‘ENZYMATIC SCREENING, BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF NOVEL PROTEASE PRODUCING BACTERIA FROM WASTE SOIL’

DISSERTATION SUBMITTED TO ST. TERESA’S COLLEGE, ERNAKULAM IN

PARTIAL FULFILLMENT OF THE REQUIREMENT

FOR THE AWARD OF

DEGREE OF MASTER OF SCIENCE IN ZOOLOGY

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CERTIFICATE

This is to certify that the dissertation entitled "**Enzymatic Screening, Biochemical and Molecular Characterization of Novel Protease Producing Bacteria from Waste Soil**", is an authentic record of original project work carried out by "**SHANA NAZRIN M N**" (SM20ZOO009), during the Academic year **2020-2022**, under my guidance in partial fulfilment of the requirement of the Degree of Master of Science Zoology from St Teresa's College, Ernakulam.

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DECLARATION

I hereby declare that this dissertation entitled **"Enzymatic Screening, Biochemical and Molecular Characterization of Novel Protease Producing Bacteria from Waste Soil"** submitted to Mahatma Gandhi University, Kottayam in the partial fulfillment for the award of Master of Science in Zoology, is a record of original project work done by me, and no part thereof has been submitted to any other course. To the best of my knowledge and belief, this project contains no material previously published or written by another person, except where due reference is made.

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ACKNOWLEDGEMENT

Foremost, I want to offer this endeavor to our God Almighty for the wisdom he bestowed upon me, the strength, peace of my mind and good health in order to finish this dissertation.

Ms. Cynthia Mathew Thaliath, Assistant Professor, Department of Zoology, St. Teresa's College (Autonomous), Ernakulam, research mentor for the continuous support and efforts, for her patience, motivation, enthusiasm and immense knowledge. Her guidance helped a lot in completion of my dissertation.

Besides my mentor, I would like to express my heartfelt gratitude to Dr. Soja Louis, Head, Department of Zoology, St. Teresa's College (Autonomous), Ernakulam, for her constant support, guidance and inspiration to complete my dissertation.

I sincerely thank our principal Dr. Lizzy Mathew, St. Teresa's College (Autonomous), Ernakulam, for providing a wonderful platform to complete my dissertation.

I profusely thank Ms. Tiya K. J and Ms. Indu Vasudevan for their valuable reinforcement and suggestion throughout.

Also, I extend my gratitude to my parents for the moral and emotional support.

Last but not the least, my profound thanks to all my friends and non- teaching staff of St. Teresa's College for the heartening and abetment for the completion of my dissertation.

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LIST OF ABBREVIATIONS

1.	µl	Microlitre
2.	mM	Millimolar
3.	G	Gram
4.	%	Percentage
5.	^0C	Degree Celsius
6.	hrs	Hours
7.	min	Minute
8.	sec	Seconds
9.	CFU	Colony-forming unit
10.	HCL	Hydrochloric acid
11.	O_2	Oxygen
12.	H_2o	Water
13.	H_2O_2	Hydrogen Peroxide
14.	NH_4H_2PO4	Ammonium dihydrogen phosphate
15.	P^H	Potential of Hydrogen
16.	SDS	Sodium Dodecyl Sulfate

17.	DNA	Deoxyribo Nucleic Acid
18.	DNTPs	Deoxy Nucleoside Triphosphates
19.	EDTA	Ethylene Diamine Tetra Acetic Acid
20.	$MgCl_2$	Magnesium Chloride
21.	Rpm	Revolutions Per Minute
22.	PCR	Polymerase Chain Reaction
23.	Taq	Thermus aquaticus
24.	TE	Tris EDTA
25.	NCBI	National Centre for Biotechnology Information
26.	BLAST	Basic Local Alignment Search Tool

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ABSTRACT

Proteases constitute a very large and complex group of enzymes, widely utilized in a host of industries. Recent years have witnessed a phenomenal increase in the use of enzymes as industrial catalysts. It represents one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes.

The current investigation intended to isolate a suitable bacterial strain for protease production. Protease producing bacteria were isolated from compost, plastic waste, poultry, agricultural and food waste containing soil, screened for protease production on skim milk agar plates and confirmed the protease production through protease assay. Among the seventy-eight bacterial isolates screened, the bacterial isolate showing highest protease production was selected and biochemically characterized using IMVIC and catalase test. Kirby Bauer antibiotic susceptibility test of the potential test isolates against various dosage of broad-spectrum antibiotics was accomplished. Molecular characterization of the six isolates namely ZKM 4, ZVP 1, ZWI 2, ZVP 8, ZVP 5, ZMC 1 involves genomic DNA extraction and PCR amplification of 16Sr DNA using universal primers and subjecting it to automated sequencing method. NCBI BLAST analysis of the six-isolate demonstrated maximum sequence similarity of 100% to *Rossellomorea marisflavi, Stenotrophomonas maltophilia, Bacillus velezensis, Bacillus subtilis, Serratia marcescens, Bacillus subtilis* respectively. Findings of the current research suggests that *Rossellomorea marisflavi* is a novel and potent proteolytic enzyme producing bacteria. Exploitation of biodiversity to provide microorganisms that produce proteases well suited for their diverse applications is considered to be one of the most promising future alternatives. In the future, protein engineering will play a primary role in producing proteases with new properties. Bacterial proteases play a vital role in different industries due to their potential, and their future use is likely to be increased. Advance strategies like protein/genetic engineering, molecular biology, and computational biology needs to be adopted to generate improved protease-producing strains.

INTRODUCTION

Soil is an important system of terrestrial ecosystem. There is a direct impact of pollutants on minerals, organic matter and microbial community of soil (Nagaraju *et al*., 2007). Soil is a specific environment, in which synthesis of chemical compounds as well as their decomposition and transformation take place. These processes ensure degradation of plant and animal residues, element cycle, formation of humus, and a proper soil structure. A majority of these enzymatic processes are primarily carried out by soil microorganisms, whose enzymatic activity has an impact on soil fertility (Nannipieri *et al*., 2002; Wolinska and Stepniewska 2011). Enzymes from the class of oxidoreductases (dehydrogenases, nitrate reductase, polyphenol oxidase, catalase, peroxidase) and hydrolases (esterases, phosphatases, proteases, cellulases, urease, and invertase) play the most important role in transformations occurring in soil (Tabatabai 1994).

Since changes in soil quality following amendment are difficult to quantify, combinations of soil biochemical, microbiological and physical properties have been investigated using traditional determinants, such as C, N, P contents, pH, texture, metabolic quotient, biomass and enzyme activities (Nannipieri, 1994; Alef and Nannipieri, 1995; Trasar-Cepeda *et al*., 1998; Leiro´s *et al*., 1999). More recently, molecular and physiological characterization of microbial community structure and diversity has advanced our understanding of soil quality (Amann and Ludwig, 2000; Doran and Zeiss, 2000; Hill *et al*., 2000; Ogram, 2000; Morris *et al*., 2002).

In recent years different kinds of wastes have been added to agricultural land. Single and repetitive applications of different amounts of sludges are reported by Banerjee *et al*. (1997) to significantly increase the amount of soil microbial biomass and enhance Nitrogen mineralisation potential but reduce the functional diversity of the microbial community at the highest rate of application (100 t haK1) (Crecchio *et al*., 2004).

Proteases are a group of enzymes catalyze hydrolysis of bonds in polypeptide chains and split them into smaller polypeptides or else free amino acids (Sharma *et al*., 2015). Proteases are the class of enzymes, which occupy key position with respect to their applications in both physiological and commercial fields. Proteases are also known as peptidyl –peptide hydrolases and are industrially useful enzymes which catalyse the hydrolysis of peptide bond from proteinand are industrially useful enzymes which catalyse the hydrolysis of peptide bonds from protein molecules. Proteases constitute 50-65% of the global industrial enzyme market, most of which are alkaline proteases (Palsaniya *et al*., 2012).

Protease has been categorized based on several standards, proteases are classified according to the position of the peptide bond cleaved into two major groups as exopeptidases and endopeptidases (El Enshasy *et al*., 2016; Prassas *et al*., 2015). They can also be classified as the acidic range (pH 2.0 to 6.0), neutral (pH 6.0 to 8.0) or alkaline proteases (pH 8.0 to 13.0) (Souza et al., 2015; 10Aladdin et al., 2017). Plants are the main source of neutral proteases and they work better at pH in the range of 7.0 or around. While microorganisms are the major sources of alkaline proteases and they work better at pH range of 8 and above (Sharma *et al*., 2015). The performance of protease is influenced by several factors, such as pH of industrial process, ionic strength, temperature and mechanical handling (Miglani *et al*.,2017).

Microorganisms are the most important sources for enzyme production. Selection of the right organism plays a key role in high yield of desirable enzymes. For production of enzymes for industrial use, isolation and characterization of new promising strains using cheap carbon and nitrogen sources is a continuous process. Habitats that contain protein are the best sources to isolate the proteolytic microorganism. Waste products of meat, poultry and fish processing industries can supply a large amount of protein rich material for bioconversion to recoverable products (Dalev, 1994; Gaustevora *et al*., 2005). They are commercially important and isolated from various living sources such as plants, animals, bacteria and fungi (Aftab *et al*., 2006).

With the advent of new frontiers in biotechnology, the spectrum of protease application has expanded into many new fields, such as clinical, medicinal and analytical chemistry (Palsaniya *et al*., 2012). Protease activities have been reported to occur partly in soil as a humocarbohydrate complex (Mayaudon *et al*., 1975; Batistic *et al*., 1980) from arable soil (Ladd, 1972; Mayaudon *et al*., 1975; Hayano *et al*., 1987); from solid municipal waste compost (Rad *et al*., 1995), and from forest or permanent grassland soils (Nannipieri *et al*., 1980, 1982, 1985).

Proteases constitute one of the most important groups of industrial enzymes and have applications in different industries for example in detergent, food, feed, pharmaceutical, leather, silk and for recovery of silver from used X-ray films (Anisworth, 1994; Fujiwara, 1993; Outtrup *et al*., 1995). This enzyme accounts for 30% of the total world enzyme production (Horikoshi, 1996). Alkaline proteases are produced by a wide range of alkalophilic microorganisms including bacteria, moulds, yeasts and also mammalian tissues have a great activity at pH around 10. The most famous of alkaline proteases are subtilisins that are produced by Bacillus licheniformis and other related species (Kalisz, 1988). Among bacteria, Bacillus sp. is a specific producer of extracellular alkaline proteases (Godfrey and Reichelt, 1985) and their enzymes are quite often added to laundry detergents to enable the release of proteinaceous soil from stains (Masse and Tilburg, 1983) and in food industries for removal of protein (Darani *et al*., 2008). Also, microbial proteins have a longer shelf life and can be stored under less-than-ideal conditions for weeks without significant loss of activity. In general, microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals (Kumar *et al*.,2014). Proteases produced from microorganisms play an important role in several industries, for example detergent, tanning, photographic industries, pharmaceutical and waste treatment etc. (Gupta *et al*.,2002). Proteases are actively involved in carbon recycling and biological transformations of soil fertility (Bolon *et al*., 2008).

Several Bacillus species involved in protease production are e.g., *B. cereus*, *B. sterothermophilus*, *B. mojavensis*, *B. megaterium* and *B. Subtilis* (Shumi *et al*., 2004). The

genus "*Bacillus*" is an important source of industrial alkaline proteases and are probably the only genera being commercialized for alkaline protease production (Ferrari *et al*.,1993). They are widely distributed in soil and water, and certain strains tolerate extreme environmental conditions including highly alkaline conditions. Screening of proteases producing Bacillus sp. from different ecological environments can result in isolation of new alkaline proteases with unique physiochemical characteristics (Singh *et al*.,1999).

AIM AND OBJECTIVES

AIM

Enzymatic screening, Biochemical and Molecular characterization of Novel Protease producing bacteria from waste soil.

OBJECTIVES

- Isolation of bacteria from waste soil samples collected from different locations.
- Screening of protease producing bacteria by skim milk hydrolysis assay.
- Screening of extracellular protease production by well diffusion assay.
- Biochemical characterization of protease producing bacteria.
- Antimicrobial susceptibility testing of protease producing bacteria.
- Molecular identification of efficient protease producing bacteria.

INNOVATION OF THE DISSERTATION

Rossellomorea marisflavi were obtained as a novel and potent proteolytic enzyme producing bacteria and hence can be assumed as the innovation of the present investigation targeting waste soil namely compost, plastic waste, poultry, agricultural and food waste containing soil etc. Extensive research can provide further insights on the properties and importance of *Rossellomorea marisflavi* (a novel proteolytic strain), *Stenotrophomonas maltophilia*, *Bacillus velezensis, Serratia marcescens, Bacillus subtilis* which can lead to better management of waste which is an underutilized prospect. Advancement in biotechnology offers a constructive position for the extraction of proteases and will continue to facilitate their applications in waste organization to provide a sustainable environment for improving the quality of human life.

REVIEW OF LITERATURE

SOIL HABITAT

Soil is one of the more complex and highly variable habitats on earth. Any organism that makes their home in soil has had to devise multiple mechanisms to cope with variability in moisture, temperature, and chemical changes so as to survive, function, and replicate. Within a distance of 1 mm, conditions can vary from acid to base, from wet to dry, from aerobic to anaerobic, from reduced to oxidized, and from nutrient-rich to nutrient-poor. Along with spatial variability there is variability over time, so organisms living in soil must be able to adapt rapidly to different and changing conditions. Variations in the physical and chemical properties of the soil are thus important determinants of the presence and persistence of soil biota (Janice *et al.,* 2006).

SOIL MICROBES

On the whole the soil is composed of five major components, these include; Mineral matter, Water, Organic matter, Air and living Organisms. The various components of the soil environment constantly change and the quantity of these constituents are not the same in all soil but vary with locality. Living portion of the soil body includes small animals and microorganisms but it is generally considered that it is microorganisms that play the most important role in the release of nutrients and carbon dioxide for plant growth. The bacteria are the most abundant group, usually more numerous than the four combined. Soil bacteria can be rod, (bacilli) cocci (spherical) spirilla (spirals), of these, bacillus is more numerous than the others. They are one of the major groups of soil bacteria population and are very widely distributed. The number and type of bacteria present in a particular soil would be greatly influenced by geographical location such as soil temperature, soil type, soil pH, organic matters contents, cultivation, aeration and moisture content (Abdulkadir *et al.,* 2012).

WASTE SOIL

Solid waste term includes all those solid and semi-solid materials that are discarded by the community. Improper management of solid wastes causes adverse effects on the ecology which may lead to possible outbreak of diseases and epidemics. Solid wastes are broadly classified into three group's namely Industrial waste, Agricultural waste, and Municipal waste apart from other categories of wastes (Dahale *et al.*,2012).

Municipal solid waste (MSW) compost is increasingly used in agriculture as a soil conditioner but also as a fertilizer. Proponents of this practice consider it an important recycling tool since MSW would otherwise be landfilled and critics are concerned with its often-elevated metal concentrations. Large amounts of MSW compost are frequently used in agriculture to meet crop N requirements and for the addition of organic matter. The main concern is loading the soil with metals that can result in increased metal content of crops. Furthermore, in some cases, metals and excess nutrients can move through the soil profile into groundwater. Municipal solid waste compost has also been reported to have high salt concentrations, which can inhibit plant growth and negatively affect soil structure (Hargreaves *et al.,* 2008).

Land application of organic wastes such as animal manure, municipal wastes, and sewage sludge could alter the soil physical properties. Repeated substantial applications of waste increase the soil organic matter percentage (Khaleel *et al.*,1981).

Valorisation of food waste offers opportunity, which can reduce the problems of its conventional disposal. Food waste is commonly disposed of in landfills or incinerated, causing many environmental, social, and economic issues. Large amounts of food waste are produced in the food supply chain of agriculture: production, post-harvest, distribution (transport), processing, and consumption. Food waste can be valorised into a range of products, including biofertilizers, bioplastics, biofuels, chemicals, and nutraceuticals. Conversion of food waste into these products can reduce the demand of fossil-derived products, which have historically contributed to large amounts of pollution. The variety of food chain suppliers offers a wide range of feedstocks that can be physically, chemically, or biologically altered to form an array of biofertilizers and soil amendments. Composting and anaerobic digestion are the main large-

scale conversion methods used today to valorise food waste products to fertilizers and soil amendments. However, emerging conversion methods such as dehydration, biochar production, and chemical hydrolysis have promising characteristics, which can be utilised in agriculture as well as for soil remediation. Valorising food waste into bio fertilisers and soil amendments has great potential to combat land degradation in agricultural areas. Biofertilizers are rich in nutrients that can reduce the dependability of using conventional mineral fertilisers. Food waste products, unlike mineral fertilisers, can also be used as soil amendments to improve productivity. These characteristics of food wastes assist in the remediation of contaminated soils (Connor *et al.*,2011).

PROTEASE

Proteases are the class of enzymes, which occupy key positions with respect to their applications in both physiological and commercial fields. Proteases are also known as peptidyl – peptide hydrolases and are industrially useful enzymes which catalyse the hydrolysis of peptide bonds from protein molecules. Proteases constitute 50-65% of the global industrial enzyme market, most of which are alkaline proteases (Palsaniya *et al.,* 2012).

Proteases refers to a group of enzymes whose catalytic function is to hydrolyze proteins. They are also called proteolytic enzymes or proteinases. Proteases are classified according to their structure or the properties of the active site. There are several kinds of proteases such as serine, metallo, carboxyl, acidic, neutral, and alkaline proteases (Sevinc *et al.,* 2011).

Proteases are classified into six types based on the functional groups in their active sites. They are aspartic, cysteine, glutamic, metallo, serine, and threonine proteases. They are also classified as exo-peptidases and endo-peptidases, based on the position of the peptide bond cleavage. Proteases are also classified as acidic, neutral or alkaline proteases based on their pH optima. Based on the sequence and structural similarities, all the known proteases are classified into clans and families and are available in the MEROPS database. Broad classifications of proteases are of endo or exo enzymes on the basis of their protein substrate site of action. Depending on their catalytic mechanism, they are further classified as serine proteases, aspartic protease, cysteine proteases or metalloproteases. The classification can be viewed from the

perspectives of their amino acid sequence and evolutionary relationship. Neutrality, alkalinity and acidity are used as another form of classification based on pH requirements and pH optimum (Soja *et al.,* 2014).

They constitute two thirds of the total enzymes used in various industries and account for at least a quarter of the total global enzyme production (Kumar *et al.,* 2002). These enzymes occupy a pivotal position due to their wide application in food processing (Pastor *et al.,* 2001), pharmaceutical industries (Anwar and Saleemuddin, 1998; Gupta *et al.,* 2002), meat tenderization process (Takagi *et al.,* 1992; Wilson *et al.,* 1992), peptide synthesis (Kumar and Hiroshi, 1999), infant formula preparation (American Academy of Pediatrics Committee on Nutrition, 1989), leather processing (George *et al.,* 1995) and in weaving processing (Helmann, 1995) (Sudipta *et al.,* 2010).

MAJOR SOURCES OF PROTEASE

Owing to the high demand of proteases in the global market, the search for proteases has tremendously increased, as they are found everywhere in nature, namely, in plants, animals, and microbes. However, production of plant proteases, such as bromelain, keratinases, and ficin, is time-consuming. The animal proteases, such as pancreatic, trypsin, pepsin, chymotrypsin, and renin are produced and prepared in pure form in large quantities. The production of proteases from animal sources is insufficient to fulfill the industrial demand worldwide; therefore, scientists have extended their research of producing proteases from bacterial sources. Owing to the broad-spectrum biochemical variety and easy genetic manipulation, microbes produce an exceptionally promising number of proteases. Among different sources, such as plants, animals, and microbes, proteases are generally produced by microbial sources. Among microbes, *Bacillus sp.* are extensively studied for protease production in a large scale, and they are exploited in various industries like leather, detergent, pharmaceuticals, and textile; some fungal species like *Aspergillus sp.* have been studied thoroughly for the production of alkaline protease. A list of microbes producing proteases is given below. Halophilic enzymes are getting more attention in biotechnological applications due to their thermal stability and ability to retain activity under high stress from organic solvents

except for pyridine, which inhibits protease activity. The enzyme activities remained the same up to 80% even at 50, 55, and 60°C for at least 30 min (Razzaq *et al.,* 2019).

MICROBIAL PROTEASE

Microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals. The yield of extracellular enzymes is influenced by the physicochemical conditions. Hence, physicochemical parameters are optimized for the maximum production of protease (Palsaniya *et al.*,2012).

Bacillus sp- MAJOR ALKALINE PROTEASE

Bacteria are the most dominant group of alkaline protease producers with the genus Bacillus being the most prominent source. A myriad of Bacillus species from many different exotic environments have been explored and exploited for alkaline protease production but most potential alkaline protease producing Bacilli are strains of *B. licheniformis, B. subtilis, B amyloliquifaciens, and B. mojavensis.* Another bacterial source known as a potential producer is Pseudomonas sp as reported by, and is considered to be another source of alkaline protease. 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 (Soja *et al.,* 2014).

PROPERTIES OF ALKALINE PROTEASE

Microbial alkaline proteases have been studied and explored and, based on their unique properties; they become useful in various industries. Information regarding their individual properties has been briefly highlighted below:

1. pH and Temperature kinetics

The thermostable nature of alkaline proteases made them detergent-compatible proteases with a high pH optimum. The laundry detergents pH is generally in the range of 8 to 12 and has uniqueness in their thermal stabilities at laundry temperatures (50–70ºC). Commercially available subtilisin-type proteases are mostly known to be active in the pH and temperature ranges 8–12 and 50–70ºC, respectively.

2. Effect of Stabilizers/Additives and Metal Ions

Some of the major commercial uses of alkaline proteases necessitate high temperatures, thus improving the thermal stability of the enzyme is distinctly advantageous. Improvement in thermal stability can be achieved either by adding certain stabilizers (PEG, polyhydric alcohols, starch) to the reaction mixture or by interfering with the tertiary structure of enzymes by protein engineering. The ion Ca 2+ is also known to play a major role in enzyme stabilization by increasing the activity and thermal stability of alkaline protease at higher temperatures. Stability of proteases can also be achieved by the use of metal ions such as Ba 2+, Mn 2+, Mg 2+, Co 2+, Fe 3+ and Zn 2+. These metal ions protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at higher temperatures.

3. Substrate Specificity

Because of their broad substrate specificity, alkaline proteases have been found to be active against a number of natural proteins and synthetic substrates. Furthermore, the literature conclusively suggests that they are more active against casein than against azocasein, hemoglobin or BSA. In addition, different types of alkaline proteases exist, i.e., collagenase, elastase, keratinase and insect cuticle-degrading protease, which are active against specific protein substrates (such as collagen, elastin, keratin, and cuticle).

4. Kinetic Parameters

Enzyme-based development process requires prior knowledge about kinetic parameters of the enzyme in question. This fundamental knowledge is extremely important with respect to efficient and sustainable process development. To be precise, kinetic properties like Vmax, Km, Kcat, and Ea are important, being not only enzyme-specific, but also substrate- and environment specific, and knowledge of these is essential for designing enzyme reactors or quantity of the enzyme under different conditions for its applications. In Terms of popularity, the synthetic substrates are more than the enzyme substrate for defining Km and Vmax as regards convenience. For an alkaline protease from B. mojavensis, the Km for casein decreased with corresponding increase in Vmax, as the reaction temperature was raised from 45 to 60ºC. In comparison, the Km and Vmax for Rhizopus oryzae alkaline protease increased with an increase in temperature from 37ºC to 70ºC (Soja *et al.*, 2014).

ACIDIC PROTEASE

Acid proteases are stable and active between pH 3.8 and 5.6 and are frequently used in soy sauce, protein hydrolysate, and digestive aids and in the production of seasoning material. The optimum pH of acidic proteases is 3–4 and the isoelectric point range is between 3 and 4.5 with a molecular weight of 30–45 kDa. Furthermore, acid proteases are also exploited for use in clearing beer and fruit juice, improving texture of flour paste, and tenderizing the fibril muscle (Zhang *et al.*, 2010).

In comparison with alkaline proteases, these extracellular acid proteases are mostly produced by fungal species, such as *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus awamori*, *Aspergillus fumigatus*, and *Aspergillus saitoi*. Most of the fungal extracellular acid proteases are known as *Aspergilla opepsins.* Aspartic proteases are acid proteases consisting of 380–420 long chains of amino acid residues constituting the active site for catalytic activity. These acidic proteases are endopeptidases and grouped into three families: pepsin (A1), retropepsin (A2), and enzymes from Pará retroviruses (A3). These three families are placed in clan AA. It is found that A1 and A2 are closely related to each other while members of the A3 family

show some relatedness to families A1 and A2. An active site cleft of the members of the pepsin family is located between lobes of a bilobal structure. A great specificity of acidic proteases is exhibited against aromatic amino acid residues located on both sides of the peptide bond. These aromatic amino acid residues with peptide bonds are similar to pepsin but less stringent in action. Broadly, acidic proteases are divided into two groups: (i) pepsin-like enzymes and (ii) rennin-like enzymes produced by Penicillium, Aspergillus, Rhizopus, Endothia, and Mucor (Razzaq *et al.*, 2019).

NEUTRAL PROTEASE

Neutral proteases are defined as, such as they are active at a neutral or weakly acidic or weakly alkaline pH. Mostly neutral proteases belong to the genus Bacillus and with a relatively low thermotolerance ranging from pH 5 to 8. They generate less bitterness in hydrolysis of food proteins due to a medium rate of reaction; therefore, they are considered more valuable in the food industry. Neutrase is incorporated in the brewing industry due to its insensitivity to plant proteinase inhibitors. On the basis of high affinity toward hydrophobic amino acids, neutral proteases are identified and characterized. During production of food hydrolysate, it is slightly advantageous to control the reactivity of neutral proteases due to low thermotolerance. A divalent metal ion is required for the activity of neutral proteases belonging to the metalloprotease type. Metalloproteases based on specificity in action are grouped into (i) neutral, (ii) alkaline, (iii) Myxobacter I, and (iv) Myxobacter II. A specificity of neutral proteases is shown for hydrophobic acids and inhibited by a chelating agent, such as EDTA (Ethylenediamine tetraacetic acid). Among different types of proteases, metalloproteases are the most diverse. Thermolysin, a well-characterized neutral protease having a single peptide without disulfide bridges, is produced by B. stearothermophilus. It has a molecular weight of 34 kDa. Between the 2-folded lobes of a protein, an essential Zn atom and four Ca atoms are embedded, exhibiting thermotolerance. This thermolysin neutral protease is very stable with a half-life of 1 h at 80°C (Razzaq *et al.*, 2019).

ACTIVITY OF PROTEASE IN HUMAN

The activities of proteases in humans include the gastrointestinal tract (i.e., digestion of ingested food proteins), hematological and immunological systems (blood-clotting, complement system, apoptosis cascades, activation of cathelicidin antimicrobial peptide pathway), and epidermal barrier function (i.e., permeability, desquamation). Depending on the amino acid sequence of a given protein, proteases can induce limited proteolysis with cleavage of a specific bond or can cause unlimited proteolysis with conversion of a protein into its component amino acids.The resultant effects induced by a protease enzyme can be (1) to inhibit the function of a physiological protein (i.e., cytokine, chemokine, enzyme) through its breakdown into inactive components, (2) to convert an inactive precursor protein into an active physiological peptide, and (3) to serve as a signaling agent to transactivate the upregulation of a specific process or pathway. Ultimately, both endogenous proteases and protease inhibitors play vital roles that are integral to maintaining the functional and structural integrity of various organ systems and their physiological functions, including skin. Abnormalities in protease function and/or activity may also be operative in certain disease states (i.e., rosacea, atopic dermatitis, psoriasis, immunobullous diseases) (Rosso *et al.*, 2013).

ISOLATION OF SOIL BACTERIA

Quantitative estimation of the number of viable microorganisms in bacteriological samples has been a mainstay of the microbiological laboratory for more than one-hundred years, since Koch first described the technique (Koch, 1883). Serial dilution techniques are routinely used in hospitals, public health, virology, immunology, microbiology, pharmaceutical industry, and food protection (American Public Health, 2005; Hollinger, 1993; Taswell, 1984; Lin and Stephenson, 1998) for microorganisms that can grow on bacteriological media and develop into colonies.The objective of the serial dilution method is to estimate the concentration (number of colonies, organisms, bacteria, or viruses) of an unknown sample by counting the number of colonies cultured from serial dilutions of the sample, and then back track the measured counts to the unknown concentration (David *et al.*, 2014).

Agar pour plate procedures are used routinely for the quantitative determination of aerobic bacteria. Koch introduced the technique in 1880 with the development of agar media, and by 1895 it was a recognized procedure. Analysis of the procedure by Breed and Dotterer in 1916 resulted in the procedures used today. In the aerobic pour plate procedure, an unknown sample is diluted many-fold and a known sample of each dilution is mixed with liquid agar in a petri dish. After incubation, that plate which has between 30 and 300 colonies is counted, and the resultant count is multiplied by the appropriate dilution to obtain the bacterial concentration in the sample. All other plates that did not have between 30 and 300 colonies were discarded. This procedure is simple, can cover a large concentration range, and at present is probably the most precise method for determining those bacteria that will grow in the agar media (Gilchrist *et al.,* 1972).

The agar-streak method described herein has been in use in this and other laboratories since 1940, but has been described only briefly in the literature. It is rapid, it does not require a sterile sample, it permits the testing of unknown substances against several bacteria or fungi at one time, and it can be utilized for testing substances in nonaqueous solutions.

SCREENING

The diffusion process may be defined as the process by which molecules intermingle as a result of their kinetic energy of random motion from high concentration areas to lower ones. The diffusion process depends on numerous factors including number, size and shape of particles. Number of particles is an important factor: the molecules diffuse faster at higher gradients of concentration. Particle volume influences diffusion rate as well: small particles will diffuse faster and large ones will diffuse slower. As the molecule radius increases, we expect diffusivity to decrease proportionally to radius-squared because of solute-solvent increased interactions. Agar well diffusion method proved to be more sensitive than disc diffusion method (Valgas *et al.,* 2007).

BIOCHEMICAL CHARACTERIZATION

The indole test screens for the ability of an organism to degrade the amino acid tryptophan and produce indole (MacWilliams *et al.,* 2012). In 1898 Voges and Proskauer characterized the fermentation of sugars by various bacterial isolates. They showed that the gas produced during fermentation was a mix of CO2 and H2 and that by adding KOH to cultures that had grown in glucose peptone media for prolonged incubation in the presence of oxygen, some organisms produced a red fluorescent color. Although the nature of the coloration was not clear at the time, this method was suggested for differentiation between bacterial isolates that produced the color and those that did not (McDevitt *et al.*, 2009). citrate is the sole source of carbon in the Simmons citrate medium while inorganic ammonium salt ($NH_4H_2PO_4$) is the sole fixed nitrogen source. When an organic acid such as citrate is used as a carbon and energy source, alkaline carbonates and bicarbonates ultimately are produced. The visible presence of growth on the medium and the change in pH indicator color due to the increased pH are the signs that an organism can import citrate and use it as a sole carbon and energy source; such organisms are considered to be citrate positive (MacWilliams *et al.,* 2009).

ISOLATION OF GENOMIC DNA AND PCR METHODS

The isolation and purification of DNA is a key step for most protocols in molecular biology studies and all recombinant DNA techniques (Sambrook *et al.,* 1989). The polymerase chain reaction (PCR) has been used as the new gold standard for detecting a wide variety of templates across a range of scientific specialties, including virology. The method utilises a pair of synthetic oligonucleotides or primers, each hybridising to one stand of a double stranded DNA (dsDNA) target, with the pair spanning a region that will be exponentially reproduced. The hybridised primer act as a substrate for a DNA polymerase (most commonly derived from the thermophilic bacterium Thermus aquatics and called Taq), which creates a complementary strand via sequential addition of deoxynucleotides. The process can be summarised in three steps:

dsDNA separation at temperature > 90 ^{0}C

I. primer annealing at 50- 75 ^{0}C, and

II. optimal extension at 72- 78 ^{0}C.

The rate of temperature change or ramp length of the incubation at each temperature and the number of times each set temperature (or cycles) is repeated are controlled by a programmable thermal cycler (Mackay *et al.*, 2002).

POLYMERASE CHAIN REACTION

Polymerase chain reaction (PCR) is a new, popular molecular biology technique for enzymatically replicating DNA without using a living organism, such as E. coli or yeast. The technique allows a small amount of the DNA molecule to be amplified many times, in an exponential manner. With more DNA available, analysis is made much easier. PCR is commonly used in medical and biological research labs for a variety of tasks, such as the detection of hereditary diseases, the identification of genetic fingerprints, the diagnosis of infectious diseases, the cloning of genes, paternity testing, and DNA computing1. The technique was developed in 1983 by Kary Mullis, PCR is now a common and important technique used in medical and biological research labs for a variety of applications. These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and paternity testing); and the detection and diagnosis of infectious diseases. In 1993, Mullis was awarded the Nobel prize in Chemistry along with Michael Smith for his work on PCR2. The PCR is commonly carried out in a reaction volume of 10-200 ml in small reaction tubes (0.2-0.5 ml volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction. Many modern thermal cyclers make use of the Peltier effect, which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current. Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration. Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermocyclers lacking a heated lid require a layer of oil on top of the reaction mixture or a ball of wax inside the tube.

PCR can be used for Diagnosis of many human diseases, broad variety of experiments and analyses3,4,5. Some examples are discussed below.

1. Infectious diseases HIV, CMV, Mycoplasma, Pneumonia, Cancer, Syphilis, fungal & Protozoal disease, hepatitis etc. 2. Diagnosis of cancer specially leukaemia and lymphomas 3. Genetic fingerprinting, paternity test (Rahman *et al.*,2013).

BLAST

BLAST is an algorithm used for the comparison of amino acid sequences of different proteins or the nucleotides sequences of nucleic acid. BLAST was invented in 1990 and has since become the defacto standard in search and alignment tool. Through a BLAST search, one can compare a query sequence with a database of sequences, and thereby identify library sequences that share resemblance with the query sequence above a certain threshold. Based on such comparison, BLAST can be used to achieve several objectives including species identification, locating domains, DNA mapping and annotation. There are several different types of BLAST programs available, and the choice of a BLAST programme depends on one's objective and the type of sequences being investigated.

ABLAST output can easily be generated by submitting a query sequence at the NCBI site http://blast.ncbi.nlm.nih.gov/ (Donkor *et al.,* 2014).

APPLICATIONS OF MICROBIAL PROTEASE

Generally microbial proteases have a large variety of applications, in various industries. These include food industries, detergent, pharmaceutical industries. The application of these enzymes varies considerably (Mienda *et al.*,2014).

Cutaneous application of proteases

Proteases (*i.e.,* collagenases) have commonly been used for several years in dermatological products applied to debride necrotic and escharotic wounds, such as decubiti and skin ulcers. This

common application may give some clinicians the impression that all proteases are caustic and highly degradative to skin tissue, which is not accurate. Over time, the diversity of proteases and their biological sources, their various properties in skin, chemical modifications of specific proteases to improve stabilization, adjustments in protease concentrations, advances in vehicles to provide better delivery and reduce irritancy, and evaluation of clinical applications have led to the development of protease formulations that appear to be applicable for dermatological usesother than wound debridement. Such applications have been designed to achieve skin cleansing, moisturization, improvements in skin surface characteristics and appearance, restoration and maintenance of SC permeability barrier function, and amelioration of cutaneous inflammation associated with an overstressed or compromised SC (Rossa *et al.,* 2013).

Leather Industry

Increased application of alkaline protease at emerging leather industries is due to the elastolytic and keratinolytic activity. These influential properties of alkaline protease are very effective in leather processing industries. The particular uses of protease are found to be relevant in the soaking, bating, and dehairing phase of preparing skin and hides. Extermination of unwanted pigments by enzymatic measures helps in clean hide production. Enzymatic proceedings of pancreatic proteases rely on the bating system. Microbial alkaline proteases have become very popular in leather industries (Takami *et al.,* 1992; Brandelli *et al.,* 2010).

Detergent Industry

Proteases have been widely used at commercial scale in the detergent industry. The various products in the detergent industry containing proteases as an essential component or ingredienthave been used for cleaning of household laundry, dentures, or contact lenses. Of the total sale of enzymes, the utilization of proteases in the detergent industry accounts for ~20%.

Photographic Industry

Alkaline proteases produced by B. subtilis, Streptomyces avermectnus, and Conidiobolus coronatus have been successfully reported to recover silver from X-ray films, ensuring that the process is eco-friendlier over the use of chemicals (Godfrey and West, 1996; Wolff *et al.,* 1996; Yang Y. et al., 2000). Silver recovery by the efficient use of thermally stable mutant alkaline protease produced by Bacillus sp. B21-2 has also been reported for its potential (Bettiol and Showell, 2002; Dhawan and Kaur, 2007; Araujo *et al.,* 2008).

Chemical Industry

Various alkaline proteases producing microorganisms, such as Bacillus pseudofirus SVB1, Aspergillus flavus, and Pseudomonas aeruginosa PseA showed substantial results in peptide synthesis due to stability in organic solvents (Nakiboglu *et al.,* 2001; Ahmed et al., 2008; Shankar et al., 2010). Some alkaline protease producing species of Bacillus and Streptomyces in the water system are active candidates for peptide and organic synthesis (Masui *et al.,* 2004; Jadhav and Hocheng, 2012; Yadav *et al.,* 2015)

Silk Degumming

A proteinaceous substance, "sericin or silk gum," must be removed by the process of degumming from raw silk in an alkaline solution of soap conventionally. Alkaline protease is the best choice to remove sericin while not attacking the fiber. It has been proven that fiber break is not amenable, and silk threads are found to be much stronger than when previous traditional treatments were used (Yadav *et al.,* 2011; da Silva *et al.,* 2017; Radha *et al.,* 2017).

Medical Field

With the passage of time, scientists have found the broad use of proteases in medical field successfully. In medicine, different formulas, such as gauze, non-woven tissues, and ointment composition containing alkaline proteases produced by B. subtilis show promising therapeutic properties (Sen *et al.,* 2011; Anbu, 2013; Awad *et al.,* 2013). Certain lytic enzyme deficiency syndromes are diagnosed to be aided by an oral administration of alkaline proteases (Gupta

and Khare, 2007; Joshi and Satyanarayana, 2013). It has been reported that fibrin degradation has been achieved by alkaline fibrinolytic proteases. The use of this fibrinolytic enzyme suggests its future application as an anticancer drug and in thrombolytic therapy (Jaouadi *et al.*, 2011, 2012). Slow-release dosage form preparation containing collagenases with alkaline proteases is extensively used in therapeutic applications. The hydrolysis of collagen by the enzyme liberates low-molecular-weight peptides without any amino acid release for therapeutic use (Romsomsa *et al.*, 2010; Suwannaphan *et al.*, 2017). For the treatment of various diseases, such as burns, carbuncles, furuncles, and wounds, a preparation of elastoterase immobilized on bandage is used (Davidenko, 1999; Palanivel *et al.*, 2013).

MATERIALS AND METHODS

1. SOIL SAMPLE COLLECTION

Materials required:

- Polythene bags
- Spatula
- Rubber gloves

Different samples of waste soils such as compost, plastic waste, poultry, agricultural and food waste containing soil etc. were collected from various localities targeting two districts of Kerala, India namely Ernakulam and Kottayam using a sterile spatula and stored in polythene bags. All the essential details such as date and location were properly labelled on the soil samples.

2. ISOLATION OF SOIL BACTERIA

I. SERIAL DILUTION

Materials required:

- Test tubes
- Conical flask
- 1ml sterile tips
- 1000 µl micropipette
- Sterile distilled water
- Test tube rack
- Soil samples

Isolation of soil bacteria from waste soil samples were carried out by Serial dilution technique by weighing 1g of soil sample and adding them to 9 ml sterile water. The water samples were then serially transferred to subsequent test tubes and made upto 10^{-6} dilutions.

II. POUR PLATE TECHNIQUE

Materials required:

- Sterile petri plate
- Nutrient agar
- Diluted samples etc.

After the serial dilution, 1ml of each dilution was transferred onto a sterile petri plate. The molten nutrient agar with an optimum temperature is poured over the sample. The plates are rotated clockwise and anti-clockwise for the evenly distribution of bacteria and nutrient agar and incubated at 370C for 24 hours.

III. QUADRANT STREAKING

Materials required:

- Nutrient agar
- Petri plates
- Inoculation loop etc.

Streak plate technique is generally used for the isolation into a pure culture of the microorganisms (mostly bacteria), from a mixed population. The inoculum is streaked over the nutrient agar surface in such a way that it thins out the bacteria. As the original sample is diluted by streaking it over successive quadrants, the number of organisms decreases. Usually, by the third or fourth quadrant, only a few organisms are transferred which will give discrete colony forming units (CFUs) after 24 hrs of incubation at 37 ^{0}C.

IV. STORAGE OF BACTERIA ISOLATES

Materials required:

- Nutrient agar
- Test tubes
- Inoculation loop
- Bacterial isolates

Nutrient agar slant is the depository for purified bacterial isolates. Bacterial colonies from the streak plates are transferred into the nutrient agar slant using sterile inoculation loop and incubated at 37 ^{0}C for 24 hours. The agar slants are then stored in the refrigerator.

3. SCREENING FOR PROTEOLYTIC BACTERIA

a) SKIM MILK PLATE ASSAY

Materials required:

- Skim milk powder
- Nutrient agar
- Petri plates
- Micropipette and tips
- Bacterial isolates etc.

Skim milk agar is a differential medium that tests the ability of an organism to produce an exoenzyme, called casease, that hydrolyzes casein. Skim milk agar medium is prepared by weighing 1% skim milk powder, autoclaving it and transferring it into a nutrient agar medium at an optimum temperature. Plate is subdivided into 20 sections and bacterial isolate is patched over the skim milk agar plate and incubated for 37 ^{0}C for 24 hours. Proteolytic activity of the bacterial isolates is determined by the appearance of well-defined clearance zones around the bacterial colonies and were further subjected to screening for extracellular protease production.

b) EXTRACELLULAR PROTEASE ACTIVITY

Materials required:

- Gel puncher
- Petri plate
- Nutrient agar
- Skim milk powder
- Bacterial supernatant etc.

Extracellular protease production of the bacterial isolates was screened using well diffusion assay on skim milk agar medium prepared by weighing 1% skim milk powder, autoclaved and dissolved in standard nutrient agar medium. After 30 minutes of solidification, the wells are being made by using a gel puncher. 20µl of crude bacterial supernatant is added to each well. The plates were incubated overnight at 37 ^{0}C for 24 hours. The zone of clearance is observed and the diameter of the clearance zone is measured and tabulated. Bacterial isolates showing promising results in the two screening protocols were further subjected to biochemical and molecular characterization. The most potent strain on the both screening procedures is further subjected to biochemical characterization.

4. BIOCHEMICAL CHARACTERIZATION

I. INDOLE TEST

Materials required:

- Tryptone broth
- Indole reagent
- Test tube
- Bacterial isolates etc.

This biochemical test screens the ability of the microorganisms to hydrolyze the amino acid tryptophan to indole. Tryptophan is hydrolyzed by the tryptophanase into three

products mainly indole, pyruvic acid, ammonium ion. Bacterial isolates are inoculated in 1.5% of tryptone broth and incubated for 370C for 24 hours.

Presence of indole is detected using the Kovac's reagent (isoamyl alcohol, para dimethylaminobenzaldehyde and concentrated HCl). After the addition of Kovac's reagent and mixing the contents, the test tube is allowed to stand. The alcohol layer gets separated from this aqueous layer and upon standing, the reddening of the alcohol layer indicates the presence of indole, positive test. The negative test is indicated by yellow coloration.

II. METHYL RED (MR) TEST

Materials required:

- MR-VP or Glucose phosphate broth
- Bacterial isolates
- Methyl red indicator
- Test tube
- Dropper
- Inoculation loop etc.

MR test detects the production of stable acid like glucose, lactic acid or formic acid as the end product of glucose fermentation. Initially glucose is glycolysis to pyruvic acid, which is further metabolized into stable acids via the mixed acid pathway. Presence of the acids creates an acidic pH in the glucose phosphate broth. The test isolates are inoculated and incubated into the medium containing glucose for 370C for 24 hours. Detection of acid is done by adding drops of methyl red indicator. If the indicator turns red, then it indicates the test isolate is a mixed acid fermenter - a positive reaction. If it's yellow coloration, then the test isolate is not an acid fermenter- a negative reaction.

III. VOGES PROSKAUER (VP) TEST

Materials required:

- MR-VP broth or Glucose phosphate broth
- Bacterial isolates
- 0.5% Barrit solution A and B
- Inoculation loop
- Test tubes
- Dropper etc.

VP test screens the ability of the microorganisms to produce acetoin (acetyl methyl carbinol) as the end product of glucose fermentation. Glucose in the MR-VP broth is glycolysed into pyruvic acid, which is further metabolized to yield acetoin. Test isolates are incubated in the MRVP broth for 370C for 24 hours. 0.5 ml of Barritt A and B solution are added. A positive VP test is represented by the development of red or pink color after 2-5 minutes. A negative VP test is represented by the development yellow brown colour.

IV. CITRATE UTILIZATION TEST

Materials required:

- Simmon's citrate agar with bromothymol blue
- Bacterial isolates
- Test tubes
- Inoculation loop etc.

Citrate agar is used to determine the ability of the microorganisms to utilize the citrate as the energy salt ($NH_4H_2PO_4$) as the source nitrogen source. Citrate Permease, an enzyme capable of converting citrate to pyruvate is produced by the bacteria which can be grown in the Simmon's citrate agar. Pyruvate can then enter the organism's metabolic

cycle for the production of energy. Test isolates are streaked on the Simmon's citrate agar and incubated for 37 ^{0}C for 24 hours. Growth in the agar is due to the utilization of citrate. When the bacteria metabolize citrate, the ammonium salt is broken down to ammonia, which increases alkalinity. This results in the shifting of pH, which turns the bromothymol blue indicator in the medium to green to blue- positive result.

V. CATALASE TEST

Materials required

- 3% hydrogen peroxide solution
- Nutrient broth
- Bacterial isolates
- Inoculation loop etc.

This test detects the presence of catalase, an enzyme that converts the hydrogen peroxide (H_2O_2) into oxygen (O_2) and water (H_2O). To the nutrient broth inoculated with bacterial suspension and incubated for 37 ^{0}C for 24 hours, 3% H_2O_2 were added. The presence of catalase in the bacterial isolates is evident by a rapid elaboration of oxygen bubbles. The lack of catalase is indicated by a weak bubble production.

5. KIRBY BAUER ANTIBIOTIC SENSITIVITY TEST

Materials required:

- Nutrient agar
- Bacterial inoculum
- Sterile petri-plates
- Sterile forceps
- L-rod
- Antibiotic disc etc.

The Kirby- Bauer test is used to determine the resistance or sensitivity of a bacteria to a particularly dosed antibiotic. The bacterial isolates are lawned using L-rod on the nutrient agar plate. The antibiotic disc of known dosage is placed on the top of the nutrient agar plate by a sterile forceps and incubated for 37 ^{0}C for 24 hours. The antibiotic diffuses from the disc into the agar in the decreasing amount as it further moves away from the disc. No growth is observed if the bacteria is killed or inhibited by the antibiotic. Zone of inhibition (clear zone) indicates the bacteria is susceptible to that particular antibiotic. The diameter of the inhibition zone was measured and the test isolates were categorized as antibiotic-sensitive or moderately sensitive or resistant.

6. MOLECULAR IDENTIFICATION OF THE BACTERIAL ISOLATES

I. GENOMIC DNA ISOLATION (Ausubel *et al.*, 1996)

Materials required:

- Bacterial isolates
- microcentrifuge tubes
- TE buffer
- Proteinase K
- 10% SDS
- Phenol:Chloroform:Isoamyl alcohol mixture
- Sodium acetate
- Isopropyl alcohol
- 70% Ethanol etc.

For molecular identification, the genomic DNA was isolated from the bacterial isolates showing antibacterial activity against the test organisms. Bacterial culture was carefully transferred into a microcentrifuge tube and allowed to spin for 10 minutes at 8000 rpm.

Supernatant was discarded. The pellet was resuspended in 875 µL of Tris-EDTA (TE) buffer. To this 5 µL Proteinase K and 100 µL of Sodium Dodecyl Sulphate (SDS) was added and incubated in a water bath at 37°C for 1 hour. Equal volume of Phenol:Chloroform:Isoamyl Alcohol (25:24:1) mixture was added and mixed properly by inverting the tubes until a white precipitate is seen and centrifuged at 8000 rpm for 5 minutes. With the help of a wide mouthed tip the upper aqueous layer was carefully collected into a fresh tube. The process was repeated twice. Equal volume of Chloroform was added and centrifuged for 5 minutes at 8000 rpm. The upper layer was collected into a fresh tube. To this 0.1 volume Sodium acetate and double volume ice cold Isopropyl alcohol was added and centrifuged at 16000 rpm for 10 minutes. The supernatant was decanted and pellet was washed in 1 ml 70% ethanol. The alcohol was decanted and the pellet was air dried. The pellet was dissolved in a minimum volume TE buffer (100µL) and stored at -20°C.

II. AGAROSE GEL ELECTROPHORESIS (Sambrook *et al.*, 2000)

Materials required:

- Electrophoresis buffer (1X TAE buffer)
- Electrophoresis grade agarose
- DNA molecular weight markers
- Horizontal gel electrophoretic apparatus
- Gel casting platform Gel comb
- DC power supply
- Ethidium bromide for staining DNA
- 10X gel loading buffer
- DNA samples.

Prepare the agarose gel by mixing 0.5g Agarose in 50 ml 1X TAE and heat the solution to dissolve the agarose. Cool the gel at room temperature. 10µl of the Ethidium bromide dye is added to the gel preparation. Pour the gel into the gel casting tray by placing the comb (to create wells) and allow it to stand for 1 hour undisturbed. Remove the comb

from the set gel and the gel is placed in the gel tank filled with 1X TAE. 5µL of each DNA sample was mixed with 1 µL of 6X gel loading dye on a parafilm. With the loaded wells with the samples the power is switched on. After the samples have reached the end of the gel, the power is switched off and bands are visualized using the Gel documentation system.

III. PCR AMPLIFICATION OF 16S rDNA

Materials required:

- PCR Thermal cycler
- Template DNA16S rDNA Forward primer, Reverse primer
- 5X assay buffer
- dNTP mix
- MgCl2
- Taq Polymerase (5U/ µl)
- Sterile water
- PCR tubes
- Microtips.

The forward and reverse primers used for the amplification of 16S rDNA sequences of the isolated DNA are given in **Table 1.**

Table 1 showing Primers used to amplify 16S rDNA

Primer	Sequence	Reference
16 SrRNA Forward	5'- AGAGTTTGATCCTGGCTCAG - 3'	Shivaji *et al.*, 2000
16 SrRNA Reverse	5'- ACGGCTACCTTGTTACGACTT – 3'	

The concentration of different PCR ingredients is given in **Table 2**

Table 2 showing PCR Master Mix Preparation

Sl.no	Ingredients	Volume in µl
1.	Molecular biology grade water	28.25
2.	5X assay buffer	10
3.	Template DNA	2.5
4.	Forward primer	2.5
5.	Reverse primer	2.5
6.	25mM $MgCl_2$	3
7.	10mM dNTP mix	1
8.	Taq Polymerase (5U/µl)	0.25
	Total	50

The contents are mixed thoroughly and placed in a thermocycler block.

PCR Program

PCR amplification was carried out in a Thermal Cycler (BioRad MJ Mini Gradient, CA, USA) using the following program **(Table 3)** with the lid temperature 105°C and volume as 50 µL.

Table 3 showing the program for PCR

Step	Temperature	Time	
Initial Denaturation	95°C	2 min	
Denaturation	95°C	30 s	35 cycles
Annealing	56°C	30sec	
Extension	72°C	2 min	
Final Extension	72°C	5 min	

The amplicon was then electrophoresed and visualized using a gel documentation system and was sent for sequencing.

IV. DNA SEQUENCING AND BIOINFORMATIC ANALYSIS

The amplified 16S rDNA was sequenced by Sanger's Dideoxy method at Agrigenome Labs, Kochi, Kerala. The identity of the sequences was determined by comparing with the sequences in the NCBI database using the online tool BLAST (Altschul *et al.*, 1990). For this, the sequences in Fasta format were pasted on the NCBI BLAST page (http://blast.ncbi.nlm.nih.gov) and nBlast was carried out.

RESULT

1. SOIL SAMPLE COLLECTION

About nine waste soil samples were collected from various locations in Ernakulam and Kottayam districts of Kerala, India which included compost soil, plastic waste soil,poultry farm soil, agricultural and food waste soil etc and were designated as in **Table 4**.

Table 4 showing sampling locations and codes

SL.NO	LOCATION	TYPE OF WASTE	SAMPLE CODE
	DISTRICT - ERNAKULAM		
1.	Kalamassery	Agricultural and plastic	ZKM
2.	Convent Junction	Plastic	ZCJ
3.	Mullassery Canal road	Agricultural	ZMC
4.	Willingdon Island	Agricultural	ZWI
5.	Marakadavu	Poultry	ZMA
6.	Varapuzha	Agricultural	ZVP
	DISTRICT - KOTTAYAM		
7.	Kalakatti	Agricultural	ZKK
8.	Kanjirapally Cooperation	Agricultural	ZKC
9.	Manjakulam	Poultry	ZMM

2. ISOLATION OF SOIL BACTERIA

Soil bacteria were isolated using serial dilution and pour plate technique and a total of 78 bacterial isolates with discrete morphology were obtained and allotted the following culture codes as represented in **Table 5.**

Table 5 representing the list of bacterial samples with the culture codes

SAMPLE NUMBER	SAMPLE CODE	SAMPLE NUMBER	SAMPLE CODE	SAMPLE NUMBER	SAMPLE CODE	SAMPLE NUMBER	SAMPLE CODE
1	**ZWI 1**	25	**ZVP 4**	49	**ZMM 9**	73	**ZMA 18**
2	**ZWI 2**	26	**ZVP 5**	50	**ZMM 10**	74	**ZMA 19**
3	**ZMC 1**	27	**ZVP 6**	51	**ZMM 11**	75	**ZMA 20**
4	**ZMC 2**	28	**ZVP 7**	52	**ZMM 12**	76	**ZMA 21**
5	**ZMC 3**	29	**ZVP 8**	53	**ZMM 13**	77	**ZMA 22**
6	**ZMC 4**	30	**ZVP 9**	54	**ZMM14**	78	**ZMA 23**
7	**ZMC 5**	31	**ZVP 10**	55	**ZMM 15**		
8	**ZMC 6**	32	**ZVP 11**	56	**ZMA 1**		
9	**ZKM 1**	33	**ZKC 1**	57	**ZMA 2**		
10	**ZKM 2**	34	**ZKC 2**	58	**ZMA 3**		
11	**ZKM 3**	35	**ZKC 3**	59	**ZMA 4**		
12	**ZKM 4**	36	**ZKC 4**	60	**ZMA 5**		
13	**ZKK 1**	37	**ZKC 5**	61	**ZMA 6**		
14	**ZKK 2**	38	**ZKC 6**	62	**ZMA 7**		
15	**ZKK 3**	39	**ZKC 7**	63	**ZMA 8**		

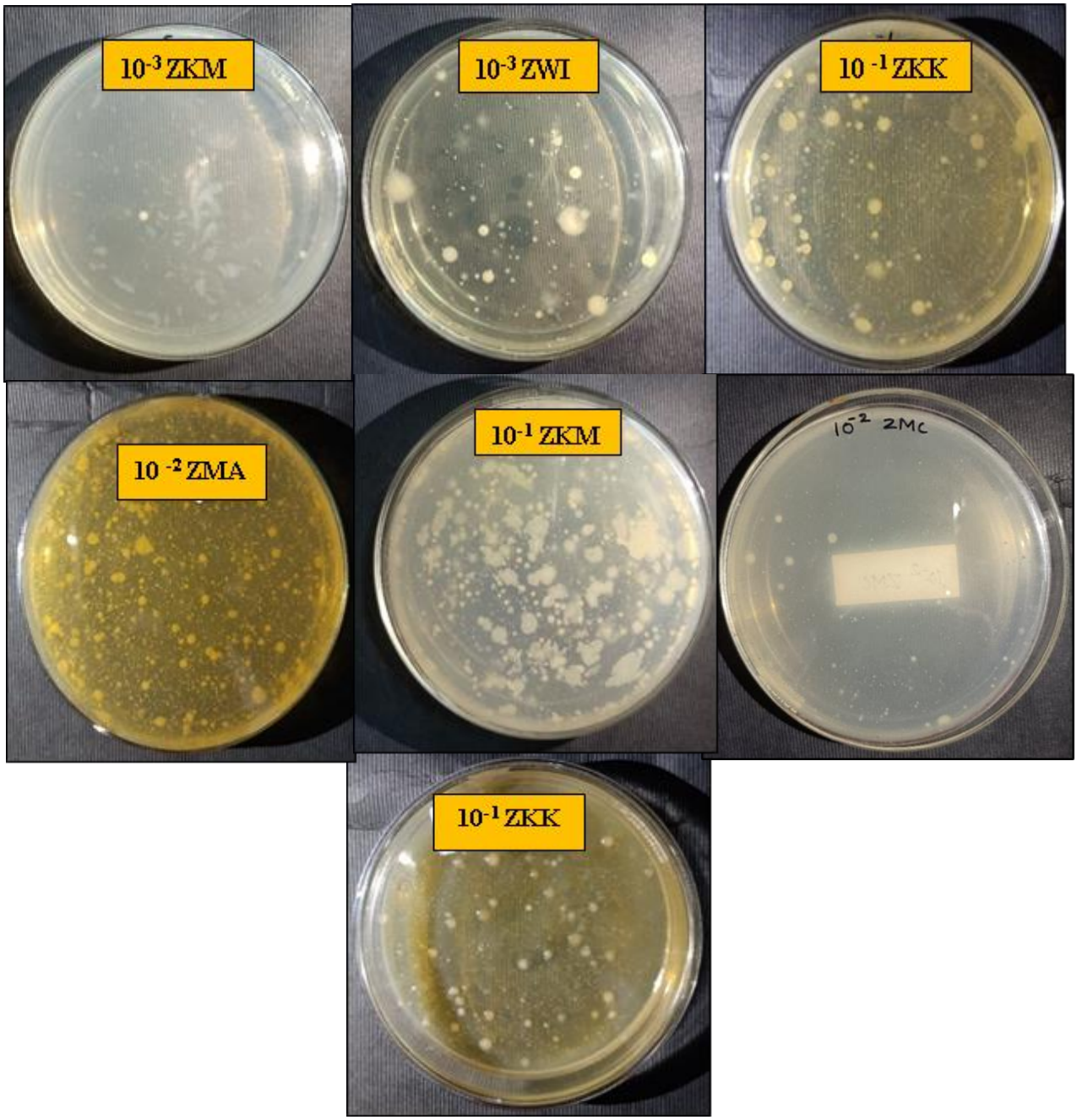


Figure 1 displaying bacterial colonies obtained after serial dilution and pour plate technique

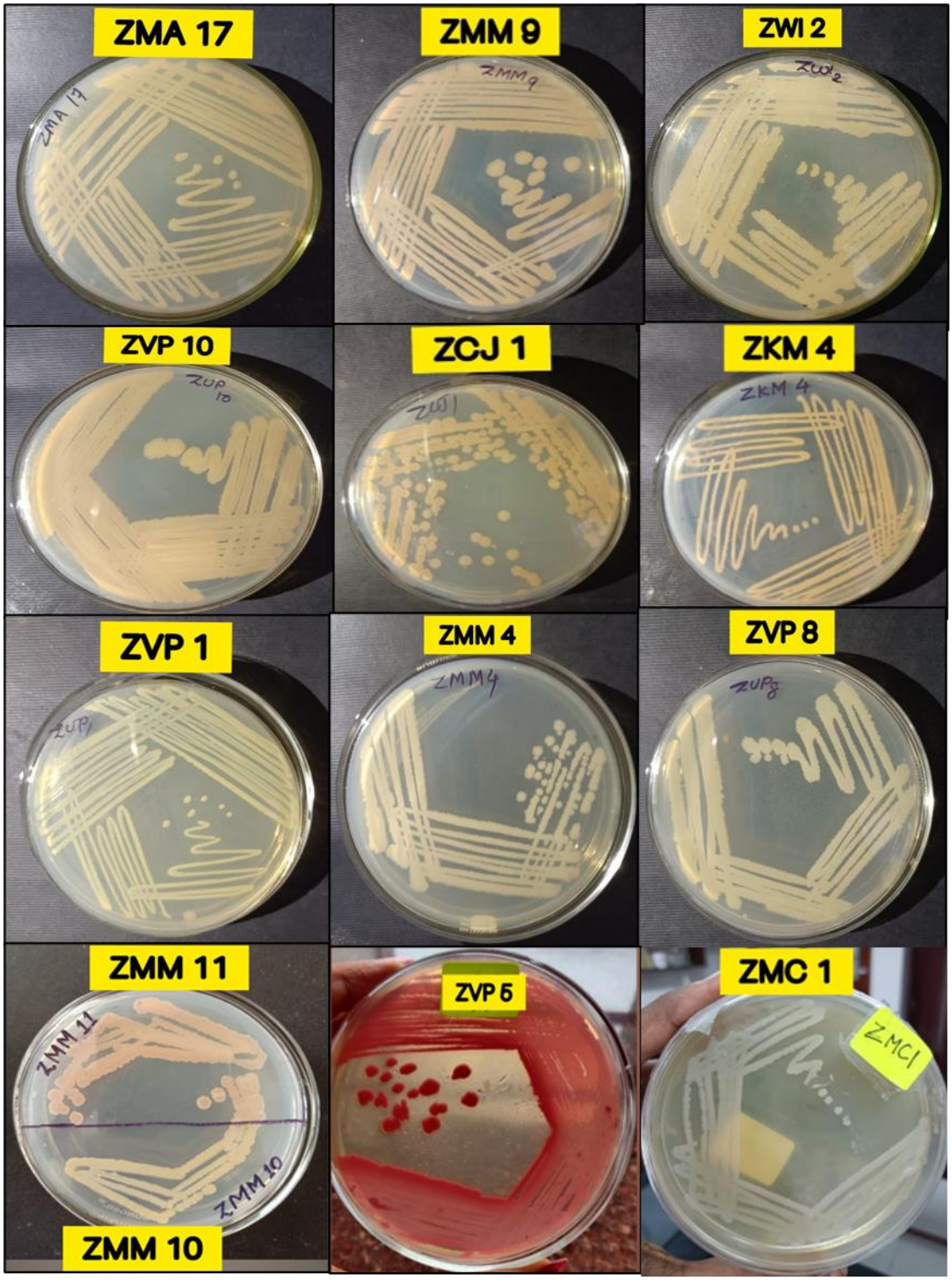


Figure 2 depicting the isolated pure bacterial culture by Quadrant streaking

3. SCREENING FOR PROTEOLYTIC BACTERIA

a) SKIM MILK PLATE ASSAY

A total of 78 isolates of bacteria were screened for proteolytic activity using patch plate technique on skim milk agar medium and proteolytic activity of the bacterial isolates were determined by the appearance of well-defined clearance zones around the bacterial colonies as indicated in **Table 6.**

Table 6 illustrating bacterial isolates subjected to proteolytic screening using skim milk plate assay

SL. NO	ISOLATES	TEST RESULTS
1.	ZWI 1	+
2.	ZWI 1	+
3.	ZMC 1	+
4.	ZMC 2	+
5.	ZMC 3	+
6.	ZMC 4	+
7.	ZMC 5	+
8.	ZMC 6	+
9.	ZKM 1	+
10.	ZKM 2	+
11.	ZKM 3	+
12.	ZKM 4	+
13.	ZKK 1	+
14.	ZKK 2	+
15.	ZKK 3	+
16.	ZKK 4	-
17.	ZKK 5	+
18.	ZKK 6	+
19.	ZCJ 1	+
20.	ZCJ 2	+
21.	ZCJ 3	-

22.	ZVP 1	+
23.	ZVP 2	-
24.	ZVP 3	-
25.	ZVP 4	-
26.	ZVP 5	+
27.	ZVP 6	-
28.	ZVP 7	-
29.	ZVP 8	+
30.	ZVP 9	+
31.	ZVP 10	-
32.	ZVP 11	+
33.	ZKC 1	+
34.	ZKC 2	+
35.	ZKC 3	-
36.	ZKC 4	-
37.	ZKC 5	+
38.	ZKC 6	+
39.	ZKC 7	+
40.	ZKC 8	+
41.	ZMM 1	+
42.	ZMM 2	+
43.	ZMM 3	+
44.	ZMM 4	+
45.	ZMM 5	-
46.	ZMM 6	+
47.	ZMM 7	+
48.	ZMM 8	+
49.	ZMM 9	+
50.	ZMM 10	+
51.	ZMM 11	+
52.	ZMM 12	+
53.	ZMM 13	+
54.	ZMM 14	+
55.	ZMM 15	+
56.	ZMA 1	+
57.	ZMA 2	+
58.	ZMA 3	-

59.	ZMA 4	-
60.	ZMA 5	-
61.	ZMA 6	+
62.	ZMA 7	+
63.	ZMA 8	-
64.	ZMA 9	-
65.	ZMA 10	-
66.	ZMA 11	+
67.	ZMA 12	+
68.	ZMA 13	+
69.	ZMA 14	-
70.	ZMA 15	-
71.	ZMA 16	-
72.	ZMA 17	-
73.	ZMA 18	-
74.	ZMA 19	+
75.	ZMA 20	-
76.	ZMA 21	-
77.	ZMA 22	-
78.	ZMA 23	+

+: Activity -: No activity

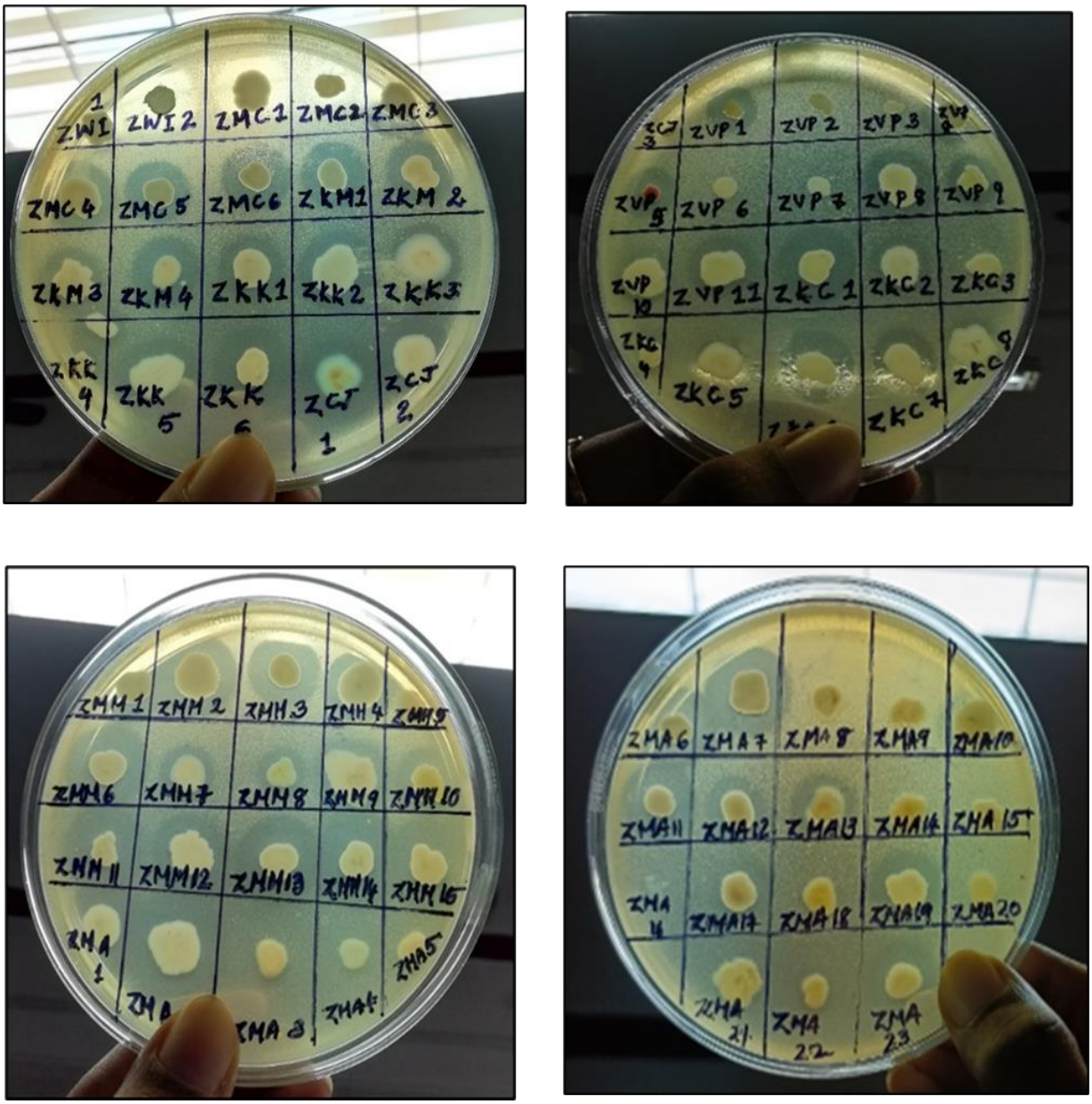


Figure 3 showing bacterial isolates with clear zones on Skim Milk agar medium

b) EXTRACELLULAR PROTEASE ACTIVITY

From the positive isolates obtained, the most potent strain defined by well manifested clear zones were chosen and subjected to extracellular protease activity by well diffusion method on skim milk agar medium. The zone of clearance was detected and the diameter of the clearance zone was measured and tabulated as in **Table 7** and portrayed in **Figure 4**. Eight isolates with remarkable protease activity were subjected to various biochemical analysis.

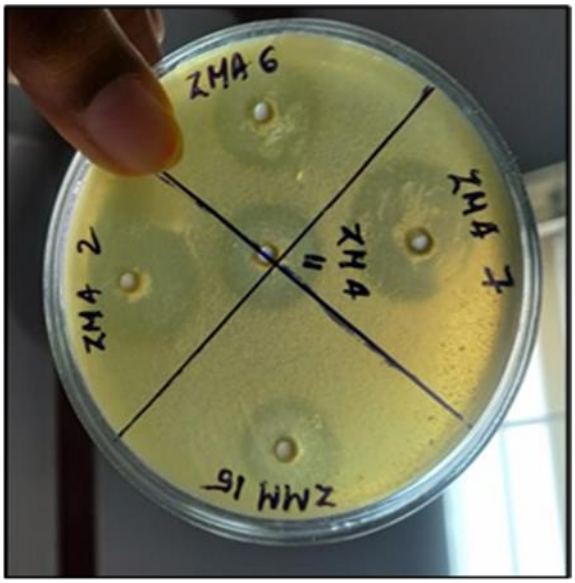
Table 7 enlisting the bacterial isolates with the extracellular protease activity

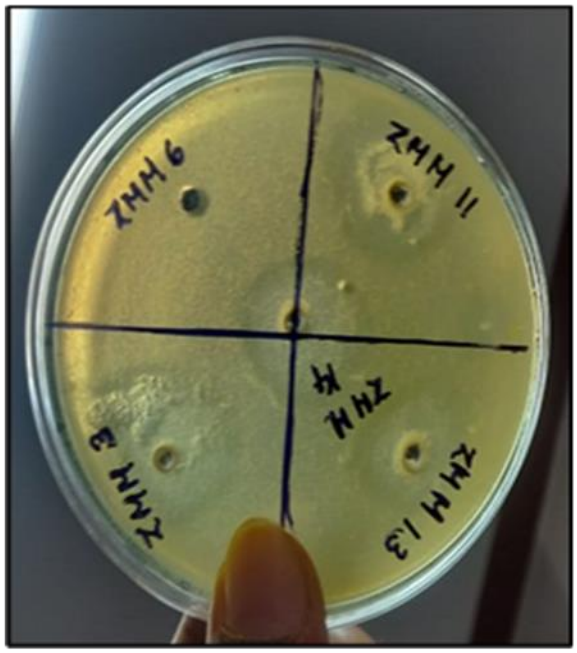
SL. NO	TEST ISOLATES	ZONE OF INHIBITION	ZONE DIAMETER (MM)
1.	ZVP 5	+	2.6
2.	ZMA 6	+	1.7
3.	ZMA 7	+	2.2
4.	ZMM 15	+	1.5
5.	ZMA 2	+	2.2
6.	ZMA 11	+	.15
7.	ZMM 6	-	-
8.	ZMM 11	+	2.1
9.	ZMM 13	+	1.9
10.	ZMM 3	+	2.2
11.	ZMM 14	+	2.0
12.	ZMC 1	+	2.3
13.	ZMC 4	+	2.7
14.	ZMC 5	+	2.2
15.	ZWI 2	+	2.4
16.	ZMC 6	+	2.1
17.	ZVP 9	+	2.2
18.	ZKC 1	+	1.9

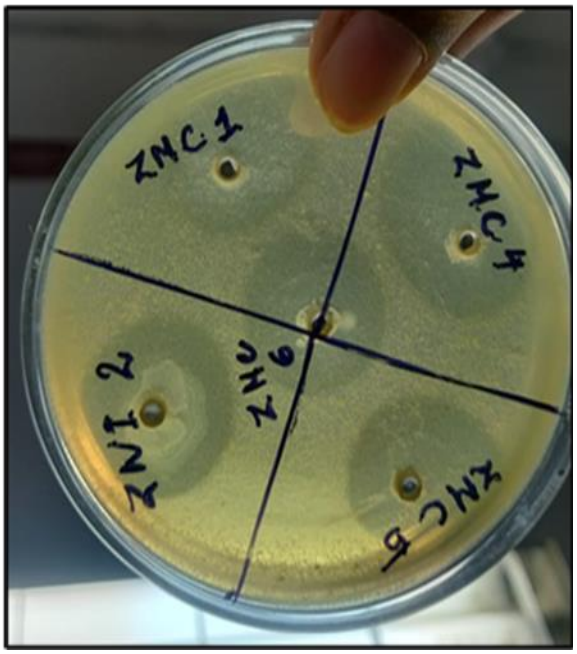
19.	ZKC 6	-	-
20.	ZMM 2	+	2.2
21.	ZKC 7	+	1.9
22.	ZVP 1	+	2.1
23.	ZMA 23	+	1.9
24.	ZCJ 1	+	2.5
25.	ZCJ 2	+	2.4
26.	ZVP 8	+	2.8
27.	ZKM 1	+	2.0
28.	ZKM 2	+	2.3
29.	ZKM 4	+	1.7
30.	ZKK 1	+	2.5
31.	ZKK 3	-	-

+: Activity -: No activity


ZVP 5


ZMA 6
ZMA 7
ZMA 2
ZMA 4
ZMM 16


ZMM 6
ZMM 11
ZMM 3
ZMM 13


ZNC 1
ZNC 4
ZNC 2
ZNC 6
ZNC 5

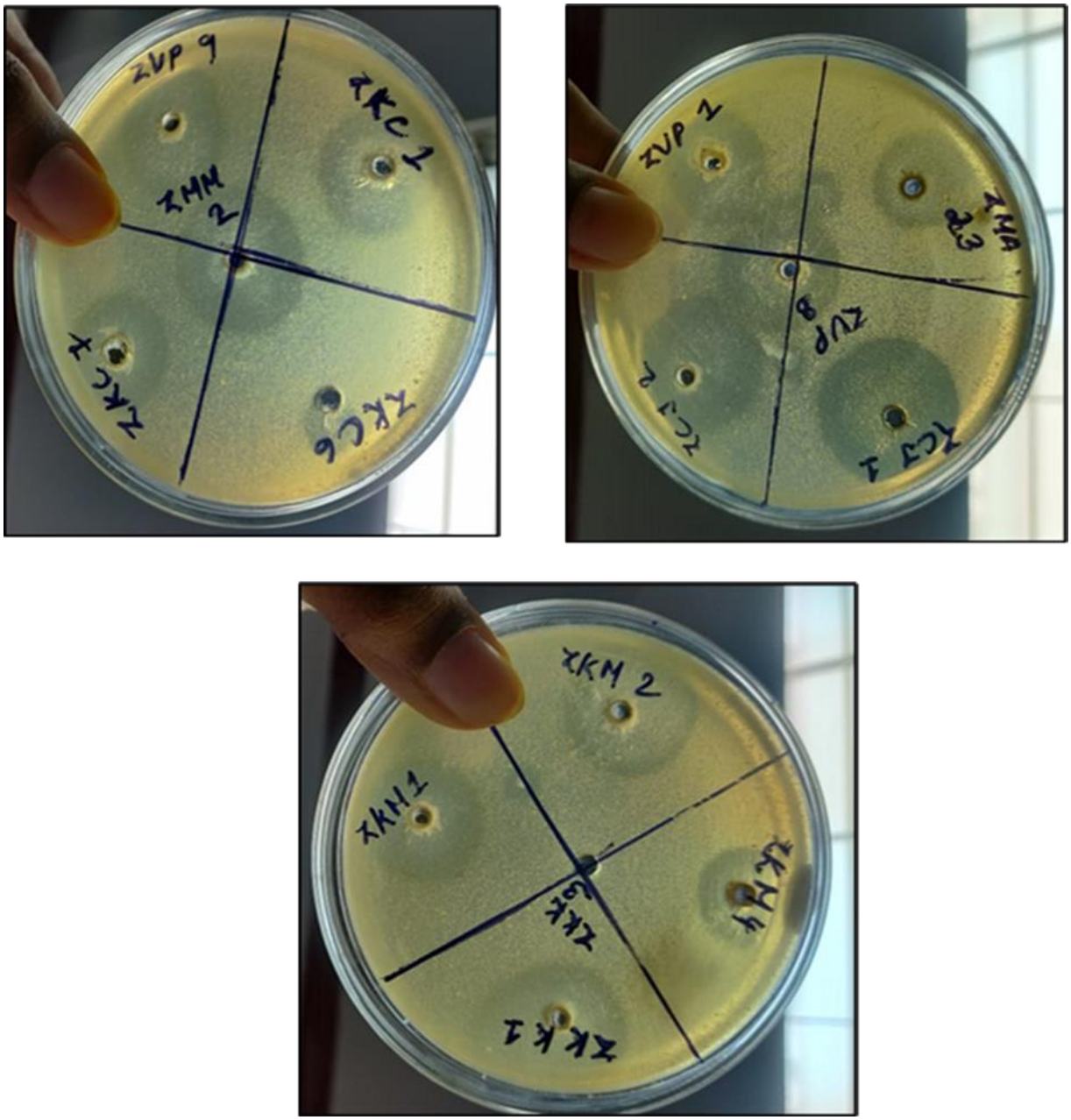


Figure 4 showing bacterial isolates with extracellular proteolytic activity by well diffusion assay

3. BIOCHEMICAL CHARACTERIZATION

I. INDOLE TEST

Presence of indole was detected by the addition of Kovac's reagent into incubated tryptophan broth with bacterial isolates. Among the eight isolates screened, all the bacterial isolates were indole test negative marked by the appearance of yellow coloration in the tryptone broth **(Figure 5)** and indicated in **Table 8**.

Table 8 showing Indole test results

SL. NO	TEST ISOLATES	RESULTS
1.	ZCJ 1	-
2.	ZVP 8	-
3.	ZVP 5	-
4.	ZKM 4	-
5.	ZKM 2	-
6.	ZVP 1	-
7.	ZMC 1	-
8.	ZWI 2	-

+: Activity -: No activity

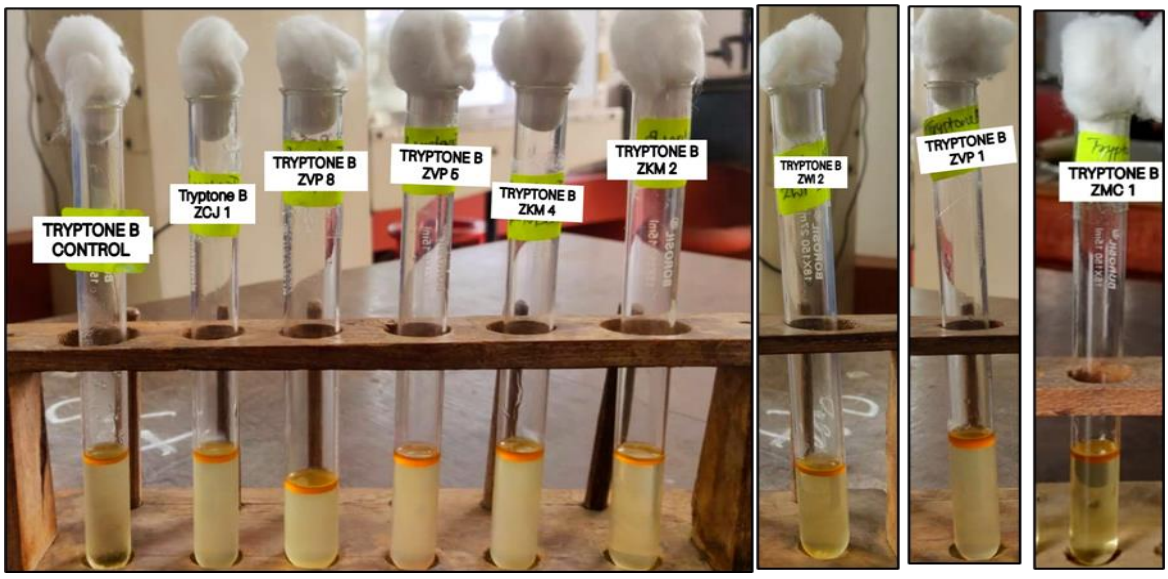


Figure 5 representing Indole production test results

II. METHYL RED (MR) TEST

Presence of stable acids like formic acid or lactic acid formed by glucose fermentation is detected by the addition of methyl red indicator to the incubated glucose phosphate broth. Among the eight isolates screened, five isolates namely ZKM 2, ZCJ 1, ZVP 8, ZVP 5 and ZMC 1 developed red colouration in the glucose phosphate broth and hence were confirmed as MR test positive and three isolates ZKM 4, ZWI 2 and ZVP 1 were confirmed as MR test negative due to the appearance of yellow colouration **(Figure 6)** and indicated in **Table 9**.

Table 9 showing methyl red test results

SL. NO	TEST ISOLATES	RESULTS
1.	ZKM 2	+
2.	ZCJ 1	+
3.	ZVP 8	+
4.	ZVP 5	+
5.	ZKM 4	-
6.	ZMC 1	+
7.	ZWI 2	-
8.	ZVP 1	-

+: Activity -: No activity

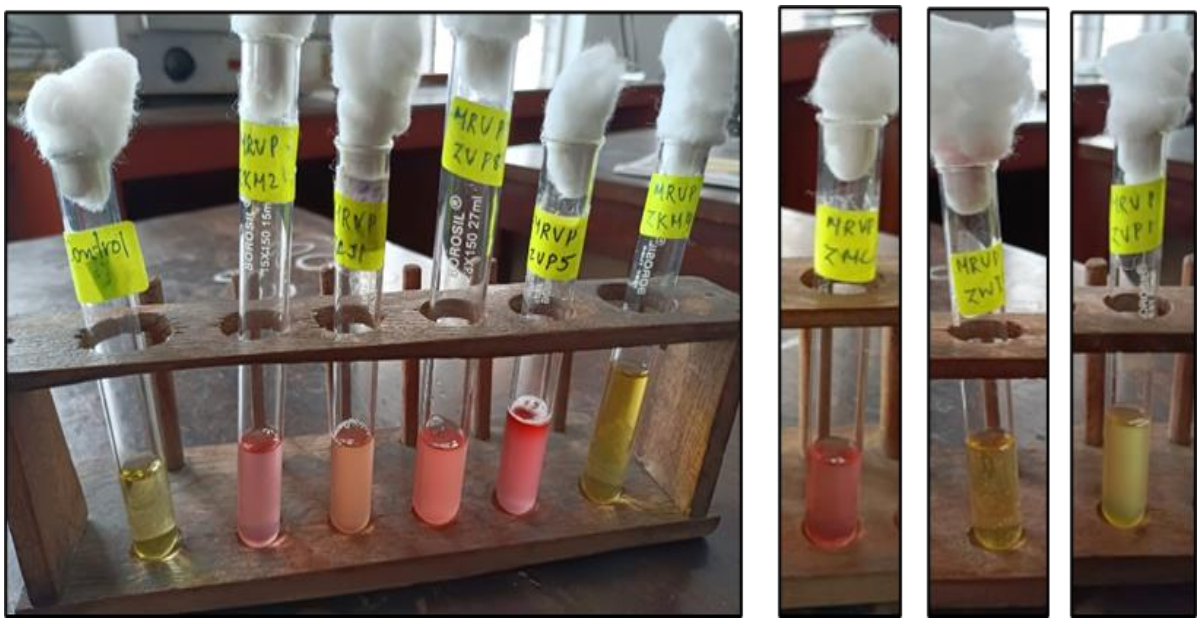


Figure 6 depicting Methyl Red (MR) test results

III. VOGES PROSKAUER (VP) TEST

Presence of acetoin from glucose is detected by the addition of 0.5% Barrit solution A and B to the incubated MRVP or glucose phosphate broth. Among the eight isolates tested, two isolates namely ZVP 5, ZVP 1 showed positive result by the development of red coloration and remaining isolates namely ZKM 2, ZKM 4, ZCJ 1, ZVP 8, ZWI 2 and ZMC 1 developed a yellow brown colouration indicating VP test negative **(Figure 7)** and depicted in the **Table 10**.

Table 10 showing Voges Proskauer test results

SL. NO	TEST ISOLATES	RESULTS
1.	ZKM 2	-
2.	ZKM 4	-
3.	ZCJ 1	-
4.	ZVP 5	+
5.	ZVP 8	-
6.	ZVP 1	+
7.	ZWI 2	-
8.	ZMC 1	-

+: Activity -: No activity

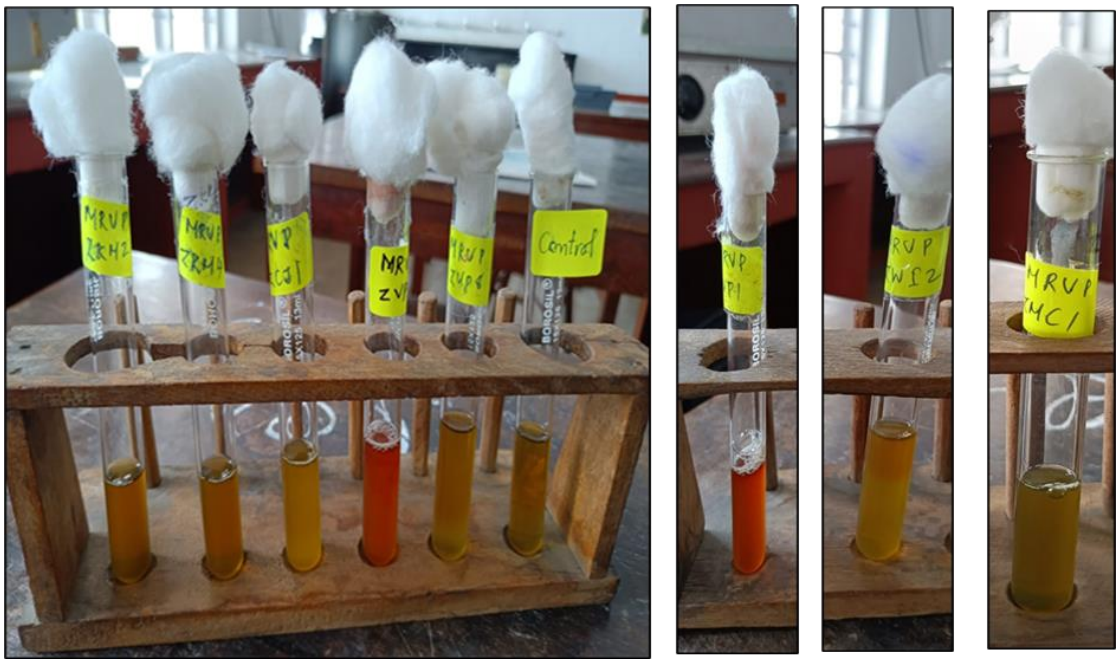


Figure 7 showing Voges Proskauer (VP) test results

IV. CITRATE UTILIZATION TEST

The Simmon's citrate agar containing the indicator Bromothymol blue was inoculated with the bacterial isolates and incubated at 370C for 24 hours. Among the eight isolates, seven isolates namely ZCJ 1, ZKM 2, ZVP 5, ZVP 8, ZMC 1, ZWI 2, ZVP 1 utilised the citrate in the Simmon's citrate agar indicated by the blue colouration and remaining isolate ZKM 4 indicated negative without the colour change **(Figure 8)** and indicated in **Table 11**.

Table 11 showing citrate utilization test results

SL. NO	TEST ISOLATES	RESULTS
1.	ZCJ 1	+
2.	ZKM 2	+
3.	ZVP 5	+
4.	ZVP 8	+
5.	ZKM 4	-
6.	ZMC 1	+
7.	ZWI 2	+
8.	ZVP 1	+

+: Activity -: No activity

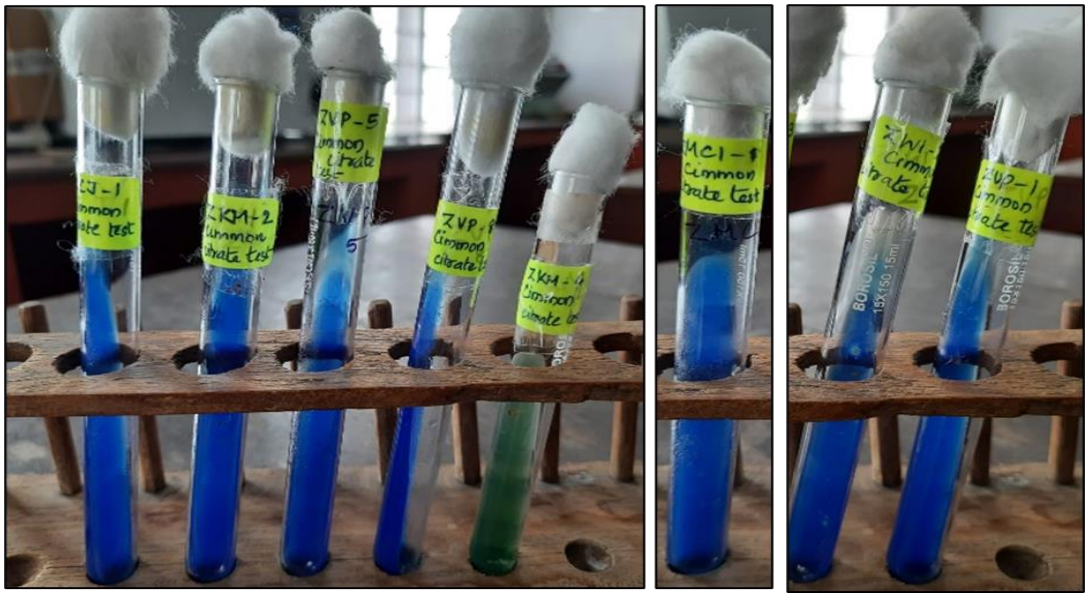


Figure 8 depicting Citrate Utilization test results

V. CATALASE TEST

To the bacterial inoculum, 3% of H2O2 were added. All bacterial isolates namely ZVP 8, ZKM 2, ZVP 5, ZCJ 1, ZKM 4, ZMC 1, ZWI 2, ZVP 1 exhibited positive results with the evolution of brisk effervescences **(Figure 9)** and indicated in **Table 12**.

Table 12 depicting Catalase test results

SL. NO	TEST ISOLATES	RESULTS
1.	ZVP 8	+
2.	ZKM 2	+
3.	ZVP 5	+
4.	ZCJ 1	+
5.	ZKM 4	+
6.	ZMC 1	+
7.	ZWI 2	+
8.	ZVP 1	+

+: Activity -: No activity

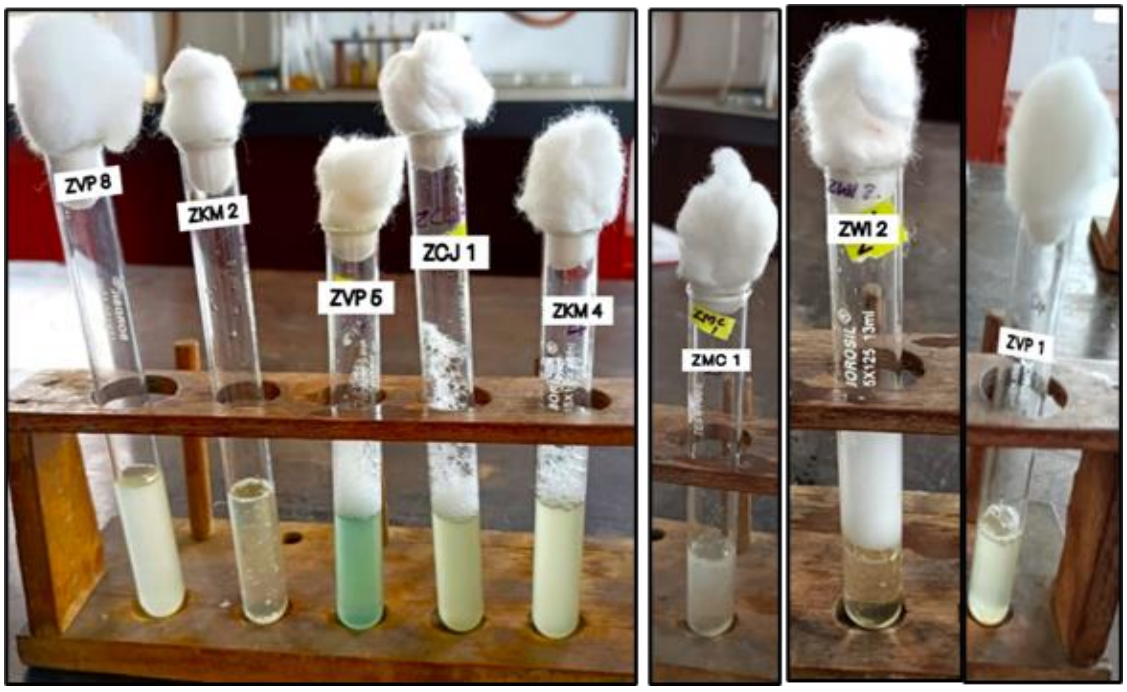


Figure 9 depicting Catalase test results

4. KIRBY BAUER ANTIBIOTIC SENSITIVITY TEST

Each bacterial isolates were swabbed onto the nutrient agar medium and the chosen antibiotics were placed on the inoculated test organism. After 24 hours of incubation the zone of clearance of each antibiotic were measured and tabulated in **Tables 13-20 and indicated in figures.**

Table 13 illustrating Kirby Bauer antibiotic sensitivity test results of ZVP 5

Sl.No	Antibiotics (Dose)	Test isolate	Zone of Diameter (mm)	Resistant	Moderately sensitive	Susceptible
1.	Streptomycin (25 µg)	ZVP 5	19	-	✓	-
2.	Chloramphenicol (30 µg)		17	-	-	✓
3.	Ampicillin (10 µg)		-	✓	-	-
4.	Gentamicin (30 µg)		21	-	-	✓
5.	Tetracycline (30 µg)		-	✓	-	-
6.	Cloxacillin (1 µg)		-	✓	-	-
7.	Penicillin (10 µg)		-	✓	-	-
8.	Erythromycin (15 µg)		-	✓	-	-
9.	Nalidixic acid (30 µg)		26	-	-	✓
10.	Cefuroxime (30 µg)		15	-	✓	-

Figure 10 depicting Kirby Bauer antibiotic sensitivity test results of ZVP 5

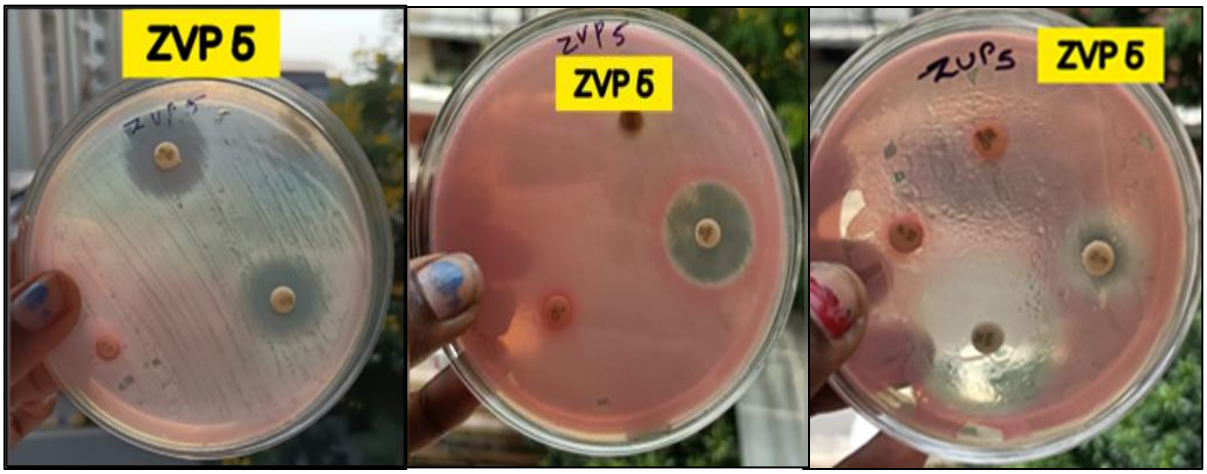


Table 14 illustrating Kirby Bauer antibiotic sensitivity test results of ZCJ 1

Sl.No	Antibiotics (Dose)	Test isolate	Zone of Diameter (mm)	Resistant	Moderately sensitive	Susceptible
1.	Streptomycin (25 µg)	**ZCJ 1**	11	✓	-	-
2.	Chloramphenicol (30 µg)		-	✓	-	-
3.	Ampicillin (10 µg)		-	✓	-	-
4.	Gentamicin (30 µg)		19	-	-	✓
5.	Tetracycline (30 µg)		-	✓	-	-
6.	Cloxacillin (1 µg)		-	✓	-	-
7.	Penicillin (10 µg)		-	✓	-	-
8.	Erythromycin (15 µg)		-	✓	-	-
9.	Nalidixic acid (30 µg)		10	✓	-	-
10.	Cefuroxime (30 µg)		-	✓	-	-

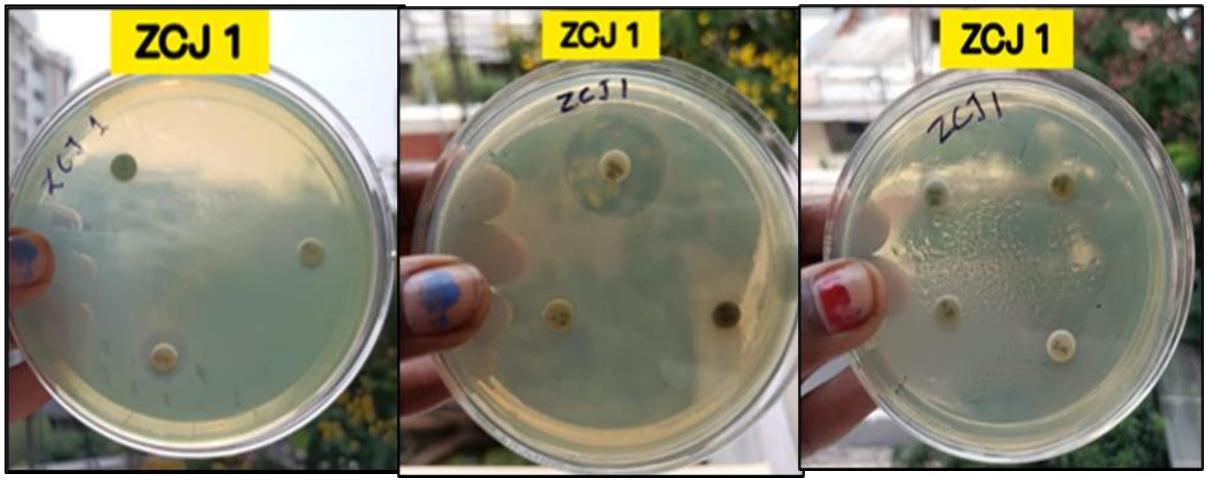


Figure 11 depicting Kirby Bauer antibiotic sensitivity test results of ZCJ 1

Table 15 illustrating Kirby Bauer antibiotic sensitivity test results of ZVP 1

Sl.No	Antibiotics (Dose)	Test isolate	Zone of Diameter (mm)	Resistant	Moderately sensitive	Susceptible
1.	Streptomycin (25 µg)	ZVP 1	14	✓	-	-
2.	Chloramphenicol (30 µg)		19	-	-	✓
3.	Ampicillin (10 µg)		11	✓	-	-
4.	Gentamicin (30 µg)		8	✓	-	-
5.	Tetracycline (30 µg)		-	✓	-	-
6.	Cloxacillin (1 µg)		-	✓	-	-
7.	Penicillin (10 µg)		-	✓	-	-
8.	Erythromycin (15 µg)		20	-	✓	-
9.	Nalidixic acid (30 µg)		22	-	-	✓
10.	Cefuroxime (30 µg)		-	✓	-	-

Figure 11 depicting the Kirby Bauer antibiotic sensitivity test results of ZVP 1

Table 16 illustrating Kirby Bauer antibiotic sensitivity test results of ZWI 2

Sl.No	Antibiotics (Dose)	Test isolate	Zone of Diameter (mm)	Resistant	Moderately sensitive	Susceptible
1.	Streptomycin (25 µg)	ZWI 2	28	-	-	✓
2.	Chloramphenicol (30 µg)		27	-	-	✓
3.	Ampicillin (10 µg)		-	✓	-	-
4.	Gentamicin (30 µg)		25	-	-	✓
5.	Tetracycline (30 µg)		11	✓	-	-
6.	Cloxacillin (1 µg)		-	✓	-	-
7.	Penicillin (10 µg)		-	✓	-	-
8.	Erythromycin (15 µg)		-	✓	-	-
9.	Nalidixic acid (30 µg)		10	✓	-	-
10.	Cefuroxime (30 µg)		-	✓	-	-

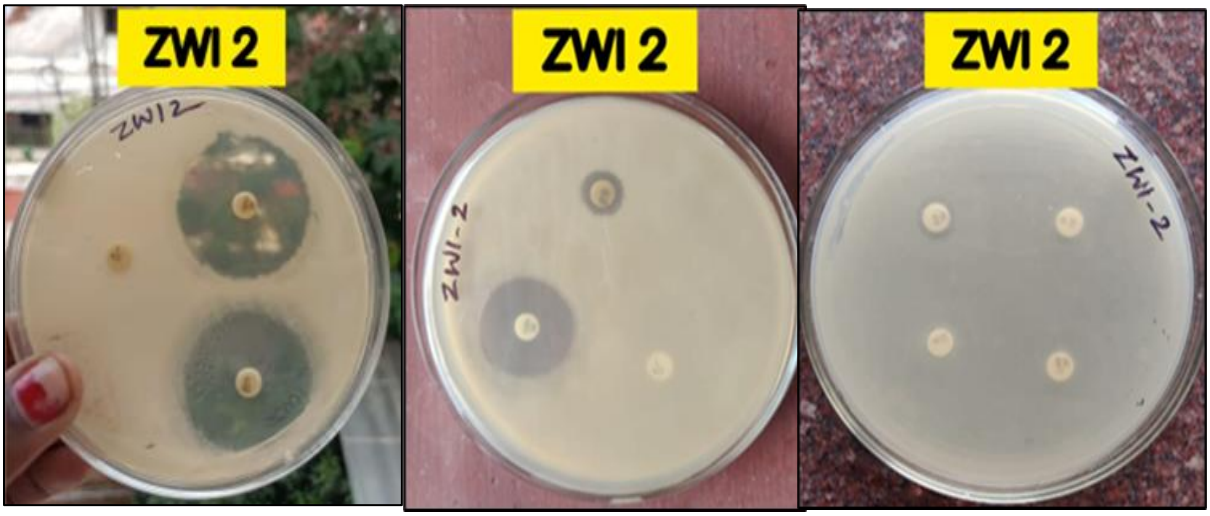


Figure 12 depicting the Kirby Bauer antibiotic sensitivity test results of ZWI 2

Table 17 illustrating Kirby Bauer antibiotic sensitivity test results of ZKM 2

Sl.No	Antibiotics (Dose)	Test isolate	Zone of Diameter (mm)	Resistant	Moderately sensitive	Susceptible
1.	Streptomycin (25 µg)	ZKM 2	13	✓	-	-
2.	Chloramphenicol (30 µg)		19	-	-	✓
3.	Ampicillin (10 µg)		-	✓	-	-
4.	Gentamicin (30 µg)		22	-	-	✓
5.	Tetracycline (30 µg)		14	✓	-	-
6.	Cloxacillin (1 µg)		-	✓	-	-
7.	Penicillin (10 µg)		-	✓	-	-
8.	Erythromycin (15 µg)		-	✓	-	-
9.	Nalidixic acid (30 µg)		9	✓	-	-
10.	Cefuroxime (30 µg)		9	✓	-	-

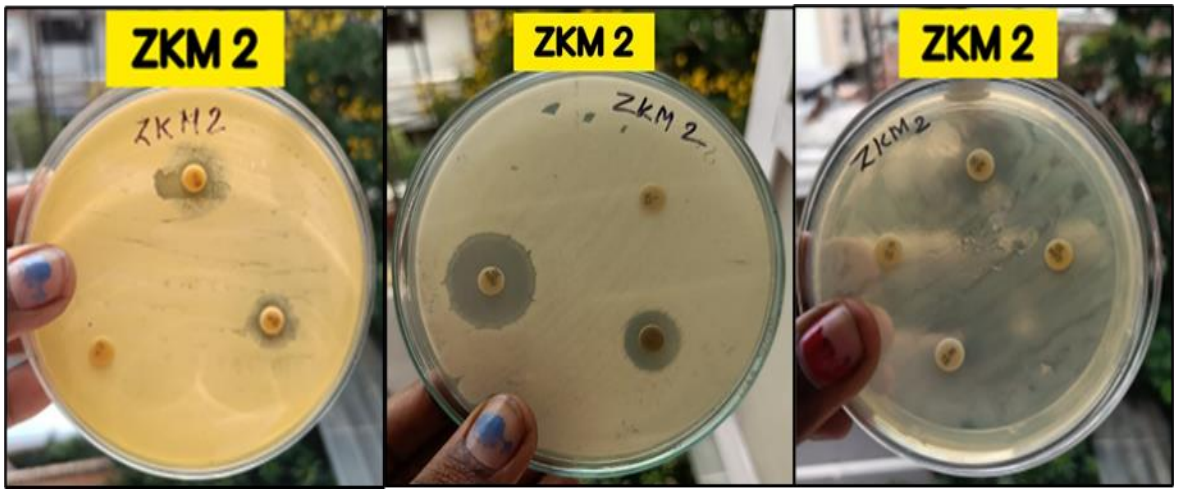


Figure 12 depicting the Kirby Bauer antibiotic sensitivity test results of ZKM 2

Table 18 illustrating Kirby Bauer antibiotic sensitivity test results of ZKM 4

Sl.No	Antibiotics (Dose)	Test isolate	Zone of Diameter (mm)	Resistant	Moderately sensitive	Susceptible
1.	Streptomycin (25 µg)	ZKM 4	29	-	-	✓
2.	Chloramphenicol (30 µg)		28	-	-	✓
3.	Ampicillin (10 µg)		-	✓	-	-
4.	Gentamicin (30 µg)		27	-	-	✓
5.	Tetracycline (30 µg)		20	-	-	✓
6.	Cloxacillin (1 µg)		-	✓	-	-
7.	Penicillin (10 µg)		-	✓	-	-
8.	Erythromycin (15 µg)		35	-	-	✓
9.	Nalidixic acid (30 µg)		35	-	-	✓
10.	Cefuroxime (30 µg)		-	✓	-	-

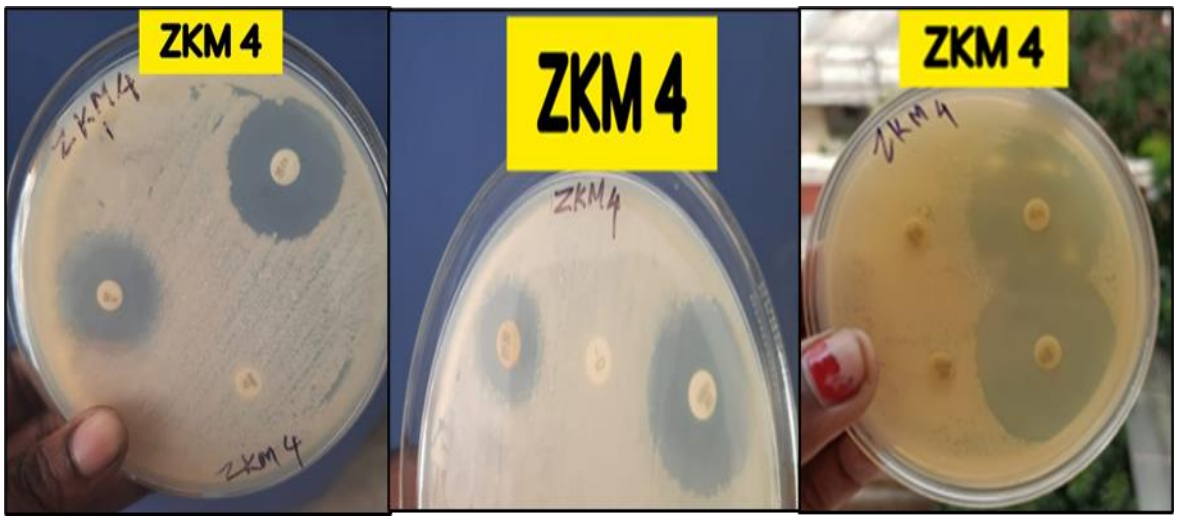


Figure 13 depicting the Kirby Bauer antibiotic sensitivity test results of ZKM 4

Table 19 illustrating Kirby Bauer antibiotic sensitivity test results of ZMC 1

Sl.No	Antibiotics (Dose)	Bacterial isolate	Zone of Diameter (mm)	Resistant	Moderately sensitive	Susceptible
1.	Streptomycin (25 µg)	ZMC 1	24	-	-	✓
2.	Chloramphenicol (30 µg)		19	-	-	✓
3.	Ampicillin (10 µg)		-	✓	-	-
4.	Gentamicin (30 µg)		23	-	-	✓
5.	Tetracycline (30 µg)		19	-	-	✓
6.	Cloxacillin (1 µg)		-	✓	-	-
7.	Penicillin (10 µg)		-	✓	-	-
8.	Erythromycin (15 µg)		26	-	-	✓
9.	Nalidixic acid (30 µg)		28	-	-	✓
10.	Cefuroxime (30 µg)		-	✓	-	-

Figure 14 depicting the Kirby Bauer antibiotic sensitivity test results of ZMC 1

Table 20 illustrating Kirby Bauer antibiotic sensitivity test results of ZVP 8

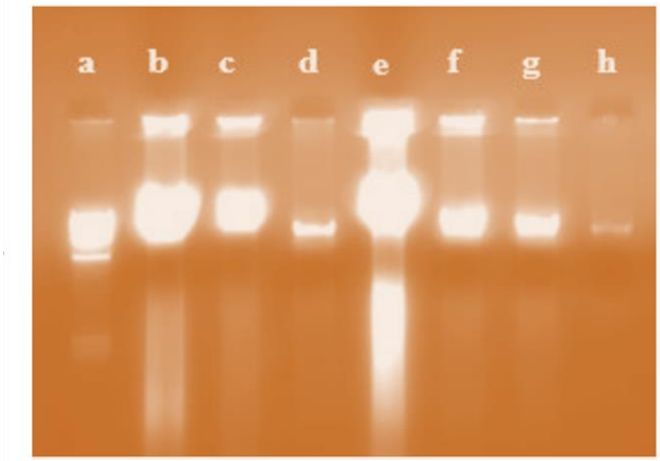
Sl.No	Antibiotics **(Dose)**	**Bacterial isolate**	Zone of **Diameter (mm)**	**Resistant**	**Moderately sensitive**	**Susceptible**
1.	Streptomycin (25 µg)	ZVP 8	22	-	-	✓
2.	Chloramphenicol (30 µg)		18	-	-	✓
3.	Ampicillin (10 µg)		-	✓	-	-
4.	Gentamicin (30 µg)		7	✓	-	-
5.	Tetracycline (30 µg)		10	✓	-	-
6.	Cloxacillin (1 µg)		-	✓	-	-
7.	Penicillin (10 µg)		-	✓	-	-
8.	Erythromycin (15 µg)		-	✓	-	-
9.	Nalidixic acid (30 µg)		24	-	-	✓
10.	Cefuroxime (30 µg)		-	✓	-	-

Figure 15 depicting the Kirby Bauer antibiotic sensitivity test results of ZVP 8

5. MOLECULAR IDENTIFICATION OF THE BACTERIAL ISOLATES

I. GENOMIC DNA ISOLATION

Genomic DNA isolated from ZKM 4, ZVP 1, ZVP 8, ZWI 2, ZMC 1, ZVP 5 was electrophoresed on agarose gel and visualised using Gel Documentation system. The DNA bands were compared with the help of 1kb DNA ladder and the band size of the DNA was found to be above 3000bp**.**



Lane 1- **1kb DNA ladder**

Lane 2- **DNA of sample of ZKM 4**

Lane 3- **DNA of sample ZVP 1**

Lane 4- **DNA of sample ZVP 8**

Lane 5- **DNA of sample ZWI 2**

Lane 6- **DNA of sample ZMC 1**

Lane 7- **DNA of sample ZVP 5**

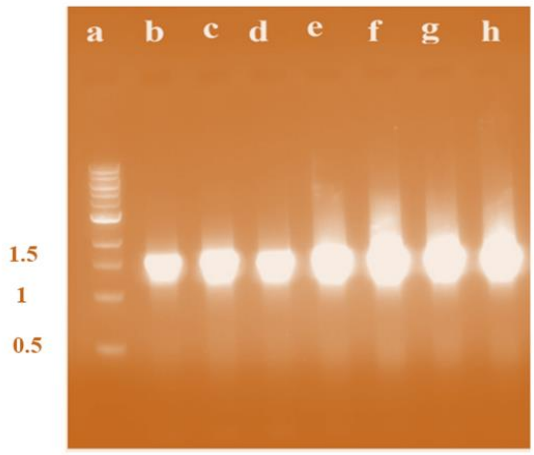
Figure 11 illustrating the Genomic DNA isolation

II. PCR AMPLIFICATION OF 16S rDNA

Amplification of the 16S rDNA is carried out with the help of Thermal Cycler (BioRad MJ Mini Gradient, CA, USA). The amplicon thus obtained was visualized by agarose gel electrophoresis.

The size of the PCR product was compared with DNA ladder and was found to be approximately 1500bp. The PCR product was then sent for sequencing.

.



Lane a- **1kb DNA ladder**

Lane b - **PCR product of sample ZKM 4**

Lane c - **PCR product of sample ZVP1**

Lane d- **PCR product of sample ZVP8**

Lane e- **PCR product of sample ZWI 2**

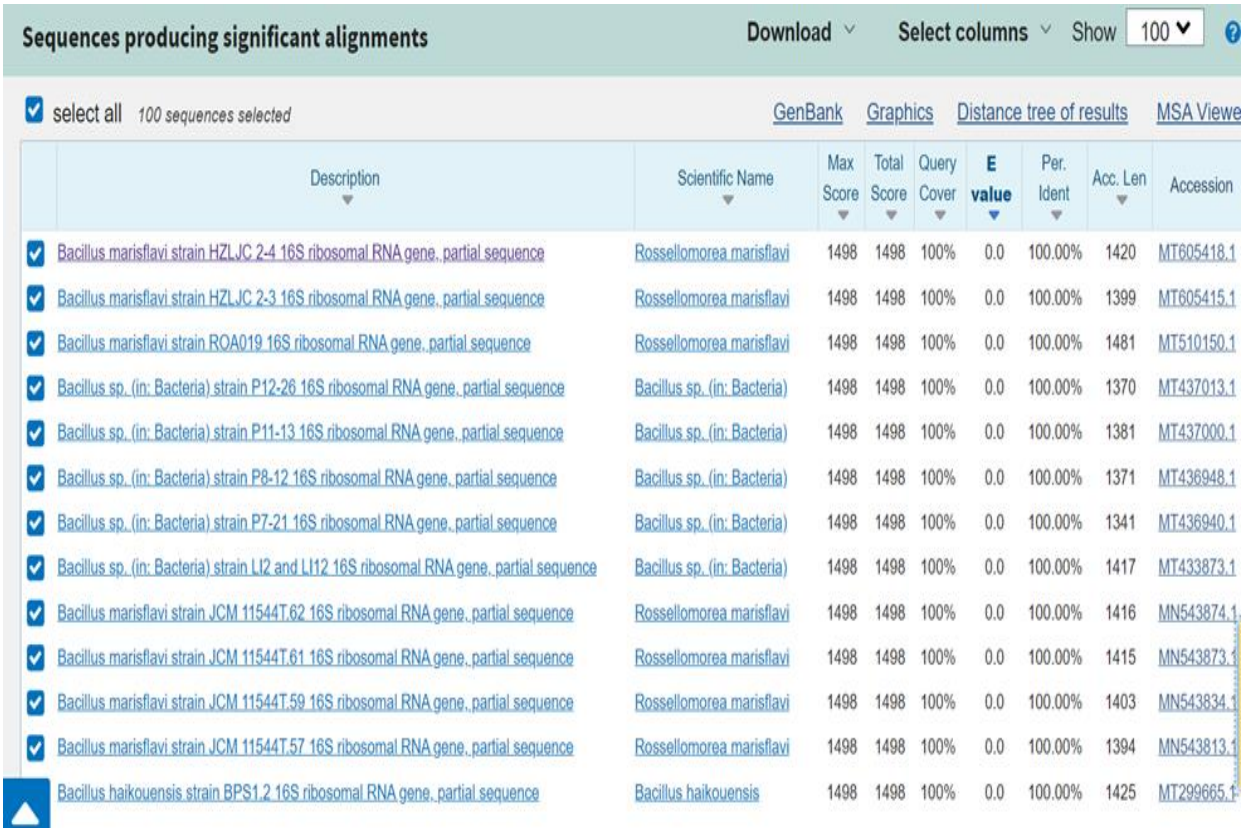
Lane f- **PCR product of sample ZMC 1**

Lane g- **PCR product of sample ZVP 5**

Figure 12 showing Agarose gel showing PCR product

III. DNA SEQUENCING AND BIOINFORMATIC ANALYSIS

After sequencing of 16S rDNA by Sanger's Dideoxy method, the identity of the sequences was determined to have 100% similarity to *Rossellomorea marisflavi*, 100% similarity to *Stenotrophomonas maltophilia*,100% identity to *Serratia marcescens*, 100% similarity to *Bacillus subtilis*, 100% identity to *Bacillus velezensis*. The NCBI hit list **Figure 13- 18** determines the bacterial identity.

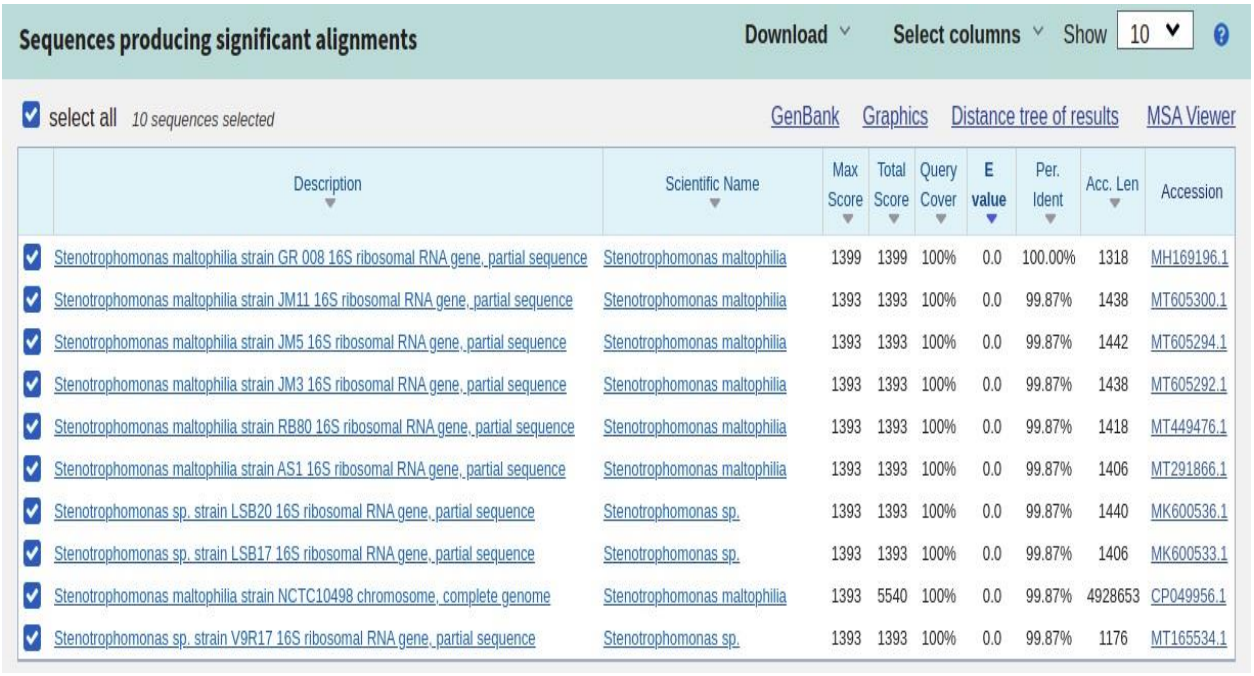


Sequences producing significant alignments — Download — Select columns — Show 100

select all 100 sequences selected — GenBank | Graphics | Distance tree of results | MSA Viewe

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus marisflavi strain HZLJC 2-4 16S ribosomal RNA gene, partial sequence	Rossellomorea marisflavi	1498	1498	100%	0.0	100.00%	1420	MT605418.1
Bacillus marisflavi strain HZLJC 2-3 16S ribosomal RNA gene, partial sequence	Rossellomorea marisflavi	1498	1498	100%	0.0	100.00%	1399	MT605415.1
Bacillus marisflavi strain ROA019 16S ribosomal RNA gene, partial sequence	Rossellomorea marisflavi	1498	1498	100%	0.0	100.00%	1481	MT510150.1
Bacillus sp. (in: Bacteria) strain P12-26 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bacteria)	1498	1498	100%	0.0	100.00%	1370	MT437013.1
Bacillus sp. (in: Bacteria) strain P11-13 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bacteria)	1498	1498	100%	0.0	100.00%	1381	MT437000.1
Bacillus sp. (in: Bacteria) strain P8-12 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bacteria)	1498	1498	100%	0.0	100.00%	1371	MT436948.1
Bacillus sp. (in: Bacteria) strain P7-21 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bacteria)	1498	1498	100%	0.0	100.00%	1341	MT436940.1
Bacillus sp. (in: Bacteria) strain LI2 and LI12 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bacteria)	1498	1498	100%	0.0	100.00%	1417	MT433873.1
Bacillus marisflavi strain JCM 11544T.62 16S ribosomal RNA gene, partial sequence	Rossellomorea marisflavi	1498	1498	100%	0.0	100.00%	1416	MN543874.1
Bacillus marisflavi strain JCM 11544T.61 16S ribosomal RNA gene, partial sequence	Rossellomorea marisflavi	1498	1498	100%	0.0	100.00%	1415	MN543873.1
Bacillus marisflavi strain JCM 11544T.59 16S ribosomal RNA gene, partial sequence	Rossellomorea marisflavi	1498	1498	100%	0.0	100.00%	1403	MN543834.1
Bacillus marisflavi strain JCM 11544T.57 16S ribosomal RNA gene, partial sequence	Rossellomorea marisflavi	1498	1498	100%	0.0	100.00%	1394	MN543813.1
Bacillus haikouensis strain BPS1.2 16S ribosomal RNA gene, partial sequence	Bacillus haikouensis	1498	1498	100%	0.0	100.00%	1425	MT299665.1

Figure 13 NCBI BLAST depicting the sequence similarity of the sample ZKM 4



Sequences producing significant alignments — Download — Select columns — Show 10

select all 10 sequences selected — GenBank | Graphics | Distance tree of results | MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Stenotrophomonas maltophilia strain GR 008 16S ribosomal RNA gene, partial sequence	Stenotrophomonas maltophilia	1399	1399	100%	0.0	100.00%	1318	MH169196.1
Stenotrophomonas maltophilia strain JM11 16S ribosomal RNA gene, partial sequence	Stenotrophomonas maltophilia	1393	1393	100%	0.0	99.87%	1438	MT605300.1
Stenotrophomonas maltophilia strain JM5 16S ribosomal RNA gene, partial sequence	Stenotrophomonas maltophilia	1393	1393	100%	0.0	99.87%	1442	MT605294.1
Stenotrophomonas maltophilia strain JM3 16S ribosomal RNA gene, partial sequence	Stenotrophomonas maltophilia	1393	1393	100%	0.0	99.87%	1438	MT605292.1
Stenotrophomonas maltophilia strain RB80 16S ribosomal RNA gene, partial sequence	Stenotrophomonas maltophilia	1393	1393	100%	0.0	99.87%	1418	MT449476.1
Stenotrophomonas maltophilia strain AS1 16S ribosomal RNA gene, partial sequence	Stenotrophomonas maltophilia	1393	1393	100%	0.0	99.87%	1406	MT291866.1
Stenotrophomonas sp. strain LSB20 16S ribosomal RNA gene, partial sequence	Stenotrophomonas sp.	1393	1393	100%	0.0	99.87%	1440	MK600536.1
Stenotrophomonas sp. strain LSB17 16S ribosomal RNA gene, partial sequence	Stenotrophomonas sp.	1393	1393	100%	0.0	99.87%	1406	MK600533.1
Stenotrophomonas maltophilia strain NCTC10498 chromosome, complete genome	Stenotrophomonas maltophilia	1393	5540	100%	0.0	99.87%	4928653	CP049956.1
Stenotrophomonas sp. strain V9R17 16S ribosomal RNA gene, partial sequence	Stenotrophomonas sp.	1393	1393	100%	0.0	99.87%	1176	MT165534.1

Figure 14 NCBI BLAST depicting the sequence similarity of the sample ZVP 1

Sequences producing significant alignments — Download — Select columns — Show 10

select all 10 sequences selected — GenBank | Graphics | Distance tree of results | MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus subtilis strain PC1 16S ribosomal RNA gene, partial sequence		1338	1338	100%	0.0	100.00%	1461	ON534347.1
Bacillus cereus strain DQ01 chromosome, complete genome		1338	18711	100%	0.0	100.00%	5322598	CP097351.1
Bacillus thuringiensis strain CPO 28.R5 16S ribosomal RNA gene, partial sequence		1338	1338	100%	0.0	100.00%	1443	ON514334.1
Bacillus thuringiensis strain CPO 28.V11 16S ribosomal RNA gene, partial sequence		1338	1338	100%	0.0	100.00%	1484	ON514326.1
Bacillus cereus strain DSC-18-02-19 16S ribosomal RNA gene, partial sequence		1338	1338	100%	0.0	100.00%	1501	ON514315.1
Bacillus albus strain GTM22 16S ribosomal RNA gene, partial sequence		1338	1338	100%	0.0	100.00%	1456	ON514248.1
Bacillus albus strain GFK31 16S ribosomal RNA gene, partial sequence		1338	1338	100%	0.0	100.00%	1465	ON514242.1
Bacillus albus strain GTM11 16S ribosomal RNA gene, partial sequence		1338	1338	100%	0.0	100.00%	1456	ON514236.1
Bacillus albus strain GTM31 16S ribosomal RNA gene, partial sequence		1338	1338	100%	0.0	100.00%	1449	ON514235.1
Bacillus albus strain GFM21 16S ribosomal RNA gene, partial sequence		1338	1338	100%	0.0	100.00%	1452	ON514231.1

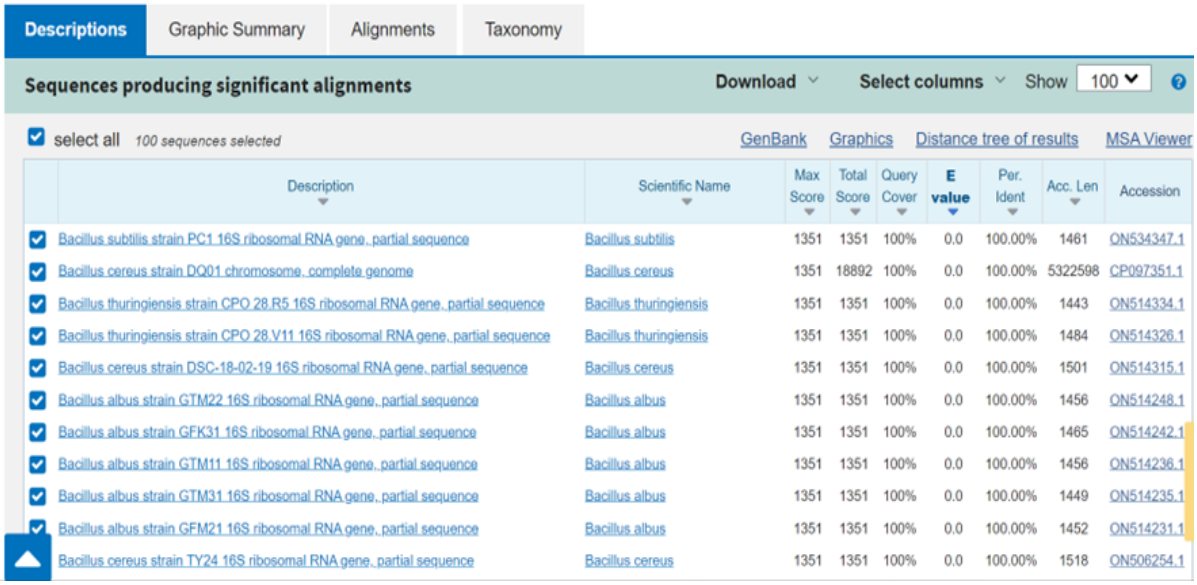
Figure 15 NCBI BLAST depicting the sequence similarity of the sample ZVP 8

Sequences producing significant alignments Download Select columns Show 10

select all *10 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus velezensis strain R-71003 16S ribosomal RNA gene, partial sequence	Bacillus velezensis	1242	1242	100%	0.0	100.00%	1449	ON358418.1
Bacterium strain SD10 16S ribosomal RNA gene, partial sequence	bacterium	1242	1242	100%	0.0	100.00%	1192	ON342885.1
Bacillus velezensis strain L1 16S ribosomal RNA gene, partial sequence	Bacillus velezensis	1242	1242	100%	0.0	100.00%	1451	ON340771.1
Bacillus subtilis subsp. subtilis strain FSLJW581 16S ribosomal RNA gene, partial sequence	Bacillus subtilis subsp. subtilis	1242	1242	100%	0.0	100.00%	1437	ON329105.1
Bacillus amyloliquefaciens strain XW-10 16S ribosomal RNA gene, partial sequence	Bacillus amyloliquefaciens	1242	1242	100%	0.0	100.00%	1449	ON326557.1
Bacillus amyloliquefaciens strain GL18 chromosome, complete genome	Bacillus amyloliquefaciens	1242	11178	100%	0.0	100.00%	3915550	CP096033.1
Bacillus siamensis strain HBUAS664829 16S ribosomal RNA gene, partial sequence	Bacillus siamensis	1242	1242	100%	0.0	100.00%	1475	ON306776.1
Bacillus amyloliquefaciens strain JDF 16S ribosomal RNA gene, partial sequence	Bacillus amyloliquefaciens	1242	1242	100%	0.0	100.00%	1447	ON287217.1
Bacillus amyloliquefaciens strain NOK109 16S ribosomal RNA gene, partial sequence	Bacillus amyloliquefaciens	1242	1242	100%	0.0	100.00%	1484	ON287186.1
Bacillus siamensis strain NOK108 16S ribosomal RNA gene, partial sequence	Bacillus siamensis	1242	1242	100%	0.0	100.00%	1426	ON287185.1

Figure 16 NCBI BLAST depicting the sequence similarity of the sample ZWI 2



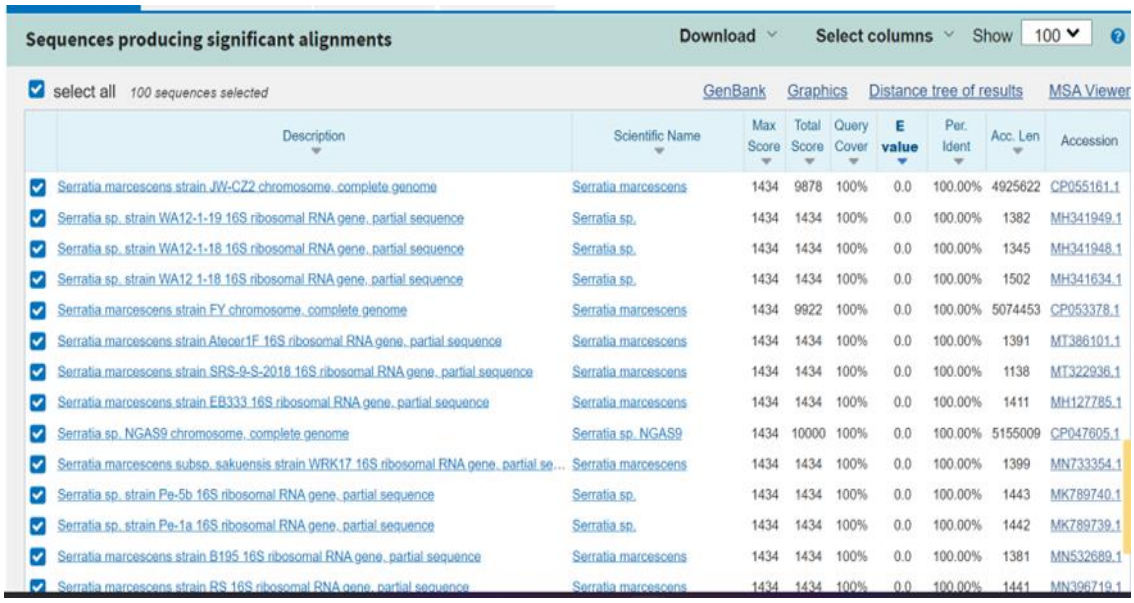
Descriptions Graphic Summary Alignments Taxonomy

Sequences producing significant alignments Download Select columns Show 100

select all *100 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus subtilis strain PC1 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	1351	1351	100%	0.0	100.00%	1461	ON534347.1
Bacillus cereus strain DQ01 chromosome, complete genome	Bacillus cereus	1351	18892	100%	0.0	100.00%	5322598	CP097351.1
Bacillus thuringiensis strain CPO 28.R5 16S ribosomal RNA gene, partial sequence	Bacillus thuringiensis	1351	1351	100%	0.0	100.00%	1443	ON514334.1
Bacillus thuringiensis strain CPO 28.V11 16S ribosomal RNA gene, partial sequence	Bacillus thuringiensis	1351	1351	100%	0.0	100.00%	1484	ON514326.1
Bacillus cereus strain DSC-18-02-19 16S ribosomal RNA gene, partial sequence	Bacillus cereus	1351	1351	100%	0.0	100.00%	1501	ON514315.1
Bacillus albus strain GTM22 16S ribosomal RNA gene, partial sequence	Bacillus albus	1351	1351	100%	0.0	100.00%	1456	ON514248.1
Bacillus albus strain GFK31 16S ribosomal RNA gene, partial sequence	Bacillus albus	1351	1351	100%	0.0	100.00%	1465	ON514242.1
Bacillus albus strain GTM11 16S ribosomal RNA gene, partial sequence	Bacillus albus	1351	1351	100%	0.0	100.00%	1456	ON514236.1
Bacillus albus strain GTM31 16S ribosomal RNA gene, partial sequence	Bacillus albus	1351	1351	100%	0.0	100.00%	1449	ON514235.1
Bacillus albus strain GFM21 16S ribosomal RNA gene, partial sequence	Bacillus albus	1351	1351	100%	0.0	100.00%	1452	ON514231.1
Bacillus cereus strain TY24 16S ribosomal RNA gene, partial sequence	Bacillus cereus	1351	1351	100%	0.0	100.00%	1518	ON506254.1

Figure 17 NCBI BLAST depicting the sequence similarity of the sample ZMC 1



Sequences producing significant alignments — Download — Select columns — Show 100

select all 100 sequences selected — GenBank — Graphics — Distance tree of results — MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Serratia marcescens strain JW-CZ2 chromosome, complete genome	Serratia marcescens	1434	9878	100%	0.0	100.00%	4925622	CP055161.1
Serratia sp. strain WA12-1-19 16S ribosomal RNA gene, partial sequence	Serratia sp.	1434	1434	100%	0.0	100.00%	1382	MH341949.1
Serratia sp. strain WA12-1-18 16S ribosomal RNA gene, partial sequence	Serratia sp.	1434	1434	100%	0.0	100.00%	1345	MH341948.1
Serratia sp. strain WA12 1-18 16S ribosomal RNA gene, partial sequence	Serratia sp.	1434	1434	100%	0.0	100.00%	1502	MH341634.1
Serratia marcescens strain FY chromosome, complete genome	Serratia marcescens	1434	9922	100%	0.0	100.00%	5074453	CP053378.1
Serratia marcescens strain Atecer1F 16S ribosomal RNA gene, partial sequence	Serratia marcescens	1434	1434	100%	0.0	100.00%	1391	MT386101.1
Serratia marcescens strain SRS-9-S-2018 16S ribosomal RNA gene, partial sequence	Serratia marcescens	1434	1434	100%	0.0	100.00%	1138	MT322936.1
Serratia marcescens strain EB333 16S ribosomal RNA gene, partial sequence	Serratia marcescens	1434	1434	100%	0.0	100.00%	1411	MH127785.1
Serratia sp. NGAS9 chromosome, complete genome	Serratia sp. NGAS9	1434	10000	100%	0.0	100.00%	5155009	CP047605.1
Serratia marcescens subsp. sakuensis strain WRK17 16S ribosomal RNA gene, partial se...	Serratia marcescens	1434	1434	100%	0.0	100.00%	1399	MN733354.1
Serratia sp. strain Pe-5b 16S ribosomal RNA gene, partial sequence	Serratia sp.	1434	1434	100%	0.0	100.00%	1443	MK789740.1
Serratia sp. strain Pe-1a 16S ribosomal RNA gene, partial sequence	Serratia sp.	1434	1434	100%	0.0	100.00%	1442	MK789739.1
Serratia marcescens strain B195 16S ribosomal RNA gene, partial sequence	Serratia marcescens	1434	1434	100%	0.0	100.00%	1381	MN532689.1
Serratia marcescens strain RS 16S ribosomal RNA gene, partial sequence	Serratia marcescens	1434	1434	100%	0.0	100.00%	1441	MN396719.1

Figure 18 NCBI BLAST depicting the sequence similarity of the sample ZVP 5

DISCUSSION

Soil is one of the best sources for isolating novel antibiotics as many scientists have chosen it for their research. It was reported that the heterogeneity of soil environment results heterogeneous population of soil bacteria. In the present study, isolation of the protease producing bacteria was performed from waste dumpsite soil.

Proteases are proteolytic enzymes that catalyze the breakdown of proteins by hydrolysis of peptide bonds. Proteolytic enzymes are ubiquitous in occurrence, being present in all living organisms and are essential for cell growth and differentiation. These are one of the most important industrial enzymes accounting for nearly 60% of the total worldwide production of enzymes. Proteases have variety of functions and important pharmaceutical biotechnological applications like, meat tenderization process infant formula preparation, diagnostic waste management and silver recovery paper.

The sole intention of the current research was to isolate protease producing bacteria from waste soil collected from various locations of Ernakulam and Kottayam district, Kerala, India. After serial dilution and pour plate technique, the discrete colony forming units (CFU) were preserved in the form of quadrant streaking and slant technique. Bacterial isolates obtained were subjected to proteolytic screening using skim milk assay and well diffusion method. Most potent isolates with prominent activity in the two screening procedures were proceeded to biochemical identification namely IMVIC and catalase test. Kirby Bauer antibiotic disc sensitivity test of the potent isolates against broad spectrum antibiotics were performed. The molecular identification of the six isolates namely ZKM 4, ZVP 1, ZWI 2, ZVP 8, ZVP 5, ZMC 1 were carried out using genomic DNA extraction, PCR amplification and the NCBI BLAST analysis reveals maximum sequence similarity of 100% to *Rossellomorea marisflavi*, *Stenotrophomonas maltophilia*, *Bacillus velezensis*, *Bacillus subtilis*, *Serratia marcescens*, *Bacillus subtilis* respectively.

In the current investigation, 78 bacterial isolates were screened for proteolytic activity and extracellular protease production using patch plate technique and well diffusion assay on 1% skim milk agar medium, autoclaved and dissolved in standard nutrient agar medium. After 30 minutes of solidification, the wells were punched on skim milk agar plate using a gel puncher. 20µl of crude bacterial supernatant were added to each well. The plates were incubated overnight at 37^0C for 24 hours. Proteolytic activity was measured and expressed on mm diameter. The same protocol was adopted in the studies conducted by Kaur *et al.,* in 1989 for screening bacterial species for proteolytic activity by well plate assay method on skim milk agar plates and expressed in terms of mm diameter of clear zones produced around the well.

Kirby Bauer antibiotic disc susceptibility test determines the resistance and susceptibility of bacteria to a particular dosed antibiotic. By the method of extracellular proteolytic screening, 31 isolates were screened and the most potent eight strains were subjected to susceptibility testing against ten antibiotics and the results were examined. The antibiotic susceptibility assay demonstrated that *Bacillus subtilis* and *Serratia sp* showed high resistivity to ampicillin, tetracycline, pencillin, cloxacillin etc. The findings of the present work are in close agreement with the studies conducted by *Senthal et al*., 2020 which suggests that the bacterial isolates *Bacillus licheniformis, Pseudomonas sp. Bacillus subtilis, Bacillus cohnii and Serratia sp*. showed high resistance to penicillin, ampicillin and tetracycline.

Molecular characterization of the most effectual protease degrading bacteria such as ZKM 4, ZWI 2, ZMC 1, ZVP 1, ZVP 5, ZVP 8 using genomic DNA extraction, PCR amplification and NCBI BLAST analysis revealed that the species shows maximum sequence similarity of 100% to *Rossellomorea marisflavi, Bacillus velezensis, Bacillus subtilis, Stenotrophomonas maltophilia, Serratia marcescens, Bacillus subtilis* respectively. The molecular characterization results suggests that the Genus *Bacillus* plays a pivotal role as protease producers which shows similarity with the work conducted by Das *et al*., in 2010 which demonstrated that Bacillus *subtilis* under study is a good producer of extracellular protease. The published report by Dunne *et al*., in 1997 also stated the ability of *S. maltophilia* W81 to protect sugar beet from Pythium-mediated damping-off due to the production of an extracellular protease.

Rossellomorea marisflavi were obtained as a novel and potent proteolytic enzyme producing bacteria as an innovation of the present investigation targeting waste soil namely compost, plastic waste, poultry, agricultural and food waste containing soil etc. which is substantiated by two screening protocol namely skim milk agar and well diffusion assay with a notable and prominent clearance zone with diameter measuring 1.7 mm.

Heterogeneity of proteases is its uniqueness, which makes them versatile biocatalyst. However, its full potential has not yet been tapped. In fact, its prospects in waste management are underutilized, especially in urban settings. The engineering of proteases for novel or combined catalytic abilities with long half-life seems to be a less addressed area. Protease-based industry looks forward to receiving engineered fusion proteases with multiple activities combined in one. Thus, the ever-growing protease market demands efficient and fast-acting proteases at cheaper price (Veloorvalappil *et al.,* 2013). Proteases have a long history of application in the food and detergent industries. Their application in the leather industry for dehairing and bating of hides to substitute currently used toxic chemicals is a relatively new development and has conferred added biotechnological importance. The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications (Rao *et al.,* 1998).

Extensive research must be carried to achieve bacterial proteases with characteristics, such as yield improvement, changing substrate specificity, enhancement of thermal stability, altering optimum pH, and prevention of auto-proteolytic inactivation.

CONCLUSION

Enzymatic screening, biochemical and molecular characterization of protease producing bacteria from the waste soil dumping yards has various applications in detergent, food industry, leather industry as well as in waste management. Proteases are a universal entity that is found everywhere, namely, in plants, animals, and microbes. They are degradative enzymes and show specificity and selectivity in protein modification. These microbial proteases are preferred to plant and animal proteases because of the presence of all desired characteristics for industrial applications. Waste soil samples collected from various locations of Ernakulam and Kottayam districts, Kerala, India were serially diluted and pour plated on nutrient agar medium. A total of 78 pure isolates obtained were subjected to proteolytic screening using skim milk assay and extracellular protease production by well diffusion method. Zone of clearance indicated protease production. Biochemical characterization, antibiotic disc susceptibility analysis and molecular identification of the potent bacterial isolates were accomplished. The molecular identification of the selected six bacterial isolates namely ZKM 4, ZVP 1, ZMC 1, ZVP 5, ZWI 2, ZVP 8 involves DNA isolation and PCR amplification of 16S rDNA. The amplified DNA fragments of the six isolates were sequenced by automated sequencing method and identity of the sequence was determined as Rossellomorea *marisflavi* (MT605418.1), *Stenotrophomonas maltophilia* (MH169196.1), *Bacillus subtilis* (ON53437.1), *Bacillus velezensis* (ON358418.1), *Bacillus subtilis* (ON534347.1), *Serratia marcescens* (CP055161.1) by NCBI BLAST analysis. The results of the current investigation support the tremendous significance of bacterial protease enzymes from waste soil which has wide applications in leather, detergent, food, meat tenderization industries, pharmaceuticals, medical diagnosis, and decomposition of gelatin on X-ray films as well as in textiles. Using Genetic Engineering, Molecular Biology and Computational Biology extensive research adopted can generate improved protease strain with newer characterization and extraction of crude bacterial proteases.

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APPENDIX

1M Tris-HCL:

Ingredients	Volume
Tris base	60.57g
Deionised water	500mL
Adjusted to desired pH using concentrated HCL	-

0.5M EDTA:

Ingredients	Volume
EDTA	18.6g
Deionised water	100mL

TE Buffer:

Ingredients	Volume
1M Tris EDTA	10mL
500 mM EDTA (pH 8.0)	2mL

50X TAE Buffer:

Ingredients	Volume
Tris base	121g
Glacial acetic acid	28.6mL
0.5 M EDTA pH 8.0	50mL

Deionised water added to make volume to 500 mL

1X TAE Buffer:

Ingredients	volume
50X TAE Buffer	10 mL
Deionised water	490mL

Skim milk agar:

Ingredients	Volume
Skim milk	1%
Nutrient broth	1.3

Deionised water added to make volume to 100 mL