

# Alkaliphilic bacteria: applications in industrial biotechnology

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**Abstract** Alkaliphiles are interesting groups of extremophilic organisms that thrive at pH of 9.0 and above. Many of their products, in particular enzymes, have found widespread applications in industry, primarily in the detergent and laundry industries. While the enzymes have been a runaway success from the industrial point of view, many more products have been reported from alkaliphiles such as antibiotics and carotenoids. Less known are their potential for degradation of xenobiotics. They also play a key role in biogeochemical cycling of important inorganic compounds. This review provides an insight into the huge diversity of alkaliphilic bacteria, the varied products obtained from them, and the need for further investigations on these interesting bacteria.

**Keywords** Alkaliphile · Enzyme · Detergents · Food industry · Biodegradation · Chemolithotrophy

## Introduction

Extremophilic microorganisms exhibit the ability to grow at the limits of environmental factors—pH, temperature, salinity, and pressure—which critically influence growth. Among these organisms, the immense potential of alkaliphiles (syn. alkalophile) has been realized since the 1960s,

primarily due to the pioneering work of Horikoshi and coworkers [104]. Products of industrial importance from alkaliphiles have been commercialized, the most successful of which have been in the detergent and food industries. It is noteworthy that industrial production of products from alkaliphiles is so far insufficient to meet the demands.

An industrial study document shows that the enzyme industry worldwide is valued at \$5.1 billion and is predicted to show an annual increase in demand of 6.3%. Specialty enzymes with process-specific characteristics and those used for animal feed processing and ethanol production are envisaged to have increased demand [269]. The study also forecasts that while developed countries are likely to show increased market share, developing countries will show the best growth.

Alkaline enzymes have a dominant position in the global enzyme market as constituents of detergents. So, it is pertinent to examine the role of alkaliphiles from which most of the commercial enzymes are obtained. This review focuses on the commercialized enzymes and other interesting products from alkaliphilic bacteria, which could be produced on an industrial scale.

Enzymes from alkaliphiles are stable in detergents due to their inherent tolerance to high pH. Before including them in commercial detergents, these enzymes are also tested for their tolerance to the presence of additives such as bleach. Enzymatic detergent formulations are more cost-effective in terms of energy requirements since they exhibit activity at lower temperatures and hence do not require high temperatures to facilitate effective washing.

Alkaliphilic microorganisms are not only found in areas having neutral or high pH but have also been isolated from acidic soil [104]. The neutral and acidic sites probably have some alkaline pockets where the alkaliphiles thrive. While these organisms can be facultative or obligate alkaliphiles,

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sub-groups can include psychro-, meso-, thermo-, and haloalkaliphiles. The true alkaliphiles, by and large, grow at and above pH of 9.0 and show optimal growth pH of 10.0. Alkaline environments can be those with high or low  $\text{Ca}^{++}$ . The thermoalkaliphiles (growing optimally at alkaline pH ranges in addition to temperatures above 50°C) and haloalkaliphiles (requiring high salinity and alkaline pH) are promising in terms of production of biomolecules suited for industrial applications. Enzymes from these microorganisms have found major commercial applications such as in laundry detergents, for efficient food processing, in finishing of fabrics, and in pulp and paper industries. The major products obtained are described in the following sections.

## Enzymes

### Protease

Proteases constitute a very important group among industrial enzymes. Their global sales amount to the order of 60% of the total enzyme market, of which alkaline proteases constitute 25% [210]. From the time Horikoshi [101] first documented that *Bacillus* sp. strain 221 was capable of secreting alkaline serine protease, many alkaline proteases from other *Bacillus* sp. have also been extensively studied, characterized, and commercialized [224].

Commercially, proteases find major uses as components of detergent formulations, contact lens solutions, in cheese production, processing of meat products, and for the recovery of silver from photographic films [81, 110, 210]. The usage of these enzymes has also contributed to making production processes more environmentally sustainable by lowering the generation of toxic waste. For instance, in contrast to conventional silver recovery techniques such as burning of films (which produces toxic fumes), utilization of the enzymatic process are eco-friendly. “Burnus,” was launched in 1913 and was the first enzymatic detergent made of sodium carbonate and pancreatic extract of trypsin, while the first detergent preparation containing bacterial enzyme (BIO-40) was marketed in 1956 [210].

To be components of detergent formulations, it is advantageous for proteases to have broad substrate specificity as well as function effectively at alkaline pH and high temperature. Proteases that can function at broad ranges of pH and temperature are also preferred since they can better withstand rigorous industrial production processes. It is desirable that proteases do not lose their activity in the presence of other constituents making up the detergent formulation such as surfactants or other additives [81, 210]. To function optimally as a detergent additive, the *pI* of the

protease should correspond to the pH of the detergent formulation [210].

Commercially used detergent proteases are serine proteases obtained from members of *Bacillus* [210]. The alkaline serine proteases, of which subtilisins are the prototypes, have been a commercial success and are important components of laundry detergents [81]; these proteases are generally active from pH 7.0–11.0. All the major subtilisins are produced by *Bacillus*.

The usage of alkaline proteases in industry has been extensively reviewed [11, 142]. A list of commercially produced alkaline proteases successfully used in detergent formulations, silk degumming, food and feed industry, photographic gelatin hydrolysis, leather dehairing, cosmetics, and pharmaceuticals can be found in the comprehensive review by Gupta’s group in 2002 [81]. A few recent reports of alkaline proteases, which show properties suitable for industrial scale-up, are shown in Table 1.

New reports of isolated proteases also show salt and organic solvent tolerance apart from being functional at low temperatures, indicating their suitability for appropriate industrial applications. The gene coding for such an alkaline protease, stable in organic solvents, was amplified from *Pseudomonas aeruginosa* strain K and cloned. Purified protease indicated activity of 1.0112 U/ml [206].

A halo-tolerant alkaliphilic isolate, *Streptomyces clavuligerus* Mit-1, showed the ability to tolerate a maximum of 15% salt and pH 11.0 [249]. The organism could also secrete alkaline protease when organic solvents were included in the medium; furthermore, the organism utilized solvents such as xylene, acetone, and butanol as sole carbon sources. A cold-active alkaline metalloprotease from *Pseudomonas lundensis* HW08 displayed the best activity at temperature and pH of 30°C and 10.4, respectively; the activity was retained even at 4°C up to a period of 60 days. The enzyme was also resistant to detergent components, suggesting its suitability for industrial use in commercial detergent formulations [270].

In the leather industry, conventional hide-processing techniques entail the usage of harmful chemicals like sodium sulfide. Utilization of keratinolytic enzymes for the dehairing step can result in improvement of leather quality as also less production of toxic waste. The non-collagenous components of skin are subjected to protease-mediated hydrolysis. This results in more rapid water absorption and lessened soaking time with the added benefit of reduced environmental pollution [210].

Keratinases are proteolytic enzymes that can break down keratin-based substrates. Most keratinases are serine or metalloproteases and function best at pH ranging from neutral to alkaline and temperatures of 40–60°C. Recent reviews have extensively covered production methods and their functional utility for diverse fields such as agriculture,

**Table 1** Production of alkaline protease from alkaliphilic bacteria: some recently published reports

Organism	Conditions for optimal production	Activity enhanced by	Activity inhibited by	Year of publication
<i>Bacillus</i> sp. GUS1 (thermophilic) [227]	pH 8.0–12.0, 70°C		Phenylmethylsulfonyl fluoride (PMSF)	2008
<i>Stenotrophomonas maltophilia</i> -MTCC 7528 (Psychrotolerant bacterium) [140]	120 h incubation, 20°C, pH 9.0	Mn <sup>2+</sup>	Metallo-protease inhibitors	2009
<i>Nocardopsis prasina</i> HA-4 [186]	55°C, two pH optima at 7.0 and 10.0	Fe <sup>2+</sup>	Ca <sup>2+</sup> and Mg <sup>2+</sup> (partial inhibition), Hg <sup>2+</sup> (complete inhibition)	2009
<i>Bacillus circulans</i> [209]	Broad temperature range, maximum 70–80°C, alkaline pH	Surfactant and oxidizing agents		2009
<i>Acinetobacter</i> , <i>Arthrobacter</i> , <i>Mycoplana</i> , <i>Pseudomonas</i> , <i>Pseudoxanthomonas</i> , <i>Serratia</i> and <i>Stenotrophomonas</i> [216]	pH 7.5, 15 to 25°C, obtained from glacier region	Ca <sup>2+</sup> , dithiothreitol and mercaptoethanol	Na <sup>+</sup> , Hg <sup>2+</sup> , Zn <sup>2+</sup> , Mn <sup>2+</sup> , PMSF, EDTA	2010
<i>Paenibacillus tezpurensis</i> AS-S24-II [207]	pH 9.5, 45–50°C, laundry-detergent-stable, Ca <sup>2+</sup> -independent, 43-kDa alkaline serine protease, improved wash performance of detergents			2010

production of pharmaceuticals, and cosmetics [35, 36, 83, 229].

A very recently published report documents the unique therapeutic potential of commercially available alkaline proteases, which caused degradation of prion protein (PrPSc) [95].

In the food industry, alkaline and neutral proteases from fungi are used in processing of soy sauce. Proteolytic modification of soy proteins by these enzymes causes improved functional properties. This consequently results in soluble hydrolysates which show better solubility as well as low bitterness [210].

These studies enlarge the potential usages of alkaline proteases. While alkaline proteases boast of a considerable market share, their potential could be much greater when considered in conjunction with their possible applications due to improved robustness. Protein engineering techniques with X-ray crystallographic data [81] could serve to drive this progress.

### Amylase

Starch is the prime component (50%) of our diet and can be commonly found in food remains on utensils and clothes. Amylases have found extensive utility in the biocatalysis of starch-based products.

Biocatalytic hydrolysis of glucosidic linkages in starch is mediated by a number of enzymes:  $\alpha$ -amylase (1,4- $\alpha$ -D-glucan glucanohydrolase; EC 3.2.1.1),  $\beta$ -amylase (1,4- $\alpha$ -D-glucan maltohydrolase; EC 3.2.1.2), glucoamylase (1,4- $\alpha$ -D-glucan glucohydrolase; EC 3.2.1.3) and  $\alpha$ -glucosidase

( $\alpha$ -1,4-glucosidase; EC 3.2.1.20). Pullulanase (pullulan  $\alpha$ -1,6-glucanohydrolase; EC 3.2.1.41) and isoamylase (glycogen  $\alpha$ -1,6-glucanohydrolase; EC 3.2.1.68) are the debranching enzymes [189].

The  $\alpha$ -amylases act on the  $\alpha$ -1,4 bonds between adjoining glucose units leading to the formation of glucose, maltose, and maltotriose. These extracellular enzymes make up around 25% of the industrial enzyme segment and are used predominantly in the food industry for operations such as brewing, baking, processing of fruit juice, and in paper and textile processing [235].  $\beta$ -amylases facilitate breakdown of the second  $\alpha$ -1,4 glycosidic bonds, resulting in maltose units. However, the  $\alpha$ -amylases can randomly cleave anywhere on the substrate and hence show faster action than  $\beta$ -amylases. Glucoamylases act on amylose and amylopectin and cleave the  $\alpha$ -1,6 glycosidic linkages and the last  $\alpha$ -1,4 glycosidic linkages resulting in release of glucose units from their non-reducing ends [199]. They function optimally at acidic pH, unlike  $\alpha$ - and  $\beta$ -amylases.

For the starch-processing and brewing industries, thermostable  $\alpha$ -amylases have been found to be especially beneficial, since these industrial processes utilize higher temperatures to facilitate quicker and enhanced activity of reactants [150, 199, 258]. Such thermostable  $\alpha$ -amylases for commercial purposes are mainly produced by members of *Bacillus*. They show advantages over fungal amylases due to their faster production time, lessened chances of contamination at higher processing temperatures, and quicker reaction rates. Known producers of amylases, some of which are used industrially, are *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, and *B. megaterium* [235].

Alkaline amylases retain activity at the pH at which detergents function (8.0–11.0) and, therefore, are of practical use in the laundry industry [110]. The first alkaline amylase was reported by Horikoshi from a *Bacillus* strain A-40-2 [102]. Subsequently there have been several other reports of alkaline amylases produced from bacteria (Table 2). However, the majority of alkaline amylases documented from alkaliphilic *Bacillus* sp. display exo-type activity. For example,  $\beta$ -amylases act on the non-reducing ends of polysaccharides to hydrolyze consecutive maltose units. Exo-activity does not contribute to effective elimination of stains when compared to endo-hydrolyzing enzymes such as  $\alpha$ -amylases; the latter cleave internal  $\alpha$ -1,4 bonds releasing smaller dextrin units that are water-soluble [109]. Hence, a combination of endo-acting enzymes along with debranching ones (such as pullulanases) contribute to effective action and are more suitable for use in detergents.

Products that contain alkaline debranching enzymes (pullulanases, amylopullulanase, neopullulanase, or iso-amylase) in combination with amylases can effectively function in stain removal [110]. Since Nakamura and his coworkers reported production of alkaline pullulanase from *Bacillus* sp. strain 202-1 [180], others have also reported of bacterial systems producing this enzyme [13, 14, 89, 127, 128]. Yang's group reported that *Halomonas* sp. 19-A and Y2, obtained using wheat straw black liquor as substrate, could produce enzymes such as carboxymethylcellulase (CMCase), xylanase, and amylase that showed high activity at alkaline pH (5.0–11.0) as well as at high NaCl concentrations (0–15%) [271]. Ballschmiter and coworkers [22], in 2005, had reported that the amylase from *Anaerobranca gottschalkii*, a thermoalkaliphile, exhibited transglycosylation on maltooligosaccharides. The same enzyme also displayed  $\beta$ -cyclodextrin glycosyltransferase (CGTase) activity. There had been no prior reports of  $\alpha$ -amylases displaying CGTase activity.

Cyclodextrins (CDs) are made of  $\alpha$ -(1,4) linked glucopyranose units in a ring-like manner. These CDs made of 6, 7, or 8 such units are termed as  $\alpha$ -,  $\beta$ -, and  $\gamma$ -cyclodextrins, respectively. Being cyclic molecules, a hydrophobic cavity is formed by them. This cavity facilitates inclusion of compounds of suitable size and polarity, which, in turn, changes the properties of these compounds and makes them more appropriate for industrial applications [225].

After the establishment of efficient industrial production of CDs using crude cyclomalto-dextrin glucanotransferases (CGTase, EC 2.4.1.19) of *Bacillus* sp. strain 38-2 by Matsuzawa and coworkers [165], there have been many other similar reports [18, 68, 164, 247, 275] documenting the production of more thermostable CGTases at pH optima ranging from 5.0 to 10.0. Industrial production of cyclodextrins has benefited from the biocatalytic process

since  $\alpha$ -,  $\beta$ -, and  $\gamma$ -CD could be produced on a large scale with a high conversion rate of substrates such as amylase and potato starch and without use of organic solvents to precipitate the CDs. The utility value attached to CGTases has also encouraged usage of CDs for improving diverse products such as food, cosmetics, and pharmaceuticals [25, 41, 242]. Some recent reports also document methods for enhanced production of CGTases, some from alkali-tolerant organisms [17, 32, 43, 44, 93, 107, 117, 148, 160, 168, 174, 175, 215, 219, 260, 261, 282].

In spite of the apparent widespread occurrence of such bacteria and efficient screening methods available, wide scope still exists for applications of amylases in other processes such as degradation of raw starch [155, 162]. Many reports have also documented raw starch degrading enzymes (RDSE) wherein the enzymes can hydrolyze raw starch substrates and reduce energy requirements, thus addressing a key shortcoming in the conventional process of conversion of starch to glucose. However, these have not been used industrially. Bioprospection studies for high-yielding strains as well as those producing amylases with better process performance characteristics can perhaps address these lacunae.

## Cellulase

Cellulose, an important component of plant cell walls, is made of D-glucose units that are joined by  $\beta$ -1,4-glycosidic bonds [116]. Extensive studies are being conducted globally on facilitating usage of cellulose-based substrates as renewable sources of bioenergy. While available as components of agricultural and municipal waste, cellulose is not effectively utilized due to the considerable expenses associated with the conversion processes [80, 130]. Cellulase is the enzyme that hydrolyzes  $\beta$ -1,4-glycosidic bonds to release glucose units. Hence, this enzyme is the crucial component that can facilitate better conversion of cellulose-containing wastes.

Many organisms such as fungi, bacteria, plants, protists, and insects possess cellulases. There are three types of cellulases that act sequentially on cellulose in a synergistic manner to ultimately generate glucose. Exo- $\beta$ -1,4-glucanases (EC 3.2.1.91) release a cellobiose unit, endo-1,4- $\beta$ -D-glucanases (EC 3.2.1.4) randomly hydrolyze the  $\beta$ -1,4 bonds releasing oligosaccharides and  $\beta$ -1,6-glucosidases (EC 3.2.1.21) cause hydrolysis of cellobiose to yield glucose [27, 28, 268]. Some cellulases show both endo and exo modes of action [55]. Exoglucanases (acting on the crystalline parts of cellulose crystal) and endoglucanases (working on the amorphous regions) proceed synergistically to hydrolyze the cellulose crystal [161]. Cellulases can be intra or extracellular. While many microorganisms possess the ability to degrade cellulose, complete hydrolysis of

**Table 2** Characteristics of alkaline amylases obtained from bacteria

Organism	Conditions for optimal production	Activity (U/mg)	Activity enhanced by	Activity inhibited by	Year of publication
<i>Bacillus</i> sp. A-40-2 [102]	55°C, 70 kDa, pH 10.5, stable with EDTA, <i>p</i> -Chloromercuribenzoate (pCMB) and diisopropyl fluorophosphate (DFP)			Urea, SDS, sodium dodecylbenzene sulfonate	1971
<i>Bacillus</i> sp. NRRL B 3881 [34]	$\beta$ -amylase, 50°C, pH 9.2, three active isoenzymes	3,483		EDTA	1972
<i>Bacillus</i> KSM-1876 [13]	Pullulanase, pH 10.0–10.5				1992
<i>Bacillus</i> sp. KSM-1378 [14]	Amylopullulanase, 210 kDa with both $\alpha$ -1,4 and $\alpha$ -1,6 hydrolytic activity, pullulanase activity optimal at 50°C and pH 9.5, $\alpha$ -amylase activity optimal at 50°C and pH 8.5		Reversal of EDTA/EGTA inhibition by $\text{Ca}^{2+}$	Both activities by DEPC, <i>N</i> -bromosuccinimide; pullulanase by $\text{Hg}^{2+}$ , $\alpha$ - and $\beta$ -cyclodextrin; amylase by EDTA, EGTA	1995
<i>Bacillus</i> sp. GM8901 [131]	$\alpha$ -amylase, 97 kDa, 60°C, pH 11.0–12.0, stable from pH 6.0–13.0	157.5	$\text{Ca}^{2+}$ , $\text{Mg}^{2+}$ , $\text{Cu}^{2+}$ , $\text{Co}^{2+}$ , $\text{Ag}^{+}$ , $\text{Zn}^{2+}$ , and $\text{Fe}^{2+}$	EDTA, PMSF	1996
<i>Bacillus</i> sp. TS 23 [155]	42 kDa, 70°C, pH 9.0, raw starch degrading amylase		$\text{Co}^{2+}$	$\text{Hg}^{2+}$ , $\text{Pb}^{2+}$ , $\text{Zn}^{2+}$ , $\text{Cu}^{2+}$ and EDTA	1996
<i>Bacillus</i> sp. TS 23 [156]	Thermotable pullulanase with both pullulanase and $\alpha$ -amylase activities, 140 kDa, 70°C, pH 8.0–9.0			$\text{Mg}^{2+}$ , $\text{Mn}^{2+}$ and $\text{Hg}^{2+}$ , EDTA	1996
<i>Bacillus</i> sp. KSM-1378 [108]	Liquefying $\alpha$ -amylase (LAMY), 53 kDa, 55°C, pH 8.0–8.5	5,000	<i>N</i> -ethylmaleimide	$\text{Ni}^{2+}$ , $\text{Cd}^{2+}$ , $\text{Zn}^{2+}$ , $\text{Hg}^{2+}$ , iodoacetate, <i>N</i> -bromosuccinimide, EDTA, EGTA	1998
<i>Bacillus</i> sp. WN11 [162]	$\alpha$ -amylase, 75–80°C, pH 5.5–9.0, raw starch degrading amylase			$\text{Hg}^{2+}$ , $\text{Cu}^{2+}$ , and $\text{Fe}^{3+}$	1999
<i>Bacillus</i> sp. KSM-K38 [84]	$\alpha$ -amylase, 55 kDa, 55–60°C, pH of 8.0 to 9.5, retained 80% activity with 1.8 M $\text{H}_2\text{O}_2$ for 1 h, resistant to chemical oxidation, stable with laundry surfactants	4,221		Linear alkylbenzene sulfonate and alkyl sulfate	2001
<i>Bacillus</i> sp. ANT-6 [39]	$\alpha$ -amylase, 94.5 kDa, 80°C, pH 9.0, stable between pH 7.0 and 11.0		$\text{CaCl}_2$ , PMSF	$\text{Zn}$ , $\text{Na}$ , <i>N</i> a-sulfide, EDTA, Urea, SDS	2003
<i>Bacillus halodurans</i> LBK 34 [88]	$\alpha$ -amylase, 60°C, pH 10.5–11.5, stable with DTT and SDS			$\text{Cu}^{2+}$ , $\text{Fe}^{2+}$ , $\text{Fe}^{3+}$ , $\text{Mn}^{2+}$ and 5 mM EDTA	2005
<i>Bacillus</i> sp. L1711 [65]	$\alpha$ -amylase, 51 kDa, 45°C, stable between 35 and 40°C, below 40°C pH optima of 9.5–10.0, above 40°C pH optima of 7.0–7.5	18.5	$\text{Na}^{+}$ , $\text{Co}^{2+}$ , EDTA	$\text{Ca}^{2+}$ , $\text{Zn}^{2+}$ , $\text{Mg}^{2+}$ , $\text{Mn}^{2+}$ , $\text{Ba}^{2+}$ , $\text{Cu}^{2+}$	2005
<i>Bacillus halodurans</i> 38C-2-1 [176]	$\alpha$ -amylase I (105 kDa) and II (75 kDa), 50–60°C, pH 10.0–11.0, not inhibited by surfactant/bleaching reagent	130, 18	$\text{Ca}^{2+}$ , $\text{Mg}^{2+}$ , PCMB, SDS	EDTA, $\text{Fe}^{2+}$ , $\text{Ni}^{2+}$ , $\text{Zn}^{2+}$ , $\text{Pb}^{2+}$ , $\text{Hg}^{2+}$	2007
<i>Bacillus</i> sp. PN5 [221]	90°C, pH 10.0, stable at 80–100°C for 1 h	65.23			2007
<i>Bacillus</i> sp. A3–15 [15]	$\alpha$ -amylase, 70°C, pH 11.0, 96% of original activity at 100°C		$\text{Ca}^{2+}$	$\text{Zn}^{2+}$ , <i>N</i> aCl, <i>N</i> a-sulfide, EDTA, PMSF, Urea, SDS	2008
<i>Bacillus flexus</i> XIU-3 [283]	40°C, pH 10.0		$\text{Co}^{2+}$ , $\text{Mg}^{2+}$		2008
<i>Halobacterium salinarum</i> MMD047 [228]	56 kDa, 40°C, pH 8.0	102.71	$\text{K}^{+}$ , $\text{Na}^{+}$ , $\text{Mg}^{2+}$ , and $\text{Fe}^{2+}$	$\text{Ca}^{2+}$ , $\text{Cd}^{2+}$ , $\text{Ni}^{2+}$ , $\text{Hg}^{2+}$ , $\text{Co}^{2+}$ , $\text{Zn}^{2+}$ , EDTA	2009

Table 2 continued

Organism	Conditions for optimal production	Activity (U/mg)	Activity enhanced by	Activity inhibited by	Year of publication
<i>Streptomyces gulbargensis</i> [53]	55 kDa, 45°C, pH 8.5–11.0	1,341.3	Ca <sup>2+</sup>	Mg <sup>2+</sup> , Mn <sup>2+</sup> , Sr <sup>2+</sup> , Pb <sup>2+</sup> , Zn <sup>2+</sup> , Cs <sup>2+</sup> , Fe <sup>2+</sup> , Fe <sup>3+</sup> and Hg <sup>2+</sup>	2009
<i>Rhizobium</i> sp. R-926 and R-991 [57]	30–50°C, pH 4.5–8.5, stable with surfactants		K <sup>+</sup> , Hg <sup>2+</sup> , Zn <sup>2+</sup> , SDS Tween-80	Co <sup>2+</sup> , Ba <sup>2+</sup> Ag <sup>+</sup> Hg <sup>2+</sup>	2010

cellulose is accomplished by only a few. Moreover, unlike fungi, bacteria lack the complete system of cellulases and possess mainly  $\beta$ -glucanase activity which only results in random cleavage of internal 1,4- $\beta$ -linkages but not of crystalline cellulose [85, 166].

Horikoshi along with his coworkers first described the production of extracellular CMCases that functioned at broad ranges of pH—5.0 to 10.0 [69, 100]. Such an enzyme used for industrial-scale production as a detergent additive was obtained from *Bacillus* sp. KSM-635 [111, 277]. The same group had also documented other *Bacillus* isolates (KSM-19, KSM-64, and KSM-520) that produced alkaline cellulases with pH optima 8.5–9.5; the enzyme activity was not inhibited by components of laundry detergents [230]. Over-expression of the enzyme from KSM-64 in *B. subtilis* resulted in production of 30 g of the enzyme per liter of production medium [243]. The result of these studies is that alkaline cellulases are now components of laundry detergents. Alkaline  $\beta$ -glucanases, as vital components of detergents, are also being marketed in many countries [112, 269].

Enzymes are also increasingly playing a key role in the finishing of fabrics and clothes. In the technique popularly called biopolishing, cellulases eliminate the rough cellulose lumps formed on cloth, thereby providing an even finish to the fabric as well as a brighter color. A similar finishing effect is produced when the enzyme is included as a laundry detergent additive. Traditionally, stonewashing of denims had been carried out using pumice stones, which damage cloth. In the currently used technology, cellulases have replaced pumice stones, resulting in the non-abrasive process known as biostoning. Biostoning of denim generally involves the use of acid and neutral cellulases, which have the undesirable property of causing back staining of indigo dye. Alkaline or alkali-stable cellulases, which can diminish indigo back staining under higher pH, are recommended for further improvement of the process [10]. In the paper recycling process, one of the processes for deinking involves cellulases that act on the paper fiber, thereby dislodging the ink and facilitating its removal by washing or flotation. This process works best under alkaline and high-temperature conditions [21]. Recent reports have documented that neutral  $\beta$ -glucanases, which show optimum pH values of 6.0–8.0, can be applied effectively for biostoning and biopolishing [28, 201] as well as for biological deinking and to improve the characteristics of recycled pulp [193]. Liu and coworkers in 2008 reported that an alkaliphilic *Bacillus* strain produced a hitherto unreported neutral  $\beta$ -glucanase along with an alkaline  $\beta$ -glucanase [157]. Since the neutral glucanase was produced at high pH conditions (9.0–10.0), there was no contamination by other microorganisms during the production process.

Alkaline black liquor, obtained during the kraft pulping process, was reported to be the source material for isolation of *Paenibacillus campinasensis* BL11 [133]. This organism produced many extracellular polysaccharide-degrading enzymes such as xylanase, pectinase, and three types of cellulases. Nizamuddin and Bajaj, in 2009, reported about *Bacillus* sp. NZ [188], which produced a thermoalkalitol-erant endoglucanase. The enzyme showed the best activity at 90°C (2,300 U/l) and was stable at alkaline pH (8.0–10.0). An endoglucanase isolated from alkaliphilic *Bacillus agaradhaerens* JAM-KU023 displayed an increase in activity when NaCl (0.2–2.0 M) was added. On inclusion of 0.2 M NaCl, the enzyme showed optimal activity at 60°C and pH range of 7.0–9.4 [94].

While bacteria produce a wide spectrum of cellulases, complete hydrolysis of cellulose requires the synergistic and sequential activity of a number of enzymes. More bioprospection studies to isolate novel cellulases appropriate for complete hydrolysis of the cellulose molecule and strengthening the focus on improvement of available cellulases using recombinant and protein engineering tools can serve to improve the spectrum of performance of these enzymes [161]. It can also provide a platform wherein such bacterial systems can find better industrial relevance due to their natural advantage of shorter growing period as compared to fungal enzymes.

## Lipase

Lipases cause hydrolysis of triglycerides releasing fatty acids and glycerol. They can also catalyze the reverse esterification reactions, thus producing glycerides from glycerol and fatty acids. Many lipases are also involved in catalysis of trans-esterification reactions and enantioselective hydrolyses [179, 187, 203, 208, 231]. Most commercial lipases are obtained from fungi (mainly *Rhizopus*, *Candida*, and *Rhizomucor*) and bacteria (*Pseudomonas* and *Chromobacterium*) [59]. Discussion about fungal lipases is beyond the scope of this review, since the primary focus is on alkaliphilic bacteria and their products.

Bacteria can produce many classes of lipases such as triacylglycerol lipase (EC 3.1.1.3) and carboxylesterase (EC 3.1.1.1). True lipases belong to the category of carboxylesterases which result in production of acylglycerols having acyl chain lengths of more than ten carbon atoms [213]. Lipases are used in many biocatalytic processes due to their functional characteristics such as activity on a broad range of substrates, non-requirement for cofactors, and stability in organic solvents [33]. Lipolytic enzymes functional in water-organic solvent mixtures broaden choices for industry since these enzymes can act on even those substrates that are only moderately soluble in water. These enzymes play a critical role in food processing, and

the pharmaceutical and laundry industries, as well as others [8, 40, 125, 190]. The smallest lipases characterized are known to be produced by *B. subtilis* and *B. pumilus* and are considered to be apt for synthesis of chiral drugs [115].

Microbial lipases functionally stable at alkaline pH are being used for organic synthesis [33] and in food and detergent industries [82]. These enzymes have also been implicated as causative factors contributing to skin infection [16]. Alkaline lipases mainly find applications in the detergent industry; however, many are inhibited by alkylbenzene sulfate or dodecyl benzene sulfonate [104]. Lipases produced by members of *Pseudomonas* have been reported to be compatible for use with detergents, pharmaceutical products, and processing of fat [139]. Activity of alkaline lipase (functioning at pH 8.0–11.0) from *Pseudomonas monteilii* TKU009 was enhanced by Triton X-100 and Tween 40 [265]. Other pseudomonads have also been shown to produce alkaline lipases [122, 212]. The lipase from *Burkholderia cepacia* LP08 functioned optimally at pH 9.0 [264]. Its activity was enhanced by common detergent additives like sodium cholate and NaCl. Similarly, a lipase from *Staphylococcus aureus*, active at pH 9.5, could function effectively even when oxidizing agents and surfactants were added [98].

A new lipase showing activity at pH 10.5 was produced by functional screening of a cosmid library [167]. Lip-EH166 was an alkaline lipase [129] obtained from metagenome of intertidal flat sediments of South Korea and active at broad pH range (5.0–11.0). It showed activity even at 5°C, thus displaying adaptation to cold though obtained from an unrelated environment.

Studies have shown that lipases from bacteria generally exhibit stability over a wide pH range from 4.0 to 11.0 [82]. Lipases possessing thermostability as well as activity in hydrophobic/hydrophilic organic solvents can be very attractive for industrial processes [59, 154]. Hence, there is a continuous search for new lipases with these desired properties. There have been many reports of lipases exhibiting organic solvent tolerance, indicating their suitability for non-aqueous catalytic processes. A thermostable alkaline lipase, active at pH 10.0, was isolated from mesophilic *Acinetobacter* sp. EH28 and was utilized for the synthesis of a flavoring substance involving organic solvents [5]. Lipase from *Burkholderia cepacia* S31 functioned optimally at pH 9.0 and temperature of 70°C [158]. It exhibited pH stability (pH 5.0–10.0) and retained excellent activity in many organic solvents. *B. multivorans* V2 was reported to produce a solvent-tolerant lipase that was an efficient biocatalyst for synthesis of ethyl butyrate ester [51]. *Amycolatopsis mediterranei* DSM 43304 produced a thermostable lipase that was stable at pH 5.0–9.0 as well in many organic solvents at a concentration of 25% (v/v) [59]. A new thermostable lipase, exhibiting tolerance

to benzene and isoamyl alcohol as well as stability in the alkaline range, was isolated from *Acinetobacter baylyi* [256].

Fang's group [66] had shown that lipase activity from *Proteus vulgaris* could be enhanced using error-prone PCR and DNA shuffling methods. Using these techniques, a mutant lipase was produced that exhibited higher activity. Couto and coworkers have recently screened a metagenomic library obtained from mangrove sediment DNA for lipase genes [49]. The activity-based screening followed by sequence analyses resulted in identification of a lipase that was suggested to belong to a new sub-family. Cost-effective methods to produce lipase using agro-industrial waste as a substrate have been reported [172]. Waste food substrate such as spoiled coconut has also been reported as the source material of *Bacillus cereus*, which produced an alkaline thermostable lipase [64].

Lipases are being used extensively in many biotechnological applications involving synthesis of biopolymers, biodiesel, pharmaceuticals, and other compounds [115]. Because of the wide-ranging variations in applications, more studies are required to focus on identification of lipases with process-specific features [82]. Technologies such as DNA shuffling [66] and activity-based screening [49] can provide advanced platforms for the search for novel lipases.

## Xylanase

Xylanases are hydrolases with a substantial market value of US \$200 million [124]. Endo-1,4- $\beta$ -D-xylanohydrolases (EC 3.2.1.8) act randomly on the xylan backbone,  $\beta$ -xylosidases (EC 3.2.1.37) release xylose monomers from the non-reducing ends of xylo-oligosaccharides while enzymes such as  $\alpha$ -L-arabinofuranosidases (EC 3.2.1.55),  $\alpha$ -glucuronidases (EC 3.2.1.139), acetylxylan esterases (EC 3.1.1.72) and ferulic acid esterases (EC 3.1.1.73) catalyze removal of the side groups [48]. Since xylan contains many side chains, several enzymes are required to act in a synergistic fashion to effect complete hydrolysis of the substrate [48]. Hemicellulose is a plant cell wall constituent composed primarily of xylan and can serve as an important renewable substrate. Biocatalytic processes, using enzymes to hydrolyse xylan, can provide cost-effective solutions for utilization of such renewable substrates as alternative energy sources [147].

Xylanases have found applications in diverse biotechnological platforms such as in the pre-bleaching of pulp, conversion of lignocellulosic biomass to serve as source of biofuel, improvement of cereal food products and animal feed-stocks, and degumming of plant fibers [121, 152, 217].

Biobleaching of alkali-treated wood pulp by xylanases has considerably stimulated the search for thermostable

and alkali-stable xylanases [104]. A very successful application of alkaline xylanase has been in pre-bleaching of kraft pulp. In this process, the enzyme serves as an eco-friendly substitute for chlorine [26]. Effective biobleaching requires thermoalkali-stable enzymes capable of functioning at high temperature (55–70°C) and alkaline pH [263]. Alkaline xylanases can also find use as bleaching agents in detergents [141].

Many bacteria (including actinomycetes) and fungi produce xylanases [113]. From the early reports of xylanases produced by alkaliphiles [73, 99, 181, 192, 211, 273], where xylanase production was studied from alkaliphilic bacteria obtained from soil, kraft pulp, alkaline soda lakes, and pulp and paper industry wastes, there have been many recent studies [74, 134, 271]. Various alkaliphilic bacteria have been shown to produce xylanase [6] with potential to remove color from paper mill effluents [19, 58, 171]. Other bacteria [20, 178] showed production of xylanases that were adaptable to and stable at broad ranges of pH.

After the first published report on alkaline xylanases and their potential for commercialization, subsequent studies have yielded considerable information, which is yet to be tapped fully [73]. Scattered information has focused mainly on identification of alkaliphiles, the enzymes produced by them, and their kinetics. Information on their adaptability to various industrial processes and successful scale up for industrial production is limited.

## Pectinase

Alkaline pectinases have found substantial applications as a biotechnological intervention during plant fiber processing for usage in textiles and during the paper-making process [97, 123]. There are three major kinds of these enzymes: pectin esterase (EC 3.1.1.11), pectin depolymerizing enzyme, and proto-pectinase [136]. Pectate lyase (Pel; pectic acid transeliminase; EC 4.2.2.2) is a type of pectin depolymerizing enzyme. Many microorganisms, predominantly those of *Bacillus* sp., have been used for alkaline pectinase production. Horikoshi first reported production of alkaline endopolygalacturonase by *Bacillus* sp. strain P-4-N [103]. Subsequently, Fogarty and coworkers [67, 126] also reported about a similar enzyme from *Bacillus* sp. strain RK9. In all of these reports, enzyme activity was optimal at pH 10.0. Kobayashi's group documented production of alkaline pectate lyase from *Bacillus* sp. strain KSM-P7. The enzyme showed effective activity at pH 10.5 [135] and also exhibited protopectinase-like activity on cotton fibers, leading to the formation of soluble pectin. The same investigative group had also characterized other types of high-alkaline pectate lyases [90, 191, 220] from alkaliphilic *Bacillus* sp. Sugar beet pulp was found to stimulate production of alkaline



pectinase in *B. gibsonii* strain S-2 [153]. The organism could grow well from pH 7.0–12.0 and temperature 4–40°C with polygalacturonase yield of 3,600 U per gram of the substrate.

Bacteria secreting alkaline pectinase were first used in the retting process of Mitsumata bast [276]. The retting process of a type of Japanese paper was improved considerably using alkaline pectic lyase (optimal pH of 9.5) from *Bacillus* sp. strain GIR 277 [104], resulting in the production of stronger paper with better quality.

Alkaliphilic bacterial strain NT-33 could degum ramie fibers [42]. Polysaccharide-degrading enzymes from alkaliphilic bacteria were studied for their effectiveness in degumming of ramie fibers and were found to be more beneficial [284]. In another study, thermoalkali-stable polygalacturonase from *Bacillus* sp. MG-cp-2 was used for similarly treating ramie and sunn hemp fibers [121]. An initial chemical treatment followed by enzyme action was found to be best since neither treatment was found to be effective when used separately.

A pectinase from *B. subtilis* SS [2] exhibiting thermoalkali-stability also displayed added properties of increasing the brightness, whiteness, and fluorescence of paper pulp, thus improving characteristics of paper. Bioscouring of cotton fabrics using pectate lyase showed that the method was as effective as the conventional method [119]. Wax and pectin removal are considered to be critical for successful scouring of fabric [1]. In this respect, alkaline pectinases showed 75% higher pectinolytic activity than the corresponding acidic pectinases.

These reports show the huge potential of these enzymes for the textile industry, which is increasingly having considerable market potential in developing economies. Playing a significant role in vital steps of fiber processing, these enzymes have contributed to the cost-effectiveness of the processes in addition to improvement of fiber quality [137]. In turn, advantages have accrued to the bast fibers in the form of better quality and financial viability of bast-processing units when compared to other types of fibers.

### Chitinase

Chitinous waste (chiefly from the seafood industry) needs to be recycled to maintain carbon–nitrogen balance in the environment and chitinases can play a key role here [182]. There are reports on alkaline chitinase production from microorganisms. The first documentation was from alkaliphilic *Nocardiosis albus* ssp. *prasina* OPC-131 [253]. This organism secreted two types of chitinases (A and B) with different pH optima—pH 5.0 and 7.0, respectively. *Bacillus* strain BG-11 showed production of chitinase, which functioned well at broad pH (7.5–9.0) and

temperature (45–55°C) ranges [29]. The enzyme also displayed stability at pH ranges of 6.0–9.0 at 50°C. Chitinase characteristics of gammaproteobacteria (obtained from an alkaline, hypersaline lake) and from a metagenomic library (estuarine bacteria) were studied using fosmids [146]; the latter are f-factor cosmids capable of containing up to 50-kb DNA. The enzymes from the alkaline lake organisms showed distinctive adaptations making them haloalkali-tolerant [146]. Another outcome of the same study was that a new sequence pertaining to a family 20 glycosyl hydrolase was obtained from a specific bacterial strain of this lake. The enzyme displayed optimal activity at pH 10.0, which was substantially higher than that of other enzymes belonging to this family. Chitinases, which function well in the alkaline pH range, could also find good applicability as biocontrol agents of agricultural pests [24].

### Catalase

Aerobic organisms utilize oxygen to facilitate efficient metabolism of nutrients. However, during this process, oxidants (collectively called reactive oxygen species or ROS) are formed. The ROS are formed due to incomplete reduction of oxygen [254]. One type of ROS produced due to aerobic respiratory processes is hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) [46]. Such oxidants can cause damage to lipids, proteins, and nucleic acids. Hence, organisms produce antioxidants in the form of enzymes and other molecules to reduce the levels of ROS [254]. Catalase (EC 1.11.1.6) is an anti-oxidant biocatalyst facilitating conversion of H<sub>2</sub>O<sub>2</sub> to O<sub>2</sub> and H<sub>2</sub>O [37], and is produced in response to oxidative stress or due to presence of ROS [45].

Catalases are classified into the following four groups: monofunctional catalases, catalase-peroxidases with dual functionality, non-heme catalases, and minor catalases [185]. The monofunctional catalases comprise the largest group and are found to be produced by the majority of aerobic Prokaryotes and Eukaryotes. Structurally, they are homotetramers with four prosthetic heme groups and range in size from 200 to 340 kDa. The catalase-peroxidases are homodimeric heme-containing proteins of sizes 120–340 kDa and display both catalase and peroxidase activities. The peroxidase (EC 1.11.1.7) function results in reduction of H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O utilizing organic substances as electron donor [79]. Non-heme catalases contain manganese (and not heme) in their active sites and have been isolated from diverse groups of bacteria [202]. The minor catalases, such as chloroperoxidase and plant peroxidases, are heme-containing proteins. Whatever low catalase activity they display is brought about by the heme [185].

Catalases find biotechnological applications in the food, medical, and textile industries [202]. In the food-processing industry, during microbial fermentation, H<sub>2</sub>O<sub>2</sub>

accumulates as a toxic by-product. Recent technologies involve the addition of glucose oxidase and catalase to foods stored in closed containers. These enzymes hinder growth of aerobes by decreasing oxygen levels, which in turn inhibit oxidation of fat and natural pigments [236]. For example, when crabs and shrimp are dipped in glucose oxidase/catalase solution, the undesirable color change from pink to yellow is prevented.

During the course of preparing textile fabrics for further processing steps, bleaching of fabric with  $H_2O_2$  is carried out before the dyeing step. The bleaching activity is enhanced under alkaline conditions and high temperatures [9, 251, 262]. Being an oxidizing agent,  $H_2O_2$  can cause degradation of reactive dyes [257] such as those of the triazinyl or vinylsulfone type. Residual peroxide on the fabric can lead to unacceptable results from the subsequent dyeing process [255]. Hence, before adding the dye, it is necessary to remove the peroxide residues from the fabric as well as the machinery. While washing the cloth with water or chemical reducers (sodium bisulfite, hydrosulfite) can reduce the quantity of residual peroxide, the process utilizes large volumes of water [92], 100 L  $kg^{-1}$  of fabric. The chemical reducers can contribute to unfavorably high salt concentrations and effluent toxicity. Catalases serve as an environmentally friendly and cost-effective alternative assisting in the removal of peroxide residues from fabric [226].

Although catalases have been studied extensively, few reports are available on thermo- and alkali-stable catalases such as that produced by *Thermoleophilum album* [7]. Yumoto's group, in 1990, had reported of alkaliphilic *Bacillus* YN-2000 secreting a catalase that showed substantial peroxidase as well as catalase activity [278]. The catalase quantity was found to be elevated when cells were grown at pH 10.0 than at lower pH ranges of 7.0–9.0. Hicks [91] showed that cells of *B. firmus* OF4, a facultative alkaliphile, showed twice the specific activity for catalase when grown at pH 10.5 compared to cells grown at pH 7.5. Nevertheless, the cells grown at pH 10.5 showed more sensitivity to exogenous hydrogen peroxide. In the facultative psychrophile *Vibrio rumoiensis* S-1T, isolated from an  $H_2O_2$ -rich location [281], the catalase activity was found to be 527,500 U  $mg$  protein $^{-1}$ , and was faster than that of bovine liver catalase. The catalase functioned optimally at broad pH ranges (6.0–10.0). Subsequently, Phucharoen and coworkers isolated an alkali- and halo-tolerant bacterium *Halomonas* sp. SK1, which produced a catalase with high specific activity (57,900 U  $mg$  protein $^{-1}$ ). This activity was twice as high as bovine liver catalase [202] and the enzyme was active over pH ranges of 5.0–11.0 with optimal activity at pH 10.0. Gudelj and coworkers [79] described a catalase-peroxidase from thermoalkaliphilic *Bacillus* sp. SF, which

exhibited stability up to pH 10.0. The enzyme was suggested to have properties suitable for management of textile bleaching waste.

Amorim and coworkers [9] analyzed the suitability of a commercial catalase from Novo Nordisk for the removal of  $H_2O_2$  residues from bleached fabric and its subsequent effect on dyeing with a bifunctional reactive dye. They concluded that use of the catalase had a noticeable and enhanced effect on the dyeing process in addition to improved color yield. There was also added simplicity of application since the catalase could be used in the dyeing bath right after bleaching, leading to lesser washing, water consumption, and effluent volume. Hence, catalases that exhibit thermoalkali stability could be a significant, viable, and practical option for textile industries.

Another study documents that an obligate alkaliphile, *Exiguobacterium oxidotolerans*, isolated from a fish-processing effluent, was able to grow at alkaline pH range (7.0–10.0). The cell extract from this bacterium showed 567 times higher catalase activity when compared to an *Escherichia coli* extract [280]. Thuy le's group demonstrated that when a catalase gene, *ohktA*, from *Halomonas* sp. SK1 was cloned and expressed in a catalase-negative *E. coli* strain, the recipient organism showed high catalase activity with a pH optimum at 10.0. To overcome the lack of thermostability of the catalase, manipulations were made to include domain II of a thermostable Mn catalase from *Thermus thermophilus*. On co-expression with a chaperone protein, a chimeric catalase stable at 37°C was obtained [250]. Catalase from *Bacillus* sp. N2a, isolated from Antarctic seawater, functioned optimally up to pH 11.0 [266].

Catalase also finds utility as a component of contact lens cleaning solutions. Acanthamoeba keratitis is a serious ocular infection that occurs due to improper cleaning and maintenance of the contact lens [52]. Cleaning solutions containing  $H_2O_2$  are effective since  $H_2O_2$  kills pathogens due to the oxidative effect produced [114]. *Acanthamoeba* cysts are killed when exposed to 3%  $H_2O_2$  for 4–6 h [54]. However,  $H_2O_2$  can cause corneal toxicity and requires neutralization before the lens can be worn [96]. Catalase, as a component of one-step or two-step cleaning solutions, plays a critical role in reduction of the  $H_2O_2$  [106]. However, these catalases do not require the necessity of being thermoalkali-stable since they function at acidic-neutral pH.

These reports show the varied properties of catalase and the potential for exploitation of thermoalkali-stable catalases in textile and food-processing industries. Genetic and protein engineering techniques can facilitate in bringing desirable changes in catalases, making them appropriate for industrial applications.

## Antibiotics

The first report of production of antibiotic in alkaline medium was by Sato's team, who found that two antifungal peptides were produced by *Paecilomyces lilacinus* 1907 at pH 10.5 [218]. Later, *Nocardiopsis dassonvillei* OPC-15, an alkaliphile, was isolated, which showed production of phenazine antibiotics at pH 10.0 [252]. Reports of antibiotic production under alkaline conditions are scarce, which could be due to the instability exhibited by antibiotics under higher pH [104]. Nevertheless, some alkaliphilic actinomycetes can grow under neutral pH. This property may be employed to induce the organism to synthesize the alkaline antibiotic and recover the same from the production medium without denaturation [104]. Dietera and coworkers [60] identified pyrocoll to be one of the metabolites produced by alkaliphilic *Streptomyces* sp. AK. Pyrocoll showed activity against some fungi, protozoa, and even various human tumor cell lines. It is an interesting point that pyrocoll is a constituent of cigarette smoke and was never reported from a biological source earlier. *Streptomyces sannanensis* strain RJT-1 produced antibiotics at optimum NaCl concentration (w/v) of 5% and pH 9.0, respectively [259]. Alkaliphilic *Streptomyces tanashiensis* strain A2D showed maximum antibiotic production at pH 8.0 with a salt concentration of 2% (w/v) [234]. Lawton and coworkers, in 2007, first reported the production of a two-peptide lantibiotic (lantionine-containing peptide antibiotic) by an alkaliphilic *Bacillus* namely *B. halodurans* C-125 [145].

A very recent report documents the production of Naphthospirozone A(1) from alkaliphilic *Nocardiopsis* sp. (YIM DT266). This metabolite showed cytotoxic and antibiotic activity [62]. Another report documents that a halotolerant alkaliphile, *Streptomyces aburaviensis* Kut-8, obtained from the saline desert region of Kutch in India secreted antibiotic from pH 7.0–9.0 [248]. These reports suggest that exploration for alkaliphilic actinomycetes can possibly provide better sources of novel antibiotics. We have not come across any report on commercialization of these antibiotics produced by alkaliphiles.

Analogs of cholic acid were reported to function effectively against bacteria [151]. In their mode of activity, these analogs resembled Polymixin B, since they could similarly permeate through the outer membranes of Gram-negative bacteria. Other such analogs developed were more effective in preventing bacterial growth when used in conjunction with hydrophobic antibiotics [151]. Kimura's group reported an alkaliphilic soil *Bacillus* that could tolerate cholic acid incorporated in growth medium [132]. To our knowledge, there are no further reports on utilization of cholic acid or its derivatives as antibiotics. The relative scarcity of reports on antibiotics from alkaliphiles makes it

crucial that extensive exploratory screening for antibiotics from alkaliphiles (including from unexplored habitats) is undertaken in conjunction with targeted designing of bioactive molecules to keep pace with ever-increasing necessity for newer antimicrobial compounds.

## Carotenoids

There is increasing demand for carotenes as diet supplements, as a source of vitamin A, and as food colorants [214]. Carotenoids have been reported to have a considerable anti-carcinogenic effect [149]. They have also been seeing an increasing market share in the nutraceuticals segment [169]. Aono and Horikoshi [12] had documented that some alkaliphilic *Bacillus* strains (A-40-2, 2B-2, 8-1, and 57-1) produced triterpenoid carotenoids and suggested that they could be of survival value. A lycopene-type of carotenoid pigment was observed to be produced at pH 10.5 by alkaliphilic *Microbacterium arborescens*-AGSB, obtained from coastal sand dunes [76]. Novel carotenoid glucoside esters have been reported from alkaliphilic heliobacteria [245]. An obligate alkaliphile, *Paracoccus bogoriensis* BOG6<sup>T</sup> was isolated from Lake Bogoria hot spring [195]. This organism was found to secrete astaxanthin, a compound which has been shown to have significant anti-oxidant potential and immunomodulatory activity [118] and inhibitory effect on some cancer cells [173, 198]. A sulfur-oxidizing alkaliphilic heterotroph, *Roseinatronobacter thiooxidans* ALG1, optimally growing at alkaline pH (8.5–10.4), was found to synthesize carotenoids in organic nitrogen-containing medium [240].

## Reduction processes

### Indigo reduction

The traditional process of indigo blue dye production is carried out by wetting dried indigo leaves, which results in increased microbial oxidation. The product formed subsequently is fermented under alkaline pH (generally above 10.0), whereby the oxidized indigo is converted to a soluble reduced form by microorganisms. Chemical methods have subsequently been favored due to complications associated with the traditional method [279]. Currently, there is an emphasis on reverting back to traditional methods due to better color of the product. Hence, there is a renewed interest in microorganisms which can reduce indigo dye at the high alkaline pH [279]. Indigo-reducing bacteria (*Bacillus* sp. and *Clostridium isatidis*) have been reported earlier [197, 244]. *Alkalibacterium psychrotolerans* IDR2-2(T), showing optimal growth at

pH 9.5–10.5 and exhibiting ability to reduce indigo dye at alkaline pH, was isolated from fermented *Polygonum tinctorium* [279]. More studies on microorganisms capable of carrying out efficient conversion of indigo can serve to improve and provide an impetus to the traditional process.

### Iron reduction

Iron reduction mediated by microbes in various environments and by diverse Fe(III)-reducing extremophilic microorganisms has been extensively documented. This process has a significant effect on decomposition of organic matter, which further impacts soil physico-chemical parameters [205, 267]. Alkaliphilic bacteria with the capability of reducing Fe(III) have been reported from alkaline locations [30, 77, 205] as well as from an industrial leachate site [274]. *Alkaliphilus metalliredigens* strain QYMF [274] and *Anaerobranca californiensis* [77] were shown to reduce Fe(III) up to a pH of 10.4. Prior to this, such reduction had been described to occur only up to pH 9.0 [30, 120]. These reports along with that of alkaliphilic *Bacillus* sp. strain SFB suggest that such Fe(III) reduction mediated by microorganisms could play a critical part in iron cycling in alkaline environments [205].

### Siderophores

Iron, though a common constituent in environment, is generally found as insoluble Fe(III) oxides, thus rendering it biologically unavailable. Anaerobic bacteria can take up and utilize Fe(II), the soluble form of iron, without the assistance of iron chelators. However, under aerobic conditions, Fe(II) can be oxidized to Fe(III) rapidly [184]. Generally, microorganisms need some quantity of iron ( $10^{-8}$  –  $10^{-6}$  M) for normal growth processes. Hence, chelators play a crucial role in making iron bioavailable for aerobic microorganisms. To obtain iron, microorganisms are known to secrete low-molecular-weight compounds called siderophores that have an affinity for Fe(III) and facilitate in solubilizing iron before transportation into the cell [272].

Siderophores have been suggested to have potential applicability in therapeutics, agriculture, and environment [47]. Biofilms, composed of bacterial aggregates, can cause problems for human health and industry [233], leading to economic losses. Iron deficiency plays an important role by stimulating bacterial aggregation and biofilm formation. These biofilms display resistance to conventional control methods such as antibiotics. Hence, siderophores are postulated to have the potential to inhibit biofilm formation by making iron unavailable to the biofilm constituents.

By producing siderophores, bacteria deprive other species of iron. On the other hand, some species are not able to produce iron-chelators but can still capture the siderophores produced by other organisms and make use of the iron chelated therein [233]. This strategy of ‘siderophore-capture’ has been used to couple antibiotics with the siderophores. This causes joint uptake and action of antibiotics in the cell along with the siderophore. While conventional antibiotics can generate mutations in target organisms and thus make them antibiotic-resistant, the joint uptake approach has the advantage that any resistance due to mutation of the transport system results in a consequent loss of iron uptake by the organism leading to its death [38, 163]. Siderophore-capturing ability of bacteria has also been shown to facilitate growth of unculturable organisms from a marine environment [63]. Ding’s group reported the design of a new siderophore analog, which was postulated to assist in a drug-delivery system linked to iron transport [61].

Plant growth is stimulated by various rhizobacteria (plant growth promoting rhizobacteria, PGPR) that produce siderophores to sequester iron [222]. Such PGPRs can potentially function as biocontrol agents since the siderophores they produce bind to iron and prevent iron utilization by pathogens, resulting in better crop yield [223]. This type of biocontrol strategy has also been suggested for possible exploitation by the wood and timber industry for wood rot management [143]. Iron has a critical role to play in wood degradation caused by white and brown rot fungi. It is a constituent of the heme enzymes employed by white rot fungi [200] and is also important for the iron/hydrogen peroxide-mediated catalysis of cellulose decomposition by brown rot fungi [177]. Hence, sequestration of iron by siderophore-producing organisms can starve wood-degrading fungi and, thereby, maintain wood quality [143]. Alkaliphilic siderophore-producing bacteria could function as biocontrol agents in alkaline areas.

The siderophore-production capability also offers the promise of an eco-friendly system for treatment of effluents contaminated with toxic components. One example is cyanide, which is produced naturally by many plants, bacteria, algae, and fungi. However, the major source of environmental toxicity from cyanide results from effluents generated by the mining and jewellery industries [105]. Cyanide toxicity results from its strong bonding with metalloproteins, especially cytochromes, which are central for respiratory processes. Biological treatments are considered to be more efficacious for the management of cyanide-containing effluent since cyanide is amenable to biodegradation [105]. Production of siderophores by cyanotrophic organisms helps to chelate iron and break down the strong cyanide-iron complex. Subsequently, the cyanide can be assimilated by the bacteria utilizing other pathways. Generally, cyanotrophs have shown the ability to

break down cyanide at neutral pH. However, cyanide can form volatile hydrocyanic acid ( $pK_a = 9.21$ ) at this pH. Hence, alkaliphilic cyanotrophs can play a vital role in effective biodegradation of cyanide [159]. In this context, Huertas and coworkers documented a protein (CNO) from *Pseudomonas pseudoalcaligenes* CECT5344 that was induced by cyanide and involved in the siderophore-bio-synthetic pathway [105, 159]. The organism could act on cyanide at a highly alkaline pH of 11.5. All of these studies have shown that due to their key role in the iron cycle, siderophores have attractive potential for development and advancement of therapeutic drugs [56], inhibition of bio-film formation [70], and bioremediation [105].

Gascoyne and coworkers had reported about alkaliphiles that produced siderophores capable of binding gallium and aluminum [71, 72]. The isolates belonged to diverse genera such as *Pseudomonas*, *Bacillus*, and *Micrococcus*. Apart from this, the authors have not come across reports of siderophore production by alkaliphiles. Considering that Fe(III) becomes unavailable for microorganisms at alkaline pH ranges, it is interesting to speculate on the mechanism by which the alkaliphilic aerobic microorganisms acquire iron [233]. It is quite possible that alkaliphiles also secrete siderophores. More studies undertaken on microorganisms from alkaline areas can shed light on their iron sequestration and recycling strategies, which can be further exploited for development of therapeutics and other industrial purposes.

### Chemolithotrophy

Organisms can metabolize inorganic substrates to obtain reducing power and subsequently synthesize cellular biomolecules utilizing aerobic/anaerobic respiratory pathways [138]. This process is called chemolithotrophy. Chemolithotrophic microorganisms are commonly used for leaching of sulfide minerals, resulting in recovery of important minerals such as copper, uranium, or gold from their ores [194]. Microbiological-assisted leaching is a promising technology for extraction of minerals from even low-grade ores [183]. It offers promise over conventional extraction methods such as pyrometallurgy or hydrometallurgy due to the utility value attached to extraction of valuable metals from low-grade ores, less investment of financial resources, lower energy costs, and largely reduced ecological damage. As an example, there is a growing global demand for nickel, which is used as a component of steel. Lowered output from conventional high-grade nickel sources has fuelled attempts to extract nickel from low-grade laterite ores. However, in the latter, nickel is not present in the discrete mineral form but as cations, making the extraction process difficult [183]. Here, microbes can

play a role since they make use of the inorganic minerals for their growth requirements and thus facilitate recovery of important metals. Hence, biohydrometallurgy, a growing interdisciplinary technology involving aspects of geomicrobiology, biochemistry, and hydrometallurgy, has rapidly gained acceptance [232], though the technology itself has been used since early times before clear enunciation of the function of microorganisms in the process [194]. The Bioshale project [50] aims to employ biotechnological intervention to facilitate mining of important metals from black shale; the latter contains large quantities of organic matter presenting complications using conventional metal extraction technology.

Earlier reports had documented microbial-assisted oxidation of inorganic sulfur-containing compounds only by neutrophiles and acidophiles. Recently, obligate chemolithotrophs, which can oxidize sulfur at alkaline pH and high salt concentrations, have also been reported. Such organisms would be vital components of the sulfur cycle in alkaline areas. Three new genera have been reported in this group - *Thialkalmicrobium* spp., *Thialkalinivibrio* spp. [237], and *Thioalkalispira* spp. [238]. *Thialkalinivibrio halophilus* strain HL 17<sup>T</sup>, growing optimally at pH 8.0–9.0, was discovered later [23]. Utility of these alkaliphiles for biohydrometallurgical processes is required to be explored further.

Reports are scarce of groups such as purple nonsulfur bacteria (PNB) from alkaline environments. *Rhodobaca bogoriensis* was the first reported alkaliphilic nonsulfur bacterium [170]. Unlike other PNB, *R. bogoriensis* exhibited the inability to grow on hydrogen and sulfide as a photolithoautotroph. Some related strains have also been isolated from soda lakes, though they are not well described. Recently, there has been a report of *R. barguzinensis*, a halotolerant alkaliphile, growing optimally at pH 9.0 [31]. This is the first report of alkaliphilic PNB from temperate areas.

Nanotechnology has become a significant influence and one of the driving forces of industrial products since the distinctive properties of nanoparticles are conferred by their size [204]. The major application platforms of nanoparticles are in electronics and information technology. In addition to conventionally used physical and chemical technology processes, deployment of microorganisms for biosynthesis of inorganic nanoparticles has added a new perspective and contributed to the field of nanobiotechnology. Chemolithotrophic energy production is one of the reasons for synthesis of nanoparticles as by-products by these organisms [138]. Microbiological systems can assure efficient and flexible processes for nanoparticle synthesis. Such biologically produced nanoparticles show biocompatibility [138], which is essential for therapeutic applications. There is one report of production of 2–5-nm-diameter ZnS particles at

high pH (7.2–8.6), within biofilms which contain members of sulfate-reducing Desulfobacteriaceae [144]. The alkali-tolerant actinomycetes *Rhodococcus* sp. was reported to produce gold nanoparticles, which were localized on the plasma membrane [4]. The thermoalkaliphilic actinomycete, *Thermomonospora* sp., was reported to synthesize extracellular gold nanoparticles [3]. Monodisperse structure of the nanoparticles was controlled by pH (9.0) and temperature (50°C). Further studies leading to biological production systems can make nanoparticles more appropriate for effective drug delivery [138].

### Biodegradation of nitriles

The nitriles are an important group of compounds, which are intermediates produced during various industrial production processes and contribute to organic synthesis. Production of acrylamide, a compound with diverse applications, from acrylonitrile using biocatalytic processes has gained importance in recent times [285]. The production process, mediated by nitrile hydratase from microorganisms, has been shown to be better than the conventional chemical process in terms of product purity as well as energy efficiency. While most members of the nitrile group are organic compounds, inorganic ones are cyanide ( $\text{N} \equiv \text{C}^-$ ) and thiocyanate ( $\text{N} \equiv \text{C-S}^-$ ) [241]. The nitrile bond being stable, these compounds are not easily degraded. Moreover, the nitriles are also toxic. For treatment of nitrile-containing waste, biodegradation methods are gaining importance. Microorganisms capable of degrading nitriles are mainly neutrophiles [241]. Biodegradation of the inorganic nitriles cyanide and thiocyanate has been reported at high pH [159, 239] by bacteria. However, the bacteria cannot degrade organic nitriles. Sorokin's group had shown that alkaliphilic *Natronocella acetinitrilica* could utilize acetonitrile and, to some extent, propionitrile as the sole carbon, nitrogen and energy source at pH 10.0 and 0.6 M of total  $\text{Na}^+$  [241].

### Alkaliphilic cyanobacteria production as food supplement

Cyanobacteria have attracted global interest for their potential use as food, source of biofuel, biofertilizer, production of vital metabolites such as vitamins, pharmaceuticals, pigments, and also for bioremediation [246]. While many have been studied, few cyanobacteria have been deployed industrially or found success in commerce. Alkaliphilic cyanobacteria such as *Spirulina platensis* grow well in highly alkaline soda lakes where pH is generally near 12.0 [78]. This organism has been a huge commercial

success and is marketed as a highly nutritious human food supplement (single-cell protein) by many companies.

### Production of other interesting compounds

#### 2-Phenylethylamine

In a study by Hamasaki et al., the authors reported that polyamines such as putrescine, spermidine, and spermine were secreted by alkaliphilic *Bacillus* sp. strain YN-2000, *B. firmus* OF-4, *B. firmus* RAB RA-1, and *B. alkalophilus* [87]. The compound 2-phenylethylamine, which is commonly used as the initial starting material for synthesis of medicinal products, was secreted by only *Bacillus* sp. strain YN-2000. Another report [86] also documented production of this compound by *B. cohnii* strains among a group of alkaliphilic *Bacillus*. It was suggested that these bacterial strains could be used for industrial-scale production of 2-phenylethylamine. The compound itself was suggested to serve as an effective chemotaxonomic marker since few bacteria have been reported to secrete it.

#### Organic acids

Metabolization of media carbohydrate by alkaliphiles leads to production of organic acids, subsequently decreasing the pH of culture media [104]. Comparative studies of organic acid production by various alkaliphilic bacilli showed that they produced many industrially important acids such as isobutyric,  $\alpha$ -oxoisocaproic and phenylacetic acid [196].

### Conclusions

It is clearly evident from the numerous publications that considerable diversity exists among alkaliphiles, but many more remain to be tapped from unexplored regions. While the alkaliphiles have yielded a rich array of products, more studies focused on specific characteristics suitable for industrial-scale use can help reap rich dividends. For industrial applications, enzymes are expected to be stable and robust enough to withstand extremes of temperature and pH. Many such enzymes have been commercially deployed. In the current scenario, the demand for commercial enzymes for specific purposes, such as incorporation into detergents, for technical processes (textile, paper, fuel ethanol production) or for food and feed processing, is much more than the production. Novozymes A/S, Genencor International, Inc. and DSM N.V. are the current market leaders in commercial enzyme production [75]. Bioprospection for novel alkaliphiles from unexplored

habitats in conjunction with high-throughput screening technologies for specific products having suitability for broad technological platforms can provide environmentally friendly and cost-effective solutions.

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