Characterization of an alkalitolerant *Bacillus* sp. for production of extracellular proteases

THESIS

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Declaration

I here declare that the work being presented in the thesis entitled "Characterization of an alkalitolerant Bacillus sp. for production of extracellular proteases" in the partial fulfillment of requirements for the award of degree of M.Sc. Biotechnology, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala is my own laboratory work carried out at the CSIR-Institute of Microbial Technology, Chandigarh, during the period of January 2019 to June 2019, under the conception and supervision of Dr. Beena Krishnan. I have not submitted the matter embodied in this thesis for the award of any other degree.

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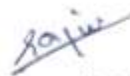

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This is to certify that the above statement made by the candidate is correct and true to the best of my knowledge.

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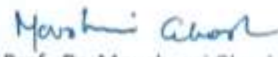

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Place: Chandigarh Rajni

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SYMBOLS AND ABRREVATIONS

A	Absorbance
BSA	Bovine serum albumin
CAGR	Compound Annual Growth Rate
DNA	Deoxyribose nucleic acid
gDNA	Genomic Deoxyribonucleic acid
hrs	Hours
kb	Kilobase
mg	Miligram
MIC	Minimum Inhibitory Concentration
mins	Minutes
ml	Millilitre
mm	Millimeter
NA	Nutrient Agar
NB	Nutrient Broth
nm	Nanometer
^{o}C	Degree celsius
OD	Optical density
PCR	Polymerase chain reaction
pH	Potential of hydrogen
16S rDNA	DNA that codes for 16S rRNA
rpm	Revolution per minute
TEM	Transmission electron microscopy
Tyr	Tyrosine
µg	Micro gram
µl	Microliter
µm	Micro meter
UV	Ultraviolet
w/o	Without
w/v	Weight/Volume
YEPD	Yeast extract peptone dextrose

Introduction

Biotechnology has gained rapid growth and development in the market as it offers various advantages over conventional methods and technologies used in industries. Industrial biotechnology introduces microorganisms and its products "enzymes" as biocatalysts to produce products with high quality and lower manufacturing cost. Enzymes are proteins that function as catalyst to accelerate the rate of reactions in cells. The microbial enzyme research involves isolation, identification, characterization, and production of these molecules from bench scale to pilot scale for their utility in industries. Enzymes possess special characteristics such as tolerance to extreme conditions, substrate specificity, high rate of reaction, biodegradability and stability at different pH and temperature (Adrio and Demain, 2014). These features allow industries to improve the product yields at lower cost (Jegannathan and Nielsen PH, 2013). Some of the other benefits associated with using enzymes includes replacing toxic chemicals like replacing acids in the starch processing industry, use of enzyme for removing stains allows washing clothes at lower temperature which leads to saving energy etc. (Gurung et al., 2013).

Enzymes that are already in use in several commercial processes includes cheese making, detergent industries, leather industries, and fermentation (Pandey et al., 1999). Industrial enzymes commonly used are proteases, carbohydrases (includes amylases, cellulases, pectinases, lactases and xylanases), lignin peroxidase, lipases, nitrite reductase, horseradish peroxidase, polyphenol oxidases, and urease. Global market for industrial enzymes was supposed to reach approximately $4.2 billion in the year 2014 and estimated rise at a compound annual growth rate (CAGR) of about 7 % over the time of 2015-2020 and reach close to $6.2 billion (Singh R et al., 2016) as shown in the Fig 1.1.

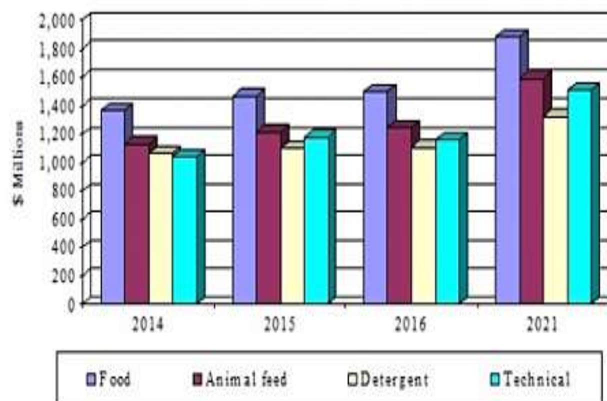


Figure 1.1: Global market for industrial enzymes (Source: http://blog.bccresearch.com/green-chemistry-boosts-global-market-growth-for-industrial-enzymes)

The enzyme market can be broadly classified into three types based on their industrial application: food enzymes, feed enzymes and technical enzymes (Saxena, 2015). World population is forecasted to increase by 6.9 billion USD to 9.1 billion USD with rise in demand of food by 70 % by 2050, a report by United Nation Department of Economic and Social Affairs(UNDESA)(http://www.un.org/waterforlifedecade/food_security.shtml).Worldwide industrial enzyme market is governed by food and beverage industries and predicted to reach a value of $2.3 billion by 2020 (Singh et al., 2016). The global enzyme market for feed enzymes was supposed $899.19 million in 2014 and estimated to reach about $1.3 billion by 2020, at a CAGR of 7.3 % from 2015 to 2020 (Singh et al., 2016). Technical enzyme category includes enzymes used in starch processing, paper and pulp processing, detergent, textile and leather industry (Saxena, 2015). Detergent industry is one of the largest industry using enzymes and contribute for 25–30 % of the total sales of enzymes and is expected to reach at a CAGR of about 11.5 % from 2015 to 2020 (Singh et al., 2016).

In the research work carried out in this thesis, we explored for the presence of two of the technical enzymes, amylases and proteases, in a alkalitolerant *Bacillus* sp. and a brief introduction to the these two widely used enzymes is described next.

1.1 Amylase

Amylases holds about 25-33% of the world's enzyme production (Souza, 2010). Amylases are one of the most important industrial enzymes and are of great interest for biotechnology. Starch is a polysaccharide consists of glucose monomers joined by α (1, 4) linkages. The simplest form of starch is the linear polymer amylose. Amylopectin is the branched form for starch with α (1, 6) linkage at the branch points. Amylases can be classified into three types: α-amylases, β-amylases and γ-amylases. Amylases are metalloenzymes with calcium ion requirement for their stability and activity. In contrast, amylases are exo-enzymes that hydrolyses the (1-4) linkage from the non-reducing end of the polysaccharide chain. Releasing two glucose units (maltose) at γ-amylase

α (1-6) and the last α (1-4) linkage of the non-reducing end of amylose and amylopectin (Sundarram et al., 2014). Enzyme industries focus more on α-amylase because it is an important enzyme in digestive and metabolic processes. α-amylases (E.C.3.2.1.1) belong to the family of endo-amylases enzymes that catalyse hydrolysis of internal α-(1, 4)-glycosidic linkages in starch and produces glucose, maltose and maltotriose units (Welker and Campbell, 1963) (Fig 1.2).

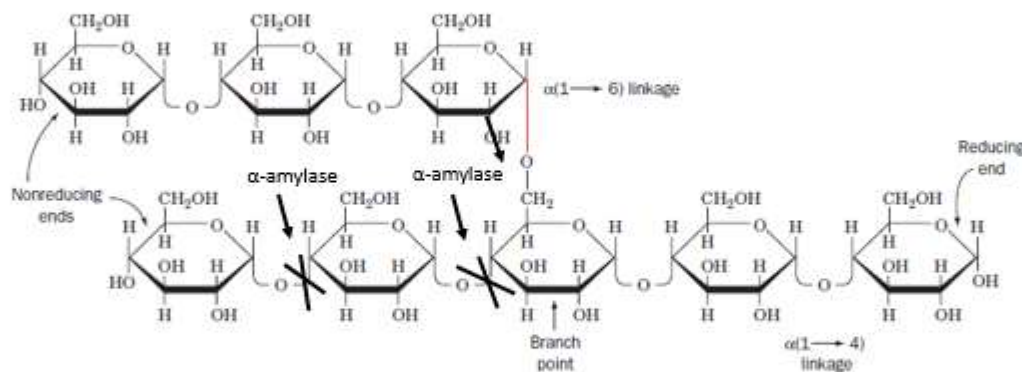


Figure 1.2: A schematic representation of starch and its hydrolysis by amylases.
(Ref: https://biochem1362.wordpress.com/tag/glycogen/)

Common sources of amylases are plants, animals, higher organisms and microorganisms (Gupta et al., 2003) mostly microbial enzymes are used as they meet the industrial demands. Some of the microorganisms that are active producers of amylases listed in Table 1.1

Table 1.1: Amylase producing microorganisms (Allam et al., 1975, Sundarram et al., 2014)

Bacteria	*Bacillus stearothermophillus, Bacillus subtilis, Bacillus circulans, Bacillus flavothermus, Bacillus alcalophilus subsp. halodurans, B. amylofaceins*
Actinomycetes	*Thermo actinomyces vulgaris, Thermonos- pora vulgaris, Thermonospora curvata*
Microscopic fungi	*Humicola insolens, Humicola lanuginosa,* T. lanuginosus *Humicola stellata, Malbrachea pulchella var. sulfurea, Mucorpusillus, Talaromyces thermophilus.* T. lanuginosus*IISc91*

Several industries use amylases such as in brewing, chemicals, in food industry, pharmaceutical etc. Amylases used in pharmaceutical industries such as in digestive syrups and tonics, hydrolyze

starch into different sugars like glucose and maltose, which have other applications. Industrial application of amylases in different industries is listed in Table 1.2.

Table 1.2: Use of amylases in various industrial sectors (Nigam, 2013)

Industrial sectors	Applications
Food industry	Production of cake, fruit juices, syrups (glucose, maltose, corn, fructose).
	In sugar syrup, viscosity is reduced.
	In fruit juices haze formation is reduced.
	For alcohol fermentation saccharification and solubilisation of starch in brewing industries to ferment alcohol.
Pharmaceutical industry	Used as a digestive aid
Paper industry	Reduction of viscosity of starch, smooth and strong coating of paper
Textile industry	As a sizing agent to strengthen the thread
Detergent industry	Useful remove residues of starchy foods

1.2 Proteases

Protease falls in the category of the largest group of industrial used enzymes worldwide and has a sale of 60% (Hamza, 2017). Functionally, proteases hydrolyze peptide bonds in proteins and they are also referred as peptidase or proteinase or proteolytic enzymes (Fig1.3)

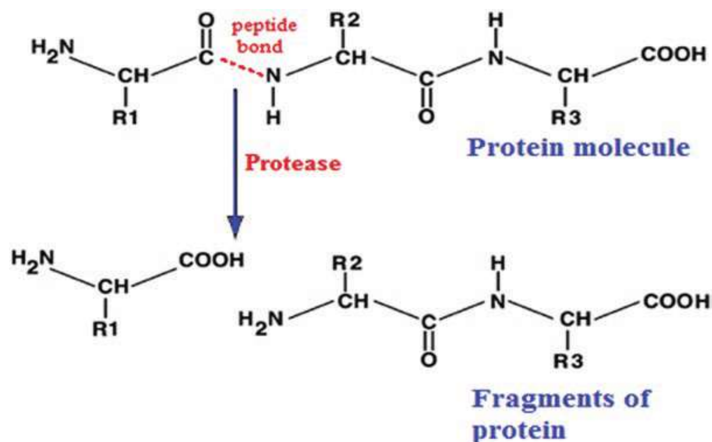


Figure1.3: Schematic representation of proteolysis reaction

(Ref: https://pubs.niaaa.nih.gov/publications/arh27-4/317-324.htm)

Proteases are vital enzymes for all living creature and therefore are present in all life forms. Examples of plant proteases are papain and bromelain. Digestive proteolytic enzymes in animals include trypsin, chymotrypsin and pepsin. Microorganisms are the largest sources of proteases one of the classification scheme for proteases is based on their optimum pH for activity. The three groups of proteases are based on this class are: acid proteases which function in a pH range about 2.0-5.0 and are produced only by fungi; neutral protease working in the pH range from 7.0-8.0 with plants as the primary source and alkaline proteases, functioning at pH above 8, majorly produced by bacteria.

Some of the microorganisms with protease are listed activity in Table 1.3.

Table 1.3: Proteases producing microbes (Hamza, 2017; Naidu, 2011)

Bacteria	*Bacillus subtilis, B. amyloliquifaciens, B. alcalophillus, B. cadolyticus, Streptococcus cremoris, Streptococcus lactic, Stretomycesgriseus, Streptomyces lividans, Enterobacteriaceae, B. megaterium, B. mojavensis*
Fungi	*Mucorpusillus, Mucormiehei, Rhizopusniveus, Rhizopuschinensis, Aspergillus awamori, Aspergillus fumigafus*
Virus	*Adenoviruss, Retroviruses, Herpesvirus, Picornviruses.*

Mostly, proteases are used in the industry of food, leather and detergent, diagnostics, waste management and recovery of silver from waste films. Proteolytic enzymes are also used in medicine as it is indicated by various clinical studies that they have huge benefits in inflammation, blood rheology, oncology and immune system regulations (Singh et al., 2016). Proteases are used in silver recovery from photographic film mostly *Bacillus* species are used in silver recovery processes such as *Bacillus licheniformis* KBDL (Hamza, 2017). The largest utility of proteases till now is in laundry detergents, where they aid in removing pretentious stains (Hamza, 2017) and *Bacillus* species play vital role in detergent industry. Some of the bacillus strains are *Bacillus* licheniformis N-29, *Bacillus subtilis,* are used in detergent industry (Hamza TA, 2017). Bacillus cereus strain AT efficiently used in dehairing processes (Hamza TA, 2017).

Based on the market demand of industrial enzymes, there is a need and interest to explore present microorganism enzymes and to develop technology for production of these proteins for industrial applications.

In view of the literature, the project was designed towards "Characterization of an alkalitolerant *Bacillus* sp. for production of extracellular proteases" with the following objectives:

Objectives:

- Characterization of an alkalitolerant *Bacillus* species (alkalitolerant *Bacillus sp.*) procured from MTCC, CSIR-IMTECH, Chandigarh.
- Standardization of alkalitolerant *Bacillus* sp. growth for maximum expression of extracellular protease(s).
- Production and quantification of an extracellular protease(s) from alkalitolerant *Bacillus* sp.

3.0 Review of literature

Microbial enzymes contribute very significantly to the biotechnology based application in industries. Many of the reactions in industry use extreme conditions of temperature, pH, and salinity. Enzymes produced by organisms classified as extremophiles, which survive under extreme conditions of temperature, pH, pressure, salinity etc. in nature find application in such difficult industrial processes.

3.1 Extremophiles

Extremophiles are primarily prokaryotes (bacteria and archaea) (Grant et al., 1998). Organisms with an optimum pH for growth at or below 3-4 are called as acidophiles such as *Bacillus acidicola*. The organisms described as alkali tolerant grow at pH values above 9 like *Clostridium paradoxum, Haloburum sp*. Organisms that require at least 1M of salt for growth are termed as halophiles such as *Halobacterium* sp. and *Halorubrum* sp (Oren, 2008). Organisms having growth temperature optimum of 80°C or higher called as hyperthermophile like Aquifex pyrophilus and P.fumarii. (Niehaus et al., 1999). Those microbes live optimally at hydrostatic pressures of 40MPa or higher termed as piezophile such as *Pyrococcus sp* and some of the organisms have a growth temperature optimum of 30°C or lower and a maximum temperature of 20°C such as *Methanogenium* known as psychrophiles (Hough et al., 1999). Extremophiles used in various industrial sectors (Table1.4) and enzymes produced by extremophiles are termed as Extremozymes (Hough et al., 1999). These are of great importance in industries as they are functional and active under adverse conditions such as proteases and cellulases produced by alkaliphiles.

Table 1.4: Extremophiles with their applications in different sectors (Rasuk et al., 2016; Otohinoyi and Ibraheem, 2015; Hegde and Kaltenegger, 2012)

Environment	Organisms	Applications
Deep sea (barophilic)	*Photobacterium sp, Pyrococcus, Pyrococcus sp.*	Food processing and antibiotic production
Snow, frozen water bodies around -17°C (psychrophiles)	*Moritella profunda, Vibrio, Ps.fluoresecne, Antartic strain TAB, Pseudoalteromomnas sp., Candida sp.*	Detergents and food applications, dephosphorylating of DNA vectors, Hydrolysing esters of medical relevance, organic synthesis related to food/feed, pharmaceuticals
Salinity conditions (halophiles)	*Streptomyces sp., Salinivibrio sp., Micrococcus sp.*	Detergent formulation, Flavor enhancing in food industries, fatty acid degradations
Soda lakes (alkaliphilic)	*Bacillus sp. halobarum sp,*	Detergent, leather and food industry
Nuclear reactor water core, submarine vent (radiation tolerant)	*D.radiodurans, Kineococcussp. Rubrabacter sp.*	Degrade or transform hazardous waste to less hazardous mixture
Sites (toxic, waste and industrial), (toxitolerant)	*Rhodococcus sp.*	Biodegradation of organic pollutants.
Dry solfataric soil (acidophile)	*Bacillus acidicola*	Starch processing, single cell protein from shellfish waste

3.1.1. Alkaliphiles

Alkaliphilic microorganisms grows well at pH 9 and above, usually in the pH range of 10-13. (Horikoshi K, 1999). Facultative alkaliphiles is a term used for alkaliphiles that grow optimally under strict alkaliphiles conditions but can also grow effectively near neutral pH, while obligate alkaliphiles grow only at pH values of ~pH 9 and above (Guffanti and Hicks, 1991). However, there is no definite characterization of an alkali tolerant organisms because some of them exhibit different range of pH and sometimes, even more than one pH for activity and growth. Growth of bacteria depends on availability of metal ions, nutrients, physical parameters such as temperature, pH etc. Alkaliphiles are widely found in nature and can be obtained from normal environments such as garden soil (Fig 1.4) and as expected these occur at higher frequencies in alkaline soil.

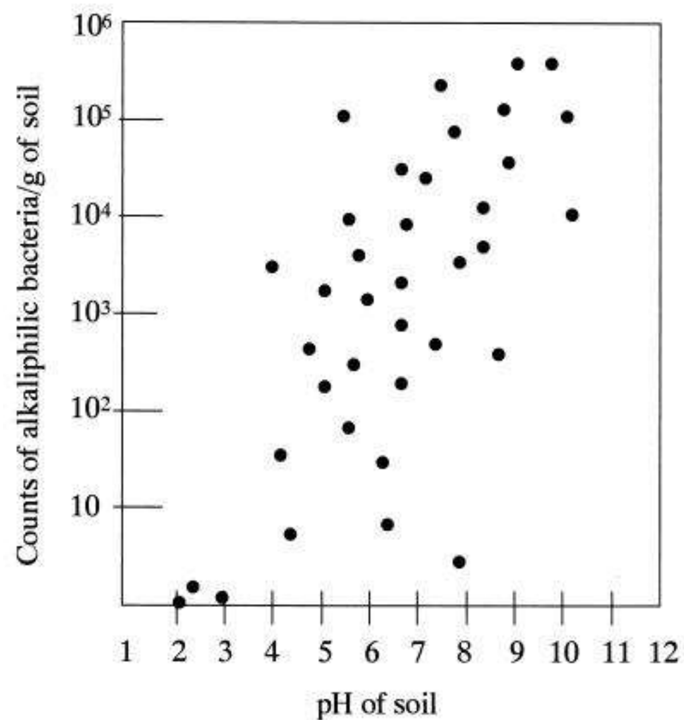


Figure 1.4: Distribution of alkaliphilic microorganisms in different soil environments (Horikoshi, 1999)

Alkaline pH in soil is caused by some biological processes and activities such as ammonification, sulphate reduction, and photosynthesis lead to generation transient alkaline conditions in soil.

Moreover, some commercial processes such as manufacturing or casting cement, electroplating, and vegetables especially potatoes, lye treatment and animal hides also contribute to alkaline conditions in soil However, from such environments comparative restricted range of microorganism can be explored like *Bacillus spp*. There are some natural occurring and most stable alkaliphiles environments on earth, where diversity of alkaliphiles can be found. One of them is soda lakes, possess a much broad range of alkaliphiles bacteria example *Arthrobacter spp*. Similarly, wide range of alkaliphiles can be isolated from $Ca(OH)_2$ enriched oligotrophic ground water. Alkaliphiles microorganisms have a great impact on the mass market products with huge number of applications in biotechnology. Commercially available enzymes in the markets are from a few bacterial taxa (notably *Bacillus spp*) (Grant et al., 1990). From the enzyme market point of view only few environments such as soda lakes or similar environment conditions are expected to yield economical efficient organisms producing new products.

3.1.2. pH hemostasis

The concentration of hydrogen ion is one of the most important environmental parameter affects the growth and activity of microorganisms. Most microorganisms can grow at pH range 4-8 and normally grow near to neutrality. Several microorganisms such as the alkaliphiles or acid tolerant ones grow outside these pH ranges, whereas their optimum pH for growth lies in between normal pH range. Surviving in an alkaliphiles condition is a challenge for bacterial cell based on the chemiosmosis principle. PMF (proton motive force) of prokaryotes possess two components, a transmembrane pH gradient (ΔpH) and electrical potential (Δψ). Generally, the former is more alkaliphiles inside the cell compared to outside and latter is more negative comparative to outside. Whereas, in alkaliphiles microorganisms the external concentration of proton ions is lower relative to inside of the cell. For maintaining pH homeostasis there is persistent availability of the efflux cation. However, it is difficult to manage high levels of PMF for the bacterial cells living in alkaliphiles conditions. Antiporter system aid the removal of cation from cytoplasm. Antiporters play crucial part for alkaliphiles, for maintaining cytoplasmic pH that is often two units below to the pH of the medium (Krulwich, 1995). Moreover, the process of capturing proton is enhanced by increasing expression of F_1F_O-ATP synthase. It has been proposed that

bacteria surviving in high pH could use sodium ions as coupling ion in addition to and instead of hydrogen ion. (Mulkidjanian et al., 2008).

3.1.3. Enzymes produced by alkaliphiles

Bacillus produces various enzymes like dispase, proteases, lipases, amylases, carbohydrate active enzymes of these various enzymes, proteases produced by alkaliphiles are the best biochemically characterized and widely used industrial enzyme.

3.1.3.1. Proteases

Proteases are also called peptidases or proteinases that decompose proteins by hydrolyzing their peptide (amide) bonds. Proteases are of great interest due to their valuable and potential industrial applications in several industrial sectors like feed, leather, textile, detergent, food and pharmaceutical industry.

3.1.3.2. Sources of proteases

Since proteases can be found in all form of life, have wide diversity of sources such as plants, animals, bacteria, archaea and viruses (Rao et al., 1998).

3.1.3.2.1. Plant proteases

Plant proteases are procured by extraction of plant materials. Accessibility of plant material depends upon various parameters such as growth conditions, suitability of weather and cultivation, availability of land for harvesting, and yield of plant. The process of obtaining plant proteases is long. They are not generally used in industries except for some of enzymes like bromelain, papain and keratinases represent well known plant proteases.

3.1.3.2.2. Animal proteases

Animal Proteases are procured by extraction of tissues. These proteases are prepared in huge quantities. Animal proteases production depends on availability of healthy tissue and livestock for slaughter and it is governed by agricultural, regulatory, ethical and political policies. The most familiar animal proteases are trypsin, chymotrypsin and pepsin.

3.1.3.2.3. Microbial proteases

Microbial proteases are always centre of attraction as cells can be cultured in bulk and in very short time by establishing fermentation methods (Kasana et al., 2011). The current enzyme market demand is primarily fulfilled by microbial proteases and not from plant and animal enzymes. Microbial proteases can be manipulated genetically to produces new enzymes with desired properties can be used in several applications). Microbial proteases can be obtained and processed from bacteria, fungi and viruses, but bacterial proteases are more preferred and extensively studied as they are easy to handle and consumes less time for production. Industrially important proteases, are mainly neutral and alkaliphiles and produced by organisms belonging to the genus *Bacillus*. Example of bacterial proteases: thrombin, plasmin, streptokinase, nattokinase (Singh et al., 2016).

Microorganisms producing proteases: *Bacillus pumilus, B. aquimaris, B. laterosporus, B. coagulans, B. amovivorus, B. flexus, B. horikoshii,Bacillus clausii, B. cereus, B. licheniformis, B. circulans B. sphaericus, B. cohnii, B. subtilis, B. sterothermophilus, B. mojavensis, B. pseudofirmus, B. megaterium, B. brevis, B. anthracis, B. thuringiensis B. coagulans, B. megaterium,, s, B. stratosphericus, B. polymyxa, B. mojavensis, B. Lentus, B. firmu , B. amyloliquifaciens, B. proteolyticus B. subtilis, B.intermedius, B. thermoruber, , B. fastidiosus, , B. pantotheneticus, B. alcalophilus, Pseudomonas aeruginosa, , P. putida, Aromonashydrophila, Serratialiquefaciens, P. fluorescens Flavobacteriumbalustinum, Exiguobacterium sp*.(Gupta et al., 2002).

3.1.4. Proteases classification

Exopeptidases enzymes: Exopeptidases cleave the peptide bonds proximal to the carboxyl or amino terminal of the substrate. Based on the site of action at the C or N terminus, proteases are categorized as carboxypeptidases and amino peptidases respectively.

Endopeptidases enzymes: Endopeptidases cleave peptide bonds distant from the termini of the substrate. Based on functional group/ their mechanism of action, these are aspartic, serine, cysteine and metallo-proteases. Other differentiation criteria can be pH. On the basis of pH, proteases are classified as acidic, neutral or alkaline proteases (Rao et al., 1998; McDonld, 1985). Further classification of exopeptidases and endopeptidases are listed in Table 1.5.

Table 1.5: Classification of proteases (Rao et al., 1998; McDonld, 1985)

Proteases	Mechanism	Examples	Common Sources
Endopeptidases			
Serine proteases	Endopeptidases have an active center serine involved in the catalytic process.	Trypsin, Chymotrypsin plasmin, thrombin Subtilases	*Digestive proteases in animals, Bacillus halodurans Bacillus* sp *etc*
Cysteine proteases	Activity depends upon presence of histidine and cysteine	Papain, Bromelain, clostripain	Papaya fruit, pineapple, *Clostridium histolyticum*,
Aspartic proteases	Depend on aspartic acid residue for their catalytic activity.	Pepsin, Renin Pepsin-likemicrobial enzymes Renin-like microbial enzymes	*Aspergillus*, *Penicillium*, *Rhizopus Endothia, Mucor* spp.
Metallo proteases	Carboxypeptidases use a metal ion in the catalytic mechanism	Thermolysin Thermolysin-like, Zn-metalloproteases	*Aspergillus, Pencillium, Pseudomonas, Streptomyces Clostridium*
Exopeptidases			
Amino-peptidases	Those acting at a free N-terminal liberate a single amino acid residue	Aminopeptidase I Aminopeptidase II	*Escherichia coli, B. stearothermophilus*
Carboxy-peptidase	Act on C-terminal of the polypeptide chain, releases single residue of amino acid or a polypeptide	Serine carboxypeptidases	*Aspergillus* spp*, Saccharomyces* spp.

3.1.5. Applications of proteases

Owing to the use of proteases in a large number of industries (Fig 1.5), the major global share of industrial enzyme market belongs to proteases (Hamza, 2017). Proteases are prepared in huge quantities and even used as crude preparations in many applications. Protease used in food and detergent industries are produced in small quantities and require maximum purification before use. Based on recent trend of developing environmental friendly processes and technologies, proteases are of more interest to explore their applications in industries like treatment of leather in leather industry and in other bioremediation processes. Proteases are used in the pharmaceutical industry for formulation of medicines such as ointments for wounds, such as pronod 153L cleaner is used to clean surgical instruments fouled by blood proteins etc. The success of detergent enzymes in detergent industry has led to exploration of detergent proteases with efficient and specific uses. For cleaning membrane systems, enzymes such as Alkazym azym, Terga-zyme, Ultrasil are used. In addition to these applications, alkaline proteases are also used in other applications, such as in isolation of nucleic acid in molecular biology, cleaning of contact lens (Nakagawa et al., 1994), controlling of pest and insects, in the process of degumming silk (Gupta et al., 2002) and packed columns of stainless steel fouled with gelatin and β-lactoglobulin can be cleaned by proteases (Sakiyama et al., 1998).

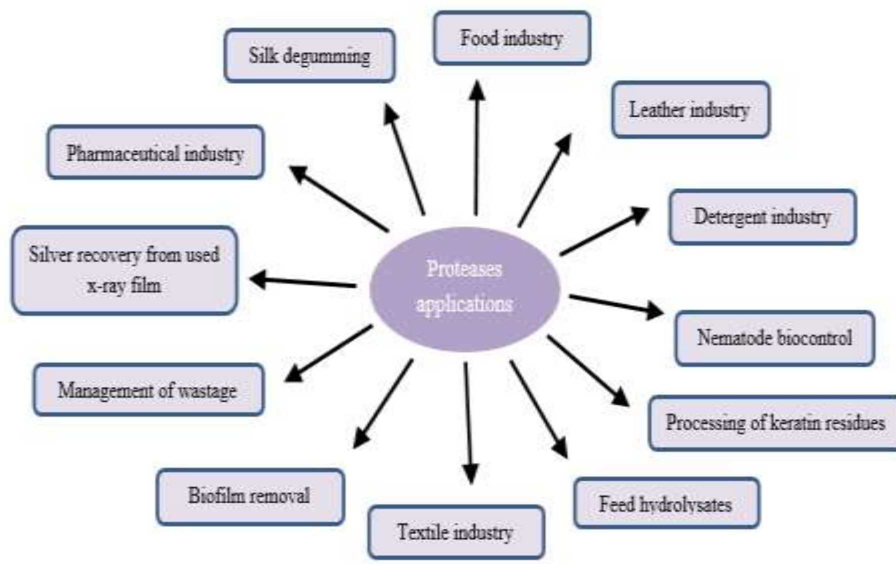


Figure 1.5: Applications of microbial proteases in different sectors (adapted from Singh, et al., 2017)

A few major uses of alkaline proteases in industrial sectors are described in the following sections.

3.1.5.1. Leather Industry

Conventionally, leather processing involves hazardous chemicals such as hydrogen sulfide, formic acid and other chemicals, contributing to environmental pollution. Using enzymes as alternatives to chemicals has proven successful in enhancing in leather quality, easy control, speed and waste reduction, thus being ecofriendly (Varela et al., 1997). Alkaline proteases possess elastolytic and keratinolytic activity and are utilized in leather-processing industries. Processing of leather includes various steps such as dehairing, soaking, bating, and tanning. Proteases find their use in the step of soaking. Earlier this step was done by an alkali and now a day, microbial alkaline are used to increases water absorption and to shorten the time requirement for the same step (Rao et al., 1998). Alkaline proteases such as *B. subtilis* IIQDB32 used in dehairing and bating stages of preparing skins and hides. As alkaline proteases speed up the process of dehairing, it swells hair roots and the consequent attack of proteases allows easy removal of the hair from the hair

follicle. Three different proteases, aquaderm for soaking, NUE for dehairing and pyrase for bating are manufactured by Novo Nordisk (Rao et al., 1998).

3.1.5.2. Food Industry

Proteases have been used for various purposes in food industry such as preparation of soya, baking, hydrolysates, and meat tenderization. Majorly, proteases are used in dairy industry for manufacturing cheese. The three main milk-coagulating enzymes used are, are used, animal rennet's, microbial milk coagulants, and genetically manipulated chymosin. Rennet is extracted from calves stomach. Animal and microbial rennet's are acid aspartate proteases. Due to shortage in calf rennet, there is a high demand for alternative way for production of cheese so, microbial milk coagulants are used. But using microbial enzymes also has its own limitations as they are inactive at higher temperatures and result in poor yield of desirable products. Functionally, proteases hydrolyze proteins; and this characteristic has been explored for the preparation of protein hydrolysates in food industries. In the formulation of dietic and healthy feeds and protein-fortified soft drinks, protein hydrolysate is used (Neklyudov et al., 2000). It has been reported that *B. subtilis* proteases are used in the production of fish hydrolysates with high nutritional value (Rebeca et al., 1991). Moreover, alkaline protease is used for the production of soy protein hydrolysates (Arai et al., 1970). In food products, the bitter taste of protein hydrolysates is a major barrier to their use. The presence of a proline residue also contributes to the bitterness. For debittering protein hydrolysate, some peptidases are of great value in food industry (Rao et al., 1998).

3.1.5.3. Photographic Industry

Silver is one of the noble metals used in bulk quantities, specifically in the photographic industry. With a rise in demand for silver in the recent days, there is interest in recovery of silver from used photographic or X-ray films. Conventionally, silver is recovered by process of burning out the X-ray films that directly contributes to environmental pollution. The waste X-ray films (1.5-2.0% silver by weight) containing black metallic silver spread in gelatin are considered to be very good source for recovery of silver compared to other available films. Films are made up of polyester and silver, cannot be efficiently recovered using conventional methods. An alternative method was developed for stripping out the silver from wasted X-ray photographic films. Alkaline

proteases have crucial part in the bioprocessing of consumed or wasted x ray films for recovery of silver. Enzyme extract produced by *B. subtilis* ATCC 6633 is used for stripping out the silver layer (Nakiboğlu et al., 2001). Polyester film can be recycled by enzymatic hydrolysis of gelatin. It has been reported, proteases can be used to degrade the gelatin in few minutes for silver recovery. About 99% pure silver recovery has been successfully achieved by using such enzyme treatments. Alkaline protease is thus an alternative enzyme for the photographic industry (Hamza, 2017).

3.1.5.4. Detergent industry

The detergent industry contributes approximately 25% of the total sale of enzyme market in world (Singh et al., 2016). Enzymes are always great interest to the detergent industry due to their ability in removing stains, especially proteinaceous stains. The detergent manufactures have great reliance on enzyme technology as it satisfies good fabric restoration, cleaning benefits, improved performance advantage over cost ratio and it also provide distinctive interests that cannot be fulfilled by other conventional detergent technologies (Rao et al., 1998, Hamza, 2017). Suitability of an enzymes for detergent industry depends on isoelectric point of an enzyme and its pH activity profile. Esperase and Savinase T, are two commercially available formulation produced by alkaline *Bacillus* spp. with very high isoelectric points (pI 11), therefore, they can efficiently withstand higher pH ranges. In addition to pH profile, few proteases are active at lower temperatures are also of interest taking consideration of recent energy crisis. A mixture of lipase, amylase, and cellulase with protease is considered in laundry detergents.

Of the various microbial protease producers, proteases produced by the *Bacillus* group form the most widely used enzymes. Some of the *Bacillus* strains producing proteases are listed below (Contesini et al., 2018).

Protease producing *Bacillus* strains	*B. koreensis BK-P21A, B. subtilis GA CAS8, B. alkalitellurisalitelluris TWI3, Bacillus sp. SM2014, B. amyloliquefaciens SYB-001, B.alveayuensis CAS 5, B. subilis KT004404, Bacillus sp. SB12, B. subtilis DR8806, B. pumilus BA06, B. megaterium RRM2, Bacillus sp. SB12, Bacillus sp. BGS, Bacillus MP 27, Bacillus sp.BBXS-2, Bacillus sp. JB-99, B. pumilus SG2, B. subtilis FBL-1, Bacillus subtilis PCSIR-5*

3.2. Alkaliphilic Bacillus

Phylogenetically, bacteria belonging to the genus *Bacillus* fall under the *Firmicutes* division of bacteria. Bacillus are found in various natural conditions such as clays, rocks, salinity environments, dust, soil, food and gastrointestinal tracts several insects and animals. *Bacillus* species are aerobic or facultative anaerobic, sporulating, have rod-shaped morphology. The genus also includes extremophiles such as thermophiles, halophilic, halotolerant, Alkaliphilic, which survive in harsh environments like hot springs, desserts, fresh water to saline water adapting to grow at different temperatures, pH values and variable salinity concentrations (Horikoshi, 1999). Alkaliphilic bacillus cell wall contains certain acidic polymers such as gluconic acid, glutamic acid, phosphoric acid, aspartic acid and galacturonic. Sodium, hydronium and hydroxide ions get easily adsorb on cell surface due to acidic nonpetidoglycan components and as a result cell grow in Alkaline conditions. Alkaliphilic *Bacillus* peptidoglycan is composed of excess of hexosamines and amino acids along with other components like muramic acid, D- and L-alanine, glucosamine, D-glutamic acid etc. (Aono and Horikoshi, 1983). For efficient solute transfer through the membrane of Alkaliphilic *Bacillus* spp, existence of sodium ion in surrounding environment is essential (Horikoshi, 1999). Due to spore forming ability of *Bacillus* species, they have been used in various fields such as, food and agriculture, pharmaceutical, and industrial processes that take advantage of their wide range of interesting characteristics along with their capability to produce industrially important enzymes, antibiotics, and other major and minor metabolites.

Materials and Methods

4.1 Bacterial strain and culture of the conditions

The bacterial strain, alkalitolerant *Bacillus* sp was procured on a streaked NA plate from MTCC, CSIR-IMTECH, Chandigarh and will be referred to as alkalitolerant *Bacillus sp.* in this report. Cells were cultured in nutrient broth (NB, Hi-Media) and NB containing 0.3% (w/v) Na_2CO_3. The -80^oC stock of the strain was prepared by mixing 500µl of the culture with 500µl of 20% glycerol solution. Subsequent experiments were performed from bacterial cells streaked on desired media plates and grown at 30^oC.

4.2 Optimization of growth medium

4.2.1. Different growth media selected for growth optimization of alkalitolerant *Bacillus sp.* (Table 1.6).

Table 1.6: Bacterial culture medium used for growing alkalitolerant *Bacillus sp.*

S.no	Media [Composition]	Concentration of additives	pH
1.	Nutrient Broth (Hi-Media) [peptone 0.5% (w/v), sodium chloride 0.5% (w/v) , meat extract B# (equivalent to beef extract) 0.15% (w/v), yeast extract 0.15% (w/v)]	-	7.5
2.	Nutrient Broth	0.3%(w/v) Na_2CO_3	9.83
3.	Reconstituted YEPD [yeast extract 0.5% (w/v), glucose 1% (w/v), peptone 0.5% (w/v) potassium dihydrogen phosphate (KH_2PO_4) 0.1% (w/v), and anhydrous magnesium sulfate (Mg_2SO_4) 0.02 % (w/v)]	-	6.59
4.	Reconstituted YEPD	0.25% (w/v) Na_2CO_3	9.56
5.	Reconstituted YEPD	1% (w/v) Na_2CO_3	10.4
6.	0.5X YEPD (Hi-Media) [yeast extract 0.5% (w/v) , glucose 1% (w/v), peptone 0.5% (w/v)]	1% (w/v) Na_2CO_3	9.57
7.	YEPD (Hi-Media)	NaOH (1M)	~9
8.	Nutrient Agar (Hi-Media) [peptone 0.5% (w/v) , HM peptone B# (equivalent to beef extract) 0.15% (w/v), yeast extract 0.15% (w/v), sodium chloride 0.5% (w/v) , agar 1.5% (w/v)]	-	
9.	Nutrient Agar	0.3%(w/v) Na_2CO_3	

Stock of 5 % (w/v) Na_2CO_3 was prepared and autoclaved separately at 121°C for 15 min. Autoclaved NA and 0.3% (w/v) Na_2CO_3 were mixed together and plates were poured.

4.2.2. Growth curve measurement

From -80°C stock, cells were inoculated in NB containing 0.3% Na_2CO_3 media and incubated at 30°C overnight at 150 rpm shaking condition. Overnight culture was used as inoculum in above mentioned media (Table 1.1, s.no 1-7) and bacterial culture growth was measured for about on by an instrument Bioscreen C (Oy growth curves AB Ltd.) at 600 nm for 18 hours at an interval of every 30 min with incubation temperature set at 30°C with mild shaking.

4.3. Molecular characterization of alkalitolerant Bacillus sp.

Overnight grown culture of alkalitolerant *Bacillus sp.* was grown in the medium NB containing 0.3% Na_2CO_3 at 30°C with 150 rpm shaking. Cells were used to perform molecular characterization test, mentioned below:

4.3.1. Gram staining

A thin smear of culture was prepared on a dry and clean slide with a sterile loop. It was dried and heat fixed. The Gram Stain kit (BD Gram Stain) was used to perform the Gram staining (Gram, (1884).) Slide was flooded with Gram Crystal Violet for 1 minute, washed. Slide was flooded with Gram iodine and washed, followed by addition of the decolorizer until the blue dye was removed from the smear. The counter strain safranin (0.5%w/v) was flooded over the smear, washed off and the dry slide was observed under the microscope using 100X oil objective.

4.3.2. KOH test

By using a sterile toothpick generous amount of bacterial cells were spotted on a glass slide and a drop of 3% KOH solution was placed on cells. Cells were mixed well by using toothpick. KOH and cell suspension was observed by raising and lowering the toothpick on the slide to observe lysis and stringiness.

4.3.3. Oxidase test

Several colonies were wiped off from a plate by the paper zone of the (diagnostic) strip (Sigma Aldrich) and same was observed on an Oxidase disc (Hi-Media). Appearance of dark blue/purple color (if any) was observed (Stainer et al., 1966).

4.3.4. Catalase test

Test was performed by placing a drop of 3% H_2O_2 (Sigma Aldrich) on bacterial colonies. Bubble formation was observed. (Beers et al., 1952)

4.3.5. Assay for extracellular enzymes

4.3.5.1. Amylase assay

Overnight grown bacterial culture of alkalitolerant *Bacillus sp.*. was spotted on the starch agar plate (Hi-Media), incubated at 30^oC for 24 hours and flooded with Gram iodine for five minutes to test for starch hydrolysis.

4.3.5.2. Protease assay

Separately autoclaved skim milk (SM) 1% (w/v) (Hi-media) was mixed with NA to prepare and poured into plates. *Bacillus sp.* was spotted on skim agar plate and incubated at 30^oC for 18-24 hours. Same assay was performed in alkaline conditions, culture of alkalitolerant *Bacillus sp.* was spotted and streaked on NA containing SM 1% (w/v) and Na_2CO_3 plate and incubated at 30^oC for 18-24 hours. Visualization of clearance zone was observed.

4.3.5.3. Gelatin assay

Overnight grown bacterial culture alkalitolerant *Bacillus sp.*. was spotted on a gelatin agar (Hi-Media) plate. Plate was incubated at 30^oC for 18-24 hours and assayed using acidic 2% Mercuric Chloride ($HgCl_2$).

4.3.6. Antibiotic assay

Sensitivity of alkalitolerant *Bacillus sp.* was tested against 12 Antibiotic (Hi-Media) Amoxicillin(25), Azithromycin(30), Chloramphenicol(50), Ciproflixacin(10), Clindamycin(10), Erythromycin(10), Lincomycin(15), Oxacillin(5), Rifampicin(30), Spiramycin(100), Streptomycin(25) and Tetracycline(10) , using disc diffusion method. Overnight grown culture in NB containing 0.3% Na_2CO_3 was used. Cell OD was adjusted to 0.2OD in same growth medium. Culture (200ul) was spread on a NA plate and the antibiotic impregnated disc was placed over the culture on the plate. The growth or presence of clearance zone was observed after 18-24 hours of incubation at 30^oC.

4.4. Transmission electron microscopy

Overnight grown culture alkalitolerant *Bacillus sp.* (0.5ml) was pelleted at 2500 rpm for 5 min. Cells were suspended and washed in 1 X PBS pH 8.0 three times at 2500 rpm for 5 mins. The washed cells were gently suspended in 1X PBS buffer pH 8.0 and used for TEM imaging. Samples were fixed on carbonated copper grids for 10-15 mins. Samples were stained with negative stain of 2% phosphotungstic acid, samples were air dried and loaded in specimen holder (Nanninga, 1968). Images were capture on JEOL-2100 200kV electron microscope.

4.5. Colorimetric assay for protease activity determination

Protease activity was assayed following method of Anson (Anson ML, 1938) with some modifications. To a microcentrifuge tube, 130 µl casein and 25 µl media supernatant was added and incubated for 20 min at 37°C. Further, 50ul of reaction mixture was added to the wells followed by addition of 125ul of sodium carbonate and incubated at 37°C for 30 min. Color was developed by adding diluted Folin and Ciocalteu's (FC) (Folin O and Ciocalteu, 1927). Absorbance was measured at 660 nm by using plate reader (Thermo Scientific Multiskan GO). Concentration of tyrosine was titrated using standard tyrosine curve. Same assay was performed in cuvette mode by taking 250ul reaction mix. (One unit of protease activity will hydrolyze casein to produce color equivalent to 1.0mmole (181mg) of tyrosine per minute at pH 7.5 at 37°C (color by Folin and Ciocalteu's reagent) under experimental conditions. Estimation of protein present in the medium was made by using the Pierce 660nm protein assay reagent following manufactures protocol with standard curve created using BSA in the range of (0-2mg/ml). Briefly, 0.75ml of the Pierce 660nm reagent was added to the sample and incubated at room temperature for 5 min. followed by measurement at 650 nm.

4.6. Extracellular protease production during growth cycle of alkalitolerant Bacillus sp.

Alkalitolerant *Bacillus sp.* cells were grown in 3 ml of NB containing 0.3% Na_2CO_3 media, for 16 hours at 30°C. The overnight grown cells were inoculated in 3ml of NB containing 0.3% Na_2CO_3 media and grown till OD600 of < 0.8 was used as inoculum for the growth curve, (size of inoculums depends upon OD of bacterial cells, in our case we preferred 0.05OD) in 100 ml of NB

containing 0.3% Na_2CO_3. Cell density was measured at different time intervals at 600 nm for growth curve.

One ml of culture after 20 hours of growth (at 21, 24, 27, 32, 42 hrs) were pelleted at 8609 g for 10 mins for determining protease activity. Separated supernatant from pellet was filtered using and 0.4µm nylon filter (Millipore Millex-HN) in the laminar air flow. Filtered supernatant was used for protease estimation using both plate based assay on NA plate containing 1% SM and Na_2CO_3 and in colorimetric assay. The plate based assay for presence of proteases activity in the medium was determined as follow, holes were punched in NA with 1% SM plates with or w/o 0.3% Na_2CO_3 and filtered supernatant (55ul) from different time interval of growth was placed into the holes. These plates were incubated at 30^0C, and proteolytic zone was observed after 18-24 hours.

4.7. Genomic DNA isolation and PCR amplification of 16S rDNA

Alkalitolerant *Bacillus sp.* grown overnight in NB containing 0.3% Na_2CO_3 media was centrifuged at 10,000 rpm for 10 mins. Pelleted cells were used to isolate genomic DNA using Thermo scientific genomic DNA kit following manufactures protocol. Nanodrop was used to measure concentration of the samples at 260 nm. Sample purity was checked from absorbance 260/280 and 260/230 ratios. Samples were run on a 0.8% agarose gel and visualized under UV transilluminator. Gel was visualized on a UV transilluminator of the gel documentation unit.

Amplification of 16 rDNA was performed on the Eppendorf gradient master cycler. The PCR amplification of 16S rDNA was standardized using Dream Taq genomic mix (Thermo) with forward (16F27, sequence: 5'-CCAGAGTTTGATCGTGGCTCAG-3') and reverse (16R1488, sequence: 5'-CGGTTACCTTGTTACGACTTCACC-3') primers and gDNA as template. The PCR condition used was:

Steps	Temperature	Time
Initial Denaturation	95^oC	5 min
Denaturation	95^oC	30 sec
Annealing	50^oC	30 sec
Extension	72^oC	1 min
Final extension	72^oC	5 min
Hold	6^oC	infinite

Number of cycles - 15

PCR product was analyzed on an agarose gel and amplified product size was compared with the known sized bands of 1 kb DNA ladder (NEB).

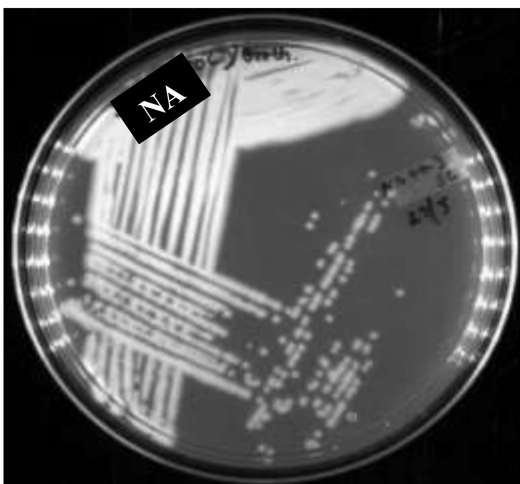
5. Results

The alkalitolerant *Bacillus* sp (alkalitolerant *Bacillus sp.*) procured from MTCC, CSIR- IMTECH, Chandigarh. Alkalitolerant *Bacillus sp.* was reported to be from Rohini, Himachal Pradesh, India, with an ability to grow at pH 9.0. Alkaliphilic microbes are known to be isolated and grown in media containing sodium carbonate, sodium bicarbonate or sodium hydroxide. The Horiskoshi media I and II containing 1% sodium carbonate is widely used for growth of alkaliphiles (Horikoshi, 1971). Alkalitolerant *Bacillus sp.* was grown initially in NB and reconstituted YEPD (similar in composition to the Horikosh I medium) in both liquid and plate culture for morphological and phenotypic characterization.

5.1. Morphological and phenotypic characterization of alkalitolerant Bacillus sp.

Morphology of the alkalitolerant *Bacillus sp.*. colony at early stage and overgrown culture of alkalitolerant *Bacillus sp.* was observed and the differences were noted. At early stage, alkalitolerant *Bacillus sp.* colony was creamish white in color, slimy and shown filamentous margin (Fig 1.6.a.) and in overgrown culture of alkalitolerant *B*. sp. (Fig 1.6.b.), the colony showed smooth and round margin with a concentric ring like pattern.

a.



b.

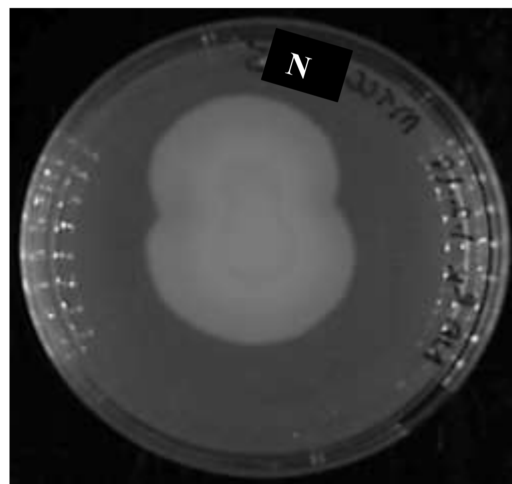


Figure 1.6: Colony characterization of *Bacillus sp.* Streaked culture of *Bacillus sp.* at early stage (**a)** and overgrown stage **(b)** on nutrient agar plate.

5.2. Optimization of growth media of alkalitolerant Bacillus sp.

Culture medium is a key factor for the growth as well as metabolite production by the microorganisms. In the present study, two media NB and YEPD, compositions with varying concentration of Na_2CO_3 or NaOH to achieve pH from 6.5-10.4 was used. The growth on alkalitolerant *Bacillus sp.* on media-agar plate indicated better growth of this strain in alkaline pH. Growth curve data in different medium shown in (Fig 1.7 and 1.8) indicates that alkalitolerant *Bacillus sp.* is a slow growing organism and cells grew to relatively higher densities in the reconstituted YEPD at alkaline pH compared to that in NB at neutral and alkaline pH. In both NB (at neutral and alkaline pH) and YEPD containing 0.25% Na_2CO_3, a sudden decrease in OD600 after about 8 hours followed by growth recovery was observed. Interestingly, cells grown only in NB (neutral and alkaline pH) and not in YEPD at alkaline pH showed protease activity. Therefore, all further experiments were carried out on cells grown in NB containing 0.3% Na_2CO_3 (~pH 9.8).

Figure 1.7: Representative growth curves for *Bacillus sp.* in various YEPD based media. Growth of alkalitolerant *Bacillus sp.* was monitored at 30°C in (**a**) reconstituted YEPD (**b**) reconstituted YEPD containing 0.25% Na_2CO_3 media **(c)** reconstituted YEPD containing 1% Na_2CO_3 media

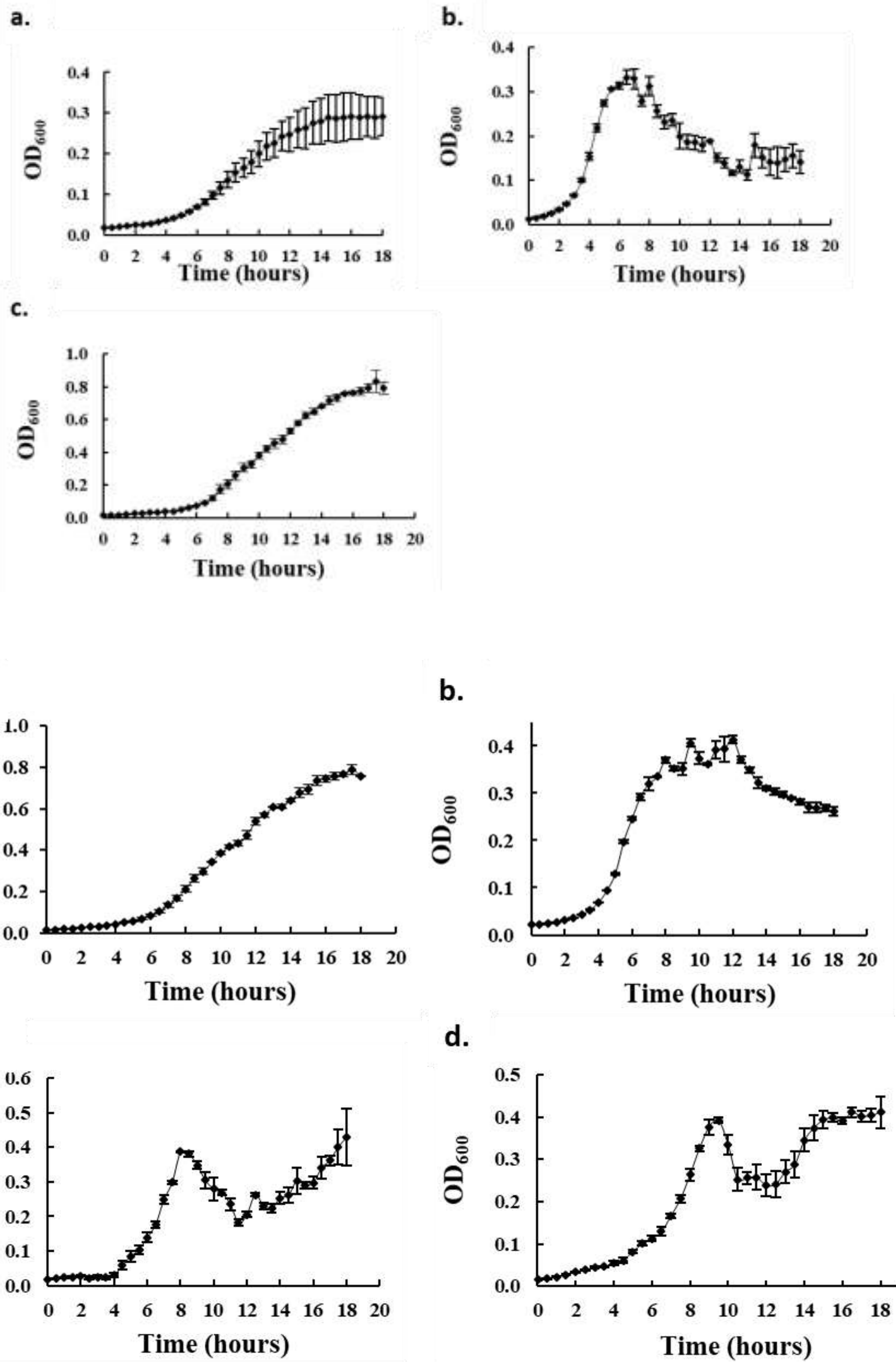


Figure 1.8: Representative growth curves for *Bacillus sp.* at 30°C in different media. **(a)** 0.5X YEPD containing 1% Na2CO3 media, **(b)** 0.5X YEPD containing 1M NaOH **(c)** NB media and **(d)** NB

containing 0.3% Na_2CO_3 media. Error bar represents standard error calculated on data collected in triplicates.

5.3. Transmission electron microscopy of alkalitolerant Bacillus sp.

TEM imaging was performed to view the cell shape and size of alkalitolerant *Bacillus sp.*. TEM images (Fig 1.9) of alkalitolerant *Bacillus sp.* indicates rod shaped bacteria cell with average dimension

a. ±0.0189) µm x 1.6 (±0.0515) µm. **b.**

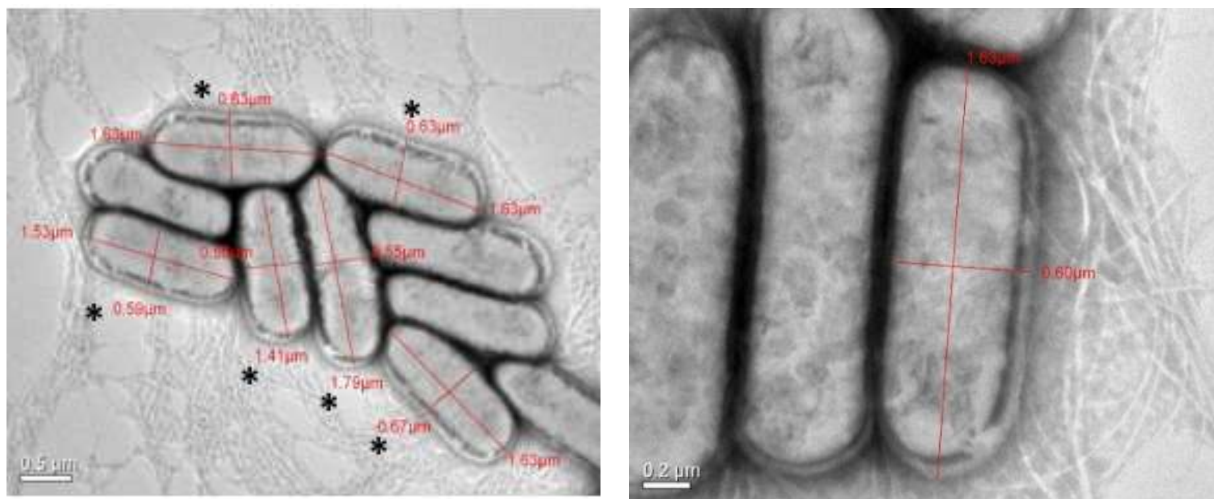


Figure 1.9: Negative stained TEM images of *Bacillus sp.*. at 20000 X **(a)** and at 60000 X **(b)** magnification. Cells marked with asterisk were used for cell dimension measurements.

The TEM images of alkalitolerant *Bacillus sp.* (Figure 2.0.a and b) also showed presence of extensive flagella around cells. All images showed cluster of cells surrounded by broken flagella.

a. **b.**

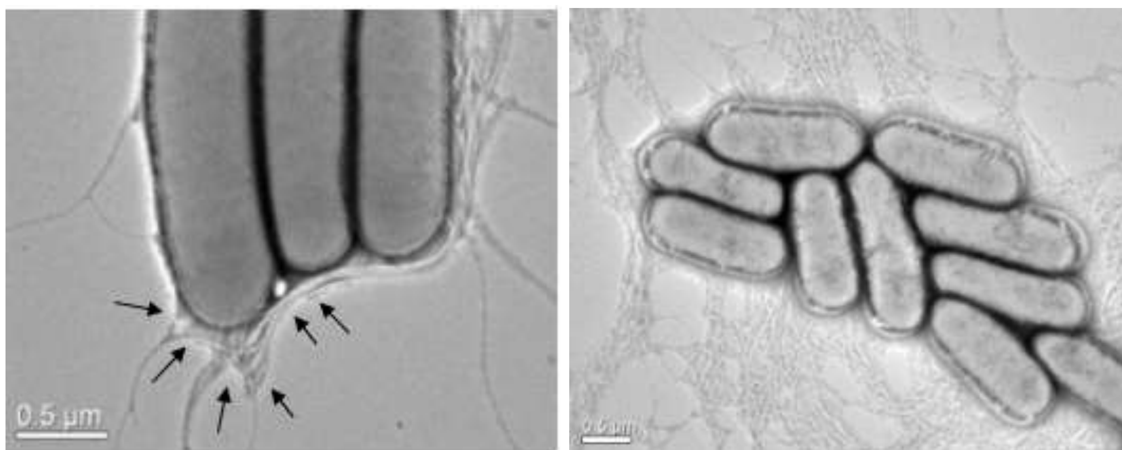


Figure 2.0: Flagella of alkalitolerant *Bacillus sp.* cells viewed in the TEM images at 60000 X (**a)** and at 20000 X **(b**) magnification.

5.4. Molecular characterization of alkalitolerant Bacillus sp.

Gram staining studies of alkalitolerant *Bacillus sp.* cells appeared purple colored rod-shaped cells confirming the organism as Gram positive (Fig 2.1). Absence of released DNA and viscosity of cells in the KOH test also confirmed that alkalitolerant *Bacillus sp.* is a Gram positive organism.

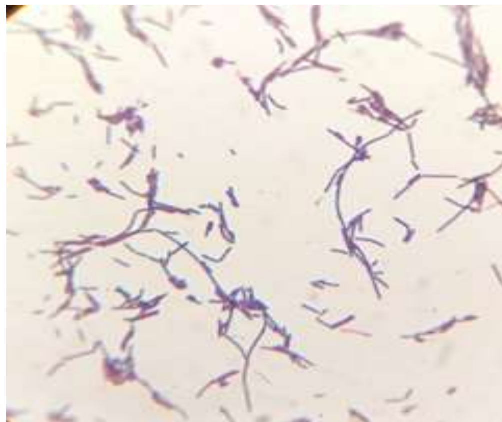


Figure 2.1: Gram stained *Bacillus sp.*

5.4.1. Catalase test

Catalase is an enzyme produced by microorganisms living in oxygen-filled environments to protect themselves from the oxidative damage of hydrogen peroxide. Catalase hydrolyses hydrogen peroxide into water and oxygen, which are nontoxic as shown in the reaction:

$$2H_2O_2 + Catalase \rightarrow 2H_2O + O_2$$

The catalase-catalyzed breakdown of H_2O_2 and rapid evolution of oxygen as evidenced by bubble formation in Figure 2.2.a. indicates alkalitolerant *Bacillus sp.* is catalase positive.

5.4.2. Oxidase test

Cytochrome systems are present in aerobic microorganisms that have ability to utilize oxygen as the final hydrogen receptor. Oxidase enzyme catalyses the oxidation of Cytochrome C. Organisms which contain Cytochrome C as part of their respiratory chain are called oxidase positive and turn the tetramethyl-p-phenylenediamene reagent into indophenol (a purple/dark blue colour product). Microorganisms lacking Cytochrome C as part of their respiratory chain do not oxidizes the reagent, leaving it colorless and called as oxidase negative. The appearance of dark blue color on disc as well as on strip test in Figure 2.2.b indicates presence of Cytochrome C oxidase in *Bacillus sp.*.

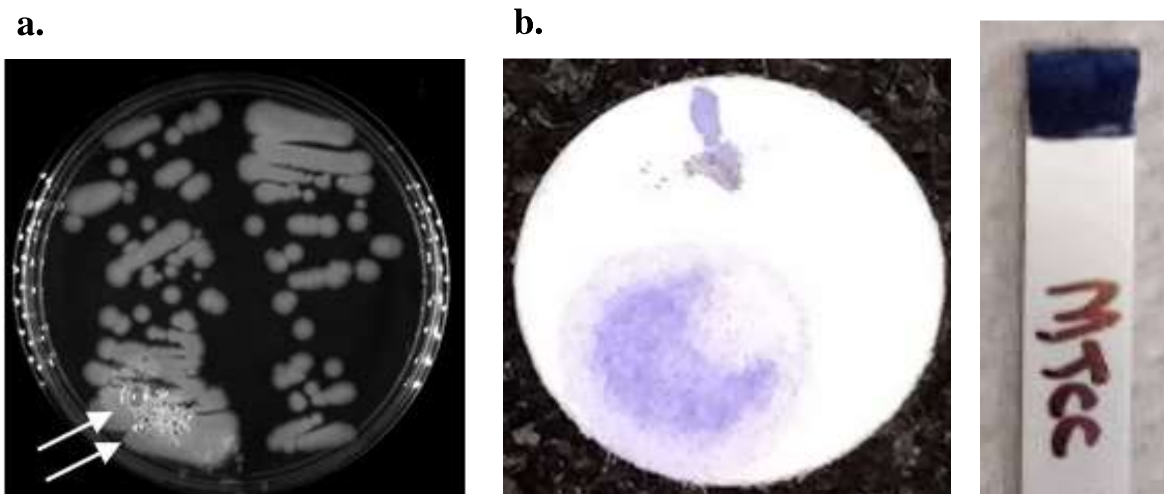


Figure 2.2: (a) Catalase test and (**b)** Oxidase test on disc and strip of alkalitolerant *Bacillus sp.* culture

5.4.3. Assay for extracellular enzymes

Plate based assay provides a sensitive, inexpensive and convenient method for screening different enzymes. Presence of amylases and proteases as extracellular enzymes (if any) produced by alkalitolerant *Bacillus sp.* were screened on different substrates containing NA plates (Fig 2.3). Direct culture of *Bacillus sp.* was spotted on starch plate and no clearance zone was seen around the spot (Fig 2.3.a) showing absence of amylase production. For protease assay, direct culture of alkalitolerant *Bacillus sp.* was spotted and clearance zone was observed around the spot culture in both SM and gelatin containing NA plates (Fig 2.3.b). Two other strains, MTCC 610 and MTCC 7914, were used as control for presence of extracellular proteases detection (Fig 2.3.c).Thus, alkalitolerant *Bacillus sp.* is a producer of protease(s) that show both caseinolytic and gelatinolytic activity.

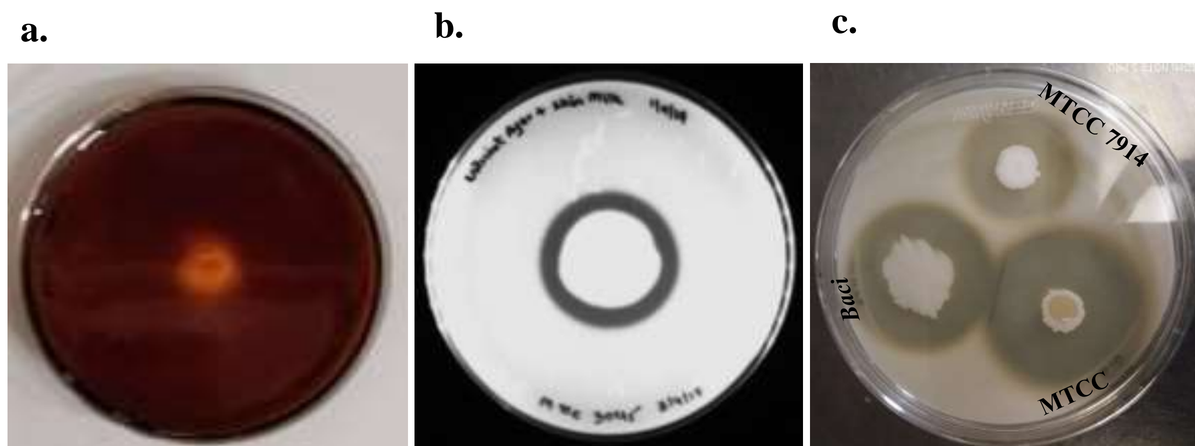
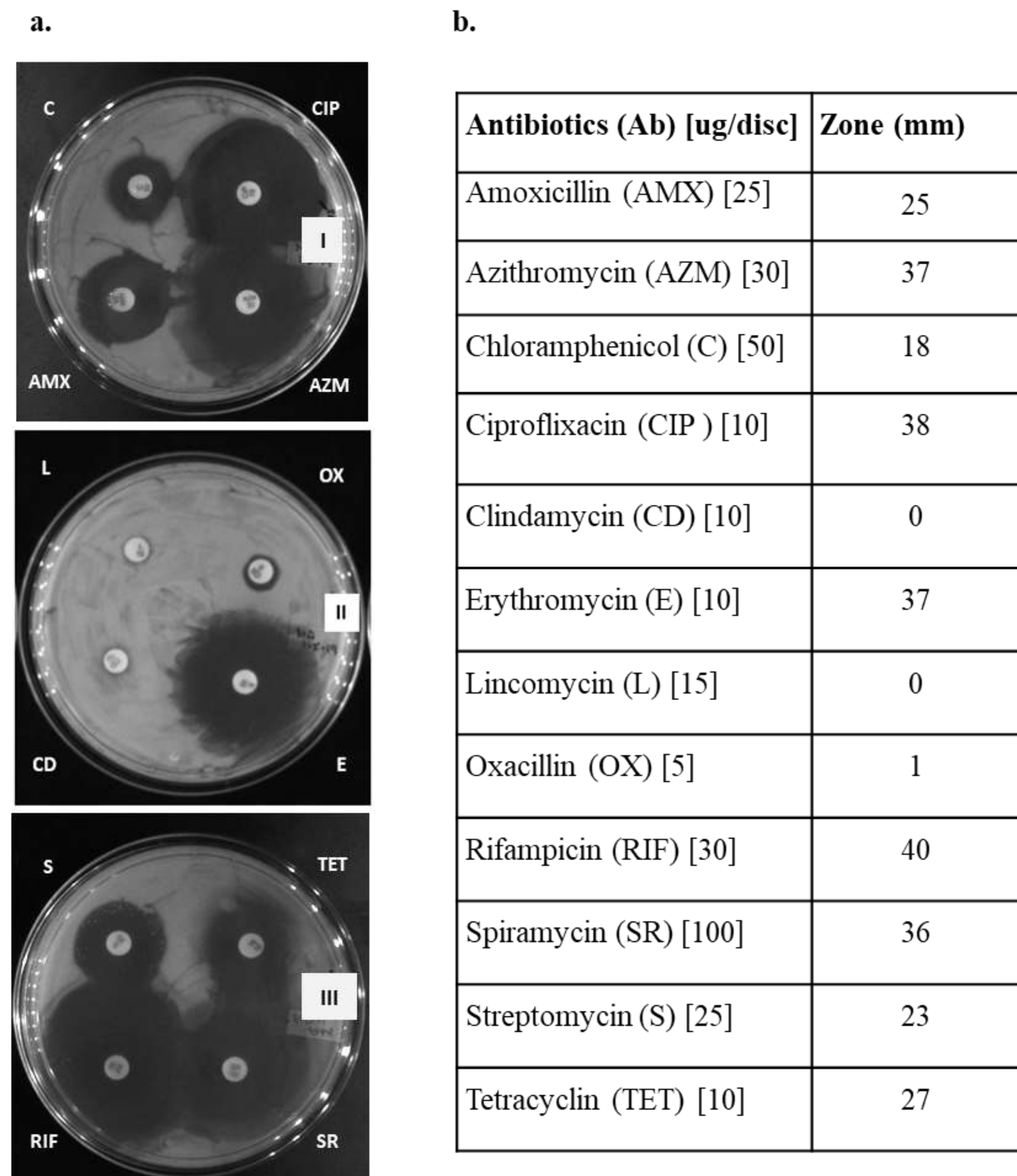


Figure 2.3: Plate assay for extracellular enzyme production by alkalitolerant *Bacillus sp.* **(a)** No clearance zone was observed on starch plate. Clearance zone was seen on nutrient agar containing skim milk **(b)** and on gelatin agar plate **(c)** MTCC 610 and MTCC 7914 served as protease producing positive control cultures.

5.4.4. Antibiotic susceptibility of alkalitolerant Bacillus sp.

As a part of molecular characterization of alkalitolerant *Bacillus sp.*, we examined the resistance (or susceptibility) of alkalitolerant *Bacillus sp.* for 12 different antibiotics using disc diffusion assay (Fig 2.4) and zone of inhibition of each antibiotic was measured by using scale reader (Table 2.0). Of the tested antibiotics, alkalitolerant *Bacillus sp.* showed resistance towards clindamycin and lincomycin and oxacillin. Lincomycin and clindamycin (a derivative of lincomycin) are inhibitors of protein synthesis, while oxacillin is a β-lactam antibiotic of the

penicillin class of antibiotics. The resistance seen in alkalitolerant *Bacillus sp.* may be due to modification of antibiotic, through efflux of the antibiotic or by altered affinity of modified protein such the penicillin binding protein for oxacillin. Future experiments will be performed to determine MIC of alkalitolerant *Bacillus sp.* for these antibiotics.



Antibiotics (Ab) [ug/disc]	Zone (mm)
Amoxicillin (AMX) [25]	25
Azithromycin (AZM) [30]	37
Chloramphenicol (C) [50]	18
Ciproflixacin (CIP) [10]	38
Clindamycin (CD) [10]	0
Erythromycin (E) [10]	37
Lincomycin (L) [15]	0
Oxacillin (OX) [5]	1
Rifampicin (RIF) [30]	40
Spiramycin (SR) [100]	36
Streptomycin (S) [25]	23
Tetracyclin (TET) [10]	27

Figure 2.4: Antibiotic susceptibility of alkalitolerant *Bacillus sp.* observed on nutrient agar plate **(a)** The measured clearance zone is tabulated in **(b)**.

5.5. Protease production

Extracellular protease enzyme by alkalitolerant *Bacillus sp.* was performed on nutrient agar containing skim milk and gelatin as substrate under neutral and alkaline pH. The cell culture of *Bacillus amyloliquifaciens* (MTCC 610) was used as a positive control. Proteolytic zone was observed around the spotted culture of both *B. amyloliquifaciens* and *Bacillus sp.* seen in the Fig 2.5.a Proteolytic zone of *Bacillus sp.* shows increased protease activity under alkaline conditions after incubation of 24 hours at 30°C (Fig 2.5.b compared with Fig 2.5.a). As expected, unlike alkalitolerant *Bacillus sp.*, MTCC 610 doesn't grow in nutrient agar containing 0.3% Na_2CO_3 plate. Protease activity observed from cells streaked in plate-based assay is confirmed to be present in the supernatant (media) obtained after separating cells by centrifugation (Fig 2.5.c), indicating that the proteolysis is due to the presence of an extracellular protease (s) secreted into the culture medium.

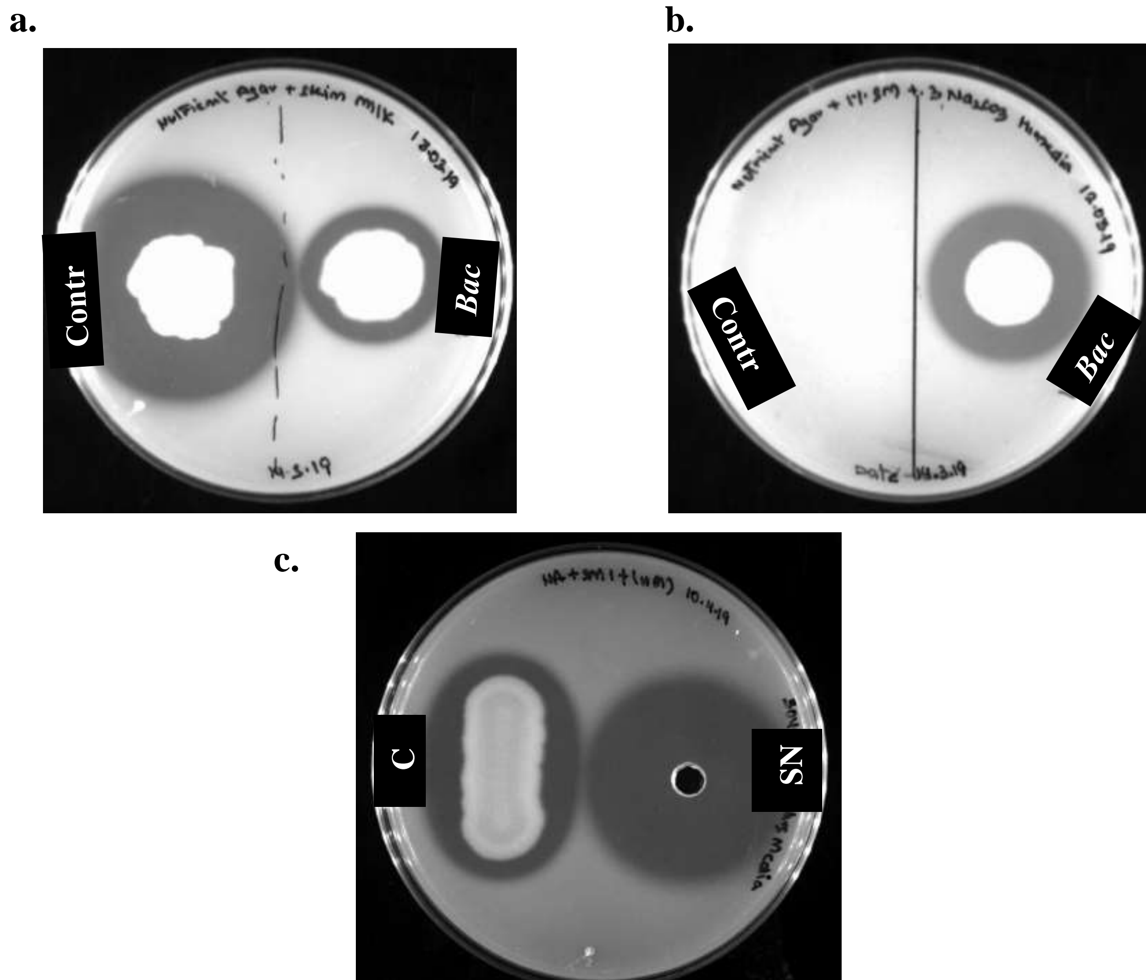


Figure 2.5: Plate assay for production of extracellular protease. Visualization of clearance zone of and *Bacillus sp.* on nutrient agar containing skim milk at neutral pH **(a)** and at alkaline pH nutrient agar containing 0.3% Na_2CO_3 **(b)** Visualization of clearance zone of alkalitolerant *Bacillus sp.* on nutrient agar containing 0.3% Na_2CO_3 **(c)** Clearance zone of spotted culture(c) and filtered medium supernatant(SN) of alkalitolerant *Bacillus sp.* on skim milk containing plate.

5.6. Growth and proteases production profile

The extent of extracellular protease production by alkalitolerant *Bacillus sp.* as a function of its growth was estimated at different intervals from 0 to 42 h was assayed at 30° C incubation. The growth of alkalitolerant *Bacillus sp.* was measured at different intervals of time with the culture grown for 27 hrs is shown in Fig. 2.6.a. The maximum production of extracellular protease by alkalitolerant *Bacillus sp.* was measured after incubation time of 24 hrs (Fig 2.6.b) As we can be seen from the protease activity data on both SM and gelatin used as substrates, the protease production occurs in the late stationary phase (Fig 2.6.c and d).

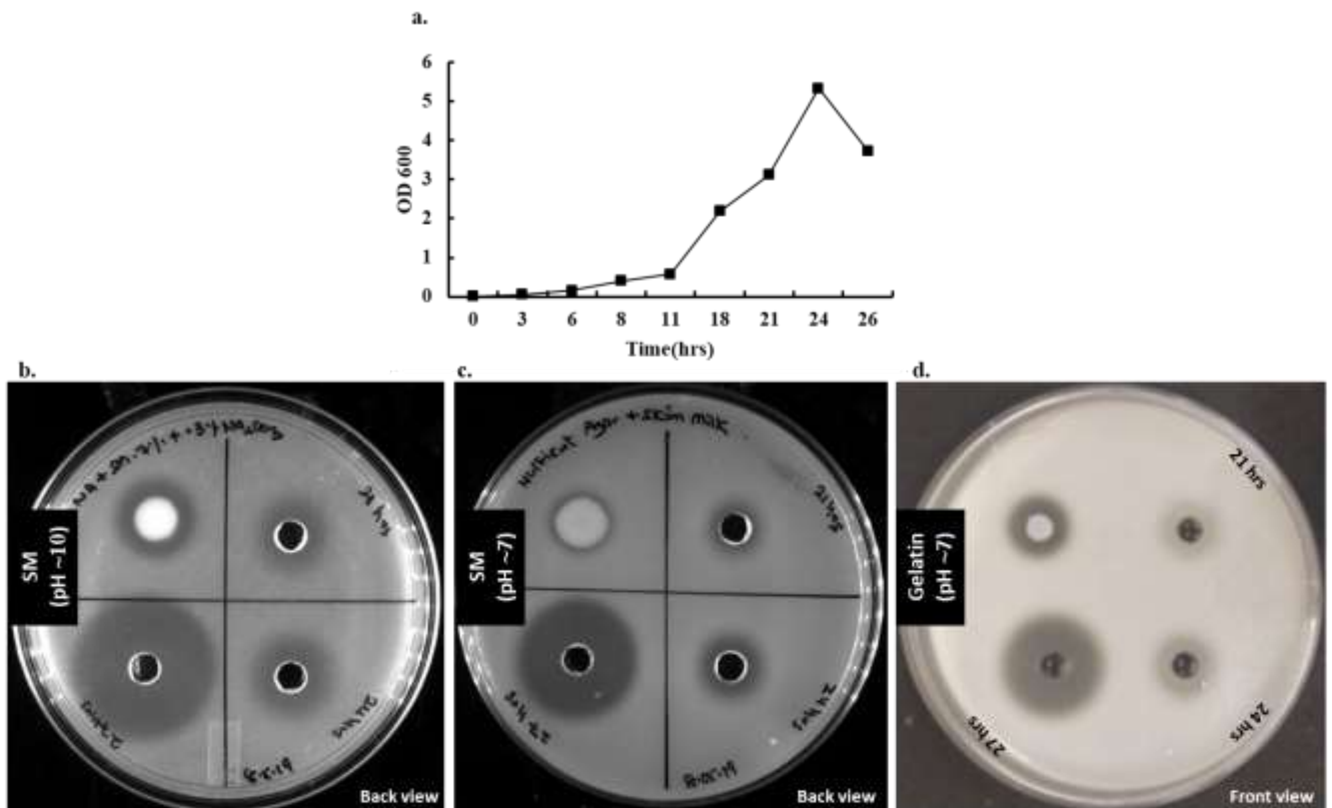
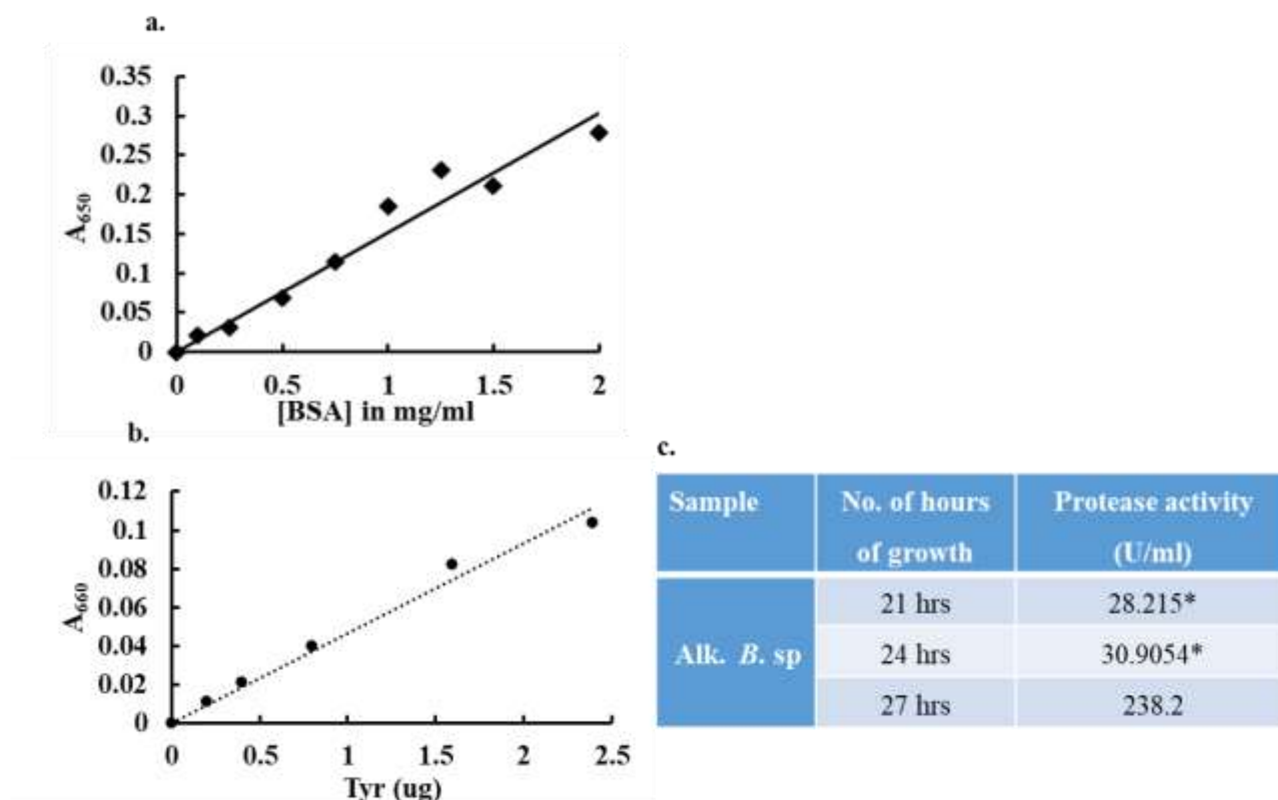


Figure 2.6: Growth and protease production profile **(a)** Growth curve of alkalitolerant *Bacillus sp.* 30°C. Proteolytic zone of alkalitolerant *Bacillus sp.* culture and filtered supernatant from cells

harvested at 21, 24 and 27 hours on nutrient agar containing Na_2CO_3 with 0.7% skim milk **(b)** Nutrient agar containing skim milk **(c)** and gelatin agar **(d)**.

5.7. Colorimetric assay for protease concentration determination

Total protein content in the supernatant medium was measured using Pierce 660nm protein assay reagent and bovine serum albumin as the standard (Fig 2.7.a). However, the amount of protein present in the supernatant medium from the 100 ml culture was observed to be very low and could not be reliably estimated. Quantitative assay for protease activity was established using tyrosine standard curve (Fig 2.7.b). The total protease activity in the medium after 27 hrs of alkalitolerant *Bacillus sp.* growth was estimated to be 238.2 U/ml.



Sample	No. of hours of growth	Protease activity (U/ml)
Alk. *B.* sp	21 hrs	28.215*
	24 hrs	30.9054*
	27 hrs	238.2

Figure 2.7: Activity determination of alkalitolerant *Bacillus sp.* produced protease. (**a)** Estimation of total protein in the medium was estimated using a calibration curve measured with bovine serum albumin (**b)** Representative of tyrosine standard curve

for protease activity determines is shown. The error bar presents standard error of data in triplicate. The calculated protease activity of the samples in tabulated in **(c)**. The protease activity in samples collected after 21 hrs and 24 hrs (marked by asterisk) was fluctuating and observed to be negligible in repeat experiments.

5.8. Alkalitolerant Bacillus sp. strain identification using 16s rDNA gene sequence analysis

16S rRNA based method was used for alkalitolerant *Bacillus sp.* strain identification. The isolated genomic DNA was checked on agarose gel and found to be acceptable purity (260/280 ratio of about 1.8) (Fig 2.8.a). Under standardized conditions, PCR amplification of 16S rDNA using primers 16F27 and 16R1488 analysed on the agarose gel shows amplified product size to be ~1.5 kb as expected (Fig 2.8.b). However, DNA sequencing on the purified 16S rDNA product was unsuccessful and needs to be repeated.

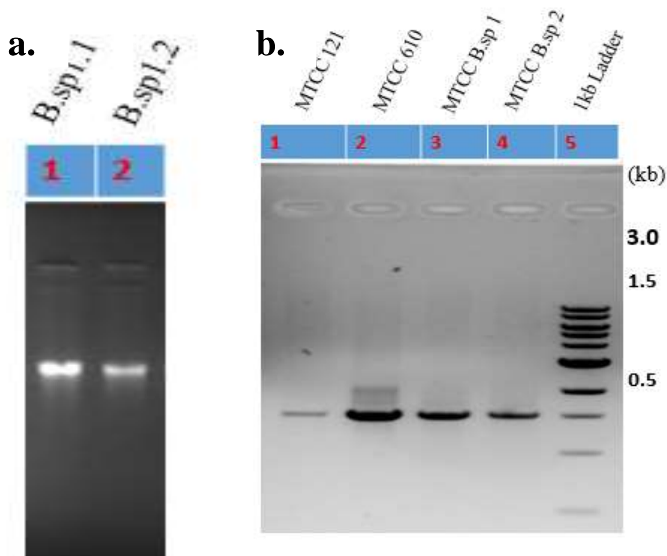


Figure 2.8: Alkalitolerant *Bacillus sp.* strain identification using 16s rDNA gene analysis (**a)** Alkalitolerant *Bacillus sp.* genomic DNA analyzed on agarose gel (**b)** PCR amplified 16S rDNA product of alkalitolerant *Bacillus sp.*, MTCC 121, and MTCC 610 (control).The 16S rDNA of all 3 organism show the expected product size of ~1.5 kb.

Discussion

Bacillus sp. obtained from MTCC, CSIR-IMTECH, was partially characterized according to the Bergey's Manual of determinative Bacteriology (Holt, 1994). Microscopic studies confirm that the cells are rod-like, flagellated Gram-positive bacilli. *Bacillus* sp. grew over a broad pH range, (pH 6.5 to 10.4) with maximum growth in media at alkaline pH, suggesting it to be an alkalitolerant bacilli. Growth curve for this bacillus showed a consistent drop in cell OD600 when cells were grown both in small (Fig.1.7 and 1.8) and larger volume (100ml, Fig.2.6) scales. The cells from late stationary phase grown cultures (3-5 ml) of alkalitolerant *Bacillus sp.* frequently showed cell cluster as strings and long undivided cells were seen under light microscope. Cell aggregation may be a possible reason for fluctuating and decreasing cell OD600. Experiments are underway to understand the significance (if any) of the OD decrease in the growth curve. Earlier reports have indicated motility in alkaliphilic bacillus to be due to the presence of peritrichous flagella (Fujinami et al., 2009). TEM images of alkaliphilic *Bacillus sp.* clearly show the presence of flagella, but the organization and location of flagella on the cell was not observed since intactness of the flagellum was lost most likely during sample preparation.

The oxidase test performed on alkalitolerant *B*. sp under aerobic conditions indicated the organism to be both catalase positive and oxidase positive (Fig.2.2). In the respiratory chain of aerobic organisms, Cytochrome C acts as an electron transporter between complex III (bc1 complex) and complex IV (cytochrome c oxidase). A typical Cytochrome C (class I) is defined as an electron transfer protein. It has been reported that a large content of membrane bound cytochrome c is present in certain alkaliphilic *Bacillus* spp, which grow better at higher pH compared to neutral pH. Increased amount of Cytochrome C increases at higher pH of the culture medium has been suggested to be an advantage for environmental adaptation of alkaliphilic Bacillus. (Goto T et al., 2005).

Alkaliphilic microorganism including *Bacillus sp.* are known to produce a variety of extracellular enzymes such as proteases, amylases, pectinases, lipases etc. Alkalitolerant *B*. sp was tested for the production of amylase and proteases(s). The starch hydrolysis test by Alkalitolerant *B*. sp

indicates absence of amylase production by this organism (Fig.2.3), while protease activity was detected on media plate containing both SM and gelatin substrates (Fig.2.3).

Protease production is an inherent capacity of all microorganisms; and large numbers of bacterial species are known to produce alkaline proteases (Gupta et al., 2002). Among various bacteria, the Bacillus species are most significant producers of specific and nonspecific alkaline proteases (Priest, 1977; Ward, 1985). Proteases may exhibit differential activity based on the substrate, for example, a thermostable protease produced Bacillus subtilis isolated from the soil samples showed higher proteolytic activity on gelatin than on skimmed milk in nutrient agar medium (Pant et al., 2015). In the case of alkalitolerant *Bacillus sp.* studied here, the clearance zone measurement indicates similar protease activity with both skim milk and gelatin as substrate. We observed that there was very little to no proteolytic activity from alkalitolerant *Bacillus sp.* grown in YEPD medium despite better growth (and cell density) of cells compared to their growth in nutrient broth, indicating that it is presence of glucose in YEPD to be the likely cause. A similar phenomenon was reported for Bacillus sp. JB-99, with the presence of glucose 1% (w/v) completely repressing the synthesis of its alkaline protease (Johnvesly and Naik, 2001). An increase in protease production with increased growth incubation is known for many bacilli proteases (Prakasham et al., 2006; Johnvesly et al., 2002; Durham et al., 1987; Gessesse, 1997). In the case of alkalitolerant *Bacillus sp.* protease production was observed only during the late stationery growth phase.

Further experiments are underway to optimize the growth and media conditions for maximal protease production and purification and characterization of the enzyme.

Summary

Extracellular enzymes are used in many environmental –friendly industrial applications as they are selective and economical. The *Bacillus* strains are the most important industrial enzyme producers because of their ability to produce and secrete large quantities of enzymes. Enzymes produced by various Bacilli strains are extensively used in several industrial like detergent, leather and food industry etc. A *Bacillus* species capable of growing at pH 9 procured from MTCC, CSIR-IMTECH was biochemically characterized and investigated for the production of extracellular proteases. *Bacillus sp.* is a flagellated, rod like, Gram positive bacterium under aerobic conditions. Growth media for *Bacillus sp.* was optimized and growth was observed in the pH range of 6.5-10 indicating that this bacillus is an alkalitolerant organism, and referred to as alkalitolerant *Bacillus sp.* in this thesis. Alkalitolerant *Bacillus sp.* produces protease in late stationary phase of growth and the protease(s) was active under both neutral and alkalitolerant pH condition (~pH 10), with higher activity observed in alkalitolerant conditions. Observation and results obtained in project indicates that alkalitolerant *Bacillus* sp. is a potential industrial enzyme producer and future work will focus on purification and characterization of the alkaliphilic protease (s).

Future direction

- Identification of alkalitolerant *Bacillus* sp. strain using 16S rRNA analysis
- Complete biochemical analysis of alkalitolerant *Bacillus* sp.
- Purification and characterization of the alkaline extracellular protease (s) produced by alkalitolerant *Bacillus* sp.

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