Isolation and identification of protease producing bacteria

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Abstract - *Microbes are utilized in numerous industrial applications in a number of ways that exploit their own metabolic capabilities to aid human benefits. Proteases, the enzymatic product of bacteria, act as an integral component in a wide variety of industrial applications including pharmaceutical, food, leather and detergent formulation. Proteases are ubiquitous as they are found in plants, animals, fungi, bacteria and viruses. Microbial sources of proteases, particularly bacteria, are of particular interest in industry. The isolation and identification of protease producing bacteria, hence, is the most basic pre-requisite in this regard for the success of any industry based on protease utilization. After their isolation from different sources, bacteria are routinely identified by morphological and biochemical characterization assays. Newer molecular techniques now allow bacterial species to be identified with authenticity by their genetic sequences. Different sources of bacteria for protease production have been exploited but soil samples, tannery and food processing industries including kitchen waste forms the basis of this report, wherein the procedures and protocols for their isolation and identification are discussed.*

Key Words: Isolation, Protease, Identification, Biochemical characterization, Morphological assay

1.INTRODUCTION

Proteases are a group of enzymes that catalyse the process of proteolysis in which proteins are broken down into single amino acids or smaller polypeptides. Proteases work to simplify proteins by disintegrating the peptide bonds by hydrolysis present within them. Protease enzymes also hold a pivotal role in biological functions, like the simplification of proteins into amino acids, their digestion followed by cell signaling (King et al., 2014; Shen et al., 2006). Proteases also cause a change by disrupting the function of a protein and breaking it down to its simpler components, which leads to the activation of a signal or a particular function in a biological pathway. Proteases occupy an important position with respect to applications in commercial as well as physiological industries. Proteases act as an integral component in a wide range of industrial applications including pharmaceutical, food, leather and detergent formulation (Mohen et al., 2005). Also, of all the total enzymes present globally, proteases account for two thirds and about quarter of the total global enzyme produced (Kumar et al., 2002). Proteases are present in all organisms including plants, animals, fungi, bacteria, and viruses. Different sources of microbial proteases from which they are isolated and identified mostly include various bacteria and fungi. Most proteases for commercial utilization, are isolated from bacteria belonging to the genus Bacillus. Bacillus megaterium, Bacillus stearothermophilus, Bacillus thuringiensis, Bacillus pumilus, Bacillus subtilis and Bacillus stearothermophilus produce thermostable proteases. Besides Bacillus species, Clostridium histolyticum and various Streptococcus sp., produce cysteine proteases and Vibrio chlorae, Pseudomonas aeruginosa and Streptomyces griseus produce metalloproteases (Gupta et al., 2002; Lakshmi et al., 2008). The proteases obtained from microbial sources hold a pivotal role in industrial applications since it leads to a great reduction in production costs (Sawant and Nagendran, 2014). The production of these protease enzymes in microbes depends on the culture media type and composition used for microbial growth, such as the ratio of carbon to nitrogen, occurrence of a metabolizable sugar such as glucose etc. (Gupta et al., 2002). Besides, protease production depends upon the physiological factors such as the optimum pH, optimum temperature, inoculation media and time of incubation (Suppiah et al., 2012). Even biological characteristics like the genome of an individual can lead to change in the biochemical behavior of the microbe and affect the production of metabolite (Varela et al., 1996). Commercially, optimization of culture media using numerous media constituents during fermentation leads to formation of a cost-effective metabolic yield (Hameed et al., 1999). Also, till date no particular media is established to maximize the production of any enzyme because the genomic diversity present in microbes causes each microbial strain to exhibit a unique environment for maximizing enzymatic production. Hence, it is quite essential to perform a series of experiments for investigating the metabolite or enzyme production pattern under different environmental aspects from new isolated microbial strains to achieve maximum production (Prakasham et al., 2006). The protease producing bacterial identification is the next step after successful isolation and is performed using various morphological and biochemical assays, supplemented by specific tests like antibiotic inhibition and serotyping tests. Recent research led to development of newer molecular diagnostic methodologies which allow microbial strain identification using their genetic sequences, directly from the source sample. Under biochemical characterization, the

classification of protease producing bacteria can be done based on cellular structure, metabolism, or differences in cellular components. By performing graham's staining technique bacteria can be characterized based on structure of cell walls possessed by them and on combining morphology with Gram-staining, the protease producing bacteria can further be classified as Gram-positive and Gram-negative cocci and Gram-positive and Gram-negative bacilli. Due to advent of biotechnology that led to advancements in molecular biology and computational science, the accurate information on phylogeny of a particular bacterial strain is possible in near future.

The literature is replete with information on isolation, purification, and characterization of bacterial strains for protease production from different sources (Banerjee et al., 1999; Joo and Chang, 2005; Sreeja et al., 2013), however the protease production by bacterial isolates from soil, kitchen waste and tannery still stand as lacunae. The prime objective of the present report is to try to gather information on isolation and identification of protease producing bacteria from these sources.

2. GENERAL TECHNIQUES

Throughout the globe, the progress in biotechnology has led to utilizing microbes for aiding human environment. For the successful usage of these microbes their isolation as well identification is necessary. Since time immemorial isolation and identification is being conducted. However due to industrialization a drastic change in methodology has been observed that has led to isolation and identification of microbes in simple ways with less time. In the review below the general isolation and identification techniques used by researchers all around the world are discussed.

General techniques in isolation of bacteria

Advancements in biotechnology led to the discovery of culturing micro-organisms in order to identify the microbes of interest to aid human needs. To identify these microbes, from samples containing high microbial content, like soil, wastes, industrial effluents, leather etc. isolation is the preliminary step. For isolating a microbe physically, it has to be cultured as a pure culture. Pure culture is defined as a culture prepared in laboratory that consists of only a single species of a particular microbe. If anyone expects to isolate for a particular microbe of interest, the microbial culture as well as the isolation techniques need to be modified and geared towards that microbe. For example, if a particular microbe is intolerant to air, it can be isolated using anaerobic isolation mechanisms only. Therefore, the development of pure culture entirely depends on the knowledge of the optimal oxygen requirements, nutritional needs and temperature for growth.

There are two universally accepted methodologies for isolating microbes:

1. Streaking by isolation of microbes on agar plates

1. Pour plate method of isolation

Streaking by isolation of microbes on agar plates involves continuous serial dilutions of sample containing microbes until the cells exist in a very low density, so that the single cells obtained thereafter can be isolated spatially to form individual bacterial colonies. On the contrary in pour plate method for isolation, you serially dilute the microbial rich sample sufficiently, and then add it to a molten cooled agar in a dish. The isolated bacterial cells will lead to formation of individual bacterial colonies on the agar. The only drawback to this methodology for isolation is that if the molten agar hot, it will denature and kill the microbes and if it is too cold, it will form a big lump in the petri dish. So due to these reasons streaking method is preferred as it yields bacterial colonies of interest on the agar surface.

General techniques in identification of bacteria

After successful isolation of the bacterial colony, its identification is also necessary in order to identify to which strain the microbe belongs to. To do this following microbial identification techniques are used:

Macroscopic and microscopic features

Macroscopic identification uses physical appearance which includes shape, size, color, and smell. For describing microscopic features for identification use of a microscope is required to infer whether they are rod, cocci, or spiral-shaped, have flagella or buds formed and have a filamentous hypha or not.

Staining for identification

Stains in cytology enable easier and clear visualization of microbes under a microscope. Staining is performed using different staining techniques.

Gram Staining - Gram staining is an important test done for bacterial identification. This purple stain test is based on the usage of a crystal violet dye (Colco, 2005). It distinguishes microbes based on the properties of their cell walls. Gram-positive microbes possess thick peptidoglycan layer, so they retain the crystal violet stain while gram-negative microbes possess a thin peptidoglycan layer, so they do not retain the stain.

Endospore Staining - It is an identification technique that involves application of a stain to the microbial sample for checking the presence of spores using a primary stain malachite green. Due to the fact that not all bacteria produce spores, this is a useful technique for identification. The various types of endospores identified are free, central, swollen and subterminal endospores. (Hussey et al., 2012)

Fungi staining **-** Fungal staining can be helpful for identifying the fungal elements and characteristics of fungal specie microbes based on cell wall staining.

Lactophenol cotton blue – this stain normally colors the carbohydrate portions present in cell walls of the fungus blue. (Heritage et al., 1996)

Periodic-acid Schiff stain (PAS stain) – this stain colors the carbohydrates along with some other components of fungal cell wall producing a magenta color in living fungi only.

Grocot's methenamine silver stain – this stain colors the cell wall of fungi brown to black, but does not distinguish whether the fungi is living or dead. (Nassar et al., 2006)

Biochemical testing for identification

Catalase testing **-** Unidentified bacterial species can be identified using the catalase activity test in which oxygen bubbles appear when hydrogen peroxide is added to a bacterial species cultured on a slide. (Mahon et al., 2011; McLeod and Gordon et al., 1923)

Oxidase testing **-** Using oxidase test, the identification of bacterial isolates is achieved by determining the cytochrome-c oxidase (CCO) activity. If cytochrome-c oxidase exists, it oxidizes a reagent called tetramethyl-p-phenylenediamine forming a purple-colored product. If cytochrome-c oxidase is absent, the reagent remains in reduced state forming a colorless product.(McFaddin, 2000)

Substrate utilization tests **-** Substrate utilization tests is usually performed to identify bacteria using a variety of substrates made from carbon and nitrogen sources. The utilization of substrates by bacterial species leads to color changes in substrates on incubation with bacteria which then generates a key of how the substrate is utilized. The key is then compared with pre-existing substrate use patterns of microbes present on various databases to generate a list of bacterial species.(Campbell et al., 2003)

Chemical/analytical identification

Fatty acid profiling and chemo-profiling **-** In bacteria, fatty acids are an integral component of their cell membranes, and depending on the species of bacteria different combinations of fatty acids are produced. Profiling of these fatty acids is done, and it is used to identify the bacterial strain by comparing them to known profiles. Besides this, bacteria also produce various secondary metabolites like antibiotics, antioxidants and immunosuppressive compounds. On the basis of type of secondary metabolites produced, different bacterial strains are identified using chemo profiling methodology of identification. (Kumari et al., 2008)

Modern methodologies

The drawbacks of the old techniques are that they identify the bacteria that are cultured *in vitro.* Additionally, some of the bacterial strains exhibit distinct biochemical characteristics that could not be identified using primitive methodologies as they did not fit in any genus or species. The advent of biotechnology has led to introduction of modern methods for identifying bacteria which reveal even the minute differences between organisms.

Microarray-Based Identification **-** Microarray-based identification of microbes is based on hybridization of amplified DNA sequences of microbes to specific oligonucleotide probes that vary from species to species. Further, each of these oligonucleotide probes are made up of different dyes that show fluorescence on hybridization. Once hybridized, the fluorescent regions on these hybrids can be compared to standard hybrids and species can be identified (Cao et al., 2011).(Figure -1)

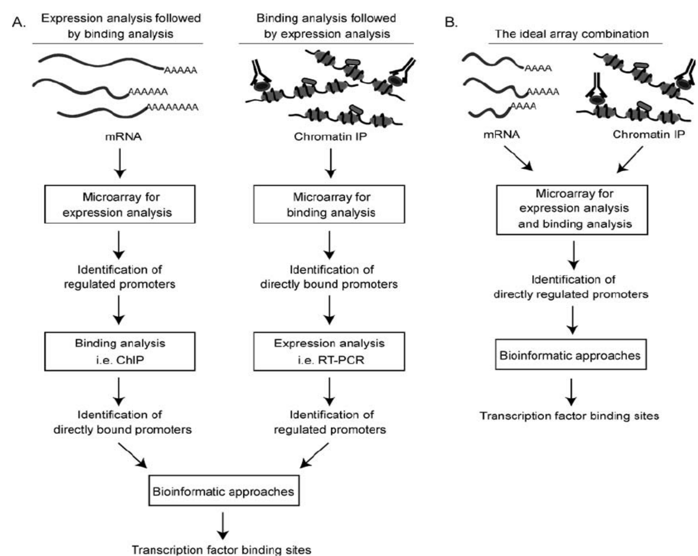

Figure-1: Microarray based identification methodology using specific oligonucleotide probes varying species to species

Next generation sequencing **-** In case of all organisms possessing DNA including various bacterial strains, the presence of 16S rRNA gene (Figure below) acts as a conserved component of the transcription machinery. (Petrosino et al., 2009). This gene acts as a target gene for sequencing DNA from bacterial strains having thousands of different species. For targeting the conserved regions in 16S rRNA gene, universal primers for PCR have been designed to amplify the target gene from a single sample. Besides this, the 16S rRNA gene is composed of both variable and conserved regions. While the role of the conserved region is to do universal amplification, the sequencing of variable regions of 16S rRNA gene allows differentiation of different bacteria leading to their identification (Figure-2).

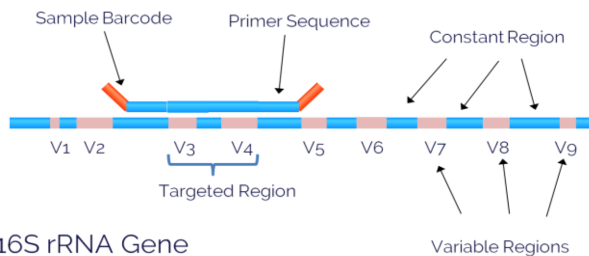

Figure-2: Regions of a 16s rRNA gene used in 16s rRNA gene sequencing technique

Different NGS methods such as 454 pyrosequencing, Illumina, SOLiD, Ion Torrent, and single-molecule real-time (SMRT) circular consensus sequencing equipment from Pacific Biosciences (Lu et al., 2009) and Oxford Nanopore have provided more pace and deep analytic power in identification of microbes (Nicholls et al., 2019).

Metagenomics - Metagenomics involves the genomic analysis of microbes that cannot be cultured(unculturable) by direct extraction of their DNA, and consequently comparing it with well-established ribosomal sequences of microbes, so that the unseen microbial population can be identified and purified. Metagenomics is also interpreted as the study of entire microbial genomes present in mixed communities, which allows them to be identified at strain-level resolution. Due to the fact that 16S or 18S or ITS sequencing is not specific enough to perform comprehensive microbiome experiments, the use of metagenomics enables the identification of the rarest of members in these unseen microbial communities. (Kumari et al., 2013)(Figure-3)

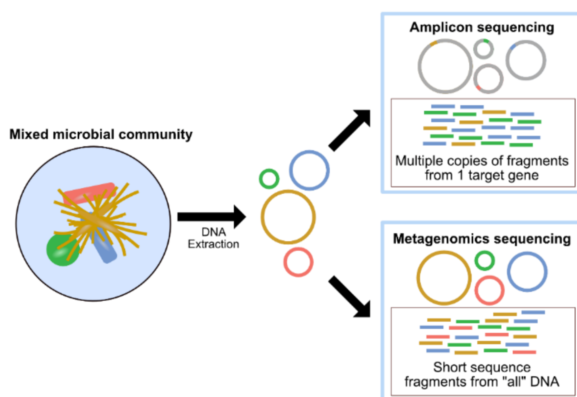

Figure-3: Role of metagenomics to identify a mixed microbial population using amplicon and metagenomics sequencing

3. METHODOLOGY

Lot of work has been carried out in the isolation, purification and characterization of bacterial strains for various purposes. However, literature is scanty as far as protease producing bacteria are specifically concerned. Literature for isolation and identification of protease producing bacteria from different sources has been thoroughly scrutinized and findings of various studies are provided here under different headings depending upon the type of source selected.

1. *Isolation and identification of protease producing bacteria from soil samples*

(Sujatha and Subash, 2017) investigated the extraction and characterization of an extracellular protease from the bacterial strain of *Bacillus subtilis isolated* from termite infested soil sample. Among the five protease producers that were isolated, one of the isolates was selected for research. This isolation was related to *Bacillus subtilis* on the basis of identification using various biochemical characterization techniques and 16S rRNA gene sequencing.

1.1 Isolation of bacteria

Termite infested soil sample was collected, and isolation was performed using serial dilutions followed by spread plate technique. The sample was first diluted up to ten-folds and 0.1 ml from 10^{-4} dilution was introduced on skim milk agar plate and incubated at room temperature for 48 hours. Post incubation, the bacterial strains producing the largest clear hydrolytic zone were selected and then sub-cultured on nutrient agar plates. An isolate 'M1' which showed maximum caseinolytic activity, was selected for purification to obtain protease.

1.2 Identification of bacteria

DNA extraction from the collected sample was performed using the CTAB method (Ausubel et al., 1992). Further the 16s rRNA sequencing was performed. Post sequencing the sequence was compared with a pre-existing gene sequence on various databases using BLAST program on NCBI webpage (Castresana, 2000), evolutionary history was traced (Saitou and Nei, 1987) and phylogenetic tree was made using MUSCLE 3.7 (Edgar, 2004) as shown in Figure 1. Based on the 16S r RNA gene sequencing methodology for identification, phylogenetic analysis was performed, and it indicated that the isolated M1 bacteria was *Bacillus subtilis. (Figure-4)*

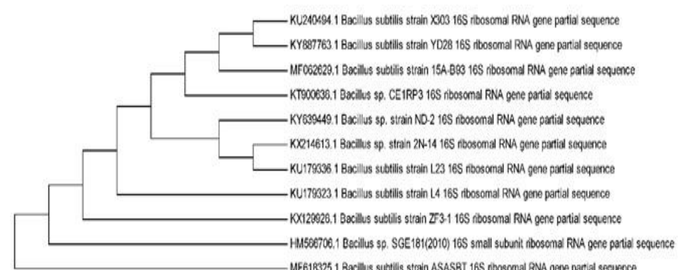

Figure-4: Phylogenetic analysis of Bacillus subtilis ASASBT 16s rRNA gene sequencing for identification

1.3 Characterization of protease enzyme from bacterial strain

Qualitative and quantitative assays of protease from isolated Bacillus subtilis

The bacteria *Bacillus subtilis* was inoculated in a culture medium and the culture was subjected to centrifugation to obtain enzyme source. **For quantitative assay, t**he activity of enzyme protease in the culture of the bacteria was determined by the methodology of Gaurav *et al.* (Pant et al., 2015) For qualitative **determination of protease, t**he protease enzyme that was extracted was cultured on a casein agar plate to confirm the presence of protease. On incubation at room temperature the clear zone was measured and presence of the enzyme was confirmed (Pant et al., 2015, Olajuyigbe and Ajele, 2005). *Bacillus subtilis* bacterial strain was further subjected to different types of culture conditions in order to identify the optimum conditions for maximizing the production of protease. Protease production was estimated at a wide pH range of 5.0 to 10.0, at varying temperatures from 20°C to 70ºC and carbon and nitrogen sources like dextrose, K_2HPO_4, sucrose, lactose, starch, gelatin, maltose, Na_2PO_4, urea and beef extracts (Abbas and Leila, 2011). **A**mmonium sulphate fractionation method based on salting out principle was used to purify the obtained proteases shown in Table 1 and Table 2 shows the purification profile using DEAE cellulose method (Simpson, 2004, Roe, 2004, Sookkheo et al., 2000).

Table-1: Purification of protease produced from Bacillus subtilis using ammonium sulphate precipitation methodology

Percentage of sample	Amount of protein (mg/ml)	Activity of protease enzyme (µ/ml)	Specific enzyme activity (µ/mg)	Amount of recovery (%)
Crude extract	452	52.5	21.5	100
$(NH_4)_2SO_4$ (0-20%)	86	25.6	27.6	24.4
$(NH_4)_2SO_4$ (20-40%)	126	38.1	27.7	36.3
$(NH_4)_2SO_4$ (40-60%)	212	68.3	29.9	65.1
$(NH_4)_2SO_4$ (60-80%)	150	42.9	26.4	40.8
$(NH_4)_2SO_4$ (80-100%)	71	13.4	17.2	12.7

Table-2: Purification of DEAE-Cellulose purified ammonium sulphate precipitated protease from the microbe

Percentage of sample	Enzyme activity	Amount of protein (mg/ml)	Specific enzyme activity (µ/mg)	Amount of recovery (%)
Crude extract	9846	456	21.5	100
$(NH_4)_2SO_4$ precipitate	6412	214	29.9	65.1
DEAE-Cellulose	3968	64	62	40.3
Gel filtration	2336	27	86	23.7
Dialyzed	4568	129	35	46.3

2. Isolation and identification of protease producing bacteria from tannery samples

(Pertiwiningrum et al., 2017) isolated and identified protease producing bacteria from solid and liquid tannery waste. The isolation of protease producing bacterial strains was performed using a sample of solid and liquid wastes taken from different waste reservoirs (three liquid waste reservoirs and one solid waste reservoir) from leather manufacturing units followed by their identification using biochemical characterization assays.

2.1 Isolation of bacteria

Numerous wastewater samples were collected from areas adjoining tanneries. Solid and liquid waste samples were obtained from four varying reservoirs. The first reservoir had tannery waste that was not processed, the second reservoir had tannery waste which was processed by aeration, and the third reservoir had tannery waste post processing. The solid waste samples were taken from the solid waste reservoir (sludge). Sample T1, T2 and T3 were isolated from liquid waste of first, second and third waste storage respectively, while sample T4 was isolated from fourth solid waste storage. For isolation of bacteria, 1mL sample was taken in test tubes. and by serial dilution the concentration was reduced from 10^{-1} to 10^{-10}. Then, 100 µm of 10^{-8}, 10^{-9} and 10^{-10} dilutions were taken, and introduced on skim media using pour plate method, followed by their incubation at 27°C for 3 days. The test was positive due to formation of a clear zone around the bacterial colonies and one colony was selected. Post purification the isolated samples were grown on agar slants, followed by incubation at 27°C for 72 hours, and stored at 5°C (Rahayu, 1991; Miyamoto et al., 2002). The isolate that showed the highest proteolytic enzyme activity was isolate T2.

2.2 Identification of bacteria

Various morphological and biochemical characterization assays including gram staining, motility test, catalase test, macroscopic and microscopic morphology, elevation convex, spherical shape, and white-coloured colonies were performed for the identification. Further characterization of isolate T2 based on microscopic morphology interpreted they were rod shaped, pink, gram-negative, non-motile and catalase positive. Based on these results, the bacterial isolate T2 was predicted to belong to genus *Bacillus*.

2.2.1 Measuring clear zone of hydrolysis diameter

Agar mediums with different pH treatment ranging from 7 to 11 were added with one drop of pre-culture followed by incubation for 3 days. Post growth of bacterial colonies, their clear-zone diameter was measured with calipers (Schmidt et al., 2011). On performing the data analysis of clear zone diameters of bacterial isolate T2 in the media with different pH determined that the clear zone diameter of pH 7 was not very different from the diameters of pH 8, pH 9 and pH 12 (Table 3). On the contrary it was different from the ones with pH 10 and 11 (Table 3). The best clear zone diameter was produced in medium with pH 11 which meant bacterial isolates T2 grew best in medium containing pH 11. Hence bacterial isolate T2 belonged to the alkaliphilic group thereby making the enzyme produced by bacteria isolates T2 as an alkaline protease enzyme.

Table-3: Measuring the colony diameter, clear-zone diameter and proteolytic index of bacterial isolate T2

Parameters	pH activity					
	7	8	9	10	11	12
Clear-zone's diameter	3.48	3.96	3.71	4.39	4.43	3.86
Colony's diameter	2.56	2.66	2.57	3.48	3.38	2.68
Proteolytic index^{ns}	2.39	2.45	2.45	2.27	2.33	2.45

**All the means having different superscripts in the same indicate significantly different (P<0.05) and ns: non-significant (P>0.05)

2.3 Characterization of protease enzyme from bacterial strain

2.3.1 Proteolytic index measurement

Proteolytic index of an enzyme can be determined using the diameter of the clear zone and the diameter of bacterial colony as it is the ratio of clear zone area's diameter to the diameter of bacterial colonies (Baehaki et al., 2011). By measuring the proteolytic index of an enzyme, it becomes easy to determine the bacterial ability for protease enzyme activity in a good quality (Syafie et al., 2013). As shown in Table 1, the proteolytic index of bacterial isolate T2 remained unaffected even on treatment with different media or different pH applied to the media.

2.3.2 Enzyme activity test

Since bacterial isolate T2 was an alkaline protease enzyme its activity test includes the blank and sample measurement as well as the standard measurement of tyrosine. To each test tube first 0.5ml buffer solution having varying pH from 7 to 12 was added followed by addition of 0.5 mL of casein as substrate and 1 mL of distilled water for blank measurement, 1 mL of the sample for sample measurement and 1 mL of tyrosine for standard measurement. All these test tubes were then incubated at room temperature for 10 minutes, followed by addition of 1ml 10% trichloroacetic acid and incubation at room temperature, and filtered with Whatman filter paper. 0.75 ml of the filtrate was taken in a test tube, and 2.5 mL 0.5M of sodium carbonate was added along with 0.5 mL folin's reagent and kept at room temperature for 15 minutes. The absorbance was examined and noted at 578 nm (Nadeem et al., 2007; Ahmed et al., 2008).

2.3.3 Protease Enzyme Characterization

The varying pH added to the culture medium had no effect on the total activity or the specific activity of the protease enzyme (Table 4). Since bacterial isolates T2 could grew best in medium containing pH 11, highest enzyme activity and specific activity could be seen at pH 11. Therefore, it was inferred that the optimum enzyme activity of isolate T2 was at pH of 11. On the contrary the enzyme activity based on varying temperature (40 – 60°C) had no difference as such (Table 5). However, the optimum enzyme activity for T2 bacterial isolate was at 40°C.

Table-4: Bacterial isolate T2 enzyme activity and specific activity of enzyme in different pH

Parameters	pH activity					
	7	8	9	10	11	12
Enzyme activity^{ns} (μ/ml)	39.4	39.1	40.8	42.6	45.1	37.6
Specific enzyme activity^{ns} (μ/mg)	37.7	37.3	39.1	40.8	43.1	36.8

** ns: non-significant (P>0.05)

Table-5: Bacterial isolate T2 enzyme activity and specific activity of enzyme on treatment with a wide range of temperatures

Parameters	Temperature		
	40°C	50°C	60°C
Enzyme activity^{ns} (μ/ml)	54.02	49.2	50
Specific enzyme^{ns} activity (μ/mg)	51.65	47.05	47.8
** ns: non-significant (P>0.05)			

3. Isolation and identification of protease producing bacteria from kitchen waste and food processing industries

(Gill et al., 2016) collected samples from kitchen waste which consisted of vegetables and fruit peels, fruit fibers, thrown away cooked food items and various food grains. PDB (protein degrading bacteria) strains were isolated using nutrient agar media by enrichment technique followed by their identification using Bergey's manual of determinative bacteriology.

3.1 Isolation of bacteria

The isolation of bacterial strains was performed using the enrichment technique. Primarily in this technique the samples are incubated in 125 ml flasks filled with 50 ml of nutrient agar medium (g/L). The composition of this nutrient agar media was 5 gm of peptone, 3 gm of yeast and beef extract in equal proportions, 15 gm of agar, 5 gm of NaCl and pH maintained at 7.4. The type of enrichment technique used for isolation of bacterial strains was serial dilution. Using serial dilution, 1 gram of kitchen waste sample per ml was added to 9 ml distilled water and dilution was performed up to factor of 10^{-6} under a laminar airflow to maintain aseptic environment. Then 0.1 ml of sample from each dilution was plated on nutrient agar followed by incubation at room temperature for 2 days. Bacterial isolates growing in nutrient agar slants were maintained at 40°C.

3.2 Identification of bacteria

Both the bacterial isolates (B1 and B2) obtained by screening were identified through morphological and biochemical characterization of cultures according to Bergey's Manual (Bergey et al., 1974) (Table-6). These isolates also underwent microscopic observation like colony size, pigmentation, form, elevation and colony color etc. (Table-7). On the basis of these identification methodologies isolate B1 was found to be *Bacillus megaterium* and B2 was found to be *Bacillus subtilis*.

Table-6: The physiological and biochemical characterization of bacterial isolate B1 and B2 form kitchen waste samples

Type of test	Bacterial isolate B1	Bacterial isolate B2
Gram staining	Gram positive	Gram positive
Endospore type	Central placed	Central placed
Morphology of colony	Rod shaped bacteria	Rod shaped bacteria
Oxidase test	✗	✓
Motility test	✓	✓
Catalase test	✓	✓
Indole production test	d	✗
Gelatin hydrolysis test	✗	✗
H2S production test	✗	✗
Lecithinase test	✗	✓
Urease test	✗	✓
Starch breakdown test	✓	✓
Methyl red test	✗	✓
Voges Proskauer test	✗	✓
Nitrate reduction test	**d**	✓
Acid production	✓	✓
Arabinose test	✓	✓
Mannitol test	✓	✗
Trealose test	✓	✓
Inositol test	✗	✗
Lactose test	✗	✗
41°C temperature test	✗	✗
50°C temperature test	✗	✗
Casein breakdown	✓	✓
Anaerobic growth	Obligate aerobic bacteria	Facultative
**✓: positive reactions, ✗: negative reactions, d: dubious reactions		

Table-7: The morphological test results of isolated bacteria

S.No.	Bacterial Isolates (B1 and B2)	Morphology observed
1)	B1 bacterial isolates	Slightly irregular shaped having an undulated margin
2)	B2 bacterial isolates	Dry, flat and irregular shaped having lobate type of margins

3.3 Characterization of protease enzyme from bacterial strain

3.3.1 Screening of protease producing microbes

Using modified basal medium (MM), the qualitative enzyme activity assay was determined. The composition of modified basal medium (g/L) was 14 gm of agar along with 6.2 g/L skim milk protein, 5 g/L of casein as substrate, 1 gm of glucose and2.5 gm of yeast extract. When the basal medium was introduced with bacterial isolates, formation of a clear zone of hydrolysis in the medium around the well, suggested that protease activity occurred (Perez et al., 2009). Two out of six isolates showed formation of transparent circular zone around bacterial colonies on skim milk agar plate and gelatin agar plate which suggested the presence of protease (Sinha et al., 2013; Alnahdi, 2012). Similar methodology for screening using skim milk and gelatin agar was used earlier by (Abirami et al., 2011; Geethanjali and Subhash, 2011; Sevinc and Demirkan, 2011; Smita et al., 2012; Sinha et al., 2013). Based on the appearance of clear zones of hydrolysis two bacterial isolates having protease production capabilities were selected. These halo zones of two bacteria are given below in Table - 8 and Figure-5.

Table-8: Analysis of the diameter of zone of hydrolysis of enzyme from kitchen wastes

S.No.	Bacterial Isolates (B1 and B2)	Diameter of hydrolysis (in mm)
1)	B1 bacterial isolates	12mm
2)	B2 bacterial isolates	14mm

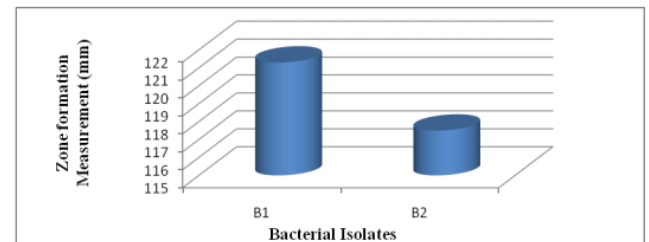

Figure-5: Zone formation measurement of the bacterial isolates from kitchen waste

3.3.2 Quantitative assay for protease activity

Using the modified methodology proposed by (Folin and Ciocalteu, 1927)protease enzyme assay was performed which exhibited as in Figure-6, that maximum enzyme activity of bacterial isolate B1 was more than B2. So suitable changes were made in the basal medium to find the best source for maximum enzyme production.

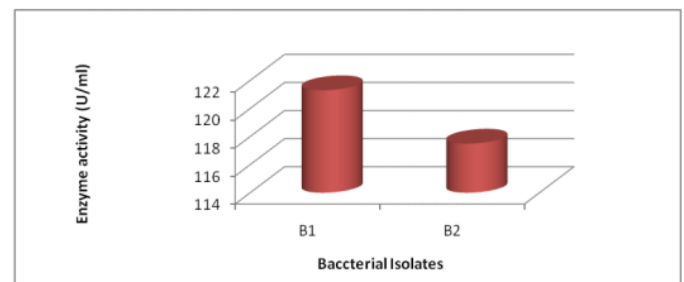

Figure-6: Qualitative enzyme activity of bacterial isolates B1 and B2

4. RESULT AND DISCUSSION

The results of the study conducted by (Sujatha and Subash, 2017) showed that the maximum enzyme activity recorded at 48 hours of incubation at room temperature was 154.13 µ/ml. The optimum pH of 7 and temperature at 40 °C showed maximum production of enzyme protease. The best carbon and nitrogen sources for production of proteases were starch and gelatin, respectively. The isolated protease was subjected to purification in four different steps namely ammonium sulphate precipitation, followed by dialysis, DEAE-Cellulose and finally sephadex G-100 chromatography which had a 4.01-fold increase in the specific activity of the enzyme protease (86.51 µ/mg) and 23.73 % increase in recovery percentage of the enzyme.

Parallelly, using the same methodology as performed by (Sujatha and Subash, 2017) research was also conducted by (Vishnu et al., 2016) and (Vijitra et al., 2019)on isolation and identification of protease producing bacteria. (Vishnu et al., 2016) isolated protease producing bacterial strains from marine soil in Tamil Nadu. Using biochemical assays for identification, it was identified as *Pseudomonas fluorescens*.

Pseudomonas fluorescens was inoculated in gelatin agar for protease enzyme production. The enzyme extract was purified using ammonium sulphate fractionation and 85% saturation was obtained. The enzyme activity was determined as 7.5 μ/ml after incubation for 1 day. The maximum extracellular protease production was found at room temperature (37°C) and the specific activity of enzyme was 49.02 μ/mg. Similarly, studies by (Vijitra et al., 2019)identified nineteen bacterial isolates having protease producing capabilities using 16S rRNA gene sequencing technique. Seventeen isolates out of nineteen were regarded as *Bacillus* spp. Based on biochemical assays only two isolates were identified as *Staphylococcus sp*. and *Enterobacter sp. Bacillus thuringiensis* was found to show the highest enzyme activity of 3.72 ± 0.08 μ/mg at optimum conditions of temperature 65°C and pH 8 post 30 minutes incubation in 0.05M PBS buffer with 1% casein as substrate. The results of the study conducted by (Pertiwiningrum et al., 2017) indicated that the morphology of the T2 isolate showed circular, convex elevation, pink, gram-negative, non-motile, gelatin negative and catalase positive. The highest recorded enzyme was achieved at a pH of 11 with enzyme specific activity as 43,19±1,69 μ /mg and total activity as 45,18±1,77 μ /ml and at optimum temperature of 40°C enzyme activity was 54,02±1,89 μ /ml and specific activity was 51,65±1,8 μ /mg. The protease enzyme activity from precipitated ammonium sulphate showed a higher result of (75,8 μ /ml) as compared to rough protease. Further the study conducted by (Gill et al., 2016) resulted in the selection of, two effective protein degrading bacteria out of the six bacterial isolated strains. Both these isolates underwent the qualitative assay and quantitative assay, and the protease enzyme activity was measured. The results from protease enzyme assay indicated that, isolate B1 exhibited the maximum enzyme activity of 121.3 μ/mL at 37°C after three days incubation. On the contrary, isolate B2 exhibited lowest enzyme activity of 117.5 μ/ml. Therefore, glucose was replaced by different sugars in the basal medium and fructose was found as the best source for production of maximum protease 125 μ/mL) by *Bacillus sp.* (Sevinc and Demirkan, 2011). Similar methodology for isolation and identification as discussed by was adopted by (Sony and Potty, 2016)for isolating and identifying protease producing bacteria from food processing industries. According to their study protease producing bacteria was isolated from soil and wastewater samples of a bakery Kollam region, India. Protease production was examined by serially diluting the samples, followed by introducing them on gelatin agar plates at 37°C for 2 days. Total 87 dissimilar bacterial colonies showed protease activity which was confirmed by the occurrence of zone around the isolates. Out of these 87 bacterial colonies, 27 showed occurrence of clear zone of hydrolysis around them. Further, out of these 27 isolates, eight were selected based on diameter of clear zone. These eight isolates were further identified using biochemical characterizations and biochemical identification using Biomerieux VITEK 2 system. These isolates were identified as *Proteus mirabilis, Cedecea davisae, Enterobacter asburiae and Staphylococcus intermedius.* Protease enzyme activity was then measured using standard methodology and enzyme was purified and used as a tool for waste recycling in food processing industries.

5. FUTURE PERSPECTIVE

Protease enzymes find application in diverse end-use markets, such as detergents formulations, pharmaceuticals and diagnostics, food processing and other industries such as textile, animal feed and chemical. Proteases are an integral component of all the industrially significant enzymes involved in most of the cellular and physiological processes. Since they are involved in physiological processes, they are abundantly found in microbes, animals and plants. However, proteases from microbes are of particular commercial interest and are preferred because of their fast growth, reduced space requirement for their growth and easy amenability and credibility to genetic manipulation. Since time immemorial protease enzymes are being extensively utilized in the food, dairy and detergent formulation industries. Apart from this, the application of proteases in therapeutics has been growing rapidly over the last decade. There is a rekindled interest in using proteases as target enzymes for the development of drugs to treat the uncontrollable fatal diseases like cancer, AIDS and malarial parasites. Also, the recent discoveries and methodologies use genetically manipulated small molecule activated (SMA) protease enzymes which leads to activation of apoptotic enzyme caspase (Gray et al., 2010) which acts as a new way for controlling the protease activity in humans and can be used in diagnostics. Further the exploitation of these proteolytic activities of proteases are opening up the vast potential for the future in diseased tissues as they offer a new way for the development of site-specific drugs (Erster et al., 2012) as well as imaging tumors (Jiang et al., 2004).

The discovery of new sequencing techniques has allowed deeper insights in studying the evolutionary relationship among proteases. Microbial proteases which, hitherto, were not amenable to biotechnological interventions are now becoming so. This understanding of evolutionary relationships among proteases would lead to isolation and identification of novel strains of bacteria producing these proteases. Therefore, the exploitation of biological resources in order to generate and extract microbes producing proteases suited for different industrial applications aiding humans is considered as the most promising future alternatives. Genetic engineering and protein engineering is now becoming possible to alter the properties of proteases and would lead to improved strains of bacteria producing these proteases that were never made in nature and that would meet the requirements of the multitude of protease applications.

With the advancements in microbiology and biotechnology, we can visualize that the utilization of proteases in the near future will be a multi-disciplinary task due to which humans could attain many dramatic successes.

6. CONCLUSION

This report is mainly focused on microbial proteases, however special emphasis is given on the isolation, purification and identification of these protease producing bacteria. Proteases from microbes play a very crucial role in numerous industrial applications such as detergent formulation, diagnostics and therapeutics, agricultural and chemical industries and are becoming an alternative in the near future. The utilization of microbes in order to produce enzymes like proteases has far too many advantages and recently has become the preferred mode for production of enzymes. However, the costs related to the production of these enzymes is quite high and costs of its procurement are even more, hence the present report highlights the potential to obtain protease producing bacteria at minimal costs which could be alternative for industrial and commercial use.

(Sujatha and Subash, 2017) investigated the extraction and characterization of an extracellular protease from termite infested soil sample. Based on 16S rRNA gene sequencing they identified the isolate to be *Bacillus subtilis*. Protease producing bacteria have been isolated and identified from tannery waste (Pertiwiningrum et al., 2017). Based on various morphological and biochemical assays, the protease producing bacteria from tannery waste was predicted to belong to genus *Bacillus* whose morphology for identification showed it to be circular, convex elevation, pink, gram-negative, non-motile, gelatin negative and catalase positive. Food processing industries & kitchen waste has also been used to isolate protease producing bacteria (Gill et al., 2016; Sony and Potty, 2016). [49] observed that two isolates had protease producing capabilities which further underwent for qualitative testing for identification and quantitative testing for determining the protease enzyme activity. Apart from this, extensive research is being conducted globally hunting for a stable enzyme having high efficiency and effectiveness in various classes of applications. According to the recent advancements in science and technology a rapidly growing trend has been observed in commercial applications of proteases and the use of microbial sources to isolate novel protease enzymes for aiding human needs. The progress and development in biotechnology is now offering a constructive position for the production of protease enzymes which would continue to accelerate their utilizations to achieve a sustainable environment.

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