Partial purification and characterization of Protease produced by *Bacillus subtilis* and *Bacillus nakamurai* isolated from the vegetable waste compost soil

By

Rajalakshmi, B.

(Reg. No: 16PBC011)

A thesis submitted to

Avinashilingam Institute for Home Science and Higher Education for Women

Coimbatore- 641043.

In partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE IN BIOCHEMISTRY

April 2018

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10/4/18

Signature of the
Head of the Department

10/04/18

Signature of the Guide

ACKNOWLEDGEMENT

It is with the choice of blessings and the divine grace of **LORD ALMIGHTY** that any human endeavour is achieved. In addition to the will of supreme divinity, the willingness of many subject experts and erudite scholars to extend their assistance and help for the completion of a work plays, indeed a vitally important role.

I take the opportunity of expressing my sincere thanks to **Padmashri Dr**. (**Thiru) P.R. Krishna Kumar**, Chancellor, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for providing the opportunity and infrastructure to undertake this investigation.

I immensely thank **Dr. Premavathy Vijayan**, Vice Chancellor, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for providing the entire facilities essential to carry out and complete the study.

I record my sincere thanks to **Dr. S. Kowsalya**, Registrar, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for timely help rendered to carry out the work.

My sincere thanks to **Dr. A. Parvathi**, Dean, Faculty of Science, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore for her guidance and affectionate support, expert suggestions throughout the project.

I record my sincere thanks to **Dr. P.R. Padma**, Professor and Head of the Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for providing the opportunity and timely help rendered to carry out the work successfully.

I express my heartfelt thanks to my **Dr. S. Annapurani**, Former Professor and Head Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for providing the opportunity and timely help rendered to carry out the work successfully.

I owe my indebtedness, profound and deepest thanks to my guide **Dr. A. Shobana,** Assistant Professor, Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for her excellent guidance, immense tolerance, meticulous care, good support, creative influence, thoughtful advice, steady encouragement, motherly love throughout the research and motivation right from the selection of topic to the compilation and completion of the work efficiently and effectively.

My special thanks to **Dr. Anitha Subash,** Professor Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, who helped me to complete the work successfully and brought out the good ideas in me.

A submit my heartfelt thanks to **Mrs. Sujatha. A, (Research scholar)** department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, who helped and supported me throughout my project.

I submit my sincere thanks to all **The Staff Members**, of Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for lending a helping hand and invaluable guidance during the course of this thesis work.

Passionately I cherish my acknowledgement to my loving **Parents**, Beloved **Brother** and all my **Friends**. As for their Unending love, Encouragement, Co-operation and Prayers during the Research work.

I acknowledge the contribution of all other unseen hands during the course of the study for help rendered in the successful completion of the study.

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INTRODUCTION

Enzymes are complex protein molecules produced by living organisms to catalyze the biochemical reactions required for life. Microorganisms requiring extreme environments for growth are called extremophiles and the enzymes they produce are called extremozymes. Thermophilic enzymes have attracted most attention during the past four decades. Such enzymes are of great industrial and biotechnological interest due to the fact that the enzymes are better suited for harsh industrial processes. Thermostable enzymes are stable and active at temperatures which are even higher than the optimum temperatures for the growth of the microorganisms (Lele *et al.,* 2016).

Proteases are hydrolytic enzymes which catalyze the cleavage of peptide bonds in proteinaceous substrates to form a polypeptide chain in a particular protein. Proteases have wide industrial applications as it accounts for two thirds of the total enzymes consumed in various industries. Proteases are routinely used in detergent, food, pharmaceuticals, leather and photographic industries (Nayab *et al.,* 2015).

Proteases contribute more than 65% of total enzyme market and have several biotechnological applications. They are also classified as exo-peptidases and endo-peptidases. Exo-peptidases involve in the catalytic process of proteins and cleave the peptide bonds at N or C terminal of polypeptide chains while endo-type hydrolyzes the peptide bonds within polypeptide chain. Different fermentation strategies have been investigated for hyper production of protease such as submerged and solid state fermentation. However, submerged fermentation has been used for the maximum production yield of enzymes due to easy nutrients and oxygen availability to the culture as well as less time required (Kamran *et al.,* 2015)

Proteases are one of the most important industrial enzymes, accounting for nearly 60% of total worldwide enzyme sales. Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases. Among bacteria, *Bacillus* strains are one of the most important producers of commercially applicable proteases among various microorganisms. These proteases have wide applications in pharmaceutical, leather, laundry, food and waste processing industries. It was reported that about 35% of the total microbial enzymes used in detergent industry are the proteases from bacteria sources. Thermophiles such as *Bacillus stearothermophilus* have been studied for their capability in producing thermostable protease (Azlina *et al.,* 2013).

Thermophilic microorganisms have gained world-wide importance due to their tremendous potential to produce thermostable enzymes that have wide applications in pharmaceuticals and industries. Several workers have reported thermophilic bacteria from diverse environmental habitats such as geothermal sites and hot springs (Panda *et al.,* 2013).

Thermophilic microbes are naturally found in shallow and deep marine hydrothermal vent environments, heated beach sediments, continental solfataric areas, geysers and hot springs. The temperatures and pressures of these habitats vary considerably. The majority of these systems are characterized by extremely low oxygen concentrations. Consequently, most of the known species of thermophiles are classified as obligate or facultative anaerobes, though aerobic and *microaerophilic* isolates are also known. The main aquatic environments are hot springs and hydrothermal vents;

hydrothermal vents being the only marine thermophilic environment (Mathai *et al.,* 2014).

Thermostable proteases are advantageous in some applications because higher processing temperatures can be employed, resulting in faster reaction rates, increase in the solubility of nongaseous reactants and products, and reduced incidence of microbial contamination by mesophilic organisms. Proteases secreted from thermophilic bacteria are thus of particular interest and have become increasingly useful in a range of commercial applications (Nascimento *et al.,* 2004).

Many *Bacillus* species produce a variety of extracellular and intracellular protease. Bacteria are the most important alkaline protease producers with the genus *Bacillus* being the most prominent source, because of their ability to produce large number of alkaline proteases having significant proteolytic activity and stability at high pH and temperature. The largest application of the proteases is in the laundry detergents, where they help in removing protein based stains from clothing during washing (Hema *et al.,* 2012).

Compost is a self -heating aerobic medium that results from microbial degradation of organic matter . When the temperature reaches 50–55° C, the microbial thermophilic community becomes dominated by members of the genus *Bacillus*. Investigation of 16S and 23S rRNA gene sequences has revealed a high heterogeneity in the genus *Bacillus* and following extensive phylogenetic analysis, the genus has undergone major taxonomic revision leading to the establishment of new genera. The genera *Geobacillus, Ureibacillus and Aneurini bacillus* are outcome of this classification (Chaebonneau *et al*., 2012).

Composting is a general treatment for recycling animal waste as plant fertilizer. During the treatment, however, a lot of nitrogen is lost, most nitrogen losses result from ammonia emission. Ammonia emission produces an unpleasant odor and is a source of irritation for neighbors of large-scale composting operations. Nitrifying bacteria oxidize ammonia and reduce the smell of ammonia, making them ideal potential organisms for animal waste bioremediation. Thermophilic and heterotrophically nitrifying bacteria isolated from compost are needed for biological deodorization of ammonia in compost.

But thermophilic nitrifying bacteria have not been isolated from compost (Shimaya *et al.,* 2011).

Composting is a biological process in which organic wastes are stabilized and converted into a product to be used as a soil conditioner and organic fertilizer. During composting, mesophilic bacterial growth is stimulated by the higher temperatures. The elevated temperature induces thermophilic bacterial growth (Zakia *et al.,* 2012).

The composts produced can be used to fertilize the soil. In Taiwan, agricultural wastes, such as fruit-vegetable refuses, and flower wastes, are often directly treated by landfilling. With the very limited land resources in Taiwan, building of new land fields or incinerators is getting more and more difficult these days. To ameliorate the situation, it is of pressing urgency to popularize the utility of agricultural wastes via composting. During composting process, the temperature rapidly increases to high level, sustains for a period of time, and then gradually cools down. Different microbial communities dominate in each composting analysis was well consistent with that of the traditional methods. Four hundred and twelve isolates were obtained. The dominant species in thermophilic composts were *Bacillus spp.*, closely related to *Bacillus licheniformis* and *Bacillus sonorensis* (Hong *et al.,* 2007).

The present study entitled "**Partial purification and characterization of Protease produced by *Bacillus subtilis* and *Bacillus nakamurai* isolated from the vegetable waste compost soil"** is focussed with the following objectives:

- Isolation and identification of thermophilic bacteria.
- Characterization of protease producing thermophilic bacteria.
- Screening of the isolates for the protease production.
- Partial purification and characterization of protease

2. REVIEW OF LITERATURE

Microbiology is the study of microorganisms, microscopic organisms that include in particular the bacteria, a large group of very small cells that have enormous basic and practical significance. Microbiology considers all aspects of microbial cells, including their structure, metabolism, diversity, genetics and evolution, ecology and roles in infectious diseases (Madigan *et al.,* 2015).

A brief review of literature on current study entitleed **"Partial purification and characterization of Protease produced by *Bacillus subtilis* and *Bacillus nakamurai* isolated from the vegetable waste compost soil"** is discussed under the following headings:

2.1. Microbiology

2.2. Microorganisms

2.3. Thermophilic bacteria

2.4. Applications of thermophilic bacteria

2.5. Enzyme

2.6. Protease

2.7. Applications of protease

2.8. Compost sample

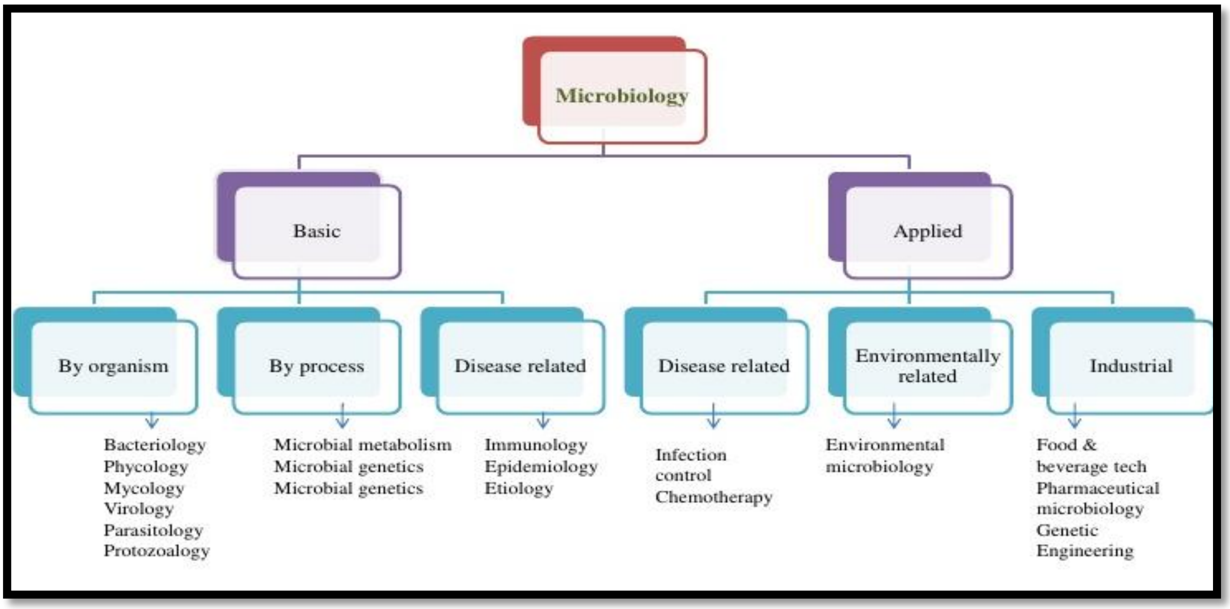
2.1. Microbiology

The English naturalist Robert Hooke (1635–1703) was an early microscopist and published the first book devoted entirely to microscopic observations of microorganisms. Hooke prepared detailed and quite accurate drawings of moulds (fungi) and many other microbes, and these were the first known description of microorganisms. As microbiology entered the twentieth century, its initial focus on basic principles, methods and medical aspects broadened to include studies of the microbial diversity of soil and water and the metabolic processes that microorganisms carry out in these habitats. Notable microbiologists of this era were Martinus Beijerinck and Sergei Winogradsky. Beijerinck’s greatest contribution was his development of the enrichment culture technique, a process in which highly selective nutrient and incubation conditions are used to isolate microbes from nature whose metabolism and other properties are best suited to the conditions employed and thus give them a competitive advantage (Sattley *et al.,* 2017).

The microbial world is the largest unexplored reservoir of biodiversity on the earth. It is an important frontier in biology under intensive investigations. The vast array of microbial activities and their importance to the biosphere and to human economics provide strong rationale for understanding their diversity, conservation and exploitation. It has been speculated that the thermophiles were among the first living organisms on this planet, developing and evolving during the primordial birthing days of the earth when surface temperatures were quite hot and thus, have been called as “Universal ancestor’’ (Verma *et al.,* 2014).

FIGURE: 1

BRANCHES OF MICROBIOLOGY



Source: https://www.quore.com/what-is-microbiology

2.2. Microorganisms

Microorganisms encompass an enormous diversity of microscopic life forms, each with distinct characteristics. On the basis of their genotypic (genetic) and phenotypic (observed) properties, all organisms are classified into one of three domains – the Bacteria, Archaea or Eukarya (Woese *et al.,* 1990).

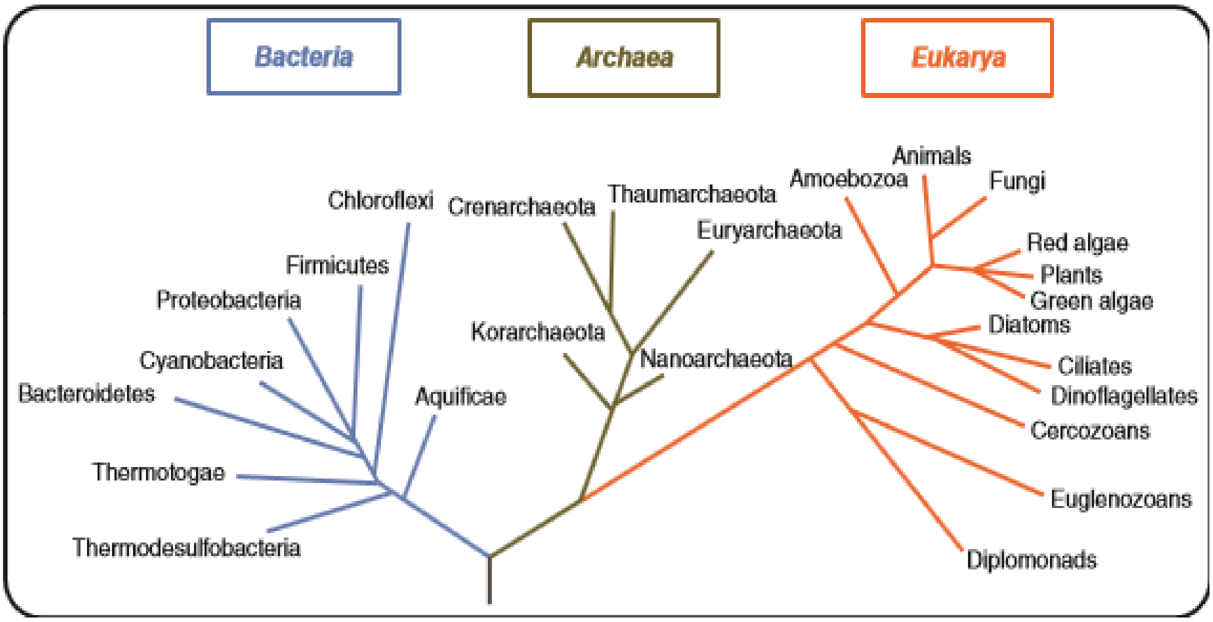
The Bacteria and Archaea are vast groups of microorganisms consisting of potentially hundreds of thousands to millions of species, most of which remain uncharacterised. These microbes are ubiquitous, inhabiting and subsist in nearly every imaginable environment on Earth. Various species thrive on or within every plant and animal, within and underneath massive glaciers, in hyper saline waters of the Great Salt Lake and the Dead Sea, and even in boiling hot springs and deep sea volcanic (hydrothermal) vents. Many microorganisms, called extremophiles, are able to thrive in environmental conditions that humans would consider punishingly harsh, and numerous members of the Archaea, in particular, excel in this lifestyle (Madigan *et al.,* 2015).

Microorganisms are known to play a vital role in technology for the production of intracellular and extracellular enzymes on an industrial scale. For maximum yield, selected organisms are grown in fermenters under optimum conditions and can be further used to make products such as cheese, bread, wine and beer. Most reaction inside living cells requires enzymes, which act as catalyst and are essential for life (Pant *et al.,* 2014).

Microorganisms produce various proteolytic enzymes. Proteases are the important group of enzymes that are highly exploited which account for 60% of the total enzyme market. The Genus *Bacillus* strains like *B. stearothermophilus, B. mojavensis, B. megaterium* and *B. subtilis* are used widely for protease production. Global demand of thermostable enzymes is ever increasing. Thermostable proteases are more advantageous than other mesophilic proteases. They have faster reaction rates, increased solubility of nongaseous reactants and products, transportation and reduced microbial contamination (Dorcas *et al.,* 2016).

FIGURE: 2

UNIVERSAL PHYLOGENETIC TREE OF THE THREE DOMAINS OF LIFE (BACTERIA, ARCHAEA AND EUKARYA)



Source: https://www.mnn.com/earth-matters/wilderness-resource/blogs/two-thirds-earths-biodiversity-bacteria

2.3. Thermophilic bacteria

Thermophilic microorganisms are not grouped into a separate taxonomic unit, but appear in various taxonomic groups and at various phylogenetic distances throughout the taxonomic system. It was shown that representatives of the genus *Bacillus* and related genera to be the thermophilic aerobes most frequently isolated from terrestrial geothermal water environments. Typically, enzymes production in the course of *Bacillus* fermentation processes occurs during a relatively short period of time, with very low cost carbon and nitrogen sources. A great part of industrially valuable enzymes are mainly produced by *bacilli* (Hovik *et al.,* 2017).

Thermophilic and hyperthermophilic organisms have the ability to thrive at high temperatures. By inference, the internal components must also be able to withstand this extreme environment . Thermophiles are microorganisms that grow fastest above 40° C. They are found within natural and anthropogenic biotopes such as hot waters, sun-heated oils, geothermal areas, heated waste dumps and cooling waters. Most thermophiles known are moderate , and show an upper temperature range of growth between 50 and 70° C, although they are still able to grow slowly at 25-40° C. In general, thermophiles are designated as microorganisms that grow optimally at 50-80° C. Owing to their close phylogenetic relationship to mesophiles and their modest thermophiles, moderate thermophiles may be secondarily adapted to hot environments. Thermophilic representatives are known within a wide range of microbial genera (Radianingtyas *et al.,* 2003).

Thermophilic microorganisms prefer living at higher temperatures in hot springs and these organisms not only survive but might even thrive in boiling water. Isolation of thermophiles has received considerable attention among the scientific community in whole world because of their biotechnological importance which is usually not denatured by high temperature and even remains active at elevated temperature. For several decades, thermophilic bacteria have attracted the interest of many scientists due to their biotechnological potential. In particular, phenotypic and genotypic characterization of thermophilic bacteria has been done for many geothermal areas in different parts of the world, including Turkey, Italy, Bulgaria, Greece, China, India and Iceland. Advances in molecular biology techniques, such as 16S rRNA sequencing have provided excellent opportunity for identification and characterization purposes of microorganism at species and subspecies levels (Verma *et al.,* 2014).

The genus *Bacillus* produces a wide variety of extracellular enzymes, including proteases. Several *Bacillus* species involved in protease production, such as *Bacillus stearothermophilus, Bacillus subtilis and Bacillus cereus*. Proteases produced by *Bacillus* sp. find a wide variety of application in detergents, leather, food and pharmaceutical industries. Optimal culture conditions for protease productivity were studied by many investigators. Recently, a thermophilic *Bacillus* strain HUTBS62 was isolated from hot-spring located near the Dead Sea, Jordan. Phenotypic characteristics and genetic polymorphism by RAPD-PCR for *Bacillus* strain HUTBS62 were determined (Aqel *et al.,* 2012).

Thermophilic bacteria has been isolated from a number of marine and terrestrial geothermally-heated habitats including hot spring hot water heaters, thermally polluted river, soil, hydrothermal vent systems sediment from volcanic islands and deep sea hydrothermal vents as well as from hot composts. Proteases are presented as one of the three largest groups of industrial enzymes because of their thermo-stability and thermo-activity. These enzymes account for about 60 % of the world enzyme market and approximately 40 % of them are of microbial origin.

Microbial proteases are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics preferred for their biotechnological applications. They have a wide range of applications in biodetergents, food, pharmaceutical, Leather, silver recovery and bioremediation processes (Ebrahimpour *et al.,* 2015).

Thermophiles can be categorized into moderate thermophiles (growth optimum, 50–60 ˚C), extreme thermophiles (growth optimum , 60–80 ℃), and hyperthermophiles (growth optimum , 80–110 ℃). Thermophiles have been isolated from different ecological zones (e.g., hot springs and deep sea) of the earth. The organisms with the highest growth temperatures (103–110 ℃) are members of the genera *Pyrobaculum, Pyrodictium*, *Pyrococcus,* and *Melanopyrus* belonging to Archaea; within Fungi, the *Ascomycetes* and *Zygomycetes* classes have high growth temperatures, while, in case of bacteria, *Thermotoga maritime* and *Aquifex pyrophilus* exhibit the highest growth temperatures of 90 and 95 ℃, respectively. Thermophilic microorganisms can be classified as Gram-positive or Gram-negative, they can exist under aerobic or anaerobic conditions, and some of them can form scientists have concentrated their studies to discover new genus and species across the world (Mohammad *et al.,* 2017).

2.4. Applications of thermophilic bacteria

The thermophilic enzymes have adapted themselves to grow in extreme conditions such as high temperature, high pressure, high salt concentrations and extreme pH. They are able to survive in such harsh conditions because of certain unique biocatalysts that they produce. Some of the enzymes obtained from such thermophiles have found use in food, pharmaceutical and chemical industries and also in environmental biotechnology. A few of them are mentioned below: Starch degrading enzymes, Xylan degrading enzymes, Chitin degradation, Alcohol dehydrogenases, DNA processing enzymes, Protein degradation, Esterases, Biofuel, etc. (Mathai *et al.,* 2014).

The potential biotechnological applications of whole-cell extremophilic biocatalysts range from the bioremediation of toxic pollutants from water and/or sediments to the production of biomolecules for medical and industrial purposes. Because of their adaptation to high concentrations of heavy metals, metalophiles/acidophiles are currently being used for bioremediation and biomining, while radiophiles are suited for application in the management of nuclear-waste-polluted environments. Applications can also be envisaged in agriculture where desert bacterial extremophiles that are able to cope with low water activity conditions can be used to improve the management of water by plants under drought stress (Rolli *et al.,* 2014).

The thermophiles and hyperthermophiles produce large amounts of starch-degrading enzymes, production is still not high enough to satisfy industrial requirements. This problem has been circumvented by cloning and expressing the genes encoding amylolytic enzymes in mesophilic hosts. In most cases, thermostable enzymes expressed in mesophilic hosts can be purified easily, and the degree of purity obtained is suitable for industrial applications (Bouzas *et al.,* 2006).

Thermophiles belonging to *Thermoanaeirobacter* and *Thermococcus* were found to hydrolyze the thermally denatured amyloid form of prion and could be used for decontamination of animal wastes (Suzuki *et al.,* 2006).

The activity of the membrane-bound proteases from thermophilic *Geobacillus colagenovorans* MO-1 towards collagen shows that their substrate binding-domains could be useful in enhancing drug delivery in some tissues. For instance, a fusion protein carrying the epidermal growth factor at the collagen binding-domain, when injected into nude mice, remained around sites of injection up to 10 days, whereas not fused substance was not detectable 24 hours after injection. Recently, thermostable protease from *Thermus* sp. is produced for cleanup of DNA before polymerase chain reaction (PCR). The application of thermostable enzyme for this purpose is caused by compatibility with an existing high-temperature process (Synowiecki *et al.,* 2010).

The potential applications of thermophilic microorganisms in industrial applications have been widely reported. Currently, only 1–2 % of the microorganisms on the earth have been commercially exploited and amongst these there are only a few examples of thermophiles. The basic advantage of stability and activity of thermozymes in one hand and a number of process advantages which could be exploited through the use of thermophilic microorganisms on the other hand project thermozymes as future biocatalyst. Energy savings through reduced cooling costs, higher saccharification and fermentation rates, continuous product removal, less energy requirement for mixing, increase in solubility of organic compounds and reduced risk of contamination are major advantages from the angle of process technology. Thermostable phytases are added to animal feeds in order to hydrolyze phytic acid (phytate), an antinutritional factor present in cereals and oil seeds, thereby releasing digestible phosphorous. Thus the need to supplement the feed with an external source of phosphorous is reduced (Dheeran *et al.,* 2014).

FIGURE: 3

APPLICATION OF THERMOPHILIC BACTERIA

Source: https://www.frontiersin.org/articles/10.3389/fmicb.2015.01014/full

2.5. Enzyme

The enzyme industry, as it is currently known, is the result of a rapid development of biotechnology, especially during the past four decades. Since ancient times, enzymes found in nature have been used in the production of food products such as cheese, beer, wine and vinegar. Enzymes which decompose complex molecules into smaller units, such as carbohydrates into sugars, are natural substances involved in all biochemical processes. Due to the enzymes' specificities, each substratum has a corresponding enzyme. Although plants, fungi, bacteria and yeasts produce most enzymes, microbial sources- produced enzymes are more advantageous than their equivalents from animal or vegetable sources. The advantages assets comprise lower production costs, possibility of large-scale production in industrial fermentors, wide range of physical and chemical characteristics, possibility of genetic manipulation, absence of effects brought about by seasonality, rapid culture development and the use of non-burdensome methods. The above characteristics make microbial enzymes suitable biocatalysts for various industrial applications (Soares *et al.,* 2007).

Enzymes are biological catalysts that allow chemical reactions to occur in living organisms at ambient conditions. One group of the enzymes that has been studied extensively is proteases or proteolytic enzymes due to their wide variety of application in various fields. Protease (peptidase or proteinase) is an enzyme that hydrolyses the peptide bonds that link amino acids together in the polypeptide chain forming the protein. Proteases differ in their ability to hydrolyze various peptide bonds. Hence, specificity associated with each enzyme differs based on catalytic site. Proteases are widely distributed in all living organisms: in plants (papaya, pineapple) in animals and mainly in microbes (bacteria, fungi and viruses). Proteases are involved in regulating practically every aspect of plant life cycles (Hussein *et al.,* 2015).

The application of enzymes in “White Biotechnology” for the synthesis of industrially important chiral compounds is becoming increasingly important for the pharmaceutical industry. Many companies who were traditionally not incorporating biocatalysis in their drug production programmes are now very keen to develop the technology. Enzyme chemistry can make reactions feasible which are currently unavailable using conventional methods. Use of enzymes for chemical processes is a route to lower energy consumption and reduced waste generation. In addition, the selectivity of enzymatic processes reduces the raw material costs and the safety issues surrounding the production of wasteful by-products. It is anticipated that optimised enzyme production through further bioprocess intensification will lead to more economically viable and cost effective, sustainable compound production (Littlechild *et al.,* 2015).

The enzyme technology is applied to pharmaceutical research, development and manufacturing and is a growing field. Therapeutic enzymes have been in use for around at least 40 years. For example, a therapeutic enzyme was described as a part of replacement therapies for genetic deficiencies in 1960s by de Duve. Attempts are made to capitalize on the advantages of enzymes as drugs at every pharmaceutical research center in the world. Regular consumption of enzymes and enzyme-rich foods contributes to vibrant health, prevention of disease, and anti-ageing process. Each cell in our body needs enzymes for its biochemical functions, and a deficiency of these enzymes will accelerate the aging process. Some of the important functions of enzymes are regulation of the growth of the body from a single cell to a mature organism, conversion of food to energy to fulfill the body's needs, and break down or buildup of certain substances within the cell (Mane *et al.,* 2015).

Enzymes are the bio-catalysts playing an important role in all stages of metabolism and biochemical reactions. Certain enzymes are of special interest and are utilized as organic catalysts in numerous processes on an industrial scale. Microbial enzymes are known to be superior enzymes obtained from different microorganisms, particularly for applications in industries on commercial scales. Though the enzymes were discovered from microorganisms in the 20th century, studies on their isolation, characterization of properties, production on bench-scale to pilot-scale and their application in bio-industry have continuously progressed, and the knowledge has regularly been updated. Many enzymes from microbial sources are already being used in various commercial processes. Selected microorganisms including bacteria, fungi and yeasts have been globally studied for the bio-synthesis of economically viable preparations of various enzymes for commercial applications (Nigam *et al.,* 2013).

FIGURE: 4

USE OF ENZYMES IN INDUSTRIAL AND OTHER APPLICATIONS

Enzyme	**Properties**	**Producer microbes**	**Applications**
Protease (Proteolytic activity)	Acidic, Nautral, Alkaline, Thermophilic, Active in presence of inhibitory compunds	Bacilli, Pseudomonas, Clostridium, Rhitropus, Penicillium, Aspergillus	Washing powder, Detergent, Tannery, Food industry, Leather processing, Pharmaceuticals, Molecular biology peptide synthersis
Keratinase (keratin–hydrolysing activity)	Specific proteolytic activity for insoluble & fibrous protein in furs, fearthers, wool, hair, thermophilic, alkalophilic, oxidation - resistant	Bacteria; Actinomycetes; Fungi	Animal feed Production; Textile processing; Detergent formulation; Leather manufacturing; Medicine
Amylase (Starch-hydrolyzing activity)	Thermotolerant, Thermpstable, Alkali resistant- Exo-, endo-, de-brancing, Cyclodextrin-Producing enzymes	Bacillus sp; Geobacillus	Starch industry (for liquefaction); paper, food industry (glucose & maltose syrups) Pharmaceutical industries; brewing Industry; textile industry; baking industry
Xylanase (Xylan-pentos polymer	Extermophilic Characteristics-Alkaliphilic, Thermophilic &	Thermoactinomy Ces thalophilus; Bacillus sp; Humicola	Pentose production-bioconversion of hemicelluloses for fule & chemicals; Fruit-

hydrolyzing activity)	Thermostable		juice clarification; paper industry- selective removals of xylans from kraft-pulp; brewing industry
Lignnase (ligninolytic complex-enzyme)	Oxidative properties in lignine peroxidise, manganese peroxidise & laccase; thermophilic	Basidiomycetes strain- steccherinum ochracsum, polypons versicolor	Denim washing; Bio- sensor; Bio bleaching of kraft-pulp; bioremediation; Pollution control;
Cellulose (cellulolytic complex enzyme)	Saccrification of crystalline & amorphous cellulose; Thermophilic; thermostable	Basidiomycetes strain- polyporus sp; Pleurous sp; Trichoderma sp; aspergillus sp;	Glucose feedstock from cellulose; bio-refinery; bio ethanol; Paper pulp industry
Lipase (lipolytic activity)	Fat- splitting stereoselectivity; Racemic- resolution activity;solvent – resistant thermotolerant	Yeast and fungal strains- candida sp; aspergillus sp;penicillium sp;	Detergent ; dairy industry- oils, fats, butter, cream, fat-spreads; feed supplement; Therapeutic agent.

Source: (Nigam *et al.,* 2013)

2.6. Protease

Proteases are a group of enzymes, whose catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids. They constitute 59% of the global market of industrial enzymes, which is expected to exceed $ 2.9 Billion by 2012 (Deng *et al.,* 2010). They have got wide range of commercial usage in detergents, leather, food and pharmaceutical industries. Sources of proteases include all forms of life, that is, plants, animals and microorganisms. Based on their acid-base behaviour, proteases are classified in to three groups, that is, acid, neutral and alkaline proteases. *Bacillus* produces a wide variety of extracellular enzymes, including proteases. Several *Bacillus* species involved in protease production are e.g. *B. cereus, B. sterothermophilus, B. mojavensis, B. megaterium and B. Subtilis.* The genus “*Bacillus*” is an important source of industrial alkaline proteases and are probably the only genera being commercialized for alkaline protease production (Hanan *et al.,* 2012).

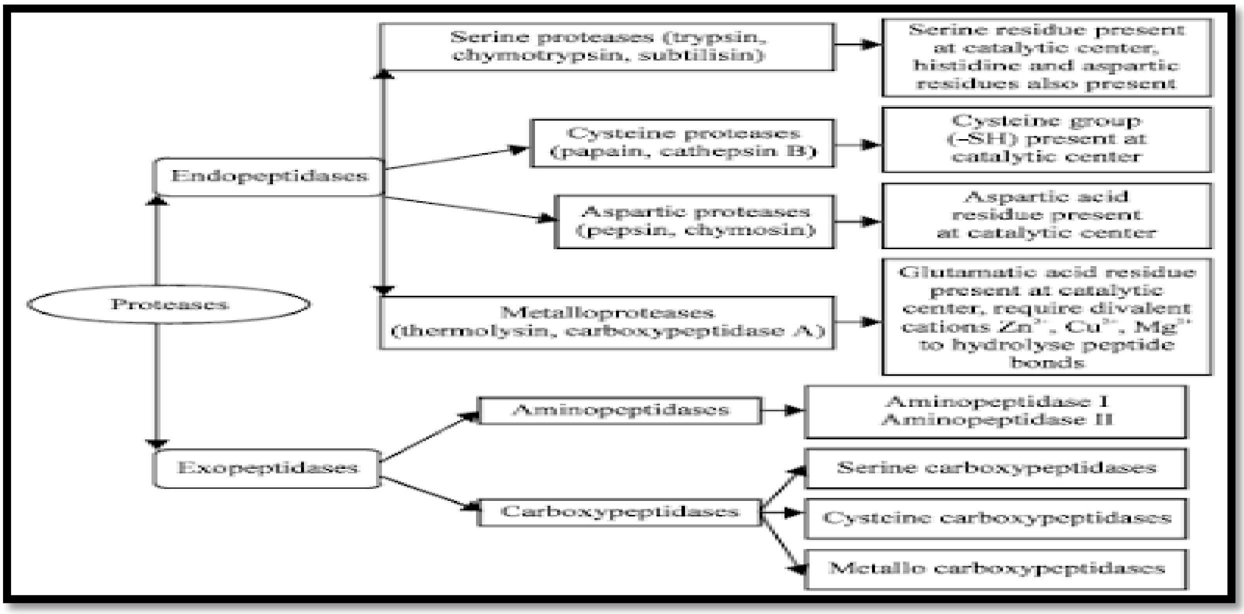
Protease constitutes a large and complex group of enzymes that plays an important nutritional and regulatory role in nature. Proteases are (physiologically) necessary for living organisms; they are ubiquitous and found in a wide diversity of sources. Protease is the most important industrial enzyme of interest accounting for about 60% of the total enzyme market in the world and account for approximately 40% of the total worldwide enzyme sale. They are generally used in detergents, food industries, leather, meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds. They also have medical and pharmaceutical applications. Microbial proteases are degradative enzymes, which catalyze the total hydrolysis of proteins. The molecular weight of proteases ranges from 18 – 90 kDa (Muthulakshmi *et al.,* 2011).

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology. Microbial sources are the best choice for economic production of proteases. As they can be cultured in large scale in relatively short time by well established fermentation reactions and also able to produce abundant and regular supply of the enzyme. As per the forecast, the global demand for enzymes will rise 7% per annum and this increasing economic importance has directed the research community to isolate hyperactive strains for the production of novel proteases and subsequent optimization of the various fermentation parameters for maximizing enzyme production. Although a wide range of micro organisms such as bacteria, fungi, actinomycetes and yeasts are known to date to produce proteases, a large potential of commercially available alkaline protease are derived from gram positive *bacillus* strains because of their ability to secret large amounts of alkaline protease having significant proteolytic activity and stability at considerable high pH and temperatures. *Bacillus* is highly favorable bacterium for protease production because it is non-pathogenic and well explored for producing various types of proteases. Among *Bacillus strains, B licheniformis*, *B subtilis, B acidophilous and B lentus* were important strains exploited industrially so far for protease production. *Bacillus* species produce two types of proteases, alkaline and neutral. Bacterial neutral proteases are active in narrow pH range (pH 5 to 8) and have relatively low thermo tolerance. This property is advantageous for controlling their activity during the production of food hydrolysis (Tennalli *et al.,* 2012).

Proteases are thus the most important group of the enzymes produced commercially and for industrial purpose. They have extensive applications in a range of industrial products and processes including detergents, food, pharmaceuticals, leather and bioremediation process. They are classified into various groups such as alkaline protease, serine protease, cystein protease, aspartic protease and metallo proteases (Sathiya *et al.,* 2013).

FIGURE: 5

CLASSIFICATION OF PROTEASES



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2.7. Applications of protease

Proteases are the most important type of enzymes in food processing and they hydrolyze the peptidic linkages in proteins. The application proteases are very diverse, including food science and technology, pharmaceutical industries, and detergent manufacturing. They are widely distributed in nature and plays important roles in biological processes. The number of industrially used proteases of plant origin is small and some cysteine proteases (CPs) such as papain, bromelain, and ficin are still being used in a variety processes. The optimum temperature and pH for enzyme activity were 70°C and 4, respectively. The protease also showed an excellent thermal stability at 60°C for 30 min. One of the main applications of proteases is for the production of cheese (Christopher *et al*., 2015).

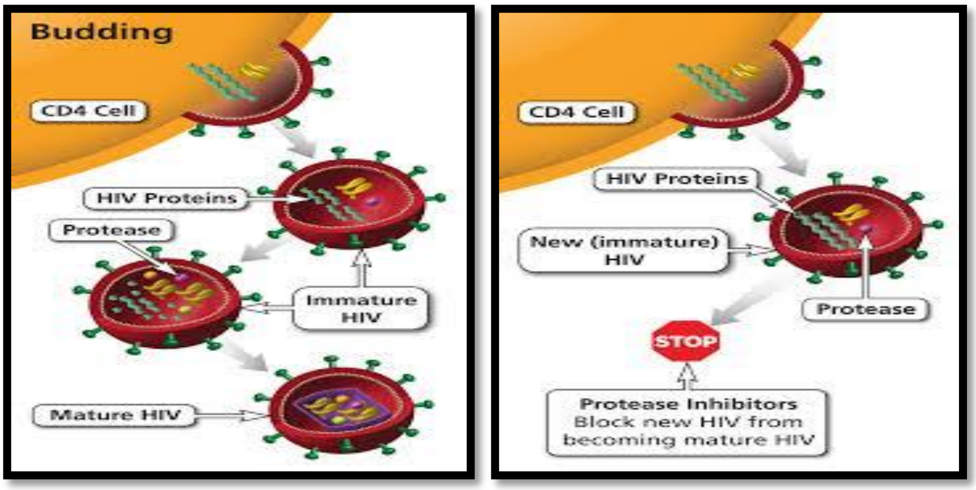
The wide diversity and specificity of microbial proteases are extensively used in diagnostic and therapeutic purposes. Bacterial and fungal proteases have contributed equally in developing effective therapeutic agents, such as anticancer, clot dissolving, antimicrobial, anti- inflammatory etc. Proteases are used in food industry for a wide range of applications. These enzymes are efficiently involved in the modification of properties of food proteins to improve nutritional value, solubility, digestibility, flavour, palatability and minimizing allergenic compounds. Besides, their basic function, they are also used to modify functional properties, such as coagulation, emulsification, foaming, gel strength, fat binding etc. of food proteins proteases used in detergent formulations are serine proteases produced by *Bacillus* strains. Alkaline proteases from fungal sources are also gaining interest due to ease in downstream processesing. In many formulations, cocktail of different enzymes including protease, amylase, cellulase and lipase are also used for improved washing effect for household purposes (Singh *et al*., 2016).

Alkaline proteases produced are of special interest as they could be used in manufacture of detergents, food, pharmaceuticals and leather. Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e.g., pH 10, and their broad substrate specificity. Their optimal temperature is around 60°C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry. *Bacillus spp*. produces the most detergent proteases currently used in the market, example is serine proteases. It is estimated that *Bacillus* spp. enzymes make up about 50% of the total enzyme market (Mienda *et al.,* 2014).

Microbial proteases are increasingly used in treatment of various disorders namely cancer, inflammation, cardiovascular disorders, necrotic wounds etc. Proteases are used an immune-stimulatory agent. Antibiotic concentration is increased at the target site when protease was concomitantly used with an antibiotic. 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. Proteases have a large variety of applications, mainly in the detergent and food industries. Proteases are envisaged to have extensive applications in leather treatment and in several bioremediation processes. Proteases that are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations; whereas those that are used in medicine are produced in small amounts but require extensive purification before they can be used (Sawant *et al.,* 2014).

FIGURE: 6

APPLICATION OF PROTEASES ENZYME IN MEDICAL INDUSTRY



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Proteases are one of the most important groups of both industrially and academically important enzymes accounting for around 65% of annual enzyme market. They have a history of applications in food and detergent industries where the alkaline proteases hold the biggest share of the enzyme market worldwide. Alkaline protease use as ingredient in detergents is largest application of this enzyme. They also have application in leather industry, medical diagnostic, recovery of silver from X-rays, food and feed industry etc (Sundus *et al.,* 2016).

2.8. Compost sample

Composting is an environmentally less burden technology because of its recycling capability of organic wastes discharged from industrial and municipal plants or livestock farming. Recent global problems of food shortage have been caused by rising cost of chemical fertilizers, and composting at low cost has been reevaluated as an important alternative fertilizer production method. High-quality compost is produced by interaction of many organisms that have suitable properties for the composting processes. Nevertheless, little information has been reported about in situ functions and roles of individual microbes in the composting processes, because many microbes related to composting are difficult to isolate and are characterized by conventional cultivation methods. Few fungus and bacterial isolates have been characterized in detail, and their roles in the composting process have been understood from physiological properties. For example, fungus *Chaetomium thermophilum* isolated from municipal waste compost produces extracellular enzymes, laccases, which are essential for formation of polyaromatic humic substances associated with phenoloxidase and peroxidase. *Bacillus licheniformis, Trichoderma viride* and complex microorganisms such as *Trichoderma sp., white-rot fungi*, *Candida rugopelliculosa*, *Bacillus casei* and *Lactobacillus buchneri* reported so far also accelerate humification of organic wastes in composting process. These characterized isolates are essential to mature compost and are useful as inoculam to increase the humification rate of composting (Awasthi *et al.,* 2012).

Composting is a preferred and environmentally sound method whereby organic waste is reduced to organic fertilizer and soil conditioners through biological processes. The high organic carbon content and biological activity of compost make it effective for applications such as erosion control and revegetatio. The composting process involves three phases, and uses diverse microflora such as bacteria, fungi and mesophilic (*Streptomyces rectus*) and *thermophilic Actinomycetes* (*Actinobifida chromogena (Thermomonospora fusca) Microbispora (Thermopolyspora) bispora*, *Therinomnonospora curvata, Thermoactinomyces sp.)* eventually converting organic waste to humus. During the first phase there is an increase in carbon dioxide along with the temperature. The substrate is reduced due to the degradation of sugar and proteins by the action of mesophilic organisms. The second phase leads to an increase of the temperature in the compost piles from 45° C to approximately 70° C and the mesophiles are replaced by thermophiles. Large numbers of pathogenic individuals are degraded during this time. The third phase begins with the decrease of temperature of the compost pile (Pan *et al.,* 2011).

The composting process results in biodegradation - induced metabolic heat production that can increase temperatures above 70° C. Only a few specialized microbes can live under these extreme environmental conditions, and the activity and diversity of composting bacterial strains are greatly suppressed at compost temperatures greater than 60° C. Strains related to *Bacillus stearothermophilus* usually dominate under these conditions. Recently, 34 thermophilic bacterial species (grown at 60° C) were isolated during aerobic composting of poultry and cattle manure, and *Geobacillus thermodenitrificans* was one of those species. It has been reported that the chemical oxygen demand, sewage sludge, total nitrogen content, and total phosphorous content of swine manure were reduced by passing through filtering system using wood chips (Yang *et al.,* 2013).

Municipal solid waste (MSW) composting is an aerobic decomposition of organic materials by a variety of microorganisms under controlled conditions to convert into humus-like product which are used in agriculture as fertilizer. Since degradation of cellulosic compounds like fruits, vegetables, kitchen refuses and industrial food processing wastes in the MSW composting process is difficult and takes considerable period of time (Nair *et al.,* 2010), the main concern for MSW composting is the shortening of composting period. In compost, many cellulose-degrading bacteria are predominant and play a key role in the process of biodegradation in thermophilic stage. They were proved to have the ability to adapt to the changing environment. Thus, their isolation and inoculation may intensify the biodegradation of recalcitrant cellulosic compounds and accelerate the process of composting (Ghaffaril *et al.,* 2011).

Vermicompost is a peat like material, exhibiting high porosity, aeration, drainage, water holding capacity, rich microbial activities and exhibit great plant growth. Vermicomposts contain rich nutrient concentrations than composts. *Bacillus subtilis* is a gram positive, spore forming, fermentative, aerobic and rod shaped bacterium. These are found mostly in soil, compost, fresh and marine water and play an important role in the decomposition of organic materials and produce secondary metabolites of commercial interest. *Bacillus subtilis* is a type of strain which is both taxonomically and metabolically diverse. These bacteria are capable of secreting antibiotics in great numbers to the exterior of the cell. Therefore, present research was carried out to discuss the *Bacillus subtilis* bacterial biodiversity of vermicomposts (Govindarajan *et al.,* 2015).

Composting is a microbial decomposition process in which easily degradable and putrescent organic waste is turned into stable material compost and current method for understanding the communities in composting ecosystem composting is a controlled decay of organic matter in a warm moist environment by action of bacteria, fungi and other organisms. Hence it is programmed molecular identification of thermophiles and parameter analysis of compost (Ilayaraja *et al.,* 2012).

The composting of manure results in a product that is more nutrient-stable and free of microbial pathogens and phytotoxins. Furthermore, composting may also decrease the concentration of excreted antimicrobials, resistance determinants, and resistant bacteria. Transportation costs are also reduced as composting significantly decreases the volume and mass of the manure. Composting is a largely aerobic and natural process involving the bio- degradation of organic matter and is often described as having three microbiological phases: 1) an initial mesophilic phase (20 to 40°C) from 1 to 3 days, 2) a thermophilic phase (35 to 65°C), and 3) a cooling phase where mesophiles increase in abundance again as the temperature falls. There is also a last stage in the composting process termed the maturation or curing phase, where microbial activity is reduced. This thermophilic phase is particularly important from an agricultural perspective as the elevated temperature is responsible for eliminating pathogens and weed seeds (Holmanl *et al.,* 2016).

Composting technology is a common treatment method for the selection of recyclable and compostable materials, which can be inoculated with preselected microorganisms. Showed that inoculation with *Bacillus shackletonni, Streptomyces* *thermovulgaris* and *Ureibacillus thermosphaericus* significantly increased the humification indices and thus improved the composting property. Which exhibited high activity of cellulase and xylanase, significantly improved the quality of compost. During the high temperature phase of the composting process, material temperature is increased by microbial-self-heating depending on the composting system, so composting process can be greatly improved by inoculation of thermophilic microorganisms (Wang *et al.,* 2014).

3. Materials and methods

The composting process results in biodegradation -induced metabolic heat production that can increase temperatures above 70℃. Only a few specialized microbes can live under these extreme environmental conditions, and the activity and diversity of composting bacterial strains are greatly suppressed at compost temperatures greater than 60℃. Strains related to *Bacillus stearothermophilus* usually dominate under these conditions. Recently, 34 thermophilic bacterial species (grown at 60℃) were isolated during aerobic composting (Gilbert *et al.,* 2008).

Proteases are one of the most important classes of industrial enzymes and comprise about 25% of the commercial enzyme in the world. They are classified as acid, neutral and alkaline proteases. These enzymes are widely used many industries (Radha *et al.,* 2011). Proteases are involved in digesting long chain into short fragments splitting the peptide bonds that link amino acid residues. Proteases are hydrolytic enzymes. These enzymes are widely used in many biotechnological industry, brewing, photographic, agriculture industry, leather, dairy industry and bioremediation processes (Motamedzadegan *et al.,* 2010).

The present study was entitled **"Partial Purification and Characterization of Protease Produced by *Bacillus subtilis* and *Bacillus nakamurai* Isolated From the Vegetable Waste Compost Soil"** from vegetable waste composting and partial purification and characterization of protease is detailed as follows. The experiment was designed as given below:

3.1 Sample collection
3.2 Composting in pits
3.3 Isolation of organism
3.4 Morphological and biochemical characterization of the bacterial isolates
3.5 Detection of protease activity of the isolates
3.6 Production medium and culture conditions
3.7 Screening of the isolates for the protease production
3.8 Partial purification of protease enzyme
    3.8.1 Precipitation by ammonium sulphate
    3.8.2 Protein estimation
    3.8.3 Protease assay

3.9 Effect of pH

3.10 Effect of Temperature

3.1 Sample collection :

Daily vegetable waste was collected for 10 to 15 days. Different methodologies were adopted to devise a standard composting method from which thermophilic microorganisms can be isolated.

3.2 Composting in pits:

A two feet deep pit was dug and 4kg vegetable waste and 1 kg dung was layered alternately. The top layer was covered with soil. Temperature was recorded using thermometer on a regular basis (Sarkar *et al.,* 2016).

3.3 Isolation of organism

The compost sample was pretreated at 60°C for 1 hour and was crushed using mortar and pestle. The sample was homogenised using distilled water and was further used for isolation of bacteria. 1 ml of homogenised sample was serially diluted (10^{-1} to 10^{-10}) and aliquots of 0.1ml were spread plated on the skimmed milk agar plates (Balachandran *et al.,* 2012). The plates were incubated at 60°C for 2 days. The isolates which exhibited protease activity on skimmed milk agar plates were subcultured on nutrient agar medium and stored at 4°C for further studies **(**Hulawale *et al.,* 2015).

3.4 Morphological and biochemical characterisation of the bacterial isolates

Isolates were characterized up to genus level based on morphological and biochemical characters, following the Bergey`s manual of systematic bacteriology.

Morphological characterisation was done by slide culture and Grams staining technique using light microscope. Morphological characters of isolates such as morphology of spore bearing hyphae with entire spore chain and structure of spore were observed.

Gram’s Staining

Bacterial characterization of gram staining procedure was determined for each isolate (David *et al.,* 2012). The detailed procedure is given in Appendix I.

Biochemical Characterization

Biochemical characterizations of (Glucose, Galactose, Xylose, Mannitol, Sucrose and Mannose) were performed in standard methods (Govindarajan *et al.,* 2015).

Indole Production Test

Indole production test is used to determine the ability of the organism to produce indole from the culture. The procedure is explained Appendix II.

Methyl Red Test

Methyl red test is employed to detect the ability of microorganisms to oxidize glucose with the production of high concentration of acid end products and the procedure is given in Appendix III.

Voges-Proskauer Test

This test is also known as the acetone production test. To detect the ability of an organism to ferment carbohydrate with the production of non acidic or neutral end product. The detailed procedure is given in Appendix IV.

Citrate Utilization Test

Some of the organisms were capable of utilizing citrate as the sole carbon source and mono ammonium phosphate as the sole source of nitrogen. The procedure is explained given in Appendix V.

Oxidase test

This test id performed to detect the ability of microorganisms on the basis of their cytochrome oxidase activity. The detailed procedure is explained in Appendix VI.

Catalase test

To detect the ability of microorganisms to oxidised H_2O_2 by producing the enzyme catalase. The detailed procedure is given in Appendix VII.

Starch hydrolysis test

To detect the amylase action of an organism by starch hydrolysis test. The procedure is explained in Appendix VIII.

Carbohydrate fermentation test

To detect the ability of an organism to fermented to specific carbohydrates. The detailed procedure is explained in Appendix IX.

Nitrate Reduction Test

This test is used to detect whether the organisms reduced the nitrates to nitrites. The procedure is explained in Appendix X.

Triple Sugar Iron Test

TSI test is used to differentiate the isolate according to the ability to ferment lactose, sucrose and glucose and production of hydrogen sulfide. The detailed procedure is explained in Appendix XI.

3.5 Detection of protease activity of the isolates

Detection of protease activity by strain fresh culture of the isolates was done. Nutrient gelatin agar plates were spot inoculated with the culture and incubated at 60°C for 24hours. After incubation plates was observed for zone of clearance around the colony.

3.6 Production medium and culture conditions

The isolate was inoculated in 250ml Erlenmeyer flasks containing 100ml of production medium, pH-7. The flask was incubated in shaker incubator at 55°C, 100 rpm for 144 hours. The aliquots were withdrawn at interval of 24 hours and centrifuged at 5000 rpm for 30 minutes. The pellet and supernatant obtained were subjected for detection of enzyme activity using enzyme assay **(**Hulawale *et al.,* 2015).

3.7 Screening of the isolates for the protease production

The bacterial isolates were screened for the yield of protease by skim milk agar plate method as described earlier. Briefly, the bacterial colonies were streaked on skimmed milk agar plate and incubated at 37℃ for 24 hours. The Formation of clear zone around the colonies indicated the proteolytic activities of bacteria resulting from milk protein hydrolysis (Nayab *et al.,* 2015).

3.8 Partial purification of protease enzyme

Ammonium sulphate precipitation is one of the most commonly used methods for large and laboratory scale protein purification and fractionation that can be used to separate proteins by altering their solubility in the presence of a high salt concentration.

Nutrient broth (pH 7) containing $CaCl_2$, $NaNO_3$ was inoculated with 24 hours old preculture. The fermented broth was harvested after 96 hours of incubation at 60°C by centrifugation at 5000 rpm 30 min, 4°C.

3.8.1 Precipitation by ammonium sulphate

Cell-free supernatant was collected after centrifugation. Different concentrations of ammonium sulphate salt from 30% to 90% of saturation were slowly added to the aliquots of supernatant. The mixture was incubated overnight at 4°C. The precipitated proteins were obtained by centrifugation at 5000 rpm for 20 min, 4°C. The resulted pellet was re-suspended in ice-cold 0.05 M phosphate buffer pH 9. The protease activity and protein concentration were determined for pellet and supernatant of each fraction **(**Hulawale *et al.,* 2015).

3.8.2 Protein assay

Total protein of the cell free filtrate was determined by the Lowry's method. Bovine serum albumin (250µg/ml) was used as a standard **(**Swamy *et al.,* 2014). The procedure is explained in Appendix XII.

3.8.3 Protease assay

Proteolytic activity in the pellet and supernatant was determined by using spectrophotometer method. 1ml of crude enzyme solution was allowed to react with 2.0 ml of 0.6% casein in phosphate buffer (50 mM, pH 9) at 45°C for 20 min. The reaction was terminated by the addition of 4 ml 10% trichloroacetic acid. The reaction mixture was allowed to stand for 15 minute before centrifugation. The mixture was then centrifuged at 5000 rpm for 15 minutes at 4°C and 1 ml of supernatant was taken as the enzyme source. The supernatant was mixed with 2.5 ml of 0.4M Na_2CO_3, 0.5 ml of 1:1 Folin Ciocalteu phenol reagent and distilled water. The reaction mixture was incubated at room temperature in the dark for 30 min. The absorbance was measured at 660nm wavelength. The amount of amino acid released was quantified from standard graph of tyrosine (Tennalli *et al.,* 2012).

3.9 Effect of pH

The activity of the protease was measured at different pH values. The pH was adjusted using the following buffers (0.05M): phosphate (pH 6.0-7.0), Tris-HCl (pH8.0), and glycine- NaOH (pH 9.0-11.0). 1ml of enzyme was incubated at 37°C for 10 minutes, and the activity of the enzyme was measured. The relative activity at each exposure was measured as per assay.

3.10 Effect of Temperature on Enzyme Activity

To determine the enzyme stability with changes in temperature, enzyme (1ml) was incubated at different temperatures ranging from 25°C to 100°C for 10 minutes and the relative protease activities were assayed under standard assay conditions (Dorcas *et al.,* 2016).

4. RESULTS AND DISCUSSION

Enzymes are among the most important products obtained for human needs through microbial sources. A large number of industrial processes in the areas of industrial, environmental and food biotechnology utilize enzymes at some stage or other. Current developments in biotechnology are yielding new applications for enzymes (Hasan *et al.,* 2006)

Proteases are a group of enzymes, whose catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids. Sources of proteases include all forms of life, that is, plants, animals and microorganisms. Based on their acid-base behavior, proteases are classified in to three groups, that is, acid, neutral and alkaline proteases. Proteases produced from microorganisms play important role in several industries example detergent, tanning, photographic industries, pharmaceutical and waste treatment etc (Deng *et al.,* 2010).

Bacterial strains having protease activity were selected and their morphological and biochemical characteristics were determined. The isolated bacterial strains were cultured in nutrient broth, the cell free extracts of the cultured bacterial strains were used for the assay of protease activities. The optimum pH and temperature for protease activity were determined.

Keeping these wide applications in mind, the present study was carried out by isolating bacteria that produce protease in compost soil sample. The findings of the present study entitled "**Partial Purification and Characterization of Protease Produced by *Bacillus subtilis* and *Bacillus nakamurai* Isolated From the Vegetable Waste Compost Soil**" are discussed in the following headings:

4.1 Bacterial strains from compost soil sample

4.2. Morphological and Biochemical characteristics of the bacterial strains

4.3 Analysis of 16S rRNA Gene Sequences

4.4 Screening of the isolates for the protease production

4.5 Detection of protease activity of the isolates

4.6 Purification by ammonium sulphate precipitation

4.7 Effect of pH

4.8 Effect of temperature

4.1 BACTERIAL STRAINS FROM VAGETABLE COMPOST SOIL SAMPLE

The vegetable compost soil sample (Figure 7) was homogenised using distilled water and was further used for isolation of bacteria. 1 ml of homogenised sample was serially diluted and seven strains were isolated by pour plate technique using nutrient agar medium. Isolation of protease producing bacteria was first carried out using nutrient agar media and further screened for protease production on screened on skim milk agar plates. Formation of clear zones around the colonies was considered as indication of protease production. The selected strains are presented in Table I and Plate 1.

The bacterial strains producing protease were maintained using nutrient agar medium for further work which is shown in Plate 3.

FIGURE 7

VEGETABLE COMPOST SOIL SAMPLE

TABLE 1

BACTERIAL STRAINS ISOLATED FROM COMPOST SOIL SAMPLE

S.No	Isolates
1	Isolate 1
2	Isolate 2
3	Isolate 3
4	Isolate 4
5	Isolate 5

4.2. MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF BACTERIAL ISOLATES

Table 2 depicts the colony morphology and biochemical characteristics of the bacterial isolates. Morphological characterization was done by slide culture and Grams staining technique using light microscope.

TABLE 2

COLONY MORPHOLOGY AND BIOCHEMICAL CHARACTERISTICS OF ISOLATED BACTERIAL STRAINS

S.NO	BIOCHEMICAL TEST	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5
1	Shape	Rod	Rod	Rod	Rod	Rod
2	Gram staining	+	+	+	+	+

3	Indole production	–	–	–	–	–
4	Methyl red	+	+	+	+	+
5	Voges proskauver	+	+	+	+	+
6	Citrate utilization	–	–	–	–	–
7	Nitrate reduction test	+	+	+	+	+
8	Carbohydrate fermentation	+	+	+	+	+
9	Starch hydrolysis	+	+	+	+	+
10	Gelatine hydrolysis	+	+	+	+	+
11	Oxidase	–	–	–	–	–
12	Catalase	+	+	+	+	+

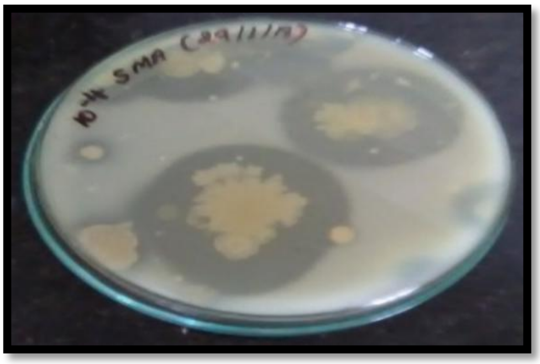
+Positive - Negative

PLATE 1

PROTEASE PRODUCING BACTERIAL ISOLATES

Isolate 1

Isolate 2



Isolate 3

Isolate 4

Isolate 5

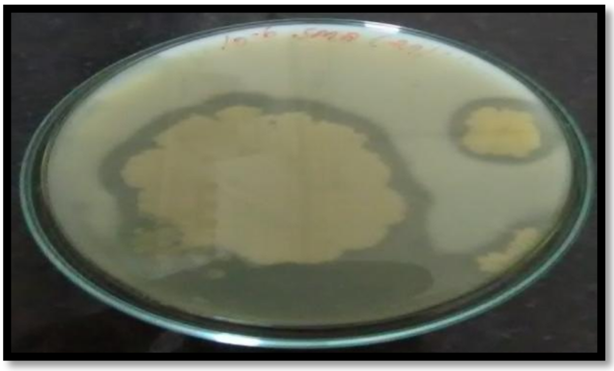


PLATE 2

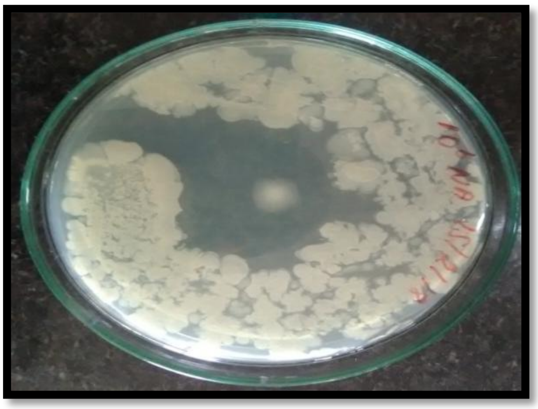
INDIVIDUAL COLONIES OF PROTEASE PRODUCING BACTERIAL ISOLATES

Isolate1

Isolate 2

Isolate 3

Isolate 4



Isolate 5

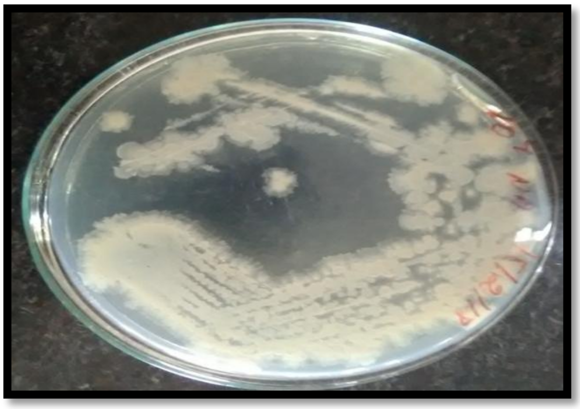


PLATE 3

GRAM STAINING

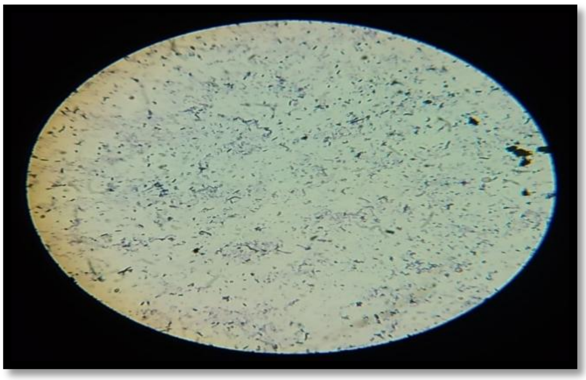
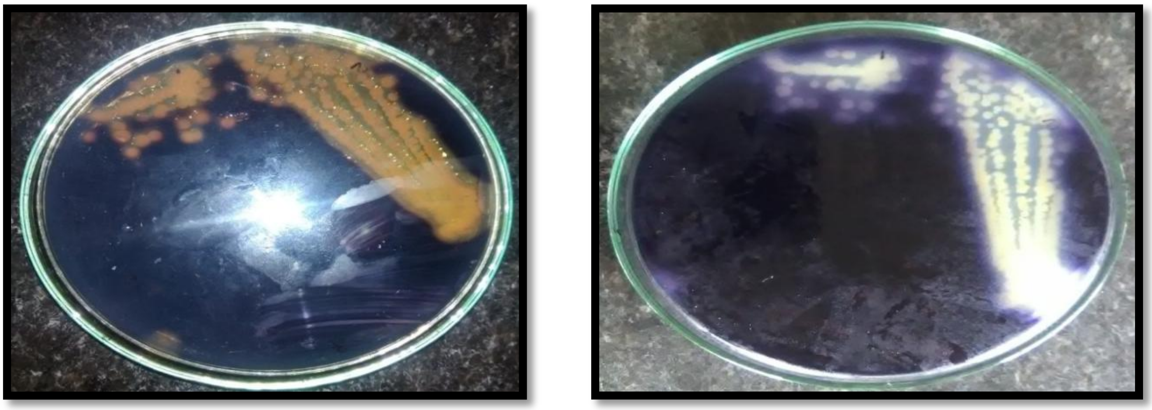


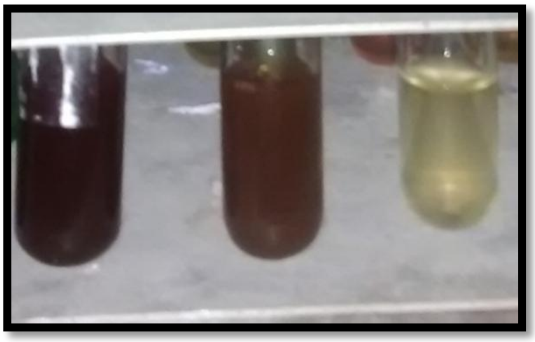
PLATE 4

BIOCHEMICAL CHARACTERIZATION OF ISOLATED BACTERIAL STRAINS



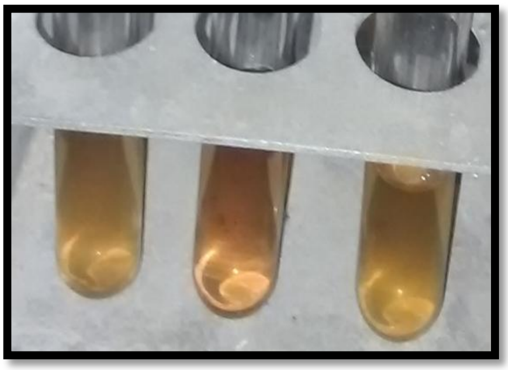
Starch hydrolysis test

Methyl red test

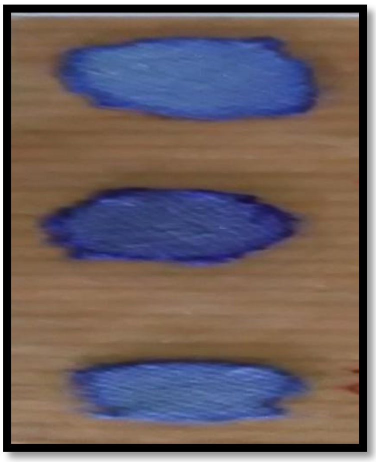


Nitrate reduction test

Voges proskauver



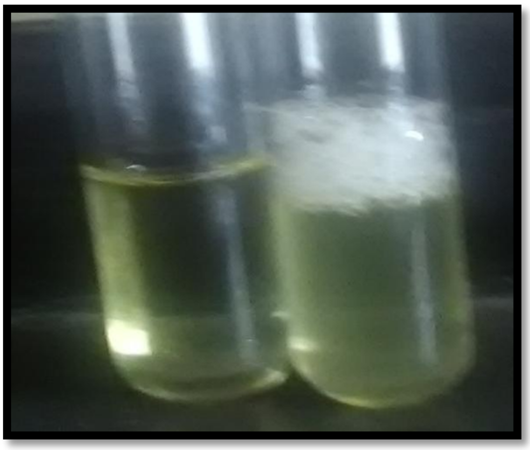
Indole test



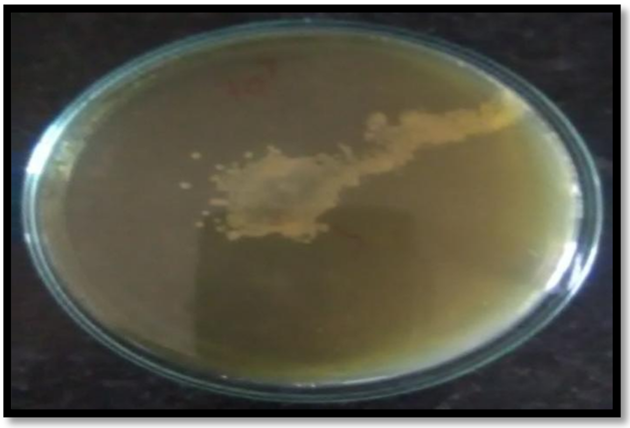
Oxidase test

Citrate utilization test

Carbohydrate fermentation test



Catalase test



Gelatin hydrolysis test

Five bacterial strains were isolated from the vegetable waste compost soil, namely, Isolate 1, Isolate 2, Isolate 3, Isolate 4 and Isolate 5 were identified as *Bacillus sp*. based on the morphological and biochemical characteristics (Plate 3 and 4).

Isolation of protease producing bacteria was first carried out using nutrient agar media and further screened for protease production on screened on skim milk agar plates. Formation of clear zones around the colonies was considered as indication of protease production. Out of 7 isolates only 5 isolates showed zone around the colonies (plate 1 and plate 2). Those bacterial isolates which showed clear zone of protease production were further explored for their culture, microscopic and biochemical characteristics.

Similar studies have been reported by Sindhu *et al.* (2006). The isolates were found to be Gram positive, rods. Catalase test was found to be positive. Based on their morphological and biochemical characteristics the bacteria were identified as belonging to the genus *Bacillus*.

Umayaparvathi *et al*. (2013) reported that microscopic observations of isolates were found to be Gram positive rod shaped bacterium; the bacterium grew aerobically and formed white colonies. The morphological and biochemical characteristic are identified as the genus *Bacillus.*

4.3 Analysis of 16S rRNA Gene Sequences

Out of five isolates, one bacterial isolates (Isolate 5) was selected and 16S rRNA gene sequence was found. The 16S rRNA sequences result is FASTA format is shown in figure 8. The obtained sequences were aligned with BLAST in NCBI data base, the bacterial isolate was confirmed as primer1 ***Bacillus subtilis*** and primer2 ***Bacillus nakamurai*** strain as shown in Figure 9. The homology among these bacteria showed a significant value of 99% homology. Neighbour-joining inferred tree based on 16S rRNA gene sequences showing the evolutionary relationship of isolates within previously characterized species (Figure 10).

Similar studies have been reported by Charbonneau *et al.* (2012). 16S rRNA genes were amplified using the universal primers. Searches for sequences similarity was performed using BLAST using blastn accessed via the National Centre for Biotechnology Information (NCBI) website.

Panda *et al.,* (2013) reported that the 16S rRNA gene from the genomic DNA of the Bacillus (based on the Biochemical and Staining properties) was enzymatically amplified by Taq DNA polymerase by using a universal bacterial primer set, were used, according to method described by William *et al.,* 1991. The obtained sequenced was blasted in NCBI data base, and phylogenetic analysis of the bacteria *Bacillus* was carried out.

FIGURE 8

ANALYSIS OF 16S rRNA GENE SEQUENCES

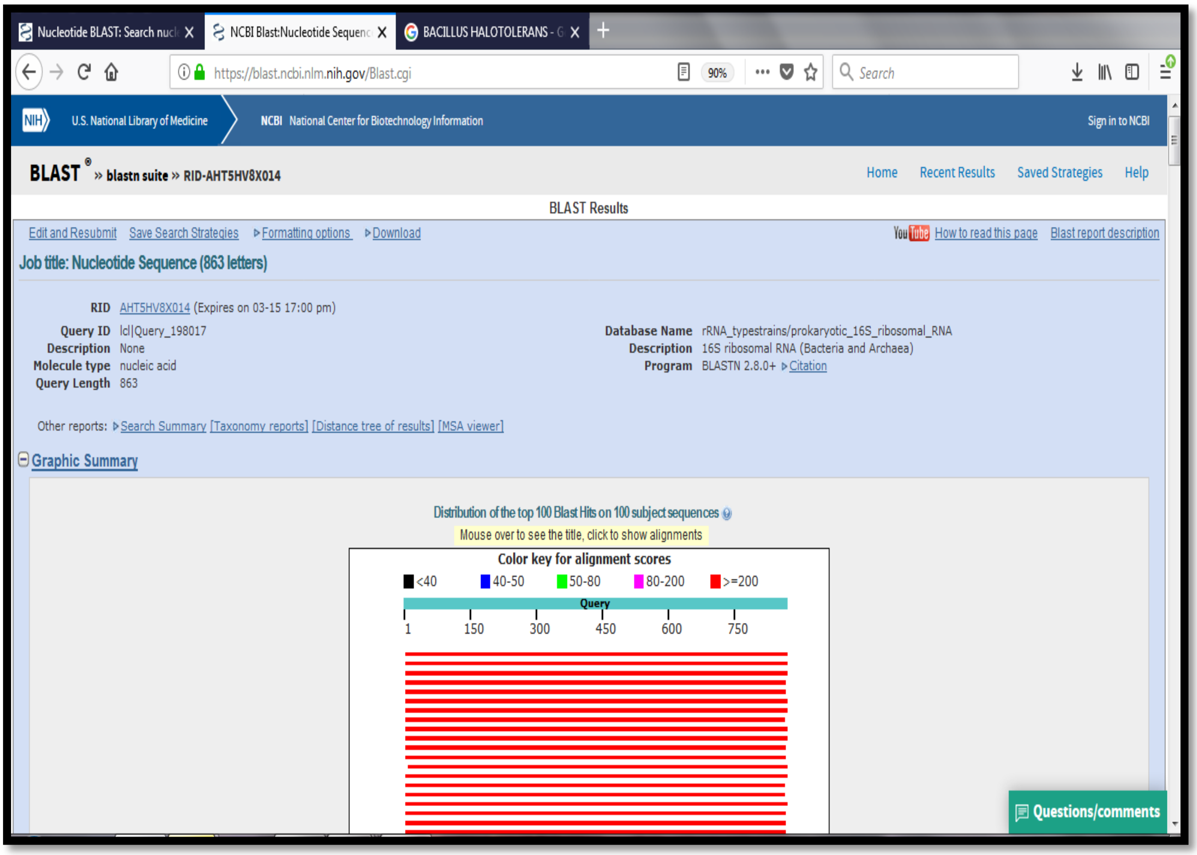
PRIMER 1(*Bacillus subtilis*)

AGCTGCTTATGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTA
GCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCC
ACGCTTTCGCTCCTCAGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTG
GTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCC
TCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCCTCCCCGGTTGAGCCGGG
GGCTTTCACATCAGACTTAAGAAACCGCCTGCGAGCCCTTTACGCCCAATAATT
CCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGC
CGTGGCTTTCTGGTTAGGTACCGTCAAGGTACCGCCCTATTCGAACGGTACTTG

TTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCGGC
GTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCG
TAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTC
GGCTACGCATCGTTGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGC
CGCGGGTCCATCTGTAAGTGGTAGCCGAAGCCACCTTTTATGTTTGAACCATGC
GGTTCAAACAACCATCCGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTT
ACAGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGCTAACATCAGGGAGC
AAGCTCCCATCTGTCCGCTCGACTTGCATGTATTAGGCACGCCGCCCGCGTTC
GTCCTGA

FIGURE 9

GRAPHICAL REPRESENTATION OF SEARCHES FOR SEQUENCES SIMILARITY WAS PERFORMED USING BLAST



BLAST® » blastn suite » RID-AHT5HV8X014

BLAST Results

Job title: Nucleotide Sequence (863 letters)

RID AHT5HV8X014 (Expires on 03-15 17:00 pm)

Query ID lcl|Query_198017
Description None
Molecule type nucleic acid
Query Length 863

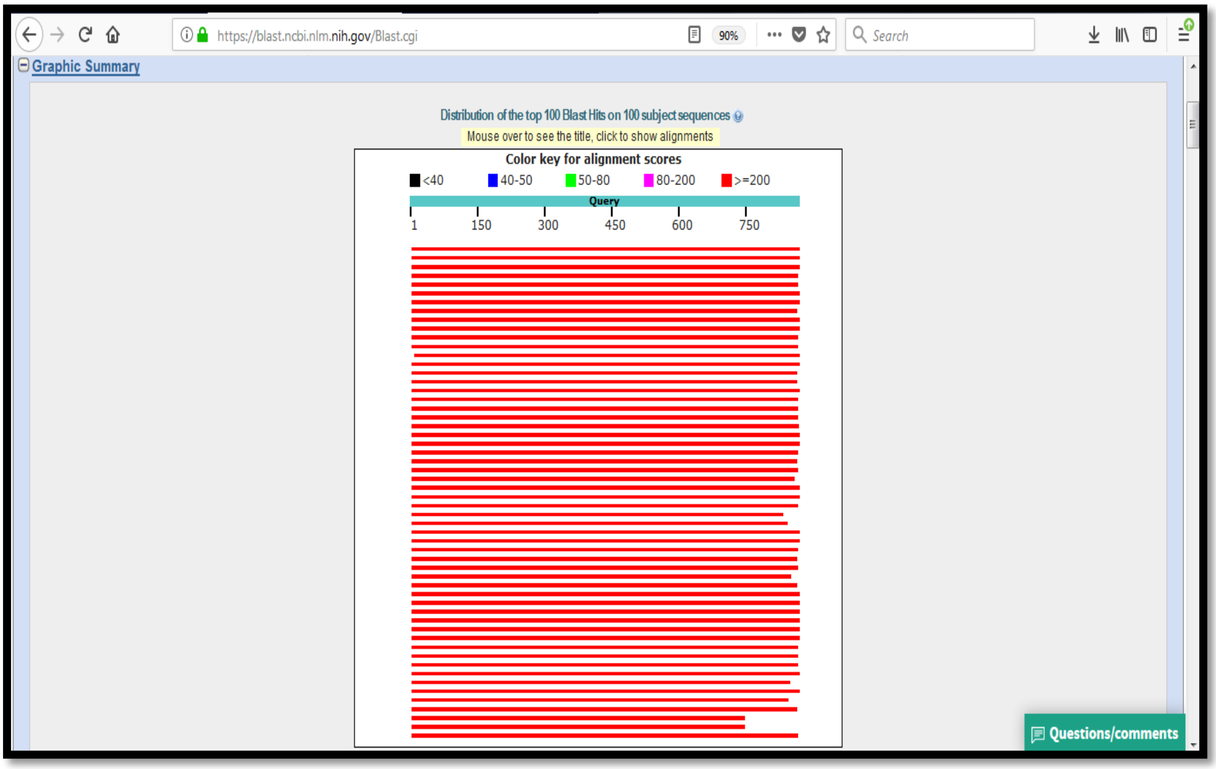
Database Name rRNA_typestrains/prokaryotic_16S_ribosomal_RNA
Description 16S ribosomal RNA (Bacteria and Archaea)
Program BLASTN 2.8.0+ Citation

Other reports: Search Summary [Taxonomy reports] [Distance tree of results] [MSA viewer]

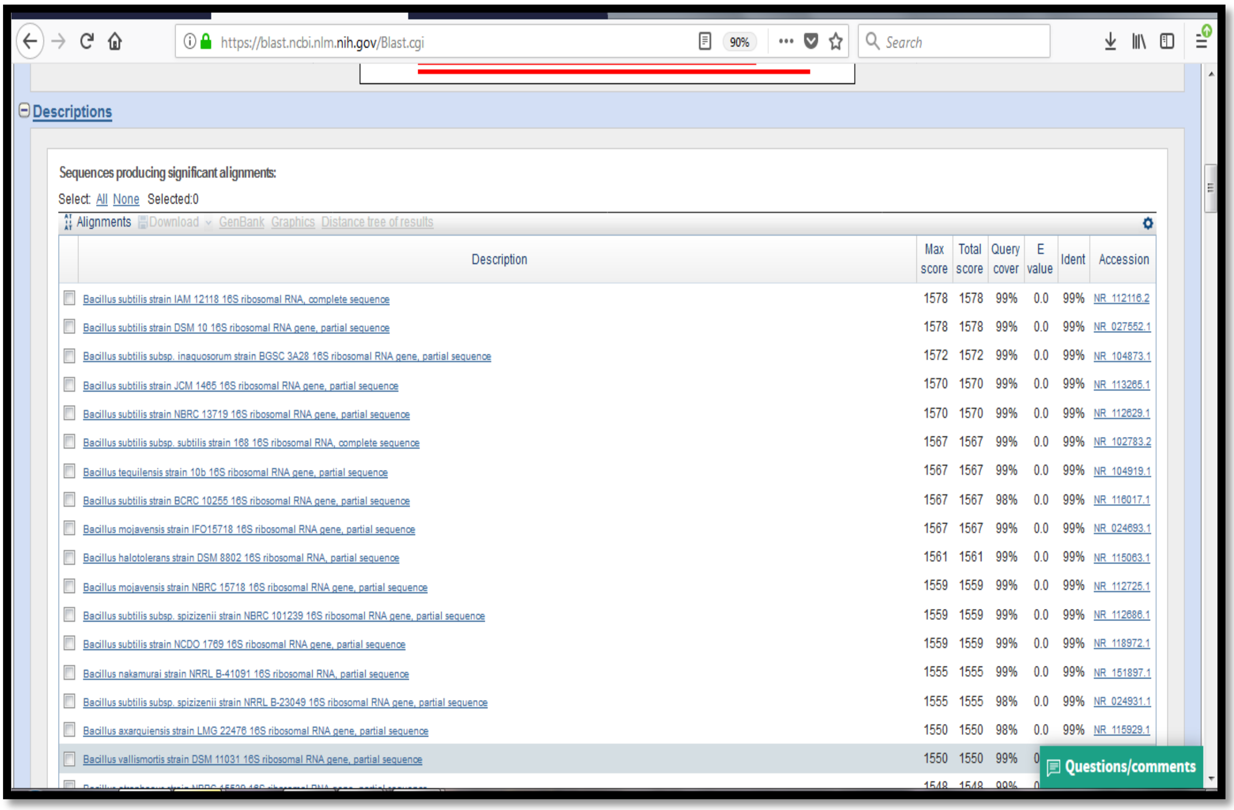
Graphic Summary

Distribution of the top 100 Blast Hits on 100 subject sequences

Mouse over to see the title, click to show alignments



Description



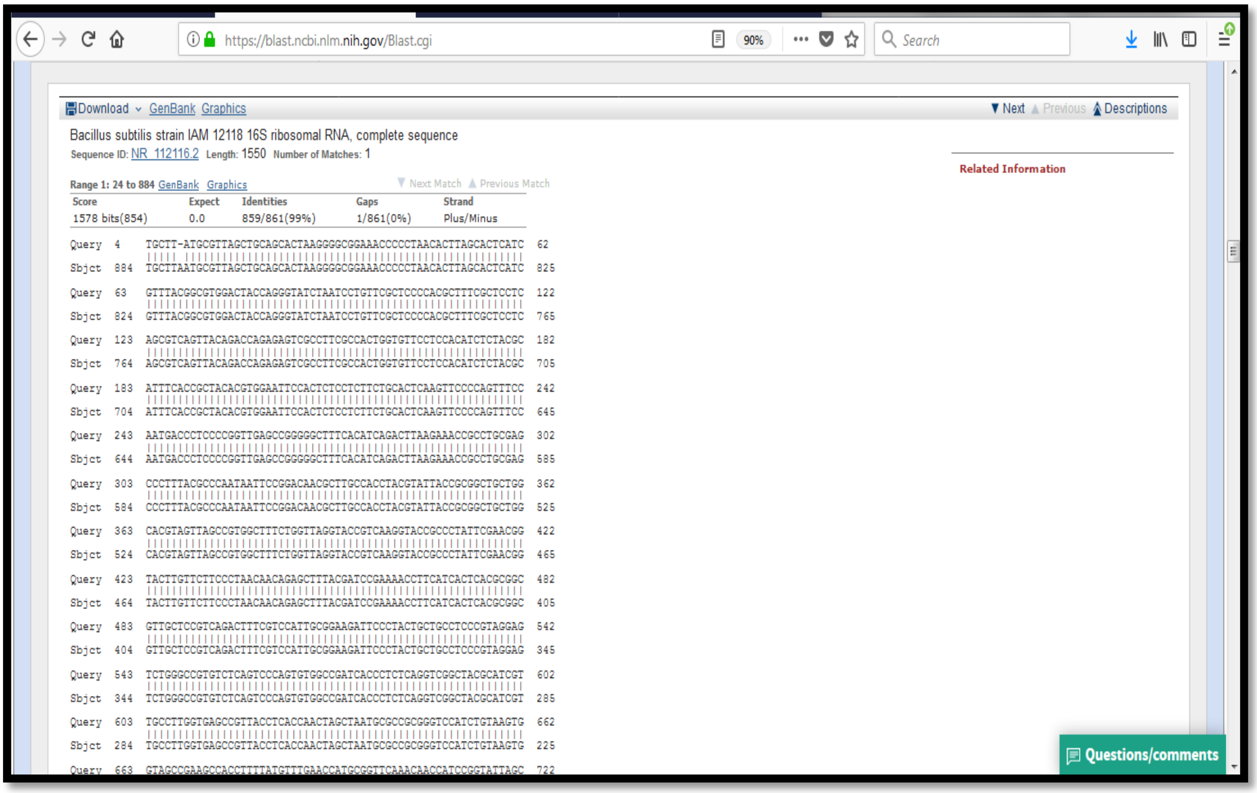
Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Bacillus subtilis strain IAM 12118 16S ribosomal RNA, complete sequence	1578	1578	99%	0.0	99%	NR_112116.2
☐	Bacillus subtilis strain DSM 10 16S ribosomal RNA gene, partial sequence	1578	1578	99%	0.0	99%	NR_027552.1
☐	Bacillus subtilis subsp. inaquosorum strain BGSC 3A28 16S ribosomal RNA gene, partial sequence	1572	1572	99%	0.0	99%	NR_104873.1
☐	Bacillus subtilis strain JCM 1465 16S ribosomal RNA gene, partial sequence	1570	1570	99%	0.0	99%	NR_113265.1
☐	Bacillus subtilis strain NBRC 13719 16S ribosomal RNA gene, partial sequence	1570	1570	99%	0.0	99%	NR_112629.1
☐	Bacillus subtilis subsp. subtilis strain 168 16S ribosomal RNA, complete sequence	1567	1567	99%	0.0	99%	NR_102783.2
☐	Bacillus tequilensis strain 10b 16S ribosomal RNA gene, partial sequence	1567	1567	99%	0.0	99%	NR_104919.1
☐	Bacillus subtilis strain BCRC 10255 16S ribosomal RNA gene, partial sequence	1567	1567	98%	0.0	99%	NR_116017.1
☐	Bacillus mojavensis strain IFO15718 16S ribosomal RNA gene, partial sequence	1567	1567	99%	0.0	99%	NR_024693.1
☐	Bacillus halotolerans strain DSM 8802 16S ribosomal RNA, partial sequence	1561	1561	99%	0.0	99%	NR_115063.1
☐	Bacillus mojavensis strain NBRC 15718 16S ribosomal RNA gene, partial sequence	1559	1559	99%	0.0	99%	NR_112725.1
☐	Bacillus subtilis subsp. spizizenii strain NBRC 101239 16S ribosomal RNA gene, partial sequence	1559	1559	99%	0.0	99%	NR_112686.1
☐	Bacillus subtilis strain NCDO 1769 16S ribosomal RNA gene, partial sequence	1559	1559	99%	0.0	99%	NR_118972.1
☐	Bacillus nakamurai strain NRRL B-41091 16S ribosomal RNA, partial sequence	1555	1555	99%	0.0	99%	NR_151897.1
☐	Bacillus subtilis subsp. spizizenii strain NRRL B-23049 16S ribosomal RNA gene, partial sequence	1555	1555	98%	0.0	99%	NR_024931.1
☐	Bacillus axarquiensis strain LMG 22476 16S ribosomal RNA gene, partial sequence	1550	1550	98%	0.0	99%	NR_115929.1
☐	Bacillus vallismortis strain DSM 11031 16S ribosomal RNA gene, partial sequence	1550	1550	99%	0		
☐		1548	1548	99%	0		

Alignment



Bacillus subtilis strain IAM 12118 16S ribosomal RNA, complete sequence

Sequence ID: NR_112116.2 Length: 1550 Number of Matches: 1

Range 1: 24 to 884 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1578 bits(854)	0.0	859/861(99%)	1/861(0%)	Plus/Minus

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Query  63   GTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTC  122
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Sbjct  824  GTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTC  765

Query  123  AGCGTCAGTTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGC  182
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  183  ATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCC  242
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Sbjct  704  ATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCC  645

Query  243  AATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGAG  302
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Sbjct  644  AATGACCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGAG  585

Query  303  CCCTTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGG  362
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Query  423  TACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCGGC  482
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Sbjct  464  TACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCATCACTCACGCGGC  405

Query  483  GTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAG  542
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Sbjct  344  TCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGT  285

Query  603  TGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTG  662
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  284  TGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTG  225

Query  663  GTAGCCGAAGCCACCTTTTATGTTTGAACCATGCGGTTCAAACAACCATCCGGTATTAGC  722
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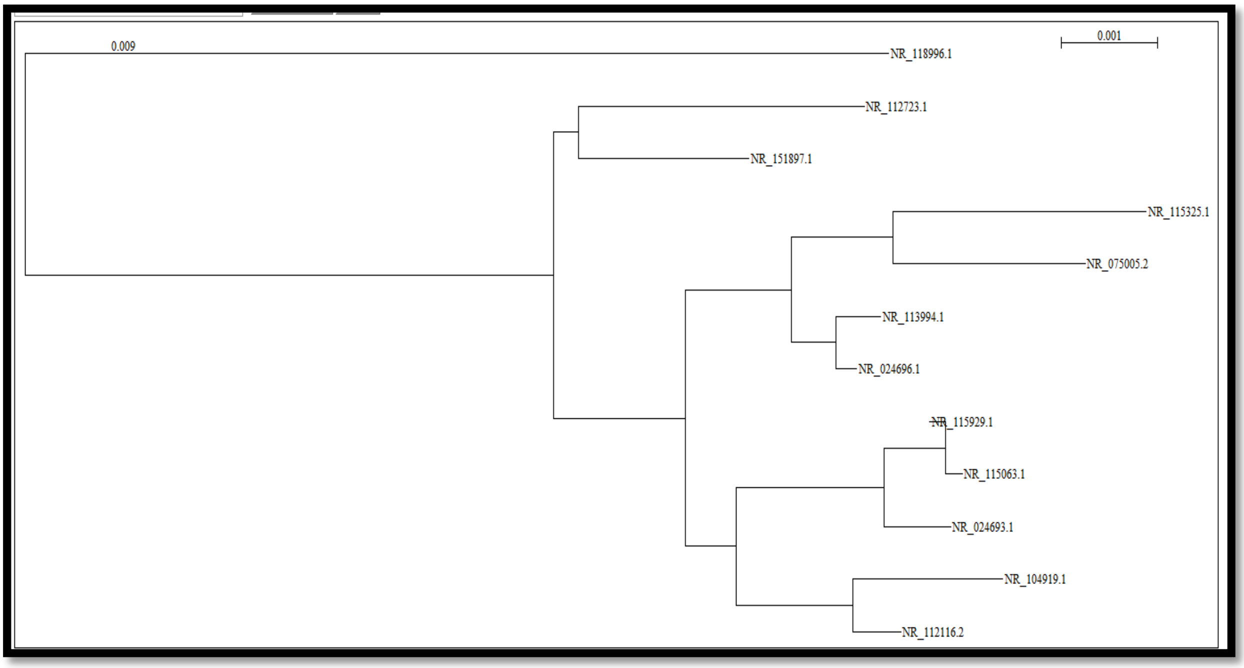
FIGURE 10

PHYLOGENETIC TREE INDICATES THE POSITION OF *Bacillus subtilis* SPECIES. STRAIN



NR_112116.2 - *Bacillus subtilis* strain

NR_104919.1- *Bacillus tequilensis* strain

NR_024693.1- *Bacillus mojavensis* strain

NR_115063.1 - *Bacillus halotolerans* strain

NR_151897.1 - *Bacillus nakamurai* strain

NR_115929.1- *Bacillus axarquiensis* strain

NR_024696.1 -*Bacillus vallismortis* strain

NR_112723.1 -*Bacillus atrophaeus* strain

NR_113994.1 -*Bacillus vallismortis* strain

NR_115325.1 -*Bacillus nematocida* strain

NR_075005.2- *Bacillus velezensis* strain

FIGURE 11

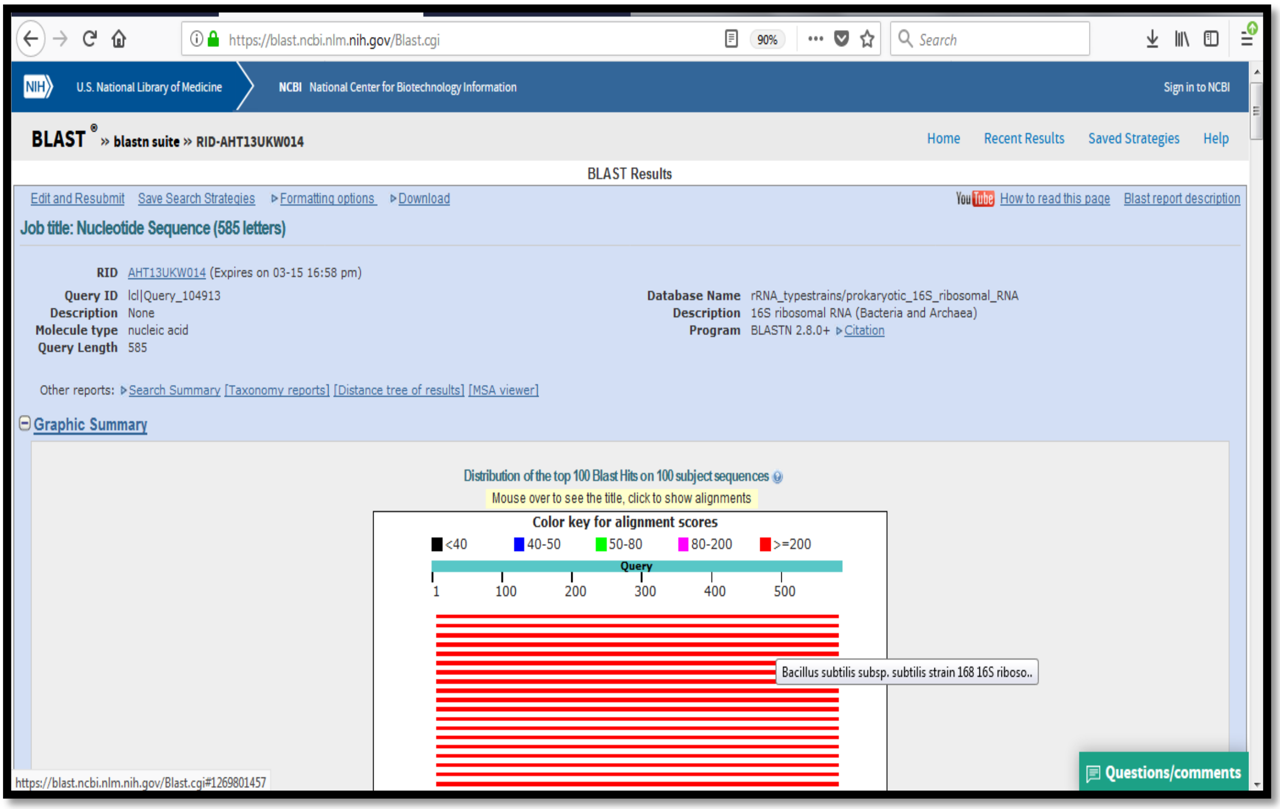
ANALYSIS OF 16S rRNA GENE SEQUENCES

PRIMER 2(*Bacillus nakamurai*)

GGCTAAGATGAGTGCTAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTA
ACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGG
AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC
GCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGT
CCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCC
AGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGT
GGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTA
CAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAA
TCTGTTCTCAGTTCGGATCGCAGTCTGGAACTCGACTGCGTGAAGCTGGAATC
CCTAGTAATCGCGGATCAGCATGCCCCGGTGAAAACGTTCCCGGGCCTTGGTC
CC

FIGURE 12

GRAPHICAL REPRESENTATION OF SEARCHES FOR SEQUENCES SIMILARITY WAS PERFORMED USING BLAST



Job title: Nucleotide Sequence (585 letters)

RID AHT13UKW014 (Expires on 03-15 16:58 pm)

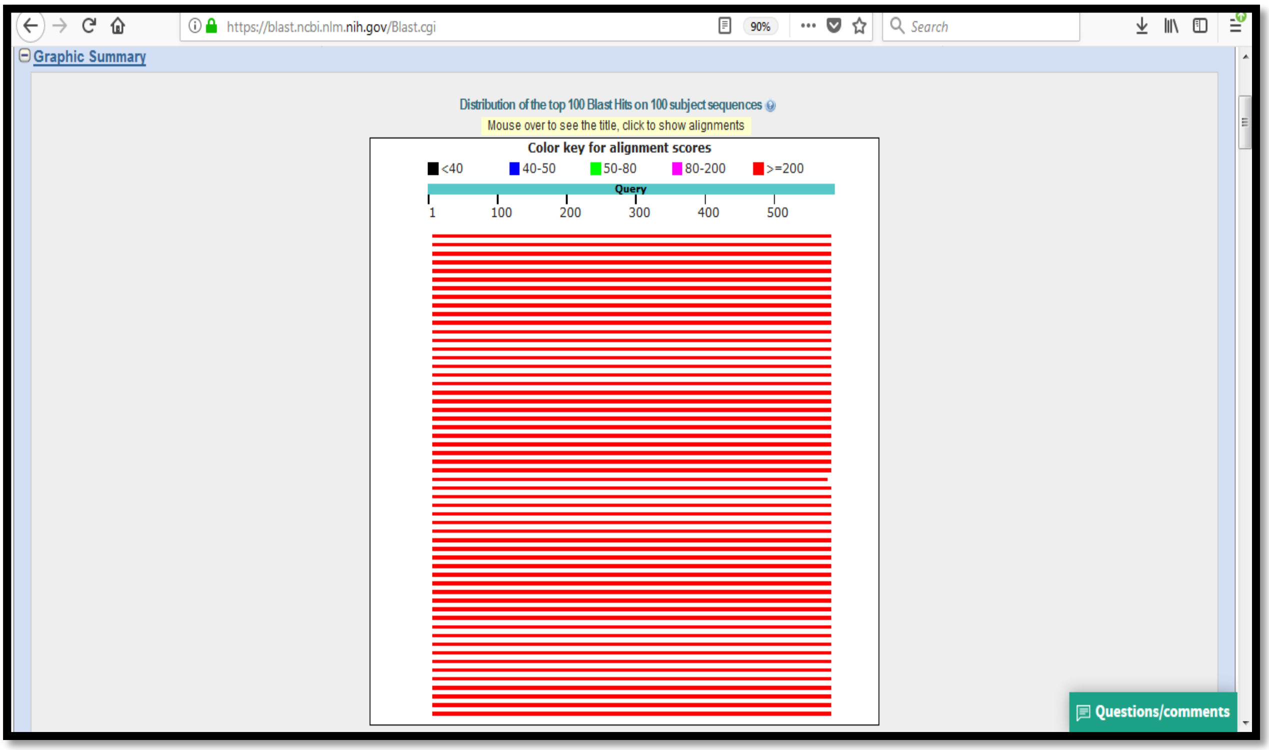
Query ID lcl|Query_104913
Description None
Molecule type nucleic acid
Query Length 585

Database Name rRNA_typestrains/prokaryotic_16S_ribosomal_RNA
Description 16S ribosomal RNA (Bacteria and Archaea)
Program BLASTN 2.8.0+ Citation

Distribution of the top 100 Blast Hits on 100 subject sequences

Mouse over to see the title, click to show alignments

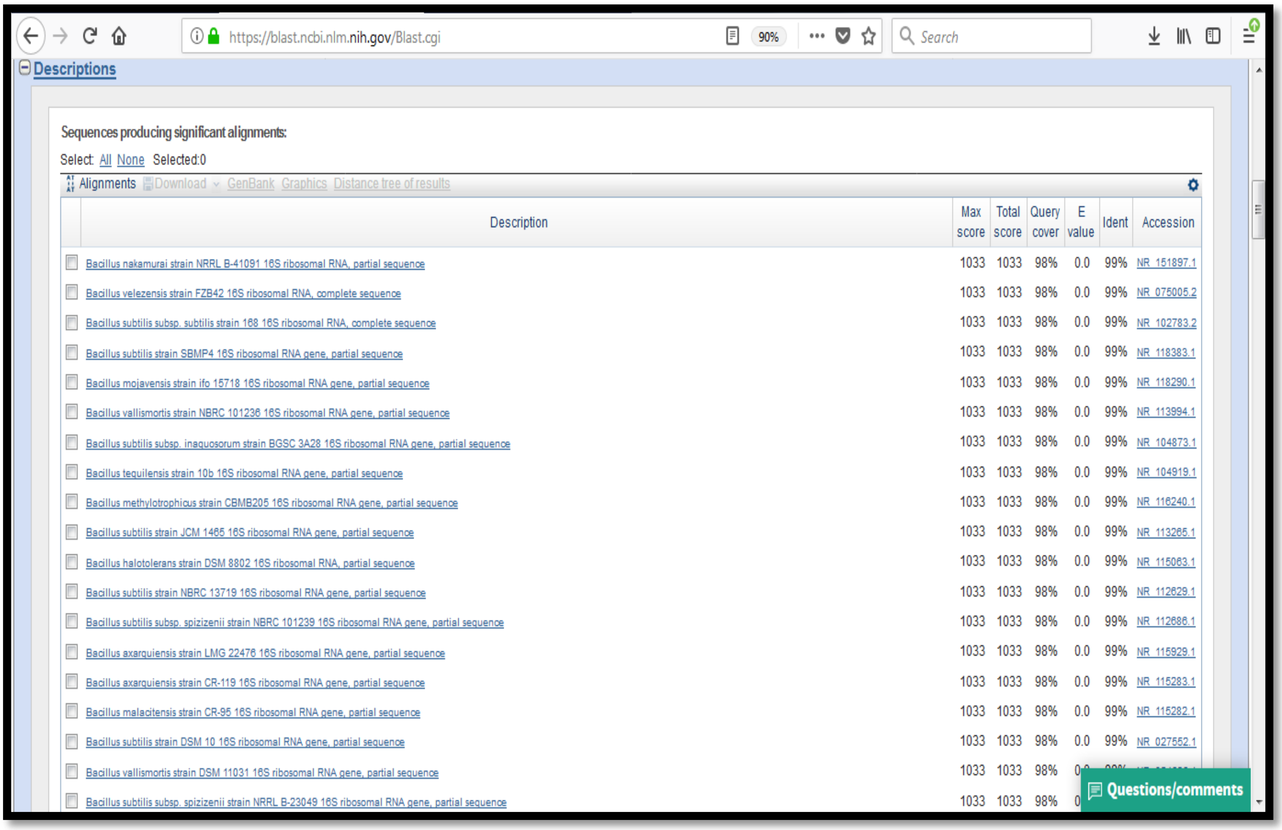
Bacillus subtilis subsp. subtilis strain 168 16S riboso..



Distribution of the top 100 Blast Hits on 100 subject sequences

Mouse over to see the title, click to show alignments

Description



Descriptions

Sequences producing significant alignments:

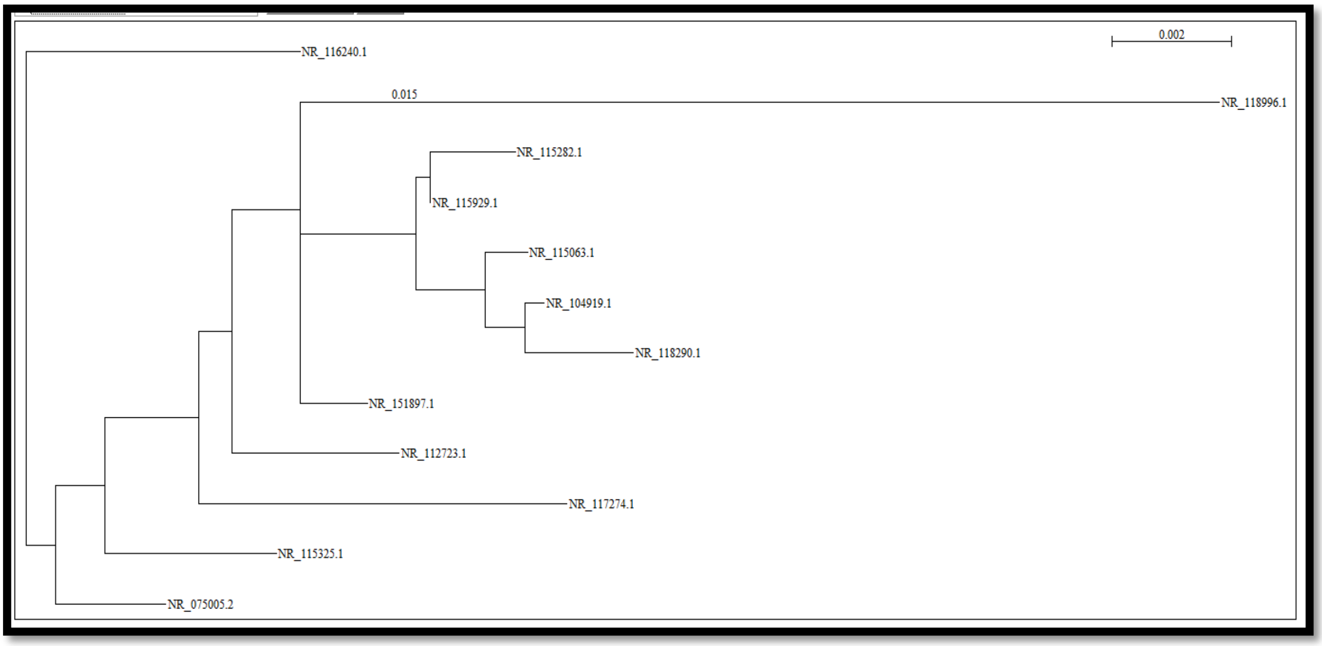
Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Bacillus nakamurai strain NRRL B-41091 16S ribosomal RNA, partial sequence	1033	1033	98%	0.0	99%	NR_151897.1
☐	Bacillus velezensis strain FZB42 16S ribosomal RNA, complete sequence	1033	1033	98%	0.0	99%	NR_075005.2
☐	Bacillus subtilis subsp. subtilis strain 168 16S ribosomal RNA, complete sequence	1033	1033	98%	0.0	99%	NR_102783.2
☐	Bacillus subtilis strain SBMP4 16S ribosomal RNA gene, partial sequence	1033	1033	98%	0.0	99%	NR_118383.1
☐	Bacillus mojavensis strain ifo 15718 16S ribosomal RNA gene, partial sequence	1033	1033	98%	0.0	99%	NR_118290.1
☐	Bacillus vallismortis strain NBRC 101236 16S ribosomal RNA gene, partial sequence	1033	1033	98%	0.0	99%	NR_113994.1
☐	Bacillus subtilis subsp. inaquosorum strain BGSC 3A28 16S ribosomal RNA gene, partial sequence	1033	1033	98%	0.0	99%	NR_104873.1
☐	Bacillus tequilensis strain 10b 16S ribosomal RNA gene, partial sequence	1033	1033	98%	0.0	99%	NR_104919.1
☐	Bacillus methylotrophicus strain CBMB205 16S ribosomal RNA gene, partial sequence	1033	1033	98%	0.0	99%	NR_116240.1
☐	Bacillus subtilis strain JCM 1465 16S ribosomal RNA gene, partial sequence	1033	1033	98%	0.0	99%	NR_113265.1
☐	Bacillus halotolerans strain DSM 8802 16S ribosomal RNA, partial sequence	1033	1033	98%	0.0	99%	NR_115063.1
☐	Bacillus subtilis strain NBRC 13719 16S ribosomal RNA gene, partial sequence	1033	1033	98%	0.0	99%	NR_112629.1
☐	Bacillus subtilis subsp. spizizenii strain NBRC 101239 16S ribosomal RNA gene, partial sequence	1033	1033	98%	0.0	99%	NR_112686.1
☐	Bacillus axarquiensis strain LMG 22476 16S ribosomal RNA gene, partial sequence	1033	1033	98%	0.0	99%	NR_115929.1
☐	Bacillus axarquiensis strain CR-119 16S ribosomal RNA gene, partial sequence	1033	1033	98%	0.0	99%	NR_115283.1
☐	Bacillus malacitensis strain CR-95 16S ribosomal RNA gene, partial sequence	1033	1033	98%	0.0	99%	NR_115282.1
☐	Bacillus subtilis strain DSM 10 16S ribosomal RNA gene, partial sequence	1033	1033	98%	0.0	99%	NR_027552.1
☐	Bacillus vallismortis strain DSM 11031 16S ribosomal RNA gene, partial sequence	1033	1033	98%	0		
☐	Bacillus subtilis subsp. spizizenii strain NRRL B-23049 16S ribosomal RNA gene, partial sequence	1033	1033	98%	0		

FIGURE 13

PHYLOGENETIC TREE INDICATES THE POSITION OF *BACILLUS NAKAMURAI* SPECIES. STRAIN



NR_151897.1- *Bacillus nakamurai* strain

NR_075005.2 - *Bacillus velezensis* strain

NR_118290.1 - *Bacillus mojavensis* strain

NR_104919.1 - *Bacillus tequilensis* strain

NR_116240.1 - *Bacillus methylotrophicus* strain

NR_115063.1 - *Bacillus halotolerans* strain

NR_115929.1 - *Bacillus axarquiensis* strain

NR_115282.1 - *Bacillus malacitensis* strain

NR_115325.1 - *Bacillus nematocida* strain

NR_117274.1 - *Bacillus siamensis* strain

NR_112723.1 - *Bacillus atrophaeus* strain

NR_118996.1 - *Bacillus licheniformis* strain

4.4 Screening of the isolates for the protease production

Bacteria were grown on skimmed milk agar and incubated for 24 hours at 37℃. Clear zone around the colony indicates the protease activity.

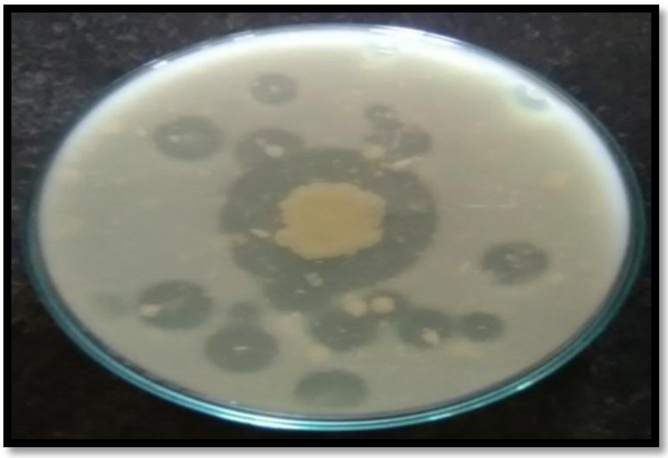
Isolation of protease producing bacteria was first carried out using nutrient agar media and further screened for protease production on skim milk agar plates. Formation of clear zones around the colonies was considered as indication of protease production and which is shown in plate 5.

Das *et al.* (2010) reported by showing the selected colonies streaked on skim milk agar plates. The plates were subjected to incubation for a period of 24 hours at 37 ° C. The plates which showed clear zone around the streaked area of test organism were selected as protease producing strain.

Similar studies have been reported by Josephine *et al.* (2012). The organism producing protease forms a clear zone around the colony as the protein near the colony is utilized. Depending upon the zone of clearance, the strain was selected for the further experimental studies.

PLATE 5

SCREENING OF PROTEASE PRODUCING BACTERIAL ISOLATES ON SKIMMED MILK AGAR



4.4 Activity of protease in the isolated bacterial strains

The isolates were cultured in production medium and the protease and activities were assayed. In quantitative analysis of enzyme, by protease assay, *Bacillus species*, showed maximum enzyme activity after 96 hours of incubation.

4.5. Purification of ammonium sulphate precipitated protease enzyme

Table 3 presents the total protein activity, specific activity, purification fold and recovery percentage of ammonium sulphate precipitated protease from vegetable waste compost soil sample. Figure 14 represent the protease activity and the specific activity of the same samples.

Total activity

It can be observed from Table 3 the protease activity of all the ammonium sulphate precipitated samples.

The observation that the 50% (34.5Units/ ml) ammonium sulphate precipitated protease from compost soil sample had higher total activity. Followed by 70% (33.18

Units/ml) ammonium sulphate precipitated protease from the sample and the lower total activity observed in 20% (23.14 Units/ml) ammonium sulphate precipitated sample.

Total protein

The total protein of the ammonium sulphate precipitated protease from vegetable waste compost soil. It was observed that the 70% (34.3 mg/ml) ammonium sulphate precipitated fraction had higher protein content followed by 40% (33.05 mg/ml). The lowest protein content was observed in 30% (13.68 mg/ml) of ammonium sulphate precipitated fraction.

Specific activity

The 50% of ammonium sulphate precipitated samples exhibited the highest specific activity (2.4 Units/mg) followed by 30% (2.1 Units/mg) ammonium sulphate precipitated fraction. The lower specific activity observed in 20% (0.87 Units/mg) of ammonium sulphate precipitated fraction.

Purification fold

The 50% of ammonium sulphate precipitated samples recorded the highest purification fold (0.8) followed by 30% (0.7) ammonium sulphate precipitated fraction and the lower purification fold in 20% (0.29) of ammonium sulphate precipitated fraction.

Recovery percentage

From Table 3 it was clear that the recovery percentage was maximum for the 50% ammonium sulphate precipitated samples as compared to the other samples. That the recovery percentage of (0.97) the 50% ammonium sulphate precipitated fraction from vegetable waste compost soil sample was lesser than the recovery percentage of the crude and followed by of 70% (0.93) and lowest recovery percentage in 20% (0.65) of ammonium sulphate precipitated fraction.

Nadeemullah *et al*. (2013) reported that the protease was purified by using ammonium sulphate fractionation. The fractionation carried out from 30% to 80%

saturation of ammonium sulphate. In ammonium sulphate precipitation, maximum enzyme purification was achieved at 60%.

Similar studies have been reported by Zhao *et al.* (2011). Where the activity of the 40-50% (31-35 U/ ml) of ammonium sulphate precipitated was higher than other ammonium sulphate precipitated samples. The protease activity of the 40-50% ammonium sulphate precipitated compost soil sample was lesser than the crude.

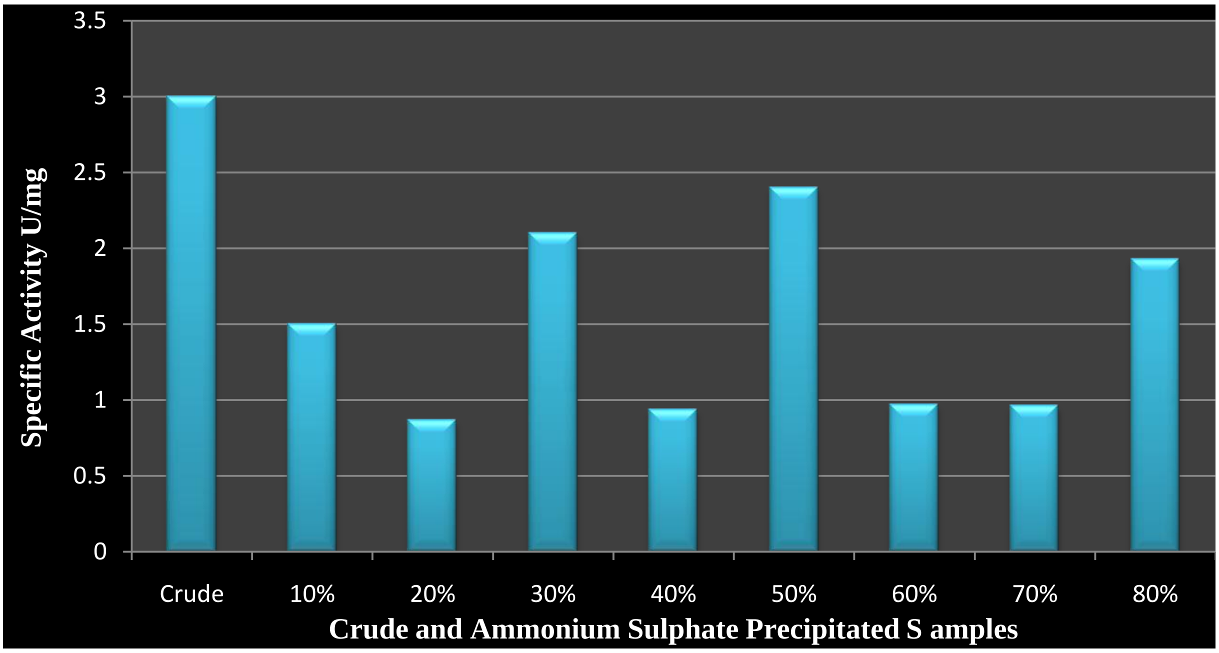
TABLE 3

Protease activities of crude and ammonium sulphate precipitated sample

S.NO	**Samples**	**Total activity (Units/ml)**	**Total protein (mg/ml)**	**Specific activity (Units/mg)**	**Recovery percentage**	**Purification fold**
1	Crude	35.46	11.8	3.0	100	1
2	Ammonium sulphate precipitated 10%	24.81	16.9	1.5	0.7	0.5
3	20%	23.14	26.59	0.87	0.65	0.29
4	30%	28.8	13.68	2.1	0.8	0.7
5	40%	31.18	33.05	0.94	0.87	0.31
6	50%	34.5	14.3	2.4	0.97	0.8
7	60%	29.84	30.47	0.97	0.84	0.32
8	70%	33.18	34.3	0.96	0.93	0.32
9	80%	31.5	16.26	1.93	0.88	0.64

FIGURE 14

EFFECT OF PROTEASE SPECIFIC ACTIVITY IN THE AMMONIUM SULPHATE PRECIPITATED EXTRACT



4.6 Effect of pH

The enzyme activity was tested using two different buffers: phosphate buffer (pH) and acetate buffers (pH) and the results were shown in figure 15.

Total activity

The protease activity of all the ammonium sulphate precipitated sample is shown in Table 4.

From table 4 it was seen that ammonium sulphate precipitated protease from compost soil sample had higher total activity at pH 10 (15.56Units/ml) followed by pH 5 (11.3Units/ml). Then the lower total activity observed in pH 7 (8.6 Units/ml) ammonium sulphate precipitated fraction.

Total protein

The total protein of the ammonium sulphate precipitated protease from vegetable waste compost soil was observed the pH 10(1.35mg/ml) ammonium sulphate precipitated had higher protein content followed by pH 6 (1.03mg/ml). Then the lowest protein content observed in pH 7 (0.47mg/ml) of ammonium sulphate fraction of sample.

Specific activity

The ammonium sulphate precipitated sample recorded the maximum specific activity at pH 7 (18.3 Units/mg) followed by pH 8 (16.3Units/mg). Lower specific activity was observed in pH 6 (9.4Units/mg) of ammonium sulphate precipitated fraction.

Purification fold

The pH 7 of ammonium sulphate precipitated samples recorded the highest purification fold of (6.1) followed by pH 5 (5.2) ammonium sulphate precipitated fraction and the lower purification fold in pH 6 (3.1) of ammonium sulphate precipitated fraction.

Recovery percentage

From Table 4 it was clear that the recovery percentage was maximum for the pH 10 (0.43) ammonium sulphate precipitated samples and followed by pH 5 (0.32). Then the lowest recovery percentage in pH 7 and 9 (0.24) of ammonium sulphate precipitated protease sample. The obtained results were presented in (Figure 16).

TABLE 4

Effect of pH

S.NO	Samples	Total activity (Units/ml)	Total protein (mg/ml)	Specific activity (Units/mg)	Recovery percentage	Purification fold
1	5	11.3	0.72	15.7	0.32	5.2
2	6	9.67	1.03	9.4	0.27	3.1
3	7	8.6	0.47	18.3	0.24	6.1

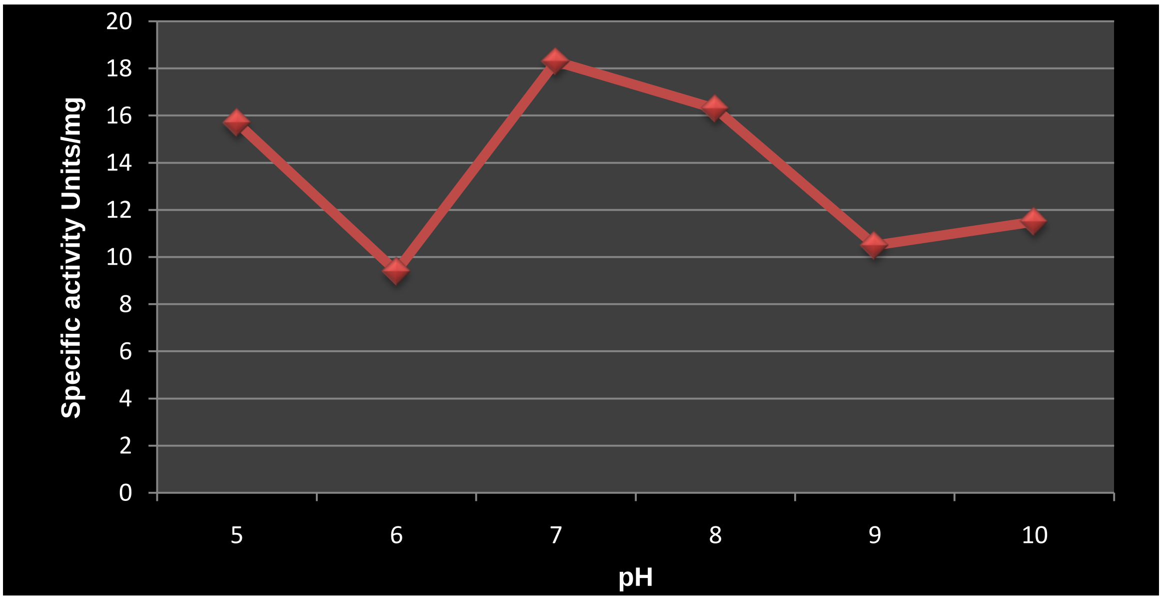
4	8	9.6	0.59	16.3	0.27	3.2
5	9	8.86	0.84	10.5	0.24	3.5
6	10	15.56	1.35	11.5	0.43	3.8

Similar studies have been reported by Qadar *et al.* (2009). The effect of some physical factors, such as pH and temperature, on the production of crude enzyme was investigated. The maximum enzymes production was obtained when pH of the culture media was maintained at 6.0 while in case *Bacillus subtilis*, the maximum production was reported at pH of 6.5.

Sinha *et al*., (2013) reported by the bacterial isolate at pH 6, 7, 8, 9, 10 were observed as 9.67(U/ml), 8.6 (U/ml), 9.6 (U/ml), 8.86 (U/ml), 15.56 (U/ml) respectively. The optimum pH for maximum production of protease was pH 10.

FIGURE 15

EFFECT OF pH ON SPECIFIC ACTIVITY IN THE PROTEASE ENZYME



4.7 Effect of Temperature on protease from the isolated bacterial strains

The present result of protease activity was studied at different temperature ranging from 20-60ºC and which shown in figure 16.

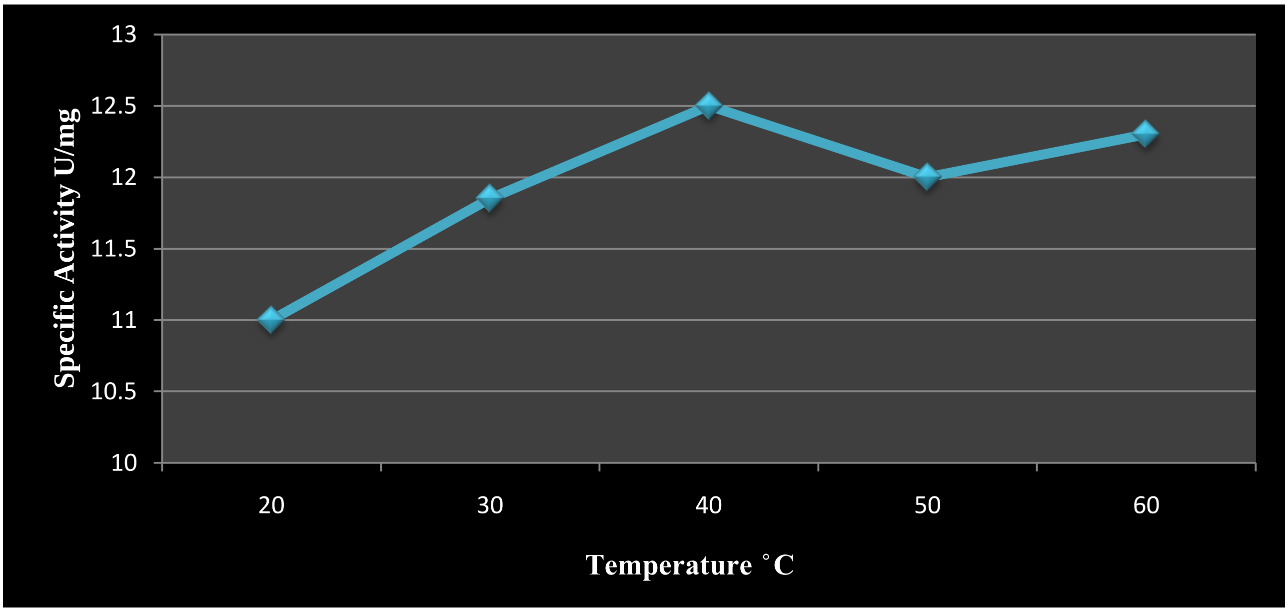
TABLE 5

Effect of Temperature on protease from the isolated bacterial strains

S.NO	Samples	Total activity (Units/ml)	Total protein (mg/ml)	Specific activity (Units/mg)	Recovery percentage	Purification fold
1	Temperature 20℃	4.84	0.44	11	0.13	3.6
2	30℃	6.4	0.54	11.85	0.18	3.95
3	40℃	10.87	0.87	12.5	0.3	4.2
4	50℃	9.26	0.77	12	0.26	4.0
5	60℃	7.85	0.64	12.3	0.2	4.1

FIGURE 16

EFFECT OF TEMPERATURE ON SPECIFIC ACTIVITY IN THE PROTEASE ENZYME



Total activity

The protease activity of all the ammonium sulphate precipitated samples is shown in Table 5.

From table 5 it was seen that ammonium sulphate precipitated protease from compost soil sample had higher total activity at 40℃ (10.87Units/ml). Then the lower total activity observed in temperature 20 ° C (4.84 Units/ml) ammonium sulphate precipitated fraction.

Total protein

The total protein of the ammonium sulphate precipitated protease from vegetable waste compost soil was observed and the temperature 40℃ (0.87mg/ml) ammonium sulphate precipitated had higher protein content. Then the lowest protein content observed in temperature 20℃ (0.44mg/ml) of ammonium sulphate fraction.

Specific activity

The ammonium sulphate precipitated samples recorded the maximum specific activity at temperature 40℃ (12.5Units/mg). Lower specific activity observed in temperature 60℃ (12.3Units/mg) of ammonium sulphate precipitated sample.

Purification fold

The temperature 40 ° C of ammonium sulphate precipitated samples recorded the highest purification fold of (4.2) ammonium sulphate precipitated samples. Then the lower purification fold in temperature 20℃ (3.6) of ammonium sulphate precipitated fraction.

Recovery percentage

From Table V it is clear that the recovery percentage was maximum for the temperature 40℃ (0.3) ammonium sulphate precipitated samples. Lowest recovery percentage in temperature 20℃ (0.13) of ammonium sulphate precipitated fraction.

Similar studies have been reported by Safey *et al*., (2004) the effect of temperature on the activity of the purified protease is shown in figure (17 and 18). The optimum incubation temperature for purified protease enzyme was 40ºC. The purified protease activity reached upto 12.5 units/mg in specific activity. While the temperature below or above 40 ºC exhibited lower activities of protease.

5. SUMMARY AND CONCLUSION

Composting is a microbial decomposition process in which easily degradable and putrescent organic waste is turned into stable material compost and current method for understanding the communities in composting ecosystem composting is a controlled decay of organic matter in a warm moist environment by action of bacteria, fungi and other organisms. Hence it is programmed molecular identification of thermophiles and parameter analysis of compost.

Thermophilic bacteria has been isolated from a number of marine and terrestrial geothermally-heated habitats including hot spring hot water heaters, thermally polluted river, soil, hydrothermal vent systems sediment from volcanic islands and deep sea hydrothermal vents as well as from hot composts . Thermophiles can be categorized into moderate thermophiles (growth optimum, 50–60℃), extreme thermophiles (growth optimum , 60–80℃), and hyperthermophiles (growth optimum, 80–110℃). Thermophiles have been isolated from different ecological zones (e.g., hot springs and deep sea) of the earth.

Enzymes are biological catalysts that allow chemical reactions to occur in living organisms at ambient conditions. One group of the enzymes that has been studied extensively is proteases or proteolytic enzymes due to their wide variety of application in various fields. The application of enzymes in “White Biotechnology” for the synthesis of industrially important chiral compounds is becoming increasingly important for the pharmaceutical industry. Many companies who were traditionally not incorporating biocatalysis in their drug production programmes are now very keen to develop the technology.

The enzyme technology is applied to pharmaceutical research, development and manufacturing and is a growing field. Therapeutic enzymes have been in use for around at least 40 years.

Protease constitutes a large and complex group of enzymes that plays an important nutritional and regulatory role in nature. Proteases are (physiologically) necessary for living organisms; they are ubiquitous and found in a wide diversity of sources. Proteases are presented as one of the three largest groups of industrial enzymes because of their thermo-stability and thermo-activity .These enzymes account for about 60 % of the world enzyme market and approximately 40 % of them are of microbial origin. Microbial proteases are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics preferred for their biotechnological applications. They have a wide range of applications in biodetergents, food, pharmaceutical, Leather, silver recovery and bioremediation processes.

The present study has been focused on the "**Partial purification and characterization of Protease produced by *Bacillus subtilis* and *Bacillus nakamurai* isolated from the vegetable waste compost soil"**

The vegetable waste compost samples were used as a source for bacteria. The bacterial colonies were isolated by culturing them in nutrient agar medium. By morphological observation, biochemical characterization and 16S rRNA gene sequences used to the bacterial isolates were identified. The protease activities were assayed and purification of ammonium sulphate precipitated protease enzyme. The effect of pH and temperature on protease activity was studied and the results are summarised below:

The five bacterial strains isolated from the vegetable waste compost soil, namely, Isolate 1, Isolate 2, Isolate 3, Isolate 4 and Isolate 5 were identified as Bacillus sp. based on the morphological and biochemical characteristics. The isolates were found to be Gram positive, rods. Catalase test was found to be positive.

The 16S rRNA sequences, result were given FASTA format. The obtained sequenced was aligned using BLAST in NCBI database, the bacterial isolate was confirmed as primer1 ***Bacillus subtilis*** and primer2 ***Bacillus nakamurai*** strain (Isolate 5).

Bacteria were grown on skimmed milk agar and incubated for 24 hours at 37℃. Clear zone around the colony indicates the protease activity.

The isolates were cultured in production medium and the protease and activities were assayed. From the results it is identified that the bacterial strain

(Isolate 5) was efficient in protease activity (35.46 U/ml) and the specific activity (3 Units/ mg of protein).

The protease activity of the all ammonium sulphate precipitated compost soil sample. The finding that the higher activity of the sample in 50% ammonium sulphate precipitated enzyme in total activity (31-35 U/ ml), specific activity (2.4 U/mg), purification fold (0.8) and recovery percentage (0.97).

The ammonium sulphate precipitated sample recorded the maximum activity of the sample in total activity at pH 10 (15.56 Units/ml), specific activity at pH 7 (18.3 Units/mg), purification fold at pH 7 (6.1) and recovery percentage at pH 10 (0.43).

The ammonium sulphate precipitated sample recorded the highest activity of the sample in total activity at temperature 40˚C (4.84 Units/ml), specific activity at temperature at 60˚C (12.3 Units/mg), purification fold at temperature 40˚C (4.2) and recovery percentage at temperature 40˚C(0.3).

FUTURE SUGGESTIONS

- Complete purification and characterization of the isolated protease.
- Immobilization of the isolated protease.
- Various application studies can be done using the isolated protease.

6. BIBLOGRAPHY

- Alnahdi, H.S. (2012). Isolation and screening of extracellular proteases produced by new Isolated *Bacillus sp, Journal of Applied Pharmaceutical Science.,* 2 (9), 71-74.
- Aqel, H., Quadan, F.A. and Yousef, T. A. (2012). A novel neutral protease from thermophilic *Bacillus* strain HUTBS62, *Journal of BioScicence Biotechnology.,* 1(2): 117-123.
- Awasthi, M.K., Bundela P.S., Pandey A.K., and Jamaluddin, M. (2012). Monitoring of microbial population and their activities during composting of organic municipal solid wastes at central india, *International Journal of Plant, Animal and Environmental Sciences*., 2 (2): 26-36.
- Azlina, I.N. and Norazila, Y. (2013). Thermostable Alkaline Serine Protease from Thermophilic *Bacillus* Species, *International Research Journal of Biological Sciences.,* 2(2), 29-33.
- Bouzas, T.M., Velázquez, J.B. and Villa, T.G. (2006). Industrial Applications of Hyperthermophilic Enzymes, *Protein & Peptide Letters*., 13, 645-651.
- Christopher, N. and Kumbalwar, M. (2015). Enzymes used in food industry a systematic review, *International Journal of Innovative Research in Science, Engineering and Technology*., 4 (10): 9830-9836.
- Charbonneau, D.M., Mouelhi, F.M., Boissinot, M., Sirois, M. and Beauregard, M. (2012). Identification of Thermophilic Bacterial Strains Producing Thermotolerant Hydrolytic Enzymes from Manure Compost, *Indian Journal of Microbiology*., 52(1):41–47.
- David, M. Charbonneau., and Mouelhi, F.M. (2012). Identification of Thermophilic Bacterial Strains Producing Thermotolerant Hydrolytic Enzymes from Manure Compost, *Indian Journal Microbiol*., 52(1):41–47.
- Deng, A., WU, J., Zhang, Y., Zhang, G. and Wen, T. (2010). Purification and characterization of a surfactant-stable high-alkaline protease from Bacillus sp. B001. *Bioresour. Technol.,* 101: 7100-7116.

- Dheeran, V. (2014). Thermozymes and their industrial application, *International Journal of Engineering Research & Management Technology.*, 1(2): 312-319.
- Dorcas, K., Dathar, V. and Pindi, P.K. (2016). Purification and characterization of protease produced by *bacillus cereus* NC77, *International Journal of Pharma and Bio Sciences*., 7(4): 898 – 903.
- Ebrahimpour, A. and Kariminik, A. (2015). Isolation, characterization and molecular identification of protease producing bacteria from tashkooh mountain located in ahvaz, iran, *International Journal of Life Sciences*., 9 (2): 39 – 42.
- Ghaffari, S., Sepahi, A.A., Razavi, M.R., Malekzadeh, F. and Haydarian, H. (2011). Effectiveness of inoculation with isolated *Anoxybacillus sp.* MGA110 on municipal solid waste composting process, *African Journal of Microbiology Research*., 5(30): 5373-5378.
- Govindarajan, B. and Prabaharan, V. (2015). *Bacillus subtilus* isolation from insecticide (monocrotophos) treated *Eudrilus eugeniae* species vermicompost in laboratory culture, *International Journal of Current Science Research*., 1(1):25-32.
- Hasan, F. Shah, A. A. and Hameed, A. (2006). Industrial application of microbial lipase. *Enzyme and Microbial technology*., 39(20): 235-251.
- Hong, S.T., Chang, G.N. and Ho, K.C (2007). Characterization of Facultative Thermophilic Microbial Community of Composts by ARDRA, *Taiwania*., 52(2):117-126.
- Hema, T.A. and Shiny, M. (2012). Production of Protease Enzyme from *Bacillus Clausii* Sm3, *IOSR Journal of Pharmacy and Biological Sciences (IOSRJPBS)*., 1(4): 37-40.
- Hovik, H. and Panosyan, M. (2017). *Thermophilic Bacilli* Isolated from Armenian Geothermal Springs and their Potential for Production of Hydrolytic Enzymes, *International Journal of Biotechnology and Bioengineering*., 3(7): 239-244.
- Holman, D.B., Hao, X., Topp, E., Yang, H.E. and Alexander, T.W. (2016). Effect of Co-Composting Cattle Manure with Construction and Demolition Waste on the Archaeal, Bacterial, and Fungal Microbiota, and on

Antimicrobial Resistance Determinants, PLOS ONE | DOI:10.1371/journal.pone.0157539., 11(6): 1-21.

- Hussein, Y., and Mohammed, I. (2015). Isolation of protease producing microorganism from food waste, *Journal of Bio.Innov.,* 4(6): 322-346.
- Hulawale, G. and Sawant, R. (2015). Identification and Characterization of Thermostable Protease Producing Organism and Partial Purification of Protease Enzyme, *International Jouranl of Current Microbiology Applied Science*., 2: 210-221
- Ilayaraja. J.J., Dhanarajan. M.S. and Mariappan. V. (2012). Analysis of Compost Parameters and a Molecular Approach on its *Thermophilic Microflora* , *International Journal of Scientific and Research Publications*., 2(8): 1-4.
- Kamran, A., Bibi, Z. and Kamal, M. (2015). Screening and enhanced production of protease from a thermophilic *Bacillus* species, *Pak. J. Biochem. Mol. Biol*., 48(1): 15-17.
- Lele, O.H. and Deshmukh, P.V. (2016). Isolation and characterization of thermophilic *Bacillus sp.*with extracellular enzymatic activities from hot spring of Ganeshpuri, Maharashtra, India, *International Journal of Applied Research.,* 2(5): 427-430.
- Littlechild, J. A. (2015). Archaeal Enzymes and Applications in Industrial Biocatalysts, *Hindawi Publishing Corporation Archaea*., 1(1): 1-11.
- Madigan, M. T. (2015). Microbiology, *In: eLS. John Wiley & Sons, Ltd.,* 4(6): 2-11.
- Mathai, S., Roy, K.R. and Rajendran, N. (2014). Importance of Marine Thermophiles in Biotechnological Applications, *International Journal of Pharmaceutical Sciences Review and Research.,* 27(2): 156-160.
- Mane, P. and Tale, V. (2015). Overview of Microbial Therapeutic Enzymes, *International Journal of Current Microbiology and Applied Sciences*., 4(4): 17-26.
- Mienda, B.S., Yahya, A., Galadima, I.A. and Shamsir, M.S. (2014). An Overview of Microbial Proteases for Industrial Applications, *Research Journal of Pharmaceutical, Biological and Chemical Sciences.,* 5(1): 388-396.

- Mohammad, B.T., Hala, I., Daghistani, A., Jaouani, A., Latif, S.A. and Kennes, C. (2017). Isolation and Characterization of Thermophilic Bacteria from Jordanian Hot Springs: *Bacillus licheniformis* and Thermomonas hydrothermalis Isolates as Potential Producers of Thermostable Enzymes, *International Journal of Microbiology*., 1(1): 1-13.
- Muthulakshmi, C., Gomathi, D., Kumar, D.G., Ravikumar, G., Kalaiselvi, M. and Uma, C. (2011). Production, Purification and Characterization of Protease by Aspergillus flavus under Solid State Fermentation, *Jordan Journal of Biological Sciences.*, 4(3): 137-148.
- Nayab, Qasim, M., Nawaz1, A., Rahman, H., Tabassum, A., Khan, T. and Mohammad, N. (2015). Isolation, Screening and Identification of Protease Producin Bacteria from Soil of Karak, Khyber Paktunkhwa, Pakistan, *Journal of Bio-Molecular Sciences (JBMS)*., 3(1): 18-24.
- Nascimento, W.C.A.D. and Martins, M.L.L. (2004). Production and Properties of An Extracellular Protease From Thermophilic *Bacillus* Sp, *Brazilian Journal of Microbiolog.,* 35:91-96.
- Nigam, P.S. (2013). Microbial Enzymes with Special Characteristics for Biotechnological Applications, *Biomolecules*., 3(3): 597- 611.
- Pant, G., Prakash, A., Pavani, J.A.V., Bera, S., Deviram, G.V.N.S., Kumar, A., Panchpuri, M. and Prasuna, R.G. (2014). Production, Optimization and Partial Purification of Protease From *Bacillus Subtilis*, *Journal of Taibah University for Science.*, 9(3): 50-55.
- Panda, M.K., Sahu, M.K. and Tayung, k. (2013). Isolation and characterization of a thermophilic *Bacillus* sp. With protease activity isolated from hot spring of Tarabalo, Odisha, India, *Iran Journal of Microbiol*., 5(2): 159-165.
- Pan, I., Dam, B. and Sen, S.K. (2011). Composting of common organic wastes using microbial inoculants, *Biotech*., 3(2): 127-134.
- Radianingtyas, H., Phillip, C. and Wright. (2003). Alcohol dehydrogenases from thermophilic and hyperthermophilic archaea and bacteria, *FEMS Microbiology Reviews*., 27(2): 593-616.

- Sathiya, G. (2013). Production of protease from *Bacillus subtilis* and its application in leather making process, *Int. J. Res. Biotechnol. Biochem*., 3(1):7-10.
- Sawant, R. and Nagendran, S. (2014). Protease: An enzyme with multiple industrial applications, *World Journal of Pharmacy and Pharmaceutical Sciences*., 3(6): 568-576.
- Sattley, W.M. and Madigan, T.M. (2015). Microbiology, *John Wiley & Sons, Ltd*., 1-9.
- Sarkar, S., Pala, S. And Chandab, S. (2016). Optimization of a Vegetable Waste Composting Process with a Significant Thermophilic Phase, *Procedia Environmental Sciences*., 35:435- 440.
- Shimaya, C. and Hashimoto, T. (2014). Isolation and characterization of novel thermophilic nitrifying Bacillus sp. from compost, *Soil Science and Plant Nutrition*., 57:150-156.
- Singh, R., Mittal, A., Kumar, M. and Mehta, P.K. (2016). Microbial Proteases in Commercial Applications, *Journal of Pharmaceutical, Chemical and Biological Sciences.*, 4(3): 365-374.
- Soares, L., Tavora, Z., Barcelos, R.P. and Baroni, S. (2007). Microorganism-Produced Enzymes in the Food Industry, *Intech Open Science/ Open Minds*., 5: 84-94.
- Sundus, H., Mukhtar, H. and Nauvaz, A. (2016). Industrial Applications and Production Sources of Serine Alkaline Proteases, *Journal of Bacteriology & Mycology: Open Access*., 3(1): 1-4.
- Suzuki, Y., Tsujimoto, Y., Matsui, H. and Watanabe, K. (2006). Decomposition of extremely hard-to-degrade animal proteins by thermophilic bacteria, *Journal of Bioscience, Bioengineering.,* 102: 73-81.
- Synowiecki, J. (2010). Some applications of thermophiles and their enzymes for protein processing, *African Journal of Biotechnology*., 9(42): 7020-7025.
- Tennalli, G., Udapudi, B. and Naik, P. (2012). Isolation of Proteolytic Bacteria and Characterization of their Proteolytic Activity, *International Journal of Advances in Engineering, Science and Technology*., 2(3): 185-192.
- Verma, A., Gupta, M. and Shirkot, P. (2014). Isolation and Characterization of Thermophilic Bacteria in Natural Hot Water Springs of Himachal Pradesh

(India), *The Bioscan An International Quarterly Journal of Life Science*., 9(3): 947-952.

- Wang, L., Wang, L., Wang, D. and Li, J. (2014). Isolation and Application of Thermophilic and Psychrophilic Microorganisms in the Composting Process, *Waste Biomass Valor.,* 5: 433–440.
- Woese, C.R., Kandler, O. and Wheelis, M.L. (1990). Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya, *Proceedings of the National Academy of Sciences USA.*, 87: 4576–4579.
- Zakia A.M. Ahmed, Z.M. Sedik, M.D. Alharery, M.A. Khalaf, Shimaa A. Nasr and Abdelrahman, H.A. (2012). Microbial Ecology of Composting Dead Poultry and Their Wastes, *Global Veterinaria*., 9 (6): 683-690.

APPENDIX I

GRAM STAINING

(David *et al.,* 2012)

AIM

To identify and differentiate the gram positive and gram negative organism using Gram's staining.

PRINCIPLE

The gram stain procedure was originally developed by the Danish physician Hans Christian Gram to differentiate *Pneumococci* from *Klebsiellapneumoniae*. In brief the procedure involves the application of a solution of iodine (potassium iodide) to cells previously stained with crystal violet or gentian violet. The procedure produces "purple coloured iodine-dye complexes" in the cytoplasm of the bacteria. The cells that are previously stained with crystal violet and iodine are next treated with a decolorizing agent such as 95% ethanol or a mixture of acetone and alcohol. The difference between gram positive and gram negative bacteria is in the permeability of the cell wall to these "purple coloured iodine dye complexes" when treated with the decolourizing solvent. While gram positive bacteria retain purple iodine – dye complexes after the treatment with the decolourizing agent, gram negative bacteria do not retain complexes when decolourized. To visualize decolourized gram negative bacteria, a red colored stain such as safranine is used after decolourization.

REAGENTS

1. Crystal violet

Solution-A

Crystal violet - 2g

Ethyl alcohol - 20ml

Dissolve the dye completely.

Solution-B

Ammonium oxalate - 0.8g

Distilled water - 80ml

Mix solution A and B

2. Gram’s Iodine
    Iodine - 1.0g
    Potassium Iodide - 2.0g
    Distilled water - 300ml
3. 95% Ethanol
4. Safranine
    Safranine (25% in
    95% Ethyl alcohol) - 10ml
    Distilled water -100ml

PROCEDURE

1. A smear was prepared following the instruction given for simple staining.
2. Crystal violet was added so that it covers the whole smear. It was allowed to act for one minute.
3. Next it was rinsed with tap water.
4. A few drops of gram’s iodine was added to cover the smear and allowed to react for 30 seconds to 1 minute.
5. Again it was rinsed with tap water.
6. The stain was decolorized with ethyl alcohol (95%) by adding the reagent drop-wise to remove the excess crystal violet from the smear.
7. It was then washed with tap water and counter stained with safranine for 45 seconds and washed again with water.
8. The slide was blotted dry and examined under oil immersion objective.

APPENDIX II

INDOLE TEST

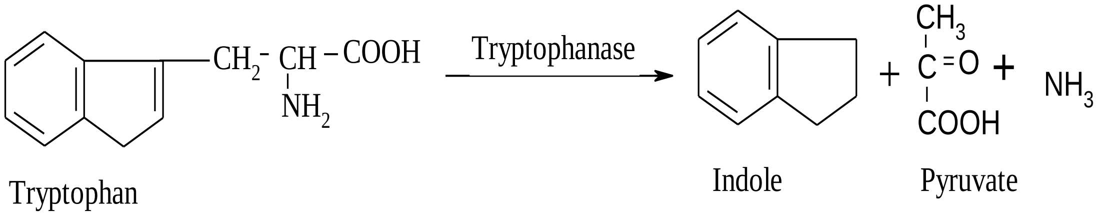
(Govindarajan *et al*., 2015)

AIM

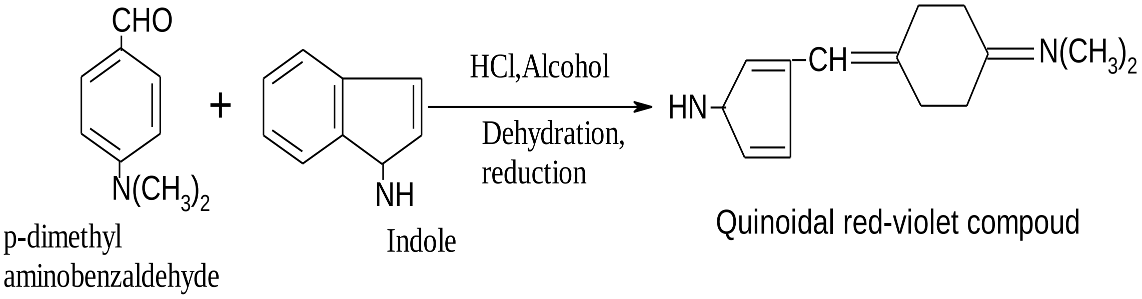
To determine the ability of the organism to produce indole from tryptophan.

PRINCIPLE

Tryptophan is oxidized with the tryptophanase resulting in the formation of indole, pyruvic acid and ammonia. The indole test is performed by inoculating a bacterium into peptone broth and the indole produced during this reaction is detected by adding Kovac's reagent (p-dimethyl aminobenzaldehyde), which produces a cherry red layer.



Indole's reaction with Kovac's reagent



REAGENTS

1. 1% Peptone broth

    Peptone – 10g/l

    NaCl – 5g/l

2.Kovac's/ Ehrlisch's reagent

5g of p-Dimethyl aminobenzaldehyde was dissolved in 75ml of amyl alcohol. 25ml of concentrated HCl was added to it and mixed.

PROCEDURE

1. Three tubes of peptone broth were prepared and sterilized in an autoclave at 15 lbs for 15 minutes.
2. To one of the tubes of peptone broth, *E.coli* was inoculated, the second tube was inoculated with *Klebsiellapneumoniae* and the third tube was kept as an uninoculated control.

3. The tubes were incubated at 37°C for 48 hours.
4. After 48 hours incubation, 1ml of Kovac’s reagent was added to each tube including the control.
5. The tubes were shaken gently with intervals of about 10 to 15 minutes.
6. The tubes were allowed to stand to permit the reagent to raise to the top.

APPENDIX III

METHYL RED TEST

AIM

To detect the ability of an organism to oxidised glucose with the production and stabilization of high concentration of acid as an end product.

PRINCIPLE

Bacteria belonging to Enterobacteriaceae ferment glucose to produce acetic acid, lactic acid, succinic acid, formic acid, ethanol, CO_2 and hydrogen. Due to the abundant acid production, the pH of the medium drops from to 10 to 4.5, which can be detected by using the methyl red as the pH indicator.

$$\text{Glucose}+H_2O \longrightarrow \text{Lactate}+CO_2+H_2\text{(pH 4)+Methyl red indicator(red colour)}$$

REAGENTS

1. MR-VP broth

    Peptone – 5g/l

    Dextrose – 5g/l

    Di potassium hydrogen phosphate – 5g/l

2. Methyl red reagent

    Methyl red – 100mg

    Ethanol (95%) – 300ml

    Distilled water – 200ml

    Transferred methyl red to ethanol, dissolved well, added water and then filtered.

PROCEDURE

1. To three tubes marked as T_1 (test organism-1), T_2 (test organism-2) and Tc (control-uninoculated), 5ml of MR-VP broth was added and sterilized.
2. T_1 was inoculated with *E.coli*, T_2 was inoculated with *Klebsiellapneumoniae* and Tc was kept as uninoculated tube (control). The tubes were incubated at 37°C for 24 hours.
3. After incubation, 5 drops of methyl red indicator was added to each tube.

APPENDIX IV

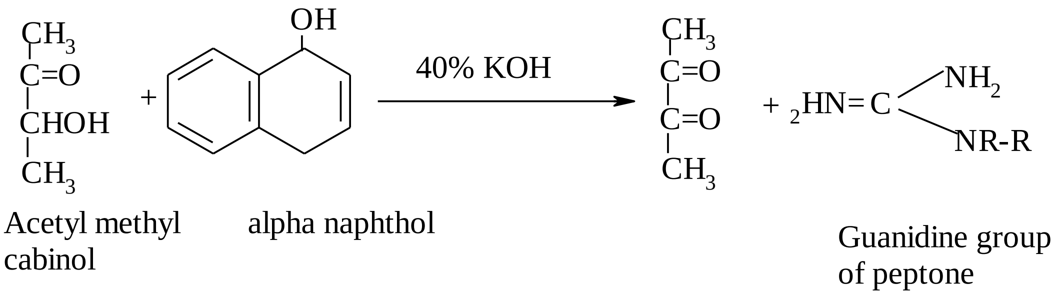
VOGES PROSKAUER TEST

AIM

To detect the ability of an organism to ferment carbohydrate with the production of non acidic or neutral end product.

PRINCIPLE

Glucose undergoes fermentation by a group of bacteria called Enterobacteriaceae to produce a non-acidic or neutral end product called acetyl methyl carbinol ($CH_3\,CO\,CHOH.CH_3$) or its reduction product 2, 3-butylene glycol. Acetyl methyl carbinol undergoes oxidation on reaction with the guanidine group present in peptone in presence of a catalyst α-naphthol and 40% KOH to form a diacetyl compound.



REAGENTS

1. MR-VP broth

Peptone – 5g/l

Dextrose – 5g/l

Di potassium hydrogen phosphate – 5g/l

2. Barritt's reagent

Solution A

α-naphthol – 5g

Ethanol (absolute) – 95ml

Dissolved α-naphthol in ethanol with constant stirring.

Solution B

KOH – 40g

Creatine – 0.3g

Distilled water – 100ml

Dissolved KOH in 75ml of distilled water and then creatine was added to the solution and made up to 100ml with distilled water.

PROCEDURE

1. To three tubes marked as T_1 (test organism-1), T_2 (test organism-2) and Tc (control-uninoculated), 5 ml of MR-VP broth was added and sterilized.
2. T_1 was inoculated with *Klebsiellapneumoniae,* T_2 was inoculated with *E.coli* and Tc was kept as uninoculated tube (control). The tubes were incubated at 37°C for 48 hours.
3. After incubation, 12 drops of α-naphthol reagent and 2-3 drops of 40% KOH were added to all the tubes.
4. The tubes were shaken gently for 30 seconds and exposed to oxygen.
5. The tubes were kept aside for 15-30 minutes and then observed.

APPENDIX V

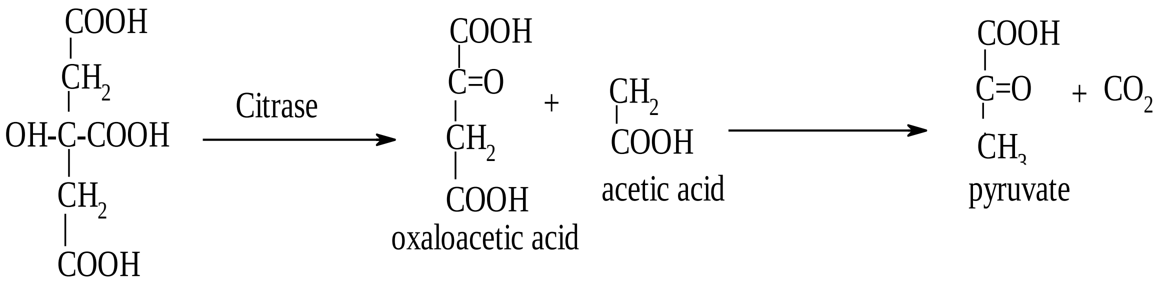
CITRATE UTILISATION TEST

AIM

To determine the ability of an organism, to use citrate as its sole carbon source using the enzyme citrase.

PRINCIPLE

Simmon's citrate agar is a defined medium containing sodium citrate as the sole carbon source and the ammonium ion as the sole nitrogen source. The pH indicator, bromothymol blue, will turn from green at neutral pH (6.9) to blue when a pH higher than 7.6 is reached (basic or alkaline). If the citrate is utilized, the resulting growth will produce alkaline products (pH >7.6), changing the color of the medium from green to blue.



$CO_2+Na^++H_2O \longrightarrow Na_2CO_3$(alkaline product)

REAGENTS

1. Simmon’s citrate agar medium

Sodium citrate – 2g/l

$MgSO_4$ – 0.2g/l

$(NH_4) H_2PO_4$ – 0.1g/l

K_2HPO_4 – 1g/l

NaCl – 5g/l

Bromothymol blue – 0.08g/l

Agar – 15g/l

PROCEDURE

1. To three tubes marked as T_1 (test organism-1), T_2 (test organism-2) and Tc (control-uninoculated), 5ml of Simmon’s citrate agar was added and sterilized.
2. The slants were made and T_1 was streaked with *E.coli,* T_2 was streaked with *Klebsiellapneumoniae* and Tc was kept as uninoculated tube (control). The slants were incubated at 37°C for 24 hours.
3. After incubation, the slants were observed for growth and formation of Prussian blue colour.

APPENDIX VI

OXIDASE TEST

AIM

To distinguish the various groups of bacteria on the basis of their cytochrome oxidase activity.

PRINCIPLE

To determine the presence of oxidase enzymes, the reagent (impregnated into strips of filter paper), which contains tetramethyl-p-phenylenediamine, serves as an alternate substrate for the cytochrome oxidase reaction. In the reduced state the reagent is colourless but when oxidized it becomes purple.

REAGENTS

1%Tetramethyl- p- phenylenediaminedihydrochloride.

PROCEDURE

1. The filter paper disc impregnated with oxidase reagent was placed aseptically on a clean sterile slide.
2. With the help of sterile glass rod, a small amount of *Pseudomonas aeruginosa* was transferred to one disc and *E.coli* to the other disc.
3. The colour change of the disc was examined.

APPENDIX VII

CATALASE TEST

AIM

To detect the ability of microorganisms to oxidize H_2O_2 by producing the enzyme catalase.

PRINCIPLE

Catalase enzyme is found in most bacteria. It catalyses the breakdown of hydrogen peroxide (H_2O_2) with the release of free Oxygen.

$$2\,H_2O_2 \xrightarrow{\text{catalase}} 2H_2O\uparrow + O_2$$

REAGENTS

3% H_2O_2

PROCEDURE

1. An aliquot of 3% H_2O_2 was added to two sterile test tubes marked T_1 and T_2 solution.
2. With the help of a sterile glass rod, a small amount of *Staphylococcus* and *Streptococcus* from nutrient agar slant or broth or trypticase soy agar (not from blood agar) were suspended into the tubes $T_1$and $T_{2.}$
3. The tubes were examined for the formation of gas bubbles.

APPENDIX VIII

STARCH HYDROLYSIS TEST

AIM

To detect the amylase action of an organism by starch hydrolysis test.

PRINCIPLE

Starch is a complex carbohydrate (polysaccharide) composed of 2 constituents, amylose, a straight chain polymer of 200 to 300 glucose units and amylopectin, a large branched polymer with phosphate groups. Starch as such cannot be transported into cell for energy production because of its high molecular weight. To assimilate starch (polysaccharide) for energy and catabolic reactions, it must be degraded into basic glucose units by amylase to maltose (disaccharide), which is then converted to glucose (monosaccharide).

$$\text{Starch} \xrightarrow[+H_2O]{\text{amylase}} \text{maltose} \xrightarrow[+H_2O]{\text{maltase}} \text{glucose}$$

The resulting low molecular weight glucose can now easily pass into the cell for energy production via glycolysis.

REAGENTS

1. Starch agar

    Peptone – 5g/l

    Beef extract – 3g/l
    Starch (soluble) – 2g/l
    Agar – 15g/l
2. Iodine solution

PROCEDURE

1. Sterile starch agar plates were prepared and marked as T_1 and $T_{2.}$
2. Culture was streaked in T_1 and *E.coli* in $T_{2.}$
3. The plates were incubated at 37°C for 48 hours.
4. The surfaces of the plates with the bacterial growth were flooded with iodine solution and the plates were observed for the starch hydrolysis.

APPENDIX IX

CARBOHYDRATE FERMENTATION TEST

AIM

To detect the ability of an organism to ferment specific carbohydrates.

PRINCIPLE

Fermentation is a metabolic process in which the final electron acceptor is an organic molecule. Each medium has a single fermentable carbohydrate added to a peptone medium. Phenol red is also added as a pH indicator. A small tube (Durham tube) is inverted and placed in each larger test tube of liquid medium. The inverted tube is able to trap any gas products.

The indicator, phenol red will turn yellow below pH 6.8 and purple pink above pH 7.4. If the organism metabolizes the carbohydrate, subsequent acid production will result in lowered pH. If the organism does not ferment the carbohydrate, the pH may remain neutral. If the organism does not ferment the carbohydrate and also utilizes the peptone, accumulation of the ammonia as a degradation product will raise the pH.

REAGENTS

Carbohydrate broth with phenol red indicator.

Trypticase - 10g/l
NaCl - 5g/l
Beef extract - 1g/l
Phenol red - 0.018g/l

PROCEDURE

1. Added the Carbohydrate broth to three tubes marked as T_1 (test organism-1), T_2 (test organism-2) and Tc (control-uninoculated), and sterilized with the Durham's tube (an inverted small tube filled with liquid without air bubbles).
2. T_1 was inoculated with *E.coli*, T_2 was inoculated with *Staphylococcus aureus* and Tc was kept as uninoculated tube (control).
3. The tubes were incubated at 37°C for 24-48 hours and examined for acid and gas production.

APPENDIX X

NITRATE REDUCTION TEST

AIM

To detect the ability of an organism to reduce nitrate to nitrite.

PRINCIPLE

Microorganisms are capable of respiring under completely anaerobic conditions by utilizing nitrate, sulphate, and carbonate as the terminal inorganic electron acceptor. The enzyme, nitrate reductase present in the organism reduces nitrate to nitrite. In some organism, nitrite is further reduced to ammonia and molecular nitrogen and this process is called denitrification.

$$NO_3^- + H^+ + 2e^- \longrightarrow 2NO_2^- + H_2O$$

Presence of nitrite is tested by addition of α-naphthylamine and sulphanilic acid in an acidic environment, with the formation of a red diazonium dye called p-sulphobenzeneazo α-naphthylamine.

REAGENTS

1. Nitrate broth

    Peptic digest of animal tissue – 5g/l

    Beef extract – 3g/l

    Potassium nitrate – 1g/l

2. α-naphthylamine and sulphanilic acid.

PROCEDURE

1. To the three tubes marked as T_1 (test organism-1), T_2 (test organism-2) and Tc (control-uninoculated), 5ml of nitrate broth was added and sterilized.
2. T_1 was inoculated with *E.coli*, T_2 was inoculated with *Pseudomonas aeruginosa* and Tc was kept as uninoculated tube (control).
3. The tubes were incubated at 37°C for 18-24 hours.
3. After 24 hours of incubation 0.5ml of α-naphthylamine reagent and 0.5ml of sulphanilic acid were added. The tubes were then observed for colour change.

APPENDIX XI

TRIPLE SUGAR IRON TEST

AIM

To detect the ability of an organism to ferment glucose, lactose, sucrose with the production of gas and hydrogen sulphide.

PRINCIPLE

Triple sugar iron agar medium is composed of three sugars, lactose, sucrose, and a small amount of glucose, iron (ferrous sulfate) and phenol red as an indicator. The phenol red indicator is employed for the detection of fermentation of sugars indicated by the change in color of the medium. Due to the production of organic acids, sodium thiosulfate, ferric and ferrous iron makes hydrogen sulfide indicator system.

Organisms that ferment glucose produce a variety of acids, turning the color of the medium from red to yellow, more amount of acid are liberated in butt, where the acid reaction is maintained because of the reduced oxygen tension and slow growth of organism. Since the substrate is present in minimal concentration, the small amount of acid produced on the slant surface is oxidized rapidly. The peptone in the medium is also used in the production of alkali. Growing bacteria also form alkaline products that utilize the large amount of acid present in the butt with appearance of alkaline slant (red) and the acid butt (yellow), which after incubation shows the organism, to be a glucose fermentor, which is able to utilize sucrose and lactose.

Bacteria that ferment lactose or sucrose (or both) in addition to glucose produce large amount of acids, enables no reversion of pH in that region and thus bacteria exhibit an acid slant and acid butt.

In some bacteria carbohydrate fermentation is not seen or does not occur, instead peptone is catabolized under anaerobic or aerobic conditions resulting in alkaline pH due to the production of ammonia. If only anaerobic degradation of peptone occurs, the alkaline reaction occurs only on the slant surface. If there is aerobic and anaerobic utilization of peptone, the alkaline reaction is present on the slant and the butt.

Gas production (carbon dioxide) is detected by the presence of crack or bubbles in the medium or the medium being pushed upwards.

When the accumulated gas escapes, the thiosulfate is reduced to hydrogen sulfide by several species of bacteria and hydrogen sulfide combines with ferric ion or ferrous salt to produce the insoluble black precipitate (ferrous sulphide). Reduction of thiosulfate proceeds only in an acid environment and blackening usually occurs in the butt of the tube.

REAGENTS

Triple sugar iron agar medium

Beef extract – 3g/l
Yeast extract – 3g/l
Peptone - 15g/l
Proteose peptone – 5g/l
Lactose – 10g/l
Sucrose – 10g/l
Glucose - 1g/l
Ferrous sulphide – 0.2g/l
Sodium chloride – 5g/l
Sodium thiosulphate – 0.3g/l
Phenol red – 0.024g/l
Agar – 12g/l

PROCEDURE

1. TSI agar was prepared, sterilized by autoclaving and the slants were made.
2. The test organisms were inoculated by stabbing in the butt and streaking the slant.
3. The tubes were incubated at 37ºC for 24-48 hours.
4. The tubes were examined for acid production, alkaline production, gas production, and hydrogen sulfide production.

APPENDIX XII

ESTIMATION OF PROTEIN BY LOWRY'S METHOD

(Swamy *et al.,* 2014)

AIM

To estimate the amount of protein in the given sample by Lowry's method.

PRINCIPLE

The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin- Ciocalteay phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids [Dunn, 13]. The Lowry method is sensitive to pH changes and therefore the pH of assay solution should be maintained at 10 - 10.5.

The Lowry method is sensitive to low concentrations of protein. Dunn [1992] suggests concentrations ranging from 0.10 - 2 mg of protein per ml while Price [1996] suggests concentrations of 0.005 - 0.10 mg of protein per ml. The major disadvantage of the Lowry method is the narrow pH range within which it is accurate. However, we will be using very small volumes of sample, which will have little or no effect on pH of the reactionmixture.

A variety of compounds will interfere with the Lowry procedure. These include some amino acid derivatives, certain buffers, drugs, lipids, sugars, salts, nucleic acids and sulphydryl reagents [Dunn, 1992]. Price [1996] notes that ammonium ions, zwitter ionic buffers, nonionic buffers and thiol compounds may also interfere with the Lowry reaction. These substances should be removed or diluted before running Lowry assays

REAGENTS

A. 2% Na_2CO_3 in 0.1 N NaOH
B. 1% NaK Tartrate in H_2O
C. 0.5% $CuSO_4.5\,H_2O$ in H_2O
D. Reagent I: 48 ml of A, 1 ml of B, 1 ml C
E. Reagent II- 1 part Folin-Phenol [2 N]: 1 part water
BSA Standard - 1 mg/ ml

PROCEDURE

- 0.2 ml of BSA working standard in 5 test tubes and make up to 1ml using distilled water.
- The test tube with 1 ml distilled water serve as blank.
- Add 4.5 ml of Reagent I and incubate for 10 minutes.
- After incubation add 0.5 ml of reagent II and incubate for 30 minutes
- Measure the absorbance at 660 nm and plot the standard graph .
- Estimate the amount of protein present in the given sample from the standard graph.