is expected that the cells activate their systems involved in repairing damages and/or recycling inactivated biomolecules. Among the damage repair systems, an increase in the level of chaperone and protein damage repair enzyme has been reported in relation to alkalinization [148, 185]. Many intracellular macromolecules that are vital for life are labile at high pH. The stability and activity of DNA, RNA, proteins, lipids, etc. can be severely affected by prolongated exposure to high pH. In general, it has been known that when cells are exposed to stress, the repair systems are often activated to mend problems suffered by the stress. Here, it may be relevant to mention the formation of isoaspartate and the associated repair system. Isoaspartate is an isomer of aspartic acid formed through the nucleophile attack of the γ-carbon in asparagine or aspartic acid residue side chains which forms a succinimide intermediate as illustrated in Fig. 11. The formation of isoaspartate affects the function and stability of many proteins [186–188]. In addition, the reaction can lead to deamidation of asparagine and formation of D-amino acids [189, 190]. If this damage remains uncorrected, the protein cannot properly perform its task. Thus, it is necessary that such protein damages must be repaired to maintain optimal cellular activities or the damaged protein should be degraded and removed. Cells produce L-isoaspartyl protein carboxyl methyltransferase (PCM), an enzyme which identifies and repairs such protein damages [191]. PCM encoding gene is widely distributed among unicellular and multicellular organisms [192], and in bacteria, it is linked to long-term stress survival [193]. High pH is known to aggravate isoaspartate

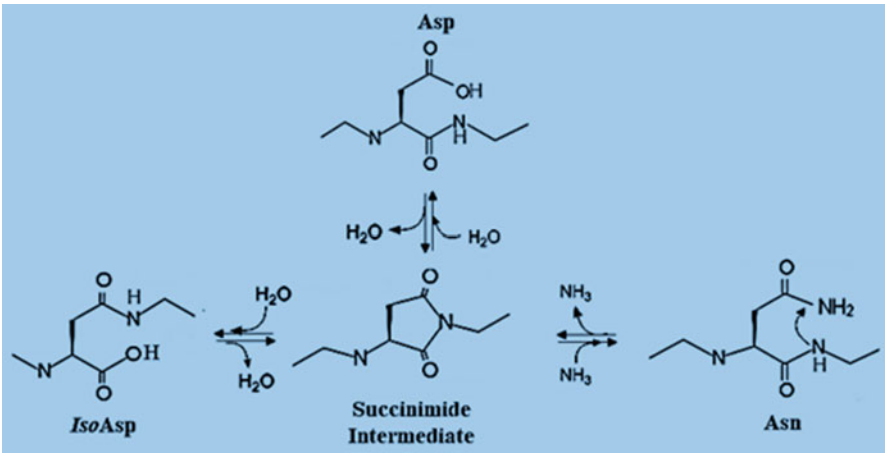


Fig. 11 Nonenzymatic conversion of Asp residue in peptide bonds to succinimide intermediate which converts to isoaspartate. The Asn residue converts to the intermediate through deamidation reaction

formation and deamidation [194–196]. Thus, the cytoplasmic protein damage is expected to increase with increasing alkalinization. Organisms that survive cytoplasmic alkalinization may have an efficient PMC that mends the damage caused by high cytoplasmic pH. Indeed, studies have indicated that protein repair mechanism of PCM is important to thrive in high pH conditions [197].

Another import repair system is the chaperon-mediated refolding of proteins. The rise in cytoplasmic pH can cause protein unfolding and aggregation. Chaperons are known to be involved in refolding proteins that are unfolded/aggregated by stresses. Studies have shown that acid stress in bacteria leads to chaperon production, which is used to adapt low pH environments [198–200]. If a parallel is drawn, alkaliphiles may also use the same strategy to alleviate high pH-induced protein unfolding/aggregation problems, especially related to sudden alkalinization. Although an increase in chaperone level has been reported in relation to alkalinization [148, 185, 201], little is known compared to its role in low pH tolerance.

Not all damages are reparable, and hence, it is possible that alkalinization may lead to accumulation of denatured biomolecules. However, for normal cellular activities, it is necessary to remove those biomolecules that are irreparably damaged. Thus, one of the relevant adaptations that alkaliphiles employ during cytoplasmic alkalinization may be enhancing the turnover rate of intracellular biomolecules. Inactivated biomolecules such as proteins should be degraded and replaced by newly synthesized active products to ensure normal physiology. An elevation in transcription of genes encoding proteases such as the ATP-dependent Clp, ATP-dependent La endopeptidase, and DnaK that are known in degrading nonfunctional proteins has been observed during alkalinization [148, 185]. However, there is no detailed study made so far on the actual involvement of these damage repair and recycling systems in high pH adaptation of alkaliphiles.

It is obvious that the data on high pH adaptations of alkaliphiles is still trickling in. However, it seems that far little is done in some areas. One of such cases is protein synthesis, which is one of the most crucial life processes vital for survival and growth. Several factors can influence this fascinating process, and pH is one of them. The optimum pH (pH 8.2–8.5) for cell-free protein translation systems of alkaliphilic origin have been reported to be only 0.5 pH units higher than that of neutralophiles [202]. However, the cytoplasmic pH of actively growing alkaliphiles can be much higher (>pH 10) such as when cells are grown close to pH 13, and hence, one expects lower rate of protein synthesis. On the other hand, alkaliphiles in general are known to grow faster than non-alkaliphiles [177], which suggest that alkaliphiles may have a very efficient protein synthesis at elevated pH. However, there is no available information how extreme alkaliphiles evolved their protein synthesis apparatus. Similarly, the adaptation of extreme alkaliphiles that shield their DNA and RNA from the effect of high pH is unknown. Since the *p*Ka of guanine (G) and thymine (T) is in the range of pH 9–10 [203], above pH 10, these residues get deprotonated and remain as negatively charged conjugate bases. This can break the hydrogen bonding between the two strands of the DNA helix and result in denaturation of DNA. This makes the DNA strand prone for damage and disrupts the replication and transcription processes. It has been reported that alkali stressed *E. coli* cells induce recA-independent DNA damage repair system [204], which suggests the possible involvement of the repair system in high pH adaptation of alkaliphiles. But the repair system is not enough by itself. There should be mechanism(s) that protect these vital macromolecules from high pH hostility.

3.5 Production of Extracellular Biomolecules Which Are Operationally Stable in Alkaline Milieu

Cells release products to their immediate environment to harvest nutrients, defense/competitional purposes, for communication, etc. At least theoretically, these products are evolved to work optimally in the host environment. Thus, products secreted by alkaliphiles are expected to be operationally stable in their high pH habitats. Among such products, enzymes have attracted a great deal of attention. Studies on alkaline active enzymes are done to understand the molecular mechanisms behind their structural and functional adaptation to high pH environment. Comparative sequence analysis and mutational studies revealed that alkaline-active enzymes exhibit reduced alkali susceptible residues and tend to increase alkali tolerant residues, especially in their exposed surfaces. The ionization state of residues such as Asp, Glu, His, Lys, and Arg side chains is determined by the pH of the environment. Thus, the distribution and frequency of these ionizable residues partly determine the pH adaptation of proteins. In line with this, Lys, Arg, Asn, His, Glu, and Asp residue content has been studied in relation to high pH adaptation [205–209]. These studies revealed the tendency of alkaline-active enzymes to have more Arg, His, and Gln in their structures. Since Arg has a higher *p*Ka than Lys,

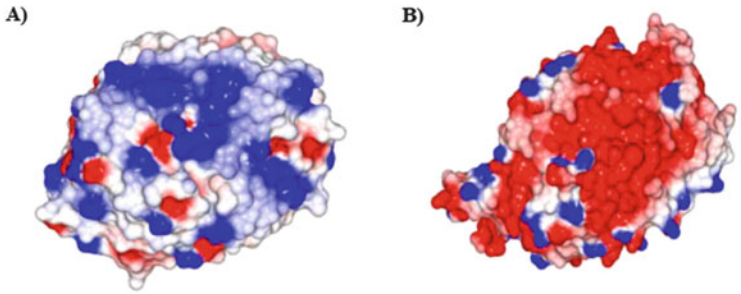


Fig. 12 The surface at the back of the catalytic cleft of an acid-active PDB 1B30 (**a**) and alkaline-active PDB 2UWF (**b**) xylanases. Negatively and positively charged surfaces are colored in red and blue, respectively

substitution of Lys by Arg may allow formation of hydrogen bonds at extremely high pH. His and Gln are largely neutral at alkaline condition, and this may be important to maintain the protein solubility in the alkaline condition. Asn is one of the most alkali susceptible residues [210, 211], and hence, its occurrence in alkaline-adapted proteins, especially on exposed surfaces, is relatively low [206]. This agrees to previous studies that involved mutational substitution of Asn with less susceptible amino acids and resulted in a better stability at high pH [212, 213].

Charged residues are known to play important roles in structural adaptation of biomolecules. Such residues are vital in high pH adaptations. Extracellular products of alkaliphiles tend to have more acidic residues on their surfaces than their non-alkaliphilic counterparts. For instance, deduced amino acid sequence analysis has shown that the externally exposed alkaliphile membrane protein loops have acidic residues, while the non-alkaliphile homologue loops have neutral/basic residues [214]. Similarly, as described above in Sect. 3.2, the surface exposed proteins and polysaccharides of alkaliphiles such as SplA, liposaccharides, and SCWPs are rich in negatively charged residues. Structural analysis of extracellular enzymes also reveals that their surface is more acidic than that of non-alkaline active counterparts. Figure 12 depicts the surface charge difference between xylanases that are optimally active at pH 5.6 [215] and 9–9.5 [216]. The alkaline active xylanase has more acidic surface than the acid-active enzyme. It seems that there is a consensus that the negatively charged surface of alkaliphiles extracellular products deters encountering negatively charged OH^- and protects the biomolecule from the aggressiveness of the high pH environment, a "Sword against sword" adaptation strategy.

When it comes to alkaline-active enzymes, it is not only their stability at high pH which is astonishing, but also their ability to optimally mediate reactions at elevated pH is intriguing. The interesting thing is that the catalytic residues and often their vicinity are highly conserved regardless of the origin of the enzyme. For instance, the endo-beta-1,4-xylanase from *Acidobacterium capsulatum* is optimally active at pH of 5 and loses its activity at or above pH 8 [217], while the xylanase from *B. halodurans* is optimally active around pH 9.5 and displayed nearly 20% of its optimal activity at pH 12 [216]. But these two enzymes belonging to the same family

(GH 10) share a similar catalytic pocket as well as identical catalytic residues (a pair of Glu). How these enzymes are able to ionize the catalytic residues in this wide range of pH and mediate the biocatalysis is fascinating.

The pH profile of enzymes such as glycosyl hydrolases is determined by the catalytic residues *pKa* values [209, 218, 219] which in turn are dependent on the microenvironment surrounding the catalytic residue. Thus, the nature of amino acids in the active site region plays a significant role in shaping the pH-activity profile of the enzymes. In general, amino acids with positive charges and hydrogen bonds lower *pKa* values, while carboxyl groups can increase or decrease the *pKa* values based on the electrostatic interaction between residues [220]. Thus, certain amino acids in the active site vicinity determine the *pKa* values by altering the active site electrostatic and dynamic aspects [221] through direct or indirect interaction with the catalytic residues. This kind of key residues, at least partially, determines the pH-dependent activities of enzymes [222], and mutational studies on such residues often shift the mutated enzyme pH-activity profile [223–225].

3.6 Adaptation to Low Nutrient Bioavailability

Nutrient bioavailability is a less studied challenge in high pH habitats. pH affects the availability of certain nutrients by determining its state (e.g., solubility), reaction with other substances, stability, etc. For instance, water in soda lakes is saturated with CO_2 that forms HCO_3^-/CO_3^{2-} which interacts with and precipitates divalent metal ions, making it less bioavailable. Thus, it is necessary for alkaliphiles to develop mechanisms that circumvent the problems related to the poor bioavailability of such nutrients. This can be achieved by deploying efficient retrieving systems for deficient nutrients or decreasing dependency on poorly available nutrients. In line with this, purification and characterization of some alkaline active extracellular enzymes revealed that the enzymes evolved some adaptive features including high affinity to metal cofactors [226] or became less dependent on it [227]. In fact, these properties are among the reasons why enzymes of alkaliphiles are desirable in detergent applications, resistant to the detergent chelator's effect.

Alkaliphiles are known to have efficient system of capturing and translocating scarce metal ions to the cytoplasm. One of the relatively well-studied scarce metals is iron. Iron is important in ATP production, and it is a crucial cofactor for enzymes involved in a variety of metabolic processes, and hence, it is essential for almost all organisms. Although it is one of the most abundant elements in nature, it is not readily available. Therefore, organisms employ different strategies to secure enough iron from their surroundings. As the solubility of iron decreases with increasing pH, it is vital for alkaliphiles to evolve a means to acquire iron. At alkaline conditions, iron exists in ferric state (Fe^{+3}) which reacts and forms the poorly soluble $Fe(OH)_3$. Thus, in alkaline environments, the bioavailability of iron is far below the requirement for living cells. At pH 10, the concentration of bioavailable iron is estimated to be approximately 10^{-23} M [228], which is much lower than the 10^{-18} M level at

pH 7 [229]. Considering the extreme scarcity of iron at high pH, one expects that alkaliphiles evolved a very efficient sequestering mechanism. Indeed, studies revealed that alkaliphiles produce very effective iron-binding chelators, siderophores. It is believed that siderophore-assisted iron acquisition is one of the critical adaptations of alkaliphiles in high pH habitats [229–231]. Studies made so far are limited to production (of siderophores). Detail biochemical characterization and structural analysis of these siderophores can be beneficial to advance our understanding on alkaliphiles adaptation and may also yield new siderophores of biotechnological importance. It is interesting that the first structural analysis proves the potential of alkaliphiles as sources of novel siderophores [229].

It is not only the metal ions' availability that is limited at highly alkaline conditions [232], other major nutrients such as nitrogen and phosphate could also be growth-limiting factors [16, 42, 233]. For example, NH_4^+ which serves as nitrogen source for a wide variety of organisms is mostly converted to volatile and toxic NH_3 and becomes unavailable in alkaline habitats of pH 10 and above. Studies have shown that the poor availability of nitrogen sources in some alkaline habitats makes the inhabitant alkaliphiles resort to utilization of certain unconventional resources such as cyanide and its derivatives as nitrogen source [230, 234].

In general, the limited work done so far indicates that there are challenges and associated adaptations regarding the bioavailability of certain nutrients in high pH habitats. It may be attractive for basic and applied areas to extend studies in this direction.

4 Adaptations of Eukaryotes to High pH Environments

There are numerous unicellular and multicellular eukaryotes such as ciliates, dinoflagellates, diatoms, fungi, green algae, invertebrates, fish, etc. that flourish in high pH habitats. Although there are very interesting studies on the diversity, taxonomy, population dynamics, ecological role, etc. of these eukaryotes [235, 236], there is very little information on how these organisms are adapted to their respective high pH habitats. Nearly all the studies regarding high pH adaptations of life have been focused on microorganisms (bacteria, archaea, and to some extent fungi). This may be due to several reasons such as their dominance/abundance, biotechnological interests, relatively easy handling, etc. On the other hand, studies on high pH adaptation of eukaryotes not only improve our understanding but also may enlighten us with new mechanisms. For instance, the cell membrane of most protozoans such as ciliates adapted to high pH environment may be exposed to the alkaline environment. It is of great interest to know how this membrane shields the cytoplasm effectively from the effect of the extreme pH. Moreover, the ciliates inner part of the cell membrane facing the cytoplasm is lined with proteinaceous structure known as pellicle. Although one expects that this pellicle may play an important role in the adaptation, there is no available information how this remarkable structure contributes in adapting alkaline habitats.

Alkaline environments, particularly the East African Rift Valley soda lakes are the most productive lakes on the planet and are supporting huge flocks of birds, especially flamingos (*Phoenicopterus roseus* and *Phoeniconaias minor*). These birds are associated with the soda lakes, wade and swim to feed on the cyanobacteria *Arthrospira* (previously known as *Spirulina*). However, these lakes are very alkaline and are hostile to practically all other forms of non-adapted life including humans. It is believed that the birds adapted to these hostile lakes have special tough skin and scales on their legs which prevent them from the alkali attack. However, detailed studies on how exactly this scale protects the birds' leg from the alkali effect are still lacking. The same holds true for other eukaryotes such as crustaceans, flagellates, insects, etc. that are thriving in high pH habitats. Relatively, fish adapted to alkaline lakes attracted attention which seems more due to economic importance than interest in basic understanding.

Although not all, some soda lakes are known for their fish. Fish such as the lake Magadi tilapia (*Alcolapia graham*) are adapted to thrive in hypersaline alkaline water that can kill other fish within minutes [237]. Studies made so far on high pH adaptation of fish revolved around two challenges, blood pH maintenance and ammonia excretion. When fish get transferred from neutral to alkaline water, the blood pH increased rapidly [238–241]. This is not mainly due to direct translocation of alkali to blood but driven by solubility of CO_2. Above pH 8.5, almost all CO_2 in water converts to bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}), and this results in a CO_2 deficiency around the gill. This creates a faster diffusion of CO_2 from the blood of the fish [242]. The rapid loss of CO_2 from the blood leads to the rise in blood pH which is known as respiratory alkalosis [238–241]. Moreover, the abundant OH^- and HCO_3^- of the alkaline environment create an electrical gradient which facilitates the exchange of blood H^+ for environmental HCO_3^- [243] and this may potentially contribute to the blood pH rise. However, studies made on tilapia that live in a pH 10 soda lake revealed that it involved mechanisms that reduce gill permeability to HCO_3^- [244]. Moreover, these fish are adapted to handle high plasma pH [242, 245]. It has also been reported that fish adapted to high pH habitats lower the blood pH to physiological range by exchanging the blood Na^+ and HCO_3^-, respectively, for H^+ and Cl^- of the aquatic body [240, 241, 246].

The other potential challenge for fish to adapt high pH environment is accumulation of ammonia in the blood caused by its unfavorable passive diffusion across the gills [240]. Since protein catabolism continuously generates ammonia [247], if it is not removed effectively, it tends to accumulate in the body. High level of NH_3 is toxic as it binds to the brain *N*-methyl-D-aspartate (NMDA) receptors and cause an over-excitation and ultimate death [248, 249]. Terrestrial animals convert NH_3 to urea or uric acid at the expense of energy and then remove it from their bodies. Whereas fish, living in aquatic environment have become ammonotelic and directly discharge NH_3 through the surface of their gills without energy expenditure. However, such removal of NH_3 at gill surface is unfavorable in high pH environment. Fish adapted to high pH habitats are able to reduce NH_3 load by converting it to urea and discharge it through urea transporter A (UT-A) at the gills surface [246, 250]. This indicates that the fish adapted to high pH aquatic environments have the necessary biochemical machineries to process NH_3 into urea and

transporting it out of the blood. Most fish do have the genes necessary for this biochemical process; however, only those fish which are adapted to alkaline habitats are able to express these genes throughout their life [240].

The work done so far has improved our understanding how fish can adapt to high pH environment. However, there are still some unanswered questions such as adaptive mechanism of gills proteins/membranes which are directly exposed to the alkaline water. Moreover, due to the external fertilization of fish reproductive process, the gametes are deposited directly into the extreme habitats. How these gamete cells survive the high pH is yet to be discovered. Studies on other eukaryotic organisms' adaptation to the high pH environment will certainly add to the existing knowledge and should be encouraged.

5 Conclusion

It has been over four decades since researchers started unraveling the secretes of high pH adaptation. Over these years, very fascinating adaptive strategies of alkaliphiles have been described in numerous publications. To thrive in high pH environments, organisms evolved multilevel adaptations that are reflected in their unique functional and structural makeups. Adaptations related to pH homeostasis and bioenergetics of alkaliphilic prokaryotes have been widely and deeply studied. However, there are still issues that are waiting for proper scientific look. One of such issues that seem overlooked is the cytoplasmic alkalinization of extreme alkaliphiles like those growing around pH 13 and the associated physiological adaptations. At the extreme pH, though it is not experimentally proven, there is a possibility that the cytoplasmic pH can drift above pH 10, and this can, at least, theoretically affect the transcription and translation processes, DNA replication, the activities and stabilities of biomolecules including enzymes, DNA and RNA, etc. However, the fact that these unique organisms are growing in the extreme habitats indicate the cytoplasmic system is functional and hence must be adapted to high pH. On the other hand, although it is unlikely, there is a possibility that these organisms manage to keep the cytoplasmic pH below pH 10. If this happens, the intracellular and extracellular pH difference can reach over 3.5 pH units for alkaliphiles thriving at pH 13.5, and this obviously requires an extremely efficient pH homeostasis even by alkaliphiles standard. Thus, these extreme alkaliphiles to thrive in their habitats should evolve either an extraordinary pH homeostasis mechanism or unique adaptation that protects their cellular activities and biomolecules from the deleterious effect of high cytoplasmic pH (>pH 10). Which one of these alternatives nature has chosen remains to be seen?

Probably one of the most studied high pH adaptations is the bioenergetics. It is widely accepted that cell membranes of alkaliphiles have low proton motive force (pmf) which makes oxidative phosphorylation-based ATP production challenging. However, it seems that alkaliphiles solved this challenge primarily by evolving efficient respiratory complexes and ATP synthase that aggregates in a patch by the cardiolipin. The respiratory complexes pump H^+ faster, and the headgroup of the

cardiolipin restricts these H^+ within the microenvironment of the patch creating high pmf. The presence of cytochrome c such as cytochrome c-550 with high electron retention capacity significantly enhance the pmf. Moreover, due to the possible interaction of cardiolipin to the respiratory complexes and ATP synthase, it can to may continuously shuttle H^+ to the synthase, which may be one of the reasons why alkaliphiles ATP synthesis is more efficient and strictly H^+-coupled. Another contribution to high pH adaptation comes from unsaturated bonds of lipids. Double bonds are known to react with radicals such as OH^- faster than single bonds. Thus, the double bonds in squalene, squalene derivatives, and unsaturated fatty acids within the lipid bilayer scavenge OH^- traversing the membrane. Although it needs to be experimentally supported, desaturases probably re-establish the double bonds lost by reacting with ingressing OH^-.

It is very clear that our understanding of high pH adaptation is expanding due to the trickling information. However, the studies are still focused on prokaryotes. Even among prokaryotes, with very few exceptions, almost all the studies are directed to Gram-positive bacteria. On the other hand, there are largely diverse Gram-negative bacteria, archaea, and eukaryotes that are known thriving in high pH habitats. It could be interesting to include these groups of organisms in future studies.

References

1. Jones BE, Grant WD, Duckworth AW, Owenson GG (1998) Microbial diversity of soda lakes. Extremophiles 2:3191–3200
2. Sorokin DY, Berben T, Melton EM, Overmars L, Vavourakis CD, Muyzer G (2014) Microbial diversity and biogeochemical cycling in soda lakes. Extremophiles 18:791–809
3. Borsodi AK, Korponai K, Schumann P, Spröer C, Felföldi T, Márialigeti K, Szili-Kovács T, Tóth E (2017) *Nitrincola alkalilacustris* sp. nov. and *Nitrincola schmidtii* sp. nov., alkaliphilic bacteria isolated from soda pans, and emended description of the genus *Nitrincola*. Int J Syst Evol Microbiol 67:5159–5164
4. Olivera N, Siñeriz F, Breccia JD (2005) *Bacillus patagoniensis* sp. nov., a novel alkalitolerant bacterium from *Atriplex lampa* rhizosphere, Patagonia, Argentina. Int J Syst Evol Microbiol 55:443–447
5. Szabo A, Korponai K, erepesi Cs K, Somogyi B, Vörös L, Bartha D et al (2017) Soda pans of the Pannonian steppe harbor unique bacterial communities adapted to multiple extreme conditions. Extremophiles 21:639–649
6. Zhang G, Yang Y, Wang S, Sun Z, Jiao K (2015) *Alkalimicrobium pacificum* gen. nov., sp. nov., a marine bacterium in the family *Rhodobacteraceae*. Int J Syst Evol Microbiol 65:2453–2458
7. Zhang YG, Lu XH, Ding YB, Wang SJ, Zhou XK, Wang HF et al (2016) *Lipingzhangella halophila* gen. nov., sp. nov., a new member of the family *Nocardiopsaceae*. Int J Syst Evol Microbiol 66:4071–4076
8. Ohkuma M, Shimizu H, Thongaram T, Kosono S, Moriya K, Trakulnaleam S et al (2003) An alkaliphilic and xylanolytic *Paenibacillus* species isolated from the gut of a soil-feeding termite. Microbes Environ 18:145–151

9. Donovan SE, Purdy KJ, Kane MD, Eggleton P (2004) Comparison of Euryarchaea strains in the guts and food-soil of the soil-feeding termite *Cubitermes fungifaber* across different soil types. Appl Environ Microbiol 70:3884–3892
10. Liang X, Sun C, Chen B, Du K, Yu T, Luang-In V, Lu X, Shao Y (2018) Insect symbionts as valuable grist for the biotechnological mill: an alkaliphilic silkworm gut bacterium for efficient lactic acid production. Appl Microbiol Biotechnol 102:4951–4962
11. Chavagnac V, Monnin C, Ceuleneer G, Boulart C, Hoareau G (2013) Characterization of hyperalkaline fluids produced by low-temperature serpentinization of mantleperidotites in the Oman and Ligurian ophiolites. Geochem Geophys Geosyst 14:2496–2522
12. Ben Aissa F, Postec A, Erauso G, Payri C, Pelletier B, Hamdi M, Fardeau M-L, Ollivier B (2015) Characterization of *Alkaliphilus hydrothermalis* sp. nov., a novel alkaliphilic anaerobic bacterium, isolated from a carbonaceous chimney of the Prony hydrothermal field, New Caledonia. Extremophiles 19:183–188
13. Mei N, Postec A, Erauso G, Joseph M, Pelletier B, Payri C et al (2016) *Serpentinicella alkaliphila* gen. nov., sp. nov., a novel alkaliphilic anaerobic bacterium isolated from the serpentinite-hosted Prony hydrothermal field, New Caledonia. Int J Syst Evol Microbiol 66:4464–4470
14. Agnew MD, Koval SF, Jarrell KF (1995) Isolation and characterisation of novel alkaliphiles from bauxite-processing waste and description of *Bacillus vedderi* sp. nov. Syst Appl Microbiol 18:221–230
15. Gee JM, Lund BM, Metcalf G, Peel JL (1980) Properties of a new group of alkalophilic bacteria. J Gen Microbiol 117:9–17
16. Kisková J, Stramová Z, Javorský P, Sedláková-Kaduková J, Pristaš P (2019) Analysis of the bacterial community from high alkaline (pH > 13) drainage water at a brown mud disposal site near Žiar nad Hronom (Banská Bystrica region, Slovakia) using 454 pyrosequencing. Folia Microbiol 64:83–90
17. Mueller RH, Jorks S, Kleinsteuber S, Babel W (1998) Degradation of various chlorophenols under alkaline conditions by Gram-negative bacteria closely related to *Ochrobactrum anthropi*. J Microbiol 38:269–281
18. Takahara Y, Tanabe O (1962) Studies on the reduction of indigo in industrial fermentation vat (XIX). Taxonomic characterisation of strain No. S-8. J Ferment Technol 40:77–80
19. Kevbrin VV (2019) Isolation and cultivation of alkaliphiles. Adv Biochem Eng Biotechnol. https://doi.org/10.1007/10_2018_84
20. Krulwich TA, Hicks DB, Swartz TH, Ito M (2007) Bioenergetic adaptations that support alkaliphily. In: Gerday C, Glansdorff N (eds) Physiology and biochemistry of extremophiles. ASM Press, Washington, pp 311–329
21. Padan E, Bibi E, Ito M, Krulwich TA (2005) Alkaline pH homeostasis in bacteria: new insights. Biochim Biophys Acta 1717:67–88
22. Slonczewski JL, Fujisawa M, Dopson M, Krulwich TA (2009) Cytoplasmic pH measurement and homeostasis in bacteria and archaea. Adv Microb Physiol 55:1–317
23. Greenwood JE, Tan JL, Ming JCT, Abell AD (2016) Alkalis and skin. J Burn Care Res 37:135–141
24. Hirata Y, Ito H, Furuta T, Ikuta K, Sakudo A (2010) Degradation and destabilization of abnormal prion protein using alkaline detergents and proteases. Int J Mol Med 25:267–270
25. Shooter KV (1976) The kinetics of the alkaline hydrolysis of phosphotriesters in DNA. Chem Biol Interact 13:151–163
26. Hunt KA, Flynn JM, Naranjo B, Shikhare ID, Gralnick JA (2010) Substrate-level phosphorylation is the primary source of energy conservation during anaerobic respiration of *Shewanella oneidensis* strain MR-1. J Bacteriol 192:3345–3351
27. Hicks DB, Liu J, Fujisawa M, Krulwich TA (2010) F_1F_0-ATP synthases of alkaliphilic bacteria: lessons from their adaptations. Biochim Biophys Acta 1797:1362–1377
28. Mitchell P (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. Nature 191:144–148

29. Chiego B, Silver H (1942) The effect of alkalis on the stability of keratins. J Invest Dermatol 5:95–103
30. Krachle RF, Krachler R, Stojanovic A, Wielander B, Herzig A (2009) Effects of pH on aquatic biodegradation. Biogeosci Discuss 6:491–514
31. Block SS (1991) Disinfection, sterilization, and preservation. Lea & Febiger, Philadelphia
32. GE Healthcare Bio-Sciences AB (2014) Use of sodium hydroxide for cleaning and sanitization of chromatography media and systems. Application note 18-1124-57 AI
33. Hobbs BC, Wilson GS (1942) The disinfectant activity of caustic soda. J Hyg 42:436–450
34. Lemire KA, Rodriguez YY, McIntosh MT (2016) Alkaline hydrolysis to remove potentially infectious viral RNA contaminants from DNA. Virol J 13:88. https://doi.org/10.1186/s12985-016-0552-0
35. Arnosti C, Bell C, Moorhead DL, Sinsabaugh RL, Steen AD, Stromberger M et al (2013) Extracellular enzymes in terrestrial, freshwater, and marine environments: perspectives on system variability and common research needs. Biogeochemistry 117:5–21
36. Bogino PC, Oliva MM, Sorroche FG, Giordano W (2013) The role of bacterial biofilms and surface components in plant-bacterial associations. Int J Mol Sci 14:15838–15859
37. Castrec J, Soudant P, Payton L, Tran D, Miner P, Lambert C et al (2018) Bioactive extracellular compounds produced by the dinoflagellate *Alexandrium minutum* are highly detrimental for oysters. Aquat Toxicol 199:188–198
38. Nwodo UU, Green E, Okoh AI (2012) Bacterial exopolysaccharides: functionality and prospects. Int J Mol Sci 13:14002–14015
39. Sebastian Engel S, Jensen PR, Fenical W (2002) Chemical ecology of marine microbial defense. J Chem Ecol 28:1971–1985
40. Waters CM, Bassler BL (2005) Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol 21:319–346
41. Boros E, Kolpakova M (2018) A review of the defining chemical properties of soda lakes and pans: an assessment on a large geographic scale of Eurasian inland saline surface waters. PLoS One 13(8):e0202205. https://doi.org/10.1371/journal.pone.0202205
42. Grant WD, Jones BE (2016) Bacteria, archaea and viruses of soda lakes. In: Schager LM (ed) Soda lakes of East Africa. Springer, Cham, pp 97–148
43. Finkelstein J (2009) Metalloproteins. Nature 460:813. https://www.nature.com/articles/460813a.pdf
44. Garland PB (1977) Energy transduction and transmission in microbial systems. In: Haddock BA, Hamilton WA (eds) 27th symposium of the Society for General Microbiology. Microbial energetics. Cambridge University Press, Cambridge, pp 1–21
45. McLaggan D, Selwyn MJ, Dawson AP (1984) Dependence on Naþ of control of cytoplasmic pH in a facultative alkalophile. FEBS Lett 165:254–258
46. Cook GM, Russell JB, Reichert A, Wiegel J (1996) The intracellular pH of *Clostridium paradoxum*, an anaerobic, alkaliphilic, and thermophilic bacterium. Appl Environ Microbiol 62:4576–4579
47. Guffanti AA, Hicks DB (1991) Molar growth yields and bioenergetic parameters of extremely alkaliphilic *Bacillus* species in batch cultures, and growth in a chemostat at pH 10.5. J Gen Microbiol 137:2375–2379
48. Sturr MG, Guffanti AA, Krulwich TA (1994) Growth and bioenergetics of alkaliphilic *Bacillus firmus* OF4 in continuous culture at high pH. J Bacteriol 176:3111–3116
49. Aono R, Ito M, Horikoshi K (1997) Measurement of cytoplasmic pH of the alkaliphile *Bacillus lentus* C-125 with a fluorescent pH probe. Microbiology 143:2531–2536
50. Olsson K, Keis S, Morgan HW, Dimroth P, Cook GM (2003) Bioenergetic properties of the thermoalkaliphilic *Bacillus* sp. strain TA2.A1. J Bacteriol 185:461–465
51. Yumoto I (2002) Bioenergetics of alkaliphilic *Bacillus* spp. J Biosci Bioeng 93:342–353
52. Krulwich TA, Guffanti AA, Ito M (1999) Mechanisms by which bacterial cells respond to pH. Novartis Foundation Symposia, vol 221. Wiley, Chichester, pp 167–182

53. Krulwich TA, Ito M, Gilmour R, Hicks DB, Guffanti AA (1998) Energetics of alkaliphilic *Bacillus* species: physiology and molecules. Adv Microb Physiol 40:401–438
54. Krulwich TA, Liu J, Morino M, Fujisawa M, Ito M, Hicks DB (2011) Adaptive mechanisms of extreme alkaliphiles. In: Horikoshi K, Antranikian G, Bull AT, Robb FT, Stetter KO (eds) Extremophiles handbook. Springer, Tokyo, pp 119–139
55. Krulwich TA, Sachs G, Padan E (2011) Molecular aspects of bacterial pH sensing and homeostasis. Nat Rev Microbiol 9:330–343
56. Paavilainen S, Helistö P, Korpela T (1994) Conversion of carbohydrates to organic acids by alkaliphilic bacilli. J Ferment Bioeng 78(3):217–222
57. Padan E, Gerchman Y, Rimon A, Rothman A, Dover N, Carmel-Harel O (1999) The molecular mechanism of regulation of the NhaA Naþ/Hþ antiporter of *Escherichia coli*, a key transporter in the adaptation to Naþ and Hþ. Novartis Foundation Symposia, vol 221. Wiley, Chichester, pp 183–196
58. Padan E, Venturi M, Gerchman Y, Dover N (2001) Na^+/H^+ antiporters. Biochim Biophys Acta 1505:144–157
59. Slonczewski JL, Rosen BP, Alger JR, Macnab RM (1981) pH homeostasis in *Escherichia coli*: measurement by 31P nuclear magnetic resonance of methylphosphonate and phosphate. Proc Natl Acad Sci U S A 78:6271–6275
60. Hamamoto T, Hashimoto M, Hino M, Kitada M, Seto Y, Kudo T, Horikoshi K (1994) Characterization of a gene responsible for the Na^+/H^+ antiporter system of alkalophilic *Bacillus* species strain C-125. Mol Microbiol 14:939–946
61. Saier MH, Reddy VS, Tsu BV, Ahmed MS, Li C, Moreno-Hagelsieb G (2016) The Transporter Classification Database (TCDB): recent advances. Nucleic Acids Res 44:D372–D379
62. Krulwich TA, Hicks DB, Ito M (2009) Cation/proton antiporter complements of bacteria: why so large and diverse? Mol Microbiol 74:257–260
63. Brett CL, Donowitz M, Rao R (2005) Evolutionary origins of eukaryotic sodium/proton exchangers. Am J Physiol Cell Physiol 288:C223–C239
64. Counillon L, Pouyssegur J (2000) The expanding family of eucaryotic Na^+/H^+ exchangers. J Biol Chem 275:1–4
65. Fliegel L (2005) The $Na^{+/}H^+$ exchanger isoform 1. Int J Biochem Cell Biol 37:33–37
66. Orlowski J, Grinstein S (2004) Diversity of the mammalian sodium/proton exchanger SLC9 gene family. Pflugers Arch 447:549–565
67. Ito M, Guffanti AA, Oudega B, Krulwich TA (1999) Mrp, a multigene, multifunctional locus in *Bacillus subtilis* with roles in resistance to cholate and to Na^+ and in pH homeostasis. J Bacteriol 181:2394–2402
68. Fuster DG, Alexander RT (2014) Traditional and emerging roles for the SLC9 Na^+/H^+ exchangers. Pflugers Arch 466:61–76
69. Padan E, Landau M (2016) Sodium-proton (Na^+/H^+) antiporters: properties and roles in health and disease. Met Ions Life Sci 16:391–458
70. Harel-Bronstein M, Dibrov P, Olami Y, Pinner E, Schuldiner S, Padan E (1995) MH1, a second-site revertant of an *Escherichia coli* mutant lacking Na^+/H^+ antiporters (*DnhaADnhaB*), regains Na^+ resistance and a capacity to excrete Na^+ in a $\Delta\mu_{H+}$-independent fashion. J Biol Chem 270:3816–3822
71. Wei Y, Liu J, Ma Y, Krulwich TA (2007) Three putative cation/proton antiporters from the soda lake alkaliphile *Alkalimonas amylolytica* N10 complement an alkali-sensitive *Escherichia coli* mutant. Microbiology 153:2168–2179
72. Krulwich TA, Ito M, Guffanti AA (2001) The Na^+-dependence of alkaliphily in *Bacillus*. Biochim Biophys Acta 1501:158–168
73. Kitada M, Kosono S, Kudo T (2000) The Na^+/H^+ antiporter of alkaliphilic *Bacillus* sp. Extremophiles 4:253–258
74. Ran S, He Z, Liang J (2013) Survival of *Enterococcus faecalis* during alkaline stress: changes in morphology, ultrastructure, physiochemical properties of the cell wall and specific gene transcripts. Arch Oral Biol l58:1667–1676

75. Harold FM, Van Brunt J (1977) Circulation of H^+ and K^+ across the plasma membrane is not obligatory for bacterial growth. Science 197:372–373
76. Speelmans G, Poolman B, Abee T, Konings WN (1993) Energy transduction in the thermophilic anaerobic bacterium *Clostridium fervidus* is exclusively coupled to sodium ions. Proc Natl Acad Sci U S A 90:7975–7979
77. Swartz TH, Ikewada S, Ishikawa O, Ito M, Krulwich TA (2005) The Mrp system: a giant among monovalent cation/proton antiporters? Extremophiles 9:345–354
78. Ito M, Morino M, Krulwich TA (2017) Mrp antiporters have important roles in diverse bacteria and archaea. Front Microbiol 8:2325. https://doi.org/10.3389/fmicb.2017.02325
79. Kajiyama Y, Otagiri M, Sekiguchi J, Kosono S, Kudo T (2007) Complex formation by the *mrpABCDEFG* gene products, which constitute a principal Na^+/H^+ antiporter in *Bacillus subtilis*. J Bacteriol 189:7511–7514
80. Morino M, Natsui S, Swartz TH, Krulwich TA, Ito M (2008) Single gene deletions of mrpA to mrpG and mrpE point mutations affect activity of the Mrp Na^+/H^+ antiporter of alkaliphilic *Bacillus* and formation of hetero-oligomeric Mrp complexes. J Bacteriol 190:4162–4172
81. Aono R, Ito M, Horikoshi K (1992) Instability of the protoplast membrane of facultative alkaliphilic Bacillus sp. C-125 at alkaline pH values below the pH optimum for growth. Biochem J 285:99–103
82. Aono R, Ito M, Machida T (1999) Contribution of the cell wall component teichuronopeptide to pH homeostasis and alkaliphily in the alkaliphile *Bacillus lentus* C-125. J Bacteriol 181:6600–6606
83. Aono R, Ogino H, Horikoshi K (1992) pH-dependent flagella formation by facultative alkaliphilic *Bacillus* sp. C-125. Biosci Biotechnol Biochem 56:48–53
84. Aono R, Ohtani M (1990) Loss of alkalophily in cell-wall-component-defective mutants derived from alkalophilic *Bacillus* C-125. Isolation and partial characterization of the mutants. Biochem J 266:933–936
85. Gilmour r, Messner P, Guffanti AA, Kent R, Scheberl A, Kendrick N, Krulwich TA (2000) Two-dimensional gel electrophoresis analyses of pH-dependent protein expression in facultatively alkaliphilic *Bacillus pseudofirmus* OF4 lead to characterization of an S-layer protein with a role in alkaliphily. J Bacteriol 182:5969–5981
86. Ito M, Hicks DB, Henkin TM, Guffanti AA, Powers B, Zvi L, Uematsu K, Krulwich TA (2004) MotPS is the stator-force generator for motility of alkaliphilic *Bacillus* and its homologue is a second functional Mot in *Bacillus subtilis*. Mol Microbiol 53:1035–1049
87. Terahara N, Kodera N, Uchihashi T, Ando T, Namba K, Minamino T (2017) Na^+-induced structural transition of MotPS for stator assembly of the *Bacillus* flagellar motor. Sci Adv 3: eaao4119
88. Fujinami S, Terahara N, Lee S, Ito M (2007) Na^+ and flagella-dependent swimming of alkaliphilic *Bacillus pseudofirmus* OF4: a basis for poor motility at low pH and enhancement in viscous media in an "up-motile" variant. Arch Microbiol 187:239
89. Chahine M, Pilote S, Pouliot V, Takami H, Sato C (2004) Role of arginine residues on the S4 segment of the *Bacillus halodurans* Na^+ channel in voltage-sensing. J Membr Biol 201:9–24
90. Koishi RXH, Ren D, Navarro B, Spiller BW, Shi Q, Clapham DE (2004) A superfamily of voltage-gated sodium channels in bacteria. J Biol Chem 279:9532–9538
91. Ito M, Xu H, Guffanti AA, Wei Y, Zvi L, Clapham DE, Krulwich TA (2004) The voltage-gated Na^+ channel NavBP has a role in motility, chemotaxis, and pH homeostasis of an alkaliphilic *Bacillus*. Proc Natl Acad Sci U S A 101:10566–10571
92. Morino M, Suzuki T, Ito M, Krulwich TA (2014) Purification and functional reconstitution of a seven-subunit mrp-type Na^+/H^+ antiporter. J Bacteriol 196:28–35
93. Fujinami S, Sato T, Trimmer JS, Spiller BW, Clapham DE, Krulwich TA et al (2007) The voltage-gated Na^+ channel NaVBP co-localizes with methyl-accepting chemotaxis protein at cell poles of alkaliphilic *Bacillus pseudofirmus* OF4. Microbiology 153:4027–4038

94. McMillan DGG, Keis S, Dimroth P, Gregory M, Cook GM (2007) A specific adaptation in the *a*-subunit of thermoalkaliphilic F_1F_O-ATP synthase enables ATP synthesis at high pH but not at neutral pH values. J Biol Chem 282:17395–17404
95. Fujisawa F, Fackelmayer OJ, Liu J, Krulwich TA, Hicks DB (2010) The ATP synthase *a*-subunit of extreme alkaliphiles is a distinct variant: mutations in the critical alkaliphile-specific residue Lys180 and other residues that support alkaliphile oxidative phosphorylation. J Biol Chem 285:32105–32115
96. Cook GM, Keis S, Morgan HW, von Ballmoos C, Matthey U, Kaim G, Dimroth P (2003) Purification and biochemical characterization of the F_1F_o-ATP synthase from thermoalkaliphilic *Bacillus* sp. strain TA2.A1. J Bacteriol 85:4442–4449
97. Dimroth P, Cook GM (2004) Bacterial Na^+- or H^+-coupled ATP synthases operating at low electrochemical potential. Adv Microb Physiol 49:175–218
98. Hicks DB, Krulwich TA (1990) Purification and reconstitution of the F_1F_O-ATP synthase from alkaliphilic *Bacillus firmus* OF4. Evidence that the enzyme translocates H^+ but not Na^+. J Biol Chem 265:20547–20554
99. Hoffmann A, Dimroth P (1990) The ATPase of *Bacillus alcalophilus*. Purification and properties of the enzyme. Eur J Biochem 194:423–430
100. Burne RA, Marquis RE (2000) Alkali production by oral bacteria and protection against dental caries. FEMS Microbiol Lett 193:1–6
101. Yokaryo H, Tokiwa Y (2014) Isolation of alkaliphilic bacteria for production of high optically pure L-(+)-lactic acid. J Gen Appl Microbiol 60:270–275
102. Wilks JC, Kitko RD, Cleeton SH, Lee GE, Ugwu CS, Jones BD, BonDurant SS, Slonczewski JL (2009) Acid and base stress and transcriptomic responses in *Bacillus subtilis*. Appl Environ Microbiol 75:981–990
103. Graham AF, Lund BM (1983) The effect of alkaline pH on growth and metabolic products of a motile, yellow-pigmented *Streptococcus* sp. J Gen Microbiol 129:2429–2435
104. Hirota K, Aino K, Yumoto I (2013) *Amphibacillus iburiensis* sp. nov., an alkaliphile that reduces an indigo dye. Int J Syst Evol Microbiol 63:4303–4308
105. Horikoshi K (2006) Alkaliphiles. Kodansha, New York
106. Aono R, Ito M, Joblin KN, Horikoshi K (1995) A high cell wall negative charge is necessary for the growth of the alkaliphile *Bacillus lentus* C-125 at elevated pH. Microbiology 141:2955–2964
107. Hancock IC, Baddiley J (1985) Biosynthesis of the bacterial envelope polymers teichoic acid and teichuronic acid. In: Martonosi NA (ed) The enzymes of biological membranes, vol 2. 2nd edn. Plenum, New York, pp 279–307
108. Ward JB (1981) Teichoic and teichuronic acids: biosynthesis, assembly and location. Microbiol Rev 45:211–243
109. Archibald AR, Baddiley J, Blumsom NL (1968) The teichoic acids. Adv Enzymol Relat Areas Mol Biol 30:223–253
110. Archibald AR, Hancock IC, Harwood CR (1993) Cell wall structure, synthesis and turnover. In: Sonenshein A, Hoch JA, Losick R (eds) *Bacillus subtilis* and other Gram-positive bacteria. American Society for Microbiology, Washington, pp 381–410
111. Araki Y, Ito E (1989) Linkage units in cell walls of Gram-positive bacteria. CRC Crit Rev Microbiol 17:121–135
112. Naumova IB, Shashkov AS (1997) Anionic polymers in cell walls of Gram-positive bacteria. Biochemistry 62:809–840
113. Aono R, Horikoshi K (1983) Chemical composition of cell walls of alkalophilic strains of *Bacillus*. J Gen Microbiol 129:1083–1087
114. Horikoshi K (1999) Alkaliphiles: some applications of their products for biotechnology. Microbiol Mol Biol Rev 63:735–750
115. Koch AL (1986) The pH in the neighborhood of membranes generating a protonmotive force. J Theor Biol 120:73–84

116. Aono R (1985) Isolation and partial characterization of structural components of the walls of alkalophilic *Bacillus* strain C-125. J Gen Microbiol 131:105–111
117. Ito M, Aono R (2002) Decrease in cytoplasmic pH-homeostastatic activity of the alkaliphile *Bacillus lentus* C-125 by a cell wall defect. Biosci Biotechnol Biochem 66:218–220
118. Corsaro MM, Gambacorta A, Iadonisi A, Lanzetta R, Naldi T, Nicolaus B et al (2006) Structural determination of the *O*-chain polysaccharide from the lipopolysaccharide of the haloalkaliphilic *Halomonas pantelleriensis* bacterium. Eur J Org Chem 2006:1801–1808
119. Silipo A, Sturiale L, Garozzo D, de Castro C, Lanzetta R, Parrilli M et al (2004) Structure elucidation of the highly heterogeneous lipid A from the lipopolysaccharide of the Gram-negative extremophile bacterium *Halomonas Magadiensis* strain 21 M1. Eur J Org Chem 2004:2263–2271
120. Messner P, Schäffer C (2003) Prokaryotic glycoproteins. In: Herz W, Falk H, Kirby GW (eds) Progress in the chemistry of organic natural products, vol 85. Springer, Wien, pp 51–124
121. Sleytr UB, Sara M, Pum D, Schuster B, Messner P, Schäffer C (2002) Self-assembly protein systems: microbial slayers. In: Steinbuchel A, Fahnestock SR (eds) Biopolymers, polyamides and complex proteinaceous matrices I, vol 7. Wiley, Weinheim, pp 285–338
122. Schäffer C, Messner P (2005) The structure of secondary cell wall polymers: how Gram-positive bacteria stick their cell walls together. Microbiology 151:643–651
123. Fujinami S, Ito M (2018) The surface layer homology domain-containing proteins of alkaliphilic *Bacillus pseudofirmus* OF4 play an important role in alkaline adaptation via peptidoglycan synthesis. Front Microbiol 9:810. https://doi.org/10.3389/fmicb.2018.00810
124. Janto B, Ahmed A, Ito M, Liu J, Hicks DB, Pagni S et al (2011) The genome of alkaliphilic *Bacillus pseudofirmus* OF4 reveals adaptations that support the ability to grow in an external pH range from 7.5 to 11.4. Environ Microbiol 13:3289–3309
125. Krulwich TA, Ito M (2013) Prokaryotic alkaliphiles. In: Rosenberg E (ed) The prokaryotes, 4th edn. Springer, Berlin, Heidelberg, pp 441–470
126. Sara M, Sleytr UB (2000) S-layer proteins: minireview. Microbiology 51:349–355
127. Yumoto I, Yamazaki K, Hishinuma M, Nodasaka Y, Suemori A, Nakajima K et al (2001) *Pseudomonas alcaliphila* sp. nov., a novel facultatively psychrophilic alkaliphile isolated from seawater. Int J Syst Evol Microbiol 51:349–355
128. Clejan S, Krulwich TA, Mondrus KR, Seto-Young D (1986) Membrane lipid composition of obligately and facultatively alkalophilic strains of *Bacillus* spp. J Bacteriol 168:334–340
129. Bodnaruk PW, Golden DA (1996) Influence of pH and incubation temperature on fatty acid composition and virulence factors of *Yersinia enterocolitica*. Food Microbiol 13:17–22
130. Banciu H, Sorokin DY, Rijpstra WIC, Damste JSS, Galinski EA, Takaichi S et al (2005) Fatty acid, compatible solute and pigment composition of obligately chemolithoautotrophic alkaliphilic sulfur-oxidizing bacteria from soda lakes. FEMS Microbiol Lett 243:181–187
131. Dunkley EA, Guffanti AA, Clejan S, Krulwich TA (1991) Facultative alkaliphiles lack fatty acid desaturase activity and lose the ability to grow at near-neutral pH when supplemented with an unsaturated fatty acid. J Bacteriol 173:1331–1334
132. Aono R, Kaneko H, Horikoshi K (1996) Alkaline growth pH-dependent increase of respiratory and NADH-oxidation activities of the facultatively alkaliphilic strain *Bacillus lentus* C-125. Biosci Biotechnol Biochem 60:1243–1247
133. Hicks DB, Plass RJ, Quirk PG (1991) Evidence for multiple terminal oxidases, including cytochrome d, in facultatively alkaliphilic *Bacillus firmus* OF4. J Bacteriol 173:5010–5016
134. Nishihara M, Morii H, Koga Y (1982) Bis(monoacylglycero)phosphate in alkalophilic bacteria. J Biochem 92:1469–1479
135. Hauß T, Dante S, Dencher NA, Haines TH (2002) Squalane is in the midplane of the lipid bilayer: implications for its function as a proton permeability barrier. Biochim Biophys Acta 1556:149–154
136. Haines TH (2001) Do sterols reduce proton and sodium leaks through lipid bilayers? Prog Lipid Res 40:299–324

137. Yumoto I, Yamazaki K, Hishinuma M, Nodasaka Y, Suemori A, Nakajima K, Inoue N, Kawasaki K (2001) *Pseudomonas alcaliphila* sp. nov., a novel facultatively psychrophilic alkaliphile isolated from seawater. Int J Syst Evol Microbiol 51:349–355
138. Gianotti A, Iucci L, Guerzoni ME, Lanciotti R (2009) Effect of acidic conditions on fatty acid composition and membrane fluidity of *Escherichia coli* strains isolated from Crescenza cheese. Ann Microbiol 59:603. https://doi.org/10.1007/BF03175152
139. Loffeld B, Keweloh H (1996) *Cis-trans* isomerization fatty acids as possible control mechanism of membrane fluidity in *Pseudomonas putida* P8. Lipids 31:811–815
140. Okuyama H, Enari D, Shibahara A, Yamamoto K, Morita N (1996) Identification of activities that catalyze the *cis-trans*isomerization of the double bond of a mono unsaturated fatty acid in *Pseudomonas* sp. strain E-3. Arch Microbiol 165:415–417
141. Yuk YG, Marshall DL (2004) Adaptation of *Escherichia coli* O157:H7 to pH alters membrane lipid composition, verotoxin secretion, and resistance to simulated gastric fluid acid. Appl Environ Microbiol 70:3500–3505
142. Koga Y, Nishihara M, Mori H (1982) Lipids of alkaliphilic bacteria: identification, composition and metabolism. J Univ Occup Environ Health 4:227–240
143. Clejan S, Krulwich TA (1988) Permeability studies of lipid vesicles from alkalophilic *Bacillus firmus* showing opposing effects of membrane isoprenoid and diacylglycerol fractions and suggesting a possible basis for obligate alkalophily. Biochim Biophys Acta 946:40–48
144. Haines TH, Dencher NA (2002) Cardiolipin: a proton trap for oxidative phosphorylation. FEBS Lett 528:35–39
145. Kitada M, Guffanti AA, Krulwich TA (1982) Bioenergetic properties and viability of the alkalophilic *Bacillus firmus* RAB as a function of pH and Na^+ contents of the incubation medium. J Bacteriol 152:1096–1104
146. Krulwich TA, Agus R, Schneier M, Guffanti AA (1985) Buffering capacity of bacilli that grow at different pH ranges. J Bacteriol 162:768–772
147. Krulwich TA, Hicks DB, Seto-Young D, Guffanti AA (1988) The bioenergetics of alkalophilic bacilli. Crit Rev Microbiol 16:15–36
148. Stolyar S, He Q, Joachimiak MP, He Z, Yang ZK, Borglin SE et al (2007) Response of *Desulfovibrio vulgaris* to alkaline stress. J Bacteriol 189:8944–8952
149. Nah T, Kessler SH, Daumit KE, Kroll JH, Leone SR, Wilson KR (2013) OH-initiated oxidation of sub-micron unsaturated fatty acid particles. Phys Chem Chem Phys 15:18649–18663
150. Eble KS, Coleman WB, Hantgan RR, Cunningham CC (1990) Tightly associated cardiolipin in the bovine heart mitochondrial ATP synthase as analyzed by 31P nuclear magnetic resonance spectroscopy. J Biol Chem 265:19434–19440
151. Fry M, Green DE (1981) Cardiolipin requirement for electron transfer in complex I and III of the mitochondrial respiratory chain. J Biol Chem 256:1874–1880
152. Paradies G, Paradies V, De Benedictis V, Ruggiero FM, Petrosillo G (2014) Functional role of cardiolipin in mitochondrial bioenergetics. Biochim Biophys Acta 1837:408–417
153. Robinson NC (1993) Functional binding of cardiolipin to cytochrome c oxidase. J Bioenerg Biomembr 25:153–163
154. von Ballmoos C, Cook GM, Dimroth P (2008) Unique rotary ATP synthase and its biological diversity. Annu Rev Biophys 37:43–64
155. Liberton M, Berg RH, Heuser J, Roth R, Pakrasi HB (2006) Ultrastructure of the membrane systems in the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803. Protoplasma 227:129–138
156. Nevo R, Charuvi D, Shimoni E, Schwarz R, Kaplan A, Ohad I, Riech Z (2007) Thylakoid membrane perforations and connectivity enable intracellular traffic in cyanobacteria. EMBO J 26:1467–1473
157. Schneider D, Fuhrmann E, Scholz I, Hess WR, Graumann PL (2007) Fluorescence staining of live cyanobacterial cells suggest non-stringent chromosome segregation and absence of a connection between cytoplasmic and thylakoid membranes. BMC Cell Biol 8:39. https://doi.org/10.1186/1471-2121-8-39

158. Belkin S, Boussiba S (1991) Resistance of *Spirulina platensis* to ammonia at high pH values. Plant Cell Physiol 32:953–958
159. Pogoryelov D, Sudhir PR, Kovacs L, Gombos Z, Brown I, Garab G (2003) Sodium dependency of the photosynthetic electron transport in the alkaliphilic cyanobacterium *Arthrospira platensis*. J Bioenerg Biomembr 35:427–437
160. Hirabayashi T, Goto T, Morimoto H, Yoshimune K, Matsyama H, Yumoto I (2012) Relationship between rates of respiratory proton extrusion and ATP synthesis in obligately alkaliphilic *Bacillus clarkii* DSM 8720. J Bioenerg Biomembr 44:265–272
161. Goto T, Matsuno T, Hishinuma-Narisawa M, Yamazaki K, Matsuyama H, Inoue N, Yumoto I (2005) Cytochrome c and bioenergetic hypothetical model for alkaliphilic *Bacillus* spp. J Biosci Bioeng 100:365–379
162. Dimroth P, von Ballmoos C, Meier T (2006) Catalytic and mechanical cycles in F-ATP synthases: fourth in the cycles review series. EMBO Rep 7:276–282
163. Hoffmann A, Dimroth P (1991) The ATPase of *Bacillus alcalophilus*. Reconstitution of energy-transducing functions. Eur J Biochem 196:493–497
164. Krulwich TA (1995) Alkaliphiles: 'basic' molecular problems of pH tolerance and bioenergetics. Mol Microbiol 15:403–410
165. Barriuso-Iglesias M, Barreiro C, Flechoso F, Martin JF (2006) Transcriptional analysis of the F_0F_1 ATPase operon of *Corynebacterium glutamicum* ATCC 13032 reveals strong induction by alkaline pH. Microbiology 152:11–21
166. Hayes ET, Wilks JC, Sanfilippo P, Yohannes E, Tate DP, Jones BD, Radmacher MD, BonDurant SS, Slonczewski JL et al (2006) Oxygen limitation modulates pH regulation of catabolism and hydrogenases, multidrug transporters, and envelope composition in *Escherichia coli* K-12. BMC Microbiol 6:89
167. Maurer LM, Yohannes E, Bondurant SS, Radmacher M, Slonczewski JL (2005) pH regulates genes for flagellar motility, catabolism, and oxidative stress in *Escherichia coli* K-12. J Bacteriol 187:304–319
168. Kosono S, Asai K, Sadaie Y, Kudo T (2004) Altered gene expression in the transition phase by disruption of a Na^+/H^+ antiporter gene (shaA) in *Bacillus subtilis*. FEMS Microbiol Lett 232:93–99
169. Ran S, Liu B, Jiang W, Sun Z, Liang J (2015) Transcriptome analysis of *Enterococcus faecalis* in response to alkaline stress. Front Microbiol 6:795. https://doi.org/10.3389/fmicb.2015.00795
170. Preiss L, Hicks DB, Suzuki S, Meier T, Krulwich TA (2015) Alkaliphilic bacteria with impact on industrial applications, concepts of early life forms, and bioenergetics of ATP synthesis. Front Bioeng Biotechnol 3:75. https://doi.org/10.3389/fbioe.2015.00075
171. Dong H, Fillingame RH (2010) Chemical reactivities of cysteine substitutions in subunit *a* of ATP synthase define residues gating H^+ transport from each side of the membrane. J Biol Chem 285:39811–39818
172. Arechaga I, Jones PC (2001) The rotor in the membrane of the ATP synthase and relatives. FEBS Lett 494:1–5
173. Liu J, Fujisawa M, Hicks DB, Krulwich TA (2009) Characterization of the functionally critical AXAXAXA and PXXEXXP motifs of the ATP synthase *c*-subunit from an alkaliphilic *Bacillus*. J Biol Chem 284:8714–8725
174. Matsuno T, Yumoto I (2015) Bioenergetics and the role of soluble cytochromes *c* for alkaline adaptation in Gram-negative alkaliphilic *Pseudomonas*. Biomed Res Int 2015:847945. https://doi.org/10.1155/2015/847945
175. Hicks DB, Krulwich TA (1995) The respiratory chain of alkaliphilic bacteria. Biochim Biophys Acta 1229:303–314
176. Muntyan MS, Bloch DA (2008) Study of redox potential in cytochrome c covalently bound to terminal oxidase of alkaliphilic *Bacillus pseudofirmus* FTU. Biochemistry (Mosc) 73:107–111

177. Matsuno T, Goto T, Ogami S, Morimoto H, Yamazaki K, Inoue N et al (2018) Formation of proton motive force under low-aeration alkaline conditions in alkaliphilic bacteria. Front Microbiol 9:2331. https://doi.org/10.3389/fmicb.2018.02331
178. Matsuno T, Yoshimune K, Yumoto I (2011) Physiological function of soluble cytochrome c-552 from alkaliphilic *Pseudomonas alcaliphila* AL15-21T. J Bioenerg Biomembr 43:473–481
179. Mulkidjanian AY, Dibrov P, Galperin MY (2008) The past and present of sodium energetics: may the sodium-motive force be with you. Biochim Biophys Acta 1777:985–992
180. Liu X, Gong X, Hicks DB, Krulwich TA, Yu L, Yu CA (2007) Interaction between cytochrome caa3 and F1F0-ATP synthase of alkaliphilic *Bacillus pseudofirmus* OF4 is demonstrated by saturation transfer electron paramagnetic resonance and differential scanning calorimetry assays. Biochemistry 46:306–313
181. Ling HL, Rahmat Z, Bakar FDA, Murad AMA, Illias RM (2018) Secretome analysis of alkaliphilic bacterium *Bacillus lehensis* G1 in response to pH changes. Microbiol Res 215:46–54
182. Saito H, Kobayashi H (2003) Bacterial responses to alkaline stress. Sci Prog 86:271–282
183. Serra-Cardona A, Canadell D, Ariño J (2015) Coordinate responses to alkaline pH stress in budding yeast. Microb Cell 2:182–196
184. Canadell D, Garcia-Martinez J, Alepuz P, Perez-Ortin JE, Arino J (2015) Impact of high pH stress on yeast gene expression: a comprehensive analysis of mRNA turnover during stress responses. Biochim Biophys Acta 1849:653–664
185. Flahaut S, Hartke A, Giard JC, Auffray Y (1997) Alkaline stress response in *Enterococcus faecalis*: adaptation, cross-protection, and changes in protein synthesis. Appl Environ Microbiol 63:812–814
186. Clarke S, Stephenson RC, Lowenson JD (1992) Lability of asparagine and aspartic acid residues in proteins and peptides. In: Ahern TJ, Manning MC (eds) Stability of protein pharmaceuticals, part A: chemical and physical pathways of protein degradation. Plenum, New York, pp 1–29
187. Shimizu T, Matsuoka Y, Shirasawa T (2005) Biological significance of isoaspartate and its repair system. Biol Pharm Bull 28:1590–159610
188. Szymanska G, Leszyk JD, O'Connor CM (1998) Carboxyl methylation of deamidated calmodulin increases its stability in *Xenopus oocyte* cytoplasm: implications for protein repair. J Biol Chem 273:28516–28523
189. Riggs DL, Gomez SV, Julian RR (2017) Sequence and solution effects on the prevalence of d-isomers produced by deamidation. ACS Chem Biol 12:2875–2882
190. Yang H, Zubarev RA (2010) Mass spectrometric analysis of asparagine deamidation and aspartate isomerization in polypeptides. Electrophoresis 31:1764–1772
191. Visick JE, Clarke S (1995) Repair, refold, recycle: how bacteria can deal with spontaneous and environmental damage to proteins. Mol Microbiol 16:835–845
192. Li C, Clarke S (1992) Distribution of an L-isoaspartyl protein methyltransferase in eubacteria. J Bacteriol 174:355–361
193. Visick JE, Cai H, Clarke S (1998) The L-isoaspartyl protein repair methyltransferase enhances survival of aging *Escherichia coli* subjected to secondary environmental stresses. J Bacteriol 180:2623–2629
194. Johnson BA, Shirokawa JM, Aswad DW (1989) Deamidation of calmodulin at neutral and alkaline pH: quantitative relationships between ammonia loss and the susceptibility of calmodulin to modification by protein carboxyl methyltransferase. Arch Biochem Biophys 268:276–286
195. Suh MJ, Alami H, Clark DJ, Parmar PP, Robinson JM, Huang ST et al (2008) Widespread occurrence of non-enzymatic deamidations of asparagine residues in *Yersinia pestis* proteins resulting from alkaline pH membrane extraction conditions. Open Proteomics J 1:106–115
196. Yan Q, Huang M, Lewis MJ, Hu P (2018) Structure based prediction of asparagine deamidation propensity in monoclonal antibodies. MAbs 10:901–912

197. Hicks WM, Kotlajich MV, Visick JE (2005) Recovery from long-term stationary phase and stress survival in *Escherichia coli* require the L-isoaspartyl protein carboxyl methyltransferase at alkaline pH. Microbiology 151:2151–2158
198. Dahl JU, Koldewey P, Salmon L, Horowitz S, Bardwell JCA, Jakob U (2015) HdeB functions as an acid-protective chaperone in bacteria. J Biol Chem 290:65–75
199. Hong W, Wu YE, Fu X, Chang Z (2012) Chaperone-dependent mechanisms for acid resistance in enteric bacteria. Trends Microbiol 20:328–335
200. Kern R, Malki A, Abdallah J, Tagourti J, Richarme G (2007) *Escherichia coli* HdeB is an acid stress chaperone. J Bacteriol 189:603–610
201. Taglicht D, Padan E, Oppenheim AB, Schuldiner S (1987) An alkaline shiftinduces the heatshock response in *Escherichia coli*. J Bacteriol 169:885–887
202. Horikoshi K, Akiba T (1982) Alkalophilic microorganisms: a new microbial world. Springer, Heidelberg
203. Verdolino V, Cammi R, Munk BH, Schlegel HB (2008) Calculation of p*K*a values of nucleobases and the guanine oxidation products guanidinohydantoin and spiroiminodihydantoin using density functional theory and a polarizable continuum model. J Phys Chem B 112:16860–16873
204. Goodson M, Rowbury RJ (1990) Habituation to alkali and increased UV-resistance in DNA repair-proficient and -deficient strains of *Escherichia coli* grown at pH 9.0. Lett Appl Microbiol 11:123–125
205. Dubnovitsky AP, Kapetaniou EG, Papageorgiou AC (2005) Enzyme adaptation to alkaline pH: atomic resolution (1.08 Å) structure of phosphoserine aminotransferase from *Bacillus alcalophilus*. Protein Sci 14:97–110
206. Mamo G, Thunnissen M, Hatti-Kaul R, Mattiasson B (2009) An alkaline active xylanase: insights into mechanisms of high pH catalytic adaptation. Biochimie 91:1187–1196
207. Shirai T, Ishida H, Noda J, Yamane T, Ozaki K, Hakamada Y, Ito S (2001) Crystal structure of alkaline cellulase K: insight into the alkaline adaptation of an industrial enzyme. J Mol Biol 310:1079–1087
208. Shirai T, Suzuki A, Yamane T, Ashida T, Kobayashi T, Hitomi J, Ito S (1997) High-resolution crystal structure of M-protease: phylogeny aided analysis of the highalkaline adaptation mechanism. Protein Eng 10:627–634
209. Zhao Y, Zhang Y, Cao Y, Qi J, Mao L, Xue Y et al (2011) Structural analysis of alkaline β-mannanase from alkaliphilic *Bacillus* sp. N16-5: implications for adaptation to alkaline conditions. PLoS One 6(1):e14608. https://doi.org/10.1371/journal.pone.0014608
210. Geiger T, Clarke S (1987) Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides: succinimide-linked reactions that contribute to protein degradation. J Biol Chem 262:785–794
211. Tyler-Cross R, Schirch V (1991) Effects of amino acid sequence, buffers, and ionic strength on the rate and mechanism of deamidation of asparagine residues in small peptides. J Biol Chem 266:22549–22556
212. Gulich S, Linhult M, Nygren PA, Hober S (2000) Stability towards alkaline conditions can be engineered into a protein ligand. J Biotechnol 80:169–178
213. Gulich S, Linhult M, Stahl S, Hober S (2002) Engineering streptococcal protein G for increased alkaline stability. Protein Eng 15:835–842
214. Krulwich TA (2005) Extreme alkaliphiles: experts at alkaline pH homeostasis and able to grow when cytoplasmic pH rises above the limit for growth of non-alkaliphiles. In: International symposium on extremophiles and their applications, pp 220–227
215. Schmidt A, Schlacher A, Steiner W, Schwab H, Kratky C (1998) Structure of the xylanase from *Penicillium simplicissimum*. Protein Sci 7:2081–2088
216. Mamo G, Hatti-Kaul R, Mattiasson B (2006) A thermostable alkaline active endo-β-1-4-xylanase from *Bacillus halodurans* S7: purification and characterization. Enzym Microb Technol 39:1492–1498

217. Inagaki K, Nakahira K, Mukai K, Tamura T, Tanaka H (1998) Gene cloning and characterization of an acidic xylanase from *Acidobacterium capsulatum*. Biosci Biotechnol Biochem 62:1061–1067
218. Yang JH, Park JY, Kim SH, Yoo YJ (2008) Shifting pH optimum of *Bacillus circulans* xylanase based on molecular modeling. J Biotechnol 133:294–300
219. Nielsen JE, McCammon JA (2003) Calculating pKa values in enzyme active sites. Protein Sci. 12:1894–1901
220. Joshi MD, Sidhu G, Nielsen JE, Brayer GD, Withers SG, McIntosh LP (2001) Dissecting the electrostatic interactions and pH-dependent activity of a family 11 glycosidase. Biochemistry 40:10115–10139
221. Nielsen JE, Borchert TV, Vriend G (2001) The determinants of alpha-amylase pH-activity profiles. Protein Eng 14:505–512
222. Coughlan S, Wang XG, Britton KL, Stillman TJ, Rice DW et al (2001) Contribution of an aspartate residue, D114, in the active site of clostridial glutamate dehydrogenase to the enzyme's unusual pH dependence. Biochim Biophys Acta 1544:10–17
223. Bai W, Cao Y, Liu J, Wang Q, Jia Z (2016) Improvement of alkalophilicity of an alkaline xylanase Xyn11A-LC from *Bacillus* sp. SN5 by random mutation and Glu135 saturation mutagenesis. BMC Biotechnol 16:77
224. Madzak C, Mimmi MC, Caminade E, Brault A, Baumberger S, Briozzo P et al (2006) Shifting the optimal pH of activity for a laccase from the fungus *Trametes versicolor* by structure-based mutagenesis. Protein Eng Des Sel 19:77–84
225. Richardson TH, Tan X, Frey G, Callen W, Cabell M, Lam D et al (2002) A novel, high performance enzyme for starch liquefaction. Discovery and optimization of a low pH, thermostable alpha-amylase. J Biol Chem 277:26501–26507
226. Aygan A, Arikan B, Korkmaz H, Dinçer S, Çolak Ö (2008) Highly thermostable and alkaline α-amylase from a halotolerant-alkaliphilic *Bacillus* sp. AB68. Braz J Microbiol 39:547–553
227. Kim DH, Morimoto N, Saburi W, Mukai A, Imoto K, Takehana T et al (2012) Purification and characterization of a liquefying α-amylase from alkalophilic thermophilic Bacillus sp. AAH-31. Biosci Biotechnol Biochem 76:1378–1383
228. Drechsel H, Jung G (1998) Peptide siderophores. J Pept Sci 4:147–181
229. McMillan DGG, Velasquez I, Nunn BL, Goodlett DR, Hunter KA, Lamont I et al (2010) Acquisition of iron by alkaliphilic *Bacillus* species. Appl Environ Microbiol 76:6955–6961
230. Luque-Almagro VM, Blasco R, Huertas MJ, Martinez-Luque M, Moreno-Vivian C, Castillo F, Roldan MD (2005) Alkaline cyanide biodegradation by *Pseudomonas pseudoalcaligenes* CECT5344. Biochem Soc Trans 33:168–169
231. Sarethy IP, Saxena Y, Kapoor A, Sharma M, Sharma SK, Gupta V, Gupta S (2011) Alkaliphilic bacteria: applications in industrial biotechnology. J Ind Microbiol Biotechnol 38:769–790
232. Sorokin DY, Kuenen JG (2005) Chemolithotrophic haloalkaliphiles from soda lakes. FEMS Microbiol Ecol 52:287–295
233. Carini SA, Joye SB (2008) Nitrification in Mono Lake, California: activity and community composition during contrasting hydrological regimes. Limnol Oceanogr 53:2546–2557
234. Luque-Almagro VM et al (2005) Bacterial degradation of cyanide and its metal complexes under alkaline conditions. Appl Environ Microbiol 71:940–947
235. Schagerl M (2016) Soda lakes of East Africa. Springer, Cham
236. Lanzén A, Simachew A, Gessesse A, Chmolowska D, Jonassen I, Øvreås L (2013) Surprising prokaryotic and eukaryotic diversity, community structure and biogeography of Ethiopian soda lakes. PLoS One 8(8):e72577
237. Kavembe GD, Meyer A, Wood CM (2016) Fish populations in East African saline lakes. Soda lakes of East Africa. Springer, Cham, pp 227–257
238. Wilkie MP, Wood CM (1991) Nitrogenous waste excretion, acid-base regulation, and ionoregulation in rainbow trout (*Oncorhynchus mykiss*) exposed to extremely alkaline water. Physiol Zool 64:1069–1086

239. Wilkie MP, Wood CM (1995) Recovery from high pH exposure in the rainbow trout: white muscle ammonia storage, ammonia washout, and the restoration of blood chemistry. Physiol Zool 68:379–401
240. Wilkie MP, Wood CM (1996) The adaptations of fish to extremely alkaline environments. Comp Biochem Physiol B Biochem Mol Biol 113:665–673
241. Yesaki TY, Iwama GK (1992) Survival, acid-base regulation, ion regulation, and ammonia excretion in rainbow trout in highly alkaline hard water. Physiol Zool 65:763–787
242. Johansen K, Maloiy G, Lykkeboe G (1975) A fish in extreme alkalinity. Respir Physiol 24:159–162
243. Wilkie MP, Wright PA, Iwama GK, Wood CM (1994) The physiological adaptations of the Lahontan cutthroat trout (*Oncorhynchus clarki* henshawi) following transfer from well water to the highly alkaline waters of Pyramid Lake, Nevada (pH 9.4). Physiol Zool 67:355–380
244. Wood CM, Bergman HL, Bianchini A, Laurent P, Maina J, Johannsson OE et al (2012) Transepithelial potential in the Magadi tilapia, a fish living in extreme alkalinity. J Comp Physiol B 182:247–258
245. Wood CM, Bergman HL, Laurent P, John MN, Narahara AB, Walsh PJ (1994) Urea production, acid-base regulation and their interactions in the Lake Magadi tilapia, a unique teleost adapted to a highly alkaline environment. J Exp Biol 189:13–36
246. Wood CM, Wilson P, Bergman HL, Bergman AN, Laurent P, Owiti G et al (2002) Ionoregulatory strategies and the role of urea in the Magadi tilapia (*Alcolapia grahami*). Can J Zool 80:503–515
247. Wilkie MP (2002) Ammonia excretion and urea handling by fish gills: present understanding and future research challenges. J Exp Zool 293:284–301
248. Randall DJ, Tsui TKN (2002) Ammonia toxicity in fish. Mar Pollut Bull 45:17–23
249. Wilkie MP, Pamenter ME, Duquette S, Dhiyebi H, Sangha N, Skelton G et al (2011) The relationship between NMDA receptor function and the high ammonia tolerance of anoxia-tolerant goldfish. J Exp Biol 214:4107–4120
250. Walsh PJ, Smith CP (2001) Urea transport. Fish Physiol 20:279–307

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Genomics of Alkaliphiles

Pedro H. Lebre and Don A. Cowan

Contents

Abstract Alkalinicity presents a challenge for life due to a "reversed" proton gradient that is unfavourable to many bioenergetic processes across the membranes of microorganisms. Despite this, many bacteria, archaea, and eukaryotes, collectively termed alkaliphiles, are adapted to life in alkaline ecosystems and are of great scientific and biotechnological interest due to their niche specialization and ability to produce highly stable enzymes. Advances in next-generation sequencing technologies have propelled not only the genomic characterization of many alkaliphilic microorganisms that have been isolated from nature alkaline sources but also our understanding of the functional relationships between different taxa in microbial communities living in these ecosystems. In this review, we discuss the genetics and molecular biology of alkaliphiles from an "omics" point of view, focusing on how metagenomics and transcriptomics have contributed to our understanding of these extremophiles.

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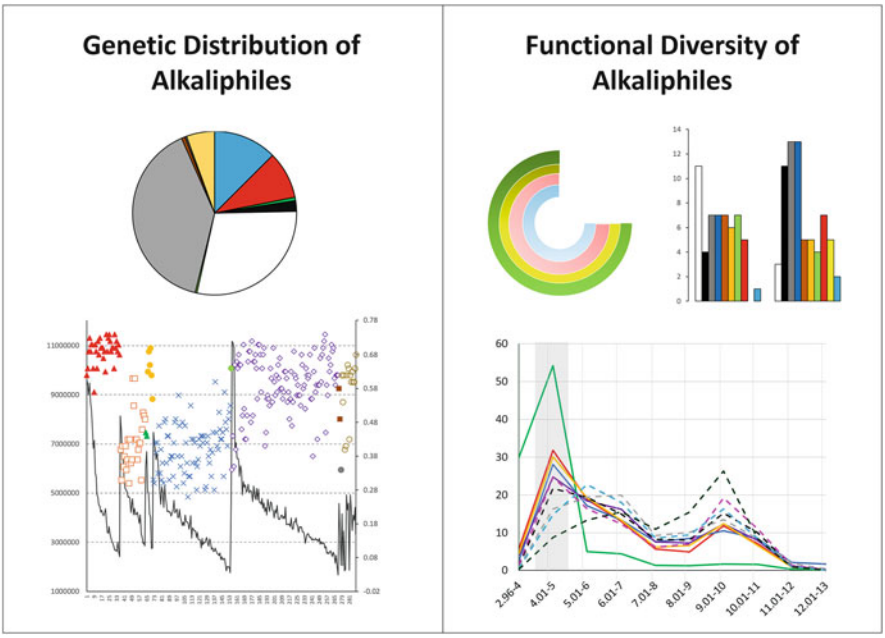
Graphical Abstract



Keywords Alkaliphiles, Bioenergetics, Genomics, Metabolic potential, Metagenomics

Abbreviations

ATP	Adenosine triphosphate
CDS	Coding sequence
DNA	Deoxyribonucleic acid
FAD	Flavin adenine dinucleotide
JGI	Joint Genome Institute
KOGs	Eukaryotic Orthologous Groups
Mb	Mega base pairs
mV	Millivolts
NAD(P)H	Nicotinamide adenine dinucleotide phosphate (reduced form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
pI	Isoelectric point
PMF	Proton motive force
PP	Pentose phosphate
SMF	Sodium motive force

1 Introduction: Alkaliphiles in the Metagenomics Era

Alkaliphiles are a diverse group of microorganisms that are defined as being able to grow in high pH environments (≥9). These organisms reside in a range of extreme environments in which high alkalinity has been established through geological processes, such as the accumulation of CO_2 and subsequent production of carbonate-/bicarbonate-rich solutions in soda lakes, as well as transient biological events such as ammonification and sulphate reduction in soils [1]. In these extreme niches, microbial communities are not only well adapted to alkalinity, but they must also cope with a range of other environmental stresses, including high salinity, low or high temperatures, and oxygen deprivation [2]. Consequently, alkaliphiles are of great scientific and biotechnological interest due to their highly selective niche specialization and their ability to produce proteins that are stable across a wide range of extreme conditions [1].

Since the first report of an alkaliphile genome sequence in 2000 [3], the number of sequenced genomes of alkaliphilic microorganisms has increased exponentially due to advances in next-generation sequencing technologies [4]. Currently, the JGI genome portal [5] lists the sequences of 288 genomes from isolated microorganisms that have been characterized to grow in alkaline conditions. These genomes are distributed across ten different phyla (Fig. 1a) and are highly variable in terms of GC content (from 26 to 74%) and genome size (from 1.6 to 11 Mb) (Fig. 1b). This high genetic variability, together with the range of habitats from which alkaliphiles have been isolated, reflects the functional diversity of alkaliphilic microorganisms. As such, no obvious trends can be found between the alkaliphilic phenotype and particular genetic features. The phylum *Firmicutes*, which includes the historically relevant species *Bacillus halodurans* C-125 and *B. pseudofirmus* OF-4 [3, 6], represents the second largest fraction of sequenced genomes (83 genomes), most of which have a GC content below 50%. By comparison, the JGI lists 114 genomes of alkaliphile *Proteobacteria*, the majority of which have a high GC content (≥50%). These include *Halomonas* sp. GFAJ-1, which is capable of thriving in arsenic-rich environments and has been associated with arsenate detoxification [7]. To date, only two genomes of alkaliphilic Cyanobacteria have been listed in the JGI database, despite the fact that members of this phylum play a crucial role as primary photoautotrophic producers in many alkaline environments [8]. The two genomes belong to the desiccation-tolerant *Chroococcidiopsis thermalis* PCC 7203, and *Arthrospira platensis* C1, which is cultivated at large industrial scale as a food product for both humans and animals [9]. All 16 publicly available alkaliphilic archaeal genomes belong to the Euryarchaeota phylum, and the majority of these organisms have been isolated from highly saline fresh water environments. Genome sizes for these archaea vary between 1.8 and 4.9 Mb, and 12 have a very high GC content (≥60%). High genomic GC content is a common feature of halophiles and has been associated with adaptation mechanisms against UV-induced thymidine dimer formation and the consequent accumulation of mutations [10]. Ten other

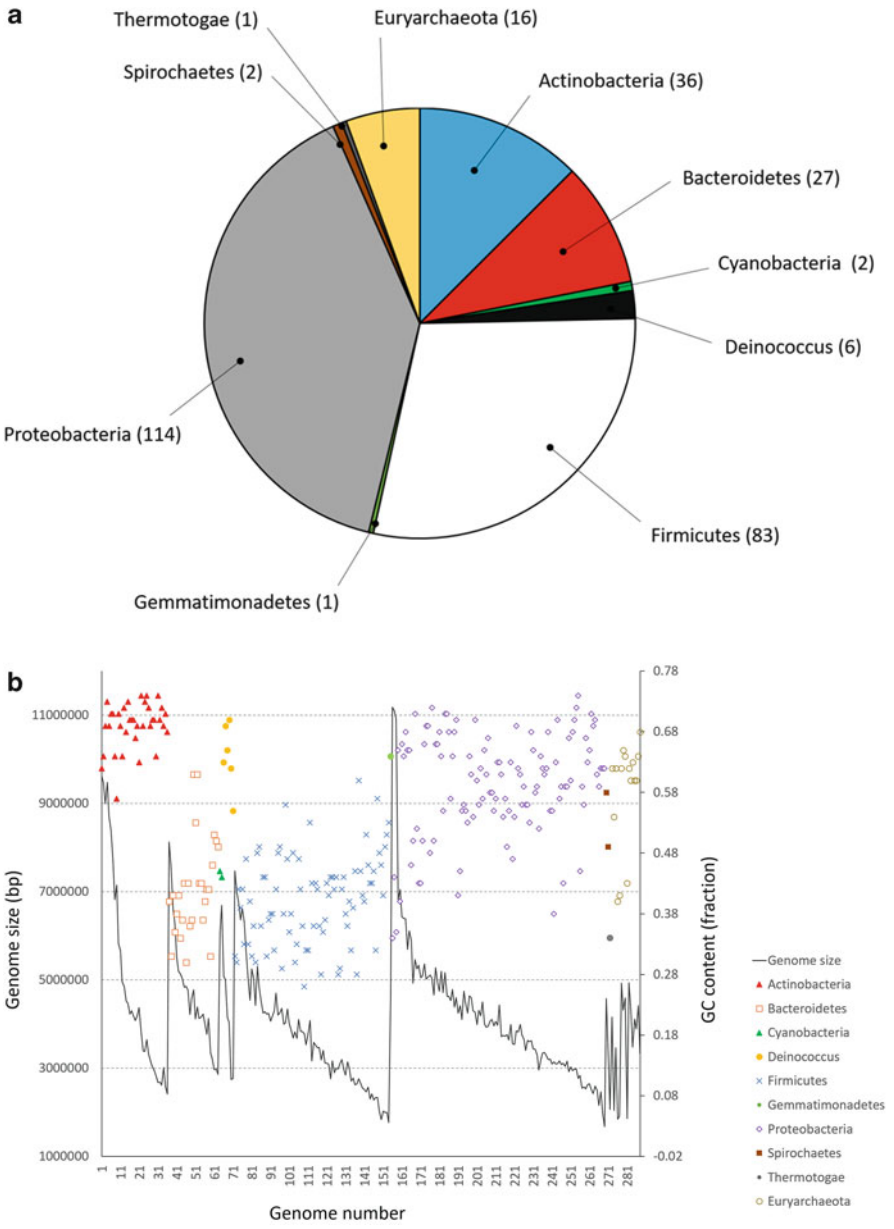


Fig. 1 (**a**) Distribution of the JGI-listed alkaliphile genomes across different phyla. The number of genomes for each phylum is indicated in brackets next to the name for that phylum. (**b**) GC content and genome size distributions of the 288 alkaliphile genomes listed in the JGI database. GC content is illustrated with the scatter plot, and units are expression as fractions. Points in the plot are colour-coded to differentiate the different phyla. Genome size distribution is represented by the line plot, and units are expressed in nucleotide base-pairs (bp)

alkaliphilic cyanobacterial genomes that are not listed in the JGI database have also been sequenced, including five additional *Arthrospira* species [11].

Compared to prokaryotic alkaliphiles, there is a dearth of knowledge on the genomics of alkaliphilic eukaryotes. Crucially, the recent drive for culture-independent techniques such as metagenomics has led to in-depth studies into the composition, functional capacities, and ecological impact of communities living in alkaline environments, including eukaryote. To date, 153 metagenomes have been obtained from alkaline environments, the majority of which were sourced from saline and alkaline water (51 metagenomes), and serpentinite rock and fluid (40 genomes). Members of the genera *Frontania* and *Lacrymaria*, both ciliates, were reported to be found in four distinct alkaline environments, while diatoms of the class Fragillariophyceae were found in both alkaline and acidic habitats [12].

2 The Genomic Features of Alkaliphilic Microorganisms

The increasing number of genomes for alkaliphiles allows for comparative genomic studies that reveal the unique genetic features of these organisms. Historically, bacteria from the genus *Bacillus* have been the target of substantial research on the adaptation to alkaliphily, with the genomes of representative strains *B. halodurans* C-125 and *B. pseudofirmus* OF4 being widely studied and characterized [13]. Both genomes share a large percentage of genes (1,510 genes, 36.8% in *B. pseudofirmus* OF4 and 38.2% in *B. halodurans* C-125), as well as 80% conserved synteny and comparable origins of replication [6]. In turn, the genomes of *B. halodur*ans C-125 and *B. subtilis* were found to share a high number of gene clusters involved in the house-keeping functions such as motility and chemotaxis, sporulation, protein secretion, main metabolic pathways, and DNA replication [3]. One big differential factor between these genomes is the number and type of transposable elements. *B. halodurans* contains 112 transposable elements divided into 27 distinct groups compared to the ten transposable elements in *B. subtilis*, and all of this share significant sequence similarity to transposases and recombinases from species such as *Rhodobacter capsulatus* and *Lactococcus lactis*. In addition, *B. halodurans* C-125 contains ten unique extracytoplasmic function σ factors that might play a role in adaptation to alkaline environments [3]. A distinct feature of *B. pseudofirmus* OF4 is the presence of two resident plasmids that contain gene clusters for metal acquisition and metal resistance, including P-type metal ATPases, copper chaperones, and cadmium resistance transporters [6]. Genomic differences between *B. pseudofirmu*s and *B. halodurans* further hint at the more alkaliphilic nature of the former. For instance, *B. pseudofirmu*s contains 13 cation/solute antiporters compared to 5 in *B. halodurans*, which might contribute to increased capacity to maintain pH homeostasis in the cytoplasm [6]. This enrichment in proton/cation transporters can also be seen in genomes from other alkaliphiles (Fig. 2). Another alkaliphilic bacillus, *Oceanobacillus iheyensis* HTE831, first isolated from deep-sea sediments on the Iheya Ridge [15], is a strict aerobe that grows optimally at pH 9.5.

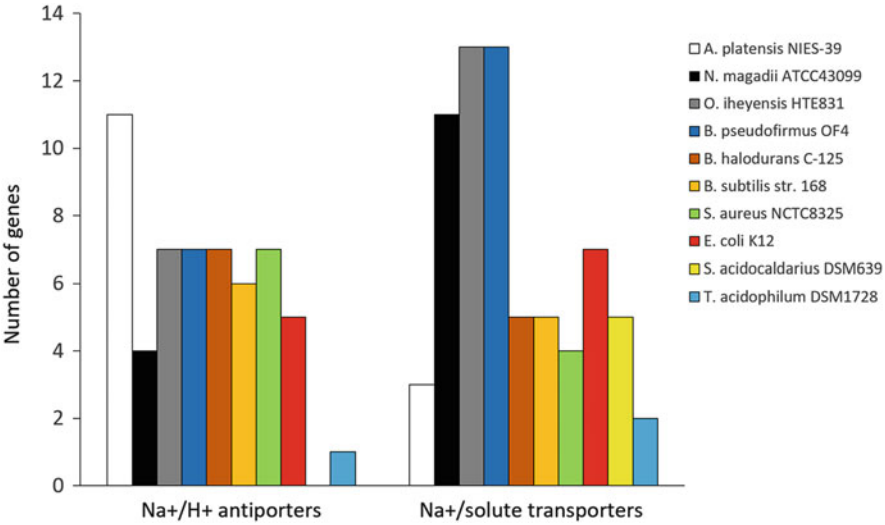


Fig. 2 Number of genes for Na^+/H^+ antiporters and Na^+/solute symporters distributed across the genomes of alkaliphiles, neutrophiles, and acidophiles. The alkaliphile genomes are generally found to have a higher number of Na^+/solute transporters, while the number of Na^+/H^+ antiporters is comparable to their neutrophilic and acidophilic counterparts, with *A. platensis* being an exception. This reverse enrichment in *A. platensis* might be accounted for by the localization of the respiratory apparatus within cytoplasmic vesicles, which might also require the active establishment of a PMF through Na^+/H^+ exchange. The genomes were obtained from the NCBI genome database (https://www.ncbi.nlm.nih.gov/genome) and annotated using the EggNOG v4.5.1 mapper [14]. The alkaliphile species used in this analysis are *A. platensis* NIES-39, *N. magadii* ATCC43099, *O. iheyensis* HTE831, *B. pseudofirmus OF*4, and *B. halodurans* C-125. The neutrophile species used are *B. subtilis* str. 168, *S. aureus* NCTC8325, and *E. coli* K12. The acidophiles used are *Sulfolobus acidocaldarius* DSM639 and *Thermoplasma acidophilum* DSM1728

This organism contains a 3.6 Mb circular genome with 35.7% GC content, with a similar tRNA arrangement to *B. subtilis* and no prophages, and encoding 3,496 CDSs. *O. iheyensis* and *B. halodurans* share 243 putative genes. Five of these genes encode branched-chain amino acid transporters, which are thought to be important in alkaliphiles due to their conversion to negatively charged glutamic acid, which in turn leads to the acidification of the cytoplasm [16]. This idea is further re-enforced by a recent transcriptomics study that shows the upregulation of branched-chain amino acid transporters in *Halomonas* sp. Y2 under alkaline stress [17].

The obligately haloalkaliphile *Natrialba magadii* is an archaeon that requires a high salt concentration (3.5 M NaCl) and pH (9.5) for optimal growth. The genome consists of four replicons, the largest of which is 3.7 Mb in size and has a high GC content (61.42%). Of the 4,212 genes coded by the combined genome, 2,387 shared orthologues with the halophilic archaeon *Haloterrigena turkmenica*, which has been isolated from a sulphate saline soil in Turkmenistan [18]. The genome of *Natrialba magadii* bears specific features that may be linked to its adaptation to its natural environment. These include the presence of genes coding for proteins involved in the intake and synthesis of osmoprotectant compounds such as trehalose and

spermidine, a large cluster of 11 genes coding for putative gas vesicle proteins that would allow for better access to surface oxygen, and genes encoding for metal transport proteins, which would may be involved in metal homeostasis in hypersaline environments [18, 19].

The genus *Arthrospira* comprises a group of non-N_2-fixing cyanobacteria that grow in carbonate/bicarbonate-rich alkaline lakes and are economically important as food sources. The draft genome of one of the two alkaliphilic cyanobacterial genomes listed in the JGI database, *Arthrospira platensis* C1, was published in 2012 [9]. This genome shares between 94.93 and 97.43% sequence identities with the other five sequenced *Arthrospira* sp., which together share a highly conserved core genome. As with many other genomes from alkaliphilic prokaryotes, *A. platensis* C1 contains several genes encoding Na^+/H^+ antiporters, particularly one NapA-type Na^+/H^+ antiporter homolog that has been associated with salt and pH homeostasis in alkaline conditions [9]. Analysis of the genome of *A. platensis* NIS-39 showed that it contained a large number of genes with adenylate and guanylate cyclase domains that are involved in cAMP and c-di-GMP signal transduction and response to external stimuli. In addition, this genome also contained seven genes for putative Na^+/H^+ antiporters, as well as seven σ factors from groups 2 and 3, which are involved in responses to environmental stress [20].

Despite the limited information on the genomics of eukaryotic alkaliphiles, several biodiversity studies have depicted a large diversity of eukaryotes in alkaline environments, including plankton diatoms, green algae, cryptophytes, and haptophytes [12, 21–23]. Two recently sequenced genomes from the closely related alkaliphilic fungi *Acremonium alcalophilum* ATCC 90507 and *Sodiomyces alkalinus* F11 provide a glimpse into the genetic basis of alkaliphily in eukaryotes [24, 25]. The genomes are 54.42 Mb and 43.45 Mb in size, respectively, and encode a comparable number of genes for hydrogen/solute symporters (25 in *S. alkalinus* vs 33 in *A. alcalophilum*), sodium/solute symporters (5 in both cases), and solute/hydrogen antiporters (10 in *S. alkalinus* vs 15 in *A. alcalophilum*). Comparison of these genomes in the JGI MycoCosm fungal genomic resource (https://genome.jgi.doe.gov/programs/fungi/index.jsf) shows that more neutrophilic fungi such as *Aspergillus oryzae* and *Plectosphaerella cucumerina* also contain comparable numbers of sodium and hydrogen transporters, and therefore these genes cannot be used as hallmarks for alkaliphily in fungi (Table 1). Instead, both *A. alcalophilum* and *S. alkalinus* show an enrichment of halotolerance proteins with FAD domains, which suggests a strong correlation between halotolerance and alkaliphily in the environments from where these fungi were isolated.

In addition to their genomic heterogeneity, several alkaliphiles have been shown to harbour extrachromosomal plasmids containing resistance genes and other genetic elements that provide an adaptive edge in the extreme environments where these organisms inhabit (Table 2). For instance, *B. pseudofirmus* OF4 contains two plasmids, pBpOF4-01 (0.28 Mb) and pBpOF4-02 (0.10 Mb), that code for genes involved in resistance to heavy metals such as cadmium, copper, and mercury [6]. Similarly, pCHRO.01 (0.37 Mb) from the alkali-tolerant *Chroococcidiopsis thermalis* PCC7203 codes for several metal efflux transporters and multicopper oxidases in addition to toxin-antitoxin systems and DNA translocation proteins. In

Table 1 The number of genes encoding proteins associated with alkaliphily in two neutrophile and two alkaliphile fungi according to the eukaryotic orthologous groups (KOGs) annotations obtained from the MycoCosm fungal genomic resource (https://genome.jgi.doe.gov/programs/fungi/index.jsf)

KOG annotation	*A. oryzae*	*P. cucumerina*	*S. alkalinus*	*A. alcalophilum*
KOG0672 halotolerance protein HAL3	3	2	19	20
KOG4126 alkaline phosphatase	3	4	4	2
KOG1397 Ca^{2+}/H^{+} antiporter VCX1 and related proteins	8	4	4	4
KOG2493 Na^{+}/P_i symporter	4	6	4	4
KOG4505 Na^{+}/H^{+} antiporter	5	5	2	3
KOG1341 Na^{+}/K^{+} transporter	5	5	2	2
KOG1650 predicted K^{+}/H^{+}-antiporter	3	2	2	1
KOG2399 K^{+}-dependent Na^{+}/Ca^{2+} antiporter	1	1	1	1
KOG0205 plasma membrane H^{+}-transporting ATPase	3	1	1	1
KOG3126 Porin/voltage-dependent anion-selective channel protein	1	1	1	1
KOG3182 predicted cation transporter	1	1	1	1

Table 2 Extrachromosomal plasmids present in representative strains of alkaliphilic bacteria and archaea

Species	Plasmids	Size (bp)	GC %	Nr. genes
Listeria monocytogenes 08-5923	pLM5578	77,054	36.59	79
Thermobacillus composti KWC4	pTHECO01	149,182	47.3	149
Kineococcus radiotolerans SRS30216	pKRAD01	182,572	69.4	183
	pKRAD02	12,917	72.3	17
Halobacillus halophilus DSM 2266	pL16	16,047	43	19
	pL3	3,329	36.5	2
Natranaerobius thermophilus JW/NM-WN-LF	pNTHE01	17,207	34.2	17
	pNTHE02	8,689	35.7	9
Chroococcidiopsis thermalis PCC 7203	pCHRO.01	370,830	45.1	335
	pCHRO.02	2,779	44.1	2
Bacillus pseudofirmus OF4	pBpOF4-01	285,222	36	260
	pBpOF4-02	105,029	35.5	113
Sinorhizobium medicae WSM419	pSMED01	1,570,951	61.5	1,441
	pSMED02	1,245,408	59.9	1,094
	pSMED03	219,313	60.1	149
Natronococcus occultus DSM 3396	Plasmid 1	12,939	54.8	20
	Plasmid 2	287,963	61.3	238

the case of *Sinorhizobium medicae* WSM419, two of its three plasmids, pSMED01 (1.57 Mb) and pSMED02 (1.24 Mb), represent a large proportion of the genome and code for a broad range of functions in addition to stress adaptation, including proteins involved in energy conversion and amino acid synthesis [26]. Alternatively, the two plasmids from the alkalithermophile *Natranaerobius thermophilus* JW/NM-WN-LF primarily code for DNA restriction and modification enzymes as well as a growth inhibition regulator and are thus not directly involved in stress adaptation [27]. Rather, the abundance of transposons, integrases, and other mobile elements in the majority of the plasmids listed in Table 1 suggests they also play an important role in both adaptive and non-adaptive plasticity of the genomes of alkaliphiles.

3 Genomic Insights into the Metabolism of Alkaliphiles

Alkaliphilic microorganism are found in a wide range of bacterial, archaeal, and eukaryotic phyla and constitute a functionally diverse group of organisms that play vastly different but interdependent ecological roles in their native habitats. For instance, communities in soda lakes are thought to be organized in a complex multilayered structure, in which the by-products and dead matter from the primary photoautotrophic cyanobacterial producers at the surface are initially used by a second layer of heterotrophic bacteria from the *Firmicutes* and *Proteobacteria* phyla. The metabolic products from these bacteria are subsequently used as substrates for anaerobic organisms that degrade organic matter, producing gases that support the growth of a fourth layer of lithotrophic organisms such as homoacetogens, hydrogenotropic sulphidogens, and anoxygenic anaerobic phototrophs [8, 28].

As primary producers, cyanobacteria play a crucial role in pioneering and maintaining communities in many extreme environments. Sequence analysis of the alkaliphilic *A. platensis* showed that it contains a full set of genes for photosystems I and II, as well as variant genes such as cytochrome c550-like and cytochrome c_6 [20]. As a photoautotroph, *A. platensis* also contains the genes for the common cyanobacterial metabolic pathways, including the pentose phosphate (PP) pathway for primary metabolism of bicarbonate ions, as well as the Calvin cycle for CO_2 fixation, which depends on the activity of two enzymes, phosphoribulokinase and RuBisCO. These two proteins are also specific to the PP pathway in light energy conditions, while under dark conditions, the glucose-6-phosphate and 6-phosphogluconate dehydrogenases are used [29]. Cyanobacteria surviving in alkaline conditions where CO_2 concentrations are limited rely on CO_2-concentrating mechanisms, which involved CO_2 and bicarbonate (HCO_3^-) uptake by NAD(P)H dehydrogenases and plasma membrane transporters, as well as HCO_3^- conversion into CO_2 catalysed in RubisCO-containing carboxysome subcellular compartments [30]. A comparative genomics study of all known alkaline cyanobacterial genomes revealed that they all contained the same RubisCO variant, RubisCO B1 [11]. In addition, all the strains analysed contained genes that encode CO_2-concentrating

mechanisms, including genes for the CO_2-uptake NAD(P)H dehydrogenase type 1 complexes NDH-1_3 and NDH-1_4, as well as the *ccmKLMNO* cluster, which codes for the structural proteins of the carboxysomes. Conversely, *A. platensis* NIES-39 and *A. platensis* sp. *paraca* were shown to contain a gene encoding the high-affinity HCO_3^- transporter StbA, in addition with the low-affinity transporter BicA, which was shared by all the genomes analysed. The presence of both transporters in these strains might provide a selective advantage in highly alkaline environments where HCO_3^- concentrations are high [11].

Alkaliphilic anaerobes thriving on the products from the primary producers can be expected to metabolize a range of complex carbohydrates. This is the case with the obligately alkaliphilic anaerobe, *Clostridium alkalicellum*, which was isolated from a cellulose-decomposing community in the Verkhnee Beloe soda lake in Russia. This bacterium was found to be very specialized, with a strictly fermentative metabolism capable of degrading xylan, cellulose, and cellobiose into hydrogen, ethanol, acetate, and lactate [31]. Other anaerobic heterotrophic bacteria found in soda lakes, such as *Halonatronum saccharophilum*, *Amphibacillus fermentum*, and *Amphibacillus tropicus*, isolated from Lake Magadi in Kenya, use a purely fermentative type of metabolism in which mono-, di-, and polysaccharides are catabolized through the fructose bisphosphate pathway into acetate, ethanol, and CO_2 [32]. Microarray studies on another hemicellulose degrading facultative anaerobe isolated from the Wudunur Soda Lake in China, *Bacillus* sp., N16-5, revealed a complex hierarchical pattern of sugar metabolism in which glucose was the preferred substrate, followed by components of complex hemicellulose polysaccharides such as xylan, pectin, and galactomannan. Glucose was found to be the primary repressor of expression of gene clusters involved in the degradation of polysaccharides, which are only partially degraded by extracellular glycoside hydrolases before being transported intracellularly by oligopeptide transporters to be further processed [33]. This is thought to be a genetic adaptation to allow responses to the carbohydrate source fluctuations in the environment [33].

Unlike acetogens from more neutrophilic habitats, acetogenic bacteria found in soda lakes cannot grow chemolithotropically on hydrogen, carbon monoxide, and carbon dioxide mixtures and are trophically limited to products from primary anaerobes in the community such as lactate, histidine, and ethanol [8]. In these bacteria, the Wood-Ljungdahl acetyl-CoA pathway, in which the CO_2 generated from the catabolism of the primary substrates is converted to acetate, is coupled with proton-dependent ATP synthesis by generating the electron and proton gradient required by F_1F_0-type ATP synthases [34]. This is the case with the bacteria *Natroniella acetigena* and *Natronincola histidinovorans*, while in *T. magadiensis* the fermentation of arginine through the ornithine cycle and subsequent conversion of carbamoyl phosphate by carbamate kinase is coupled to ATP synthesis [34]. Genome analysis of the strictly anaerobic and sulphate-reducing bacterium *Thermodesulfovibrio* sp. N1 showed that it lacked the complete TCA and Wood-Ljundahl pathways and would therefore not be capable of fully oxidizing organic substrates [35]. Instead, it can reversibly decarboxylate pyruvate to acetyl-CoA, which is further converted into acetate by the acetyl-CoA synthetase, generating ATP in the process. Several other dehydrogenases present in the periplasmic space,

such as four [NiFe]-family dehydrogenases that allow for growth with H_2 as an energy source, and a formate dehydrogenase that uses formate as a proton donor, contribute to the proton motive force (PMF) used to drive proton-coupled ATP synthesis [35].

As noted above, alkaline ecosystems also harbour metabolically versatile chemoautotrophic microorganisms that can feed on a variety of organic and inorganic substrates. *Alkalilimnicola ehrlichii* MLHE-1, a facultative Gram-negative chemoautotroph isolated from the alkaline and hypersaline Mono Lake in the USA, is capable of growing both aerobically and anaerobically with inorganic electron donors such as arsenite, hydrogen, and nitrate as the electron acceptor while also growing heterotrophically on organic acids [36, 37]. Genome analysis of this bacterium revealed that, in addition to the RuBisCO genes *cbb*L and *cbb*S, which allow for CO_2 fixation through the Calvin cycle, it also contained the CO dehydrogenase operon *coxFEDLSM*, which allows for CO metabolism [37]. Three bacterial strains (A1, B1, and H1) isolated from a serpentinizing site in the USA and phylogenetically related to the genera *Hydrogenophaga* and *Malikia* were also genetically characterized [38]. Metabolic profiling of these strains revealed that they have genes required for chemoautotrophic growth on H_2, specifically group 2b and 3d [NiFe]-hydrogenases, as well as genes encoding for carboxysome shell proteins, which suggest the ability to employ CO_2-concentrating mechanisms for carbon fixation. In addition, strain A1 also contained genes encoding for benzene and phenylalanine/phenylacetate degradation, which would facilitate growth in environments with aromatic compounds.

The recent sequencing of the genome of the alkaliphile fungi *S. alkalinus*, originally extracted from soda lakes, also contributed to our understanding of the role that eukaryotic alkaliphiles play in these complex trophic systems. Metabolic profiling of the genome revealed the capacity to grow on xylan from maize, pectins, and monosaccharides [24]. In addition, S. *alkalinus* was found to express a narrow range of peptidases with strong protease activity at alkaline conditions, which support the hypothesis that it used protein-rich microscopic crustaceans and prokaryotes as primary food sources [24].

4 The Bioenergetics of Alkaliphiles

The bioenergetics of aerobic alkaliphiles revolves around the control of the PMF and SMF across the cytoplasmic membrane. pH homeostasis is regulated by coupling the outwards pumping of protons through the respiratory chain to generate a PMF, with the export of sodium by Na^+/H^+ antiporters using the PMF. This interplay maintains the conditions required for ion gradient-coupled bioenergetics processes while achieving the acidification of the cytoplasm [39].

The sodium cycle, in which a sodium motive force (SMF) is established across the membrane to drive bioenergetics processes, has been historically associated with adaptation to alkaline environments where establishing a proton motive force is extremely energy demanding [40]. However, the existence of alkaliphiles that can grow in alkaline conditions using protons as the coupling ion has since discredited the idea of sodium cycling being an obligate requirement for alkaline adaptation [41, 42]. Nonetheless, the sodium cycle plays an important role in the maintenance of pH homeostasis in a restricted number of alkaliphiles. One of the main antiporters involved in this cycle is the Mrp-antiporter, which was first identified in *B. halodurans* [43]. In *Bacillus pseudofirmus* OF4 the operon for the *mrp* Na^+/H^+ antiporter contains seven genes, all of which were found to be essential for the Na^+ exclusion and antiport activity [44]. Two other major components of this cycle are the sodium/solute symporter and the voltage-gate channel associated with a sodium dependent flagellar motor [45], which is found exclusive in alkaliphiles [46]. Studies on non-alkaliphilic mutant strains of *B. halodurans* C-125 have revealed the importance of Na^+/H^+ antiporters, as the alkaliphilic nature of the strain was restored after cloning in a 3.7 kb stretch of DNA containing an Na^+/H^+ antiporter gene [3, 47]. In the Gram-negative bacterium *Alkalimonas amylolytica*, isolated from Lake Chahannor in China, the monovalent sodium/proton antiporter NhaD provided sodium (lithium)/proton antiport activity at pH values above 9 and over a broad range of sodium concentrations, but activity was severely reduced under more neutrophilic conditions [48]. It is also notable that the genome of the alkaliphile *O. iheyensis* contains 18 genes coding for C4-dicarboxylate carriers, 7 of which are also shared by *B. halodurans* [49] that are commonly associated with Na^+ and H^+ symport activity.

A recent transcriptomics study of the alkaliphile *Halomonas* sp. Y2 revealed that it differentially expresses distinct Na^+/H^+ antiporters in response to different stresses. For instance, expression of the Mrp transporter did not vary across different pHs, while its absence negatively impacted the organism's resistance to Na^+, Li^+, and K^+ ions. By comparison, the Ha-NhaD2 transporter, which is homologous to the NhaD antiporter from *Alkalimonas amylolytica*, was upregulated with increased pH, suggesting that it plays a more significant role in pH homeostasis than Mrp, under alkaline stress [17]. In addition to the canonical Na^+/H^+ antiport, some members of this superfamily also show functional versatility. This is the case of the antiporter Ap-NapA1-2 from the halotolerant alkaliphilic cyanobacterium *Aphanothece halophytica*, which shows the capacity to replace proton uptake by potassium uptake [50].

Due to the reversed proton gradient in alkaliphiles, where the cytoplasmic pH is more acidic than the alkaline environment, processes that require a PMF, such as motility, are severely compromised [51]. To circumvent this limitation, alkaliphiles use SMF-driven motors to achieve motility [52]. In *B. halodurans* C-125 and

B. pseudofirmus OF4, MotPS functions as the sole sodium-dependent stator for the flagellar motor [3], while *O. iheyensis* also contains the proton-coupled MotAB stator [15]. The difference between these species might be due to the more halotolerant nature of the latter. Conversely, *Bacillus clausii* KSM-K16 contains set genes with high homology to MotAB from *B. subtilis* that can couple both sodium and protons for motility [53]. Another sodium channel, NaChBac, was found to play a major role in the pH homeostasis of *B. halodurans* C-125 and *B. pseudofirmus* OF4, particularly in conditions where sodium and solute concentrations are low [54, 55].

5 The Alkaliphilic Respiration Chain

As noted above, the bioenergetics of alkaliphiles depends on the delicate balance between pH homeostasis and the PMF. The acidification of the cytoplasm has the detrimental effect of significantly reducing the PMF across the membrane. For instance, the PMF of the facultative alkaliphile *Bacillus* sp. TA2.A1 shifts from −164 to −78 mV when pH is changed from 7.5 to 10 [56]. Despite this apparent limitation, aerobic alkaliphiles are adapted to cope with a reduced proton electrochemical gradient [41]. Examples of high pH-specific adaptations include the overexpression of cytochromes in some strains when grown in high pH environments [57] and the fact that the redox midpoint potential of these cytochromes is markedly lower than for their neutrophilic counterparts, as is the case with the cytochrome-b from *Bacillus firmus* RAB [58]. The respiratory chain of *B. pseudofirmus* OF4 is similar to that in other *Bacillus* species, containing two NADH dehydrogenases, a succinate dehydrogenase, as well as cytochrome *bd* and cytochrome caa_3. Other than the elevated cytochrome expression, the other components of the respiratory chain maintain constant expression levels across different pHs [41].

The archaeon *Natrialba magadii* contains a full arsenal of proteins that allow for efficient respiration and oxidative phosphorylation. These include the operon atpHIKECFAB, encoding a proton-coupled ATP synthase, genes encoding for type II and mitochondrial NADH dehydrogenases, as well as genes for the cytochrome c-type terminal oxidase subunits I and II and for the cytochrome ubiquinol oxidase I on II. Conversely, the *N. magadii* genome does not encode a cytochrome bc1, which is predicted to couple reduced quinone to the electron carrier halocyanin in halophilic archaea. The presence of several two-component signal transduction systems as well as three rhodopsin homologue genes and two loci encoding for chemoreceptors suggests that *N. magadii* is capable of sensing and responding to a variety of light and chemical signals [18].

6 The Link Between Respiration and ATP Synthases

Despite the low bulk PMF generated in high pH environments, alkaliphilic *Bacillus* species exclusively use proton-coupled F_1F_0-ATP synthases, contrary to some anaerobic and fermentative bacteria which use Na^+-coupled ATP synthases. Evidence showing that respiration supports maximal ATP synthesis at high pH, while artificially imposed diffusion gradients fail to energize the ATP synthase, suggests that the cytochrome complexes involved in the generation of bulk electrochemical gradient during respiration play a crucial role in the oxidative phosphorylation in alkaliphiles [39]. Models suggest that physical interactions between the terminal oxidases of the respiration chain, such as cytochrome-caa_3, and the ATP synthase would allow for protons to be retained in the membrane instead of dissipating into the extracellular environment [59]. The operon coding for the ATP synthase in *B. pseudofirmus* OF4, the *atp* operon, contains an extra gene upstream of the conventional genes coding for the ATP synthase complex and the putative chaperone *atp*I. This gene, *atp*Z, has several homologues in other *Firmicutes* and, together with *atp*I, is hypothesized to form a divalent cation channel for the import of Mg^{2+}. Magnesium is required for the formation of the transition state between ADP and ATP in the F_1 catalytic moiety of the ATP synthase and might contribute to charge compensation during pH cytoplasmic homeostasis in alkaliphiles [60, 61]. In addition, structural studies have shown that proton-coupled synthases from alkaliphiles contain specific amino acid compositions that allow ATP synthesis at high pH values. For example, a lysine residue at position 180 of the alpha subunit of the ATP synthase from *Bacillus* sp. TA2.A1 was shown to facilitate proton capture at high pH [62]. Multiple alignment studies between the alpha subunits of alkaliphiles and neutrophiles identified additional motifs that are distinct in alkaliphiles, in particular two conserved methionines (M171 and M184) in the membrane portion close to the proton pathway that affect growth on malate when replaced by the neutrophilic counterparts [63]. The ATP synthase of *B. pseudofirmus* OF4 contains a consensus alanine-rich motif AxAxAxA in the N-terminal helix of the c-subunit that is conserved across alkaliphilic *Bacilli* and is suggested to play an important role in the correct assembly of the c-subunit rotor, as indicated by the inability of mutants with a glycine GxGxGxG substitution to produce ATP [64]. Another example of how the respiration chain is linked with ATP production in alkaliphiles comes from the cyanobacterium *A. platensis*, in which the F_0F_1-ATP synthase is co-localized with the photosynthetic complex in thylakoid vesicles in the cytoplasm [65, 66]. Since the cytoplasmic pH of this cyanobacterium is actively maintained at neutral pH through the activity of Na^+/H^+ antiporters, the PMF across the thylakoid membranes is much more akin to that found in neutral environments and thus favourable for oxidative phosphorylation [67, 68].

Due to the "reverse" transmembrane pH characteristic of alkaline habitats, it was expected that alkaliphiles would express sodium-coupled ATP synthases to take advantage of the greater SMF generated during pH homeostasis. However, only alkaliphilic anaerobes such as *Clostridium paradoxum* have been found to contain a

Na^+-coupled ATP synthase. This enzyme exhibits low ATP synthesis activity and is thought to rather play a role in the establishment of an SMF for sodium-coupled solute uptake and motility [69]. A reason for the preferential use of proton-coupled ATP synthases in most alkaliphiles might relate to the overwhelming pressure to maintain pH homeostasis, as the proton uptake during ATP synthesis contributes to the acidification of the cytoplasmic environment [67]. This suggestion is also re-enforced by the fact that most alkaliphile ATP synthases exhibit very low or absent hydrolytic activity. For instance, the ATP synthase from the thermoalkaliphilic *Bacillus* sp. TA2.A1 contains two salt bridges in the beta subunit that prevent the protein from rotating in the ATP hydrolytic direction, thus preventing the outwards flux of protons to the bulk medium [70].

6.1 NADH and Succinate Dehydrogenases

Studies of *B. pseudofirmus* have demonstrated that it contains two distinct types of NADH dehydrogenases, NDH-2A and NDH-2B [41]. NDH-2A shows significant orthology with the type 2 NDH from *Bacillus subtilis*, YjlD (37% identity), while NDH-2B shows significant homology (56%) to a putative NADH dehydrogenase from *Halobacillus dabanensis* that has been shown to couple Na^+/H^+ antiport activity to the respiration chain [64]. Both dehydrogenases are localized in the cytoplasm and show very different spectra of activity, with NDH-2A being predominantly active against NADH, while NDH-2B also shows activity against NADPH and d-NADH with ferricyanide being the primary acceptor. It is hypothesized that NDH-2A plays the primary role in providing the entry point for electrons to the respiratory chains [64].

Bacillus YN-1 was found to contain a type 2 NDH homodimer which contains one FAD and shows homology to a thioredoxin reductase from *Escherichia coli* [71]. In turn, the NDH-2 from the thermoalkaliphilic bacterium *Caldalkalibacillus thermarum* is an FAD and NADH utilizing homodimer composed of subunits with both membrane-anchoring and catalytic domains, where binding sites for NADH and quinone do not overlap [72].

6.2 Cytochromes

In addition to the antiporters, *B. halodurans* also contains two bo3-type cytochrome c oxidase genes that are absent from the neutrophilic *B. subtilis* [13]. C-type cytochromes from alkaliphiles can be distinguished by being low midpoint potential electron carriers (+50 to +100 mV) compared to their neutrophilic counterparts (+180 to +250 mV) and by having low isoelectric points [41]. *B pseudofirmus* was shown to express four distinct heme-containing membrane peptides, all of which have counterparts in *B. subtilis* [41]. One of these is subunit II of the terminal oxidase

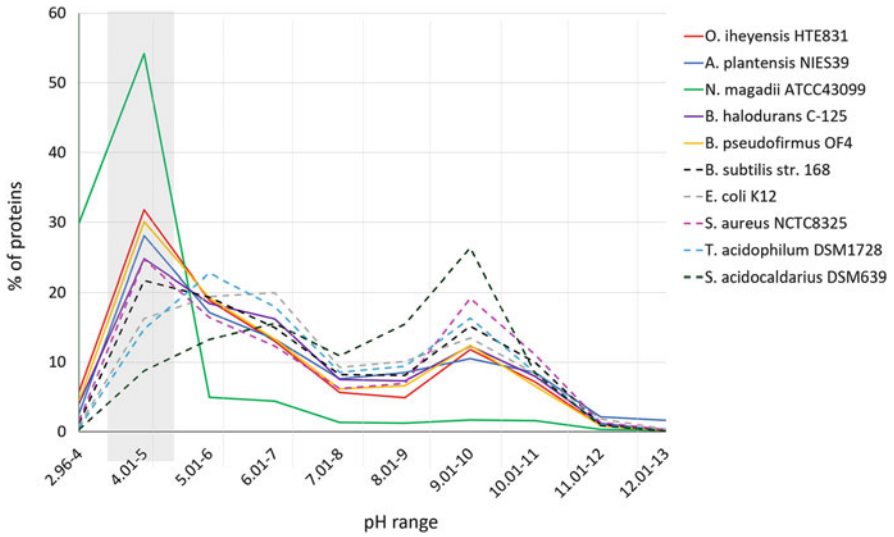


Fig. 3 Protein *pI* distribution across alkaliphilic, neutrophilic, and acidophilic genomes. Alkaliphile genomes are represented by the solid plot-lines, while neutrophile and acidophile genomes are represented by the dashed plot-lines. The alkaliphilic *pI* skew towards low pH values is illustrated by the gray area across the plot. The pI values for proteins of each genome were calculated using the Sequence Manipulation Suite server [76]

cytochrome caa_3, which in *B. firmus* RAB and *B. pseudofirmus* OF4 is composed of three subunits (I–III). In *B. pseudofirmus* OF4, the cytochrome-caa_3 complex was coded by the *cta* operon, with *cta*C-F coding for the four subunits [73], while *cta*A and *cta*B are involved in heme A synthesis [74, 75]. In *B. pseudofirmus* OF4, c-type cytochromes are found to be upregulated at high pHs [41]. Another terminal oxidase found in *B. pseudofirmus* is cytochrome-bd, which is only expressed during stationary phase at high pHs and therefore probably does not play a role in alkaliphily [41]. By comparison, *Bacillus* YN-2000 expresses cytochrome-aco as its terminal oxidase, in which heme C and the protoheme O are directly involved in the transfer of electrons from cytochrome-c553 to oxygen, while heme A might operate as an electron reservoir [2].

Another defining feature of the surface proteins of alkaliphiles is enrichment of neutral and acidic residues. For example, the exposed loops of the membrane-bound cytochrome-caa3 in *B. pseudofirmus* have a lower number of basic residues than its neutrophilic homologues [41]. Analysis of predicted protein isoelectric point (pI) distribution across the genomes of alkaliphiles also shows a skew towards lower pH values, indicating a preference for acidic amino acids (Fig. 3).

While Gram-positive alkaliphiles contain a single cytoplasmic membrane in which most cytochromes are embedded, Gram-negative alkaliphiles have a periplasmic space between the outer and inner membranes and are thus able to express soluble cytochromes. The facultative alkaliphile *Pseudomonas alcaliphila* AL 15-21^T expresses three soluble cytochrome c proteins, namely, cytochrome-c552,

c-554, and c-551, with cytochrome-c552 making up more than 60% of the total soluble cytochrome c content [77]. The midpoint potential of cytochrome-c552 in this bacterium is similar to that of neutrophilic cytochromes-c (+218 mV at pH 9 and 10), which suggests that the bioenergetic state of the periplasm of *Pseudomonas alcaliphila* is akin to of its more neutrophilic counterparts. Despite this, cytochrome-c552 was shown to play an important role for growth of this bacteria at high pH by working as a proton reservoir in the periplasmic space to compensate for the low proton concentrations in the extracellular medium [77].

7 Concluding Remarks

Alkaliphiles exist in one of the accepted "extreme environments," where selective pressures preclude most other organisms. In alkaline niches such as soda lakes and saline freshwater, a combination of abiotic pressures, including low CO_2 and metal ions concentrations, low proton gradient, and high salinity, impose a thermodynamic burden on essential biological functions such as carbon fixation, motility, and oxidative phosphorylation. Despite apparent thermodynamic limitations, alkaliphiles comprise a diverse group of microorganisms with divergent genetic origins, metabolic requirements, and functional capabilities. This genetic diversity, observed across eukaryotic, archaeal, and bacterial phyla, suggests that alkalinity is not a "narrow" driver of the evolution of organisms, in that alkaliphilicity is a derived property across a very wide taxonomic space.

Alkaliphiles share two main features: the ability to maintain pH homeostasis and the ability to perform bioenergetics processes in an environment with an inverse, or "reversed", chemical gradient. Accordingly, many alkaliphile genomes are enriched in genes for cation/proton antiporters and cation/substrate symporters. The enrichment of such transporters reflects the need to balance both PMF and SMF in order achieve pH homeostasis and fuel energetically expensive processes such as substrate acquisition and chemotaxis. In addition, alkaliphile genomes encode a large number of membrane-localized proton and electron-retaining proteins such as cytochrome oxidases, which allow for the maintenance of a PMF across the membrane that favours efficient proton-coupled ATP synthesis. Some level of metabolic specialization can also be observed as a possible response to secondary pressures exerted in an alkaline environment. For instance, alkaliphilic cyanobacteria express genes involved in CO_2-concentration mechanisms, which allow for efficient carbon fixation in environments where the levels of CO_2 are low. Other alkaliphilic bacteria resort to using other gases and inorganic compounds such as H_2 and sulphites as energy sources, although such trophic characteristics are not limited to high pH environments. In turn, heterotrophic aerobes and aerobes utilize on the products of the primary autotrophs. As is typical of all complex communities, microbial communities in alkaline niches live as multilayered tiers of microorganisms that are functionally and metabolically interdependent, where such interdependency is expressed as a high level of genetic diversity.

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References

1. Grant WD, Mwatha WE, Jones BE (1990) Alkaliphiles: ecology, diversity and applications. FEMS Microbiol Rev 75:255–270
2. Yumoto I, Fukumori Y, Yamanaka T (1991) Stopped-flow and rapid-scan studies of the redox behavior of cytochrome aco from facultative alkalophilic *Bacillus*. J Biol Chem 266 (2):14310–14316
3. Takami H, Takaki Y, Maeno G, Sasaki R, Masui N, Fumie F et al (2000) Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with *Bacillus subtilis*. Nucleic Acids Res 28(21):4317–4331
4. Koboldt DC, Steinberg KM, Larson DE, Wilson RK, Mardis ER (2013) The next-generation sequencing revolution and its impact on genomics. Cell 155(1):27–38
5. Nordberg H, Cantor M, Dusheyko S, Hua S, Poliakov A, Shabalov I et al (2014) The genome portal of the department of energy Joint Genome Institute: 2014 updates. Nucleic Acids Res 42: D26–D31
6. Janto B, Ahmed A, Ito M, Liu J, Hicks DB, Pagnl S et al (2011) Genome of alkaliphilic *Bacillus pseudofirmus* OF4 reveals adaptations that support the ability to grow in an external pH range from 7.5 to 11.4. Environ Microbiol 13(12):3289–3309
7. Wu S, Wang L, Gan R, Tong T, Bian H, Li Z et al (2018) Signature arsenic detoxification pathways in *Halomonas sp*. strain GFAJ-1. MBio 9(3):e00515–e00518
8. Zavarzin GA, Zhilina TN, Kevbrin VV (1999) The alkaliphilic microbial community and its functional diversity. Microbiology 68(5):503–521
9. Cheevadhanarak S, Paithoonrangsarid K, Prommeenate P, Kaewngam W, Musigkain A, Tragoonrung S et al (2012) Draft genome sequence of *Arthrospira platensis* C1 (PCC9438). Stand Genomic Sci 6:43–53
10. Paul S, Bag SK, Das S, Harvill ET, Dutta C (2008) Molecular signature of hypersaline adaptation: insights from genome and proteome composition of halophilic prokaryotes. Genome Biol 9:R70
11. Klanchui A, Cheevadhanarak S, Prommeenate P, Meechai A (2017) Exploring components of the CO_2-concentrating mechanism in alkaliphilic cyanobacteria through genome-based analysis. Comput Struct Biotechnol J 15:340–350
12. Amaral-Zettler LA (2012) Eukaryotic diversity at pH extremes. Front Microbiol 3:441
13. Takami H (2011) Genomic and evolution of alkaliphilic Bacillus species. In: Horikoshi H (ed) Extremophiles handbook. Springer, Tokyo, pp 183–211
14. Huerta-Cepas J, Forslund K, Coelho LP, Szklarczyk D, Jensen LJ, von Mering C et al (2017) Fast genome-wide functional annotation through orthology assignment by eggNOG-mapper. Mol Biol Evol 34(8):2115–2122
15. Takami H, Takaki Y, Uchiyama I (2002) Genome sequence of *Oceanobacillus iheyensis* isolated from the Iheya ridge and its unexpected adaptive capabilities to extreme environments. Nucleic Acids Res 30(18):3927–3935
16. Schadewaldt P, Hummel W, Wendel U, Adelmeyer F (1995) Enzymatic method for determination of branched-chain amino acid aminotransferase activity. Anal Biochem 230(2):199–204
17. Cheng B, Meng Y, Cui Y, Li C, Tao F, Yin H et al (2016) Alkaline response of a halotolerant alkaliphilic *halomonas* strain and functional diversity of its Na+(K+)/H+ antiporters. J Biol Chem 291(50):26056–26065
18. Siddaramappa S, Challacombe JF, DeCastro RE, Pfeiffer F, Sastre DE, Gimenez MI et al (2012) A comparative genomics perspective on the genetic content of the alkaliphilic haloarchaeon *Natrialba magadii* ATCC 43099^T. BMC Genomics 13:165

19. Saunders E, Tindall BJ, Fahnrich R, Lapidus A, Copeland A, del Rio TG et al (2010) Complete genome sequence of *Haloterrigena turkmenica* type strain ($4k^T$). Stand Genomic Sci 2(1):107–116
20. Fujisawa T, Narikawa R, Okamoto S, Ehira S, Yoshimura H, Suzuki I et al (2010) Genomic structure of an economically important Cyanobacterium, *Arthrospira* (*Spirulina*) *platensis* NIES-39. DNA Res 17:85–103
21. Grum-Gzhimaylo AA, Georgieva ML, Bondarenko SA, Debets JM, Bilanenko EN (2016) On the diversity of fungi from soda soils. Fungal Divers 76(1):27–74
22. Keresztes ZG, Felfoldi T, Somogyi B, Szekely G, Dragos N, Marialigeti K et al (2012) First record of picophytoplankton diversity in Central European hypersaline lakes. Extremophiles 16(5):759–769
23. Lanzen A, Simachew A, Gessesse A, Chmolowska D, Jonassen I, Ovreas L (2013) Surprising prokaryotic and eukaryotic diversity, community structure and biogeography of Ethiopian Soda Lakes. PLoS One 8(8):e72577
24. Grum-Grzhimaylo AA, Falkoski DL, van den Heuvel J, Valero-Jimenez CA, Min B, Choi IG et al (2018) The obligate alkalophilc soda-lake fungus Sodiomyces alkalinus has shifted to a protein diet. Mol Ecol 27:4808–4819
25. Pereira EO, Tsang A, McAllister TA, Menassa R (2013) The production and characterization of a new active lipase from Acremonium alcalophilum using a plant bioreactor. Biotechnol Biofuels 6:111
26. Reeve W, Chain P, O'Hara G, Ardley J, Nandesena K, Brau L et al (2010) Complete genome sequence of the Medicago microsymbiont *Ensifer* (*Sinorhizobium*) *medicae* strain WSM419. Stand Genomic Sci 2(1):77–86
27. Zhao B, Mesbah NM, Dalin E, Goodwin L, Nolan M, Pitluck S et al (2011) Complete genome sequence of the anaerobic, halophilic alkalithermophile Natranaerobius thermophilus JW/NM-WN-LF. J Bacteriol 193(15):4023–4024
28. Sorokin DY, Berben T, Melton ED, Overmars L, Vavourakis CD, Muyzer G (2014) Microbial diversity and biogeochemical cycling in soda lakes. Extremophiles 18(5):791–809
29. Cogne G, Gros JB, Dussap CG (2003) Identification of a metabolic network structure representative of Arthrospira (spirulina) platensis metabolism. Biotechnol Bioeng 84(6):667–676
30. Price GD, Badger MR, Woodger FJ, Long BM (2008) Advances in understanding the cyanobacterial CO2-concentrating-mechanism (CCM): functional components, Ci transporters, diversity, genetic regulation and prospects for engineering into plants. J Exp Bot 59(7):1441–1461
31. Zhilina TN, Kevbrin VV, Tourova TP, Lysenko AM, Kostrikina NA, Zavarzin GA (2005) *Clostridium alkalicellum* sp. nov., an obligately alkaliphilic cellulolytic bacterium from a soda lake in the Baikal region. Microbiology 74(5):555–566
32. Garnova ES, Krasil'nikova EN (2003) Carbohydrate metabolism of the Saccharolytic alkaliphilic anaerobes *Halonatronum saccarophilum*, *Amphibacillus fermentum*, and *Amphibacillus tropicus*. Mikrobiologiia 72(5):627–632
33. Song Y, Xue Y, Ma Y (2013) Global microarray analysis of carbohydrate use in alkaliphilic hemicellulolytic bacterium *Bacillus sp.* N16-5. PLoS One 8(1):e54090
34. Detkova EN, Pusheva MA (2006) Energy metabolism in halophilic and alkaliphilic acetogenic bacteria. Microbiology 75(1):1–11
35. Frank YA, Kadnikov VV, Likina AP, Banks D, Beletsky AV, Sen'kina EI et al (2016) Characterization and genome analysis of the first facultatively alkaliphilic *Thermodesulfovibrio* isolated from the deep terrestrial subsurface. Front Microbiol 7:2000
36. Oremland RS, Hoeft SE, Santini JM, Bano N, Hollibaugh RA, Hollibaugh JT (2002) Anaerobic oxidation of arsenite in Mono Lake water and by a facultative, arsenite-oxidizing chemoautotroph, strain MLHE-1. Appl Environ Microbiol 68:4795–4802
37. Hoeft SE, Blum JS, Stolz JF, Tabita FR, Witte B, King GM et al (2007) Alkalilimnicola ehrlichii sp. nov., a novel, arsenite-oxidizing haloalkaliphilic gammaproteobacterium capable of chemoautotrophic or heterotrophic growth with nitrate or oxygen as the electron acceptor. Int J Syst Evol Microbiol 57(Pt 3):504–512

38. Susuki S, Kuenen JG, Schipper K, van der Velde S, Ishii S, Wu A et al (2014) Physiological and genomic features of highly alkaliphilic hydrogen-utilizing Betaproteobacteria from a continental serpentinizing site. Nat Commun 5:3900
39. Krulwich TA, Hicks DB, Swartz TH, Ito M (2007) Bioenergetic adaptations that support alkaliphily. In: Gerday C, Glansdorff N (eds) Physiology and biochemistry of extremophiles. ASM Press, Washington, pp 311–329
40. Skulachev VP (1995) Membrane-linked energy transductions. Bioenergetic functions of sodium H^+ is not unique as a coupling ion. FEBS J 151(2):199–208
41. Hicks DB, Krulwich TA (1995) The respiratory chain of alkaliphilic bacteria. Biochim Biophys Acta 1229:303–314
42. Mulkidjanian AY, Galperin MY, Koonin V (2009) Co-evolution of primordial membranes and membrane proteins. Trends Biochem Sci 34(4):206–215
43. Ventosa A, Nieto JJ, Oren A (1998) Biology of moderately halophilic aerobic bacteria. Microbiol Mol Biol Rev 62(2):504–544
44. Ito M, Guffanti AA, Krulwich TA (2001) Mrp-dependent Na^+/H^+ antiporters of *Bacillus* exhibit characteristics that are unanticipated for completely secondary active transporters. FEBS Lett 496:117–120
45. Krulwich TA, Ito M, Guffanti AA (2001) The Na(+)-dependence of alkaliphily in Bacillus. Biochim Biophys Acta 1505(1):158–168
46. Ito M, Xu H, Guffani AA, Wei Y, Zvi L, Clapham DE et al (2004) The voltage-gates Na^+ channel NavBP has a role in motility, chemotaxis, and pH homeostasis of an alkaliphilic *Bacillus*. Proc Natl Acad Sci U S A 101:10566–10571
47. Horikoshi K (1999) Alkaliphiles: some applications of their products for biotechnology. Microbiol Mol Biol Rev 63(4):735–750
48. Liu J, Xue Y, Wang Q, Wei Y, Swartz TH, Hicks DB et al (2005) The activity profile of the NhaD-type $Na^+(Li^+)/H^+$ antiporter from the soda Lake Haloalkaliphile *Alkalimonas amylolytica* is adaptive for the extreme environment. J Bacteriol 187(22):7589–7595
49. Janausch I, Zientz GE, Tran Q, Kroger HA, Unden G (2002) C4-dicarboxylate carriers and sensors in bacteria. Biochim Biophys Acta 1553:39–56
50. Wutipraditkul N, Waditee R, Incharoensakdi A, Hibino T, Tanaka Y, Nakamura T et al (2005) Halotolerant cyanobacterium *Aphanothece halophytica* contains NapA-Type Na^+/H^+ antiporters with novel ion specificity that are involved in salt tolerance at alkaline pH. Appl Environ Microbiol 71(8):4176–4184
51. Krulwich TA (1985) Alkaliphiles: "basic" molecular problems of the pH tolerance and bioenergetics. Mol Microbiol 15(3):403–410
52. Hirota N, Kitada M, Imae Y (1981) Flagellar motors of alkalophilic *Bacillus* are powered by an electrochemical potential gradient of Na^+. FEBS Lett 132(2):278–280
53. Terahara N, Krulwich TA, Ito M (2008) Mutations alter the sodium versus proton use of a *Bacillus clausii* flagellar motor and confer dual ion use on *Bacillus subtilis* motors. Proc Natl Acad Sci U S A 105(38):14359–14364
54. Fujinami S, Terahara N, Krulwich TA, Ito M (2009) Motility and chemotaxis in alkaliphilic *Bacillus* species. Future Microbiol 4(9):1137–1149
55. Ren D, Navarro B, Xu H, Yue L, Shi Q, Clapham DE (2001) A prokaryotic voltage-gated sodium channel. Science 294(5550):2372–2375
56. Olsson K, Keis S, Morgan HW, Dimroth P, Cook GM (2003) Bioenergetic properties of the Thermoalkaliphilic *Bacillus* sp. strain TA2.A1. J Bacteriol 185(2):461–465
57. Guffanti AA, Finkelthal O, Hicks DB, Falk L, Sidhu A, Garro A (1986) Isolation and characterization of new facultatively alkalophilic strains of *Bacillus* species. J Bacteriol 167(3):766–773
58. Kitada M, Lewis RJ, Krulwich TA (1983) Respiratory Chain of the alkalophilic bacterium *Bacillus firmus* RAB and its non-alkalophilic mutant derivative. J Bacteriol 154(1):330–335
59. Krulwich TA, Ito M, Hicks DB, Gilmour R, Guffanti AA (1998) pH homeostasis and ATP synthesis: studies of two processes that necessitate inward proton translocation in extremely alkaliphilic *Bacillus* species. Extremophiles 2:217–222

60. Hicks AB, Wang Z, Wei Y, Kent R, Guffani AA, Banciu H et al (2003) A tenth *atp* gene in the conserved atpI gene of a Bacillus atp operon have a role in Mg^{2+} uptake. PNAS 100(18):10213–10218
61. Ko YH, Hong S, Pedersen PL (1999) Chemical mechanism of ATP synthase. Magnesium plays a pivotal role in formation of the transition state where ATP is synthesized from ADP and inorganic phosphate. J Biol Chem 274(41):28853–28856
62. McMillan DGG, Keis S, Dimroth P, Cook GM (2007) A specific adaptation in the alpha subunit of thermoalkaliphilic F1F0-ATP synthase enables ATP synthesis at high pH but not at neutral pH values. J Biol Chem 282:17395–17404
63. Preiss L, Hicks DB, Suzuki S, Meier T, Krulwich TA (2015) Alkaliphilic bacteria with impact on industrial applications, concepts of early life forms, and bioenergetics of ATP synthesis. Front Bioeng Biotechnol 3:75
64. Liu J, Fujisawa M, Hicks DB, Krulwich TA (2009) Characterization of the functionally critical AXAXAXA and PXXEXXP motifs of the ATP synthase c-subunit from an alkaliphilic *Bacillus*. J Biol Chem 284:8714–8725
65. Liberton M, Howard Berg R, Heuser J, Roth R, Pakrasi HB (2006) Ultrastructure of the membrane systems in the unicellular cyanobacterium *Synechocystic sp*. strain PCC6803. Protoplasma 227(2–4):129–138
66. Pogoryelov D, Yu J, Meier T, Vonck J, Dimroth P, Muller DJ (2005) The c15 ring of the Spirulina platensis F-ATP synthase: F1/F0 symmetry mismatch is not obligatory. EMBO Rep 6 (11):1040–1044
67. Hicks DB, Liu J, Fujisawa M, Krulwich TA (2010) F1F0-ATP synthases of alkaliphilic bacteria: lessons from their adaptations. Biochim Biophys Acta 1797:1362–1377
68. Pogoryelov D, Sudhir PR, Kovacs L, Gombos l, Brown I, Garab G (2003) Sodium dependency of the photosynthetic electron transport in the alkaliphilic cyanobacterium *Arthrospira platensis*. J Bioenerg Biomembr 35(5):427–437
69. Ferguson SA, Keis S, Cook GM (2006) Biochemical and molecular characterization of a Na^+-translocating F1F0-ATPase from the thermoalkaliphilic bacterium Clostridium paradoxum. J Bacteriol 188(14):5045–5054
70. Stocker A, Keis S, Vonck J, Cook GM, Dimroth P (2007) The structural basis for unidirectional rotation of thermoalkaliphilic F1-ATPase. Structure 15(8):904–914
71. Xu X, koyama N, Cui M, Yamagishi A, Nosoh Y, Oshima T (1991) Nucleotide sequence of the gene encoding NADH dehydrogenase from an alkalophile, *Bacillus sp*. strain YN-1. J Biochem 109:678–683
72. Heikal A, Nakatani Y, Dunn E, Weimar MR, Day CL, Baker EN, Lott JS, Sazanov LA, Cook GM (2014) Structure of the bacterial type II NADH dehydrogenase: a monotopic membrane protein with an essential role in energy generation. Mol Microbiol 91(5):950–964
73. Quirk PG, Hicks DB, Krulwich TA (1993) Cloning of the cta operon from allkaliphilic *Bacillus firmus* OF4 and characterization OF the pH-regulated cytochrome caa_3 oxidase it encodes. J Biol Chem 268(1):678–685
74. Brown KR, Allan BM, Do P, Hegg EL (2002) Identification of novel hemes generated by heme A synthase: evidence for two successive monooxygenase reactions. Biochemist 41(36):10906–10913
75. Throne-Holst M, Hederstedt L (2000) The *Bacillus subtilis* ctaB paralogue, yjdK, can complement the heme A synthesis deficiency of a CtaB-deficient mutant. FEMS Microbiol Lett 183(2):247–251
76. Stothard P (2000) The sequence manipulation suite: JavaScript programs for analysing and formatting protein and DNA sequences. BioTechniques 28:1102–1104
77. Matsuno T, Yumoto I (2015) Bioenergetics and the role of soluble cytochromes c for alkaline adaptation in gram-negative alkaliphilic *Pseudomonas*. Biomed Res Int 2015:847945

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Metabolites Produced by Alkaliphiles with Potential Biotechnological Applications

Elvira Khalikova, Susanne Somersalo, and Timo Korpela

Contents

Abstract Alkaliphiles are a diverse group of relatively less known microorganisms living in alkaline environments. To thrive in alkaline environments, alkaliphiles require special adaptations. This adaptation may have evolved metabolites which can be useful for biotechnological processes or other applications. In fact, certain metabolites are found unique to alkaliphiles or are effectively produced by alkaliphiles. This probably aroused the interest in metabolites of alkaliphiles. During recent years, many alkaliphilic microbes have been isolated, especially in countries having alkaline environments, like soda lakes. Even if the number of such isolated

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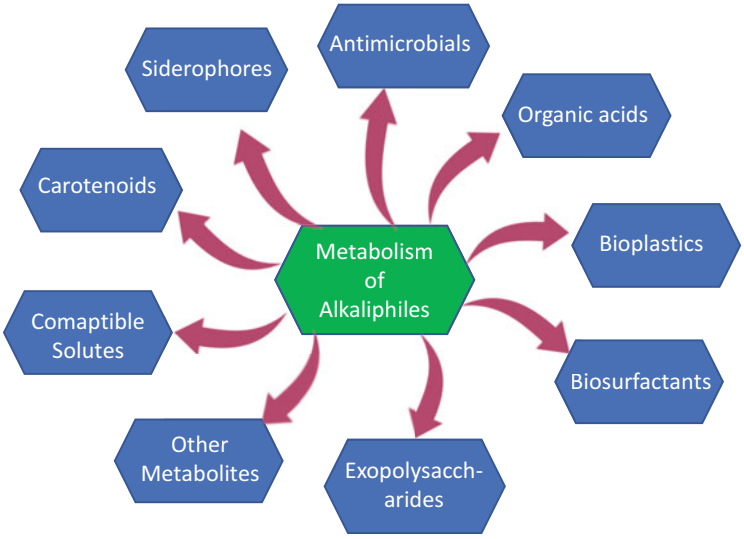
alkaliphiles is large, their metabolites have not yet been extensively analyzed and exploited. This is expected to come in the years ahead. So far, the focus of interests in metabolites from alkaliphiles falls into categories such as organic acids, ingredients for foodstuffs and cosmetics, antibiotics, and substances which modify properties of other materials used in industry. This chapter deals with biotechnologically important metabolites of alkaliphiles including compatible solutes, biosurfactants, siderophores, carotenoids, exopolysaccharides, and antimicrobial agents. It also covers the promising potential of alkaliphiles as sources of bioplastic raw materials. Moreover, an overview of the patent literature related to alkaliphiles is highlighted.

Graphical Abstract



Keywords Alkaliphilic microbes, Antimicrobial compounds, Bioplastics, Carotenoids, Compatible solutes, Exopolysaccharides, Metabolites, Patents, Siderophores, Sugar metabolism in *Bacillus*

Abbreviations

CD	Cyclodextrin
CGTase	Cyclomaltodextrin glucanotransferase
CMC	Critical micelle concentration

CSs	Compatible solutes
EMP	Embden–Meyerhof–Parnas
EPS	Exopolysaccharides
HMP	Hexose monophosphate
MRSA	Methicillin-resistant *Staphylococcus aureus*
NAD(P)	Nicotinamide adenine dinucleotide (phosphate)
NMR	Nuclear magnetic resonance
OFAT	One-factor-at-a-time technique
PHA	Polyhydroxyalkanoate
PHB	Poly-beta-hydroxybutyrate
pH_i	Intracellular pH
pH_o	Outside (extracellular) pH
TCA	Tricarboxylic acid
WIPO	World Intellectual Property Organization

1 Introduction

The group of alkaliphilic microbes growing well above pH 9 is practically as diverse as microbes living in neutral habitats: there are facultative and obligate, aerobic and anaerobic, methanogens, cyanobacteria, and sulfur-oxidizing alkaliphiles. However, the diversity of alkaliphiles on earth may account for only a few percentages of the total microbes. Some alkaliphiles are adapted to other environmental extremes (dual or poly extremophiles) like groups referred to as halo-, thermo-, psychrophilic, and alkaliphilic photosynthetic microbes as reviewed by Horikoshi [1]. To keep the present overview focused to biotechnological aspects, alkaliphiles are dealt here with seemingly as one group, despite their enormous diversity.

The intracellular pH (pH_i) of alkaliphiles can be 1–3 units lower than that of the outside (extracellular) pH (pH_o). Therefore, the chemical milieu for biomolecules inside and outside of the microbial cell can be very different. The pH optimums of many internal enzymes of alkaliphiles are, however, higher than those from neutralophiles, showing that also internal metabolism has adapted to functioning at higher pH, up to 9.5 [1] which means that the inside proton concentration can be >100 times less than that of the neutral (and hydroxide ions correspondently higher) condition. Most enzyme reactions involve protons in one or another form. Thus, enzymes of internal metabolism must have adapted to function in a low proton concentration. Without such adaptation, they should have had to accept drastically lower specific activity, which should be seen in retardation of the growth and other metabolic activities; the fact that does not seem to be valid. The cells of alkaliphiles shall communicate chemically with their environment. This means that nutrients must be transferred from higher to lower pH and products to opposite direction. In the growth medium, there lie potential problems, as well. For example, amino groups are fully deprotonated at pH, say, 10–12 and exist as very reactive nucleophiles,

which can react with carbonyl groups. Sulfur compounds are sensitive to oxidation and to other reactions at high pH. Sugar rings can open, decompose, and react with amino compounds. Nucleic acids are unstable and hydrolyze in alkali. Racemization of many compounds occurs in alkali, especially in the presence of metal ions. The very basic thing: the stability of nutrients in alkaline environment has been widely neglected. The same stability problem exists for products that are secreted by alkaliphile.

At least few people working in microbiology laboratory have inadvertently autoclaved medium adjusted to the final pH of the growth medium (around pH 10–11) and found that the alkaliphiles do not grow after inoculation. This shows that fatal chemical reactions have occurred in the nutrient medium by the combination of alkali and heat. This may be partly due to difficulty in transporting chemically altered nutrients such as amino acids, sugars, and other nutrients (e.g., vitamins) from the medium to cells through specific cellular channel ports. Since growth at high pH reduces contamination, it has been considered as an advantage which avoids sterilization. However, it works only rarely. There are still enough microbial species that can grow alkaline niche that challenges this biotechnological advantage.

The adaptation of alkaliphilic microbes to live in alkaline conditions is extensively reviewed in this book and elsewhere. The secreted enzymes of alkaliphiles have found a significant role in biotechnology, and there are a large number of patented discoveries/inventions related to alkaliphilic proteins, genes coding them, as well as to various methods using those enzymes. Small-molecule primary and secondary metabolites are also of interests because the alkaline adaptation may have demanded to develop special metabolic pathways and adaptations to operate in alkaline condition including protection of the cells. A competition between alkaliphilic microbial species exists which necessitates production of a set of antibiotics against competitors. Alkaliphiles may even produce substances which are anti-bacteriophages and other viruses. Alkaliphiles are potential sources of new metabolites which may not be found in microbes growing in acidic and neutral environments. Certainly, this potential has not been fully explored yet. Some usual metabolites are advantageously produced by alkaline fermentation. Environmental technologies, e.g., bioremediation, can exploit the capability of alkaliphiles to metabolize harmful substances in alkaline conditions.

The present discussion aims at compounds and processes related to metabolites from alkaliphiles which are, or may become, of biotechnological interest. It is attempted to answer what metabolites are unique to alkaliphiles. General aspects of the central metabolism of alkaliphiles are examined first. Specific fields which may have future applications in industrial scale are dealt with. The field of the potential applications is wide; therefore, it is focused to those which are putatively the most potential. It is recommended to see further details from other chapters of this book and from cited reviews.

2 Basic Sugar Metabolism

Whereas alkaliphilic microbes are studied rather extensively for their extracellular enzymes and bioenergetics, their physiology and intracellular enzymes have been neglected. Features of the intermediary metabolism are valuable to know, since they aid in characterizing the microbe, the enzyme composition, the metabolic stage of the cell, and the possibilities for metabolic engineering. A question remains also whether there are any fundamental differences in the basic metabolism of alkaliphiles as compared to neutralophiles. In spite of protection of the cell from the extracellular pH, the intracellular pH is still 1–2.5 units higher than that of the neutral-growing microbes, and hence, there could differences in the basic metabolism between these organisms.

One research theme in the studies of alkaliphiles is to understand what kind of metabolic pathways alkaliphiles are exploiting. Is there any adaptation of those pathways compared to neutralophiles? Rather, few studies have been conducted to study the intermediary metabolism of alkaliphiles.

The majority of studied alkaliphiles belong to genus *Bacillus*, which consists of aerobes and facultative anaerobes. Utilization of sugars by *Bacillus circulans* var. *alkalophilus* was studied in comparable growth conditions [2]. Based on measurement of several enzyme activities, the alkaliphile employed EMP (glycolysis) and HMP (hexose monophosphate) pathways for glucose catabolism. The glucose oxidase activity was remarkably high. The net reaction of the EMP pathway is:

$$\text{Glucose} + 2\text{ADP} + 2\text{P}_i + 2\text{NAD}^+ \rightarrow 2\,\text{pyruvate} + 2\,\text{ATP} + 2\,\text{NADH} + 2\text{H}^+ + 2\text{H}_2\text{O}.$$

The first reaction of the HMP path is the phosphorylation of glucose. The net reaction of HMP is:

$$3\ \text{glucose 6-P} + 6\ \text{NADP}^+ + 5\ \text{NAD}^+ + 5\text{P}_i + 8\ \text{ADP} \rightarrow 5\ \text{pyruvate} + 3\ \text{CO}_2 + 6\ \text{NADPH} + 5\ \text{NADH}^+ + 8\text{ATP} + 2\text{H}_2\text{O} + 8\ \text{H}^+$$

The HMP reaction works reversibly and enables to change five hexose monophosphates to six pentose phosphates even if its energy efficiency is less [3]. Both reactions produce protons. A large number of neutral *Bacillus* strains use EMP pathways 60–100% and HMP pathway 0–40%. *B. circulans* var. *alkalophilus* used 90% EMP and 10% HMP pathways [2]. No principal differences seem to exist between alkaliphilic and neutralophilic *Bacillus* in the intermediary sugar catabolism by evaluating by this limited data.

With *B. circulans* var. *alkalophilus*, the appearance of (1-^{14}C) glucose in acetic acid proved the prevalence of the EMP pathway because through other pathways, the 1-^{14}C flows to tricarboxylic acid (TCA) cycle and/or it forms CO_2. Only about 2% of total CO_2 was formed from (6-^{14}C) glucose. This shows that the TCA cycle exists, but it was not actively employed for the sugar catabolism. This was consistent with the finding that this bacterium can grow only very slowly in acetate [4].

Production of acids from a large number of sugars, sugar polymers, and sugar alcohols (1% w/v each) in otherwise identical media composition was studied with four *Bacillus* strains – *B. circulans* var. *alkalophilus* (facultative alkaliphile), *B. alkalophilus* spp. *halodurans*, and *Bacillus* sp.17-1 – all of which were able to grow also in neutral pH. The fourth *B. alcalophilus* was an obligate alkaliphile [5]. The acids were studied with different chromatographic techniques together with acid-base titrations to assess total acid and basic compounds. In general, all the alkaliphiles produced acetic acid (4.5–5 g/L at maximum). Among the tested bacteria, formic acid was produced only by *B. circulans* var. *alkalophilus* (up to 2 g/L). Acetoin, butanediol, or ethanol were not detected, which is a difference to neutrophilic *Bacillus* species. The studied alkaliphiles differed from each other in amounts of production of less common branched-chain organic acids. They were probably generated through amino acid metabolism. Since the amino acid source was the same, the acid composition reflects differences in the amino acid metabolism. The high pH and high buffering capacity of the medium favored the formation of acids and oxoacids. At low pH, hydroxy acids seem to be advantageous. At high pH, the protons formed via NAD(P) from substrates can be easily secreted to medium, while at low pH, it is easier to transfer protons to form hydroxy acids (like lactate) to avoid the over-acidification [5]. The keto acids are useful compounds for biomaterials since they contain the reactive carbonyl groups.

Alkaliphiles have exceptionally effective respiratory chain. Non-alkaliphilic *Bacillus* mutants have less cytochromes and cytochrome species than the corresponding alkaliphilic cells [6, 7]. NADH oxidase activity (nmol/min/mL) was 5–20 times higher than the activities of the key enzymes in the glycolysis pathway [2]. An interesting feature of alkaliphiles is their ability to fluctuate the medium pH when grown in carbohydrate-containing media [8]. In standard experiments, the starting pH of growth was 10.3. During the logarithmic growth, the pH dropped sharply to 8 and then slowly increased back to 9.5 even with no growth (even a decrease in turbidity measured at A_{600nm}; Fig. 1). This phenomenon has been often neglected even if it is likely to be typical to alkaliphiles. It is tempting to explain that the pH drop during the growth in sugar media originates from secretion of organic acids into medium, while the pH increase could be uptaking the acids (i.e., change the catabolism from sugars to acids). The first evidence against that explanation was that TCA cycle was functioning very slowly with the studied alkaliphilic *Bacillus* sp. Moreover, the microbe grew very poorly in 1% acetate, glycerol, or citrate. Because of an obvious conflict against previous conception for the origin of the pH changes, the growth was studied with accurate sampling and measurement of acids. To get accurate timing, an automatic sampling device was developed [8, 9]. During the middle of the pH drop, there occurred a sharp peak in reducing sugars with starch substrate. Maximum of secreted acetic acid appeared into the medium 3–5 h later than the pH minimum (Fig. 1). It is possible to speculate that the microbe produced also other than acetic and formic acids. Back-titration curve of the cultivation medium to original starting pH, however, showed similar timing profile as the pH drop curve. It was possible to trace all carbon in acetic acid, formic acid, and CO_2 and protons, i.e., to make credible total inventory of the sugar metabolism. The slow

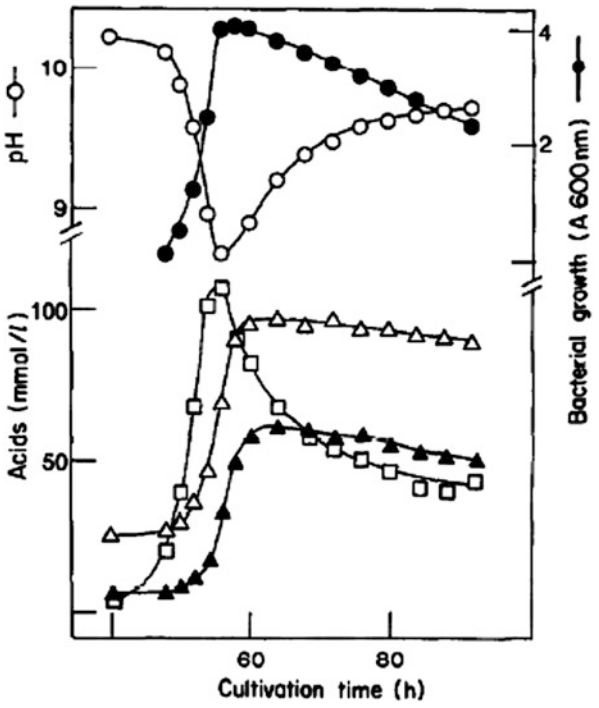


Fig. 1 Changes of metabolites and pH in the cultivation medium of *Bacillus circulans* var. *alkalophilus* grown in a basal growth medium containing 1% (w/v) starch as the carbon source. The acid metabolites were followed with (1) consumption of the titrants in the pH region 3.5–5.5 (a measure of carboxylic acids, shown as open triangles), (2) back-titration to the initial pH with NaOH (open squares), and (3) measurement of acetic acid by gas chromatography (filled triangles). The growth curve (filled circles) and pH of the medium (open circles) are shown. Reproduced from the Ph.D. thesis of Paavilainen [2]

increase of the pH to about 9.5 was not due to metabolizing of acids or production of bases [10].

The energetics of alkaliphiles is considered in more detail elsewhere in this book. However, it is evident that full picture is not yet clear. Major focus on protons involved in the energy metabolism is focused to near-membrane environment of the reactions ([11]; see Figs. 2 and 3 and text and references). The metabolic feature of re-increasing the pH is certainly unique, at least for a representative group of alkaliphilic *Bacillus* species. Related results were obtained with other sugars than starch and with four randomly selected alkaliphiles [5]. Descriptively, in normal conditions, alkaliphiles constitutively pump protons into the cells. When there appears a metabolic boost, the generated electrons flow through the cytochrome system. The proton pumps are regulated to cease their function from outside to inside and allow leaking protons out to avoid over-acidification. When the metabolic boost is over, the proton pumps continue their function, and/or protons are oxidized in cytochrome oxidase system to produce water [11, 12].

The ability of alkaliphiles to produce acids from sugars was ingeniously exploited in lowering sugar-containing alkaline wastewaters from pH 12.0 to 7.5 by a facultative alkaliphile *Exiguobacterium* sp. as fast as in 2 h [13, 14]. This bacterium was able to grow also in nonsugar substrates like glycerol. The wastewater studied was generated in soft drink beverage industry mainly from washing liquids containing basic components like caustic soda and NaOH. It is evident that related processes

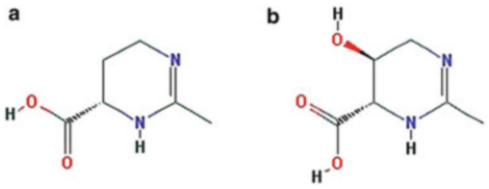


Fig. 2 Structures of ectoine (**a**) and hydroxyectoine (**b**), as examples of compatible solutes. For additional structures of CSs, see the cited reviews above

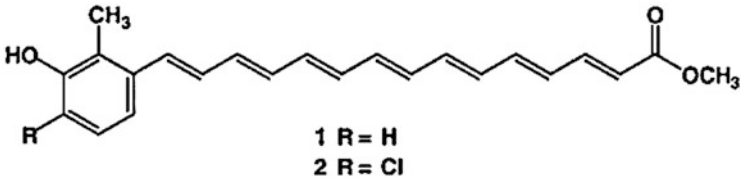


Fig. 3 Structure of the two species of the yellow pigment from *Thioalkalivibrio versutus* ALJ 15 grown at pH 10 and 2 M Na^+ ion concentration: *1* natronochrome, *2* chloronatronochrome

can be applied widely in food industries. The neutralized wastewater can be introduced to communal drainage systems for further bioprocessing.

Lactic acid has many applications in food chemistry, medicine, organic syntheses, and as monomers for bioplastics. In a previous study [5], none of four different alkaliphilic *Bacillus* species grown in various sugars produced lactic acid. However, Yokaryo and Tokiwa [15] isolated alkaliphiles including *Bacillus* sp. producing lactic acid. *Enterococcus casseliflavus* sp. produced L(+)-lactic acid 103 g/L from 129 g/L of glucose with optical purity of 99.5%. During the conversion, the pH was maintained at 8. The stereoisomers and their ratios appeared to be stable and typical for each strain. D-Lactate with high optical purity was efficiently produced in fed-batch fermentation in non-sterile conditions by an engineered alkaliphilic *Bacillus* sp. with maximum concentration of 144 g/L. The yield was even better than in sterile conditions implying to a synergy of a microbial association. Moreover, cheap nitrogen source was used. The exopolysaccharide production was disrupted by gene engineering to lower viscosity and energy consumption. The use of NaOH to maintain the culture pH instead of $CaCO_3$ avoided precipitation. Polymer-grade D-lactate was also produced by an engineered alkaliphilic *Bacillus* under non-sterile conditions [16].

The metabolic engineering of alkaliphiles has advanced only slowly because of the technical difficulties. Could alkaliphiles have benefits as the host organisms? Are the metabolic rates high enough? Are there specific reactions which are faster than in neutral conditions? Rationally thinking, the growth rates of alkaliphiles should be lower than in neutral conditions, but it does not seem to be always true. Functioning in non-sterile conditions is clearly beneficial, but such processes may be exceptions.

Natural plasmids have been found from alkaliphiles, but their exploitation has not yet been wide. May be plasmids have a role in the pH adaptation like that of lactic acid bacteria? The enzymes found from alkaliphiles have been usually cloned and expressed in standard neutral-growing host organisms. The motivation for the efforts for metabolic engineering in alkaliphiles would increase when industrially feasible unique metabolites will be found from alkaliphiles. Expression of monomers for biopolymers, like bioplastics, and specialty chemicals, is a potential field for genetic engineering. Even if the basic metabolism of alkaliphiles has not yet demonstrated exceptional principal differences compared to that of neutralophiles, it still needs to be considered that only a minor part of alkaliphiles has been relatively studied in detail. Bioinformatics and fast sequencing will, undoubtedly, allow the emergence of new discoveries soon. These questions will be dealt with in detail in other chapters in this book.

3 Bioplastics

Bioplastics are biopolymers, the name referring to plasticity of a material. Plastics can be molded to hard or flexible forms like mulches, bottles, utensils, parts of machines, construction materials, foams, and so on. The industry is so vast and established that existing practices are not easy to change. The term bioplastic involves several meanings which are not always recognized. The term can be used for plastics made of renewable biological materials. They may or may not be biodegradable. If not, they are called durable. Durable (bio- or oil-based) plastics can break down to small particles but may practically never disappear from nature, and those micro- and nanoparticles are widely recognized as a serious threat for life on earth. If the cycling of plastics is not perfect, as seems to be the fact in the real life, it means that the nature will be increasingly polluted with small particles of durable plastics. That process will continue hundreds of years even after dumping of the plastics is stopped. The composites of biodegradable and durable plastics have also been produced. These composites seemingly disappear rather fast, but the microscopic durable particles stay in soil. Thus, such composites are, even in a short period, more dangerous to nature and human health than durable plastic. For recycling, it is important to be able to divide the waste plastics into biodegradable and durable fractions. The sustainable long-term solution may be to use fully biodegradable plastics. The current problem related to the use of biodegradable plastic is high price and sometimes the nonoptimal mechanical properties. The European Commission is presently formulating its plastics strategy wherein different measures will be included to avoid drawbacks made by plastics (https://www.european-bioplastics.org). As can be seen from the European dialogues on plastics, the question is not black-and-white. EU, however, recognizes the benefits of bioplastics. The production of bioplastics is highly energy-consuming, and therefore, its circulation back to industry shall be organized. Whatsoever, biodegradable bioplastics will be extremely big business in the future and also attractive topic to

scientists. Even in the short term, bioplastics will have increasingly diverse high-value applications. There is plenty of written material on bioplastics, including text books. Therefore, here, the discussion is confined to the materials that are potentially produced by alkaliphilic microbes. The term “bioplastic” is used here in the narrow meaning for biodegradable bio-based plastics.

Even at the time when the general problems of plastics were not comprehended, many proposals for bioplastics were presented. Polylactic acid is one of the most intensively studied materials obtained from the precursor lactic acid by fermentation. Several chemical ester polymerization routes are known. L(+)-Lactic acid can be produced by alkaliphiles maintained at pH 8 during the sugar-lactate conversion with excellent yield and optical purity. The high optical purity is advantageous for properties of polylactic acid used for bioplastics [15]. Lactic acid fermentation is profoundly studied in neutral and acidic media in industrial level for the use of lactic acid in food industry. In food and feed industry, there are strict limitations on microbial species that may be employed. However, for bioplastics production, the microbial selection can be wider, and also nutrients of less value can be used. Commercial bioplastics based on lactic acid are available for various purposes. Their properties vary depending on the optical purity of the precursor and polymerization methods. Examples of alkaliphilic producers of optically pure L and D-lactates were described in the first section above describing the sugar metabolism. As stated, alkaliphilic microbes can produce high-quality lactates with very high yields from sugar. Strain improvement and genetic engineering may be still exploited with process optimization with cheap substrates to get lactic acid. Keeping lactic acid as the only precursor of polymers may also be a too trivial approach. There are other biological compounds which can be produced industrially (or be side-products) and polymerized with chemical bonds which enzymes can break.

Polyhydroxyalkanoates (PHAs) are accumulated intracellularly as energy storage compounds up to 10–75% of cell weight, especially under unbalanced nutritional conditions [17, 18]. The polymers are formed from hydroxy acid monomers (hydroxy butyrate, -valerate, etc.) by biological esterification. Due to the bifunctional structure of the monohydroxy acids, linear polymers are formed. The amount of complex hydroxy acids (more hydroxy groups per molecule) in the structure defines the grafting degree and increases the spectrum of properties of the PHAs. PHAs, as a group, have a great variety of characteristics, and due to their sustainability, biodegradability, and biocompatibility, many industries are interested in them, for example:

- Single use packaging for foods, beverages, and consumer products
- Medical applications like sutures, bone marrow scaffolds, and bone plates
- Agricultural foils and films

Some of PHAs exhibit features that are similar to those of the conventional synthetic plastics but with a significantly lower environmental impact on the production process. However, many challenges have restrained PHAs’ applications, including complicated genetic engineering processes for producing new PHAs and inadequate mechanical strength. The high production cost and poor processability of

PHAs limit their competitive forces to substitute traditional synthetic plastics. Modification of PHAs to achieve better performance has attracted increasing attention from industry and scientific research organizations and has gained excellent achievements in materials and medical applications [19]. In spite of formal and temporary obstacles, PHAs with various compositions of esters have established their position in the bioplastic market. The present production of bioplastics is made with genetically engineered microbes grown in neutral conditions. Haloalkaliphilic microbes can accumulate >50% of their dry weight with PHA-related compounds. The alkaline microbial producers have been reviewed by Zhao et al. [18]. Recently, a wide systematic approach was described to improve PHA biosynthesis by engineering microorganisms [20]. Plastics have got so prevalent position in human life that it is unlikely that it can be easily substituted. The biodegradable plastics shall find their specific place in the consumer use. Bioplastics can be based also on composite materials from different sources, like microbial and plant polymers. Considerable technological efforts exist in this field, for example, in composites of cellulose and polylactic acid (https://makingoftomorrow.com/biocomposites-insights-patent-landscape). The composites allow using of cheaper raw materials together with the binder plastics. It is illustrating that the total number of patents on bio-composites is about 14,400 after the year 2000 (see the above presentation).

4 Compatible Solutes (CSs)

Microorganisms living in extreme environments must often adapt to fast changes in physical (heat, cold, radiation, compression, shearing) and chemical conditions (ionic strength, oxygen, pH) to maintain cellular functions like turgor pressure, cell volume, and ionic strength in tolerable limits. Genetic studies show that alkaliphiles do have many arrangements for that in different levels of the cell physiology [21]. That is, microbes can react to several simultaneous stress factors. One part of that is considered to be achieved by regulation of small molecular compound called osmolytes or osmoprotectants. They are inert molecules not affecting the overall cellular functions, although they can modulate individual events like protein folding, enzyme activities, and a multitude of bioaffinity events. Because these compounds may have also other functions than regulation of osmotic pressure, the term compatible solute (CS) has been adapted. This is more exact term than osmolyte when the function is not known. Molecular structures of CSs can be anionic, cationic, or neutral, and their molecular sizes can be different. The structures and functions of CSs related to halophilic microbes are reviewed by Roberts [22]. In a way, CSs could act as "molecular relaxants" when bound to a protein, for example, they could maintain flexible structure of the protein by the ability of CSs to have various tautomeric or resonance structures (e.g., ectoines). The cited review also shows extensive list of CSs and discusses their biosynthesis. Although the review focuses to halophiles, the major part of it is also relevant to alkaliphiles. Zhao et al. [18] have reviewed the most known CSs from haloalkaliphiles including ectoines and betaines.

It may be difficult to know the exact function of CSs in cells. Roberts [22] classified poly-beta-hydroxybutyrate (a PHB) as belonging to CSs. It might act so, if the concentration of monomers could be regulated enzymically for increasing the ionic strength and vice versa. However, this and also some other CSs could be used as energy or phosphorus storages. The most advantageous way for a cell is to exploit them for dual- or multi-purposes, as osmo- and/or pH-regulators as and energy/nutrient storage and even maintaining the dynamics of proteins structures. It is peculiar that CSs tend to occur in cells at high intracellular concentrations. For getting relevant dynamic data, effective analysis methods are needed. Because of the high concentrations, nondestructive NMR methods of studying compatible solutes inside cells in different conditions can be advantageously applied [22]. Bound/solid and free molecules may be differentiated, while the intracellular pH data could be simultaneously monitored. The NMR methods for measuring intracellular pH are based on the chemical shift value of an atom of a known compound near an ionizable functional group. It is compared with the chemical shift of the same molecule in solution in different pHs. The chemical shift values should be evaluated critically because the molecules may not be free inside the cell but bound to other molecules. The binding can affect the pK_a values (and through it to chemical shifts of the indicator atom). Then the intracellular pH can be misinterpreted. The proton activity and the whole concept of pH are also strongly dependent on the polarity of the solvent/microenvironment. For instance, protonation of vitamin B6 bound to its apoenzyme was misinterpreted in a series of publications because of assuming that the situation is similar to free vitamin B6 in solution [23]. The same potential source of errors exists with the usual pH-indicator dyes when measuring internal pH of a cell.

Numerous CSs are already commercially available, and it is believed that CSs have a significant commercial potential. However, finding a suitable compound to a certain application from a long list is not easy. Further fundamental research is required to find out general guidelines how to use a certain CS. Biological antifreeze compounds are known for a long time and are likely to share related features with CSs for the interaction with water molecules. Crystal structures (and NMR studies) of proteins harboring CSs probably unveil their mechanisms of action and thus help obtaining reason-based means to test a CS for a specific purpose. After the basic knowledge is available, molecular modeling studies may be exploited, as well. Structures of two alkaliphile-produced compatible solutes, ectoine and hydroxyectoine (Fig. 2), are already known in molecular level for their effects on protein stability and folding (see [18] and references therein).

Ectoine is commercialized and used as an active ingredient in skin care. It stabilizes proteins and other cellular structures and protects the skin from stresses like UV irradiation and dryness. In addition to their uses in research, medicine, and agriculture sectors, ectoines could also be used for formulating various products with other CSs.

5 Biosurfactants

There is a widespread concern about accumulation of chemically synthetized surfactants in different environments, suggesting an immediate need for biodegradable surfactants. Although there are many known biodegradable surfactants, they are far expensive than the chemical surfactants driven from petrochemicals, and this limits their application. Many biosurfactant candidates with suitable ionic and dissolving properties exist from a multitude of microbial sources. Thus, it is important to produce these biodegradable surfactants at competitive price. First attempts for genetically modified super bio-producers have been made. Moreover, the development of fermentation processes that use waste materials is undergoing. It is evident that prices of biosurfactants will go down when the production volumes will grow.

Surfactants used in detergent formulations are released into the environment by the wastewater pathway. It may be possible to recover such surfactants from wastewater. However, as far as the authors know, attempts to recover any surfactants back from wastewaters have not been tried. It is probably advantageous if the spectrum of surfactant structures in wastewaters is heterogenic. The European Detergent Regulation 684/004 (https://eur-lex.europa.eu/eli/reg/2004/648/oj) demands that all types of commercially available surfactants must be ultimately biodegradable under aerobic conditions which is one of the most important mechanisms for removal of chemicals released into the environment. Half of the total surfactant consumption belongs to household applications, which is the largest market for surfactants. Anaerobic biodegradability is not required so far, with exception of eco-labeled products. Although the natural environment is predominately aerobic, there are some more or less strict anaerobic compartments such as river sediments, subsurface soil layer, and anaerobic sludge digesters of wastewater treatment plants. In this environment, anaerobic biodegradation may contribute to avoid accumulation of anthropogenic chemicals [24]. All biosurfactants are surface-active compounds and are synthetized by microbes such as bacteria, fungi, and yeasts. Biosurfactants are classified to low-molecular-weight (usually 500–1,500 Da) agents and high-molecular-weight agents called bio-emulsifiers. These two groups are further divided into subgroups based on their components, like glycolipids, lipoproteins, phospholipids, etc. Undoubtedly, biosurfactants are produced by a wide range of organisms, but microbial sources are the most promising for large-scale production.

Biosurfactants are of major commercial interest, and they can have a critical micelle concentration (CMC) even distinctly lower than those of the chemical surfactants. Normally, biosurfactants are nontoxic and harmless to the environment because they degrade quickly in the nature. They have large diversity and exhibit a variety of properties and are therefore flexible, and a suitable agent can be found to any application in concern. Properties, structures, and applications of biosurfactants are extensively summarized with illustrative introduction to the micellar structures by Santos et al. [25].

Biosurfactants are highly attractive to be used in industrial products like cosmetics, pharmaceuticals, agrochemicals, food, medicine, and detergents. Oil industry is a potential big user of biosurfactants as emulsifiers. Bioremediation processes could benefit from biosurfactants and even can be produced in situ. Some biosurfactants have also antibiotic and/or insecticidal properties. The commercial applications and production of biosurfactants have been reviewed by Fakruddin [26].

Biosurfactants produced and secreted by alkaliphiles are ideal for detergent formulations which are alkaline. The positive image of eco-friendly products may justify the higher price, as well. Many reports describe the biosurfactant production by microbes in moderate pH environments [25–28]. So far, a few alkaliphilic biosurfactant producers have been isolated from different but rather harsh habitats such as oil-contaminated saline sites and wastewaters [29–31], alkaline and saline soda lakes [32–34], hot spring [35], Red Sea water [36], and PAH-contaminated soil [37]. Their potential to synthesize surfactants was mainly identified by the hemolytic emulsification activity and reduction in surface tension. *Achromobacter xylosoxidans* bacterium from Lonar Lake, India, and archaea *Natronolimnobius innermongolicus* WN23 from soda lakes, Egypt, had high degradative potential for oil and had the emulsification index values 58% and 55%, respectively [32, 34]. Biosurfactant crude extracts produced by *Archaea* WN23 and WN26 were stable in a broad pH range of 5–12 and up to 35% (w/v) of NaCl. The change in the pH did not affect the stability of the emulsion [32].

The influence of media compositions and cultivation conditions on production of biosurfactants by microorganisms have been extensively studied. Corn powder, an unconventional carbon source, increased biosurfactant production by alkaliphilic *Klebsiella* sp. up to 15.4 g/L against 10.0 g/L with starch [38]. Yield of biosurfactant of *Cronobacter sakazakii* in a sucrose-containing medium was 3.15 g/L after 72 h [31]. In addition to production studies, analysis of the biosurfactants has been considered. The chemical characteristics of two purified biosurfactants were analyzed with spectroscopic methods [31, 39]. The compounds were heteropolysaccharide-protein complexes comprised of glucose, galactose, mannose, xylose, arabinose, and uronic acid. These compounds were high-molecular-weight thermally stable biopolymers which exhibited degradation above 260°C. Furthermore, it had low viscosity with pseudoplastic rheological behavior and significant emulsification activity with oils and hydrocarbons. The isolated biosurfactant of strain *Klebsiella* sp. RJ-03 is compatible with detergents and resulted in enhanced oil removing efficiency from soil and cotton cloths [40].

Maximum biosurfactant production by an *Exiguobacterium* sp. was found to take place using coconut oil and brake oil as carbon sources. The best growth was achieved at pH 10, 50°C, and in the presence of 4% (w/v) of NaCl. It was suggested that this microbe might be used for remediation of polluted sites in marine environment [33]. Similar results were found with biosurfactant production by alkali-halothermophilic bacteria *Virgibacillus salarius*. The highest biosurfactant synthesis was obtained in the presence of 4% (w/v) of NaCl and cultivated at pH 9 and 45–50°C using frying oil waste as carbon source. The structural analysis confirmed the

lipopeptide nature of the surfactant [30]. Biosurfactant production by marine alkaliphilic bacterium *Pseudomonas aeruginosa* was strongly dependent on the cultivation pH. The strain was able to grow and reduce the surface tension of the culture broth to 28 mN/m when cultured using sunflower oil as the sole carbon source and peptone as the nitrogen source in pH 9 medium and at 30°C [36].

Ochrobactrum intermedium produces a thermostable lipase and biosurfactants and known to be resistant to alkaline washing powders. Biosurfactant produced by *O. intermedium* exhibited high stability at pH 10–13 and temperature of 70–90°C. The biosurfactant was stable in the presence of various metal ions, detergents, and organic solvents and exhibited good antimicrobial activity [35]. The biosurfactants produced by the marine bacteria *Bacillus amyloliquefaciens* and *Bacillus thuringiensis* showed high emulsifying indexes. These biosurfactants were stable at moderate temperature (30°C), high alkalinity (pH 11), and high salt concentration (15%, w/v). Characterization of a partially purified biosurfactant from the most active strain, *B. amyloliquefaciens*, indicated that the biosurfactant is a non-anionic didemnin surfactant [29].

The lipopeptide biosurfactant produced by a strain of *Hydrogenophaga* sp. under iron-reducing condition facilitated anaerobic degradation of pyrene and benzopyrene. Temperature and initial pH value of medium for both aerobic and anaerobic growth were set at 28°C and pH 9.0, respectively. This indigenous alkaliphilic organism seems to be suitable for bioremediation of petroleum- contaminated alkaline sediment [37].

6 Siderophores

Iron (Fe) in its different ionic forms is biologically active compound which have various functions in living organisms. Two readily convertible ferrous Fe(II) and ferric Fe(III) forms allow iron to play pivotal role in numerous electron transfer processes. The precisely tuned steric and electronic environments within enzyme active sites permit even more highly oxidized Fe(IV) and Fe(V) or reduced Fe(I) states to function as intermediates of enzyme catalyzes. Iron ions have been studied for decades in bioorganic chemistry, and their coordination chemistry has solid background as can be found in textbooks. Iron is an essential nutrient for all known life forms.

Under iron-depleted conditions, microorganisms secrete small molecules known as siderophores (Greek: "iron carrier"). These compounds have high affinity for iron and are known to be iron-chelating agents. Siderophores may serve as iron transporters through the cell membrane of microbes. This is the traditional function, but studies have shown that the role of siderophores is far more complex. At present, nearly 500 structures of siderophores are reported from various microorganisms [41]. The paper by Johnstone and Nolan [42] also reviews structural families of siderophores. Typical metal-binding motifs of siderophores are catecholates, hydroxamates, and alpha-hydroxycarboxylic acids. In addition to iron ions, many

other metal ions including ions of Zn, Cu, Mo, V Cd, Ni, Pb, Mn, Al, Th, U, and Pu can bind to siderophores [43]. That makes it possible to develop various environmental applications and bioleaching processes. In the framework of the new approach on the development of diagnostic systems, siderophores can be applied as biosensors and nanosensors [43, 44]. Since iron is required by all living systems, it is also biologically competed element among the microbes. On the other hand, free iron can be biologically harmful, and its existence must be strictly controlled. Free soluble Fe(II) may react with oxygen and produce damaging oxygen species. In human body, for example, extra iron ions are bound to specific protein carriers [45], and concentration of free iron is extremely low in serum, $<10^{-24}$ M Fe(III) [46]. The repertoire of siderophores varies among microbial species and even among strains. There is an increasing amount of data suggesting that siderophores impact on microbial pathogenesis with different mechanisms. Siderophores can also modulate the host response [46]. In pathogenic interactions, siderophores are involved in iron acquisition from the host and are sometimes necessary for full virulence. To protect themselves against iron theft, mammalian hosts have developed siderophore-binding molecules, siderocalins, which may also trigger immunity. As to plants, fewer data are available, but the plant-microbe relation is surely important as it is also with mammals. In plants, siderophores can trigger immunity in several contexts through induced systemic resistance. However, the underlying mechanisms are not yet well understood [47].

Bacteria are known in transporting extracellular siderophores charged with metal ions to their intracellular environment. Thus, infection of antibiotic-resistant pathogens can be prevented through siderophores: some bacteria can link antibacterial agent into a siderophore to generate a sideromycin. A competing bacterium, attempting to steal iron from another one, may uptake sideromycin and get the toxin inside the cell, acting like the "Trojan horse" [48]. To date, siderophore-producing bacteria and fungi that thrive at normal pH are well studied and reviewed [41, 49]. The information on the nature of iron-chelating molecules produced by extremophiles is scanty, in particular that of alkaliphiles [50]. The biochemical and molecular mechanisms used by alkaliphilic bacteria to acquire iron are not well understood. One aspect is that in alkaline, environment iron and some other metal ions precipitate as hydroxides. Therefore, especially in alkaline milieu, the availability of iron can become growth limiting. May be iron hydroxides require different binding compounds. Siderophores should therefore be exceptionally important for alkaliphiles. It was demonstrated that alkaliphilic *Bacillus* sp. strains, *Caldalkalibacillus thermarum*, *B. halodurans* C-125, *B. pseudofirmus*, and *B. alcalophilus*, were sensitive to artificial iron (Fe^{3+}) chelators even though these microbes produce siderophores. The siderophores contained catechol and hydroxamate moieties, and their synthesis was stimulated by manganese (II) salts and suppressed by $FeCl_3$ addition. Purification and mass spectrometric characterization of *C. thermarum* siderophores failed to identify any matches to previously observed fragmentation spectra of known siderophores, suggesting novel structures [51].

An alkaliphilic bacterium, *Alkaliphilus metalliredigens*, was isolated from leachate ponds at the US Borax Company, Boron, California. It was able to grow with metals Fe(III)-citrate, Fe(III)-EDTA, Co(III)-EDTA, and Cr(VI) as the electron acceptors, at pH values up to 11 in the presence of elevated salt levels in anaerobic conditions. This metal-reducing bacterium might be unique for bioremediation of metal-contaminated alkaline environments and for mobilizing metal ions in the process of bioleaching in anaerobic conditions [52]. An alkaliphilic bacterial isolate *Halomonas* sp. SL01 was found to produce relatively high concentrations of siderophores in liquid medium (up to 40 μM). Structure of the purified siderophore was determined using LC/MS and fatty acid methyl ester (FAME) GC. Two distinct new families of amphiphilic siderophores were produced by the isolate SL01. All the siderophores were 989–1,096 Da in size and consisted of a conserved peptide-head group, which binds iron together with coordinated fatty acids. These siderophores resembled amphiphilic aquachelin siderophores produced by *H. aquamarina* strain DS40M3, a marine bacterium, as well as siderophores from *Halomonas* sp. SL28 which also produced amphiphilic siderophores. The names of halochelins B, C, D, E, and F were proposed for the siderophores produced by *Halomonas* sp. SL01 [53].

The alkaliphilic strain *Pseudomonas pseudoalcaligenes* CECT5344 isolated from sludge of the Guadalquivir River (Cordoba, Spain) can metabolize cyanide, cyanate, some metal-cyanide complexes, and different nitriles (cyanohydrins) as nitrogen source under alkaline conditions, thus preventing volatile HCN formation. This strain was able to tolerate pH of 11.5 and up to 100 mM cyanate. Although it has been discussed that the synthesis of siderophores for iron acquisition should be necessary for an efficient cyanide assimilation process, it seems that *Pseudoalcaligenes* CECT5344 does not produce siderophores and lacks putative genes involved in the synthesis of these ferric ion-specific chelators [54]. However, quantitative proteomic analysis revealed that the cyanotrophic strain possesses the ferric-uptake system FhuC (ATP-binding component of a hydroxamate-type siderophore import system) [55]. It can use Prussian blue as the source of nitrogen and iron generating decolored halos around the colonies when growing on agar plates [54].

Two siderophore-producing strains VITVK5 and VITVK6 were found and characterized [56]. The bacterial isolates showed a close resemblance to species of the genera *Bacillus* and *Enterobacter* sp., respectively. Both strains grew and produced siderophores in the pH range of 4.0–10 and temperature 25–45°C and optimally produce siderophores at pH 8 and at 37°C with glucose or sucrose as the carbon source and $NaNO_3$ as the nitrogen source. The strains VITVK5 and VITVK6 are promising candidates for the production of the siderophores because of simple nutrient requirement. The authors considered them to suit for many applications in medicine and industry [56].

7 Carotenoids

Carotenoids constitute a structurally diverse group of colored lipidic pigments, which are widespread in nature and play crucial roles in many physiological processes. Carotenoids represent a long aliphatic polyene chain composed of "n" isoprene units (C_5H_8), which are sometimes terminated by rings, and may or may not have additional oxygen atom attached. They are initially synthesized with a chain of 30 or 40 carbon atoms from prenyl pyrophosphates, and they may be extended at a later stage up to 50 carbon atoms [57]. There are two general classes of carotenoids: carotenes and xanthophylls. The familiar carotenes are β-carotene and lycopene. Both are strict hydrocarbon carotenoids and do not possess any substituent (or even oxygen) in their structures. Xanthophylls or oxycarotenoids, which belong to the second group, are oxygen-containing molecules. Lutein and zeaxanthin are two xanthophylls with –OH groups in their structures, whereas canthaxanthin and echinenone contain =O groups. Astaxanthin has both –OH and =O groups in its structure [58].

Carotenoids have a wide range of application in the health care and nutraceutical industry; they are produced as food colorants, feed supplements, and nutraceuticals, for cosmetic and for pharmaceutical purposes. Carotenoids are essential for humans and must be supplied through diet. The growing importance of carotenoid has also led to an increase in the demand for carotenoids in the global market, which was estimated to be ~1.24 billion USD in 2016, and is projected to increase to ~1.53 billion USD by 2021, at a compound annual growth rate (CAGR) of 3.8% from 2016 to 2021 [58].

Carotenoids are essential components of all photosynthetic organisms due to their eminent photoprotective and antioxidant properties. However, their occurrence is not restricted to plants, algae, and cyanobacteria, as some fungi and non-photosynthetic bacteria can synthesize carotenoids as well [59]. Recently, microbial sources of carotenoids have received attention due to the stringent rules and regulations applied to chemically synthesized/purified pigments (like replacing synthetic astaxanthin for salmon feed). Microorganisms for the industrial production of carotenoids are preferred over other natural sources (such as vegetables and fruits) due to problems of seasonal and geographic variability in production of the latter [60].

According to the Carotenoids Database providing chemical information on 1,117 natural carotenoids with 683 source organisms, carotenoids seem to have been spread largely in bacteria, as they produce C30, C40, C45, and C50 carotenoids (based on the molecular carbon number), with the widest range of end groups, and they share a small portion of C40 carotenoids with eukaryotes. Archaea share an even smaller portion with eukaryotes. Eukaryotes have evolved a considerable variety of C40 carotenoids. Considering carotenoids, eukaryotes seem more closely related to bacteria than to archaea [61].

Carotenoids of diverse structures have been also found among alkaliphilic bacteria. Previous reviewers have reported about triterpenoid carotenoids from yellow

pigmented alkaliphilic *Bacillus* sp. [1]. A lycopene type of carotenoid pigment was produced by alkaliphilic *Microbacterium arborescens* AGSB, obtained from coastal sand dunes. Novel carotenoid glucoside esters have been reported from alkaliphilic Heliobacteria [62]. Later, the same type of carotenoid, OH-diaponeurosporene glucosyl esters, was found in two other novel taxa of *Heliobacteria*, *Heliorestis acidaminivorans* sp. nov. strain HR10BT, and *Candidatus Heliomonas lunata* strain SLH [63]. An isolate from Lake Bogoria hot spring, obligate alkaliphile *Paracoccus bogoriensis* BOG6T was found to secrete astaxanthin with yield of 0.4 mg/g of wet cells indicating a potential for application in commercial production of carotenoids [64]. A sulfur-oxidizing alkaliphilic heterotroph, *Roseinatronobacter thiooxidans* ALG1, synthesized carotenoids in organic nitrogen-containing medium [62]. Cis- and trans-canthaxanthin (cx) isomers with different antioxidant activities were isolated from orange-pigmented mesophilic bacterium *Dietzia* sp. K44 with optimum growth at pH 8. It was suggested that 9-cis-cx is more effective as an antioxidant than all-trans-cx [65].

Detailed pigment analysis of alkaliphilic purple sulfur bacterium *Thiorhodospira sibirica* gen. nov. sp. nov from Siberian soda lakes revealed carotenoids of the spirilloxanthin series with anhydrorhodovibrin (37.6%), followed in quantity by rhodopsin (31.5%), spirilloxanthin (21.8%), didehydrorhodopin (6.5%), and lycopene (2.7%) [66]. Carotenoids of spirilloxanthin series are not uncommon among the phototrophic purple sulfur bacteria. The spirilloxanthin was the main pigment in a new bacterium from a soda lake in Mongolia *Ectothiorhodosinus mongolicum* gen. nov., sp. nov. [67], in an alkaliphilic and halophilic *Ectothiorhodospira variabilis* sp. nov. [68], in a new species of the genus *Ectothiorhodospira*, *Ect. magna* sp. nov. The presence of carotenoids of the spirilloxanthin series was supported by the maximum light absorption within a range of 485–514 nm and a shoulder at 545 nm. A similar in vivo pigment absorption spectrum with three longwave maxima is typical of many *Ectothiorhodospiraceae* species [69]. Carotenoids of *Thioalkalicoccus limnaeus* gen. nov., sp. nov., isolated from cyanobacterial mats of soda lakes in certain ecoregions called steppe of the southeast Siberia, Russia, demonstrated the spectral characteristics similar to 3,4,3H,4H tetrahydrospirilloxanthin [70]. The presence of both spheroidene and spirilloxanthin carotenoids was reported for *Rubribacterium polymorphum* gen. nov., sp. nov., an alkaliphilic nonsulfur purple bacterium from an eastern Siberian soda lake [71, 72] and for two nonsulfur purple alkaliphilic isolates of a new genus and species of the family *Rhodobacteraceae*, *Rhodobaculum claviforme* gen. nov., sp. nov. [73]. Carotenoids of the spheroidene series were found in the cells of *Roseibacula alcaliphilum* gen. nov. sp. nov. A new alkaliphilic aerobic anoxygenic phototrophic bacterium from a meromictic soda lake Doroninskoe (East Siberia, Russia) contains bacteriochlorophyll α and carotenoids [74].

The fact that the pigments of a new facultative alkaliphile strain of the genus *Roseococcus*, *Rsc. suduntuyensis* sp. nov. had different retention times in chromatography indicates that carotenoids differed from spirilloxanthin. In these carotenoids of the strain, the spirilloxanthin methoxy groups are replaced by glycosides. Similar replacements were observed in the carotenoids of the type strain of *Rsc.*

thiosulfatophilus [71, 72]. Accumulation of demethylspheroidene and demethylspheroidenone carotenoids in substantial amounts was observed in the cells of a novel alkaliphilic purple nonsulfur bacterium *Rhodobaca bogoriensis* strain LBB1. That is regarded to be unique among anoxygenic phototrophs, as these two carotenoids are known intermediates of the spheroidene pathway, but their content is typically low in organisms that express enzymes of this pathway [75]. A novel type of membrane-bound yellow pigments was detected in the extremely salt-tolerant alkaline strain of *Thioalkalivibrio*, whose specific concentration correlated with increasing salinity of the growth medium. In contrast to carotenoids, the new pigments possess considerably less carbon atoms (i.e., total number of carbon atoms is 23) and have a straight polyene instead of an isoprenoid chain. This polyene chain is terminated with a hydrophilic methylated phenol and a carboxymethyl group. The compound (2) differs from the compound (1) by the presence of chloride in the phenol group, which makes it even more unusual. The names natronochrome and chloronatronochrome were suggested to the compounds (1) and (2) in Fig. 3, respectively [76].

Marine alkali-tolerant strain 11shimoA1 (JCM19538), *Jejuia pallidilutea*, synthesized a novel monocyclic carotenoid, 2′-isopentenylsaproxanthin, as well as zeaxanthin. Saproxanthin and myxol, which have monocyclic carotenoids with a γ-carotene skeleton, have been reported to show a stronger antioxidant activity than those with β-carotene and zeaxanthin. The strain 11shimoA1 produced more 2′-isopentenylsaproxanthin in the alkaline medium (pH 9.2) than in medium with a pH of 7.0. These carotenoids are likely to play some roles in the adaptation of the bacterium to the environmental conditions [77].

8 Exopolysaccharides

Microorganisms synthetize a wide spectrum of multifunctional polysaccharides including intracellular, cell wall, and extracellular polysaccharides. Extracellular polysaccharides, exopolysaccharides, or EPSs can be found in capsular materials or as a dispersed slime with no obvious association to any one particular cell common to the microbes. EPSs are composed of sugar monomers and some noncarbohydrate substituents. Attachment to material surfaces (biofilms) is one function of EPSs. EPSs could serve also as protective barrier to hostile environment and an external energy storage for microbes.

Microbial sugar polymers and their derivatives form a very complicated and less known class of chemical compounds. The statistical variability of the chemical bonds can be enormous. There are analytical methods to study EPSs, but they are laborious and demand specialists even though a great progress has taken place during recent years for introducing advanced mass spectrometric and NMR methods. Pathogenic microbes require first to make a firm adherence to host organs in order to colonize. Pathogens have not only carbohydrates to attach but also protein chains which are synthetized as monomers and joined together with “lock strands”

attachment to get long fibers possibly capped by a specific attachment protein [78]. Without the property to the firm attachment, a microbe cannot be pathogenic, as known from pathogenic and nonpathogenic *E. coli* strains. EPSs could provide an evident survival mechanism to extremophiles for making capsules around the cells and for binding to surfaces for propagation. Structures of EPSs vary and constitute a wide spectrum of properties.

EPSs biosynthesis can take place either fully extracellularly or partly intracellularly and partly extracellularly. Glycosyltransferase enzymes make the complicated transfers of sugar units in the growing polymer system. EPSs and their structures, analysis, biosynthesis, and properties from extremophiles are reviewed in detail [79]. Alkaliphiles have unusual structures of EPSs. Many haloalkaliphiles strains produce more than one different EPS [79]. Natural or chemically functionalized carbohydrates are important biotechnological products in industry as gelling agents, thickeners, or edible films. EPSs also have multifarious biotechnological applications in food and pharmaceutical industries. Microbial EPSs could add to the spectrum of related gum-like substances of non-microbial origin. Food industry is strictly limited to certain accepted nonpathogenic producer strains. This limitation does not apply to other technical applications where the product repertoire may thus be much bigger.

Cyclodextrins (CDs) are ring-shaped glucose oligomers (6, 7, or 8 glucose units) derived from starch by the enzyme cyclomaltodextrin glucanotransferase, CGTase ([80]; a review). Formally, CGTases are messengers of the cells, like any enzymes or EPSs aimed at acquiring better living conditions for a microbe. There was for a long time conception that there are separate enzymes for the different CD rings. It was convincingly shown that there is only one enzyme by purifying CGTase from an alkaliphilic *Bacillus* to an extremely homogenous stage on the immobiline electrofocusing. There were microheterogenic fractions (one or two charge differences), but the CD profile was the same [81, 82]. However, the reaction conditions affect the equilibrium between acyclic and cyclic forms of CDs, a low water activity (studied in water-ethanol mixtures) favoring the cyclic forms [83]. The function of a transglycosylation reaction could be illustrated with using pure maltose as the substrate for the enzyme CGTase. A decreasing concentration series of higher polymerization degree malto-oligomers up to maltoheptaose was obtained. The amount of glucose increased when the polymerization degrees increased. That is, in a microbial medium, glucose could be ingested by the microbe producing the enzyme, and CDs can serve as a prolonged supply of glucose. The reaction micromilieu also affects the results. Novel types of sugar syrups containing CDs were obtained by adding immobilized CGTase enzyme to sugar syrup solutions. If free soluble enzyme was added, the reaction was negligible [81, 82]. Glycosyltransferases (exemplified here by the CGTase) can dynamically modify sugar polymers to get specific and extremely complex EPSs. These carbohydrates can serve as nutrient storages, means to cell protection, and adhesion to surfaces. The strategic advantage is in the microbe's individual structure of EPSs. The structures of different microbes can originate from only slightly different compositions of enzymes and/or their

specificities. The microbial strain producing its specific EPS has also enzymic tools to digest it back to monomers.

It is rather easy to isolate CGTases from soil microbes, i.e., it is not a rare enzyme. It is costly to a microbial cell to synthetize an enzyme, and thus the enzyme must have an ecological meaning. There have been repeated questions about the benefits to produce CDs. One answer is that CDs can act as complexing agents to small-molecular hydrophobic compounds (like siderophore for metal ions). For example, lipids or vitamins could be "wrapped" inside CDs. Protein side chains can complex with CDs (a correlation to compatible solutes). CDs could be functional also in microbial signaling between them and between other life forms. CDs in soil can retard germination of seeds and distort growth of micropropagated plants [84]. Typical symptoms of imbalance of growth factors were found (e.g., callus formation) suggesting that CDs interfere with them. The retardation of germination of seeds was suggested to be due to disturbances in gibberellin signaling in grain and not being due to the amylase inhibition as thought earlier (solid-bound CDs had similar effect as free). For a bacterium, postponing of the germination of a seed and/or inducing abnormal morphological changes would increase probability of decay of a seed or plantlet and thus create access to nutrients. Thus, there exist various reasons why CDs can be ecologically advantageous.

EPSs produced by alkaliphiles are far from explored. Those studied exopolysaccharides-producing alkaliphiles belong mainly to haloalkaliphiles and representatives of genus *Bacillus*. Like others, alkaliphilic producers of EPSs have been found from different sources, some being exotic like a shallow hydrothermal vent [85], radioactive hot spring [86], or saline lake Cape Russell in Antarctica [87], or they were even scraped off from the deteriorating marble of Moscow Kremlin masonry [88].

Optimization of the bioprocess plays a crucial role in reducing the production cost of EPSs. The production depends on factors like physiological properties of employed microbial species and cultural condition. A maximum yield of EPSs of 18.5 g/L was observed when a strain of *Bacillus* sp. isolated from sugarcane field in Bhilai, India, was cultured at 37°C. The strain was able grow in a 4% NaCl, in a pH range of 3–10 and at a temperature range of 27–70°C [89]. The very high pH range of cultivation implies to a significant role of EPSs in protection of the microbe. EPS production of 12.9 g/L was also achieved by *Halobacillus trueperi* when cultivated at pH 9 and 35°C for 72 h [90]. When grown at 37°C and pH 8, a strain of *Exiguobacterium aurantiacum*, a member of the *Bacilli* class, isolated from Marchica lagoon in Morocco synthesized maximum of EPS, 2.6 g/L. Chemical analysis showed that the temperature and pH have no effect on the protein content of EPS, while the carbohydrate amount varied [91].

The EPSs of alkaliphiles represent a diverse group of biopolymers probably for their benefits in adaptation to alkaline environments. An exopolysaccharide obtained from an alkaliphilic *Bacillus* spp. was found along with D-galactopyranuronic acid (GalpA), 2,4-diacetamido-2,4,6-trideoxy-D-glucopyranose (QuipNAc4NAc), 2-acetamido-2-deoxy D-mannopyranuronic acid (ManpNAcA), and one uncommon unit of D-galactopyranuronic acid with the carboxyl group amide linked to

glycine [GalpA(Gly)]. The following linear structure of the repeating unit was established: →3)-α-D-GalpA(Gly)-(1→4)-β-D-ManpNAcA-(1→4)-α-D-GalpA-(1→3)-α-D-QuipNAc4NAc-(1→ [92]. The gross chemical composition of the EPS, produced by halophilic bacterium *Vagococcus carniphilus* from alkaline Lonar Lake, India, indicated content of 20% of protein and 75% neutral sugars. The monosaccharide composition revealed the presence of galactose and mannose with an additional three unidentified compounds. FTIR analysis confirmed the presence of an alkyl groups in the polysaccharide and a 75% similarity with standard dextran. The organism produced 1.1 g/L of EPS under optimized conditions [93].

Isolated from the radon hot spring, a thermophilic, endospore-forming, and radiation-resistant bacterium *Geobacillus* sp. produced an exopolysaccharide with the average molecular weight 3.2×10^6 Da and exhibited maximum growth at 60°C and pH 8.0. The EPS was a heteropolysaccharide, composed of mainly D-mannose, D-glucose, and rhamnose. The uronic acids and acetyl contents were at 18.6% and 6.1%, respectively. The EPS demonstrated good antioxidant activities and restrained proliferation of hepatoma carcinoma cells [86]. A thermophilic haloalkaliphilic *Bacillus licheniformis* strain, produced a novel 1,000 kDa exopolysaccharide. In particular, EPS1 displayed a high carbohydrate content (99%) with fructose and fucose as the major monosaccharides, low content of protein (1.2%), and 0.5% of uronic acid. Due to its structure, it could be a convenient thickener in industrial products. Under optimal growth conditions (50°C; pH 8 and 5% (w/v) NaCl), a 366 mg/L EPS production was achieved after 48 h of cultivation. The EPS was stable and exhibit decomposition at very high temperature, 240°C. Screening for biological activity showed its cytoprotective effect against Avarol in the brine shrimp test, indicating a potential use in the development of novel drugs [85]. Another thermostable EPS that degrades at a very high temperature (290°C) has been reported from haloalkaliphilic *Bacillus* sp. The amount of neutral sugars, uronic acid, and amino sugars were 52.4, 17.2, and 2.4%, respectively. Heteroglucan with Mw 2.2×10^6 Da had considerable flocculation activity and high gel strength [94]. The EPS produced by haloalkaliphilic *Methylophaga murata* Kr3 was composed of carbohydrate and protein moieties that could stabilize the cells and prevent them from drying [88]. Strain Kr3T represents a promising candidate for cost-effective large-scale production of EPS as the result of the ability to use methanol, methylamine, trimethylamine, and fructose as carbon and energy sources under a wide range of pHs and salinities.

The limitation for commercial applications of alkaliphiles EPS is associated with the lack of proper large-scale production. Overcoming such constrains by process development and with more proper bioreactor design specific for alkaliphiles will help in the commercialization of EPSs from alkaliphiles. Most probably, metabolic engineering of the alkaliphiles will be also required. Abundant EPSs production is often induced by special factors in the nutritional conditions (often a lack of a nutrient). This is controversial in increasing the production. While EPSs protect microbes, they can also prevent them to access to low-molecular opportunistic nutrient sources.

9 Antimicrobial Compounds

Discovery of antibiotics was a success story of mankind in many respects, enabling various basic medical studies, sophisticated surgical operations, and cancer therapies. Peak of the discovery of new antibiotics was soon after developing large-scale production of penicillin. Several classes of antibiotics were found in the 1950s and 1960s. After that "golden age," the main work was focused on chemical derivatization of known antibiotics. After the 1960s, the number of new useful antibiotics has diminished even when the needs have increased. The overuse of antibiotics for human and animal health has caused generation of multiresistant microbes. The rise in drug resistance for curing of microbial infections is not only life-threatening but also has a wider socioeconomic impact on mankind (http://www.un.org/sustainabledevelopment/sustainable-development-goals). Due to international efforts, the number of new drugs in pipelines has slightly increased during the last few years, but these efforts are far from adequate (WHO/EMP/IAU/2017.11). The cited WHO report lists the state of art in the main antibiotic classes as well as the priority for the pathogens. A new antibiotic is considered to be novel, if it (1) has no cross-resistance to existing antibiotics, (2) belongs to a new chemical class, (3) has a new physiological target, and (4) has new mechanism of action. Although many antibiotics have been proven to be efficient against Gram-positive bacteria, the situation with the Gram-negative pathogens is worse because of their complex membrane system. The WHO report focused on bacterial pathogens, but related problems exist as to other microbial pathogen groups and parasites.

How could alkaliphilic microbes serve as sources of novel antibiotics? Like other microbes, alkaliphiles certainly produce bioactive agents for competitive advantage. However, the research on the alkaliphiles was not conducted at the time of the "golden age" of antibiotics. Moreover, the pH_o (extracellular) is much higher than pH_i (intracellular); thus, the antibiotics must be alkali resistant and be able to bind or enter the cell. The entrance of the small-molecule antibiotics to bacterial cells might also be provided by cell wall-hydrolyzing enzymes which may weaken the cell wall (a role of some secreted enzymes of alkaliphiles?). One aspect is that the producer of an antibiotic must be itself "immune" to it. This might sometimes offer a key to study the mechanism of action of the produced antibiotics.

The maintenance of internal pH of alkaliphiles shall operate safely to avoid large pH fluctuations inside the cell. The maintenance system is based on cell wall and cell membrane and system containing ionic channels. Presumably, these channels are especially important for the surveillance of alkaliphiles. The diffusion rate of protons is extremely high, and their leakage to outside of the cell can be fatal. In all cells, the entrance of nutrients and ions through pores and channels is a weak point. There are many known antibiotics acting as ionophores and disturbing this traffic (e.g., bacteriocins, gramicidin, and valinomycin). It is conceivable that alkaliphiles have developed different strategies for protecting and attacking (microbe-microbe competition) the pores and channels in the cell membrane. Therefore, novel antibiotics

could be found among alkaliphiles. Other possible targets can be cell wall and synthesis of specific lipids found in alkaliphiles.

There are relatively many publications on isolation of antimicrobial compounds from various classes of alkaliphiles. The majority of the studies have only dealt with detection of the antimicrobial activity but not the analysis of the product structures or the mechanism of action. Seemingly, there is not so much practical value finding the same molecular structure from an alkaliphile which is already known from other sources and probably studied already including its production. However, there remains a probability that the main antibiotic components are synthetized with a set of different isomers, and it appears that one or more of the isomers are more active and/or more specific than the known drug. A well-known example is the fungal antibiotic cyclosporine, having many structural isomers (A, B, C, D) with a set of biological activities. The isomer spectrum is commonly specific to the producer strain. The antibiotics are often produced with special multienzyme complexes giving rise to a great number of variations of the products. Less accurate specificities of the enzymes in the complex add to the variation. The origin of the generation of the isomeric structures by the microbes may have the purpose of offering a wider-spectrum antibiotic. In spite of the possibility of finding new isomers from alkaliphiles, the most desirable target is a new antibiotic having the above-described innovative features (WHO/EMP/IAU/2017.11).

The methods for screening of antibiotics from alkaliphiles are often straightforward. Antibiotics are generally more or less hydrophobic. After cultivation, growth medium with or without cells are subjected to two-phase extraction or treated with hydrophobic polymers. Another way is to dry the medium and extract with organic solvents. After evaporation of the solvent, and redissolving in an aqueous phase, extracts can be used for preliminary testing the antimicrobial activities on Petri dishes inoculated with an indicator microbe. The raw extracts with antimicrobial activities are then studied further with chromatographic fractioning, and finally, the structures are determined with NMR, HPLC-MS, and other spectroscopic methods. A typical isolation and identification process for antifungal cyclic lipopeptides from a neutral-growing *Paenibacillus* sp. is described by Aktuganov et al. [95].

Most of the antibiotics from non-extreme terrestrial microorganisms are originated from actinomycetes. The genus *Streptomyces* is the well-known source of naturally occurring antimicrobial substances [96]. In recent years, the rate of discovery of novel compounds from microorganisms thriving in moderate environment has decreased, and repeated appearance of the same compounds has made them less attractive for screening programs [97]. Currently, research efforts have focused on microbes living in extreme habitats. Great taxonomical variety of alkaliphilic and moderately haloalkaliphilic bacterial isolates exhibiting antimicrobial activities were obtained from Lonar Lake, India [98–100]. Haloalkaliphilic actinomycetes such as *Nocardiopsis* sp. and *Streptomyces* sp. from the mud soil of solar salt works in India, in vitro, had effectively suppressed the growth of most of the pathogenic bacteria, as well as pathogenic fungi [101]. The haloalkaliphilic and alkaliphilic actinomycetes from saline desert of Kutch, India, produced the antimicrobial compounds against Gram-positive and Gram-negative bacteria [102, 103]. Alkaliphilic fungi which can

grow at pH 11 and exhibiting antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis* were also isolated [104]. The same researchers isolated other alkaliphiles which have shown significant activity against methicillin-resistant *S. aureus* (MRSA) [105]. Facultative anaerobic haloalkaliphilic strains, *Alkalibacterium kapii* ALK 6, *Marinilactibacillus psychrotolerans* ALK 9, and *Facklamia tabacinasalis* ALK 1, were isolated from a smear ecosystem originating from a commercial Raclette-type cheese and exhibited strong anti-listerial activity in situ on cheese surface [106].

Physicochemical parameters like temperature, pH, incubation period, and medium nutrients play a role in enhancing cell growth and increasing the yield of the target product. The traditional "one-factor-at-a-time" (OFAT) technique was used to increase the production of antibacterials of haloalkaliphilic actinomycetes such as *Streptomyces sannanensis* strain RJT-1 [107], *Streptomyces tanashiensis* strain A2D [108], *Streptomyces aburaviensis* Kut −8 [102, 103], *Streptomyces* sp. EWC [109], and *Streptomyces* spp. AJ8 [110]. Important media components affecting the antibacterial activity of the cyanobacteria, *Synechocystis aquatilis*, were evaluated with the help of statistical Plackett-Burman design [111]. In the study of Souagui et al. [112], antifungal production from *Streptomyces* sp. SY-BS5 was simultaneously optimized by the OFAT and response surface methodology, using a Box-Behnken design. In addition to these preliminary screening studies described above, there are reports on the production and detailed chemical characterization of a range of antibiotics produced by different alkaliphilic bacteria as described below. Some of them may be of less practical value, because of repetition of known molecules.

An aminoglycoside antibiotic substance isolated from *Bacillus alkalophilishaggy* JY-827 showed not only an excellent dental anticaries activity but also no toxicity against gingival cells when it was introduced into toothpaste. This substance was suggested to replace chlorhexidine used in toothpastes and thus have a high potential for commercial applications [113]. Dieter and co-workers identified pyrocoll (which is known as a synthetic compound and constituent of cigarette smoke) to be one of the metabolites produced by alkaliphilic *Streptomyces* sp. AK 409 from a steel waste tip soil. The compound showed biological activity against various *Arthrobacter* strains, filamentous fungi, several pathogenic protozoa, and some human tumor cell lines [114]. Two metabolites from the culture filtrate of alkaliphilic *Streptomyces sanglieri* AK 623 were structurally elucidated as lactonamycin Z, a new derivative of lactonamycin, and its aglycone, lactonamycinone. Lactonamycin Z showed weak activity against Gram-positive bacteria, but it strongly inhibited the proliferation of gastric adenocarcinoma cells [115]. By using genetic data, instead of isolation, a two-component new lantibiotic, haloduracin, was identified in the genome of the Gram-positive alkaliphilic *Bacillus halodurans* C-125. Haloduracin was active against a large number of Gram-positive bacteria, including many lactococci and representative of *Listeria*, *Streptococcus*, *Enterococcus*, *Bacillus*, and *Pediococcus* species. However, haloduracin did not owe activity against Gram-negative bacteria [116]. Later on, an optimized production of haloduracin by another strain of *Bacillus halodurans* was achieved by solid state fermentation [117].

Two alkaliphiles, *Nocardiopsis dassonvillei* WA52 from Wadi Araba, Egypt [118] and alkaliphilic *Streptomyces werraensis* isolated from Rajkot, India [119] were found to be new producers of the macrolide antibiotic, erythromycin. A new pyranonaphthoquinone antibiotic, griseusin D was isolated from the cultural fluid of the alkaliphilic *Nocardiopsis* sp. It displayed strong cytotoxicity against human leukemia cells (HL60) and modest cytotoxicity against human lung adenocarcinoma cell lines (AGZY) and exhibited weak antifungal activity against *Alternaria alternata* [120]. Two other griseusins, F and G, were obtained from an alkalophilic *Nocardiopsis* sp. (YIM DT266) isolated from tin mine tailings [121]. Both these griseusins displayed antibacterial activity toward *Micrococcus luteus*, *S. aureus* ATCC 29213, and *B. subtilis*. Naphthospironone A, a novel polycyclic metabolite with an unprecedented spiro[bicyclo[3.2.1]octene-pyran]dione ring system, was isolated from alkalophilic *Nocardiopsis* sp. YIM DT266. Its complete structure with the absolute stereochemistry was elucidated with spectroscopy, X-ray crystallography, and optical rotation data. Naphthospironone A exhibited antibiotic bioactivities with moderate cytotoxicity [122]. A broad-spectrum antifungal compound was produced by salt-tolerant alkaliphilic *Streptomyces violascens* IN2–10. It was partially characterized, and the results indicate that it belongs to the heptaene group of polyene class antibiotics [123]. Two new pyrones named Nocardiopyrones A and B along with other identified compounds, (three known pyridinols and 1-acetyl-β-carboline) were obtained from *Nocardiopsis alkaliphila* YIM-80379 that was isolated from a desert soil sample [124]. Nocardiopyrone A was active against *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, and *Escherichia coli*. Nocardiopyrone B was active against *P. aeruginosa*, *E. aerogenes*, *E. coli*, *Staphylococcus aureus*, and *Candida albicans*.

A novel angucycline-type antibiotic, warkmycin, was isolated from the culture filtrate of alkaliphilic *Streptomyces* strain Acta 2930. Its chemical structure was elucidated by HR-MS and one-dimensional and two-dimensional NMR. The compound inhibits the growth of Gram-positive bacteria and shows a strong antiproliferative activity against mouse fibroblast cell line NIH-3T3 and human cancer cell lines HepG2 and HT29 [125]. Secondary metabolites isolated from haloalkaliphilic *Streptomyces castaneoglobisporus* AJ9 were structurally characterized by GC-MS analysis and confirmed as the benzoic acid derivatives of 2-hydroxy-4-methoxy-6-methyl-, methyl ester. The metabolites significantly suppressed bacterial, fungal, and viral pathogens in vitro and were able to kill the L 929 cancer cells with an efficiency of 76% [126]. There exists a considerable interest for using bacteriophages as antibacterial agents in medicine and agriculture. Bacteriophages are abundant and extremely diversified and are surviving well under unfavorable conditions including alkaline pH. However, phages do not seem to be stable for extended period in pH values of above 9 [127]. Phages that exist in alkaline environments should have a means to survive in the high pH habitat for long time. However, these phages are not yet well studied, and exceptions may be found which can be potentially used to control certain microbes. Because the structures of phages contain a minimum of components (DNA or RNA and capsid), they can be used to

study the effects of alkaline environment. Possibly, phages could also serve as gene vectors for alkaliphiles.

10 Patent Landscape of Alkaliphilic Microbes

Patent searches were done from various databases with a goal to understand patent activities in the area of alkaliphilic microorganisms. The search was focused to alkaliphiles in biodegradation, environmental remediation, and in production of useful compounds for industrial and pharmaceutical uses. Unfortunately, only seldom in the patent literature a microbe is characterized as being "an alkaliphile" or being "alkaliphilic." Therefore, such terms are not effective. One should search databases microbe by microbe, area of use, or their combination.

Illustratively, with the terms "alkaliphilic," "alkalophilic," "alkalophile," or "alkaliphile," altogether 241 patent publications were found since 1966 from the database of World Intellectual Property Organization (WIPO) (http://www.patentscope.int/). Almost the same number (247) of hits is received by starting with broad search with the term "extremophile" and then limiting the search with negative limitations "not algae," "not plant," "not thermophile," and "not acidophile." A large number (135) of these publications, claimed novel alkaline-active enzymes and their production for industrial use. Major part of the publications related to industrial enzymes is published before 2012. Among the applicants, Novo Nordisk AS and Genencor Int. are prevailing. The enzymes from alkaliphiles are discussed in more details elsewhere in this book.

Of the 241 patent publications, 50 disclose the use of alkaliphilic microbes in wastewater treatment. All these patents have been filed since 2008. Twenty-one of the 241 disclosed methods and compositions for agriculture, including the use of microbe compositions as fertilizers or insecticides, as well as accelerators of composting. Fourteen deals about treatment of polluted soil. Figure 4 illustrates patent publications on alkaliphilic microbes according to the filing country.

Searching the patent databases (WIPO database at www.patentscope.wipo.int, US Patent Office's database at www.USPTO.gov; European Patent Office's database at www.espacenet.com; and Google patents) with terms such as "bioplastics," "biodegradable," and "bioremediation" was also done. The search terms combined with names of microbes give accurate results. However, it was still difficult to have information related to alkaliphilic microbes in general. Based on the patent literature retrieved, there is industrial interest in alkaliphilic microbes beyond the enzyme industries (Fig. 5). One branch of inventions originates from the genomic studies and another the use of alkaliphilic microbes in environmental applications.

Due to the fact that alkaliphilic bacteria have to keep the inside of the cell pH lower than the outside pH, the ion channels of alkaliphiles possess not only academic but also industrial interest. Examples of patents on this field are US patent 7,790,845 for calcium-selective ion channel cloned and characterized from *Bacillus halodurans*, US patent 5,346,815 for a sodium-binding protein of *Bacillus*

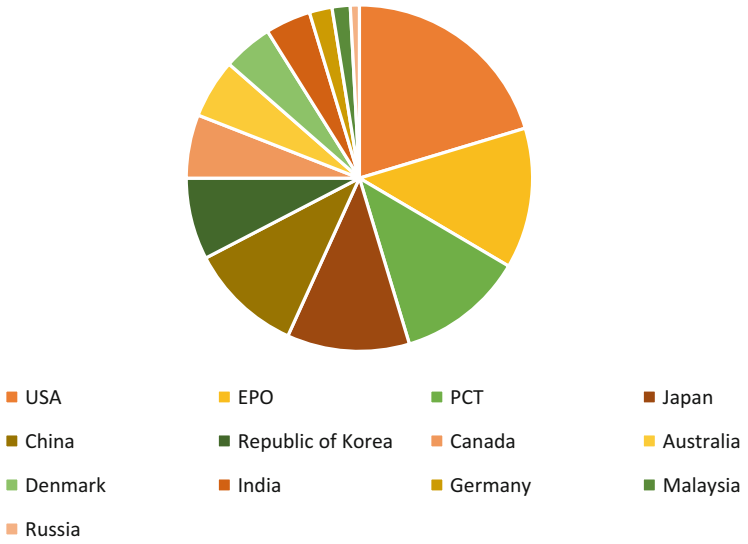


Fig. 4 Illustrates proportional distribution of the filing countries

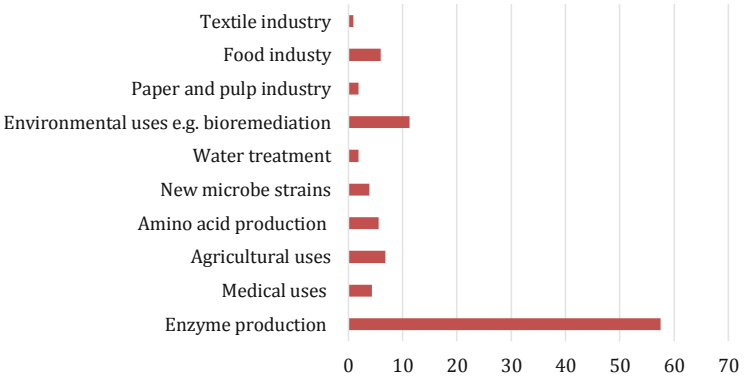


Fig. 5 The patents percentual distribution to different areas of applications

pseudofirmus OF 4, and Chinese patent application CN103087165 for sodium hydrogen pump protein of *Bacillus pseudofirmus* OF 4.

Alkaliphiles in the treatment of polluted water and soil are noticeable. Examples of alkaliphilic microorganisms in bioremediation are patent publications WO2007085039 and US20180050943 for ALCOA, an Australian well-known company specialized in bauxite mining and alumina refinery. Methods are described for bioremediation of red muds, which are highly alkaline waste composed mainly of iron oxide generated during the refinery of alumina. The French Institute de Recherche pour le Development (IRD) has been granted a US patent

(US7,862,721) on method for decontaminating heavy metal-containing flows by culturing alkaliphilic sulfate-reducing bacteria. Geo Fossil Fuels, LLC is a Colorado-based energy company engaging in microbial enhanced oil recovery. An example of their portfolio is US patent 9,290,688 for a method of enhanced oil recovery using natural or genetically engineered obligatory alkaliphilic microbes. US patent application publication 20,170,313,948 claims a process for biodegradation of hydrocarbons in an oily sludge. The method applies a mixture of soil bacteria cultivations, including alkaliphiles. *Exiguobacterium* isolates capable of neutralizing wastewater have been protected (e.g., US 7,396,671) for Council of Scientific and Industrial Research (India).

Alkaliphilic bacteria in agriculture are exemplified by the Chinese patent application CN102515977 that discloses a water-holding root-promoting halophilic alkalophilic microorganism and a root dipping liquid comprising such microbe. Japanese patent application 200,184,685 discloses a material comprising alkaliphilic bacterial to improve soil by converting alkaline soil to become more suitable for agriculture.

Patents related to the development of bioplastics are exemplified by the US patent 8,841,113 issued in 2014 for Japanese National Institute of Advanced Industrial Science and Technology, in which a novel alkaliphilic bacterium (a strain of *Enterococcus casseliflavus*) was patented for producing lactic acid. Japanese patent publication JP2000245491 discloses a method to prepare raw material for bioplastics by culturing *Streptococcus lactobacillus* in distillery waste of Imojotyu (liquor distilled from sweet potato).

Getting an integral picture of patents around alkaliphilic microbes is not straightforward (excluding the enzymes) due to the variety of the microbes. The examples above are intended to give an idea of the areas of patent activities. Since the patent literature becomes public only 18 months after the filing, the most recent patent applications are not yet even public. Within next few years, one can expect a boom in the number of patent publications in areas such as treating or processing microplastic waste.

References

1. Horikoshi K (1999) Alkaliphiles: some applications of their products for biotechnology. Microbiol Mol Biol Rev 63:735–750. http://www.currentscience.ac.in/Volumes/114/04/0845.pdf
2. Paavilainen S (1995) Carbohydrate catabolism in alkaliphilic bacilli. Academic Dissertation, University of Turku, Finland. Annales Universitatis Turkuensis. ISBN 951-29-0628-7
3. Doelle H (1975) Bacterial metabolism, 2nd edn. Academic Press, New York
4. Paavilainen S, Oinonen S, Korpela T (1999) Catabolic pathways of glucose in *Bacillus circulans* var. *alkalophilus*. Extremophiles 3:269–276
5. Paavilainen S, Helistö P, Korpela T (1994) Conversion of carbohydrates to organic acids by alkaliphilic bacilli. J Ferment Bioeng 78:217–222

6. Goto T, Matsuno T, Hishinuma-Narisawa M, Yamazaki K, Matsuyama H, Inoue N, Yumoto I (2005) Cytochrome c and bioenergetic hypothetical model for alkaliphilic *Bacillus* spp. J Biosci Bioeng 100(4):365–379. https://doi.org/10.1263/jbb.100.365
7. Matsuno T, Yumoto I (2015) Bioenergetics and the role of soluble cytochrome c for alkaline adaptation in alkaliphilic *Pseudomonas*. Biomed Res Int 2015:847945
8. Mäkelä M, Paavilainen S, Korpela T (1990) Growth dynamics of cyclomatodextrin glucanotransferase-producing *Bacillus circulans* var. *alkalophilus*. Can J Microbiol 36:176–182
9. Mäkelä M, Paavilainen S, Korpela T (1990) A device for automatic sampling of microbial culture fluids. Lab Pract 37:69–70
10. Paavilainen S, Mäkelä M, Korpela T (1995) Proton and carbon inventory during the growth of an alkaliphilic *Bacillus* indicates that protons are independent from acid anions. J Ferment Bioeng 80:429–433
11. Preiss L, Hicks D, Suzuki S, Meier T, Krulwich T (2015) Alkaliphilic bacteria with impact on industrial applications, concepts of early life forms, and bioenergetics of ASTP synthesis. Front Bioeng Biotechnol 3:1–16
12. Hirabayashi T, Goto T, Morimoto H, Yoshimune K, Matsyama H, Yumoto I (2012) Relationship between rates of respiratory proton extrusion and ATP synthesis in obligately alkaliphilic *Bacillus clarkii* DSM 8720. J Bioenerg Biomembr 44:265–272
13. Kulshreshtha N, Kumar A, Dhall P, Gupta S, Gopal B, Pasha S, Singh V, Kumar R (2010) Neutralization of alkaline industrial waste waters using *Exiguobacterium* sp. Int Biodeter Biodegr 64:191–196
14. Kulshreshtha N, Kumar A, Gobal B, Pasha S, Kumar R (2012) Usefulness of organic acids produced by *Exiguobacterium* sp. 12/1 on neutralization of alkaline waste water. Sci World J 2012:345101
15. Yokaryo H, Tokiwa Y (2014) Isolation of alkaliphilic bacteria for production of high optically pure L-(+)-lactic acid. J Gen Appl Microbiol 60:270–275
16. Assavvasirinda N, Ge D, Yu B, Xue Y, Ma Y (2016) Efficient fermentative production of polymer-grade D-lactate by an engineered alkaliphilic *Bacillus* sp. strain under non-sterile conditions. Microb Cell Fact 15(1):3. https://doi.org/10.1186/s12934-015-0408-0
17. Kanekar P, Joshi A, Kelkar A, Boregave S, Sarnaik S (2008) Alkaline Lonar lake, India – a treasure of alkaliphilic and halophilic bacteria. In: Sengupta M, Dalawani R (eds) Proceedings of Taal 2007: the 12th world lake conference, pp 1765–1774
18. Zhao R, Yan Y, Chen S (2014) How could haloalkaliphilic microorganisms contribute to biotechnology? Can J Microbiol 60:717–727
19. Li Z, Yang J, Loh XJ (2016) Polyhydroxyalkanoates: opening doors for a sustainable future. NPG Asia Mater 8(4):e265
20. Chen G, Jiang X (2018) Engineering microorganisms for improving polyhydroxyalkanoate biosynthesis. Curr Opin Biotechnol 53:20–25
21. Janto B, Ahmed A, Ito M, Liu J, Hicks DB, Pagni S, Fackelmayer OJ, Smith TA, Earl J, Elbourne LD, Hassan K, Paulsen IT, Koplsto AB, Tourasse NJ, Ehrlich GD, Boissy R, Ivey DM, Li G, Xue Y, Ma Y, Krulwich TA (2011) Genome of alkaliphilic *Bacillus pseudofirmus* OF4 reveals adaptations that support the ability to grow in an external pH range from 7.5 to 11.4. Environ Microbiol 13:3289–3309
22. Roberts MF (2005) Organic compatible solutes of halotolerant and halophilic microorganisms. Saline Syst 1(5):1–36
23. Korpela T, Mattinen J, Himanen JP, Mekhanic M, Torchinsky Y (1987) Phosphorus -31 nuclear magnetic resonance of aspartate aminotransferase from chicken heart cytosol. Biochem Biophys Acta 915:299–304
24. Meretting-Bruns U, Jelen E (2009) Anaerobic biodegradation of detergent surfactants. Materials 2:181–206. https://doi.org/10.3390/ma2010181. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5445686/

25. Santos DKF, Rufino RD, Luna JM, Santos VA, Sarubbo LA (2016) Biosurfactants: multifunctional biomolecules of the 21st century. Int J Mol Sci 17(3):401. https://doi.org/10.3390/ijms17030401
26. Fakruddin M (2012) Biosurfactant: production and application. J Pet Environ Biotechnol 3(4):124. https://doi.org/10.4172/2157-7463.1000124
27. Sáenz-Marta CI, Ballinas-Casarrubias M, Rivera-Chavira BE, Nevárez-Moorillón GV (2015) Biosurfactants as useful tools in bioremediation. In: Shiomi N (ed) Advances in bioremediation of wastewater and polluted soil, 2nd edn. IntechOpen, Rijeka. https://doi.org/10.5772/60751. https://www.intechopen.com/books/advances-in-bioremediation-of-wastewater-and-polluted-soil/biosurfactants-as-useful-tools-in-bioremediation
28. Vijayakumar S, Saravanan V (2015) Biosurfactants-types, sources and applications. Res J Microbiol 10(5):181–192. https://doi.org/10.3923/jm.2015.181.192
29. Barakat KM, Hassan SWM, Darwesh OM (2017) Biosurfactant production by haloalkaliphilic *Bacillus* strains isolated from Red Sea, Egypt. Egypt J Aquat Res 43:205–211. https://doi.org/10.1016/j.ejar.2017.09.001
30. Elazzazy AM, Abdelmoneim TS, Almaghrabi OA (2015) Isolation and characterization of biosurfactant production under extreme environmental conditions by alkali-halo-thermophilic bacteria from Saudi Arabia. Saudi J Biol Sci 22:466–475. https://doi.org/10.1016/j.sjbs.2014.11.018
31. Jain RM (2013) Application of alkaliphilic bacteria in bioremediation. PhD thesis, Maharaja Krishnakumarsinhji Bhavnagar University. http://hdl.handle.net/10603/36099
32. Selim SA, Saher MEA, Hagagy NI, Hassanin AAI, Khattab RM, El-Meleigy ESA, Aziz MHA, Maugeri TL (2012) Oil-biodegradation and biosurfactant production by haloalkaliphilic *Archaea* isolated from Soda lakes of the Wadi An Natrun, Egypt. J Pure Appl Microbiol 6(3):1011–1020
33. Shende AM (2013) Studies on biosurfactant from *Exiguobacterium* sp. Sci Res Rep 3(2):193–199
34. Tambekar DH, Dose PN, Gunjakar SR, Gadakh PV (2012) Studies on biosurfactant production from Lonar Lake's *Achromobacter xylosoxidans* bacterium. Int J Adv Pharm Biol Chem 1(3):415–419. www.ijapbc.com
35. Zarinviarsagh M, Ebrahimipour G, Sadeghi H (2017) Lipase and biosurfactant from *Ochrobactrum intermedium* strain MZV101 isolated by washing powder for detergent application. Lipids Health Dis 16:177. https://doi.org/10.1186/s12944-017-0565-8
36. Dukhande M, Ward M (2016) Production of biosurfactant by a marine alkaliphilic strain of *Pseudomonas aeruginosa* and effect of various physico-chemical parameters on its production. Int J Sci Res 5(5):1233–1238. https://www.ijsr.net/archive/v5i5/v5i5.php
37. Yan Z, Zhang Y, Wu H, Yang M, Zhang H, Hao Z, Jiang H (2017) Isolation and characterization of a bacterial strain *Hydrogenophaga* sp. PYR1 for anaerobic pyrene and benzo[a] pyrene biodegradation. RSC Adv 7:46690–46698. https://doi.org/10.1039/c7ra09274a
38. Jain RM, Mody K, Joshi N, Mishra A, Jha B (2013) Production and structural characterization of biosurfactant produced by an alkaliphilic bacterium, *Klebsiella* sp.: evaluation of different carbon sources. Colloids Surf B Biointerfaces 108:199–204. https://doi.org/10.1016/j.colsurfb.2013.03.002
39. Jain RM, Mody K, Mishra A, Jha B (2012) Isolation and structural characterization of biosurfactant produced by an alkaliphilic bacterium *Cronobacter sakazakii* isolated from oil contaminated wastewater. Carbohydr Polym 87:2320–2326. https://doi.org/10.1016/j.carbpol.2011.10.065
40. Jain RM, Mody K, Mishra A, Jha B (2012) Physicochemical characterization of biosurfactant and its potential to remove oil from soil and cotton cloth. Carbohydr Polym 89:1110–1116. https://doi.org/10.1016/j.carbpol.2012.03.077
41. Ali SS, Vidhale NN (2013) Bacterial siderophore and their application: a review. Int J Curr Microbiol App Sci 2(12):303–312. http://www.ijcmas.com
42. Johnstone T, Nolan E (2015) Beyond iron: nonclassical biological functions of bacterial siderophores. Dalton Trans 44:6320–6339

43. Ahmed E, Holmström S (2014) Siderophores in environmental research. Microb Biotechnol 7(3):196–208. https://doi.org/10.1111/1751-7915.12117
44. Nosrati R, Dehghani S, Karimi B, Yousefi M, Taghdisi SM, Abnous K, Alibolandi M, Ramezani M (2018) Siderophore-based biosensors and nanosensors; new approach on the development of diagnostic systems. Biosens Bioelectron 117(15):1–14. https://doi.org/10.1016/j.bios.2018.05.057
45. Hagan A (2017) Siderophores: bacterial iron scavengers? Microbiol Sci. https://www.asm.org/index.php/general-science-blog/item/6411
46. Behnsen J, Raffatellu M (2016) Siderophores: more than stealing iron. MBio 7(6):e01906–e01916. https://doi.org/10.1128/mBio.01397-16
47. Aznar A, Dellagi A (2015) New insights into the role of siderophores as triggers of plant immunity: what we can learn from animals. J Exp Bot 66:3001–3010. https://academic.oup.com/jxb/article/66/11/3001/467272
48. Wencewicz TA, Miller MJ (2017) Sideromycins as pathogen-targeted antibiotics. In: Fisher J, Mobashery S, Miller M (eds) Antibacterials. Topics in medicinal chemistry, vol 26. Springer, Cham. https://doi.org/10.1007/7355_2017_19
49. De Serrano LO (2017) Biotechnology of siderophores in high-impact scientific fields. Biomol Concepts 8(3–4):169–178. https://doi.org/10.1515/bmc-2017-0016
50. De Serrano LO, Camper AK, Richards AM (2016) An overview of siderophores for iron acquisition in microorganisms living in the extreme. Biometals 29:551–571. https://doi.org/10.1007/s10534-016-9949-x
51. McMillan DGG, Velasquez I, Nunn BL, Goodlett DR, Hunter KA, Lamont I, Sander SG, Cook GM (2010) Acquisition of iron by alkaliphilic *Bacillus* species. Appl Environ Microbiol 76(20):6955–6961. https://doi.org/10.1128/AEM.01393-10
52. Ye Q, Roh Y, Carroll SL, Blair B, Zhou J, Zhang CL, Fields MW (2004) Alkaline anaerobic respiration: isolation and characterization of a novel alkaliphilic and metal-reducing bacterium. Appl Environ Microbiol 70(9):5595–5602. https://doi.org/10.1128/AEM.70.9.5595–5602.2004
53. Figueroa LS, Schwarz B, Richards AM (2015) Structural characterization of amphiphilic siderophores produced by a soda lake isolate, *Halomonas* sp. SL01, reveals cysteine-, phenylalanine- and proline-containing head groups. Extremophiles 19:1183–1192. https://doi.org/10.1007/s00792-015-0790-x
54. Cabello P, Luque-Almagro VM, Olaya-Abril A, Saez LP, Moreno-Vivian C, Roldan MD (2018) Assimilation of cyanide and cyano-derivatives by *Pseudomonas pseudoalcaligenes* CECT5344: from omic approaches to biotechnological applications. FEMS Microbiol Lett 365:fny032. https://doi.org/10.1093/femsle/fny032
55. Ibañez MI, Cabello P, Luque-Almagro VM, Saez LP, Olaya A, Sanchez de Medina V et al (2017) Quantitative proteomic analysis of *Pseudomonas pseudoalcaligenes* CECT5344 in response to industrial cyanide-containing wastewaters using Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS). PLoS One 12(3):e0172908. https://doi.org/10.1371/journal.pone.0172908
56. Kumar SV, Menon S, Agarwal H, Gopalakrishnan D (2017) Characterization and optimization of bacterium isolated from soil samples for the production of siderophores. Resour Effic Technol 3(4):434–439. https://doi.org/10.1016/j.reffit.2017.04.004
57. Sandmann G (2015) Carotenoids of biotechnological importance. Adv Biochem Eng Biotechnol 148:449–467. https://doi.org/10.1007/10_2014_277
58. Sathasivam R, Ki J-S (2018) A review of the biological activities of microalgal carotenoids and their potential use in healthcare and cosmetic industries. Mar Drugs 16(26):19. https://doi.org/10.3390/md16010026
59. Avalos J, Pardo-Medina J, Parra-Rivero O, Ruger-Herreros M, Rodríguez-Ortiz R, Hornero-Méndez D, Limón MC (2017) Carotenoid biosynthesis in fusarium. J Fungi 3:39. https://doi.org/10.3390/jof3030039

60. Gharibzahedi SMT, Razavi SH, Mousavi M (2014) Carotenoid production from hydrolyzed molasses by *Dietzia natronolimnaea* HS-1 using batch, fed-batch and continuous culture. Ann Microbiol 64:945–953. https://doi.org/10.1007/s13213-013-0728-4
61. Yabuzaki J (2017) Carotenoids database: structures, chemical fingerprints and distribution among organisms. Database 2017:bax004. https://doi.org/10.1093/database/bax004
62. Sarethy IP, Saxena Y, Kapoor A, Sharma M, Sharma SK, Gupta V, Gupta S (2011) Alkaliphilic bacteria: applications in industrial biotechnology. J Ind Microbiol Biotechnol 38:769–790. https://doi.org/10.1007/s10295-011-0968-x
63. Asao M, Takaichi S, Madigan MT (2012) Amino acid-assimilating phototrophic heliobacteria from soda lake environments: *Heliorestis acidaminivorans* sp. nov. and 'Candidatus Heliomonas lunata'. Extremophiles 16:585–595. https://doi.org/10.1007/s00792-012-0458-8
64. Osanjo GO, Muthike EW, Tsuma L, Okoth MW, Bulimo WD, Lünsdorf H, Abraham W-R, Dion M, Timmis KN, Golyshin PN, Mulaa FJ (2009) A salt lake extremophile, *Paracoccus bogoriensis* sp.nov., efficiently produces xanthophyll carotenoids. Afr J Microbiol Res 3(8):426–433. http://www.academicjournals.org/ajmr
65. Venugopalan V, Subhash KT, Pradip N, Saradhi PP, Das RH, Gautam HK (2013) Characterization of canthaxanthin isomers isolated from a new soil *Dietzia* sp. and their antioxidant activities. J Microbiol Biotechnol 23(2):237–245. https://doi.org/10.4014/jmb.1203.03032
66. Bryantseva I, Gorlenko VM, Kompantseva E, Imhoff JF, Süling J, Mityushina L (1999) *Thiorhodospira sibirica* gen. nov., sp. nov., a new alkaliphilic purple sulfur bacterium from a Siberian soda lake. Int J Syst Bacteriol 49:697–703
67. Gorlenko VM, Bryantseva IA, Panteleeva EE, Tourova TP, Kolganova TV, Makhneva ZK, Moskalenko AA (2004) *Ectothiorhodosinus mongolicum* gen. nov., sp. nov., a new purple bacterium from a soda lake in Mongolia. Microbiology 73(1):66–73
68. Gorlenko VM, Bryantseva IA, Rabold S, Tourova TP, Rubtsova D, Smirnova E, Thiel V, Imhoff JF (2009) *Ectothiorhodospira variabilis* sp. nov., an alkaliphilic and halophilic purple sulfur bacterium from soda lakes. Int J Syst Evol Microbiol 59:658–664. https://doi.org/10.1099/ijs.0.004648-0
69. Bryantseva IA, Tourova TP, Kovaleva OL, Kostrikina NA, Gorlenko VM (2010) *Ectothiorhodospira magna* sp. nov., a new large alkaliphilic purple sulfur bacterium. Microbiology 79(6):780–790. https://doi.org/10.1134/S002626171006010X
70. Bryantseva IA, Gorlenko VM, Kompantseva EI, Imhoff JF (2000) *Thioalkalicoccus limnaeus* gen. nov., sp. nov., a new alkaliphilic purple sulfur bacterium with bacteriochlorophyll b. Int J Syst Evol Microbiol 50:2157–2163
71. Boldareva EN, Moskalenko AA, Makhneva ZK, Tourova TP, Kolganova TV, Gorlenko VM (2009) *Rubribacterium polymorphum* gen. nov., sp. nov., a novel alkaliphilic nonsulfur purple bacterium from an eastern Siberian soda lake. Microbiology 78(6):732–740. https://doi.org/10.1134/S0026261709060101
72. Boldareva EN, Tourova TP, Kolganova TV, Moskalenko AA, Makhneva ZK, Gorlenko VM (2009) *Roseococcus suduntuyensis* sp. nov., a new aerobic bacteriochlorophyll α-containing bacterium isolated from a low-mineralized soda lake of Eastern Siberia. Microbiology 78(1):92–101. https://doi.org/10.1134/S0026261709010123
73. Bryantseva IA, Gaisin VA, Gorlenko VM (2015) *Rhodobaculum claviforme* gen. nov., sp. nov., a new alkaliphilic nonsulfur purple bacterium. Microbiology 84(2):247–255. https://doi.org/10.1134/S0026261715020022
74. Nuyanzina-Boldareva EN, Gorlenko VM (2014) *Roseibacula alcaliphilum* gen. nov. sp. nov., a new alkaliphilic aerobic anoxygenic phototrophic bacterium from a meromictic soda lake Doroninskoe (East Siberia, Russia). Microbiology 83(4):381–390. https://doi.org/10.1134/S0026261714040134
75. Takaichi S, Jung DO, Madigan MT (2001) Accumulation of unusual carotenoids in the spheroidene pathway, demethylspheroidene and demethylspheroidenone, in an alkaliphilic purple nonsulfur bacterium *Rhodobaca bogoriensis*. Photosynth Res 67:207–214. https://doi.org/10.1023/A:1010666406176
76. Banciu H, Sorokin DY, Rijpstra WIC, Damste JSS, Galinski EA, Takaichi S, Muyzer G, Kuenen JG (2005) Fatty acid, compatible solute and pigment composition of obligately

chemolithoautotrophic alkaliphilic sulfur-oxidizing bacteria from soda lakes. FEMS Microbiol Lett 243:181–187
77. Takatani N, Nishida K, Sawabe T, Maoka T, Miyashita K, Hosokawa M (2014) Identification of a novel carotenoid, 2′-isopentenylsaproxanthin, by *Jejuia pallidilutea* strain 11shimoA1 and its increased production under alkaline condition. Appl Microbiol Biotechnol 98:6633–6640. https://doi.org/10.1007/s00253-014-5702-y
78. Zav'yalov V, Zavialov A, Zav'yalova G, Korpela T (2010) Adhesive organelles of Gram-negative pathogens assembled with the classical chaperone/usher machinery: structure and function from a clinical standpoint. FEMS Microbiol Rev 34(3):317–378. https://doi.org/10.1111/j.1574-6976.2009.00201.x
79. Nicolaus B, Kambourova M, Oner E (2010) Exopolysaccharides from extremophiles: from fundamentals to biotechnology. Environ Technol 31:1145–1158
80. Korpela T, Mattsson P, Hellman J, Paavilainen S, Mäkelä M (1989) Cyclodextrins: production, properties and applications in food chemistry. Food Biotechnol 2:199–210
81. Mattsson P, Korpela T, Mäkelä M (1990) Methods to produce starch syrups containing cyclodextrins. Finnish patent no. 8116
82. Mattsson P, Meklin S, Korpela T (1990) Analysis of cyclomaltodextrin glucanotransferase isoenzymes by isoelectric focusing in immobilized pH gradients. J Biochem Biophys Methods 20:237–246
83. Mattsson P, Korpela T, Paavilainen S, Mäkelä M (1991) Enhanced conversion of starch to cyclodextrins in ethanolic solutions by *Bacillus circulans* var *alkalophilus* cyclomaltodextrin glucanotransferase. Appl Biochem Biotechnol 30:17–28
84. Salminen L, Uosukainen M, Mattsson P, Korpela T (1990) Action of cyclodextrins on germinating seeds and on micropropagated plants. Starch 42:350–353
85. Spano A, Gugliandolo C, Lentini V, Maugeri TL, Anzelmo G, Poli A, Nicolaus B (2013) A novel EPS-producing strain of *Bacillus licheniformis* isolated from a shallow vent off Panarea Island (Italy). Curr Microbiol 67:21–29. https://doi.org/10.1007/s00284-013-0327-4
86. Wang L, Zhang H, Yang L, Liang X, Zhang F, Linhardt RJ (2017) Structural characterization and bioactivity of exopolysaccharide synthesized by *Geobacillus* sp. TS3-9 isolated from radioactive radon hot spring. Adv Biotechnol Microbiol 4(2):555634. https://doi.org/10.19080/AIBM.2017.04.555635
87. Poli A, Esposito E, Orlando P, Lama L, Giordano A, de Appolonia F, Nicolaus B, Gambacorta A (2007) *Halomonas alkaliantarctica* sp. nov., isolated from saline lake Cape Russell in Antarctica, an alkalophilic moderately halophilic, exopolysaccharide-producing bacterium. Syst Appl Microbiol 30(1):31–38. https://doi.org/10.1016/j.syapm.2006.03.003
88. Doronina NV, Lee TD, Ivanova EG, Trotsenko YA (2005) *Methylophaga murata* sp. nov.: a haloalkaliphilic aerobic methylotroph from deteriorating marble. Microbiology 74(4):440–447
89. Shukla P, Patel N, Mohan RR, Shukla J, Verma S et al (2011) Isolation and characterization of polyhydroxyalkanoate and exopolysaccharide producing *Bacillus* sp. PS1 isolated from sugarcane field in Bhilai, India. J Microb Biochem Technol 3:33–35. https://doi.org/10.4172/1948-5948.1000048
90. Arun J, Sathishkumar R, Muneeswaran T (2014) Optimization of extracellular polysaccharide production in *Halobacillus trueperi* AJSK using response surface methodology. Afr J Biotechnol 13(48):4449–4457. https://doi.org/10.5897/AJB2014.14109
91. Dah Dossounon YD, Lee K, Belghmi K, Benzha F, Blaghen M (2016) Exopolysaccharide (EPS) production by *Exiguobacterium aurantiacum* isolated from Marchica lagoon ecosystem in Morocco. Am J Microbiol Res 4(5):147–152. https://doi.org/10.12691/ajmr-4-5-4
92. Corsaro MM, Grant WD, Grant S, Marciano CE, Parrilli M (1999) Structure determination of an exopolysaccharide from an alkaliphilic bacterium closely related to *Bacillus* spp. Eur J Biochem 264:554–561
93. Joshi AA, Kanekar PP (2011) Production of exopolysaccharide by *Vagococcus carniphilus* MCM B-1018 isolated from alkaline Lonar Lake, India. Ann Microbiol 61(4):733–740. https://doi.org/10.1007/s13213-010-0189-y

94. Kumar CG, Joo H-S, Choi J-W, Koo Y-M, Chang C-S (2004) Purification and characterization of an extracellular polysaccharide from haloalkaliphilic *Bacillus* sp. I-450. Enzym Microb Technol 34(7):673–681. https://doi.org/10.1016/j.enzmictec.2004.03.001
95. Aktuganov G, Jokela J, Kivelä H, Khalikova E, Melentjev A, Galimsianova N, Kuzmina L, Kouvonen P, Himanen J-P, Susi P, Korpela T (2014) Isolation and identification of cyclic lipopeptides from *Paenibacillus ehimensis* strain IB-X-b. J Chromatogr B 973:9–16
96. Watve MG, Tickoo R, Jog MM, Bhole BD (2001) How many antibiotics are produced by the genus Streptomyces? Arch Microbiol 176:386–390
97. Sarker AK, Haque MA, Saha U, Rahman MA, Islam MAU (2015) Evaluation of antibacterial, antifungal and cytotoxic potentials of crude metabolite of ANAM-39, a marine bacterium isolated from Sundarbans, Bangladesh. Bangladesh Pharm J 18:103–109. https://doi.org/10.3329/bpj.v18i2.24306
98. Borgave SB, Joshi AA, Kelkar AS, Kanekar PP (2012) Screening of alkaliphilic, haloalkaliphilic and alkalithermophilic actinomycetes isolated from alkaline soda lake of Lonar, India for antimicrobial activity. Int J Pharm Bio Sci 3(4):258–274
99. Shinde VA, Patil RB, Pawar PV (2017) Comparative study of antimicrobial potentials of phospholipid compound produced by halophilic and alkaliphiles *Bacillus subtilis* isolated from alkaline meteorite crater Lonar lake, India. Int J Life Sci 5(3):420–424
100. Tambekar DH, Dhundale VR (2013) Screening of antimicrobial potentials of haloalkaliphilic bacteria isolated from Lonar lake. Int J Pharm Chem Biol Sci 3(3):820–825. www.ijpcbs.com
101. Jenifer JA, Donio MBS, Viji VT, Velmurugan S, Babu MM, Albin Dhas S, Citarasu T (2013) Halo-alkaliphilic actinomycetes from solar salt works in India: diversity and microbial activity. Blue Biotechnol J 2(1):137–151
102. Thumar J, Pethani B, Vasoya A (2010) Desert actinomycetes as the potential source of new generation of antimicrobial agents: extraction and partial characterization. Int J Sci Comput 6(2):144–147
103. Thumar JT, Dhulia K, Singh SP (2010) Isolation and partial purification of an antimicrobial agent from halotolerant alkaliphilic *Streptomyces aburaviensis* strain Kut-8. World J Microbiol Biotechnol 26:2081–2087
104. Kishor PB, Padalia U (2015) In-vitro antimicrobial activity of fungi from extreme environment. Int J Life Sci A5:92–94. http://www.ijlsci.in
105. Kishor BP, Padalia U (2018) In-vitro antibacterial property of metabolites of alkaliphilic isolates against methicillin resistant *S. Aureus* (MRSA). IJSART 4(2):127–131. www.ijsart.com
106. Roth E, Schwenninger SM, Eugster-Meier E, Lacroix C (2011) Facultative anaerobic halophilic and alkaliphilic bacteria isolated from a natural smear ecosystem inhibit Listeria growth in early ripening stages. Int J Food Microbiol 147(1):26–32. https://doi.org/10.1016/j.ijfoodmicro.2011.02.032
107. Vasavada SH, Thumar JT, Singh SP (2006) Secretion of a potent antibiotic by salt-tolerant and alkaliphilic actinomycete *Streptomyces sannanensis* strain RJT-1. Curr Sci 91:1393–1397
108. Singh LS, Mazumder S, Bora TC (2009) Optimisation of process parameters for growth and bioactive metabolite produced by a salt-tolerant and alkaliphilic actinomycete, *Streptomyces tanashiensis* strain A2D. J Mycol Med 19:225–233. https://doi.org/10.1016/j.mycmed.2009.07.006
109. Kumar V, Gusain O, Thakur RL, Bisht GS (2013) Isolation, purification and partial characterization of an antibacterial agent produced by halotolerant alkaliphilic *Streptomyces* sp. EWC 7(2). Proc Natl Acad Sci India Sect B Biol Sci 83:199–206. https://doi.org/10.1007/s40011-012-0117-y
110. Jenifer JA, Donio MBS, Michaelbabu M, Vincent SGP, Citarasu T (2015) Haloalkaliphilic *Streptomyces* spp. AJ8 isolated from solar salt works and its' pharmacological potential. AMB Expr 5:59–68. https://doi.org/10.1186/s13568-015-0143-2
111. Deshmukh DV, Puranik PR (2010) Application of Plackett-Burman design to evaluate media components affecting antibacterial activity of alkaliphilic cyanobacteria isolated from Lonar Lake. Turk J Biochem 35(2):114–120

112. Souagui Y, Tritsch D, Grosdemange-Billiard C, Kecha M (2015) Optimization of antifungal production by an alkaliphilic and halotolerant actinomycete, *Streptomyces* sp. SY-BS5, using response surface methodology. J Mycol Med 25(2):108–115. https://doi.org/10.1016/j.mycmed.2014.12.004
113. Chun JY, Ryu IH, Park JS, Lee KS (2002) Anticaries activity of antimicrobial material from *Bacillus alkalophilshaggy* JY-827. J Microbiol Biotechnol 12:18–24
114. Dieter A, Hamm A, Fiedler HP, Goodfellow M, Müller WEG, Brun R, Beil W, Bringmann G (2003) Pyrocoll, an antibiotic, antiparasitic and antitumor compound produced by a novel alkaliphilic *Streptomyces* strain. J Antibiot 56:639–646. https://doi.org/10.7164/antibiotics.56.639
115. Fiedler HP, Goodfellow M (2004) Alkaliphilic streptomycetes as a source of novel secondary metabolites. Microbiol Aust 25:27–29
116. Lawton EM, Cotter PD, Hill C, Ross RP (2007) Identification of a novel two-peptide lantibiotic, haloduracin, produced by the alkaliphile *Bacillus halodurans* C-125. FEMS Microbiol Lett 267:64–71. https://doi.org/10.1111/j.1574-6968.2006.00539.x
117. Danesh A, Mamo G, Mattiasson B (2011) Production of haloduracin by *Bacillus halodurans* using solid-state fermentation. Biotechnol Lett 33:1339–1344
118. Ali MI, Ahmad MS, Hozzein WN (2009) WA52 – A Macrolide antibiotic produced by alkalophile Nocardiopsis Dassonvillei WA52. Aust J Basic Appl Sci 3:607–616
119. Sanghvi GV, Ghevariya D, Gosai S, Langa R, Dhaduk N, Kunjadia PD, Vaishnav DJ, Dave GS (2014) Isolation and partial purification of erythromycin from alkaliphilic Streptomyces werraensis isolated from Rajkot, India. Biotechnol Rep 1–2:2–7
120. Li YQ, Li MG, Li W, Zhao JY, Ding ZG, Cui XL, Wen ML (2007) Griseusin D, a new pyranonaphthoquinone derivative from a alkaphilic *Nocardiopsis* sp. J Antibiot 60(12):757–761
121. Ding ZG, Zhao JY, Li MG, Huang R, Li QM, Cui XL, Zhu HJ, Wen ML (2012) Griseusins F and G, spironaphthoquinones from a tin mine tailings-derived alkalophilic *Nocardiopsis* species. J Nat Prod 75:1994–1998
122. Ding ZG, Li MG, Zhao JY, Ren J, Huang R, Xie MJ, Cui XL, Zhu HJ, Wen ML (2010) Naphthospironone A: an unprecedented and highly functionalized polycyclic metabolite from an alkaline mine waste extremophile. Chem Eur J 16:3902–3905. https://doi.org/10.1002/chem.200903198
123. Bisht GS, Bharti A, Kumar V, Gusain O (2013) Isolation, purification and partial characterization of an antifungal agent produced by salt-tolerant alkaliphilic *Streptomyces violascens* IN2-10. Proc Natl Acad Sci India Sect B Biol Sci 83:109–117. https://doi.org/10.1007/s40011-012-0086-1
124. Wang Z, Fu P, Liu P, Wang P, Hou J, Li W, Zhu W (2013) New pyran-2-ones from alkalophilic actinomycete, *Nocardiopsis alkaliphila* sp. Nov. YIM-80379. Chem Biodivers 10:281–287. https://doi.org/10.1002/cbdv.201200086
125. Helaly SE, Goodfellow M, Zinecker H, Imhoff JF, Süssmuth RD, Fiedler HP (2013) Warkmycin, a novel angucycline antibiotic produced by *Streptomyces* sp. Acta 2930. J Antibiot (Tokyo) 66:669–674. https://www.nature.com/articles/ja20137
126. Jenifer A, Remya R, Velmurugan S, Michaelbabu M, Citarasu T (2018) *Streptomyces castaneoglobisporus* AJ9, a haloalkaliphilic actinomycetes isolated from solar salt works in southern India and its pharmacological properties. Indian J Mar Sci 47:475–488
127. Jonczyk E, Klak M, Miedzybrodzki R (2011) The influence of external factors on bacteriophages – a review. Folia Microbiol (Praha) 56:191–200

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Alkaliphilic Enzymes and Their Application in Novel Leather Processing Technology for Next-Generation Tanneries

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Contents

Abstract Leather manufacturing involves conversion of raw skin and hides into leather (stable material) through series of mechanical and chemical operations. The leather industry has attracted public outcry due to severe environmental degradation, pollution and health and safety risks. Currently the industry faces serious sustainability challenge due to extensive use of toxic chemicals and generation of hazardous waste.

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This chapter describes the polluting chemicals consumed in different stages of conventional leather processing and the nature of waste generated. In order to overcome the hazards caused by toxic chemicals in tanneries and protect the environment, enzymes have been identified as a realistic alternate for chemicals used in beam house operation and waste management. Alkaline active proteases of alkaliphiles offer advantages over the use of conventional chemical catalysts for numerous reasons, for example, they exhibit high catalytic activity and high degree of substrate specificity, can be produced in large amounts and are economically viable. This is because the enzymes of these alkaliphiles are capable of catalysing reactions at the extremes of pH, temperature and salinity of leather-manufacturing processes.

The chapter describes how alkaliphilic enzyme can effectively be used in soaking, dehairing, bating and degreasing operations to prevent waste generation, help in recovery of valuable by-products, reduce cost and increase leather quality. It is worth noting that protease has the capability to replace sodium sulphide in the dehairing process. In addition, alkaline proteases have shown remarkable ability in bioremediation of waste generated during the industrial processes. Intensive efforts are being directed towards chemical-based industries to use viable clean technology in their operation to reduce their negative impact on the environment. Similarly, leather industry should adopt the use of eco-friendly reagents such as enzymes to achieve long-term sustainability and clean environment and avert health hazards. Application of enzyme technology in clean leather processing strongly depends on legislation, political will and allocation of financial resources in research, development and implementation of this potentially powerful technology.

Graphical Abstract

Keywords Alkaline active protease, Biodegradation and sustainability, Clean environment, Leather manufacturing, Pollution

Abbreviations

BOD	Biological oxygen demand
COD	Chemical oxygen demand
CTLSs	Chrome-tanned leather shavings
DHA	Docosahexaenoic acids
DPA	Docosapentaenoic acid
EC	Enzyme commission
EPA	Eicosapentaenoic
FPH	Fish protein hydrolysate
GRAS	Generally recognized as safe
PTM	Posttranslational modification
PUFAs	Polyunsaturated fatty acids
TAG	Triacylglycerols
TDS	Total dissolved solids
WE	Wax esters
WS	Wax ester synthase

1 Introduction

Leather is appreciated and treasured as one of the uttermost important ancient natural products still existing in the industrial era [1]. Leather-making operation involves conversion of raw skin and hide, a highly spoilable material from meat industry, into leather, a stable material through series of mechanical and chemical operations. The industry serves a paramount role in global economic development by providing necessities such as leather clothing, boots, balls, saddles, hunting accessories, tent coverings, containers, boat coverings, dog chews, drum heads, bookbindings, lacing, shoes, purses, gloves, luggage bags, coats, clothing accessories, automotive interiors, upholstery for boats, aircraft, furniture and other garments. In spite of the industry making important contribution to the socio-economic development of developed and developing countries, through employment, production of valuable products and export earnings, the industry has attracted public outcry due to severe environmental degradation. Chemical-based industries are the prime targets of the environmental activist for their crusade against pollution, and leather industries have not been left out of such environmental protection campaigns. Close surveillance from environmental control authorities and increased public awareness has pressurized tanneries to search for eco-friendly processing methods [2].

2 Conventional Leather Processing and Environmental Pollution

The essential protein component of leather, collagen, exists in hides and skins is association with various globular proteins specifically, globulin, albumin, mucoids and fibrous proteins such as α-keratin, elastin, and reticular fibres [3]. During leather preparation processes, the hair or scales attached to the skins/hide is often removed. In addition, the non-collagenous constituents such as fat, blood remains and flesh are also removed in pre-tanning and tanning operations. The degree of their removal and the method used determine the leather product quality. Conventional leather-manufacturing processes use huge quantities of fresh water, lime, acid, pigments, sodium sulphide, heavy oils, fungicide, salts, soap, dyes, solvents and energy. When all these chemicals are mixed at different stages of leather processing, they form a complex and highly toxic waste difficult to treat. Table 1 gives an example of overall consumption levels in a tannery [4].

Health problems, pollution and environmental degradation arising from tanneries originate from the nature of the process, raw materials and chemicals consumed in processing. The level of consumption and emission in tanneries vary significantly owing to the variability of tanneries in terms of the type (primitive to modern), kind of skins/hide processed, volumes processed, chemicals used and quality specifications of the final product. Different forms of waste in quantity and quality are generated during chemical transformation of hides and skins into leather. The use of certain procedure in production and abatement may also result in some cross-

Table 1 Main and auxiliary process chemicals for a conventional process for salted, bovine hides

Chemical consumption	Process step	Approximate weight (%)
Standard inorganic chemicals (without salt from curing, acids, bases, sulphides, chemicals containing ammonium)	Soaking, bating, liming, unhairing, deliming, fat liquoring	40
Standard organic (acids, bases, salts)	Unhairing, liming, pickling, deliming	7
Tanning chemicals (chromium, vegetable and alternative tanning agents)	Tanning and pre-tanning	23
Dyeing agents and auxiliaries	Dyeing	4
Fat-liquoring agents	Pickling	8
Finishing chemicals (pigments, special effect chemicals, binders and cross-linking agents)	Finishing, dyeing	10
Organic solvents	Degreasing	5
Surfactants	Degreasing	1
Biocides	Preservation, soaking	0.2
Enzymes	Bating, unhairing, soaking	1
Others (sequestering agents, wetting agents, complexing agents)	Stripping and bleaching	1
Total		100

media effects [5]. The quantity of chemicals utilized varies considerably depending on the quality description of the final product, the pelts treated and the procedures employed in processing. Consumption of chemicals can therefore only be given within a broad range. In addition, lack of standards in terms of quality of chemicals supplied to tanneries by different manufacturers in developing countries poses safety challenge and difficulty in finding the environmental impact of the used chemicals. Lack of material safety data sheets on most chemicals of which some are toxic to humans and environment has been a major challenge for tanneries [5].

Water used must also be taken into account when comparing consumption and emission figures. An average of 30–35 m^3 of wastewater is generated per ton of raw hide processed [6]. About 20–50% of the pelt weight is added as inorganic standard chemicals and about 3–40% as organic chemicals [5]. The inability by tanneries to treat waste and uncontrolled discharge or disposal of untreated wastes is the major course of environmental degradation facing tanneries across the globe. Huge quantities of claimable by-products are generated in the leather industry but cannot be recycled due to cross contamination with toxic chemicals. For example, when 1,000 kg raw hide is processed using traditional leather processing method, 30 m^3 of water are required and yields 150 kg hair-free leather, 30 m^3 wastewater and 700 kg solid trash [7]. In another study undertaken by Black et al. [5], processing 1,000 kg of salted raw hide using conventional leather-manufacturing method requires 22 m^3 water, 425 kg of process chemicals and 9.2 GJ of energy. The yields consist of 145 kg leather, 22 m^3 wastewater, 391 kg by-product, 906 kg solid waste, 277 kg of sludge from wastewater and 1.5 kg air emission. Disposal of toxic solid waste and huge quantities of highly polluted wastewaters is a dilemma for tanneries. Figure 1 gives an input/output overview from an advanced tannery producing upholstery leather [5].

Pre-tanning and tanning processes contribute 80–90% of the total pollution load in tanneries [8]. The waste generated by depilation/dehairing process accounts for 60% of the suspended solid, 68% of salt, 83% of BOD, 73% of COD and 76% of toxic chemicals [9]. The probable escape to the atmosphere is emissions from wet processing, finishing and effluent treatment. They include dust particles, sulphur dioxide, aerosols, hydrogen sulphide, ammonia, fume of formic acid, water vapour, carbon dioxide, chlorine, solvent vapours, volatile organic compounds (VOCs), etc. Air emissions have a negative environmental effect since some of the gases initiate photochemical reactions, increase greenhouse gases, destroy ozone layer, reduce visibility in the urban area and form acid rain or fog [10]. Traditionally, tanneries have been condemned for the discharge of noxious niff rather than any other air emissions, although the emissions of organic solvents have been a major problem. Air emissions do not only have negative health effects on tannery workforce but affect people several kilometres beyond the tannery site. Tannery ventilation requirement for health and safety of employees may limit the degree of air pollution in the buildings but not the environment outside the building [5].

The main liquid and solid outputs from leather-making process arise from decaying flesh, suspended and dissolved solids, fats, dyestuffs, organic matters, colouring pigments, dissolved lime, splitting, soluble proteins, shaving, toxic

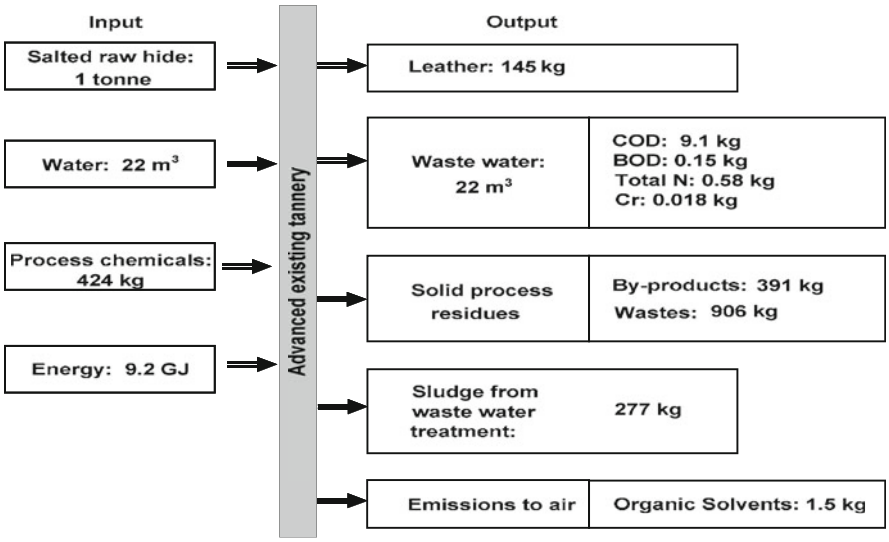

Fig. 1 Input/output overview from an advanced existing tannery for bovine salted hides per tonne of raw hide treated, producing upholstery leather (some chromium tanned) [5]

chemicals, heavy metals like chromium, etc [10]. Currently, chemical precipitation methods are normally employed for the removal of chromium from the effluent, but that leads to the formation of chrome-bearing solid wastes [11]. Leather processing can be divided into four vital subprocesses, viz. pre-tanning or beam house operations, tanning, post-tanning and finishing. Figure 2 schematically summarizes the possible steps in the production of leather through chromium tanning process and waste generated although there may be a significant variation between tanneries, depending on the kind of hide processed and quality of leather.

Legal requirements are slowly gaining momentum across the globe in forcing chemical-based industries including tanneries to apply innovative techniques in order to attain eco-friendly processing, waste reduction and waste recycling. The attention of chemical-based manufacturing industries has focused towards remodelling the processing procedures by incorporating recovery systems and improving effluent treatment methods to make processing eco-friendly and less costly and improve product quality. In order to overcome the hazards caused by excessive use of toxic chemicals in tanneries and protect the environment, enzymes have been identified as a realistic alternate for beam house operation and waste management.

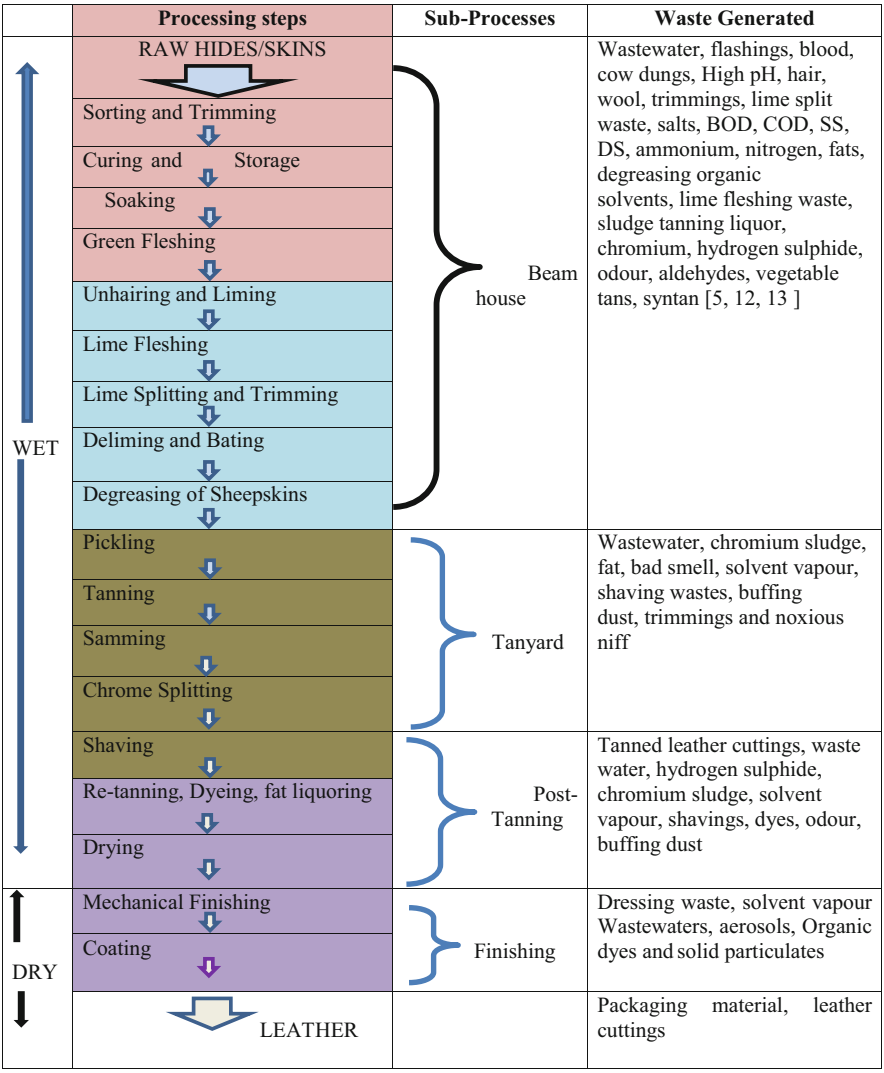

Fig. 2 Process steps in leather making (chromium tanning) and waste generated

3 Use of Enzymes in Tannery Processing

The leather industry faces serious sustainability issues due to pollution and environmental and negative health effect. New processing is a must! On this account, tanneries need to embrace cleaner production, prevent or reduce waste formation and the inevitable small amounts of waste generated disposed in an environmentally friendly way [12]. Application of neutral and alkaline-stable enzymes in tanneries

could be the most effective way in reducing the use of toxic chemicals for soaking, dehairing and bating. The bio-based leather processing aims at use of enzymes instead of chemicals, thereby consuming much less energy, chemicals and water. It can significantly contribute to both economic development and cutting down environmental pollution.

Enzymes are biocatalysts that are suitable for the practice of green chemistry and can be utilized to achieve eco-friendly industrial processing. Enzymes have been utilized broadly in manufacturing of household products. Amongst the largest groups of industrial enzymes, viz. proteases, amylases and lipases, proteases account for about 59–65% of the total worldwide enzyme sale [13, 14]. Alkaline active proteases have been used in products such as cosmetics, synthesis of oligopeptides, drugs, detergents, fertilizers, cloth and in processing food and leather [15–19]. Keratinolytic protease is one of the marvellous biocatalysts able to hydrolyse and break disulphide bonds found in hair with little damage to skin/hide grain [7].

Enzymatic leather processing is a transformative and ambitious technology which simultaneously addresses major economic, social and environmental challenges affecting the tannery. The 'greening' of the leather processing industry, with the elimination of toxic chemicals has the potential to revive collapsed leather industries severely restricted by pollution and health concerns. Use of enzymes may drastically reduce the pollution load of the effluents, help in hair recovery and at the same time enable recycling (such as wastewater) since there is no cross contamination by toxic chemicals. Enzymes are not persistent and can be readily deactivated and biodegraded. One of the major hindrances to the uptake of enzyme technology for the leather industry just like most industries is the technical limitations around tailoring an enzymatic process to the already existing standardized industrial chemical processes. However, with the advent of technology, it has been possible to design an enzymatic process that easily fits into the already existing factory operational parameters. Successful research has been carried out in processing of cow-, goat-, sheep- and fishskins using proteases, though their use has not been fully exploited due to the following technical limitations.

1. Limited number of people with product knowledge on enzyme production, application and specificity.
2. High initial cost of equipment for enzyme production.
3. Enzymes are limited in the activity range especially with regard to pH and temperature. This requires application of more than one enzyme for complete processing.
4. If not well controlled, one can risk destroying the valuable grain surface.

4 Protease

Protease (peptidases or proteolytic enzymes) constitutes a large group of enzymes that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein

[20, 21]. Breakdown of peptide bonds helps in the protein degradation into their constituent amino acids, smaller peptides or it can be specific, leads to selective protein cleavage for post-translational modification (PTM) and processing [22]. Proteases are categorized as peptidases or peptide hydrolases (EC 3.4) and comprise a large family of enzymes, divided into endopeptidases (EC 3.4.21–99) and exopeptidases (EC 3.4.11–19) and grouped depending on the position of the peptide bond to be cleaved. Protease can also be classified according to the pH range where they have optimal activity: acidic (pH 2.0–6.0), neutral (pH 6.0–8.0) and alkaline (pH 8.0–13.0) [13, 23, 24].

The growing realization of a diverse array of biological, economic and technical challenges has generated renewed interest in the study of proteolytic enzymes for different industrial applications. The most common proteases of animal origin include pepsin, pancreatic, rennins, trypsin and chymotrypsin. Plant origin proteases consist of papain, bromelain and keratinases. Use of plant proteases is controlled by land availability for agriculture, labour, government policies and climate, while the recovery of animal protease in bulk is determined by the presence of livestock for slaughter, which in turn is dictated by environmental, political, religious belief and government policies. The failure of plant and animal protease to quench market demands has led to an increased interest in microbial proteases [25]. Microbial sources of protease are widely preferred for industrial application because of the following advantages:

- Require limited space for their production.
- Broad biochemical diversity.
- Rapid growth of the microorganisms and faster production.
- Flexibility in production.
- Ease of genetic manipulation to generate enzymes for different characteristics and applications.
- The enzyme can be easily recovered.
- Microorganisms can secrete large amount of enzymes for large-scale applications.
- Economical.

Microbial proteases are derived from a wide variety of microorganisms, which include bacteria, yeasts and fungi. Proteases of industrial value are mainly obtained from microorganisms, and these are *Bacillus* species from the bacterial kingdom and *Aspergillus* from the fungal kingdom because they are known to be generally recognized as safe (GRAS) [26]. Proteases have been used in laundry and detergent industries for over 50 years to facilitate release of proteinaceous materials in stains and account for about 25% of total worldwide sales of enzymes [27]. Detergent industry heavily uses thermostable alkaline proteases from thermophiles as an additive [28]. Other enzymes used in detergents include amylases, mannanase, cellulase and lipases. The use of different enzymes as detergent additives arises from the fact that proteases can hydrolyse proteinaceous stains, cellulases are effective in cleaning, colour clarification and anti-redeposition (cotton), mannanases are perfect for stain removal, amylases are effective against starch and other carbohydrate stains, while lipases are effective against oily or fat stains [29, 30]. An ideal enzyme for detergent should have broad substrate specificity, be stable at high pH

and temperatures, be able to withstand oxidizing and chelating agents and be effective at low enzyme levels in detergent solutions. The leading enzyme suppliers and detergent manufacturers are actively pursuing the development of new enzyme activities that address consumer needs for improved cleaning, fabric care and antimicrobial properties [31]. As the detergent industry grow both in terms of size and complexity cleaning properties, new applications and demand of the enzyme will continue to expand.

Use of enzyme in processing of hide and skin has been practiced since ancient times. Traditionally, biocatalysts found in dog's dung were used to soak hides and skins to make them pliable by extracting protein, oil and fat constituents. Use of this method was not only unhygienic but unsustainable due to high market demand and health concern. The reasoning behind use of proteolytic enzyme from dog's dung lies in the fact that the protein is the major constituent of hair found on skins and hides. Hair is composed of α-keratin fibres and insoluble protein molecules containing a large fraction of cysteine residues and having α-helix conformation. The α-keratin is arranged in piles of fibrils. Different skin layers are composed of collagens, α-keratin and some elastin. Proteases can hydrolyse the protein fraction of dermatan sulphate, making the collagen more reachable by water and reducing the attachment of the basal layer [32]. Extremophiles can survive under extreme conditions. These include temperature (−2 to 12°C, 60 to 110°C), high pressure, radiation, salinity (2–5 M NaCl) and pH (<2, >9) [33]. Alkaline lipases from *Bacillus* strains, which grow under highly alkaline conditions in combination with other alkaline or neutral proteases, are currently being utilized in leather industry for assisted dehairing of animal hides and skin [33].

5 Enzymes from Extremophiles Microorganisms

The demand for industrial enzymes that can withstand harsh operating conditions such as high pH, temperature, salinity and pressure has greatly increased over the past decade. This has led to extensive research in exploring extremophilic microorganisms in search for novel enzymes. As a result enzymes from thermophiles and alkaliphiles have become the subject of special interest for biotechnological applications due to their efficiency and high stability at adverse operational and/or storage conditions [34]. This is because enzymes obtained from alkaliphiles are stable when added to detergents due to their inherent tolerance to high pH and can also generally function in the presence of bleaching chemicals [35]. Extreme environmental conditions require optimized interactions within the protein, at the protein–solvent boundary or with the influence of extrinsic factors such as metabolites, cofactors and compatible solutes [36]. Factors that contribute to the remarkable stability of extremozymes include an increased number of ion pairs, reduction in the size of loops and in the number of cavities, reduced ratio of surface area to volume, changes in specific amino acid residues, increased hydrophobic interaction at subunit

interfaces, changes in solvent-exposed surface areas, increase in the extent of secondary structure formation and truncated amino and carboxyl termini [37].

6 Application of Protease in Cleaner Leather Processing

Pre-tanning operations in conventional leather processing generate significantly high pollution problems as compared to the post-tanning operation. Responsible chemicals for pollution in the pre-tanning processes include lime, detergents, sodium sulphide, caustic soda, sodium chloride, inorganic salt, acids and degreasing solvents. In addition, pre-tanning operations consume large amounts of energy and huge quantities of water. The sludge generated from flashings, fat waste and digested hair results in further environmental pollution. In addition, tanneries incur high costs in treatment of the waste as well in purchasing the same chemicals. Significant efforts have been made in the past decades to render pre-tanning operations in tanneries cleaner. The best strategy in reducing or eliminating pollution problems in leather industry is by targeting and replacing toxic polluting chemicals by eco-friendly chemicals and recycling some of the waste such as water.

7 Enzymatic Leather Processing

The following section summarizes the major operations performed in beam house, tanyard, post-tanning and finishing areas in leather production. All the progressive steps in the ongoing processing of hides and skins involve application of enzyme either directly or indirectly to facilitate the processing and production of desired quality. Enzymes are mainly applied in soaking, dehairing, bating, degreasing and waste treatment in effort to avert negative effect associated with use of chemicals.

7.1 Hide and Skin Sorting, Trimming and Storage

7.1.1 Sorting and Trimming

Hides and skins of good quality are sorted into different grades, types and weight. This procedure may be performed at the tannery or slaughterhouse. Unsuitable materials may be sold to other tanners or if in extremely poor conditions, discarded as waste. Selected hides/skins are then trimmed to remove spoiled parts such as edges, legs, tails, face, udders, etc. from the raw skin. This stage generates huge putrescible wastes, which require to be discarded following the set regulation on animal by-products/waste disposal. Proteolytic and lipolytic enzymes can be applied

at this step to digest the spoiled parts of the hides/skins including those in poor condition and recover valuable products from the hydrolysate.

7.1.2 Curing and Storage

Curing process is performed to prevent degradation and restrains microbial attack on hides/skins before processing. For fresh hide processing immediately after skinning, this step can be omitted. Curing for long-term storage of up to 6 months includes drying, salting and brining [5]. Long-term preservation methods are used when the tannery anticipates shortage of the products in the market or when trading the hides and skins especially for intercontinental trading. Short-term curing involves preserving for 1–6 days using methods such as cooling, refrigeration and addition of biocides. These methods are used when raw materials are directly received from local sources. The raw materials are generally stored as they are received by the tannery on pallets in ventilated or air conditioned and/or cooled areas, depending on the method of curing chosen. The stored hides/skins are then taken to the beam house for processing.

7.2 Preparation for Skins and Hides for Tanning

The process in the beam house of the tannery involves removal of the skins or hides from storage and their preparation for tanning and is often carried out in processing mixer vessels or rotating drums.

7.2.1 Enzymatic Soaking

Soaking allows the hides/skins to rehydrate and helps in opening up the contracted fibre structure [15]. Soaking is necessary for solubilisation and removal of interfibrillary material and cleaning the hides and skins from dirt (salt, soil particles, dung, blood remains and soluble proteins on the surface of the skin). Soaking is mostly executed in two steps. First there is a dirt soak which removes salt and dirt. The second soak is also known as main soak which is longer and may last between 8 and 72 h. Due to the long soaking period in the main soak, in traditional leather processing method, putrefying bacteria thrive, and biocides are added to curtail their activity and growth [38].

Application of alkaline protease in the dirt soak and main soak reduces processing time and initiates the fibre opening of the hide and skin tissue. Enzymatic soaking is successfully carried out under alkaline conditions using proteolytic enzymes that are optimally active in alkaline conditions. The advantages of enzymatic soaking include:

- Loosening of the scud
- Partial dehairing during soaking process

- Solubilisation and removal of non-collagen protein
- Reduced time of soaking and faster opening of the fibre structure
- Production of leather with less wrinkled grain
- Improves softness and elasticity of the leather
- Increases the yield of hair, wool, scales and fur
- Reduces the amount of water used and wastewaters generated
- Energy efficient

7.2.2 Enzymatic Dehairing

Dehairing is the main and most polluting operations in the beam house. The aim of dehairing is to remove the hair, scales, epidermis and to some extent the interfibrillary proteins from the skin. Five methods of dehairing are generally used, i.e. (1) clipping process, (2) scalding process, (3) sweating process, (4) chemical process and (5) enzymatic process [3]. Clipping process is achieved by cuttings the wool of the sheep when the animal is either alive or dead. Scalding is the process of treating carcasses or skin with hot water or steam to loosen the hair or feather (of birds) in the follicle to aid their removal. Pork and poultry carcasses are both commonly subjected to a scalding operation during processing [39]. Sweating process is applied if the wool is of greater value than the skins and it involves hanging up soaked skins in dark humid rooms or in piles inside suitable chambers under controlled conditions of temperature and humidity for about 4–5 days where bacteria attacks keratin cells of hair and epidermis, until wool is loose. Sweating process may result in serious damage to the raw hide surface [40].

Of these, the most commonly practiced method of dehairing of hides or skins is the chemical process using high concentration of lime and sodium sulphide which creates extremely alkaline environments which result in pulping of hair and its subsequent removal. High alkalinity also helps in opening the fibre structure and hide plumping. The time span of the process may fluctuate from 18 to 168 h depending on the method used [41]. Even though this process is very efficient in hair removal, its inherent drawback has to be taken into account, noteworthy amongst these are:

- High concentration of lime blended with sodium sulphide accounts for generation of 70–80% of the total BOD and COD of effluent from all the leather-making processes. About 75% of the organic waste from a tannery is from the beam house and 70% of this waste is from dissolved hair rich in nitrogen [3].
- Sodium sulphide and hydrogen sulphide generated is highly toxic with extremely unpleasant odour. It can cause major health and pollution problems in the sewers if left untreated.
- Long processing time compromises the quality of the final leather and affects the costs.
- The process yields greenhouse and toxic gases such as carbon dioxide, hydrogen sulphide, ammonia and organic solvents.

- Workers' health is put at risk due to exposure to severe alkaline condition and toxic sulphide.
- The process consumes huge quantities of water and energy and generates a lot of wastewaters. Water polluted with these chemicals and the solubilized hair leads to an increase in alkalinity, organic nitrogen, BOD, TDS, COD and air pollution by hydrogen sulphide [15].
- Generate large quantities of solid wastes and obnoxious odour.

Enzymatic dehairing is suggested as being eco-friendly, more efficient and superior to chemical process. Dehairing, descaling and dewooling are the prominent stages where proteolytic enzymes can effectively be used in the leather processing and cut over 80% of the pollution load in the industry. Proteolytic enzymes can be used in dehairing to circumvent use of sodium sulphide and its negative environmental effect. Proteolytic enzymes are more efficient in dehairing and offer an environmentally friendly alternative to the conventional chemical process. Research by Wanyonyi et al. [15] demonstrated the technical and economic feasibility of enzymatic hair removal, in which the lime and sulphide chemicals were completely replaced by crude alkaline protease. Figure 3 shows a piece of hide successfully dehaired by alkaline proteases within 3 h as compared to a negative control which remained with hair when treated in distilled water under the same environmental conditions [42].

Skin (or hide) is usually considered to comprise of three layers. On the surface of the intact skin lies the keratinous epidermal layer comprising the epidermis and its appendages (hairs, hair root sheaths, etc.). Immediately below it, the basement membrane is to be found, attaching the epidermal layer to the underlying dermis (or corium). During leather processing, the corium is transformed into leather [43]. The proteolytic enzymes remove the hair by attacking the proteinaceous matter adjacent to the base of the hair root [44]. The ability of protease to digest the basal

Fig. 3 Photograph of hide completely dehaired by crude alkaline protease within 3 h (pH 12 and 37°C); negative control where distilled water under the same condition remained with hair

cells of the hair follicle and the cells of the Malpighian layer is found to be more efficient, and it happens without disturbing the native state of skin. The loosening of hair and its removal from the skin are accomplished after swelling and subsequent protease attack on the outermost sheath leading to breakdown of the inner root sheath (parts of the hair that are not keratinized). In enzymatic dehairing, the epidermal layer is removed from the dermis without damaging the hair and the grain layer because they are easily degraded and dissolved. Another component of raw skin (or hide) is the subcutaneous layer (connective tissue and fat) which is removed by fleshing. That layer, however, is of no relevance to dehairing except when applying the painting method [43]. Application of proteases facilitates faster dehairing process without damage to the fibrous collagen and hair or wool. It is worth noting that fully developed keratin in hair, nails and the upper part of the epidermal layer is highly resistant to chemical or biological attack, due to stable disulphide bonds (–S–S–), except from sulphide which breaks down the disulphide bonds.

Enzyme concentration, pH and temperature also play a significant role in enzymatic dehairing. pH range of 9.0–12.5 provides optimal conditions for alkaline protease activity and effectively aids in skin swelling and loosening of hair and removal. Dehairing is accomplished between 3 and 12 h when temperature range is 30–40°C. At temperature below 30°C, the duration of enzyme exposure and concentration needs to be increased for complete dehairing [15]. Further, below 15°C no appreciable dehairing within practicable time is observed. Studies have further shown that about 2% (w/w) of crude enzyme was sufficient for dehairing but 3% (w/w) of the enzyme was preferred because at this concentration even the tough hair at the neck areas was completely eliminated [44].

Enzymatic hair loosening and removal provide the necessary conditions for hair, wool, fur and scales recovery while still in good condition. Techniques commonly employed in enzymatic dehairing processes include paint, dip and spray methods. In the paint method, the enzyme solution is mixed with an inert substance like kaolin; a thin paste is made after adjusting to the prerequisite pH before applying on the flesh side of hides or skins. The skins are then piled, covered with polythene sheets and kept till dehairing takes place. In the dip method, the hides or skins are submerged in the enzyme solution at the right temperature and pH in a pit or rotating drum till all hair loosen and fall off [3]. In the spray method, a concentrated solution of enzyme is sprayed on the flesh side at a high pressure forcing its entry into the skin [3]. Difficult areas to dehair such as backbone and neck can be sprayed with more amount of enzyme, thereby accelerating the process. After dehairing, the enzyme may be reused in the second or third pile of skin or incorporated in the soaking solution till its activity diminishes. The major benefits of enzymatic dehairing include:

- Remarkable reduction or even total elimination of toxic sodium sulphide in dehairing.
- Applying enzyme can reduce the total pollution load of a tannery by over 80%.
- The process makes it possible to recover valuable hair, wool, fur and scales while still in good saleable condition.

- Enzymatic dehairing process takes short period as compared to chemical dehairing, i.e. the lime–sodium sulphide process takes about between 48 and 72 h, while the enzymatic dehairing process is accomplished within 3 and 12 h [15].
- The process eliminates the problem of waste disposal because by-products like hairs, wool, flesh, etc. can be recovered during or after dehairing.
- Enzymes are not persistent and can be readily inactivated and biodegraded.
- The process simplifies pre-tanning operations by cutting down one step, viz., bating since proteolytic enzymes are capable of removing all the hair.
- Use of enzymes greatly cuts down pungent smell and air emission.
- The process results in significant reduction in total solids, dry sludge and chemical oxygen demand in the effluent due to reduced use of chemicals.
- Treating hide/skins with enzymes produces soft, tough and pliable leather of high quality with greater surface area.
- Using the enzyme-based process creates an ecologically conducive atmosphere for the workers.

7.2.3 Fleshing

Fleshing is the mechanical scraping of the unwanted organic material from the hide and skins (connective tissue, flesh, fat, etc.). This process can be executed before soaking, after soaking, after liming or after pickling. In conventional leather processing, the process of fleshing is called green fleshing if the process is done before liming and dehairing. If fleshing is effected after liming and dehairing, it is called lime fleshing [5]. Sheepskins may be fleshed in the pickled state. Fleshing operations give rise to an effluent containing fatty and fleshy matter in suspension. If green fleshing is adopted, proteolytic and lipolytic enzymes can be utilized to hydrolyse fleshy matter to generate valuable products and recover biomolecules since the waste are not contaminated with sulphide.

7.2.4 Splitting

The uneven thickness of hides and skins is balanced out by mechanical splitting horizontally into a grain layer and flesh layer. Splitting is undertaken using splitting machines, fitted with band knife. Splitting can either be done in limed state or in the tanned condition [5]. This process generates huge quantities of flesh and fat waste, which can be solubilized and hydrolysed by use of proteolytic and lipolytic enzymes as described in fleshing to section.

7.2.5 Deliming

In enzymatic leather processing, this stage is eliminated since no lime is used. However, lowering of the pH of the hide/skin by soaking in acidified water is necessary since proteolytic dehairing is commonly done under alkaline environment. Lowering of pH is critical in preparing of the pelt for the subsequent operation since alkalinity has detrimental effect on the tanning process. The conventional deliming process is executed to eradicate the liming agent from the pelts by lowering the pH to between 8 and 9 [38]. The process involves gradual lowering of the pH using weak acidic solutions, fresh water or ammonium chloride salt. Acidification liquids which may still contain sulphide generate poisonous hydrogen sulphide gas. The process may also involve increase in temperature, removal of residual chemicals and degraded skin components. The quality of the final leather determines the extent of deliming. After deliming, the hides and skins are ready for vegetable tanning. Chrome tanning requires delimed hides and skins to be further processed through pickling and bating [5]. Once the skins and hides have been delimed, they must be taken to the next process immediately since removal of alkali provides favourable conditions for putrefying bacteria to thrive.

7.2.6 Bating

Application of protease in dehairing eliminates bating process since the hair roots are fully removed. However, the traditional chemical dehairing method leaves the skin surface with some attached hair roots and epidermal pigments which are undesirable for certain types of leather. Removal of these remnants is effected by the bating process. In the current bating process, commercially available proteolytic enzymes are used. Bating is stopped by lowering the temperature and pH and diluting the enzyme solution when the appropriate level of softening is achieved. This assessment is hard to standardize although it is based on the empirical evaluation by experienced personnel [1].

7.3 Tanyard Operations

The tanyard operations are commonly carried out in a section of the plant known as the tanyard. The operations are often carried out in the same processing vessels, with changes of float and chemicals. In chromium tanning, the vessels are usually rotating drums.

7.3.1 Degreasing

Domestic sheepskins normally contain large amount of natural grease (natural fat content is estimated to be between 10% and 20% on dry weight) which must be removed by degreasing operation. Excess fat is eliminated from fatty skins to avert the development of insoluble chrome soaps or block fat spumes formation in subsequent stage. Excess grease on the skin prevents even penetration of dye or tan, causing difficulties in the finishing processes and creating dark and greasy smudge on surface of the finished leather. Three methods are usually employed for degreasing, i.e.:

- Use of aqueous medium with organic solvent and non-ionic surfactant
- Use of aqueous medium with non-ionic surfactant
- Degreasing in solvent medium

Application of proteolytic enzyme in dehairing greatly helps in the removal and recovery of grease/fat/oil in the skin/hide by dissolving protein matter surrounding fatty tissues. Tanneries currently apply alkaline lipases at different steps of processing to eliminate natural fats from skins [44]. Lipases can remove fats and grease from skins and hides, particularly those with a moderate fat content. By combining the effects of alkaline protease, alkaline lipase and acid active lipases in dehairing, one may establish an effective method of degreasing prior to tanning and also improving the brightness and uniformity in dyeing [44]. The main advantages of using lipases are a more uniform colour and a cleaner appearance. Lipases also improve the production of hydrophobic (waterproof) leather; makers of leather for car upholstery have commented that ‘fogging’ is reduced [45].

7.3.2 Tanning

Tanning process introduces a tanning agent into the skins/hides, thereby stabilizing collagen fibres in the skin such that the hide is no longer susceptible to putrefaction or rotting. Collagen fibres are stabilized by cross-linking action of the tanning agents. The dimensional stability, resistance to mechanical action and heat resistance increase upon the tanning treatment. There are different tanning materials and methods, and one’s choice depends mainly on characteristics of the desired finished leather, cost and availability of the chemicals and the type of hides and skins. The majority of tanning agents fall into one of the following groups:

- Vegetable tannins
- Syntans
- Aldehydes
- Mineral tannages
- Oil tannage

A high percentage (80–90%) of all the leather manufactured today is tanned using basic chromium sulphate ($Cr(OH)SO_4$) because chrome tanning produces light and inexpensive leather of high thermal and bacterial resistance [44]. Although proteolytic and other enzymes are not used in the tanning process, their applications in the previous stage significantly influence the quality of tanned leather.

7.3.3 Shaving

The shaving process is carried out to reduce or standardize the thickness of hide or skin. Machines with a rapidly revolving cylinder are used to cut fine, thin fragments from the flesh side of the skin to achieve uniformity. This process can be undertaken on tanned or crusted leather. Small pieces of leather waste which are cut off are called shavings. Solubilizing of the chrome shaving using proteolytic enzyme assists in chrome recovery and solid waste management.

8 Protease in Bioremediation of Waste

Effluent discharges from tanneries create health hazards and environmental problems. When raw hides and skins are processed to leather, a number of by-products such as claws, scales, trimmings, tails, fleshings, pelt cuts, gluestock and tanned material such as cuts, buffing dust, dyed and chrome shavings are obtained [3]. As a result, the industry generates huge quantities of proteinaceous waste, chromium containing waste and wastewaters which pose serious environmental and pollution problems. Currently, the most common way practiced in the management of solid wastes is by tanneries disposing them in landfills. The disposal of untreated wastes into land and water bodies from tanneries results in air and water pollution as well as emission of greenhouse gases like methane and carbon dioxide [46]. Chromium leaching into the soil and groundwater makes it unfit for cultivation and other uses. Discharge of coloured wastewater contaminated with organic dyes from tanneries into natural streams has caused severe pollution problems, such as increased toxicity, BOD, COD and TDS of the effluent and also reduced light penetration, which has adverse effects on photosynthesis [47, 48].

Exploitation of enzymes in treatment of chrome-tanned leather shavings (CTLSs) at a commercial level is not fully developed. Studies have shown that protein is the main component of most of the CTLSs generated in tanneries [12]. However, due to non-biodegradability of CTLSs, their disposal poses a major problem for the leather industry. Enzymatic hydrolysis of CTLS is a viable method and provides a 50–60% yield of hydrolysate, which shows low ash content and a low content of chromic

compounds [49]. Protein hydrolysate produced by enzymatic hydrolysis of CTLS wastes reacted with polyvinyl alcohol for producing biodegradable plastics [49].

9 Value-Added Product from Tannery Waste

Tannery wastes can be an important source of proteins and lipids, and there are reports on efforts made to recover these biomolecules [50]. The decaying of proteins and lipids is one of the major sources of offensive odours associated with tannery waste due to oxidation of disulphide bridges in proteins and unsaturated fatty acids present in these lipids. The protein-based components that can be recovered from tannery waste include protein hydrolysates, peptides and amino acids, collagen and gelatin and fish meal. The lipid-based compounds that can be recovered are oils, omega-3 fatty acids, phospholipids, squalene, vitamins, cholesterol, etc. that are required by many industries including food, agriculture, aquaculture and pharmaceuticals [51]. Proteinaceous fish meal is the main ingredient in fish feed formulation, because of its high protein content and an adequate profile of indispensable amino acids which is a requirement for the growth of fish, especially the carnivorous fish [52].

Tannery waste can also be utilized in the production of organic fertilizers and composts which have significant benefits over chemical-based products. Potential for biofuel recovery from such waste has been reported [53]. The recovery of components with potential biological activities and functionalities provides a means for value addition to the tannery processing waste and also adds to plant economy. Currently, by-products from tannery processing if not dumped are used to make low-valued products. Hides and skin from mature animals is tanned into leather at a small percent for making different products. Fish frames and heads are used for human consumption in the local community or used to make animal feeds (at small scale). Oil obtained from the by-products can be utilized for value-added products such as soap, biofuel and cosmetics.

10 Novel Biotechnological Approaches for Transforming Tannery Waste into Bioproducts Using Biocatalysts

Today tannery biowaste poses environmental risks while being an important potential feedstock resource for producing a wide range of bioproducts. The potential to exploit tannery biowastes as a raw material for bioproducts/energy requires the application of new technologies to arrive at novel and economically viable solutions. Enzymes are becoming a key element in the toolbox for the chemist. In particular, biocatalytic transformation of tannery waste is promising in production of bioproducts for use in areas such as pharmaceutical and food chemicals where target molecules are selective

and complex. While new chemical catalysts are becoming available, the unique properties of proteolytic and lipolytic enzymes as biocatalysts offer green alternatives such as reduced use of organic solvents, efficient use of reagents and elimination of chemical catalysts. Enzyme technology could be the most useful technology for adding value to by-products and solving the waste problem in the tannery industry globally. A few reports are available on recovery of lipids by enzymatic hydrolysis of skin and hides and subsequent enrichment of omega-3 fatty acids using lipases [54, 55]. In the case of fishskins, omega-3 fatty acids devoid of most of the saturated fatty acids are preferred over the native fish oils since they keep the daily intake of lipids as low as possible. Omega-3 polyunsaturated fatty acids (PUFAs) are gaining recognition as important components of the human diet. They have been implicated in lowering the incidence of certain cardiovascular diseases, improving neural and retinal development in infants and slowing the growth of cancerous cells. In addition, several studies have shown fish diet containing PUFA before the period of gonad maturity affect fecundity, fertility, hatching rate and quality of eggs [56]. Two important PUFAs, eicosapentaenoic (20:5, n-3; EPA) and docosahexaenoic acids (22:6, n-3; DHA), are mainly found in sheep- and goatskins and cold water fish oils and also in substantial amounts in oil of Nile perch, a warm water fish [54]. In addition, fishskins contain docosapentaenoic acid (DPA) that also has been reported to be present in seal oil [54, 57]. Hence PUFA concentrates from fishskins would in addition to DHA and EPA present in cold water fish oil also contain DPA making them unique.

11 Bioinnovation Potential of Tannery Waste Biomass

Tannery waste is an attractive biowaste stream as it is produced in significant quantities globally. It is therefore possible to apply a cascading approach, giving priority to the transformation towards bioproducts while also permitting a possible conversion to energy, e.g. to bioparaffin and biodiesel. By adopting enzyme technology for recovery of protein hydrolysate, oil from skin and hide waste, it would therefore also be possible to recover other functional ingredients such as collagen. Since enzymes work best at low temperatures ($\leq$55°C), the process minimizes oxidation of unsaturated omega-3 fatty acids and results in fish protein hydrolysate (FPH) with higher degree of hydrolysis, and the process is more energy efficient as compared to conventional chemical treatment and cooking methods. Soluble proteins have been used in aquaculture, in preparation of fortified animal feeds and protein supplements. Due to its high protein content, sludge emanating from such a process can be a source of plant nutrients for food production or as animal feed. Use of raw tannery waste may causes problems of odour and attracting wild/domestic animals; thus it needs to be composted prior to its use.

The by-products from tannery processing are a potential source of collagen, the especially skin. Collagen is the main component found in skin, and gelatin can be obtained through partial hydrolysis of collagen. Gelatin from warm water fishskins is

reported to have properties similar to that of gelatin derived from porcine [58]. Use of enzymes to extract gelatin should be targeted in enzymatic tannery processing. The proposed gelatin production method produces gelatin with very low colour and high gel strength over a very wide range of viscosities. If further improvement in gel strength is needed, transglutaminase may be used for cross-linking some smaller molecules, thereby improving the gel strength. The high purity, enzyme-extracted gelatin is produced with a dramatic reduction in gelatin production cycle due to elimination of liming step. Further, the low-temperature enzyme method of producing gelatin results in reduction of unit production costs due to increased yield, reduced chemical cost, reduced water usage, reduced utility cost and reduced emission of greenhouse gases and climate change.

Gelatin is an attractive molecule to be used in cultivation of mammalian cells. Use of bovine or porcine gelatin is nowadays restricted, and there seems to be great potential for other sources of gelatin material including fish-skin-derived gelatin. The gelatin produced should be evaluated with respect to this area of application. Furthermore, the gelatin can be modified with cross-linking functional groups for the preparation of chirally pure hydroxyalkanoic to be used as carriers of bioactive agents in various forms (hydrogels, capsules, microspheres, films, etc.). Addition of functional groups and blending with other biopolymers containing polysaccharides and lipids will confer to the gelatin buffering capacity and hydrophobicity, thus protecting the sensitive bioactive agents during gastric transit.

Tannery waste biomass is an abundant feedstock globally and has the potential to be exploited as a rich source of fatty acids for use in the biosynthesis of wax esters and lipid-based biopolymers. Wax esters are esters of long-chain aliphatic alcohols and fatty acids and are used as high-performance lubricants for engines, transmission and hydraulic systems. They are also used in cosmetics, foods and pharmaceuticals. Currently, these compounds are produced at a scale of 3 million tons per year from mineral oils. The last few years have seen a push towards the production of biodegradable lubricants from renewable sources. However, so far, the only natural sources of wax esters are whale sperm oil and jojoba oil, which are too expensive for wide range use. As an alternative, researchers have recently begun to explore lipid sources for the synthesis of bio-WEs. Synthesis of jojoba oil-like wax esters such as palmityl oleate, palmityl palmitoleate and oleyl oleate from oleate requires first the CoA activation of a fatty acid catalysed by an acyl-CoA synthetase and in a second step esterification with a fatty alcohol catalysed by a WE synthase (WS). Genes encoding these two enzyme functions have only recently been identified [59]. Both WSs not only catalyse esterification of fatty acids with long-chain alcohols but also esterification of diacylglycerol which results in the production of both triacylglycerols (TAGs) and WEs. The ratio of produced WEs (the desired product) and TAGs depends on the fatty acids used.

Despite several reports indicating more valuable products can be obtained from such by-products, there is no information on an integrated approach of recovery oil, omega-3 fatty acids, biodiesel, protein hydrolysate, amino acids, enzymes, gelatin, biofertilizer, etc. at large scale from such by-products globally. Such an approach is

feasible in the tannery industry and will greatly add value to the tannery residues while addressing growing environmental concerns. This is an incentive to the tannery and other agroprocessing industries to generate more revenue and also present products of more value to the consumer.

12 Conclusions

The industry is celebrated for playing a paramount role in global economic development by providing valuable leather products, employment and foreign exchange. However, the industry faces serious sustainability issue due to pollution and environmental and negative health effect. Tanneries need to embrace cleaner production, prevent or reduce waste formation and the inevitable small amounts of waste generated be disposed of in an environmentally friendly way. Enzymes have been identified as a realistic alternate for toxic chemicals used in beam house operation.

Proteolytic and lipolytic enzymes in particular can be effectively used in soaking, dehairing, bating and degreasing operation to and help in waste reduction and recovery of valuable by-product, reduce cost and increase leather quality. We have demonstrated that proteases and lipases from extremophilic microorganisms have the capability to replace toxic sodium sulphide used in dehairing process. In addition, studies have shown that proteolytic enzyme can be used in bioremediation of generated waste. Exploitation of enzymes in treatment of chrome-tanned leather shavings (CTLSs) at a commercial level is not fully developed. Leather industry should adopt use of eco-friendly enzyme to achieve long-term sustainability and clean environment and avert health hazards. However, adoption of new technology by stakeholders in leather industry is critical but is difficult to implement due to resources involved. Implementation of enzyme-based technology in leather processing is strongly dependent on the legislation (nationwide and international), the political will and the financial resources put into research, development and implementation of this powerful technology.

References

1. Beghetto V, Zancanaro A, Scrivanti A, Matteoli U, Pozza G (2013) The leather industry: a chemistry insight part I: an overview of the industrial process. Sci Ca'Foscari 1:13–22
2. George N, Chauhan PS, Kumar V, Puri N, Gupta N (2014) Approach to ecofriendly leather: characterization and application of an alkaline protease for chemical free dehairing of skins and hides at pilot scale. J Clean Prod 79:249–257
3. Kamini NR, Hemachander C, Mala JGS, Puvanakrishnan R (1999) Microbial enzyme technology as an alternative to conventional chemicals in leather industry. Curr Sci 77:80–86

4. Rydin S, Frendrup W (1993) Possibilities for a reduction of the pollution load from tanneries. Nordic Council of Ministers, Copenhagen
5. Black M, Canova M, Rydin S, Scalet BM, Roudier S, Sancho LD (2013) Best available techniques (BAT) reference document for the tanning of hides and skins. European Commission Database, p 46. http://eippcb.jrc.ec.europa.eu/reference/BREF/TAN_Published_def.pdf
6. Islam BI, Musa AE, Ibrahim EH, Sharafa SAA, Elfaki BM (2014) Evaluation and characterization of tannery wastewater. J For Prod Ind 3:141–150
7. Fang Z, Yong Y-C, Zhang J, Du G, Chen J (2017) Keratinolytic protease: a green biocatalyst for leather industry. Appl Microbiol Biotechnol 101(21):7771–7779
8. Thanikaivelan P, Rao JR, Nair BU, Ramasami T (2004) Progress and recent trends in biotechnological methods for leather processing. Trends Biotechnol 22(4):181–188
9. Saravanabhavan S, Thanikaivelan P, Rao JR, Nair BU, Ramasami T (2004) Natural leathers from natural materials: progressing toward a new arena in leather processing. Environ Sci Technol 38(3):871–879
10. Hashem A, Arefin S, Jor A (2015) Gaseous air pollutants and its environmental effect-emitted from the tanning industry at Hazaribagh, Bangladesh. Am J Eng Res 4:138–144
11. Aravindhan R, Madhan B, Rao JR, Nair BU, Ramasami T (2004) Bioaccumulation of chromium from tannery wastewater: an approach for chrome recovery and reuse. Environ Sci Technol 38(1):300–306
12. Ozgunay H, Colak S, Mutlu MM, Akyuz F (2007) Characterization of leather industry wastes. Pol J Environ 16(6):867–873
13. Rao MB, Tanksale AM, Ghatge MS, Deshpande VV (1998) Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 62(3):597–635
14. Aqel H (2012) Phenotypic and protease purification of two different thermophilic *Bacillus* strains HUTBS71 and HUTBS62. Ann Biol Res 3(4):1747–1756
15. Wanyonyi WC, Onyari JM, Shiundu PM, Mulaa FJ (2016) New eco-friendly and clean method of processing hides and fish skins into leather using alkaline protease enzyme. Patent. (KE/P/2015/2231)
16. Dayanandan A, Kanagaraj J, Sounderraj L, Govindaraju R, Rajkumar GS (2003) Application of an alkaline protease in leather processing: an ecofriendly approach. J Clean Prod 11(5):533–536
17. Han W, He M (2010) Short-term effects of exogenous protease application on soil fertility with rice straw incorporation. Eur J Soil Biol 46(2):144–150
18. Valdez-Peña AU, Espinoza-Perez JD, Sandoval-Fabian GC, Balagurusamy N, Hernandez-Rivera A, De-la-Garza-Rodriguez IM, Contreras-Esquivel JC (2010) Screening of industrial enzymes for deproteinization of shrimp head for chitin recovery. Food Sci Biotechnol 19 (2):553–557
19. Paul T, Das A, Mandal A, Halder SK, Jana A, Maity C, Das Mohapatra PK, Pati BR, Mondal KC (2014) An efficient cloth cleaning properties of a crude keratinase combined with detergent: towards industrial viewpoint. J Clean Prod 66:672–684
20. Gabres CA, Undan JR, Jhane M, Valentino G (2016) Proteolytic enzyme like activity of fungal endophytes and their effects in the proximate composition of dried bamboo leaves. Int J Biol Pharm Allied Sci 5(6):1298–1306
21. Suseela L, Anjali CH, Muralidhar P (2017) Enhanced production of alkaline protease by Aspergillus niger DEF 1 isolated from dairy form effluent and determination of its fibrinolytic ability. Afr J Microbiol Res 11(11):440–449
22. de Souza PM, Bittencourt ML, Caprara CC, de Freitas M, de Almeida RPC, Silveira D, Fonseca YM, Ferreira Filho EX, Pessoa Junior A, Magalhães PO (2015) A biotechnology perspective of fungal proteases. Braz J Microbiol 46(2):337–346
23. Gupta R, Beg Q, Lorenz P (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 59(1):15–32

24. Sabotič J, Kos J (2012) Microbial and fungal protease inhibitors – current and potential applications. Appl Microbiol Biotechnol 93(4):1351–1375
25. Sawant R, Nagendran S (2014) Protease: an enzyme with multiple industrial applications. World J Pharm Sci 3:568–579
26. Joo H-S, Chang C-S (2006) Production of an oxidant and SDS-stable alkaline protease from an alkaophilic *Bacillus clausii* I-52 by submerged fermentation: feasibility as a laundry detergent additive. Enzyme Microb Technol 38(1–2):176–183
27. Ahmed I, Zia MA, Iftikhar T, Muhammad H, Iqbal N (2011) Characterization and detergent compatibility of purified protease produced from *Aspergillus niger* by utilizing agro wastes. Bioresources 6:4505–4522
28. Lagzian M, Asoodeh A (2012) An extremely thermotolerant, alkaliphilic subtilisin-like protease from hyperthermophilic *Bacillus* sp. MLA64. Int J Biol Macromol 51(5):960–967
29. Anwar A, Saleemuddin M (1997) Alkaline proteases: a review. Bioresour Technol 64:175–183
30. Kiran I, Ilhan S, Caner N, Iscen CF, Yildiz Z (2009) Biosorption properties of dried *Neurospora crassa* for the removal of Burazol Blue ED dye. Desalination 249(1):273–278
31. Vojcic L, Pitzler C, Körfer G, Jakob F, Martinez M, Maurer K-H, Schwaneberg U (2015) Advances in protease engineering for laundry detergents. N Biotechnol 32(6):629–634
32. De Souza FR, Gutterres M (2012) Application of enzymes in leather processing: a comparison between chemical and coenzymatic processes. Braz J Chem Eng 29(3):473–482
33. Sanchez S, Demain AL (2017) Useful microbial enzymes – an introduction. Biotechnology of microbial enzymes. Elsevier, Amsterdam, pp 1–11
34. Golaki BP, Aminzadeh S, Karkhane AA, Yakhchali B, Farrokh P, Khaleghinejad SH, Tehrani AA, Mehrpooyan S (2015) Cloning, expression, purification, and characterization of lipase 3646 from thermophilic indigenous *Cohnella* sp. A01. Protein Expr Purif 109:120–126
35. Chinnathambi A (2015) Industrial important enzymes from alkaliphiles – an overview. Biosci Biotechnol Res Asia 12(3):2007–2016. http://www.biotech-asia.org/?p=3705
36. Ladenstein R, Antranikian G (1998) Proteins from hyperthermophiles: stability and enzymatic catalysis close to the boiling point of water. In: Antranikian G (ed) Biotechnology of extremophiles. Springer, Berlin, pp 37–85
37. Demirjian DC, Morís-Varas F, Cassidy CS (2001) Enzymes from extremophiles. Curr Opin Chem Biol 5:144–151
38. Soerensen NH, Hoff T, Oestergaard PR, Cassland P (2016) Dehairing of skins and hides. Patent. EP2585618B1
39. Irshad A, Arun TS (2013) Scalding and its significance in livestock slaughter and wholesome meat production. Int J Livest Res 3(2):45–53
40. Madhavi J, Srilakshmi J, Rao MVR, Rao KRSS (2011) Efficient leather dehairing by bacterial thermostable protease. Int J Biosci Biotechnol 3(4):11–27
41. Rose C, Suguna L, Rajini R, Samivelu N, Rathinasamy V, Ramalingam S, Iyappan K, Parvathaleswara T, Ramasami T (2004) Process for lime and sulfide free unhairing of skins or hides using animal and/or plant enzymes. Patent. US7198647B2
42. Wanyonyi WC (2015) Isolation and characterization of biomass modifying enzymes for bioremediation and production of 'green specialty products'. PhD thesis, University of Nairobi, Kenya
43. Frendrup W, Buljan J (2000) Hair-save unhairing methods in leather processing. United Nations Industrial Development Organization report. US/RAS/92/120. http://footwearsinfoline.tripod.com/hairsave_unhairing.pdf
44. Choudhary RB, Jana AK, Jha MK (2004) Enzyme technology applications in leather processing. Indian J Chem Technol 11:659–671
45. Hasan F, Shah AA, Hameed A (2006) Industrial applications of microbial lipases. Enzyme Microb Technol 39(2):235–251
46. Sundar VJ, Gnanamani A, Muralidharan C, Chandrababu NK, Mandal AB (2011) Recovery and utilization of proteinous wastes of leather making: a review. Rev Environ Sci Bio 10 (2):151–163

47. Bulut Y, Aydın H (2006) A kinetics and thermodynamics study of methylene blue adsorption on wheat shells. Desalination 194(1–3):259–267
48. Wanyonyi WC, Onyari JM, Shiundu PM (2013) Adsorption of methylene blue dye from aqueous solutions using *Eichhornia crassipes*. Bull Environ Contam Toxicol 91(3):362–366
49. Pati A, Chaudhary R, Subramani S (2014) A review on management of chrome-tanned leather shavings: a holistic paradigm to combat the environmental issues. Environ Sci Pollut R 21 (19):11266–11282
50. Rustad T (2003) Utilisation of marine by-products. J Environ Agric Food Chem 2(4):458–463
51. Wasswa J, Tang J, Gu X, Yuan X (2007) Influence of the extent of enzymatic hydrolysis on the functional properties of protein hydrolysate from grass carp (*Ctenopharyngodon idella*) skin. Food Chem 104(4):1698–1704
52. Nyina-wamwiza L, Xu XL, Blanchard G, Kestemont P (2005) Effect of dietary protein, lipid and carbohydrate ratio on growth, feed efficiency and body composition of pikeperch *Sander lucioperca* fingerlings. Aquacult Res 36(5):486–492
53. Mshandete A, Kivaisi A, Rubindamayugi M, Mattiasson B (2004) Anaerobic batch co-digestion of sisal pulp and fish wastes. Bioresour Technol 95(1):19–24
54. Mbatia B, Adlercreutz D, Adlercreutz P, Mahadhy A, Mulaa F, Mattiasson B (2010) Enzymatic oil extraction and positional analysis of ω-3 fatty acids in Nile perch and salmon heads. Process Biochem 45(5):815–819
55. Mbatia B, Adlercreutz P, Mulaa F, Mattiasson B (2010) Enzymatic enrichment of omega-3 polyunsaturated fatty acids in Nile perch (*Lates niloticus*) viscera oil. Eur J Lipid Sci Technol 112(9):977–984
56. Nyina-Wamwiza L, Wathelet B, Richir J, Rollin X, Kestemont P (2009) Partial or total replacement of fish meal by local agricultural by-products in diets of juvenile African catfish (*Clarias gariepinus*): growth performance, feed efficiency and digestibility: locals by-products in diets of *C. gariepinus*. Aquacult Nutr 16(3):237–247
57. Ogwok P, Muyonga JH, Sserunjogi ML, Amegovu AK, Makokha V (2009) Variation in chemical composition of oils from Nile Perch (*Lates niloticus*) belly flaps with capture site and season. J Aquat Food Prod Technol 18(4):331–344
58. Karim AA, Bhat R (2009) Fish gelatin: properties, challenges, and prospects as an alternative to mammalian gelatins. Food Hydrocoll 23(3):563–576
59. Sabirova JS, Haddouche R, Van Bogaert IN, Mulaa F, Verstraete W, Timmis KN, Schmidt-Dannert C, Nicaud JM, Soetaert W (2011) The 'LipoYeasts' project: using the oleaginous yeast Yarrowia lipolytica in combination with specific bacterial genes for the bioconversion of lipids, fats and oils into high-value products: bioconversion of lipids by *Yarrowia lipolytica*. J Microbial Biotechnol 4(1):47–54

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Starch-Modifying Enzymes

Suhaila Omar Hashim

Contents

Abstract Starch is a carbohydrate polymer found abundantly on earth. It is synthesized in plants as a short-term storage compound for respiration in the leaves and for long-term storage in the tubers, seeds and roots of plants. A wide variety of enzymes modify or convert starch into various products. The classes of enzymes that act on starch include endoamylases, exoamylases, debranching enzymes and transferases. Starch-modifying enzymes of microbial origin are utilized in a wide variety of industrial applications. Alkaline-active amylases are diverse in terms of optimum reaction conditions, substrate and product specificity. Amylases that are active at lower temperatures and alkaline conditions are most suited for detergent

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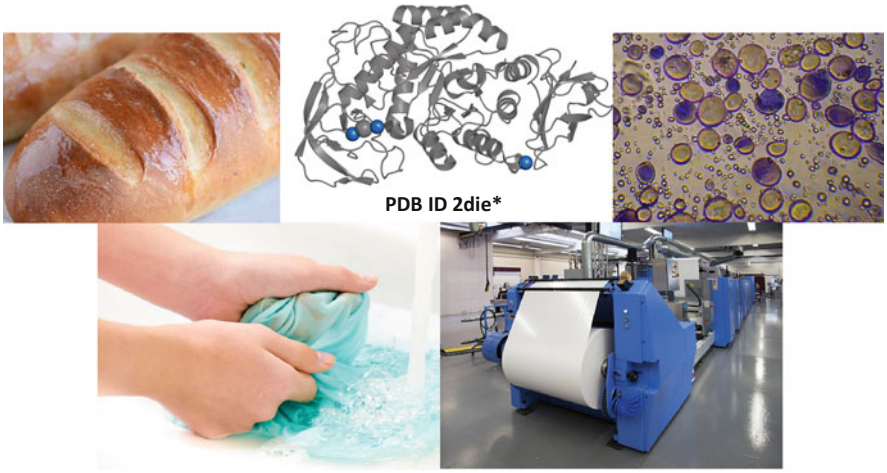
formulation. Other notable starch-modifying enzymes from alkaliphiles include maltooligosaccharide-forming amylases and cyclodextrin glycosyltransferases (CGTases), which produce a variety of maltooligosaccharides and cyclodextrins, respectively. Such compounds are used in the food, fine chemical, pharmaceutical and cosmetic industries, among others. Alkaline-active amylases are also applicable in the paper, textile and leather industries and also in bioremediation and alkaline waste water treatment. Their application in these fields is further enhanced through stabilization and improving their specificity and catalytic action by employing nanotechnology and genetic engineering.



Graphical Abstract **Alkaline alpha-amylase* AmyK from *Bacillus* sp. KSM-1378. Shirai T, Igarashi K, Ozawa T, Hagihara H, Kobayashi T, Ozaki K, Ito S (2007) Proteins 66:600–610. Source: Protein Data Bank in Europe (PDBe)

Keywords Alkaline, Alkaliphiles, CGTase, Cyclodextrins, Detergent, Industry, Leather, Maltooligosaccharides, Nanotechnology, Paper, Starch, Textile, Waste water treatment, α-Amylase

Abbreviations

AKD	Alkylketene dimer
ASA	Alkenylsuccinic anhydride
BAA	*Bacillus amyloliquefaciens* amylase
CDs	Cyclodextrins
CGTases	Cyclodextrin glycosyltransferases
DDM	Dodecyl-β-d-maltoside
DDMO	Dodecyl-β-d-maltooctaoside

DE	Dextrose equivalent
DP	Degree of polymerization
EC	Enzyme Commission
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
kDa	Kilodalton
MNPs	Magnetic nanoparticles
SEN	Single-enzyme nanoparticle

1 Starch

Starch is one of the most abundant carbohydrate polymers on earth. It is produced by plants as a result of photosynthesis, a process that involves conversion of energy from sunlight into chemical energy. Starch is synthesized in the chloroplasts in leaves of plants as a short-term storage compound for respiration during dark periods. It is also synthesized as a long-term storage compound in the tubers, seeds and roots of plants such as maize, tapioca, potato, rice and wheat.

Starch is a polymer of glucose units linked to one another via glycosidic bonds, which are stable at high pH but hydrolyse at low pH. It is made up of two polymers, amylose and amylopectin. Amylose is a linear polymer consisting of up to 1,000 glucose units linked to each other via α-1-4 glycosidic bonds. It is insoluble in cold water. The number of glucose residues or degree of polymerization (DP) varies with origin – amylose from potato starch has a higher DP than that from maize or wheat. The average amylose content in starch varies between 0 and 75%, with typical values ranging between 20 and 29%.

Amylopectin, on the other hand, is a branched, water-soluble polymer, made up of short, α-1-4-linked linear chains of 10–60 glucose units and α-1-6-linked side chains with 15–45 glucose units. The complete amylopectin molecule contains an average of 2,000,000 glucose units, making it one of the largest molecules in nature. The molecular structures of amylose and amylopectin are shown in Fig. 1 below.

Starch is a major component of the human diet. It is chemically and enzymatically processed into a wide range of products including glucose syrup, fructose, maltodextrins, cyclodextrins and starch hydrolysates. These products have a variety of applications in the food industry, while the sugars generated from starch can be fermented to produce ethanol. In the food industry, starch contributes greatly to the textural properties of many foods. It is also used in industrial applications as a thickener, colloidal stabilizer, gelling agent, bulking agent and water retention agent [1].

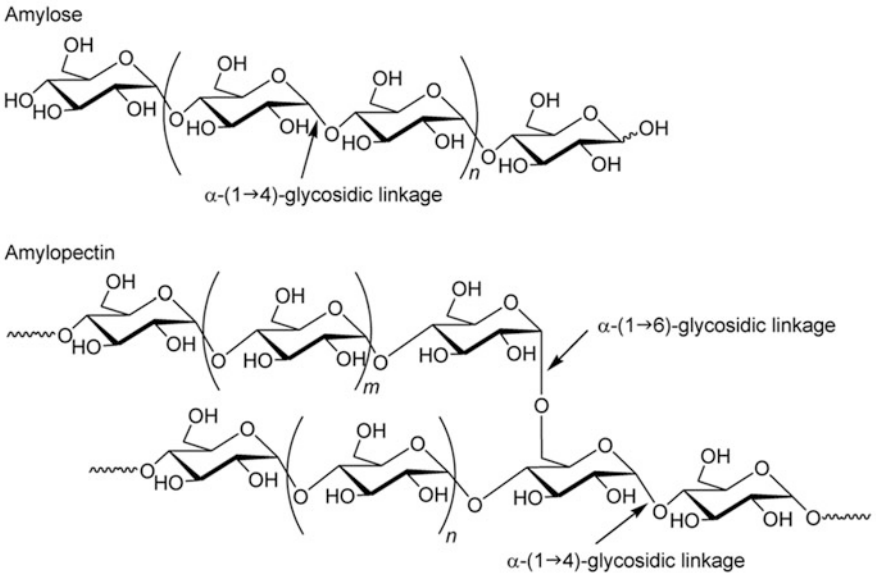


Fig. 1 Molecular structure of amylose and amylopectin. (Source: https://chemistry.stackexchange.com/questions/58080/bonding-between-amylopectin-and-amylose)

2 Scope of Enzymes that Act on Starch

Enzymes that act on starch are a very diverse group that exhibit a wide variety of substrate and product specificity. They are divided into four groups based on the reactions they catalyse and the products formed by their action on starch. These include (1) endoamylases, (2) exoamylases, (3) debranching enzymes and (4) transferases [2–5]. Starch-modifying enzymes can be obtained from animals, plants and microorganisms. However, it is those from fungal and bacterial origin that are widely applied in the industries.

2.1 Endoamylases

This group of enzymes cleaves the α-1-4 glycosidic bonds present in the inner part of the amylose and amylopectin polymers, to produce linear and branched oligosaccharides of varying lengths referred to as α-limit dextrins. The products formed are in the α-anomeric configuration as a result of the retaining mechanism of bond cleavage. An example of endoamylase is α-amylase (EC 3.2.1.1). Endoamylases are further divided into two categories according to the degree of hydrolysis of substrates: liquefying (30–40%) and saccharifying (50–60%) amylases [6].

Endoamylases are among the most important starch hydrolysing enzymes. They are of great significance for biotechnology with approximately 25% of the world enzyme market [7, 8]. Amylases of microbial origin are commercially available and have replaced the chemical hydrolysis of starch in starch processing industry [9]. The industrial application of amylases has been extensively discussed in several reviews [2, 6, 8, 10–16].

α-Amylases have been used in a wide range of applications such as in food, fermentation, textile, paper, detergent, pharmaceutical and fine chemical industries. With the advances in biotechnology, the amylase applications have expanded in many fields such as clinical, medicinal and analytical chemistry, as well as their widespread application in starch saccharification and in the textile, food, brewing and distilling industries [10–12].

2.2 Exoamylases

Exoamylases act on the external glucose residues of amylose and amylopectin. These enzymes may either exclusively cleave only the α-1-4 glycosidic bonds to produce maltose and β-limit dextrin, such as the β-amylase (EC 3.2.1.2), or cleave both α-1-4 and α-1-6 glycosidic bonds to produce only glucose, such as amyloglucosidase or glucoamylase (EC 3.2.1.3) and α-glucosidase (EC 3.2.1.20). β-Amylase and glucoamylase further convert the anomeric configuration of their products from α- to β-, via an inverting mechanism of bond cleavage. Glucoamylase and α-glucosidase differ in their substrate preferences, with the former hydrolysing long-chain polysaccharides more effectively, while the latter preferentially hydrolyses shorter maltoligosaccharides.

Also included in this group of enzymes are cyclodextrin glycosyltransferases (EC 2.4.1.19), enzymes with an additional transglycosylation function, maltogenic α-amylase (EC 3.2.1.133) and maltooligosaccharide-forming amylases such as the maltotetraose-forming enzyme from *Pseudomonas stutzeri* (EC 3.2.1.60) and the maltohexaose-forming amylase from *Klebsiella pneumonia*e (EC 3.2.1.98).

2.3 Debranching Enzyme

This group of enzymes exclusively hydrolyse the α-1-6 glycosidic bonds of amylopectin. They include isoamylase (EC 3.2.1.68) and pullulanase (EC 3.2.1.41) type I, which, in addition to amylopectin and glycogen, hydrolyse pullulan, a polymer consisting of repeating units of maltotriose that are linked via α-1-6 glycosidic bonds. The end products formed are long linear polysaccharides. Pullulanase type II hydrolyses both α-1-4 and α-1-6 glycosidic bonds to produce maltose and maltotriose. These enzymes are also referred to as α-amylase-pullulanase or amylopullulanase. Neopullulanases (EC 3.2.1.135) hydrolyse the α-1-4 glycosidic

bond of pullulan to produce panose (6-alpha-D-glucosylmaltose). They also catalyse transglycosylation reactions, with the formation of new α-1-4 or α-1-6 glycosidic bonds.

2.4 Transferases

Transferases cleave the α-1-4 glycosidic bond of a donor molecule and transfer part of the donor molecule to a glycosidic acceptor with the formation of a new glycosidic bond. Examples of this group of enzymes include amylomaltase (EC 2.4.1.25) and cyclodextrin glycosyltransferases or CGTases (EC 2.4.1.19), which form new α-1-4 glycosidic bonds, and 1,4-α-glucan branching enzymes (EC 2.4.1.18), which form new α-1-6 glycosidic bonds. CGTases make cyclic oligosaccharides with 6, 7 or 8 glucose residues by intramolecular transglycosylation. They also produce highly branched high-molecular-weight dextrins, referred to as glycosyltransferase limit dextrins. Amylomaltase, on the other hand, performs transglycosylation reactions resulting in the formation of linear products as opposed to cyclic products [66].

3 Starch-Modifying Enzymes from Alkaliphiles

Alkaliphiles are an important source of alkaline-active enzymes. Members of the genus *Bacillus* from the alkaliphilic microbial community are major producers of alkaline-active enzymes of industrial importance such as α-amylases. Most of these enzymes are secreted outside the cells, thus making their purification easier. Furthermore, the high growth rate of strains within this genus gives *Bacillus* species an added advantage in the production of enzymes of industrial interest. Currently, industrially important amylases are produced by recombinant DNA technology, and the source of genes encoding commercial amylases are from *Bacillus* species. Considerable diversity of α-amylases from alkaliphilic *Bacillus* species has been reported, in terms of activity and stability features of the enzymes, substrate specificity and products formed.

The first report of an alkaline-active α-amylase was from alkaliphilic *Bacillus* sp. strain A-40-2 [17]. The enzyme had a molecular weight of 70 kDa and was most active at pH 10.0–10.5, retaining 50% of activity between pH 9 and 11.5. It was classified as a saccharifying amylase, as it hydrolysed 70% of starch to produce mainly glucose, maltose and maltotriose. Several alkaline-active amylases from *Bacillus halodurans* species have been reported, including *Bacillus* sp. A-59 by Horikoshi and co-workers [18]. This species was reported to produce α-amylase, pullulanase and α-glucosidase [20]. Other alkaline-active enzymes that modify starch including maltooligosaccharide-forming amylases, cyclodextrin glycosyltransferases, pullulanases and isoamylases have also been reported extensively and discussed in the context of their industrial applications in subsequent sections.

4 Biotechnological Application of Alkaline-Active Starch-Modifying Enzymes

Alkaline-active starch-modifying enzymes have a diverse range of properties in terms of reaction conditions for activity, substrate and product specificity. For instance, they act on starch, amylose, amylopectin and pullulan to produce a wide range of products including glucose, maltose, maltooligosaccharides, cyclodextrins and branched dextrins. The optimum temperature of activity of these enzymes varies from moderate to high, while others are stable in the presence of various additives. These properties make these enzymes promising in industrial applications.

4.1 Detergent Industry

Detergents have been formulated to contain enzymes in order to enhance their performance in the removal of tough stains and also make the detergents environment friendly. Amylases are the second most important enzymes used in detergent industries, and about 90% of all liquid detergents are containing these enzymes [9, 10, 19]. The enzymes in the detergents aid in degrading starchy food residues from potato, custard, gravies, chocolate and so forth to dextrins and other smaller oligosaccharides, thereby facilitating dirt removal [20, 21]. Amylases from alkaliphiles that are active at lower temperatures and alkaline conditions are most suited for this application. In addition, they are also required to be stable against chelating agents, surfactants and the prevailing oxidizing washing environment [13, 22]. As metalloenzymes, most α-amylases contain at least one calcium ion per enzyme molecule, which is essential for activity and stability [23]. The amount of bound calcium may vary from one to about ten [6, 24]. As such, these enzymes are generally inhibited by chelating agents such as zeolites, EDTA and EGTA [25]. Several amylases from alkaliphiles have been reported to exhibit stability under these conditions, including stability in the presence of ionic and non-ionic surfactants such as sodium dodecyl sulphate, Triton X-100 and Tween 20 as well as in the presence of EDTA and oxidizing agents such as hydrogen peroxide and sodium perborate. These amylases are from *Bacillus licheniformis* MTCC1483 [26], *Bacillus licheniformis* NH1 [9], thermophilic *Bacillus* sp. A3-15 [27], *Bacillus* sp. PN 5 [28], *Bacillus* sp. ANT-6 [29], *Bacillus* sp. TS-23 [30], *Bacillus* sp. KSM-1378 [31], *Bacillus* sp. L1711 [32] and *Bacillus* sp. KSM-K38, which were found to have remarkable properties such as stability in the presence of up to 100 mM EDTA and EGTA and excess (1.8 M) of H_2O_2 [25].

Alkaline-active debranching enzymes in combination with α-amylases have been proposed to be very effective for the removal of starch stains [33]. This is because these enzymes can catalyse hydrolysis of both α-1-6 and α-1-4 bonds in starch, thereby complementing the α-amylase activity and enhancing the efficiency of breakdown of starch-based stains. Alkaline-active debranching enzymes that have been described include alkaline-active pullulanases from *Bacillus* sp. 202-1 [34],

Bacillus sp. S-1 [35, 36], *Micrococcus* sp. Y-1 [35], *Bacillus* sp. KSM-1876 [37], amylopullulanase from *Bacillus* sp. KSM-1378 [38], neopullulanase from *Bacillus* sp. KSM-1876 [39] and an isoamylase of alkaliphilic *Bacillus* sp. KSM-3309 [40].

The demand for amylases with high specific activity and activity at alkaline conditions for the detergent industry has prompted the engineering of existing enzymes to produce enzymes with the desired properties. Genes encoding wild-type and mutant *Bacillus amyloliquefaciens* amylase (BAA) were subjected to error-prone PCR and gene shuffling. One of the mutants resulted in a fivefold higher activity at pH 10, thus producing a more suitable enzyme for application in the detergent industry [41].

4.2 Production of Maltoligosaccharides

Maltoligosaccharides is a generic term that is used to refer to saccharides derived from glucose, with a degree of polymerization of two to ten glucose residues. The composition of saccharides obtained after starch hydrolysis is highly dependent on the effect of temperature, the conditions of hydrolysis and the origin of enzyme, enzyme properties such as substrate specificity, thermostability and pH optimum [12]. The discovery of microbial enzymes that produce specific oligosaccharides such as maltotriose, maltotetraose, maltopentaose and maltohexaose has made possible the manufacture of syrups with high content of each maltooligosaccharide. These syrups are prepared from liquefied starch using the specific enzyme in combination with a debranching enzyme, such as pullulanase or isoamylase. Such syrups have low sweetness, provide resistance to retrogradation of starch gels and prevent crystallization of sucrose. These products are now used in powdering materials (fillers), saccharides for dry milk, flavour enhancers for various foods, liquid diets for patients and viscosity-increasing agents in the food, beverage, cosmetic, pharmaceutical and fine chemicals industries [42].

There are various reports of alkaline-active maltooligosaccharide-forming amylases in the literature. These vary in the composition of the saccharides formed, which is dependent on the substrate used and the hydrolysis conditions. For instance, Amy 34, a maltohexaose-forming amylase from *Bacillus halodurans* LBK 34, produced maltohexaose as the main initial product of starch hydrolysis, while the major product formed from amylose, amylopectin and maltodextrin was maltotetraose [43]. Other maltooligosaccharide-forming amylases that have been described from alkaliphiles include the maltotetraose-forming alkaline α-amylase from an alkaliphilic *Bacillus* strain GM8901 [44], maltopentaose-forming amylase from *Bacillus* sp. TS-23 [30], maltohexaose-forming amylases from alkaliphilic *Bacillus* sp. H-167 [45, 46], *Bacillus* sp. 707 [47], maltohexaose and maltoheptaose-forming amylase from *Alcaligenes latus* D2271 [42], wherein it is reported that it is feasible to increase the yield of maltohexaose and maltoheptaose by the enzyme when the debranching enzymes pullulanases and isoamylases are included during the hydrolysis. Examples of alkaliphilic *Bacillus* species that produce maltooligosaccharides are summarized in Table 1. Majority of these

Table 1 Some examples of alkaline-active maltooligosaccharide-forming α-amylases from alkaliphiles

Organism	Molecular wt (kDa)	pH optimum	pH stability	Temperature optimum (°C)	Temperature stability (°C)	Major hydrolysis product	Reference
Bacillus sp. A-40-2	B	10–10.5	9.0–11.5	N.D.	N.D.	G1, G2, G3	[17]
Bacillus sp. ANT-6	94.5	10.5	7.0–11.0	80	50–100	ND	[29]
Bacillus strain GM8901	97	11.0–12.00	6.0–13.0	60	Up to 50	G4 via G6 and G5 intermediates	[44]
Bacillus sp. IMD 370	159	10.0	8.5–10.5	40	Up to 45	G4 and less	[121]
Bacillus sp. strain XAL601	224	9.0	–	70	–	G2–G4	[108]
Bacillus sp. H-167	102	10.5	7.0–12.0	60	50–55	G6>G4>G2>G5>G3.G1	[45]
Bacillus isolate KSM-K38	55	8.0–9.5	6.0–11.0	55–60	Up to 30	G3, G6, with G7 and G2 as intermediates	[25]
Bacillus strain KSM-1378	53	8.0–8.5	6.0–10.0	55	Up to 45	G3, G5, G6, G2	[31]
Bacillus sp. TS-23	65	9.0	7.0–10.0	60	30–90	G4	[62]
Bacillus sp. TS 23	65	9.0	–	60	–	G5	[30]
Bacillus clausii BT-21	101	9.5	N.D	55	Up to 55	G6, G4 and G2	[122]
Bacillus coagulans R_3	N.D.	10.5	7.0–11.0	85	Up to 75	G1–G4 and higher	[123]
Bacillus halodurans LBK 34	119	10.5–11.5	9.0	60	Up to 55	G6 and G4.G2.G5>G3 and G1	[43]

enzymes produce more than one maltooligosaccharide, although they preferentially produce one type as the predominant or initial product. Maltotetraose syrup (G4 syrup) is produced by starch hydrolysis using maltotetraose-forming amylases (G4 amylase). Commercial thermostable α-amylase of alkaliphilic *B. licheniformis* or *B. subtilis* strains has been used in the production of maltotetraose syrup [16].

The production of a mixture of maltooligosaccharides, or commonly referred to as "maltooligomer mix", a new commercial product, is produced by starch hydrolysis using α-amylase, β-amylase and pullulanase. Maltooligomer mix tastes less sweet than sucrose and has a lower viscosity than corn syrup because of its low content of high-molecular-weight dextrins. Thus, maltooligomer mix is mainly used as a substitute for sucrose and other saccharides. It is also used for preventing crystallization of sucrose in foods [2]. Maltooligosaccharide-forming amylases have potential application in the production of these maltooligomer mixtures of varying compositions.

A recent review on maltooligosaccharide-forming amylases mentions the benefits of maltooligosaccharides in human health [48]. Maltooligosaccharides reach the intestine without being digested and are used as substrates by the intestinal α-glucosidase which is derived from erythrocytes, thus providing continuous energy supply. They are therefore suitable for use by athletes and special patients. Additionally, pancreatic α-amylases digest dietary starch to produce maltose and maltooligosaccharides, which are then digested by α-glucosidases from the intestinal enterocytes to produce glucose. Maltooligosaccharides have been implicated in glycaemic control responses as they have been shown to signal intestinal enterocytes to promote faster cell differentiation [49] in [48].

4.3 Production of Cyclodextrins

Cyclodextrins (CDs) are non-reducing cyclic oligosaccharides composed of D-glucose units linked by α-1,4 glycosidic bonds. The most common are CDs made of 6, 7 or 8 of such units and are referred to as α-, β- and γ-cyclodextrins, respectively (Fig. 2). These cyclic molecules have an interior hydrophobic cavity and a hydrophilic exterior, which enables the inclusion of various hydrophobic molecules within the hydrophobic cavity, commonly referred to as "guest" molecules. This property makes these compounds more suitable for industrial application in analytical chemistry, agriculture, pharmaceutical, food, cosmetic, textile and other industries [50–53]. β-Cyclodextrin has a low solubility in water, which makes its separation and purification relatively easy compared to α- and γ-cyclodextrin [54]. On the other hand, compared to α- and β-cyclodextrins, γ-cyclodextrin has a larger internal cavity, higher water solubility and more bioavailability. As such, it has wider applications in many industries, especially in the food and pharmaceutical industries [55]. On the basis of the size of their cavities, α-cyclodextrin can form inclusion complexes only with low-molecular-weight molecules or compounds with aliphatic side chains, and β-cyclodextrin can complex aromatics or heterocycles,

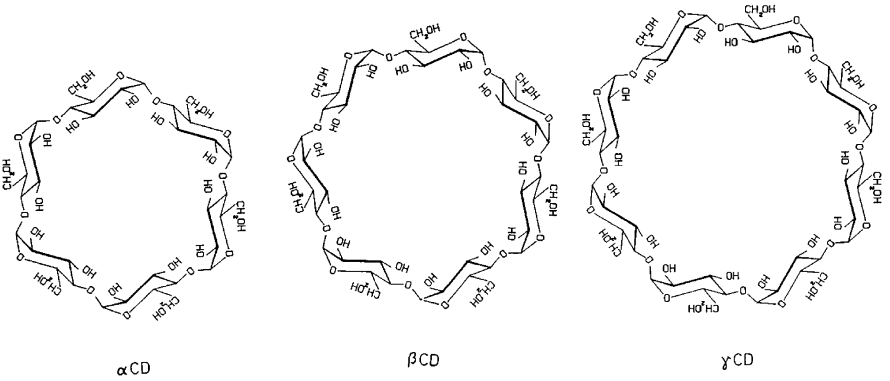


Fig. 2 Chemical structure of the three main types of cyclodextrins. Source: József Szejtli, 2004, IUPAC, Pure and Applied Chemistry 76(10), 1825–1845. (Reprinted with kind permission from IUPAC/Walter de Gruyter GmbH)

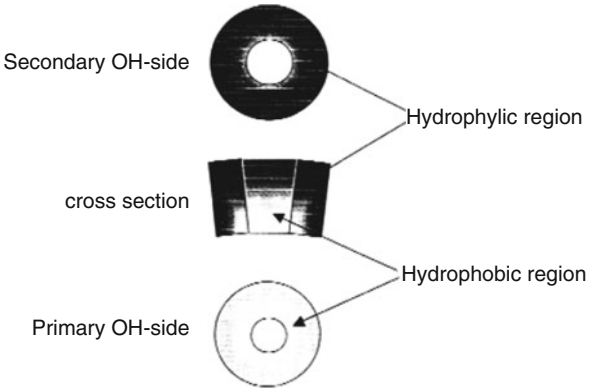


Fig. 3 Hydrophilic and hydrophobic regions of cyclodextrins. Adopted from József Szejtli, 2004, IUPAC, Pure and Applied Chemistry 76(10), 1825–1845. (Reprinted with kind permission from IUPAC/Walter de Gruyter GmbH)

while γ-cyclodextrin can accommodate a wider variety of large organic compounds such as macrocycles (molecules and ions containing twelve or more membered rings) and steroids [51].

The spatial arrangement of the glucose molecules of γ-cyclodextrin forms a toroid or cone shape with a cavity (Fig. 3). The smaller opening of the cavity consists of the primary hydroxyl groups, while the larger opening of the cavity consists of the secondary hydroxyl groups of the glucose residues.

Cyclodextrin glycosyltransferases (CGTases) produce CDs by their trans-glycosylation (cyclization) reaction on starch. In contrast to amylases, which generally hydrolyse glycosidic bonds in the starch, CGTases catalyse transglycosylation as a major reaction with hydrolysis being a minor activity [54, 56]. However, it is

worth noting that maltooligosaccharide-forming amylases are thought to be the evolutionary link between α-amylases and CGTases and are referred to as "intermediary" enzymes, thereby exhibiting features from both groups of enzymes [57]. CGTases and α-amylases are the most closely related enzymes within the α-amylase family, differing in their product specificity and reaction, whereby α-amylases carry out hydrolysis while CGTases perform transglycosylation reactions [3, 57]. An α-amylase from *Anaerobranca gottschalkii*, a thermoalkaliphile, was reported to exhibit transglycosylation on maltooligosaccharides and also displayed CGTase activity [58], while some amylases have been reported to hydrolyse CDs with varying rates of hydrolysis [59–62].

Initial industrial production of cyclodextrins from starch employed the use of a CGTase from a neutralophilic *Bacillus macerans* strain. However, the enzyme did not yield much product due to the low conversion rate, and the use of toxic solvents for precipitating the cyclodextrins posed a major production problem [63]. These problems were overcome by the use of a CGTase from the alkaliphilic *Bacillus* sp. strain 38-2, which resulted in yields of cyclodextrins at 85–90% from amylose and 70–80% from potato starch on a laboratory scale, allowing the direct crystallization of cyclodextrins from the hydrolysate without the use of organic solvents [64]. As a result, the cost of β-CD reduced from $1,000/kg to $5/kg and that of α-CD to within $15/kg [63].

Over the years, several other reports describing CGTase production from alkaliphiles have been published, with a majority of the CGTases reported to produce mixtures of α-, β- and γ-cyclodextrin at varying ratios [65–72]. However, in a number of reports, the ratio of the final products was found to change depending on the reaction time, type and concentration of substrate [68, 73]. Production of mixtures of cyclodextrins makes the separation of the individual cyclodextrins costly, and hence CGTases that produce predominantly a single type of cyclodextrin are of more interest [74]. All known wild-type CGTases essentially produce a mixture of α-, β- and γ-cyclodextrins, and as such, they have been further classified into α-, β- and γ-CGTases according to their major cyclodextrin products [75].

Examples of alkaliphilic microorganisms that predominantly produce one type of cyclodextrin include the CGTase from the thermoalkaliphilic anaerobic *Anaerobranca gottschalkii* which preferentially produces α-cyclodextrin [68], β-cyclodextrin producing CGTases from *Bacillus agaradhaerens* LS-3C [74], *Bacillus firmus* [76], *Amphibacillus* sp. NPST-10 [77], *Bacillus clausii* E16a [78], *Bacillus pseudalcaliphilus* 20RF [67] and γ-cyclodextrin producing CGTases from *Bacillus* sp. G-825-6 [73], *Brevibacterium* sp. No. 9605 [79] and *Bacillus clarkii* [80, 81]. In many of these reports, the reaction conditions and choice of substrate were found to influence the type of cyclodextrin produced. Furthermore, genetic manipulation of CGTases to enhance production of a specific cyclodextrin has also been conducted on several enzymes [82–86].

There are also reports of production of large ring cyclodextrins – cyclodextrins with greater than nine glucose units (CD_9) from various microorganisms, including alkaliphiles [87]. However, only a few have been characterized, owing to their low yields and difficulty in isolation from commercial cyclodextrin mixtures [88]. The

amount and size of large ring cyclodextrins formed was found to depend on the enzyme CGTase used, reaction time and type of substrate used. These large ring cyclodextrins have unique structures and geometry of their cavities, which are more flexible and have been proposed to find applications as novel host compounds in molecular recognition processes [89].

CGTases have also been applied in the synthesis of alkyl glycosides with long carbohydrate groups for use as surfactants. Surfactants with long-carbohydrate-chain alkyl groups are generally preferred due to their attractive properties. However, they are difficult to synthesize. Several studies have shown the possibility of using CGTase for the production of surfactants with longer carbohydrate chains. In one study, the commercially available surfactant, dodecyl-β-d-maltoside (DDM), was converted to dodecyl-β-d-maltooctaoside (DDMO), in a single step by using *B. macerans* CGTase as catalyst and α-cyclodextrin (α-CD) as glycosyl donor, resulting in high yields of up to 80% of the DDMO [90]. In a follow-up study, the enzyme was immobilized on Eupergit C and used in a packed-bed reactor for continuous production of long-carbohydrate-chain alkyl glycosides from α-cyclodextrin and n-dodecyl-(1,4)-beta-maltopyranoside (C(12)G(2)beta), a commercially available surfactant [91]. Yields of up to 50% of the product, n-dodecyl-(1,4)-beta-maltooctaoside (C(12)G(8)beta), were achieved with the immobilized enzyme. An investigation of the transglycosylation reaction between an alkyl polyglycoside and α-cyclodextrin by the *Bacillus macerans* CGTase resulted in production of alkyl glucopyranosides glycosylated with 6 (primary coupling products) or 12 (secondary coupling products) glucose residues, with glycosylation of both α- and β-anomers [92]. Thus CGTases have shown to be important enzymes for synthesis and biotransformation applications such as in the production of surfactants with superior properties.

4.4 Food Industry

Starch is an important constituent of the human diet. It contributes greatly to the textural properties of many foods and is widely used in food and industrial applications as a thickener, colloidal stabilizer, gelling agent, bulking agent and water retention agent. It is chemically and enzymatically processed into a variety of products such as starch hydrolysates, glucose syrups, fructose, maltodextrin and cyclodextrin derivatives for use in the food industry.

Industrial starch processing involves three stages, gelatinization, liquefaction and saccharification. In gelatinization, starch slurry consisting of 30–40% dry solids is injected with pressurized steam (jet cooking) at 105°C for 5 min, resulting in a highly viscous suspension of dissolved starch. After gelatinization, the starch slurry is cooled to the desired temperature for liquefaction. Liquefaction is the partial hydrolysis of the starch slurry and results in reduced viscosity of the starch hydrolysate. It is carried out using amylases which hydrolyse the starch slurry to produce dextrins. Liquefaction is typically carried out at 95–100°C for 1–2 h, in the

presence of a thermostable α-amylase from *Bacillus licheniformis* (e.g. Termamyl from Novozymes) or *Bacillus amyloliquefaciens*, although the *B. licheniformis* enzyme is preferred due to its higher temperature stability. Furthermore, due to the enzyme's superior thermal stability, gelatinization and liquefaction can be combined, whereby the enzyme is added to the starch slurry prior to gelatinization, with a minimum adjustment of pH and calcium ion concentration in order to accommodate the enzyme.

Liquefaction is stopped once the required dextrose equivalent (DE) value is obtained, typically between 8 and 12. The DE value is defined as the amount of reducing sugars in a product and often expressed as percentage on dry basis relative to pure glucose (dextrose), with glucose having a DE of 100 and that of starch at near zero. The maximum DE value that can be obtained by *Bacillus* amylases used in this process is 40. The final stage is saccharification, which involves the production of glucose and maltose via further enzymatic hydrolysis. This involves the use of exo-acting enzymes such as β-amylase, pullulanase or glucoamylase. The liquefied starch with a DE of 8–12 is pumped into a large stirred vessel and after adjusting the pH to 4–5 and the temperature to about 60°C. The exoamylases are added to further degrade the liquefied starch into maltodextrins and maltose or glucose syrups. For the production of high-glucose syrup, the enzymes glucoamylase and pullulanase are used during saccharification, while, for the production of maltose syrup, fungal α-amylase is used at a pH of 5.5 at 55°C.

The conditions applied for liquefaction and saccharification largely depend on the enzyme used for these processes. Several alkaliphiles have been reported to produce thermostable, liquefying and saccharifying amylases suitable for starch processing and have been proposed for potential application in these processes. These include a novel liquefying α-amylase (LAMY) from the alkaliphilic *Bacillus* isolate, KSM-1378 [31]; saccharifying amylase from *Bacillus* sp. A3-15 [27], *Bacillus* sp. PN-5 [28], *Bacillus* sp. BCC 01-50 [93], *Bacillus* species IMD 435 which produce glucose and maltose from both soluble starch and raw corn starch [94] and *Bacillus subtilis* JS-2004 [95]; and several pullulanases that have been reported [35–40, 96].

The baking industry is another large consumer of starch and starch-modifying enzymes. It is one of the oldest industries to use α-amylases. These enzymes give the baked products, such as bread, a higher volume, better colour and a softer crumb, as they degrade the starch into smaller dextrins, thereby allowing the yeast to continuously ferment the dough during production [10]. In addition, the small oligosaccharides and sugars such as glucose and maltose produced by these enzymes enhance the Maillard reactions responsible for the browning of the crust and the development of an attractive baked flavour.

During storage of bread, the crumb becomes dry and firm, the crust loses its crispness and the flavour of the bread deteriorates. These undesirable changes are referred to as staling. Additives such as sugars, salts, milk powder, emulsifiers, monoglycerides/diglycerides, sugar esters, lecithin, granulated fat and anti-oxidants (ascorbic acid or potassium borate) have been conventionally used to prevent staling [97]. Many starch-modifying enzymes are now used as anti-staling agents. These

include α-amylases [98, 99], β-amylases [100], maltogenic amylases [101], branching enzymes [102], debranching enzymes [103] and amyloglucosidases [104]. These may be used separately or in combination [103]. Maltooligosaccharide-forming amylases have also been shown to reduce the staling of bread as they produce maltotriose (G3), maltotetraose (G4) and maltopentaose (G5), which retain water molecules, thereby preventing starch-starch interactions and inhibiting starch recrystallization [105, 106]. However, excessive use of α-amylase results in stickiness in bread [101]. This is circumvented by the use of intermediate temperature-stable (ITS) α-amylases, which are inactivated before the completion of the baking process [10].

4.5 Alkaline-Active Raw Starch-Degrading Amylases for Pulp and Paper Industry

In the pulp and paper industry, the primary role of amylases is in the production of suitable starch for coating and surface sizing of the paper. It is important at this point to distinguish between internal sizing and surface sizing during the production of paper. Internal sizing refers to the process of adding hydrophobic substances to the pulp slurry in order to produce paper that is fluid resistant, while surface sizing involves the application of a viscous solution to the surface of the paper [107]. Paper sizing is primarily done to protect the paper against mechanical damage, enhance the stiffness and strength of the paper and improve the erasability of the paper [10].

Earlier, internal sizing was conducted at acidic conditions using rosin- and aluminium-based compounds. Since the 1980s, there has been a significant shift towards using sizing chemicals such as alkylketene dimer (AKD) and alkenylsuccinic anhydride (ASA) at alkaline conditions [107]. This is because paper produced from sizing at acidic conditions was found to deteriorate very rapidly, while at alkaline conditions, a more durable product is produced.

For surface sizing and coating of paper, starch has been found particularly useful, and the process is well described in a review by Gupta et al. [10]. Starch is added to the paper in the size press, and the paper picks up the starch by passing through two rollers that transfer the starch slurry. The temperature of this process lies in the range of 45–60°C. A constant viscosity of the starch is required for reproducible results at this stage. The viscosity of the raw or native starch is too high for paper sizing and is adjusted either chemically or by partially degrading the polymer with α-amylases in a batch or continuous process. The hydrolysis conditions depend on the source of starch and the α-amylase used. Since alkaline conditions are preferred for the internal sizing process, it is therefore advantageous to use alkaline-active amylases for the production of starch with suitable viscosity. Furthermore, α-amylases that are capable of hydrolysing raw starch are preferred, as this will reduce production costs by avoiding expensive chemically modified starches. Raw starch-degrading amylases have been reported from alkaliphiles. The enzyme from *Bacillus* sp. IMD 435 was reported to hydrolyse

both soluble starch and raw corn starch to produce glucose and maltose [94]. The thermophilic and alkaliphilic *Bacillus* sp. strain XAL601 was reported to produce an amylopullulanase which could hydrolyse raw starch under a broad range of conditions [108]. From *Bacillus* sp. TS-23, the amylase was reported to hydrolyse raw starch with concomitant production of maltotriose, maltotetraose and maltopentaose [62]. Such alkaline-active, raw starch-degrading amylases are therefore most suitable for application in surface sizing and coating of paper using starch.

4.6 Textile Industry

During the process of weaving the yarn in the textile industry, the warp threads are exposed to extensive mechanical strain. To avoid breakage, the warp threads are coated using a gelatinous compound in a process referred to as "sizing". Starch, either native or modified, is commonly used for sizing as it is a cheap, readily available polymer and can easily be removed from the fabric after the weaving process. Subsequently, after sizing with starch, the coated warp threads are not able to absorb water or finishing agents such as dyes and other chemicals used in the textile industry. As such, the starch needs to be removed from the warp. This is done by a process known as "desizing". The process was conventionally carried out using oxidizing agents such as ammonium persulfate or hydrogen peroxide at high pH and temperature. This process has limitations as it does not efficiently remove the starch and resulted in a fabric with reduced tensile strength. Furthermore, use of chemicals is costly and harmful to the environment and would subsequently require effluent treatment and disposal. An alternative is the use of alkaline-active amylases that catalyse hydrolysis of starch to water-soluble dextrins which are then removed by washing at mildly alkaline conditions. These enzymes efficiently remove the starch coating without damaging the fabric. The enzyme PrimaGreen®ALL from DuPont™ has been engineered to desize textiles throughout a much broader spectrum of operating conditions including temperature ranging from 20 to 105°C and pH of 6–10.

4.7 Alkaline Waste Water Treatment

Various industries generate waste waters that are toxic, rich in heavy metals and dyes, highly acidic or highly alkaline. The paper and pulp, textile and potato processing industries generate alkaline waste water with pH greater than 10. In a study conducted in India, alkaliphilic microorganisms were isolated from textile waste water and were found to neutralize the alkaline industrial waste waters [109]. It was postulated that these microbes were able to neutralize the pH by producing acids through their carbohydrate metabolism by alkaline-active amylases. Thus, these enzymes are suitable combined with microorganisms that can metabolize

the hydrolysis products from starch for neutralizing alkaline waste waters from the pulp and paper and textile industries, which are not only alkaline but also contain starch. Apart from neutralization of the pH of alkaline waste waters, these enzymes break down the polysaccharides present in the industrial effluents, thereby reducing the biochemical oxygen demand (BOD), chemical oxygen demand (COD) and suspended solids.

An area that is gaining popularity in waste water treatment is nanotechnology, whereby nanoparticles are used for degradation of contaminants to harmless products like water and CO_2 [110]. Bioremediation of waste water can be achieved by using a combination of enzyme technology and nanotechnology known as SEN, that is, single-enzyme nanoparticle [111]. A SEN is an enzyme covered by a protective thick cage that is a few nanometres. Typically, either cell-free crude extracts or purified enzymes are used for SEN formulation. Nanotechnology has been successfully applied in immobilization and stabilization of amylolytic enzymes including α-amylases, β-amylases, glucoamylases and pullulanases [112]. Immobilization improves enzyme performance and helps to minimize steric hindrances during binding of substrate to the active site of the enzyme. It also results in enzymes with very high resistance to inactivation, high activity and stability, thus enabling their repeated and continuous use in applications in other areas than waste water treatment, such as food, fuel, textile, paper and pulp, detergent, environmental, medical and analytical fields [112]. A *Bacillus alcalophilus* alkaline-active α-amylase was immobilized onto nanosized supermagnetic iron oxide magnetic nanoparticles (MNPs) [113]. The immobilized enzyme exhibited significantly higher specific activity, stability and reusability as compared to the free enzyme. Such enzyme formulations would be suitable for application where alkaline conditions prevail, such as in alkaline waste water treatment, detergent, textile, paper and pulp industries.

4.8 Leather Industry

Conventional leather processing or beam-house operations involve the use of chemicals such as lime and sodium sulphide for the dehairing of animal skins at alkaline conditions. The liming-reliming processes contribute 60–70% of the total pollution load in leather processing [114]. There have been eco-friendly initiatives to address this through the use of enzymes to replace the toxic chemicals for these processes. Proteases and lipases have been applied for the unhairing and degreasing/defleshing of the animal skins and hides with several advantages. These include preservation of skin colour and significant reduction of BOD and COD values of effluents [115, 116]. Furthermore, enzymatically processed skins produced crust leather that exhibited similar physical and tactile properties as compared to conventional crust leather produced by chemicals. In a recent study, an α-amylase was used for fibre splitting of goatskins, with the aim of reducing pollution from beam-house processes [114]. The use of the enzyme resulted in a lower pollution load (COD,

TS), with significant release of inter-fibrillary materials. The enzyme was produced using solid-state fermentation with wheat bran as the substrate and showed equivalent fibre splitting with lower cost than chemical-based processes and commercially used enzyme powder products. Alkaline-active amylases produced from such inexpensive substrates are thus good candidates for the leather processing and can also be used in treatment of alkaline waste water generated from the leather processing.

5 Conclusion

Alkaline-active starch-modifying enzymes have significant real and potential industrial applications. A lot of alkaline-active amylases have been heterologously expressed in recombinant hosts in order to improve yields and optimize their properties. An example is the recombinant alkaline-active and thermotolerant amylase from *Bacillus halodurans* MS-2-5 which was cloned and expressed in *E. coli* with 104-fold yield as compared to that of the parent strain, MS-2-5 [117]. In another strategy employing the atmospheric and room temperature plasma (ARTP) mutation breeding technique, it was found that some *Bacillus subtilis* mutants produced higher yields of recombinant alkaline α-amylase (AMY) as compared to expression of the amylase in the wild-type *Bacillus subtilis* host [118].

Studies have also been conducted to continuously improve the properties of these enzymes. Through site-directed mutagenesis, the oxidative stability of alkaline-active amylase from *Alkalimonas amylolytica* was enhanced, while some mutants also exhibited extended pH stability of up to pH 12 [119]. Such mutants would be more suitable for the detergent and textile industries. The specific activity and catalytic efficiency of alkaline-active α-amylase (AmyK) from *Alkalimonas amylolytica* was further enhanced by using a novel protein engineering strategy that involved the integration of terminal truncation and N-terminal oligopeptide fusion [120]. This was done by partially truncating the C- or N-terminus of AmyK, followed by fusion of an oligopeptide at the N-terminus of the truncated AmyK. The truncation-fusion mutants were found to have higher catalytic efficiency than AmyK. Higher flexibility around the active site was proposed to be the possible reason for the improved catalytic efficiency of AmyK amylase. With enhanced catalytic activity and stability through genetic engineering and nanotechnology, greater potential can be realized from these robust enzymes.

References

1. Singh J, Kaur L, McCarthy OJ (2007) Factors influencing the physico-chemical, morphological, thermal and rheological properties of some chemically modified starches for food applications – a review. Food Hydrocoll 21(1):1–22

2. van der Maarel MJEC, van der Veen B, Uitdehaag JCM, Leemhuis H, Dijkhuizen L (2002) Properties and applications of starch-converting enzymes of the alpha-amylase family. J Biotechnol 94:137–155
3. Janecek S (1997) alpha-Amylase family: molecular biology and evolution. Prog Biophys Mol Biol 67:67–97
4. Janecek S (2000) Amylolytic enzymes: their specificities, origins and properties. Biologia-Bratislava 55:605–615
5. Kuriki T, Imanaka T (1999) The concept of the alpha-amylase family: structural similarity and common catalytic mechanism. J Biosci Bioeng 87:557–565
6. Vihinen M, Mäntsälä P (1989) Microbial amylolytic enzymes. Crit Rev Biochem Mol Biol 24:329–418
7. Rajagopalan G, Krishnan C (2008) Alpha-amylase production from catabolite derepressed *Bacillus subtilis* KCC103 utilizing sugarcane bagasse hydrolysate. Bioresour Technol 99:3044–3050
8. Reddy NS, Nimmagadda A, Sambasiva Rao KRS (2003) An overview of the microbial α-amylase family. Afr J Biotechnol 2:645–648
9. Hmidet N, El-Hadj Ali N, Haddar A, Kanoun S, Alya S-K, Nasri M (2009) Alkaline proteases and thermostable α-amylase co-produced by *Bacillus licheniformis* NH1: characterization and potential application as detergent additive. Biochem Eng J 47:71–79
10. Gupta R, Gigras P, Mohapatra H, Goswami VK, Chauhan B (2003) Microbial α-amylases: a biotechnological perspective. Process Biochem 38:1599–1616
11. Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R (2000) Advances in microbial amylases. Biotechnol Appl Biochem 31(Pt 2):135–152
12. Kandra L (2003) α-Amylases of medical and industrial importance. J Mol Struct (THEOCHEM) 666–667:487–498
13. Kirk O, Borchert TV, Fuglsang CC (2002) Industrial enzyme applications. Curr Opin Biotechnol 13:345–351
14. de Souza PM, de Oliveira Magalhães P (2010) Application of microbial α-amylase in industry – a review. Braz J Microbiol 41:850–861
15. Mobini-Dehkordi M, Javan FA (2012) Application of alpha-amylase in biotechnology. J Biol Today's World 1:15–20
16. Vaidya S, Srivastava PK, Rathore DP, Pandey AK (2015) Amylases: a prospective enzyme in the field of biotechnology, vol 41, pp 1–18
17. Horikoshi K (1971) Production of alkaline enzymes by alkalophilic microorganisms part II. Alkaline amylase produced by *Bacillus* No. A-40-2. Agric Biol Chem 35:1783–1791
18. Yamamoto M, Tanaka Y, Horikoshi K (1972) Alkaline amylases of alkalophilic bacteria. Agric Biol Chem 36:1819–1823
19. Mitidieri S, Souza Martinelli AH, Schrank A, Vainstein MH (2006) Enzymatic detergent formulation containing amylase from *Aspergillus niger*: a comparative study with commercial detergent formulations. Bioresour Technol 97:1217–1224
20. Mukherjee AK, Borah M, Rai SK (2009) To study the influence of different components of fermentable substrates on induction of extracellular α-amylase synthesis by *Bacillus subtilis* DM-03 in solid-state fermentation and exploration of feasibility for inclusion of α-amylase in laundry detergent formulations. Biochem Eng J 43:149–156
21. Olsen HS, Falholt P (1998) The role of enzymes in modern detergency. J Surfactant Deterg 1:555–567
22. Chi M-C, Chen Y-H, Wu T-J, Lo H-F, Lin L-L (2010) Engineering of a truncated α-amylase of *Bacillus* sp. strain TS-23 for the simultaneous improvement of thermal and oxidative stabilities. J Biosci Bioeng 109:531–538
23. Vallee BL, Stein EA, Sumerwell WN, Fischer EH (1959) Metal content of alpha-amylases of various origins. J Biol Chem 234:2901–2905
24. Janeček Š, Baláž Š (1992) α-Amylases and approaches leading to their enhanced stability. FEBS Lett 304:1–3

25. Hagihara H, Igarashi K, Hayashi Y, Endo K, Ikawa-Kitayama K, Ozaki K, Kawai S, Ito S (2001) Novel α-amylase that is highly resistant to chelating reagents and chemical oxidants from the alkaliphilic *Bacillus* isolate KSM-K38. Appl Environ Microbiol 67:1744–1750
26. Dahiya P, Rathi P (2015) Characterization and application of alkaline α-amylase from *Bacillus licheniformis* MTCC1483 as a detergent additive. Int Food Res J 22:1293–1297
27. Arikan B (2008) Highly thermostable, thermophilic, alkaline, SDS and chelator resistant amylase from a thermophilic *Bacillus* sp. isolate A3-15. Bioresour Technol 99:3071–3076
28. Saxena RK, Dutt K, Agarwal L, Nayyar P (2007) A highly thermostable and alkaline amylase from a *Bacillus* sp. PN5. Bioresour Technol 98:260–265
29. Burhan A, Coral G, Colak O, Aygan A, Gulnaz O (2003) Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. Isolate ANT-6. Process Biochem 38:1397–1403
30. Lo H-F, Lin L-L, Chen H-L, Hsu W-H, Chang C-T (2001) Enzymic properties of a SDS-resistant *Bacillus* sp. TS-23 α-amylase produced by recombinant Escherichia coli. Process Biochem 36:743–750
31. Igarashi K, Hatada Y, Hagihara H, Saeki K, Takaiwa M, Uemura T, Ara K, Ozaki K, Kawai S, Kobayashi T, Ito S (1998) Enzymatic properties of a novel liquefying α-amylase from an alkaliphilic *Bacillus* isolate and entire nucleotide and amino acid sequences. Appl Environ Microbiol 64:3282–3289
32. Bernhardsdotter ECMJ, Ng JD, Garriott OK, Pusey ML (2005) Enzymic properties of an alkaline chelator-resistant α-amylase from an alkaliphilic *Bacillus* sp. isolate L1711. Process Biochem 40:2401–2408
33. Ito S, Kobayashi T, Ara K, Ozaki K, Kawai S, Hatada Y (1998) Alkaline detergent enzymes from alkaliphiles: enzymatic properties, genetics, and structures. Extremophiles 2:185–190
34. Nakamura N, Watanabe K, Horikoshi K (1975) Purification and some properties of alkaline pullulanase from a strain of *Bacillus* No. 202-1, an alkalophilic microorganism. Biochim Biophys Acta 397:188–193
35. Kim C-H, Choi H-I, Lee D-S (1993) Pullulanases of alkaline and broad pH range from a newly isolated alkalophilic *Bacillus* sp. S-1 and a *Micrococcus* sp. Y-1. J Ind Microbiol 12:48–57
36. Kim C-H, Choi H-I, Lee D-S (1993) Purification and biochemical properties of an alkaline pullulanase from Alkalophilic *Bacillus* sp. S-l. Biosci Biotechnol Biochem 57:1632–1637
37. Ara K, Igarashi K, Saeki K, Kawai S, Ito S (1992) Purification and some properties of an alkaline pullulanase from alkalophilic *Bacillus* sp. KSM-1876. Biosci Biotechnol Biochem 56:62–65
38. Ara K, Saeki K, Igarashi K, Takaiwa M, Uemura T, Hagihara H, Kawai S, Ito S (1995) Purification and characterization of an alkaline amylopullulanase with both alpha-1,4 and alpha-1,6 hydrolytic activity from alkalophilic *Bacillus* sp. KSM-1378. Biochim Biophys Acta 1243:315–324
39. Igarashi K, Ara K, Saeki K, Ozaki K, Kawai S, Ito S (1992) Nucleotide sequence of the gene that encodes a neopullulanase from an alkalophilic *Bacillus*. Biosci Biotechnol Biochem 56:514–516
40. Ara K, Saeki K, Ito S (1993) Purification and characterization of an alkaline isoamylase from an alkalophilic strain of *Bacillus*. J Gen Microbiol 139:781–786
41. Bessler C, Schmitt J, Maurer K-H, Schmid RD (2003) Directed evolution of a bacterial α-amylase: toward enhanced pH-performance and higher specific activity. Protein Sci 12:2141–2149
42. Nakano M, Chaen H, Sugimoto T, Miyake T (2001) Maltohexaose and maltoheptaose-forming amylase, and its preparation and uses. Patent no. US6242224B1
43. Hashim SO, Delgado OD, Martínez MA, Kaul R-H, Mulaa FJ, Mattiasson B (2005) Alkaline active maltohexaose-forming α-amylase from *Bacillus halodurans* LBK 34. Enzyme Microb Technol 36:139–146

44. Kim TU, Gu BG, Jeong JY, Byun SM, Shin YC (1995) Purification and characterization of a maltotetraose-forming alkaline (alpha)-amylase from an alkalophilic *Bacillus* Strain, GM8901. Appl Environ Microbiol 61:3105–3112
45. Hayashi T, Akiba T, Horikoshi K (1988) Production and purification of new maltohexaose-forming amylases from alkalophilic *Bacillus* sp. H-167. Agric Biol Chem 52:443–448
46. Hayashi T, Akiba T, Horikoshi K (1988) Properties of new alkaline maltohexaose-forming amylases. Appl Microbiol Biotechnol 28:281–285
47. Kanai R, Haga K, Akiba T, Yamane K, Harata K (2004) Biochemical and crystallographic analyses of maltohexaose-producing amylase from alkalophilic *Bacillus* sp. 707. Biochemistry 43:14047–14056
48. Pan S, Ding N, Ren J, Gu Z, Li C, Hong Y, Cheng L, Holler TP, Li Z (2017) Maltooligosaccharide-forming amylase: characteristics, preparation, and application. Biotechnol Adv 35:619–632
49. Chegeni M, Hamaker B (2015) Induction of differentiation of small intestinal enterocyte cells by maltooligosaccharides. FASEB J 29:596.14
50. Shieh WJ, Hedges AR (1996) Properties and applications of cyclodextrins. J Macromol Sci Part A 33:673–683
51. Del Valle EMM (2004) Cyclodextrins and their uses: a review. Process Biochem 39:1033–1046
52. Astray G, Gonzalez-Barreiro C, Mejuto JC, Rial-Otero R, Simal-Gándara J (2009) A review on the use of cyclodextrins in foods. Food Hydrocoll 23:1631–1640
53. Amiri S, Amiri S (2017) Cyclodextrin applications. Cyclodextrins. Wiley-Blackwell, Chichester, pp 269–300
54. Biwer A, Antranikian G, Heinzle E (2002) Enzymatic production of cyclodextrins. Appl Microbiol Biotechnol 59:609–617
55. Li Z, Wang M, Wang F, Gu Z, Du G, Wu J, Chen J (2007) γ-Cyclodextrin: a review on enzymatic production and applications. Appl Microbiol Biotechnol 77:245–255
56. van der Veen BA, van Alebeek GJ, Uitdehaag JC, Dijkstra BW, Dijkhuizen L (2000) The three transglycosylation reactions catalyzed by cyclodextrin glycosyltransferase from *Bacillus circulans* (strain 251) proceed via different kinetic mechanisms. Eur J Biochem 267:658–665
57. Janeček Š (1995) Close evolutionary relatedness among functionally distantly related members of the (α/β)8-barrel glycosyl hydrolases suggested by the similarity of their fifth conserved sequence region. FEBS Lett 377:6–8
58. Ballschmiter M, Armbrecht M, Ivanova K, Antranikian G, Liebl W (2005) AmyA, an alpha-amylase with beta-cyclodextrin-forming activity, and AmyB from the thermoalkaliphilic organism *Anaerobranca gottschalkii:* two alpha-amylases adapted to their different cellular localizations. Appl Environ Microbiol 71:3709–3715
59. Brumm PJ, Hebeda RE, Teague WM (1991) Purification and characterization of the commercialized, cloned *bacillus megaterium* α-amylase. Part I: purification and hydrolytic properties. Starch-Stärke 43:315–319
60. Brumm PJ, Hebeda RE, Teague WM (1991) Purification and characterization of the commercialized, cloned *Bacillus megaterium* α-amylase. Part II: transferase properties. Starch-Stärke 43:319–323
61. Saha BC, Zeikus JG (1992) Cyclodextrin degrading enzymes. Starch-Stärke 44:312–315
62. Lin LL, Chyau CC, Hsu WH (1998) Production and properties of a raw-starch-degrading amylase from the thermophilic and alkaliphilic *Bacillus* sp. TS-23. Biotechnol Appl Biochem 28(Pt 1):61–68
63. Horikoshi K (1999) Alkaliphiles: some applications of their products for biotechnology. Microbiol Mol Biol Rev 63:735–750
64. Matzuzawa M, Kawano M, Nakamura N, Horikoshi K An improved method for the preparation of schardinger β-dextrin on an industrial scale by cyclodextrin glycosyl transferase of an alkalophilic *Bacillus* Sp. (ATCC 21783). Starch-Stärke 27:410–413

65. Nakamura N, Horikoshi K (1976) Purification and properties of cyclodextrin glycosyltransferase of an alkalophilic *Bacillus* sp. Agric Biol Chem 40:935–941
66. Kim MH, Sohn CB, Oh TK (1998) Cloning and sequencing of a cyclodextrin glycosyltransferase gene from Brevibacillus brevis CD162 and its expression in *Escherichia coli*. FEMS Microbiol Lett 164:411–418
67. Atanasova N, Kitayska T, Bojadjieva I, Yankov D, Tonkova A (2011) A novel cyclodextrin glucanotransferase from alkaliphilic *Bacillus pseudalcaliphilus* 20RF: purification and properties. Process Biochem 46:116–122
68. Thiemann V, Dönges C, Prowe SG, Sterner R, Antranikian G (2004) Characterisation of a thermoalkali-stable cyclodextrin glycosyltransferase from the anaerobic thermoalkaliphilic bacterium *Anaerobranca gottschalkii*. Arch Microbiol 182:226–235
69. Cao X, Jin Z, Wang X, Chen F (2005) A novel cyclodextrin glycosyltransferase from an alkalophilic *Bacillus* species: purification and characterization. Food Res Int 38:309–314
70. Moriwaki C, Ferreira LR, Rodella JRT, Matioli G (2009) A novel cyclodextrin glycosyltransferase from *Bacillus sphaericus* strain 41: production, characterization and catalytic properties. Biochem Eng J 48:124–131
71. Vassileva A, Atanasova N, Ivanova V, Dhulster P, Tonkova A (2007) Characterisation of cyclodextrin glucanotransferase from *Bacillus circulans* ATCC 21783 in terms of cyclodextrin production. Ann Microbiol 57:609–615
72. Yim DG, Sato HH, Park YH, Park YK (1997) Production of cyclodextrin from starch by cyclodextrin glycosyltransferase from *Bacillus firmus* and characterization of purified enzyme. J Ind Microbiol Biotechnol 18:402–405
73. Hirano K, Ishihara T, Ogasawara S, Maeda H, Abe K, Nakajima T, Yamagata Y (2006) Molecular cloning and characterization of a novel γ-CGTase from alkalophilic *Bacillus* sp. Appl Microbiol Biotechnol 70:193–201
74. Martins RF, Hatti-Kaul R (2002) A new cyclodextrin glycosyltransferase from an alkaliphilic *Bacillus agaradhaerens* isolate: purification and characterisation. Enzyme Microb Technol 30:116–124
75. Penninga D, Strokopytov B, Rozeboom HJ, Lawson CL, Dijkstra BW, Bergsma J, Dijkhuizen L (1995) Site-directed mutations in tyrosine 195 of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 affect activity and product specificity. Biochemistry 34:3368–3376
76. Moriwaki C, Costa GL, Pazzetto R, Zanin GM, Moraes FF, Portilho M, Matioli G (2007) Production and characterization of a new cyclodextrin glycosyltransferase from *Bacillus firmus* isolated from Brazilian soil. Process Biochem 42:1384–1390
77. Ibrahim ASS, Al-Salamah AA, El-Tayeb MA, El-Badawi YB, Antranikian G (2012) A Novel Cyclodextrin Glycosyltransferase from Alkaliphilic *Amphibacillus* sp. NPST-10: Purification and Properties. Int J Mol Sci 13:10505–10522
78. Alves-Prado HF, AAJ Carneiro, FC Pavezzi, E Gomes, M Boscolo, CML Franco, R da Silva (2007) Production of cyclodextrins by CGTase from *Bacillus clausii* using different starches as substrates. In: Biotechnology for fuels and chemicals, Humana Press, Totowa, NJ, pp 123–133
79. Mori S, Hirose S, Oya T, Kitahata S (1994) Purification and properties of cyclodextrin glucanotransferase from *Brevibacterium* sp. No. 9605. Biosci Biotechnol Biochem 58:1968–1972
80. Takada M, Nakagawa Y, Yamamoto M (2003) Biochemical and genetic analyses of a novel γ-cyclodextrin glucanotransferase from an Alkalophilic *Bacillus clarkii* 7364. J Biochem 133:317–324
81. Takada M, Ide T, Yamamoto T, Unno T, Watanabe Y, Sone H, Yamamoto M (2003) Novel cyclodextrin glucanotransferase, process for producing the same and process for producing cyclodextrin by using this enzyme. Patent no. US20030194796A1

82. Matioli G, Zanin GM, de Moraes FF (2000) Enhancement of selectivity for producing γ-cyclodextrin. In: Twenty-first symposium on biotechnology for fuels and chemicals, Humana Press, Totowa, NJ, pp 955–962
83. van der Veen BA, Uitdehaag JC, Dijkstra BW, Dijkhuizen L (2000) Engineering of cyclodextrin glycosyltransferase reaction and product specificity. Biochim Biophys Acta 1543:336–360
84. Parsiegla G, Schmidt AK, Schulz GE Substrate binding to a cyclodextrin glycosyltransferase and mutations increasing the γ-cyclodextrin production. Eur J Biochem 255:710–717
85. Lee K-W, Shin H-D, Lee Y-H (2003) Catalytic function and affinity purification of site-directed mutant β-cyclodextrin glucanotransferase from alkalophilic *Bacillus firmus* var. *alkalophilus*. J Mol Catal B: Enzym 26:157–165
86. Wang L, Duan X, Wu J (2016) Enhancing the α-cyclodextrin specificity of cyclodextrin glycosyltransferase from *Paenibacillus macerans* by mutagenesis masking subsite -7. Appl Environ Microbiol 82:2247–2255
87. Feng T, Zhuang H, Ran Y (2011) The application of cyclodextrin glycosyltransferase in biological science. J Bioequiv Bioavailab 3:202–206
88. Taira H, Nagase H, Endo T, Ueda H (2006) Isolation, purification and characterization of large-ring cyclodextrins (CD_{36}–CD_{39}). J Incl Phenom Macrocycl Chem 56:23–28
89. Zheng M, Endo T, Zimmermann W (2002) Synthesis of large-ring cyclodextrins by cyclodextrin glucanotransferases from bacterial isolates. J Incl Phenom 44:387–390
90. Svensson D, Ulvenlund S, Adlercreutz P (2009) Efficient synthesis of a long carbohydrate chain alkyl glycoside catalyzed by cyclodextrin glycosyltransferase (CGTase). Biotechnol Bioeng 104:854–861
91. Svensson D, Adlercreutz P (2011) Immobilisation of CGTase for continuous production of long-carbohydrate-chain alkyl glycosides Control of product distribution by flow rate adjustment. J Mol Catal B: Enzym 69:147–153
92. Svensson D, Adlercreutz P (2011) Characterisation of a glycosylated alkyl polyglycoside produced by a cyclodextrin glycosyltransferase by HPLC-ELSD and -MS. J Chromatogr B 879:1857–1860
93. Simair AA, Khushk I, Qureshi AS, Bhutto MA, Chaudhry HA, Ansari KA, Lu C (2017) Amylase production from thermophilic *Bacillus* sp. BCC 021-50 isolated from a marine environment. Fermentation 3:1–12
94. Hamilton LM, Kelly CT, Fogarty WM (1999) Production and properties of the raw starch-digesting α-amylase of *Bacillus* sp. IMD 435. Process Biochem 35:27–31
95. Asgher M, Javaid Asad M, Rahman S, Legge R (2007) A thermostable α-amylase from a moderately thermophilic *Bacillus subtilis* strain for starch processing. J Food Eng 79:950–955
96. Asha R, Nyonzima FN, Sunil SM (2013) Purification and properties of pullulanase from *Bacillus halodurans*. Int Res J Biol Sci 2:35–43
97. Spendler T, Jorgensen OB (1997) Use of a branching enzyme in baking. Patent no. PCT/DK1997/000202
98. Cole MS (1982) Antistaling baking composition. Patent no. US05862653
99. Stefanis VAD, Turner EW (1981) Modified enzyme system to inhibit bread firming method for preparing same and use of same in bread and other bakery products. Patent no. US4299848A
100. Würsch P, Gumy D (1994) Inhibition of amylopectin retrogradation by partial beta-amylolysis. Carbohydr Res 256:129–137
101. Olesen T (1991) Antistaling process and agent. Patent no. PCT/DK1990/000244
102. Okada S, Kitahata S, Yoshikawa S, Sugimoto T, Sugimoto K (1984) Process for the production of branching enzyme, and a method for improving the qualities of food products therewith. Patent no. US4454161A
103. Carroll JO, Boyce COL, Wong TM, Starace CA (1987) Bread antistaling method. Patent no. US06760877
104. Vidal FD, Gerrity AB (1979) Antistaling agent for bakery products. Patent no. US05788193

105. Min B-C, Yoon S-H, Kim J-W, Lee Y-W, Kim Y-B, Park KH (1998) Cloning of novel maltooligosaccharide-producing amylases as antistaling agents for bread. J Agric Food Chem 46:779–782
106. Auh JH, Lee SY, Seung SY, Son HJ, Lee JW, Lee SI, Kim YB, Park KH (2005) A novel maltopentaose producing amylase as a bread antistaling agent. Food Sci Biotech 14:681–684
107. Hubbe MA (2004) Acidic and alkaline sizing for printing, writing and drawing papers. In: The Book and Paper Group Annual, American Institute for Conservation, Washington, pp 139–151
108. Lee SP, Morikawa M, Takagi M, Imanaka T (1994) Cloning of the aapT gene and characterization of its product, alpha-amylase-pullulanase (AapT), from thermophilic and alkaliphilic *Bacillus* sp. strain XAL601. Appl Environ Microbiol 60:3764–3773
109. Kumar R, Sharma A, Dhall P, Kulshreshtha NM, Kumar A (2011) Neutralization of alkaline waste waters using a blend of microorganisms. Int J Biotechnol Bioeng 5:224–227
110. Fulekar MH (2010) Nanotechnology: importance and applications. K. International Publication House, New Delhi
111. Watlington K (2005) Emerging nanotechnologies for site remediation and wastewater treatment. US Environmental Protection Agency, Washington
112. Husain Q (2017) Nanomaterials as novel supports for the immobilization of amylolytic enzymes and their applications. Biocatalysis 3:37–53
113. Mukherjee AK, Kumar TS, Rai SK, Roy JK (2010) Statistical optimization of *Bacillus alcalophilus* α-amylase immobilization on iron-oxide magnetic nanoparticles. Biotechnol Bioproc Eng 15:984–992
114. Pandi A, Ramalingam S, Rao JR, Kamini NR, Gowthaman MK (2016) Inexpensive α-amylase production and application for fiber splitting in leather processing. RSC Adv 6:33170–33176
115. Gupta RK, Prasad D, Sathesh J, Naidu RB, Kamini NR, Palanivel S, Gowthaman MK (2012) Scale-up of an alkaline protease from *Bacillus pumilus* MTCC 7514 utilizing fish meal as a sole source of nutrients. J Microbiol Biotechnol 22:1230–1236
116. Saran S, Mahajan RV, Kaushik R, Isar J, Saxena RK (2013) Enzyme mediated beam house operations of leather industry: a needed step towards greener technology. J Clean Prod 54:315–322
117. Murakami S, Nagasaki K, Nishimoto H, Shigematu R, Umesaki J, Takenaka S, Kaulpiboon J, Prousoontorn M, Limpaseni T, Pongsawasdi P, Aoki K (2008) Purification and characterization of five alkaline, thermotolerant, and maltotetraose-producing α-amylases from *Bacillus halodurans* MS-2-5, and production of recombinant enzymes in *Escherichia coli*. Enzyme Microb Technol 43:321–328
118. Ma Y, Yang H, Chen X, Sun B, Du G, Zhou Z, Song J, Fan Y, Shen W (2015) Significantly improving the yield of recombinant proteins in *Bacillus subtilis* by a novel powerful mutagenesis tool (ARTP): alkaline alpha-amylase as a case study. Protein Expr Purif 114:82–88
119. Yang H, Liu L, Wang M, Li J, Wang NS, Du G, Chen J (2012) Structure-based engineering of methionine residues in the catalytic cores of alkaline amylase from *Alkalimonas amylolytica* for improved oxidative stability. Appl Environ Microbiol 78:7519–7526
120. Yang H, Liu L, Shin H, Chen RR, Li J, Du G, Chen J (2013) Integrating terminal truncation and oligopeptide fusion for a novel protein engineering strategy to improve specific activity and catalytic efficiency: alkaline α-amylase as a case study. Appl Environ Microbiol 79:6429–6438
121. Tigue MAM, Kelly CT, Doyle EM, Fogarty WM (1995) The alkaline amylase of the alkalophilic *Bacillus* sp. IMD 370. Enzyme Microb Technol 17:570–573
122. Duedahl-Olesen L, Kragh KM, Zimmermann W (2000) Purification and characterisation of a malto-oligosaccharide-forming amylase active at high pH from *Bacillus clausii* BT-21. Carbohydr Res 329:97–107
123. Bezbaruah RL, Gogoi BK, Pillai KR, Nigam JN (1991) Amylase production by three *Bacillus* strains active at alkaline pH. J Basic Microbiol 31:13–20

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Alkaline Active Hemicellulases

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Contents

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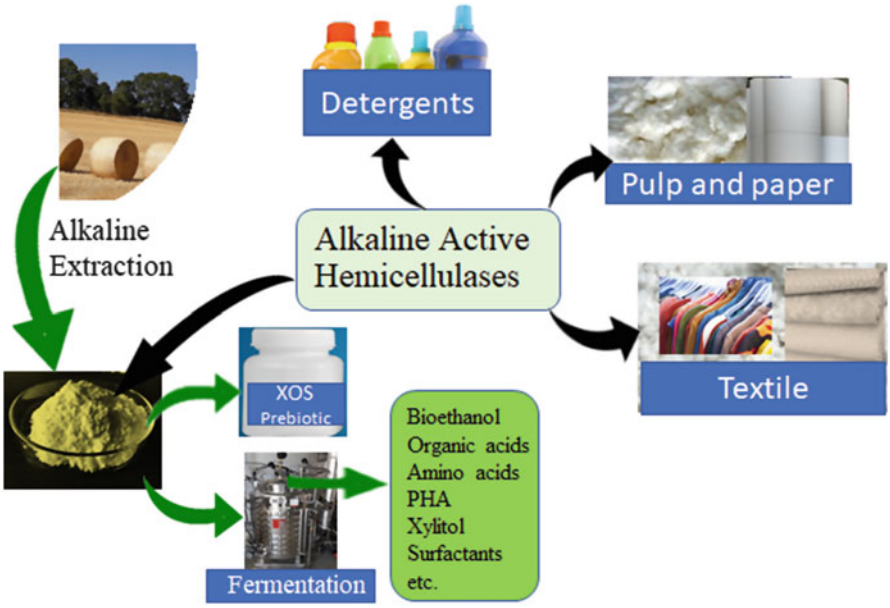
Abstract Xylan and mannan are the two most abundant hemicelluloses, and enzymes that modify these polysaccharides are prominent hemicellulases with immense biotechnological importance. Among these enzymes, xylanases and mannanases which play the vital role in the hydrolysis of xylan and mannan, respectively, attracted a great deal of interest. These hemicellulases have got applications in food, feed, bioethanol, pulp and paper, chemical, and beverage producing industries as well as in biorefineries and environmental biotechnology. The great majority of the enzymes used in these applications are optimally active in mildly acidic to neutral range. However, in recent years, alkaline active enzymes have also become increasingly important. This is mainly due to some benefits of utilizing alkaline active hemicellulases over that of neutral or acid active enzymes. One of the advantages is that the alkaline active enzymes are most suitable to applications that require high pH such as Kraft pulp delignification, detergent formulation, and cotton bioscouring. The other benefit is related to the better solubility of hemicelluloses at high pH. Since the efficiency of enzymatic hydrolysis is often positively correlated to substrate solubility, the hydrolysis of hemicelluloses can be more efficient if performed at high pH. High pH hydrolysis requires the use of alkaline active enzymes. Moreover, alkaline extraction is the most common hemicellulose extraction method, and direct hydrolysis of the alkali-extracted hemicellulose could be of great interest in the valorization of hemicellulose. Direct hydrolysis avoids the time-consuming extensive washing, and neutralization processes required if non-alkaline active enzymes are opted to be used. Furthermore, most alkaline active enzymes are relatively active in a wide range of pH, and at least some of them are significantly or even optimally active in slightly acidic to neutral pH range. Such enzymes can be eligible for non-alkaline applications such as in feed, food, and beverage industries.

This chapter largely focuses on the most important alkaline active hemicellulases, endo-β-1,4-xylanases and β-mannanases. It summarizes the relevant catalytic properties, structural features, as well as the real and potential applications of these remarkable hemicellulases in textile, paper and pulp, detergent, feed, food, and prebiotic producing industries. In addition, the chapter depicts the role of these extremozymes in valorization of hemicelluloses to platform chemicals and alike in biorefineries. It also reviews hemicelluloses and discusses their biotechnological importance.

Graphical Abstract



Keywords Alkaliphile, Bioethanol, Biomass, Biorefinery, Delignification, Enzyme, Feed, Hemicellulose, Hydrolases, Mannooligosaccharides, Paper and pulp, Platform chemicals, Prebiotic, Waste management, Xylooligosaccharides

Abbreviations

CBM	Carbohydrate-binding module
DP	Degree of polymerization
EC	Enzyme Commission
GH	Glycoside hydrolase
ISO	International Organization for Standardization
MOS	Mannooligosaccharides
PDB	Protein Data Bank
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
XOS	Xylooligosaccharides

1 Introduction

Enzymes catalyze reactions with great specificity and rate enhancements. These catalytic properties, together with their environmentally benign and easy disposal nature, provide tremendous opportunities for industries. Often, industrial applications demand extreme conditions like high or low pH, elevated temperature, high solvent concentration, etc. On the other hand, nature provides, with some notable exceptions, enzymes that operate best under rather mild conditions. In industrial processes, the use of robust enzymes that directly mediate the catalysis without any pre-adjustment(s) such as cooling and neutralization has enormous economic and technical advantages over the use of enzymes which are labile to the industrial application conditions. Thus, there has always been interest in finding enzymes that are compatible with industrial application conditions. This impetus forged with the basic research interest geared for the exploration of life that can serve as sources of enzymes that are amenable to industrial applications. The major search target for such robust enzymes has been extremophiles, organisms that thrive in extreme environments. The assumption is that those who dwell in extreme habitats evolved enzymes that are operationally stable in the extreme environments in which they are thriving. Thus, it seems ideal to look for thermostable enzymes from thermophiles, acid active enzymes from acidophiles, alkaline active enzymes from alkaliphiles, cold active enzymes from psychrophiles, and so on. Indeed, it has been proven that extremophiles produce fascinating enzymes which are able to mediate reactions under extreme conditions at which biocatalysts from their mesophilic counterparts are often neither active nor stable. Over the years, a range of enzymes has been reported from extremophiles. Characterization studies of these enzymes revealed the suitability of some of these enzymes in industrial applications. The discovery of industrially amenable enzymes of extremophiles not only substitutes some of the toxic and hazardous industrial processes but also paved the way to envision new industrial processes. This has significantly contributed to the expansion of industrial biocatalysis.

Plant biomass modifying enzymes are among the extremozymes that attracted a great deal of interest from researchers, industrialists, and environmentalists. Enzymes which act on various constituents of plant biomass have got applications such as in food, pulp, detergent, textile, and bioethanol industries [1–4]. In addition, there is a recent global trend toward greener and sustainable processes which is driven by the growing concern of economic, environmental, and health issues related to heavy use of petroleum and its derivatives [5, 6]. Huge amount of lignocellulosic materials is generated from agricultural and agroindustry processes each year. This biomass can potentially serve as renewable feedstock to produce chemicals, materials, and energy carriers that may be of substitute to petroleum and its derivatives [7–9]. The valorization of the biomass to these entities involves hydrolysis which can be done either by chemical or biotechnological approaches. However, the biotechnological route is preferable due to its greener, clean, specific, and benign nature. Moreover, it is free from undesirable products such as fermentation inhibitors. Thus, the robust extremozymes can be instrumental in valorization of plant biomass to various products. In this regard, the emergence and expansion of the

biorefinery concept and some of the valorization shining showcases [10, 11] are expected to give a great momentum for the sustainable development effort and consequentially raise the demand for plant biomass degrading extremozymes.

Hemicelluloses, which are structurally and compositionally diverse polysaccharides, are among the major constituents of plant biomass. Due to their complex natures, several enzymes are involved in the hydrolysis of hemicelluloses. These enzymes are often referred to as hemicellulases. Hemicellulases have been in the toolbox of biotechnology for many years and are applied such as in food, feed, pulp, and beverage productions [12–14]. In recent years, such enzymes have also been considered in valorization of hemicelluloses [15–17], which is believed to have enormous economic and environmental potential. This remarkable application potential of hemicellulases has led to intensive screening studies that result in the discovery of a range of hemicellulases from various extremophiles. Alkaliphiles, which are the most important sources of alkaline active enzymes, are among these extremophile groups that have been intensively screened.

Alkaline active hemicellulases are highly desired for high pH applications such as in pulp and paper, detergent, and textile industries. In addition, these extremozymes seem to have greater potential in valorization of hemicelluloses to various value-added products than the non-alkaline active counterparts. It is believed that the use of alkaline active hemicellulases in valorization processes is advantageous due to better solubility of hemicelluloses at alkaline condition and possibility of direct hydrolysis of alkali-extracted hemicelluloses. Alkaline active xylanases and mannanases which, respectively, hydrolyze the main chains of xylan and mannan are the most studied alkaline active hemicellulases. The studies have shown the immense biotechnological importance of these enzymes, and this chapter primarily focuses on these two groups of extremozymes, which play crucial roles in the hydrolysis of the two abundant hemicelluloses.

2 Hemicelluloses

A broad and inexplicit definition of hemicellulose refers to plant cell wall polysaccharides that occur in close association with cellulose [18] or are noncellulosic cell wall polysaccharides extracted from plant specimen [19]. However, since the term is introduced long before the structure was determined, there has been a controversy regarding these definitions. One of the arguments is that the definitions do not exclude plant cell wall polysaccharides such as pectin which is not hemicellulose. Currently, hemicelluloses are defined as a group of cell wall polysaccharides that are neither cellulose nor pectin and have equatorially configured β-(1→4)-linked glucose, mannose, or xylose backbones [20, 21]. This definition clearly excludes pectin and encompasses xyloglucans, xylans, mannans, heteromannans, and even mixed-linkage β-(1-3,1-4)-glucans. However, it seems excluding carbohydrates such as laminarin, arabinogalactan, and curdlan which have β-(1→3)-linked backbone but are considered as hemicelluloses. This shows that even the widely accepted

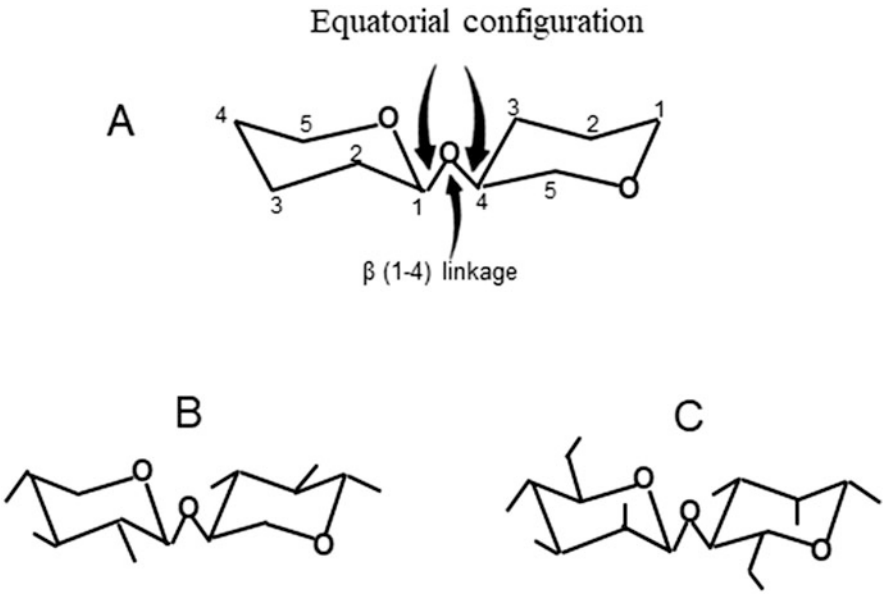


Fig. 1 The equatorial configuration of the repeating disaccharide units (**a**) of hemicelluloses such as xylan (**b**) and mannan (**c**)

definition is generic and it does not clearly define hemicellulose. In this chapter, hemicellulases are defined as cell wall polysaccharides which are neither cellulose nor pectin and are composed of equatorially configured β-(1-4 and/or 1-3) xylose, glucose, and/or mannose backbones.

The structural analysis of hemicelluloses revealed that the backbones of hemicelluloses are composed of repeating disaccharide units. Figure 1 shows the repeating units of the hemicellulose xylan and mannan with equatorial configuration (i.e., perpendicular to the main sugar structure orientation) of the β-(1→4) linkage.

Hemicelluloses are the second most abundant polysaccharides in nature which are amorphous heterogeneous polysaccharides containing more than 20 different types of monomers including xylose, arabinose, mannose, galactose, glucose, fucose, glucuronic acid and galacturonic acid in various arrangements and proportions, often with 100–200 degree of polymerization (DP). In most plants, hemicellulose constitutes about 15–35% of the dried biomass weight [22]. On average, it accounts for about 26% of hardwood, 22% of softwood, and 30% of various agricultural residues dry weight [7, 23]. However, the amount and type of hemicellulose varies depending on maturity, botanical source, type of tissues, growth conditions, and other factors [23]. It can compose up to 30% of the dry weight of primary cell walls, even higher in secondary walls, and much higher in some specialized walls such as those of storage cell walls of seeds [24]. Straw and grass are among the most common and abundant plant biomass containing high amount of hemicelluloses. The hemicellulose content of wheat straw is roughly 23–38%, while barley straw has

about 24–37% [7, 25]. The hemicellulose fraction of grass and switchgrass can reach up to 50% of the total dry weight [7].

Xylan and mannan are the most abundant hemicelluloses which are the dominant constituents of hardwood and softwood hemicelluloses, respectively. Thus, biotechnological applications involving enzymatic modification of hemicelluloses often revolve around xylan and mannan hydrolysis.

2.1 Xylan

Xylan is an important hemicellulose. It is the second most abundant polymer in nature and roughly accounts for one-third of the renewable biomass available on Earth. It constitutes about 30% of the cell wall material of annual plants, 15–35% of hardwoods, and 7–10% of softwoods [26, 27]. Xylan often consists of a β-1,4-linked D-xylose backbone which can be substituted with different side groups such as L-arabinose, D-galactose, acetyl, feruloyl, *p*-coumaroyl, and glucuronic acid residues which are attached at positions C-2 and C-3 of xylose residues (Fig. 2).

Xylan is known to occur in several structural varieties in terrestrial plants and algae. It has also been found in other organisms. A glucuronoxylan was reported from unicellular flagellate *Herpetomonas samuelpessoai* [28], a high molecular weight glycan containing mixed linked xylan from a parasitic protozoan *Leptomonas samueli* [29], and the β-1,4-D-xylan containing exopolysaccharide from the bacterium *Pasteurella multocida* [30].

Its structure and composition vary depending on the botanical source and even in between different tissues of the same plant [31]. The diversity is attributed to the difference in the nature of substituents, the frequency and position of substitution,

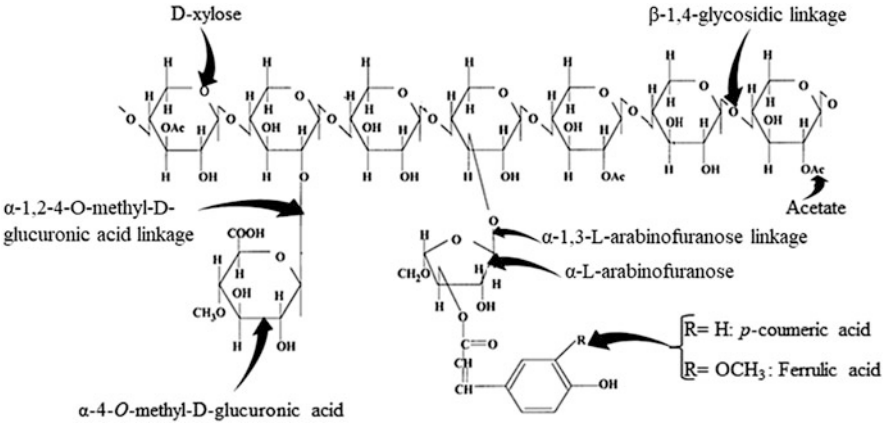


Fig. 2 Simplified illustration of xylan structure

the type of glycosidic linkages, etc. Based on the nature of substituents found on the backbone, xylans are categorized as linear homoxylan, arabinoxylan, glucuronoxylan, and glucuronoarabinoxylan. However, in each category there exists microheterogeneity with respect to the degree and nature of branching.

Homoxylans are xylans which are linear and unsubstituted. Homoxylans with β-1,3-linkages substitute cellulose in the cell wall structure of the green algae *Caulerpa* sp. and *Bryopsis maxima* [32]. Similarly, homoxylans with mixed β-1-3-/β-1-4-linkages serve as cell wall components of red seaweeds of the *Palmariales* and *Nemaliales* [33]. Higher plants possess xylans containing β-1-4-linked xylose units as the backbone which is usually substituted with sugar units and *O*-acetyl groups and are known as heteroxylans. The only exception of heteroxylans with mixed β-1-3/β-1-4-linkages in the main chain has been isolated from the seeds of *Plantago* species [34]. However, unsubstituted linear forms have been obtained from guar seed husk, esparto grass, and tobacco stalks.

Arabinoxylans contain a higher amount of arabinose, whereas glucuronoxylans contain more glucuronic acid attached to the backbone. The glucuronoxylans exist as *O*-acetyl-4-*O*-methylglucuronoxylan in hardwoods and as arabino-4-*O*-methylglucuronoxylan in softwoods [35]. Xylans in cereals, grasses, and annual plants are typically arabinoxylans [35]. Glucuronoarabinoxylans contain both arabinose and glucuronic acid. Arabinoxylans, glucuronoxylans, and arabino-glucuronoxylans are branched, and the degree of branching, the type of side-chain residues, the type of linkage, etc. result in diverse features that make these xylans very heterogeneous.

Arabinose can be attached to the xylan backbone by α-1,2- or α-1,3-linkages. This side-chain residue can also contain xylose and galactose which can be linked either to arabinose or xylose. In various plants, xylan exists in acetylated form [35]. Acetyl residues may be attached to the C-2 or C-3 of the xylose residue or to both sites [36] in the backbone of xylan, and the degree of acetylation differs strongly among xylans of different origin. Glucuronic acid and its 4-*O*-methyl ether are attached to the xylan backbone via an α-1,2-linkage, whereas aromatic (feruloyl and *p*-coumaroyl) residues have so far only been found attached to the C-5 of terminal arabinose residues.

2.2 Mannan

Mannan is another important hemicellulose which is found almost in all plant cell wall. One of its distinguishing features is its backbone which can be entirely mannan or a mix of mannose and glucose [37]. Like xylan, it is also diverse and exists in different forms including as linear mannan, galactomannan, glucomannan, and glucogalactomannan.

Linear mannan is a polymer containing more than 95% mannose and can be considered as “pure” mannan. It occurs in some seeds such as tagua palm seed, coffee beans, and orchid tubers. Galactomannan is characterized by β-(1–4)-mannan

backbone having single galactose branches linked α-(1–6) [38]. The mannose-to-galactose ratio and the structure vary from source to source [39]. It is a common hemicellulose in legumes seeds. Fenugreek, guar gum, tara gum, and locust bean gums are some of the most known galactomannan hemicelluloses. Guaran (or guar gum) and locust bean gums are widely used as thickeners in the food industry.

Glucomannan has a backbone composed of mannose and glucose. The mannose-to-glucose ratio and the presence of side group vary among glucomannans from different botanical sources. Hardwood glucomannan has no attached side group, and the most common mannose-to-glucose ratio is about 2:1. On the other hand, glucomannan from softwood contains side groups, and the common mannose-to-glucose ratio is about 3:1. The side group is galactose which is attached to mannose residue at position C-6 or at C-3 of glucose. Glucomannans are found in iris seeds, lily bulbs, bluebell seeds, orchids, etc. Galactoglucomannan is basically a lower DP glucomannan which consists of about ten times more galactose. Glucomannans and galactoglucomannans are the main hemicelluloses in softwoods and hardwoods. Hardwoods contain about 3–5% glucomannans, whereas softwoods contain mainly galactoglucomannans [40].

2.3 Other Hemicelluloses

Xyloglucan is a common hemicellulose in non-graminaceous plants and accounts for up to 20% of the dry weight of the cell wall [41]. It has celluloselike backbone consisting of β-1-4-linked glucose residues to which xylose units are attached at position 6 of some of the backbone glucose residues and are highly branched unlike cellulose [41]. To the xylose residues attached to backbone glucose, residues such as arabinose and galactose can be attached and form di- or tri-glycosyl side chains [42]. Other hemicelluloses which consist of hexose backbones are also known. Depending on the nature of the sugar backbone, these hemicelluloses are divided into different groups. Among these groups, arabinogalactans, β-glucan, lichenan, and laminarin are well-characterized. Arabinogalactan has a backbone of β-1,3-linked D-galactose residues and is substituted with β-1,6 side chains. It is the dominant hemicellulose in larchwood, an exception among softwoods where galactoglucomannan is the dominant hemicellulose [27]. β-glucan and lichenan are glucan-based hemicelluloses. The gramineae seed β-glucan consists of linear polymer of mixed β-1,3-/β-1,4-linked glucan in a DP range of 25–75 [43]. On the other hand, lichenan has a similar structure to that of β-glucan, but with DP in the range of 100–200. Lichenan constitutes about 10% of the lichen Icelandic moss *Cetraria islandica* [44]. Laminarin possesses β-1,3-linked glucose backbone with an average DP of 20–25 [33]. The brown alga *Phaeophyta* spp. is the commercial source of this hemicellulose. A microbial hemicellulose, curdlan (β-1,3-glucan) has been reported from *Agrobacterium* sp. and the alkali-tolerant *Alcaligenes faecalis* [45, 46].

3 Biotechnological Importance of Hemicelluloses

Hemicelluloses have got several applications in their native or modified forms as shown in Table 1. Moreover, in recent years, there has been an increasing interest in hemicellulose hydrolysates due to their outstanding potential applications. The hemicellulose hydrolysates, such as xylooligosaccharides (XOS) and manno-oligosaccharides (MOS), have been reported to function as potential: prebiotics revitalizing the growth of intestinal bifidobacteria [47, 48], anticarcinogenic agents [49], immune boosters [50], antioxidants [51], and antimicrobial compounds [52]. In addition, these oligosaccharides serve in food industry as thickeners, antifreeze, and

Table 1 Applications of intact and modified hemicelluloses in various industrial sectors

Industry	Hemicellulose	Application
Chemicals	Carboxymethyl xylans	Flocculants
	Xylan	Xylitol, furfural
Cosmetic	Arabinoxylan	Emulsifiers, thickeners, stabilizers
Detergents	Carboxymethyl xylans	Detergency promoters, additives
Fermentation	Xylan and mannan	Production of enzymes
	Hemicellulose hydrolysates	Fermentation feedstock for production of different chemicals
Food	Arabinoxylans	Additives, thickeners, emulsifiers, gelling agents, adhesives, adsorbents, stabilizers, etc.
	Glucomannans	Caviar substituent
	Gums	Stabilization of fruit drinks, thickeners, gelling agents, etc.
	Xylan and mannan hydrolysate	Fat substitute, antifreeze agents, thickeners
	Xylan	Production of sweetener
Film	Carboxymethyl xylan	production of different films
Hydrogels	Xylan	Drug delivery and water remediation
Mining	Arabinogalactans	For processing of iron and copper ores
	Gur gum	Oil drilling
Paper	Carboxymethyl xylans	Adhesive in coating paper
Pharmaceutical	Xylan sulfate derivatives	Antitumoral and lypolemic activity
	Arabinoxylan	Emulsifiers, thickeners and stabilizers
	Arabinogalactans	Tablet binder and emulsifier
	Gums	Stabilizing agent such as barium sulfate suspension for X-ray diagnostic preparation
Plastic	Xylan	Oxygen tight packaging film, additive
Prebiotics	Xylan and mannan	Production of prebiotic oligosaccharides

fat substitutes, while in pharmaceutical industry, together with other ingredients, they are used for direct tableting [47]. Monomeric sugars obtained from hemicellulose degradation have been used as fermentative substrate for production of different chemicals and biofuels [6, 42, 53–55]. Hydrolysis of hemicelluloses can be achieved either through enzymatic or chemical means. Chemical hydrolysis, which commonly involves acids, is very efficient and inexpensive. However, it is unspecific, results in undesirable products, has corrosive effects, and generates waste and fermentation inhibitors. Moreover, this hydrolysis route often produces monomers as major products and suffers thereby from poor product diversity. On the other hand, enzymatic saccharification is attractive as it is specific, biocompatible, and environmentally benign and offers a large variety of products. This makes hemicellulose-degrading enzymes, which are collectively known as hemicellulases, the focus of intense research.

4 Hemicellulases

A vast array of microorganisms producing hemicellulose-modifying enzymes have been isolated from various habitats and studied. As hemicelluloses constitute a heterogeneous group of polysaccharides with diverse compositions, structures, linkages, etc., nature has evolved a suite of enzymes that degrades it. Enzymes that belong to this group are either glycoside hydrolases that hydrolyze glycosidic bonds or carbohydrate esterases which hydrolyze ester linkages. Thus, as shown in Fig. 3, the complete hydrolysis of hemicelluloses requires a concerted action of enzymes that cleave the main chain as well as the substituent linkages. Endo- and exo-hemicellulases attack the same types of linkages in the backbone of hemicelluloses. But, the endo-glycoside hydrolases cleave the internal glycosidic linkages, while the exo-glycoside hydrolases release mono- or disaccharides either from the reducing or the nonreducing end of the polysaccharide. Hemicellulases such as endo-β-1,4-xylanases hydrolyze the main chain of xylan, and the exo-acting β-xylosidases release xylose from the reducing end of the backbone. Although the endo-xylanases are often perceived acting randomly, it seems that these enzymes attack xylan not at random but in an orderly fashion. Factors such as the degree of substrate branching, the length of the branch, the nature of substituents, etc. determine which bond has to be cleaved [36]. The backbone-hydrolyzing hemicellulases, specifically the endo-glycoside hydrolases, are crucial for hemicellulose hydrolysis. However, the accessibility of the glycosidic bonds in the backbone structures could be limited due to the presence of substituents. Thus, debranching enzymes which are often referred to as accessory enzymes remove the substituents and facilitate the hemicellulose complete hydrolysis by the endo- and exo-hemicellulases.

The complete hydrolysis of the common native xylan, at least theoretically, requires the action of several enzymes including arabinofuranosidases, α-glucuronidases, α-galactosidases, acetyl xylan esterases, ferulic acid esterase, *p*-coumaric acid esterase, endo-xylanase, and β-xylosidase. Arabinose and methyl

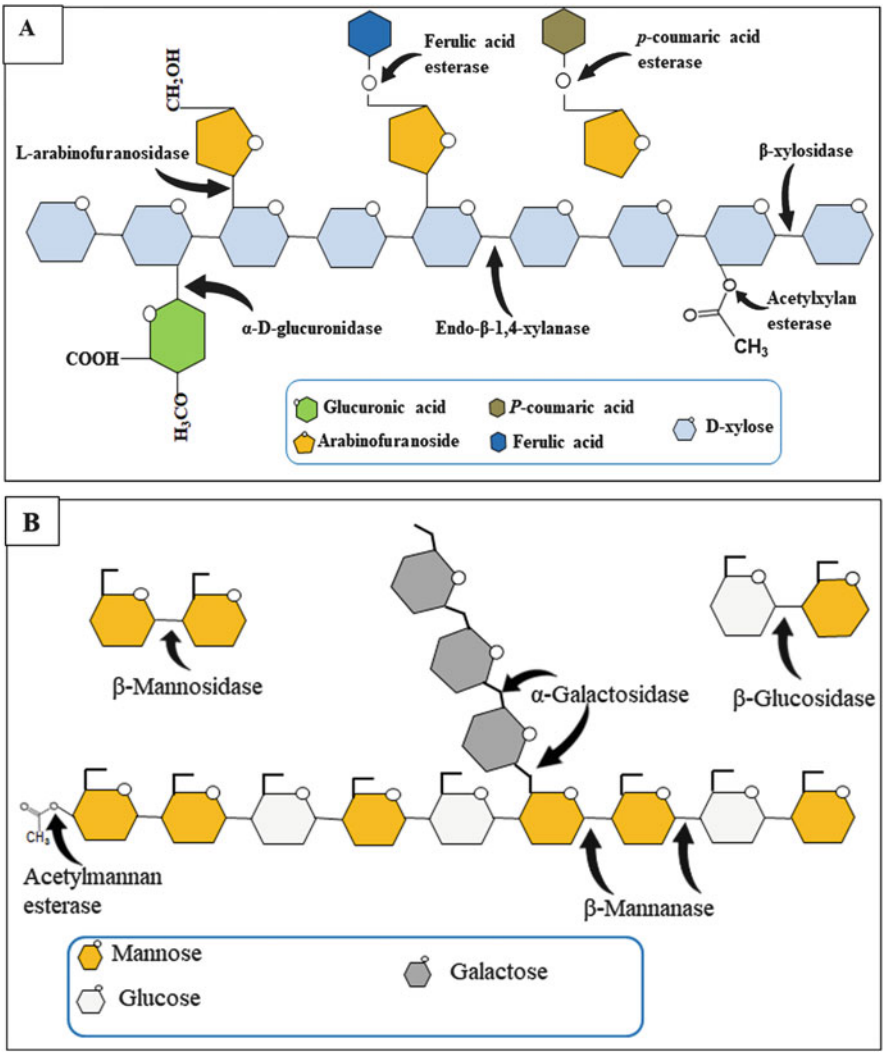


Fig. 3 Some of the important hemicellulases involved in xylan (**a**) and mannan (**b**) degradation

glucuronic acid groups can be cleaved from the backbone by α-arabinofuranosidase and α-glucuronidase, respectively. Acetylxylan esterase deacetylates xylose residues, while the ferulic acid esterase cleaves the ester linkage between arabinose and ferulic acid residues. In case of mannan, the backbone-hydrolyzing enzymes are β-mannanase, β-glucosidase, and β-mannosidase, whereas enzymes such as acetylmannan esterase and α-galactosidase remove side-chain substituents attached to the backbone of mannans.

Most of the accessory enzymes remove substituents from oligomeric substrates released by the endo-enzymes, xylanases and mannanases [56]. Some accessory enzymes that can act on intact hemicelluloses have been known. However, even these enzymes prefer oligomeric substrates over intact (polymeric) hemicelluloses [57].

Although complete hydrolysis of hemicelluloses requires both main chain- and side group-cleaving enzymes, the type of the hemicellulose, the method employed to extract the hemicellulose, and the enzymatic reaction conditions determine which enzyme may be required for complete hydrolysis. This is mainly related to the difference in the presence or absence of side groups and the stability of the chemical bonds that exist in the hemicelluloses. It is obvious that the set of enzymes required for complete hydrolysis of homoxylan and heteroxylan are different. Similarly, the extraction methods used to recover hemicelluloses from lignocellulosic biomass are known to affect the nature of the hemicellulose, and this can influence which suite of enzymes to be considered for complete digestion. For instance, the alkaline extraction, which is the prominent method of hemicellulose extraction, is often accompanied by deacetylation and removal of the uronic acid groups [58–60]. Thus, in principle, complete hydrolysis of alkali-extracted hemicelluloses does not require acetylmannan esterase and acetylxylan esterase, α-glucuronidase, and to some extent ferulic acid esterase. Thus, hydrolysis of alkali-extracted xylan is more attractive, not only for it requires relatively fewer number of enzymes for complete conversion to its monomers but also for its efficiency. This has been demonstrated recently by Lyczakowski and coworkers [61] who have shown an efficient hydrolysis of glucuronic acid-free xylan. Moreover, accomplishing hydrolysis at alkaline condition avoids extensive washing and subsequent pH readjustment of alkali-extracted hemicellulose, while it benefits from the better solubility of hemicellulose which in turn enhances the digestion efficiency.

5 Alkaline Active Hemicellulases

Alkaliphiles, organisms thriving in alkaline habitats, are the main sources of alkaline active enzymes. Several alkaliphiles are known in producing intracellular as well as extracellular hemicellulases. However, it is the extracellular enzymes that are often alkaline active as it is evolved to work in the alkaline habitat. On the other hand, since the cytoplasmic pH is often in the neutral to slightly alkaline pH range, intracellular hemicellulases are often active around neutral condition [62]. Extremely alkaline active hemicellulases have also been reported from non-alkaliphilic organisms. The β-mannanases of *Bacillus subtilis* subsp. *inaquosorum* CSB31 [63] and *Streptomyces* sp. CS147 (Mn147) [64], which are optimally active at pH 12.5 and pH 11, respectively, can be examples of alkaline active enzymes of non-alkaliphilic origin. Similarly, *Anoxybacillus kamchatkensis* [65] and *Actinomadura* sp. Cpt20 [66] which are adapted to grow around pH 7

produce alkaline active xylanases that are optimally active at pH 9 and 10, respectively.

5.1 Properties

In general, the properties of enzymes are known to dictate their application potential. Thus, enzyme studies often involve characterization which reveal their properties. Studies have shown that alkaline active hemicellulases of a certain group (e.g., xylanases) exhibit astonishingly diverse properties. Probably, the only property that is shared among alkaline active hemicellulases such as xylanases could be their significant activity and stability in the alkaline pH range. The degree of substrate specificity, the actual pH profile for activity and stability, the effect of temperature on the activity and stability, molecular weight, end-product profile, etc. vary from source to source. Some of the relevant properties of xylanases and mannanases are discussed below.

5.1.1 Xylanases

Probably, xylanases are the most studied alkaline active hemicellulases. This may be primarily driven by the application potential in pulp and paper industry. Since the first xylanase from an alkaliphilic strain, *Bacillus* sp. C-59-2, was reported in 1973 [67], numerous alkaline active xylanases have been reported from alkaliphilic organisms. This includes the xylanases produced by the alkaliphilic strains of *Bacillus* [68–70], *Micrococcus* [71], *Streptomyces* [72], *Alkalitalea* [73], *Arthrobacter* [74], *Staphylococcus* [75], *Enterobacter* [76], etc. Alkaline active xylanases have also been reported from alkaliphilic fungal strains such as *Aspergillus nidulans* KK-99 [77] and *Penicillium citrinum* [78]. Alkaliphiles isolated from soda lake and soil samples have been the major sources of alkaline active xylanases. However, such xylanases have also been reported from insect gut microbes [68, 79] and from metagenome libraries [80, 81].

Several xylanases from alkaliphilic strains have been purified and characterized. Some of these enzymes are found to be optimally active around neutrality, and few of them are even in the acidic range (Table 2). However, these enzymes, although they are optimally active at neutral to acidic conditions, often exhibit reasonably high activity at alkaline condition. Thus, these xylanases are active in a wide range of pH. For instance, the alkaliphilic strain *Bacillus firms* K-1 produces a xylanase that is optimally active at pH 5 but displays activity in the pH range of 3–12 [85]. Surprisingly, only few xylanases are known to be optimally active above pH 9 when assayed at >50°C. These include *Actinomadura* Cpt20 [66], XylB from *Bacillus* sp. AR-009 [87], and *Bacillus halodurans* xylanases [69, 98]. The most alkaline active xylanase ever reported is the one from *Streptomyces* sp. CS802 which is optimally active at pH 12 and stable even at pH 13 [72]. Although it is assayed at

Table 2 Alkaline active xylanases from different organisms and their properties

Alkaliphile	pH optimum for activity	Active in the pH range	Optimum temperature (°C)	Molecular weight (kDa)	References
Alkalibacterium sp. SL3	9	5–12	55	149	Wang et al. [82]
Actinomadura sp. strain Cpt20	10	–	80	20	Taibi et al. [66]
Arthrobacter sp. MTCC 5214	9	5–10	100	22	Khandeparkar and Bhosle [74]
Aspergillus nidulans KK-99	8	4.0–9.5	55	–	Taneja et al. [77]
Bacillus sp. TAR1	9	4–11	70	40	Nakamura et al. [83]
Bacillus sp. 41M-1 xylJ	9	4–11	50	36	Nakamura et al. [84]
Bacillus firms K-1	5	3–12	60	–	Mongkorntanyatip et al. [85]
Bacillus halodurans S7	9–9.5	5–12	75	43	Mamo et al. [69]
B. halodurans PPKS-2	11	6–12	70	24	Prakash et al. [86]
Bacillus sp. AR-009 xylB	9–10	5–11	75	48	Gessesse [87]
Bacillus licheniformis Alk-1	9	4–11	60	46	Raj et al. [70]
B. firmus xyn10A	7–9	4–12	70	45	Chang et al. [88]
B. firmus xyn11A	5–7	4–11	60	23	Chang et al. [88]
B. pumilus 13a	9	8–11[a]	60	–	Duarte et al. [89]
Enterobacter sp. MTCC 5112	9	–	100	43	Khandeparkar and Bhosle [76]
Geobacillus thermoleovorans	8.5	5–11	80	–	Sharma et al. [90]
Kocuria sp. Mn22	8.5	5.5–12	55	40	Chanjuan et al. [91]
Microcella alkaliphila	8	5–11	65	150	Kuramochi et al. [92]
Micrococcus	7.5–9	5.5–11	55	56	Gessesse and Mamo [71]
Paenibacillus	6–7	5–10.5	–	–	Ohkuma et al. [79]
Paenibacillus barcinonensis	9.5	–	60	–	Valenzuela et al. [93]
Paenibacillus macquariensis	8.6	4–11	50	31	Sharma et al. [94]
Penicillium citrinum	8.5	–	50	25	Dutta et al. [78]

(continued)

Table 2 (continued)

Alkaliphile	pH optimum for activity	Active in the pH range	Optimum temperature (°C)	Molecular weight (kDa)	References
Streptomyces sp. CS802	12	7–13	–	–	Simkhada et al. [72]
Streptomyces sp. QG-11-3	8.6	5–10	60		Beg et al. [95]
Staphylococcus sp. SG-13	7.5 and 9.2	4–10.5	50	60	Gupta et al. [75]
Stenotrophomonas maltophilia	9	–	80	142	Raj et al. [96]
Thermobifida halotolerans YIM 90462^T	9	–	70	34	Zhang et al. [97]

[a]The effect of pH on the enzyme activity is done only in the pH range of 8–11

lower temperature (37°C), the *B. halodurans* PPKS-2 xylanase displayed optimum activity at pH 11 making it the second most alkaline active xylanase so far [86]. However, the pH optima of glycosyl hydrolases are often affected by the assay temperature [89, 99], and hence, the *B. halodurans* PPKS-2 enzyme might not display the same pH profile if assayed at higher temperature. Other alkaline active xylanases of *B. halodurans* strain are known to be optimally active around pH 9–9.5 [69, 98]. On the other hand, based on the molecular weight, the *B. halodurans* xylanases which are optimally active around pH 9.5 belong to family 10 xylanases, while the low molecular weight (24 kDa) of the *B. halodurans* PPKS-2 xylanase suggests that it belongs to family 11 xylanase.

Most alkaline active xylanases are optimally active in the temperature range of 40–75°C. The highest optimal temperature for activity of an alkaline active xylanases, 100°C, is reported for *Arthrobacter* [74] and *Enterobacter* [76] strains. The size of these extremozymes are in the range of 20–149 kDa, the majority are within 20–50 kDa range (Table 4). Larger xylanases such as those from *Alkalibacterium* sp. SL3 [82], *Microcella alkaliphila* [92], and *Stenotrophomonas maltophilia* [96] contain non-catalytic domains which are often used to bind substrates.

Xylan is a complex heteropolysaccharide, and its complete hydrolysis requires the action of different enzymes. An efficient xylan backbone hydrolysis by endo-xylanases requires the removal of side chains, which can prevent the full enzyme access to the backbone linkages probably by steric hindrance [35]. For this reason, xylanolytic microorganisms produce a suite of auxiliary enzymes that are needed to make the backbone accessible. For example, the genome search of one of the most studied alkaliphile, *B. halodurans* C-125, revealed the presence different enzymes that are necessary for complete degradation of xylan (Table 3).

Table 3 Hemicellulose-degrading enzymes identified in the genome sequence of *B. halodurans* C-125

Enzyme	Number	Gene name	GenBank ID	Family	Leader peptide
Endo-β-1,4-xylanase	2	BH0899	BAB04618.1	GH11	Yes
		BH2120	BAA00055.1	GH10	Yes
Exo-β-1,4-xylosidase	3	BH1068	BAB04787.1	GH39	Not detected
		BH3683	BAB07402.1	GH43	Not detected
		BH2114	BAB05833.1	GH52	Not detected
Rex exo-oligoxylanase[a]	1	BH2105	BAB05824.1	GH8	Not detected
α-L-Arabinofuranosidase	2	BH1861	BAB05580.1	GH51	Not detected
		BH1874	BAB05593.1	GH51	Not detected
Acetylxylan esterase	2	BH3326	BAB07045.1	CE7	Not detected
		BH1115	BAB04834.1	CE12	Not detected
α-Glucuronidase	1	BH1061	BAB04780.1	GH67	Not detected
α-Galactosidase	3	BH2223	BAB05942.1	GH36	Not detected
		BH2228	BAB05947.1	GH4	Not detected
		BH1870[b]	BAB05589.1	GH27	Not detected
β-Glucosidase	1	BH1923	BAB05642.1	GH1	Not detected

[a]Reducing end xylose-releasing exo-oligoxylanase
[b]Only the first part clearly belongs to galactosidase

Endo-xylanases are the major enzymes that hydrolyze the main chain of xylan. Since xylan cannot be directly transported to cell, the initial hydrolysis happens extracellularly. Therefore, the endo-β-1,4-xylanases should be secreted extracellularly, a phenomenon that often requires leader peptide as shown in Table 3. In the early phase of the hydrolysis, these enzymes form xylooligosaccharides from xylan, and toward the end xylobiose, xylotriose, and xylotetraose accumulate as major end products, while xylose appears upon prolonged incubation. The smaller oligos with side chains can be transported to the cell and probably the completion of the hydrolysis accomplished within the cell. Thus, most of the accessory enzymes might be cell associated, and this may one of the reasons why there is no detectable leader peptide for the *B. halodurans* accessory enzymes (Table 3). However, the absence of the leader peptide cannot confirm that the enzymes are intracellular.

5.1.2 Mannanases

Mannan and heteromannan (glucomannan and galactomannan) are among the important hemicelluloses, and there has been biotechnological interest in enzymes that modify these carbohydrates. Among the enzymes, β-mannanases which break the β-1,4-mannosidic linkages in the main chain of mannan and heteromannan have attracted a great deal of interest and got several applications in food, detergent, and pulp and paper industries [113–115]. The requirement of alkaline active mannanases such as in detergent formulation and Kraft pulp production has contributed to initiation of the search for alkaline active mannanases. Today, several alkaline active

Table 4 Alkaline active mannanases

Organism	Optimum pH	Activity range	Optimum temperature (°C)	Molecular weight (kDa)	References
Bacillus JAMB-750	10	5–10.5	55	130	Takeda et al. [100]
Bacillus JAMB-602	9	6.5–10	65	50	Takeda et al. [101]
B. halodurans PPKS-2	11	5–13	70	22	Vijayalaxmi et al. [102]
B. clausii S10	9.5	6–11.5	75	34	Zhou et al. [103]
Bacillus sp. N16-5 (ManA)	9.5	5–11	70	55	Ma et al. [104]
B. nealsonii PN-11	8.8	7.2–9.2	65	50	Chauhan et al. [105]
Streptomyces sp. CS428	12.5	3–13.5	60	35	Pradeep et al. [106]
Bacillus sp. M1	9	6–10	60	58	Akino et al. [107]
Bacillus sp. M2	9	6–10	60	59	Akino et al. [107]
Bacillus sp. M3	8.5	5–10	65	42	Akino et al. [107]
B. subtilis subsp. *inaquosorum* CSB31	12.5	4–13	60	47	Regmi et al. [63]
B. cereus N1	10	–	50	63	El-Sharouny et al. [108]
B. circulans CGMCC1554	7.6	6–9	60	32	Yang et al. [109]
B. circulans CGMCC1416	7.6	5–10	58	31	Li et al. [110]
Bacillus sp. 22	8.8	7–9.2	70	38	Kumar et al. [111]
Bacillus sp. JB-99	10	5–11	65	–	Virupakshi et al. [112]

β-mannanases from a range of organisms have been reported (Table 4). The great majority of alkaliphiles from which alkaline active mannanases have been reported belong to the genus *Bacillus*. These includes *Bacillus agaradhaerens* [116], *Bacillus clausii* [103], *Bacillus circulans* [109, 110], and several other unidentified *Bacillus* strains [104, 117–119].

Characterization studies of *Bacillus* mannanases revealed that most of these enzymes are optimally active around acidic to neutral pH [114]. However, as shown in Table 4, some mannanases from alkaliphilic and alkali-tolerant *Bacillus* strains exhibit optimal activity in the alkaline range (pH 8.5–12.5). Moreover, similar to alkaline active xylanases (Table 2), the majority of the alkaline active

mannanases are also active and stable in a wide range of pH, pH 6–11 [119, 120]. The two most alkaline active mannanases which are optimally active at pH 12.5 are reported from *Streptomyces* sp. and *B. subtilis* subsp. *inaquosorum* CSB31.

The optimum temperature for the activity of alkaline active mannanases falls in the range of 50–75°C, which is within the range (40–75°C) known for most mannanases [121]. The size of these enzymes is in the range of 30–60 kDa (Table 4). However, mannanases with very low or high molecular weights have also been reported. The mannanase from *B. halodurans* PPKS-2 has a molecular weight of 22 kDa [102], while that of *Bacillus* JAMB-750 is 130 kDa [100].

5.2 Multiplicity of Xylanases and Mannanases

Several xylan-degrading organisms produce multiple xylanases. For example, *Streptomyces* sp. B-12-2 produces five endo-xylanases [122]. *Aspergillus niger* and *Trichoderma viride* exhibited, respectively, up to 15 and 13 xylanases in their culture supernatant [123]. *Phanerochaete chrysosporium* produced about 30 xylanases [124]. Alkaliphiles are also known in producing multiple xylanases. For instance, *B. halodurans* C-125 produces two extracellular xylanases [98] and an intracellular xylanase [62]. Two extracellular endo-xylanases are reported from an alkaliphilic *Bacillus* strain [87]. Three xylanases, two extracellular and one cell associated, are produced by the alkaliphilic *Bacillus* sp. K-1 [125]. Multiplicity is also a feature of mannanases. For instance, the fungus *Sclerotium rolfsii* produces two β-mannosidases and five β-mannanases [126], and the alkaliphilic *Bacillus* sp. AM-001 produces three mannanases [107].

There are many factors which lead to the appearance of multiple xylanases or mannanases in the cultures of microorganisms. Multiplicity may be the result of having distinct xylanase or mannanase encoding genes such as in *B. halodurans* and *Bacillus* sp. AM-001 or due to posttranslational processing such as glycosylation, differential mRNA processing, and post-secretional modification by proteolytic digestion [12, 36, 123].

As aforementioned, xylans and mannans are complex polysaccharides, and their glycosidic linkages may not be equally accessible to xylan-degrading enzymes. Thus, the complete hydrolysis of these hemicelluloses may be difficult to be achieved by a single xylanase or mannanase. This implies that an efficient hydrolysis of these polysaccharides requires the action of multiple xylanases or mannanases which work synergistically with overlapping but different specificities. The better substrate hydrolysis achieved by the enzymes' synergy can be correlated to substrate utilization efficiency of the organism producing the enzymes. This may offer a competitive advantage to the producer organism. Moreover, organisms may be adapted to grow under different environmental conditions. In such scenarios, an organism may produce a single enzyme (e.g., a mannanase) that works efficiently under broad conditions at which the organism is adapted to grow or it may produce different enzymes (e.g., mannanases) that work optimally at the different growth

conditions at which the organism able to grow. For instance, an organism may be adapted to grow in a pH range of 7–11. In this case, the organism either produce an enzyme (e.g., mannanase) that work effectively at pH 7–11 or produce two different enzymes (e.g., mannanases), which are optimally active in neutral or alkaline range. This can be exemplified by *B. halodurans* xylanases. However, it may be relevant to have a glance at the different families to which xylanases and mannanases belong to before discussing the example.

Based on the catalytic domain amino acid sequence homology, carbohydrate-modifying enzymes are grouped into families. The most informative and updated classification of hemicellulases and other carbohydrate active enzymes is available on the Carbohydrate-Active Enzymes (CAZy) server (http://afmb.cnrs-mrs.fr/CAZy). The great majority of xylanases are confined to glycoside hydrolase family 10 (GH10) and family 11 (GH11). Thus, there had been a notion that xylanases are restricted to GH10 and GH11 families. However, at the time of writing this review, xylan-degrading enzymes are also found in GH families 5, 8, 30, 43, 51, 198, and 141. Unlike xylanases that are distributed in 9 families, mannanases, at least at the moment, are restricted into 4 families, GH families 5, 26, 113, and 134. GH5 and GH26 are the two families where most of the mannanases belong to. The alkaline β-mannanases from the alkaliphilic microorganisms *Bacillus* sp. N16-5 and *Bacillus* sp. JAMB-602 belong to family 5, and the enzymes from *Bacillus* sp. strain JAMB-750 are categorized with family 26.

The enzymes that belong to a family may have distinct properties. For instance, the GH11 xylanases are smaller in size (<30 kDa) compared to the GH10 xylanases which characteristically have a molecular weight of over 30 kDa. Moreover, GH11 xylanases are more specific than GH10 xylanases. Substrate specificity studies on GH10 xylanases show that they are versatile and even can act on cellulosic substrates. However, xylan-specific GH10 xylanases have also been reported [69, 127]. Such differences in physical and catalytic properties among hemicellulases that belong to different families can contribute to the efficiency of hemicellulose hydrolysis. If *B. halodurans* is taken as an example, it produces three types of xylanases that belong to different families. These enzymes differ in many aspects [62, 98], and some of these differences are summarized in Table 5. These xylanases are believed to work synergistically and offer a competitive advantage to the organism.

Table 5 Features of *B. halodurans* xylanases

	Xylanase family		
Characteristic	GH8	GH10	GH11
Molecular weight (kDa)	45	43	16
pH optimum	6.2–7.3	6–10	7
Temperature optimum (°C)	50	70	70
Location	Intracellular	Extracellular	Extracellular
Mechanism	Inverting	Retention	Retention
Protein fold	$(\alpha/\alpha)_6$	$(\beta/\alpha)_8$	β-jelly roll

As shown in Table 5, the GH10 xylanase is active in a wide range of pH, and hence when the cells grow under alkaline condition, this enzyme can primarily degrade xylan, whereas when the growth condition is near neutral, both GH10 and GH11 xylanases may be involved in the degradation of xylan, which might compensate the effect of lower substrate solubility (at neutral condition), a phenomenon that can possibly reduce catalytic efficiency. Moreover, the difference in the properties of the two xylanases may have another competitive advantage in hydrolyzing xylan from different sources. GH11 xylanase prefers to hydrolyze not close to branching site, while GH10 xylanase hydrolyzes xylan even close to branching position. GH11 xylanases tend to release bigger fragments than GH10 xylanases. GH10 xylanases can attack smaller xylooligosaccharides which cannot be digested by family 11 xylanase. On the other hand, the smaller GH11 xylanase can diffuse easily and accesses xylan that cannot be reached by the bigger GH10 xylanase. This suggests that the two enzymes work synergistically to bring better xylan degradation. Xylooligosaccharides released by the two extracellular endo-xylanases will be taken up by the cells and further hydrolyzed by GH8 xylanase inside the cell. Thus, the multiplicity is expected to offer some competitive advantage, and this may be one of the reasons why hemicellulose-degrading organisms tend to produce multiple xylanases and mannanases having diverse physicochemical properties, structures, specific activities, and even end-product profiles, thereby increasing the efficiency and extent of hydrolysis.

5.3 Modular Organization

Many glycoside hydrolases and glycosyltransferases have modular structural architecture containing catalytic and non-catalytic domains connected to each other via short linker segments. The non-catalytic domains include structures that bind to cellulose, xylan, chitin, dockerin, etc. and other domains for which the function has yet to be established. The non-catalytic domains are commonly involved in carbohydrate binding and hence often referred to as carbohydrate-binding modules (CBMs). Like other glycoside hydrolases, modularity has been reported in alkaline active hemicellulases [82, 92, 128]. CBM structures are known to bind to insoluble substrates such as crystalline cellulose and insoluble xylan. This binding enhances the hydrolysis of such poorly soluble substrates by keeping the catalytic domain of the enzyme in close proximity to the substrate and by weakening the intermolecular interaction of the substrate molecules [129, 130]. However, in addition to its catalytic role, thermal stabilization effect has been widely reported [129]. On the other hand, there is little information if CBM structures are involved in enhancing enzyme activity and stability at high pH. But mutational studies on xynAS27 from *Streptomyces* sp. S27 revealed the possible roles of the non-catalytic domain on the activity and stability of alkaline active hemicellulases in alkaline milieu [128]. However, it is not clear if this role is direct or indirect. The great majority of alkaline active xylanases and mannanases from genus *Bacillus* are non-modular with some

exceptions such as the mannanases of *Bacillus* sp. JAMB-602 and *Bacillus* JAMB-750 [118] (http://www.cazy.org/CBM23_characterized.html) and the *Bacillus* sp. SN5 xylanase [131].

5.4 Structural Features

In the glycoside hydrolase classification system, related families of enzymes that share common ancestry, structural fold, catalytic residues, and mechanism of catalysis are grouped in clans [132]. In this system, xylanases are grouped in four different clans (GH-A, GH-C, GH-F, and GF-M), while all known families of mannanases, except GH134, belong to clan GH-A (Table 6). Among xylanases, those that belong to GH98 and GH141 are not yet a member of any known clan, and the same holds true for GH134 mannanases (Table 6). Most of the hemicellulases belong to the clan GH-A, which also contains other families with $(\alpha/\beta)_8$-barrel fold, a structure most common among all known enzyme folds. Roughly, one out of ten enzymes has an $(\alpha/\beta)_8$-barrel fold [143], and in addition to GH-A, members of the clans GH-D, GH-H, and GH-K exhibit this folding motif. The $(\alpha/\beta)_8$-barrel is composed of eight parallel β-strands that makes the inner β-barrel sheet that is

Table 6 Xylanases and mannanases that belong to different clans and their protein fold

Enzyme	Family	Clan	Fold	PDB code (example)	References
Endo-β-1,4-xylanase (EC 3.2.1.8)	5	GH-A	$(\beta/\alpha)_8$	1NOF	Larson et al. [133]
	8	GH-M	$(\alpha/\alpha)_6$	1H12	Van Petegem et al. [134]
	10	GH-A	$(\beta/\alpha)_8$	2UWF[a]	Mamo et al. [135]
	11	GH-C	β-jelly roll	2NQY[a]	Unpublished
	30	GH-A	$(\beta/\alpha)_8$	3KL5	St John et al. [136]
	43	GH-F	5-blade β-propeller	3CU9[b]	Alhassid et al. [137]
	51	GH-A	$(\beta/\alpha)_8$	3UG3[b]	Im et al. [138]
	98	–	–	2WMI[b]	Higgins et al. [139]
	141	–	(β)-helix	5MQP[b]	Unpublished
Endo-β-1,4-mannanase (EC 3.2.1.78)	5	GH-A	$(\beta/\alpha)_8$	3JUG[a]	Zhao et al. [140]
	26	GH-A	$(\beta/\alpha)_8$	4YN5[a]	Unpublished
	113	GH-A	$(\beta/\alpha)_8$	5YLI[a]	You et al. [141]
	134	–	–	5JTS	Jin et al. [142]

[a]Enzymes from alkaliphiles
[b]Representative structures that are neither xylanase nor mannanase

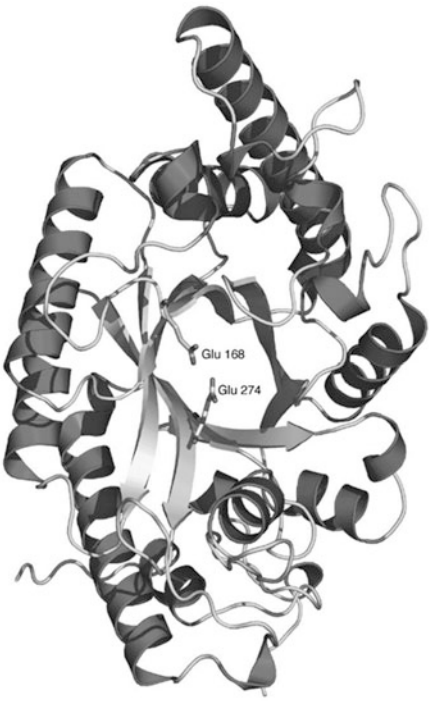


Fig. 4 The $(\beta/\alpha)_8$ fold of a xylanase (PDB 2UWF) depicting the most common fold among hemicellulases. The Glu residues shown in the structure are the catalytic residues

surrounded by eight α-helices as in Fig. 4. However, this fold includes several enzymes in which the barrels are deviated from the ideal $(\alpha/\beta)_8$-barrel geometry and topology. GH11 xylanases belong to clan GH-C and have β-jelly roll fold. β-jelly roll fold consists of eight β-strands. GH8 xylanases belong to clan GH-M and composed of α-helixes. The GH43 xylanases are members of GH-F clan and adopt the 5-blade β-propeller fold. Table 6 summarizes the clans to which the different families of hemicellulases belong to and the structural folds with representative structures deposited in PDB.

Solving the three-dimensional structure of alkaline active hemicellulases would help to decipher the structural basis for high pH catalytic adaptation, which, in addition to expanding the basic understanding of protein structure function, helps to engineer enzymes to be operationally stable at elevated pH. In line with this, the three-dimensional structures of an alkaline active GH10 xylanases from *Bacillus* sp. NG-27 [144] and *B. halodurans* S7 [135] are determined. The structure of an alkaline active family GH11 xylanase from an alkaliphilic *Bacillus* sp. SN5 has also been determined [145]. In a similar way, the structures of alkaline active GH5 β-mannanases from *Bacillus* sp. N16-5, *Bacillus agaradhaerens*, and *Bacillus* sp. JAMB-602 have been solved [140].

Comparative analysis of the structures revealed that alkaline active enzymes deploy different strategies to be operationally stable at high pH. Compared to the

respective non-alkaline counterparts, the alkaline active hemicellulases display a high percentage composition of acidic amino acids, fewer solvent exposed polar residues, acidic surfaces, and fewer solvent exposed alkali labile residues [135, 140, 144].

Three different types of active site topological arrangements are recognized in GHs [146]. A tunnel is uniquely suited for processive exo-attack as in the case of β-glucosidase. Another active site topology is a cleft which allows endo-attack like endo-xylanases. The third topology is a crater/pocket that mostly fits for degradation of substrates by end-on-attack like GH8 xylanases. GH10 xylanases have clefts in the large radius face of their structure. At least in this group of enzymes, the cleft topology does not seem conserved. For instance, GH10 xylanases from *P. simplicissimum* (PDB 1B30) and *T. aurantiacus* (PDB 1K6A) have very shallow clefts, while xylanases of *B. halodurans* S7 (PDB 2UWF) and *G. stearothermophilus* (PDB 1HIZ) have relatively deep clefts [135]. The variation in the cleft topology can affect enzyme properties. As aforementioned, generally, GH10 xylanases show broader substrate specificity when compared to GH11 xylanases. One of the possible reasons for the difference in the substrate specificity could be the depth of their active site clefts [147]. GH11 xylanases have deeper cleft than GH10 xylanases. However, there are some xylan-specific GH10 xylanases such as the alkaline active xylanase from *B. halodurans* S7. Such GH10 xylanases have deeper catalytic cleft which might be the reason behind their substrate specificity [135].

Analysis of the GH5 mannanases three-dimensional structures has indicated the tendency of increasing structural compactness with an increase in the enzymes' optimum pH for activity [140]. There seems to be rearrangement of secondary structures around the active site region, which distinctly shape the catalytic cleft of the alkaline active mannanases. This unique microenvironment created by the rearrangement of loops, helices, and β-strands affects the catalytic amino acids protonation state, which determines the optimum pH for the mannanase activity. Mutating residues that exist in the microenvironment can affect the pH profile of mannanases, and indeed this has been proven [140].

5.5 *Mechanism of Catalysis*

There is a wide variety of glycosidic bonds in organic molecules. These bonds are vital in formation of carbohydrate polymers such as xylan and mannan. There exists a range of enzymes whose function is to cleave these bonds. Generally, hydrolysis of this bond by an enzyme carried out with one of the two stereochemical outcomes, net inversion or retention of the anomeric configuration. Based on this, glycosidases (including xylanases and mannanases) are classified as inverting or retaining. The inverting mechanism involves transition states and a pair of carboxylic acids at the active site, and the reaction occurs via a single displacement mechanism wherein one carboxylic acid acts as a general base and the other as a general acid. The average

distance between the two catalytic carboxylic acid residues is about 9.5 Å [12]. In retaining mechanism, the two carboxylic acid residues of the catalytic amino acids are positioned approximately 5.5 Å apart. These carboxylic acids are involved in intermediate formation. In the first step of the catalysis, one of the carboxylates protonates the substrate and acts as a general acid catalyst, while the other carboxylic acid performs a nucleophilic attack which results in the departure of the leaving group and the formation of glycosyl-enzyme intermediate. In the second step, the first carboxylate group now functions as a general base, taking a proton from a nucleophilic water molecule which attacks the anomeric carbon. This leads to a second substitution in which the anomeric carbon again passes via a transition state to give rise to a product with β configuration. Thus, the overall result is retention of the configuration at the anomeric center. These catalytic properties of hemicellulases are shown in Table 7.

GH10 and GH11 xylanases catalyze the hydrolysis of glycoside bonds by retention double displacement mechanism, in which a covalent glycosyl-enzyme intermediate is formed and subsequently hydrolyzed via transition states. Several models have been proposed to explain the mechanism of xylanase action. A suitable enzyme mechanism that combines the classic concepts listed above and facts derived from experimental results is proposed by [148]. According to this proposal, xylan is recognized and bound by xylanase as a left-handed threefold helix. Then, the xylosyl residue at subsite −1 is distorted and pulled down toward the catalytic residues, and the glycosidic bond is strained and broken to form the enzyme-substrate covalent intermediate. The intermediate is attacked by an activated water molecule, following the classic retaining glycosyl hydrolase mechanism, and the product is released.

Table 7 The catalytic mechanisms and active site residues of xylanases and mannanases

Hemicellulase	GH Family	Mechanism	General acid/ base residue	Nucleophile	Displacement
Endo-β-1,4-xylanase	5	Retaining	Glu	Glu	Double
	8	Inverting	Glu	Asp	Single
	10	Retaining	Glu	Glu	Double
	11	Retaining	Glu	Glu	Double
	30	Retaining	Glu	Glu	Double
	43	Inverting	Glu	Asp	Single
	51	Retaining	Glu	Glu	Double
	98	Inverting	Glu	Asp + Glu	–
	141	Unknown	Asp	Asp	Unknown
Endo-β-1,4-mannanase	5	Retaining	Glu	Glu	Double
	26	Retaining	Glu	Glu	Double
	113	Retaining	Glu	Glu	Double
	134	Inverting	–	–	–

Xylanases that belong to GH8, GH43, and GH98 display an inverting mechanism of catalysis. Unlike other families, at the time of writing, the GH134 family comprises only β-mannanases which are distinct from other β-mannanases by its inverting catalytic mechanism [149]. However, the catalytic residues and the displacement mechanism of this enzyme is yet to be identified.

6 Applications of Alkaline Active Hemicellulases

With the growing interest in "greener" industrial processes and the discovery of more amenable enzymes that fit to industrial requirements, the application of enzymes is expanding both in volume and diversity. This seems reflected in the ever-increasing market value of industrial enzymes. It is believed that hydrolases are the major commercial enzymes which roughly account for 75% of the total industrial enzyme market [150]. Hemicellulases are among these commercially important hydrolases. The annual market value of xylanases alone is estimated to be over 200 million USD [151], and recently the compound annual growth rate of the global xylanase market has been projected to be about 6.6% [152]. Although neutralophiles have been the dominant sources for commercial hemicellulases, in recent years alkaliphiles and alkali-tolerant organisms are also becoming important. Xylanases and mannanases which are significantly active and stable in high pH conditions are the ideal choices for applications such as biobleaching of Kraft pulp and detergent formulation which require alkaline conditions. Moreover, as shown in Tables 2 and 4, most alkaline active hemicellulases are often active in a wide range of pH, from slightly acidic to highly alkaline milieu. Thus, potentially, at least some of these hemicellulases of alkaliphiles can be used in areas where enzymes of neutralophiles are being used like in food, beverage, and feed industries. Below, some of the important real and potential applications of alkaline active hemicellulases are discussed.

6.1 Pulp and Paper

The pulp and paper industries use different methods of recovering cellulose fibers from lignocellulosic biomass, most of which is from wood. Chemical pulping which includes Kraft, sulfite, and semichemical processes is the dominant method among the pulping processes. Kraft pulping accounts for over 90% of the total chemical pulping. The Kraft process which is also called the sulfate process involves an alkaline treatment with solutions of sodium sulfide and sodium hydroxide at about 170°C for 2 h resulting in the degradation and solubilization of lignin. The resulting pulp from this alkaline cooking has brownish color primarily due to the residual lignin and lignin derivatives which are covalently attached to the hemicellulose fraction. Removal of this lignin involves a multistage bleaching process, which involves elemental chlorine. Although chlorine-based bleaching of pulp is effective,

it results in chlorinated organic by-products which are reported to have highly persistent toxic and mutagenic effects [153]. Because of the growing public concern about environment and strict legislations regarding pollution, the search for alternative ways to reduce or avoid the release of chlorogenic compounds with Kraft mill bleaching effluent has been promoted. Today, alternative environmentally benign bleaching chemicals like H_2O_2, O_2, and O_3 are substituting the toxic chlorine-based bleaching chemicals.

Enzymatic treatment of Kraft pulp prior to bleaching hydrolyzes the xylan component of wood, which in turn facilitates lignin removal, a phenomenon that has shown a substantial reduction in the use of bleaching chemicals [70, 154, 155]. For instance, a ton of xylanase-treated pulp consumes 5–7 kg less chlorine dioxide for bleaching [156]. Biobleaching of pulp with xylanase, in addition to improving the quality of the pulp and reduce the environmental impact, downsizes the process cost [157]. For example, the biobleaching of hardwood Kraft pulp to 89% ISO brightness reduces the bleaching cost by about 2.3 USD per ton of dried pulp [158]. Thus, considering that the price of xylanase treatment is below 2 USD per ton [159], the biobleaching has some economical gain. Thus, the use of xylanases in pre-bleaching of Kraft pulp has been considered as one of the greatest success stories of enzymes in pulp and paper industry, and today, there are more than 20 mills in North America and Scandinavia alone that employs xylanase treatment [56]. Progress in enzyme production technology has significantly diminished the production cost, which subsequently reduced the enzyme selling price. This cost reduction is expected to attract more mills to consider using enzymes in Kraft pulp bleaching. As shown in Table 8, a number of companies are producing and marketing xylanases for this purpose.

Although removal of xylan and the associated lignin from the pulp are believed to facilitate the bleaching, it was not always the case. This observation led to several studies which tried to decipher the mechanism(s) by which xylanases enhance pulp bleaching. The findings of the studies center around these three major mechanisms by which xylanases enhance pulp bleaching: (1) improving lignin extraction,

Table 8 Some suppliers of commercial bleaching enzymes and the trade names

Product name	Supplier	Country
Bleachzyme	Bicon	India
Cartazyme	Sandoz, Charlotte, N.C. and Basle	Switzerland
Ecopulp	Rohn Enzyme 0Y, Primalco	Finland
Ecopulp	AlkoRajamaki	Finland
Ecozyme	Thomas Swan	UK
Irgazyme	Genecor	Finland
Optipulp	Solvay Interox	USA
Pulpzyme HB	Novo Nordisk	Denmark
SP431	Novo Nordisk	Denmark
GS-35, HS70	Iogen	Canada
VAI xylanase	Voestalpine	Austria

(2) altering the carbohydrate-lignin associations, or (3) cleaving the redeposited xylan.

Hexenuronic acid which absorbs UV is formed during Kraft pulping from 4-methyl-glucuronic acid residues of xylan [160]. Xylan hydrolysis allows easy removal of such groups and decreases the amount of bleaching chemicals that would be needed to bleach those residues. Moreover, hexenuronic acid removal by xylanases helps to prevent brightness reversion of the treated Kraft pulps [161].

After pulping, a considerable amount of xylan is present in the pulp, some of which is reprecipitated on the surface of the fiber and decreases the accessibility of lignin. Hydrolysis of this xylan is believed to render the fiber structure more permeable and allows the alkali or bleaching agents to have better access to lignin. Moreover, the increased permeability allows the passage of lignin or lignin-carbohydrate molecules efficiently in subsequent processes [162], and this enables the removal of lignin and achieves a better bleaching efficiency with reduced amount of bleaching chemicals. On the other hand, there is evidence that xylan does not necessarily reprecipitate on the surface of all pulps [163] and the demonstration of xylan removal does not always correlate with bleaching efficacy and varies from enzyme to enzyme [164]. This shows that the exact mechanism of xylanase-mediated bleaching is not fully understood, and hence, at this point, it is difficult to predict if bleaching will be enhanced by a given xylanase or not. However, some properties of xylanases are considered desirable. Among these traits, the temperature and pH profiles of the enzymes are known to be relevant to this application.

The Kraft process results in pulp that is alkaline and hot, and hence an ideal bleaching enzyme should be operationally stable under these conditions and could be used directly without any temperature or pH adjustment. However, the great majority of xylanases are neither active nor stable at alkaline condition and high temperature. In fact, the first-generation xylanases used in pulp treatment were not active at alkaline conditions. Thus, pulp cooling and pH adjustment were necessary prerequisites. The pulp pH is often adjusted to around neutral range with sulfuric acid, and this has been associated with the accelerated corrosion of equipment used in enzymatic treatment of pulp [165]. On the other hand, the use of alkaline active and thermostable enzymes potentially avoids the cooling and pH adjustment steps, which concomitantly minimizes not only equipment corrosion and the related maintenance cost but also the time required to make the adjustments. This has led to the continuous search and development of alkaline active thermostable enzymes that are more suitable for direct Kraft pulp treatment, and xylanases such as Ecopulp TX-200C that are operationally stable around pH 10 and 90°C [56] are the results of this effort. In addition to activity and stability at high pH and temperature, the molecular weight and specificity of the enzyme affect the biobleaching efficiency. Compared to GH10 xylanases, those that belong to family GH11 are more effective in biobleaching of pulp [166]. It is believed that this is due to smaller size which allows GH11 xylanases diffuse effectively in the pulp and remove the xylan which cannot be accessed by the bigger GH10 xylanases. Moreover, most GH10 xylanases show catalytic promiscuity and degrade cellulose, a property that can deteriorate the quality of the pulp.

In addition to xylanases, other hemicellulases have also been tested as bleaching aids. Among these enzymes, mannanases have been shown to facilitate bleaching, eliminating residual lignin, and increasing paper brightness [111, 167] in a similar way to that of xylanases but by removing the mannan component of the wood. However, compared to that of xylanases, the effect of mannanases is often less pronounced [1, 168].

6.2 Detergent

Enzymes, which degrade organic dirt, are known to boost the cleaning performance of detergents. This performance-enhancing nature has been the major impetus for developing a variety of enzymes for detergent applications. Today, this sector has emerged as one of the most important commercial segments absorbing nearly 30% of the total enzyme market value. Due to the alkaline nature of detergents, enzymes intended for this application should be primarily operationally stable in high pH condition. Alkaliphiles, which are the prominent sources of alkaline active enzymes, have been serving as the major sources of detergent enzymes. Traditionally, alkaline active proteases, amylases, and cellulases have been the dominant detergent enzymes. However, in recent years, alkaline active hemicellulases such as mannanases are also emerging as detergent enzymes.

Cleaning botanical and mold dirt is a challenge, especially when the washing temperature is low and the cycles are shorter. Stains from tomato, orange, tea, coffee, banana, mango, broccoli, grass, etc. contain hemicelluloses and are often difficult to effectively clean. However, the use of detergents containing enzymes that degrade these hemicelluloses can remove such though stains with remarkable efficiency [169, 170]. For instance, mannan is common in food such as those prepared from legume seeds and food containing gelling agents and thickeners like locust bean gum and guar gum. Mannans in such food attach dirt due to its sticky nature and make stains which are not easy to clean. But the use of alkaline active mannanases containing detergent on the spot can degrade the mannan and remove the attached dirt and clean the stains efficiently [171]. Hence, it is beneficial to use detergents containing mannanases to effectively clean dishes and clothes soiled with such food. Today, mannanases are known in the detergent industry as stain removers. The use of alkaline active xylanases in detergents would also improve the removal of botanical dirt such as grass, fruit, and vegetable stains [2]. In fact, it has been observed that the use of alkaline active xylanases boosts the washing performance of detergents [154, 170, 172].

6.3 Textile and Fiber Processing

Another area of application for alkaline active hemicellulases is in the textile and associated industries. Cotton fibers are important textile input, and processing of these fibers involves several steps. Scouring is one of the steps that involve removal of noncellulosic materials from the cotton. The conventional scouring of cotton is done at high pH and temperature, a process which is not only expensive but also polluting. Moreover, the harsh scouring process affects the quality of the fiber. Thus, it was imperative to look for economical and benign alternatives. With this impetus, bioscouring, the enzyme-assisted scouring processes that alleviate the problems related to the conventional process, has emerged as promising alternative [173]. Xylanases have been successfully tried as cotton scouring agents [174–176]. The results of these evaluation studies indicate that the use of xylanases as bioscouring agents allows to reduce the concentration of NaOH, lower the treatment temperature, and shorten the treatment duration. In addition to these advantages, the xylanase treatment improves the quality of the fiber. Unlike the conventional processing, the enzyme bioscouring does not compromise the fiber strength, while it improves the fiber water absorption capacity, and this facilitates the spinning process. Furthermore, the enzymatic treatment makes the fiber soft and smooth [2, 177].

In a similar way to cotton fiber processing, alkaline active xylanases and mannanases can be used to process other plant fibers. For instance, dried ramie stems can be soaked with hemicellulases to recover intact long cellulose fibers [178, 179]. Together with pectinases, hemicellulases have been used to degum bast fibers such as hemp, flax, and jute [19]. Since the lignin does not undergo oxidation (which often leads to darkening) during enzymatic treatment, a strong bleaching step is not a necessity. Alkaline soaking is preferable as it facilitates the removal of noncellulosic materials from the fiber and inhibits the growth of cellulose-degrading fungi. Thus, the use of alkaline active hemicellulases is ideal for these applications.

6.4 Xylo- and Mannooligosaccharides

Oligosaccharides are short-chain sugar molecules containing two to ten monosaccharide units. The XOS and MOS, which are, respectively, obtained from xylan and mannan, have many applications as discussed above in Sect. 3. Although these oligosaccharides can be produced through acid or enzymatic routes, the enzymatic process is the most desirable route. The use of alkaline active xylanases and mannanases in production of these interesting saccharides has advantages over that of neutral or acid active enzymes, mainly due to the better solubility of the hemicelluloses in alkaline condition, a case demonstrated by Samanta et al. [180]. Moreover, since hemicelluloses are often extracted with alkaline extraction method, it

requires extensive washing and neutralization before it is hydrolyzed by neutral or acid active enzymes. This is not only cost incurring but also is time-consuming. On the other hand, the use of alkaline active enzymes allows direct hydrolysis of alkali-extracted hemicellulose. This has motivated the evaluation of alkaline active hemicellulases in production of the oligosaccharides. Alkaline active xylanases have been successfully used to produce XOS [181–184]. Similarly, production of MOS by alkaline active mannanases has been reported [103, 105, 106].

6.5 *Hemicellulose Biorefinery*

Biorefinery is a concept of an integrated process which valorizes biomass to different value-added products in a way a petroleum refinery produces various petrochemicals from crude oil. Biorefineries primarily separate the major constituents of plant biomass cellulose, hemicellulose, and lignin. As illustrated in Fig. 5, the hemicellulose biorefinery valorizes hemicelluloses to various value-added products. The world is producing huge amount of agricultural and forestry residues, which are rich in hemicelluloses. These residues are considered as waste and often disposed of by burning [185]. Therefore, the use of these residues in the production of value-added goods has both economic and environmental advantages. Valorization of hemicelluloses often involves hydrolysis process that releases monomeric units from the polysaccharides, which can be chemically or biologically converted to target products. As acid hydrolysis results in fermentation inhibitors, and it is not benign to the

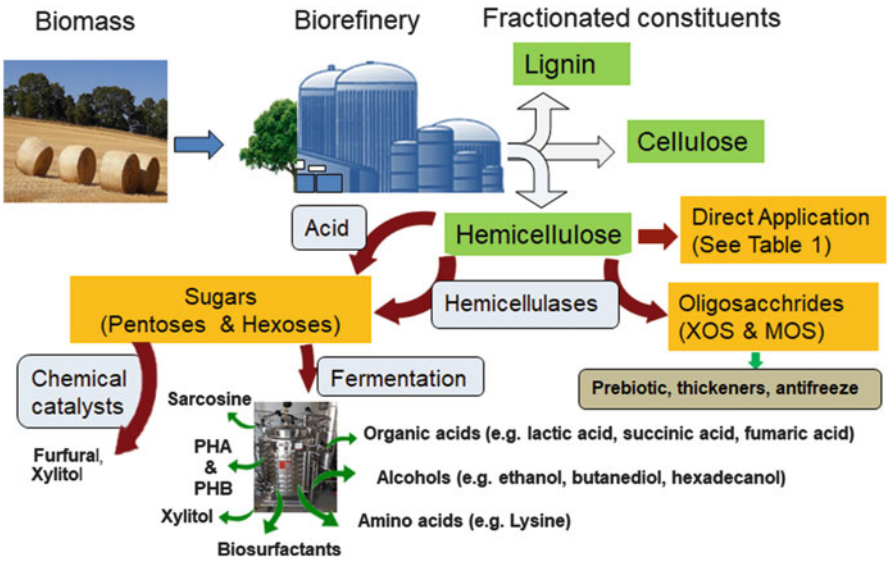


Fig. 5 Biorefinery and the valorization of hemicellulose to different products

environment and handling, the enzymatic hydrolysis has become attractive, especially if the hydrolysate is considered as fermentation feedstock. Hence, xylanases and mannanases are used to convert the polymeric xylan and mannan to fermentable sugars in hemicellulose biorefineries. In addition to bioethanol production [17, 186, 187], hemicellulose hydrolysates have been used as fermentation feedstocks to produce a variety of chemicals such as xylitol [188], 2,3-butanediol [16, 189], 3-hydroxypropionic acid [190], succinic acid [191], lactic acid [192], amino acids [193], 1-hexadecanol [15], biosurfactants [194], etc. Some of these chemicals produced from hydrolysates are platform chemicals, chemicals that subsequently can be converted to a range of other high-value chemicals. This widens the potential product portfolio from hemicelluloses. Moreover, with advances in metabolic engineering and microbiology, it is possible to get more organisms that can industrially valorize hemicellulose hydrolysates to different products, and this will significantly contribute to further expand the product portfolio from hemicelluloses.

In addition to sorted hemicelluloses, hydrolysis of hemicelluloses in unsorted biomass can increase productivity by improving cellulose accessibility to cellulases. This synergy can enhance the total conversion of biomass to fermentable sugars. An increase in the amount of fermentable sugars would ultimately increase the amount of fermentation products from the biomass.

The use of alkaline active hemicellulases in hemicellulose biorefineries has advantages over that of neutral active counterparts for the same reasons described in the above, better solubility of hemicelluloses at high pH and direct hydrolysis. Often hemicelluloses are extracted from lignocellulosic biomass by alkaline extraction methods and hence the use of alkaline active enzymes allow direct conversion of alkali extracted hemicellulose to its monomeric sugars, which avoids the time consuming and corrosive (addition of acid) pH adjustment. The other benefit is the high solubility of hemicelluloses at high pH which facilitates an efficient hydrolysis of the hemicellulose. There is another advantage of direct hydrolysis of alkali-extracted hemicelluloses in biorefineries, and that is its alkalinity. Often, microbial fermentation is a companied by pH drop, and to maintain the pH of the culture within the tolerance limit of the microorganism, it is a standard protocol that bases are pumped to the fermentation tank. The base consumption is high, especially when the target product is organic acid such as lactic acid or succinic acid. Thus, the use of alkaline hydrolysate can buffer the pH drop, which can substantially cut the base consumption. This may require starting the cultivation around neutral condition (as most of the industrial fermentation is accomplished around neutral to mildly acidic conditions) with enough substrate to induce acid production and then feed the major substrate, the alkaline hydrolysate.

6.6 Animal Feed

Cereal grains such as barley and wheat, which contain a lot of arabinoxylans, are among the major ingredients in poultry diets. For instance, in barley arabinoxylans

constitute 4–8% of the kernel and about 25 and 70% of the cell wall polysaccharides of the endosperm and aleurone layer, respectively [162]. These hemicelluloses have little nutritional significance for nonruminant organisms (e.g., chicken and pigs) as they lack the appropriate digestive enzymes. Undigested arabinoxylans increase the viscosity of the digesta in the gut and are known to have anti-nutritive effects. The high viscosity in the chicken intestine impairs penetration of digestive enzymes and ultimately decreases nutrient absorption. This results in an inefficient use of the feed and poor growth of the chicken. Moreover, the high viscosity may also support pathogenic conditions, especially in broiler chicks [35]. Addition of hemicellulases to the feed releases oligosaccharides which have prebiotic effect and stimulate the growth of beneficial bacteria in the gut, and this promotes the chickens' health [195, 196]. The use of xylanases, preferably together with mannanases, in feed formulation alleviates the anti-nutritive effects imposed by the arabinoxylans [196, 197]. Moreover, xylanases help in the degradation of the cereal cell walls and improve nutrient accessibility [198]. The enzymes eliminate sticky excreta problems and significantly contribute to reducing waste problems by lowering the water content of the droppings. In addition, the efficient use of the grain decreases the manure output, which is beneficial to the environment.

The ruminant animal feed nutritional quality can be improved with addition of xylanases in the feed. This is believed to be due to an increase in cellulose accessibility to ruminant digestion by removing the xylan component of the feed. It makes the digestion complete and improves the feed conversion efficiency [199]. In a similar way, most feed ingredients such as soybean meal, copra meal, palm kernel meal, and legumes contain mannan. Because of its indigestive nature, it negatively affects the growth performance of the animals. Thus, addition of mannanases to the feed can improve the feed digestibility by degrading the mannan and improve productivity [200].

Hemicellulases used as feed additives are known to be active around moderately acidic pH, especially if it is meant to be active in the digestive tract of the animals where the pH is about 5. However, if the enzyme is intended to improve the feed quality before feeding (during pelleting and storage), it should not be necessarily active at low pH. This has brought the possibility of using enzymes that are active in the neutral range. Even, the potential of alkaline active hemicellulases as feed additives has been positively evaluated [201]. Thus, alkaline active hemicellulases which are active in a wide range of pH can be considered for feed applications.

6.7 Food

In bread-making, xylanases improve dough development time, consistency, extensibility, and resistance to breakdown. It improves the desirable texture, loaf volume, and shelf life of the baked bread [162, 202]. However, all xylanases are not useful for this purpose. Xylanases that digest and solubilize water-unextractable arabinoxylans with only little effect on water-extractable arabinoxylan are the most preferred.

Family 8 xylanases such as from *Pseudoalteromonas haloplanktis* and the alkaliphile *B. halodurans* had a more positive effect in baking than the commonly used commercial enzyme [203, 204]; however, it is not yet clear if this is due to selective action on the water-unextractable hemicellulose. The use of xylanases is also beneficial in biscuit baking due to its ability to make cream crackers lighter and improve its texture and tastiness [202].

The use of hemicellulases in food processing can improve the nutritional quality of food. For instance, due to their bioactive nature, XOS and MOS are being used as dietary supplement that infer prebiotic, antioxidant, and potential immune-modulating properties [47, 48]. These oligosaccharides can be obtained directly by treating xylan- or mannan-containing food with xylanase or mannanase. Moreover, the nutritional quality of cereal-based foods can be improved upon using xylanases that modify the dietary fiber. Water-soluble dietary fibers have beneficial health effects such as lowering plasma cholesterol which is associated to coronary heart disease and type 2 diabetes [205, 206]. Xylanases improve the level of water-soluble arabinoxylans in fiber-enriched bread [207] and pasta [208] and hence infer health benefits.

Hemicellulases are important processing aids in industrial wheat gluten and starch separation processes. Xylanases which act on soluble arabinoxylan rapidly decrease the viscosity of wheat flour slurries and have shown excellent performance in the wheat separation process, which results in higher gluten and starch yields and improved gluten quality [209]. Hemicellulases have also been successfully tried in plant oil extraction [210, 211].

6.8 Beverages

In brewing industries, the viscosity created by soluble arabinoxylans may pose wort filterability and haze formation problems. The use of xylanases that degrade water-soluble arabinoxylans decreases wort viscosity and leads to an increased filtration rate and reduced beer haze formation [162]. Xylanases together with cellulase and pectinase are used to clarify must and juices and liquefy fruits and vegetables [19]. This enzymatic treatment helps to stabilize the fruit pulp, reduce viscosity, and remove substances that may cause cloudiness in the concentrate [156, 202]. Mannanases are used to reduce the viscosity of coffee extracts in production of soluble coffee and clear fruit juices and wines [114].

6.9 Other Applications

Xylanases and mannanases have also been tried in many other applications. Xylanases have been considered in the synthesis of fine chemicals [212, 213]. Since xylan is readily soluble in alkaline condition, a better synthetic efficiency is

expected at higher pH using alkaline active xylanases. Hemicellulases are also important in waste management. In addition to agricultural and municipal solid waste residues, different agro-industries, biorefineries, paper and pulp production plants, etc. generate huge amounts of hemicellulose-containing waste. Therefore, xylanases and mannanases can be used in managing such waste [162, 214]. This avoids burning, release to aquatic bodies, or damping of hemicellulose-containing waste, which makes it an environmentally sound method of disposal. Hemicellulases have also been used to induce the synthesis of phytoalexins and acylated sterol glycosides by tobacco cell suspension [215]. Moreover, xylanases have been successfully demonstrated in rhizosecretion of a target protein in simple hydroponic medium [216] and also in making protoplasts [19]. In oil drilling, guar gum is used to flood production wells to fracture the bedrock and enhance oil recovery. Reduction of the viscosity by hydrolyzing the guar gum eases the oil flow. Due to the high temperature and alkaline conditions of the wells, thermostable alkaline active mannanases have been developed to reduce the polymer viscosity and improve oil flow [217].

7 Conclusion

Several xylanases and mannanases have been studied and considered in a growing number of applications. Applications such as detergent formulation, pulp biobleaching, and textile fiber recovery and polymer (guar gum)-assisted oil recovery require enzymes which are active and stable in the alkaline range. This has led to the search for alkaline active hemicellulases primarily from alkaliphiles. Indeed, a variety of alkaline active hemicellulases have been purified and characterized from alkaliphiles culture. Although the studies revealed the presence of very interesting enzymes that are suitable for high pH applications, it seems that there has been very little progress regarding the commercial utilization of alkaline active hemicellulases. One of the reasons that possibly hinder the industrial application of these extremozymes could be their limited availability in market. Commercial scale development and production of alkaline active hemicellulases may facilitate wider application of these enzymes in various sectors. Enzymes of alkaliphiles should not be restricted only for high pH applications; there are many hemicellulases which are significantly active in neutral to low pH conditions. The use of such enzymes in non-alkaline applications should be encouraged, and this may contribute to their commercialization.

A variety of hemicellulases are known to be involved in complete degradation of hemicelluloses, and alkaliphiles are known to produce repertoire of these enzymes. However, only xylanases and mannanases are attracting due attention. So far, very little is known about the properties of other hemicellulases from alkaliphiles. Thus, it would be very interesting to study these hemicellulases.

The economical, health, and environmental concerns associated with the use of petroleum and its derivatives brought the campaign for utilization of biomass for

production of biofuels, chemicals, and materials. The emerging biorefinery concept is intended to facilitate the transfer of petro-based economy to more sustainable bio-based economy, which relies largely on lignocellulosic biomass. Thus, valorization of at least the major polysaccharide constituents of plant biomass should be within the scope of the sustainable development scenario. However, despite the recent activities, the major effort and success have been focused on cellulose and mostly sidelined the valorization of hemicelluloses. This might be partly due to the inability of the common industrial strains such as yeast in metabolizing the pentose sugars of hemicelluloses. In the last decade, there has been an impressive range of studies which aim to develop industrial strains and processes that can valorize hemicelluloses. The list of organisms that able to metabolize hemicellulose hydrolysates and produce a variety of chemicals is growing with time. Indeed, with the emergence of systems metabolic engineering which integrates genetic engineering, systems biology, and synthetic biology disciplines, the repertoire of chemical that can be produced from hemicellulose hydrolysates will be astonishing. Thus, the future of alkaline active xylanases and mannanases is expected to be remarkable.

References

1. Bhat MK (2000) Cellulases and related enzymes in biotechnology. Biotechnol Adv 18:355–383
2. Burlacu A, Cornea CP, Israel-Roming F (2016) Microbial xylanase: a review. Sci Bull Ser F Biotechnol 20:335–342
3. Li X, Chang SH, Liu R (2018) Industrial applications of cellulases and hemicellulases. In: Fang X, Qu Y (eds) Fungal cellulolytic enzymes. Springer, Singapore
4. Mamo G, Faryar R, Nordberg Karlsson E (2013) Microbial glycoside hydrolases for biomass utilization in biofuels application. In: Gupta VK, Tuhoy MG (eds) Biofuel technologies: recent developments. Springer, Berlin, pp 171–188
5. Horváth IT (2018) Introduction: sustainable chemistry. Chem Rev 118:369–371
6. Kumar R, Singh S, Singh OV (2008) Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. J Ind Microbiol Biotechnol 35:377–391
7. Isikgor FH, Becer CR (2015) Lignocellulosic biomass: a sustainable platform for the production of bio-based chemicals and polymers. Polym Chem 6:4497–4559
8. Kohli K, Prajapati R, Sharma BK (2019) Bio-based chemicals from renewable biomass for integrated biorefineries. Energies 12:233
9. Roddy DJ (2013) Biomass in a petrochemical world. Interface Focus 3:20120038. https://doi.org/10.1098/rsfs.2012.0038
10. Ravella SR, Gallagher J, Fish S, Prakasham RS (2012) Overview on commercial production of xylitol, economic analysis and market trends. In: da Silva S, Chandel A (eds) D-xylitol. Springer, Berlin, pp 291–306
11. Robak K, Balcerek M (2018) Review of second generation bioethanol production from residual biomass. Food Technol Biotechnol 56:174–187
12. Collins T, Gerday C, Feller G (2005) Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol Rev 29:3–23
13. Srivastava P, Appu Rao AR, Kapoor M (2014) Structural insights into the thermal stability of endo-mannanase belonging to family 26 from *Bacillus* sp. CFR1601. FASEB J 28:580.2

14. Suurnäkki A, Tenkanen M, Buchert J, Viikari L (1997) Hemicellulases in the bleaching of chemical pulps. Adv Biochem Eng Biotechnol 57:261–287
15. Guo W, Sheng J, Zhao H, Feng X (2016) Metabolic engineering of Saccharomyces cerevisiae to produce 1-hexadecanol from xylose. Microb Cell Factories 15:24. https://doi.org/10.1186/s12934-016-0423-9
16. Kim SJ, Sim HJ, Kim JW, Lee YG, Park YC, Seo JH (2017) Enhanced production of 2,3-butanediol from xylose by combinatorial engineering of xylose metabolic pathway and cofactor regeneration in pyruvate decarboxylase-deficient *Saccharomyces cerevisiae*. Bioresour Technol 245(Pt B):1551–1557
17. Rodrussamee N, Sattayawat P, Yamada M (2018) Highly efficient conversion of xylose to ethanol without glucose repression by newly isolated thermotolerant *Spathaspora passalidarum* CMUWF1–2. BMC Microbiol 18:73
18. Stephen AM (1983) Other plant polysaccharides. In: Aspinall GO (ed) The polysaccharides, vol 2. Academic Press, London, pp 97–193
19. Beg QK, Kapoor M, Mahajan L, Hoondal GS (2001) Microbial xylanases and their industrial applications: a review. Appl Microbiol Biotechnol 56:326–338
20. Pauly M, Gille S, Liu L, Mansoori N, de Souza A, Schultink A, Xiong G (2013) Hemicellulose biosynthesis. Planta 238:627–642
21. Scheller HV, Ulvkov P (2010) Hemicelluloses. Annu Rev Plant Biol 61:263–289
22. Limayem A, Ricke SC (2012) Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects. Prog Energy Combust Sci 38:449–467
23. Kuhad RC, Singh A, Eriksson KEL (1997) Microorganisms and enzymes involved in the degradation of plant fiber cell walls. In: Eriksson K-EL (ed) Biotechnology in the pulp and paper industry. Springer, Berlin, pp 45–125
24. Reid JG (2000) Cementing the wall: cell wall polysaccharide synthesising enzymes. Curr Opin Plant Biol 3:512–516
25. Sun RC, Lawther JM, Banks WB (1995) Influence of alkaline pre-treatment on the cell wall components of wheat straw. Indust Crops Prod 4:127–145
26. Prade RA (1996) Xylanases: from biology to biotechnology. Biotechnol Genet Eng Rev 13:101–131
27. Timel TE (1967) Recent progress in the chemistry of wood hemicelluloses. Wood Sci Technol 1:45–70
28. Previato LM, Gorin PAJ, Previato JO (1979) Investigations on polysaccharide components of cells of *Herpetomonas samuelpessoai* grown on various media. Biochemistry 18:149–154
29. Jones C, Wait R, Previato JO, Mendonça-Previato L (2000) The structure of a complex glycosylphosphatidyl inositol-anchored glucoxylan from the kinetoplastid protozoan *Leptomonas samueli*. Eur J Biochem 267:5387–5396
30. Rosner H, Grimnecke HD, Knirel YA, Shaskov AS (1992) Hyaluronic acid and a (1,4)-beta-D-xylan, extracellular polysaccharides of *Pasteurella multocida* (Carter type A) strain 880. Carbohydr Res 223:329–333
31. Ebringerova A, Heinze T (2000) Naturally occurring xylans structures, isolation procedures and properties. Macromol Rapid Commun 21:542–556
32. Yamagaki T, Maeda M, Kanazawa K, Ishizuka Y, Nakanishi H (1996) Structures of *Caulerpa* cell wall microfibril xylan with detection of β-l,3-xylooligosac-charides as revealed by matrix-assisted laser desorption ionization/time of flight/mass spectrometry. Biosci Biotechnol Biochem 60:1222–1228
33. Painter TJ (1983) Algal polysaccharides. In: Aspinall GO (ed) The polysaccharides. Academic, San Diego, pp 196–285
34. Samuelson AB, Lund I, Djahromi JM, Paulsen BS, World JK, Knutsen AH (1999) Structural features and anti-complementary activity of some heteroxylan polysaccharide fractions from the seeds of *Plantago major* L. Carbohydr Polym 38:133–143
35. Kulkarni N, Shendye A, Rao M (1999) Molecular and biotechnological aspects of xylanases. FEMS Microbiol Rev 23:411–456

36. Sunna A, Antranikian G (1997) Xylanolytic enzymes from fungi and bacteria. Crit Rev Biotechnol 17:39–67
37. Moreira L (2008) An overview of mannan structure and mannan-degrading enzyme systems. Appl Microbiol Biotechnol 79:165–178
38. Blibech M, Maktouf S, Chaari F, Zouari S, Neifar M, Besbes S, Ellouze-Ghorbel R (2013) Functionality of galactomannan extracted from Tunisian carob seed in bread dough. J Food Sci Technol 52:1–7
39. McCleary BV, Allan HC, Dea ICM, Rees DA (1985) The fine structures of carob and guar galactomannans. Carbohydr Res 139:237–260
40. Puls J, Schuseil J (1993) Chemistry of hemicelluloses: relation between hemicellulose structure and enzyme required for hydrolysis. In: Coughlan MP, Hazlewood GP (eds) Hemicellulose and hemicellulases. Portland Press, London and Chapel Hill, pp 1–27
41. Rose JK (2003) The plant cell wall. CRC Press, Boca Raton
42. Gírio F, Fonseca C, Carvalheiro F, Duarte L, Marques S, Lukasik R (2010) Hemicelluloses for fuel ethanol: a review. Bioresour Technol 101:4775–4800
43. Willkie KCB (1979) The hemicelluloses of grasses and cereals. Adv Carbohydr Chem Biochem 36:215–264
44. Gorin PAJ, Barreto-Bergter E (1983) The chemistry of polysaccharides of fungi and lichens. In: Aspinall GO (ed) The polysaccharides, vol 2. Academic Press, London, pp 365–409
45. Lee JH, Lee IY (2001) Optimization of uracil addition for curdlan (beta-1,3-glucan) production by *Agrobacterium* sp. Biotechnol Lett 23:1131–1134
46. Phillips KR, Lawford HG (1983) Theoretical maximum and observed product yields associated with curdlan production by *Alcaligenes faecalis*. Can J Microbiol 29:1270–1276
47. Moure A, Gullón P, Domínguez H, Parajó JC (2006) Advances in the manufacture, purification and applications of xylo-oligosaccharides as food additives and nutraceuticals. Process Biochem 41:1913–1923
48. Singh S, Ghosh A, Goyal A (2017) Manno-oligosaccharides as prebiotic-valued products from agro-waste. In: Varjani S, Parameswaran B, Kumar S, Khare S (eds) Biosynthetic technology and environmental challenges. Energy, environment, and sustainability. Springer, Singapore
49. Ando H, Ohba H, Sakaki T, Takamine K, Kamino Y, Moriwaki S et al (2004) Hot-compressed-water decomposed products from bamboo manifest a selective cytotoxicity against acute lymphoblastic leukemia cells. Toxicol In Vitro 18:765–771
50. Nawaz A, Bakhsh Javaid A, Irshad S, Hoseinifar SH, Xiong H (2018) The functionality of prebiotics as immunostimulant: evidences from trials on terrestrial and aquatic animals. Fish Shellfish Immunol 76:272–278
51. Yuan X, Wang J, Yao H (2005) Antioxidant activity of feruloylated oligosaccharides from wheat bran. Food Chem 90:759–764
52. Christakopoulos P, Katapodis P, Kalogeris E, Kekos D, Macris BJ, Stamatis H, Skaltsa H (2003) Antimicrobial activity of acidic xylo-oligosaccharides produced by family 10 and 11 endoxylanases. Int J Biol Macromol 31:171–175
53. Gupta A, Das SP, Ghosh A, Choudhary R, Das D, Goyal A (2014) Bioethanol production from hemicellulose rich *Populus nigra* involving recombinant hemicellulases from *Clostridium thermocellum*. Bioresour Technol 165:205–213
54. He YC, Jiang CX, Jiang JW, Di JH, Liu F, Ding Y, Qing Q, Ma CL (2017) One-pot chemoenzymatic synthesis of furfuralcohol from xylose. Bioresour Technol 238:698–705
55. Wischral D, Arias JM, Modesto LF, de França Passos D, Pereira Jr N (2019) Lactic acid production from sugarcane bagasse hydrolysates by Lactobacillus pentosus: integrating xylose and glucose fermentation. Biotechnol Prog 35(1):e2718. https://doi.org/10.1002/btpr.2718
56. Viikari L, Suurnäkki A, Grönqvist S, Raaska L, Ragauskas A (2009) Forest products: biotechnology in pulp and paper processing. In: Schaechter M (ed) Encyclopedia of microbiology. Academic Press, New York, pp 80–94
57. Poutanen K, Sundberg M (1988) An acetyl esterase of *Trichoderma reesei* and its role in the hydrolysis of acetyl xylans. Appl Microbiol Biotechnol 28:419–424

58. Marques G, Gutiérrez A, Jdel Río JC, Evtuguin DV (2010) Acetylated heteroxylan from *Agave sisalana* and its behavior in alkaline pulping and TCF/ECF bleaching. Carbohydr Polym 18:517–523
59. Reinoso FAM, Rencoret J, Gutiérrez A, Milagres AMF, del Río JC, Ferraz A (2018) Fate of *p*-hydroxycinnamates and structural characteristics of residual hemicelluloses and lignin during alkaline-sulfite chemithermomechanical pretreatment of sugarcane bagasse. Biotechnol Biofuels 11:153
60. Sporck D, Reinoso FAM, Rencoret J, Gutiérrez A, del Rio JC, Ferraz A, Milagres AMF (2017) Xylan extraction from pretreated sugarcane bagasse using alkaline and enzymatic approaches. Biotechnol Biofuels 10:296
61. Lyczakowski JJ, Wicher KB, Terrett OM, Faria-Blanc N, Yu X, Brown D et al (2017) Removal of glucuronic acid from xylan is a strategy to improve the conversion of plant biomass to sugars for bioenergy. Biotechnol Biofuels 10(224):2017
62. Honda Y, Kitaoka M (2004) A family 8 glycoside hydrolase from *Bacillus halodurans* C-125 (BH2105) is a reducing end xylose-releasing exo-oligoxylanase. J Biol Chem 279:55097–55103
63. Regmi S, Yoo HY, Choi YH, Choi YS, Yoo JC, Kim SW (2017) Prospects for bio-industrial application of an extremely alkaline mannanase from *Bacillus subtilis* subsp. *inaquosorum* CSB31. Biotechnol J 12(11). https://doi.org/10.1002/biot.201700113
64. Yoo HY, Pradeep GC, Lee SK, Park DH, Cho SS, Choi YH et al (2015) Understanding β-mannanase from *Streptomyces* sp. CS147 and its potential application in lignocellulose based biorefining. Biotechnol J 10:1894–1902
65. Yadav P, Maharjan J, Korpole S, Prasad GS, Sahni G, Bhattarai T, Sreerama L (2018) Production, purification, and characterization of thermostable alkaline xylanase from *Anoxybacillus kamchatkensis* NASTPD13. Front Bioeng Biotechnol 6:65
66. Taibi Z, Saoudi B, Boudelaa M, Trigui H, Belghith H, Gargouri A, Ladjama A (2012) Purification and biochemical characterization of a highly thermostable xylanase from *Actinomadura* sp. strain Cpt20 isolated from poultry compost. Appl Biochem Biotechnol 166:663–679
67. Horikoshi K, Atsukawa Y (1973) Xylanase produced by alkalophilic *Bacillus* no C-59-2. Agric Biol Chem 37:2097–2103
68. Aizawa T, Urai M, Iwabuchi N, Nakajima M, Sunairi M (2010) *Bacillus trypoxylicola* sp. nov., xylanase-producing alkaliphilic bacteria isolated from the guts of Japanese horned beetle larvae (*Trypoxylus dichotomus septentrionalis*). Int J Syst Evol Microbiol 60:61–66
69. Mamo G, Hatti-Kaul R, Mattiasson B (2006) A thermostable alkaline active endo-β-1-4-xylanase from bacillus halodurans S7: purification and characterization. Enzym Microb Technol 39:1492–1498
70. Raj A, Kumar S, Singh SK, Prakash J (2018) Production and purification of xylanase from alkaliphilic *Bacillus licheniformis* and its pretreatment of eucalyptus Kraft pulp. Biocatal Agric Biotechnol 15:199–209
71. Gessesse G, Mamo G (1998) Purification and characterization of an alkaline xylanase from alkaliphilic *Micrococcus* sp. AR-135. J Ind Microbiol Biotechnol 20:210–214
72. Simkhada JR, Yoo HY, Choi YH, Kim SW, Yoo JC (2012) An extremely alkaline novel xylanase from a newly isolated *Streptomyces* strain cultivated in corncob medium. Appl Biochem Biotechnol 168:2017–2027
73. Zhao B, Chen S (2012) *Alkalitalea saponilacus* gen. nov., sp. nov., an obligately anaerobic, alkaliphilic, xylanolytic bacterium from a meromictic soda lake. Int J Syst Evol Microbiol 62:2618–2623
74. Khandeparkar RDS, Bhosle NB (2006) Isolation, purification and characterization of the xylanase produced by *Arthrobacter* sp. MTCC 5214 when grown in solid-state fermentation. Enzym Microb Technol 39:732–742

75. Gupta S, Bhushan B, Hoondal GS (2000) Isolation, purification and characterization of xylanasefrom *Staphylococcus* sp. SG-13 and its application in biobleaching of Kraft pulp. J Appl Microbiol 88:325–334
76. Khandeparkar R, Bhosle N (2006) Purification and characterization of thermoalkalophilic xylanase isolated from the *Enterobacter* sp. MTCC 5112. Res Microbiol 157:315–325
77. Taneja K, Gupta S, Kuhad RC (2002) Properties and application of a partially purified alkaline xylanase from an alkalophilic fungus *Aspergillus nidulans* KK-99. Bioresour Technol 85:39–42
78. Dutta T, Sengupta R, Sahoo R, Ray SS, Bhattacharjee A, Ghosh S (2007) A novel cellulase free alkaliphilic xylanase from alkali tolerant *Penicillium citrinum*: production, purification and characterization. Lett Appl Microbiol 44:206–211
79. Ohkuma M, Shimizu H, Thongaram T, Kosono S, Moryia K, Trakulnaleamsai S et al (2003) An alkaliphilic and xylanolytic *Paenibacillus* species isolated from the gut of a soil-feeding termite. Microbes Environ 18:145–151
80. Verma D, Kawarabayasi Y, Miyazaki K, Satyanarayana T (2013) Cloning, expression and characteristics of a novel alkalistable and thermostable xylanase encoding gene (Mxyl) retrieved from compost-soil metagenome. PLoS One 8:e52459
81. Wang G, Huang X, Ng TB, Lin J, Ye XY (2014) High phylogenetic diversity of glycosyl hydrolase family 10 and 11 xylanases in the sediment of lake Dabusu in China. PLoS One 9: e112798
82. Wang G, Wu J, Yan R, Lin J, Ye X (2017) A novel multi-domain high molecular, salt-stable alkaline xylanase from *Alkalibacterium* sp. SL3. Front Microbiol 7:2120
83. Nakamura S, Nakai R, Wakabayashi K, Ishiguro Y, Aono R, Horikoshi K (1994) Thermophilic alkaline xylanase from newly isolated alkaliphilic and thermophilic *Bacillus* sp. strain TAR1. Biosci Biotechnol Biochem 58:78–81
84. Nakamura S, Wakabayashi K, Nakai R, Aono R, Horikoshi K (1993) Purification and some properties of an alkaline xylanase from alkaliphilic *Bacillus* sp. strain 41M-1. Appl Environ Microbiol 59:2311–2316
85. Mongkorntanyatip K, Limsakul P, Ratanakhanokchai K, Khunrae P (2017) Overexpression and characterization of alkaliphilic *Bacillus firmus* strain K-1 xylanase. Agric Nat Resour 51:437–444
86. Prakash P, Jayalakshmi SK, Prakash B, Rubul M, Sreeramulu K (2012) Production of alkaliphilic, halotolerant, thermostable cellulase free xylanase by *Bacillus halodurans* PPKS-2 using agro waste: single step purification and characterization. World J Microbiol Biotechnol 28:183–192
87. Gessesse A (1998) Purification and properties of two thermostable alkaline xylanases from an alkaliphilic *Bacillus* sp. Appl Environ Microbiol 64:533–3535
88. Chang P, Tsai WS, Tsa CL, Tseng MJ (2004) Cloning and characterization of two thermostable xylanases from an alkaliphilic *Bacillus firmus*. Biochem Biophys Res Commun 319:1017–1025
89. Duarte MC, Pellegrino AC, Portugal EP, Ponezi AN, Franco TT (2000) Characterization of alkaline xylanases from *Bacillus pumilus*. Braz J Microbiol 31:90–94
90. Sharma A, Adhikari S, Satyanarayana T (2006) Alkali-thermostable and cellulase-free xylanase production by an extreme thermophile *Geobacillus thermoleovorans*. World J Microbiol Biotechnol 23:483–490
91. Chanjuan L, Hong Y, Shao Z, Lin L, Huang X, Liu P et al (2009) Novel alkali-stable, cellulase-free xylanase from deep-sea *Kocuria* sp. Mn22. J Microbiol Biotechnol 19:873–880
92. Kuramochi K, Uchimura K, Kurata A, Kobayashi T, Hirose Y, Miura T et al (2016) A high-molecular-weight, alkaline, and thermostable beta-1,4-xylanase of a subseafloor *Microcella alkaliphila*. Extremophiles 20:471–478
93. Valenzuela SV, Díaz P, Javier Pastor FI (2010) Recombinant expression of an alkali stable GH10 xylanase from *Paenibacillus barcinonensis*. J Agric Food Chem 58:4814–4818

94. Sharma M, Mehta S, Kumar A (2013) Purification and characterization of alkaline xylanase secreted from *Paenibacillus macquariensis*. Adv Microbiol 3:32–41
95. Beg QK, Bhushan B, Kapoor M, Hoondal GS (2000) Production and characterization of thermostable xylanase and pectinase from *Streptomyces* sp. QG-11-3. J Ind Microbiol Biotechnol 24:396–402
96. Raj A, Kumar S, Singh SK (2013) A highly thermostable xylanase from *Stenotrophomonas maltophilia*: purification and partial characterization. Enzyme Res 2013:429305. https://doi.org/10.1155/2013/429305
97. Zhang F, Chen JJ, Ren WZ, Lin LB, Zhou Y, Zhi XY et al (2012) Cloning, expression, and characterization of an alkaline thermostable GH11 xylanase from Thermobifida halotolerans YIM 90462T. J Ind Microbiol Biotechnol 39:1109–1116
98. Honda H, Kudo T, Ikura Y, Horikoshi K (1985) Two types of xylanases of alkalophilic *Bacillus* sp. No. C-125. Can J Microbiol 31:538–542
99. Herlet J, Kornberger P, Roessler B, Glanz J, Schwarz WH, Liebl W, Zverlov VV (2017) A new method to evaluate temperature vs. pH activity profiles for biotechnological relevant enzymes. Biotechnol Biofuels 10:234
100. Takeda N, Hirasawa K, Uchimura K, Nogi Y, Hatada Y, Usami R et al (2004) Purification and enzymatic properties of a highly alkaline mannanase from alkaliphilic *Bacillus* sp. strain JAMB-750. J Biol Macromol 4:67–74
101. Takeda N, Hirasawa K, Uchimura K, Nogi Y, Hatada Y, Akita M et al (2004) Alkaline mannanase from a novel species of alkaliphilic *Bacillus*. J Appl Glycosci 51:229–236
102. Vijayalaxmi S, Prakash P, Jayalakshmi SK, Mulimani VH, Sreeramulu K (2013) Production of extremely alkaliphilic, halotolerant, detergent, and thermostable mannanase by the free and immobilized cells of *Bacillus halodurans* PPKS-2. Purification and characterization. Appl Biochem Biotechnol 171:382–395
103. Zhou C, Xue Y, Ma Y (2018) Characterization and high-efficiency secreted expression in *Bacillus subtilis* of a thermo-alkaline β-mannanase from an alkaliphilic *Bacillus clausii* strain S10. Microb Cell Factories 17:124
104. Ma Y, Xue Y, Dou Y, Xu Z, Tao W, Zhou P (2004) Characterization and gene cloning of a novel β-mannanase from alkaliphilic *Bacillus* sp. N16-5. Extremophiles 8:447–454
105. Chauhan PS, Sharma P, Puri N, Gupta N (2014) Purification and characterization of an alkali-thermostable β-mannanase from *Bacillus nealsonii* PN-11 and its application in mannooligosaccharides preparation having prebiotic potential. Eur Food Res Technol 238:927–936
106. Pradeep GC, Cho SS, Choi YH, Choi YS, Jee JP, Seong CN et al (2016) An extremely alkaline mannanase from *Streptomyces* sp. CS428 hydrolyzes galactomannan producing series of mannooligosaccharides. World J Microbiol Biotechnol 32:84. https://doi.org/10.1007/s11274-016-2040-5
107. Akino T, Nakamura N, Horikoshi K (1988) Characterization of three β-mannanases of an alkalophilic *Bacillus* sp. Agric Biol Chem 52:773–779
108. El-Sharouny EE, El-Toukhy NM, El-Sersy NA, El-Gayar AA (2015) Optimization and purification of mannanase produced by an alkaliphilic-thermotolerant *Bacillus cereus* N1 isolated from Bani Salama Lake in Wadi El-Natron. Biotechnol Biotechnol Equip 29:315–323
109. Yang P, Li Y, Wang Y, Meng K, Luo H, Yuan T et al (2009) A novel beta-mannanase with high specific activity from *Bacillus circulans* CGMCC1554: gene cloning, expression and enzymatic characterization. Appl Biochem Biotechnol 159:85–94
110. Li Y, Yang P, Meng K, Wang Y, Luo H, Wu N et al (2008) Gene cloning, expression, and characterization of a novel beta-mannanase from *Bacillus circulans* CGMCC 1416. J Microbiol Biotechnol 18:160–166
111. Kumar D, Angural S, Rana M, Kaur G, Puri N, Gupta N (2017) Cloning, characterization and its potential in pulp bio bleaching by alkali thermostable β-mannanase from *Bacillus* sp. 22. Eur J Pharm Med Res 4:584–592

112. Virupakshi S, Babu G, Naik GR (2005) Partial purification and characterization of thermostable alkaline β-mannanase from *Bacillus* sp. JB-99 suitable for the pulp bleaching. J Microbiol Biotechnol 15:689–693
113. Chauhan PS, Puri N, Sharma P, Gupta N (2012) Mannanases: microbial sources, production, properties and potential biotechnological applications. Appl Microbiol Biotechnol 93:1817–1830
114. Dhawan S, Kaur J (2007) Microbial mannanases: an overview of production and applications. Crit Rev Biotechnol 27:197–216
115. Kansoh AL, Nagieb ZA (2004) Xylanase and mannanase enzymes from *Streptomyces galbus* NR and their use in biobleaching of softwood Kraft pulp. Antonie Van Leeuwenhoek 85:103–114
116. Bettiol JP, Showell MS (2002) Detergent compositions comprising a mannanase and a protease. US Patent No. 6,376,445
117. Akino T, Nakamura N, Horikoshi K (1987) Production of β-mannosidase and β-mannanase by an alkalophilic *Bacillus* sp. Appl Microbiol Biotechnol 26:323–327
118. Akita M, Takeda N, Hirasawa K, Sakai H, Kawamoto M, Yamamoto M et al (2004) Crystallization and preliminary X-ray study of alkaline mannanase from an alkaliphilic *Bacillus* isolate. Acta Crystallogr D Biol Crystallogr 60:1490–1492
119. Hatada Y, Takeda N, Hirasawa K, Ohta Y, Usami R et al (2005) Sequence of the gene for a high-alkaline mannanase from an alkaliphilic *Bacillus* sp. strain JAMB-750, its expression in *Bacillus subtilis* and characterization of the recombinant enzyme. Extremophiles 9:497–500
120. He X, Liu N, Li W, Zhang Z, Zhang B, Ma Y (2008) Inducible and constitutive expression of a novel thermostable alkaline β-mannanase from alkaliphilic Bacillus sp. N16-5 in Pichia pastoris and characterization of the recombinant enzyme. Enzyme Microb Technol 43:13–18
121. Luo Z, Miao J, Li G, Du Y, Yu X (2017) A recombinant highly thermostable β-mannanase (ReTMan26) from thermophilic Bacillus subtilis (TBS2) expressed in Pichia pastoris and its pH and temperature stability. Appl Biochem Biotechnol 182:1259–1275
122. Elegir G, Szakacs G, Jeffries TW (1994) Purification, characterization and substrate specificities of multiple xylanases from *Streptomyces* sp. strain B-12-2. Appl Environ Microbiol 60:2609–2615
123. Biely P, Markovik O, Mislovicova D (1985) Sensitive detection of endo-1,4,-β-glucanases and endo-1,4-β-xylanases in gels. Anal Biochem 144:147–151
124. Dobozi MS, Szakacs G, Bruschi CV (1992) Xylanase activity of *Phanerochaete chrysosporium*. Appl Environ Microbiol 58:3466–3471
125. Lee YS, Ratanakhanokchai K, Piyatheerawong W, Kyu KL, Rho M, Kim YS et al (2006) Production and location of xylanolytic enzymes in alkaliphilic *Bacillus* sp. K-1. J Microbiol Biotechnol 16:921–926
126. Gübitz GM, Hayn M, Sommerauer M, Steiner W (1996) Mannan-degrading enzymes from *Sclerotium rolfsii*: characterisation and synergism of two endo β-mannanases and a β-mannosidase. Bioresour Technol 58:127–135
127. Shrinivas D, Savitha G, Raviranjan K, Naik GR (2010) A highly thermostable alkaline cellulase-free xylanase from thermoalkalophilic *Bacillus* sp. JB 99 suitable for paper and pulp industry: purification and characterization. Appl Biochem Biotechnol 162:2049–2057
128. Li N, Shi P, Yang P, Wang Y, Luo H, Bai Y et al (2009) A xylanase with high pH stability from *Streptomyces* sp. S27 and its carbohydrate-binding module with/without linker-region-truncated versions. Appl Microbiol Biotechnol 83:99–107
129. Maharjan A, Alkotaini B, Kim BS (2018) Fusion of carbohydrate binding modules to bifunctional cellulase to enhance binding affinity and cellulolytic activity. Biotechnol Bioprocess Eng 23:79–85
130. Mamo G, Hatti-Kaul R, Mattiasson B (2007) Fusion of carbohydrate binding modules from *Thermotoga neapolitana* with a family 10 xylanase from *Bacillus halodurans* S7. Extremophiles 11:169–177

131. Bai W, Xue Y, Zhou C, Ma Y (2015) Cloning, expression, and characterization of a novel alkali-tolerant xylanase from alkaliphilic *Bacillus* sp. SN5. Biotechnol Appl Biochem 62:208–217
132. Henrissat B, Bairoch A (1996) Updating the sequence-based classification of glycosyl hydrolases. Biochem J 316:695–696
133. Larson SB, Day J, Barba de la Rosa AP, Keen NT, McPherson A (2003) First crystallographic structure of a xylanase from glycoside hydrolase family 5: implications for catalysis. Biochemistry 42:8411–8422
134. Van Petegem F, Collins T, Meuwis MA, Gerday C, Feller G, Van Beeumen J (2003) The structure of a cold-adapted family 8 xylanase at 1.3 A resolution. Structural adaptations to cold and investigation of the active site. J Biol Chem 278:7531–7539
135. Mamo G, Thunnissen M, Hatti-Kaul R, Mattiasson B (2009) An alkaline active xylanase: insights into mechanisms of high pH catalytic adaptation. Biochimie 91:1187–1196
136. St John FJ, Hurlbert JC, Rice JD, Preston JF, Pozharski E (2011) Ligand bound structures of a glycosyl hydrolase family 30 glucuronoxylan xylanohydrolase. J Mol Biol 407:92–109
137. Alhassid A, Ben-David A, Tabachnikov O, Libster D, Naveh E, Zolotnitsky G et al (2009) Crystal structure of an inverting GH 43 1,5-alpha-L-arabinanase from *Geobacillus stearothermophilus* complexed with its substrate. Biochem J 422:73–82
138. Im DH, Kimura KI, Hayasaka F, Tanaka T, Noguchi M, Kobayashi A et al (2012) Crystal structures of glycoside hydrolase family 51 alpha-L-arabinofuranosidase from *Thermotoga maritima*. Biosci Biotechnol Biochem 76:423–428
139. Higgins MA, Whitworth GE, El Warry N, Randriantsoa M, Samain E, Burke RD et al (2009) Differential recognition and hydrolysis of host carbohydrate-antigens by *Streptococcus Pneumoniae* family 98 glycoside hydrolases. J Biol Chem 284:26161–26171
140. Zhao Y, Zhang Y, Cao Y, Qi J, Mao L, Xue Y et al (2011) Structural analysis of alkaline β-mannanase from alkaliphilic *Bacillus* sp. N16-5: implications for adaptation to alkaline conditions. PLoS One 6(1):e14608
141. You X, Qin Z, Yan Q, Yang SQ, Li Y, Jiang ZQ (2018) Complex structure of GH113 beta-1,4-mannanase. J Biol Chem 293:11746–11757
142. Jin Y, Petricevic M, John A, Raich L, Jenkins H, Portela De Souza L et al (2016) A beta-mannanase with a lysozyme-like fold and a novel molecular catalytic mechanism. ACS Cent Sci 2:896–903
143. Farber GK, Petsko GA (1990) The evolution of α/β barrel enzymes. Trends Biochem Sci 15:228–234
144. Manikandan K, Bhardwaj A, Gupta N, Lokanath NK, Ghosh A, Reddy VS et al (2006) Crystal structures of native and xylosaccharide-bound alkali thermostable xylanase from an alkalophilic *Bacillus* sp. NG-27: structural insights into alkalophilicity and implications for adaptation to polyextreme conditions. Protein Sci 15:1951–1960
145. Bai W, Zhou C, Xue Y, Huang CH, Guo RT, Ma Y (2014) Three-dimensional structure of an alkaline xylanase Xyn11A-LC from alkalophilic *Bacillus* sp. SN5 and improvement of its thermal performance by introducing arginines substitutions. Biotechnol Lett 36:1495–1501
146. Davies G, Henrissat B (1995) Structures and mechanisms of glycosyl hydrolases. Structure 3:853–859
147. Biely P, Vrsanska M, Tenkanen M, Kluepfel D (1999) Endo-β-1,4-xylanase families: differences in catalytic properties. J Biotechnol 57:151–166
148. Leggio LL, Jenkins J, Harris GW, Pickersgill RW (2000) X-ray crystallographic study of xylopentaose binding to *Pseudomonas fluorescens* xylanase A. Proteins Struct Funct Genet 41:362–373
149. Shimizu M, Kaneko Y, Ishihara S, Mochizuki M, Sakai K, Yamada M et al (2015) Novel β-1,4-mannanase belonging to a new glycoside hydrolase family in *Aspergillus nidulans*. J Biol Chem 290:27914–27927
150. Li S, Yang X, Yang S, Zhu M, Wang X (2012) Technology prospecting on enzymes: application, marketing and engineering. Comput Struct Biotechnol J 2:e201209017

151. Sarethy IP, Saxena Y, Kapoor A, Sharma M, Sharma SK, Gupta V, Gupta S (2011) Alkaliphilic bacteria: applications in industrial biotechnology. J Ind Microbiol Biotechnol 38:769–790
152. Sharma K, Thakur A, Goyal A (2019) Xylanases for food applications: enzymes in industrial food processing. In: Parameswaran B, Varjani S, Raveendran S (eds) Green bio-processes: enzymes in industrial food processing. Springer, Singapore, pp 99–116
153. Pokhrel D, Viraraghavan T (2004) Treatment of pulp and paper mill wastewater – a review. Sci Total Environ 333:37–58
154. Dhiman SS, Sharma J, Battan B (2008) Industrial applications and future prospects of microbial xylanases: a review. Bioresources 3:1377–1402
155. Sindhu I, Chhibber S, Caplash N, Sharma P (2006) Production of cellulase-free xylanase from *Bacillus megaterium* by solid state fermentation for biobleaching of pulp. Curr Microbiol 53:167–172
156. Polizeli MLTM, Rizzatti ACS, Monti R, Terezni HF, Jorge JA, Amorim DS (2005) Xylanases from fungi: properties and industrial applications. Appl Microbiol Biotechnol 67:577–591
157. Valls C, Vidal T, Roncero MB (2010) Boosting the effect of a laccase-mediator system by using a xylanase stage in pulp bleaching. J Hazard Mater 177:586–592
158. Spence K, Tucker J, Hart P (2009) Comparison of various hardwood Kraft pulp pre-bleaching techniques. TAPPI J 8:10–14
159. Sing G, Capalash N, Kaur K, Puri S, Sharma P (2016) Enzyme applications in pulp and paper industry. In: Dhillon SG, Kaur S (eds) Agro-industrial wastes as feedstock for enzyme production: apply and exploit the emerging and valuable use options of waste biomass. Academic Press, London, pp 157–172
160. Jiang ZH, Van Lierop B, Berry R (2000) Hexenuronic acid groups in pulping and bleaching chemistry. TAPPI J 83:167–175
161. Buchert J, Bergnor E, Lindblad G, Viikari L, Ek M (1997) Significance of xylan and glucomannan in the brightness reversion of Kraft pulps. TAPPI J 80:165–171
162. Subramaniyan S, Prema P (2002) Biotechnology of microbial xylanases: enzymology, molecular biology, and application. Crit Rev Biotechnol 22:33–64
163. Suurnäkki A, Heijnesson A, Buchert J, Westermark U, Viikari L (1996) Effect of pulp surfaces on enzyme-aided bleaching of Kraft pulps. J Pulp Pap Sci 22:J91–J96
164. Wedin H, Antonsson S, Ragnar M, Lindström M (2012) Influence of xylan content on the oxygen delignification performance of eucalypt Kraft pulps as studied using prehydrolysis and xylanase treatments. Bioresources 7:5527–5541
165. Tolan JS, Olson D, Diners RE (1996) Survey of mill usage of xylanase. In: Jeffries TW, Viikari L (eds) Enzymes for pulp and paper processing. ACS symposium series 655. American Chemical Society, Washington, pp 23–35
166. Georis J, Giannotta F, Buyl ED, Granier B, Frère JM (2000) Purification and properties of three endo-beta-1,4-xylanases produced by *Streptomyces* sp. strain S38 which diver in their ability to enhance the bleaching of Kraft pulps. Enzym Microb Technol 26:178–186
167. Bhoria P, Singh G, Sharma JR, Hoodal GS (2009) Biobleaching of wheat straw-rich-soda pulp by the application of alkalophilic and thermophilic mannanase from *Streptomyces* sp. PG-08-3. Afr J Biotechnol 11:6111–6116
168. Montiel MD, Rodríguez J, Pérez-Leblic MI, Hernández M, Arias ME, Copa-Patiño JL (1999) Screening of mannanase in actinomycetes and their potential application in the biobleaching of pine Kraft pulps. Appl Microbiol Biotechnol 52:240–245
169. Baeck AC, Busch A, Alfons, IM, Herbots J, Moese RL (1998) Detergent compositions comprising xylan degrading alkaline enzyme and dye transfer inhibiting polymers. European Patent Office. EP0964910A1
170. Kumar BK, Balakrishnan H, Rele MV (2004) Compatibility of alkaline xylanases from an alkaliphilic *Bacillus* NCL (87-6-10) with commercial detergents and proteases. J Ind Microbiol Biotechnol 31:83–87

171. Bettiol JLP, Cooremans SPG, Johnstone R, Sreekrishna K, Saunders CW, Maurice I et al (2002) Laundry detergent compositions comprising a saccharide gum degrading enzyme. United States patent US 6,486,112 B1
172. Herbots IMAJ, Moese RL, Baeck AC, Busch A (1998) Cleaning compositions comprising xylan degrading alkaline enzyme and bleaching agent. Patent WO 983943A1
173. Shahid M, Mohammad F, Chen G, Tang RC, Xing T (2016) Enzymatic processing of natural fibres: white biotechnology for sustainable development. Green Chem 18:2256–2281
174. Kalantzi S, Kekos D, Mamma D (2019) Bioscouring of cotton fabrics by multienzyme combinations: application of Box–Behnken design and desirability function. Cellulose 26:2771–2790
175. Singh A, Kaur A, Patra AK, Mahajan R (2018) A sustainable and green process for scouring of cotton fabrics using xylano-pectinolytic synergism: switching from noxious chemicals to eco-friendly catalysts. 3 Biotech 8(4):184
176. Wang Q, Fan X, Hua Z, Gao W, Chen J (2007) Influence of combined enzymatic treatment on one-bath scouring of cotton knitted fabrics. Biocatal Biotransform 25:9–15
177. Dhiman SS, Sharma J, Battan B (2008) Pretreatment of fabrics by alkalothermophilic xylanase from *Bacillus stearothermophilus* SDX. Enzym Microb Technol 43:262–269
178. Brühlmann F, Leupin M, Erismann KH, Fiechter A (2000) Enzymatic degumming of ramie bast fibers. J Biotechnol 76:43–50
179. Wang Y, Shu T, Fan P, Zhang H, Turunen O, Xiong H, Yu L (2017) Characterization of a recombinant alkaline thermostable β-mannanase and its application in eco-friendly ramie degumming. Process Biochem 61:73–79
180. Samanta AK, Jayapal N, Kolte AP, Senani S, Sridhar M, Suresh KP et al (2012) Enzymatic production of xylooligosaccharides from alkali solubilized xylan of natural grass (*Sehima nervosum*). Bioresour Technol 112:199–205
181. Faryar R, Linares-Pastén J, Immerzeel P, Mamo G, Andersson M, Stålbrand H et al (2014) Production of prebiotic xylooligosaccharides from alkaline extracted wheat straw using the K80R-variant of a thermostable alkali-tolerant xylanase. Food Bioprod Process 93:1–10
182. Haddar A, Driss D, Frikha F, Ellouz SC, Nasri M (2012) Alkaline xylanases from *Bacillus mojavensis* A21: production and generation of xylooligosaccharides. Int J Biol Macromol 51:647–656
183. Shen R, Li HQ, Zhang J, Xu J (2016) Effects of impurities in alkali-extracted xylan on its enzymatic hydrolysis to produce xylo-oligosaccharides. Appl Biochem Biotechnol 179:740–752
184. Zhu Y, Li X, Sun B, Song H, Li E, Song H (2012) Properties of an alkaline-tolerant, thermostable xylanase from *Streptomyces chartreusis* L1105, suitable for xylooligosaccharide production. J Food Sci 77:C506–C511
185. Cassou E (2018) Field burning: agricultural pollution. World Bank, Washington. https://openknowledge.worldbank.org/handle/10986/29504. License: CC BY 3.0 IGO
186. Singla A, Paroda S, Dhamija SS, Goyal S, Shekhawat K, Amachi S, Inubushi K (2012) Bioethanol production from xylose: problems and possibilities. J Biofuels 3:39–49
187. Zhao J, Xia LM (2010) Ethanol production from corn stover hemicellulosic hydrolysate using immobilized recombinant yeast cells. Biochem Eng J 49:28–32
188. deAlbuquerque TL, SilvaJr IJ, Macedo GR, PonteRocha MV (2014) Biotechnological production of xylitol from lignocellulosic wastes: a review. Process Biochem 49:1779–1789
189. Li H, Zhang G, Dang Y (2016) Adaptive laboratory evolution of *Klebsiella pneumoniae* for improving 2,3-butanediol production. Bioengineered 7:432–438
190. Kildegaard KR, Wang Z, Chen Y, Nielsen J, Borodina I (2015) Production of 3-hydroxypropionic acid from glucose and xylose by metabolically engineered *Saccharomyces cerevisiae*. Metab Eng Commun 2:132–136
191. Bradfield MF, Nicol W (2016) Continuous succinic acid production from xylose by *Actinobacillus succinogenes*. Bioprocess Biosyst Eng 39:233–244

192. Ye L, Zhou X, Hudari MS, Li Z, Wu JC (2013) Highly efficient production of L-lactic acid from xylose by newly isolated *Bacillus coagulans* C106. Bioresour Technol 132:38–44
193. Gopinath V, Meiswinkel TM, Wendisch VF, Nampoothiri KM (2011) Amino acid production from rice straw and wheat bran hydrolysates by recombinant pentose-utilizing *Corynebacterium glutamicum*. Appl Microbiol Biotechnol 92:985–996
194. Faria NT, Santos MV, Fernandes P, Fonseca LL, Fonseca C, Ferreira FC (2014) Production of glycolipid biosurfactants, mannosylerythritol lipids, from pentoses and d-glucose/d-xylose mixtures by *Pseudozyma* yeast strains. Process Biochem 49:1790–1799
195. Liu WC, Kim IH (2017) Effects of dietary xylanase supplementation on performance and functional digestive parameters in broilers fed wheat-based diets. Poult Sci 96:566–573
196. Mendes AR, Ribeiro T, Correia BA, Bule P, Maçãs B, Falcão L et al (2013) Low doses of exogenous xylanase improve the nutritive value of triticale-based diets for broilers. J Appl Poult Res 22:92–99
197. Kiarie E, Romero LF, Ravindran V (2014) Growth performance, nutrient utilization, and digesta characteristics in broiler chickens fed corn or wheat diets without or with supplemental xylanase. Poult Sci 93:1186–1196
198. Munyaka PM, Nandha NK, Kiarie E, Nyachoti CM, Khafipour E (2016) Impact of combined β-glucanase and xylanase enzymes on growth performance, nutrients utilization and gut microbiota in broiler chickens fed corn or wheat-based diets. Poult Sci 95:528–540
199. Romero JJ, Macias EG, Ma ZX, Martins RM, Staples CR, Beauchemin KA, Adesogan AT (2016) Improving the performance of dairy cattle with a xylanase-rich exogenous enzyme preparation. J Dairy Sci 99:3486–3496
200. Tewoldebrhan TA, Appuhamy JADRN, Lee JJ, Niu M, Seo S, Jeong S, Kebreab E (2017) Exogenous β-mannanase improves feed conversion efficiency and reduces somatic cell count in dairy cattle. J Dairy Sci 100:244–252
201. Panwar D, Srivastava PK, Kapoor M (2014) Production, extraction and characterization of alkaline xylanase from *Bacillus* sp. PKD-9 with potential for poultry feed. Biocatal Agric Biotechnol 3:118–125
202. Mandal A (2015) Review on microbial xylanases and their applications. Int J Life Sci 4:178–187
203. Collins T, Hoyoux A, Dutron A, Georis J, Genot B, Dauvrin T et al (2006) Use of glycoside hydrolase family 8 xylanases in baking. J Cereal Sci 43:79–84
204. Dutron A, Georis J, Genot B, Dauvrin T, Collins T, Hoyoux A et al (2004) Use of family 8 enzymes with xylanolytic activity in baking. World Intellectual Property Organization, PCT, WO 2004/023879 A1
205. McRae MP (2017) Dietary fiber is beneficial for the prevention of cardiovascular disease: an umbrella review of meta-analyses. J Chiropr Med 16:289–299
206. Yang J, Summanen PH, Henning SM, Hsu M, Lam H, Huang J et al (2015) Xylooligosaccharide supplementation alters gut bacteria in both healthy and prediabetic adults: a pilot study. Front Physiol 6:216. https://doi.org/10.3389/fphys.2015.00216. eCollection 2015
207. Laurikainen H, Harkonen T, Autio K, Poutanen K (1998) Effects of enzymes in fibre-enriched baking. J Sci Food Agric 76:239–249
208. Ingelbrecht JA, Moers K, Abecassis J, Rouau X, Delcour JA (2001) Influence of arabinoxylans and endoxylanases on pasta processing and quality. Production of high-quality pasta with increased levels of soluble fiber. Cereal Chem 78:721–729
209. Christophersen C, Andersen E, Jacobsen TS, Wagner P (1997) Xylanases in wheat separation. Starch-Starke 49:5–12
210. Cruz F, Migo V, Valencia AS, Demafelis R, Alfaf GC, Alcantara JA (2007) Enzyme mixtures for the extraction of oil from the seeds of vutalao (*Calophyllum inophyllum*). Crop Prot Newslett 32:17–30
211. Ricochon G, Muniglia L (2010) Influence of enzymes on the oil extraction processes in aqueous media. OCL 17:356–359

212. Mamo G, Kasture S, Faryar R, Hashim S, Hatti-Kaul R (2010) Surfactants from xylan: production of *n*-octyl xylosides using a highly thermostable xylanase from *Thermotoga neapolitana*. Process Biochem 45:700–705
213. Morrill J, Månberger A, Rosengren A, Naidjonoka P, von Freiesleben P, Krogh KBRM et al (2018) β-Mannanase-catalyzed synthesis of alkyl mannooligosides. Appl Microbiol Biotechnol 102:5149–5163
214. Pangsri P, Pangsri P (2017) Mannanase enzyme from *Bacillus subtilis* P2-5 with waste management. Energy Procedia 138:343–347
215. Moreau RA, Powell MJ, Whitaker BD, Bailey BA, Anderson JD (1994) Xylanase treatment of plant cells induces glycosylation and fatty acylation of phytosterols. Physiol Plant 91:575–580
216. Borisjuk NV, Borisjuk LG, Logendra S, Petersen F, Gleba Y, Raskin I (1999) Production of recombinant proteins in plant root exudates. Nat Biotechnol 17:466–469
217. Zhang B, Huston A, Whipple L, Barrett H, Wall M, Hutchins R, Mirakyan A (2013) A superior, high-performance enzyme for breaking borate crosslinked fracturing fluids under extreme well conditions. SPE Prod Oper 28:210–216

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Alkaliphiles: The Emerging Biological Tools Enhancing Concrete Durability

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Contents

Abstract Concrete is one of the most commonly used building materials ever used. Despite it is a very important and common construction material, concrete is very sensitive to crack formation and requires repair. A variety of chemical-based techniques and materials have been developed to repair concrete cracks. Although the use of these chemical-based repair systems are the best commercially available choices, there have also been concerns related to their use. These repair agents suffer

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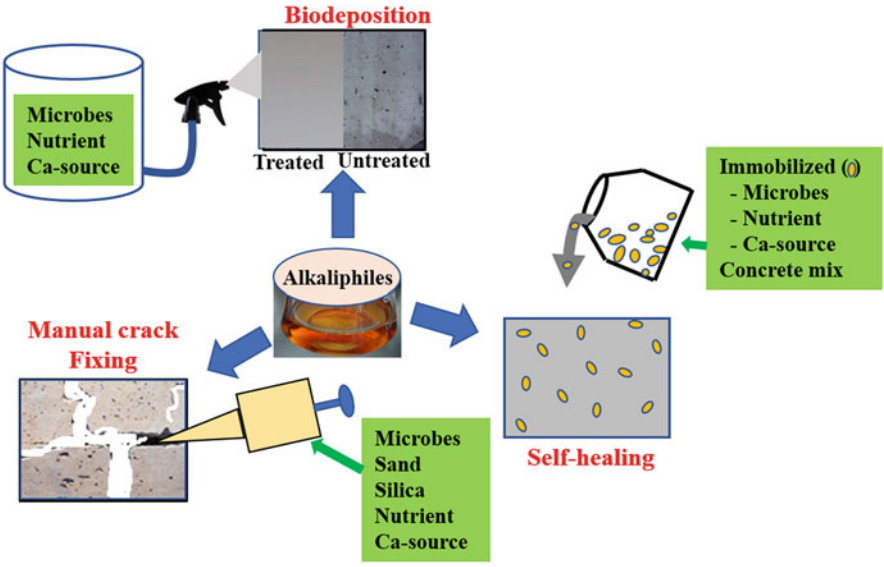
from inefficiency and unsustainability. Most of the products are expensive and susceptible to degradation, exhibit poor bonding to the cracked concrete surfaces, and are characterized by different physical properties such as thermal expansion coefficients which are different to that of concrete. Moreover, many of these repair agents contain chemicals that pose environmental and health hazards. Thus, there has been interest in developing concrete crack repair agents that are efficient, long lasting, safe, and benign to the environment and exhibit physical properties which resemble that of the concrete. The search initiated by these desires brought the use of biomineralization processes as tools in mending concrete cracks. Among biomineralization processes, microbially initiated calcite precipitation has emerged as an interesting alternative to the existing chemical-based concrete crack repairing system. Indeed, results of several studies on the use of microbial-based concrete repair agents revealed the remarkable potential of this approach in the fight against concrete deterioration. In addition to repairing existing concrete cracks, microorganisms have also been considered to make protective surface coating (biodeposition) on concrete structures and in making self-healing concrete.

Even though a wide variety of microorganisms can precipitate calcite, the nature of concrete determines their applicability. One of the important factors that determine the applicability of microbes in concrete is pH. Concrete is highly alkaline in nature, and hence the microbes envisioned for this application are alkaliphilic or alkali-tolerant. This work reviews the available information on applications of microbes in concrete: repairing existing cracks, biodeposition, and self-healing. Moreover, an effort is made to discuss biomineralization processes that are relevant to extend the durability of concrete structures.

Graphical Abstract



Keywords Alkaliphiles, Bioconstruction, Biodeposition, Biomineralization, Calcite, Cement, Concrete, Construction biotechnology, Crack repair, Extremophiles, Self-healing, Silicate

Abbreviations

ABC	ATP-binding cassette
ACDC	Activated compact denitrifying core
BCM	Biologically controlled mineralization
BIM	Biologically induced mineralization
CERUP	Cyclic EnRiched Ureolytic Powder
EPS	Extracellular polymeric substances
MICCP	Microbial-induced calcium carbonate precipitation
PCB	Polychlorinated biphenyl
RH	Relative humidity

1 Introduction

Among all materials ever used in construction, concrete has a unique combination of desirable properties. It is cheap, easy to cast, resistant to fire, exhibits enormous compressional strength, and its raw materials are relatively abundant. These make concrete one of the most common and indispensable building materials that the world has seen. A wide variety of constructions such as residential and commercial buildings, roads, dams, bridges, railways, airports, and subways use concrete. Although concrete has high compressional strength, its tensile strength is relatively low, and hence it is usually reinforced with high tensile strength materials such as steel [1]. Reinforced concrete is used widely because of its strength, durability, price, and beauty. However, due to its inherent property (heterogeneity) and non-favorable environments (such as earthquake and high external loads), even reinforced concrete structures are subject to a deterioration phenomenon known as cracking. If the cracks remain open, the concrete will be degraded and the steel corrode, which lead to an eventual collapse of the structure. Thus, if the service time of the concrete structure must be extended, cracks should be repaired.

Over the years, different concrete crack repair methods have been developed. Once the presence of crack is identified and repair is a necessity, the crack repair agent will be applied either topically or injected into the crack. The topical method applies fixing agents such as epoxy and cementitious or caulk product on top of the crack. Although this contributes to the appearance and to some extent slows down the cracking process, it will not last long before the crack transmits itself through the seal and the deterioration resumes. The other method of application involves injection of epoxy, polyurea, urethane, sand, silica fillers, or another repair agent into the

crack. At present, the use of these chemical-based concrete crack repair agents is the most important approach and applicable to many existing concrete structures. However, it is known that these chemical-based repair agents suffer from various limitations such as poor weather resistance, sensitivity to moisture, unsustainability, poor bonding with concrete, susceptibility to degradation, delamination with age, and/or have different thermal expansion coefficient than that of the concrete [2–5]. Moreover, most of the repair agents contain chemicals that pose environmental and health hazards. Thus, there has been an interest to develop concrete crack repair agents that are efficient, long lasting, and safe. These desires have brought the use of microorganisms as concrete crack repair agents into focus.

Studies made so far on development of microbial-based repair agents primarily focus on calcite ($CaCO_3$) precipitation (crystallization). Concrete deterioration is often associated with penetration of water, which often carries aggressive substances such as chlorides and sulfates. In addition to cracks, concrete is intrinsically porous in nature and often has many openings on its surface that may let in water to the structure. When microbial-based preparations are applied on concrete, the microbes deposit calcite that fills the cracks, surface pores, and cavities. This deposition of calcite blocks the water ingress to the concrete structure. The water ingress prevention in turn protects the concrete from deterioration, and hence the application of calcite precipitating microbes is expected to extend the durability of concrete structures.

Calcite precipitating organisms are ubiquitous and use different mechanisms to precipitate $CaCO_3$ such as through degradation of urea, dissimilatory sulfate reduction, metabolism of organic acids, ammonification, methane oxidation, etc. [6]. However, only some of these calcite precipitating mechanisms are suitable for concrete crack repair applications. For example, organisms that precipitate calcite through methane oxidation or ammonification cannot be considered for this application. Even among microbes with suitable mechanisms of calcite precipitation, only few have the potential to effectively treat concrete. Several factors determine the efficiency of microbially driven concrete crack repair. The nature of concrete is one of the most important factors that dictate which organisms to be considered. Concrete is very alkaline (pH of about 12.5) in nature; therefore microbes intended for concrete crack repair application should be able to thrive in high pH environment and precipitate copious amount of calcite under the harsh condition. Thus, alkaliphiles that precipitate calcite are the most desirable organisms for this application.

The application of alkaliphiles in concrete goes beyond the repair of existing cracks. It has also a great potential in making more durable concrete. Concrete repair has been neither cheap nor easy. Annually, the world spends billions of dollars to repair concrete infrastructures. For example, in Europe, it is estimated that half of the annual construction budget allocated by EU countries is consumed by the repair works [7]. Moreover, in addition to direct costs, there are punishingly high indirect costs [1] related to disuse of the facility, road blockage, occurrence of traffic jams, overall inconvenience, etc. The use of more durable concrete is expected to substantially cut down these repair costs and the associated inconveniences. This has triggered the effort to find ways in making more durable concrete.

The standard procedure used in mending concrete follows monitoring, detecting, and repairing steps. Thus, the cracks must be discovered to perform the repair. However, this is not an easy task. High tensile stresses due to external loads, volume shrinkage that imposes deformations (e.g., due to temperature gradient or confined shrinkage), or volume expansion (initiated by reinforcement corrosion, alkali silica reaction, or sulfate attack) often result in micro-cracks in the concrete structure [8, 9]. Some of these micro-cracks (with width of less than 300 μm) may autogenously heal [10, 11], but the rest requires human intervention. However, the micro-cracks could be too small for visual detection or be located deep inside the concrete structure, making early detection and subsequent repair almost impossible. On the other hand, if these cracks, even the smallest ones, are not repaired in time, they often expand further and can reach to the reinforcement bar [12]. The cracks provide passage for water and gases that may contain aggressive substances such as chlorine, sulfate, carbon dioxide, and oxygen that attack both the concrete and its reinforcement [9, 12]. This attack potentially impairs the strength and durability of concrete structures. Thus, if prolonging the service life of the concrete structures is of interest, it is ideal that the micro-cracks which are precursors for concrete structural failures [13] should be repaired in time. However, repair of these small cracks is difficult not only due to invisibility but also by inaccessibility. Even if the micro-cracks are discovered, proper application of the repair agents to these cracks is very difficult. The repair agents are applied from outside and should penetrate to reach the internal cracks. Although this approach is quite efficient for repairing large cracks, it is not suitable for small and deep cracks, which often remain inaccessible to the applied repair agent. Therefore, there has been interest for alternative repair methods that potentially are suitable to reach all sorts of cracks in concrete structures. This challenge has partly contributed to the emergence of the idea of developing concretes with self-healing properties [1, 14–17]. Currently, one of the rapidly expanding research areas in the field of concrete materials is the development of self-healing concrete, and the use of microbes as self-healing agents has become a very attractive strategy [18, 19]. In fact, the potential of microbes in making smart concrete that autonomously heals its crack is enormous. The conventional, concrete repair is labor intensive, and its direct and indirect costs are very high. Thus, the use of concrete that fixes its crack independent of human intervention is expected to have remarkable economic, environmental, and technical advantages.

Microbial-based self-healing concrete potentially seals cracks without human intervention they are formed anywhere in the structure and when they are yet small. Thus, in addition to alleviating inaccessibility, it avoids the difficult and costly monitoring and detection of cracks in traditional crack repair procedure. In this approach, the repair agents are added during concrete mixing and casting, and upon crack formation, the repair agents seal the crack autonomously [20]. Alkaliphiles have also been tried as surface coating agents for concrete structures. The application of microbial-based preparation on the surface forms calcite which coats and plugs openings (i.e., pores and cracks). This limits the water and aggressive substance ingress to the subsurface of the concrete structure [21].

This emerging construction biotechnology that uses microorganisms to extend the durability of concrete structures relies on one of nature's intriguing phenomena, biomineralization.

2 Biomineralization

The use of biological agents in sealing concrete cracks seems to be one of the recent alternative methods of combating concrete degradation. This approach is based on biomineralization, a biochemical process in which organisms stimulate the formation of minerals [22]. The process is broadly classified into two groups: biologically controlled mineralization (BCM) and biologically induced mineralization (BIM) [23–25]. In BCM process, cell-associated minerals are synthesized within specific conditions. It happens when cations interact with macromolecules such as lipids, proteins, polysaccharides, etc. that initiate mineral crystal growth. On the other hand, in BIM process, the minerals generally form by nucleation and grow extracellularly through chemical reactions involving metabolic byproducts. Cell walls or exopolymers of bacteria such as slimes, sheaths, biofilms, etc. can serve as mineral nucleation and growth sites [26–28]. One of the most interesting features of biomineralization is its ability of making minerals in highly remarkable precision and reproducibility that are very difficult to achieve under the conventional synthetic process. A classic example could be the bio-fabrication of biosilica glass, which organisms such as diatoms, sponges, radiolarians, choanoflagellates, and plants form in an astonishing variety that cannot be achieved by means of the existing chemical methods [29]. The traditional synthetic process of nanoscale materials is known to be energy intensive, involve stringent synthetic conditions (like high temperature, pressure, or pH), and often release toxic by-products [30]. Moreover, not only the quantities produced are small but also usually irreproducible due to the difficulties of controlling agglomeration [31]. On the other hand, biologically synthesized materials often have properties that surpass the properties of the analogous products made by the synthetic route [32]. Unlike the traditional chemical synthetic process, biomineralization happens under mild conditions with the help of macromolecules such as proteins [33]. Thus, biominerals are usually composites in nature due to the presence of organic molecules; however, these materials are often not only as hard as the pure mineral but are also tougher [32].

Different organisms are known to be involved in mineral formation. In higher organisms, biomineralization involves production of cystolith inclusions in leaves and strong body parts like bones, teeth, and shells. Currently, there are over 60 different mineral groups that are formed by organisms [34, 35] and deposited in their body for various biological functions. Algal and diatoms silicates, invertebrate carbonates, and vertebrate calcium phosphates and carbonates can be mentioned as examples. As in the case of higher organisms, lower group of organisms, especially prokaryotes such as bacteria, are highly potent agents of biomineralization. These organisms form an enormous variety of minerals such as carbonates,

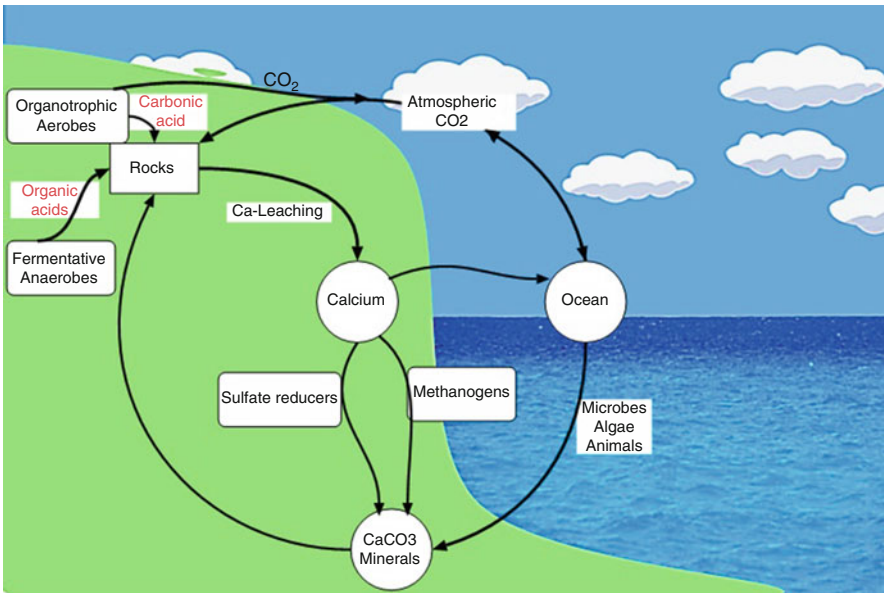


Fig. 1 Part of the calcium cycle showing the different groups of microbes involved in solubilization and precipitation of calcium minerals

phosphates, oxides, sulfides, and silicates [36, 37]. Microbes are also involved in mineralization of gold, copper, iron, uranium, etc., and in fact some of these processes are being used at commercial level in a sector called microbial mining [38]. In addition, siliceous sinter, diatomaceous earth, opal, pyrite, marcasite, travertine, etc. have also biogenic origin.

Microbe-driven biomineralization is an important biogeochemical phenomenon and plays vital roles in different geochemical cycles. One of these geochemical cycles that heavily depend on biomineralization is the calcium cycle. The cycle starts by leaching calcium-containing rocks mainly by organotrophic organisms (Fig. 1). The respiration of these organotrophic aerobes release CO_2 to the soil, and in the presence of moisture, it results in leaching with carbonic acid and soda salinization [39]. On the other hand, in anoxic conditions, the organic acid production by fermentative anaerobes is believed to be the main factor of the calcium leaching [40]. The leached calcium precipitate as calcium carbonate mainly by sulfate reducers and to some extent by methanogens [41]. The carbonate precipitation is important in maintaining neutral conditions in various ecosystems, which ensures a conducive environment for a variety of life forms. Moreover, this cycle profoundly influences the fate of the global inorganic carbon and serves as a sink to atmospheric CO_2, and hence it plays a crucial role in checking the increase in the greenhouse effect.

Microorganisms mediate mineral production in two different ways: passive or active processes [23, 24]. The passive biomineralization (BIM) is the process driven

by microbial cell surface which serves as nucleation and crystal growth sites. This process happens due to the nature of cell surfaces. Bacterial cell walls and external sheaths are rich in chemically reactive negatively charged sites that readily bind to dissolved mineral-forming elements. The adsorption substantially minimizes the activation energy barrier which normally inhibits the spontaneous nucleation and crystal growth. The mineral crystallization on the surface of the cells leads to complete encrustation. Part of the iron oxides, phosphates, carbonates, and clays precipitation happens in this way. The hot spring bacteria-induced amorphous silica precipitation is a good example of the passive type of microbial biomineralization. On the other hand, the active biomineralization (BCM) process is a complex process arbitrated by the metabolic activity of organisms. The nucleation as well as the growth of the mineral particles are often controlled by intricate metabolic activities. In this scenario, the organisms produce minerals that are unique to the species, and it happens without the influence of the environment. This biomineralization process can happen in two distinct ways, directly through enzymatic modification of minerals or by triggering change in the vicinity of minerals that leads to precipitation. The enzyme-mediated reductive uraninite precipitation by some metal-reducing bacteria [42], the accumulation of tiny magnetite particles by magnetotactic bacteria [36], and silica deposition by coccolithophores and diatoms [28, 43] are examples of this type of biomineralization process. Bacteria-mediated gold and silver accumulation is also a reductive precipitation of an active biomineralization process [44, 45].

In some cases, the active biomineralization process is related to complex metabolic activities that can change the solution chemistry which tends to promote mineral oversaturation and its subsequent precipitation. One example is the metabolic activity of photosynthetic cyanobacteria that increases the pH of the aquatic environment in which they are living [46]. The rise in pH of the water leads to precipitation of carbonate minerals like calcite ($CaCO_3$) and strontianite ($SrCO_3$) [47]. In a similar way, the metabolic activities of microorganisms induce production of mackinawite, pyrite, and other sulfide minerals [48].

Microbial-based carbonate precipitation plays a vital role in metal coprecipitation which has cementation function in natural systems including soil, caves, and aquatic bodies [6]. This process, which occurs in different geological sceneries, can be mimicked and implemented in different biotechnological applications including metal remediation, enhanced oil recovery, carbon sequestration, and construction restoration [49–52]. The major focus of this chapter is on calcite precipitation by this intriguing natural process and its role in enhancing the durability of concrete structures. In addition to calcite, silica precipitation has also potential in concrete application. Thus, microbially induced calcite and silicate precipitation are discussed below.

2.1 *Microbial-Induced Calcium Carbonate Precipitation (MICCP)*

Calcium carbonate is the prominent global reservoir of carbon. The two carbonates, limestone and dolomite alone, account nearly 42% of the global carbon reserve [53]. It is believed that a significant amount of the calcium carbonate available in earth's surface is of biogenic origin [54]. Stromatolites and whiting events can be good examples of massive MICCP phenomena [55, 56] that contribute to the biogenic surface calcium carbonate deposit. As shown in Table 1, MICCP is driven by microbial activities such as denitrification [65], photosynthesis [66], ammonification [67], ureolysis [63], sulfate reduction [68], and methane oxidation processes [60]. In addition to these metabolic activities, microbial surfaces including cell walls and extracellular polymeric substances serve as calcium carbonate nucleation and crystal growth sites.

Microbial-mediated $CaCO_3$ formation is a type of biomineralization process which is dependent on environmental conditions [69]. There is no specialized means or specific molecular activities involved in production of $CaCO_3$ [70]. In fact, calcium carbonate mineralization is a straightforward simple chemical process which is governed primarily by (1) calcium concentration, (2) pH, (3) concentration of dissolved inorganic carbon, and (4) availability of nucleation sites [71]. In addition to these precipitation factors, factors such as salinity and composition of the culture medium are also reported to influence calcium carbonate precipitation [72–74]. Microorganisms contribute to carbonate precipitation by changing almost any of these four precipitation factors. Often, the microbes alter one or more of the factors at a time to induce the precipitation process [71]. It is believed that the major role of microbes is their ability to create conducive pH through their metabolic activities [71].

An interesting study made on molecular level with *Bacillus subtilis* that precipitates calcite revealed the presence of a gene cluster involved in calcite precipitation [70, 75]. This cluster contains five genes named *lcf*A, *ysi*A, *ysi*B, *etf*B, and *etf*A. Mutational studies on these genes resulted in five constructs, FBC1–FBC5. Except FBC1 (which contains a mutated *lcf*A), the mutants were unable to form calcite crystals. This shows that at least these four genes are somehow relevant for the precipitation of calcite. Closer analysis of the *etf*A gene indicated that its product resembles an a-subunit of prokaryotic heterodimeric flavoproteins which is involved in electron transport during fatty acid metabolism [70]. Further studies on this mutant demonstrated that inactivation of *etf*A led to a decrease in the pH of the precipitation medium, which is the reason why this mutant does not precipitate calcite. This agrees with the observation that the same mutant can produce calcite in buffered medium [76]. Some heterotrophic as well as autotrophic pathways are known to raise the pH of an environment [77], and this includes the metabolic processes indicated in Table 1. One of the heterotrophic pathways that raise pH is the dissimilatory sulfate reduction process of the sulfur cycle accomplished under anoxic conditions by sulfate-reducing bacteria. The other heterotrophic pathway

Table 1 Metabolic pathways that are relevant to MICCP

Metabolism	Reactions	By-product	References
Aerobic oxidation of organic compounds	1. $CH_3COO^- + 2O_2 \rightarrow HCO_3^- + CO_2 + H_2O$ 2. $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2+}$ 3. $2H^+ + CO_3^{2+} + 2OH^- + Ca^{2+} \rightarrow CaCO_3$	–	[57]
Denitrification	1. $CH_2COO^- + 2.6H^+ + 1.6NO_3^- \rightarrow 2CO_2 + 0.8\ N_2 + 2.8H_2O$	Complete reaction: H_2O, N_2, and CO_2	[58, 59]
	2. $Ca^{2+} + CO_2 + 2OH^- \rightarrow CaCO_3 + H_2O$	Incomplete reaction: H_2O, NO, N_2O	
Methane oxidation	Anaerobic oxidation: $CH_4 + SO_4^{2-} + Ca^{2+} \rightarrow CaCO_3 + H_2S + H_2O$ Aerobic oxidation: $CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$	H_2S	[60]
Photosynthesis	$2HCO_3^- + Ca^{2+} \rightarrow CH_2O + CaCO_3 + O_2$	O_2	[61, 62]
Sulfate reduction	$SO_4^{2-} + 2[CH2O] + OH^- + Ca^{2+} \rightarrow CaCO_3 + CO_2 + 2H_2O + HS^-$	CO_2, H_2O, and HS^-	[62]
Ureolytic	$CO(NH_2)_2 + 2H_2O + Ca^{2+} + Cell \rightarrow 2NH_4^+ + Cell\text{-}CaCO_3$	NH_4^+	[63, 64]

Adapted with modification from Zhu and Dittrich [6]

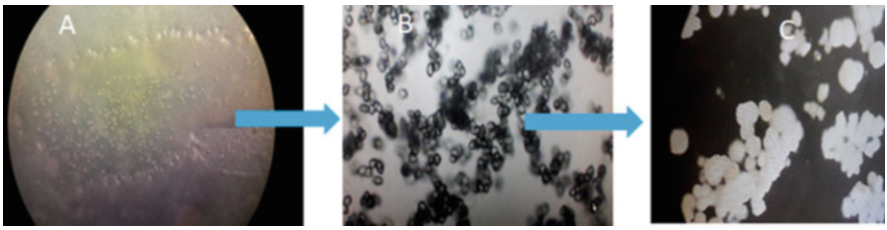


Fig. 2 Calcite precipitation by unidentified alkaliphilic *Bacillus* strain. The alkaliphile precipitated calcite crystals on calcium lactate-containing agar plate, 40× magnification field of vision (**a**). (**b**) Shows the calcite crystals in a sample taken from the agar plate under 100× magnification, and picture (**c**) is the calcite dried on microscopic slide

involves the nitrogen cycle including oxidative deamination of amino acids under aerobic condition, the anaerobic or microaerophilic dissimilatory nitrate reduction, and the aerobic assimilatory ureolytic process that can raise pH and facilitate carbonate precipitation. Another interesting microbial activity that simultaneously increases the pH and the concentration of dissolved inorganic carbon is the metabolism of organic acids [78]. In addition to the metabolic activities, carbonate precipitation can also be achieved by ion exchange process through cell membrane by unknown mechanism [69, 79].

The bacterial surface is known to serve as a nucleation site for calcite precipitation [80]. The cell wall has negatively charged residues which can bind positively charged metal ions such as calcium [41, 81]. The bound metal ions (calcium) attract anions like carbonate, and the reaction leads to precipitation (i.e., calcium carbonate) (Fig. 2). The anion could be originated from bacterial metabolism or abiotic source [81]. Studies revealed that specific bacterial exopolysaccharides and glycoproteins play a remarkable role in the overall mineral morphology of bacterially induced carbonate precipitation [82, 83]. Several factors can influence the type and amount of carbonate precipitation. For instance, the extracellular polymeric substances produced by a strain of bacteria can vary depending on the medium composition or the culture conditions [84–86], and this influences the carbonate formation.

MICCP is ubiquitous in nature, and a highly diverse group of calcium carbonate precipitating microorganisms has been reported (Table 2). Indeed, under proper conditions most bacterial strains are capable of inducing carbonate precipitation [75]. In nature, MICCP is believed to play an important role in the cementation of soils, caves, sediments, open-water system, and aquifers [6, 52, 129, 130]. This mineralization process is inspired to coprecipitate metal ions, sequester CO_2, and cement sands/soils/minerals and proposed to be a promising technology in remediation of metal pollution, soil reinforcement, oil recovery enhancement, and concrete crack healing [3, 6, 49–51, 118, 131, 132]. MICCP forms composite material by binding, for example, sand and gravel together, an ability that can help to mend concrete cracks like the biocalcification process that heals broken bones. Indeed, results of MICCP studies on concrete generated encouraging results such as sealing

Table 2 Some microbial and algal genera represented by carbonate precipitating members

Genera	References	Genera	References
Acinetobacter	[87]	*Nostoc*	[88]
Aerobacter	[89]	*Oscillatoria*	[88]
Arthrobacter	[90–93]	*Phormidium*	[94, 95]
Bacillus	[96–105]	*Planktothrix*	[88]
Calothrix	[106]	*Prochlorococcus*	[107]
Chroococcus	[108]	*Proteus*	[89, 109]
Deleya	[110]	*Pseudanabaena*	[111]
Desulfovibrio	[112, 113]	*Pseudomonas*	[114]
Diaphorobacter	[115]	*Rivularia*	[108]
Dichothrix	[107]	*Scenedesmus*	[116]
Halomonas	[72]	*Schizothrix*	[61, 117]
Homoeothrix	[108]	*Scytonema*	[61]
Kocuria	[118]	*Shewanella*	[119]
Leptolyngbya	[111]	*Spirulina*	[88]
Lysinibacillus	[120]	*Sporosarcina*	[121–123]
Micrococcus	[124]	*Stenotrophomonas*	[125]
Myxococcus	[124, 126, 127]	*Synechococcus*	[94]
Nocardia	[128]	*Trichodesmium*	[95]

of cracks, compressive strength enhancement, and recovery of strength after crack formation and healing [18, 133, 134].

2.2 Microbe-Mediated Silicate Precipitation

Another important biomineralization process relevant to concrete durability is the microbial-based silicate precipitation. Silicon (Si) is the second-most abundant element accounting nearly 29% of the earth's mass [135]. It exists mainly in silica (SiO_2) form that occurs as monosilicic acid ($Si(OH)_4$) in aqueous solution. Like calcium carbonate precipitation, biomineralization of silicate seems an important process in nature. A large amount of silica is known to be mineralized in the body of organisms ranging from terrestrial plants to marine organisms such as diatoms. This silica formed in the body of organisms is known as biogenic silica. Organisms synthesize this remarkable substance by extracting and processing naturally occurring silica or silicon from their respective environments and often using it for structural or defensive purposes. The biosilicification process could be simple accumulation of silica in the body of organisms, or it could be very complex process performed by specifically evolved metabolic activities. For instance, in marine organisms, silica deposition is known to be chemically and spatially controlled and involves silica-depositing proteins such as silicatein and silaffin [136, 137]. Unlike the manmade and the natural geological fabrication processes of silicates that require extreme temperature, pressure, or pH, organisms produce remarkably diverse

nanostructured silicates at near-neutral pH, atmospheric pressure, and room temperature [138]. Efforts have been made to unravel natures secrete in synthesizing complex biogenic silica using biological machineries [139]. Although Schröder et al. [33] demonstrated the possibility of producing biosilica glass using sponge enzymes, there has been little progress made so far. Understanding and use of biosilicification process could be of immense potential application in concrete.

Eukaryotic organisms are not the only group known with ability of biomineralization of silica. Prokaryotes also demonstrate this process. In fact, it is not unusual to find bacteria associated with clay-like silicate minerals in hot springs [47, 140–143] and surface sulfide springs [41]. Clay-like bacterial surface precipitates have also been reported even in places such as freshwater lakes and rivers with low concentrations of dissolved silica [41]. It is believed that microbes are instrumental in the formation of these minerals.

In microbial mats, silica seems abundant, and it exists on the microbes' surface forming the mats' micro-laminated layers [144]. This silica biomineralization in microbial mats seems linked to Fe precipitation. In line with this, the biomineralization of silica by *Thermus thermophilus* TMY has been studied, and the results indicated that the microbe produces a silica-induced protein [138]. Analysis of the protein amino acid sequence revealed that it is related to solute-binding protein of the Fe^{3+} ATP-binding cassette (ABC) transporter. There is a possibility that this silica-induced protein contributes to silica mineralization by mediating protein-Fe-silica interaction. However, some researchers argue that the contribution of bacteria like *Thermus* in biomineralization of silica is not significantly important. According to these researchers, silica and iron precipitation is dominated by non-microbially mediated process. They suggested that microorganisms contribute only marginally to silicification [145] which is an inorganically controlled process [146].

As there is controversy over how important microorganisms are in silica biomineralization, there has also been disagreement regarding silica sorption capacity of bacterial cell wall and exopolysaccharides. Studies made on silica precipitation by the cyanobacteria *Calothrix* sp. indicated that the silica precipitation role of the cell surface is limited [146]. The conclusion drawn from this study is that precipitation of silica is largely non-biogenic, and the microbial surfaces have a minor effect on silica nucleation. However, there is evidence that bacterial surfaces are good sorption interfaces to bind silicate ions [140]. Although bacterial cell surface has net negative charges, there are also positively charged amine groups. At near-neutral pH, these positively charged residues can interact with silicate anions. However, the available amine groups may not be enough to adsorb large amount of silicate that leads to precipitation. On the other hand, more silicate binding can occur due to cross bridging involving metal ions which link silicate anions to the negatively charged groups within the cell wall matrix [147]. The binding of silicate anions results in deposition of poorly ordered silicate mineral on the surface of the bacteria, which eventually become clay-like crystalline particles. The formation of these particles increases the sorption interface for metal ions and hence increases the overall metal-binding ability of the bacteria [148, 149]. However, it seems that the bacterial surface has a greater affinity for metal ions than the clay particles [150–152].

In the last two decades, study results that support microbe's contribution to silica precipitation are trickling in. Even a mechanism how amorphous silica biogenically precipitated has been proposed using *Shewanella oneidensis* MR-1 as a model organism [153]. Silicate modifying proteins of bacterial origin are also reported [154]. These authors reported that a strain of *Shewanella* sp. isolated from a hot-spring leaches silica and transforms it to Gehlenite which significantly improves the concrete strength by filling the micropores. Moreover, unidentified microbe affiliated to *Thermoanaerobacter thermohydrosulfuricus* produces bioremediase, an enzyme with silica leaching activity. Both the isolated bioremediase and the microbe were able to increase the compressional and tensile strength of cementitious materials by more than 20% [155, 156]. The patent filed by Chattopadhyay and Mandal [157] presents a very interesting result on the ability of the protein in releasing silica. This may initiate further studies on microbial-based precipitation of calcite for concrete application. Since there is a lot of silica in concrete, the modification of the silica could be interesting in reconfiguring the structural integrity of the concrete matrix.

3 Microorganisms in Concrete Application

The deterioration of concrete structures and its associated economic impact has drawn a lot of attention in handling concrete degradation. A variety of methods have been developed and used for maintenance of concrete structures. However, these methods have limitations such as inefficiency and unsustainability, which triggered the search for better ways of managing concrete degradation. In recent years, the use of microorganisms to repair concrete cracks emerged as an attractive alternative. Studies made so far revealed the advantages of using microorganisms in the fight against deterioration of concrete. Microbially precipitated calcite has been regraded as the most environmental friendly and economical material for repair or protection of concrete. The use of microorganisms in the construction sector has been broadly discussed in many reviews [18, 19, 57, 64, 158–161]. This chapter provides an overview on the potential application of microorganisms in enhancing the durability of concrete, specifically through their use in existing crack repair, making protective surface coating, and self-healing concrete.

3.1 Microbes in Repairing Existing Concrete Cracks: Manual Crack Fixing

The main reason of reduced durability of concrete structures is crack formation, a phenomenon which makes easy path for water and dissolved aggressive substances to the concrete structure. Thus, in concrete repair, it is mandatory to stop water

ingress. There are many alternative chemical-based products which upon application to cracks can stop water ingress and the subsequent physical deterioration. However, it has been shown that some of these conventional crack repair methods such as the application of chemicals and polymers are sources of health- and environmental risks, and more importantly, they are effective only for short term due to several factors such as difference in the thermal expansion coefficient of the repairing material and susceptibility to biological and chemical degradation [2–5]. Thus, treatment methods that are environmentally benign, safe, long lasting, and sustainable are in high demand. The biological way of mending concrete cracks by biogenic $CaCO_3$ precipitation is potentially cheap, exhibit compatible properties to concrete, and it is environmentally benign and safe.

Biomineralization can be used in two ways to fix cracks in concrete. In one of these methods, microbial cells together with nutrients, calcium source, and urea (in most studies) are added into the concrete during mixing and casting. Later, when cracks happen, the organisms are activated and form calcite that seals the cracks, a method widely known as self-healing. This will be discussed below under Sect. 3.3. In the second approach, which is a passive way, after localizing cracks during inspection, the microbial agents are applied on the identified crack surface. This method is sometime referred to as bio-based manual crack fixing or bioremediation of concrete. The results among the first studies on using bacteria to repair concrete crack was encouraging [122, 162, 163]. Artificially made cracks on mortar cubes were filled with *Sporosarcina pasteurii* (formerly *Bacillus pasteurii*) cells, nutrients, and sand. Then, the cube was immersed in urea-$CaCl_2$ solution for 28 days. Cubes with their cracks filled by the biomineralization process exhibited a significant increase in compressive strength compared to specimens without bacteria [163]. Similar results of higher compressive strength of microbial-treated concrete over untreated samples have been widely reported [158, 164–166]. The interesting observation is that the $CaCO_3$ precipitate serve as special cement that glue the sand particles together, which indicates the potential of the microbially induced mineralization in making better repair. The glue property may be partly attributed due to the bacterial exopolysaccharides [124]. In addition to increasing the compressional strength, the precipitated calcite effectively mends cracks. The sealed cracks exhibit reduction in water permeability (10^{-10}–10^{-11} m/s coefficient) and crack bridging which was demonstrated by an increase in ultrasonic pulse velocity [4, 9]. It has also been reported that the microbial-based crack fixing resulted in a 60% strength regain in mortar prisms. The presence of calcite in the repair material has also been confirmed by thermogravimetry [4], scanning electron microscopy (SEM) [167], and X-ray diffraction (XRD) [168] analyses. Over the years, several research publications appeared which report on the use of different microorganisms, different sources of calcium and nutrients, cell protection methods, etc. A summary of the efficiency of some of these microbial-based concrete crack repair studies reported by different researchers is given in Table 3, which is partly adapted and modified from Achal and Mukherjee [64].

In general, the results from studies made so far clearly indicate the great potential of microbial-induced calcite precipitation for repairing concrete cracks. However, there are still some issues that need to be properly addressed. For instance, analysis

Table 3 A summary of some studies made on microbial-based concrete crack fixing

Microorganism	Specimen (volume/ dimension in mm)	Crack size in mm	Efficiency	References
Bacillus pseudofirmus	Concrete prisms (40.9 × 40.9 × 160)	Micro-cracks (≤0_25)	Cracks effectively sealed, water ingress restricted	[169]
Sporosarcina pasteurii	Cement mortar (70.6 mm^3)	Depth = 18.8 Width = 3	Crack plugging, improvement in compressive strength	[168]
Bacillus sp. CT-5	Cement mortar (70.6 × 70.6 × 70.6)	Depth = 13.4–17.2 Width = 3	Cracks healed, compressive strength improved	[170]
Bacillus sphaericus	Concrete prisms (160 × 160 × 70)	Depth = 20 Width = 0.3	Reduction in water permeability, crack bridging,	[4, 13]
Bacillus Alkalinitrilicus	Reinforced mortar (40 × 40 × 160)	Multiple cracks Width upto 1	Oxygen diffusion barrier	[10]
S. pasteurii	Cement mortar (50.8 × 50.8 × 50.8)	Depth = 25.4 Width = 3.175	Improvement in compressive strength	[122]
S. pasteurii (encapsulated)	Cement mortar (50.8 × 50.8 × 50.8)	Depth = 25.4 Width = 3.18	Improvement in compressive strength	[51]
B. megaterium, *B. licheniformis*	Concrete beam (500 × 100 × 100)	Depth = 10 Width = 0.3	High strength regain and complete crack healing	[164]
B. pseudofirmus, *B. cohnii*	Cement mortar (40 × 40 × 40)	–	Reduction in water permeability	[171]
B. sphaericus	Reinforced prism (40 × 40 × 360)	Depth = 20 Width = 0.3–0.5	Higher strength regain and reduction in water permeability	[9]
B. sphaericus	Reinforced prism (30 × 30 × 360)	Multiple cracks Width = 0.2–0.22	Reduction in water permeability	[172]

of the microbially treated concrete revealed that the repair is done only at the surface layer, and often the part of the crack deep inside the concrete remains unsealed [121]. One of the factors that contributed to this phenomenon is the oxygen limitation in the deeper zone of the crack that hampers the activities of the microbes and

the subsequent calcite precipitation. Since the organisms used in these studies such as *S. pasteurii* are aerobic and accumulates the calcite relatively faster at the surface where there is no oxygen limitation. The other reason is directly related to the calcite accumulated at the crack surface, which becomes a barrier for the ongoing diffusion of calcium, urea, and even water. Hence, the calcite precipitation in the deeper part of the crack will dwindle and with it diminishes the healing efficiency. To tackle this problem, Bang et al. [162] investigated the use of polyurethane-immobilized *B. pasteurii* to repair cracks. The pores in polyurethane foam can both increase the surface area and minimize the diffusion limitation for calcium, urea, water, and oxygen and thus promote the formation of calcite in the deeper part of the crack. *B. pasteurii* cells were immobilized on polyurethane strips and manually placed in artificial cracks (width = 3.18 mm, depth = 25.4 mm) in the cement mortar. This preparation was immersed in the urea-$CaCl_2$ solution for 28 days. Although a remarkable compressive strength increase was achieved in the specimens with the immobilized cells compared to the cell-free controls, it was observed that the calcite precipitation did not improve the tensile strength and the stiffness of the treated specimen. This may be due to the lack of chemical bonding between the polyurethane matrix and the concrete crack surface. The polyurethane and the associated precipitated calcite simply plugged the crack but did not make the desired kind of sealing. Thus, it is a possibility that the concrete treated with microbial agents may not gain its full strength. It will be beneficial to look for a means that can improve calcite strength and reduce its brittleness such as through composite formation.

Another issue that needs attention is the filling material that can be used with the microbial-based agent in repairing concrete cracks. Although the filling materials play an important role, little has been done on its screening and selection. Even among the limited work done, it seems that some of them led to ambiguity. For example, *S. sphaericus* immobilized on silica gel was manually injected into concrete cracks, and the specimens were immersed into urea and calcium source solution [4, 13]. The permeability of water was greatly reduced in the samples treated with silica gel-immobilized cells as well as specimens treated with cell-free silica gel. This indicates that the effect that resulted in reduced permeability was the filling of the crack by silica gel. However, the authors believe that the calcite precipitated inside the silica gel can enhance the durability of the repair material. Researchers have also tried concrete crack fixing without any filling materials, by immersing the cracked concrete specimens into a microbial medium which consisted of bacterial cells, urea, and $CaCl_2$ for 28 days. In such a way, it was possible to fill the entire crack by the precipitating calcite [173]. Although the authors have shown the crack sealing potential of calcite without any other filler, they failed to mention the size of the cracks in their specimen. However, considering that it happened without other filling materials, one can assume that the cracks were narrow and shallow.

Almost all the studies that have been reported are lab-scale investigations, often carried out in ideal conditions where there is no limitation of calcium supply, urea, or nutrients to support the activities of the microorganisms. It is obvious that there is a lot of data trickling in from such studies and enriching the field with substantial amount of information. However, it is very important to make field trial studies that

can facilitate the actual development of products for real commercial applications. One such kind of interesting study has been reported by Wiktor and Jonkers [174]. This on-site application study of microbial-based crack-fixing was done using two solutions, A and B. Solution A contains alkaliphilic bacteria, sodium-silicate (alkaline buffer), and sodium-gluconate (carbon source for bacteria growth), and solution B contains alkaliphilic bacteria and calcium nitrate. The use of these two solutions is believed to make the preparation work both in the presence and absence of oxygen. That means the crack surface can possibly be filled by the aerobic denitrification, while the anaerobic denitrification process seals the deep part of the crack with little or no oxygen. Sodium silicate maintains an alkaline condition in the system and forms a gel when it is injected or sprayed to the crack. The formation of the gel allows a rapid sealing of the crack (within a few hours) and creates conducive environment for the microbe to accumulate calcite. The formation of the gel gives enough time for the precipitation of a substantial amount of $CaCO_3$ to seal the crack. Considering that one of the limitations of the microbial-based repair systems is its slow performance, the gel formation can offer a rapid plugging effect even though it is temporary. The authors successfully used their preparation to treat damaged ramp of parking garages by spraying the repair solution directly to the cracked concrete, which is an attractive method of application from technical as well as economic point of view. The water permeability and freeze-thaw resistance tests were used to assess the crack-sealing efficiency and improvement of frost salt scaling, respectively. The use of this system successfully sealed the cracks and stopped the leaking. In addition, the treatment of the concrete with the repair system significantly improved the freeze/thaw resistance of concrete.

An additional field trial worth to mention is the study made on 16 m^2 wall which is divided into two [175]. Part of the concrete cover was removed, and the reinforcement bars were exposed. One part of this wall was treated with concrete containing bioagent, while the other half was repaired without bioagent. The main interest in this study was to observe the effect of adding the bioagent on application (spraying), consistency, and appearance of the concrete. The outcome of this study shows there is no major difference in the application and consistency of the microbe containing and the microbe free concrete preparation. Brownish spots on bacterial-based preparation were observed which the authors claim that it faded with time.

Results generated from numerous studies undeniably indicated that the use of microbial-based $CaCO_3$ precipitation system with suitable filling materials has a promising potential to be used for manual repair of already existing cracks. However, there is a need for optimization of the repairing agent composition (bacterial load, calcium source concentration, the bacterial nutrient concentration, the ratio between filling materials and the bioactive agents, etc.) to get an effective calcite precipitation throughout the filling material and greatly enhance the repairing efficiency. Moreover, it is necessary to consider during screening if the repair agent bonds to the crack walls. If the bonding between the cracked surface and the repair agent is good, the cracks not only seal but also heal, and that is the grand objective of repairing concrete cracks. It is also necessary to consider that the whiteness of the calcite may not redeem aesthetically, and hence it will be rewarding to develop a coloring means to adjust the color to the desired appearance.

3.2 *Use of Microbes for Concrete Surface Coating: Biodeposition*

Most deterioration of concrete structures is due to water coming from surface to the interior through cracks and openings. Therefore, diminishing the uptake of water is a very important measure in protecting concrete against severe damage [176]. The environment and the concrete permeation properties determine the risk of damage and the rate at which the damage can develop. Without repair, the service time of a concrete structure is expected to be relatively short if the environment is moist, aggressive substances such as acid and chlorides are present, and the concrete is permeable to liquid. In such environments, surface treatments that reduce the infiltration of water and dissolved aggressive substances into concrete play a major role in extending the service time of concrete structures. An array of chemical-based products is available on the market to choose from to treat concrete surfaces which includes water repellents, coatings, and pore blockers. Although these chemical-based surface-treating agents are useful in minimizing the concrete water uptake, they suffer from some setbacks such as (1) they are subject to chemical, physical, and biological degradation; (2) their thermal expansion coefficients are different to that of concrete; and (3) some of these materials contain solvents that pollute the environment [97, 177]. These setbacks have led to the search for alternative methods that are environmentally benign. It is within this framework that MICCP has been proposed as a novel and eco-friendly strategy for surface treatment of concrete structures [178, 179].

The process of microbially induced precipitation of $CaCO_3$ on concrete or other building material surfaces is known as biodeposition. Initially, biodisposition was used for surface protection and consolidation of historical buildings and repair of limestone monuments [96, 100, 124]. The main desire to use biodeposition in treating these valuable specimens emanates from the limitation of the existing traditional treatment methods and incompatibility problems with organic coatings and consolidants [180–182]. The pioneer strains for these applications were mostly isolated from carbonate producing environmental samples such as calcareous sludge [100] and calcareous stones [183, 184].

The biodeposition process was initiated by applying the microbial agent and the precipitation precursors on the stone surface by immersion, spraying, or brushing [18]. The biodeposition forms a layer of $CaCO_3$ precipitate which serves as a barrier to resist degradation or as a binder to consolidate the loose particles on the surface of the stones [124]. The precipitate is found firmly attached to the stone surface and brought a consolidating effect [2, 3, 185]. It is believed that the remarkable cohesion of the precipitate may be due to incorporation of organic materials in the $CaCO_3$ crystals that influence the crystals' epitaxial growth [124]. It was in 1993 the first on-site microbially mediated bioconsolidation treatment was done on 50 m^2 of the Thouars Saint-Medard Church tower [97]. Later, biodeposition was also used for on-site conservation of decayed stones [126, 186]. Analysis of the treated limestone revealed that it was evenly strengthen at thickness of 30 mm with at least a similar performance to that of the conventional surface treatments such as ethyl silicates

[179]. The treatment was solvent free and resulted in a significant protective and consolidating effect. The drilling resistance achieved was more than doubled just with two rounds of spray applications in a day. However, in these studies, hygroscopic salts such as calcium chloride, calcium acetate, and calcium formate have been used, and this created a concern that this may later damage the stone. It is believed that substitution of the calcium salt with less hygroscopic sources could alleviate this concern. So far, the biodeposition application in this regard is limited to the on-site bioconsolidation of calcareous cultural heritage buildings. However, the findings of these application studies clearly indicated that the technique has improved the surface strength, reduced the water permeability, and enhanced the freeze-thaw resistance, which positively contribute to the stone protection [97, 124, 185, 187]. Moreover, these effects are long lasting as shown in treated limestones that did not require new treatment for more than a decade [77]. A comparison of different biodeposition methods have been extensively reviewed by De Muynck et al. [18].

The encouraging results of biodeposition applications on stone surfaces led to use of microorganisms on the treatment of concrete surfaces. This is indeed very attractive as degradation of concrete structures often starts from the surface. Surface treatment of concrete is believed to be an effective way in enhancing the durability of concrete structures. Although the principle is the same as for the treatment of stone surfaces, the nature of stone and concrete is somehow different; hence, the biodeposition process should be done in a way that suits the concrete nature. One of the differences is the high alkalinity of concrete. Thus, the use of microorganisms that are tolerant to high pH is a requirement. *S. pasteurii* and *B. sphaericus* are alkali-tolerant and are the most commonly used organisms in concrete biodeposition studies. The other difference is the porosity. Compared to porous limestone, concrete is more compact and hence less efficient in retaining microorganisms on its surface layer. However, a number of studies have successfully applied the biodeposition treatment on concrete surfaces [2, 3, 187–189].

The microbial agent and the precipitating precursor substances can be applied in two steps [2, 3, 190] or in a single step [187]. In the two-step immersion process, concrete specimens were first immersed in the bacterial culture, and then the specimens were wiped with a towel to remove the excess surface liquid and immersed in solution of urea and Ca^{2+} for 72 h for deposition of $CaCO_3$. In the single-step process, the concrete specimens were immersed in medium containing the bacterial culture, urea, and Ca^{2+}. In this single-step immersion procedure, the precipitation of $CaCO_3$ on the top surface of the specimens formed into a dense and coherent layer which has a thickness of 150–290 μm. However, the $CaCO_3$ deposition was not only restricted to the concrete surface but it had also been observed in the bulk solution. On the other hand, in the two-step immersion system, the deposition of $CaCO_3$ was performed by the bacteria that had been adsorbed on the concrete surface.

In both cases, the presence of $CaCO_3$ layer on the concrete surface dramatically reduced the capillary water absorption and gas permeability, which concomitantly enhanced the resistance to carbonation, chloride penetration, and freezing and

thawing [2, 3, 187, 190]. For instance, De Muynck et al. [3] have shown that the biodeposition of $CaCO_3$ on concrete reduced the water absorption by 65–90% depending on the porosity of the specimens. Achal et al. [170] have demonstrated the difference in absorption of water between mortar cubs treated by *Bacillus* sp. CT5 cells and the control without the bacterial cells. The reduced water absorption by *Bacillus* sp. CT5 treated specimens is accompanied by a decrease in rate of carbonation and chloride migration by about 25–30% and 10–40%, respectively. A similar observation made by Li and Qu [191] confirms that the biodeposited calcite on concrete surface reduces capillary water uptake, leading to the carbonation rate constant to be decreased by 25–40%. A good acid resistance has also been reported upon biodeposition of $CaCO_3$ on the concrete surface [187, 190]. Further studies in this line could be of great interest for concrete structures intended to be erected in low pH environments and regions prone to acid rain.

The effect of concrete surface treatment with pure and mixed ureolytic bacterial cultures on durability (i.e., based on capillary water uptake and gas permeability) was studied [2]. The authors concluded that the use of pure cultures led to a more pronounced decrease in water uptake. They also indicated that the durability performance achieved with *B. sphaericus* cultures was comparable to that of the conventional water repellents (silanes and siloxanes). Another interesting observation mentioned in their study is that the biologically produced calcium carbonate is less soluble when compared to the inorganically precipitated calcite. This indicates a higher performance potential of the calcite obtained through microbial precipitation. Another treatment was made by Okwadha and Li [188] who used *S. pasteurii* strain ATCC 11859 cells to create a biosealant on concrete surface contaminated by PCB. The treatment resulted in water permeability reduction by up to five orders of magnitude. Moreover, the biodeposition on the PCB-contaminated concrete exhibited a remarkable resistance to carbonation. Another example is the one that results in a significant reduction in water absorption and chloride permeability of concrete with biodeposited calcite layer [192].

The results from studies cited above are very interesting and were comparable to those of the conventional surface treatment substances such as acrylate, silane, siloxane, silicone, and silicate [175]. However, the procedure used in the biodeposition process, immersion, is not feasible in real application. Spraying the bacterial culture and the precipitating precursor seems to be more attractive in the real world. Although it might be labor intensive, application of the biodepositing agents by brushing the surface of the concrete structures could also be another alternative approach. Thus, field trial studies on these methods of applications would be interesting.

The biodeposition process forms a layer of $CaCO_3$ crystals, and this deposited layer functions as protective coating barrier which minimizes the penetration of aggressive substances into the concrete structure. The protection is expected to enhance the durability of concrete structures. However, the nature of the deposited layer determines the effectiveness of the biodeposited matrix. The thickness, density, cohesion, and the layer bond with the concrete matrix are some of the factors that determine the effectiveness of the layer [57]. The layer thickness is determined by

the amount of $CaCO_3$ precipitated, the higher the amount of $CaCO_3$ formed, the thicker the layer becomes to effectively decrease the water absorption by the concrete [193]. The rate of precipitation and organic matter presence are known to affect the deposit density and cohesion [124]. As loose layers are not effective [124], the layer not only be thicker but also should be dense and coherent to be protective enough.

Another interesting application of the biodeposition is improving the concrete aesthetically, which is so far given little attention. It is not uncommon to see unevenness on finished concrete surfaces. This phenomenon has effect on the appearance of the structure. The use of calcite precipitating microbes in addition to filling small cracks and pores on the surface can improve the smoothness of the surface by depositing calcite layers. In fact, a very encouraging smoothing effect has been reported by Richardson et al. [194]. Their study showed a reduction in differential surface topography using Alicona scanner. These authors also indicated that if the white-colored calcite layer is not aesthetically pleasing, it is possible to get the desired color by using suitable pigment additives.

3.3 Microbial-Based Self-Healing Concrete

A passive way of fixing concrete cracks involves constant inspection. When only the cracks are noticed, any of the available and appropriate method(s) will be implemented to repair and maintain the concrete structure. This is an expensive process, and countries often allocate a considerable annual budget for repair and maintenance of existing concrete structures [2, 195]. Despite that concrete production cost is in the range of \$65 and \$80 per m^3, the direct cost of concrete cracks repair and maintenance is estimated to be around \$147 per m^3 of concrete [196]. The indirect costs are even much higher at least for structures such as bridge repair. According to the FHWA [195] report, bridge maintenance indirect cost includes a traffic tie-up or detour that can result in wear and tear on automobiles, increased gasoline use, delays in product transport, missed appointments, and other inconveniences that incur economic loss. The report stated that the indirect cost of steel bridge maintenance can be tenfold higher than the direct cost; thus one can assume a similar trend for maintenance of concrete bridges. This (the sum of the direct and indirect costs) clearly indicates the enormous amount of resources consumed annually and globally to repair concrete structures. Therefore, preventive approaches to confine and heal crack at early stage are vital, and the use of self-healing durable concretes can substantially reduce the maintenance and repair cost of concrete structures.

Studies on the use of microbial-based preparations to manually repair existing cracks revealed many interesting results as discussed in Sect. 3.1. This approach is notable not only by its potential for long-lasting effect but also for being environmentally benign. Furthermore, it prevails the conventional repair techniques due to its bonding capacity and compatibility with concrete content [165]. These desired

features have brought a great deal of attention on using microbes to develop self-healing concrete. Like the other two applications of microbes discussed above, the bio-based self-healing concrete is based on $CaCO_3$ precipitation. In this case, dormant microbe(s) (normally spores), precipitation precursors, and nutrients are added into the concrete during the mixing and casting process. When cracking occurs, the microorganisms in the crack zone become activated by moisture and air (oxygen) coming through the crack and initiate precipitation of $CaCO_3$ which heals the concrete cracks in situ. Unlike the biodeposition and manual crack-fixing applications, in self-healing system the microorganisms should remain dormant but viable for a long time, survive the concrete mixing process, tolerate the drastic conditions of the concrete environment, and be activated when crack occurs.

The success of developing bio-based self-healing concrete depends on several factors such as the organism nature, the way cells are added to the concrete, type of calcium precursor used, etc. Concrete is known to be a harsh environment for organisms to survive. It is alkaline, nutrient poor, anoxic, and dry. Moreover, the mixing is very rough. These conditions have a great impact on survival of the microorganisms. The alkaline environment makes alkaliphiles the primary choice. Since it is difficult to estimate the timing at which crack occurs (which can be after days or several years), it is preferable to have cells that can last for long time. This favors the use of endospore-forming bacteria. It is generally known that spores, the dormant state of some microbes, have much longer survival time than vegetative cells, from several years to hundreds of years [197] and get activated when the environment becomes conducive. Often, the presence of moisture, air, and nutrients triggers the germination (activation) of spores. Therefore, spore-forming alkaliphilic bacteria, which become active upon concrete cracking and deposit good amount of calcite, are most favorable for this application.

The harsh concrete environment can severely affect the survival rate of microbes. Studies, as summarized in Table 4, have shown the rapid loss of viability when the microbes are added directly to the mixing concrete. This potentially hinders the long-term concrete crack-healing efficiency. Thus, it is imperative to enhance the cells viability to improve the long-term efficiency and applicability of such preparations

Table 4 The viability of vegetative cells and spores directly added to cement and mortar mix

Microbe state	Organism	Material	Viability (% survived)	References
Vegetative	*S. pasteurii*	Cement pest	20% after 1 day 1% after 7 days 0.4% after 28 days	[198]
	Shewanella	Mortar	Survive for 7 days	[154]
	B. megaterium	Mortar	1% after 3 days 0.1% after 28 days	[199]
Spore	*B. cohnii*	Concrete	1–2% after 9 days	[104, 200]
	B. sphaericus	Concrete	Lost after 2 days	[201]
	B. cohnii	Cement	0.5–2.5% after 9 days	[169]
	B. pseudofirmus	Cement	1–4% after 42–93 days	[169]
	B. halodurans	Cement	2% after 10 days	[200]

in self-healing concretes. Spores are known to be more resistant to harsh conditions than vegetative cells, and hence the survival rate of spores in concrete is expected to be better than that of vegetative cells. However, the survival study results indicate that the survival of spores in concrete was not significantly higher than that of vegetative cells (Table 4). Thus, it is mandatory to protect the cells/spores from the harsh environment of the concrete to enhance their viability.

Certain additives such as fly ash and silica fume are known to improve the viability of cells in mortar preparation [202–204]. Even if such additions improve viability, direct addition of cells/spores and their nutrients pose two more problems. One of the limitations is that direct addition of the healing agents (microbes and their nutrients) may lead to undesirable foaming during mixing which affects the quality of the concrete [172]. The other challenge is that the microbes added to the concrete during mixing can be activated by the presence of air, moisture, and nutrients, which may lead to untimely and undesirable calcite precipitation that depletes the resources (calcium source and microbial nutrients). These problems related to direct addition of bioagents and nutrients to the concrete mix triggered the search for a compatible protection system. Over the years, an array of methods and materials have been tried to protect the microbes and improve their viability and restrain their activity during mixing and casting. Immobilization of cells/spores has emerged as a prominent protection system. Three different methods of immobilizations: (1) encapsulation in porous solids such as diatomaceous earth and clay [10], (2) microencapsulation in gels such as in silica gel [172], and (3) use of pellets and flakes [205] have been tried.

The encapsulation method showed good results in self-healing efficiency with respect to crack filling and the amount of calcium carbonate precipitation. This may be due to uniform distribution and protection of the bioagent [165, 206]. Figure 3 depicts how encapsulated cells self-heal concrete crack. Several materials and even air voids have been considered to immobilize cells intended for self-healing concrete application (Table 5). A good protection system should be flexible so that it cannot

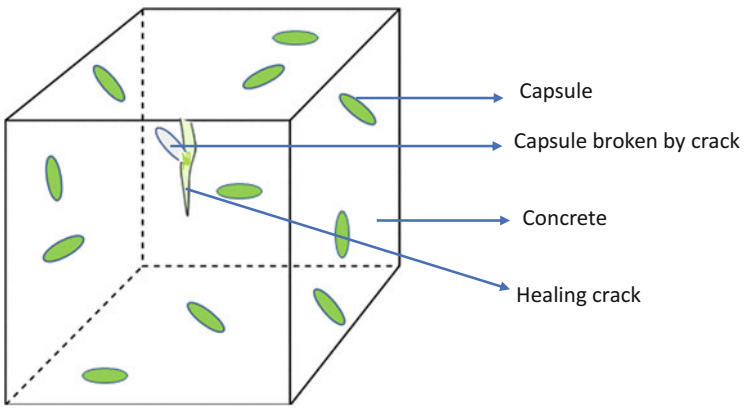


Fig. 3 An illustration of capsule containing immobilized bioagent and nutrient in a concrete cube. The healing agent from the broken capsule was released when crack happens and heals the crack

Table 5 Different materials used to immobilize biological agents, nutrients, and calcium sources

Material	Organism	References
Air voids	*B. sphaericus*	[201]
Bacteria self-immobilization	Community	[115]
Alginate	*B. sphaericus*	[207]
Ceramsite carrier	*B. mucilaginosus*	[208]
Diatomaceous earth	*B. sphaericus*	[209]
Expanded clay	*B. alkalinitriculus*	[10]
Expanded perlite	*B. pseudofirmus*	[210]
Glass tubes	*S. pasteurii*	[51]
Granular activated carbon	*Diaphorobacter nitroreducens*	[115]
Graphite nanoplatelets	*B. subtilis*	[211]
Iron oxide nanoparticles	*Bacillus* sp.	[212]
Melamine formaldehyde-based microencapsulation	*Bacillus* spp.	[172, 213]
Metakaolin	*B. sphaericus*	[115, 214]
Polyurethane	*B. sphaericus*	[9]
Salt encapsulation	ACDC denitrifying community	[215]
Silica gel	*B. sphaericus*	[216]
Silica gel or polyurethane in glass tubes	*B. sphaericus*	[9]
Superabsorbent polymers	*B. sphaericus*	[11, 217]
Zeolite	*Diaphorobacter nitroreducens*	[115]

be broken during mixing, remain intact physically and chemically during and after mixing, be brittle enough to release its content during crack formation, not negatively affect the structural and physical properties of the concrete structure, be cheap, and be readily available.

As shown in Table 1, there are different microbial activities or physiological processes that can precipitate calcite. However, not all calcite precipitating processes are equally applicable in making self-healing concrete, and hence only some of them have been considered. The studies made so far on developing microbial-based self-healing concrete can be categorized into three systems based on the physiological process that mediate the calcite precipitation: ureolytic, oxidation and denitrification.

3.3.1 Ureolytic-Based Self-Healing System

The ureolytic process of calcite precipitation is one of the most studied applications of microbes in concrete. This process releases NH_4^+ and CO_3^{2-} from urea in the presence of water as shown in Fig. 4. The NH_4^+ contributes to alkalinity, while the CO_3^{2-} interacts with Ca^{2+} to form calcite. Most published studies on the use of microbes as agents for manual concrete crack repair, protective surface biodeposition, and self-healing are based on this system. The ureolytic *Bacillus* and *Sporosarcina*

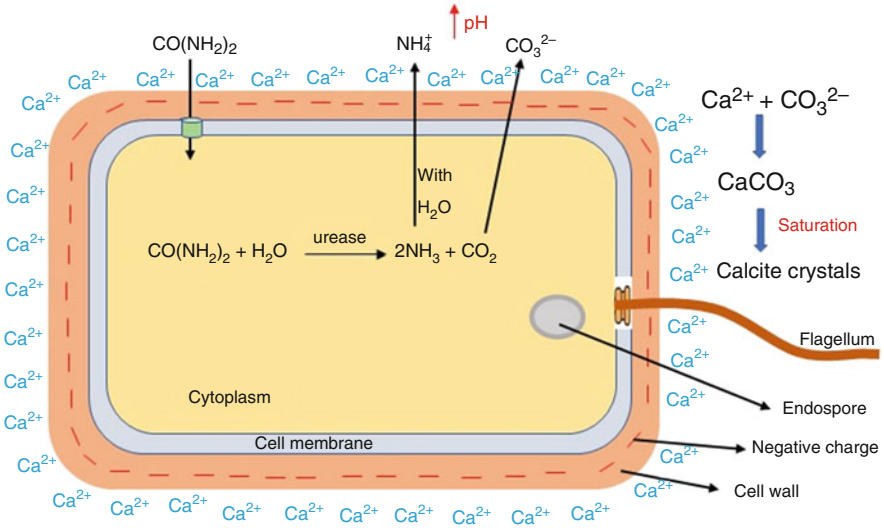


Fig. 4 Ureolytic-based calcite precipitation. An illustration of an alkaliphilic *Bacillus* cell with ample negative charges (−) in the cell wall which potentially attracts Ca^{2+}. The cell also releases CO_2 that forms CO_3^{2-} due to the NH_4^+ generated from the urea that increases the pH of the surrounding. This facilitates the formation of $CaCO_3$ in the vicinity of the cell surface; the repetition of the process increases the saturation and then to precipitation

strains are the most common biological agents used in these studies [165], which may be primarily due to the formation of resistant spores, alkaliphilicity, high carbonate productivity, and ability of precipitating high amount of calcite [218].

As the survival rate of unprotected cells or spores is low (Table 4), recent studies use protected cells/spores as self-healing agents [161]. The efficiency of the system significantly varies depending on the method of protection used. The type of the cell immobilization material and the method of immobilization not only affect the healing efficiency but also affects the strength regain [9]. For instance, this study revealed that calcite precipitation was higher in silica gel immobilized cells than those immobilized by polyurethane. However, the strength regain was better when polyurethane was used as immobilizing matrix than silica gel. The concrete treated with polyurethane-immobilized bacteria regained 50–80% of its initial strength which is much better than the 5% strength regain achieved using silica gel immobilized bacteria. The authors also reported a lower water permeability coefficient (10^{-10}–10^{-11} m/s) in the mortar treated by polyurethane-immobilized cells. On the other hand, the water permeability coefficient of the specimen treated with silica gel immobilized cells was in the range of 10^{-7}–10^{-9} m/s. Another material used in protecting the bioagents is diatomaceous earth. Concrete treated by cells immobilized in diatomaceous earth resulted in about a 70% reduction in water permeability [209]. This material has another important feature; it is the only cell carrier used that has a positive impact on the strength of the concrete [219, 220]. The observed improvement in the concrete strength is believed to be due to the

pozzolanic nature of diatomaceous earth. Since nutrients and microbes (depending on the amount and the type used) are known to reduce the concrete strength [172], the use of diatomaceous earth can compensate the potential concrete strength loss emanating from incorporation of nutrients. This remarkable material has small pores, and the microbes probably adsorb on the surface instead of getting inside the pores [209], which makes the protective effect of diatomaceous earth debatable. Thus, it is assumed that it protects only a portion of the cells/spores used, and this may be the reason why the healing is slow; a major crack healing was observed after 40 days [209].

The effect of microencapsulated cells/spores on self-healing of concrete has also been studied. Encapsulation of cells by impermeable microcapsules greatly enhanced the concrete self-healing efficiency [172]. In this study, it was observed that the microencapsulated *B. sphaericus* healed a larger crack area than the control without bacteria. The encapsulated spore specimen healed 49–80 mm^2 of crack area, whereas the non-bacterial specimen healed only 13–58 mm^2 of crack area autogenously. The average healed crack area in the bacterial-based system was about 50% higher than that of the non-bacterial specimen. The maximum crack width healed by the bacterial-based system was about 970 μm, which is about four times the value of the non-bacterial specimens. The water permeability in bacteria-containing samples was about ten times lower than that of the bacteria-free references.

Studies made on using microencapsulated spores revealed that liquid water is important for efficient healing. For instance, concrete samples maintained at 95% relative humidity did not show any self-healing [221]. Liquid water is essential for bacterial activities, and it is needed for germination of spores, hydrolysis of urea by urease, reaction of carbonate and calcium, and redissolution of nutrients to make them available for the microbes. Due to these important roles of water in microbial-based self-healing process, studies on self-healing have been done by incubating cracked specimens at full submersion or wet-dry cycles. However, from application point of view, such submersion may not be realistic. On the other hand, using carriers which have the capacity to take up water or moisture from the surroundings and can keep the water for a long time could be very interesting, and this has brought the use of hydrogels as water reservoir for microbial-based self-healing system into focus [11, 217]. Indeed, the use of hydrogels resulted in attractive results. When Pluronic-based hydrogel-encapsulated microbe-based self-healing system was used, significant crack closure happened after 7 days. A 0.5 mm crack width was almost completely healed within 7 days [11], and the healing ratio was about 20–60% higher than for the specimens without bacteria. Hydrogels can take up moisture from the surroundings, and this could be desirable for concrete self-healing in the absence of liquid water (but this has yet to be proven). In principle, hydrogels can be used as carriers for protection of spores during concrete mixing process and serve as water reservoir for spore germination and bacterial activity when cracking occurs. The hydrogel should be easy/safe to make and should not affect the property of the concrete. Unfortunately, unless it is prepared separately, the use of Pluronic-based hydrogel requires UV curing which is not good for viability of spores and genetic stability. Such methods should be avoided. The other disadvantage of this hydrogel is that it resulted in a drastic reduction in comprehensive strength, a staggering 50%

[11, 207]. This has led to search for another kind of hydrogel, and a methacrylate modified alginate-based hydrogel has been tried [207]. This hydrogel absorbs water which is higher than its weight just at 98% relative humidity (RH) and nearly 50% of its weight at 90% RH. This means it can feed water to fuel the cells activity even in the absence of liquid water. The *B. sphaericus* spores encapsulated in the modified-sodium alginate hydrogel precipitated a large amount of $CaCO_3$, indicating the efficiency of protection. However, the authors reported that this alginate-based hydrogel also reduces the strength of the concrete and the preparation involves UV treatment. Thus, although the studies indicated the potential of hydrogels, it seems that an ideal hydrogel for practical application has yet to come.

The microbial-based self-healing systems described above are based on pure microbial cultures (axenic cultures); however, recently non-axenic cultures have also been considered [222]. The use of non-axenic cultures eliminates the need for sterile production conditions and lowers the production cost. A further cost reduction can be achieved by minimizing the immobilization cost. Lately, self-protected non-axenic mixed cultures which is referred to as Cyclic EnRiched Ureolytic Powder (CERUP) have been studied [222]. CERUP is an ureolytic community protected by its high salt content and obtained from side streams of a vegetables processing plant. The efficiency of this non-axenic culture is comparable to that of the benchmark *B. sphaericus*. Direct addition of the self-protected CERUP at levels of 0.5% and 1% of the cement weight was sufficient, and this healed the concrete crack effectively within 28 days [222]. The use of this non-axenic self-immobilized preparation substantially reduces the production cost. In fact, the cost analysis revealed that it is 40 times cheaper than the operational expense cost of the axenic *B. sphaericus* culture [222]. A self-immobilized bacterial preparation has also been tried successfully in self-healing concrete [115].

Even though most of the studies made so far on development of microbial-based self-healing concrete are based on this (ureolytic) system, it suffers from potential risks which pose a serious threat on its future commercial-scale applicability. The ureolytic activity produces ammonium ions (NH_4^+) which can result in nitrogen oxides emission into the atmosphere. It is estimated that remediation of one m^2 of concrete needs about 10 g of urea which releases about 4.7 g of nitrogen [18]. Nitrate can also be formed in this process. Ammonium and nitrate can be toxic and hazardous to human health at high concentrations [65]. Moreover, ammonium that stays in the concrete is a risk for bacteria-mediated nitrification into nitric acid which can lead to deterioration of building materials [223]. These risks resulted in a dramatic shift from the once dominant ureolytic-based approach to the other two systems described below.

3.3.2 Oxidation of Organic Compound-Based Self-Healing System

This system is based on the precipitation of calcite through consumption of organic compounds by microbial agents added to the concrete [104, 171, 200, 224]. Aerobic oxidation of organic acids by microorganisms produces CO_2 which forms CO_3^{2-} in an alkaline environment. In the presence of a calcium source, the

carbonate interacts with the calcium and results in precipitation of $CaCO_3$. Since direct addition of spores and nutrient to concrete significantly reduce the viability of spores as well as the concrete strength, Wiktor and Jonkers [10] used porous expanded clay for immobilization of the microbial agents. The clay aggregate, in addition to immobilizing the microbial agents and the nutrient-calcium sources such as Ca-lactate, serves as a structural constituent of concrete. However, a slight decrease in the concrete strength has been observed. The concrete specimens with expanded clay immobilized spores and nutrients were immersed in water for 100 days. The oxygen consumption study revealed that the immobilization enhanced the spore viability. The immobilized spores exhibited remarkably efficient healing after 40 days. The study reported that the maximum crack width healed was 0.46 mm, which was about two times more than the reference specimens. The interesting observation the authors stated is that there was no difference in the healing efficiency in the first 20 days between the samples with microbial agents and the reference samples without the bioagent. The possible reasons could be that it took relatively longer time for the spores to germinate and become active cells, or most of the spores lost viability, and the remaining few cells had to propagate to reach critical mass that produces extra $CaCO_3$ to seal the cracks.

In general, this approach of using aerobic oxidation of organic acids to precipitate calcite is interesting as it does not have any undesirable by-product and it is more sustainable. When crack happens, the immobilized microbes released by breakage of their protective capsules or porous materials. The released microbes will be activated by the incoming water and produce $CaCO_3$ that heals the cracks. However, the system is not as efficient as the ureolytic process and requires relatively high concentrations of calcium source [225] which could possibly lead to undesirable buildup of high level of salts in concrete. Furthermore, the efficiency of this approach can be limited in healing deep cracks or concretes with low oxygen environments such as underground structures. On the other hand, this system is the first one tried in field application [175]. The irrigation canals in Ecuadorian highland had a cracking problem, and the use of microbial-based self-healing concrete has been proposed to solve the daunting problem. In 2014, self-healing concrete mix prepared from locally available ingredients and healing agents was used to make new concrete lining of canals, and the result is very encouraging.

3.3.3 Denitrification-Based Self-Healing System

The third system that has been used in developing self-healing concrete is the dissimilatory nitrate reduction process. This process is primarily accomplished by facultative heterotrophic microbes which enzymatically reduce nitrate to N_2 (Fig. 5). The heterotrophs require organic carbon source to fuel its metabolic activity and get O_2 for respiration either from air (atmospheric or water) or by reducing nitrate (upon depletion of O_2). Thus, denitrification occurs during anoxic condition at which nitrate is used as electron acceptor. The identification process, in the presence of organic carbon, generates CO_2 and OH^- (Fig. 5), which leads, respectively, to formation of CO_3^{2-} and alkalinity, conditions necessary for calcite precipitation.

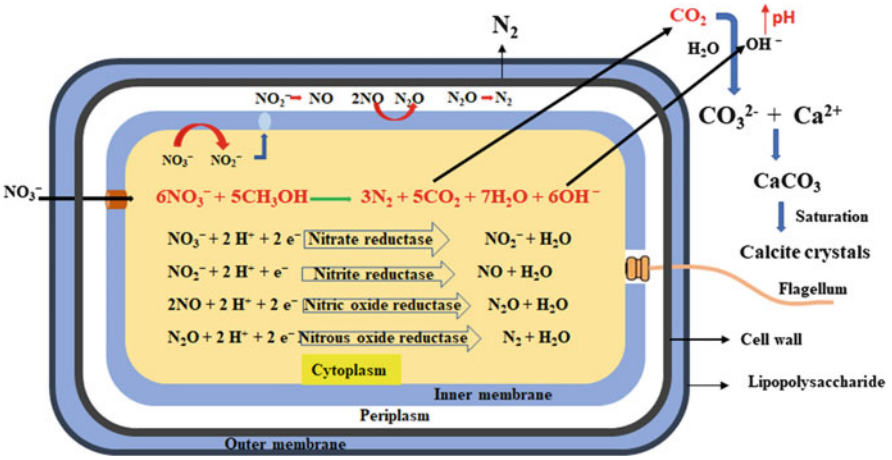


Fig. 5 An illustration of a gram-negative bacterium depicting the sequential nitrate reduction to N_2 and the concomitant release of CO_2 and OH^- from an organic compound

Unlike the other two systems, the ureolytic and oxidation of organic compounds, this system is not dependent on oxygen. Thus, it offsets the limitation of the other two systems that tend to heal the crack surfaces and leave the deeper part of the crack open due to poor oxygen availability. The system uses NO_3^- or NO_2^- as electron acceptor, and when the reduction is complete, it releases N_2.

A wide variety of microbes are known to reduce NO_3^- or NO_2^- during oxidation of organic compounds. Among such organisms *Diaphorobacter nitroreducens* and *B. sphaericus* have been considered as concrete self-healing agents [115]. The self-healing performance of concrete specimens containing *D. nitroreducens* was investigated by Ersan et al. [59, 115, 215]. In these studies, expanded clay was used to immobilize *D. nitroreducens* cells. The cells immobilized together with Ca-formate and Ca-nitrate were added into concrete during mixing and casting. The concrete was cracked and immersed in water for 4 weeks; cracked specimens with bacteria healed cracks up to 0.35 mm in width and absorbed 51% less water than the reference samples without cells. In addition to axenic cultures, non-axenic cultures have also been tried. Among the non-axenic cultures, activated compact denitrifying core (ACDC) is the most studied. ACDC is a denitrifying microbial community protected by various bacterial partners and obtained in a sequential batch reactor by applying selective stress conditions [215, 226]. Direct addition of the granulated axenic culture (70% biomass and 30% salt in composition) to concrete mix heals cracks of up to 0.5 mm in width within 3–14 weeks [215]. Moreover, concrete specimens containing this self-protected non-axenic granular culture were able to heal the inner cracks and significantly reduced the capillary water absorption compared to the specimens without the biological agent.

In addition to its ability of self-healing concrete cracks, the use of denitrifiers in concrete makes nitrite (NO_2^-) as a by-product which can serve as corrosion inhibitor for the concrete reinforcement steel, and this plays a vital role for the durability of

concrete structures. Studies made on the effect of NO_2^- on corrosion have been made using steel coupons. Results from the studies indicated effective corrosion inhibition of steel coupons due to the NO_2^- accumulation during biological NO_3^- reduction by denitrifiers [227]. In line with this, non-axenic culture of ACDC reduced corrosion in corrosive electrolyte solution (0.05 M NaCl) by producing about 57 mM NO_2^- in 1 week [226]. Moreover, corrosion experiments have been carried out by immersing cracked concretes specimens containing an embedded steel rod in 0.4 M chloride solution that mimics the chloride concentrations in seawater. Moreover, a 0.3 mm crack was completely healed. The use of ACDC system that generates corrosion inhibitor could postpone the time to corrosion initiation by a factor of 2.4 [19].

The solubility of the electron acceptor is an important parameter in determining the efficiency of the system. Since the solubility of O_2 in water (9.1 mg/L at 20°C) is up to 105 times lower than that of NO_3^-, the nitrate reduction seems more effective, and it is expected to be accompanied by an efficient calcite precipitation. The higher solubility of nitrate may favor the use of NO_3^- as an electron acceptor instead of O_2 during microbial oxidation of organic matter. Even enhanced $CaCO_3$ precipitation can be potentially achieved in nutrient-poor environments which makes the denitrification-based self-healing system feasible for self-healing concrete [59, 227]. This self-healing system is particularly attractive for applications in anoxic zones or healing deep cracks. However, it has been shown that the $CaCO_3$ precipitation efficiency of the denitrification system is much lower than that of ureolysis process [65]. Moreover, if the reduction process is incomplete, it may produce the nasty by-product N_2O, especially under aerobic condition [228]. Thus, it may be necessary to study the microbes intended for this application if they produce these highly potent ozone-depleting greenhouse gas during the healing process. So far, there is no available information in this regard.

4 Factors That Determine the Efficiency of Microbes in Enhancing Concrete Durability

The success of microbial preparations in maintenance and repair of concrete structures primarily relies on the efficiency of calcite precipitation. Usually, the more the precipitate is, the more efficient it becomes. To effectively seal cracks or coat the surface of a structure, it is important that the calcite precipitated is enough. The efficiency of MICCP is affected by several factors such as the type of organism used, the cell load, pH of the precipitation environment, temperature, concentration of calcium and carbonate, etc. [229–232]. These factors are important in microbial-based concrete, manual repair, self-healing, and biodeposition applications, and are discussed below.

4.1 Type of Microorganism and Cell Load

Nearly all organisms studied in treatment of concrete are those which precipitate calcite. This efficiency of microorganisms can vary depending on the mechanism of calcite induction, the organisms ability to raise the pH of the environment which in turn increases the carbonate concentration, the amount and nature of exopolysaccharide secreted by the organism, etc. Among the studies in this line, a great deal of attention had been given to urease producing organisms which are capable of precipitating high amounts of calcium. *Arthrobacter nicotianae* [125], *B. megaterium* [233, 234], *B. subtilis* [233], *Bacillus* sp. [235], *B. thuringiensis* [125, 233], *Sporosarcina pasteurii* [133, 236, 237], *Deleya halophila* [110], *Halomonas eurihalina* [72], *Kocuria flava* [118], *Lysinibacillus sphaericus* [233], *Methylocystis parvum* [223], *Myxococcus xanthus* [99], *Proteus mirabilis* [89], *Pseudomonas denitrificans* [238], *Sporosarcina ginsengisoli* [131], and *Stenotrophomonas maltophilia* [125] could be mentioned as examples. Abiotic factors such as the type and amount of nutrients available and degree of aeration are also known to influence calcite precipitation [239, 240].

The intended application dictates the type of microorganisms that have to be considered. For instance, for concrete surface treatment applications, organisms that can precipitate enough amount of calcium carbonate at reasonable pace and tolerate the pH of the concrete may be good enough. But microorganisms planned for self-healing concrete application need to be more resilient. At least in addition to carbonate precipitation and tolerance to high pH, organisms envisioned for self-healing application should be able to remain dormant preferably for a long time (spore former) and withstand the dryness and the anoxic nature of the concrete. Not only the type of microorganism but also the amount of cells used per unit volume affects the level of calcite precipitation, which in turn influences the efficiency of the treatment on concrete. Among urease producers, it has been shown that 10^6–10^8 cells per ml resulted in enough amount of calcite precipitate [241]. The amount of cells used influences the rate of urea hydrolysis more than the initial urea concentration. The mere increase of cell load from 10^6 to 10^8 cells/mL resulted in 30% more CO_3^{2-} in solution [241]. However, it is worth to note that high rate of calcite precipitation does not necessarily lead to the target effect on treated concrete structures. For instance, in a two-step immersion system for surface treatment of concrete (as described in Sect. 3.2), the bacteria that biodeposit calcite are only the ones retained on the concrete surface [3, 190]. On the other hand, in a one-step immersion system [187], potentially the entire amount of bacteria added to the mixture act on the urea that leads to rapid decomposition which is accompanied by fast $CaCO_3$ precipitation. However, this fast precipitation of calcite results in loose $CaCO_3$ particles instead of a dense and cohesive $CaCO_3$ layer [124]. The loose biodeposited matrix cannot serve the purpose and hence is not desirable. A similar observation, fast precipitation of $CaCO_3$, has been made when using urease instead of cells [242]. Another important issue related to microbial-induced calcite precipitation is the nature of the cell surface (cell wall and exopolysaccharides). It has been known that bacterial cells serve as nucleation sites for $CaCO_3$ precipitation [133],

and the availability of nucleation sites is important for calcite precipitations [230]. Comparative study on induced calcite precipitation at alkaline condition (pH 9, buffer and medium), in the absence or presence of cells has shown that 98% of the Ca^{2+} precipitated in the presence of microbial cells, but only 35 and 54% of the Ca^{2+} was precipitated in the buffered water and medium, respectively. This difference is due to the bacterial cells which provide the nucleation sites for $CaCO_3$ precipitation and its ability to maintain an alkaline environment for induction and further growth of calcite crystals [93]. Hence, it is possible to extrapolate that any factor that influences the amount and composition of the exopolysaccharide produced by the microbe has effect on the efficiency of calcite precipitation. Culture conditions (such as media composition, degree of aeration, pH of media, and cultivation temperature) are among such factors. The density of negative charges in microbial cell wall/EPS matrix which serves as the nucleation sites by attracting Ca^{2+} could also play a role in the efficiency of calcite precipitation. Alkaliphiles as a way of adaptation to the high pH environment evolved cell wall/EPS matrices with high density of anions, and this can be considered one benefit in using alkaliphiles in concrete application. It is not only the ureolytic process that is affected by the type and amount of cells, but the efficiency of the processes oxidizing organic compounds and denitrifying nitrates is also affected by it. The microbes should be at least tolerant to high pH and able to generate enough calcite under the circumstances [175].

4.2 pH

Calcite precipitation is influenced by pH [243]. This is mainly due to better solubility of CO_2 and high stability of carbonate at elevated pH. Moreover, in the case of urease-driven precipitation, the optimum pH for urease activity is in the alkaline range. Most of the microbial ureases studied are optimally active around pH 8 [133, 236]. Degradation of urea by urease generates NH_4^+ which raises the pH of the medium. The increase in pH or maintenance of the alkaline condition is ideal for calcite precipitation, because at low pH, the carbonate tends to dissolve rather than precipitate [244]. The CO_3^{2-} that ends up in the calcite precipitation is CO_2 driven. Since the solubility of CO_2 increases with pH, the CO_3^{2-} concentration rises concomitantly, and in the presence of Ca^{2+}, this facilitates the formation of $CaCO_3$. The CO_2 can be atmospheric or generated by cellular activities [230]. Most microbial-based calcite precipitation occurs under alkaline conditions from pH 8.7 to 9.5 [133, 245, 246]. However, it is not clear if this is due to the activity of urease or influenced by another factor. Salt concentration and temperature have a clear effect on the activity of urease. From this point of view, urease-producing alkaliphilic and halophilic microorganisms could be of interest for concrete applications. Recently, Stabnikov et al. [247] investigated whether haloalkaliphilic ureolytic bacteria are active at high concentrations of salt and high pH, conditions that are suitable for manufacturing biocement.

4.3 Temperature

The influence of temperature on bacterial and enzymatic activity, reactant solubility, and reaction rate is well known. A modest increase in temperature is often accompanied by an increase in bacterial growth and activity. The activity is related to the effect of temperature on the chemical and enzymatic reaction rates, and hence temperature directly affects MICCP. For instance, most ureases are optimally active in the range of 20–37°C, and their activity on urea is temperature dependent [241, 245, 248]. An increase in treatment temperature (up to its optimum) increases the rate of calcite precipitation [249]. On the other hand, the efficiency of calcite precipitation can drop with temperature. Thus, temperature is one of the important parameters that determine the success of microbial-based applications in concrete.

It appears that with rise in temperature, the rate of calcite precipitation increases, and with decreasing temperature, the rate of calcite formation falls. Both fast and slow rate of calcite deposition is not desirable. If $CaCO_3$ precipitates too fast, it will loosely accumulate, and this will not bring the desired effect [124]. If it is slow, it will take very long time to seal the cracks and that may incur loss. Thus, it is important to consider a moderate pace with lasting instead of transient carbonation process to achieve an effective treatment that brings the desired quality on the concrete. As different countries have different environmental temperature and humidity, it is very important to formulate the most suitable microbial agents that can optimally work in specific region of the world. In cold environments, it may be good to use organisms that are active at relatively low temperature. Whereas, since tropical regions are warmer in most of the year, the use of microbes that optimally grow around 30°C could be rewarding.

4.4 Urea, Ca^{2+}, and CO_3^{2-} Concentrations

From a chemical perspective, the concentrations of Ca^{2+} and CO_3^{2-} directly determine the amount of $CaCO_3$ precipitation [232]. Concrete contains a high amount of calcium; however, the amount of Ca^{2+} leached from the concrete is very small and often not enough to make good precipitation [250]. Thus, it is necessary to add an external Ca^{2+} source to the mixture. Different calcium sources like Ca-lactate, Ca-formate, Ca-gluconate, and Ca-nitrate have been considered. Even $CaCl_2$ has been used in many laboratory studies [165] despite the fact that it cannot be applied to concrete. In ureolytic process, urea is the source of $CO_3^{2-}$and hence can influence the precipitation process. However, the efficiency of $CaCO_3$ precipitation relies more on Ca^{2+} than urea concentration [241]. This could be partly due to the fact that unlike urea, the calcium added seems not metabolically utilized, but it accumulates outside the cell and remain readily available for precipitation of $CaCO_3$ [251]. Moreover, the activity of urease is stimulated by the presence of Ca^{2+}. Compared to Ca^{2+}-free system, a tenfold increase in the activity of urease has

been reported in the presence of 30 mM Ca^{2+} [252]. On the other hand, a higher concentration of calcium is inhibitory to urease [253].

A lot of concentration optimization studies have been done to identify the ideal concentrations for efficient calcite precipitation. However, the results are quite variable, and the optimum concentration of urea and Ca^{2+} was in the range of 50–500 mM, while the best urea to Ca^{2+} concentration ratio reported varies between 1:1 and 1:5 [18, 241, 254]. The contradicting optimal concentrations could be due to the difference in the nature of the strains, temperature, and media composition. Moreover, the optimal dosage of Ca^{2+} and urea can vary not only on the type of bacteria used but also on the amount of the microbial cells applied in the study. The other factor that possibly contributes to the observed variation regarding concentrations is the type of the calcium source. Achal and Pan [235] studied the effect of Ca^{2+} sources on carbonate precipitation using various calcium sources in urea – nutrient broth medium – and found out that calcium chloride was the best to produce calcite. However, due to the chloride ion undesirable effect, calcium chloride cannot be considered for concrete application.

4.5 Calcium Carbonate Crystal Morphology

The literature in this field uses the term "precipitation" for $CaCO_3$ crystal formation. A similar approach is adopted throughout this chapter. However, since this section deals about crystal morphologies, the term crystal is used instead of precipitate in this section for simplicity.

A relevant issue worth to mention in relation to microbially crystallized calcium carbonate is the crystal morphology. $CaCO_3$ is a polymorphic material with three different anhydrous crystalline morphs (Table 6). These crystals are calcite, aragonite, and vaterite, in order of increasing solubility and decreasing thermodynamic stability. The difference among these crystalline forms is the carbonate ions distribution with respect to the calcium ions [255].

Based on the properties of the crystal forms, calcite, the thermodynamically stable form, is the most desired crystal morph for concrete application. Moreover, compared to the other crystal morphs, calcite exhibits higher bonding strength with cement hydrates [256]. Fortunately, calcite is the primary product in many

Table 6 Properties of the anhydrous $CaCO_3$ crystal morphs

Type of crystal	Shape of the crystal	Specific gravity (g/cm^3)	Stability
Calcite	Hexagonal (rhombohedral)	2.71	Most stable form at room temperature and the least soluble crystal form
Aragonite	Orthorhombic	2.93	Stable at high temperature and high pressure
Vaterite	Hexagonal	2.65	Least stable anhydrous crystal polymorph, loosely packed, and most soluble crystal form

microbially driven $CaCO_3$ crystallization processes [133, 223, 241, 257]. However, formation of other crystal forms has also been noted. For instance, Rivadeneyra et al. [110] reported that *Deleya halophila* predominantly produce aragonite. In the case of *Proteus mirabilis*, the $CaCO_3$ produced is complex and has even an unusual morphology, vaterite hollow spheres [89]. This type of strain-based variation could be due to the difference in the nature of the extracellular polymeric substances (EPS) which dictates the formation of specific $CaCO_3$ crystal [105, 258] through peculiar Ca^{2+}-binding pattern that promotes the crystal formation [239]. Medium composition, calcium source, and the crystallizing conditions have also a profound impact on $CaCO_3$ crystal morphology [71, 239, 259, 260]. For instance, the type of crystal biodeposited by a *Myxococcus* strain can be vaterite or calcite depending on the composition of the medium [67, 261], which can possibly affect the nature of the EPS among other things. Calcium chloride characteristically induces formation of calcite [236, 262, 263]. On the other hand, calcium acetate induces a lettuce/lamellar shape crystal of vaterite, while calcium lactate and calcium gluconate result in a more complex form, a spherical-shape vaterite [264]. This possibly indicates that the microbes do not genetically or directly determine which crystal form to precipitate.

5 Conclusion

Construction is one of the biggest pillars of the world economy, and in the year 2020, the value of the global construction output is forecasted to reach about $10 trillion and projected to grow to $15.5 trillion by 2030. Concrete, being one of the most vital construction materials, has been in use for nearly two millennia and probably will remain as the dominant construction material in the foreseeable future. Thus, its role in the world economy is very prestigious. However, this remarkable construction material is prone to crack formation, which if not repaired will lead to deterioration and collapse of the structure. The world spends billions of dollars to protect and repair existing concrete structures. A variety of chemicals such as concrete admixtures, concrete adhesives, concrete sealants, and protective coatings are available in the market. Although these chemicals are used to repair and enhance the durability of concrete structures, it suffers from limitations. On the other hand, the use of biological means to improve the durability of concrete seems to have some advantages over the chemical-based approach. It is safe; the healing quality is remarkable, sustainable, and greener. Since the concrete industry has a negative image due to its high CO_2 footprint, consumption of resources, and generation of waste, the use of greener biological processes to improve concrete properties may also contribute to change the public opinion.

The last decade witnessed a tremendously growing interest in developing biologically based concrete: crack repair, self-healing, and protective coatings. Almost all the studies made in this regard focused on microbially induced calcite precipitation. The results of the laboratory scale studies made so far show promising results. The general trend indicates that the microbial-based system, as measured by the strength gain, reinforcement corrosion resistance, reduced water permeability, and

chloride mobility, has a great potential to emerge as an alternative approach in making durable concrete. However, it is not yet out of the woods. There are critical challenges that must be properly addressed to ensure the competitiveness of this approach. One of the most important limitation is the high production cost. It is vital to substantially cut down the production cost to enhance the applicability of this technology among others through strain selection, use of cheap media, and efficient and affordable cultivation-lyophilization and immobilization processes. For instance, the production cost of axenic cultures and the subsequent encapsulation is expensive; thus the use of non-axenic self-protected cultures may be the way forward. However, the efficiency of the non-axenic self-protected cultures needs to be further studied and proven. It should be noted that most of the studies reported are done in a very ideal laboratory conditions which is different from the real concrete environment. Thus, it is very difficult to expect a similar efficiency as achieved in laboratories in in situ application. What is achieved can be considered as a remarkable proof of concept, but it needs more field trial studies to close the gap between the laboratory ideal condition and the reality. It is delighting to see that a first step in commercialization has been taken by the company Basilisk. Such steps will lead forward to forge far efficient products that live up to its expectation.

Bacterial strains belonging to genus *Bacillus* are the gold standard microbes in this area of applications. However, fungi and algae could also be of interest as their use may offset some of the limitations related to bacterial applications. For example, nutrient and precipitation precursor translocation could be a benefit of using fungal strains. Moreover, the effort on developing biological agents for concrete application is almost entirely focused on calcite precipitation. But it may be beneficial to look other ways as well. One interesting approach could be silicate precipitation.

References

1. Van Tittelboom K, De Belie N (2013) Self-healing in cementitious materials – a review. Materials (Basel) 6:2182–2217
2. De Muynck W, Cox K, De Belie N, Verstraete W (2008) Bacterial carbonate precipitation as an alternative surface treatment for concrete. Constr Build Mater 22:875–885
3. De Muynck W, Debrouwer D, De Belie N, Verstraete W (2008) Bacterial carbonate precipitation improves the durability of cementitious materials. Cem Concr Res 38:1005–1014
4. Van Tittelboom K, de Belie N (2010) Self-healing concrete: suitability of different healing agents. Int J 3R's 1:12–21
5. Dhami N, Mukherjee A, Reddy MS (2012) Biofilm and microbial applications in biomineralized concrete. In: Seto J (ed) Advanced topics in biomineralization. IntechOpen, New York, pp 137–164. https://doi.org/10.5772/31124
6. Zhu T, Dittrich M (2016) Carbonate precipitation through microbial activities in natural environment, and their potential in biotechnology: a review. Front Bioeng Biotechnol 4:1–21
7. Cailleux E, Pollet V (2009) Investigations on the development of self-healing properties in protective coatings for concrete and repair mortars. In: Proceedings of 2nd international conference on self-healing materials, Chicago, IL, 28 June–1 July
8. Alonso C, Andrade C, Rodriguez J, Diez JM (1998) Factors controlling cracking of concrete affected by reinforcement corrosion. Mater Struct 31:435–441

9. Wang J, Tittelboom KV, De Belie N, Verstraete W (2012) Use of silica gel or polyurethane immobilized bacteria for self-healing concrete. Constr Build Mater 26:532–540
10. Wiktor VAC, Jonkers HM (2011) Quantification of crack-healing in novel bacteria based self-healing concrete. Cem Concr Compos 33:763–770
11. Wang J, Snoeck D, Van Vlierberghe S, Verstraete W, De Belie N (2014) Application of hydrogel encapsulated carbonate precipitating bacteria for approaching a realistic self-healing in concrete. Constr Build Mater 58:110–119
12. Zhang P, Wittmann FH, Haist M, Müller HS, Vontobel P, Zhao TJ (2014) Water penetration into micro-cracks in reinforced concrete. Restor Build Monum 20:85–94
13. De Belie N, De Muynck W (2008) Crack repair in concrete using biodeposition. In: Alexander MG, Beushausen HD, Dehn F, Moyo P (eds) Proceedings of ICCRR, Cape Town, South Africa. CRC Press, Leiden, pp 291–292
14. Wu M, Johannesson B, Geiker M (2012) A review: self-healing in cementitious materials and engineered cementitious composite as a self-healing material. Constr Build Mater 28:571–583
15. Mihashi H, Nishiwaki T (2012) Development of engineered self-healing and self-repairing concrete-state-of the-art report. J Adv Concr Technol 10:170–184
16. Snoeck D, de Belie N (2015) From straw in bricks to modern use of microfibers in cementitious composites for improved autogenous healing – a review. Constr Build Mater 95:774–787
17. Yıldırım G, Keskin ÖK, Keskin SB, Sahmaran M, Lachemi M (2015) A review of intrinsic self-healing capability of engineered cementitious composites: recovery of transport and mechanical properties. Constr Build Mater 101:10–21
18. De Muynck W, De Belie N, Verstraete W (2010) Microbial carbonate precipitation in construction materials: a review. Ecol Eng 36:118–136
19. De Belie N (2016) Application of bacteria in concrete: a critical review. RILEM Tech Lett 1:56–61
20. Sierra-Beltran MG, Jonkers HM, Schlangen E (2014) Characterization of sustainable bio-based mortar for concrete repair. Constr Build Mater 67:344–352
21. De Belie N (2010) Microorganisms versus stony materials: a love-hate relationship. Mater Struct 43:1191–1202
22. Hamilton WA (2003) Microbially influenced corrosion as a model system for the study of metal microbe interactions: a unifying electron transfer hypothesis. Biofouling 19:65–76
23. Lowenstam HA (1981) Minerals formed by organisms. Science 211:1126–1131
24. Lowenstam HA, Weiner S (1989) On biomineralization. Oxford University Press, New York
25. Frankel RB, Bazylinski DA (2003) Biologically induced mineralization by bacteria. Rev Mineral Geochem 54:95–114
26. Beveridge TJ (1989) Role of cellular design in bacterial metal accumulation and mineralization. Annu Rev Microbiol 43:147–171
27. Konhauser KO (1998) Diversity of bacterial iron mineralization. Earth Sci Rev 43:91–121
28. Bäuerlein E (2003) Biomineralization of unicellular organisms: an unusual membrane biochemistry for the production of inorganic nano- and microstructures. Angew Chem Int Ed 42:614–641
29. Schröder HC, Wang X, Tremel W, Ushijima H, Müller WE (2008) Biofabrication of biosilica-glass by living organisms. Nat Prod Rep 25:455–474
30. Tamerler C, Sarikaya M (2006) Molecular biomimetics: linking polypeptides to inorganic structures. In: Rehm B (ed) Microbial bionanotechnology: biological self-assembly systems and biopolymer-based nanostructures. Horizon Bioscience, Wymondham, pp 190–221
31. Legros M, Hemker KJ, LaVan DA, Sharpe WN, Rittner MN, Weertman JR (1996) Microtensile testing of nanocrystalline Al/Zr alloys. In: Komarneni S, Parker JC, Wollenberger HJ (eds) Nanophase and nanocomposite materials. Materials research society symposium proceedings, vol 457, pp 272–278. https://doi.org/10.1557/PROC-457-273
32. Meyers MA, Chen PY, Lin AYM, Seki Y (2008) Biological materials: structure and mechanical properties. Prog Mater Sci 53:1–206

33. Schröder HC, Brandt D, Schlossmacher U, Wang X, Tahir MN, Tremel W, Belikov SI, Müller WE (2007) Enzymatic production of biosilica glass using enzymes from sponges: basic aspects and application in nanobiotechnology (material sciences and medicine). Naturwissenschaften 94:339–359
34. Bäuerlein E (2000) Biomineralization: from biology to biotechnology and medical application. Wiley, Weinheim
35. Weiner S, Dove PM (2003) An overview of biomineralization processes and the problem of the vital effect. Rev Mineral Geochem 54:1–26
36. Bazylinski DA, Frankel RB, Konhauser KO (2007) Modes of biomineralization of magnetite by microbes. Geomicrobiol J 24:465–475
37. Ngwenya B (2016) Bacterial mineralisation. Reference module in materials science and materials engineering. Elsevier, Amsterdam. https://doi.org/10.1016/B978-0-12-803581-8.02248-7
38. Schippers A, Hedrich S, Vasters J, Drobe M, Sand W, Willscher S (2014) Biomining: metal recovery from ores with microorganisms. Adv Biochem Eng Biotechnol 141:1–47
39. Zavarzin GA (2002) Microbial geochemical calcium cycle. Microbiology 71:1–17
40. Zavarzina DG, Pchelintseva NF, Zhilina TN (1996) Calcium leaching by primary anaerobes. Mikrobiologiya 65:604–608
41. Douglas S, Beveridge TJ (1998) Mini review: mineral formation by bacteria in natural microbial communities. FEMS Microbiol Ecol 26:79–88
42. Suzuki Y, Kelly SD, Kemner KM, Banfield JF (2005) Direct microbial reduction and subsequent preservation of uranium in natural near-surface sediment. Appl Environ Microbiol 71:1790–1797
43. Bauerlein E (2004) Biomineralization. Progress in biology, molecular biology and application. Wiley, Weinheim
44. Reith F, Etschmann B, Grosse C, Moors H, Benotmane MA, Monsieurs P, Grass G, Doonan C, Vogt S, Lai B, Martinez-Criado G, George GN, Nies DH, Mergeay M, Pring A, Southam G, Brugger J (2009) Mechanisms of gold biomineralization in the bacterium *Cupriavidus metallidurans*. Proc Natl Acad Sci U S A 106:17757–17762
45. Iravani S (2014) Bacteria in nanoparticle synthesis: current status and future prospects. Int Sch Res Notices 2014:359316. https://doi.org/10.1155/2014/359316
46. Miller AG, Colman B (1980) Evidence of HCO_3 transport by the blue-green alga (cyanobacterium) *Coccochloris peniocystis*. Plant Physiol 65:397–402
47. Schultze-Lam S, Beveridge TJ (1994) Nucleation of celestite and strontianite on a cyanobacterial S-layer. Appl Environ Microbiol 60:447–453
48. Hamilton TL, Bryant DA, Macalady JL (2016) The role of biology in planetary evolution: cyanobacterial primary production in low-oxygen Proterozoic oceans. Environ Microbiol 18:325–340
49. Dejong JT, Fritzges MB, Nüsslein K (2006) Microbially induced cementation to control sand response to undrained shear. J Geotech Geoenviron Eng 132:1381–1392
50. Sen R (2008) Biotechnology in petroleum recovery: the microbial EOR. Prog Energy Combust Sci 34:714–724
51. Bang SS, Lippert JJ, Yerra U, Mulukutla S, Ramakrishnan V (2010) Microbial calcite, a bio-based smart nanomaterial in concrete remediation. Int J Smart Nano Mater 1:28–39
52. Chou CW, Seagren EA, Aydilek AH, Lai M (2011) Biocalcification of sand through ureolysis. J Geotech Geoenviron Eng 137:1179–1189
53. Ehrlich HL (1996) Geomicrobiology. Marcel Dekker, New York
54. Gadd GM (2010) Metals, minerals and microbes: geomicrobiology and bioremediation. Microbiology 156:609–643
55. Grotzinger JP, Knoll AH (1999) Stromatolites in Precambrian carbonates: evolutionary mileposts or environmental dipsticks? Annu Rev Earth Planet Sci 27:313–358

56. Thompson JB, Schultze-Lam S, Beveridge TJ, Des Marais DJ (1997) Whiting events: biogenic origin due to the photosynthetic activity of cyanobacterial picoplankton. Limnol Oceanogr 42:133–141
57. Wang JY, Ersan YC, Boon N, De Belie N (2016) Application of microorganisms in concrete: a promising sustainable strategy to improve concrete durability. Appl Microbiol Biotechnol 100:2993–3007
58. Hamdan N (2013) Carbonate mineral precipitation for soil improvement through microbial denitrification. MSc dissertation, Arizona State University, Tempe
59. Erşan YÇ, De Belie N, Boon N (2015) Microbially induced $CaCO_3$ precipitation through denitrification: an optimization study in minimal nutrient environment. Biochem Eng J 101:108–118
60. Reeburgh WS (2007) Oceanic methane biogeochemistry. Chem Rev 107:486–513
61. Merz MUE (1992) The biology of carbonate precipitation by cyanobacteria. Facies 26:81–102
62. Baumgartner LK, Reid RP, Dupraz C, Decho AW, Buckley D, Spear JR, Przekop KM, Visscher PT (2006) Sulfate reducing bacteria in microbial mats: changing paradigms, new discoveries. Sediment Geol 185:131–145
63. Fujita Y, Ferris FG, Lawson RD, Colwell FS, Smith RW (2000) Calcium carbonate precipitation by ureolytic subsurface bacteria. Geomicrobiol J 17:305–318
64. Achal V, Mukherjee A (2015) A review of microbial precipitation for sustainable construction. Constr Build Mater 93:1224–1235
65. Van Paassen LA, Daza CM, Staal M, Sorokin DY, van der Zon W, van Loosdrecht MCM (2010) Potential soil reinforcement by biological denitrification. Ecol Eng 36:168–175
66. Dupraz C, Visscher PT, Baumgartner LK, Reid RP (2004) Microbe-mineral interactions: early carbonate precipitation in a hypersaline lake (Eleuthera Island, Bahamas). Sedimentology 51:745–765
67. González-Muñoz MT, Rodriguez-Navarro C, Martínez-Ruiz F, Arias JM, Merroun ML, Rodriguez-Gallego M (2010) Bacterial biomineralization: new insights from *Myxococcus*-induced mineral precipitation. Geol Soc Spec Publ 336:31–50
68. Braissant O, Decho AW, Dupraz C, Glunk C, Przekop KM, Visscher PT (2007) Exopolymeric substances of sulfate-reducing bacteria: interactions with calcium at alkaline pH and implication for formation of carbonate minerals. Geobiology 5:401–411
69. Rivadeneyra MA, Delgado R, Del Moral A, Ferrer MR, Ramos-Cormenzana A (1994) Precipitation of calcium carbonate by *Vibrio* spp. from an inland saltern. FEMS Microb Ecol 13:197–204
70. Barabesi C, Galizzi A, Mastromei G, Rossi M, Tamburini E, Perito B (2007) *Bacillus subtilis* gene cluster involved in calcium carbonate biomineralization. J Bacteriol 189:228–235
71. Hammes F, Verstraete W (2002) Key roles of pH and calcium metabolism in microbial carbonate precipitation. Rev Environ Sci Biotechnol 1:3–7
72. Rivadeneyra MA, Delgado G, Ramos-Cormenzana A, Delgado R (1998) Biomineralization of carbonates by *Halomonas eurihalina* in solid and liquid media with different salinities: crystal formation sequence. Res Microbiol 149:277–287
73. Knorre H, Krumbein KE (2000) Bacterial calcification. In: Riding EE, Awramik SM (eds) Microbial sediments. Springer, Berlin, pp 25–31
74. Rivadeneyra MA, Parraga J, Delgado R, Ramos-Cormenzana A, Delgado G (2004) Biomineralization of carbonates by *Halobacillus trueperi* in solid and liquid media with different salinities. FEMS Microbiol Ecol 48:39–46
75. Boquet E, Boronat A, Ramos-Cormenzana A (1973) Production of calcite (calcium carbonate) crystals by soil bacteria is a general phenomenon. Nature 246:527–529
76. Marvasi M, Visscher PT, Perito B, Mastromei G, Casillas-Martinez L (2010) Physiological requirements for carbonate precipitation during biofilm development of *Bacillus subtilis etf*A mutant. FEMS Microbiol Ecol 71:341–350
77. Castanier S, Le Metayer-Levrel G, Orial G, Loubiere JF, Perthuisot JP (1999) Bacterial carbonatogenesis and applications to preservation and restoration of historic property. In:

Ciferri O, Tiano P, Mastromei G (eds) Proceedings of microbes and art: the role of microbial communities on the degradation and protection of cultural heritage. CNR, Florence, pp 203–218
78. Braissant O, Verrecchia E, Aragno M (2002) Is the contribution of bacteria to terrestrial carbon budget greatly underestimated? Naturwissenschaften 89:366–370
79. Castanier S, Le Metayer-Levrel G, Perthuisot JP (1999) Ca-carbonates precipitation and limestone genesis – the microbiogeologist point of view. Sediment Geol 126:9–23
80. Morita RY (1980) Calcite precipitation by marine bacteria. Geomicrobiology 2:63–82
81. Ehrlich HL (1998) Geomicrobiology: its significance for geology. Earth Sci Rev 45:45–46
82. Braissant O, Cailleau G, Dupraz C, Verrecchia EP (2003) Bacterially induced mineralization of calcium carbonate in terrestrial environments: the role of exopolysaccharides and amino acids. J Sediment Res 73:485–490
83. Ercole C, Cacchio P, Botta AL, Centi V, Lepidi A (2007) Bacterially induced mineralization of calcium carbonate: the role of exopolysaccharides and capsular polysaccharides. Microsc Microanal 13:42–50
84. Dupraz C, Visscher PT (2005) Microbial lithification in marine stromatolites and hypersaline mats. Trends Microbiol 13:429–438
85. Wright DT, Oren A (2005) Nonphotosynthetic bacteria and the formation of carbonates and evaporites through time. Geomicrobiol J 22:27–53
86. Wright DT, Wacey D (2005) Precipitation of dolomite using sulphate-reducing bacteria from the Coorong Region, South Australia: significance and implications. Sedimentology 52:987–1008
87. Ferrer MR, Quevedo-Sarmiento J, Rivadeneyra MA, Bejar V, Delgado G, Ramos-Cormenzana A (1988) Calcium carbonate precipitation by two groups of moderately halophilic microorganisms at different temperatures and salt concentrations. Curr Microbiol 17:221–227
88. Susana GF, Maria CI, Humberto JS (2002) Effect of a cyanobacterial community on calcium carbonate precipitation in Puente del Inca (Mendoza, Argentina). Acta Bot Croat 61:1–9
89. Chen L, Shen Y, Xie A, Huang B, Jia R, Guo R, Tang W (2009) Bacteria-mediated synthesis of metal carbonate minerals with unusual morphologies and structures. Crys Growth Des 9:743–754
90. Van Waasbergen LG, Balkwill DL, Crocker FH, Bjornstad BN, Miller RV (2000) Genetic diversity among arthrobacter species collected across a heterogeneous series of terrestrial deep-subsurface sediments as determined on the basis of 16S rRNA and recA gene sequences. Appl Environ Microbiol 66:3454–3463
91. Cacchio P, Ercole C, Cappuccio G, Lepidi A (2003) Calcium carbonate precipitation by bacterial strains isolated from a limestone cave and from a loamy soil. Geomicrobiol J 20:85–98
92. Fredrickson JK, Zachara JM, Balkwill DL, Kennedy D, Li SW, Kostandarithes HM, Daly MJ, Romine MF, Brockman FJ (2004) Geomicrobiology of high-level nuclear waste-contaminated vadose sediments at the Hanford site, Washington State. Appl Environ Microbiol 70:4230–4241
93. Barrientos-Díaz L, Gidekel M (2008) Characterization of rhizospheric bacteria isolated from *Deschampsia antarctica* Desv. World J Microbiol Biotechnol 24:2289–2296
94. Aloisi G (2008) The calcium carbonate saturation state in cyanobacterial mats throughout Earth's history. Geochim Cosmochim Acta 72:6037–6060
95. Kremer B, Kazmierczak J, Stal LJ (2008) Calcium carbonate precipitation in cyanobacterial mats from sandy tidal flats of the North Sea. Geobiology 6:46–56
96. Tiano P, Biagiotti L, Mastromei G (1999) Bacterial bio-mediated calcite precipitation for monumental stones conservation: methods of evaluation. J Microbiol Methods 36:139–145
97. Le Metayer-Levrel G, Castanier S, Orial G, Loubiere JF, Perthuisot JP (1999) Applications of bacterial carbonatogenesis to the protection and regeneration of limestones in buildings and historic patrimony. Sediment Geol 126:25–34

98. Castainer S, Le MG, Perthuisot JP (2000) Bacterial roles in the precipitation of carbonate minerals. In: Riding RE, Awramik SM (eds) Microbial sediments. Springer, Heidelberg, pp 32–39
99. Ben Chekroun K, Rodríguez-Navarro C, González-Muñoz MT, Arias JM, Cultrone G, Rodríguez-Gallego M (2004) Precipitation and growth morphology of calcium carbonate induced by *Myxococcus Xanthus*: implications for recognition of bacterial carbonates. J Sediment Res 74:868–876
100. Dick J, Windt W, Graef B, Saveyn H, Meeren P, De Belie N, Verstraete W (2006) Biodeposition of a calcium carbonate layer on degraded limestone by *Bacillus* species. Biodegradation 17:357–367
101. Baskar S, Baskar R, Mauclaire L, Mc Kenzie JA (2006) Microbially induced calcite precipitation in culture experiments: possible origin for stalactites in Sahastradhara caves, Dehradun, India. Curr Sci India 90:58–64
102. González-Muñoz MT (2008) Bacterial biomineralization applied to the protection-consolidation of ornamental stone: current development and perspectives. Coalition 15:12–18
103. Jroundi F, Merroun ML, Arias JM, Rossberg A, Selenska-Pobell S, González-Muñoz MT (2007) Spectroscopic and microscopic characterization of uranium biomineralization in *Myxococcus xanthus*. Geomicrobiol J 24:441–449
104. Jonkers HM, Thijssen A, Muyzer G, Copuroglu O, Schlangen E (2010) Application of bacteria as self-healing agent for the development of sustainable concrete. Ecol Eng 36:230–235
105. Tourney J, Ngwenya BT (2009) Bacterial extracellular polymeric substances (EPS) mediate $CaCO_3$ morphology and polymorphism. Chem Geol 262:138–146
106. Riding R (2009) An atmospheric stimulus for cyanobacterial-bioinduced calcification ca. 350 million years ago? Palaios 24:685–696
107. Planavsky N, Reid RP, Lyons TW, Myshrall KL, Visscher PT (2009) Formation and diagenesis of modern marine calcified cyanobacteria. Geobiology 7:566–576
108. Kamennaya NA, Ajo-Franklin CM, Northen T, Jansson C (2013) Cyanobacteria as biocatalysts for carbonate mineralization. Minerals 2:338–364
109. Whiffin VS (2004) Microbial $CaCO_3$ precipitation for the production of biocement. Dissertation, School of Biological Sciences and Biotechnology, Murdoch University, Perth
110. Rivadeneyra MA, Ramos-Cormenzana A, Delgado G, Delgado R (1996) Process of carbonate precipitation by *Deleya halophila*. Curr Microbiol 32:308–313
111. Zippel B, Dynes JJ, Obst M, Lawrence JR, Neu TR (2010) EPS composition and calcification potential of tufa-dominating cyanobacteria investigated by Scanning Transmission X-ray Microscopy (STXM) and Laser Scanning Microscopy (LSM). Earth Environ Sci 1:26–27
112. Atlan G, Delattre O, Berland S, LeFaou A, Nabias G, Cot D et al (1999) Interface between bone and nacre implants in sheep. Biomaterials 20:1017–1022
113. Gauri KL, Bandyopadhyay JK (1999) Carbonate stone: chemical behaviour, durability and conservation. Springer, New York
114. May E (2005) Biobrush research monograph: novel approaches to conserve our European heritage. EVK4-CT-2001-00055
115. Ersan YC, Da Silva FB, Boon N, Verstraete W, De Belie N (2015) Screening of bacteria and concrete compatible protection materials. Constr Build Mater 88:196–203
116. Santomauro G, Baier J, Huang W, Pezold S, Bill J (2012) Formation of calcium carbonate polymorphs induced by living microalgae. J Biomater Nanobiotechnol 3:413–420
117. Foster JS, Green SJ, Ahrendt SR, Golubic S, Reid RP, Hetherington KL, Bebout L (2009) Molecular and morphological characterization of cyanobacterial diversity in the stromatolites of Highborne Cay, Bahamas. ISME J 3:573–587
118. Achal V, Pan X, Zhang D (2011) Remediation of copper-contaminated soil by *Kocuria flava* CR1 based on microbially induced calcite precipitation. Ecol Eng 37:1601–1605
119. Ghosh P, Mandal S, Chattopadhyay BD, Pal S (2005) Use of microorganism to improve the strength of cement mortar. Cem Concr Res 35:1980–1983

120. Park S, Park Y, Chun W, Kim W, Ghim S (2010) Calcite forming bacteria for compressive strength improvement in mortar. J Microbiol Biotechnol 20:782–788
121. Day JL, Ramakrishnan V, Bang SS (2003) Microbiologically induced sealant for concrete crack remediation. In: Proceedings of the 16th engineering mechanics conference, Seattle, WA, pp 1–8
122. Ramachandran SK, Ramakrishnan V, Bang SS (2001) Remediation of concrete using microorganisms. ACI Mater J 98:3–9
123. Ramakrishnan V (2007) Performance characteristics of bacterial concrete – a smart biomaterial. In: Proceedings of the 1st international conference on recent advances in concrete technology, Washington, DC, pp 67–78
124. Rodriguez-Navarro C, Rodriguez-Gallego M, Ben Chekroun K, Gonzalez-Munoz MT (2003) Conservation of ornamental stone by *Myxococcus xanthus*-induced carbonate biomineralization. Appl Environ Microbiol 69:2182–2193
125. Park JM, Park SJ, Ghim SY (2013) Characterization of three antifungal calcite-forming bacteria, *Arthrobacter nicotianae* KNUC2100, *Bacillus thuringiensis* KNUC2103, and *Stenotrophomonas maltophilia* KNUC2106, derived from the Korean islands, Dokdo and their application on mortar. J Microbiol Biotechnol 23:1269–1278
126. Jroundi F, Fernandez-Vivas A, Rodriguez-Navarro C, Bedmar EJ, Gonzalez-Munoz MT (2010) Bioconservation of deteriorated monumental calcarenite stone and identification of bacteria with carbonatogenic activity. Microb Ecol 60:39–54
127. Ettenauer J, Piñar G, Sterflinger K, González-Muñoz MT, Jroundi F (2011) Molecular monitoring of the microbial dynamics occurring on historical limestone buildings during and after the *in situ* application of different bio-consolidation treatments. Sci Total Environ 409:5337–5352
128. Ercole C, Bozzelli P, Altieri F, Cacchio P, Santacecilia A, Del Gallo M (2012) Exopolymeric substances involved in calcium carbonate biomineralization and their use to preserve and restore stone monuments. Environ Eng Manag J 11:85–90
129. Riding R (2000) Microbial carbonates: the geological record of calcified bacterial-algal mats and biofilms. Sedimentology 47:179–214
130. Banks ED, Taylor NM, Gulley J, Lubbers BR, Giarrizzo JG, Bullen HA, Hoehler TM, Barton HA (2010) Bacterial calcium carbonate precipitation in cave environments: a function of calcium homeostasis. Geomicrobiol J 27:444–454
131. Achal V, Pan X, Zhang D (2012) Bioremediation of strontium (Sr) contaminated aquifer quartz sand based on carbonate precipitation induced by Sr resistant *Halomonas* sp. Chemosphere 89:764–768
132. Achal V, Pan X, Zhang D, Fu Q (2012) Bioremediation of Pb-contaminated soil based on microbially induced calcite precipitation. J Microbiol Biotechnol 22:244–247
133. Stocks-Fischer S, Galinat JK, Bang SS (1999) Microbiological precipitation of $CaCO_3$. Soil Biol Biochem 31:1563–1571
134. Mitchell AC, Ferris FG (2006) The influence of *Bacillus pasteurii* on the nucleation and growth of calcium carbonate. Geomicrobiol J 23:213–22694
135. Wedepohl KH (1995) The composition of the continental crust. Geochim Cosmochim Acta 59:1217–1232
136. Cha JN, Shimizu K, Zhou Y, Christiansen SC, Chmelka BF, Stucky GD, Morse DE (1999) Silicatein filaments and subunits from a marine sponge direct the polymerization of silica and silicones *in vitro*. Proc Natl Acad Sci U S A 96:361–365
137. Kröger N, Deutzmann R, Sumper M (1999) Polycationic peptides from diatom biosilica that direct silica nanosphere formation. Science 286:1129–1132
138. Doi K, Fujino Y, Inagaki F, Kawatsu R, Tahara M, Ohshima T, Okaue Y, Yokoyama T, Iwai S, Ogata S (2009) Stimulation of expression of a silica-induced protein (Sip) in *Thermus thermophilus* by supersaturated silicic acid. Appl Environ Microbiol 75:2406–2413

139. Wang X, Schröder HC, Wiens M, Schloßmacher U, Müller WE (2012) Biosilica: molecular biology, biochemistry and function in demosponges as well as its applied aspects for tissue engineering. Adv Mar Biol 62:231–271
140. Konhauser KO, Ferris FG (1996) Diversity of iron and silica precipitation by microbial mats in hydrothermal waters, Iceland: Implications for Precambrian iron formations. Geology 24:323–326
141. Konhauser KO, Fyfe WS, Ferris FG, Beveridge TJ (1993) Metal sorption and mineral precipitation by bacteria in two Amazonian river systems, Rio Solimoìes and Rio Negro, Brazil. Geology 21:1103–1106
142. Konhauser KO, Schultze-Lam S, Ferris FG, Fyfe WS, Longsta FJ, Beveridge TJ (1994) Mineral precipitation by epilithic biofilms in the Speed River, Ontario, Canada. Appl Environ Microbiol 60:549–553
143. Fortin D, Davis B, Beveridge TJ (1996) Role of *Thiobacillus* and sulfate reducing bacteria in iron biocycling in oxic and acidic mine tailings. FEMS Microbiol Ecol 21:11–24
144. Peng X, Zhou H, Yao H, Li J, Tang S, Jiang L, Wu Z (2007) Microbe-related precipitation of iron and silica in the Edmond deep-sea hydrothermal vent field on the Central Indian Ridge. Chin Bull 52:3233–3238
145. Konhauser KO, Jones B, Phoenix VR, Ferris G, Renaut RW (2004) The microbial role in hot spring silicification. Ambio 33:552–558
146. Yee N, Phoenix VR, Konhauser KO, Benning LG, Ferris FG (2003) The effect of cyanobacteria on silica precipitation at neutral pH: implications for bacterial silicification in geothermal hot springs. Chem Geol 199:83–90
147. Urrutia-Mera M, Beveridge TJ (1993) Mechanism of silicate binding to the bacterial cell wall in *Bacillus subtilis*. J Bacteriol 175:1936–1945
148. Urrutia MM, Beveridge TJ (1994) Formation of fine-grained metal and silicate precipitates on a bacterial surface (*Bacillus subtilis*). Chem Geol 116:261–280
149. Urrutia MM, Beveridge TJ (1995) Formation of short range ordered aluminosilicates in the presence of a bacterial surface (*Bacillus subtilis*) and organic ligands. Geoderma 65:149–165
150. Burne RV, Moore LS (1987) Microbialites, organo-sedimentary deposits of benthic microbial communities. Palaios 2:241–254
151. Walker SG, Flemming CA, Ferris FG, Beveridge TJ, Bailey GW (1989) Physicochemical interaction of *Escherichia coli* cell envelopes and *Bacillus subtilis* cell walls with two clays and ability of the composite to immobilize heavy metals from solution. Appl Environ Microbiol 55:2976–2984
152. Flemming CA, Ferris FG, Beveridge TJ, Bailey GW (1990) Remobilisation of toxic heavy metals adsorbed to bacterial wall-clay composites. Appl Environ Microbiol 56:3191–3203
153. Furukawa Y, O'Reilly SE (2007) Rapid precipitation of amorphous silica in experimental systems with nontronite (NAu-1) and *Shewanella oneidensis* MR-1. Geochim Cosmochim Acta 71:363–377
154. Ghosh S, Biswas M, Chattopadhyay BD, Mandal S (2009) Microbial activity on the microstructure of bacteria modified mortar. Cem Concr Compos 31:93–98
155. Biswas M, Majumdar S, Chowdhury T, Chattopadhyay B, Mandal S, Halder U, Yamasaki S (2010) Bioremediase a unique protein from a novel bacterium BKH1, ushering a new hope in concrete technology. Enzym Microb Technol 46:581–587
156. Majumdar S, Sarkar M, Chowdhury T, Chattopadhyay B, Mandal S (2012) Use of bacterial protein powder in commercial fly ash pozzolana cements for high performance construction materials. Open J Civil Eng 2:218–228
157. Chattopadhyay B, Mandal S (2013) Hot spring bacterial strain BKH1 and protein isolated therefrom, concrete compositions, and uses thereof. US 8476039 B2
158. Siddique R, Chahal NK (2011) Effect of ureolytic bacteria on concrete properties. Constr Build Mater 25:3791–3801

159. Kumar VR, Bhuvaneshwari B, Maheswaran S, Palani GS, Ravisankar K, Iyer NR (2011) An overview of techniques based on biomimetics for sustainable development of concrete. Curr Sci 101:741–747
160. Sarayu K, Iyer NR, Murthy AR (2014) Exploration on the biotechnological aspect of the ureolytic bacteria for the production of the cementitious materials – a review. Appl Biochem Biotechnol 172:2308–2323
161. De Belie N, Gruyaert E, Al-Tabbaa A, Antonaci P, Baera C, Bajare D, Darquennes A, Davies R, Ferrara L, Jefferson T, Litina C, Miljevic B, Otlewska A, Ranogajec J, Roig-Flores M, Paine K, Lukowski P, Serna P, Tulliani JM, Vucetic S, Wang J, Jonker HM (2018) A review of self-healing concrete for damage management of structures. Adv Mater Interfaces 5:1–28
162. Bang SS, Galinat JK, Ramakrishnan V (2001) Calcite precipitation induced by polyurethane immobilized *Sporosarcina pasteurii*. Enzym Microb Technol 28:404–409
163. Ramakrishnan V, Ramesh KP, Bang SS (2001) Bacterial concrete. In: Wilson AR, Asanuma H (eds) Smart materials. Proceedings of the Society of Photo-Optical Instrumentation Engineers (SPIE) 4234, pp 168–176
164. Krishnapriya S, Venkatesh Babu DLV, Arulraj GP (2015) Isolation and identification of bacteria to improve the strength of concrete. Microbiol Res 174:48–55
165. Seifan M, Samani AK, Berenjian A (2016) Bioconcrete: next generation of self-healing concrete. Appl Microbiol Biotechnol 100:2591–2602
166. Irwan JM, Anneza LH, Othman N, Alshalif AF, Zamer MM, Teddy T (2017) Mechanical properties of concrete with *Enterococcus Faecalis* and calcium lactate. Procedia Eng 171:592–597
167. Ramakrishnan V, Deo KS, Duke EF, Bang SS (1999) SEM investigation of microbial calcite precipitation in cement. In: Proceeding of the 21st international conference on cement microscopy, Las Vegas, NV, pp 406–414
168. Achal V, Mukherjee A, Reddy MS (2010) Biocalcification by *Sporosarcina pasteurii* using corn steep liquor as nutrient source. Ind Biotechnol 6:170–174
169. Sharma TK, Alazhari M, Heath A, Paine K, Cooper RM (2017) Alkaliphilic *Bacillus* species show potential application in concrete crack repair by virtue of rapid spore production and germination then extracellular calcite formation. J Appl Microbiol 122:1233–1244
170. Achal V, Mukerjee A, Reddy MS (2013) Biogenic treatment improves the durability and remediates the cracks of concrete structures. Constr Build Mater 48:1–5
171. Jonkers HM, Schlangen E (2009) A two component bacteria-based self-healing concrete. In: Alexander NG, Beaushausen H-D, Dehn F, Moyo P (eds) Proceedings of 2nd international conference on concrete repair, rehabilitation and retrofitting II (ICCRRR-2), Cape Town, 2008 November 24–26. Taylor & Frances, London, pp 215–220
172. Wang JY, Soens H, Verstraete W, De Belie N (2014) Self-healing concrete by use of microencapsulated bacterial spores. Cem Concr Res 56:139–152
173. Sarode DD, Mukherjee A (2009) Microbial precipitation for repairs of concrete structures. In: Grantham M, Majorana C, Salomoni V (eds) Concrete solutions. CRC Press, Boca Raton, pp 191–198
174. Wiktor V, Jonkers HM (2015) Field performance of bacteria-based repair system: pilot study in a parking garage. Case Stud Constr Mater 2:11–17
175. Tziviloglou E, van Tittelboom K, Palin D, Wang J, Sierra Beltran MG, Ersan YC, Mors M, Wiktor VAC, Jonkers HM, Schlangen E, de Belie N (2016) Bio-based self-healing concrete: from research to field application. Adv Polym Sci 273:346–385
176. Basheer L, Kropp J, Cleland DJ (2001) Assessment of the durability of concrete from its permeation properties: a review. Constr Build Mater 15:93–103
177. Perez JL, Villegas R, Vale JF, Bello MA, Alcade M (1995) Effects of consolidant and water repellent treatments on the porosity and pore size distribution of limestones. In: Proceedings of international colloquium: methods of evaluating products for conservation of porous building materials in monuments, ICCROM, Rome, pp 203–211

178. Adolphe JM, Loubiere JF, Paradas J, Soleilhavoup F (1990) Procédé traitement biologique d'une surface artificielle. European patent 90400G97.0 (after French patent 8903517, 1989)
179. De Muynck W, Boon N, De Belie N (2014) From lab scale to in situ applications – the ascent of a biogenic carbonate based surface treatment. In: Quattrone M, John VM (eds) XIII international conference on durability of building materials and components (DBMC), 2014 September 2–5, São Paulo, Brazil, pp 728–735
180. Moropoulou A, Kouloumbi N, Haralampopoulos G, Konstanti A, Michailidis P (2003) Criteria and methodology for the evaluation of conservation interventions on treated porous stone susceptible to salt decay. Prog Org Coat 48:259–270
181. Vintzileou E, Miltiadou-Fezans A (2008) Mechanical properties of three leaf stone masonry grouted with ternary or hydraulic lime-based grouts. Eng Struct 30:2265–2276
182. Kalagri A, Miltiadou-Fezans A, Vintzileou E (2010) Design and evaluation of hydraulic lime grouts for the strengthening of stone masonry historic structures. Mater Struct 43:1135–1146
183. Jimenez-Lopez C, Rodriguez-Navarro C, Pinar G, Carrillo-Rosua FJ, Rodriguez-Gallego M, Gonzalez-Munoz MT (2007) Consolidation of degraded ornamental porous limestone stone by calcium carbonate precipitation induced by the microbiota inhabiting the stone. Chemosphere 68:1929–1936
184. Jimenez-Lopez C, Jroundi F, Pascolini C, Rodriguez-Navarro C, Pinar-Larrubia G, Rodriguez-Gallego M, Gonzalez-Munoz MT (2008) Consolidation of quarry calcarenite by calcium carbonate precipitation induced by bacteria activated among the microbiota inhabiting the stone. Int Biodeterior Biodegrad 62:352–363
185. De Muynck W, Leuridan S, Van Loo D, Verbeken K, Cnudde V, De Belie N, Verstraete W (2011) Influence of pore structure on the effectiveness of a biogenic carbonate surface treatment for limestone conservation. Appl Environ Microbiol 77:6808–6820
186. Tiano P, Cantisani E, Sutherland I, Paget JM (2006) Biomediated reinforcement of weathered calcareous stones. J Cult Herit 7:49–55
187. Qian CX, Wang JY, Wang RX, Cheng L (2009) Corrosion protection of cement-based building materials by surface deposition of $CaCO_3$ by *Bacillus pasteurii*. Mater Sci Eng C 29:1273–1280
188. Okwadha G, Li J (2011) Biocontainment of polychlorinated biphenyls (PCBs) on flat concrete surfaces by microbial carbonate precipitation. J Environ Manag 92:2860–2864
189. Achal V, Mukherjee A, Goyal S, Reddy MS (2012) Corrosion prevention of reinforced concrete with microbial calcite precipitation. ACI Mater J 109:157–163
190. Nosouhian F, Mostofinejad D, Hasheminejad H (2015) Influence of biodeposition treatment on concrete durability in a sulphate environment. Biosyst Eng 133:141–152
191. Li P, Qu W (2012) Microbial carbonate mineralization as an improvement method for durability of concrete structures. Adv Mater Res 365:280–286
192. Achal V, Mukherjee A, Reddy MS (2011) Effect of calcifying bacteria on permeation properties of concrete structures. J Ind Microbiol Biotechnol 38:1229–1234
193. De Muynck W, Verbeken K, De Belie N, Verstraete W (2010) Influence of urea and calcium dosage on the effectiveness of bacterially induced carbonate precipitation on limestone. Ecol Eng 36:99–111
194. Richardson A, Coventry K, Pasley J (2016) Bacterial crack sealing and surface finish application to concrete. In: Claisse P, Ganjian E, Naik T (eds) Fourth international conference on sustainable construction materials and technologies, Las Vegas, 7–11 Aug
195. FHWA (Federal Highway Administration) (2002) Corrosion cost and preventive strategies in the United States. Publication No. FHWA-RD-01-156. http://impact.nace.org/documents/ccsupp.pdf
196. Silva FP, Boon N, De Belie N, Verstraete W (2015) Industrial application of biological self-healing concrete: challenges and economical feasibility. J Commer Biotechnol 21:31–38
197. Setlow P (1994) Mechanisms which contribute to the long-term survival of spores of Bacillus species. J Appl Bacteriol 76:S49–S60

198. Basaran Z (2013) Biomineralization in cement based materials: inoculation of vegetative cells. Dissertation, University of Texas, Austin
199. Achal V, Mukherjee A, Reddy MS (2010) Isolation and characterization of urease producing and calcifying bacteria from cement. J Microbiol Biotechnol 20:1571–1576
200. Jonkers HM, Schlangen E (2007) Self-healing of cracked concrete: a bacterial approach. In: Proceedings of the 6th international conference on fracture mechanics of concrete and concrete structures. Italy, vol 3, pp. 1821–1826
201. Silva FB (2015) Up-scaling the production of bacteria for self-healing concrete application. Dissertation, Ghent University, Ghent
202. Chahal N, Siddique R, Rajor A (2012) Influence of bacteria on the compressive strength, water absorption and rapid chloride permeability of fly ash concrete. Constr Build Mater 28:351–356
203. Chahal N, Siddique R (2013) Permeation properties of concrete made with fly ash and silica fume: influence of ureolytic bacteria. Constr Build Mater 49:161–174
204. Farmani F, Bonakdarpour B, Ramezanianpour AA (2015) pH reduction through amendment of cement mortar with silica fume enhances its biological treatment using bacterial carbonate precipitation. Mater Struct 48:3205–3215
205. Jonkers HM, Mors RM (2012) Full scale application of bacteria-based self-healing concrete for repair purposes. In: Alexander MG, Beushausen HD, Dehn F, Moyo P (eds) Proceedings 3rd international conference on concrete repair, rehabilitation and retrofitting, 2012 September 3–5, Cape Town, South Africa. Taylor & Francis, London, pp 967–971
206. Vijay K, Murmu M, Deo SV (2017) Bacteria based self-healing concrete – a review. Constr Build Mater 152:1008–1014
207. Wang J, Mignon A, Snoeck D, Wiktor V, Van Vliergerghe S, Boon N, De Belie N (2015) Application of modified-alginate encapsulated carbonate producing bacteria in concrete: a promising strategy for crack self-healing. Front Microbiol 6:1088
208. Chen H, Qian C, Huang H (2016) Self-healing cementitious materials based on bacteria and nutrients immobilized respectively. Constr Build Mater 126:297–303
209. Wang J, De Belie N, Verstraete W (2012) Diatomaceous earth as a protective vehicle for bacteria applied for self-healing concrete. J Ind Microbiol Biotechnol 39:567–577
210. Alazhari M, Sharma T, Heath A, Cooper R, Paine K (2018) Application of expanded perlite encapsulated bacteria and growth media for self-healing concrete. Constr Build Mater 160:610–619
211. Khaliq W, Ehsan MB (2016) Crack healing in concrete using various bio influenced self-healing techniques. Constr Build Mater 102:239–357
212. Seifan M, Sarmah AK, Samani AK, Ebrahiminezhad A, Ghasemi Y, Berenjian A (2018) Mechanical properties of bio self-healing concrete containing immobilized bacteria with iron oxide nanoparticles. Appl Microbiol Biotechnol 102:4489–4498
213. De Belie N, Wang J, Soens H (2013) Microcapsules and concrete containing the same, UK Patent application 1303690.0 & 1314220.3, US application AEC/PM334564US. Applicants: Devan Chemicals NV, Universiteit Gent
214. Li M, Zhu X, Mukherjee A, Huang M, Achal V (2017) Biomineralization in metakaolin modified cement mortar to improve its strength with lowered cement content. J Hazard Mater 329:178–184
215. Ersan YC, Gruyaert E, Louis G, Lors C, De Belie N, Boon N (2015) Self-protected nitrate reducing culture for intrinsic repair of concrete cracks. Front Microbiol 6:1228
216. Van Tittelboom K, De Belie N, De Muynck W, Verstraete W (2010) Use of bacteria to repair cracks in concrete. Cem Concr Res 40:157–166
217. Wang J, Dewanckele J, Cnudde V, Van Vlierberghe S, Verstraete W, De Belie N (2014) X-ray computed tomography proof of bacterial based self-healing in concrete. Cem Concr Compos 53:289–304
218. Wang J, Jonkers HM, Boon N, De Belie N (2017) *Bacillus sphaericus* LMG 22257 is physiologically suitable for self-healing concrete. Appl Microbiol Biotechnol 101:5101–5114

219. Tagnit-Hamou A, Petrov N, Luke K (2003) Properties of concrete containing diatomaceous earth. ACI Mater J 100:73–78
220. Bakr HEGMM (2010) Diatomite: its characterization, modifications and applications. Asian J Mater Sci 2:121–136
221. Wang J, De Belie N (2014) Effect of water availability on microbial self-healing of concrete. In: Justness H (ed) Concrete innovation conference, 2014 June 11–13, Norway, Oslo, pp 1–8
222. Silva FP, Boon N, De Belie N, Boon N, Verstraete W (2015) Production of non-axenic ureolytic spores for self-healing concrete applications. Constr Build Mater 93:1034–1041
223. Ganendra G, De Muynck W, Ho A, Arvaniti EC, Hosseinkhani B, Ramos JA, Rahier H, Boon N (2014) Formate oxidation driven calcium carbonate precipitation by *Methylocystis parvus* OBBP. Appl Environ Microbiol 80:4659–4667
224. Jonkers HM, Schlangen HEJG (2008) Development of a bacteria-based self-healing concrete. In: Walraven JC, Stoelhorst D (eds) Tailor made concrete structures. Taylor & Francis, London, pp 425–430
225. Burbank MB, Weaver TJ, Green TL, Williams B, Crawford RL (2011) Precipitation of calcite by indigenous microorganisms to strengthen liquefiable soils. Geomicrobiol J 28:301–312
226. Erşan YC, Hernandez-Sanabria E, De Belie N, Boon N (2016) Enhanced crack closure performance of microbial mortar through nitrate reduction. Cem Concr Compos 70:159–170
227. Ersan YC, Verbruggen H, De Graeve I, Verstraete W, De Belie N, Boon N (2016) Nitrate reducing $CaCO_3$ precipitating bacteria survive in mortar and inhibit steel corrosion. Cem Concr Res 83:19–30
228. Takaya N, Catalan-Sakairi MAB, Sakaguchi Y, Kato I, Zhou Z, Shoun H (2003) Aerobic denitrifying bacteria that produce low levels of nitrous oxide. Appl Environ Microbiol 69:3152–3157
229. Mortensen BM, Haber MJ, DeJong JT, Caslake LF, Nelson DC (2011) Effects of environmental factors on microbial induced calcium carbonate precipitation. J Appl Microbiol 111:338–349
230. Ng SW, Lee ML, Hii SL (2012) An overview of the factors affecting microbial-induced calcite precipitation and its potential application in soil improvement. World Acad Sci Eng Technol 62:723–729
231. Qabany AA, Soga K, Santamarina C (2012) Factors affecting efficiency of microbially induced calcite precipitation. J Geotech Geoenviron Eng 138:992–1001
232. Anbu P, Kang CH, Shin YJ, So JS (2016) Formations of calcium carbonate minerals by bacteria and its multiple applications. Springerplus 5:250
233. Dhami NK, Reddy MS, Mukherjee A (2013) Biomineralization of calcium carbonate polymorphs by the bacterial strains isolated from calcareous sites. J Microbiol Biotechnol 23:707–714
234. Dhami NK, Reddy MS, Mukherjee A (2014) Synergistic role of bacterial urease and carbonic anhydrase in carbonate mineralization. Appl Biochem Biotechnol 172:2552–2561
235. Achal V, Pan X (2014) Influence of calcium sources on microbially induced calcium carbonate precipitation by *Bacillus* sp. CR2. Appl Biochem Biotechnol 173:307–317
236. Gorospe CM, Han SH, Kim SG, Park JY, Kang CH, Jeong JH, So JS (2013) Effects of different calcium salts on calcium carbonate crystal formation by *Sporosarcina pasteurii* KCTC 3558. Biotechnol Bioprocess Eng 18:903–908
237. Kang CH, Han SH, Shin YJ, Oh SJ, So JS (2014) Bioremediation of Cd by microbially induced calcite precipitation. Appl Biochem Biotechnol 172:1929–1937
238. Karatas I, Kavazanjian JE, Rittmann BE (2008) Microbially induced precipitation of calcite using *Pseudomonas denitrificans*. In: Proceedings of 1st bio-geo engineering conference, TU Delft and Deltares, Delft, pp 58–66
239. Dhami NK, Reddy MS, Mukherjee A (2013) Biomineralization of calcium carbonates and their engineered applications: a review. Front Microbiol 4:1–13

240. Seifan M, Samani AK, Berenjian A (2017) New insights into the role of pH and aeration in the bacterial production of calcium carbonate ($CaCO_3$). Appl Microbiol Biotechnol 101:3131–3142
241. Okwadha GDO, Li J (2010) Optimum conditions for microbial carbonate precipitation. Chemosphere 81:1143–1148
242. Krajewska B (2018) Urease-aided calcium carbonate mineralization for engineering applications: a review. J Adv Res 13:59–67
243. Declet A, Reyes E, Suraz OM (2016) Calcium carbonate precipitation: a review of the carbonate crystallization process and applications in bioinspired composites. Rev Adv Mater Sci 44:87–107
244. Loewenthal RE, Marais GVR (1976) Carbonate chemistry of aquatic systems: theory and application, vol 1. Ann Arbor Science, Ann Arbor
245. Ferris FG, Phoenix V, Fujita Y, Smith RW (2004) Kinetics of calcite precipitation induced by ureolytic bacteria at 10 to 20°C in artificial groundwater. Geochim Cosmochim Acta 67:1701–1710
246. Dupraz S, Menez B, Gouze P, Leprovost R, Benezeth P, Pokrovsky OS, Guyot F (2009) Experimental approach of CO_2 biomineralization in deep saline aquifers. Chem Geol 265:54–62
247. Stabnikov V, Jian C, Ivanov V, Li Y (2013) Halotolerant, alkaliphilic urease-producing bacteria from different climate zones and their application for biocementation of sand. World J Microbiol Biotechnol 29:1453–1460
248. Mitchell AC, Ferris FG (2005) The coprecipitation of Sr into calcite precipitates induced by bacterial ureolysis in artificial groundwater: temperature and kinetics dependence. Geochim Cosmochim Acta 69:4199–4210
249. De Muynck W, Verbeken K, De Belie N, Verstraete W (2013) Influence of temperature on the effectiveness of a biogenic carbonate surface treatment for limestone conservation. Appl Microbiol Biotechnol 97:1335–1347
250. Revertegat E, Richet C, Gegout P (1992) Effect of pH on the durability of cement pastes. Cem Concr Res 22:259–272
251. Silver S, Toth K, Scribner H (1975) Facilitated transport of calcium by cells and subcellular membranes of *Bacillus subtilis* and *Escherichia coli*. J Bacteriol 122:880–885
252. Hammes F, Boon N, de Villiers J, Verstraete W, Siciliano SD (2003) Strain-specific ureolytic microbial calcium carbonate precipitation. Appl Environ Microbiol 69:4901–4909
253. Nemati M, Greene EA, Voordouw G (2005) Permeability profile modification using bacterially formed calcium carbonate: comparison with enzymic option. Process Biochem 40:925–933
254. Wang JY, Van Tittelboom K, De Belie N, Verstraete W (2010) Potential of applying bacteria to heal cracks in concrete. In: Zachar J, Claisse P, Naik TR, Ganjian E (eds) 2nd international conference on sustainable construction materials and technologies. 2010 June 28–30, Ancona, Italy. UWM Center for By-Products Utilization, Milwaukee, pp 1807–1818
255. Somani RS, Patel KS, Mehta AR, Jasra RV (2006) Examination of the polymorphs and particle size of calcium carbonate precipitated using still effluent (i.e. CaCl + NaCl Solution) of soda ash manufacturing process. Ind Eng Chem Res 45:5223–5230
256. Bentz DP, Ardani A, Barrett T, Jones SZ, Lootens D, Peltz MA, Sato T, Stutzman PE, Tanesi J, Weiss WJ (2015) Multi-scale investigation of the performance of limestone in concrete. Constr Build Mater 70:1–10
257. Spanos N, Koutsoukos PG (1998) The transformation of vaterite to calcite: effect of the conditions of the solution in contact with the mineral phase. J Cryst Growth 191:783–790
258. Kawaguchi T, Decho AW (2002) A laboratory investigation of cyanobacterial extracellular polymeric secretion (EPS) in influencing $CaCO_3$ polymorphism. J Cryst Growth 240:230–235
259. Ferrer MR, Quevedo-Sarmiento J, Bejar V, Delgado R, Ramos-Cormenzana A, Rivadeneyra MA (1988) Calcium carbonate formation by *Deleya halophila*: effect on salt concentration and incubation temperature. Geomicrobiol J 6:49–57

260. Shirakawa MA, Cincotto MA, Atencio D, Gaylarde CC, John VM (2011) Effect of culture medium on biocalcification by *Pseudomonas Putida, Lysinibacillus Sphaericus* and *Bacillus Subtilis*. Braz J Microbiol 42:499–507
261. Rodriguez-Navarro C, Jroundi F, Schiro M, Ruiz-Agudo E, González-Muñozb MT (2012) Influence of substrate mineralogy on bacterial mineralization of calcium carbonate: implications for stone conservation. Appl Environ Microbiol 78:4017–4029
262. De Yoreo JJ, Vekilov PG (2003) Principles of crystal nucleation and growth. Rev Mineral Geochem 54:57–93
263. Favre N, Christ ML, Pierre AC (2009) Biocatalytic capture of CO_2 with carbonic anhydrase and its transformation to solid carbonate. J Mol Catal B Enzym 60:163–170
264. Tai CY, Chen FB (1998) Polymorphism of $CaCO_3$ precipitated in a constant-composition environment. AIChE J 44:1790–1798

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