‘ENZYMATIC SCREENING, BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF EFFICIENT AMYLASE PRODUCING BACTERIA FROM WASTE SOIL’

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

FULFILLMENT OF THE REQUIREMENT

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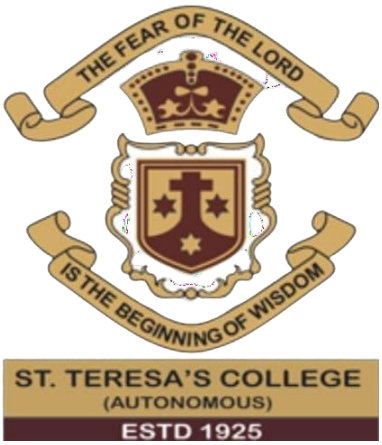

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CERTIFICATE

This is to certify that the dissertation entitled '**Enzymatic screening, Biochemical and Molecular Characterization of efficient Amylase producing bacteria from the waste soil'** is an authentic record of original project work carried out by "**T.H.DHIVYA**" (Reg:-No SM20ZOO010), during the Academic year **2020-2022**, under the guidance of Ms. Cynthia Mathew Thaliath Assistant Professor, Department of Zoology,St Teresa's College, for the partial fulfilment of the requirement of the Degree of Master of Science in 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 efficient Amylase 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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T.H. DHIVYA

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LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

µl	:	Micro litre
mL	:	Milli litre
Mm	:	Milli molar
g	:	Gram
%	:	Percentage
^{0}	:	Degree Celicus
hrs	:	Hours
min	:	Minute
S	:	Seconds
CFU	:	Colony- forming unit
HCl	:	Hydrochloric Acid
BTB	:	Bromothymol blue
O_2	:	Oxygen
H_2O	:	Water
H_2O_2	:	Hydrogen peroxide

pH	:	Potential of Hydrogen
SDS	:	Sodium Dodecyl Sulfate
DNA	:	Deoxyribo Nucleic Acid
dNTPs	:	Deoxy Nucleoside Triphosphates
EDTA	:	Ethylene Diamine Tetra Acetic Acid
Mgcl2	:	Magnesium Chloride
PCR	:	Polymerase Chain Reaction
Rpm	:	Revolutions Per Minute
Taq	:	Thermus aquaticus
TBE	:	Tri Borate EDTA
TE	:	Tris EDTA
NCBI	:	National Centre for Biotechnology Information

CONTENTS

ABSTRACT

Microbes from the waste soil have the potential to produce the amylase enzymes. Amylases are the hydrolytic enzymes that hydrolyses the starch into simple sugars. The bacterial microbes are considered as the best source of amylase production, which can be widely used in the industrial process.

The objective of the present investigation was to screen the amylase producing bacteria from the waste soil samples. The soil samples were collected from the agricultural and food waste, poultry, waste dumping yards from the diverse locations of Ernakulam and Kottayam districts, Kerala, India. The bacterial strains were isolated by serial dilution and pour plate techniques. The starch hydrolysis test of the isolated bacterial strain was performed by the method of patch plating in starch agar medium and the extracellular amylase production by well diffusion assay. These two techniques demonstrated the screening of effective amylase producing bacteria from the total strains of soil bacteria isolated. The selected bacterial samples with amylase production were further considered for its disc susceptibility against broad spectrum antibiotics, biochemical and molecular characterization. The genomic DNA of the isolates namely ZCJ3, ZVP1, ZVP 8, ZVP10, ZWI4, ZMM9 were identified by 16S rDNA amplification and the BLAST analysis reveals maximum sequence similarity of 100% to *Arthrobacter sp, Stenotrophomonas maltophilia, Bacillus cereus, Bacillus subtilis, Bacillus velezensis, Bacillus paramycoides respectively*.

Currently amylase represents one of the most important enzyme group within the field of biotechnology. Amylase is potentially useful in various pharmaceutical, fine chemical industries. The use of these enzymes has widened in clinical research, medical chemistry and analytical chemistry. Microbial production of amylase is more effective than those of other sources as the technique is easy, cost effective and fast and can be modified to obtain the desired characteristics of the enzymes. Screening of soil microorganisms with higher amylase could therefore facilitate the discovery of novel amylase suitable for new biotechnological and industrial applications.

INTRODUCTION

Soil is a complex system that comprises a variety of microhabitats with different physicochemical gradients and discontinuous environmental conditions. Microorganisms adapt to microhabitats and live together in consortia with more or less sharp boundaries, interacting with each other and with other parts of the soil biota (Torsvik *et al.,* 2002). Many microorganisms that live in the soil play an indispensable role in maintaining life on the planet by degrading or chemically modifying molecules **(**Bala *et al.,*2013). Soil is a medium that is used as a place to live and grow bacteria in a complex manner. Bacteria can live in the soil by utilizing all the nutrients in it (Kaur *et al.,* 2012). Microbial population in the soil counts for a huge mass of organic matter on earth (Begum *et al.,* 2017). Microbial diversity in soil ecosystems exceeds, by far, that of eukaryotic organisms. One gram of soil may harbour up to 10 billion microorganisms of possibly thousands of different species. Microbial diversity describes complexity and variability at different levels of biological organization. It encompasses genetic variability within taxons (species), and the number (richness) and relative abundance (evenness) of taxons and functional groups (guilds) in communities (Torsvik *et al.,* 2002).

Waste generation and its control have taken an important role in our environment. With the doubling of population and changing lifestyle pattern of the inhabitants the quantity of municipal waste generated is increasing at an alarming rate. Most of this waste is subjected to dumping in a specified disposal yard. The greatest challenge to the environmentalists is the eco-friendly management of this waste and application of microorganisms in this context has advanced over other available technologies. Organic waste is consumed by the bacteria, used as nutrients by the bacteria, and is no longer present to produce odours, sludge, pollution or unsightly mess.

When bacteria consume waste, they convert the waste into safe by-products and in due course of this conversion they actually produce several metabolites to break down the complex waste into simple compounds. Soil microorganisms are increasingly becoming an important source in the search for industrially important molecules. In soil 80 to 99% of microorganisms remain unidentified whereas these biological communities are known to play a dominant role in maintaining a sustainable biosphere. Bacteria are present in diverse ecological habitats. They are considered highly valuable as they are used in fermentation processes, much as brewing, baking, cheese and butter manufacturing, chemical manufacturing such as ethanol, acetone, organic acid, enzymes, perfumes etc., microbial mining and they produce various antibiotics, vaccines, steroids as well as other therapeutically useful compounds with diverse biological activities. Hence there is an immense possibility to screen effective bacterial strains from waste dump sites with valuable applications (Saha *et al.*,2014).

Enzymes are catalysts for biochemical reactions. They speed up reactions by providing a substitute reaction pathway of lower activation energy. The molecules at the beginning of the process upon which enzymes may act are called substrates and the enzyme converts these into different molecules, called products, which initiate and accelerate thousands of biochemical reactions in living cells. Enzymes are among the most important products obtained for human needs through microbial sources (Sundarapandiyan *et al.,* 2017).

The amylases are hydrolytic enzymes which promote the decomposition of starch. They attack glycogen as well as certain dextrin (Kindle, 1983). Amylase is an industrially important enzyme used for the hydrolysis of dietary starch into disaccharides and trisaccharides and ultimately to glucose (Sreelekshmi *et al.,* 2019**)**. Plants, animals and certain microorganisms produce this amylase and are used for various biochemical reactions. The most stable and reliable source of amylase is obtained from microbes. The microbial amylases could be potentially useful in various pharmaceutical, fine-chemical industries etc., with the event of new frontiers in biotechnology, the use of amylase has widened in clinical research, medical chemistry and starch analytical chemistry. Amylases are also used in baking, brewing, textile, detergent, paper and distilling industries (Ashwini *et al.*,2011).

The enzyme amylase, also addressed as 'glycoside hydrolases', breaks down the carbohydrate molecules into smaller products (Gurung *et al.*,2013). Their main substrate is starch which is split into its smaller components such as dextrin, maltose, maltotriose, and glucose. The enzyme basically hydrolyses the α-1, 4 - glycosidic bond that holds the glucose units together. Amylase is produced most widely by microorganisms such as bacteria and fungi compared to plants and animals (Islam *et al*.,2017). Starch is the major polysaccharide made of amylose and amylopectin (Monnet *et al.*,2010). Starch is the primary storage polysaccharide in plants and an important constituent of the human diet. Starch can be converted into various useful products by the application of enzymes (Souza *et al.,* 2010). Amylase is an important starch degrading enzyme which is widely distributed in the world of microbial, plant and animals (Banks *et al.,* 1975). Amylase hydrolyzes the bonds between adjacent glucose units to yield various other products (Dhanya *et al.*,2009).

The most important amylolytic enzymes include alpha-amylase, beta-amylase, glucoamylase, alpha-glucosidase and the pullulan-degrading enzymes (Vihinen *et al.,* 1989). Due to the availability of substrates such as starch,pullulan and glycogen as important sources of energy, many microorganisms,plants and animals, have evolved to produce a large variety of amylolytic enzymes that can hydrolyze such sources of carbon (Meisam, *et al.*,2014). The α-amylase family, *i.e*. the clan GH-H of glycoside hydrolyses, is the largest family of glycoside hydrolases, transferases and isomerases comprising nearly 30 different enzyme specificities (Henrissat.,1991). A large variety of enzymes are able to act on starch. These enzymes can be divided basically into four groups: endoamylases, exoamylases, debranching enzymes and transferases. 1. Endoamylases: cleave internal α-1,4 bonds resulting in α-anomeric products, 2. Exoamylases: cleave α-1,4 or a-1,6 bonds of the external glucose residues resulting in a- or b-anomeric products, 3. debranching enzymes: hydrolyze α-1,6 bonds exclusively leaving long linear polysaccharides, and 4. transferases: cleave α-1,4 glycosidic bond of the donor molecule and transfer part of the donor to a glycosidic acceptor forming a new glycosidic bond(Maarel *et al.*,2002).

The α-amylase family can roughly be divided into two groups: the starch hydrolyzing enzymes and the starch modifying, or transglycosylation enzymes. The enzymatic hydrolysis is preferred to acid hydrolysis in the starch processing industry due to a number of advantages such as specificity of the reaction, stability of the generated products, lower energy requirements and elimination of neutralization steps (Satyanarayana *et al.*,2005). The rate of hydrolysis of starch by α-amylase depends on many process conditions such as temperature, pH, nature of substrate, substrate concentration, enzyme concentration, presence of Ca2+ ions and other stabilizing agents(Sivaramakrishnan *et al.*,2006).

Amylases from plant and microbial sources have been employed for centuries as food additives. Barley amylases have been used in the brewing industry. Fungal amylases have been widely used for the preparation of oriental foods. In spite of the wide distribution of amylases, microbial sources, namely fungal and bacterial amylases,are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization (Burhan *et al.*,2003).

So, isolation and characterization of amylase producing bacteria from waste soil will have a paramount role in various pharmaceutical, fine-chemical industries, clinical research, medical chemistry and starch analytical chemistry, thereby contributing to better application of microbes isolated from the waste soil.

AIM AND OBJECTIVES

AIM

Enzymatic screening, Biochemical and Molecular characterization of efficient amylase producing bacteria from waste soil.

OBJECTIVES

- Isolation of bacteria from waste soil collected from various locations.
- Screening of amylase producing bacteria using starch hydrolysis assay.
- Screening of extracellular amylase production by well diffusion assay.
- Biochemical characterization of amylase producing bacterial isolates.
- Antibiotic disc susceptibility analysis of potential amylase producing bacteria.
- Molecular characterisation of the amylase producing bacteria.

INNOVATION OF THE DISSERTATION

The isolated different forms of amylolytic bacterial strains from the waste soil have the tremendous potential to produce the amylase enzymes which can be widely used in the field of biotechnology to meet the demands of industries in the production of fermented foods and beverages.Apart from these applications it has high potentiality in the manufacturing of paper and pulp industry, textile industry and in pharmaceutical applications etc. Hence the amylolytic enzymes can be used in the better utilization and management of waste and its remnants. Soil harbours a rich collection of microbes, which are capable of producing the amylase enzymes.

In order to increase sustainable development in the present era, these enzymes can be used, as they are the biodegradable ones and can be produced even from the biodegradable sources. Amylase enzymes are enlisted in the various fields of manufacturing or production of goods, and in the pharmaceutical or diagnostic aids. The amylase enzyme has an ability to produce an alternative form of energy as it is involved in the production of biofuel with the starch as a raw material. Screening of soil microorganisms with higher amylase could facilitate the discovery of novel amylase suitable for new biotechnological and industrial applications.

REVIEW OF LITERATURE

SOIL HABITAT

Soil habitat is defined as the totality of living organisms inhabiting soil including plants, animals, and microorganisms and their abiotic environment. The exact nature of the habitat in which the community of organisms is living is determined by a complex interplay of geology, climate, and plant vegetation (Voroney, 2007).

SOIL AS A MICROBIAL HABITAT

Soils are the naturally occurring physical covering of the earth's surface, and represent the interface of three material states: solids (geological and dead biological materials), liquids (water), and gases (air in soil pores). Soils are the foundation of all terrestrial ecosystems and are home to a vast diversity of bacteria, archaea, fungi, insects, annelids, and other invertebrates as well as plants and algae. Soils also play critical roles in buffering and filtering freshwater ecosystems. Over time these factors interact so that soils develop characteristic horizons. The profile of a soil reflects the decomposition and incorporation of organic materials into the mineral matrix, the formation of humus, and the processes of mineral weathering. Decomposition and weathering are mediated by soil microbes (Aislabie *et al.*, 2013). Soil is one of the more complex and highly variable habitats on earth. Any organisms that make their home in soil have 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 (Thies *et al.*, 2006).

SOIL MICROBES AND ITS IMPORTANCE

Microbes are the unseen majority in soil and comprise a large portion of life's genetic diversity. Soil microbes are important regulators of plant productivity, especially in nutrient poor ecosystems where plant symbionts are responsible for the acquisition of limiting nutrients. Mycorrhizal fungi and nitrogen-fixing bacteria are responsible for 5–20% (grassland and savannah) to 80% (temperate and boreal forests) of all nitrogen, and up to 75% of phosphorus, that is acquired by plants annually. Free-living microbes also strongly regulate plant productivity, through the mineralization of, and competition for, nutrients that sustain plant productivity. Soil microbes, including microbial pathogens, are also important regulators of plant community dynamics and plant diversity, determining plant abundance and, in some cases, facilitating invasion by exotic plants. Conservative estimates suggest that *c.* 000 plant species are completely dependent on microbial symbionts for growth and survival pointing to the importance of soil microbes as regulators of plant species richness on Earth. Overall, this review shows that soil microbes must be considered as important drivers of plant diversity and productivity in terrestrial ecosystems (Heijden *et al.*, 2008).

ENZYMES

Enzymes are biological catalysts, which initiate and accelerate thousands of biochemical reactions in living cells. Major sources of enzymes are the biological organisms, plants, animals and microorganisms (bacteria and fungi).Of these sources, microbial enzymes account for the major volume. However, about less than 50 species are actually used to produce the entire microbial enzyme. The potential obviously exists to search for the species producing novel enzymes or enzymes with better properties and yield (Verma *et al*.,2011).

STARCH AND AMYLASE

Starch is a heterogeneous polysaccharide composed of two high molecular – weight components, called amylose and amylopectin. α Amylose consists of long unbranched chains on which all the D-glucose units are bound in α (1-4) linkages (Patel, 2001). The chains are poly disperses and vary in Molecular weight from a few thousand to 500,000. Amylose is not truly soluble in water but forms hydrated micelles, which give a blue color with iodine. In such micelles, the polysaccharide chain is twisted into a helical coil. Amylopectin is highly branched, the average length of the branched is from 24 to 30 glucose resides, depending upon the species. The backbone glycosidic linkage is α (1-4) but the branch points are α (1-6) linkage. Amylopectin yields colloid or micelle solutions, which give a red – violet color with iodine. Its molecular weight may be as high as 100 million (Madhav *et al.,* 2011)

Amylases are enzymes which hydrolyze starch molecules to give diverse products including dextrin and progressively smaller polymers composed of glucose units (Windish *et al.*,1965). These enzymes are of great significance in present day biotechnology with applications ranging from food, fermentation, textile to paper industries. Although amylases can be derived from several sources, including plants, animals and microorganisms, microbial enzymes generally meet industrial demands. Today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in the starch processing industry (Pandey *et al.,* 2000).

Amylases can be divided into two categories, endoamylases and exoamylases. Endoamylases catalyze hydrolysis in a random manner in the interior of the starch molecule. This action causes the formation of linear and branched oligosaccharides of various chain lengths. Exoamylases hydrolyze from the non-reducing end, successively resulting in short end products. Today a large number of enzymes are known which hydrolyze starch molecule into different products and a combined action of various enzymes is required to hydrolyze starch completely.

A number of reviews exist on amylases and their applications; however, none specifically covers α-amylases at length. α-Amylases are one of the most popular and important forms of industrial amylases and the present review highlights the various aspects of microbial α-amylases (Naidu *et al.*, 2013).

MICROORGANISMS AND AMYLASES

Amylases can be derived from several sources, such as plants, animals, bacteria and fungi. Because of the short growth period, biochemical diversity and the ease with which enzyme concentrations might be increased by environmental and genetic manipulation, the enzymes from microbial sources generally meet industrial demands (Naidu *et al.,* 2013). The majority of enzymes used to date have been obtained from mesophilic microorganisms. The applications of these enzymes are restricted because of their limited stability to extreme temperature, pH and ionic strength (Utong *et al.,* 2006). Therefore, efforts were made on the enzymes of thermophilic and halophilic bacteria, which could be used in many harsh industrial processes where the concentrated salt solution and high temperatures used would inhibit many enzymatic conversions (Naidu *et al.,* 2013).

The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and microbes are easy to manipulate to obtain enzymes of desired characteristics. α -Amylase has been derived from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from fungal and bacterial sources have dominated applications in industrial sectors (Manonmani *et al.,* 1999). Several *Bacillus sp.* and thermostable *Actinomycetes* including *Thermomonospora* and *Thermoactinomyces* are versatile producers of the enzymes (Massoud *et al.,*1999).

ISOLATION OF SOIL BACTERIA

Serial Dilution techniques are widely used in various areas of microbiology, when numerous media are used for the differential enumeration of mixed microbial populations (Stotzky *et al*; 1965). Serial dilution is a common practice to determine microbial counts for both liquid and solid specimens. The identifying a method for bacterial enumeration, used first for the study of water quality *About Detection Methods for Microorganisms in Water* in 1883. The objective of the serial dilution is to estimate the number of colonies on agar plate cultivated from serial dilution by total dilution of tube (used to make plate for colony count) multiplied by the volume plated (Reynolds ;2005).

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.,* 1973).

SCREENING OF AMYLASE PRODUCING BACTERIA

Bacterial isolates were screened for amylolytic properties by starch hydrolysis test on starch agar plate. The microbial isolates were streaked as a line on the starch agar plate and plates were incubated at 37°C for 24 hr. After incubation 1 % iodine solution (freshly prepared) was flooded on the starch agar plate. Presence of blue colour around the growth indicates negative result and a clear zone of hydrolysis surrounding the growth indicates positive result.

The isolates produced clear zones of hydrolysis were considered as amylase producers (Hamilton *et al.*, 1999).

Production or secretion of amylase can be screened by different common methods, including solid-based or solution based techniques. The solid-based method is carried out on nutrient agar plates containing starch as the substrate, whereas solution-based methods include the dinitro salicylic acid (DNS) and Nelson-Somogyi (NS) techniques. In the solid-agar method, the appropriate strain (fungi or bacteria) is pinpoint-inoculated onto the starch-containing agar at the center of the petri plate. After an appropriate incubation period, the plate is flooded with iodine solution, which reveals a dark bluish color on the substrate region and a clear region (due to hydrolysis) around the inoculum, indicating the utilization of starch by the microbial amylase (Gopinath *et al.*,2003).

BIOCHEMICAL CHARACTERIZATION OF BACTERIA

Proteins are molecules essential to the structure and function of all living organisms. They are made up of chains of any number of amino acids linked by peptide bonds and folded in a variety of complex structures. Proteins may be broken down into amino acids and peptides by hydrolysis, using strong acids, bases or proteolytic enzymes, in order to provide nutrients in forms that cells may easily utilize. Protein hydrolysates, also called peptones, are the result of the hydrolysis process on protein material (Power *et al.*, 2009).

The citrate test screens a bacterial isolate for the ability to utilize citrate as its carbon and energy source. Simmons citrate medium is employed as an agar slant, and the solid surface makes it easier to determine whether or not a significant amount of growth has occurred. Because citrate utilization requires oxygen, the slant style will also increase the amount of growth. The increase in pH of the medium that occurs upon citrate breakdown can be detected by the concomitant change in the bromothymol blue (BTB) color (Williams,2009).

Indole nitrite medium was developed to serve the dual role of detecting indole production and nitrate reduction of a wide range of microorganisms. Due to its nutritive content, the medium will support the growth of aerobes, microaerophiles and facultative and obligate anaerobes.

The casein peptone contains tryptophan, which is attacked by certain microorganisms, resulting in the production of indole, detectable by the addition of chemical reagents to 18- to 48-hour cultures. Potassium nitrate serves as the substrate for determining the ability of microorganisms to reduce nitrates to nitrites (Power *et al.,* 2009). Indole nitrite medium can be used for nitrite tests with members of the Enterobacteriaceae but is not recommended for the indole test with these organisms since they reduce nitrate to nitrite, which prevents the detection of indole. Tryptophan (Trypticase™) 1% Solution is the medium of choice for indole test with enteric bacilli (Power *et al.,* 2009).

DNA ISOLATION

The process of DNA isolation includes cell lysis and homogenization which typically involves heating, detergents, and/or mechanical force followed by the stepwise removal of all non-DNA constituents and the eventual elution of DNA in a suitable storage buffer (Whitehouse and Hottel, 2006, Mahmoudi *et al*., 2011). Numerous commercial DNA extraction kits are available that enable the processing of high volumes of samples with relatively lower cost and time consumption in comparison with previous established methods (Whitehouse and Hottel, 2006; Dauphin *et al*., 2009).

Electrophoresis belongs to the most commonly used method for the preparation of nucleic acids and proteins, for further analysis. With the help of electrophoresis, it is possible to separate mixtures of DNA fragments (and other polar macromolecules), which cannot be separated by other methods (for instance by centrifugation in a dense gradient). Electrophoresis is the movement of electrically charged particles (molecules) towards an electrode with an opposite polarity (charge). The most commonly used carriers are polyacrylamide or agarose gel, which forms a complicated network structure of polymeric molecules with pores. The size of the pores in the resulting gel can be influenced by the composition of the solution and the concentration of the polymer from which the gel is

prepared. The main carrier of the nucleic acids charge, which enables the movement of the DNA in a electric field, are the negatively charged phosphate groups, so the sample application hollows have to be on the side of the cathode and molecules will be pulled towards the anode (Bohmer *et al;* 2010).

POLYMERASE CHAIN REACTION (PCR)

PCR is a nucleic acid amplification technique developed in the 1980's that has become a cornerstone of many molecular analyses employed across nearly the entire spectrum of biological sciences. PCR proceeds via the cyclical denaturation of DNA molecules by heating, followed by cooling which triggers hybridization with target sequenced primers (most often targeting genes coding for 16S rRNA in prokaryotes) that are assembled into complementary strands, or copies, of the original fragment by DNA polymerase (Mullis *et al*., 1986).

DNA SEQUENCING

DNA sequencing is one of the most important platforms for the study of biological systems today. Sequence determination is most commonly performed using dideoxy chain termination technology. (Mizusawa *et al;* 1986).

BASIC LOCAL ALIGNMENT SEARCH TOOL (BLAST)

A new approach to rapid sequence comparison, basic local alignment search tool (BLAST), directly approximates alignments that optimize a measure of local similarity, the maximal segment pair (MSP) score. Basic local alignment search tool (BLAST) is a sequence similarity search program that can be used via a web interface or as a stand-alone tool to compare a user's query to a database of sequences (Altschul *et al.,* 1990).

BLAST employs a measure based on well-defined mutation scores. The method will detect weak but biologically significant sequence similarities, and is more than an order of magnitude faster than existing heuristic algorithms (Stephen *et al*., 1990).

INDUSTRIAL APPLICATINS OF α-AMYLASE

α-Amylase is gaining increased attention due to its starch hydrolyzing properties and the activities that can be carried out owing to this property. There are many potentials and widely used applications of this enzyme on the industrial front. Enzymes have replaced the previously used chemical methods of hydrolysis in various industrial sectors to make the process environment friendly and make processes easier (Sundarram *et al.,* 2014).

a. Production of Fructose and Glucose by Enzymatic Conversion of Starch

Starch is used in the production of fructose and glucose syrups (Maarel *et al.,* 2002). This process involves three steps: Gelatinization, Liquefaction, and Saccharification. Gelatinization involves the dissolving of starch granules in water to form a viscous starch suspension. The amylose and amylopectin are dispersed into the water on dissolution. Liquefaction of starch is its partial hydrolysis into short chain dextrins by α-Amylase resulting in reduction of the viscosity of the starch suspension. Saccharification is the production of glucose and fructose syrup by further hydrolysis. This is carried out by glucoamylase which acts as an exo-amylase by cleaving the α-1, 4 glycosidic linkages from the non reducing terminal. The action of pullulanase along with glucoamylase yields high glucose syrup. This high glucose syrup can then be converted into high fructose syrup by isomerization catalysed by glucose isomerase. The fructose syrup obtained is used as a sweetener, especially in the beverage industry (Sundarram *et al.,* 2014).

b. Bakery Industry

α-Amylase is added to the dough in the bread baking process. This causes the starch to hydrolyze into small dextrins which can further be fermented by yeast. This increases the rate

of fermentation. Also, the starch hydrolysis decreases the viscosity of the dough, thus improving its texture and increasing loaf volume by rising of dough (Sundarram *et al.*, 2014).

c. Detergent Industry

The use of enzymes in detergents has increased with the changing methods of dishwashing and laundry. Consumers prefer to use cold water and mild conditions which requires the detergent to work in those imitations. Earlier the chemicals used in detergents caused harm when ingested and the conditions of dishwashing were very harsh. Hence enzymes showed the industry an alternative path. The enzymes are environmentally safe and work at mild conditions. α-Amylase is used to digest the starch containing food particles into smaller water soluble oligosaccharides. Starch can attract soil particles onto the clothes. Hence removal of starch is also important to maintain the whiteness of clothes. The stability of α-Amylase at low temperature and alkaline pH contributes to its extensive use in detergents (Sundarram *et al.*, 2014).

d. Desizing of Textiles

Modern production processes in the textile industry can cause breaking of the warp thread. To strengthen the thread, sizing agents are used which strengthen the thread by forming a layer on it and can be removed after the fabric is woven. Starch is a preferred sizing agent as it is easily available, cheaper and can be easily removed from the fabric. The layer of starch is subjected to hydrolysis in the desizing process where α-Amylase is employed to cleave starch particles randomly into water soluble components that can be removed by washing. The enzyme acts specifically on the starch molecules alone, leaving the fibers unaffected (Sundarram *et al.*, 2014).

e. Paper Industry

Like textiles, paper is also treated with sizing agents to protect it from mechanical strain during processing. The sizing also contributes to better quality of the paper in terms of strength, smoothness, writing and erasability. Starch is commonly used as the sizing agent. The role of α-Amylase in the paper industry is the partial hydrolysis of starch to make it less viscous in a batch or a continuous process. This is owing to the highly viscous nature of natural starch making it unsuitable for coating on paper (Sundarram *et al.,* 2014).

f. Fuel Alcohol Production

Among biofuels, ethanol is most widely used. As starch is an economical starting material, it is used for the production of ethanol as a biofuel. This is done in a series of steps. Firstly, the starch is subjected to liquefaction to form a viscous starch suspension. This is followed by the saccharification process where the starch is hydrolyzed by α-Amylase to yield fermentable sugars. These sugars are then fermented by yeast to produce alcohol. As an improvisation of this process, protoplast fusion between the amylolytic yeast *Saccharomyces fibuligera* and *S. cerevisiae* was performed to obtain a new yeast strain that can directly produce the biofuel from starch, eliminating the need for a saccharification step (Chi *et al., 2009).*

MATERIALS AND METHODS

1. COLLECTION OF SOIL SAMPLES

Materials Required: Sterile polythene bags, spatula, and gloves etc.

The waste soil samples, namely the poultry farm soil, food and agricultural waste soil, plastic waste soil,compost soil etc were collected aseptically at a depth of 4cm from different locations of the Ernakulam and Kottayam districts of Kerala, India. Samples were properly labeled and brought to the laboratory in sterile conditions and kept in sealed bags for further studies.

2. ISOLATION OF SOIL BACTERIA

Materials Required: Soil samples, nutrient agar, nutrient broth, petri plates, test tubes, sterile distilled water, conical flask, 1ml pipette sterile tips and sterile loop etc.

a. Serial dilution

Soil bacteria was isolated using serial dilution and pour plate method on nutrient agar medium. Weigh one gram of waste soil sample and transfer it to 9ml sterile distilled water and mix well. Make serial dilutions from 10^{-} 1 to 10^{-6} dilutions.

b. Pour plating

Transfer 1ml from each dilution to the sterile petri plates, pour the nutrient agar medium and mix the plates well in clock and anticlockwise direction for uniform spreading of the sample. Incubate the petri plates for 24 to 48 hours at 37^{o}C until the bacterial colonies appear.

c. Quadrant Streaking

Bacterial isolates were inoculated on nutrient agar media using a sterile loop and incubated at 37°C for 24 hours so that the individual cells or colony forming units (CFUs) can become isolated and grow into individual, pure colonies.

d. Preservation of bacterial isolates

Nutrient agar slants were used for the storage of the purified bacterial isolates. Bacterial isolates were inoculated onto the nutrient agar slants using a sterile loop. The slants were incubated for 24 hours and stored in the refrigerator at 4°C.

3. SCREENING OF AMYLASE PRODUCING BACTERIA (Sharma *et al.*,2015*)*

Materials required: Bacterial isolates, sterile petri plates, nutrient agar, soluble starch, Gram's iodine, Potassium iodide (KI), and sterile loop etc.

Starch hydrolysis assay

Amylolytic properties of the isolated bacteria were screened using Nutrient agar supplemented with 2% starch solution. Isolated bacterial colonies were patched onto starch agar plates and incubated at 37°C for 48 hours. After incubation, amylase producing bacteria were identified by flooding the plates with Gram's iodine solution (prepared by dissolving 0.1 g of iodine crystals and 1.0 g of potassium iodide in 100 mL of distilled water, stored at room temperature).

Starch reacted with iodine to form a dark blue starch-iodine complex that covered the entire agar. The positive colonies demonstrate a region of clear zone of hydrolysis around the colonies when flooding with Gram's iodine solution. The negative colonies show no zone of hydrolysis around them against a blue-black coloration on starch agar.

4. SCREENING FOR EXTRACELLULAR AMYLASE PRODUCTION

Materials Required: Sterile petri plates, sterile tips, nutrient agar, soluble starch, bacterial supernatant, gel puncher etc.

Bacterial isolates were screened for extracellular activity by a well diffusion method on starch agar plate. On the starch agar plates, punch the well using the gel cutter and to these wells, 20µl each of culture supernatant were transferred. The plates were incubated for 24-48 hours at 37°C. After incubation, the zone of clearance was examined and the diameter of the clearance zone was measured.

5. BIOCHEMICAL CHARACTERIZATION OF AMYLASE PRODUCING BACTERIA

A. INDOLE TEST

Materials Required: Bacterial isolates, Tryptone broth, Indole reagent,Test tubes etc.

If the bacterial isolate possesses enzyme tryptophanase, they degrade the amino acid tryptophan to indole, pyruvic acid and ammonia. The test organism was inoculated in peptone broth and incubated for 2-3 days at 37°C. Development of red colour in the alcohol layer in addition to kovac's reagent indicates a positive reaction.

B. METHYL RED TEST

Materials Required: Bacterial isolates, Methyl red solution, etc.

Fermentation of glucose by the bacteria leads to the formation of acid, which changes the pH of the medium. The pH falls and is maintained below 4.5. This test detects the production of the acid with the help of the indicator dye such as methyl red. The dye changes colour when pH increases or decreases. The test organism was inoculated in glucose phosphate broth and after 2-3 days of incubation, five drops of 0.04% solution of methyl red were added to it. Positive tests give bright red colour and yellow colour indicates negative test.

C. VOGES PROSKAUER TEST

Materials Required: Bacterial isolates,MRVP broth, Barrit solution A and B etc.

Fermentation of carbohydrates by bacteria results in the production of acetyl methyl carbinol (acetoin). In the presence of alkali and atmospheric oxygen, acetoin is oxidized to diacetyl, which reacts with peptone of the broth to give a red colour. The test organism was inoculated in glucose phosphate broth and incubated at 37^{o}C for 48 hours,to this 0.5 ml of Barrit solution A and B is added. Positive result is indicated by the appearance of pink colour in the broth in 2-5 minutes.

D. CATALASE TEST

Materials Required: 3% hydrogen peroxide solution, nutrient broth, Bacterial isolates, droppers, test tubes, sterile loop etc.

A drop of 3% H_2O_2 was added to the nutrient broth inoculated with bacterial isolate. Prompt effervescence indicates catalase production.

E. CITRATE UTILIZATION TEST

Materials Required: Simmon citrate agar, test tubes, bacterial isolates, sterile loops etc.

This test is used to study the ability of an organism to utilize citrate present in Simmon's media as a sole source of carbon for the growth. The bacterial colony was directly inoculated on simmon's citrate agar containing bromothymol blue as indicator. Positive test is indicated by the appearance of growth with blue colour. Negative test shows no growth with the original green colour retained.

6. ANTIBIOTIC DISC SUSCEPTIBILITY TEST OF BACTERIAL ISOLATES AGAINST BROAD SPECTRUM ANTIBIOTICS (Banjara *et al.*, 2012*)*

Materials required: Sterile petri plates, Nutrient broth, Nutrient agar, Bacterial samples, antibiotic discs, L-rod, test tubes etc.

Inoculate the bacterial isolates in the nutrient broth and shake thoroughly and incubate at 37°C for 24 hours. Prepare a nutrient agar media, to the surface of this nutrient agar media swab the test organisms with an L-rod after immersing it in the bacterial broth culture. Antibiotic discs are placed on the nutrient agar medium using sterile forceps. Incubate the plates at 37°C. The bacterial isolates that show an inhibition zone were noted and the diameter of the zone were measured.

7. 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.

1) Transfer 2ml bacterial culture into a microcentrifuge tube and spin for 10 minutes at 8000 rpm.

2) Decant the supernatant completely and rinse the pellet with sterile distilled water.

3) Resuspend the pellet in 875 µl of Tris-EDTA (TE) buffer. Add 5µl Proteinase K and 100µl 10% sodium dodecyl sulphate (SDS). Incubate in a water bath at 37oC for 1hr.

4) Add equal volume of Phenol-Chloroform-Isoamyl Alcohol (25:24:1).

a. Phenol causes severe burns, take extra care and wear gloves.

b. Mix properly by inverting the tube and centrifuge at 8000rpm for 10 mins.

5) With the help of a wide mouthed tip carefully collect the upper aqueous layer into a fresh tube. Be sure not to carry over any phenol during pipetting.

6) Repeat the process twice (optional).

7) Add equal volume of chloroform and spin for 5minutes at 8000 rpm.

8) Collect the upper layer into a fresh tube.

9) Add 0.1 volume 3M sodium acetate (pH 5.2) and double volume ice cold isopropyl alcohol.

10) Spin at 10000 rpm for 10 min.

11) Decant the supernatant.

12) Add 1ml 70% ethanol and spin for 5 mins.

13) Decant the alcohol and air dry the pellet in a covered tray.

14) Dissolve the pellet in minimum volume TE (50µl) and store at -20ºC.

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 16SrDNA

Materials required: PCR Thermal cycler, template DNA, 16SrDNA Forward primer, Reverse primer, 5x assay buffer, dNTP mix, $MgCl_2$, 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
16S Forward	5' GAGTTTGATCCTGGCTCAG 3'	Shivaji *et al*., 2000
16S 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 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	30 sec	
Extension	72°C	2 min	
Final Extension	72°C	5 min	

The amplicon, then electrophoresed and visualized using a gel documentation system and 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. COLLECTION OF SOIL SAMPLES

A total of nine waste soil samples were collected from different locations in Ernakulam and Kottayam districts of Kerala, India, which includes the samples from food and agricultural waste dumping regions, waste disposal sites, poultry waste soil, plastic waste etc. The samples were then named with a three-letter code indicating the place of location.

Table 4: Sampling locations and their codes

SAMPLE NO.	LOCATION	SAMPLE CODE
1.	Poultry farm premises from Marakadavu, Ernakulam.	**ZMA**
2.	Agricultural waste from Varapuzha, Ernakulam.	**ZVP**
3.	Waste dumping area in Town hall, Kanjirapally.	**ZKC**
4.	Poultry farm waste soil from Manjakulam, Kottayam	**ZMM**
5.	Waste soil from the canal premises of Kalaketty, Kottayam	**ZKK**
6.	Waste dumping regions in Convent junction, Ernakulam.	**ZCJ**
7.	Waste dumping Yard, Kalamassery, Ernakulam	**ZKM**
8.	Waste dumping yard, Willington Island, Ernakulam	**ZWI**
9.	Waste soil from the Mullassery canal, Ernakulam	**ZMC**

2. ISOLATION OF SOIL BACTERIA

The soil bacteria were isolated using serial dilution and pour plate technique as depicted in **Figure 1.**The isolated bacterial samples were streaked onto nutrient agar medium for the growth of bacteria as individual pure colonies as indicated in **Figure 2.**A total of 78 bacterial isolates were obtained with distinct morphology, and designated as in (**Table 5**).

Table 5- List of bacterial isolates obtained

Sample number	Sample code	Sample number	Sample code	Sample number	Sample code	Sample number	Sample code
1	**ZWI 1**	13	**ZVP 1**	25	**ZKC 2**	37	**ZMA 6**
2	**ZWI 2**	14	**ZVP 2**	26	**ZKC3**	38	**ZMA 7**
3	**ZMC 1**	15	**ZVP 3**	27	**ZKC 4**	39	**ZMA 8**
4	**ZMC 2**	16	**ZVP 4**	28	**ZKC 5**	40	**ZMA 9**
5	**ZMC 3**	17	**ZVP 5**	29	**ZKC 6**	41	**ZMA 10**
6	**ZMC 4**	18	**ZVP 6**	30	**ZKC 7**	42	**ZMA 11**
7	**ZMC 5**	19	**ZVP 7**	31	**ZKC 8**	43	**ZMA 12**
8	**ZMC 6**	20	**ZVP 8**	32	**ZMA 1**	44	**ZMA 13**
9	**ZKM 1**	21	**ZVP 9**	33	**ZMA 2**	45	**ZMA 14**
10	**ZKM 2**	22	**ZVP 10**	34	**ZMA 3**	46	**ZMA 15**
11	**ZKM 3**	23	**ZVP 11**	35	**ZMA 4**	47	**ZMA 16**
12	**ZKM 4**	24	**ZKC 1**	36	**ZMA 5**	48	**ZMA 17**

49	**ZMA 18**	61	**ZMM 7**	73	**ZKK 4**
50	**ZMA 19**	62	**ZMM 8**	74	**ZKK 5**
51	**ZMA 20**	63	**ZMM9**	75	**ZKK 6**
52	**ZMA 21**	64	**ZMM 10**	76	**ZCJ 1**
53	**ZMA 22**	65	**ZMM 11**	77	**ZCJ 2**
54	**ZMA 23**	66	**ZMM 12**	78	**ZCJ 3**
55	**ZMM 1**	67	**ZMM 13**		
56	**ZMM 2**	68	**ZMM 14**		
57	**ZMM 3**	69	**ZMM 15**		
58	**ZMM 4**	70	**ZKK 1**		
59	**ZMM 5**	71	**ZKK 2**		
60	**ZMM 6**	72	**ZKK 3**		

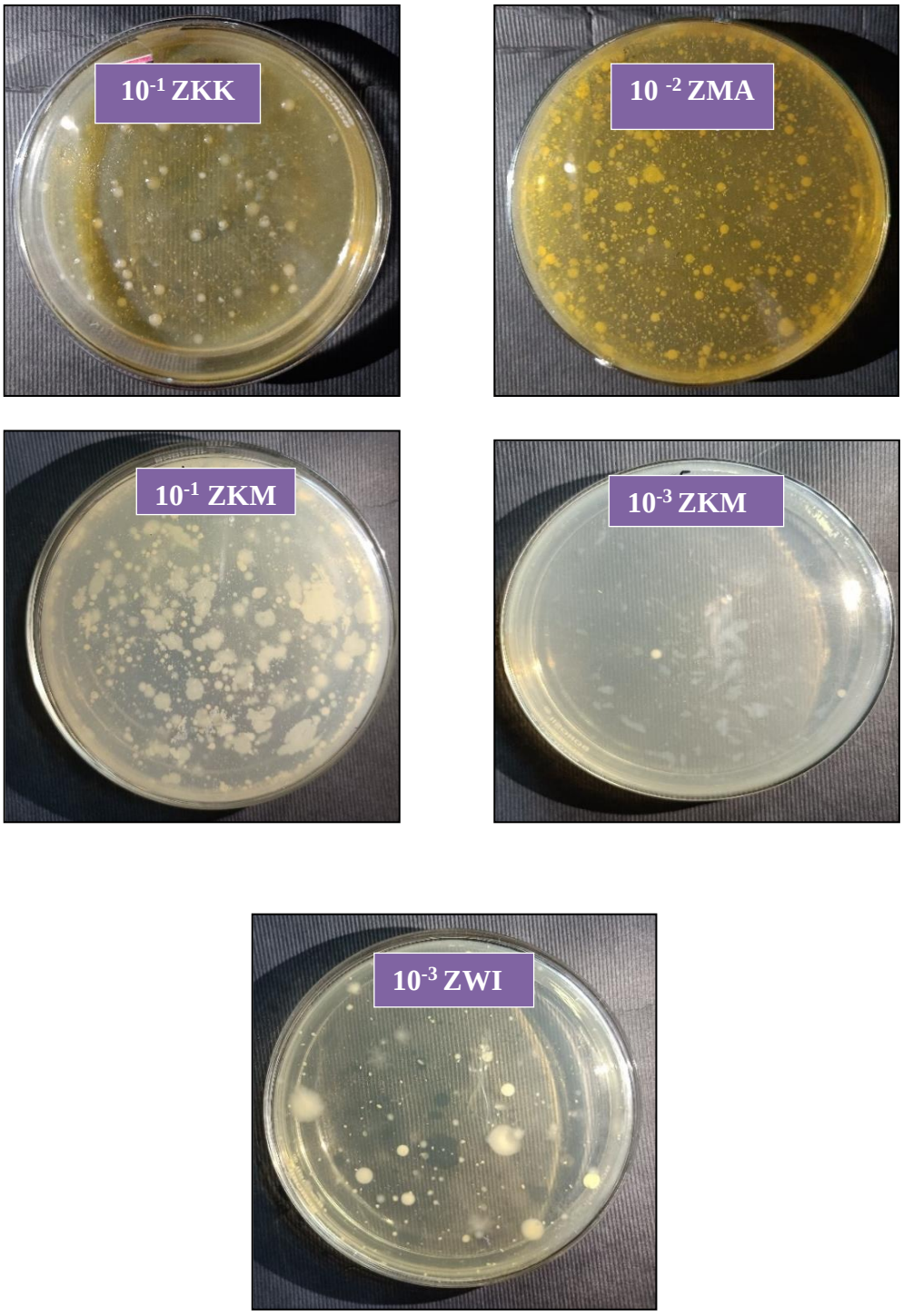


Fig 1- Bacterial colonies obtained after serial dilution and pour plate technique

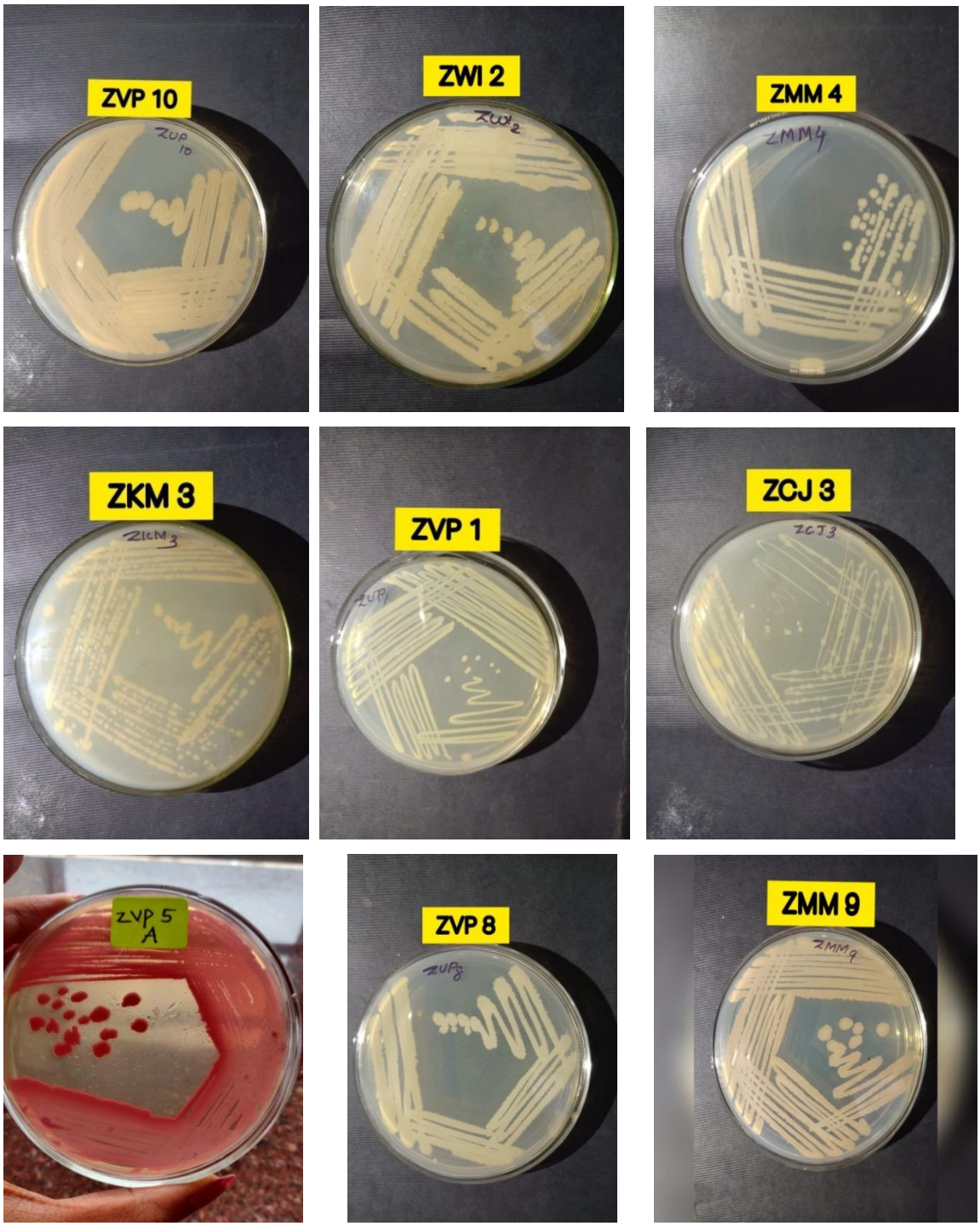


Fig 2–Pure colonies of bacterial isolates obtained by quadrant streaking

3. SCREENING OF AMYLASE PRODUCING BACTERIA

The 78 bacterial isolates were screened for amylase production using patch plate technique. The bacterial isolates patched on the starch agar plates, produced definite clear zones on flooding the plates with Gram's iodine solution and hence indicate the amylase production as illustrated in **Figure 3**. Among the total of 78 bacterial isolates screened, 34 isolates were confirmed to have amylase activity as represented in (**Table 6**). Those isolates with prominent zone of clearance on the starch agar medium were further screened for their extracellular amylase production by disc diffusion assay.

Table 6- List of amylase producing bacteria

Sample no.	Bacterial Strain	Result	Sample no.	Bacterial Strain	Result	Sample no.	Bacterial Strain	Result
1	ZWI 1	-ve	15	ZVP 1	+ve	29	ZMM 3	-ve
2	ZWI 2	+ve	16	ZVP 2	-ve	30	ZMM 4	+ve
3	ZMC 1	-ve	17	ZVP 3	-ve	31	ZMM 5	-ve
4	ZMC 2	+ve	18	ZVP 4	-ve	32	ZMM 6	-ve
5	ZMC 3	+ve	19	ZVP 5	-ve	33	ZMM 7	-ve
6	ZMC 4	+ve	20	ZVP 6	-ve	34	ZMM 8	-ve
7	ZMC 5	-ve	21	ZVP 7	-ve	35	ZMM 9	+ve
8	ZMC 6	-ve	22	ZVP 8	+ve	36	ZMM 10	-ve
9	ZKM 1	+ve	23	ZVP 9	-ve	37	ZMM 11	-ve
10	ZKM 2	+ve	24	ZVP 10	+ve	38	ZMM 12	-ve
11	ZKM 3	+ve	25	ZVP 11	+ve	39	ZMM 13	+ve
12	ZKM 4	+ve	26	ZKC 1	-ve	40	ZMM 14	-ve
13	ZKK 1	+ve	27	ZKC 2	+ve	41	ZMM 15	-ve
14	ZKK 2	+ve	28	ZKC 3	-ve	42	ZMA 1	+ve

43	ZKK 3	-ve	63	ZKC 8	+ve
44	ZKK 4	-ve	64	ZMM 1	-ve
45	ZKK 5	+ve	65	ZMM 2	+ve
46	ZKK 6	+ve	66	ZMA 18	-ve
47	ZCJ 1	-ve	67	ZMA 19	-ve
48	ZCJ 2	+ve	68	ZMA 20	-ve
49	ZCJ 3	+ve	69	ZMA 21	-ve
50	ZMA 9	-ve	70	ZMA 22	-ve
51	ZMA 10	+ ve	71	ZMA 23	+ ve
52	ZMA 11	+ ve	72	ZMA 2	+ve
53	ZMA 12	+ ve	73	ZMA 3	-ve
54	ZMA 13	-ve	74	ZMA 4	-ve
55	ZMA 14	- ve	75	ZMA 5	-ve
56	ZMA 15	- ve	76	ZMA 6	+ve
57	ZMA 16	- ve	77	ZMA 7	-ve
58	ZMA 17	+ ve	78	ZMA 8	-ve
59	ZKC 4	-ve			
60	ZKC 5	+ve			
61	ZKC 6	-ve			
62	ZKC 7	+ve			

+ve : Activity **-ve : No activity**

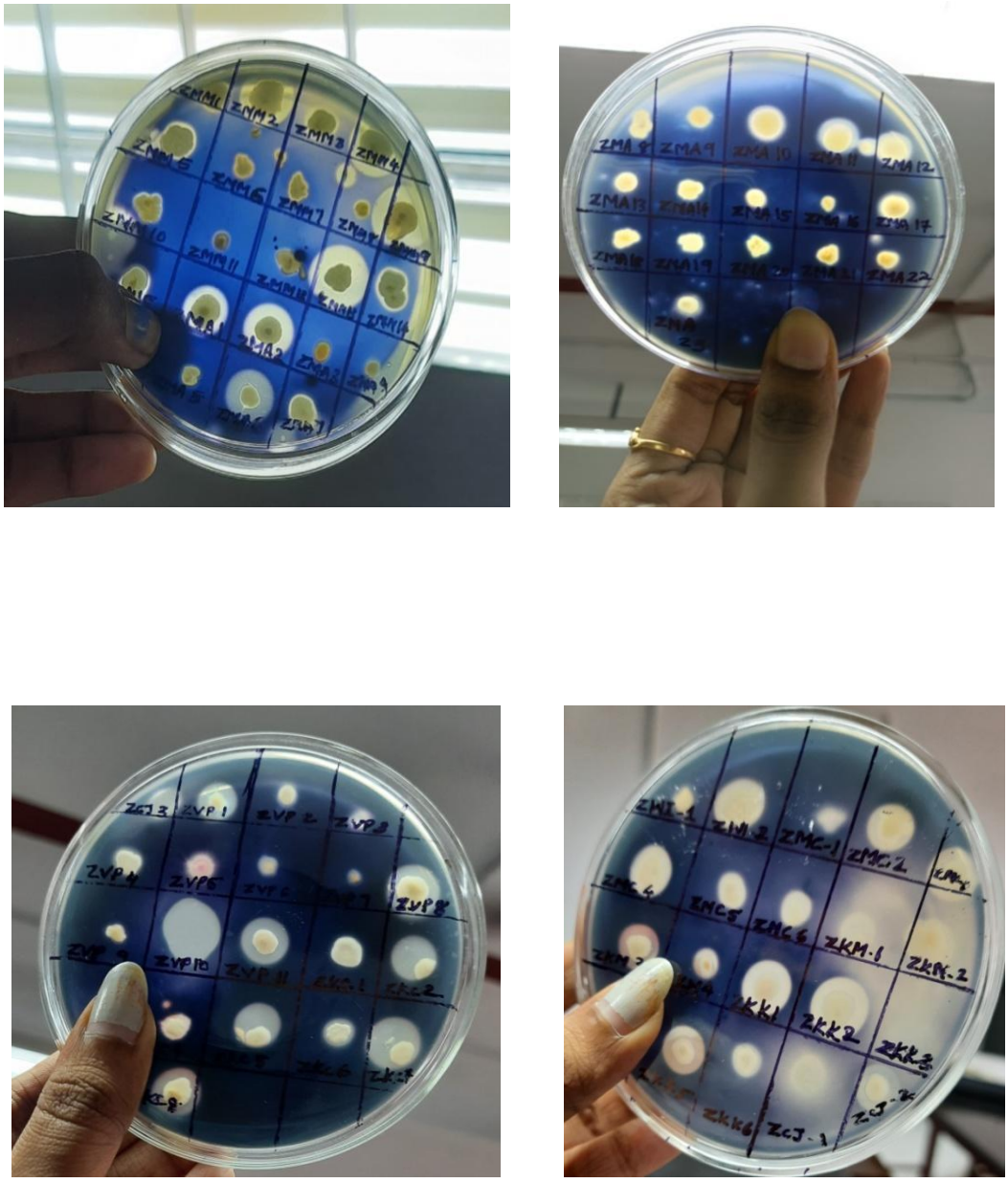


Fig 3- Starch agar plate showing the clear zones around bacterial colonies

4. SCREENING FOR EXTRACELLULAR AMYLASE PRODUCTION

The bacterial isolates that exhibit the clear zones during starch hydrolysis test for amylase production were further screened for their extracellular activity by the well diffusion method on starch agar plate as shown in **Figure 4.** The diameter of clear zones was measured and tabulated as in (**Table 7**).

The amylase producing bacterial samples with significant and notable results in both the screening procedures were further analysed for their biochemical and molecular characterization.

Table 7 – Diameter of clearance zone by amylase producing bacteria

Sl. No.	Bacterial isolates	Zone diameter (in cm)
1.	ZMM 2	2
2.	ZMM4	2.5
3.	ZVP 1	2.3
4.	ZMC 2	1.5
5.	ZKK 5	1.3
6.	ZCJ 3	2.8
7.	ZCJ 2	1.8
8.	ZMM 9	2
9.	ZKC 7	1.7
10.	ZWI 2	1.6
11.	ZKK 1	2
12.	ZMA 2	1.4
13.	ZMA 12	2
14.	ZMM 13	2
15.	ZVP 10	2.6
16.	ZKK 2	2
17.	ZMA 6	2
18.	ZMA 11	1.8
19.	ZMC 4	2
20.	ZKM 3	1.7
21.	ZVP 11	2
22.	ZVP 8	2.1
23.	ZKC 2	2
24.	ZMA 17	1.4

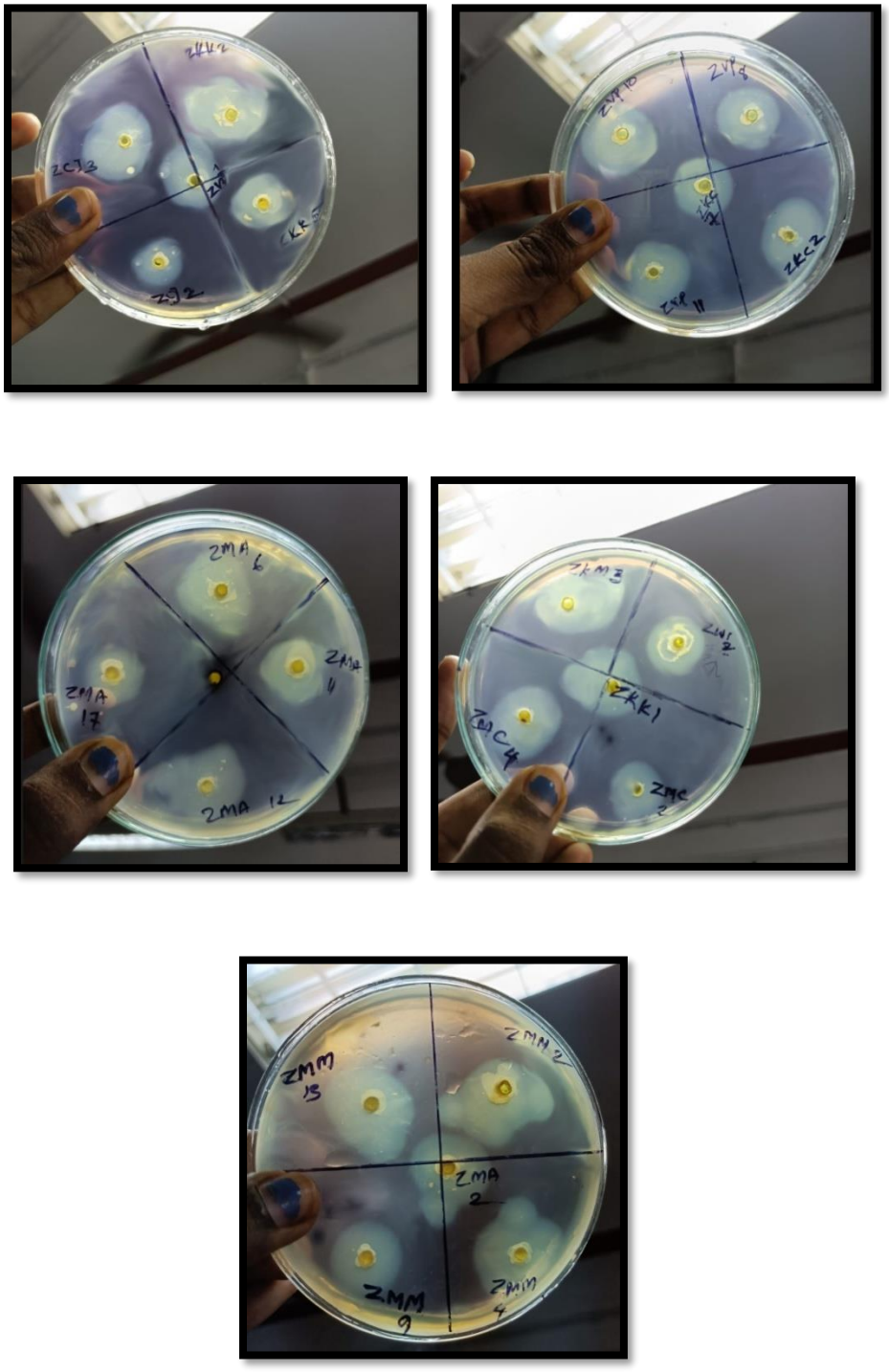


Fig 4- Amylase producing bacteria with the clear zones on starch agar medium

5. BIOCHEMICAL CHARACTERIZATION OF AMYLASE PRODUCING BACTERIA

A. INDOLE TEST

All the test isolates screened revealed negative result for the indole test as displayed in **Figure 5.**Non - appearance of red colouration on addition of Kovac's reagent, indicates the absence of enzyme tryptophanase in bacterial isolates which on presence degrade the amino acid tryptophan,and indicated in (**Table 8**).

Table 8 –Indole Test result

Bacterial isolates	Result
ZCJ 3	-
ZVP 1	-
ZVP 8	-
ZVP 10	-
ZMM 4	-
ZMM9	-
ZWI 2	-
ZKM3	-

-: No activity

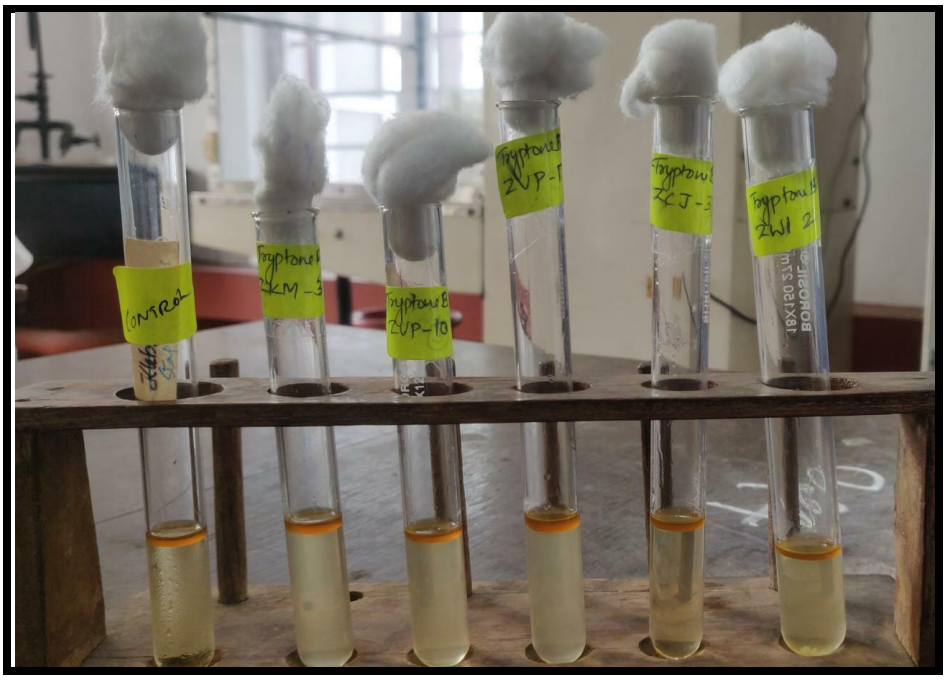


Fig 5- Indole Test result

B. METHYL RED TEST

Methyl red test detects the production of acid by the bacteria as the fermentation of glucose occurs; this is indicted upon addition of the indicator solution methyl red. Out of the eight test organisms screened, the six bacterial isolates namely ZCJ 3, ZVP 8, ZVP 10, ZMM 4, ZMM 9, ZKM 3 were acid fermenters or methyl red test positive giving pink colouration in the glucose phosphate broth and two bacterial isolates namely ZWI 2, ZVP 1gives a yellow colour which indicates the negative result for the testas shown in **Figure 6**and depicted in the **(Table9).**

Table 9 - Methyl red test results

Bacterial isolates	Result
ZCJ 3	+
ZVP 1	-
ZVP 8	+
ZVP 10	+
ZMM 4	+
ZMM9	+
ZWI 2	-
ZKM3	+

+ : Activity -: No activity

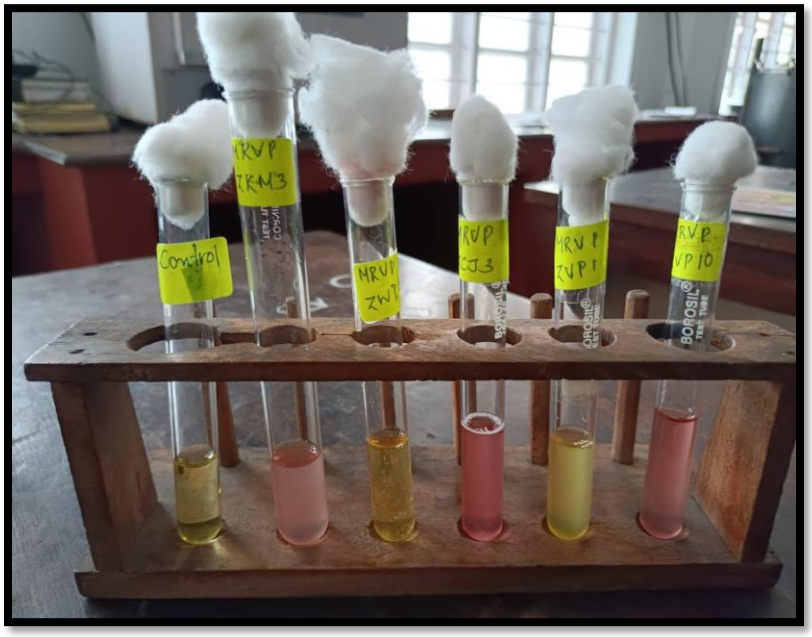


Fig 6- Methyl red test result

C. **VOGES PROSKAUER TEST**

VP test screens the ability of the microorganisms to produce acetoin (acetyl methyl carbinol) as the end product of glucose fermentation. On addition of 0.5 ml of Barritt A and B solution, to the glucose phosphate broth containing inoculated test bacterial samples, the development of red colour indicates VP test positive and a yellow colouration gives a negative result. Out of the eight test organisms screened, the bacterial isolateZVP 1 were VP test positive giving red colouration in the glucose phosphate broth and seven bacterial isolates namely ZWI 2, ZKM 3, ZCJ 3, ZVP 8, ZVP 10, ZMM 4, ZMM 9 gives a yellow colour which indicates the negative result for the testas shown in **Figure 7** and depicted in the **(Table10).**

Table 10 –Voges Proskauer test results

Bacterial isolates	Result
ZCJ 3	-
ZVP 1	+
ZVP 8	-
ZVP 10	-
ZMM 4	-
ZMM9	-
ZWI 2	-
ZKM3	-

+ : Activity -: No activity

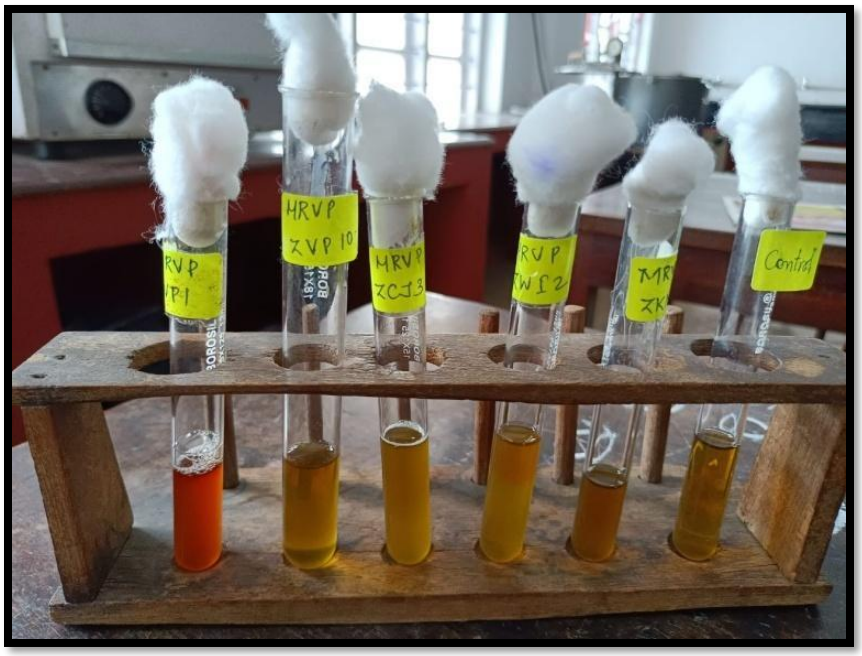


Fig 7- Vokes-Proskaur test result

D.CATALASE TEST

On addition of a drop of 3% H_2O_2 to the nutrient broth inoculated with the bacterial isolate, development of prompt effervescence indicates catalase production. Out of the eight test bacterial isolates tested, all the bacterial isolates were confirmed to be catalase test positive as depicted in (**Table 11)**by the appearance of prompt effervescence as shown in **Figure 8**.

Table 11–Catalase test results

Bacterial isolates	Result
ZCJ 3	+
ZVP 1	+
ZVP 8	+
ZVP 10	+
ZMM 4	+
ZMM9	+
ZWI 2	+
ZKM3	+

+ : Activity

Fig 8- Catalase test result

D.CITRATE UTILIZATION TEST

The organism capable of utilizing citrate present in Simmon's media as a sole source of carbon for their growth is indicated by the appearance of growth in the medium with blue colour. Negative test shows no growth with the original green colour retained. Out of the eight test organisms screened, the seven bacterial isolates ZWI 2, ZKM 3, ZCJ 3, ZVP 8, ZVP 10, ZMM 4, ZVP 1 were test positive and bacterial isolate namely ZMM 9 were test negative as indicated in **Figure 9** and depicted in (**Table 12**).

Table 12 –Simmon'sCitrate test results

Bacterial isolates	Result
ZCJ 3	+
ZVP 1	+
ZVP 8	+
ZVP 10	+
ZMM 4	+
ZMM9	-
ZWI 2	+
ZKM3	+

+ : Activity **- : No activity**

Fig 9- Simmon's Citrate test result

6. ANTIBIOTIC DISC SUSCEPTIBILITY TEST OF BACTERIAL ISOLATES AGAINST BROAD SPECTRUM ANTIBIOTICS

The test for the effect of antibiotics of the selected bacterial isolates was conducted. Ten different antibiotics were selected and this was done by the lawn culture technique. The zone of inhibition is produced on the plates by the bacterial samples as shown in **Figures 10-17** and diameter of the zones of each antibiotic disc are measured as depicted in the (**Table 13-20**) and based on which interpretation of the result susceptibility or resistant is made.

Table 13- Effect of antibiotics for the bacterial sample ZCJ3

Sl.No	Antibiotics(Dose)	Bacterial isolate	Diameter (mm)	Sensitive	Moderately sensitive	Resistant
1.	Erythromycin(15µg)	ZCJ 3	43	✓	-	-
2.	Cloxacillin(1µg)		-	-	-	✓
3.	Chlorampenicol(30µ)		18	✓	-	-
4.	Gentamicin(30µg)		22	✓	-	-
5.	Nalidixic Acid(30µg)		13	–	-	✓
6.	Streptomycin(25µg)		21	✓	-	-
7.	Tetracycline(30µg)		9	-	-	✓
8.	Cefuroxime(30µg)		-	-	-	✓
9.	Ampicillin(10µg)		-	-	-	✓
10.	Pencillin(10µg)		-	-	-	✓

Table 14- Effect of antibiotics f or the bacterial sample ZVP 1

Sl.No	Antibiotics(Dose)	Bacterial isolate	Diameter (mm)	Sensitive	Moderately sensitive	Resistant
1.	Erythromycin(15µg)	ZVP 1	20	-	✓	-
2.	Cloxacillin(1µg)		-	-	-	✓
3.	Chlorampenicol(30µ)		19	✓	-	-
4.	Gentamicin(30µg)		35	✓	-	-
5.	Nalidixic Acid(30µg)		22	✓	-	-
6.	Streptomycin(25µg)		14	-	-	✓
7.	Tetracycline(30µg)		14	-	✓	-
8.	Cefuroxime(30µg)		-	-	-	✓
9.	Ampicillin(10µg)		11	-	-	✓
10.	Pencillin(10µg)		-	-	-	✓

Table 15- Effect of antibiotics for the bacterial sample ZVP 8

Sl.No	Antibiotics(Dose)	Bacterial isolate	Diameter (mm)	Sensitive	Moderately sensitive	Resistant
1.	Erythromycin(15µg)	ZVP 8	-	-	-	✓
2.	Cloxacillin(1µg)		-	-	-	✓
3.	Chlorampenicol(30µ)		18	✓	-	-
4.	Gentamicin(30µg)		7		-	✓
5.	Nalidixic Acid(30µg)		24	✓	-	-
6.	Streptomycin(25µg)		22	✓	-	-
7.	Tetracycline(30µg)		10	-	-	✓
8.	Cefuroxime(30µg)		-	-	-	✓
9.	Ampicillin(10µg)		-	-	-	✓
10.	Pencillin(10µg)		-	-	-	✓

Table 16- Effect of antibiotics for the bacterial sample ZVP 10

Sl.No	Antibiotics (Dose)	Bacterial isolate	Diameter (mm)	Sensitive	Moderately sensitive	Resistant
1.	Erythromycin(15µg)	ZVP 10	27	✓	-	-
2.	Cloxacillin(1µg)		-	-	-	✓
3.	Chlorampenicol(30µ)		20	✓	-	-
4.	Gentamicin(30µg)		10	-	-	✓
5.	Nalidixic Acid(30µg)		28	✓	-	-
6.	Streptomycin(25µg)		23	✓	-	-
7.	Tetracycline(30µg)		10	-	-	✓
8.	Cefuroxime(30µg)		-	-	-	✓
9.	Ampicillin(10µg)		-	-	-	✓
10.	Pencillin(10µg)		-	-	-	✓

Table 17- Effect of antibiotics for the bacterial sample ZMM 4

Sl.No	Antibiotics(Dose)	Bacterial isolate	Diameter (mm)	Sensitive	Moderately sensitive	Resistant
1.	Erythromycin(15µg)	ZMM 4	33	✓	-	-
2.	Cloxacillin(1µg)		-	-	-	✓
3.	Chlorampenicol(30µ)		22	✓	-	-
4.	Gentamicin(30µg)		23	✓	-	-
5.	Nalidixic Acid(30µg)		25	✓	-	-
6.	Streptomycin(25µg)		14	-	-	✓
7.	Tetracycline(30µg)		14	-	✓	-
8.	Cefuroxime(30µg)		-	-	-	✓
9.	Ampicillin(10µg)		-	-	-	✓
10.	Pencillin(10µg)		-	-	-	✓

Table 18- Effect of antibiotics for the bacterial sample ZMM 9

Sl.No	Antibiotics(Dose)	Bacterial isolate	Diameter (mm)	Sensitive	Moderately sensitive	Resistant
1.	Erythromycin(15µg)	ZMM 9	30	✓	-	-
2.	Cloxacillin(1µg)		-	-	-	✓
3.	Chlorampenicol(30µ)		17	-	✓	-
4.	Gentamicin(30µg)		22	✓	-	-
5.	Nalidixic Acid(30µg)		26	✓	-	-
6.	Streptomycin(25µg)		21	✓	-	-
7.	Tetracycline(30µg)		17	✓	-	-
8.	Cefuroxime(30µg)		-	-	-	✓
9.	Ampicillin(10µg)		-	-	-	✓
10.	Pencillin(10µg)		-	-	-	✓

Table 19- Effect of antibiotics for the bacterial sample ZWI 2

Sl.No	Antibiotics (Dose)	Bacterial isolate	Diameter (mm)	Sensitive	Moderately sensitive	Resistant
1.	Erythromycin(15µg)	ZWI 2	-	-	-	✓
2.	Cloxacillin(1µg)		-	-	-	✓
3.	Chlorampenicol(30µ)		27	✓	-	-
4.	Gentamicin(30µg)		25	✓	-	-
5.	Nalidixic Acid(30µg)		10	-	-	✓
6.	Streptomycin(25µg)		28	✓	-	-
7.	Tetracycline(30µg)		11	-	-	✓
8.	Cefuroxime(30µg)		-	-	-	✓
9.	Ampicillin(10µg)		-	-	-	✓
10.	Pencillin(10µg)		-	-	-	✓

Table 20- Effect of antibiotics for the bacterial sample ZKM 3

Sl.No	Antibiotics(Dose)	Bacterial isolate	Diameter (mm)	Sensitive	Moderately sensitive	Resistant
1.	Erythromycin(15µg)	ZKM 3	17	-	✓	-
2.	Cloxacillin(1µg)		-	-	-	✓
3.	Chlorampenicol(30µ)		23	✓	-	-
4.	Gentamicin(30µg)		20	✓	-	-
5.	Nalidixic Acid(30µg)		13	-	-	✓
6.	Streptomycin(25µg)		18	-	✓	–
7.	Tetracycline(30µg)		13	-	✓	-
8.	Cefuroxime(30µg)		-	-	-	✓
9.	Ampicillin(10µg)		-	-	-	✓
10.	Pencillin(10µg)		-	-	-	✓

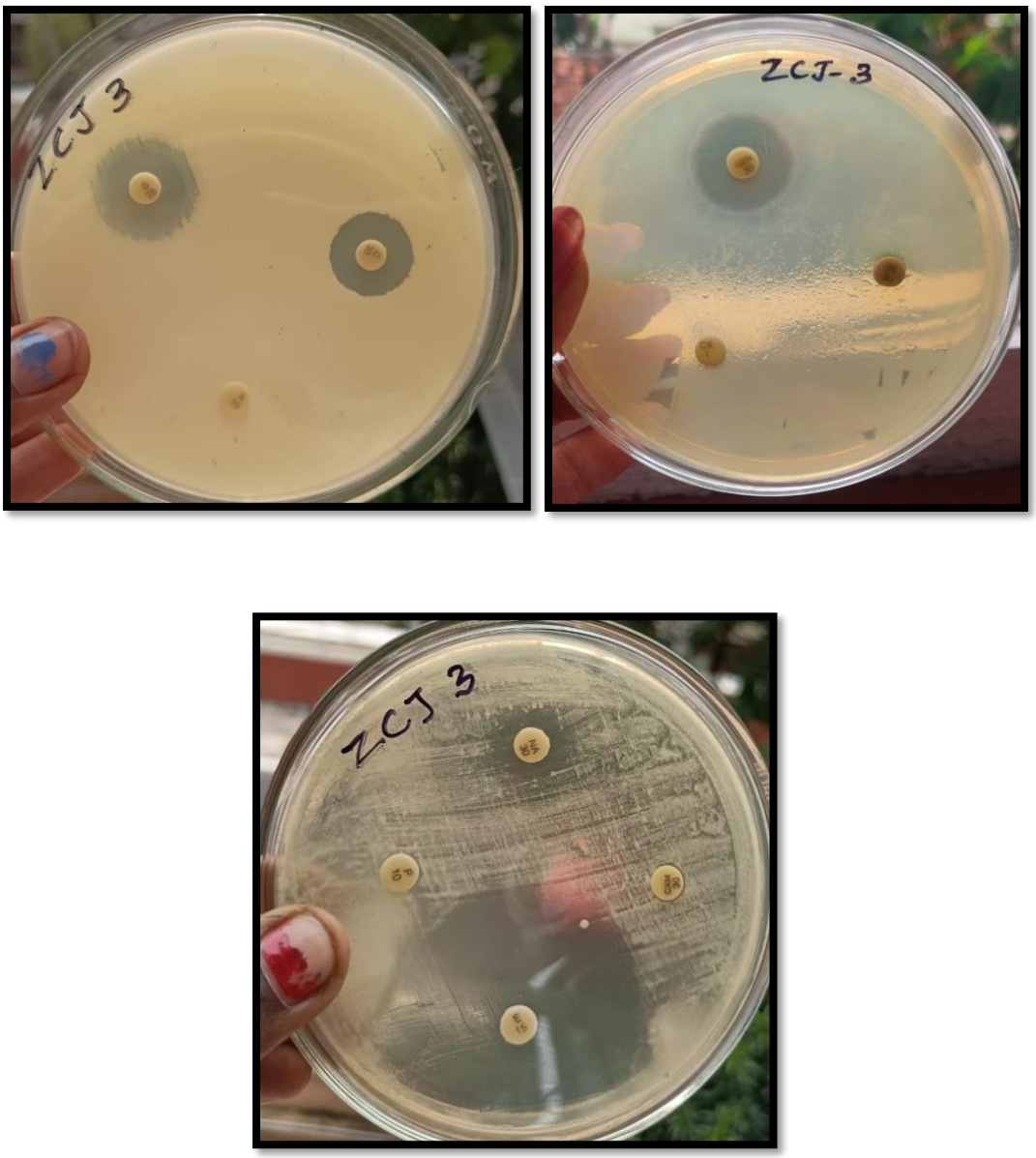


Fig 10- Antibiotic test result for bacterial sample ZCJ 3

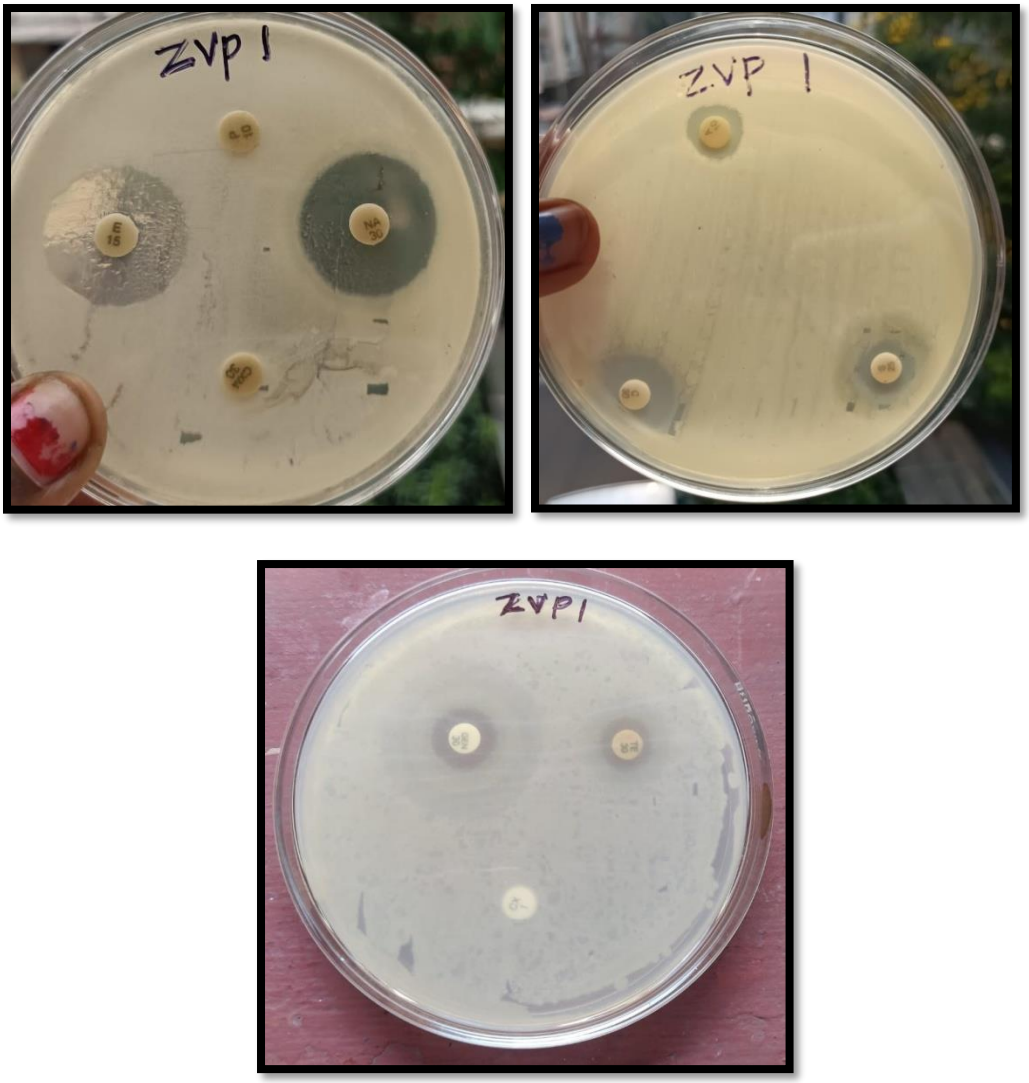


Fig 11- Antibiotic test result for bacterial sample ZVP 1

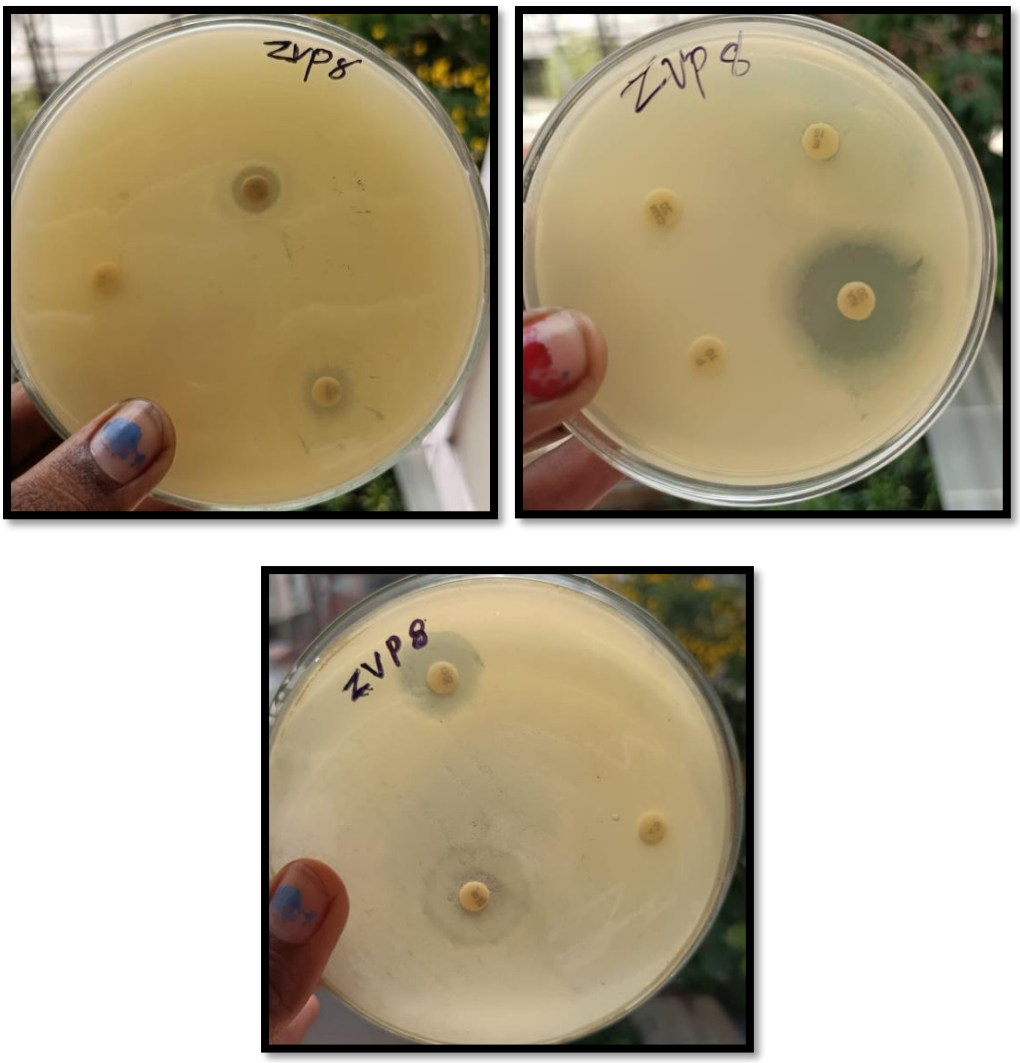


Fig 12- Antibiotic test result for bacterial sample ZVP 8

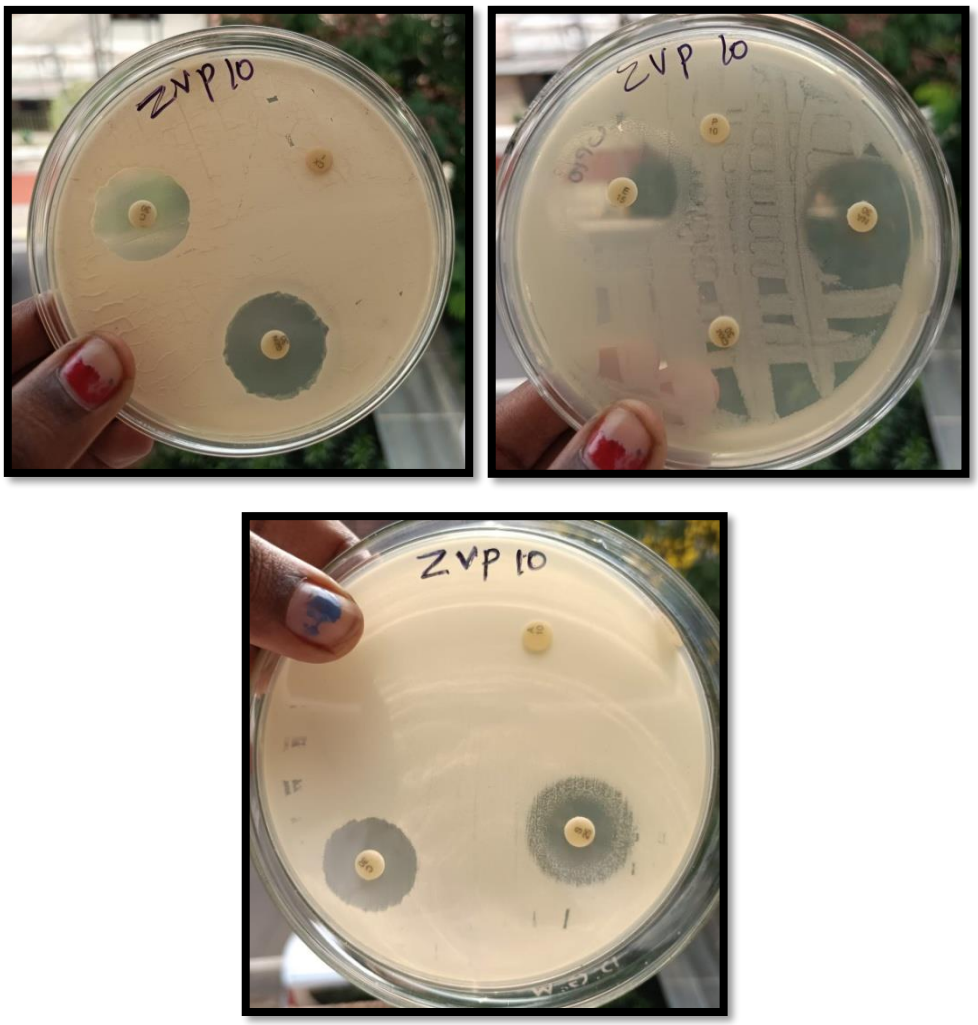


Fig 13- Antibiotic test result for bacterial sample ZVP 10

Fig 14- Antibiotic test result for bacterial sample ZMM4

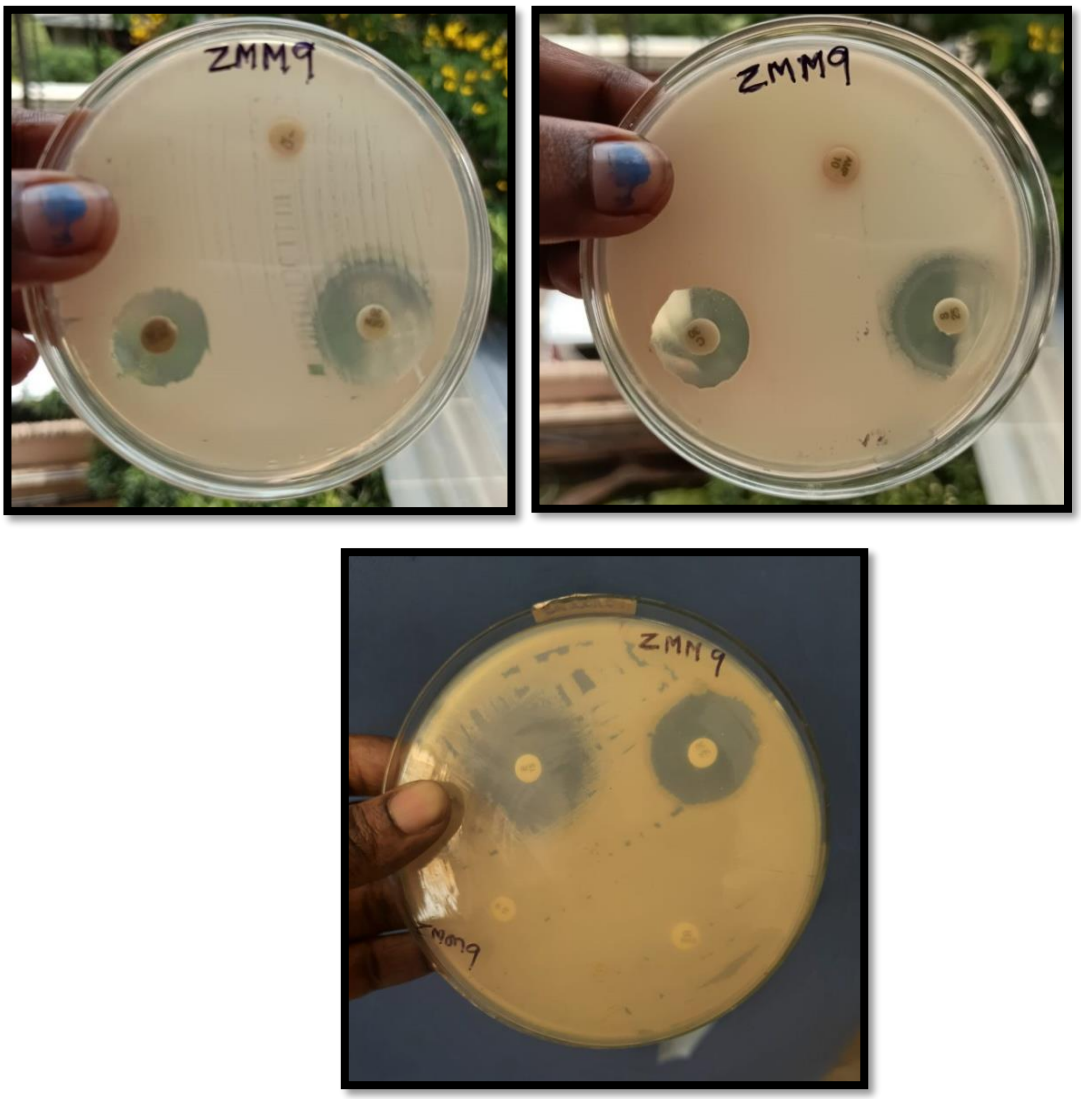


Fig 15- Antibiotic test result for bacterial sample ZMM 9

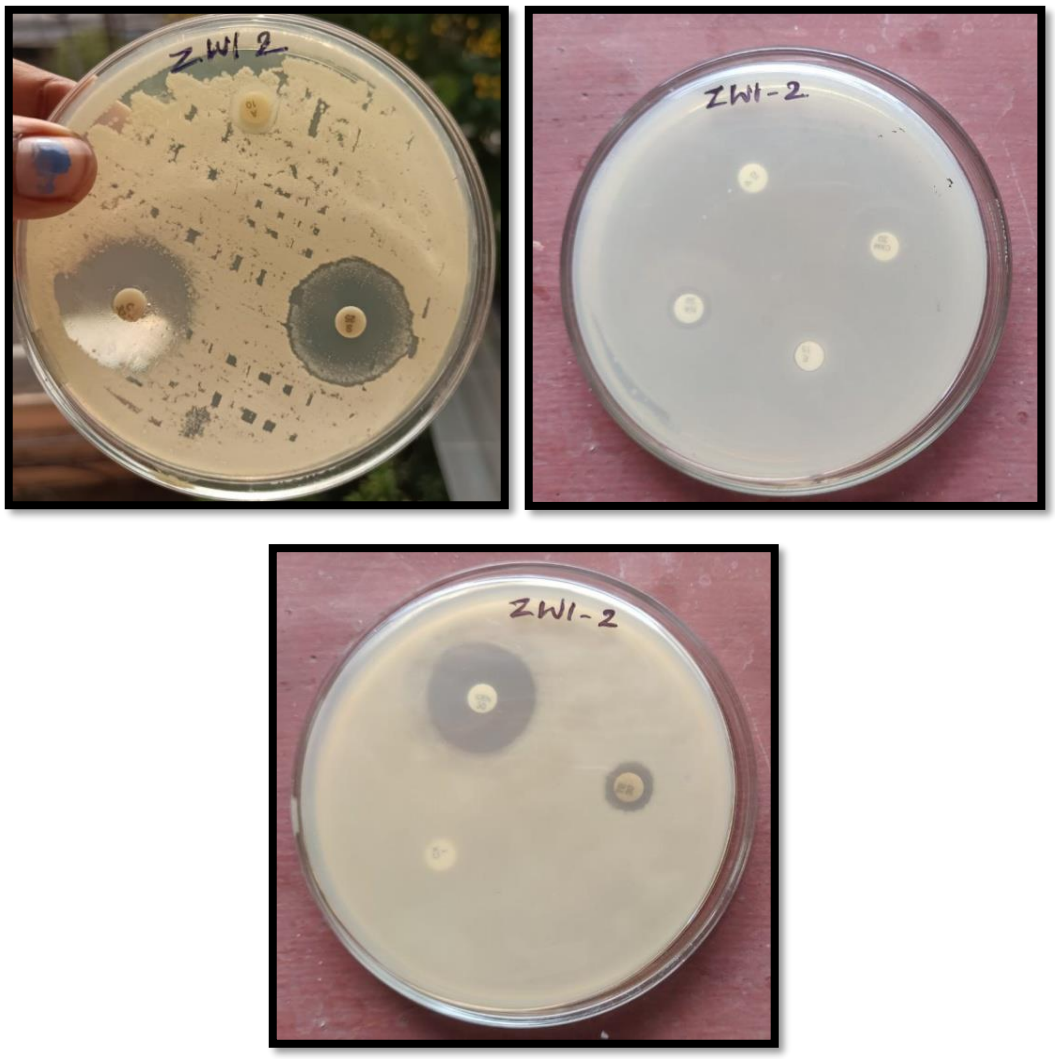


Fig 16- Antibiotic test result for bacterial sample ZWI 2

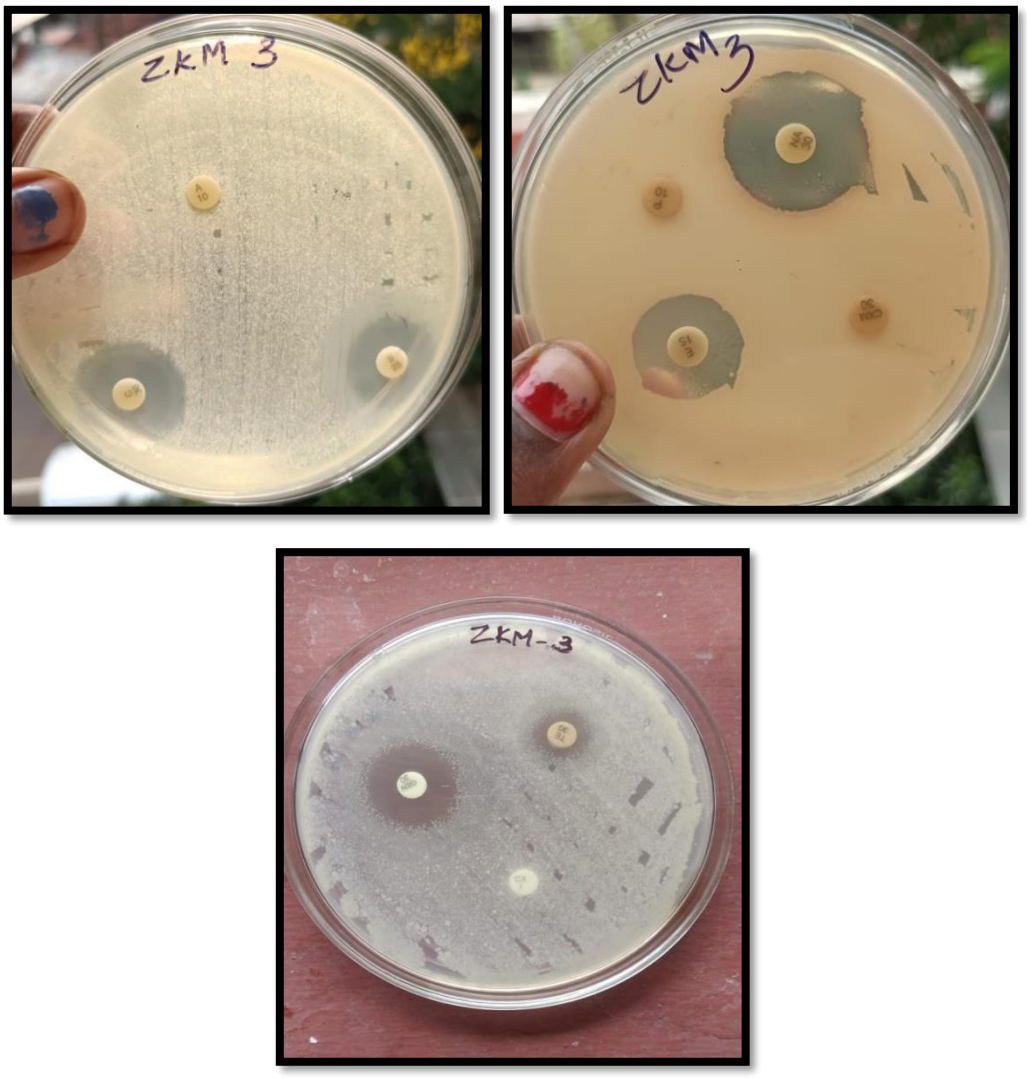
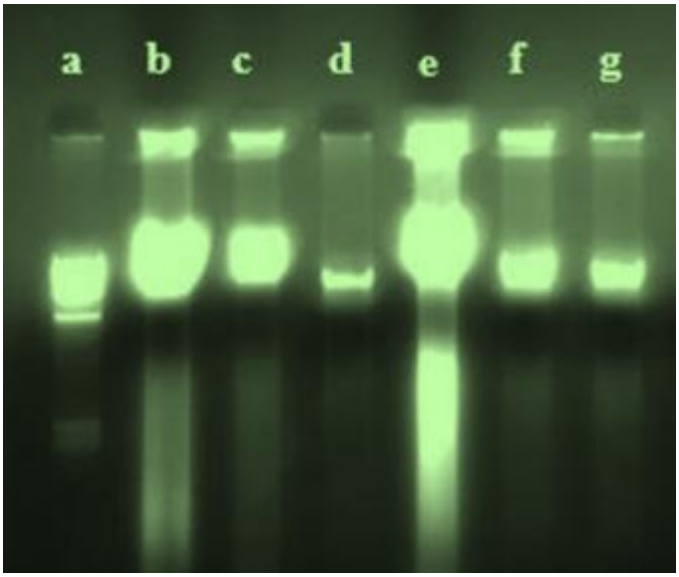


Fig 17- Antibiotic test result for bacterial sample ZKM 3

7. MOLECULAR IDENTIFICATION OF THE BACTERIAL ISOLATES

I.GENOMIC DNA ISOLATION

Genomic DNA isolated from ZCJ3, ZVP1, ZVP8, ZVP10, ZMM4, ZMM9, was electrophoresed on agarose gel and visualised using Gel Documentation system. The DNA bands were compared with the help of 1 kb DNA ladder and the band size of the DNA was found to be above 3000bp, depicted in **FIGURE 18.**



Lane a- 1kb DNA ladder

Lane b - DNA of sample ZCJ3, Lane c - DNA of sample ZVP1

Lane d- DNA of sample ZVP8, Lane e - DNA of sample ZVP10

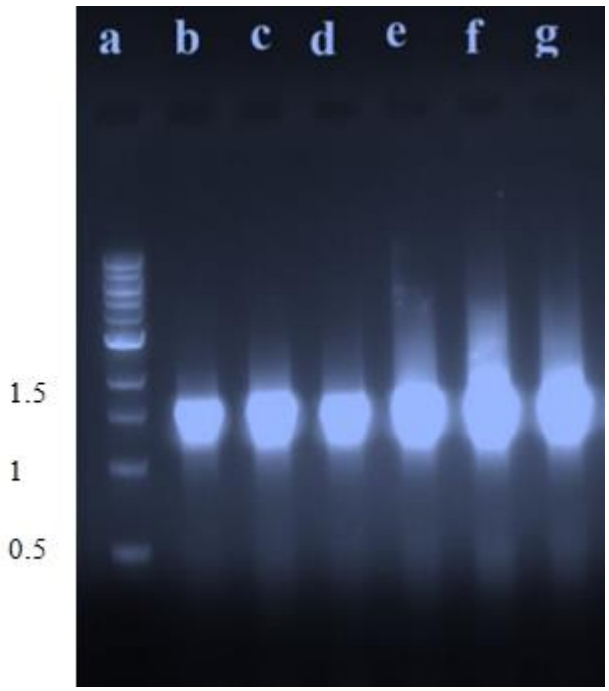
Lane f- DNA of sample ZMM9, **Lane g- DNA of sample ZWI 2**

Fig 18- Agarose gel showing isolated genomic DNA

II.PCR AMPLIFICATION OF 16SrDNA

Amplification of the 16Sr DNA 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 ZCJ3,

Lane c - PCR product of sample ZVP1, Lane d- PCR product of sample ZVP8,

Lane e- PCR product of sample ZVP10, Lane f- PCR product of sample ZMM9,

Lane g- PCR product of sample ZWI 2

Fig 19- Agarose gel showing PCR product

III.DNA SEQUENCING AND BIOINFORMATIC ANALYSIS

After sequencing of 16Sr DNA by Sanger's Dideoxy method, the identity of the sequences of was determined to have **100%** similarity to *Arthrobacter sp (*MK966335.1*),* **99.8%** identity to the *Bacillus paramycoides* (MT611845.1),**100%** similarity to *Stenotrophomonas maltophilia (*MH169196.1),**100%** identity to *Bacillus cereus* (MK606066.1),**100%** similarity to *Bacillus subtilis* (ON534347.1), **100%** identity to *Bacillus velezensis* (CP097288.2).The NCBI hit list **FIGURE 20- 25** determines the bacterial identity.

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
Arthrobacter sp. strain BUUCLN104 16S ribosomal RNA gene, partial sequence	Arthrobacter sp.	1495	1495	100%	0.0	100.00%	1326	MK966335.1
Arthrobacter sp. strain PM6 16S ribosomal RNA gene, partial sequence	Arthrobacter sp.	1472	1472	100%	0.0	99.51%	1472	KX890309.1
Arthrobacter crystallopoietes strain CS18KG0LA394 16S ribosomal RNA gene, partial sequ...	Arthrobacter crystallopoietes	1463	1463	100%	0.0	99.26%	1338	OM971448.1
Arthrobacter sp. strain PM19 16S ribosomal RNA gene, partial sequence	Arthrobacter sp.	1461	1461	100%	0.0	99.26%	1343	KX890311.1
Arthrobacter sp. strain BAB-6459 16S ribosomal RNA gene, partial sequence	Arthrobacter sp.	1456	1456	100%	0.0	99.14%	1340	MF289524.1
Arthrobacter sp. strain JMC01-13 16S ribosomal RNA gene, partial sequence	Arthrobacter sp.	1452	1452	99%	0.0	99.25%	1380	OK576568.1
Arthrobacter sp. strain NQ13_32 16S ribosomal RNA gene, partial sequence	Arthrobacter sp.	1452	1452	99%	0.0	99.25%	1310	MW806490.1
Arthrobacter crystallopoietes strain 10-CTC-2 16S ribosomal RNA gene, partial sequence	Arthrobacter crystallopoietes	1447	1447	99%	0.0	99.13%	1361	MH894256.1
Bacterium strain BS0788 16S ribosomal RNA gene, partial sequence	bacterium	1447	1447	99%	0.0	99.13%	1274	MK823976.1
Arthrobacter sp. Marseille-P4198 partial 16S rRNA gene, strain Marseille-P4198	Arthrobacter senegalensis	1447	1447	99%	0.0	99.13%	1522	LS999985.1
Arthrobacter sp. strain CAU 1506 16S ribosomal RNA gene, partial sequence	Arthrobacter sp.	1447	1447	99%	0.0	99.13%	1491	MK053925.1
Arthrobacter sp. strain A24 16S ribosomal RNA gene, partial sequence	Arthrobacter sp.	1447	1447	99%	0.0	99.13%	1427	MG598447.1
Arthrobacter sp. strain P798-52 16S ribosomal RNA gene, partial sequence	Arthrobacter sp.	1447	1447	99%	0.0	99.13%	1300	KY910132.1
Arthrobacter sulfonylureivorans strain S4-C24 chromosome, complete genome	Arthrobacter sulfonylureivorans	1447	5788	99%	0.0	99.13%	3972796	CP093326.1

Fig 20- NCBI BLAST list depicting the sequence similarity of the sample ZCJ3

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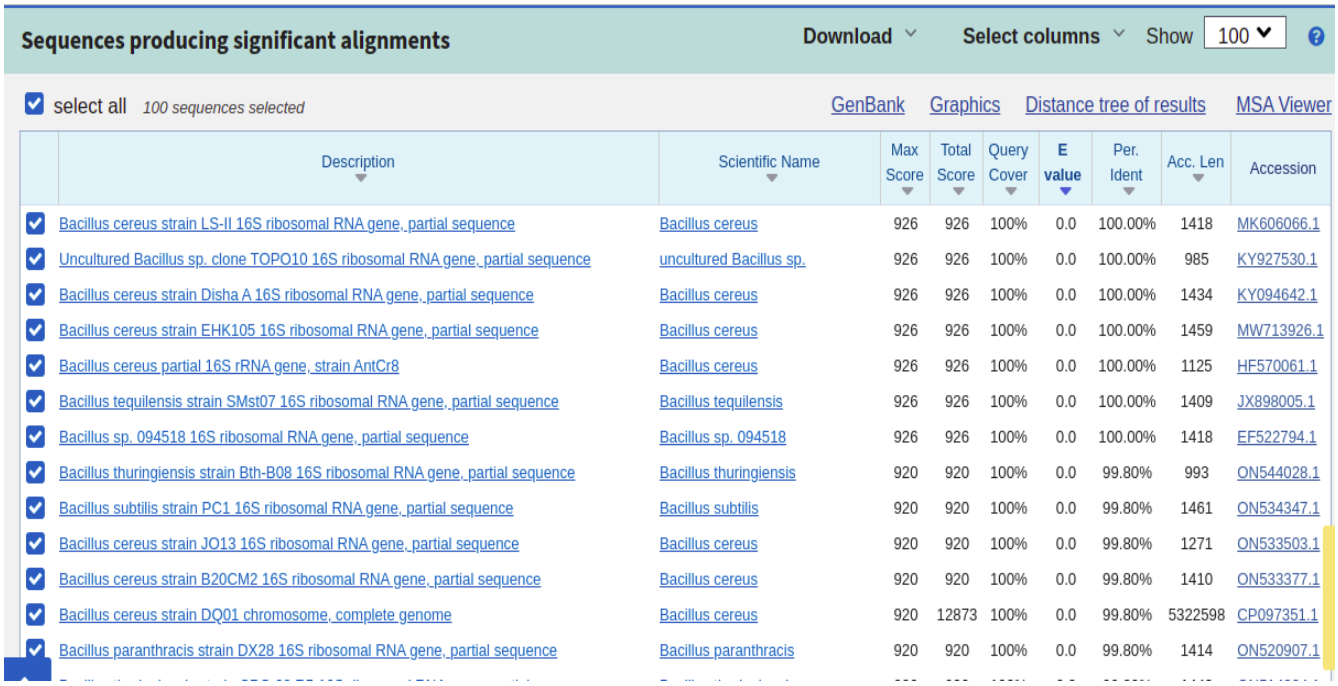
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Stenotrophomonas maltophilia strain GR 008 16S ribosomal RNA gene, partial sequence	Stenotrophomonas maltophilia	1399	1399	100%	0.0	100.00%	1318	MH169196.1
Stenotrophomonas maltophilia strain JM11 16S 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
Stenotrophomonas sp. strain V9R9 16S ribosomal RNA gene, partial sequence	Stenotrophomonas sp.	1393	1393	100%	0.0	99.87%	1209	MT165529.1
[Pseudomonas] geniculata strain NRB093 16S ribosomal RNA gene, partial sequence	Stenotrophomonas geniculata	1393	1393	100%	0.0	99.87%	1365	MK542999.1
Stenotrophomonas maltophilia strain Os_Ep_PSA_55 16S ribosomal RNA gene, partial s...	Stenotrophomonas maltophilia	1393	1393	100%	0.0	99.87%	1410	MN932365.1
Stenotrophomonas maltophilia strain 480 16S ribosomal RNA gene, partial sequence	Stenotrophomonas maltophilia	1393	1393	100%	0.0	99.87%	1418	MT050413.1
Stenotrophomonas maltophilia strain XS 8-4 16S ribosomal RNA gene, partial sequence	Stenotrophomonas maltophilia	1393	1393	100%	0.0	99.87%	1441	MT000012.1
Stenotrophomonas maltophilia strain CPBW01 chromosome, complete genome	Stenotrophomonas maltophilia	1393	5573	100%	0.0	99.87%	4444327	CP047310.1

Fig 21- NCBI BLAST list depicting the sequence similarity of the sample ZVP 1

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	1338	1338	100%	0.0	100.00%	1461	ON534347.1
Bacillus cereus strain DQ01 chromosome, complete genome	Bacillus cereus	1338	18711	100%	0.0	100.00%	5322598	CP097351.1
Bacillus thuringiensis strain CPO 28.R5 16S ribosomal RNA gene, partial sequence	Bacillus thuringiensis	1338	1338	100%	0.0	100.00%	1443	ON514334.1
Bacillus thuringiensis strain CPO 28.V11 16S ribosomal RNA gene, partial sequence	Bacillus thuringiensis	1338	1338	100%	0.0	100.00%	1484	ON514326.1
Bacillus cereus strain DSC-18-02-19 16S ribosomal RNA gene, partial sequence	Bacillus cereus	1338	1338	100%	0.0	100.00%	1501	ON514315.1
Bacillus albus strain GTM22 16S ribosomal RNA gene, partial sequence	Bacillus albus	1338	1338	100%	0.0	100.00%	1456	ON514248.1
Bacillus albus strain GFK31 16S ribosomal RNA gene, partial sequence	Bacillus albus	1338	1338	100%	0.0	100.00%	1465	ON514242.1
Bacillus albus strain GTM11 16S ribosomal RNA gene, partial sequence	Bacillus albus	1338	1338	100%	0.0	100.00%	1456	ON514236.1
Bacillus albus strain GTM31 16S ribosomal RNA gene, partial sequence	Bacillus albus	1338	1338	100%	0.0	100.00%	1449	ON514235.1
Bacillus albus strain GFM21 16S ribosomal RNA gene, partial sequence	Bacillus albus	1338	1338	100%	0.0	100.00%	1452	ON514231.1
Bacillus cereus strain TY24 16S ribosomal RNA gene, partial sequence	Bacillus cereus	1338	1338	100%	0.0	100.00%	1518	ON506254.1
Bacillus thuringiensis strain BG15 16S ribosomal RNA gene, partial sequence	Bacillus thuringiensis	1338	1338	100%	0.0	100.00%	1326	ON506253.1
Bacillus thuringiensis strain BG12 16S ribosomal RNA gene, partial sequence	Bacillus thuringiensis	1338	1338	100%	0.0	100.00%	1326	ON506252.1

Fig 22- NCBI BLAST list depicting the sequence similarity of the sample ZVP8



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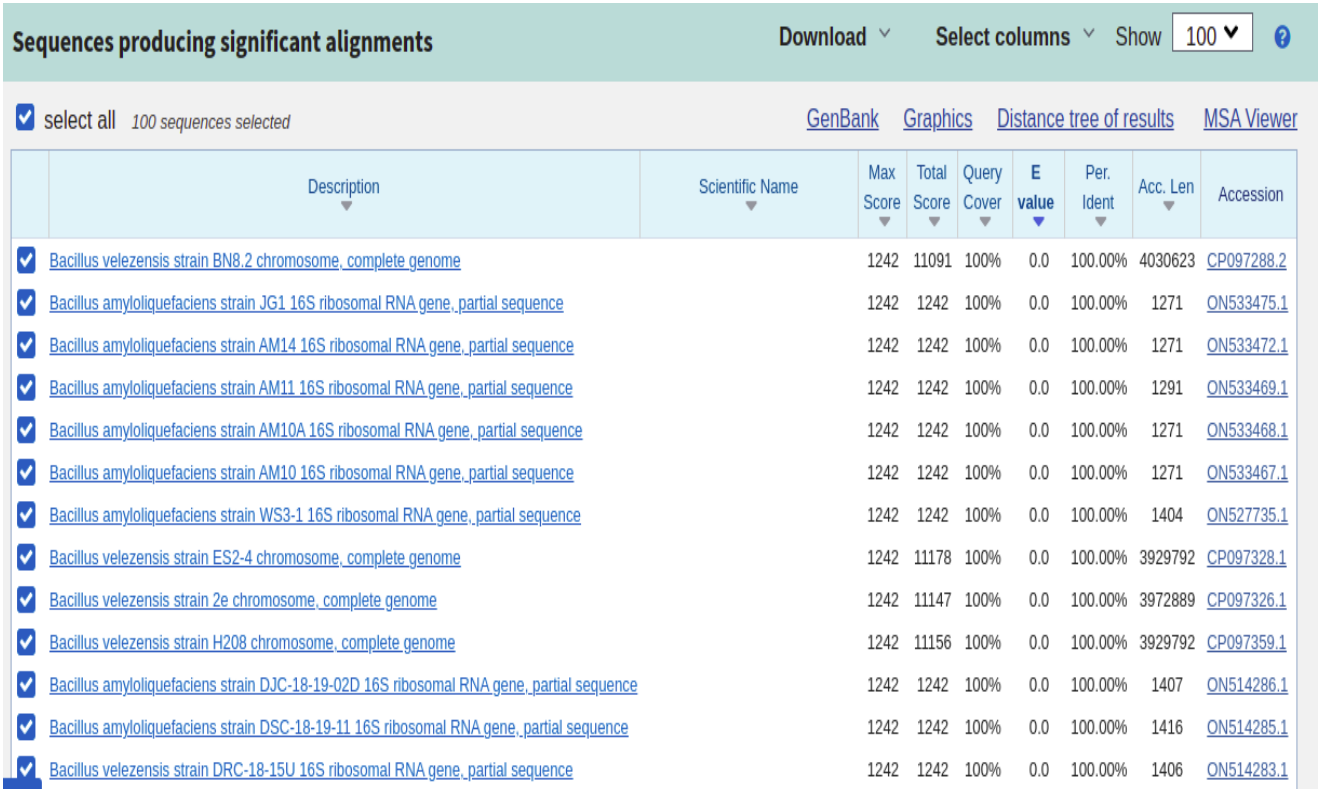
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus cereus strain LS-II 16S ribosomal RNA gene, partial sequence	Bacillus cereus	926	926	100%	0.0	100.00%	1418	MK606066.1
Uncultured Bacillus sp. clone TOPO10 16S ribosomal RNA gene, partial sequence	uncultured Bacillus sp.	926	926	100%	0.0	100.00%	985	KY927530.1
Bacillus cereus strain Disha A 16S ribosomal RNA gene, partial sequence	Bacillus cereus	926	926	100%	0.0	100.00%	1434	KY094642.1
Bacillus cereus strain EHK105 16S ribosomal RNA gene, partial sequence	Bacillus cereus	926	926	100%	0.0	100.00%	1459	MW713926.1
Bacillus cereus partial 16S rRNA gene, strain AntCr8	Bacillus cereus	926	926	100%	0.0	100.00%	1125	HF570061.1
Bacillus tequilensis strain SMst07 16S ribosomal RNA gene, partial sequence	Bacillus tequilensis	926	926	100%	0.0	100.00%	1409	JX898005.1
Bacillus sp. 094518 16S ribosomal RNA gene, partial sequence	Bacillus sp. 094518	926	926	100%	0.0	100.00%	1418	EF522794.1
Bacillus thuringiensis strain Bth-B08 16S ribosomal RNA gene, partial sequence	Bacillus thuringiensis	920	920	100%	0.0	99.80%	993	ON544028.1
Bacillus subtilis strain PC1 16S ribosomal RNA gene, partial sequence	Bacillus subtilis	920	920	100%	0.0	99.80%	1461	ON534347.1
Bacillus cereus strain JO13 16S ribosomal RNA gene, partial sequence	Bacillus cereus	920	920	100%	0.0	99.80%	1271	ON533503.1
Bacillus cereus strain B20CM2 16S ribosomal RNA gene, partial sequence	Bacillus cereus	920	920	100%	0.0	99.80%	1410	ON533377.1
Bacillus cereus strain DQ01 chromosome, complete genome	Bacillus cereus	920	12873	100%	0.0	99.80%	5322598	CP097351.1
Bacillus paranthracis strain DX28 16S ribosomal RNA gene, partial sequence	Bacillus paranthracis	920	920	100%	0.0	99.80%	1414	ON520907.1

Fig 23- NCBI BLAST list depicting the sequence similarity of the sample ZVP10



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Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus velezensis strain BN8.2 chromosome, complete genome		1242	11091	100%	0.0	100.00%	4030623	CP097288.2
Bacillus amyloliquefaciens strain JG1 16S ribosomal RNA gene, partial sequence		1242	1242	100%	0.0	100.00%	1271	ON533475.1
Bacillus amyloliquefaciens strain AM14 16S ribosomal RNA gene, partial sequence		1242	1242	100%	0.0	100.00%	1271	ON533472.1
Bacillus amyloliquefaciens strain AM11 16S ribosomal RNA gene, partial sequence		1242	1242	100%	0.0	100.00%	1291	ON533469.1
Bacillus amyloliquefaciens strain AM10A 16S ribosomal RNA gene, partial sequence		1242	1242	100%	0.0	100.00%	1271	ON533468.1
Bacillus amyloliquefaciens strain AM10 16S ribosomal RNA gene, partial sequence		1242	1242	100%	0.0	100.00%	1271	ON533467.1
Bacillus amyloliquefaciens strain WS3-1 16S ribosomal RNA gene, partial sequence		1242	1242	100%	0.0	100.00%	1404	ON527735.1
Bacillus velezensis strain ES2-4 chromosome, complete genome		1242	11178	100%	0.0	100.00%	3929792	CP097328.1
Bacillus velezensis strain 2e chromosome, complete genome		1242	11147	100%	0.0	100.00%	3972889	CP097326.1
Bacillus velezensis strain H208 chromosome, complete genome		1242	11156	100%	0.0	100.00%	3929792	CP097359.1
Bacillus amyloliquefaciens strain DJC-18-19-02D 16S ribosomal RNA gene, partial sequence		1242	1242	100%	0.0	100.00%	1407	ON514286.1
Bacillus amyloliquefaciens strain DSC-18-19-11 16S ribosomal RNA gene, partial sequence		1242	1242	100%	0.0	100.00%	1416	ON514285.1
Bacillus velezensis strain DRC-18-15U 16S ribosomal RNA gene, partial sequence		1242	1242	100%	0.0	100.00%	1406	ON514283.1

Fig 24- NCBI BLAST list depicting the sequence similarity of the sample ZWI 2

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 paramycoides strain 2883 16S ribosomal RNA gene, partial sequence	Bacillus paramycoides	1530	1530	100%	0.0	99.88%	1454	MT611845.1
Bacillus anthracis strain FDAARGOS_695 chromosome	Bacillus anthracis	1530	16765	100%	0.0	99.88%	5135792	CP054816.1
Bacillus anthracis strain FDAARGOS_702 chromosome	Bacillus anthracis	1530	16765	100%	0.0	99.88%	5272559	CP054800.1
Bacillus anthracis strain FDAARGOS_703 chromosome	Bacillus anthracis	1530	16765	100%	0.0	99.88%	5261520	CP054797.1
Bacillus thuringiensis strain FDAARGOS_791 chromosome, complete genome	Bacillus thuringiensis	1530	21405	100%	0.0	99.88%	5281841	CP054568.1
Bacillus sp. (in: Bacteria) strain AJLP17 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bacteria)	1530	1530	100%	0.0	99.88%	1224	MT559525.1
Bacillus thuringiensis strain FDAARGOS_792 chromosome, complete genome	Bacillus thuringiensis	1530	21372	100%	0.0	99.88%	5251676	CP053938.1
Bacillus thuringiensis strain FDAARGOS_794 chromosome, complete genome	Bacillus thuringiensis	1530	19858	100%	0.0	99.88%	5214223	CP053934.1
Bacillus cereus strain FDAARGOS_797 chromosome, complete genome	Bacillus cereus	1530	21399	100%	0.0	99.88%	5413450	CP053931.1
Bacillus cereus strain FDAARGOS_780 chromosome, complete genome	Bacillus cereus	1530	19864	100%	0.0	99.88%	5271040	CP053997.1
Bacillus cereus strain FDAARGOS_781 chromosome, complete genome	Bacillus cereus	1530	19864	100%	0.0	99.88%	5271029	CP053991.1
Bacillus thuringiensis strain FDAARGOS_795 chromosome, complete genome	Bacillus thuringiensis	1530	19875	100%	0.0	99.88%	5228070	CP053980.1
Bacillus cereus strain FDAARGOS_802 chromosome, complete genome	Bacillus cereus	1530	21399	100%	0.0	99.88%	5342923	CP053965.1

Fig 25- NCBI BLAST list depicting the sequence similarity of the sample ZMM9

DISCUSSION

Amylases are a group of enzymes that hydrolyse α-1,4 glycosidic linkages of starch to yield dextrin and different monomeric products. Amylases have potential application in a wide number of industrial processes such as food, fermentation and pharmaceutical industries (Souza *et al.,* 2010). Today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry. The amylases of microorganisms have a broad spectrum of industrial applications as they are more stable than when prepared with plant and animal α- amylases(Tanyildizi*et al., 2005*).

The present investigation aimed on the isolation, screening of the amylase producing bacteria from the waste soils. The soil samples were collected from different waste soil localities in the Ernakulam and Kottayam districts. The bacterial strains were isolated from the soil samples by serial dilution and pour plate techniques. The starch hydrolysis test for Amylase production from the seventy-eight bacterial strain isolated was performed by the method of patch plating in starch agar medium and the extracellular amylase production by well diffusion assay. The selected bacterial samples with amylase production were further considered for its antibiotic sensitivity, biochemical and molecular characterization. BLAST results of the potent isolates revealed maximum sequence similarity to *Arthrobacter sp, Stenotrophomonas maltophilia, Bacillus cereus, Bacillus subtilis, Bacillus velezensis,* and *Bacillus paramycoids* respectively.

The present study researched on the isolation of the bacterial forms capable of producing amylase enzymes from the waste soil such as compost waste soil, agricultural and food waste and dumping yard soils, as these soils are abundant in amylase producing microorganisms. Previous studies conducted by Pranay *et al.,* 2019 have also pointed out that soil rich in garbage waste and agriculture fields, garbage from household, vegetable wastes, or industrial wastes decomposes are a potent source of Amylase producing bacteria.

In the current investigation, primary screening of amylase producing bacteria were carried out by starch hydrolysis test and extracellular well diffusion assay using 2% starch on nutrient agar plates and subsequent flooding of Gram's Iodine solution. The appearance of clear zones on the starch agar medium indicated amylase production. The same methodology has been adopted for the primary screening of Amylase producing bacterial strains in recent studies by Pranay *et al.,* 2019.

The antibiotic sensitivity profile of the potent amylase producing bacteria against a wide range of broad-spectrum antibiotics were conducted. The findings of the sensitivity test determined the resistance of each bacterial isolates to the specific antibiotics as majority of the bacterial isolates especially the *Bacillus* strains of the bacteria such as *Bacillus subtilis, Bacillus paramycoides* exhibited multiple drug resistance to the antibiotics such as penicillin, ampicillin, tetracycline 'The result of the present study is correlating with the previous study conducted by Senthil *et al.,* 2020 which also reveals that the *Bacillus subtilis*is highly resistant to the antibiotics namely penicillin, ampicillin, tetracycline. Similar study investigated by Sarwan in 2022 also reported that *Bacillus paramycoides*are resistant to the antibiotic penicillin.

Zone of clearance on starch agar medium by well diffusion assay for amylase production indicated higher rate of enzymatic activity by the test isolates and hence were further subjectedto molecular characterization. The molecular characterization involves genomic DNA isolation, PCR amplification and the NCBI BLAST output of the effectual bacterial strains manifesting amylase activity were disclosed as *Bacillus sp, Arthrobacter sp, Stenotrophomonas maltophilia,* of which Genus *Bacillus* ranked the primary position when compared to *Arthrobacter sp, Stenotrophomonas maltophilia* respectively.

In a recent study conducted by Pranay *et al.,* 2019, an attempt was made to isolate and screen efficient amylolytic strains of *Bacillus sp.* Initial screening based on the starch hydrolysis test resulted in the selection of 72 amylolytic bacterial strains. Molecular identification based on 16S rDNA sequence revealed that three most efficient strains [BCM36 (KR1), BCM33 (KR2), and BCM25 (KR3)] belonged to *Bacillus sp.* Similar study conducted by Luang-In *et al., in 2019 a*ll 13 amylase-positive bacterial strains were subjected to 16S rRNA gene sequencing for strain identification. The BLAST results displayed that all amylase-positive isolates belong to the Genus *Bacillus*, except for one isolate belonging to *Enterobacter*. In the light of all these studies which have significant similarity with the current study, the Genus *Bacillus* can be regarded as an effective amylase degrading bacterium.

Arthrobacter sp showed the highest measure of the diameter of clearance zone for amylase production in both the screening test. The study forms a comparison with an early study conducted by Smith *et al.,*2005 which reported the isolation and production of amylase from the *Arthrobacter psychrolactophilus* using the enzymatic assay. The production of extracellular amylase by the psychrotrophic bacterium *A. psychrolactophilus. A. psychrolactophilus* apparently produced a single type of extracellular amylase, as evidenced by a single band of amylolytic activity following native PAGE and staining for amylolytic activity. The molecular mass of the amylase was estimated using purified preparations, which gave rise to two major raw starch-binding protein bands on SDS gels (105 kDa, 26 kDa). Amylase producing bacterial isolates *Stenotrophomonas maltophilia* in the current findings were also stated as efficient amylase producers, isolated from rhizospheric soil in a previously published report by Oo*et al.*,2020.

The present research highlights the efficacy of the soil bacteria isolated from waste as a potential source of amylases enzymes which has profound implications in agriculture, food processing and textile industries in the future. Thus, this will reduce the cost of industrial enzyme and dependent of these enzymes from the external sources. Further new insights about bacterial amylases can be contributed by extensive research.

CONCLUSION

The microbes in the soil are highly potent in producing various hydrolytic enzymes. Amylases are the hydrolytic enzymes which are capable of decomposing the starch, the abundant distribution of this amylase producing microbes have generated an interest among the biologist to apply in the field of biotechnology in which it ranges in the production of food and beverages, textile and paper industry and other industrial fields etc. The present study focused on Enzymatic screening, biochemical and molecular characterization of efficient amylase producing bacteria from the waste soil samples collected from diverse locations of Ernakulam and Kottayam districts of Kerala, India. Starch hydrolysis test of the bacterial samples were performed by the method of patch plating on starch agar medium and extracellular amylase production by well diffusion assay. Among the 78 isolates screened, the most potent isolates which revealed efficient amylolytic activity were further subjected to biochemical, antibiotic and molecular characterization. Molecular identification of the selected six bacterial isolates namely ZCJ3, ZVP1, ZVP8, ZVP10, ZWI2 , ZMM9 are done by the DNA isolation, and PCR amplification of 16srRNA gene. The amplified DNA fragments were sequenced and the bacterial strains were identified as *Arthrobacter sp, Stenotrophomonas maltophiliaBacillus cereus, Bacillus subtilis, Bacillus velezensis, Bacillus paramycoides* by NCBI BLAST analysis.

Present study confirms that the soil harbors plentiful microbes that are capable of producing the amylase enzymes which has tremendous applications in the field biotechnology for the production of foods and beverages, pharmaceutical etc. Apart from these applications, it can be applied for the well utilization and management of waste and its remnants.

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APPENDIX

1. 2% STARCH AGAR MEDIUM

Ingredients	volume
Nutrient agar	1.3g
Starch	2g
Deionised water	100mL

2. 1M Tris-HCL:

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

3. 0.5M EDTA:

Ingredients	Volume
EDTA	18.6g
Deionised water	100mL

4. TE Buffer:

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

5. 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

6. 1X TAE Buffer:

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

7. 6X Gel- loading buffer:

0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol FF
40%(w/v) sucrose in water
Stored at 4^{o}C