

# Alkalophilic Protease Producing Bacteria and Some Biotechnological Potentials

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## ABSTRACT

**Background:** Use of extremophiles is a hot topic in the field of biotechnology for their immense potential and applications in multiple industries.

**Objectives:** The present review aims to sum up the potential applications of alkalophilic protease-producing bacteria and their optimized growth requirement. The isolation, characterization, and optimization of various isolates (especially of genus *Bacillus*) from different harsh niches, including soil samples from deserts and soil having decaying matters, wastewaters from industries, soda lakes, and alkaline springs have been reported in this review.

**Methodology:** All the relevant papers published from 2013-2020 were looked over numerous sources like Google Scholar, Medline, PubMed, Research Gate, Science Direct, Scopus and Web of Science.

**Results:** Most of the microbial life found in extreme alkaline habitats are found to form a variety of enzymes and an array of other substances of biotechnological interests. These enzymes, especially proteases, are exploited in industries globally because of their ability to withstand rigorous industrial reactions and conditions.

**Conclusion:** Though a number of alkalophilic protease-producing bacteria have been isolated, still a large number of these microorganisms are unidentified. The current demand for biotechnological products from them appeals to the need for isolation of unidentified bacteria.

### Keywords

Alkalophilic, Biotechnological potential, Extremophiles, Industries, Protease.

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## INTRODUCTION

Over the past few decades, scientists have become interested in the extraordinary organisms that can survive in severe and harsh environments where no life form can

sustain except extremophiles. Microenvironments or niches inhabited by these organisms are once considered hostile for life which could have extreme pH conditions,

ice, hot springs, concentrated salt solutions, high pressure, toxic waste deposits, organic solvents, heavy metals, oxygen scarcity, variety of radiations and various combinations thereof. The power to adapt extreme niches could be imagined by the fact that some extremophiles have been isolated from pH ranges 0-12.8, from ice-cold habitats (-20°C) to hydrothermal vents (122°C), 100-110MPa pressure as well as from deep-sea (10km) and earth's crust at the depth of 6.7km. Additionally, extremophiles adapted for more than one extreme conditions are referred to as polyextremophiles<sup>1,2</sup>.

### Extremophiles: A Hot Debate

Since the discovery of extremophiles, these organisms are of the most concerning issue of biotechnology because of their adaptability and survival in harsh conditions. Because of such complex physiology, they are exploiting their potential at the industrial level. The discovery of renowned enzyme *Taq* polymerase from an extremophile *Thermus aquaticus* employed in PCR is one of the examples, which bring a revolution in the field of gene cloning. After this discovery, extremophiles laid down the base of a large group of extremophilic researchers as well as was thrown into gear a new area of research which resulted in the "International Society for Extremophiles", and even a journal founded by Koki Horikoshi namely "Extremophiles". The importance of extremophiles could be realized by the fact that separate conferences on extremophiles are conducted in different parts of the world<sup>3</sup>.

Recently, some books have been published regarding the whole spectrum of extremophiles which deals with the lifestyle, biochemistry, genomics, physiology, and even the regulation of their metabolism. Research on extremophiles is plentiful and is as diverse as the extremophilic microbes themselves, so the information regarding extremophiles is beyond the scope of this review which restricts us, to sum up with few examples<sup>4,5,6</sup>.

### Classification of Extremophiles

The classification is based upon the circumstances in which they survive and grow. These include psychrophiles, halophiles, alkaliphiles, acidophiles, basophils, thermophiles, and hyperthermophiles which show optimal growth at low temperatures, high salt

concentrations, basic or acidic pH, under pressure and high or very high temperatures, respectively<sup>7,8</sup>.

### Alkaliphiles / Alkalophiles

Alkaliphiles, show the ability to grow in various habitats having a pH range of 9.0 to 11.0 and show optimum growth at pH 10.0. They could be isolated from common habitats like garden soil perhaps due to momentary alkaline conditions resulting from biological activities including ammonification or sulfate reduction. In contrast, other potential sources of alkaliphiles include soil samples from deserts and soil having decaying matters, wastewaters from industries, soda lakes, and alkaline springs<sup>9,10</sup>. This class of extremophiles is of keen interest because of their bioenergetic, environmental, and industrial potentials<sup>11</sup>. These microbes are found to produce a rich collection of products including cyclodextrin, wood pulp, WA 52 (macrolide antibiotic) sugar cleaning agents<sup>12,13,14,15</sup>, and are sources of active enzymes e.g. proteases, amylases, cellulases, and xylanases which work optimally in alkaline conditions. These enzymes have a major impact and biotechnological potential in various commercial applications and industrial processes e.g. production of detergents<sup>16,17</sup>. Industrial synthesis of products from alkaliphiles is so far inadequate to meet up the demands. Apart from significant diversity, many more of them remain to be hidden from unexplored remote environments. The aims current work is to go over the potential applications of alkalophilic protease producing bacteria and their optimized growth requirements.

### Alkaliphiles: A Rich Source of Proteases

Enzymes are bio-catalysts that have marvellous applications in several industries including food, detergent formulations, metal recovery, leather processing etc. Extremophilic enzymes can withstand and catalyze reactions in extreme environments and found to have potential applications in above-mentioned industries<sup>18</sup>. Up to now, a vast array of more than three thousand (3000) enzymes have been reported and exploited in various industrial applications. This immense number is still not enough to meet the current demand because most known enzymes do not survive in extreme industrial reaction conditions, which results in drawing the attention for the isolation and optimization of microbes which can flourish

in that hostile environment. Microorganisms or microbial sources account for 90% of the enzymes produced for industrial purposes<sup>19</sup>.

Proteases (EC 3.4.21-24 and 99; peptidyl-peptide hydrolases) are among three major classes of enzymes which have been studied extensively and play a fundamental role in cellular metabolic processes including cell growth and differentiation. Proteases hydrolyze proteins, termed as proteolysis; results in the formation of protein, peptide fragments, and free amino acids<sup>20,21,22</sup>. These enzymes are not only biologically important but also industrially. Proteases are ruling and exploiting globally on an industrial scale<sup>23</sup> as they can withstand severe and harsh conditions like a wide range of pH, extreme temperature, high salts, organic solvents, detergents, and denaturing agents<sup>24</sup>. Proteases play a significant role in various industrial applications viz, brewing, meat, photographic, leather, dairy<sup>25</sup>, detergent, paper, and pulp and silk industries<sup>26</sup>, food, and pharmaceutical as well as in bioremediation processes<sup>27,28,29</sup>.

Proteases account for about the 2/3 (60-65%) share of the total enzyme market worldwide and this value is thought to be increased in near future. The dominance of this enzyme could be estimated by the fact that protease sales globally were estimated to be \$1 billion in 1998<sup>30</sup>.

### **Bacillus sp. and Alkalophilic Enzyme Production**

The major assemblage of alkaliphiles is found in the bacterial genera *Bacillus*, *Micrococcus*, and *Pseudomonas* but major contributions is of *Bacillus*. Important roles played by *Bacillus* spp. have a long history of more than 1000 years<sup>11</sup>. The alkaline proteases produced by different *Bacillus* spp. are of commercial importance as these can survive a wide range of temperature and pH for multiple applications. A variety of them has been isolated and characterized<sup>31,32,33</sup>. A huge list of microbial strains of *Bacillus* spp. i.e. *Bacillus pseudofirmus*, *Bacillus subtilis*, *Bacillus licheniformis*, and *Bacillus cereus* has been exploited at the industrial scale for their catalytic role (Table 1)<sup>34,35,36</sup>. *Bacillus flexus*, *Bacillus cereus*, *Bacillus pseudofirmus*, *Bacillus pseudoalkalophilus*, make up a toolbox of industrial enzymes and are alkalophilic protease producers showing optimum growth at pH 10<sup>37</sup>.

**Table 1. Different Alkaline Protease-Producing Bacillus Species Exploited at the Industrial Scale for Their Catalytic Role.**

S. No.	<i>Bacillus</i> sp.	Reference
1.	<i>B. stearothermophilus</i>	33
2.	<i>B. alcalophilus</i> TCCC11004	34
3.	<i>B. subtilis</i> AKRS3	35
4.	<i>B. pseudofirmus</i> SVB1	36
5.	<i>B. cereus</i> , <i>B. flexus</i> , <i>B. pseudoalcalophilus</i>	37
6.	<i>B. licheniformis</i>	38
7.	<i>B. thermoruber</i> BT2T	39
8.	<i>Bacillus</i> sp. strain B18'	40

The optimum enzyme activity of extracellular protease production from *Bacillus cereus* strain CA15 was noted in the medium containing skimmed milk and starch 1% with 0.6% MgSO<sub>4</sub>.7H<sub>2</sub>O at pH 8.0 and 35°C. This bacterium was found to be stable against various commonly available detergents and also showed maximum enzyme production in the stationary phase<sup>41</sup>. A similar attempt was done in which 39 isolates were collected from industrial waste from Lahore-Pakistan. The screening was performed on Luria broth medium agar with pH ranging from 8 to 10. Thirty-two (32) isolates were selected but only 20 were found to meet the above-mentioned criteria. Among 20 isolates, BCTL-147 was characterized as genus *Alcaligenes* which was then optimized for growth at pH 10 and 35°C<sup>42</sup>. In a study, *Bacillus halodurans* were used to optimize the production of the alkaline enzyme at different conditions of pH value, temperature, aeration, and incubation time along with different sources and concentrations of carbon, nitrogen, and metals. The optimum activity was observed at 37°C with 200 rpm agitation in a medium containing (g/L) lactose 15, soybean 6 together with a 5mM mixture of trace elements like Mg, Ca, and Mn<sup>43</sup>. Recently, 12 bacteria were isolated from Lonar soda lake, District Buldhana, Maharashtra-India. These isolates LAP42 were characterized as gram-positive rods at optimum growth conditions of pH 10 and 30°C in 1% NaCl. The crude enzyme was found to work

optimally at similar pH but at 65°C, augmented with 5mM CaCl<sub>2</sub> and MgSO<sub>4</sub>. This enzyme was well-suited to use in the detergent industry<sup>44</sup>. In Hungary, from alkaline soda lakes, three strains of halophilic and alkaliphilic nature (K1-5<sup>T</sup>, K1-10, and B1-1) were isolated, optimized, and characterized with maximum growth at basic pH 9.0-10.0 and 3-7% (w/v) sodium chloride. These strains were gram-positive straight rods, aerobic, catalase-positive and oxidase-negative. Multi-phasic characterization of the isolates indicates a novel species of *Bacillus*, *Bacillus aurantiacus* sp. nov.,<sup>45</sup>. Similarly, from dairy industry soil (pH 9.86) a *Bacillus* sp. was isolated. Upon optimization and characterization, the strain was found to produce extracellular alkaline protease optimally at pH 8-11, temperature ranging after 25-50°C and was somewhat halophilic. 16S rDNA sequencing confirmed the isolated species as *Bacillus flexus* and it was 99% identical with related existing strain viz., *Bacillus flexus* accession No. JN033557.1, FJ948078.1, EF157300.1 retrieved from the

databases Gene bank, NCBI, and Ribosomal Database Project<sup>46</sup>. Eighteen isolates were screened for alkaline protease production from various microenvironments, only six out of eighteen isolates showed efficient enzyme production. Among them, two isolates were recognized as *Bacillus pumilus* p1 and *Staphylococcus auricularis* p18 which showed noteworthy enzyme activity. Upon characterization, they showed optimal enzyme-producing activity at 72 hours incubation period, pH 8.0-9.0 and at 45°C. Nitrogen sources were optimized and reported as 0.9% and 0.5% for peptone and yeast extract, respectively for both strains. *Bacillus pumilus* and *Staphylococcus auricularis* showed 0.065 U/ml and 0.038 U/ml enzyme activity, respectively when assayed using the tyrosine-casein method. The potent applications were found as dehairing and depilating of raw leather, metal recovery (Ag) from X-ray photographic films and bacterial bio-film degradation<sup>47</sup>.

**Table 2. Some Examples of Alkaline Protease-Producing *Bacillus* Species and Their Cultivation Media.**

S. No.	Species	Medium	References
1	<i>Bacillus</i> sp. JB-99	Citric acid, 10.0(g/l); NaNO <sub>3</sub> , 10.0(g/l); K <sub>2</sub> HPO <sub>4</sub> , 5.0(g/l); MgSO <sub>4</sub> .7H <sub>2</sub> O, 0.3(g/l); CaCl <sub>2</sub> .2H <sub>2</sub> O, 0.2(g/l); NaCl, 5.0(g/l) and Na <sub>2</sub> CO <sub>3</sub> , 10.0(g/l) at pH 10.0.	49
2	<i>Bacillus pseudofirmus</i> AL-89	Casein, 10(g/l); peptone, 5(g/l); yeast extract, 1(g/l); K <sub>2</sub> HPO <sub>4</sub> , 1(g/l); MgSO <sub>4</sub> .7H <sub>2</sub> O, 0.2(g/l); CaCl <sub>2</sub> , 0.1(g/l); Na <sub>2</sub> CO <sub>3</sub> , 10(g/l); and for agar plates 15 g/l agar was included.	50
3	<i>Bacillus</i> sp. I-312	Soybean meal, 15(g/l); wheat flour, 10(g/l); fructose, 5(g/l); K <sub>2</sub> HPO <sub>4</sub> , 4(g/l); Na <sub>2</sub> HPO <sub>4</sub> , 1(g/l); CaCl <sub>2</sub> , 0.05(g/l); Na <sub>2</sub> CO <sub>3</sub> , 8(g/l) at 32 °C for 48 hours incubation period with agitation of 250 rpm.	51
4	<i>Bacillus</i> sp. Ve1	Gelatin, 10(g/l); casein enzymatic hydrolysate, 10(g/l); NaCl (w/v), 100(g/l) at pH 9.	52
5	<i>Bacillus</i> sp. NPST-AK15	Fructose, 20(g/l); yeast extract, 7.5(g/l); K <sub>2</sub> HPO <sub>4</sub> , 1.0(g/l); Mg <sub>2</sub> SO <sub>4</sub> .7H <sub>2</sub> O, 0.2(g/l); NaCl 50(g/l) and Na <sub>2</sub> CO <sub>3</sub> , 10(g/l).	53
6	<i>Bacillus</i> No. 221	Glucose, 10(g/l); polypeptone, 5(g/l); Difco yeast extract, 5(g/l); K <sub>2</sub> HPO <sub>4</sub> , 1(g/l); MgSO <sub>4</sub> .7H <sub>2</sub> O, 0.2(g/l); Na <sub>2</sub> CO <sub>3</sub> , 10(g/l).	54
7	<i>Bacillus alcalophilus</i> subsp. halodurans KP1239	1% sodium citrate, 0.3% yeast extract and 0.3% KH <sub>2</sub> PO <sub>4</sub> , pH 7.6 after 24h of cultivation	55
8	<i>Bacillus licheniformis</i>	Corn starch, 100(g/l); Amylase (Optitherm-L420), 0.4(g/l); Na-caseinate, 27.0(g/l); Soy flour, 23.0(g/l); (NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub> , 0.5(g/l); Na <sub>2</sub> HPO <sub>4</sub> .2H <sub>2</sub> O, 0.3(g/l); Corn steep liquor, 0.7(g/l); Antifoam agent Ke2111, 4.0(g/l); KH <sub>2</sub> PO <sub>4</sub> , 0.3(g/l); MnSO <sub>4</sub> .H <sub>2</sub> O, 0.02(g/l); FeSO <sub>4</sub> .7H <sub>2</sub> O, 0.05(g/l) and MgSO <sub>4</sub> .7H <sub>2</sub> O, 0.05(g/l).	56
9	<i>Bacillus sphaericus</i>	Glucose, 10.0(g/l); biopeptone, 5.0(g/l); yeast extract, 5.0(g/l); KH <sub>2</sub> PO <sub>4</sub> , 1.0(g/l); MgSO <sub>4</sub> .7H <sub>2</sub> O, 0.2(g/l) and Na <sub>2</sub> CO <sub>3</sub> , 10.0(g/l).	57

In a related study, a thermophilic *Bacillus* sp. GUS1 was identified from a soil sample from a citrus garden. Detection by SDS-PAGE and zymogram analysis confirmed the production of three proteases which were stable in the alkaline pH range (8.0-12.0), and optimum temperature (70°C). The enzymes retained 100% of their activities at these extreme values of pH and temperature. Addition of phenylmethylsulfonyl fluoride (PMSF) inhibited the presence of 2-mercaptoethanol however, iodoacetate does not affect the enzyme activities. SDS and EDTA affected the protease activities somewhat, but the presence of Ca<sup>2+</sup> showed no effect on their activity. This work inferred that these proteases were not metalloproteases, but Ca<sup>2+</sup>-independent serine alkaline proteases<sup>48</sup>. EDTA resistance and not requiring Ca<sup>2+</sup> ions are advantageous properties of these enzymes to be used as additives in the detergent industry. A number of researchers have previously utilized various *Bacillus* strains and different cultivation media to maximize the yield of alkaline protease (Table 2).

## CONCLUSION

From the above given account, it can be inferred that microorganisms, especially extremophiles, are amazing creatures which are immense sources of a variety of useful substances. Particularly, the alkalophilic protease producing bacteria which are significant contributors of biocatalysts with wide-ranging applications across several industries i.e. brewing, meat, photographic, leather, dairy, detergent, paper, and pulp, silk, food and pharmaceutical industries as well as in bioremediation processes. These enzymes are important because of their stability and ability to withstand the harsh conditions of industries. The use of protease, produced from alkalophilic bacteria, in the industry is still increasing, and there is a need to further explore the hostile environments for the isolation, characterization, and optimization of strains that give higher yields to meet with the ever-growing demands.

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## LIST OF ABBREVIATIONS

16S rDNA	Ribosomal deoxyribonucleic acid where S is Svedberg (a unit)
EDTA	Ethylenediaminetetraacetic acid
LB medium	Luria broth medium
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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