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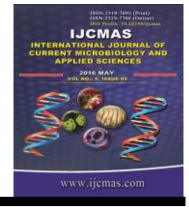

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Protease Production from Polyextremophilic Bacteria

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A B S T R A C T

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.

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, psychrohalophiles, 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, pectinases, 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, bioremediation, 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 *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,*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 e.g. ice cream
Alkaliphiles and Acidophiles	**Applications**
Proteases, cellulases, lipases and pullulanases	Detergents
Elastases, keritinases	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

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)
Aspegillus clavutus	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)
Thermophillic 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 an 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 aerugenosa 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 dyein (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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