Protease Production from Polyextremophilic Bacteria

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ABSTRACT

Extremophilic organisms which are capable of surviving in two different extreme environmental conditions simultaneously are known as polyextremophiles. These organisms are known to be potent producers of bioactive compounds namely enzymes such as proteases which are rapidly used in the growing industrial sector uses such as in the detergent industry, dairy industry, silver recovering, leather industry, etc. These polyextremophilic bacteria have not been explored much, only 2-5% of them are known, the rest remains to be discovered, thus we aim to isolate proteolytic enzymes from polyextremophiles since the isolated enzyme will be stable at more than one extreme condition, which would therefore be greatly employed in the industrial sector where these enzymes will be stable at different conditions simultaneously.

Keywords
Extremophilic, Polyextremophiles, Proteases, Industrial sector

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Introduction

Extremophiles are those groups of microorganisms that have evolved to exist in a variety of extreme environments where normal life is not possible. They may be unicellular or multicellular organisms which fall into different categories such as thermophiles, psychrophiles, halophiles, barophiles, acidophiles, alkaliphiles and others (Rothschild et al., 2001). In order to adapt in such harsh conditions of temperature, pH, etc. microbes modify their cellular and molecular components and sustain well (Bertemont and Gerday, 2011).

Extremophiles are of wide industrial importance as a number of industrially active enzymes (amylases, proteases, lipases, cellulases, xylanases, etc.), secondary metabolites (antibiotics, phenols, alkaloids) and pigments are being produced by them which are of wide industrial importance, some examples of the biotechnological products derived from these extremophiles are given in Table 1 (Bertus van den Burg, 2003).

The extremophiles that can tolerate more than one factor of harsh conditions are called polyextremophiles. They have specialized cell wall architecture that makes them susceptible to more than one form of environmental stress, some common examples include thermoacidophiles, psychrophalophiles, thermoalkaliphiles, etc. However, only 2-5% of these polyextremophilic bacteria have been explored, the rest are still in dark, these
bacteria are known as producers of a great range of bioactive compounds (enzymes, antibiotics, pigments), thus they are a major area of research.

Polyextremophiles are the producers of industrially important enzymes such as *Bacillus* sp. which produces alkaline and serine proteases, amylases, pectinas, cellulases, lipases and xylanases (Martins et al., 2001).

Thus, polyextremophiles are a rich source of industrially important enzymes which are rapidly used to boost up the industrial sector and proteases being one of them. A number of polyextremophilic bacteria are known to survive in different extreme environmental conditions, some of the major types of polyextremophiles are described below.

**Types of Polyextremophiles**

The extremophiles that can tolerate more than one factor of harsh conditions are called polyextremophiles. They are unicellular or multicellular organisms that are present worldwide in different extreme environments where normal life is not possible i.e. they are found where others cannot survive. The polyextremophiles are divided into different categories depending on the habitats where they are found, some of them have been described in the text below.

Psychrohalophiles are readily found in environments where high saline conditions accompany cold atmospheres. These are found in habitats such as Arctic and Antarctic lakes and oceans. In India they are found mainly in the lakes of Jammu and Kashmir. A psychrophilic and slightly halophilic methanogen *Methanococcoides burtonii* was isolated from perennially cold, anoxic hypolimnion of Ace Lake, Antarctica (Franzmann et al., 1992).

Thermoacidophiles prefer temperatures of 70-80°C and pH between 2 and 3. They live mostly in hot springs or within deep ocean vent communities. The most thermophilic of the extreme thermoacidophiles, *Acidianus infernus*, grows at temperatures up to 95°C but at pH as low as 1.0 (Huber et al., 2006). Furthermore, several new species in known genera of *Sulfolobales* (Acidianus, Metallosphaera) have also been reported, as well as a new member of the Thermoplasmatales, *Thermogymnomonas acidicola* have been reported (Yoshida et al., 2006; Itoh et al., 2007; Plumb et al., 2007 and Kozubal et al., 2008).

Recently, an extracellular thermostable acid protease from a thermoacidophilic archaeon *Thermoplasma volcanium* was discovered (Semra et al., 2007).

Haloalkaliphiles require both alkalinity (pH 9) and salinity up to saturation 33% (wt/vol) for survival (Horikoshi, 1999). Some of the common habitats of these haloalkalophiles are Wadi Natrun Lakes of Egypt, Lake Magadi in Kenya, and the Great Basin lakes of the western United States (Shiladitya et al., 2012). In India they are usually found in Lake Lonar. Some examples of haloalkalophiles include *Natronobacterium magadii* (Lodwick et al., 1994), *Alcalilimnicola halodurans* (Yakimov et al., 2001), etc.

Halothermophiles are defined as an organism requiring at least 1.5 M NaCl and a temperature at or above 50°C for optimal growth. Only a small number of halothermophiles have been validly described so far. Some of them are *Haloarcula quadrata* (Oren et al., 1999), *Haloterrigena thermotolerans* (Montalvo-Rodriguez et al., 2000), and *Halobacterium salinarum* (Grant, 2001). Habitats for these microorganisms include Great Salt Lake in
the western United States and the Dead Sea in the Middle East.

Thermoalkaliphiles are a group of organisms which require both high temperatures as well as very high pH ranges. Few of them which have been recently discovered are *Bacillus clausii* (Kazan et al., 2005), *Bacillus licheniformis* (Olajuyigbe et al., 2005) and *Bacillus circulans* (Jaswal et al., 2007). They are found in various geothermally heated regions of the Earth, such as hot springs like those in Yellowstone National Park and deep sea hydrothermal vents. In India a great number of thermophiles can be extracted from arid and semiarid regions of Gujarat, Karnataka and Rajasthan.

Oligotroph is an organism that can live in environment that offers low levels of nutrients; they may be contrasted with copiotrophs, which prefer nutritionally rich environments. Pelagibacter ubique, which is the most important organism in the oceans and lichens with their extremely low metabolic rate. Lake Vostok in Antartica, sand plains and lateritic soils of South Western America and Indian Ocean are certain examples of oligotrophic habitats.

**Proteases**

Proteases (E.C.3.4.21.14) are those groups of hydrolytic enzymes that act on proteins and break them into peptides and amino acids, thus also known as proteolytic enzymes; they perform proteolysis by degradation of complex substances into simpler ones (Swapna et al., 2011). They are the most important industrial enzymes constituting upto 60-65% of world’s total enzyme market (Woods et al., 2001) and are of great application in detergents, food processing, silk gumming, feather processing, food processing, pharmaceuticals, remediation, biosynthesis and biotransformation (Gupta et al., 2002; Bhaskar et al., 2007; Jellouli et al., 2009 and Sareen and Mishra, 2008).

Proteases are produced from different kinds of microorganisms; from bacteria (Najafi et al., 2005; Nadeem et al., 2009 and Pawar et al., 2009), fungi (Charles et al., 2008 and Sindhu et al., 2009), yeast (Chi et al., 2007) and actinomycetes (Thumar and Singh, 2007; Vonothini et al., 2008 and Vishalakshi et al., 2009) in addition to its production from plants (papain and ficin) and animals (trypsin and chymotrypsin). Some of the different types of proteases are described in Table 2.

A number of bacterial species have been found to produce proteases, these could be exploited on a commercial level to boost up the industrial sector, and some of the bacterial strains which produce protease have been mentioned in Table 3.

**Industrial Uses of Proteases**

**Food and Feed Industry**

Proteases are often used for purposes such as cheese making, baking, preparation of soya hydrolysates, and meat tenderization. These enzymes are used to improve the extensibility and strength of the dough. Chymosin is usually preferred due to its high specificity for casein, which is responsible for its excellent performance in cheese making (Saraswathy et al., 2014).

**Leather Industry**

In leather industries, proteases are used to speed up the process of dahairing. Complete removal of hair has been achieved through enzymes without chemical assistance (Thangam et al., 2001; Dayanandana et al.,
2003 and Macedo et al., 2005). Similar findings about dehairing have also been reported with protease produced by a mutant strain of \textit{B. pumilus} BA06 (Wang et al., 2007). The use of enzyme based leather dehairing technology has been considered as an environment friendly alternative to the conventional chemical process (Dayanandan et al., 2003; Arunachalam et al., 2009).

**Table 1** Industrially Important Products Derived from Extremophiles.

| Thermophiles and Hyperthermophiles | Applications |
|------------------------------------|--------------|
| DNA polymerases                    | DNA amplification by PCR |
| Lipases, pullulanases and proteases| Detergents |
| Amylases                           | Baking and brewing |
| Xylanases                          | Paper bleaching |

| Halophiles                        | Applications |
|------------------------------------|--------------|
| Bacteriorhodopsin                 | Optical switches and photocurrent generators |
| Lipids                            | Liposomes for drug delivery and cosmetics |
| Compatible solutes e.g. Ectoin    | Protein, DNA and cell protectants |
| g-Linoleic acid, b-carotene and cell extracts, \textit{e.g. Spirulina and Dunaliella} | Health foods, dietary supplements, food colouring and feedstock |

| Psychrophiles                     | Applications |
|-----------------------------------|--------------|
| Alkaline phosphatase              | Molecular biology |
| Proteases, lipases, cellulases and amylases | Detergents |
| Polyunsaturated fatty acids       | Food additives, dietary supplements |
| Ice nucleating proteins           | Artificial snow, food industry \textit{e.g. ice cream} |

| Alkaliphiles and Acidophiles      | Applications |
|-----------------------------------|--------------|
| Proteases, cellulases, lipases and pullulanases | Detergents |
| Elastases, keratinases            | Hide dehairing |
| Cyclodextrins                     | Foodstuffs, chemicals and pharmaceuticals |
| Acidophiles                       | Fine papers, waste treatment and degumming |
| Sulphur oxidizing acidophiles     | Recovery of metals and desulphurication of coal |
| Acidophiles                       | Organic acids and solvents |
Table 2: Types of Proteases

| Types of Proteases | Uses | Brands |
|--------------------|------|--------|
| Serine proteases   | Uses a serine alcohol | Trypsin, Chymotrypsin, Elastase, Proteinase Thrombin |
| Threonine proteases| Uses a threonine secondary alcohol | Ornithine acetyltransferase |
| Cysteine proteases | Uses a cysteine thiol | Calpains, Cathepsins, Caspases, Papain |
| Aspartate proteases| Uses an aspartate carboxylic acid | Pepsin, Renin |
| Glutamic acid proteases | Uses a glutamate carboxylic acid | Eqolisins |
| Metalloproteases   | Uses a metal ion | Thermolysin, collagenase, Carboxypeptidases |

Table 3: Major Bacteria Producing Proteases

| Name of organism               | References                |
|--------------------------------|---------------------------|
| *Streptomyces microflavus*     | Rifaat *et al.*, (2006)    |
| *Aspergillus clavatus*         | Hajji *et al.*, (2007)     |
| *Bacillus circulans*           | Jaswal *et al.*, (2007)    |
| *Salinivibrio* sp. Strain AF-2004 | Heidari *et al.*, (2007)  |
| *Lactobacillus helveticus*     | Valasaki *et al.*, (2008)  |
| Thermophilic bacteria          | Tyagi *et al.*, (2008)     |
| *Pseudomonas aeruginosa*       | Tang *et al.*, (2010)      |
| *Streptomyces* isolate EGS-5   | Ahmad (2011)              |
| *Gammaproteobacter*            | Fulzele *et al.*, (2011)   |
| *Bacillus licheniformis*       | Sathyavrathan *et al.*, (2013) |

Medicinal Industry

Microbial proteases are increasingly used in treatment of various disorders such as cancer, inflammation, cardiovascular disorders, necrotic wounds, etc (Chanalia *et al.*, 2011). Proteases are also used as immunostimulants (Biziulenvicius, 2006). Proteases are used extensively in the pharmaceutical industry for preparation of medicines such as ointments for debridement of wounds. It is also used in denture cleaners and as contact-lens enzyme cleaners (Gupta *et al.*, 2002)

Detergent Industry

Enzymes have been added to laundry detergents since last 50 years to facilitate the release of proteinaceous material in stains such as those of milk and blood. Proteases isolated from *Pseudomonas aeruginosa* PD100 was used to remove blood stains from coton cloths in the absence of detergents (Najafi *et al.*, 2005). They are also used to remove proteins from cloths spoiled with blood, meat, sweat, etc (Kumar *et al.*, 2008).

Silk Degumming

Enzymatic degumming involves the proteolytic degradation of sericin. Enzymatic action modifies the surface of wool and silk fibres to provide them a new and unique finishing. The traditional process
are generally expensive and therefore an alternative method suggested is the use of enzyme preparations, such as protease, for degumming the silk prior to dyeing (Johnny et al., 2012).

**Silver Recovery**

Recovery of silver by burning the films causes environmental pollution and health risks. On the other hand, protease breaks the gelatin layer embedded with silver in films creating pollution free stripping. The amount of silver varies from 5-15 g/kg of film. The enzymatic method although being slow is free from pollution and cost-effective too (Vaishali, 2013).

**Peptide Synthesis**

Recently the application of proteases in synthesis of oligopeptides has received great attention as an alternative to chemical approach (Ma et al., 2007; Wang et al., 2009). Proteases have been used successfully for the synthesis of dipeptides (Barros et al., 1999) and tripeptides (So et al., 2000).

**Proteases from Polyextremophiles**

We have recently discussed about the multiple applications of proteases in different industrial sectors, however, if an enzyme is designed to combat in a variety of conditions then that will prove as a boon for the industrial sector. A number of polyextremophiles have been identified in the last few years which are a rich source of industrially important enzymes and other secondary metabolites but a very little work has been done on protease production from polyextremophiles, thus they could be cultured and grown for isolation of proteases which are of industrial use.

In conclusion, they are the unique microorganisms, with great potential for microbiology and biotechnological exploitation. Proteases play a decisive role in detergent, pharmaceutical, leather, food and agricultural industries. However, only 10% of the polyextremophiles have been discovered, the rest remains to be discovered, they are of great industrial application and could be exploited in number of industries to boost up the industrial sector, thus a lot of work remains to be done on them. Properties of proteases such as alkaline pH, thermostability, solvent and detergent resistance makes the enzyme very useful for various industrial applications. Thus it is desirable to search for new proteases with novel properties from as many extremophilic sources as possible.

A large number of proteases have already been discovered, but we are looking towards those proteolytic enzymes which can work simultaneously at two different conditions i.e., at high temperature and high pH (Thermoalkaliphiles), high temperature and low salt concentrations (Halothermophiles), etc.

Thus, these polyextremophiles which are capable of working at two different conditions are a novel source of industrial enzymes with potent industrial applications. Thus, the emphasis is towards isolation of proteases producing polyextremophiles which can act simultaneously under two different extreme conditions.

Looking into the commercial success of this enzyme class, researchers have now started aiming at the discovery and engineering of novel enzymes that are more robust with respect to their pH and temperature kinetics. Hence, although microbial proteases already play an important role in several industries, their potential is much greater and their
applications in future processes are likely to increase in the near future.

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