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

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

PARTIAL FULFILLMENT OF THE REQUIREMENT

FOR THE AWARD OF

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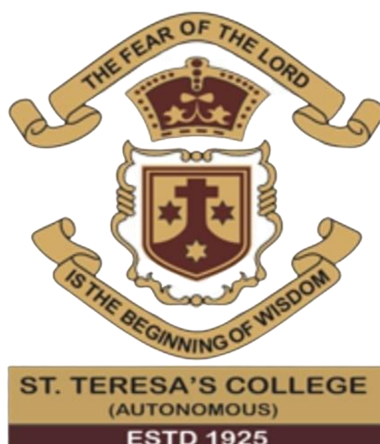

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CERTIFICATE

This is to certify that the dissertation entitled "**Enzymatic Screening, Biochemical and Molecular Characterization of Novel Cellulase Producing Bacteria from Waste Soil**", is an authentic record of original project work carried out by "**SANDRA S K**" (SM20ZOO008), 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 Cellulase 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.

SANDRA S K

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LIST OF ABBREVIATIONS

1.	µl	Microlitre
2.	mM	Millimolar
3.	G	Gram
4.	%	Percentage
5.	^{0}C	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

Cellulose is the major biopolymer and most abundant biomass on the earth. Microorganisms bring about most of the cellulose degradation occurring in nature. Cellulolytic microorganisms such as Fungi and Bacteria are responsible for much of the cellulose degradation in soils. However, relatively little is known about cellulase producing bacteria despite of their extremely high natural diversity, which endows then with the capability to produce stable enzymes.

The present investigation was undertaken to isolate and screen the cellulase producing bacteria from waste soil. Different types of waste soil samples such as agricultural, compost, farmyard manure (FYM), and waterway were collected from various agro-climatic locations in different areas of two distinct districts namely Kottayam and Ernakulam, Kerala, India. Serial dilution agar plating method was used for the isolation of soil bacteria. The seventy-eight isolates were screened for cellulolytic activity using Congo red stain on Carboxymethylcellulose (CMC) agar medium and thirty-five isolates were found to hydrolyze Carboxymethylcellulose. Extracellular cellulase production was quantitatively analysed by well diffusion assay. Out of these 35 isolates, only seven showed potential cellulase production. Biochemical characterization and Kirby Bauer disk diffusion susceptibility test of the most potent seven isolates were conducted and selected for molecular identification. The Genomic DNA of the isolates namely ZMA 17, ZVP 1, ZMM 4, ZMM 9, ZVP 5, ZVP 10 & ZMC 1were identified by 16S amplification method. BLAST analysis of the 16S rDNA of seven isolates revealed that the selected isolates showed maximum similarity of 100%, 100%, 100%, 99.76%, 100%, 100% & 100% to *Elizabethkingia sp. Stenotrophomonas maltophilia, Bacillus subtilis, Bacillus paramycoides, Serratia marcescens, Bacillus cereus & Bacillus subtilis* respectively. Thus, the overall findings of the current study indicate the importance of Cellulose degradation and its subsequent utilizations for global carbon sources. The value of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial interest.

INTRODUCTION

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). Each soil is a unique product of the combination of geological parent material, glacial and geomorphological history, the presence and activity of biota, and the history of land use and disturbance regimes. Soils also play critical roles in buffering and filtering freshwater ecosystems. Consequently, soils are extremely important to human societies. We depend on soils for the production of food, building materials, and other resources; indeed, soils influence most ecosystem services on which we depend (Dominati *et al*. 2010).

Soil microbes, bacteria, archaea, and fungi play diverse and often critical roles in these ecosystem services. The vast metabolic diversity of soil microbes means their activities drive or contribute to the cycling of all major elements (e.g. C, N, P), and this cycling affects the structure and the functions of soil ecosystems. Microbes exist throughout the soil profile; however, they are most abundant in surface soils, the rhizosphere of plants, and around macrospores (Bundt *et al*., 2001; Fierer *et al*., 2007). Microorganisms are capable of degrading various wastes and therefore have been developed to be of use in recycling domestic solid waste as well as toxic substances, the paramount role of microorganisms in the global recycling of carbon and other elements has long been recognized (Magnelli and Forchiassin, 1999) For example, Microorganisms are important in conversion of lignocellulose wastes into valuable products like biofuels produced by fermentation (Shin, 2000).

Soil enzymes such as amylase, arylsulphatases, glucosidase, cellulose, chitinase, dehydrogenase, phosphatase, protease and urease regulate ecosystem functioning and in particular play a key role in nutrient cycling. Soil enzymes play key biochemical functions in the overall process of organic matter decomposition in the soil system (Burns, 1983; Sinsabaugh *et al*., 1991). They are important in catalysing several important reactions necessary for the life processes of micro-organisms in soils and the stabilisation of soil structure, the decomposition

of organic wastes, organic matter formation and nutrient cycling (all soils contain a group of enzymes that determine soil metabolic processes (McLaren, 1975) which, in turn, depend on its physical, chemical, microbiological and biochemical properties. The enzyme levels in soil systems vary in amounts primarily due to the fact that each soil type has different amounts of organic matter content, composition and activity of its living organisms and intensity of the biological processes (Stevenson, 1986).

1. Introduction to cellulose

Cellulose is the most abundant organic compound in the biosphere, comprising almost 50% of the biomass synthesised by photosynthetic fixation of CO2 (Eriksson *et al.,* 1990). Growth and survival of micro-organisms important in most agricultural soils depends on the carbon source contained in the cellulose occurring in the soils (Deng and Tabatabai, 1994). It is the dominant waste material from agricultural industry in the form of stalks, stems and husk, there has been great interest in utilizing cellulose as an energy resource and feed (Balachandrababuet *et al.*, 2012). The cellulose is composed of D-glucose units linked together to form linear chain via ß-1, 4-glycosidic linkages (Salmon and Hudson, 1997).

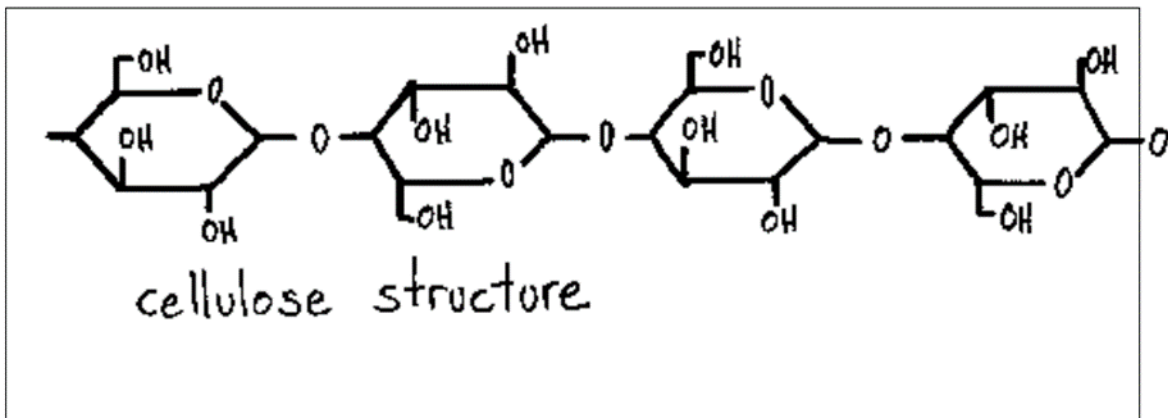


FIGURE 1: Structure of cellulose

Cellulose is the most abundant biomass on the earth (Venkata *et al.*, 2013). Plant biomass contains cellulose as the major component. Cellulose accounts for 50% of the dry weight of

plant biomass and approximately 50% of the dry weight of secondary sources of biomass such as agricultural wastes (Haruta *et al*., 2003).

Celluloses are observed as the most important renewable resource for bioconversion. It has been become the economic interest to develop an effective method to hydrolyse the cellulosic biomass (Saraswati *et al*., 2012).

There are two types of bonds in cellulose molecules: one which that forms between the C30H group and the oxygen in pyranose ring and those that forms between the C_6 OH group of one molecule and the oxygen of glucosidic bond of another molecule. They stabilize the cellulose structure according to the considerable cohesive energy in the cellulose network. Hydrogen bonding, electrostatic interactions, and Van der Waals dispersion forces play an important role in determining the cellulose crystal structure during the cellulose-water interactions.

Cellulose degradation and its subsequent utilizations are important for global carbon sources. The value of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial interest (Bhat *et al*., 2000). There has been much research aimed at obtaining new microorganisms producing cellulase enzymes with higher specific activities and greater efficiency (Subramaniyan *et al*., 2000).

2. Cellulose degrading enzyme

Cellulolytic enzymes play an important role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded by cellulolytic fungi, bacteria, actinomycetes and protozoa. Bioconversion of cellulose to soluble sugars and glucose is catalysed by a group of enzymes called cellulases. Microorganisms including fungi, bacteria and actinomycetes produce mainly three types of cellulase components—endo-1,4-β-D-glucanase, exo-1,4-β-D-glucanase and β-glucosidase—either separately or in the form of a complex. Cellulose is commonly degraded by an enzyme called cellulase. This enzyme is produced by several microorganisms, commonly by bacteria and fungi (Immanuel *et al*., 2006).

Cellulase is an important and essential kind of enzyme for carrying out the depolymerisation of cellulose into fermentable sugar (Xing-hua *et al*., 2009). Cellulases are the inducible bioactive compounds produced by microorganisms during their growth on cellulosic matters (Lee and Koo, 2001). Cellulose degrading microorganisms can convert cellulose into soluble sugars either by acid or enzymatic hydrolysis. Thus, microbial cellulose utilization is responsible for one of the largest materials flows in the biosphere (Lynd *et al.*, 2002).

3. Cellulose degrading microorganisms

Cellulolytic enzymes are synthesized by a number of microorganisms. Fungi and bacteria are the main natural agents of cellulose degradation (Lederberg, 1992). The cellulose utilizing population includes aerobic and anaerobic mesophilic bacteria, filamentous fungi, thermophilic and alkaliphilic bacteria, actinomycetes and certain protozoa (Alexander, 1961).

Microorganisms bring about most of the cellulose degradation occurring in nature. Many microorganisms have been reported with cellulosic activities including many bacterial and fungal strains both aerobic and anaerobic. *Chaetomium, Fusarium, Myrothecium, Trichoderma, Penicillium, Aspergillus, and so forth,* are some of the reported fungal species responsible for cellulosic biomass hydrolysation. Cellulolytic bacterial species include *Trichonympha, Clostridium, Actinomycetes, Bacteroides succinogenes, Butyrivibrio fibrisolvens, Ruminococcus albus, and Methanobrevibacter ruminantium* .

The cellulase was first discovered in 1983 from the anaerobic, thermophilic spore-forming *Clostridium thermocellum* (Maki *et al*., 2011). Cellulose is commonly degraded by cellulase. Cellulolytic enzyme system consists of three major components such as endoglucanases, exoglucanases and ß-glycosidase. Cellulases have a potentiality to use in biotechnology and in industry such as, starch processing, alcoholic beverage, malting and brewing, clarify of juice, pulp bleaching, textile industry and animal feed (Sreeja *et al*., 2013).

Certain cellulase producing bacteria also inhabit the other factors which are responsible for decomposition of organic matter and composting (Shankar *et al*., 2011). Beyond free bacterial

cellulases, is the opportunity for whole cells in bacterial co-culture and strains with multiple exploitable characteristics to reduce the time and cost of current bio-conversion processes. It is also noticeable that the final product of cellulose degradation by cellulase enzyme is glucose which is soluble sugar. So, isolation and characterization of cellulase producing bacteria will continue to be an important aspect of biofuel research, biodegradation and bioremediation.

AIM AND OBJECTIVES

AIM

Enzymatic screening, Biochemical and Molecular characterization of novel cellulase producing bacteria from waste soil.

OBJECTIVES

- Isolation of bacteria from waste soil samples collected from diverse locations.
- Screening of cellulase using CMC agar plate assay.
- Screening of extracellular cellulase production by well diffusion assay,
- Biochemical depiction of cellulase producing bacterial isolates.
- Kirby-Bauer disk diffusion susceptibility testing of potential cellulase producing bacteria.
- Molecular characterization of the isolate possessing cellulase activity.

INNOVATION OF THE INVESTIGATION

Elizabethkingia sp. were obtained as novel and potent cellulase enzyme producing bacteria and hence can be regarded as an innovation of the present investigation. Isolated dissimilar forms of cellulase producing bacteria from the waste soil which can lay open to various applications like decomposition of wastes and residues, miscellaneous food applications, cellulase enzyme in textile industries, cellulase enzyme in detergent, pulp and paper industry etc. There has been much research aimed at obtaining new microorganisms producing cellulase enzymes with higher specific activities and greater efficiency.

REVIEW OF LITERATURE

Soil Habitat

Microbial interactions occur in habitats much smaller than those generally captured in homogenized soil cores sampled across a plot or field. Hofmockel *et al*., 2018 studied soil aggregates to examine soil microbial community composition and structure of both bacteria and fungi at a microbial-relevant scale. Bacteria and fungi exhibited similar patterns of community structure and diversity among soil aggregates, regardless of land management. Results show micro aggregates support highly diverse microbial communities, including several unidentified genera. Isolating aggregates with a microbial sensitive approach provides new opportunities to explore soil microbial communities and the factors shaping them at relevant spatial scales.

Soil Microorganisms

The roles of microbes in the ecosystem services provided by soils to humans. The diversity of microbes in soil is enormous and they drive many soil services. Deslippe *et al.,* 2013 examined the functional, metabolic, and phylogenetic diversity of soil bacteria, archaea, and fungi. The roles of these soil microbes are highlighted in the cycling of major biological elements (C, N, P), in the recycling of wastes, and the detoxification of environmental pollutants. Microbes play a pivotal role in the cycling of nitrogen; they exclusively mediate nitrogen fixation, denitrification, and nitrification. He also discussed about the recent theoretical advances in understanding of ecosystem processes that were made possible through explicit consideration of the roles of soil microbes.

In terrestrial ecosystems, soil microorganisms play an important role in the cycling of elements and stabilization of soil structure. Soil microbes must be considered as important drivers of plant diversity and productivity in terrestrial ecosystems. Moreover, soil microbes represent the unseen majority in soil and comprise a large portion of the genetic diversity on Earth (Whitman *et al.,* 1998). For instance, it has been estimated that one gram of soil contains as many as 10^{10} – 10^{11} bacteria (Devine *et al.,* 2003), 6000-50,000 bacterial species (Curtis *et al.,* 2002) and up to

200 m fungal hyphae. However, while it is widely recognized that microbes perform crucial role in biogeochemical cycling, the impact of microbes on plant productivity and diversity is still poorly understood (Leake *et al.,* 2004).

Cellulose

Cellulose is a crystalline polymer, an unusual feature among biopolymers. Cellulose chains in the crystals are stiffened by inters and intra chain hydrogen bonds and the adjacent sheets which overlie one another are held together by weak Vander Waals forces. In nature, cellulose is present in a nearly pure state in a few instances whereas in most cases, the cellulose fibres are embedded in a matrix of other structural biopolymers, primarily hemicelluloses and lignin. An important feature of this crystalline array is the relative impermeability of not only large molecules like enzymes but in some cases even small molecules like water. There are crystalline and amorphous regions, in the polymeric structure and in addition there exists several types of surface irregularities (Cowling *et al.,* 1975; Fan *et al.,* 1980).

Cellulose as a material has been widely used for centuries in all kinds of practical applications. The modern history of cellulose chemistry actually began in 1837 when Anselme Payen chemically identified cellulose from plants (Hon, 1994). Since then, the establishment of its chemical and physical structures has undergone multitudinous periods of struggle. Higher plant tissues such as trees, cotton, cereal straw represent the main sources of cellulose *i.e.* it makes up 35-50% of dry plant biomass (Lynd *et al.,* 1999). Higher algae tissue such as *Microdicyan* and *Valoniaventricosa* are representatives of lower plants that synthesize cellulose (Fierobe, *et al.,* 2002). In addition to plants, non-photosynthetic organisms such as bacteria *i.e.* aerobic Acetobacterxylinum, marine invertebrates from the ascite family *i.e*. tunicates, fungi, slime moulds and amoebae also produce cellulose (Tomme *et al.,* 1995; Lynd *et al,* 2002). Bacteria has high growth rate as compared to fungi has good potential to be used in cellulose production. Some bacterial species viz., *Cellulomonas* species, *Pseudomonas* species, *Bacillus* species and *Micrococcus* have cellulolytic property (Nakamura and Kappamura, 1982).

The production of cellulase has been reported from a wide variety of bacteria (Immanual *et al.,* 2006) and fungi (Selvakumar *et al.,* 1999; Anita *et al.,* 2009). However, filamentous fungi are preferred for commercial enzyme production, because the level of the enzymes produced by these cultures is higher than those obtained from yeast and bacteria (Bakri *et al.,* 2003). Almost all fungi of genus *Aspergillus* synthesize cellulase, therefore this genus has the potential to dominate the enzyme industry. *Aspergillus* and *Trichoderma* spp. are well known efficient production of cellulases (Gielkens *et al.,* 1993).

Lin *et al,* (2011) studied cellulolytic bacterium strain Pa5, showing high level production of exo-cellobiase was obtained and it was identified as *Bacillus subtilis* based on morphology, physiological and biochemical characteristics and homology of 16s rDNA gene sequence. The cellulases mixture from strain Pa5 has a relative high heat and pH stability. Furthermore, strain Pa5 was selected for performing the solid-state fermentation(SSF) with okara (the insoluble residue of the production of soymilk from soybean seeds) as sole substrate and the results revealed that the optimal incubation temperature, initial moisture content, loadage of the substrate, and inoculum size for both carboxymethyl cellulase (CMC) and cellobiase production were 30°C, 71.5%, 10 g dry matter per 250 ml flask, and 1 ml of inoculum at the density of 1×10^5 colony-forming units/ml per 10 g of dried okara, respectively. 48 h after the solid-state fermentation under the above conditions, the maximum values of carboxymethyl cellulase and cellobiase productivities were 48.42 ± 0.87 and 154.50 ± 2.15 IU/g of dried okara, respectively.

Balamurugan *et al* (2011) performed experiment where cellulose degrading bacteria of tea garden soil were isolated, screened in vitro and its characterization, in relation to cellulase activity, was studied. Among the 25 isolates, the five strains showed higher enzyme activity when compared to other strains. Cellulase activity was expressed at a higher level by strain CDB12 when blotting paper was used as a cellulose source in comparison with the other two substrate sources incorporated with minimal salt medium and followed by CDB13 and CDB21 in blotting paper. Maximum growth of cellulose degradation bacteria (CDB) was recorded at 30°C and pH 7.0. Among the carbon sources tested, maximum growth was observed in glucose amended mineral salts medium followed by fructose and maltose. Ammonium sulphate, ammonium nitrate and potassium nitrate were good nitrogen sources for better survival of CDB

isolates. The biomass was continuously removed and placed as such into the tea field, then native and proven CDB strains were applied and they played an important role on the degradation of harvested biomass, which required replenishment to maintain the sustainable productivity of tea.

Mechanism of cellulose hydrolysis

Cellulases are naturally produced by a wide spectrum of bacteria and fungi. In nature, complete cellulose hydrolysis is mediated by a combination of three main types of cellulases: (1) endoglucanases (EC 3.2.1.4), (2) exoglucanases, including cellobiohydrolases (CBHs) (EC 3.2.1.91), and (3) β -glucosidase (BG) (EC 3.2.1.21). These enzymes usually exist as cellulosomes attached to the cell wall of bacteria. Exoglucanases are further grouped into glucanohydrolases (cellodextrinases) and cellobiohydrolases. These categories are based on their structural properties and mode of action (Henrissat *et al.,* 1998; Henrissat & Davies, 2000). Endoglucanase, or CMCase, randomly cut β -1,4-bonds of cellulose chains, generating new ends. Whereas, Exoglucanases act in a processive manner on the reducing or nonreducing ends of cellulose polysaccharide chains, liberating either cellobiose or glucose as major products (Sukumaran *et al*., 2005).

This enzyme system displays synergy, a phenomenon in which the collective enzyme activity is higher than the sum of activities of individual enzymes. There have been four forms of synergy reported. Exo-exo synergy between exoglucanase attacking the reducing and the non-reducing ends of cellulose; endo-exo synergy between endoglucanases and exoglucanases; Exo-β glucosidase synergy and intramolecular synergy between the catalytic domain and the CBMs (Din *et al.,* 1994; Driskill *et al.,*1999). Synergy among exoglucanase (CBH) and endoglucanase is vital for complete hydrolysis of cellulosic materials. A number of studies have been conducted to investigate the optimal ratio between exoglucanase and endoglucanase for a maximum synergy degree, and so on.

Cellulolytic anaerobes have an extra cytoplasmiccellodextrinase for hydrolyzing cellodextrins and intracellular cellodextrin and cellobiosephosphorylases (CdP and CbP). These

phosphorylases catalyse Pi mediated phosphorylation of cellodextrins and cellobiose respectively to yield glucose 1 monophosphate (G-1-P) which is converted to Glucose 6 Phosphate (G-6-P), the entry point to Embden-Meyerhoffpathway (Lynd *et al.,* 2002). Other bacteria produce intracellular β glucosidases which cleave cellobiose and cellodextrins to produce glucose which is assimilated by the microbes (Karmakar & Ray, 2011). Simultaneous presence of extracellular cellodextrinases, intracellular CbP and CdP activities, and intracellular β glucosidases in cellulolytic microorganisms suggest that metabolism of cellobiose and cellodextrins probably occurs through several pathways. (i) Extracellular hydrolysis of the substrates i.e. cellobiose and cellodextrins and subsequent uptake and metabolism. (ii) Direct uptake followed by intracellular phosphorolytic cleavage and subsequent catabolism. (iii) Direct uptake by the organism followed by hydrolytic cleavage and metabolism (Lynd *et al.*, 2002). Cellulosic substrates occurring in nature contain hemicellulose and lignin which impedes the access of cellulase components to beta (1-4) glucosidic linkages thus other hydrolytic enzymatic activities distinct to those of cellulases are required. Enzymatic cleavage of the β 1 - 4-glucosidic linkages in cellulose proceeds through an acid hydrolysis mechanism, using a proton donor and nucleophile or base (Lynd *et al.,* 2002).

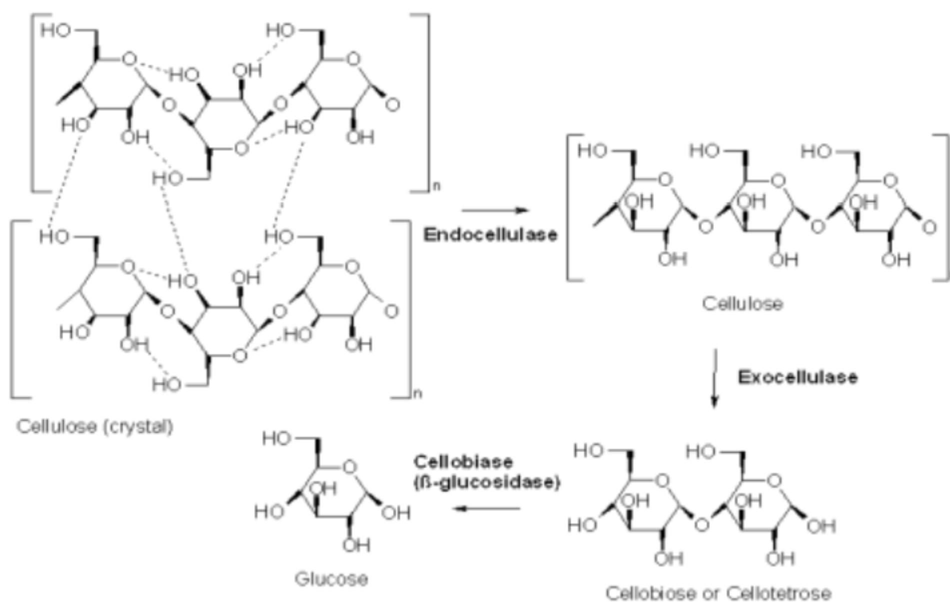


FIGURE 2: A schematic representation of cellulolysis

Hydrolysis of cellulose

The three types of reactions catalyzed by cellulases: (1) Breakage of the non-covalent interactions present in the amorphous structure of cellulose by endoglucanase (2) Hydrolysis of chain ends to break the polymer into smaller sugars by exoglucanase (3) Hydrolysis of disaccharides and tetrasaccharides into glucose by β-glucosidase (Adapted from Karmakar and Ray, 2011).

Enzyme system

Enzymes are delicate protein molecules necessary for life. Cellulase are used in enzymatic hydrolysis of cellulosic substances (Kubicek *et al.,* 1993). Despite a worldwide and enormous utilization of natural cellulosic sources, there are still abundant quantities of cellulosic sources, cellulose containing raw materials and waste products that are not exploited or which could be used more efficiently (Sonia *et al.,* 2013). Cellulose utilization takes place in both aerobic and anaerobic microorganisms. Cellulase enzyme systems are generally classified into two; complexed (Shoham *et al.,*1999; Schwarz, 2001) and non-complexed (Stutzenberger, 1990; Teeri, 1997). This classification is dependent on whether the microorganism is aerobic or anaerobic (Lynd *et al.,* 2002).

Non-complexed systems

Components are normally found in aerobic cellulose degraders *i.e.* both fungi and bacteria (Rapp & Beerman, 1991). Cellulases from aerobic fungi have received more study than have those of any other physiological group, and fungal cellulases currently dominate the industrial applications of cellulases. In particular, the cellulase system of *T. reesei* (teleomorph:*Hypocrea jecorina*, initially called *Trichoderma viride*) has been the focus of research for 50 years (Mandels&Weber,1969). *T. reesei* produces at least two exoglucanases (CBHI and CBHII), five endoglucanases (EGI, EGII, EGIII, EGIV, and EGV), and two β-glucosidases (BGLI and BGLII (Kubeck & Penttila, 2005).

Most aerobic bacteria species are found in soil. They fall in genera that are known for non-growth associated metabolism (secondary metabolism) that include formation of dormant states *(Bacillus, Miromonospora* and *Thermobifida* and production of secondary metabolites such as antibiotics (*Bacillus* and *Micromonospora* (Lynd *et al.,* 2002). Most aerobic bacteria adhere to cellulose but the physical contact is not necessary for cellulose hydrolysis (Kauri & Kushner, 1985).

Complexed system

Microorganisms producing complexed cellulase systems (cellulosomes) are typically found in anaerobic environments, where they exist in consortia with other microorganisms, both cellulolytic and noncellulolytic (Schwarz, 2001). Cellulosomes are protuberances produced on the cell wall of cellulolytic bacteria when growing on cellulosic materials. These protuberances are stable enzyme complexes that are firmly bound to the bacterial cell wall but flexible enough to also bind tightly to microcrystalline cellulose. Cellulosomes from different clostridia (*Clostridium thermocellum, Clostridium cellulolyticum, Clostridium cellulovorans,* and *Clostridium josui*) and *Ruminococcus* species in the rumen have been studied in detail. The cellulosome of the thermophilic *C. thermocellum* is discussed and briefly compared to those of the mesophilic *C. cellulolyticum, C. cellulovorans,* and *R. albus* (Schwarz, 2001). The cellulosome structure of *C. thermocellum* was resolved through a combination of biochemical, immunochemical, ultrastructural, and genetic techniques (Lame *et al.,* 1994).

The cellulosome is thought to bring enzyme activity in close proximity to the substrate thus facilitating optimum synergy by the cellulases present in the cellulosome and also to minimize the distance over which hydrolysis products diffuse thus allowing for efficient uptake of oligosaccharides by the cell (Bayer *et al.,* 1994; Schwarz, 2001).

Cellulolytic Microorganisms

Ability to degrade cellulose is widely distributed in several fungal and bacterial genera. In addition to these two, the domain eubacteria have a considerable distribution of cellulytic capability. Members in the aerobic order *Actinomyctes* and anaerobic order *Clostridiales*. Fungi are the main agents of decomposition of organic matter in general and especially cellulosic substrates (Lynd *et al.,* 2002; Montegut *et al.,* 1991) and it's no surprise that cellulolytic capability is distributed across the entire kingdom from the advanced Basidiomycetes to the primitive *Chytridomycetes* (Lynd *et al.,* 2002). *Chytridomycetes* are known to degrade cellulose in gastrointestinal tracts of ruminant animals (Orpin, 1977).

Cellulolytic capability is however not exclusive to microorganisms. Species such as termites and cray fish produce their own cellulases that are different from those produced by their indigenous micro flora (Orpin, 1977). There is a broad distribution of cellulolytic capability and it's possible that a primordial ancestor acquired it early in the evolutionary development. This however may not be the case because cellulose biosynthesis capability evolved much later with the development of land plants, algae amongst others (Lynd *et al.,* 2002).

Bacteria

The bacterial systems have also been investigated for saccharification of the biomass and might have advantage because of the fast growth rate of bacteria. It has also been reported that the enzyme preparation from cellulolytic bacteria can effectively saccharify different cellulosic substrates (Choundry *et al.,* 1980, 1981; Bynd *et al.,* 1987; Rajoka *et al.,* 1984; Waldron *et al.,* 1986). *Cytophaga hutchinsonii* revealed an unusual collection of genes for an organism that can attack crystalline cellulose. Location, formation and biosynthetic regulation of cellulases in were demonstrated on different substrates (Louime *et al.,* 2006). Acetivibrrio cellulolytieus has been studied by many workers investigated that both endoglucanase and exo-glucanase can be regulated by induction and catabolite repression. It differs from most of the cellulolytic organisms in its ability to utilize only cellulose, cellobiose or salicin for growth

(Saddler *et al.,* 1980). This organism has attracted special interest because of high specific activities for endo- and exo glucanase (Mackenzie *et al.,* 1985). The endocellulase activity of the culture broth was determined during growth of *Acinetobacter anitratus* and *Branhamella* species by measuring release of reducing sugars from CMC (Ekperigin *et al.,* 2006). The enzyme preparations from *A. cellulolyticus* have the ability to saccharify cellulose the condition for maintaining high specific activity has also been determined. Cellulase system of Pseudomonas sp. has been fairly well studied (Yamane *et al.,* 1970). Yoshikawa *et al.* (1974) studied the biogenesis of multiple cellulose components of *P. fluorescens.* Cellulose with special emphasis on effects of culture conditions on the multiplicity of cellulose titres produced by the organism. Tewari et al., (1977) reported production, purification and properties of cellulase (extracellular) from *Pseudomonas sp*. Localization of cellulase components in Pseudomonas sp., isolated from activated sludge have been investigated (Ramasamy *et al.,* 1980). Endo-glucanase is the major components of cellulase complexes with three being from *P. fluorescens* varieties. cellulose (Yashikawa *et al.,* 1974) and four or more by Pseudomonas sp. (Ramasamy *et al.,* 1980). Gene for galactose dehydrogenase has been cloned in E. coli (Buckle et al., 1988). Pseudomonas fluorescens, Bacillus subtilis, E. coli and Serratia marcescens, cellulase producing bacteria was isolated from soil and optimization of the fermentation medium for maximum cellulase production was studied and found *Pseudomonas fluorescens* as best cellulase producer (Sethi *et al.,* 2013). The cellulolytic enzymes of *Bacillus* species have been the focus of many due to their potential use in the conversion of agricultural wastes into useful products (Ozaki *et al.,* 1990). It was reported that *Bacillus subtilis* CBTK 106 can produce a considerable amount of cellulase activity (Krishna, 1999).

Table 1: Bacteria with cellulolytic capability (Adapted from Kuhad *et al.,* 2011).

	Aerobic bacteria *Acinetobacter junii, A. amitratus; Acidothermus cellulolyticus; Anoxybacillus sp; Bacillus subtilis, B.pumilus, B. licheniformis, B. amyloliquefaciens, B. circulans, B. flexus; Bacteroides sp; Cellulomonas biazotea; cellvibrio gilvus; Eubacterium cellulosolvens,*

Bacteria	*Geobacillus sp; Microbispora bispora; Paenibacillus curdlanolyticus; Pseudomonas cellulose; Salinivibrio sp; Rhodothermus marinus.* ***Anaerobic bacteria*** *Acetovibrio cellulolyticus; Butyrivibrio fibrisolvens; Clostridium thermocellum; C. cellulolyticum; C. acetobutylium; C. papyrosolvens; Fibrobacter succinogenes; Ruminoccus albus*

Application of Cellulases in the Industries

For years, cellulases have been a target for academic and industrial research and are currently being applied in many industries (Singh *et al.,* 2007).

Cellulases application in the Wine and Brewing industry

Enzyme technology plays a crucial role in the beer and wine industries. Wine making requires the extraction of juice from grapes and subsequent fermentation by yeast while beer brewing involves malting of barley and fermentation of the resulting wort (Bhat, 2000). Brewing of beer is based on activity of the enzymes activated during malting and fermentation stages. Malting is dependent on seed germination, which initiates biosynthesis and activation of α and β amylases, carboxy peptidases and β glucanase that hydrolyses the seed reserves (Bamforth, 2009). Under optimal conditions all the three enzymes act in synergy to produce high quality malt. However due to seasonal variations and or poor harvest, brewers end up using poor quality barley which contains low levels of endogenous β glucanase activity. This results in the presence of a 6 -10 % of non starch polysaccharide mainly soluble β glucan which forms a gel during the brewing process leading to poor filtration of the wort, slow run off time, low extract yield and development of a haze in the final product (Galante *et al.,* 1998b). The viscosity of the wortis usually reduced by addition of microbial β glucanases, which hydrolyse β glucan. Commonly used microbial β glucanases are obtained from *Penicillium emersonii, Aspergillus niger*, *Bacillus subtilis* and *Trichoderma reesei* (Galante *et al.,* 1998b).

A study carried out by Oksanen *et al*., (1985) observed that endoglucanase II and cellobiohydrolase II of the Trichoderma cellulase system were responsible for most activity in reduction of the degree of polymerization and wort viscosity thus they are best suited for the production of high quality beer from low quality barley. In wine making, pectinases, β glucanases and hemicellulases comprise the main exogenous enzymes added. These enzymes give a better skin maceration, improved colour extraction, easy must clarification and filtration and improved wine quality and stability (Galante *et al.,* 1998b). In order to improve the wine's aroma, β-glucosidase is added to modify glycosylated precursors that are naturally present (Caldini *et al.,* 1994; Gunata *et al.,* 1990).

Cellulases in the Food industry

Industries producing fruit juices in the 1930s encountered challenges such as low yield and a poor clarity of the product (Uhlig, 1998). Research on industrially suitable enzymes such as cellulases, hemicellulases and pectinases from food grade microorganisms such as Aspergillus niger, Trichoderma sp. and increased knowledge of fruit components led to the overcoming of these challenges and led to improved methods of extraction, clarification and stabilization (Singh *et al.,* 2007). Cellulases along with xylanases and pectinases are the macerating enzymes that serve to increase the yield and process performance without any additional cost. Macerating enzymes are usually used in two steps; after crushing, the fruit pulp is macerated to either partial or complete liquefaction. After the extraction, pectinases are then used for its clarification and this lowers viscosity of fruit juice prior to its concentration and further increases the filtration rate and the stability of the juice. Macerating enzymes also improve the cloud stability, texture, decrease viscosity and facilitate easy concentration of nectars and purees (Grassin and Fauquembergue, 1996).

There is a growing demand for natural pigments for food colorants such as carotenoids. In their natural state, carotenoids remain bound to proteins thus preventing pigment oxidation. When solvents are used to extract carotenoids, they disrupt that association thus making the pigments insoluble in water and oxidation. This can be prevented by use of enzymatic methods. Cellulases hydrolyze cellulose in the cell walls hence the structural rigidity is interfered with

exposing intracellular materials for extraction. These pigments remain bound to proteins and are more stable than those obtained through traditional methods that involve use of solvents (Bassi *et al.,* 1993).

Cellulases in the Bio-refinery

Bioconversion of lignocellulosic biomass to produce biofuel is the most popular area of cellulase application being investigated recently (Sukumaran *et al.*, 2005). Potential lignocellulosic feedstocks sources include agricultural crop residues such as straw, the perennial prairie grass, municipal waste, packaging and construction debris, agricultural or forest processing by products e.g. food processing residues, pulping liquor from paper mills and forest woody biomass either logging residues from conventional harvest operations or removal of excess biomass from timberlands (NREL, 2006). Lignocellulosic biomass consists of cellulose tightly linked to lignin and hemicellulose (Kuila *et al.,* 2011).

Cellulases in Agriculture

Cellulolytic fungi including, *Trichoderma sp., Geocladium sp., Chaetomium sp. and Penicillium sp.* are known to play an important role in agriculture by facilitating enhanced seed germination, rapid plant growth and flowering, improved root system and increased crop yields (Bailey and Lumsden, 1998; Harman and Kubicek, 1998). Cellulases and related enzymes from certain fungi are capable of degrading the cell wall of plant pathogens hence controlling plant diseases. The β-1, 3- glucanase and N-acetyl- glucosaminidase From *Trichoderma harzianum* were reported to synergistically inhibit the spore germination and germ tube elongation of *B. cinerea* (Lorito *et al.*, 1994; Bhat, 2000). The β-1,3-glucanase from *Trichoderma harzianum* CECT 2413 induced morphological changes such as hyphal tip swelling, cytoplasm leakage and the formation of numerous septae and also inhibited the growth of *R. solani and Fusarium sp.* (Benitez *et al.,*1998).

Cellulases are also important in soil quality improvement. In order to reduce overreliance on mineral fertilizers, farmers incorporate straw in soil. Microbial routes to hasten straw

decomposition was using organisms such as *Aspergillus, Chaetomium and Trichoderma* and *Actinomycetes* have shown promising results (Ortiz Escobar and Hue, 2008; Tejada *et al.,* 2008).

Cellulases in Pulp and Paper industry

Application of enzyme preparations comprising cellulases, xylanases and lignases in the pulp and paper industry has increased in the last decade (Mai *et al.,* 2004; Karmakar and Ray, 2011). Pulping starts with the conversion of woody raw material into a flexible fiber that can be made into paper. Depending on the application of the paper, various methods of pulping can be used (Bajpai, 2012). Mechanical pulping usually involves mechanical grinding of the woody material to give fibers that can be used in the production of different grades of paper. This method is usually characterized by high energy consumption and gives paper with incompletely ground fiber bundles, low strength and tends to yellow with time due to little removal of lignin a weakness associated with the process (Bhat, 2000). Bio pulping using cellulases and allied enzymes reduces the energy required to achieve the desired strength and freeness of the pulp hence it's a better alternative to mechanical pulping (Karmakar and Ray, 2011). Cellulases containing enzyme mixtures are also useful in the hydrolysis of fines small particles produced during refining of primary or secondary fibers. These particles usually reduce the drainage rate of pulp during the paper making process. Hydrolysis of these particles improves the pulps drainage property which in turn determines the paper mill 's speed. Addition of these preparations before refining is either done to improve the beatability response or modify the fiber properties (Noe *et al.,* 1986; Pommier *et al.,* 1989; Pommier *et al.,* 1990).

Deinking process is crucial during paper recycling. Enzymatic deinking using cellulases reduces the need for deinking chemicals and also results to little or no loss in paper strength. Enzymatic deinking is usually combined with mechanical agitation in order to improve the efficacy of the process (Karmakar and Ray, 2011).

Cellulases in the Detergent industry

Recent innovations in the detergent industry have seen the incorporation of enzymes such as cellulases, proteases and lipases in detergents (Singh *et al.,* 2007). Due to repeated washing, cotton and cotton blend fabrics become dull and fluffy due to the presence of detached micro fibrils. Cellulase containing detergents are capable of degrading the cellulose micro fibrils to restore a smooth surface and original color to the garment. In addition, the degradation softens the fabric and removes dirt particles trapped in the micro fibril network (Sukumaran et al., 2005; Singh *et al.,* 2007). Cellulase preparations from *H. insolens* that are active under mild alkaline conditions (pH 8.5 - 9) and temperatures over 50 °C are added to detergents. Such cellulases active under alkaline conditions increase the cleaning capacity of detergents by selective contraction fibers hence facilitating the removal of oil from inter fiber space (Karmakar and Ray, 2011).

Cellulases in the Textile industry

The textile industry has been revolutionized by introduction of enzymes that are slowly replacing the conventional chemical processes, which are generally severe and lead to fiber damage (Bhat, 2000; Kuhad *et al.,* 2011). Cellulases have the ability to modify cellulosic fiber in a controlled and desired manner thus improving the fabric quality (Mojsov, 2012). They are mostly used during wet processing to improve fabric properties. Processes that involve cellulase activity include biostoning of jeans and biopolishing cellulosic fibers. Denim is heavy grade cotton and when dyed, the dye is mainly adsorbed on the surface of the fiber. When cellulases are used during the biostoning process, they break off small fiber ends on the yarn surface to loosen the dye, which is consequently easily removed during the wash cycle by mechanical abrasion. This enzyme-based treatment replaced pumice stone biostoning hence less damage to the fiber, increased productivity and a safe working environment (Christian *et al.,* 2006; Karmakar *et al.,* 2011).

Fading can be achieved without loss of fabric strength. Fabrics made from cellulosic fibers such

as cotton, linen, ramie, viscose and lyocell are normally characterized by short fibers protruding from the surface (fuzz formation) and pilling'*i.e.* loosened fuzz attached to the surface. This often decreases their market value and in order to prevent this, a process called biopolishing is done. Biopolishing is usually done during the wet processing stage and includes scouring, bleaching, dyeing and finishing. Cellulase mixtures usually rich in endoglucanases are used in this process to remove the small protruding fibers from the fabric surface without using chemicals. The fabric attains a smooth and a glossy appearance, improved brightness and uniformity. Biopolishing is a key procedure in the production of high-quality garments (Bhat, 2000; Kuhad *et al.,* 2011).

Thus, many researches have been conducted to find out industry beneficiary strain to increase the yield of cellulose production.

MATERIALS AND METHODS

1.ASSORTMENT OF SOIL SAMPLES

Materials Required:

- Germ-free poly bags
- Spatula
- Disposable nitrile gloves etc.

Different types of waste soil samples such as agricultural, compost, farmyard manure (FYM), and waterway were collected from various agro-climatic locations were collected from different areas of two distinct districts namely Kottayam and Ernakulam. Using a sterile spatula, the soil from approximately 4cm depth was collected and stored in poly bags. The collected waste soil samples were properly labelled according to the area, date of collection and tenfold serial dilutions of each soil sample were prepared in sterilized distilled water.

2. ISOLATION OF BACTERIA

a. Serial dilution

Materials Required:

- Conical flask
- 1ml sterile pipette tips
- 1000µl micropipette
- Test tubes
- Test tube stand
- Sterile distilled water
- Soil samples
- Media plates
- Wire loop etc.

Bacterial samples were isolated from soil by using serial dilutions and pour plate technique. One gram of waste soil was measured and mixed well with 9ml of sterile distilled water. The waste soil suspension was shaken vigorously under room temperature and tenfold serial dilution was carried out up to the 10^{-6} dilution. 1ml of each dilution were pour plated on nutrient agar media. Inoculated plates were incubated for 24hrs day and morphologically distinct colonies were selected for further analysis.

a. Pour plating

Materials Required:

- Sterile petri plates
- Nutrient agar
- Diluents etc.

1ml of each dilution were pour plated on nutrient agar media. The plates were rotated clockwise and anticlockwise for evenly distribution of bacteria. Inoculated plates were inoculated for 24hrs at 37^0C and morphologically distinct colonies were selected for further analysis.

b. Quadrant Streaking

Materials Required:

- Nutrient agar plates
- Wire loop etc.

Bacterial colonies were purified by repeated streaking on nutrient agar plates using a wire loop. The purified colonies were preserved for 24 hours so that the colony forming units (CFUs) can become isolated and grow into individual, pure colonies.

c. Preservation of bacterial isolates

Materials Required:

- Purified bacterial colonies
- Wire loop

- Nutrient agar
- Test tubes

Nutrient Agar Slant is used for cultivation of a wide variety of less fastidious microorganisms. It is used for the storage of the purified bacterial isolates. Purified 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 CELLULOLYTIC BACTERIA

a. CMC AGAR PLATE ASSAY

Pure bacterial isolates were individually sub cultured in Carboxymethyl Cellulose (CMC) agar plates by patch plate technique (Gulati *et al*., 2008). 0.26% of CMC powder is weighed and autoclaved, and poured into sterilized petri plates. Each plate was sub divided into 20 sections and the bacterial colonies were sub cultured. The plate was inoculated at 37 ^{0}C for 24 hrs.

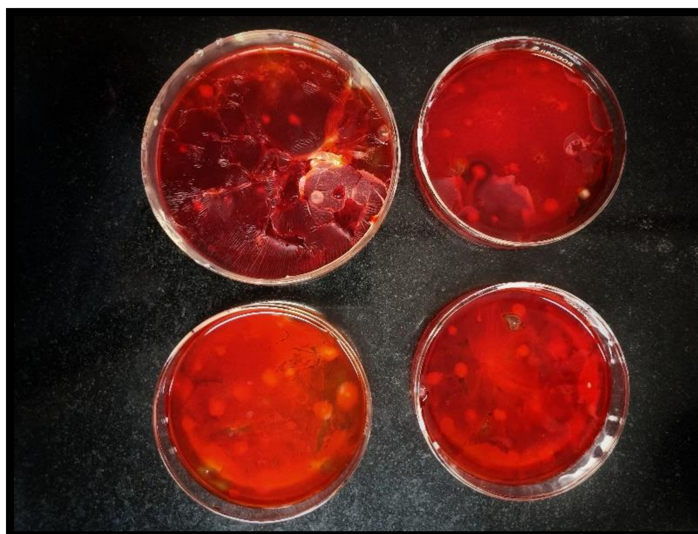


FIGURE 3: CMC agar plates flooded with Congo red

After inoculation for 24 hours, CMC agar plates were flooded with 0.1 % Congo red and allowed to stand for 15 minutes at room temperature. One molar NaCl was thoroughly used for counterstaining the plates (Lisdiyantie *et al*.,2012). Clear zones appearing around growing bacterial colonies indicates cellulose hydrolysis. The bacterial colonies showing well prominent clear zone were selected for identification and further analysis.

b. EXTRACELLULAR CELLULASE PRODUCTION

Materials Required:

- Sterile petri plates
- Sterile tips
- CMC agar powder
- Agar-agar
- Bacterial supernatant
- Gel puncher etc.

Bacterial isolates were screened for extracellular activity by a well diffusion method on CMC agar plates. Using 0.3% of agar-agar and 2.6 % of CMC powder, the plates were made and using a gel puncher, the wells were made on the plates after solidification of CMC agar plates. 20µl of crude bacterial supernatant were transferred into each well. The plates were incubated at 37°C for 24 hrs.

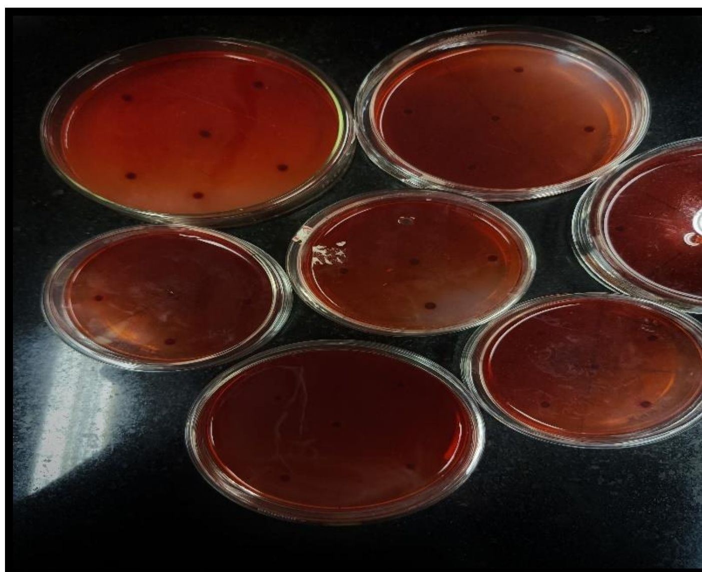


FIGURE 4: Well diffusion CMC agar plates flooded with Congo red

After incubation, the CMC plates were flooded with1% Congo red. One molar NaCl was thoroughly used for counterstaining the plates (Lisdiyantie *et al.*,2012). The zone of clearance was observed and the diameter of the clearance zone was measured.

4. IDENTIFICATION OF CELLULOLYTIC BACTERIA

Identification of cellulolytic bacteria was carried out, which was based on biochemical tests and molecular identification.

BIOCHEMICAL CHARACTERIZATION

a. INDOLE TEST

Materials Required:

- Bacterial isolate
- Tryptone broth
- Indole reagent
- Test tubes etc.

Indole test is used to determine the ability of an organism to split amino acid tryptophan to form the compound indole.

Procedure:

1) Inoculate the tryptophan broth with bacterial culture or emulsify isolated colonies of the test organism in tryptophan broth.
2) Incubate at 37°C for 24-28 hours.
3) Add 0.5 ml of Kovac's reagent to the broth culture.

Expected results:

Positive: Pink colored ring after addition of appropriate reagent

Negative: No color change even after the addition of appropriate reagent

b. MR-VP test

Methyl red test and Voges-Proskauer test both are done in methyl red–Voges-Proskauer (MR-VP) broth, but the reagents that added are different.

METHYL RED (MR) TEST

Materials Required:

- Methyl red indicator
- MRVP or Glucose phosphate broth
- Bacterial isolates
- Wire loop

Procedure:

The test bacterium 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.

Expected results:

- Positive methyl red test is indicated by the development of red colour after the addition of methyl red reagent.
- A negative methyl red test is indicated by no colour change after the addition of methyl red Positive tests give bright red colour and yellow colour indicates negative test.

VOGES PROSKAUER TEST

Materials Required:

- Bacterial isolates,
- MR VP broth,
- 0.5% of Barrit solution A and B

Procedure:

The test organism was inoculated in glucose phosphate broth and incubated at 37°C for 48 hours, to this 0.5 ml of Barrit solution A and B is added.

Expected results:

- Negative test is indicated by lack of color change after the addition of Barritt's A and Barritt's B reagents.
- A positive Voges-Proskauer test is indicated by the development of red-brown color after the addition of Barritt's A and Barritt's B reagents.

c. CATALASE TEST

Materials Required:

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

The enzyme catalase converts hydrogen peroxide into water and oxygen, thus helping an organism cope with toxic O_2 species. The catalase test is used to detect an organisms' ability to produce catalase.

Procedure:

A drop of 3% hydrogen peroxide was added to nutrient broth inoculated with bacterial isolate.

Expected results:

- Prompt effervescence indicates catalase production.
- No effervescence indicates absence of catalase.

e. CITRATE UTILIZATION TEST

Materials Required:

- Simmon citrate agar with bromothymol blue
- Bacterial isolates
- Sterile loops etc.

Procedure:

Citrate utilization test is used to determine the ability of bacteria to utilize sodium citrate as its only carbon source and inorganic ammonium dihydrogen phosphate (NH4H2PO4) as the sole nitrogen source. The pure bacterial isolates were directly inoculated on simmon's citrate agar containing bromothymol blue as indicator.

Expected results:

- Negative citrate utilization test is indicated by the lack of growth and colour change in the tube
- A positive citrate result as indicated by growth and a blue colour change

5.KIRBY BAUER DISK DIFFUSION SUSCEPTIBILITY TEST

Materials required:

- Sterile petri plates
- Nutrient broth
- Nutrient agar
- Bacterial samples
- Antibiotic discs
- L-rod
- Test tubes etc.

Kirby-Bauer disk diffusion susceptibility test is a standardized technique for testing rapidly growing pathogens. Briefly, a standardized bacterial inoculum is swabbed onto the surface of agar media with an L-rod after immersing it in the bacterial broth culture. Antibiotic discs are placed on the nutrient agar medium using sterile forceps. Based on the zone diameter the test organisms were categorized as antibiotic sensitive/ resistant or moderately sensitive.

6. MOLECULAR IDENTIFICATION OF THE BACTERIAL ISOLATES

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

Materials required:

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

Genomic DNA isolation is the first and the most important requirement in carrying out molecular biology techniques (Atashpaz *et al*., 2010). Genomic DNA was isolated and purified using phenol:chloroform:isoamyl alcohol method.

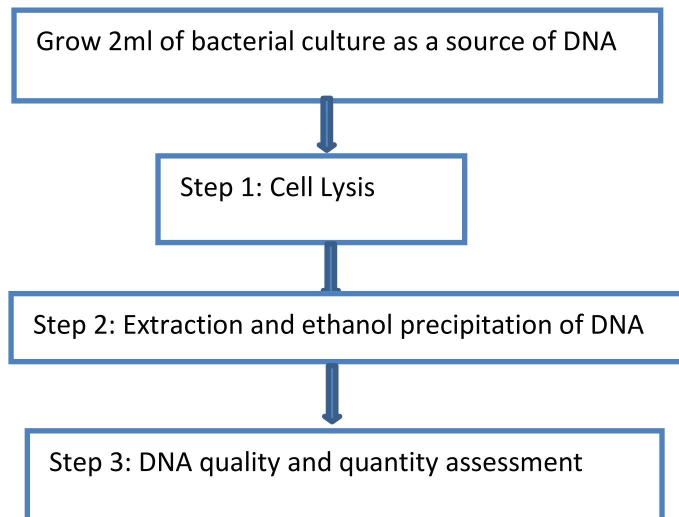


FIGURE 5: Flowchart showing complete genomic dna isolation protocol

1)Directly take about 2ml of overnight bacterial culture that was grown in LB medium and transfer it to a 1.5 ml of Eppendorf tube and centrifuge it at 8000rpm for 10 minutes for extracting the cell pellet.

2) Then discard the supernatant and rinse the pellet with sterile distilled water.

3) Resuspend the pellet in about 875 µl of Tris-EDTA (TE) buffer (lysis buffer). Add 5µl Proteinase K and 100µl 10% sodium dodecyl sulphate (SDS) and vortex it completely for proper mixing. Incubate it for 1hr in a water bath at 37 ^{0}c.

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 til they are properly mixed and centrifuge at 8000rpm for 10 minutes which will lead to the formation of white layer in aqueous phenol/chloroform interface.

5) Now carefully transfer the aqueous phase to a new tube through a 1ml pipette. Repeat the above steps till the white layer disappears.

6) For removing phenol take equal volume of chloroform and add to the aqueous layer and spin at maximum speed for 5 minutes.

7)Now transfer the aqueous layer to a new tube.

8) Collect the upper layer into a fresh tube.

9) Next 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) Rinse the DNA pellet with 1ml 70% ethanol and spin for about 5 minutes.

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

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

2. 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.

1) 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.
2) 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.
3) Remove the comb from the set gel and the gel is placed in the gel tank filled with 1X TAE.
4) 5µL of each DNA sample was mixed with 1 µL of 6X gel loading dye on a parafilm.
5) With the loaded wells with the samples the power is switched on.
6) After the samples have reached the end of the gel, the power is switched off and bands are visualized using the Gel documentation system.

3. PCR AMPLIFICATION OF 16SrDNA

Materials required:

- PCR Thermal cycler
- Template DNA
- 16SrDNAForward primer
- Reverse primer
- 5x assay buffer
- dNTP mix
- $MgCl_2$,
- Taq Polymerase (5U/ µl)
- Sterile water
- PCR tubes
- Micro tips.

The forward and reverse primers used for the amplification of 16S rDNA sequences of the isolated DNA are given in Table 2.

Table 2 showing Primers used to amplify 16S rDNA

Primer	Sequence	Reference
16S Forward	5’ GAGTTTGATCCTGGCTCAG 3’	Shivaji *et al.*,
16S Reverse	5’ ACGGCTACCTTGTTACGACTT 3’	2000

The concentration of different PCR ingredients is given in Table 2

Table 3 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 4)** with the lid temperature 105°C and volume as

Table 4 showing the program for PCR

Step	Temperature	Time	
Initial Denaturation	95°C	2 min	
Denaturation	95°C	30 s	35
Annealing	56°C	30 sec	cycles
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.

4. 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. ASSORTMENT OF SOIL SAMPLES

Present study was conducted by the collection of various soil samples from different localities of two districts namely Ernakulum and Kottayam, Kerala, India. About nine soil samples were collected from areas including waste dumping sites, agricultural wastes, poultry farming premises etc. and were designated as in (**Table 5**).

Table 5: Sampling sites and codes

DISTRICT - ERNAKULAM			
SAMPLE NO	**LOCATION**	**TYPE OF SITE**	**SAMPLE CODE**
1.	Kalamassery	Garbage dumping area	**ZKM**
2.	Mullassery Canal	Septage and Plastic dumping area	**ZMC**
3.	Willington Island	Plastic waste	**ZWI**
4.	Varapuzha	Agricultural and kitchen waste	**ZVP**
5.	Convent Junction	Municipal waste segregation ground	**ZCJ**
6.	Marakadavu	Poultry farm premises	**ZMA**
DISTRICT - KOTTAYAM			
7.	Kalaketty	Septage and Garbage dumping area	**ZKK**
8.	Manjakulam	Household Poultry farm premises	**ZMM**
9.	Kanjirappally Town Hall	Municipal waste segregation ground	**ZKC**

2. ISOLATION OF BACTERIA

Bacterial samples were isolated from various waste soil samples using serial dilution and pour plate technique as depicted in (**Figure 6**). A total of 78 bacterial samples which were morphologically distinct were streaked onto nutrient agar plates for further studies as represented in (**Figure 7**). Even the isolates were preserved on nutrient agar slants at 4 ^{0}C for further analysis. The isolates with discrete morphology were assigned the following culture code as indicated in (**Table 6**).

Table 6: List of bacterial isolates obtained

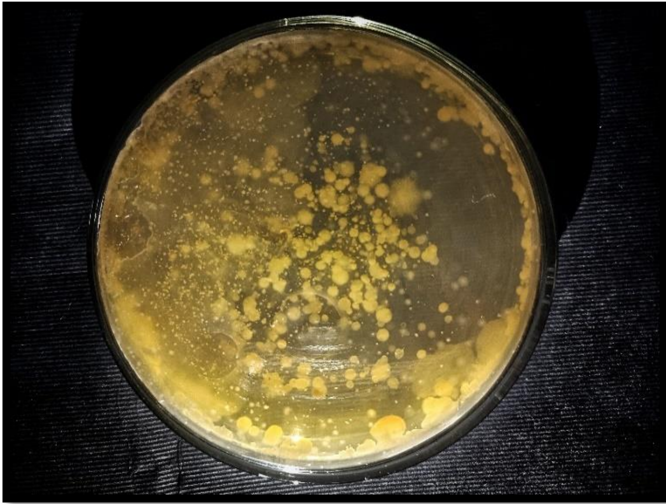
Sl. No	Sample code	Culture code	Sl. No	Sample Code	Culture code	Sl. No	Sample code	Culture code
1		ZKM 1	9		ZMC 5	17		ZVP 5
2	ZKM	ZKM 2	10		ZMC 6	18		ZVP 6
3		ZKM 3	11	ZWI	ZWI 1	19		ZVP 7
4		ZKM 4	12		ZWI 2	20		ZVP 8
5		ZMC 1	13		ZVP 1	21		ZVP 9
6		ZMC 2	14	ZVP	ZVP 2	22		ZVP 10
7	ZMC	ZMC 3	15		ZVP 3	23		ZVP 11

<table>
<tr><td>8</td><td rowspan="13">ZMA</td><td>ZMC 4</td><td>16</td><td rowspan="3"></td><td>ZVP 4</td><td>24</td><td rowspan="3">ZCJ</td><td>ZCJ 1</td></tr>
<tr><td>25</td><td>ZCJ 2</td><td>38</td><td>ZMA 12</td><td>51</td><td>ZKK 2</td></tr>
<tr><td>26</td><td>ZCJ 3</td><td>39</td><td>ZMA 13</td><td>52</td><td>ZKK 3</td></tr>
<tr><td>27</td><td>ZMA 1</td><td>40</td><td rowspan="10"></td><td>ZMA 14</td><td>53</td><td rowspan="10">ZMM</td><td>ZKK 4</td></tr>
<tr><td>28</td><td>ZMA 2</td><td>41</td><td>ZMA 15</td><td>54</td><td>ZKK 5</td></tr>
<tr><td>29</td><td>ZMA 3</td><td>42</td><td>ZMA 16</td><td>55</td><td>ZKK 6</td></tr>
<tr><td>30</td><td>ZMA 4</td><td>43</td><td>ZMA 17</td><td>56</td><td>ZMM 1</td></tr>
<tr><td>31</td><td>ZMA 5</td><td>44</td><td>ZMA 18</td><td>57</td><td>ZMM 2</td></tr>
<tr><td>32</td><td>ZMA 6</td><td>45</td><td>ZMA 19</td><td>58</td><td>ZMM 3</td></tr>
<tr><td>33</td><td>ZMA 7</td><td>46</td><td>ZMA 20</td><td>59</td><td>ZMM 4</td></tr>
<tr><td>34</td><td>ZMA 8</td><td>47</td><td>ZMA 21</td><td>60</td><td>ZMM 5</td></tr>
<tr><td>35</td><td>ZMA 9</td><td>48</td><td>ZMA 22</td><td>61</td><td>ZMM 6</td></tr>
<tr><td>36</td><td>ZMA 10</td><td>49</td><td>ZMA 23</td><td>62</td><td>ZMM 7</td></tr>
</table>

37		ZMA 11	50	ZKK	ZKK 1	63		ZMM 8
64		ZMM 9	69		ZMM 14	74		ZKC 4
65		ZMM 10	70		ZMM 15	75		ZKC 5
66		ZMM 11	71	ZKC	ZKC 1	76		ZKC 6
67		ZMM 12	72		ZKC 2	77		ZKC 7
68		ZMM 13	73		ZKC 3	78		ZKC 8

(a)

(b)



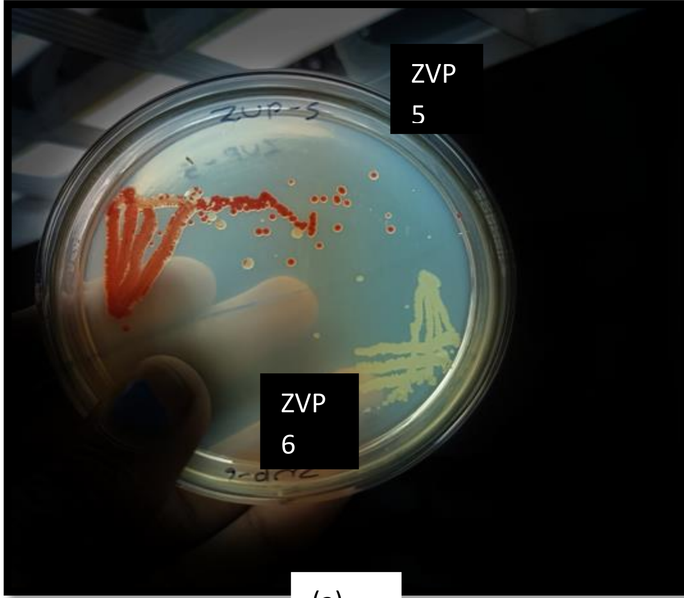
(c)

(d)

(e)

(f)

FIGURE 6: Bacterial colonies obtained from pour plate technique.



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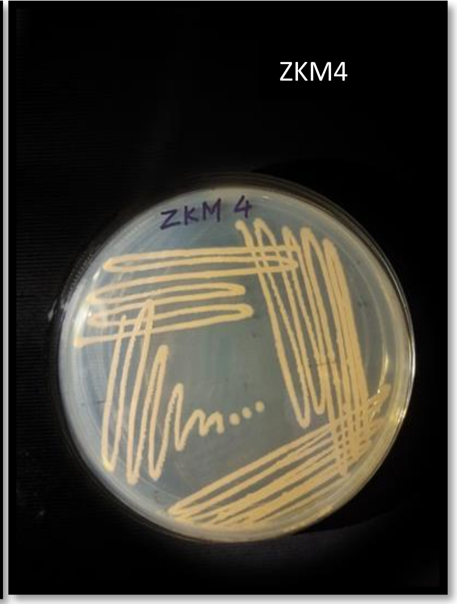
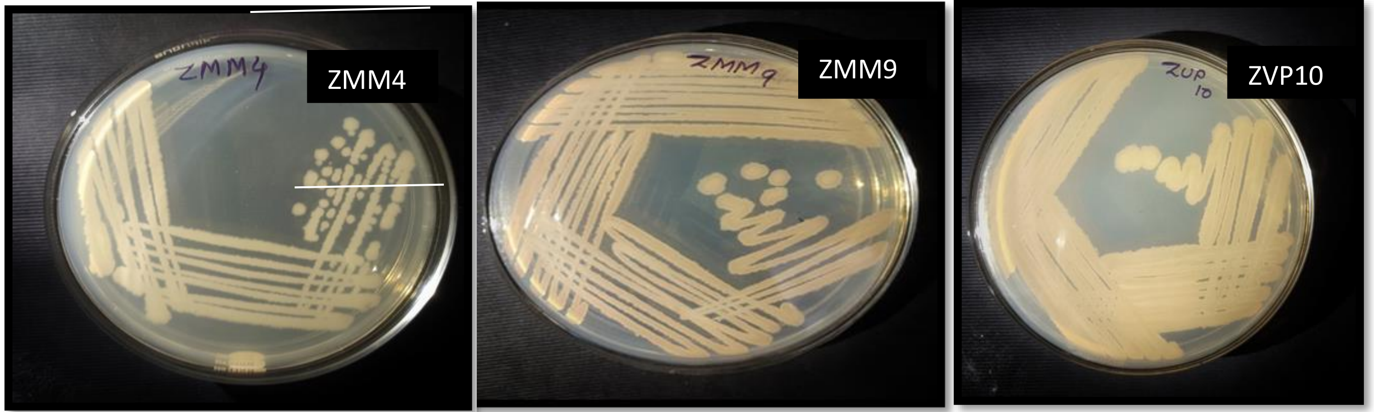


(c) (d) (e)



(f) (g) (h)

(i) (j)

FIGURE 7: Pure culture of bacterial isolates by quadrant streak plate method.

3. SCREENING OF CELLULOLYTIC BACTERIA

a) CMC AGAR PLATE ASSAY

The isolated 78 bacterial colonies were patched on carboxy methyl cellulose medium and after incubation, the plates were flooded with Congo red. Out of the 78 isolates, 35 isolates showed degradation which was confirmed by the presence of well-defined clear zone and cellulase activity as illustrated in (**Table 7**). The isolates with prominent clear zone around the bacterial colonies is indicated in (**Figure 8**) and were employed for further analysis by the well diffusion method.

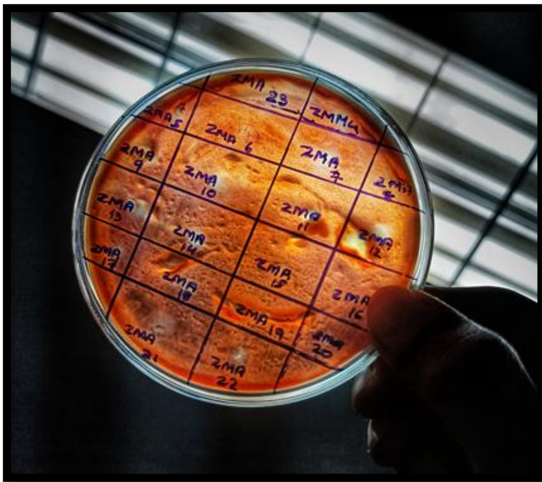
Table 7: List of Cellulolytic bacteria obtained

Sample No	Bacterial Isolate	Result	Sample No	Bacterial Isolate	Result	Sample No	Bacterial Isolate	Result
1	ZWI 1	+	11	ZKM 3	_	21	ZCJ 3	_
2	ZWI 2	_	12	ZKM 4	+	22	ZVP 1	+
3	ZMC 1	+	13	ZKK 1	+	23	ZVP 2	+
4	ZMC 2	_	14	ZKK 2	+	24	ZVP 3	+
5	ZMC 3	+	15	ZKK 3	+	25	ZVP 4	+
6	ZMC 4	+	16	ZKK 4	+	26	ZVP 5	+
7	ZMC 5	+	17	ZKK 5	+	27	ZVP 6	+

8	ZMC 6	+	18	ZKK 6	+	28	ZVP 7	+
9	ZKM 1	+	19	ZCJ 1	+	29	ZVP 8	+
10	ZKM 2	+	20	ZCJ 2	+	30	ZVP 9	+
31	ZVP 10	+	47	ZMM 7	_	63	ZMA 8	+
32	ZVP 11	+	48	ZMM 8	+	64	ZMA 9	+
33	ZKC 1	+	49	ZMM 9	+	65	ZMA 10	+
34	ZKC 2	+	50	ZMM 10	+	66	ZMA 11	+
35	ZKC 3	_	51	ZMM 11	+	67	ZMA 12	+
36	ZKC 4	+	52	ZMM 12	_	68	ZMA 13	+
37	ZKC 5	+	53	ZMM 13	_	69	ZMA 14	+
38	ZKC 6	_	54	ZMM 14	_	70	ZMA 15	+
39	ZKC 7	_	55	ZMM 15	_	71	ZMA 16	_
40	ZKC 8	+	56	ZMA 1	+	72	ZMA 17	+
41	ZMM 1	+	57	ZMA 2	+	73	ZMA 18	+
42	ZMM 2	+	58	ZMA 3	+	74	ZMA 19	+

43	ZMM 3	+	59	ZMA 4	+	75	ZMA 20	+
44	ZMM 4	+	60	ZMA 5	+	76	ZMA 21	+
45	ZMM 5	+	61	ZMA 6	+	77	ZMA 22	+
46	ZMM 6	+	62	ZMA 7	+	78	ZMA 23	+

+: Activity **-: No Activity**



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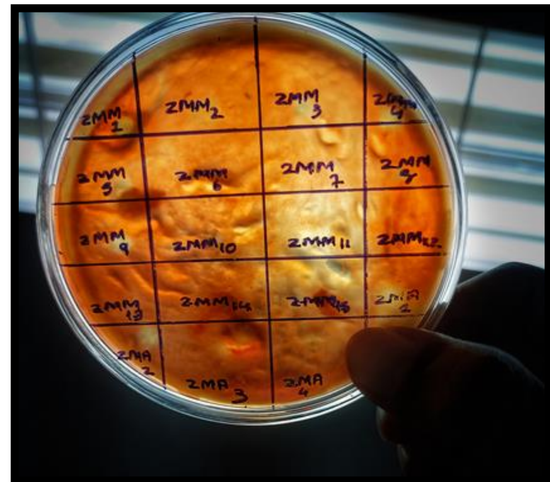


(b)

(c)

(d)

FIGURE 8: CMC agar plates with prominent clear zones upon addition of Congo Red

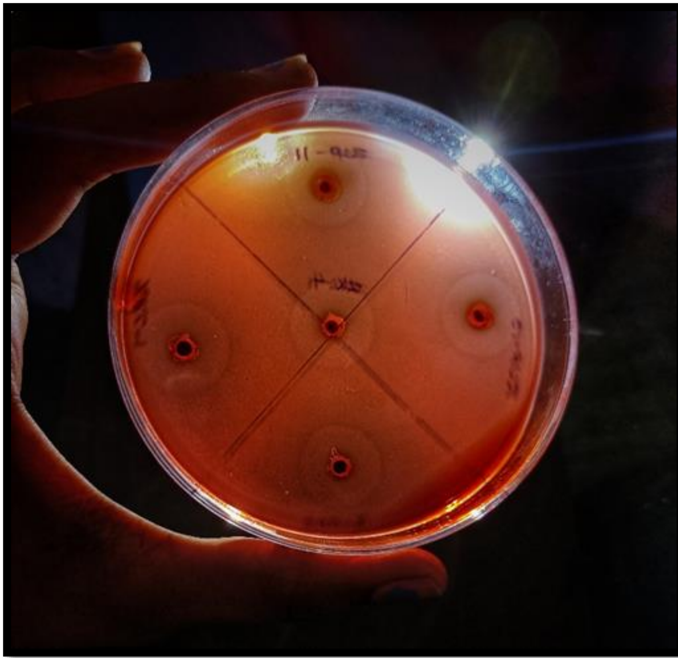
b. SCREENING FOR EXTRACELLULAR CELLULASE PRODUCTION

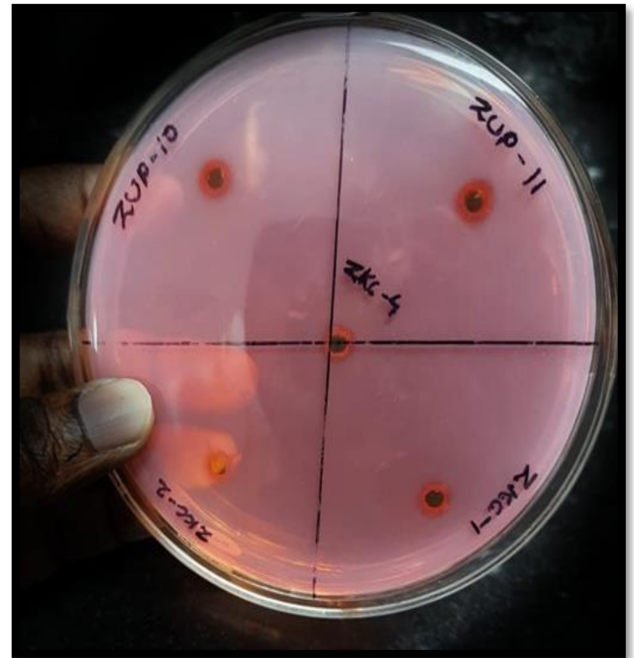
The pure bacterial isolates which showed potent clear zones on carboxy methyl cellulose agar medium using patch plate technique were further screened for extracellular cellulase activity by well diffusion method on CMC plate. Isolates which showed hydrolysis zone on agar plates, containing CMC as core carbon source, after the addition of Congo red stain and counter stain NaCl, the zone diameter were measured and enlisted as in **Table 8** and were subjected to further analysis.

Table 8: CMC agar hydrolysis of potential isolates with zone diameter.

Sample No	Sample Code	Zone Diameter (z)	Sample No	Sample Code	Zone Diameter (z)
1	ZKC 1	1.8	18	ZMC 1	1.8
2	ZKC 2	1.9	19	ZMC 3	1.9
3	ZVP 10	1.9	20	ZMC 4	1.7
4	ZVP 11	1.5	21	ZKK 2	1.8
5	ZKC 4	1.6	22	ZMA 10	1.4
6	ZMA 11	1.5	23	ZMA 5	1.4
7	ZMA 7	1.5	24	ZMM 3	1.3
8	ZVP 9	1.2	25	ZKK 5	1.5
9	ZCJ 2	0.9	26	ZVP 8	1.2
10	ZVP 5	1.3	27	ZMM 9	1.7
11	ZMA 1	1.5	28	ZMA 2	1.5
12	ZMA 3	1.6	29	ZMA 4	1.5
13	ZMA 13	1.6	30	ZMA 17	2
14	ZMA 22	0.7	31	ZMA 23	2
15	ZMM 4	1.9	32	ZKC 5	1.8

16	ZMM 1	1.6	33	ZMM 2	1.8
17	ZMM 5	1			





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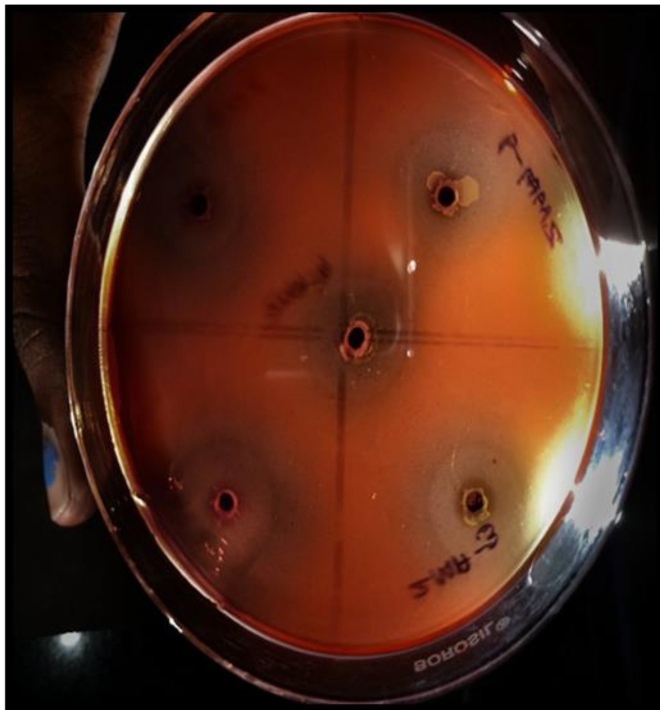

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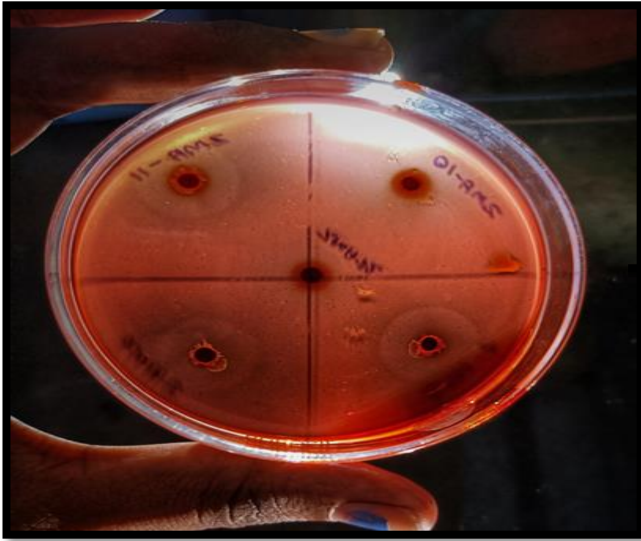

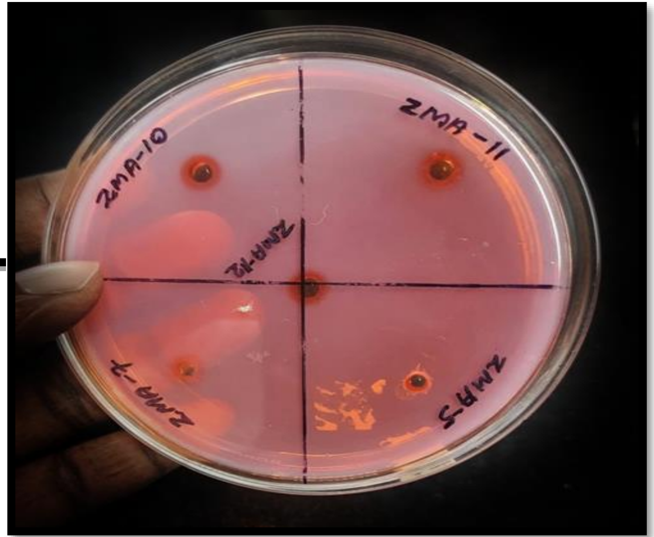

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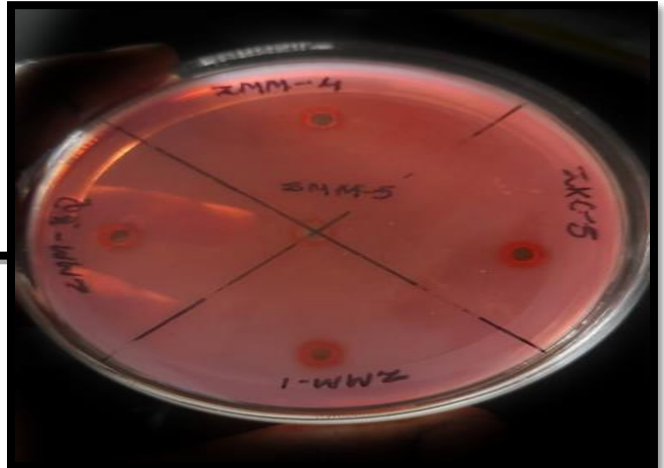

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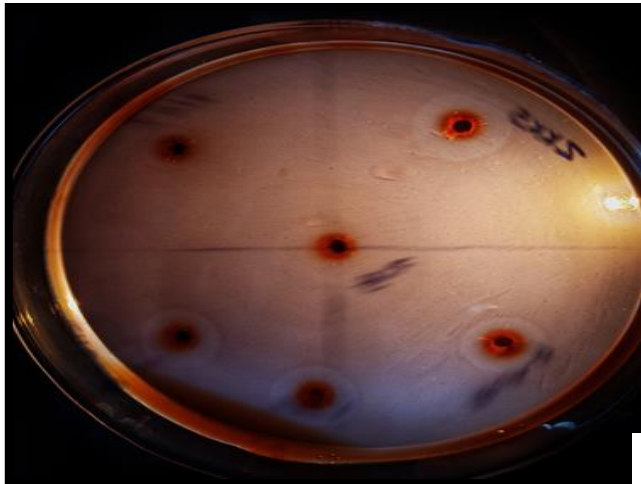

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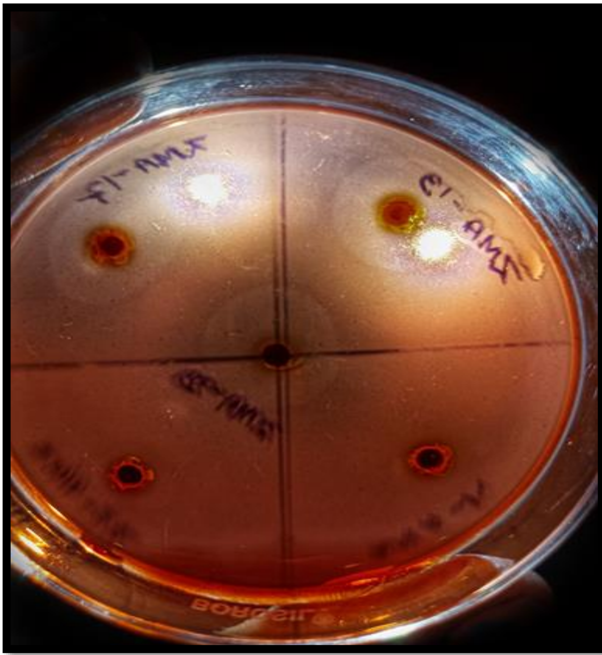
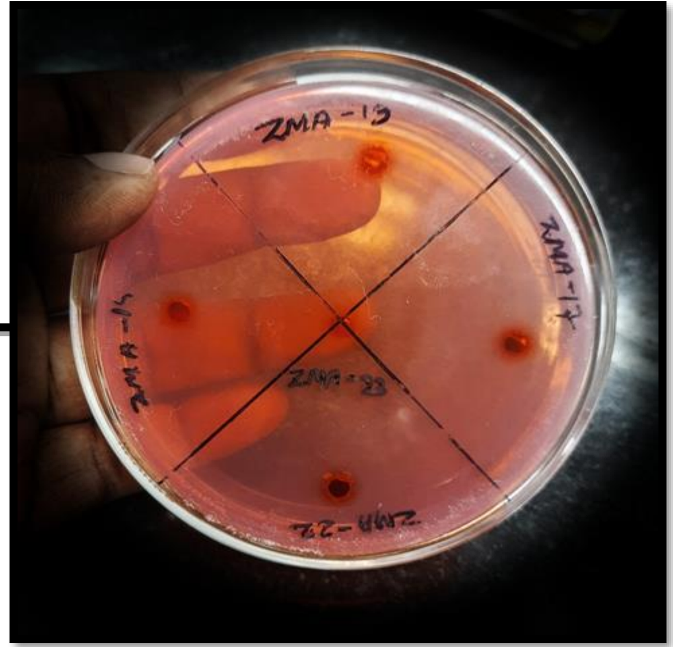
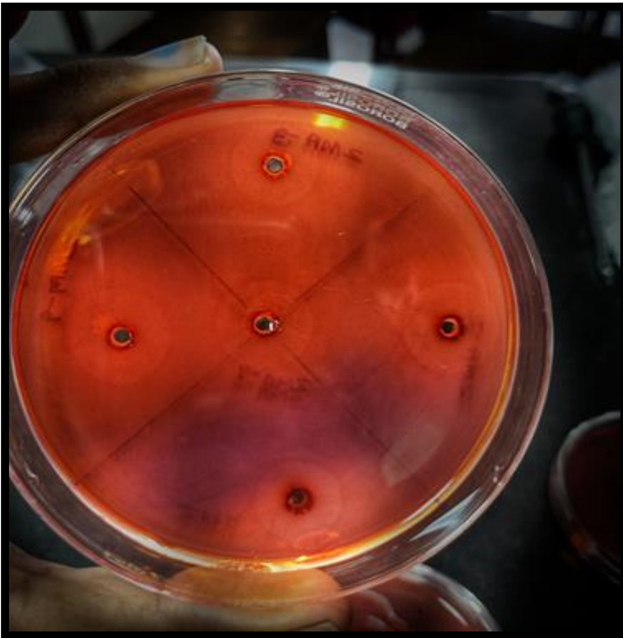




(f)



(g)

FIGURE 9: Extracellular screening of cellulase production by bacterial isolates.

4. IDENTIFICATION OF CELLULOLYTIC BACTERIA

I. BIOCHEMICAL CHARACTERIZATION:

a. INDOLE TEST

Presence of indole was detected by the addition of Kovac's reagent into incubated tryptophan broth with bacterial isolates.

There was no visible change in the Tryptone broth for all the samples tested expect ZMA 17 which showed positive indole test result by the appearance of pink colour as presented in the **FIGURE 10**. ZVP 5, ZMM 9, ZVP 10, ZVP 1, ZMM 4, ZMC 1, all these isolates remain unchanged after incubation which indicated that all of these isolates were indole negative.

Table 9: Indole test results obtained.

SL NO.	TEST ISOLATES	RESULT OBTAINED
1	ZMA 17	+
2	ZMC 1	_
3	ZMM 4	_
4	ZMM 9	_
5	ZVP 5	_
6	ZVP 10	_
7	ZVP 1	_

+: **Activity** **-: No Activity**

FIGURE 10: Indole test results - ZMA 17, ZMC 1, ZMM 4, ZMM9, ZVP 1, ZVP 5 & ZVP 10

b. MR-VP TEST

METHYL RED (MR) TEST

In the Methyl red test, the test bacterial isolates were grown in broth medium containing glucose. If the bacteria have the ability to utilize glucose with production of a stable acid the colour of Methyl red changes from yellow to red when added into the broth culture. In MR test, the isolates showed in **FIGURE 11**- ZMA 17, ZMC 1, ZVP 1, ZMM 4, ZMM9, ZVP 10 & ZVP 5 changed the color of the solution to red color. So, it indicated that these isolates are positive.

Table 10: MR test results obtained

SL NO.	TEST ISOLATES	RESULTS OBTAINED
1	**ZMA 17**	+
2	**ZMC 1**	+
3	**ZMM 4**	+
4	**ZMM 9**	+
5	**ZVP 5**	+
6	**ZVP 10**	+
7	**ZVP 1**	_

+: Activity -: No Activity

FIGURE 11: MR test results- ZMA 17, ZMC 1, ZVP 1, ZMM 4, ZMM9, ZVP 5 & ZVP 10

VOGES PROSKAUER TEST

The Voges-Proskauer test is performed to determine if an organism produces acetylmethyl carbinol from glucose fermentation. The test isolates were inoculated in glucose phosphate broth and Barrit solution A and B was added. In VP test, the isolates revealed different result as shown in **FIGURE 12**- ZVP 1, ZVP 5 & ZVP 10 changed the color of solution to red-brown, whereas ZMA 17, ZMC 1, ZMM 4 & ZMM 9 remain unchanged. So it indicated that ZVP 5 & ZVP 10 is VP test positive.

Table 11: VP test results obtained

SL NO.	TEST ISOLATES	RESULTS OBTAINED
1	**ZMA 17**	_
2	**ZMC 1**	_
3	**ZMM 4**	_
4	**ZMM 9**	_
5	**ZVP 5**	+
6	**ZVP 10**	_
7	**ZVP 1**	+

+: Activity -: No Activity

FIGURE 11: VP test results- ZMA 17, ZMC 1, ZVP 1, ZMM 4, ZMM9, ZVP 5 & ZVP 10

c. CATALASE TEST

Catalase test was performed by inoculating the test organism for about 18-24hrs in nutrient broth and 3% hydrogen peroxide was added. The evolution of oxygen bubbles as depicted in **FIGURE 13** indicated that the isolates ZMA 17, ZMC 1, ZVP 1, ZMM 4, ZMM9, ZVP 5 & ZVP 10 were catalase positive. All six isolates were able to produce enzyme catalase and converts hydrogen peroxide into water and oxygen.

Table 12: Catalase test results obtained

SL NO.	TEST ISOLATES	RESULTS OBTAINED
1	**ZMA 17**	+
2	**ZMC 1**	+
3	**ZMM 4**	+
4	**ZMM 9**	+
5	**ZVP 5**	+
6	**ZVP 10**	+
7	**ZVP 1**	+

+: **Activity** **-: No Activity**

FIGURE 13: Catalase test results- ZMA 17, ZMC 1, ZVP 1, ZMM 4, ZMM9, ZVP 5 & ZVP 10

d. CITRATE UTILIZATION TEST

Simmon citrate agar is a selective and differential medium based on citrate utilization.The medium tests the ability of organisms to utilize citrate as sole carbon source.Positive reaction is indicated by the growth of bacteria with an intense blue colour in the slant. In **FIGURE 14**, the results of citrate utilization test are presented. ZMA 17, ZMC 1, ZVP 1, ZMM 4, ZVP 5 & ZVP 10 changed the color of the slant to blue, were ZMM 9 remain unchanged. So it indicated that ZMM 9 is negative and others are citrate positive respectively.

Table 13: Citrate test results obtained

SL NO.	TEST ISOLATES	RESULT OBTAINED
1	**ZMA 17**	+
2	**ZMC 1**	+
3	**ZMM 4**	+
4	**ZMM 9**	_
5	**ZVP 5**	+
6	**ZVP 10**	+
7	**ZVP 1**	+

+: Activity -: No Activity

FIGURE 14: Citrate utilization test results- ZMA 17, ZMC1, ZVP 1, ZMM 4, ZMM9, ZVP 5 & ZVP 10

5. KIRBY BAUER DISK DIFFUSION SUSCEPTIBILITY TEST

Pure bacterial isolates were swabbed on the nutrient agar and the selected antibiotic disc were placed. After 24 hours of incubation the zone of inhibition of each antibiotic against the test isolates were measured and charted (**Table 14).**

Table 14: Antibiotic test results obtained

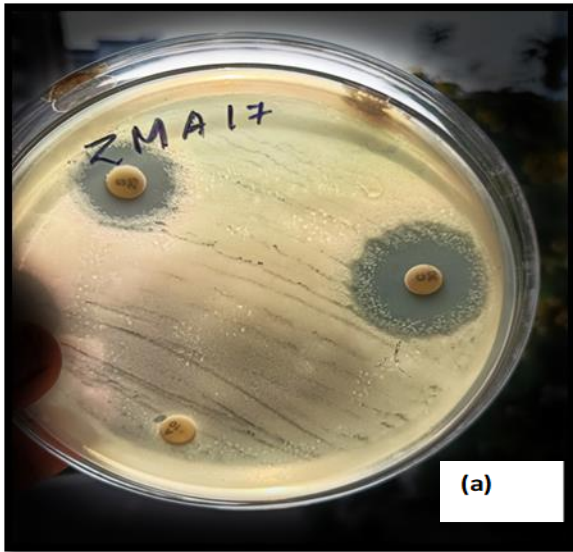
SL NO.	ANTIBIOTICS (DOSE)	TEST ISOLATE	DIAMETER (Z)(MM)	SENSITIVE	MODERATELY SENSITIVE	RESISTANT
1	**Streptomycin (S^{25})**	**ZMA 17**	**18**	✓	_	_
2	**Chloramphenicol (C^{30})**		**24**	✓	_	_
3	**Ampicillin (AMP^{1O})**		_	_	_	_
4	**Gentamicin (GEN^{30})**		**24**	✓	_	_
5	**Tetracycline (TE^{30})**		**10**	✓	_	_
6	**Cloxacillin (CX^{1})**		_	_	_	✓
7	**Penicillin (P^{10})**		_	_	_	✓
8	**Erythromycin (E^{15})**		**15**	_	✓	
9	**Nalidixic acid (NA^{30})**		_	_	_	✓
10	**Cefuroxime (CXM^{30})**		_	_	_	✓
11	**Streptomycin (S^{25})**		**24**	✓	_	_
12	**Chloramphenicol (C^{30})**		**19**	✓	_	_

13	**Ampicillin (AMP^{10})**	**ZMC 1**	_	_	_	_
14	**Gentamicin (GEN^{30})**		**23**	✓	_	_
15	**Tetracycline (TE^{30})**		**19**	✓	_	_
16	**Cloxacillin (CX^{1})**		_	_	_	✓
17	**Penicillin (P^{10})**		_	_	_	✓
18	**Erythromycin (E^{15})**		**26**	_	_	✓
19	**Nalidixic acid (NA^{30})**		**28**	✓	_	_
20	**Cefuroxime (CXM^{30})**		_	_	_	✓
21	**Streptomycin (S^{25})**	**ZMM 4**	**14**	_	_	✓
22	**Chloramphenicol (C^{30})**		**22**	✓	_	
23	**Ampicillin (AMP^{10})**		_	_	_	✓
24	**Gentamicin (GEN^{30})**		**23**	✓	_	_
25	**Tetracycline (TE^{30})**		**14**	_	_	✓
26	**Cloxacillin (CX^{1})**		_	_	_	✓
27	**Penicillin (P^{10})**		_	_	_	✓
28	**Erythromycin (E^{15})**		**33**	✓	_	_
29	**Nalidixic acid (NA^{30})**		**25**	✓	_	_
30	**Cefuroxime (CXM^{30})**		_	_	_	_
31	**Streptomycin (S^{25})**		**21**	✓	_	_

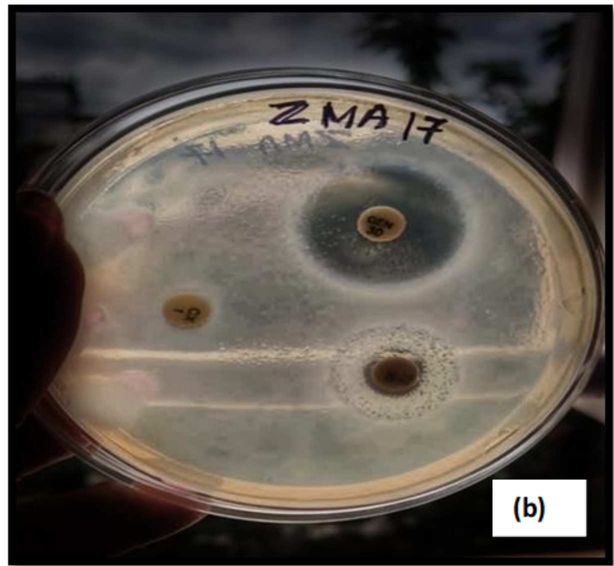
32	**Chloramphenicol (C^{30})**		**17**	_	✓	_
33	**Ampicillin (AMP^{1O})**	**ZMM 9**	_	_	_	_
34	**Gentamicin (GEN^{30})**		**22**	✓	_	_
35	**Tetracycline (TE^{30})**		**17**	✓	_	_
36	**Cloxacillin (CX^{1})**		_	_	_	_
37	**Penicillin (P^{10})**		_	_	_	✓
38	**Erythromycin (E^{15})**		**30**	✓	_	_
39	**Nalidixic acid (NA^{30})**		**26**	✓	_	_
40	**Cefuroxime (CXM^{30})**		_	_	_	✓
41	**Streptomycin (S^{25})**	**ZVP 5**	**19**	_	✓	_
42	**Chloramphenicol (C^{30})**		**17**	_	✓	_
43	**Ampicillin (AMP^{1O})**		_	_	_	✓
44	**Gentamicin (GEN^{30})**		**14**	_	✓	_
45	**Tetracycline (TE^{30})**		_	_	_	✓
46	**Cloxacillin (CX^{1})**		_	_	_	✓
47	**Penicillin (P^{10})**		_	_	_	✓
48	**Erythromycin (E^{15})**		_	_	_	✓
49	**Nalidixic acid (NA^{30})**		**26**	✓	_	_
50	**Cefuroxime (CXM^{30})**		**15**	_	_	_

51	**Streptomycin (S^{25})**		**23**	✓	–	–
52	**Chloramphenicol (C^{30})**		**20**	✓	–	–
53	**Ampicillin (AMP^{10})**	**ZVP 10**	–	–	–	✓
54	**Gentamicin (GEN^{30})**		**10**	–	–	✓
55	**Tetracycline (TE^{30})**		**10**	–	–	✓
56	**Cloxacillin (CX^{1})**		–	–	–	✓
57	**Penicillin (P^{10})**		–	–	–	✓
58	**Erythromycin (E^{15})**		**27**	✓	–	–
59	**Nalidixic acid (NA^{30})**		**28**	✓	–	–
60	**Cefuroxime (CXM^{30})**		–	–	–	✓
61	**Streptomycin (S^{25})**	**ZVP 1**	**14**	–	–	✓
62	**Chloramphenicol (C^{30})**		**19**	✓	–	
63	**Ampicillin (AMP^{10})**		**11**	–	–	✓
64	**Gentamicin (GEN^{30})**		**35**	✓	–	–
65	**Tetracycline (TE^{30})**		**14**	–	✓	–
66	**Cloxacillin (CX^{1})**		–	–	–	✓
67	**Penicillin (P^{10})**		–	–	–	✓
68	**Erythromycin (E^{15})**		**20**	-	✓	–
69	**Nalidixic acid (NA^{30})**		**22**	✓		
70	**Cefuroxime (CXM^{30})**		–	–	–	✓

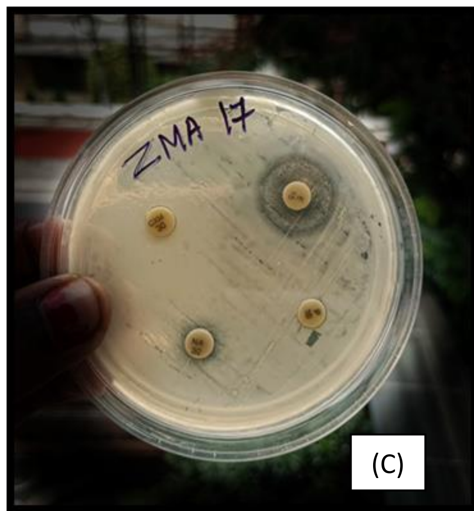
------ Test results for ZMA 17------



(a)

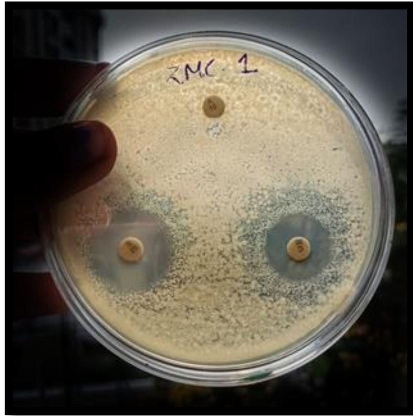


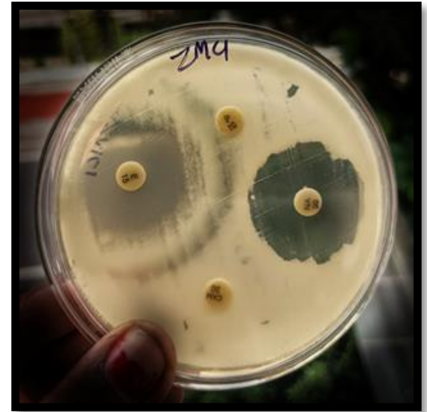
(b)



(C)

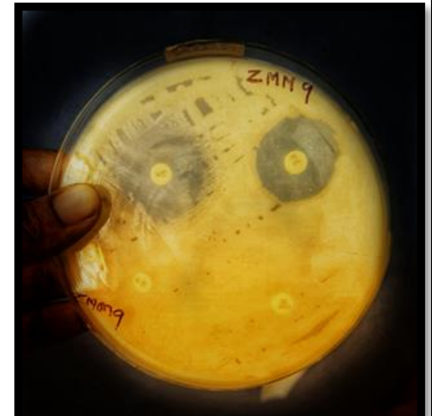
------Test results for ZMC 1------



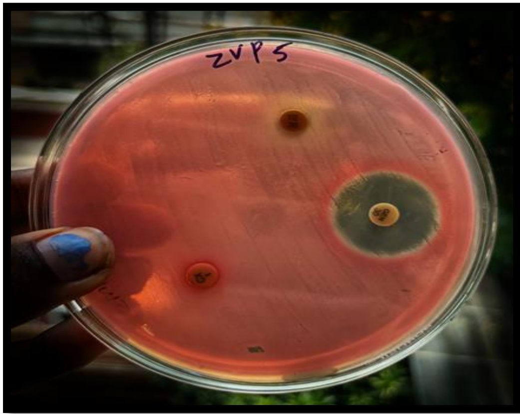


------Test results for ZMM 4 ------

------Test results for ZMM 9 ------



------Test results for ZVP 5 ------



------Test results for ZVP 10 ------

(a)

(b)

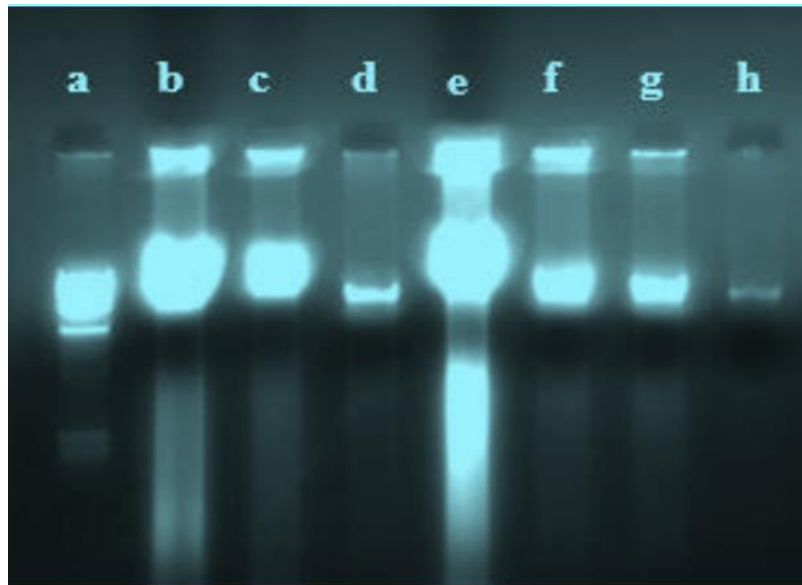
(c)

FIGURE 15: Antibiotic test results ZMA 17, ZMC1, ZVP 1, ZMM 4, ZMM9, ZVP 5 & ZVP 10

6. MOLECULAR IDENTIFICATION OF THE BACTERIAL ISOLATES

I.GENOMIC DNA ISOLATION

Genomic DNA isolated from ZMA17, ZVP1, ZVP10, ZMC1, ZMM4, ZMM9 & ZVP5 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 16**.



Lane a**- 1kb DNA ladder**

Lane b **- DNA of sample ZMC1,** Lane c **- DNA of sample ZMM4**

Lane d**- DNA of sample ZVP5,** Lane e **- DNA of sample ZMA17**

Lane f**- DNA of sample ZMM9,** Lane g**- DNA of sample ZVP1**

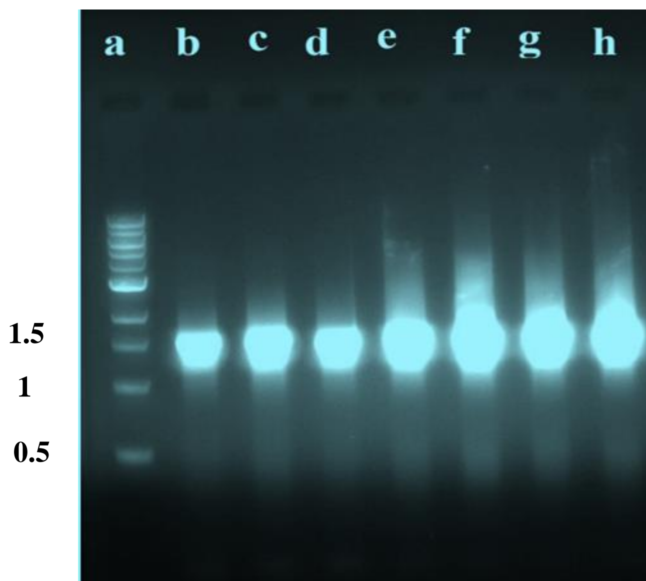
Lane h- **DNA of sample ZVP10**

FIGURE 16 - Agarose gel showing isolated genomic DNA

II. PCR AMPLIFICATION OF 16S rDNA

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 ZVP10,**

Lane c **- PCR product of sample ZVP1,** Lane d**- PCR product of sample ZMC1,**

Lane e**- PCR product of sample ZMM4,** Lane f**- PCR product of sample ZMM9,**

Lane g**- PCR product of sample ZMA17,** Lane h**- PCR product of sample ZVP5**

FIGURE 17 - 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 ZMA17, ZMC1, ZMM4, ZMM9, ZVP10, ZVP5 & ZVP1 was determined to have 100% similarity to *Elizabethkingia sp.* (CP040694.1) 99.76% similarity to the *Bacillus paramycoides* (MT611845.1), 100% similarity to *Stenotrophomonas maltophilia* (MH169196.1), 100% similarity to *Bacillus subtilis* (ON534347.1), 100% similarity to *Bacillus cereus* (MK606066.1) & 100% similarity to *Bacillus subtilis* (ON534347.1), 100% similarity to *Serratia marcescens* (CPO55161.1). The NCBI hit list **FIGURE 18- 24** determines the bacterial identity.

------Test result for ZMA17-------

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Elizabethkingia sp. JS20170427COW chromosome, complete genome	Elizabethkingia sp. JS201...	444	2221	100%	3e-120	100.00%	2691279	CP040694.1
uncultured bacterium partial 16S rRNA gene	uncultured bacterium	422	422	100%	1e-113	98.33%	1439	LR651512.1
Chryseobacterium piscium strain ENR6J 16S ribosomal RNA gene, partial sequence	Chryseobacterium piscium	416	416	100%	6e-112	97.92%	1362	KY852467.1
Uncultured Soonwooa sp. clone PY11-05-282 16S ribosomal RNA gene, partial sequence	uncultured Soonwooa sp.	416	416	100%	6e-112	97.92%	489	KF009004.1
Chryseobacterium piscium strain C-B13F 16S ribosomal RNA gene, partial sequence	Chryseobacterium piscium	416	416	100%	6e-112	97.92%	1386	KJ806508.1
Uncultured bacterium clone leachfield16Sseq296 16S ribosomal RNA gene, partial sequence	uncultured bacterium	416	416	98%	6e-112	98.31%	910	KJ489975.1
Soonwooa buanensis strain T0.1-25 16S ribosomal RNA gene, partial sequence	Soonwooa buanensis	416	416	100%	6e-112	97.92%	1388	MN330202.1
Soonwooa buanensis strain C7b-4m 16S ribosomal RNA gene, partial sequence	Soonwooa buanensis	416	416	100%	6e-112	97.92%	939	HQ663901.1
Uncultured bacterium clone LaYa5b-51 16S ribosomal RNA gene, partial sequence	uncultured bacterium	416	416	100%	6e-112	97.92%	883	GU291585.1

FIGURE 18- NCBI BLAST list depicting the sequence similarity of the sample ZMA17

------Test result for ZMC1------

	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 19- NCBI BLAST list depicting the sequence similarity of the sample ZMC1

------Test result for ZMM4------

	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	1336	1336	100%	0.0	100.00%	1461	ON534347.1
☑	Bacillus cereus strain DQ01 chromosome, complete genome	Bacillus cereus	1336	18685	100%	0.0	100.00%	5322598	CP097351.1
☑	Bacillus thuringiensis strain CPO 28.R5 16S ribosomal RNA gene, partial sequence	Bacillus thuringiensis	1336	1336	100%	0.0	100.00%	1443	ON514334.1
☑	Bacillus thuringiensis strain CPO 28.V11 16S ribosomal RNA gene, partial sequence	Bacillus thuringiensis	1336	1336	100%	0.0	100.00%	1484	ON514326.1
☑	Bacillus cereus strain DSC-18-02-19 16S ribosomal RNA gene, partial sequence	Bacillus cereus	1336	1336	100%	0.0	100.00%	1501	ON514315.1
☑	Bacillus albus strain GTM22 16S ribosomal RNA gene, partial sequence	Bacillus albus	1336	1336	100%	0.0	100.00%	1456	ON514248.1
☑	Bacillus albus strain GFK31 16S ribosomal RNA gene, partial sequence	Bacillus albus	1336	1336	100%	0.0	100.00%	1465	ON514242.1
☑	Bacillus albus strain GTM11 16S ribosomal RNA gene, partial sequence	Bacillus albus	1336	1336	100%	0.0	100.00%	1456	ON514236.1
☑	Bacillus albus strain GTM31 16S ribosomal RNA gene, partial sequence	Bacillus albus	1336	1336	100%	0.0	100.00%	1449	ON514235.1
☑	Bacillus albus strain GFM21 16S ribosomal RNA gene, partial sequence	Bacillus albus	1336	1336	100%	0.0	100.00%	1452	ON514231.1
☑	Bacillus cereus strain TY24 16S ribosomal RNA gene, partial sequence	Bacillus cereus	1336	1336	100%	0.0	100.00%	1518	ON506254.1
☑	Bacillus thuringiensis strain BG15 16S ribosomal RNA gene, partial sequence	Bacillus thuringiensis	1336	1336	100%	0.0	100.00%	1326	ON506253.1

FIGURE 20- NCBI BLAST list depicting the sequence similarity of the sample ZMM4

-------Test result for ZMM9-------

	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

FIGURE 21 - NCBI BLAST list depicting the sequence similarity of the sample ZMM9

-------Test result for ZVP1-------

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

FIGURE 22- NCBI BLAST list depicting the sequence similarity of the sample ZVP1

-------Test result for ZVP5-------

	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

FIGURE 23- NCBI BLAST list depicting the sequence similarity of the sample ZCJ3

-------Test result for ZVP10-------

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
Bacillus thuringiensis strain CPO 28.R5 16S ribosomal RNA gene, partial sequence	Bacillus thuringiensis	920	920	100%	0.0	99.80%	1443	ON514334.1

FIGURE 24 - NCBI BLAST list depicting the sequence similarity of the sample ZVP10

DISCUSSION

Cellulose is the most abundant organic compound in the biosphere, comprising almost 50% of the biomass synthesised by photosynthetic fixation of CO_2 (Eriksson *et al.,* 1990). This abundance confers to cellulose an immense potential as a renewable source of energy. Cellulose is commonly degraded by an enzyme called cellulase. This enzyme is produced by several microorganisms, commonly by bacteria and fungi (Immanuel *et al.,* 2006). The latter is more environmentally friendly, and gives a pure product with consumption of less energy (Bakare, 2005; Fennington *et al.,* 1982).

A large number of microorganisms are capable of degrading cellulose, only a few of them produces significant quantities of cell-free bioactive compounds capable of completely hydrolyzing crystalline cellulose in vitro. Numerous investigations have reported the degradation of cellulosic materials, but few studies have examined which microorganisms had met the industrial requirement (Lee and Koo, 2001). For understanding the mechanism of cellulose degradation by cellulase, it is necessary to isolate, purify and characterize this enzyme. Therefore, the present investigation was designed to isolate and screen the Cellulase producing bacteria from soil. Diverse types of waste soil samples such as agricultural, compost, farmyard manure (FYM) were collected from various agro-climatic locations of two distinct districts namely Kottayam and Ernakulam, Kerala, India. Serial dilution agar plating method was used for the isolation of soil bacteria. The seventy-eight isolates were screened for cellulolytic activity using Congo red stain on Carboxymethylcellulose (CMC) agar medium and thirty-five isolates were found to hydrolyze Carboxymethylcellulose. Extracellular cellulase production was quantitatively analysed by well diffusion assay. Out of these 35 isolates, only seven showed potential cellulase production. Biochemical characterization and Kirby Bauer disk diffusion susceptibility test of the most potent seven isolates were conducted and selected for molecular identification. The genomic DNA of the isolates namely ZMA17, ZMM9, ZMM4, ZMC1, ZVP5, ZVP1 & ZVP 10 were identified by 16S amplification method. BLAST analysis of the 16S rDNA of six isolates revealed that the selected isolates showed maximum similarity of 100%, 100%, 99.76%, 100%, 100%, 100% & 100% to *Elizabethkingia sp., Stenotrophomonas*

maltophilia, Bacillus paramycoides, Bacillus subtilis, Bacillus subtilis, Serratia marcescens, Bacillus cereus respectively.

In the current investigation, initially 78 bacterial isolates were primarily isolated from waste soil were preliminary subjected to screen using patch plate technique and well diffusion assay on CMC agar plate by staining the colonies with Congo red and counter stain NaCl. Cellulolytic activity was confirmed by the appearance of clear zone upon the addition of bacterial supernatant to the wells punched on CMC agar medium after sufficient hours of incubation and addition of Congo red and counter stain. The same methodology was approved by the studies conducted by Cintia *et al.,*2013 followed by the isolation of microorganisms with specific capacities. The strains were screened for cellulase activity on MM with 1% CMC (carboxymethyl-cellulose) as substrate. After incubation, the plates were stained with Congo red dye and washed. A positive cellulase activity was detected by the formation of a yellow halo (8 mm or more) against a red background.

Antibiotic susceptibility broad-spectrum analysis aids in determining how efficiently efficient will an antibiotic is towards the testing a species. By the method of extracellular screening of cellulase activity, 35 isolates were screened and the most potent seven strains were then analysed for their sensitivity to ten antibiotics and the results were examined. The antibiotic susceptibility assay result assay revealed that *Bacillus subtilis,* and *Serratia sp.* showed high resistivity to ampicillin, tetracycline, penicillin, cloxacillin etc. The findings of the antibiotic susceptibility test in the present work shows correlation with recent studies conducted by Senthil *et al.,* 2020 which suggests that the bacterial isolates *Bacillus licheniformis, Pseudomonas sp. Bacillus subtilis, Bacillus cohni*i and *Serratia sp.* showed high resistivity to penicillin, ampicillin and tetracycline.

In the current research, the molecular characterization and the BLAST analysis of the six potent cellulase producing bacteria reveals maximum similarity of 100%, 100%, 100%, 100%, 96.76%, 100% & 100% to to *Elizabethkingia sp., Stenotrophomonas maltophilia, Bacillus paramycoides, Bacillus subtilis, Bacillus subtilis, Serratia marcescens, Bacillus cereus* respectively. *Bacillus cereus* produced clear prominent zone with a diameter of 1.9 cm after

extracellular screening of cellulase activity. According to Barbosa *et al.* (2005), *B. cereus* is known as one of the potential bacteria for cellulase production and was found mostly to produce extracellular endoglucanase type cellula*se* by the development of visible clearance zone measuring a diameter of 0.4cm. However, the current findings clearly substantiate the high cellulolytic potential of the bacterial strain *Bacillus cereus.*

Many efforts were taken to generate microorganisms with high ability to produce cellulase that can degrade native cellulose (Aristidou and Penttila, 2000). Among all isolated strains, the three cellulolytic bacterial strains the maximum enzyme activity were showed in *Bacillus cereus*, followed by *Bacillus subtilis*, and *Bacillus thuringiensis*. Mukesh Kumar *et al.,* (2012) reported cellulase activity in *Bacillus cereus* and in both studies the strain was confirmed by 16s rDNA method. Venkata *et al.,* (2013) also concluded the *Bacillus cereus* as a promising bacterium to produce cellulose. Hence, the results from these studies proposed the potential of *B. cereus* strains as cellulase producers.

In the present study *Bacillus subtilis* also has been identified as a cellulase producer. Similarly, Kim, *et al.,* (2012), Ramalingam and Ramasamy in 2013 also reported the cellulase activity of in *Bacillus subtilis* which have high growth rate as compared to fungi, good potential to be used in cellulose production. However, the application of bacteria in producing cellulase is not widely used. (Sonia *et al.,* 2013).

Another species recognized to be cellulase producing bacteria was *Bacillus paramycoides* which is able to produce different enzymes at a time such as cellulases, protease and amylases (Jyoti *et al.,* 2022). *Bacillus* have wide range of applications in various fields of sciences for conserving environment as well as at the point of view with industrial enzymes. Cellulase, proteases and amylase are all important enzymes among industrial enzymes with their numerous applications. Study has proved that microbes especially Genus *Bacillus* are able to produce multiple enzymes at a time.

Cellulolytic bacteria identified as cellulase producer isolated from soil waste in the current investigation *Elizabethkingia sp*. showed comparatively higher clearance zone formation with an overall diameter of 2cm indicating high cellulase production. Soil waste when used as a

substrate results in increased enzyme production. However, yet studies had not been conducted relevant to this, *i.e.* the isolation of cellulase producing *Elizabethkingia sp*. from waste soil.

Further studies and extensive research can bring more insights about the properties of this cellulase producing bacterium and its enzyme characterization.

Current study has explored seven different bacterial strains having the potential to produce cellulase. Different types of cheap and ubiquitous soil wastes were used. All of these seven strains showed maximum enzyme activity in media consisting of CMC medium. Thus, these strains and media could be exploited for the production of cellulase in industrial-scale operations. Further studies were in progress in the purification and application of cellulase in different commercial fields. The purified cellulase can be used for various purposes in detergent industries, food industries, and pharmaceutical industries. The high activity and stability of cellulase enzymes between neutral to alkaline pH and high temperature will be of use in various industrial and biotechnological applications. These outcomes have enlarged the possibility of discovering environmentally significant microbes from agro waste dumping sites and this isolate might be essential basis to explore the industrially valuable bacterial metabolites.

CONCLUSION

Soil microorganisms are vital members of the environment for the maintenance and balance of nutrients. Soil harbours many microorganisms with better advantages over other sources for industrial use. Cellulose is the most abundant biopolymer in terrestrial ecosystems and is degraded by microbial communities in soils. However, relatively little is known about the diversity and function of soil microorganisms that might participate in the overall degradation of this biopolymer. Cellulases are the enzymes responsible for the degradation of cellulosic polymers. It is also noticeable that the final product of cellulose degradation by cellulase enzyme is glucose which is soluble sugar. The present investigation focused on Screening, Biochemical and Molecular characterization of cellulase producing bacteria from waste. A total of seventy-eight isolates were obtained from different types of waste soil samples such as agricultural, compost, farmyard manure (FYM), and waterway collected from various agro-climatic locations in different areas of two distinct districts namely Kottayam and Ernakulam using serial dilution and pour plating method. CMC agar plate assay and Well diffusion assay were employed for the screening of potential cellulase producing bacteria.The bacteria with notable cellulase activity were further proceeded for antibiotic susceptibility test, biochemical and molecular characterization. The molecular characterization of the potent six strains namely ZMC1, ZMA 17, ZMM 4, ZMM 9, ZVP1, ZVP 5 & ZVP 10 were confirmed by BLAST analysis of the 16S rDNA sequence showed maximum similarity of 100%, 100%, 100%, 99.76%, 100%, 100% & 100% to *Elizabethkingia sp. Stenotrophomonas maltophilia, Bacillus subtilis, Bacillus paramycoides, Serratia marcescens, Bacillus cereus & Bacillus subtilis* respectively. Current study is only the beginning to unfold many more studies, however needed before industrial application of these isolates. These include enzyme activity assays of the purified specific cellulases for comparison with the results in this study and with those that have been purified. These studies would shed more light on whether to use the whole organism in the industry or harvest the enzymes and carry out downstream processes or purify the gene to know whether the gene is to be added to the genetic pool for protein engineering and directed

evolutionary studies to come up with super enzymes. So, isolation and characterization of cellulase producing bacteria will remain to be an important aspect of biofuel research, biodegradation and bioremediation.

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APPENDIX

1. 1M Tris-HCL:

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

2. 0.5M EDTA:

Ingredients	Volume
EDTA	18.6g
Deionised water	100mL

3. TE Buffer:

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

4. 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

5. 1X TAE Buffer:

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

6. CMC agar medium

Ingredients	Volume
CMC powder	1g
Agar-Agar	3g
Distilled water	100mL

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°C