ISOLATION AND MOLECULAR CHARACTERIZATION OF *Cellulomonas spp*. FROM DIFFERENT SOIL HABITATS

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PAL 0225

DEPARTMENT OF PLANT BIOTECHNOLOGY

UNIVERSITY OF AGRICULTURAL SCIENCES

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in

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Affectionately

Dedicated to

My **P**arents,

Wife and

Daughter

DEPARTMENT OF PLANT BIOTECHNOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
GKVK CAMPUS, BENGALURU - 560065

CERTIFICATE

This is to certify that the thesis entitled **"ISOLATION AND MOLECULAR CHARACTERIZATION OF *Cellulomonas spp*. FROM DIFFERENT SOIL HABITATS"** submitted in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (Agriculture)** in **PLANT BIOTECHNOLOGY** to the University of Agricultural Sciences, GKVK, Bengaluru is a record of bonafide research work done by **Mr. KHETA RAM TAK** during the period of his study in this University under my guidance and supervision and the thesis work has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

Bengaluru
August, 2012

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Bengaluru
August, 2012 **(Kheta Ram Tak)**

"ISOLATION AND MOLECULAR CHARACTERIZATION OF *Cellulomonas spp*. FROM DIFFERENT SOIL HABITATS"

THESIS ABSTRACT

Cellulose is one of the most dominant waste material obtained from agriculture industry in the form of stalks, stems and husk and is one of the most abundant renewable sources. Although, generally considered a plant material, cellulose is also produced by some bacteria. Hence, the present work envisaged isolation of *Cellulomonas*, a cellulose degrading bacteria, from natural forest soil, paper mill industry soil and cultivable agricultural field soil, isolation and characterization of *Cellulomonas sp*. from different sources and diversity analysis of *Cellulomonas sp.* using RAPD marker. Maximum count of cellulose degrading bacteria was noticed in the soil sample collected from the region of natural forest, Kodagu. All the *Cellulomonas* isolates which used in the study were rod shaped and gram positive in character. Maximum colony diameter (3.6 mm) in Congo-Red Agar medium of *Cellulomonas* was noticed in case of soil collected paper mill Industrial, Davangere), whereas, maximum zone of clearance (17.0 mm) was observed in sample B (Grassland, Natural forest soil, Madikeri) as well as in sample C (Soil collected from paper mill Industrial, Davangere). Highest hydrolysis capacity (6.61) was observed in sample B. Maximum extent of degradation of filter paper was noticed in the sample collected from the region of natural forest, Madikeri. The RAPD banding patterns of all the *Cellulomonas* isolates indicates distinguished into one cluster and one isolate with linkage distance from 1.75 to 2.25 and the second group with linkage distance from 1.75 to 1.90.

Signature of the Student
(KHETA RAM TAK)

Signature of the Major Advisor
Dr. K. M. HARINIKUMAR

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Introduction

I. INTRODUCTION

Soil is a natural body consisting of soil horizons of minerals constituents of variable thicknesses. It is a mixture of minerals and organic constituents that are in solid, gaseous and aqueous phases. Micro-organisms, affect chemical exchanges between roots and soil, act as a reserve of nutrients. Soil may contain upto one million species of microbes per gram, making soil the most abundant ecosystem on the earth. These micro-organisms decompose dead plant parts which come to the soil.

The micro-organisms inhabiting in the soil can be classified into bacteria, actinomycetes, fungi, algae and protozoa. Bacteria are the most dominant group of micro-organism in the soil and probably it is equal to one half of the microbial biomass in the soil. They are present in all types of soil but their population decreases as the depth of the soil increases (Nandkar and Jachak, 2011).

Cellulose is probably the most abundant biological compound on terrestrial earth. It is the dominant waste material from agriculture industry in the form of stalks, stems and husk and is one of the most abundant renewable sources. Cellulose occurs in almost pure form in cotton fiber and in combination with other materials, such as lignin and hemicelluloses, in wood, leaves, stalks *etc*. Although generally considered a plant material, cellulose is also produced by some bacteria (Immanuel *et al.,* 2006).

The organisms varying widely in morphological and physiological properties have been associated by various workers with the natural decomposition of cellulose, namely certain, aerobic and anaerobic forms of bacteria, fungi and some species of actinomycetes.

Several studies were carried out to produce cellulolytic enzymes in bio-waste degradation process by several microorganisms including fungi such as *Trichoderma sp*., *Penicillium sp*., *Aspergillus sp.* (Mandels 1975), (Brown *et al.,* 1987), (Lakshmikant and Mathur, 1990) *etc*. Similarly cellulolytic property of bacterial species like *Pseudomonas, Cellulomonas, Cellulovibrio* and *Sporocytophaga sp.* was also reported (Nakamura and Kappamura, 1982). The specific cellulolytic activity shown by the bacterial species is found to be depending on the source of occurrence (Sexana *et al*., 1993). The cellulase system of the mesophilic cellulolytic aerobe, *Cellulomonas fimi* is one of the first studied and has since been one of the most studied bacterial cellulase systems (Whittle *et al*., 1982; O'Neill *et al*., 1986 and Shen *et al*., 1995). Many spore-forming bacteria have been isolated from various factors that have a feedstock from cattle waste (Sharma and Hobson, 1985), cow manure (Palop *et al*., 1989), woody biomass (Sleat and Mah, 1984), municipal solid waste (Cailliex *et al*., 1992) *etc*. Similarly many strains of cellulolytic anaerobic bacteria have been reported from various sources as human colon (Skinner, 1960), estuarine sediments (Madden, *et al*., 1982), freshwater sediments (Leschine and Canale Parola, 1984), decomposing vegetation (Madden, 1983). Because of the common occurrence of these bacteria in various natural environments, they are responsible for huge amount of cellulose decomposition.

Cellulase yields appear to depend on a complex relationship involving a variety of factors like inoculum size, Carbon source cellulose quality, pH value, temperature, presence of inducers, medium additives, aeration and growth time. The particle size of cellulose can affect cellulase production by microorganisms (Greaves, 1971). The bioconversion of various complex cellulosic waste materials such as bagasse (Kansoh *et al*., 1999), Corncob (Ojumu *et al*., 2003) and saw dust (Solomon *et al*., 1999) have been reported. Coir dust and fibres are also major bio-wastes of coir industries of India that discarded along with coir retting effluent to estuarine environment.

The distinctive feature of bacteria of the genus *Cellulomonas* is its ability to utilize cellulose as substrate since they are morphologically similar to *Arthrobacter*. They do not undergo the distinctive rod-coccus morphogenesis however cells in stationary phase are markedly shorter than growing cells and a small proportion may be truly coccoidal. They are facultative anaerobe, although fermentative growth is very poor. They are motile by one or a few subpolar or lateral flagella.

Cellulases are used in the manufacturing of pharmaceutics, beverage, paper and textile industries. Bacterial and fungal cellulases are used in animal feed industry, grain alcohol fermentation, brewing, malting and extraction of fruit and vegetable juices (Deshpande *et al.,* 1984; Kubicek *et al.,* 1993).

Genetic diversity can be estimated at molecular level. One such method is protein analysis using electrophoresis or direct amino acid sequencing. Electrophoresis analysis of protein has been long a valuable tool in systematic and population genetics studies of bacteria, plant and fungi. The biochemical characteristics are useful in distinguishing two different genera or species. Further it can also identify different compound produced by different strain (Dodd *et al.,* 1996).

Another method of species characterization is DNA fingerprinting. DNA fingerprinting techniques are used for an extremely wide variety of applications. Molecular analysis of genomic DNA of the organism is useful for distinguishing the bacterial strains better at intra-species level. These techniques provide valuable information on the magnitude of genetic variability within and between organisms of different species. With the advent of molecular DNA technique, several arbitrary primers based Randomly Amplified Polymorphic DNA (RAPD) technique has been used for typing and identification of number of closely related species of bacteria and assessment of their genetic relationships. These results are usually consistent with those of DNA-DNA homology studies and can be used to estimate the genetic distance (Bert *et al.,* 1996; Kumar *et al.,* 2005).

RAPD investigation used the polymerase chain reaction (PCR) to amplify DNA sample with short oligonucleotide primer that anneal randomly throughout the genome. The result is a distinctive set of amplification products. Differences are visualized by staining the gel after electrophoresis of PCR amplification products. Individuals or population are characterized by set of banding patterns they produce for a number of different primers. RAPD analysis is faster, technically less demanding and more economical than the other genomic typing method like Restriction Fragment Length Polymorphism (RFLP) and Amplified Fragment Length Polymorphism (AFLP). Unlike conventional PCR data on DNA sequence of the organisms are not a pre-requisite for RAPD analysis (Seppala *et al.,* 1996). Further, this technique elucidates the biodiversity in a group of isolates (Hansel *et al.,* 1998).

Hence, *Cellulomonas*, a cellulose degrading bacteria, has been used in this present research study to carry out several investigations which includes its (*Cellulomonas*) isolation, identification and molecular characterization. Therefore, the present investigations were undertaken with the following objectives:

1. Isolation and Characterization of *Cellulomonas spp*. from different sources.
2. Genetic diversity Analysis of *Cellulomonas spp.* using RAPD Marker technique.

Review of Literature

II. REVIEW OF LITERATURE

Cellulomonas is an aerobic bacteria which efficiently degrade the cellulose and hemicellulose into disaccharide (cellobiose). It found on cellulose rich material like decaying leaf and other agro-waste material (Dworkin *et al*., 2006).

2.1 History of *Cellulomonas*

Stackebrandt and Prauser, 1991 defined the *Cellulomonas* and put in the *Cellulomonas* genera which comes under Cellulomonadaceae family. The rationale for the establishment of this family was based mainly on comparative analysis of 16S rRNA cataloguing data of the type species of some genera of the order Actinomycetales. Phylogenetically, neighboring taxa to Cellulomonadaceae were *Promicromonospora*, *Cellulosimicrobium*, *Rarobacter, Arthrobacter*, *Renibacterium*, *Micrococcus*, *Stomatococcus*, *Dermatophilus* and *Microbacterium* (Dworkin *et al*., 2006).

Jones, 1975 *Cellulomonas* and *Ropionibacterium* were distinct taxa more closely related to *Corynebacterium* than to either *Arthrobacter* or *Lactobacillus.* The cellulolytic forms of Nocardia have the some distinct characteristic features than *Cellulomonas,* so they were put in different genus. Members of the Cellulomonadaceae were phenotypically characterized by a combination of phenotypic characters that distinguish them from the adjacent taxa: *Microbacterium Dermatophilus Brevibacterium* and other members.

Stackebrandt *et al.,* 1997 described the family Cellulomonadaceae, on the basis of phylogenetic position and the distribution of signature nucleotides of the 16S rRNA gene of all validly described Actinomycete genera, which is a member of the suborder Micrococcineae, order Actinomycetales.

Cellulomonadaceae family has two genera: *Cellulomonas* and *Oerskovia* (Table 1). Both genera were united under *Cellulomonas* when phylogenetic relatedness based on 16S rDNA catalogues was considered more significant than differences in chemotaxonomic properties, such as the amino acid composition of peptidoglycan.

2.1.1 Taxonomy

Domain	**Bacteria**
Phylum	Actinobacteria
Subclass	Actinobacteridae
Order	Actinomycetales
Suborder	Micrococcineae
Family	Cellulomonadaceae
Genus	*Cellulomonas*
Species	*Fimi*

2.1.2 Habitat

A large amount of cellulose finds into the soil as the main component of crop residues. Under normal conditions of temperature and moisture, this cellulose decomposes almost completely and quite rapidly by the action of aerobic mesophilic population of microorganisms that are efficient in utilizing cellulose. In cellulose degrading microorganisms, *Cellulomonas* is playing a vital role in degrading the cellulose and hemicellulose.

The main habitat of *Cellulomonas* is organically rich soil, forest soils. Emphasis placed on the cellulolytic activity of these organisms has resulted in the successful isolation of *Cellulomonas* strains from rumen, activated sludge, and cellulose-enriched environments such as bark and wood, coffee cherries, soils enriched by flax or sisal fibers, and sugar (Dworkin *et al.,* 2006), refuse of a landfill (Pourcher *et al.,* 2001) and from an agricultural encatchment (Ulrich and Wirth, 1999). *Cellulomonas hominis* is the first representative of the genus isolated from human clinical samples (cerebrospinal fluid); (Funke *et al.,* 1995). This strain of *C. hominis* did not show any cellulolytic activity.

Table 1: Twenty two species of *Cellulomonas spp*. reported was

(Reference: - http://www.bacterio.cict.fr/c/*Cellulomonas*.html)

Sl No	**Name of species**	**Sl No**	**Name of species**
1.	*Cellulomonas aerilata*	12.	*Cellulomonas flavigena*
2.	*Cellulomonas biazotea*	13.	*Cellulomonas gelida*
3.	*Cellulomonas bogoriensis* sp. nov.	14.	*Cellulomonas hominis* sp. nov
4.	*Cellulomonas cartaez* sp. nov.	15.	*Cellulomonas humilata* comb. nov.
5.	*Cellulomonas cellulans* comb. nov.	16.	*Cellulomonas iranensis* sp. nov
6.	*Cellulomonas cellasea*	17.	*Cellulomonas persica* sp. nov.
7.	*Cellulomonas chitinilytica* sp. nov.	18.	*Cellulomonas phragmiteti* sp. nov
8.	*Cellulomonas composti* sp. nov.	19.	*Cellulomonas terrae* sp. nov.
9.	*Cellulomonas denverensis* sp. nov.	20.	*Cellulomonas turbata* comb. Nov
10.	*Cellulomonas fermentans* sp. nov.	21.	*Cellulomonas uda*
11.	*Cellulomonas fimi*	22.	*Cellulomonas xylanilytica* sp. nov.

Han and Srinivasan, (1968) isolated cellulose-decomposing aerobic and mesophilic bacterium from the soil of sugarcane fields. The organism was identified as a member of the genus *Cellulomonas*. The isolate excreted cellulase into the menstruum, and it hydrolyzed various cellulosic materials producing cellobiose as the final breakdown product in the menstruum. Stewart and Leatherwood *et al.* (1976) isolated *Cellulomonas sp.* from the soil which hydrolyzed cellulose, as shown by clear-zone formation on cellulose agar medium.

Choi *et al.* (1978) isolated *Cellulomonas* strain (CSI-I) from soil which could readily degrade cotton wool. Production of cell-bound and extracellular carboxymethylcellulase (CMCase), β-glucosidase and avicelase during growth on different substrates was determined. The mutant (CSl-7) which was able to degrade cotton wool more rapidly than the parent revealed large differences in the levels of cell-bound and extracellular CMCase when compared to *Cellulomonas* strain (CSI-L).

2.1.3 Isolation and Identification

Stackebrandt and Keddie, 1986 has described a suitable procedure to enrich *Cellulomonas* in mineral-based medium containing a low concentration (0.05–0.1%) of yeast extract to which filter paper is added as principal carbon source. Isolates was tested on plates containing Avicel, Solka Floc, CF11 cellulose, carboxymethyl cellulose, or phosphoric acid-treated cellulose. Cellulose degradation can be visualized owing to the formation of clearing zones. This method was not selective for *Cellulomonas* and isolates must be screened for typical coryneform morphology. Cells were routinely grown in shake flasks at 30°C. Moderate growth occurred on meat extract agar, peptone agar, or medium based on yeast extract or peptone at around neutral pH. Growth promoting factors (GPF) in yeast extract were thiamine and biotin. *Cellulomonas persica* and *C. iranensis* were grown on trypticase soy agar at 30°C. *Cellulomonas hominis* can be cultured on Columbia agar with 5% sheep blood in a 5% CO_2 atmosphere or on trypticase soy yeast extract medium at 37°C.

Choi *et al.* (1978) studied most cellulolytic bacteria caused little or no degradation of either highly crystalline cellulose such as cotton wool or lignocellulosic material which had not been subjected to some pretreatment. Generally, bacterial growth and cellulolytic properties were studied using highly processed essentially pure cellulose or filter paper (FP). The potential of bacterial systems for the degradation of highly crystalline or natural cellulosic material may not have been fully explored.

Summers *et al.* (1979) studied the mutant strain of *Cellulomonas sp*. (ATCC 21399) under glucose and zinc limitation at a variety of growth rates in continuous culture. The growth characteristics and macromolecular composition of the population varied with the limitation imposed and the growth rate. The maximum population growth rate in zinc-limited cultures was directly proportional to the zinc concentration and demonstrated a traditional steady-state function.

Bagnara *et al.* (1985) isolated new mesophilic, cellulolytic microorganism from a municipal dumping ground. The isolation of this bacterium was performed under anaerobic conditions, but its growth was similar under both anaerobic and aerobic conditions. Nucleic acid hybridization tests between the DNA of the isolate and the DNA of *Cellulomonas uda* ATCC 21399 showed some partial (37 to 40%) relatedness. This information, in addition to other data, suggests that this organism belongs to the genus *Cellulomonas,* and it is proposed here as a new species, *Cellulomonas fermentans.*

Rodriguez *et al.* (1988) investigated the effect of physicochemical parameters on the cellulolytic activity of *Cellulomonas sp*. IIbc grown on sugarcane bagasse pith, and the optimum ranges for enzyme activity. The cellulases were more stable when incubated at the optimum growth temperature (32°C) than under optimum activity conditions (45°C for β-glucosidases and 50°C for CMC- and FP-cellulases).

Angelo *et al.* (1990) isolated a cellulolytic bacterium from leaf litter. Its nutritional characteristics and most of its morphological features closely resembled those of ATCC 482, which is considered to be the type species of *Cellulomonas flavigena*.

Malekzadeh *et al.* (1993) isolated three strains of cellulose-degrading, aerobic, mesophilic bacteria from forest soils and they were identified as members of the genus *Cellulomonas* on the basis of their cultural, biochemical and physiological characteristics. The isolates were able to use urea as N source in cellulose fermentation. All the three strains were motile with one to four peritrichous flagella.

Maki *et al.* (2011) reported that screening wide variety of bacteria in the environment permits for more efficient cellulases to help overcome current challenges in biofuel production. The study was focused 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.

Cells in young broth cultures were typically coryneform with a snapping division and transformed to short rods after one week (Figure 1). On yeast extract-glucose agar, *C. flavigena* forms smooth, glistening, yellow colonies about 5 mm in diameter. *C. flavigena* was described as non-motile, but according to Thayer *et al.* (1984) *C. flavigena* cells possessed polar multitrichous flagella. *C. flavigena* was efficiently grown

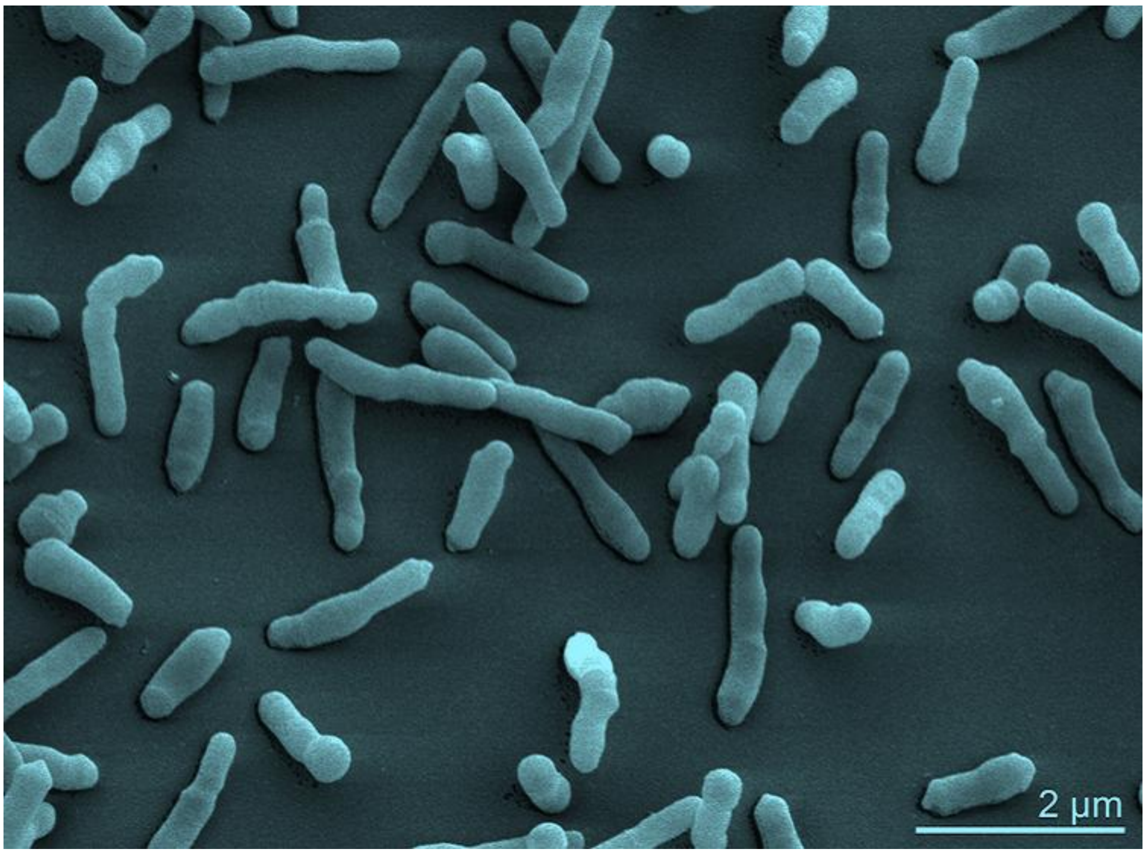


Figure 1: Scanning electron micrograph of *C. flavigena* 134T (Abt *et al.,* 2010).

under aerobic conditions with an optimal growth temperature of 30°C and an optimal pH of 7 (Abt *et al.,* 2010).

Upadhyaya *et al.* (2012) isolated several strains of *Cellulomonas* from the gut of termite in three different medium. Each strain showed significant cellulolytic activity in congo red assay. Several biochemical tests were carried out to identify the bacteria.

Shanmugapriya *et al.* (2012) reported that identity bacteria has high growth rate as compared to fungi and has good potential to be used in cellulase production. Cellulolytic property of some bacterial genera such as *Cellulomonas sp.*, *Pseudomonas sp.*, *Bacillus sp. and Micrococcus sp.* were reported.

2.1.4 Identification methods for bacteria

Thayer *et al.* (1984) observed motile cells by phase-contrast microscopic examination of hanging drop suspensions from cultures. The percentage of motile cells showed variation greatly. The cultures of *C. biazotea*, *C. fimi*, and *C. uda* contained relatively few motile cells. This evidence indicated that the majority of cells have polar multitrichous flagella which were the appropriate morphological description for members of this genus.

Gomes *et al.* (1992) optimized the medium composition on a shake-flask scale using the Graeco-Latin square technique. The temperature and time for optimal growth and production of the enzymes in shake cultures were optimized using a central composite design. The temperature optima for maximal production of filter paper cellulase (FPase), xylanase and β-gluosidase were 32.8^{0}C, 34.7^{0}C and 31.1^{0}C, respectively, and the optimum times for reduction of these enzymes were found to be 144, 158 and 170 h respectively. The optimized culture medium and conditions (33^{0}C) gave 0.55 unit of FPase, 188.1 units of xylanase and 3.37 units of β-glucosidase per milliliter of culture filtrate at 144 h of shake culture. Among the different carbon sources tested, the maximum enzyme activities were observed with sulphite pulp and all three enzymes were produced irrespective of the carbon sources used.

Kim *et al.* (2008), on the basis of 16S rRNA gene sequence data, found that the strain to be a member of the genus *Cellulomonas,* which currently contains 14 species. Further study of this strain, based on a polyphasic approach that included chemotaxonomic, physiological and DNA-DNA hybridization analysis, confirmed its position as a representative of novel species within the genus *Cellulomonas.*

2.1.5 Cellulose Degrading Microorganism

A wide variety of aerobic, facultative anaerobic and anaerobic bacteria and fungi have the capacity to degrade cellulose. Microbial cellulose degradation is easy to observe but many cellulose degrading bacteria are difficult to purify. Moreover, conventional enrichment procedures as applied to soil yielded cellulose degrading fungi, but bacteria brought into pure culture from such enrichments are normally not cellulose degraders (Lynch *et al.,* 1981). Cellulose degradation occurs naturally in complex microbial communities that include many non-cellulolytic micro-organisms and many cellulose degraders are relatively slow growing. They have compared different cellulosic substrates for purification methods with a view to obtain cellulose degrading colonies very early before extensive overgrowth by other bacteria (Ramasamy *et at.,* 1981).

Mullings *et al.* (1984) developed new procedures for the isolation and purification of aerobic and facultative anaerobic bacteria which was able to utilize cellulose as sole source of carbon and energy. Wood pulp medium was used for the enrichment, and bacterial cellulose, obtained from cultures of *Acetobacter aceti subsp. xylinus,* was employed as carbon substrate during purification and for the rapid screening of colonies for cellulolytic activity.

Li and Gao, (1997) isolated a new facultative anaerobic, Gram-negative bacterium, *Cytophuga sp.* LX-7, degrading crystalline cellulose completely from soil by dilution plating on cellodextrin agarose plates. The strain could excrete extracellularly all three types of cellulase and cellulosic substrates were the strongest inducer of endocellulase with CMC-liquefying activity production. An enzyme which degrades crystalline cellulose was detected in cultures of cellulose by measuring the formation of soluble carbohydrate but was not detected by determining the reducing sugar released. The strain synthesized cell-bound cellobiose oxidizing enzyme.

Wierzba and Nabrdalik, (2007) studied the attempt at conducting biodegradation of bottom sediments of Turawa Lake. In the research, cellulolytic bacteria strains were employed for the biodegradation of cellulose which results in the 19% reduction of the total cellulose content. At first, cellulolytic bacteria which originating from the bottom sediments, were isolated and selected. Next step involved evaluation of the cellulolytic activity of bacterial strains and selection of the three most vigorous, to be used in the process of bottom sediments biodegradation. The amount of decomposed cellulose was assessed with the use of anthrone method and changes in bacterial enumeration were determined with the index method.

Lignocellulosic biomass is a renewable and abundant resource with great potential for bioconversion to value-added bioproducts. The novel cellulases from strains like *Cellulomonas flavigena* and *Terendinibacter turnerae,* produce multifunctional cellulases with broader substrate utilization. Anaerobic bacteria like the *Clostridia* offer potential due to species capable of producing compound multienzyme complexes called cellulosomes which provide synergy and close proximity of enzymes to substrate that results in increases the activity towards crystalline cellulose. *Clostridium thermocellum has* ability to ferment sugars to ethanol; its amenability to co-culture and, recent advances in genetic engineering, offer a promising future in biofuels (Maki *et al.,* 2009).

Singh *et al.* (2010) studied the microorganisms which are major key players for sustaining the soil quality and sustainable agriculture by the degradation of cellulose of the legume plant residues which used as biofertilizer. The bacterial isolates viz., *Serratia* sp. (MSK1 and MSK24) and *Pseudomonas* sp. (MSK13) exhibiting cellulase activity of 3.83, 4.21 and 4.52 mM glucose ml^{-1} h^{-1} respectively were introduced as inoculants.

Lennox *et al.* (2010) isolated microorganisms from sawdust have been demonstrated to be effective in its degradation. Eight bacteria and eight fungi were isolated from wet decaying sawdust. Among the bacteria, *Cellulomonas sp.* was found to be the most effective degrading agent based on its high percentage degradation (18.3%). This was followed by M*icrococcus sp*. (16.0%) and *Pseudomonas sp.* (14.6%), *Cytophaga sp.* and *Bacillus sp*. had the lowest percentage degradation of 0.2 and 7.7%, respectively. Okeke & Lu, (2011) reported that enzymes of the cellulase complex (exoglucanase, endoglucanase, and β-glucosidase) act in concert to degrade biomass cellulose to soluble sugars. Members of the genera- *Cellulomonas*, *Clostridium, Bacillus*, *Thermomonospora, Ruminococcus, Bacteriodes, Erwinia, Acetovibrio, Microbispora*, and *Streptomyces* are some bacteria reported to produce cellulases.

Barman *et al.* (2011) conducted the investigation to find out the effective cellulolytic bacteria for biodegradation of solid kitchen and agricultural wastes as organic manure or compost. Bacterial strains of

Moraxella sp., Cellulomonas sp. and *Planococcus sp*. were isolated from soil and cultured on nutrient agar medium. In comparison to *Cellulomonas sp*. and *Planococcus sp*., inoculation of *Moraxella sp*. enhanced the degradation of kitchen and agricultural waste, shown by the increased CO_2 release (54.3 and 37.62 mg), crude fiber loss (46.86% and 45.11%), total sugar reduction (72.52% and 74.27%), fat reduction (65.20% and 61.22%). Inoculation of *Cellulomonas sp.* strain (53.89% and 77.96%) showed high protein reduction in comparison with inoculation of *Moraxella sp.* strain (20.04% and 63.42%) for kitchen and agricultural wastes.

2.1.6 *Cellulomonas* as Cellulose Degrading Microorganism

Singh and Jain (1986) studied the cellulolytic bacteria for the degradation of cellulose constitutes which are the major biodegradable part of lignocellulosic cattle which results in the production of acetate and other volatile fatty acids as a result of fermentation of its hydrolysis products. Therefore, to obtain higher biogas yields, cellulose needs to be hydrolyzed at an efficient rate which might be achieved by efficient cellulolytic bacteria (Oyeleke and Okusanmi, 2008). Prasertsan and Doelle, (1986) studied cellulolytic materials can be converted to valuable products either by microbial, enzymic or chemical saccharification. Bioconversions are achieved mainly by fungi, bacteria or actinomycetes. Examples of cellulolytic bacteria are *Cellulomonas sp*., *Clostridium thermocellum* and *Acetovibrio cellulolyticus.* Cellulose degradation involves a complexity of cellulase enzymes. Rodriguez *et al.,* (1996) studied the regulation of cellulolytic enzymes in bacteria different species.

Langsford *et al.* (1984) cultured *Cellulomonas fimi*. It was concluded that the cellulase system of *C. fimi* was composed of only three enzymes and these enzymes had a great affinity for, and were stabilized by binding to an insoluble cellulosic substrate. Enzymes which accumulated free in the culture medium were subject to limited proteolysis and de-glycosylation which generated a variety of products, some of which retained enzymatic activity.

Many micro-organisms secrete cellulases under appropriate conditions, thereby allowing the utilization of cellulose as a carbon source. Mutants with an improved ability to degrade cellulose can be isolated from a given organism. In the strains of *Cellulomonas,* for example, mutants have been isolated which are more efficient than the parent strains in the degradation of crystalline cellulose. These can be resistant to catabolite repression (Stewart & Leatherwood, 1976), end-product inhibition (Choudhury *et al.,* 1980), or can produce increased levels of cellulases (Choi *et al.,* 1978). Rapp and Wagner, 1986 studied xylan degradation and production of II-xylanase and D-xylosidase activities cultures of *Cellulomonas uda* grown on purified xylan from birchwood. Xylanase activity was found to be associated with the cells, although in various degrees.

Dermoun *et al.* (1988) had shown that *Cellulomonas uda* solubilize microcrystalline celluloses to a great extent under anaerobic growth conditions. During cellulose degradation, the actual substrate for growth is cellobiose (Gong and Tsao, 1979), the main product of cellulolysis by *Cellulomonas* species. Intergeneric hybrids have been constructed *between Cellulomonas* and *Zymomonas* so as to enhance the industrial potential of this organism (Chaudhary *et al.,* 1997).

Hsing and Canale-Parola, (1992) studied on the bacterial degradation of plant cell wall polysaccharides. They observed that growing cells of motile cellulolytic bacteria accumulated without attachment, near cellulose fibers present in the cultures. They studied primarily the responses toward cellobiose and hemicellulose hydrolysis products. They found that cellobiose, cellotriose, D-glucose, xylobiose, and D-xylose, as well as other sugars that are hemiceliulose components, served as chemo-attractants for *C. gelida*, as determined by a modification of Adler's capillary assay.

Rajoka *et al.* (1997) studied that *Cellulomonas sp*. caused rapid degradation of plant lignocellulosic biomass by the production of cellulases and xylanases. Disaccharides and oligosaccharides arise slowly during the hydrolysis of cellulose or lignocellulosic biomass and are inducers of xylanases and their quantity in the fermentation mash may influence the yield of enzymes. Mayorga-Reyes and Ponce-Noyola, (1998) studied the biosynthesis, characterization and regulation of cellulases and xylanases from *Cellulomonas flavigena* which has the roles in the bio-conversion of agricultural wastes.

Chakraborty *et al.* (2000) isolated cellulose degrading bacteria from the intestinal fluid of the silver cricket *Lepisma sp.* and culture was developed anoxigenically in the cellulose degrading medium. The cellulolytic activity of the microbe was examined in a broth culture using Whatman 42 filter paper as the source of insoluble cellulose. Activity was measured spectrophotometrically at 620 nm; following Anthrone reaction of the culture filtrate and the sugar produced was quantified as a factor of time, pH optimum between 7 and 8 and temperature profile optimum was between 30 and 37^{0}C. The microbes appeared as white colonies on a solid medium. Morphologically the bacterium is a gram-positive nonspore forming rod which was tentatively identified as a new strain of *Cellulomonas sp*.

Hoorebeke *et al.* (2010) reported that the cellobiose phosphorylase enzyme from *Cellulomonas uda* (CPCuda) which belongs to glycoside hydrolase family 94 and catalyzes the reversible breakdown of cellobiose [β-D-glucopyranosyl-(1,4)-D-glucopyranose] to D-glucose-1-phosphate and D-glucose. Hernandez *et al.* (2011) studied to evaluate the enzyme extracts from *Cellulomonas flavigena* cultured at different temperatures using sugarcane bagasse as substrate to determine the xylanolytic activity and the degradation of the enzyme extracts protein under *in vitro* rumen conditions.

2.2 Molecular Characterization of *Cellulomonas sp*.

2.2.1 Molecular markers

Biological diversity is the variability among all living organisms existing on earth in various ecosystems and ecological complexes. This diversity is the basis for continuous evolution of life forms and in turn maintaining the life sustaining systems of the biosphere. Various methods which have been used to study genetic diversity in micro-organisms are biochemical tests, REstriction Analysis of Chromosomal DNA (REAC), Restriction Fragment Length Polymorphism (RFLP), Randomly Amplified Polymorphic DNA (RAPD), Pulsed-Field Gel Electrophoresis (PFGE), Amplified Fragment Length Polymorphism (AFLP), Variable Number of Tandem Repeats (VNTR), repetitive sequence element based strategies like REP (Repetitive Extragenic Palindrome)-PCR and ERIC (Enterobacterial Repetitive Intergenic Consensus)-PCR, Box-PCR based finger printing, Multi-Locus Enzyme Electrophoresis (MLEE), and single locus and Multi-Locus Sequence Typing (MLST) (Suman *et al.,* 2001; Muthukumaraswamy *et al.,* 2002).

2.2.1.1 DNA based markers

The molecular markers are one of the versatile tools in molecular biology and genetic engineering. With the advent of molecular markers, new generation of markers have been introduced over the last two decades, which has revolutionized the entire scenario of biological sciences. Ever since their development, they are constantly being modified to enhance their utility and to bring about automation in the process of genome analysis. The discovery of Polymerase Chain Reaction (PCR) was a landmark in this effort and proved to be a unique process that brought a new class of DNA markers. Molecular markers discriminate organisms at the level of DNA and are inherited in simple Mendelian fashion (Waltson, 1993). There are different types of DNA markers and many more are being discovered. There are two important classes of markers *viz.,* Hybridization based markers and PCR based markers.

2.2.1.1.1 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism is the original DNA marker, and was developed in the late 1970s (Botstein *et al.,* 1980). Their development was facilitated by the discovery of restriction enzymes. Genetic differences between chromosomes can result in differences in the lengths of particular restriction fragments. Substitutions occurring in DNA can result in sequence difference within a particular recognition sequence leading to either loss or gain of a particular restriction site and a length difference in the fragment produced. Alternatively, insertions or deletions of DNA segments between two restriction sites may occur changing the length of a particular fragment.

RFLP process allows the detection of these length polymorphisms in particular restriction fragments via hybridization with labeled probes. RFLP analysis of PCR-amplified 16S rRNA genes is especially applicable for determination of inter and intra-generic relationship among rhizobial species. This method is also suitable for grouping strains at the species level or higher, and it can be used to detect potential new taxa or phylogenetic classification of a vast number of rhizobia. However, 16S rDNA analysis is based on the features of only one gene, whereas REP-PCR and RAPD generates fingerprints of the entire genome (Bradic *el al.,* 2003).

2.2.1.1.2 Amplified Fragment Length Polymorphism (AFLP)

AFLP is a technique based on the detection of genomic restriction fragments by PCR amplification and can be used for DNA of any origin or complexity. The number of fragments detected in a single reaction can be 'tuned' by selection of specific primer sets. AFLP technique is reliable since stringent reaction conditions are used for primer annealing. This technique adapts the combination of RFLP and PCR techniques (Vos *et al.,* 1995).

AFLP is a dominant marker and useful in detection of polymorphism between closely related genotypes. High resolution genotyping method of AFLP analysis was used to study the genetic relationship within and between natural populations of five *Fusarium sp.* The total of 80 AFLP markers were obtained using four primer combinations, with an average of 20 polymorphic markers were observed per primer pair. The phenetic dendrogram generated by UPGMA as well as principal coordinate analysis (PCA) grouped all the *Fusarium sp*. into five major clusters. No clear trend was detected between clustering in the AFLP dendrogram and geographic origin, host genotype of the tested isolates with a few exceptions. The results provided the high discriminatory power of AFLP analysis, suggesting the possible applicability of this method to the molecular characterization of *Fusarium* (Abdel-Satar *et al.,* 2003).

2.2.1.1.3 Sequence Tagged Sites (STS)

RFLP probes specifically linked to desired trait can be converted into PCR-based STS markers based on nucleotide sequence to obtain specific amplicon. Using this technique, tedious hybridization procedures involved in RFLP analysis can be avoided. This approach is extremely useful for studying the relationship between various species (De-Bustos *et al.,* 1999).

2.2.1.1.4 Microsatellite markers

Litt and Luty, (1989) coined the term microsatellites, which are also called as Simple Sequence Repeats (SSRs). They consist of tandem repeats of simple motif sequences, usually one to five bases, which are amplified by PCR. Tandem repeats of many simple sequence motifs, in particular the dinucleotide repeats are abundant in most eukaryotic genomes, and are distributed throughout these genomes in dispersed locations. These microsatellite repeats are flanked by unique sequences, occurring only once in the genome. Microsatellites are highly informative owing to their high degree of polymorphism and co-dominance.

2.2.1.1.5 Randomly Amplified Polymorphic DNA (RAPD)

Randomly amplified polymorphic DNA (RAPD) involved the use of random primers in PCR reactions (Welsh and McClelland, 1990; Williams *et al.,* 1990). It has been used increasingly to distinguish closely related organisms (Bassom *et al.,* 1992) based on polymorphism among RAPD products. This RAPD technology is a very useful, simple, fast and informative. Further, the technique gives an opportunity to get information about the biodiversity in a group of isolates to distinguish them from one another.

RAPD with incorporation of fluorescent deoxynucleotides was used to examine the genetic diversity among *Beauveria bassiana* isolates from Argentina and Brazil. High resolution DNA fingerprints was generated by PAGE of amplified products and automated laser fluorescence analysis. Each isolate displayed a distinct genotype. Cluster analysis showed a high level of variability among these genotypes. A phenetic group of 80% similarity represented mainly the isolates exhibiting high virulence against the sugarcane borer, *Diatraea saccharalis* (Marcelo *et al.,* 1998).

Archana *et al.* (2007) conducted study for the analysis of the genetic diversity of rhizobacterial isolates of *Bacillus* with antifungal activity using PCR-based RAPD technique. Twenty isolates were selected and characterized by 18 RAPD primers. The fingerprints obtained in all the 18 primers indicated the polymorphic bands. From the dendrogram generated using the NTSYSpc programme, the genetic diversity was evident based on the percentage similarity between the isolates. Five major clusters were obtained at 61% similarity level.

2.2.2 Applications

Torre and Campillo, (1984) identified two bacterial species from a mixed culture of *Cellulomonas flavigena* and *Xanthomonas sp.* and were isolated by batch-culture enrichment techniques. The capacity of both bacteria to grow as pure culture in a mineral medium with alkaline pretreated sugarcane bagasse or cellobiose was tested. *C. flavigena* as pure culture was able to grow on both substrates only when yeast extract or biotin and thiamine were added to the culture medium, while *Xanthomonas sp.* could not grow on sugarcane bagasse, but assimilated cellobiose if yeast extract was supplied. It was concluded that the interaction was favorable to both species. The mixed culture had the capacity to degrade a number of different agricultural wastes and to use them as the sole carbon and energy source for the production mainly of biomass.

Meinke *et al.* (1991) determined the nucleotide sequence of the CenB gene and used to deduce the amino acid sequence of endoglucanase B (CenB) of *Cellulomonas fimi*.

Rodriguez *et al.* (1993) reported the biomass production of *Cellulomonas* was optimal with 1% (w/v) bagasse pith pre-treated with either 0.2 M NaOH for 1 h at 80^0C or 0.4 M NaOH for 40 h at 28 to 30^0C.

Growth was similar to that obtained with a more severe treatment used as control and compared well with other reports for cellulolytic bacteria cultivated on pre-treated bagasse pith.

Shen *et al*. (1995) studied the cellulase system of *C. fimi* which resembles those of fungi in comprising multiple endoglucanases and cellobiohydrolases. Ponce-Noyola and Torre, (1995) investigated the genetic improvement of *Cellulomonas* for cellulase and single-cell protein production in several laboratories and hyper producing mutant strains have been obtained.

Sangkharak *et al.* (2011) studied to develop high sugar production by optimization of enzymatic hydrolysis process as well as applications of enzyme for ethanol production using effects of substrate concentration. *Cellulomonas sp*. strain TSU-03 produced highest activity of xylanase and endoglucanase at 1860.1 and 388.5 U mg^{-1} protein respectively. At 50^0C, cellulase was highly stable and loosing less than 20 percent of initial activity after 24 hours of incubation. Cellulase production from strain TSU-03 can be an advantage as the activity of enzymes is the highest value ever reported from *Cellulomonas sp*.

Material and Methods

III. MATERIAL AND METHODS

The present research was undertaken to isolate, identify and characterize *Cellulomonas spp.* from different sources. The material and methods employed for achieving the objectives were detailed below:

3.1 Isolation and Characterization of *Cellulomonas sp*. from different sources

3.1.1 Isolation of *Cellulomonas sp.* from different soils

500 g each soil samples were collected from different sites by scraping off surface material with a sterile spatula from 2 to 5 cm below the soil surface. First soil sample was collected from the forest of Virajapate (Coorg district). Second soil sample was collected from the grass land in madikeri (Coorg district), third and fourth sample were collected from 30 and 50 mts away from a paper mill industry. Fifth and sixth samples were collected from sugarcane and paddy rhizosphere from nagarakera and kebbahally respectively. The soil samples were collected (Plate 1) in sterile polyethylene bags, labeled and stored at 4^0C until further use. Sample was collected from different places for different strains of *Cellulomonas* (Table 2).

3.1.1.1 Medium used for Culture

Basal salt medium (Appendix I) was used for isolation of cellulose degrading bacteria. Basal Salt Medium contains filter paper (Whatman filter paper No.1) 2gm/1000 ml for the isolation of cellulolytic bacteria. All components were individually weighed and the pH was adjusted to 7.0 and pH was checked before the addition of agar. All components were autoclaved together for 20 min at 121^0C at 15 lbs pressure. For isolation of bacteria, medium was supplemented with 0.02 % bavistin to inhibit the growth of fungi (Gupta *et al.,* 2012).

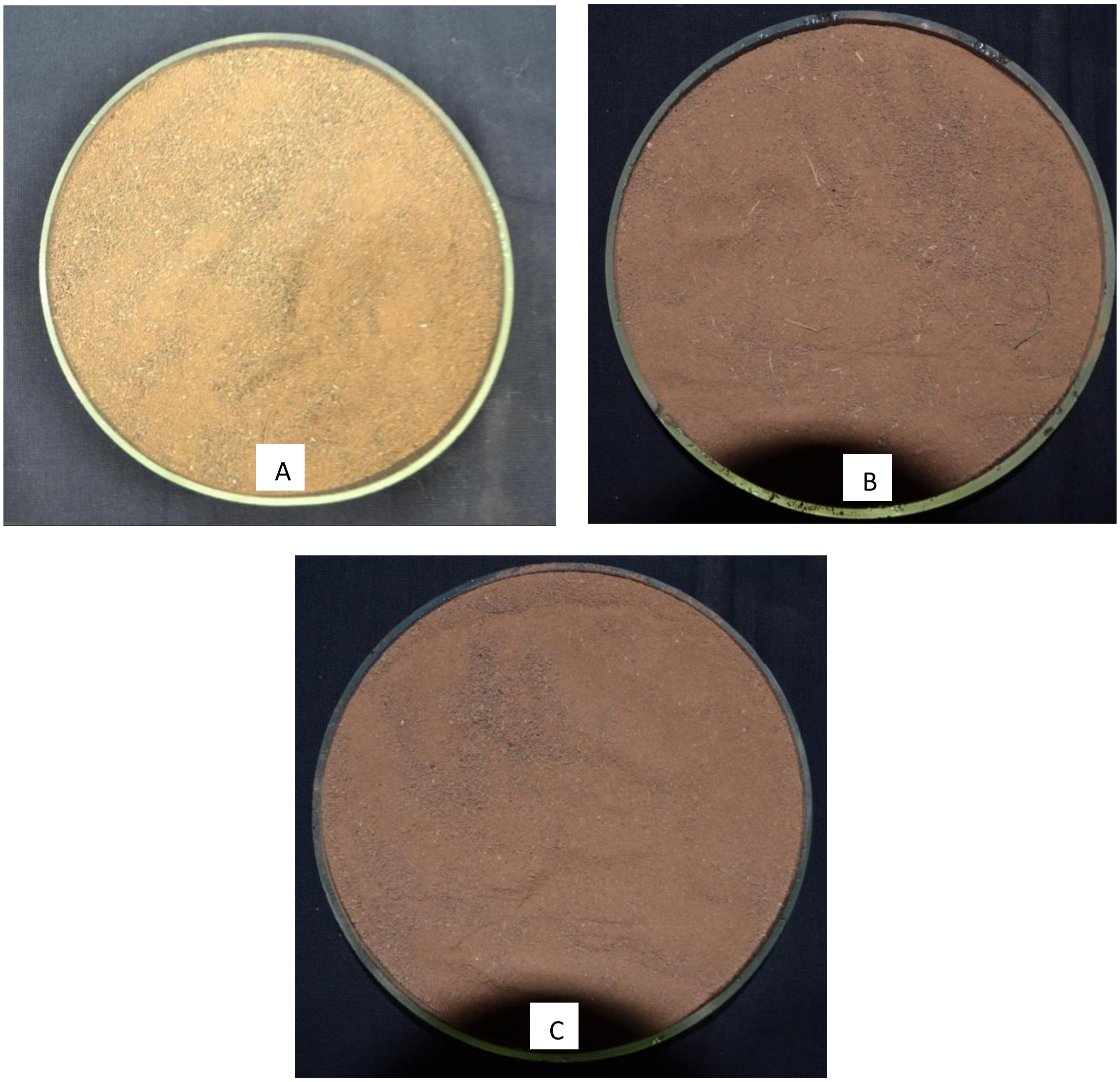


Plate 1: Soil sample collected from different locations for isolation of *Cellulomonas sp.* A: Soil sample collected from natural forest, B: Soil sample collected from industrial area, C: Soil sample collected from agricultural land

Table 2: List of different location where soil samples were collected for isolation of *Cellulomonas spp*.

Sl. No.	Name of the place	District	Habitat	Code
1.	Virajpate	Madikeri	Natural forest near tress	A
2.	Madikeri	Madikeri	Natural forest soil with grassland	B
3.	Paper Mill	Davangere	Soil near paper mill Industry (30m)	C
4.	Paper Mill	Davangere	Soil near paper mill Industry (50m)	D
5.	Nagarakera	Mandya	Soil from cultivable agricultural land grown with cowpea	E
6.	Kebbahally	Mandya	Soil from cultivable agricultural land grown with paddy	F

3.1.1.2 Pour plate method

Cellulose degrading bacteria were isolated from natural forest soil, industrial soil and agricultural waste soil. The isolates were cultured using Basal salt medium (Appendix I). Samples were diluted and transferred to sterile petriplate containing Basal salt medium and incubated for 3 days at 30^{0}C. The result showed different colonies containing cellulose degrading organisms. Each colony was counted by bacterial colony counter (counting small embedded colonies).

Approximately 1 ml of diluted (10^{-1}, 10^{-2}) samples were transferred to sterilized and labeled petriplates. Fifteen ml of melted and cooled upto soft hand touch (45 ^{0}C), medium poured into petriplates.

a) Procedure

1. One gram of soil sample was added to 10 ml of water in a test tube. That was taken as stock.
2. Mixed thoroughly to obtain uniform suspension and serial dilutions were carried out as mentioned upto 10^{-2}.
3. About 0.1 ml suspension was pipetted to labeled petriplates and poured the melted and cooled approximately 15 ml of Basal salt medium into petriplate.
4. The plates were incubated for 3 days at 30^{0}C till a discrete colony appeared.

b) Colonies were calculated in Colony Forming Unit (CFU)/g

$$\text{CFU/g} = \text{No. of CFU/Plate} \times \frac{\text{dilution factor}}{\text{aliquot taken}}$$

Bacterial colonies capable of utilizing cellulose as sole source of carbon were isolated on cellulose agar medium.

3.1.1.3 Purification of *Cellulomonas sp.* using streak plate method

a) Principle

The streak plate method is a rapid qualitative isolation method of microorganisms. The technique is commonly used for isolation of discrete colonies and it requires the number of microbial cell in the dilution was reduced. It was essentially a dilution technique that involves spreading a loopful of culture over the surface of cellulose agar plate. The resulting dilution of the population size ensures that the individual cells will be sufficiently far apart on the surface of the agar medium for easy separation of the different species present. Although different types of procedures were performed, the four ways or quadrant streak is mostly commonly done.

b) Procedure

1. The inoculating loop sterilized in the Bunsen burner. The loop was put onto the flame until it was red hot. Allowed it to cool.
2. An isolated colony picked from the Basal salt agar plate culture and spread it over the first quadrant (approximately 1/4 of the plate) using close parallel streaks. The loop flamed in the Bunsen burner.
3. The plate turned 90^0 and lightly swiped the loop 1 to 2 times through the inoculated area, then streaked into the next quadrant without overlapping the previous streaks. The loop flamed in the Bunsen burner.
4. The plate turned 90^0, overlapped the previous area 1 to 2 times, and streaked into the next quadrant as in step 4.
5. The loop flamed in the Bunsen burner.
6. The remaining plates were repeated with step 1 to 5.
7. The plates were inverted and incubated at 37^0C for 24 hr.

3.1.2 Characterization of *Cellulomonas sp*. from different sources

3.1.2.1 Colony characters

It was essential to record all the characters of the colony in order to familiarize with the colony which helped in instant detection of contamination or mutation in the colony.

Observations to be recorded were as follows: 1) Color, 2) Elevation, 3) Periphery, 4) Surface and 5) Growth of the colony.

3.1.2.2 Microscopic studies

3.1.2.2.1 Identification of isolated bacteria by Gram staining

The staining technique is based on the difference between the cell wall compositions of different bacteria. Bacterial cell wall may have higher lipid content or the protein content. The stains used in Gram staining have different affinity for these components and they bind with them reversibly or irreversibly. Hence, the stain bind irreversibly to the cell wall of Gram positive bacteria and cannot be decolourised by alcohol where as the stain bind reversibly to the cell wall of Gram negative bacteria and give it away when washed with water and alcohol. Then they take up the secondary stain (counter stain) and cells become pink in colour.

a) Principle

The difference in cell wall composition and affinity of stain to different cell walls was the principle.

b) Procedure

1. A clean microscope slide smeared and heat fixed with *Cellulomonas sp*. bacterial culture.

2. Two to three drops of Crystal Violet stain were put on the smear and allowed to stand for 30 seconds.
3. The Crystal violet stain rinsed off with slow running tap water.
4. Two to three drops of the Gram stain (potassium iodide) were put on the smear and allowed to stand for 30 seconds, then rinse off with tap water.
5. Two to three drops of 95% ethanol was added and allowed for 10 to 15 seconds. Wash with tap water.
6. The Saffranin (counter stain) placed on the smear and allowed to react for 20 seconds. Wash with tap water.
7. Observe the specimen under microscope (Olympus BX 51, Germany) at 100x (oil emersion) and determined whether the bacterial culture is positive or negative in Gram reaction.

3.1.2.3 Biochemical analysis

A) Confirmation of cellulose-degrading bacteria (*Cellulomonas sp.*)

Confirmation of cellulose-degrading ability of bacterial isolates was performed by streaking on the cellulose Congo-Red agar medium (Appendix I). The use of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic bacteria. Colonies showing discoloration of Congo-Red were taken as positive cellulose-degrading bacterial colonies (Gupta *et al.,* 2012).

Only positive cellulose-degrading bacterial isolates were collected for further study. Cellulose-degrading potential of the positive isolates was also qualitatively estimated by calculating hydrolysis capacity (HC).

$$\text{Hydrolysis Capacity (HC)} = \frac{\text{Diameter of clearing zone}}{\text{Diameter colony}}$$

B) Filter Paper Assay

The selected *Cellulomonas* isolates were cultured at 30^{0}C at 150 rpm in Basal salt medium (Appendix I) containing Whatman filter paper No.1 (1 × 6 cm strip, 0.105 g per 40mL) and pH was adjusted at 7. After three days of incubation period, the Broth culture was subjected to centrifugation at 5000 rpm for 15 min at 4^{0}C. Supernatant was discarded and Pellet recovered after centrifugation of broth culture was subjected to gravimetric analysis in order to determine the residual cellulose of filter paper.

a) Gravimetric Analysis

Gravimetric method of analysis was based on the measurement of mass of the residues filter paper. There are two gravimetric methods as precipitation method and volatilization method. In laboratory, precipitation method was used, in which the precipitate (cellulose) was converted to an insoluble product, filtered, washed and heated. The mass of the resulting residue was determined (Crampton *et al.,* 1938).

b) Procedure

1. The filter paper to be used for cellulose degradation by *Cellulomonas* was weighed and immersed in a broth.
2. The broth inoculated with *Cellulomonas sp.* followed by incubation for three days at 37^{0}C and 150 rpm.
3. After three days, the broth was centrifuged in centrifuge tube at 5000 rpm for 15 min at 4^{0}C.

4. Supernatant was discarded and precipitate was used for further analysis.
5. The mixture was filtered by using muslin cloth to separate the precipitate from the supernatant and the precipitate was obtained.
6. Acetic acid-nitric acid cell lysis buffer added to obtained precipitate for the removal of organism cell impurities (Add 15 ml of 80% acetic acid and 1.5 ml conc. $HN0_3$).
7. Centrifuged at 1000 rpm for 15 min.
8. Supernatant discarded and precipitate washed with 70 percent alcohol.
9. The precipitate was dried in hot air oven at 52^0C for 2 days.
10. The precipitate weighed.

c) Calculation

$$\text{Percent degradation of FP} = \frac{\text{Mass of precipitated FP}}{\text{Initial mass of FP}} \times 100$$

3.1.3 Preservation of cellulolytic bacterial strains

In order to identify cellulose degrading bacteria, the confirmed colonies were preserved in half strength of Nutrient Agar (NA) medium (Appendix I) after Gram staining was completed.

3.2 Diversity analysis of *Cellulomonas sp.* using RAPD

3.2.1 Isolation of total DNA from the isolates

a) Reagent preparation

Reagents required for isolation of DNA from *Cellulomonas sp*. was given in appendix- II

b) Procedure

DNA extraction protocol was followed according to Sambrook *et al.* (1989). Bacterial isolates were inoculated into Basal salt medium and incubated at 30^0C for three days under shaking at 150 rpm for growth. About 1.5 ml of culture was taken in microcentrifuge tube, spun for 7 minutes at 8,000 rpm and supernatant was discarded. In the microcentrifuge tube containing pellet, 567 µl of TE Buffer, 3 µl of 20 mg /ml proteinase-k, and 30 µl of 10 percent SDS were added and incubated for one hour at 37^0C. Again 100 µl of 5 M NaCl and 80 µl of CTAB solution were added and incubated for ten minute at 65^0C. Further it was extracted with equal volume of Chloroform: Isoamyl alcohol and the upper aqueous phase was transferred to the fresh tube and to this equal volume of Phenol: Chloroform: Isoamyl alcohol was added and subjected to centrifugation at 8,000 rpm for 5 min at 4^0C. It was washed with chloroform: Isoamyl alcohol until the clear supernatant was obtained. Then equal volume of chilled propanol was added, mixed gently and kept at -20^0C overnight for precipitation of DNA. Later centrifuged at 10,000 rpm for 20 minutes at 4^0C to pellet the DNA. The pellet was washed with 70 percent ethanol and air-dried. The DNA was dissolved in TE buffer and stored at 4^0C.

b) Quantification of total DNA By spectrophotometer

Total DNA was quantified by taking the OD readings by using the Nanodrop spectrophotometer at 260 nm as outlined by Sambrook and Russel (2001).

$$\text{Quality of sample} = \frac{\text{OD at 260nm}}{\text{OD at 280nm}}$$

If the value was between 1.4-1.6, it indicates DNA (can be used for PCR). 1.6 or more indicates RNA and <1.4 indicates protein.

3.2.2 PCR amplification condition

3.2.2.1 Selection of primers

For the selection of RAPD primers that can amplify informative sequences, primer screening was carried out using DNA obtained from the bacterial isolates (*Cellulomonas sp*.); four primers which were producing sharp, intense bands selected for the RAPD analysis.

Table 3: RAPD primers with sequences selected for analysis of *Cellulomonas sp.*

Sl. No.	Primer No.	Sequence
1.	Random primer 1	5'-CCA GGA GGA C-3'
2.	Random primer 2	5'-AGG TGA CCG T-3'
3.	Random primer 3	5'-GTG AGG CGT C-3'
4.	Random primer 4	5'-GGA GGG TGT T-3'

3.2.2.2 PCR reaction mixture

Template DNA	-	30 ng/µl
dNTPs	-	2 mM
Taq polymerase	-	3 U/µl
Primer	-	100 µM

10X *Taq* assay buffer A: 50mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris.HCl (pH 9.0), Gelatin 0.1%, 0.5% Triton-X100 and 0.05% NP_40.

30 ng of genomic DNA was used as the template for the standardization of PCR reactions and the PCR conditions were optimized to produce the reproducible and fine fingerprints. PCR reactions were performed in a final volume of 25 µl containing 30 ng of template DNA, 0.75 µl of 2 mM dNTPs each, 2.5 µl of 10X taq buffer, 0.36 µl of 3 unit/ µl of *Taq*

DNA polymerase, 3 µl of 10 picomoles primer. Amplifications were achieved in MWG-Biotech primus thermocycler with the program consisting initial denaturation of 94^0C for 3 min followed by 45 cycles each consisting of denaturation at 94^0C for 1 min, primer annealing temperature at 37^0C for 1 min, primer extension at 72^0C for 3 min, and a final extension of 72^0C for 10 min. These reactions were repeated to check the reproducibility of the amplification.

3.2.3 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to resolve the amplified products using 1.4 percent agarose in 1X TBE buffer, 0.5 µg/ml of ethidium bromide and loading buffer (0.25% Bromophenol Blue in 40% sucrose). 5 µl of the loading dye was added to 25 µl of PCR products and loaded to the agarose gel. Electrophoresis was carried at 65 V for 4.5 hour. The gel was visualized under UV light and documented using Gel Documentation unit.

3.2.4 Analysis of RAPD data

The bands were manually scored '1' for the presence and '0' for the absence and the binary data were used for statistical analysis. The scored band data (Presence or absence) was subjected to cluster analysis-using STATIST1CA. The dendrogram was constructed by Ward's method of clustering using minimum variance algorithm. The dissimilarity matrix was developed using Squared Euclidean Distance (SED), which estimated all the pair wise differences in the amplification product (Sokal and Sneath, 1973). Only clear and unambiguous bands were taken into account and the bands were not scored if they were faint or diffused, as such fragments posses poor reproducibility. The band sizes were determined by comparing with the 1000 bp DNA ladder, which was run along with the amplified products. The Genetic distance was computed as:

$$\Sigma^{n}=I \quad dj^{2} \qquad \text{where } dj = (X_{ik}-X_{jk})$$

Where X_{ik} refers to binary code of i^{th} tree for allele "k" and X_{jk} refers to the binary code of the j^{th} tree for allele "k". Dendrogram was computed based on Ward's method of clustering, using minimum variance algorithm (Ward, 1963).

Experimental Results

IV. EXPERIMENTAL RESULTS

This chapter describes the result of various experiments conducted on "isolation and molecular characterization of *Cellulomonas* from different sources".

4.1 Isolation of *Cellulomonas* from different sources

4.1.1 Pour Plate Method

Maximum count of cellulose degrading bacteria (7.2 x 10^3 CFU/g) was noticed in the soil sample collected from the regions of natural forest (Madikeri) followed by soil samples from paper mill industry (Davangere) (6.7 x 10^3 CFU/g) and lowest in soil of cultivable agricultural land, Kebbahally, Mandya (1.0 x 10^3) (Table 4).

4.1.2 Purification of *Cellulomonas sp.* using Streak Plate Method

The result of the study shown that 40-70 single *Cellulomonas* colony was identified (Plate 2a, 2b). Based on characters, cellulolytic bacterial colonies (creamy white colonies with zone of clearance) were selected and further purified on Cellulose Agar Medium. These colonies were further streak on Cellulose Agar Medium and incubated for 3 days at 30^0C.

4.1.3 Microscopic observation

4.1.3.1 Colony character

Colony characters were used for primary identification of cellulolytic bacteria which produced different types of colonies on Cellulose Agar medium. Some colonies with pale yellow color were spreaded along the plate which was not isolated and the other colonies with creamy white color with zone of clearance were produced (Plate 3, 4, 5, 6, 7, 8) which referred for selection. Based on these characters,

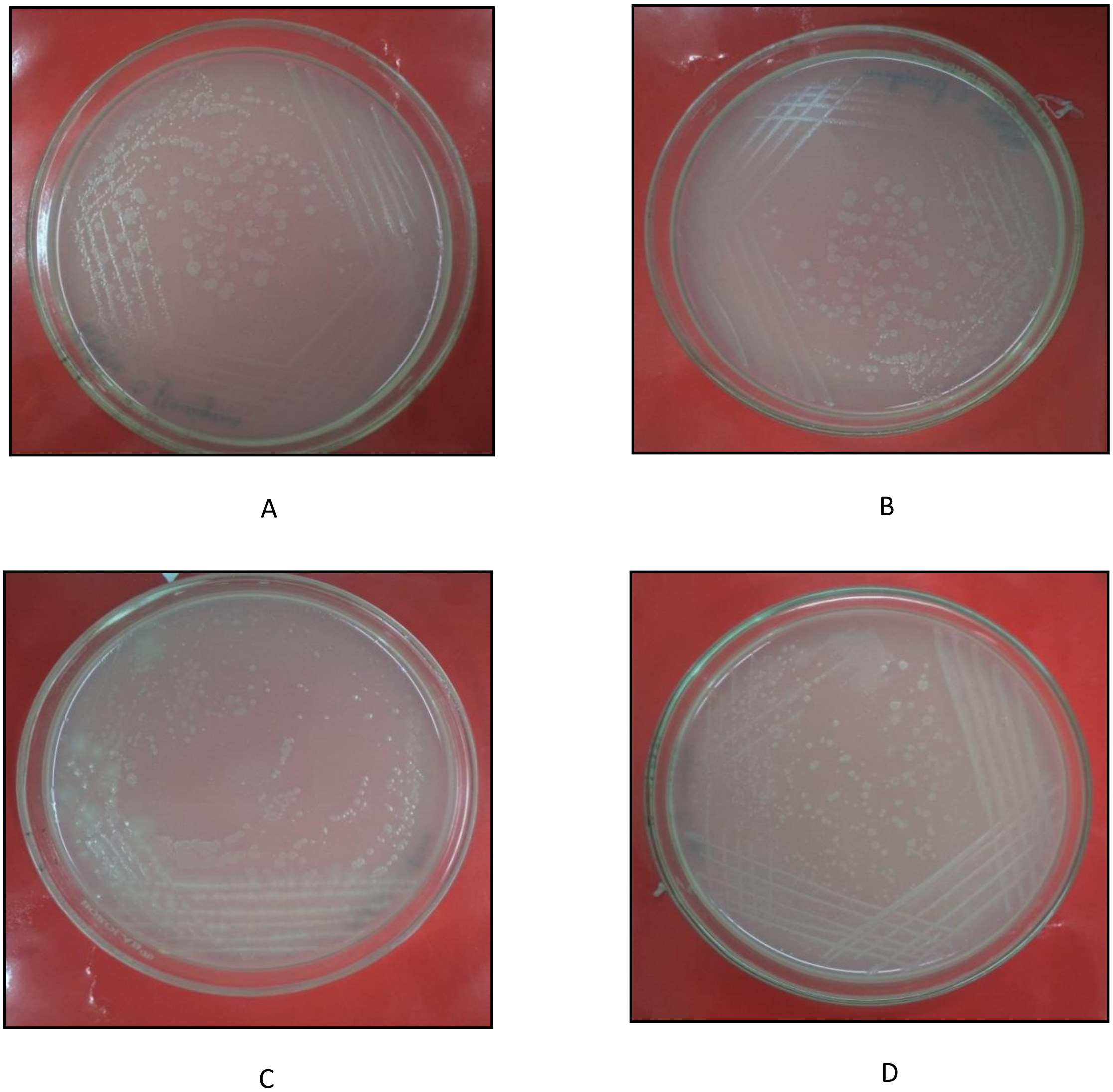


Plate 2a: Purification of *Cellulomonas sp.* (creamy white colonies with zone of clearance) using streak plate method *on* Cellulose Agar Media.

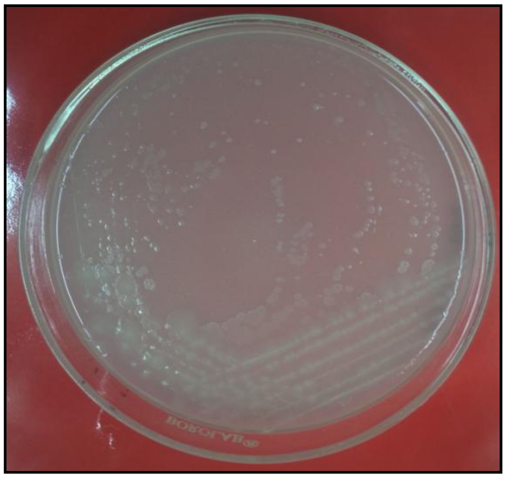
Legend:

A : Soil collected from natural forest near tress, Madikeri

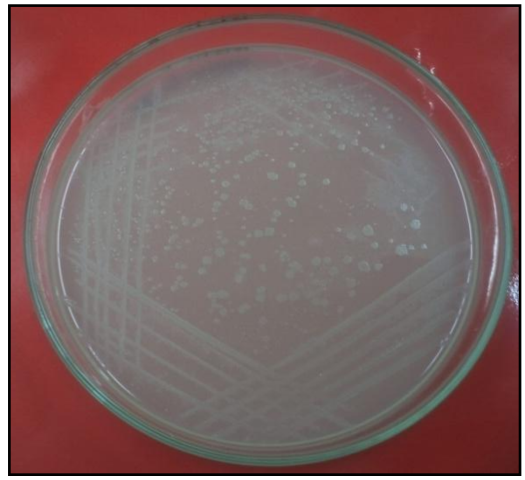
B : Soil collected from natural forest soil with grassland, Madikeri

C : Soil collected from 30 meter away from paper mill Industrial, Davangere

D : Soil collected from 50 meter away from paper mill Industrial, Davangere



E



F

Plate 2b: Purification of *Cellulomonas sp.* (creamy white colonies with zone of clearance) using streak plate method *on* Cellulose Agar Media.

Legend:

E : Soil of cultivable agricultural land grown with cowpea, Nagarakera, Mandya

F : Soil of cultivable agricultural land grown with paddy, Kebbahally, Mandya

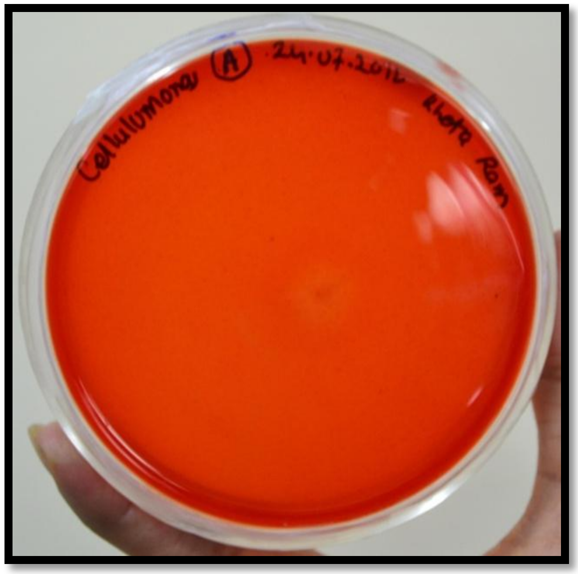


Plate 3: **Zone of clearance of Cellulose Congo-Red Agar plate for isolate A: Soil collected from natural forest near tress, Madikeri, after 72 hours of incubation. The formation of clearing zone around colonies confirms the secretion of the extracellular cellulase.**

Plate 4: **Zone of clearance of Cellulose Congo-Red Agar plate for isolate B: Soil collected from natural forest soil with grassland, Madikeri, after 72 hours of incubation. The formation of clearing zone around colonies confirms the secretion of the extracellular cellulase.**

Plate 5: Zone of clearance of Cellulose Congo-Red Agar plate for isolate, C: Soil collected from 30 meter away from paper mill Industrial, Davangere after 72 hours of incubation. The formation of clearing zone around colonies confirms the secretion of the extracellular cellulase.

Plate 6: Zone of clearance of Cellulose Congo-Red Agar plate for isolate, D: Soil collected from 50 meter away from paper mill Industrial, Davangere after 72 hours of incubation. The formation of clearing zone around colonies confirms the secretion of the extracellular cellulase.

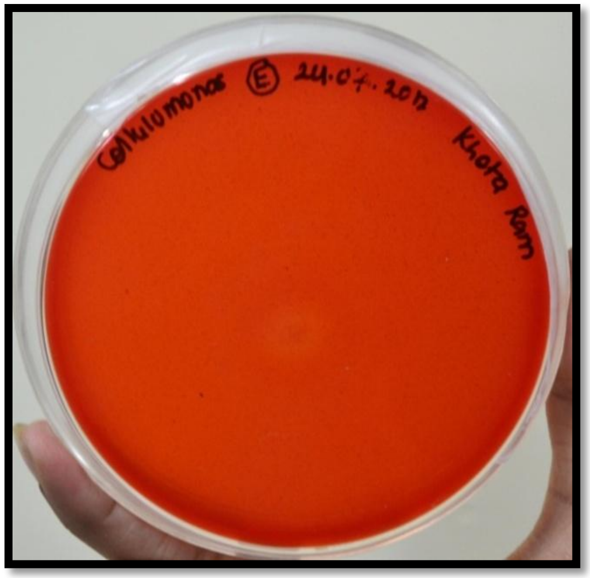


Plate 7: Zone of clearance of Cellulose Congo-Red Agar plate for isolate E: Soil of cultivable agricultural land grown with cowpea, Nagarakera, Mandya, after 72 hours of incubation. The formation of clearing zone around colonies confirms the secretion of the extracellular cellulase.

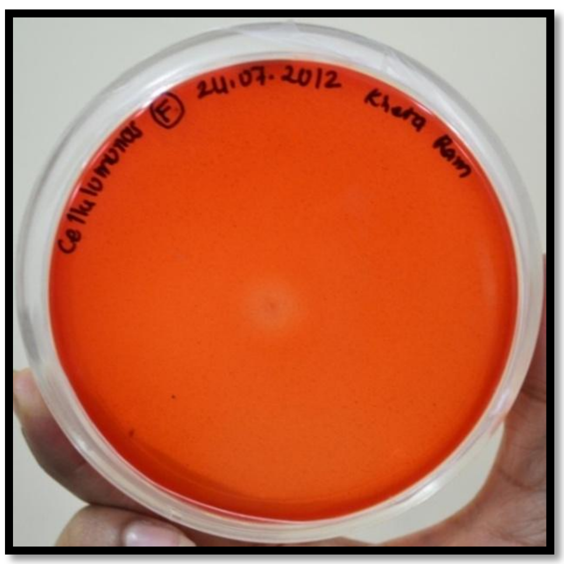


Plate 8: Zone of clearance of Cellulose Congo-Red Agar plate for isolate F: Soil of cultivable agricultural land grown with paddy, Kebbahally, Mandya after 72 hours of incubation. The formation of clearing zone around colonies confirms the secretion of the extracellular cellulase.

cellulolytic bacterial colonies were selected and further purified on Cellulose agar medium (Table 5).

4.1.3.2 Gram Staining

Gram Staining and microscopic study were carried out for the analysis morphologically and phenotypic characteristics of *Cellulomonas spp.* All the 6 *Cellulomonas sp.* isolates produced violet colour when subjected to gram staining. This indicates that 100 percent of the isolates used in the study were gram positive (Plate No. 9)

4.1.4 Biochemical Analysis

4.1.4.1 Congo red cellulose agar medium

The experiment was carried out to study of zone of clearance around *Cellulomonas* colony. *Cellulomonas* was inoculated on modified Cellulose Congo-Red agar medium (Appendix I) and incubated for 3 days at 30^0C. The result (Table 6) showed that cellulose degradation was directly related to Zone of clearance. Maximum colony diameter (3.6 mm) of *Cellulomonas* was noticed in case of sample D, while maximum zone (17.0 mm) of clearance observed in sample B as well as C. Hydrolysis capacity was highest in sample B (6.61)(Figure 2).

4.1.4.2 Filter paper assay for cellulose degradation

Maximum extent of degradation of filter paper was noticed in the sample B (52.9 %), whereas lowest percentage of the same was observed in the case of sample D (24.5 %) (Table 7, Figure 3 and Plate 10).

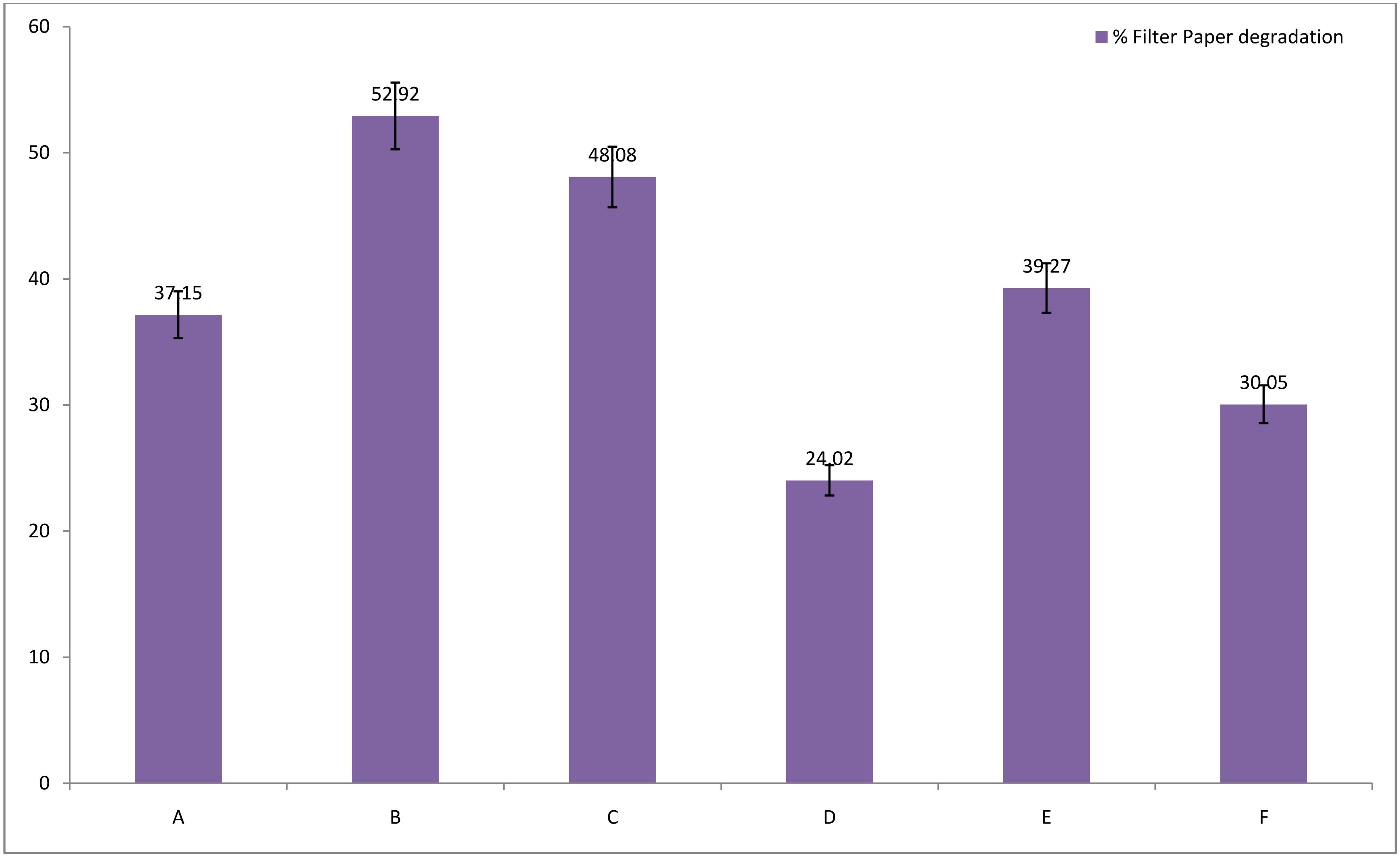


Figure 2: Percent Filter Paper degradation by *Cellulomonas spp.* isolate

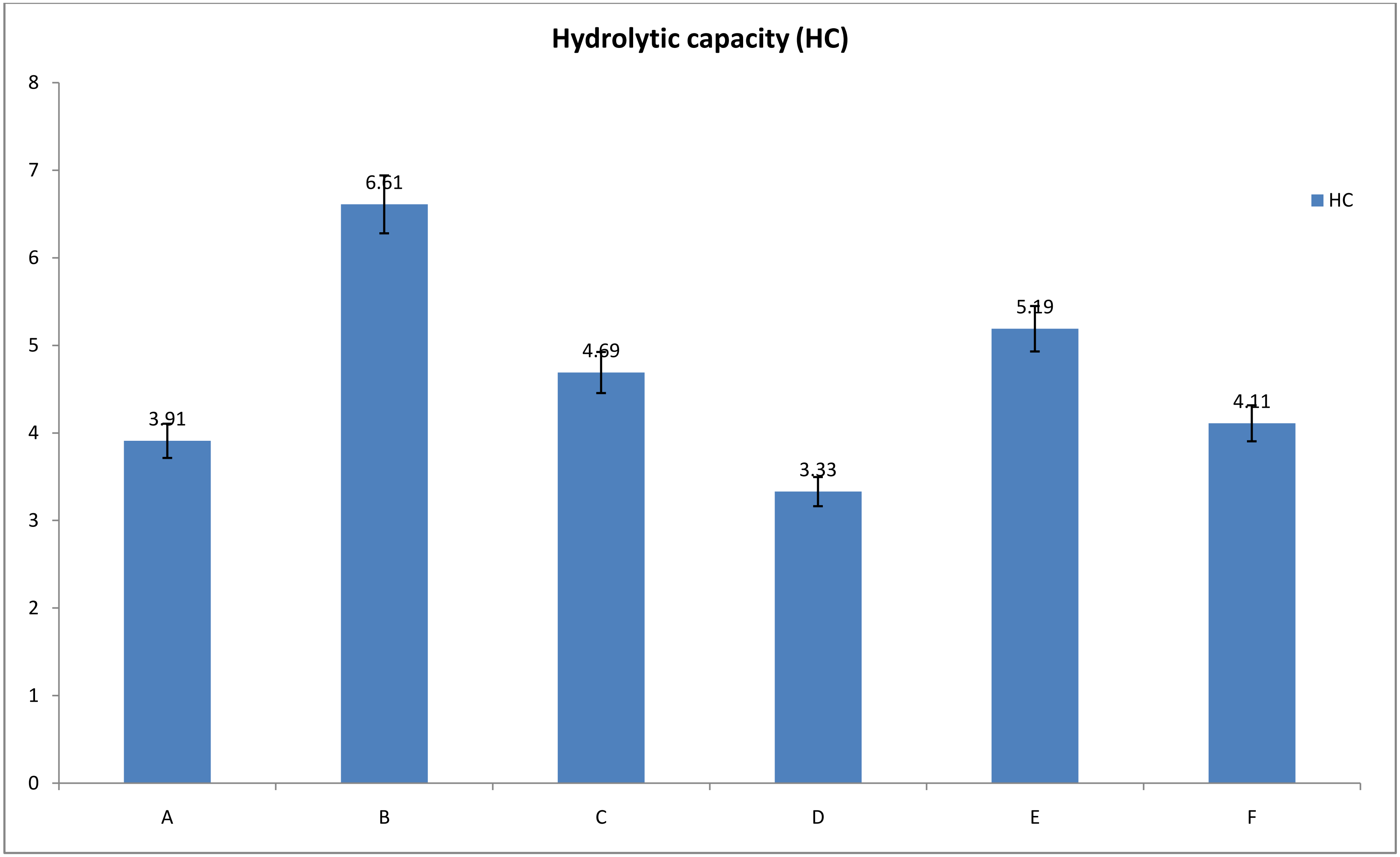
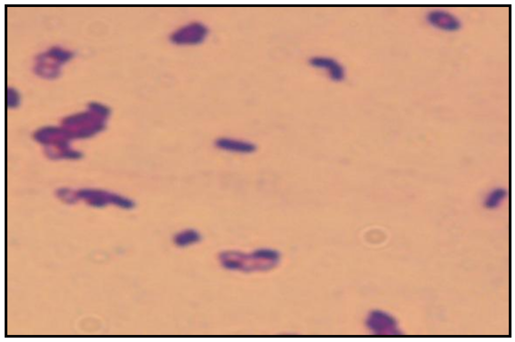
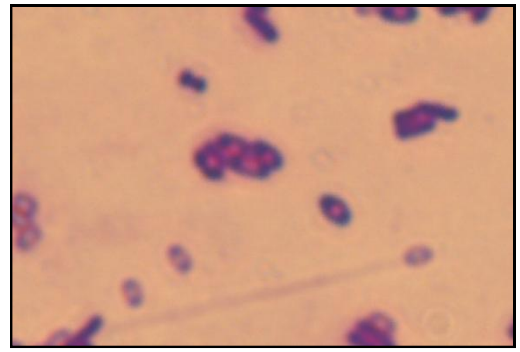


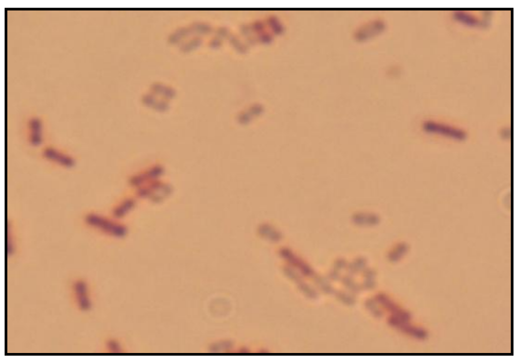
Figure 3: Hydrolytic capacity by *Cellulomonas sp.* isolate



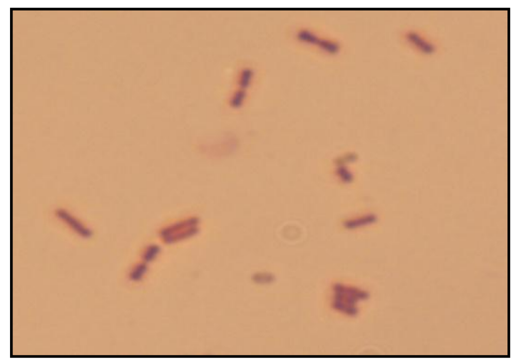
A



B



C



D

Plate 9a: *Cellulomonas* observed under microscope (Olympus BX 51, Germany) at 1000x (oil emersion)

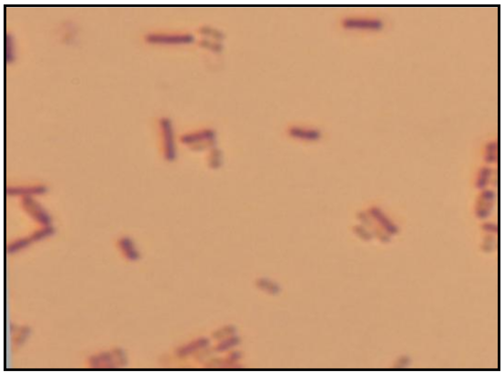
Legend:

A : Soil collected from natural forest near tress, Madikeri

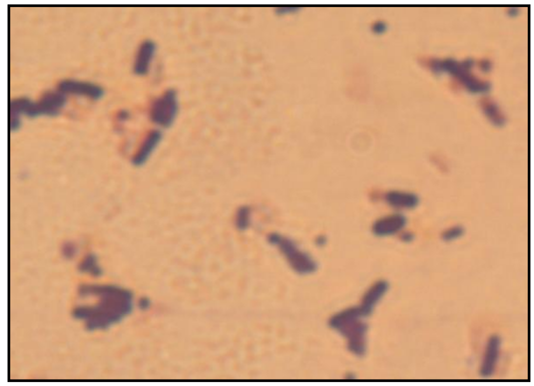
B : Soil collected from natural forest soil with grassland, Madikeri

C : Soil collected from 30 meter away from paper mill Industrial, Davangere

D : Soil collected from 50 meter away from paper mill Industrial, Davangere



E



F

Plate 9b: *Cellulomonas* observed under microscope (Olympus BX 51, Germany) at 100x (oil emersion)

Legend:

E : Soil of cultivable agricultural land grown with cowpea, Nagarakera, Mandya

F : Soil of cultivable agricultural land grown with paddy, Kebbahally, Mandya

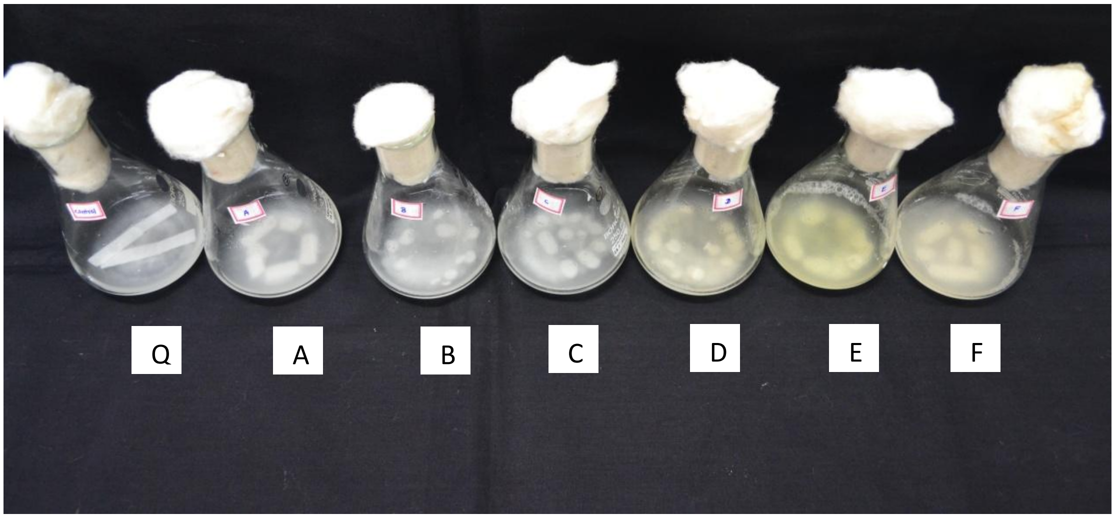


Plate 10: Growth of *Cellulomonas* on filter paper for biochemical analysis on cellulose degradation, Q (Control

Legend:

Q : Control

A : Soil collected from natural forest near tress, Madikeri

B : Soil collected from natural forest soil with grassland, Madikeri

C : Soil collected from 30 meter away from paper mill Industrial, Davangere

D : Soil collected from 50 meter away from paper mill Industrial, Davangere

E : Soil of cultivable agricultural land grown with cowpea, Nagarakera, Mandya

F : Soil of cultivable agricultural land grown with paddy, Kebbahally, Mandya

Table 4: Population of cellulose degrading micro-organisms per gram soil sample from soil habitats

Sl. No.	Sample No.	CFU/ g (x 10^2)
1.	A	46.0[c]
2.	B	72.0[a]
3.	C	62.0[b]
4.	D	39.0[d]
5.	E	42.0[dc]
6.	F	10.3[e]
	S-Em+/-	1.58
	CD @ 5%	4.87

Legend:

A : Soil collected from natural forest near tress, Madikeri

B : Soil collected from natural forest soil with grassland, Madikeri

C : Soil collected from 30 meter away from paper mill Industrial, Davangere

D : Soil collected from 50 meter away from paper mill Industrial, Davangere

E : Soil of cultivable agricultural land grown with cowpea, Nagarakera, Mandya

F : Soil of cultivable agricultural land grown with paddy, Kebbahally, Mandya

Note: Similar alphabets don’t differ significantly.

Table 5: Colony Characteristics of *Cellulomonas sp.* on Cellulose Agar Medium isolated from different habitats observed after 72 hrs

Sl. No.	Sample No.	Colony color	Colony Periphery	Colony Elevation	Colony surface
1.	A	Creamy white	Irregular	flat	Rough
2.	B	Creamy white	Irregular	flat	Rough
3.	C	Creamy white	Irregular	flat	Rough
4.	D	Creamy white	Irregular	flat	Rough
5.	E	Creamy white	Irregular	flat	Rough
6.	F	Creamy white	Irregular	flat	Rough

Legend:

A : Soil collected from natural forest near tress, Madikeri

B : Soil collected from natural forest soil with grassland, Madikeri

C : Soil collected from 30 meter away from paper mill Industrial, Davangere

D : Soil collected from 50 meter away from paper mill Industrial, Davangere

E : Soil of cultivable agricultural land grown with cowpea, Nagarakera, Mandya

F : Soil of cultivable agricultural land grown with paddy, Kebbahally, Mandya

Table 6: Hydrolysis capacity of *Cellulomonas* isolates from soil habitats

Sl. No.	Sample	Average colony diameter (mm)	Average zone of clearance (mm)	Hydrolysis capacity (HC)
1.	A	3.3	13.0	3.91
2.	B	2.6	17.0	6.61
3.	C	3.3	17.0	4.69
4.	D	3.6	13.1	3.33
5.	E	2.6	13.1	5.19
6.	F	3.3	12.1	4.11
	CD @ 5%			1.72
	S-Em+/-			0.56

Legend:

A : Soil collected from natural forest near tress, Madikeri

B : Soil collected from natural forest soil with grassland, Madikeri

C : Soil collected from 30 meter away from paper mill Industrial, Davangere

D : Soil collected from 50 meter away from paper mill Industrial, Davangere

E : Soil of cultivable agricultural land grown with cowpea, Nagarakera, Mandya

F : Soil of cultivable agricultural land grown with paddy, Kebbahally, Mandya

Table 7: Percent filter paper (FP) degradation by various *Cellulomonas spp.* isolate

Sl. No.	Sample	Initial wt. of FP (mg)	wt. of FP after degradation (mg)	Percent degradation filter paper
1.	A	105	68	37.15[dc]
2.	B	102	48	52.92[a]
3.	C	103	54	48.08[b]
4.	D	102	79	24.02[f]
5.	E	101	61	39.27[c]
6.	F	102	72	30.05[e]
	S-Em+/-			1.29
	CD @ 5%			3.99

Legend:

A : Soil collected from natural forest near tress, Madikeri

B : Soil collected from natural forest soil with grassland, Madikeri

C : Soil collected from 30 meter away from paper mill Industrial, Davangere

D : Soil collected from 50 meter away from paper mill Industrial, Davangere

E : Soil of cultivable agricultural land grown with cowpea, Nagarakera, Mandya

F : Soil of cultivable agricultural land grown with paddy, Kebbahally, Mandya

Note: Similar alphabets don't differ significantly.

4.2 Standardization of protocol for RAPD analysis

4.2.1 Amplification conditions

For fingerprinting and diversity analysis, PCR amplification conditions were optimized based on the protocol outlined by Abbas *et al.* (1996) with minor modifications.

In order to obtain higher amplification rate and reproducible banding patterns, reaction trials were optimized for different durations for hot start, temperatures for denaturation, primer annealing and primer extension steps. The PCR reaction was evaluated for 30, 40 and 45 cycles using standard buffer as outlined in Material and Methods. The optimum conditions for each cycle of PCR were developed for obtaining high amplification levels. The optimum PCR conditions consisted of the following steps.

Initial strand separation or hot start at 94^0C for three minute followed by 45 cycles

i. Denaturation at 94^0C for 3 minutes.
ii. Primer annealing at 37^0C for 30 seconds,
iii. Primer extension at 72^0C for 1 minute and
iv. Final extension period at 72^0C for 10 minute.

4.3.2 Reaction parameters

In order to produce informative and reproducible RAPD fingerprints, it is important to optimize the concentration of PCR mixture. Hence, different concentrations of template DNA (10-15 ng, 25-30 ng, 40-50 ng) were tried with similar amplification conditions. A concentration of 25-30 ng of template DNA and 2 mM of dNTPs per reaction were found to be optimum for obtaining intense, clear and reproducible banding patterns in

Cellulomonas isolates. In all these reaction cases, 4 µl of 10 picomoles of primer and 0.36 µl of 3 unit/µl of *Tag* polymerase per reaction were used. However, small fluctuation in the concentration of template DNA was affected the amplification i.e. with too little DNA (10-15 ng) causing either reduced or no amplification of small fragments (Table 8).

4.3.2 RAPD Characterization

The total 20 RAPD bands produced from the selected 4 primers were used for fingerprinting and estimation of genetic diversity among six isolated samples of *Cellulomonas sp*. For the purpose of illustration, the RAPD fingerprints or electrophore gram generated for 6 *Cellulomonas sp.* isolates using four primers were presented in plates 11-14.

The numbers of bands were scored for each primer that varied from 1 to 7 with an average 5 bands per primer. Out of 20 amplification bands, 5 bands (25%) were monomorphic, 3 bands (15%) were unique and 12 bands (60%) were shared polymorphic, which were informative in revealing the relationship among the genotype (Table 9).

4.3.3 Cluster analysis and genetics distance of 6 *Cellulomonas sp.* isolates

The cluster analysis based on 20 RAPD bands revealed that the six C*ellulomonas sp.* isolates were examined. The dendrogram (Figure 4) obtained from RAPD analysis clearly indicated that all the six different *Cellulomonas* isolates formed two major clusters. Among the two isolates, A, E, F formed the first cluster and isolates B, D, C formed the second major cluster. In first group, there was one cluster and one isolate with linkage distance from 1.75 to 2.25 and second group with linkage distance 1.75 to 1.90. This indicated the genetic similarity and difference between the *Cellulomonas* isolates which selected for investigation.

Table 8: Optimum concentration and conditions of PCR for RAPD analysis

Sl. No.	Variable	Conditions/ concentration	
		Evaluated	Optimum
1.	**PCR amplification**		
A.	Hot start (94^0C)	2min, 3min, 4min.	3min,
B.	Denaturation (94^0C)	30sec, 1min, 1.5min.	1min,
C.	Annealing (37^0C)	1min, 1.5min, 2.0min	1min,
D.	Extension(72^0C)	1min, 1.5min, 2.0min, 3min	2min,
E.	Number of cycles	35,40, 45 cycles	45 cycles
2.	**RAPD Protocol**		
A.	Template DNA	10-15ng,25-30ng,40-50ng	25-30ng
B.	DNTPs	1.5mM, 2mM, 2.5mM	2mM

Table 9: Oligonucleotide primer that showed genetic variation among the *Cellulomonas sp.* isolates

Primers	Number of amplified fragment	Number of Polymorphic bands		Number of Monomorphic bands
		Shared	Unique	
Random primer 1	6	3	0	3
Random primer 2	5	2	1	2
Random primer 3	7	5	2	0
Random primer 4	2	2	0	0
Total	20	12	3	5
Percentage		60%	15%	25%

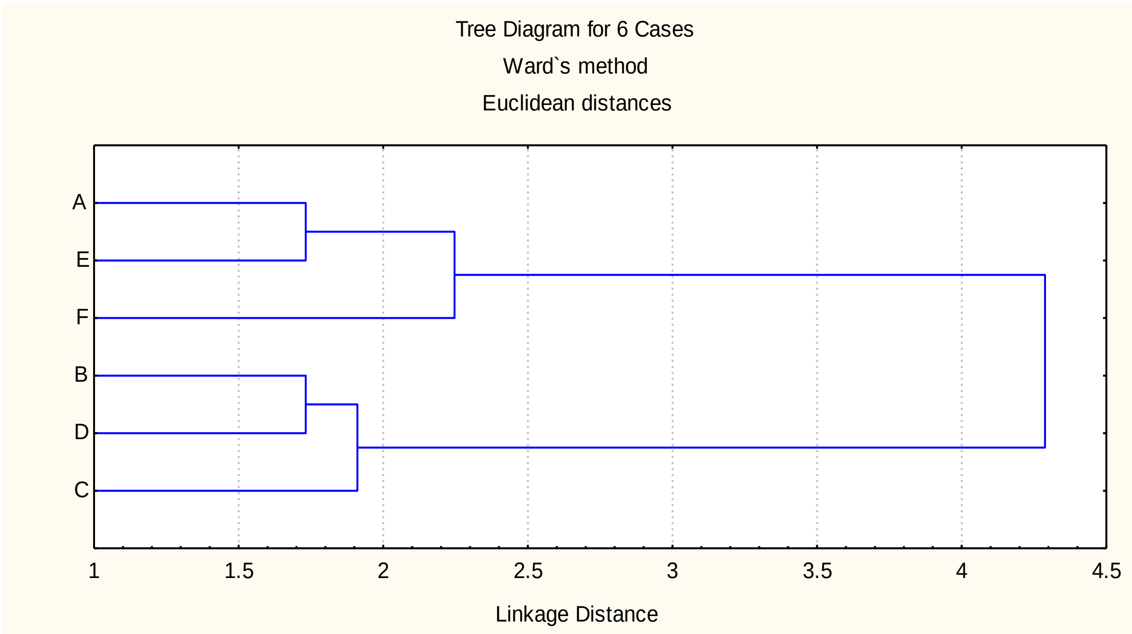


Figure 4: Dendrogram based on RAPD profile of 6 *Cellulomonas sp.* isolated from three different sites with two samples from each site

Legend:

A : Soil collected from natural forest near tress, Madikeri

B : Soil collected from natural forest soil with grassland, Madikeri

C : Soil collected from 30 meter away from paper mill Industrial, Davangere

D : Soil collected from 50 meter away from paper mill Industrial, Davangere

E : Soil of cultivable agricultural land grown with cowpea, Nagarakera, Mandya

F : Soil of cultivable agricultural land grown with paddy, Kebbahally, Mandya

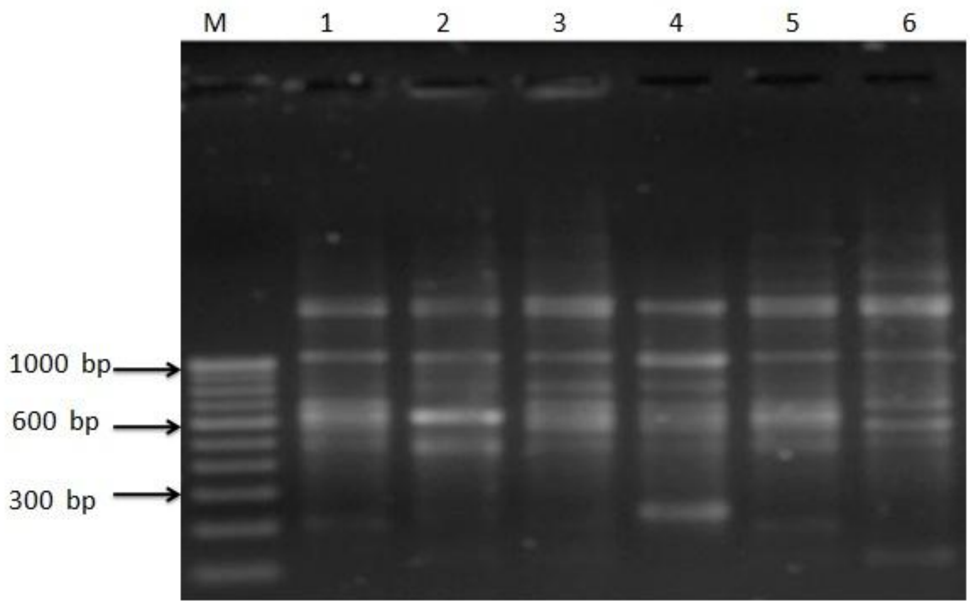


Plate 11: RAPD GEL profile of cellulolytic bacteria isolates generated using 10-mer random primer no. 1. M: marker lane (100 to 1000 bp) and lane 1 to 6 represent.

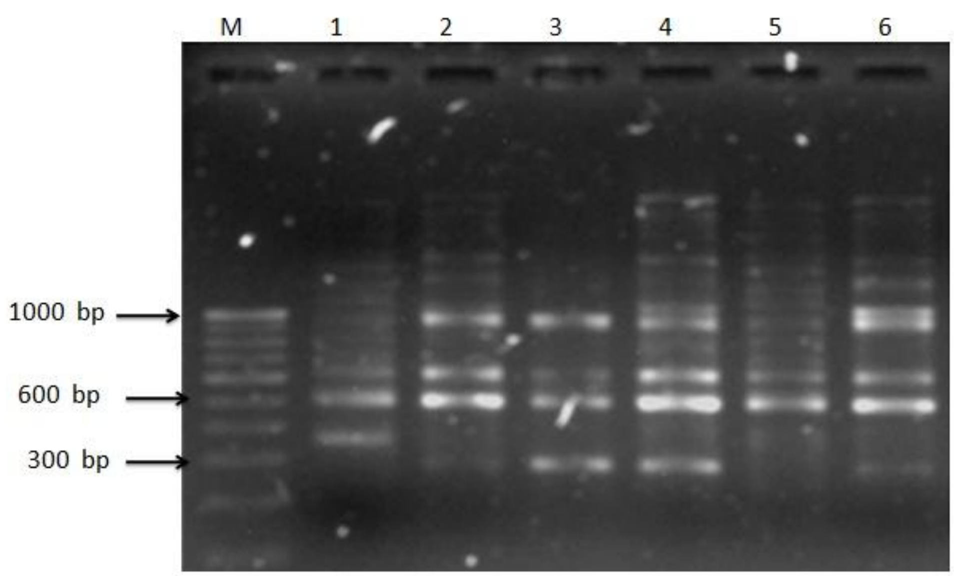


Plate 12: RAPD GEL profile of cellulolytic bacteria isolates generated using 10-mer random primer no. 2. M: marker lane (100 to 1000 bp) and lane 1 to 6 represent isolates

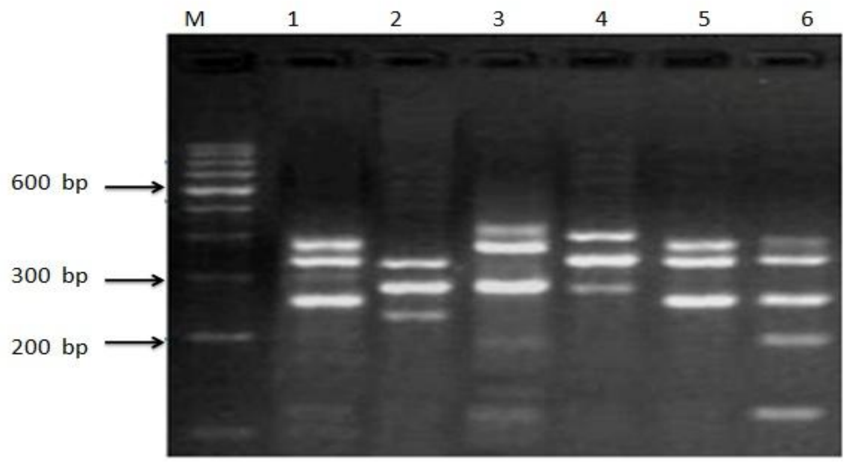


Plate 13: RAPD GEL profile of cellulolytic bacteria isolates generated using 10-mer random primer no. 3. M: marker lane (100 to 1000 bp) and lane 1 to 6 represent isolates

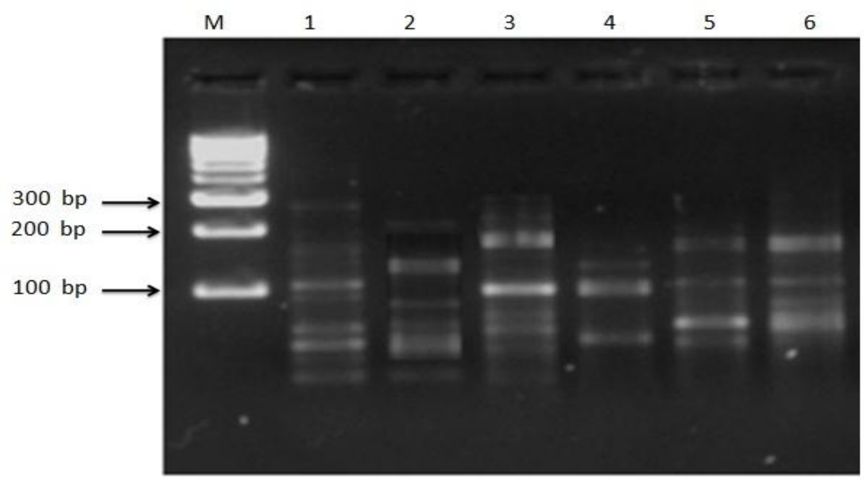


Plate 14: RAPD GEL profile of cellulolytic bacteria isolates generated using 10-mer random primer no. 4. M: marker lane (100 to 1000 bp) and lane 1 to 6 represent

A: Soil collected from natural forest near tress, Madikeri; B: Soil collected from natural forest soil with grassland, Madiker; C: Soil collected from 30 meter away from paper mill Industrial, Davangere; D: Soil collected from 50 meter away from paper mill Industrial, Davangere; E: Soil of cultivable agricultural land grown with cowpea, Nagarakera, Mandya; F: Soil of cultivable agricultural land grown with paddy, Kebbahally, Mandya

Discussion

V. DISCUSSION

Cellulomonas is an aerobic bacterium, which degrade the cellulose and hemicellulose into disaccharide (cellobiose). It finds on cellulose rich materials like decaying leaf, agro-waste material *etc*. (Dworkin *et al*., 2006).

Cellulose is the most abundant biopolymer on the earth (Levy and Shoseyov, 2002). Cellulose constitutes one-third to one-half of the approximately 150 billion ton of organic matter synthesized annually (Shewale and Sadana, 1978; Bayer and Moray, 1994). Degradation of cellulose is commonly catalyzed by an enzyme called cellulase. This enzyme is produced by several microorganisms, mainly by bacteria and fungi (Bahkali, 1996; Immanuel *et al.,* 2006; Mangelli and Forchiassin, 1999; Shin *et al.,* 2000). Cellulolysis is a relatively slow process as compared with most other enzymatic reactions (Stewart and Leatherwood, 1976). Cellulolysis is the biological process controlled and processed by the enzymes of cellulase system. Cellulase enzyme system comprises three classes of soluble extracellular enzymes: 1,4-β-endoglucanase, 1,4-β-exoglucanase and β-glucosidase (β-D-glucoside glucohydrolase or cellobiase). Endoglucanase is responsible for random cleavage of β-1,4-glycosidic bonds along the cellulose chain. Exoglucanase is necessary for cleavage of the non-reducing end of a cellulose chain and splitting of the elementary fibrils from the crystalline cellulose, and β-1, 4-glucosidase hydrolyses cellobiose and water-soluble cellodextrin to glucose (Shewale, 1982; Woodward and Wiseman, 1983).

5.1 Isolation and Characterization of *Cellulomonas sp*. from different sources

Cellulomonas sp. isolated from soil collected from the natural forest, paper mill industry and cultivable agricultural field and studies were conducted to isolate *Cellulomonas* from these soil samples (Kellerman *et al.,* 1913), cellulose-enriched environments such as bark and wood (Przybyl, 1979 and Deschamps, 1982), sugar fields (De Leon and Joson, 1980), activated sludge (Ramasamy *et al.,* 1981), paper mill samples and sewage samples (Mullings and Paris, 1984), rumen (Lee and Lee, 1986), forest soils (Malekzadeh *et al.,* 1993), coffee cherries (Silva *et al.,* 2000), soils enriched by flax or sisal fibers (Lednicka *et al.,* 2000), organically rich soils (Collins and Pascual, 2000) and agricultural soil samples (Sangkharak *et al.,* 2011).

Isolation of *Cellulomonas* was carried out on Basal Salt Medium supplemented with cellulose powder (2 g/l) and similar medium used by Yoon *et al.,* 2008; Gupta *et al.* 2012 and Whittle *et al.* 1982 for isolation of cellulolytic microorganisms. Medium was supplemented with 0.02 % bavistin to inhibit the growth of fungi and same study carried by Lednicka, 2000, medium supplemented with 0.02 % cycloheximide to inhibit the growth of fungi.

Result of pour plate method showed that the population of cellulolytic bacteria in the soil was varied between 7.2×10^3 and 1.0×10^3/g of soil sample. The population of cellulolytic bacteria was observed less in samples due antifungal agent (bavistin) compared to non treated sample bavistine may be checked the growth of bacteria. Singh and Jain, 1986 studied the population of cellulolytic bacteria and found that bacteria population varied between 2.0×10^3 and 1.4×10^5/g slurry (Singh and Jain, 1986).

Colony formation on Cellulose Congo-Red Agar Medium observed after incubation period of 72 hours at 30^0C and observed clear zones (where the medium pigment turned red to light red colour). Stewart and Leatherwood, (1976) studied colony formation after 24 to 48 h of incubation and observed clear zones. Teather and Wood, (1982);

Prasertsan and Doelle, (1986); Singh *et al*. (2010) observed clear zones after 2 to 3 days. The colour of the colony varied from cream to yellow depending on the growth medium and on the age of the culture.

Cellulomonas is gram positive bacteria but it is very sensitive to the counter stain and discoloring agent therefore, it sometime appears pink coloured. Abt *et al.* (2010) concluded that *Cellulomonas fimi* was stained Gram positive with a very fast rate of decolorization.

The ratio of diameter of clearing zone and colony is known as hydrolysis capacity (HC). In case of hydrolysis capacity of *Cellulomonas sp.,* cellulose degradation is directly related to zone of clearance as same observations were noticed by Gupta *et al.* (2012).

Maximum extent of degradation of filter paper was noticed in the sample B (52.9 %), whereas lowest was observed in case of sample D (22.5 %). The cellulolytic bacterial colonies were identified by the circular clearings formed by the action of cellulase enzyme, secreted by the bacteria. The number of clearings gives a direct estimation of the number of cellulolytic bacteria in the sample (Gylswyka, 1970).

5.2 RAPD characterization

The total 20 RAPD bands were produced from the selected 4 primers which were used for fingerprinting and estimation of genetic diversity among six isolated samples of *Cellulomonas sp*. The number of bands scored for each primer varied from 1 to 7 with an average of 5 bands per primer. Out of 20 amplification bands, 5 bands (25%) were monomorphic, 3 bands (15%) were unique and 12 bands (60%) were shared polymorphic which were informative in revealing the relationship among the isolates. Statistical analysis of RAPD data enabled to the classify the six *Cellulomonas sp.* isolates into two major isolates A, E, F formed the first cluster and isolates B, D, C formed the second major cluster. This indicated the genetic similarity and difference between the *Cellulomonas sp.* isolates which were used for investigation.

Kumar *et al.* (2008) carried out RAPD analysis in seventy isolates of *Bacillus thuringiensis,* isolated from cotton fields. When the RAPD banding pattern data was subjected to dendrogram construction, the 70 isolates fell into two separate clusters, cluster I and cluster II, which included 26 and 44 *B. thuringiensis* isolates, respectively. Those two main clusters were further divided into four sub-clusters at Euclidian distance of 150 and 80 % similar index.

Archana *et al*. (2007) carried out genetic diversity evaluation of *Bacillus* isolates using Random Amplified Polymorphic DNA (RAPD) molecular marker. Twenty isolates were selected and characterized by various biochemical tests and enzyme production. Out of twenty RAPD screened, 18 primers were found to be polymorphic. The fingerprints obtained in all the 18 primers remarkably indicated the polymorphic bands. From the dendrogram generated using the NTSYSpc programme, the genetic diversity was evident based on the percentage similar between the isolates. Five major clusters were obtained at 61% similarity level.

Future line of work

These *Cellulomonas spp.* isolate further should be confirm by 16 sRNA Analysis. *Cellulomonas sp.,* the best isolates in this study should be tested for their efficiency to produce exocellulase to degrade cellulose entity from various agricultural biomasses, paper mill industrial, cotton wool mills and sugarcane industrial byproducts into disaccharides (cellobiose). Further, it can be used as a substrate in the production of bioethanol with the help of yeast (*Saccharomyces cerevisiae*). In future, existing *Cellulomonas sp*. strains would be used in the transformation procedures for the production of more exocellulase enzyme which perform better and efficient bacterial degradation of lignocelluloses from biomass.

We tried with 16s rRNA for identification of *Cellulomonas*. However, we did not get results. We tried to assess the variability among the isolates in biochemical/cultural/functional characters. But, we did not get the significance difference among the isolates.

Summary

VI. SUMMARY

Cellulose is found in ample as agriculture waste. Cellulose occurs in almost pure form in cotton fiber and in combination with other materials, such as lignin and hemicelluloses in wood, plant leaves, stalks *etc*. Apart from plant sources, cellulose is also produced by some bacteria (Immanuel *et al.,* 2006). *Cellulomonas*, an aerobic bacterium, is involved in degradation of cellulose and hemicellulose into disaccharides (cellobiose).

Cellulomonas fimi is the first studied bacteria for its cellulolytic properties and has since been one of the most studied bacterial cellulase systems (Whittle *et al*., 1982; O'Neill *et al*., 1986; Shen *et al*., 1995). Because of the common occurrence of these bacteria in various natural environments, they are responsible for huge amount of cellulose decomposition.

The present work envisaged isolation of *Cellulomonas* from natural forest soil, paper mill industry soil and cultivable agricultural field soil, isolation and characterization of *Cellulomonas sp*. from different sources and diversity analysis of *Cellulomonas sp.* using RAPD marker. The results obtained are summarized herein.

1. Maximum count of cellulose degrading bacteria was noticed in the soil sample collected from the region of natural forest, Kodagu.

2. All the *Cellulomonas* isolates which used in the study were rod shaped and gram positive in character.

3. Maximum colony diameter (3.6 mm) in Congo-Red Agar medium of *Cellulomonas* was noticed in case of sample D (Soil near

paper mill Industrial, Davangere), while maximum zone of clearance (17.0 mm) was observed in sample B (Grassland, Natural forest soil, Madikeri) as well as in sample C (Soil collected from paper mill Industrial, Davangere), while highest hydrolysis capacity (6.61) was observed in sample B.

4. Maximum extent of degradation of filter paper was noticed in the sample collected from the region of natural forest, Madikeri.

5. The RAPD banding patterns of all the *Cellulomonas* isolates indicates distinguished into one cluster and one isolate with linkage distance from 1.75 to 2.25 and the second group with linkage distance from 1.75 to 1.90.

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Appendices

APPENDIX- I

MEDIUM PREPARATION

1: Chemical composition of Basal Salt Medium (Gupta *et al.* 2012)

Sl. No.	Ingredients	Quantity (gram per liter)
1.	$NaNO_3$	2.5
2.	KH_2PO_4	2.0
3.	$MgSO_4$	0.2
4.	NaCl	0.2
5.	$CaCl_2.6H_2O$	0.1
6.	pH	6.8-7.2

Note: - Basal Salt Medium contains filter paper (Whatman filter paper no.1) 2 gm/1000 ml for the isolation of cellulolytic bacteria. These cultures were incubated for 3 days in a shaker incubator at 30°C at 100 rpm.

2: Chemical composition of Cellulose Agar Medium (Gupta *et al.* 2012)

Sl. No.	Ingredients	Quantity (gram per liter)
1.	KH_2PO_4	0.5
2.	$MgSO_4$	0.25
3.	Cellulose	2.0
4.	Agar	15
5.	Gelatin	2.0
6.	pH	6.8-7.2

3: chemical composition of Cellulose Congo-Red agar medium (Gupta *et al.* 2012)

Sl. No.	Ingredients	Quantity (gram per liter)
1.	KH_2PO_4	0.5
2.	$MgSO_4$	0.25
3.	Cellulose	2
4.	Agar	15
5.	Congo-Red	0.2
6.	Gelatin	2
7.	pH	6.8-7.2

4: Chemical composition of Half Strength Nutrient Agar (NA) Medium

Sl. No.	Ingredients	Quantity (gram per liter)
1.	Peptone	2.5
2.	Beef extract	1.5
3.	Sodium Chloride	2.5
4.	Glucose	2.5
5.	Agar-agar	17
6.	pH	6.8-7.2

APPENDIX- II

Preparation of DNA Extraction Reagents

i) Cetyl-Trimethyl Ammonium Bromide (CTAB) / NaCl solution:

4.1 g of NaCl was dissolved in 80 ml of water to which 10 g of CTAB was added slowly, in order to increase the rate of dissolving it was heated to 65°C. Finally the volume was made up to 100 ml by adding sterile distilled water.

ii) NaCl (5M)

The stock solution of NaCl was prepared by dissolving 29.2 g salt in 80 ml water. Volume was adjusted to 100 ml with water and later it was sterilized by autoclaving and stored at room temperature.

iii) Tris base (1M, pH 8.0)

The stock solution of Tris base was prepared by dissolving 11 g of Tris base in 80 ml of water then the pH was adjusted to 8.0 with concentrated HCl. It was sterilized by autoclaving and stored at room temperature.

iv) EDTA (0.5M)

EDTA of 18.61 g was added in 80 ml water and stirred vigorously on a magnetic stirrer. The pH was adjusted to 8.0 with NaoH pellets and then it was sterilized by autoclaving and stored at room temperature.

v) Chloroform: IsoamyJ alcohol (24:1 v/v)

Chloroform of 96 ml was mixed with 4 ml of isoamyl alcohol and stored under refrigerator conditions.

vi) Phenol: chloroform: Isoamyl alcohol (25:24:1 v/v)

Equilibrated phenol of 50 ml was mixed with 48 ml of chloroform and 2 ml of isoamyl alcohol and stored at 4°C.

vii) TE (Tris EDTA pH 8.0) buffer

Tris base (IM, pH 8.0) of 1 ml was mixed with 0.2 ml of EDTA (0.5 M, pH 8.0) and finally the volume was adjusted to 100ml with sterile water and finally this solution was sterilized by autoclaving.

viii) RNAase

RNAase of 10 mg was dissolved in 1 ml of Tris EDTA buffer (pH 7.8) and it was heated to near boiling point. This was finally stored under freezing temperature.

ix) TBE (5X, pH 8.0)

Tris base of 27 g and 13.75 g of boric acid were dissolved in 200 ml of sterile water to which 10 ml of EDTA (0.5 M) was added and finally volume was adjusted to 500 ml by adding sterile water.

x) Ethidium Bromide (lOOmg / ml)

Ethidium bromide of 1 g was added to 100 ml of sterile water and was stirred using magnetic stirrer until the dissolution of dye. Later the container was wrapped in aluminum foil and stored at room temperature.

xi) 70 Per cent ethanol

Absolute alcohol of 70 ml was mixed with 30 ml of distilled water and it was stored at room temperature.