

SCREENING OF CELLULASE DEGRADING BACTERIA

Source: Mangrove soil, Marsh land, Rumen of Goat.

Submitted in partial fulfilment of the requirements for the award of Bachelor of Technology
degree in Biotechnology

by

JERFIN JOHN J.A. (3877006), RUJA BISHOYI (38770014)



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

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JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI – 600 119

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Jeppiaar Nagar, Rajiv Gandhi Salai, Chennai – 600 119
www.sathyabama.ac.in



DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Jerfin John J.A.(3877006), Ruja Bishoyi(38770014)** who carried out the project entitled "**Screening of Cellulase Bacteria**" under our supervision from December 2020 to March 2021

Internal Guide

Dr. S USHA NANDHINI

External Guide

Dr. ANEESH NAIR

Head of the Department

Dr. V RAMESH KUMAR

Submitted for Viva voce Examination held on _____

Internal Examiner

External Examiner

DECLARATION

I **Jerfin John J.A.(3877006)** and **Ruja Bishoyi(38770014)** hereby declare that the Project Report entitled “**Screening of Cellulase producing Bacteria**” done by us under the guidance of **Dr. S Usha Nandhini** (Sathyabama institute of science and technology) and **Dr.Aneesh Nair** at Biozone Research and Development Center,. Zameen pallavarm is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

DATE:

PLACE: Chennai

SIGNATURE OF THE CANDIDATE

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ABSTRACT

The aim of current work is to illustrate the screening of cellulase degrading bacteria from soil. Soil from Pulicat marshland, Tamilnadu, India were used for the screening of cellulose degrading bacteria by serial dilution and pour plating method. Bacteria were further characterized by biochemical tests that is congo red assay. Different production variables like pH, temperature, incubation time, autoclaving, sterilization time, substrate concentration were optimized. Serial dilution of saline water was done in 24 test tubes and soil samples were mixed in the mother – test tubes gently. The isolated strains of bacteria were subjected to screening for their cellulolytic activity. To screen for cellulolytic organisms, the isolates were grown in Bushnell haas agar plate consisting of magnesium sulphate (0.02%), Calcium Chloride (0.002%), Monopotassium Phosphate (0.1%), Dipotassium Phosphate (0.1%), Ammonium Nitrate (0.1%), ferric chloride (0.005%), Agar (2%), CMC (%). The spread plates were incubated at 37°C for 2 days. Simultaneously, 2 more BHA plates were prepared for further congo red assay, and wells were made. Then, isolated bacterial colonies were inoculated in the bushnell haas broth for the growth and kept for incubation at 32°C for 24 hour. After the growth the bacteria were filled in the well on the BHA plates and kept for incubation for 48 hours at 30°C. To visualize the hydrolysis zones, the plates were flooded with an aqueous solution of congo-red for 15 minutes and washed with NaCl. Cellulolytic organisms produced a clear zone around the colonies because of the digestion of the CMC. The zone of digestion was measured and documented.

Keywords: Cellulase degrading bacteria, cellulolytic activity, Bushnell has agar, Hydrolysis zones, Bacterial colony.

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LIST OF SYMBOLS AND ABBREVIATIONS

CMC – Carboxymethylcellulose

BHA – Bushnell Haas agar

kDa – Kilodalton

CBH – Cellulobiohydrolases

BG – β - Glucose

EG – Endoglucanases

UDP – Uridine Disphosphate Glucose

GLC – Gas Liquid Chromatography

NWCMP – National Wetland conservation and management programme

HC – Hydrolysis Capacities

DNS – Dinitro salicylic

DEAE – Diethylaminoethyl cellulose

SDS-PAGE – Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis.

BLAST – Basic Local Alignment Search Tool

NCBI – National Center For Biotechnology Information

GPC – Gel Permeation Chromatography

GC-MS – Gas Chromatography – Mass Spectrometry

NB – Nutrient Broth

NA – Nutrient Agar

MA – Minimal Agar

CHAPTER 1

INTRODUCTION

1.1 AN OVERVIEW OF CELLULASE

Cellulases are the enzymes that are produced predominantly by fungi, bacteria, and protozoans that catalyze cellulolysis, the decomposition of cellulose and of some related polysaccharides. These are the enzymes that hydrolyze β -1,4 linkages in cellulose chains. The pure forms of cellulase have a molecular weight of 40 kDa. Cellulolytic enzyme system consists noncatalytic carbohydrate-binding modules (CBM's) and/or other functionally known or unknown modules, which may be located at the N- or C-terminus of a catalytic module. In nature, complete cellulose hydrolysis is mediated by a combination of three main types of cellulases endoglucanases, exoglucanases, including cellobiohydrolases (CBHs), and β -glucosidase (BG). To hydrolyze and metabolize insoluble cellulose, the microorganisms must secrete the cellulases (possibly except BG) that are either free or cell-surface-bound. Cellulases are increasingly being used for a large variety of industrial purposes—in the textile industry, pulp and paper industry, and food industry, as well as an additive in detergents and improving digestibility of animal feeds. Endoglucanases, hydrolyze internal glycosidic bonds in cellulose. EGs generally have cleft-like active sites, and they are described as hydrolyzing and then dissociating, though some bacterial EGs are known to act processively on crystalline cellulose (Li *et al.*, 2007).

Cellulose is commonly degraded by cellulase. The further improvement on cellulase performance needs the better understanding of cellulose hydrolysis mechanisms as well as the relationship of cellulase molecular structure, function, and substrate characteristics. Cellulase enzymes have a significant role in the biomass derived biofuels production process. Enzyme cost is one of the major challenges currently faced by biofuels industries. The biomass to biofuels production process is an environmentally friendly and sustainable approach. For developing economically viable cellulases, various approaches have been tried. Among these, synthetic biology is one of the potential techniques and is a sustainable approach to enhance the production of cellulase enzymes. Using recombinant expression technology, highly active and stable cellulases at low cost can be produced. Serious and in-depth

investigations are under process to develop sustainable recombinant technology and other glycosyl-hydrolase enzymes. Cellulase production from bacterial strains were also studied though less in number than fungal cellulase due to lower penetration ability and unavailability to use a wide range of substrates for cellulase production (Wei *et al.*, 2009).

Among bacterial species, both aerobic and anaerobic organisms are documented for a fairly good amount in all types of cellulase production and the difference lies only in terms of their mode of action to degrade substrate, the types of cellulase systems found in them, cell mass, and product yield. *Bacillus* sp., *Cellulomonas*, and *Clostridium* are the well-known cellulase producing bacterial species (Rajoka and Malik 1997; Thomas *et al.*, 2014).

1.2 SIGNIFICANCE OF CELLULASE

Cellulase is an enzyme that breaks down the cellulose molecule into monosaccharides such as beta-glucose. Cellulose is the most abundant natural organic compound on earth. On average, cellulose accounts as 50% of the dry weight of plant biomass. Cellulose is a molecule, consisting of hundreds – and sometimes even thousands – of carbon, hydrogen and oxygen atoms. Cellulose is the main substance in the walls of plant cells, helping plants to remain stiff and upright.

Humans cannot digest cellulose, but it is important in the diet as fiber. Fiber assists your digestive system – keeping food moving through the gut and pushing waste out of the body. Animals, such as cows, sheep and horses, can digest cellulose, which is why they can get the energy and nutrients they need from grass. Cellulose has many uses. In cotton, it makes clothes like t-shirts and jeans. Paper-making needs huge quantities of cellulose, obtained mainly from wood. Cellulose is an example of a natural polymer. A polymer is a long and repeating chain of the same molecule stuck together. Cellulose is a long-chain polymer of glucose molecules joined together. As the plant adds one glucose molecule to the polymer, one molecule of water is released. You can see this mechanism in the video opposite. Cellulose

polymerisation occurs as a plant grows and creates new cells. Cellulose is a generous biological compound on terrestrial and aquatic biodiversity and is the major element of plant biomass. It is a natural substance that forms the cell walls of all plants and trees and is used in making plastics, paper, etc. It is the paramount waste material from agricultural industry in the form of stalks, stems and husk, there has been great delight in making use of cellulose as an energy resource and feed. The cellulose is composed of D-glucose units linked together to form linear chain via β -1,4-glycosidic linkages (Salmon and Hudson, 1997).

1.3 PRODUCTION OF CELLULASE

As endoglucanases create new ends for CBHs to bind to, it is not surprising that significant synergism is observed between EGs and CBHs. EGs are found among numerous GH families, with fungal examples predominantly in GH5, 7, 12, and 45. Exoglucanases i.e β -1,4-Exoglucanases or cellobiohydrolases (CBHs) are of significant importance to cellulolytic systems, facilitating the production of mostly cellobiose which can readily be converted to glucose by β -glucosidases. These enzymes largely derive from the GH families 6, 7, 9, and 48. CBHs attach to the ends of cellulose microfibrils and then processively slide down the strands cleaving off cellobiose as they progress. The processive nature of CBHs is mediated by tunnel-like active sites, which can only accept a substrate chain via its terminal regions.

These exo-acting CBH enzymes function by threading the cellulose chain through the tunnel, removing cellobiose units in a sequential manner.

Beta-glucosidase is composed of two polypeptide chains. These two chains are chiral in nature meaning that the chains are asymmetric and not superimposable. Each chain is made up of 438 amino acids and constitute a subunit of the enzyme. Each of these subunits contain an active site. The active site serves as the location where an enzyme and substrate bind and an enzymatic reaction can occur. The active site has three potential components: the pocket, the cleft, and the tunnel. The pocket structure is beneficial for recognition of monosaccharide like glucose. The

cleft is allowed for binding of sugars to form polysaccharides. The tunnel allows for the enzyme to attach to polysaccharide and then release product while still attached to the sugar. Engineering cellulolytic enzymes with improved catalytic efficiency and enhanced thermo-stability is important to commercialize lignocelluloses biorefinery. Individual cellulase can be enhanced by using either rational design or directed evolution. Cellulases have a potential use in biotechnology and industry such as, starch processing, alcoholic beverage, malting and brewing, clarify of juice, pulp bleaching, textile industry and animal feed (Sreeja *et al.*, 2013).

When compared to the anaerobic bacteria, aerobic bacteria can perform better cellulase production of around 90%–95% of bacterial cellulose degradation. Bacterial cellulase production have been mostly recorded under submerged fermentation conditions, whereas solid-state fermentation for bacterial cellulases are growing. Moreover, like fungi, bacterial cellulase production is also based on, and recorded, at significant parameters of growth temperature of bacterial microorganisms (Kuhad *et al.*, 2016; Desvaux *et al.*, 2006).

1.4 INDUSTRIAL BENEFITS OF CELLULASE

Cellulases are currently the third largest industrial enzyme class in sales volume and are likely to become the largest when and if the cellulosic ethanol industry matures. Cellulases also are key enzymes in recycling plant cell walls in nature. They are very diverse enzymes, because there are at least 10 different protein folds for cellulases, and in addition there is extensive sequence variation in each of the 12 cellulase families. This diversity probably result from two factors; one is the difficulty in hydrolyzing crystalline cellulose, which is reported to have a half-life greater than 107 years at pH 7, and the other is the extreme heterogeneity of plant cell walls, which are the main substrates of cellulolytic organisms. Thus, cellulolytic organisms may evolve a set of cellulases that are optimal for the types of cell walls that they use. There is evidence for positive selection for some cellulase genes, which is consistent with cellulases being evolved for specific substrates.

Microbial cellulases, a major group of industrial enzymes, have a wide range of

applications as far as biotechnological, environmental issues, industrial products, and processes are concerned. Enzyme hydrolysis of lignocellulose by cellulases owns many advantages compared with chemical treatment, such as low yield of by-products, mild reaction conditions, noncorrosion, and low energy demand. Lignocellulose has a very stable internal structure with strong resistance. Microorganism able to use the carbohydrates in lignocellulosic biomass has to secrete a series of enzymes to degrade the lignocellulose with compact structure into the available monosaccharide.

General cellulase is actually a mixture of several enzymes called cellulase system made up with a variety of protein components. Cellulases hydrolyze cellulose β -1,4-glycosides and break long-chain cellulose into cellobiose and glucose for next process, fermentation by the microorganism.

1.5 AN OVERVIEW OF CELLULOSE

Cellulose is composed of a linear chain of β -1,4 linked d-glucose units with a degree of polymerization ranged from several hundreds to over ten thousand, which is the most abundant organic polymer on the earth. It is used in the diet as a fibre supplement, also to produce paperboard and paper products, in the production of rayon, helps as an additive in various food items and used as a preservative in cheese as it plays the role of an anti-clumping agent. There are four different assortments of cellulose: cellulose I, II, III, and IV. Naturally occurring cellulose is known as cellulose I, which exists in parallel strands without in-heme sheet hydrogen bonding. Cellulose II is thermodynamically more stable and exists in antiparallel strains with intersheet hydrogen bonding. Cellulose is the richest carbohydrate present in nature. It is insoluble in water. It is a crystalline solid having a white powdery appearance. It has high tensile strength due to firm hydrogen bonds between the individual chains in cellulose microfibrils. For industrial use, cellulose is mainly acquired from wood pulp and cotton. Some animals, particularly herbivorous mammals and termites, can digest cellulose with the help of symbiotic micro-organisms that live in their guts, such as *Trichonympha*.

Cellulose is synthesized by a large number of living organisms ranging from the bacterium *Acetobacter xylinum* to forest trees. *A. xylinum* produces abundant amounts of cellulose and this bacterium has been used as a model system for studies on cellulose biosynthesis and structure of the cellulose product. Cellulose is synthesized by the enzyme cellulose synthase, a membrane protein that catalyzes the direct polymerization of glucose from the substrate UDP-glucose into a cellulose product. Genes for cellulose synthases have been identified from many bacteria, *Dictyostelium discoideum*, and higher plants. Analysis of the predicted protein sequences has allowed identification of conserved residues in cellulose synthases from different organisms. The conserved residues are found in the globular region of the cellulose synthases. Using site-directed mutagenesis experiments Michael Smith have shown that the conserved amino acid residues are required for cellulose synthase activity in *A. xylinum*.

1.6 SIGNIFICANCE OF CELLULOSE

Cellulose is one of the most abundant biomaterials on the earth. It is generally synthesized by plants, but it is also produced by some bacteria. Like starch, cellulose is a homopolymer of glucose, and yet unlike starch, glucose monomers are joined by β -1,4 linkages. Cellulose, a tough, fibrous, and water-insoluble polysaccharide, plays an integral role in keeping the structure of plant cell walls stable. Cellulose chains are arranged in microfibrils or bundles of polysaccharide that are arranged in fibrils (bundles of microfibrils), which in turn make up the plant cell wall. This arrangement not only aids in the stability of plant structures but also suggests that cellulose is a biomaterial with high strength and other superior mechanical properties. It is not only plants that synthesize cellulose. Bacteria are capable of producing the polysaccharide as well. Bacterial cellulose synthesis has been most extensively studied in *Acetobacter xylinum*. It is thought that the biological role of cellulose produced by bacteria is to aid in flocculation or to maintain certain environment, such as aerobic conditions or allowing attachment to plants. Bacterial cellulose is arranged similarly to plant cellulose, as polysaccharide chains form microfibrils and bundles of microfibrils form ribbons. Unlike plant-based cellulose, bacterial cellulose is highly pure and does not need to be separated from lignin in processing.

1.6.1SYNTHESIS OF CELLULYTIC BACTERIA

Also, unlike plant cellulose, bacterial cellulose has superior water retention properties; plant cellulose exhibits water retention values of 60%, whereas bacterial cellulose can exhibit retention of water up to 1000% of the cellulose sample weight. Superior water retention of bacterial cellulose allows the polymer to possess high crystallinity, yet it is smooth and moldable, thus adding to its suitability in medical applications, such as a structural component of artificial organs and blood vessels.

Cellulose synthase is a complex of enzymes that spans the cell membrane in plants and, in the case of bacteria, spans the entire cell wall. In both plants and bacteria, UDP-glucose (UDP-GLC) is the key intermediate in cellulose synthesis. The cellulose synthase complex uses the glucose moiety from UDP-GLC, transports the glucose across the cell membrane or cell wall, and adds the monomer to the nascent extracellular cellulose chain. Applications of plant-based and bacterial cellulose are many, and, as can be expected, some applications are more suitable for one type of cellulose over the other. Cellulose is the main constituent of paper and paper products as well as textiles made from cotton, linen, and other plant fibers. Microcrystalline cellulose is used as a filler, both in the pharmaceutical and food industries. Cellulose can be converted into cellophane or nitrocellulose for use in photographic and household applications, respectively, as well as gunpowder.

1.7 ECONOMICAL BENEFITS OF CELLULOSE

Nanocellulose, or nanocrystalline cellulose, is a material that is becoming increasingly more valued for several applications. Nanocellulose is produced by acid treatment of plant or bacterial cellulose. As the name implies, nanocellulose exhibits nanoscale structures, such as fibers or crystals. The properties of nanocellulose make it suited for applications such as photonics or optoelectronics. Crystallized nanocellulose has been proposed as a raw material for food packaging and electronics. In addition, nanocellulose can be derivatized with different chemical groups, which confers different properties to the polymer. Esterification of nanocellulose increases the hydrophobicity and strength of the material, while

cationization confers pH sensitivity to nanocellulose, as well as allows for CO₂-controlled flocculation. Currently, like with many biopolymers, nanocellulose applications are limited because of availability and cost. With such promising applications, it seems as though nanocellulose is a potential value target for metabolic engineering of microorganisms. There are several types of natural wetlands such as swamps, fens, bogs, marshland, and tidal freshwater areas. Land that is used in this project for soil sample collection is marshland. Swamps and marshes have open flowing water and are distinguished in terms of vegetation, soil type, and wild life. A marshland is a type of wetland where water covers ground for long periods of time. Unlike swamps, which are dominated by trees, marshes are usually treeless and dominated by grasses and other herbaceous plants. For example, The Okavango Marshes east of the Kalahari Desert in Botswana are perhaps the best example of marshes formed in an interior, closed basin that has no drainage. Other basins without outlets like that of the Great Salt Lake in Utah have accumulated too much salt or marsh growth. A marsh is a wetland that is dominated by herbaceous rather than woody plant species. Marshes can often be found at the edges of lakes and streams, where they form a transition between the aquatic and terrestrial ecosystems. They are often dominated by grasses, rushes or reeds. If woody plants are present, they tend to be low growing shrubs, and then sometimes called carrs. This form of vegetation is what differentiates marshes from other types of wetland such as swamps, which are dominated by trees, and mires, which are wetlands that have accumulated deposits of acidic peat.

1.8 PRODUCTION OF HALOPHILIC BACTERIA

Marshes provide habitats for many kinds of invertebrates, fish, amphibians, waterfowl and aquatic mammals. This biological productivity means that marshes contain 0.1% of global sequestered terrestrial carbon. Moreover, they have an outsized influence on climate resilience of coastal areas and waterways absorbing high tides and other water changes due to extreme weather. Though some marshes are expected to migrate upland, the majority of natural marshlands will be threatened by sea. sea level rise and associated erosion. Marshes differ depending mainly on their location and salinity. Both of these factors greatly influence the range and

scope of animal and plant life that can survive and reproduce in these environments. The three main types of marsh are salt marshes, freshwater tidal marshes, and freshwater marshes. These three can be found worldwide and each contains a different set of organisms. Salt marshes, Saltwater marshes are found around the world in mid to high latitudes, wherever there are sections of protected coastline. They are located close enough to the shoreline that the motion of the tides affects them and, sporadically, they are covered with water. Salt marshes are dominated by specially adapted rooted vegetation, primarily salt-tolerant grasses. Salt marshes are most commonly found in lagoons, estuaries, and on the sheltered side of shingle or sandspit. The currents there carry the fine particles around to the quiet side of the spit and sediment begins to build up. These locations allow the marshes to absorb the excess nutrients from the water running through them before they reach the oceans and estuaries. These marshes are slowly declining. Coastal development and urban sprawl has caused significant loss of these essential habitats.

However, without the stresses of salinity at work in its saltwater counterpart, the diversity of the plants and animals that live in and use freshwater tidal marshes is much higher than in salt marshes.

Pulicat land is the Asia's second largest marsh land, where the soil sample collected. Reason behind or target behind using this Pulicat marsh land is its halophilic stress. Pulicat wetland is a freshwater marsh in the city of Chennai, India. It is situated adjacent to the Bay of Bengal, about 20 kilometres (12 mi) south of the city centre, and has a geographical area of 80 square kilometres (31 sq mi). Pulicat marshland is the only surviving wetland ecosystem of the city and is among the few and last remaining natural wetlands of South India. It is one of the 94 identified wetlands under National Wetland Conservation and Management Programme (NWCMP) operationalised by the Government of India in 1985–86 and one of the three in the state of Tamil Nadu, the other two being Point Calimere and Kazhuveli. It is also one of the prioritised wetlands of Tamil Nadu. The topography of the swamp is such that it always retains some storage, thus forming an aquatic ecosystem. A project on 'Inland Wetlands of India' commissioned by the Ministry of Environment and Forests, Government of India had prioritised Pulicat marsh as one of the most significant

wetlands of the country. The marsh contains several rare or endangered and threatened species and acts as a forage and breeding ground for thousands of migratory birds from various places within and outside the country. The number of bird species sighted in the wetland is significantly higher than the number at Vedanthangal Bird Sanctuary.

CHAPTER 2

LITERATURE SURVEY

Kledson *et al.*, studied on the isolation, selection, and partial characterization of bacterial cellulases present in the intestinal tract of the sugarcane borer and (b) to identify cellulase-producing bacteria by analyzing the 16S *rDNA* gene. Cellulase production and purification assays was experimented and resulted in similar electrophoretic profiles between four bacterial strains. Those strains were identified as *Klebsiella pneumoniae*, *Klebsiella* sp. and *Bacillus* sp. *K. pneumoniae* was the main cellulase-producing microorganism. The results which they got shown the possibility of finding cellulolytic microorganisms that inhabit in the gut of herbivorous animals, especially those that are predators of important crops of economic value (Kledson Lopes Barbosa *et al.*, 2020)

Behera *et al.*, conducted a study to isolate cellulose degrading bacteria from mangrove soil of Mahanadi river delta, Odisha, India and also to evaluate their cellulase production ability. Their results showed that in total fifteen cellulose degrading bacteria were isolated based on their halo zone formation on congored agar medium. Their maximum carboxymethylcellulose hydrolysis capacities (HC value) were ranged from 1.18 to 2.5 cm. A CMCase test of these fifteen isolates showed that their enzyme activity ranged from 2.471 to 98.253 U/ml/min. From the morphological, and biochemical characterisation, the isolates were identified as *Micrococcus* spp., *Bacillus* spp., *Pseudomonas* spp., *Xanthomonas* spp. and *Brucella* spp. (Behera *et al.*, 2014)

Azhar A. Hussain *et al* proofs that the cellulase activity of the isolated bacteria was determined by DNS (dinitrosalicylic) acid method. The highly cellulolytic isolates were identified on the basis of Gram staining, morphological cultural characteristics, and biochemical tests. The isolates were also identified with 16S *rDNA* analysis. The phylogenetic analysis of their 16S *rDNA* sequence data showed that isolates 99% similarity with *Anoxybacillus flavithermus*, *Bacillus megaterium*, *Bacillus amyloliquefaciens* and *Bacillus subtilis*. Cellulase production by these strains was optimized by controlling different environmental and nutritional factors such as pH, temperature, incubation period, different volumes of media, aeration rate and carbon

source. In the study they have done results in, fifty four bacterial cultures were isolated from soil according to their cellulase productivity. All strains were Gram positive, spore formers and showed different reactions for biochemical tests. (Azhar A. Hussain *et al*, 2016)

LI-JUNG YIN *et al* conducted a study which shows that the cellulase-producing bacterium was isolated from soil and identified as *Cellulomonas* sp. YJ5. Maximal cellulase activity was obtained after 48 h of incubation at 30 °C in a medium containing 1.0% carboxymethyl cellulose (CMC), 1.0% algae powder, 1.0% peptone, 0.24% (NH₄)₂SO₄, 0.20% K₂HPO₄, and 0.03% MgSO₄ · 3 H₂O. After the preparation of the media cellulase was purified after Sephacryl S-100 chromatography twice with a recovery of 27.9% and purification fold of 17.5. It was, with N-terminal amino acids of AGTKTPVAK, stable at pH 7.5-10.5 and 20-50 °C with optimal pH and temperature of 7.0 and 60 °C, respectively. Cu²⁺, Fe²⁺, Hg²⁺, Cr³⁺, and SDS highly inhibited, but cysteine and β-mercaptoethanol activated, its activity. The Substrate specificity indicated it to be an endo-β-1,4-glucanase. The cellulase-producing bacteria surviving after 7 days of incubation in a selective medium containing 1.0% of CMC at 37 °C were isolated from soil. After being transferred to CMCB, only 18 colonies had CMC-degrading abilities among the total 50 colonies of these strains (data not shown). The strain with the highest hydrolytic activity was subjected to identification (LI-JUNG YIN *et al* ,2010)

Miranda L Mark *et al* made a study on the isolation of efficient cellulase producing bacteria found in organic fertilizers and paper mill sludges which can be considered for use in large scale biorefining. Pure isolate cultures were screened for cellulase activity. Isolates produced halos greater in diameter than the positive control (*Cellulomonas xylanilytica*), suggesting high cellulase activities. A portion of the 16S rDNA genes of cellulase positive isolates were amplified and sequenced, then BLASTed to determine likely genera. Phylogenetic analysis revealed genera belonging to two major Phyla of Gram positive bacteria: Firmicutes and Actinobacteria. All isolates were tested for the visible degradation of filter paper; Isolates with (*Paenibacillus* species) were observed to completely break down filter

paper within 72 and 96 h incubation, respectively, under limited oxygen condition. Isolates with cellulolytic growth were selected for the FP assay for quantification of total cellulase activities. It was shown that 1% (w/v) CMC could induce total cellulase activities of 1652.2 ± 61.5 and 1456.5 ± 30.7 μM of glucose equivalents for the samples, respectively. CMC could induce cellulase activities 8 and 5.6X greater than FP, therefore CMC represented a good inducing substrate for cellulase production. The genus *Paenibacillus* are known to contain some excellent cellulase producing strains, Isolates with superior cellulase activities and represent excellent candidates for further cellulase analysis and characterization. Carboxymethyl cellulase activity A total of 53 isolates were described based on size, colour, and morphology, labelled for database. From the database 30 of 53 isolates were removed due to similar size, colour and morphological characteristics (Miranda L Mark *et al*, 2011)

Farjana Islam *et al* performed isolation, screening and purification of cellulase from bacteria present in sugar industry waste (molasses)., three bacterial strains produced clear transparent zone into carboxymethyl cellulose (CMC) agar plate were identified as cellulase producing bacteria. Different culture parameters such as pH, temperature, incubation period, substrate concentration and carbon sources were optimized for enzyme production. According to the morphological and biochemical tests, the isolated strains were identified as *Paenibacillus* sp., *Bacillus* sp. and *Aeromonas* sp. The first strain *Paenibacillus* sp. showed high potentiality for maximum cellulase production ($0.9 \mu\text{mol ml}^{-1} \text{ min}^{-1}$) at pH 7.0 after 24 h of incubation at 40 °C in a medium containing 1.0% CMC. Then *Paenibacillus* sp. was selected for enzyme purification by ammonium sulphate precipitation, DEAE-cellulose and CM-cellulose column chromatography, respectively. In last step of purification, specific activity, recovery and purification fold were 2655 U/mg, 35.7% and 9.7, respectively. The molecular weight of the purified cellulase was found to be 67 kDa by SDS-PAGE, had an optimal pH and temperature at 7.0 and 40 °C. According to substrate specificity, the purified cellulase had high specificity on CMC substrate which indicated it to be an endo- β -1,4-glucanase. To characterize the purified enzyme specificity, they have used qualitative tests like CMC test and filter paper degradation test. CMC agar is used to identify isolates with cellulase activity on soluble cellulose such as CMC thus representing mainly endoglucanase and

beta- glucosidase activities. Secondly, they have screened isolates displaying cellulase activity on CMC for activity on crystalline insoluble cellulose such as filter paper (Farjana Islam *et al*,2018)

Joseph Vimal *et al* proofs that the isolate was tentatively identified to be *Bacillus* species based on cultural, morphological, biochemical. Further, the genomic DNA was isolated and amplified with universal primers 27F and 1492s specific for 16S rRNA. The amplified 16S rRNA PCR product of 1500bp was sequenced and the unknown organism was identified using the maximum aligned 16SrRNA sequences available in the GenBank of NCBI through BLAST search. The samples showed (100% and 99% respectively) homology to *Bacillus subtilis* strain. To analysis the evolutionary relationships, phylogenetic analysis was performed with the program MEGA 6.0 using the 16SrRNAsequence. The isolates were then evaluated by submerged fermentation process for maximum cellulase production. The various process parameters like pH, temperature, incubation period, substrate concentration and inoculum volume were then optimized for the maximum production of cellulase by the isolates. The cellulase production started at the optimum temperature (30°C – 40 °c). The period of incubation was 96 hours. The CMCase activity was assessed based on the formation of zone of hydrolysis or the halo zone formed due to the secretion of cellulase and subsequent hydrolysis of cellulose incorporated in the medium by the bacterial isolates. The ratio obtained by dividing zone diameter by colony diameter gives a measurement of cellulase reactivity. Among the stains they have used the *Bacillus cereus* strain was found to be the most active cellulase producer (Joseph Vimal *et al*, 2016)

Lekh Ram *et al* has taken a total of 21 fungal isolates were isolated from these soil samples, out of which four isolates were showing the cellulase activity. The fungal isolate isolated from Paper Industry soil sample was noticed to show maximum zone of hydrolysis of carboxymethyl cellulose. From their particular sample soils Fungi grown on the selective media supported the growth of the fungi by using cellulose as the carbon source. Then the isolated strains were carefully identified by morphological Characteristics include colour of the colony and growth pattern studies. Some of them were observed under microscope to examine the colour and

spores. After the observation under microscope efficient cellulase producing fungi isolates were finally selected based on the zone of the clearing around the fungi on carboxyl methyl cellulase agar (CMC agar) plates. The appearance of the clear zone around the colony when the Congo red solution was added. The formation of zones were the strong evidence that the fungi produced cellulase in order to degrade cellulose. The plates which produced zones of hydrolysis in the CMC agar was taken for the final result (Lekh Ram *et al*, 2018)

Puspita Lisdiyanti *et al* conducted a study that the cellulolytic bacteria were isolated from peat soils . The soils were tested for their cellulase activities, and the potential isolates were identified into genus or species level. The ability of degradation of cellulose was screened using 1% CMC (carboxy methyl cellulose) as a substrate and the cellulase activities of crude enzyme were determined using DNS (3,5-dinitro salicylic acid) method. Twenty eight bacterial isolates were isolated from peats Congo red (sodium diphenyl-bis-alpha-naftilamin sulfonate) solution. All of cellulase-producing bacteria were inoculated to the CMC broth medium containing 1% (b/v) CMC as inducer component and 0.2% (b/v) yeast extract and 0.1% (b/v) D-glucose as initiation of growth phase. After the concentration of glucose is low, the bacteria will use cellulose as carbon source to produce cellulase. Apparently, IS value is not show correlation with cellulase activity. Production of extracellular depends greatly on the composition of medium and substrate concentration of oil by using CMC solid medium. All the isolates which they had capability to degrade CMC and showed the cellulase activity when screened by Congo red. They had a clear zone formation differed for each bacteria, which indicates that each bacteria is able to produce in different cellulase complex (Puspita Lisdiyanti *et al.*, 2012)

Nitin Trivedi *et al* has preferred to use an extracellular alkali-halotolerant cellulase from the strain *Bacillus flexus* NT isolated from *Ulva lactuca*. Then it was purified to homogeneity with a recovery of 25.03% and purity fold of 22.31. The molecular weight of the enzyme was about 97 kDa and the Vmax and Km was 370.17 U/ml/min and 6.18 mg/ml respectively. The optimal temperature and PH for the enzyme activity was 10 and 45 °C. The sample plates which kept for incubation showed maximum zone of clearance on CMC agar plates after flooding with Lugol's iodine

and selected for next process. The strain was found to be gram positive, short rods, aerobic and motile in nature. The phylogenetic analysis of the strain with 16S rDNA gene sequence exhibited its maximum homology (99%) with *Bacillus flexus* strain. The stabilized activity of the enzyme even at high pH of 9.0–12.0 and residual activity of about 70% at salt concentration (NaCl 15%) revealed for its alkali-halotolerance nature. The enzymatic hydrolysis of the CMC was confirmed with GPC and GC-MS analysis (Nitin Trivedi *et al.*, 2010)

C.-J. Duan *et al* preferred Rumen contents as sampled from four slaughtered buffalos that were fed with hay and leaves of sugarcane. The samples were collected and stored at 80°C until the DNA extraction was performed. To obtain the bacterial fraction attached to cellulose particles, the rumen samples were diluted in potassium-phosphate buffer (PPB; 0.18 mol/l), pH 6.5, then they have mixed them gently to allow cellulose particles to settle. To determine the cellulase activities, a standard reaction containing 0.6 lg of recombinant C67-1 and 1% CMC in 0.5 ml. citrate / phosphate buffer, pH 4.5, was incubated at 45°C for 10 min. Activities of the sample were determined under a range of pH values between pH 3 and 8 in the same buffer, and a range of temperatures between 15 and 70°C. pH and thermal stabilities were assayed. Substrate specificity of the samples were tested against a panel of different substrates in standard reactions with CMC replaced by lichenan, barley-glucan, laminarin, oat spelt xylan, avicel or acid-swollen cellulose that was prepared by the method of Wood (1971) or using filter paper (Whatman). Km and Vmax values for C67-1 were determined under optimal condition at CMC concentrations ranging from 8 to 50 mg/ml. Cellulase activities, including activities of endoglucanase, exoglucanase and b-glucosidase, were present. Then the sample were taken for enzyme quantification assay (C.-J. Duan *et al.*, 2008)

Prabesh Khatiwada *et al* proofed that cellulolytic bacteria were isolated and screened from municipal solid wastes and rice straw wastes using Carboxy Methyl Cellulose (CMC) agar medium as a selected medium. The clear zones were produced by the bacterial isolates on CMC agar medium supplemented with 1% CMC was considered as indicative of extracellular cellulase activity. The size of transparent zone diameter was considered as proportional to the level of cellulase

production. The samples of bacterial isolates were identified as *Bacillus* sp., *Pseudomonas* sp. and *Serratia* sp. based on morphological, cultural and biochemical characteristics. A basal medium was prepared containing CMC, KH_2PO_4 , K_2HPO_4 , MgSO_4 , $(\text{NH}_4)_2\text{SO}_4$, CaCl_2 and FeSO_4 at pH 7.0 was used for cellulase production. Appropriate dilutions of each sample were inoculated on CMC agar plates. Using of CMC as the sole carbon source plays a pivotal role for achieving the highest level of cellulase production because CMC induces cellulase gene expression. Isolated bacterial colonies with higher cellulase activity were further screened to obtain the pure culture. The pure culture produced clear zone when it was flooded with Gram's iodine solution due to hydrolysis of CMC. After the Gram's iodine procedure performed a bluish-black complex formed with cellulose but not with hydrolyzed cellulose, clear zone around the cellulase-producing bacterial colonies were formed within 3 to 5 minutes. Clear zone producing bacterial isolates were then subjected to various morphological, cultural and biochemical tests. Based on the biochemical tests, morphological and cultural characteristics, the sample results were identified as *Bacillus* sp., *Serratia* sp. and *Pseudomonas* spp. optimal cellulase production at 42 h of cultivation. Along with cellulase production, bacterial cell growth was also observed by counting the colony forming units. Then the zoned pure colonies were induced for Enzyme quantification assay (Prabesh Khatiwada *et al.*, 2006)

Yagya Prasad Paudel & Wensheng Qin conducted a study on the isolation of cellulase bacteria from Rotting wood sample . Sixty bacterial samples were collected from different locations . The CMCase activity of strain sample was found maximum at pH 6 which was significantly different to other pH tested during the experiment The enzyme showed significant decrease after this pH retaining 38 % of its activity at pH 8. A similar result was also reported in *Bacillus* sp. Microbial cellulase activity has been maintained by temperature. The optimal temperatures are different in different bacteria. The bacterial strain sample showed cellulase activity from 30 to 70 °C. The maximum enzyme activity was found at 50 °C, and this activity was significantly different to the CMCase activity at 30, 60 and 70 °C . A time course of the bacterial strain and enzyme production was performed over a period of 120 h. The strain samples showed growth after 3 days of incubation. Also, the cellulase yield reached

a maximum at 72 h of incubation which was significantly different to the cellulase production at 24, 96 and 120 h. Seventeen strains showed cellulase activity in CMC agar plate assay. This method of isolation was found easy for preliminary screening of cellulolytic bacteria. The strains were compared with a cellulase-producing positive control (*C. xylanilytica*) and negative control (*E. coli*) with no cellulase activity. After the congo red assay the zoned colonies were identified. The *Bacillus* strains are related to Gram-positive Firmicutes and the strains *Pseudomonas*, *Rahnella* and *Buttiauxella* are related to Gram-negative Proteobacteria. The bacterial strain sample showed the largest diameter of halo region and was selected for further enzyme assay. The morphological examination showed the colonies of the strain sample as a rough opaque and grey. The other bacterial colonies also exhibited similar morphological features (Yagya Prasad Paudel & Wensheng Qin.,2015)

B.C.Behera *et al* studied on the Cellulose degrading from soil using the CMC agar medium 15 morphologically distinct bacterial isolates which were giving halo zones in CMC agar medium were selected and named as Cellulose degrading bacteria, The clearing zone size and colony diameter of these isolates were measured when incubated aerobically at 37°C; the result showed that maximum clearing zone ranged from 0.9 and 2.1 cm and the average HC value, i.e. ratio of zone size to colony diameter ranged from 1.18 to 2.5 cm, demonstrating that all the isolates have the ability to degrade the carboxymethyl cellulose and indicating high ability of Cx cellulase production. The colony characteristics of the isolates were found variable. The size of the vegetative cell of the isolates was 0.8 to 2.1 µm. Microscopic observation was required to reveal the size of the isolates that most of them are rod shaped and motile. The isolates were found variable towards Gram's stain. All the isolates were sensitive to penicillin G, polymyxin B, Erythromycin, Gentamycin and Ciprofloxacin and resistant to Nystatin. Except one sample all the isolates were sensitive to norfloxacin and bacitracin. And some isolates are sensitive to chloramphenicol. But only two of the isolates were only resistant to Tetracycline. On the basis of their morphological characterisation, antibiotic sensitivity test and biochemical characterisation these isolates were identified as *Bacillus brevis*, *Bacillus alcalophilus*, *Xanthomonas* spp. , *Micrococcus* spp , *Brucella* spp ,

Micrococcus spp , Xanthomonas spp , Brucella spp , Bacillus subtilis , Pseudomonas stutzeri , Pseudomonas spp, Bacillus spp , Pseudomonas spp , Bacillus spp and Micrococcus spp .also reported the cellulytic activity of Pseudomonas spp, Bacillus spp, Bacillus polymyxa, and Bacillus brevis (B.C.Behera *et al.*, 2013)

Rajib Kumar Shil *et al* conducted research for cellulolytic bacteria from the gut of three different phytophagous insects were studied to isolate novel cellulolytic organism for biofuel industry. Fifteen different isolates were obtained from the gut of velox, miliaris and P.quatuordecimpunctata. The phytophagous guts of each insect were inoculated in 5 mL of basal salt media (2.5 g NaNO₃, 0.2g MgSO₄, 0.2 g NaCl, 0.1 g CaCl₂.6H₂O in a liter water) containing filter paper (5 pieces of Whatman filter paper no.1, each of 5 mg and area 0.49 cm²) for isolating the cellulolytic microorganisms. Plates were prepared with basal salt media and 5 pieces of Whatman filter paper but no gut extract for checking the degradation/disappearance of filter paper. The tubes were incubated aerobically and shaken at 180 rpm at 37oC in a shaker incubator. After the filter paper was visibly degraded, indicating the presence of cellulases, or after 4-6 weeks , serial dilutions of the cultures (10 µL of each) were transferred to solid medium (CMC agar plate). As a control of microbial contamination plates were opened and kept inside the UV chamber for 15 mins. This was done to check the contamination from within the hood. All the plates were incubated at 37oC until colonies were visible. Then the Bacterial colonies seemed to be different based on the colony size, shape and colour found in the CMC plate for total count of cellulolytic bacteria or for the isolation of bacteria capable of filter paper digestion were transferred to two sets of CMC plates by tooth pick -one for identification of isolates and other for examining their cellulolytic potential using the Congo-Red overlay method (Teather and Wood 1982). For the Congo-Red method, plates were flooded with 0.1% Congo red (Sigma-Aldrich) for 10-15 min before de-staining with 1M NaCl solution for 15-20 min for several times or until the clear zones around the colonies were visualized. Colonies showing discoloration of Congo red were taken as positive cellulose degrading microbial colonies. Identification of Potential Cellulolytic Microbes , which produced clear zones, were identified based on the cultural, morphological and biochemical characteristics. Then the formed zones were taken for Quantitative assay (Rajib Kumar Shil *et al.*,2014)

Vinod VISWANATH *et al* conducted a study on bacterial isolate from sugarcane bagasse, was found to be the best cellulase producer. This isolate was identified as *Pseudomonas fluorescens* by 16S rRNA. The plant which they chose is Cellulose rich plant waste like sugar cane bagasse, banana peel and vegetable waste were screened for isolation of cellulolytic organism. Colonies with hydrolytic zone on CMC agar was taken as cellulase positive. The size of transparent zone (mm) was considered as proportionate to the level of cellulase activity. Accordingly, maximum cellulase activity was observed for the isolate from sugarcane bagasse. Quantitative estimation also exhibited the same trend. Cellulase production was maximum by the isolate obtained from sugar cane bagasse which contained high cellulose (59%) ; when compared to banana peel and cucumber (13%) . The higher cellulose content in sugar cane would have triggered the cellulase production of the isolate in a better way. Circular moist translucent surface colonies in nutrient agar on microscopic examination revealed the presence of short gram negative rods .The isolate was which they found had to be catalase , oxidase and citrate positive. The isolate was negative for Indole, Methyl red and Voges proskauer. The results were suggestive of *Pseudomonas* sp. Isolation of *Pseudomonas* sp. from agri-waste . The sequence of the partial nucleotide sequence (1.5 Kbp) of 16S rRNA gene amplified from the genomic DNA conformed to *Pseudomonas fluorescens* (99%) (Vinod VISWANATH, A. K BEENA, Jeffy D SILVA .,2018)

CHAPTER 3

AIM & OBJECTIVE

3.1 AIM

Keeping the introduction in vision, the present study aimed to isolate and characterize bacterial isolates with high cellulase-producing ability from various sources.

- I. Pulicat lake.
- II. Pallavaram marsh lands.
- III. Rumen of Goat.

3.2 OBJECTIVE

- To collect samples from different locations.
- To Isolate pure colonies.
- To screen cellulolytic bacteria
- Enzyme quantitative assay

CHAPTER 4

MATERIALS & METHODS

4.1 MATERIALS

4.1.1 EQUIPMENTS REQUIRED

- Soil sample
- Lumen of Goat
- Distilled water
- Saline water
- Nutrient and minimal agar
- Nutrient and minimal broth
- Congo red solution
- Test tubes
- Test tubes stand
- Conical flask
- Petri dish
- Tissue paper
- Centrifuge tube
- Micro pipette
- Inoculation loop
- L- rod
- Laminar air flow
- Burner
- Shaker incubator

4.2 METHODOLOGY

4.2.1 Collection of samples.

Samples were collected from different locations .

- Puliact lake
- Pallavarm marsh land
- Rumen of Goat

Sample 1(Pulicat lake)

Sample soils were collected from the Mangrove soils of Pulicate lake . The samples were collected in Eight 15ml centrifuge tubes .Each tubes contained 14gms of soil sample in it. Samples were collected in two aspect ratios . each samples were collected from different location in pulicat lake.,So totally 4 locations in pullicat lake. From each location two samples were collected one will be from the surface of the soil and the next one will be 15cms deep the soil .In total eights samples were collected and sealed it in a air tight bag.



fig 4.1



fig 4.2

Sample 2 (Pallavaram marsh land)

Sample soils were collected from the Marsh lands of Pallavaram.The samples were collected in Four 15ml centrifuge tubes. Each tubes contained 14gms of soil samples. Each samples were collected from different locations in pallavaram marsh lands . From each locations one sample were collected from the surface of the soil. Totally four samples were collected in tubes from different locations and sealed it in a air tight bag.



Fig 4.3

Sample 3 (Rumen of Goat)

This sample is collected from the Rumen of Goat. 10gms of two samples were collected from the rumen of Goat. Two samples were collected in a air tight bags.



fig 4.4

4.2.2 Preparation of Media and plating.

Sample 1

Nutrient agar was used as media for the pullicat lake soil sample. 200ml of Nutrient was prepared. Nutrient agar (2.8g) Distilled water(200ml) pH of 7.3 was maintained. Sterilization of Nutrient Agar media was done with Autoclave at 121°c 15 psi for 30 mis and 15 mins for the pressure to be released. The media was kept in Laminar chamber for 10 mins for an optimal temperature. The Nutrient agar was plated in four petri plates. Each plate containing 50ml of Nutrient agar.

Sample 2

Minimal agar + CMC was used as media for Pallavaram marsh land soil sample. Yeast extract (0.2%)0.3mg, KH_2PO_4 (0.1%) 0.15mg, MgSO_4 (0.5%) 0.75mg, CMC (0.5%) 0.75mg, Agar agar 4g in 80 ml of distilled water. Sterilization of Minimal agar+ CMC was done with Autoclave at 121°c 15 psi for 30 mis and 15 mins for the pressure to be released. The media was kept in Laminar chamber for 10 mins for an optimal temperature. The Minimal agar+CMC was plated in two petri plates each containing 40ml of Minimal agar+ CMC. Two wells were prepared in each minimal agar plates. Totally 4 wells prepared.

Sample 3

Bushnell haas agar+ CMC were used as media for Rumen of Goat. Magnesium sulphate 0.02mg, calcium chloride 0.002mg, monopotassium phosphate 0.1mg, Dipotassium phosphate 0.1mg, Ammonium nitrate 0.1g, Ferric chloride 0.005mg, Agar 2g and CMC 1g with 100ml of distilled water. Sterilization of Bushnell haas

agar+ CMC was done with Autoclave at 121°C 15 psi for 30 min and 15 mins for the pressure to be released. The media was kept in Laminar chamber for 10 mins for an optimal temperature. The Bushnell haas agar+CMC was plated in two petri plates each containing 50ml of Minimal agar+ CMC. Two wells were prepared in each minimal agar plates. Totally 4 wells prepared.

4.2.3 Inoculation of samples in media

Sample 1

After the preparation of media Saline was prepared for serial dilution NaCl 0.95g in 300ml of distilled water. Serial dilution was done with 24 test tubes each test tubes were containing 9µl of saline for 4 mother samples. Each mother sample were diluted in 6 test tubes of saline. Each test tubes was marked as 10^{-1} to 10^{-6} . From each mother sample 10^{-3} and 10^{-6} were taken and spread plated in the Nutrient agar media. And the incubation was done for 24hrs in +/- 30°C. After the 24 the bacterial growth was seen. Then pure colonies were isolated. Another two Nutrient agar plates were prepared. Nutrient agar (2.8g) Distilled water(100ml) pH of 7.3 was maintained. Sterilization of Nutrient Agar media was done with Autoclave at 121°C 15 psi for 30 min and 15 mins for the pressure to be released. The media was kept in Laminar chamber for 10 mins for an optimal temperature. The Nutrient agar was plated in two petri plates. Each plate containing 50ml of Nutrient agar after that pure colonies were taken in inoculation loop and streaked in the new Nutrient agar plate.



fig 4.5

Sample 2

After the preparation of Minimal agar plates. The four samples collected from Pallavaram marsh land was inoculated in four test tubes containing Nutrient broth (0.39g), distilled water (30ml). Nutrient broth(7.5ml) was present in each test tubes. 2.5g of sample were added in each Nutrient broth. After 48hrs of incubation at 30°C the bacterial growth was seen in the nutrient agar. 10µl of each Nutrient broth was

added to each wells of the Minimal agar plates and those plates were incubated for 72hrs in 42°c.

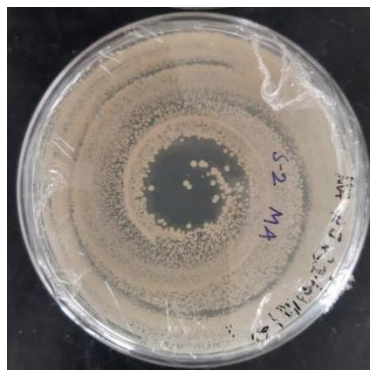


fig 4.6

Sample 3

After the preparation of Bushnell haas agar. The samples was washed with distilled for several times. Then the samples were crushed in the presence of phosphate-Buffered saline Distilled H₂O (8ml), Nacl (0.08mg), Kcl (0.002mg), Na₂HPO₄(0.014mg), kH₂PO₄ (0.002mg).Then the PBS were collected in two 5ml eppendorf tubes. The PBS were loaded in the each wells of Bushnells haas agar and incubates for 92hrs at 42°c.



fig 4.7



fig 4.8

4.2.4 Congo red assay and Quantitative assay

Sample 1

After 24hrs of incubation the growth of the bacterias have been seen. The grown plates are flooded with congo red stain. Congo red (0.5g), distilled water (50ml).The stain is flooded above the surface of the media for 15mins.Then the flooded stain is washed with Nacl (5.84g), distilled water (100ml) for 2 times .

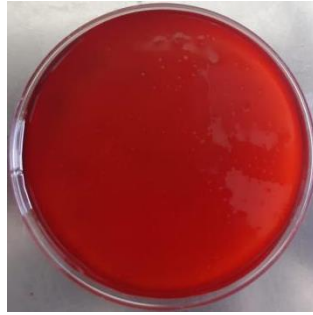


Fig 4.9

Sample 2

After 72hrs of incubation the growth of the bacteria have been observed. The grown plates are flooded with congo red stain. Congo red (0.1g), distilled water (50ml).The stain is flooded above the surface of the media for 15mins.Then the flooded stain is washed with Nacl (2.92g), distilled water (100ml) for 2 times .

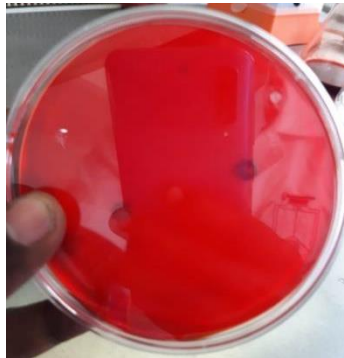


fig 4.10

Sample 3

After 92hrs of incubation the growth of the bacteria have been observed. The grown plates are flooded with congo red stain. Congo red (0.1g), distilled water (50ml).The stain is flooded above the surface of the media for 15mins.Then the flooded stain is washed with Nacl (2.92g), distilled water (100ml) for 2 times.

5.0 RESULT AND DISCUSSION

Serial Dilution of samples and preparation of saline was done. Then Nutrient Agar media was prepared for pour plating. Spread plating was done on the plates for the uniform microbial growth in the plates and to isolate pure colonies after the growth. After the incubation, isolated colonies were resuspended and quadrant streaking was done over the nutrient agar plates and incubated for further growth. Then Minimal Agar Media was prepared. The samples were spread over the minimal agar using sterilized L-rod and incubated for 48 hrs at 37 °C. Simultaneously, preparation of Bushnell-Haas Agar was done followed by spread plating and microbial growth. At last, Congo red solution was prepared using 0.1 g of Congo red powder and 10 ml of distilled water and plates were flooded. After 15 minutes of flooding, plates were de-stained using NaCl solution. As a result the isolated organisms from the sample shown positive results as zone formation on the plates were observed and the organism isolated were cellulolytic.



fig 5.1

6.0 SUMMARY AND CONCLUSION

Cellulose is commonly degraded by cellulase. Cellulases are the enzymes that are produced predominantly by fungi, bacteria, and protozoans that catalyze cellulolysis, the decomposition of cellulose and of some related polysaccharides. These are the enzymes that hydrolyze β -1,4 linkages in cellulose chains. Cellulase enzymes have a significant role in the biomass derived biofuels production process. From the present study it can be concluded that all the samples that were aimed for isolation and characterization of bacterial isolates with high cellulase-producing ability from various sources like Pulicat lake, Pallavaram marsh lands and Rumen of Goat could efficiently produce cellulase in the medium which could probably help taxonomists, enzymologists, and even some industrialist in their own researches. Screening of cellulase producing bacteria can be useful in production of more cellulase in order to be helpful in industrial use especially for textile wet processing, biostoning of denim fabric, biopolishing of textile fibers, softening of garments, and removal of excess dye from the fabrics etc.

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