STUDIES ON WAX DEGRADING MICROORGANISMS AND ITS EFFECT ON DECOMPOSITION OF SUGARCANE BAGASSE

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Thesis submitted in part fulfillment of the requirements for the degree of

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CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON WAX DEGRADING MICROORGANISMS AND ITS EFFECT ON DECOMPOSITION OF SUGARCANE BAGASSE"** submitted in part fulfillment of the requirements for the degree of **MASTER OF SCIENCE (AGRICULTURE) IN AGRICULTURAL MICROBIOLOGY** to the Tamil Nadu Agricultural University, Coimbatore is a record of bonafide research work carried out by **Mr. V. MUTHUVELU** under my supervision and guidance and no part of the thesis has been submitted for the award of any degree, diploma, fellowship or other similar titles and that the work has not been published in part or full in any scientific or popular journal or magazine.

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"THE INFLUENCE OF THE TEACHER NEVER ENDS" -Henry Adams

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(V.MUTHUVELU)

ABSTRACT

STUDIES ON WAX DEGRADING MICRORGANISMS AND ITS EFFECT ON DECOMPOSITION OF SUGARCANE BAGASSE

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The organic residual sources such as crop residues and agro-industrial waste are rich in nutrients which would aid in increasing the productivity by supplementing nutrients for plant and facilitate to meet out the growing demand for food. The agricultural crop residues production has increased gradually from 602.3 million tones to 889.1 million tones in India. Agriculture residues are predominantly constituted with cellulose, hemicellulose, lignin, pectin, cutin and other waxes, fats, oils etc. Soil organic matter constituent about 2-6% of fats, resins, waxes.

Biodegradation of these ubiquitous and abundant plant polysaccharides and neutral lipids, waxes, fats with suitable microorganism facilitates nutrient release from crop residues and enhances the soil health and soil fertility. The wax degrading bacterial isolates and actinomycetes cultures of enriched automobile waxy waste were isolated using the five media such as beeswax mineral salt medium, mineral salt medium I, mineral salt medium II, inorganic basal medium and modified mineral salt medium of 0.5, 1, 2 and 5 per cent paraffin wax and beeswax. Wax degrading bacterial isolates were shown maximum in mineral salt medium with 0.5 per cent beeswax and paraffin wax. Wax degrading actinomycetes culture was recorded higher in mineral salt medium with 0.5 per cent paraffin wax than beeswax. Seventeen wax degrading bacterial isolates and

five actinomycetes cultures were selected and characterized by morphological, colony and biochemical characterization and tentatively identified as genus of *Bacillus* sp, *Serratia* sp, *Pseudomonas* sp and *Streptomyces* sp. Efficient wax degrading bacterial isolates such as *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12, *Bacillus* sp. WDB17 and actinomycetes cultures such as *Streptomyces* sp. WDA2, *Streptomyces* sp. WDA3 and *Streptomyces* sp. WDA4 were screened in coconut oil, n- hexadecane, beeswax and paraffin wax.

Automobile waxy waste with 75 per cent cow dung recorded maximum wax degrading bacterial population of 40 x 10^3 g^{-1} on 240^{th} day, whereas, in MPN method automobile waxy waste with 75 per cent cow dung was shown higher population of 72 x 10^4 ml^{-1} which is 10 fold higher than wax degrading bacteria in beeswax with mineral salt medium I.

Wax degrading bacterial isolates *Bacillus* sp. WDB11 and actinomycetes culture *Streptomyces* sp. WDA3 were recorded higher growth, biomass and growth yield in n-hexadecane. The specific growth rate of the wax degrading bacterial isolate *Bacillus* sp. WDA12 was found rapidly in mineral salt medium I with 1 per cent (v/v) n- hexadecane. The inoculation of wax degrading bacterial isolates *Bacillus* sp. WDB12 in one per cent n- hexadecane was observed maximum of 39 x 10^6 ml^{-1} on 9^{th} day and decline further. Similar trend was observed in wax degrading actinomycetes cultures. Emulsifying activity was shown higher in wax degrading bacterial isolates *Bacillus* sp. WDB11 and actinomycetes culture *Streptomyces* sp. WDA2 on 15^{th} day.

The maximum biosurfactant activity of wax degrading bacterial isolates and actinomycetes cultures was observed on 15^{th} day. Among the wax degrading bacterial isolates higher amylase and cellulase activity of 3.97 and 3.89 unit ml^{-1} was recorded in *Bacillus* sp WDB12 on 12^{th} day whereas, wax degrading actinomycetes cultures *Streptomyces* sp. WDA3 recorded maximum amylase activity of 5.24 unit ml^{-1}. Xylanase activity was shown maximum in *Bacillus* sp. WDB7 and *Streptomyces* sp. WDA2. The wax degrading bacterial isolates *Bacillus* sp. WDB12 and *Streptomyces* sp. WDA3 were observed highest lipase activity of 2.99 and 3.07 unit ml^{-1}. The beeswax degradation was registered in wax degrading bacterial isolates and actinomycetes culture. Wax degrading

bacterial isolate *Bacillus* sp. WDB12 observed higher beeswax degradation of 0.05 mg, whereas, wax degrading actinomycetes culture *Streptomyces* sp. WDB3 was recorded maximum beeswax degradation of 0.06 mg. *Streptomyces* sp.WDA2 exhibits antagonistic activity with *Bacillus* sp.WDB3 and *Bacillus* sp.WDB17.

Bagasse incorporated with soil and 10 per cent microbial consortium registered highest bacterial, fungal and actinomycetes population of 71 x 10^6 g^{-1}, 36 x 10^4 g^{-1} and 19 x 10^3 g^{-1} on 75^{th} and 105^{th} day, whereas, wax degrading bacterial and actinomycetes population recorded maximum of 39 x 10^6 g^{-1} and 15 x 10^3 g^{-1} in sterilized bagasse with 10 per cent microbial consortium.

Enzyme activity such as amylase, cellulase and dehydrogenase was increased in bagasse incorporated with soil and 10 per cent microbial consortium and recorded maximum of 3.08 unit ml^{-1}, 3.36 unit ml^{-1} and 1.56 µg of TFP formed g^{-1} h^{-1} on 75^{th} day of decomposition. Higher xylanase activity of 2.91unit ml^{-1} was recorded in sterilized bagasse with 10 per cent microbial consortium. Sterilized bagasse with 10 per cent microbial consortium recorded very low water repellency of 0.8 MED and followed by bagasse incorporated with soil and 10 per cent microbial consortium.

Significant reduction in organic carbon content and change in pH from acidic to neutral was observed in bagasse incorporated with soil and microbial consortium during incubation. Higher nitrogen content, phosphorous of 0.56 and 0.05 per cent were recorded in bagasse incorporated with soil and 10 per cent microbial consortium, whereas maximum potassium content was observed higher in bagasse incorporated with 10 per cent microbial consortium.

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Dedicated to

World of Microbiologist and Indian Farming Community….

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CHAPTER I

INTRODUCTION

Nutrient in soil is depleted through continuous cultivation, it could be replenished through inorganic fertilizers and organic sources. The organic sources, biomass, crop residues, farmyard manure, poultry and piggery waste, sewage and municipal waste, forest litter and agro-industrial waste and agricultural residue includes all leaves, straw and husks left in the field after harvest, hulls and shells removed during processing of crop at the mills, as well as animal dung. The single largest category of crops is cereals, with global production of 1800 Tg in 1985 (FAO, 1986). The agricultural crop residues production has increased gradually from 602.3million tonnes to 889.1 million tonnes in India (Iyer *et al.,* 2002). The solid waste generated in urban areas is commonly termed as municipal solid waste (MSW) is about 27.4 million tones in India and this can be effectively decomposed and utilized for crop growth. The organic wastes available in India are rice straw, rice husk and rice bran, wheat and other cereal straw, sugarcane trash, tobacco waste, cotton waste, pulses, oil seeds waste, groundnut shell, maize cob, banana stem of 10.6, 14, 18.2, 0.06, 4.5, 17.5, 11.4, 1.8, 2.1 and 10.6 million tones respectively.

The crop residues, agro-industrial waste are decomposed municipal waste contains carbohydrates, polysaccharides, proteins, lipids, fats, waxes and resins. The wax content of plant and municipal waste are resistant to enzyme and microbial degradation. Agriculture residues contain cellulose, hemicellulose, lignins, pectin, chitin and waxes. Plants waxes are wax esters are accomplished by significant amount of unesterified fatty alcohol and fatty acids as well as hydrocarbons such as n- alkanes are predominant with carbon atoms in the range C_{25}-C_{35} of which C_{29} and C_{31} are most abundant in higher plants. When these waxes are broken down they release some wax ester and alcoholic compounds such as policosanol (PC) is the common name that refers to a mixture of long chain (20–36 carbon) aliphatic primary alcohols with 20-36 carbon atoms. Seeds of sunflower, soyabean, celery seed, sesamum seeds, sugarcane contain wax content of about 3, 0.5, 0.3 and 1-2 per cent respectively.

Plant waxes and their biodegradable products are major contributors to water repellency (Franco *et al.,* 2000*a*). The occurrence of water repellency has been associated with particular vegetation types (Doerr *et al.,* 2000). Plants commonly associated with water repellency are certain evergreen types such as eucalyptus and pines shrubs trees with a considerable amount of resins, waxes or aromatic oils and in grassland eg. *Lupinus cosentinii* (Carter *et al.,* 1994). Inoculation of wax degrading bacteria under laboratory condition resulted in significant increase in soil wet ability (Roper, 2004). Wax degrading microorganisms degraded waxes through pseudosolubilization, adherence on waxes and adsorption of wax by direct contact and also production of biosurfactant such as glycolipids, lipoaminoacids, lipopepides and emulsifying activity. Cameotra (1995) reported biosurfactant production in *Serratia marcesens* and observed growth in n- hexadecane.

Water repellency is caused by uneven filtration of water and this cause delayed germination of crop, poor establishment and increased risk for wind and water. Waxes in soil are responsible for water repellency and are degraded by *Rhodococcus* sp. and *Streptomyces* sp. These microorganisms produce biosurfactant to improve their ability to utilize wax and their compounds.

The accumulation of hydrophobic waxes on soil particles of humic acids and fulvic acid and other long chain organic compounds on/ between soil particles all are contributing to negative impacts phenomenon. Wax products are policosanol are separated from beeswax and sugarcane extracts used in prevention and treatment of cardiovascular disease. The plant waxes product policosanol and triacontanol from wheat germ oil and sunflower seed could be obtained from establishment of distribution patterns and extraction procedures. Bioconversion of wax containing organic waste is gaining importance to reduce pollution and water repellency in the environment. The present investigation has been taken up with aim of understanding the

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CHAPTER II

REVIEW OF LITERATURE

Manures, green manure and crop residues are effectively utilized in crop production to attain maximum grain yield. Decomposition and recycling of crop residues, rural urban wastes, wastes from agricultural residues play major role in promoting available major nutrients for plant growth. In India, estimates revealed that by the end of year 2020 India need approximately about 280 million tones of food grain to feed their own growing population. To produce 280 million tones of food grains, 20 million tones of nitrogen, phosphorous and potassium are required In integrated nutrient management a variety of organic sources are available in cropping systems. They are biomass, crop residues, farmyard manure, poultry and piggery dropping, slaughter house waste, human habitation refuse, forest litter and agricultural residues. These organic sources provide partially required nutrients for plant growth. Lal (1995) reported that 295.2 million ton of crop residues are produced per year and 74 million tones of major nutrients of NPK can be obtained by decomposting with suitable decomposers.

Agricultural residues can be divided into two groups: crop residues and agro-industrial residues. Every year billion tons of crop residues are added in the field after crops have been harvested and thrashed or left after pastures are grazed. Agro-industries account for production of large quantity of wastes such as coir industry waste, paper and dairy industry waste, biscuit industry waste, fruit pulp industry waste, oil refineries and breweries wastes, stems, leaves and flowers from aromatic oil extraction units. Apart from sugar and alcohol as primary products, sugar industries and fermentation units also produce many by-products such as press mud, bagasse, distillery waste, boiler ash and fermentation yeast sludge. All these wastes serve as an excellent source of nutrient (Kitturmath *et al*., 2007). Agriculture residues consist of cellulose, hemicellulose, lignin, pectin, cutin and other waxes, fats, oils etc.

Municipal solid waste (MSW) refers to the materials discarded in the urban area which encompasses household refuse, institutional wastes, street sweepings, commercial wastes, as well as construction and demolition debris. In developing countries, MSW also contains varying amounts of industrial wastes from small industries, as well as dead

animals, and fecal matter (Holmes, 1984). Chemical characterization of municipal solid waste showed that it contains majority of lipids, carbohydrates, proteins 5-10 %, natural fibers, synthetic organic materials (5-7%) and non combustibles (30-50%) of dry solid (Bhinde and Sundersan, 1983).

2.1. Agricultural residues

Every year billions tons of agricultural waste are generated in the developing and developed countries. Agricultural residue includes all leaves, straw and husks left in the field after harvest, hulls and shells removed during processing of crop at the mills, as well as animal dung. The single largest category of crops is cereals, with global production of 1800 Tg in 1985 (FAO, 1986). The agricultural crop residues production has increased gradually from 602.3 million tones to 889.1 million tones in India (Iyer *et al.*, 2002). Wheat, rice, maize, barley, millet and sorghum account for 28%, 25%, 27%, 10%, and 6%, respectively.

Cellulose, hemicellulose, lignin are the most abundant compounds in agriculture residues (Singh, 1995). Hemicelluloses are the second most abundant polysaccharides in paddy straw. Hemicellulose is a branched or linear heteropolymer which contains various pentoses as xylose and arabinose, hexoses as mannose, glucose and galactose and uronic acids as glucuronic and galacturonic acids. Due to the heterogenity of the sugar units, several different exo- and endo-enzymes are involved in the hydrolysis of hemicellulose molecules to oligomers and monomers (Sylvia *et al*., 1999).

Lignin is recalcitrant because its biodegradation is a complex oxidative process (Atlas and Bartha, 1998).Chitin is a polymer of N-acetyl-glucosamine linked by -1, 4-bonds. Chitin is hydrolysed to oligomers and further to monomers (Atlas and Bartha, 1998; Tabatabai and Fu 1992). Proteins are hydrolysed by proteinases into peptides. The peptides formed are hydrolysed by peptidases into single amino acids.

Wheat straw also contains low molecular weight substances that are removable by neutral solvents. These materials are referred to as extractives, and may include a diverse group of substances, such as phenolics, sugars, fats, fatty acids, waxes, etc. Esters, such as waxes, are hydrolysed by esterases that cleave the ester linkages between the organic acid and the alcohol (Manafi *et al*., 1991).

Biodegradation of these ubiquitous and abundant neutral lipids such as waxes, fats with suitable microorganism facilitates nutrient from crop residues and maintain sustainable soil health and soil fertility. Therefore it is obviously, of enormous ecological significance and evidence to date suggests that microorganisms play a major role in accomplishing this task.

2.2. Waxes

The name wax is derived from the Anglo-Saxon word "weax" for beeswax. In plants the outer covering consists of a hydroxy fatty acid polymer called cutin and healed wound surfaces of plants are covered with an analogous substance suberin. These substances are frequently mixed with other lipids and form a complex mixture called epicuticular wax. Cutin is a lipid polymer containing C_{16} and C_{18} families of acids. The former is more abundant in growing parts; the later is present in the cuticle of slower-growing plants. These acids may be saturated or unsaturated and mono- or di-hydroxylated. Cutin are a polyester structure where cross-linking depends on the availability of secondary hydroxyl groups. In contrast suberins are β-hydroxy acids and dicarboxylic acids, with more than twenty carbon atoms.

Plant leaf surfaces are coated with a thin layer of waxy material which is microcrystalline in structure and forms the outer boundary of the cuticular membrane and it is the interface between the plant and the atmosphere. It serves many purposes, for example to limit the diffusion of water and solutes, while permitting a controlled release of volatiles that may deter pests or attract pollinating insects. The wax provides protection from disease and insects, and helps the plants resist drought. As plants cover much of the earth's surface, it seems likely that plant waxes are the most abundant of all natural lipids (Kolattukudy *et al.,* 1976).

The lipid types in plant waxes are highly variable with composition and major component of waxes. The major constituents of plant waxes are n-alkanes ($CH_3(CH_2)_x CH_3$), alkyl esters ($CH_3(CH_2)_xCOO(CH_2)_yCH_3$), fatty acids ($CH_3(CH_2)_xCOOH$), fatty alcohols $CH_3(CH_2)_yCH_2OH$, fatty aldehydes ($CH_3(CH_2)_yCHO$), ketones ($CH_3(CH_2)_xCO(CH_2)_yCH_3$), secondary fatty alcohols ($CH_3(CH_2)_xCHOH(CH_2)_yCH_3$), β-Diketones ($CH_3(CH_2)_xCOCH_2CO(CH_2)_yCH_3$) with carbon atom of 21-35, 34 -62, 16-32, 22-32, 22-32,

23-33, 23-33, 27-33 respectively. The waxes also conatins triterpenols constituted by sterols, α-amyrin, β-amyrin, uvaol, lupeol, erythrodiol and triterpenoid acids ursolic acid, oleanolic acid, etc.

In addition, hydroxy-β-diketones, oxo-β-diketones, alkenes, branched alkanes, acids, esters, acetates and benzoates of aliphatic alcohols, methyl, phenylethyl and triterpenoid esters also present in the plant leaf wax. The lipid class and the nature and proportions of waxes vary greatly according to the plant species and the site of wax deposition in leaf, flower, fruit etc.

Hamilton (1995) reported that hydrocarbons, wax esters, aldehydes, ketones primary and secondary alcohols, acids of grape leaf, rape leaf, apple fruit, rose flower, pea leaf, sugarcane stem. He found that apple fruit contain maximum of wax ester 18 % and followed by rape leaf 16%.

2.3. Waxes in agricultural residues

Agriculture residues contain cellulose, hemicellulose, lignins, pectin, chitin and waxes and percentage of these constituent are varied with plant species. El marsy (1983) reported that rice straw consists of 43.30% cellulose, 26.40% hemicellulose, 16.29% lignin, 12.26% ash and 2.18% waxes. Kim and Dale (2004) reported that an corn stover accumulated 80,000 ton of Ca, K, Mg, P and 77,000 tons of lipids, waxes, fats and 2,00,000 tons of protein (excluding cell protein) per year. Natural waxes contain a wide variety of simple components such as hydrocarbons, esters, fatty acids, ketones, mono-di-tri acylglycerols and sterol esters (Kolatttukudy *et al.*, 1976).

In sunflower waxes are mainly located in the hull with concentration of 3% depending on the hybrid and origin of seeds (Morrison, 1982), which is composed of long chain fatty esters, free fatty acids and free fatty alcohols. Though the wax is present in all parts of the seed, a high content of wax is found in the hull portion (Morrison *et al.*, 1981). Soyabean wax (*Gylcine soja*), celery seed (*Apium graveolens* L.) and sesame seed (*Sesamum indicum* l.) were contain waxes about 0.5, 0.2 and 0.3 per cent respectively.

Palms (*Copernicia cerifera*) waxes are more abundant on the leaves than on other parts. A semi-refined sugarcane wax contains 70-72% wax esters of which 20% myricyl

palmitate, 1-2% myricyl myricinate, 37-38% stigmasteryl palminate, 13% phytosteryl dihydroxypalmitate and 14% free fatty acids (Warth, 1947). Mitsui (1937) has reported that stigmasterol constituted about 2% of the total wax of sugarcane and that sitosterol accounts for an additional 0.77%. Chargaff (1935) have demonstrated waxes in alkanet root (*Anchusa tinctoria* Lam.) and found that wax is composed chiefly of carnaubyl cerotate.

Pollard *et al*. (1933) had demonstrated the presence of n-octacosanol in wax extracted of the wheat leaves. Chibnall (1933) showed that lucerne leaf or alfalfa (*Medicago sativa*) is source of n-triacontanol ($CH_3(CH_2)_{28}CH_2OH$) and referred as "melissyl" or myricyl alcohol. Hilditch (1945) had reported the presence of n-triacontanol in apple cuticle wax, sugarcane wax, beeswax and carnauba wax respectively. Kahlon *et al.* (1992) reported that corn wax from (*Zea mays* L.) is composed of hentriacontane, myricyl tetraconsanate and myricyl isobehenate, sitosterol and stigmasterol, whereas sunflower seed wax dominated with ceryl cerotate.

Ghosh and Sengupta (1967) reported that lac mud constituent about 8 % of stick lac, lac dye (10-15%), lac wax (8-10%) and lac resin (10-12%). Power and Rogerson (1910) first identified hentriaconatone ($C_{31}H_{64}$) incarnatyl alcohol, ($C_{34}H_{69}OH$) a phytosterol "trifolianol" and palmitic, steric, oleic, linoleic and isolinolenic acids from the wax of the crimson clover flower (*Trifolium incarnatum* L.)

2.4. Wax products

The majority of wax products are separated from beeswax and sugarcane extracts. Policosanol (PC) is the common name that refers to a mixture of long chain (20-36 carbon) aliphatic primary alcohols with 20-36 carbon atoms. The mixture contains mainly docosanol (C_{22}), tetracosanol (C_{24}), hexacosanol (C_{26}), octacosanol (C_{28}), and triacontanol (C_{30}). The role of PC in prevention and treatment of cardiovascular disease was well documented by (Varady *et al.,* 2003).

Taylor *et al.* (2003) reported the beneficial health effects of octacosanol (C_{28}). Carbajal *et al*. (1998) shown that policosanol decreases platelet aggregation, endothelial damage, and foam cell formation. The effectiveness of policosanol as a lipid-lowering agent, in several different populations has been extensively studied by (Castano *et al.,* 1996;

Gouni-Berthold and Berthold, 2002; Mas *et al.,* 1999; Menendez *et al.,* 1994). Policosanol lowers cholesterol levels by inhibiting cholesterol biosynthesis and enhancing LDL decatabolism (Menendez *et al.,* 1994). Policosanol improves protection of lipoproteins against lipid peroxidation, both lipid and protein moieties (Menendez *et al.,* 1999). Wheat germ oil and seed of sunflower containing policosanol (PC) and triacontanol respectively. Commercial policosanol (PC) could be obtained from wheat by establishment of distribution pattern and extraction procedure.

D-003 is a mixture of very long-chain aliphatic acids purified from sugarcane (*Saccharum officinarum*, L) wax, containing octacosanoic acid as the most abundant component and low concentration of tetracosanoic, pentacosanoic, hexacosanoic, heptaco -sanoic, onacosanoic, triacontanoic, hentriacontanoic, dotriacontanoic, tritiacontanoic, tetratriacontanoic, pentatriacontanoic and hexacotriacontanoic (Mas, 2004).

2.5. Wax degrading microorganism

Various biological studies have been carried out to identity the major microbiological agents responsible for biodegradation of waxes. The sole agents for decomposition of carbonaceous material are heterotrophic microorganisms (Singh and Jin, 2008). Foster (1962) reported that most of the microbes used in investigation on hydrocarbon degradation and assimilation have been obtained by direct enrichment technique. Utilization of hydrocarbon as a sole source of carbon and energy by microorganisms in fertile soil was reported by Perry and Scheld (1968) and also found that soil from oil fields contain higher number of total microbial populations which grow on petroleum hydrocarbons.

Jobson *et al*. (1972) demonstrated the utilization of two crude oil by pure and mixed bacterial cultures obtained by enrichment technique and they found that the bacterial isolates obtained with a high quality crude oil was not effective in utilizing a lower quality crude oil as sole carbon source. Raymond *et al*. (1970) observed significant increase in hydrocarbon utilizing microorganisms in soil applied with pure hydrocarbon n-hexadecane or the hydrocarbons of lubricating oils was also supported by Reisfeld *et al*. (1972)

Ridgway *et al*. (1990) documented hydrocarbon utilizing eubacteria genera are *Pseudomonas, Alcaligenes, Micrococcus, Nocardia, Cornebacteria, Arthtobacter, Bacillus, Streptomycetes, Rhodococcus, Proteus*. Becker and Dott (1995) characterized 48 aerobic heterotrophic bacterial isolates from soil of oil contaminated sites and also found that strains of the same species showed different degradation abilities for hydrocarbon substrates. Roper (2004) isolated 37 isolates of wax degrading microorganisms from soil with whale oil, sheep camp, abattoir, water repellent soil, sewage effluent, composted animal manure, soil containing sewage, animal fats.

2.6. Biodegradation of n-alkanes

Kennedy and Finnerty (1979) reported that there are two problems associated with the biodegradation of hydrocarbons by microorganisms. First, the insoluble substrate cannot enter the cells and second, the cells do not produce the necessary enzymes needed to metabolize the hydrocarbons. Microbes able to grow on hydrocarbons have shown the ability to accumulate the paraffinic substrate intracellularly in inclusion bodies. The uptake of the solid n-alkanes by microorganisms is believed to occur through transport across the cell membrane. This passage through the cells is a crucial step for the catabolism of these chemicals. Miller and Bartha (1989) had shown that long chains are limiting factor for hydrolysis of hydrocarbon. However, microorganisms must have the phenotype allowing long chain hydrocarbon and degrading by producing enzymes.

Three mechanisms are generally accepted as possible mechanisms for the uptake of insoluble hydrocarbon of bacteria and yeast (Wang and Ochoa, 1972).

1. The cells utilize the hydrocarbon dissolved in the aqueous phase.
2. The cells utilize " solubilized / pseudosolubilised or accomdated" submicron droplets of hydrocarbons
3. The cells utilize the substrate through a divert contact with large hydrocarbon drops.

Katinger (1973) reported that transfer of insoluble hydrocarbon to microbial occurred through direct contact between the organisms and the insoluble hydrocarbons and it depends on interfacial area between the cells and hydrocarbon. Erikson and Nakahara (1975) observed that the interfacial area between water and oil was proceeded

with some fermentations proceeded due to extracellular products such as lipids (may be in the form of micellus), fatty acids or the cells themselves which were directly responsible for pseudosolubilization of the hydrocarbon substrate.

Velankar *et al.* (1975) reported that rate of degradation depends on chain length of hydrocarbon. EDTA can inhibit pseudosolubilization because it can bind the Ca^{++} ions needed for pseudosolubilization activity. Also, in the case of pseudosolubilization the agitation rates have no effect on growth rates. Cells that were able to grow on hydrocarbons demonstrated the ability to strongly adhere to the hydrocarbons (Kennedy and Finnerty, 1979).

Gutierrez and Erickson (1977) reported that surface active materials have been shown to increase the specific growth rate of bacterial cells in hydrocarbons. The bacterial cells adsorb to the surface of large insoluble hydrocarbon by which most substrate is transported inside the cells (Zilber *et al*., 1979).

Adherence is directly related to the hydrophobicity of the microorganisms and is important for hydrocarbon degrading microbes in the environment and thin fimbriae believed to help cells to adhere. Cell surface hydrophobocity determines, adherence to the oil but it does not determine the ability to grow on it (Rosenberg and Rosenberg, 1985). Lipopolysacharide moiety on the cell surface was also believed to be involved in the affinity of the cells for alkane (Britton, 1984).

Husain *et al*. (1997) showed that a *Pseudomonas nautica* strain adapted to grow on solid n- alkane (eicosane) by morphological changes in filamentous structure allowed a three fold increase in the adherence of cells.

2.7. Biosurfactant activity of microorganism

Microbial surfactants include a structurally diverse group of compounds containing a hydrophilic and a lipophilic moiety with in the same molecule. The common lipophilic part is built up by the hydrocarbon chain of a fatty acid, while the polar or hydrophilic group is derived from the ester or alcohol groups of neutral lipids or the carboxylate group of fatty acids or amino acids or phospholipids or glycolipids. The structures and physico- chemical properties of microbial surface compounds are varied with bacteria, yeast and fungi (Fiechter, 1992; Desai and Desai, 1993).

Due to their amphiphilic character of different degrees of polarity and hydrogen bonding surfactants partition preferentially attached at the interface between phases of oil or water, air or water and solid or water interfaces. The formation of an orderly molecular film lowers the interfacial energy at the interface (interfacial tension) and it is the unique properties of surfactant molecules (Georgiou *et al*., 1992).

Rosenberg *et al*. (1979) isolated *Acinetobacter calcoaceticus* as a hydrocarbon degrading microorganisms which produced a potent extra cellular bioemulsifer. Kaplan and Rosenberg (1982) suggested that *Acinetobacter calcoaceticus* BD 413 either produced a modified extracellular polysaccharide or excreted an additional substance that was responsible for the emulsifying activity. Person and Molin (1987) reported that production of biosurfactant by strains of *Pseudomonas* and *Vibrionaceae*.

Singer and Finnertly (1990) isolated biosusrfactant producing *Rhodococcus* sp. from soil enriched with oil. Vander vegt *et al*. (1991) have identified five biosusrfactant producing *Streptococcus* sp. and biosusrfactant production was assayed by axis symmetric drop shape analysis method. Abu-ruwaida *et al*. (1991) reported the production of surface active compounds in gram positive, non fermentative, rod shaped bacterium *Rhodcoccus* isolated from soil enriched with kerosene and n-paraffin. Rocha *et al*. (1992) isolated two strains of biosusrfactant producing bacteria from injection water and crude oil-associated water in Venezuelan oil fields and identified as *Pseudomonas aeruginosa.*

Cameotra (1995) documented *Serratia marcescens* from Gujarat oil field which produced a biosusrfactant of (2.6g/l) when grown on n- hexadecane. Dezeil *et al*. (1996) isolated *Pseudomonas* strain from soil contained with petroleum and detected the production of biosusrfactant while growing on naphthalene and phenanthrene. Valreads *et al*. (1996) observed the production of biosusrfactant by fifteen isolates of *Lactobacillus* in the mid exponential and stationary growth phase.

Karpenko *et al*. (1996) studied biosurfactant activity of *Pseudomonas* sp. S-27 and found that rhamnolipids reduces biosusrfactant activity of *Pseudomonas*. Carrillo *et al*. (1996) reported that haemolysis has been used as initial selection criteria for the primary isolation of surfactant producing bacteria. The further studies clearly

indicated that strains, has haemolytic activity been directly proportional to production of biosurfactant. Haemolysis and biosurfactant productions are probably closely associated phenomena.

2.8. *Invitro* enzymes activity

The enzymes are extra cellular microbial proteins which hydrolysis cellulose, hemicellulose, lignin, starch, pectin, cutin and other degraded polymers. Sylvia *et al*. (1999) stated that extracellular hydrolytic enzymes convert the detrital carbon and organic compounds of phosphorus, nitrogen and sulphur into available form of nutrients to plants and microbes in soils.

Starch is a storage polysaccharide made up of repeating unit of linear α-1,4-linked glucose units or α-1,6-branched chains. Amylases (endo-1,4-α-D -glucan glucohydrolase EC 3.2.1.1) are extra-cellular endo enzymes that randomly cleave the 1,4- α-D -glucosidic linkages between adjacent glucose units in the linear amylose chain (Robinson, 1987; Boopathy, 1994). Anthony and Murray (1996) demonstrated the amylase activity of many microorganisms and found that fungi had been found to be good sources of amylolytic enzymes. Evidences of amylase in yeast, bacteria and mould have been reported and their properties documented (Adebiyi and Akinyanjus, 1998; Akpan *et al.*, 1999; Buzzini and Martini, 2002). *Bacillus coagulans* and *Bacillus subtilis* were shown to produce α-amylase by utilizing wheat bran and rice husk in solid state fermentation (Babu and Satyanarayana, 1995).

Cellulase is also known as (1-4) α D- glucan hydrolase. Cellulase hydrolyse β (1-4)- D- glucan links in cellulose to cellobiose (β-D- glucoside- D- glucose). Celluloytic enzymes hydrolyse cellulose to oligomers or monomers of glucose (Tomme *et al*., 1995). All lignocellulosics wastes can be converted into products that are of commercial interest such as ethanol, glucose, and single cell protein. Cellulase enzyme are effectively hydrolyzed for the bioconversion of lignocellulosic substrates such as bagasse, corncob and sawdust to useful products (Kansoh *et al.,* 2001). Economical production of cellulase and xylanase from *Bacillus* sp. utilizing agro-byproducts such as wheat bran, rice husk, rice bran, rice straw and sugarcane bagasse is well documented (Poorna and Prema, 2007; Techapun *et al*., 2003).

Although much attention was paid initially to fungal cellulases, more recently bacterial cellulases have been studied more extensively particularly those of *Cellulomonas fimi*, *Clostridium thermocellum* and *Thermomonospora fusca.* Cellulase produced by thermophiles are of greater importance for applications than those from mesophiles, as they are likely to be more thermostable (Hazlewood *et al.,* 1988).

Beguin and Aubert (1994) demonstrated higher cellulase enzyme production in aerobic and facultative anaerobic cellulolytic bacterium *Pseudomonas* and *Cellulomonas* respectively.

Xylanase is directly involved in the degradation of the major polymeric constituents of plant litter and is a useful indicator for organic matter losses (Rodriguez, 1982; Sinsabaugh *et al*., 1994). The enzyme complex consists of endo - and exoxylanases degrading the hemicellulose xylan into readily a vailable compounds xylose and other carbohydrates. Kanosh *et al.* (2001) isolated twenty-four different strains of *Streptomyces* spp. from Egyptian soil that produce extracellular xylanases and obtained optimum production after five days of incubation at 30^{o}C

Lipases catalyse both hydrolysis and synthesis of esters from glycerol and long-chain fatty acids. Fat and oil (i.e. "grease", defined as plant oil/animal fats) are the stable organic compounds in wastewater. Fat and oil have been discharged from food industries, restaurants, slaughter house, household (Becker *et al*., 1999; Stoll and Gupta, 1997) Fairolnzia *et al*. (2007) reported that a thermophilic bacterium *Bacillus* sp. strain L2 produces an extracellular lipase enzyme and also factors affecting the secretion of extracelullar lipase.

Microbial lipases are produced by yeast, fungi, and bacteria as extracellular, intracellular and cell-bound enzyme. Chigusa *et al*. (1996) isolated yeast culture from food and oil wastewater and demonstrated that inoculation of mixed yeast culture recorded a reduction of 94% fat in waste water of soyabean oil producing industry. Yeast produced extracellular lipase in *Candida deformans*, C. *rugosa* (Rao *et al*., 1993) where as *Pseudomonas aeruginosa* EF2, *P*. *fragi* CRDA 323 (Pabai *et al*., 1995) and *Alcaligenes* sp. produced intracellular lipase (Kokusho *et al*., 1982).

Large *et al*. (1999) recorded lipase activity in *Streptomyces lividans, Streptomyces clavuligerus, Streptomyces coelicolor,* and *Streptomyces rimosus* and a wild-type and recombinant strain of *Saccharopolyspora erythraea.* Haba *et al*. (2000) isolated *Pseudomonas, Bacillus, Candida, Rhodococcus* and *Staphylococcus* that grow on the waste oils and produce lipolytic activity.

2.9. Waxes in soil

Soil organic matter is a complex mixture of organic substances inherited from the decay of plants and microorganisms (Schnitzer, 1972). This can be separated into two groups of compounds: 1) Humic substances, a series of brown to black, high-molecular weight polymers formed by secondary synthesis reactions, 2) Non humic substances, consisting of amino acids, carbohydrates, lipids etc (Dubach and Mehta, 1963). The lipid fraction of the soil has been largely ignored (Stevenson, 1970). In normal aerobic soil, the lipids probably exist largely as remnants of microbial tissue, low and variable quantities of these constituents may be associated with undecomposed plant residues and the bodies of living and dead micro faunal organisms. Plant wax n-alkanes may be enclosed with in the soil matrix by different processes involving van der waals forces. Wax n- alkanes adsorbed on soil humic substances by hydrophobic–hydrophobic interactions (Bollag *et al*., 1983). The acclumation of hydrophobic waxes on soil particles (Franco *et al*., 2000*a*) of humic and fulvic acid soil coatings (Karnok *et al*., 1993) and other long chained organic compounds on between soil particles (Doerr *et al*., 2000) are all accepted as factors contributing to their negative impact phenomenon. Soil organic matter constituent about 2-6% of fats, resins, waxes**.**

Greig-Smith (1912) studied the water repellency of soil particles by wax-like substances and explained "soil exhaustion" on the accumulation of fats and waxes which he termed "agroire." Waxes protecting the surfaces of leaves, needles, trunks and fruit of many higher plants are particularly resistant to decomposition; these should be capable of surviving essentially unchanged over long geological periods (Bergmann, 1963).

Soil waxes are chiefly esters of the higher members of the homologous series of n-acids and n-alcohols, is in agreement with the animal and plant waxes. Carbon atom of animal and plant waxes are saturated straight-chain compounds containing primarily C-even acids and alcohols.

Soil organic matter is accumulated with 2% waxes content which deposit along with minerals and which cause water repellency. A pigment identified as a hexachloropolynuclear quinone of the dihydroxyperylenequinone or dihydroxydinapthyl- quinone type (chemical formula of $C_{20}H_4O_5C_{16}$) was isolated from the original extract containing the wax; this material was responsible for the green color of the soil (Stevenson, 1970).

2.9.1. Water Repellency

Water repellency results from the drying of amphiphilic compounds produced by roots, decomposed organic matter and soil biota, particularly fungi. These compounds are strongly hydrophilic when wet, but below a critical moisture threshold, the hydrophilic ends of amphiphilic compounds bond strongly with each other and soil particles, leaving an exposed hydrophobic surface that induces water repellency (Wallach *et al*., 2005)

Tillman *et al*. (1989) introduced the concept of 'subcritical' water repellent soil, where water infiltration is impeded by repellency despite the soil appearing to wet readily. This will be referred to a 'water resistance' from herein and it is expected to influence almost all surface soil. Severe soil water repellency is widespread and affects land used for agriculture, amenity surfaces such as parks and golf courses, and coastal dune sands (Wallis and Horne, 1992). Ritsema *et al*. (1997) reported that repellency causes uneven infiltration of water into soil.

Kostka (2000) reported that multimillion dollar was invested to provide wetting's agents to over come water repellency in amenity surfaces particularly golf courses. Franco *et al.* (2000*b*) reported the most problematic areas of south-western Australia, where over two million hectare soil is affected by water repellency and recorded very low yield. Wallach *et al*. (2005) reported that increased use of effluent water is increases water repellency in some arid regions that are reliant on irrigation. Water repellency reduces crop and pasture yields through uneven as well as delayed germination, poor stand establishment and increased risk of erosion from wind and water (King, 1981; Tate *et al.,* 1989). Water repellency is primarily caused by a skin around soil particles of waxes consisting of branched and unbranched C_{16} to C_{36} fatty acids, and their esters, alkanes, alcohols, and sterols (Roberts and Carbon, 1972; Ma'shum *et al.,* 1988; Franco *et al.,* 2000*b*; Morley *et al.,* 2005).

Soils which have hydrophobic properties can resist or retard surface water infiltration (Brandt, 1969). McGhie and Posner (1981) reported that severe water repellency has been induced by intermixing as little as 2–5% by weight of organic matter to hydrophilic sand. Hunt and Gilkes (1992) reported that susceptibility of a soil being or becoming water repellent will be determined not only by the presence of hydrophobic material, but also soil texture. Coarsely textured sandy soils that contain less than 5% clay are very susceptible to becoming water repellent. Water repellency can occur in fine texture soils as well as strongly aggregated soil structure. The repellency also occurs when the aggregates become coated with hydrophobic material. Repellency is affected by hydrophobic organic compounds deposited on soil minerals or aggregate surfaces (Doerr *et al.,* 2000). Another common cause of water repellency is the accumulation of hydrophobic waxes or humic substances on soil particles or hydrophobic compounds released from plant roots was observed by McKenna *et al.* (2002) or hydrophobic compounds released from plant roots. Soil water content is considered to be one of the most important factors in temporal variations in water repellency (Doerr *et al.,* 2000). They become water repellent when they desiccate during dry periods in summer, with water repellency often vanishing when the soil becomes wet in winter times (Dekker and Ritsema, 1994). Plants associated with water repellency in soil are *Chamaecystitus proliferus*, *Hordeum vulgare*, *Lupinus cosentinii*, *Medicago sativa*, *Trifolium subterranum* (Carter *et al*., 1994). Plant derived organic matter may act as a source of hydrophobic waxes which can coat sand grains, thereby influencing the hydraulic behaviour of the soil (Nicolau *et al*., 1996). Hallet *et al.* (2001) found that stimulating the microbial biomass with nutrients greatly enhances the repellency of agricultural soils.

2.9.2. Crop growth

Waksman *et al.* (1928) reported the approximate composition of prairie soil humus which contains 1.2-6.3 % of fats, waxes and resins. Ashworth (1942) found that changes in fats, waxes and resins during decomposition in compost heaps were small and fluctuating. Low concentrations of borneol ($C_{10}H_{18}O$) and camphor ($C_{10}H_{18}O$) were found to be highly toxic. Turpentine, ($C_{10}H_{16}$), also inhibited plant growth. Dihydroxystearic acid, $CH_3 (CH_2)_7$-CHOHCHOH $(CH_2)_7$COOH, was toxic to plants and that it was one of the compounds responsible for the low fertility of worn-out soils.

Wheat seedlings showed abnormal growth when grown on soils containing small quantities of dihydroxystearie acid. The conditions under which dihydroxystearic acid accumulated were deficiency of lime, lack of good oxidation (poor aeration) and poor tilth.

Prescott and Piper (1932) concluded that many soils from the mallee area in South Australia contained substances which prevented seed germination due to oils from eucalyptus trees. Ashworth (1942) emphasized that "better quality" soils are associated with a low average content of fats and waxes.

McCalla *et al.* (1963) studied the phytotoxic materials in the stubble-mulch system of farming and found that ethanol-soluble substances in wheat straw, which had a depressing effect on germination as well as root and shoot growth. Sandy soils are often made unproductive for cropping because the individual sand grains become coated with organic constituents these coatings confer water-repelling properties to the sand. The compounds responsible for this phenomenon are unknown, but participation of lipids is suspected. Lipids such as paraffin hydrocarbons, phospholipids, fats, waxes, fatty acids, and terpenoids are present in soil. Some of the lipids known to occur in soil have phytotoxic properties like depressing effect on seed germination and on root and shoot growth. Waxes and similar materials may be responsible for the difficultly wettable condition of certain sand (Stevenson, 1970).

Ries *et al*. (1977) stated that triacontanol (CH_3 $(CH_2)_{28}CHOH$) is a straight chain fatty alcohol of thirty carbon atoms (C_{30}) has been recognized as prominent chemical for plant growth promotion of many agricultural and horticultural crops. It exists as constituent of cuticular waxes (Kolattukudy and Watson, 1973). It has great stimulatory effect on various processes including growth (Misra and Srivastava, 1991), protein content (Janardhar, 1992). Triacontanol is a natural growth promoting substance and enhanced growth rates and yield of many crops (Ivanov and Angelov, 1997). It also stimulates photosynthesis and several enzyme activities. Triacontanol also increase dry weight, carbon dioxide fixation, reducing sugars and free amino acids leading to the enhancement of plant growth and crop yield.

2.10. Sugarcane bagasse

Sugarcane bagasse is one of the largest agro-industrial byproducts from sugarcane industry with average production of 45 million tones every year. The direct incorporation of raw materials such as bagasse into the soil is not usually suitable because that may cause undesirable effects such as phytotoxicity and soil nitrogen immolibization. Chemical composition of bagasse is cellulose 42%, xylan 25%, lignin 25% and waxes 1%. Sugarcane bagasse was usually burnt or used by the sugar factories as a fuel for boilers, to produce gur, desichini and paper, chip- board. The conversion of bagasse into value- added compost that has the potential to improve productivity of crops and reduces the problem of environmental pollution (Negro *et al.,* 1999)

2.10.1. Microbial population in sugarcane population

Bagasse constituent offers unique challenges in processes of biodegradation. Biodegradation is considered to be one of the most suitable ways of converting bagasse into products that are beneficial for plant growth through microbial process. The aerobic *Achrmonobacter, Cytophaga, Flavobacterium, Bacillus, Pseudomonas, Azotobacter, Micrococcus, Verticillium*, *Aspergillus*, *Streptomyces* and *Ralstonia eutropha* aerobic bacterium exhibits strongly biological oxidation of aromatic phenolic compounds in bagasse (Louie *et al.*, 2002).

Hydrolysis of bagasse is done slowly by native microorganisms even when all the enrichment conditions maintained at an optimum level because of the unfavorable C: N ratio and high lignin content of lignocellulose (Li-xin *et al.,* 2002). Zayed and Abdel-motaal (2005) reported that the inoculation of cellulose degrading microorganisms *Trichoderma viridae* and *Aspergillus niger* increased decomposition rate and obtained well decomposed compost within 105 days. They also reported that incorporation of FYM with cellulolytic fungi *Trichoderma viridae* and phosphate solubilizer. *Aspergillus niger* were increased total bacterial population, fungi and phosphate solubilizers in decomposed bagasse.

2.10.1. Enzyme activity of bagasse

Enzymes are microbiological proteins which are involved in the recycling of nutrients in organic residues and influence the availability of nutrients to plants. Enzyme

activity is considered as an index of microbial activity in the soil. The physical, chemical and biological properties of bagasse are influenced by enzyme activity. The changes in the soil enzyme activity and the level of enzyme activity can be used as indicator of soil fertility (Burns, 1982). It has been now recognized that biochemical reactions involving plant nutrient transformations and organic matter decomposition are catalysed by the enzyme existing outside microbial cells and plant roots (Subramanian and Kannaiyan, 1989). The composting of agricultural wastes was quickened by inoculating microbial culture (Son, 1995).

Subramanian and Kannaiyan (1989) have estimated amylase, invertase and dehydrogenase and urease activity in soil incorporated with azolla and found that enzyme activity was increased a 20^{th} day and 40^{th} day. Different sources of lignocellulose such as wheat bran, rice husk and sugarcane bagasse have been utilized to produce various extracellular enzymes (Parajo *et al*., 1998) and (Pessoa *et al*., 1997). Sugarcane bagasse is one of the high-volume low-value agro-byproducts and therefore it is a feasible growth substrate for economical fermentation process (Pandey *et al*., 2000).

Ross (1966) reported that amylase activity was inducible and negatively correlated with soil depth and organic matter content. Soil cellulase activity is known to be influenced by addition of organic matter content (Abdel ghaffer *et al*., 1977). Biological oxidation of organic compounds is generally dehydration process and there are many dehydrogenases (enzyme catalyzing dehydrogenation) which are highly specific. The dehydrogenase activity is commonly used as an indicator of biological activity in soil. They play significant role in the biological oxidation of soil (Casida, 1977). Benekiser *et al*. (1984) stated that dehydrogenase is a useful indicator of microbial metabolism levels and it acts as a parameter for the activity of soil microflora and for microbial biomass.

2.10.2. Nutrient status of bagasse

Bagasse consists of 71% of organic matter, with total N:P:K content of 0.36, 0.03 and 0.15% respectively, fiber content 40-53%, cellulose content 30-39%, hemicellulose content of 24-30% , lignin 1-22% and wax content of 0.8-1%. The ammonical nitrogen and nitrogen content was increased in bagasse during decomposition. The maximum

available phosphorous content of 180mg/kg was recorded in bagasse on 75^{th} days. The addition of cellulolytic bacteria, farm yard manure increased the available phosphorous to 810 mg/kg. (Zayed and Abdel-Motaal, 2002) C: N ratio of bagasse 70:1 was reduced to the addition of FYM which available carbon source as well as source of heterotrophic microflora. The decrease in organic carbon content and changes in pH from alkaline to neutral was observed during decomposition of bagasse.

CHAPTER III

MATERIALS AND METHODS

3.1. General

3.1.1. Location

The experiments were conducted at the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore.

3.1.2. Glasswares

All the glasswares used for the experiments were cleaned first with soap water and then soaked in 6.5 per cent chromic acid for 2 h and finally washed with water. The glasswares thus cleaned were rinsed once with distilled water and sterilized before use.

3.1.3. Chemicals used in the study

The chemicals used were of analytical grade obtained from Hi-media, Fischer and Merck.

3.1.4. Method of sterilization

Glasswares were sterilized in a hot air oven at 180° C for three hours. All growth media, broth and water blanks were sterilized in an autoclave at 121^{o}C for 15 min. Vitamin solutions, growth factors and antibiotics were filter sterilized using sartorius 0.22 µm nitrocellulose membrane filters. Isolation, purification, inoculation and other microbiological works were carried out in laminar air flow chamber.

3.1.5. Composition of media, buffer and solutions

The composition of different culture media and reagents used in this study are given in appendix.

3.1.6. Purification and maintenance

The bacterial isolates were purified and single colony was transferred to nutrient agar slants and maintained under refrigerated condition. The bacterial isolates were sub cultured once in a month. Actinomycetes isolates were maintained on nutrient agar slants at 4˚C. The slant cultures were used for further study.

3.2. Enrichment of automobile waxy waste (AWW)

Automobile waxy waste samples were collected from nearby automobile work shop and enriched with following treatments.

T_1 : Automobile waxy waste

T_2 : Automobile waxy waste + 25% soil

T_3 : Automobile waxy waste + 50% soil

T_4 : Automobile waxy waste + 75% soil

T_5 : Automobile waxy waste + 25% FYM

T_6 : Automobile waxy waste + 50% FYM

T_7 : Automobile waxy waste + 75% FYM

T_8 : Automobile waxy waste + 25% cow dung

T_9 : Automobile waxy waste + 50% cow dung

T_{10} : Automobile waxy waste + 75% cow dung

Enriched automobile waxy waste with 25% of soil, FYM and cow dung samples were drawn on 30^{th} day and used for isolation of wax degrading bacteria and actinomycetes. Enriched automobile waxy waste samples were drawn at 0, 30, 60, 90, 120, 150, 180, 210 and 240^{th} day and used for enumeration of wax degrading bacteria.

3.2.1. Isolation of wax degrading bacteria from enriched automobile waxy waste (Allen, 1953)

Ten gram of enriched automobile waxy waste sample was suspended in 100 ml of sterile water in 250 ml Erlenmeyer flask and suspended samples of enriched automobile waxy waste were serially diluted. One ml aliquots were with drawn and plated in beeswax mineral salt medium (Mckenna *et al*., 2002), mineral salt medium I (Akit *et al*., 1981), mineral salts medium II (Skinner *et al*., 1952), inorganic basal medium (Sorkoh *et al*., 1995), modified mineral salt medium (Sorkoh *et al*., 1995) (Appendix I) with 0.5%, 1%, 2%, 5% of paraffin wax and beeswax respectively. Morphologically distinct colonies were picked up, purified by incubating at 28^{o}C for 7 days and maintained on nutrient agar slants.

3.2.2. Isolation of wax degrading actinomycetes from enriched automobile waxy waste (Allen, 1953)

Isolation of wax degrading actinomycete cultures from enriched automobile waxy waste was carried out as described in 3.2.1.

3.3. Identification of wax degrading bacteria and actinomycete

Wax degrading bacterial isolates and actinomycete cultures of enriched automobile waxy waste were identified by morphological, cultural and physiological characteristics as described in Bergey's Manual of Systematic Bacteriology (Williams *et al.,* 1984).

3.3. Characterization of bacterial isolates

3.3.1. Morphological characters

The morphological characters of bacterial isolates were identified by following the method of Gerhardt *et al*. (1981).

3.3.2. Colony characters

The colony characters *viz.*, margin, elevation, colour, shape, surface of bacterial isolates were observed on agar medium as described by Gerhardt *et al*. (1981).

3.3.3. Gram staining

The Gram's reaction of bacterial isolates was performed by following the method of Gerhardt *et al*. (1981).

3.3.4. Spore staining

The presence of endospore in bacterial isolates were observed by spore staining as per the standard method (Hariigan and Mc cance, 1966).

3.3.5. Biochemical characters

3.3.5.1. Starch hydrolysis (Beisher, 1991)

Bacterial isolates were streaked on starch agar petri plate and incubated for 48 h. The petri plates were flooded with iodine solution for 30 seconds after incubation and

drained. The positive of amylase activity was indicated by the formation of yellow zone around the colonies.

3.3.5.2. Cellulose degradation (Aneja, 1996)

The bacterial isolates were streaked on Czapex mineral salt agar medium (Appendix I) containing carboxy methyl cellullose (CMC) and incubated at 35^{o}C for 2-5 days. The positive result was indicated by the formation of clear zone around the colonies when flooded with hexadecyl trimethyl ammonium bromide after incubation.

3.3.5.3. Gelatin hydrolysis (Lysenko, 1961)

Bacterial isolates were tested for the production of gelatinase which is a proteolytic exo enzyme and capable of hydrolyzing gelatin. The bacterial isolates were inoculated into nutrient gelatin deep tubes and incubated for 48 h at 28 ^{o}C. Then the tubes were placed in a refrigerator at 4^{o}C for 30 minutes and observed for gelatin liquefaction. The production of gelatinase and ability to hydrolyze gelatin was indicated by the gelatin liquefaction.

3.3.5.4. Hydrogen sulfide production test (Seeley and Vandemark, 1981)

Sulfide indole motility (SIM) agar deep tubes were stab inoculated with bacterial isolates and incubated at 35°C for 48 h. Black colouration along the line of stab inoculation indicated H_2S production.

3.3.5.5. Catalase test (Smibert and Kreig, 1981).

Catalase test of bacterial isolates were performed by adding one ml of 0.5 per cent hydrogen peroxide to agar slant culture and observed for appearance of gas bubbles.

3.3.5.6. Citrate utilization test (Simmons, 1976)

The citrate utilization test was performed by streaking bacterial isolates in Simmon's citrate slants and incubated at 37^{o}C for 48 h. The change in coloration of the medium from green to blue was positive for test after incubation.

3.3.5.7. Indole production test (Gillus, 1956)

The bacterial isolates were inoculated into glucose tryptone broth taken and incubated for 48 h. 0.3 ml of Kovac's reagent was added and mixed well after incubation.

The reddening of the alcohol layer within a few minutes indicated positive for indole production by the bacterial isolates.

3.3.5.8. Methyl Red-Voges Proskauer test

MR-VP tests were performed to differentiate bacteria that produce acid from those that produce acetoin, a neutral product. The bacterial isolates were inoculated into MR-VP broth and incubated at 35^{o}C for 48 h. The change in colour of broth from yellow to red by the addition of methyl red indicator after incubation was indicated as positive methyl red test. Voges Proskauer tests were recorded positive by the development of red colour in MRVP broth by addition of Baritts' reagent of Omeara (1931).

3.3.5.9. Urease test (Chistensen, 1946)

Urease test of bacterial isolates were performed in urea broth containing the pH indicator phenol red. The bacterial isolates were inoculated and incubated for 24 h. The development of red colour in the broth was indicated for positive urease test.

3.3.5.10. Nitrate reduction (Neyra *et al.*, 1977)

Nitrate reductase activities of bacterial isolates were tested. The bacterial isolates were inoculated into 0.1 per cent nitrate broth (nutrient broth with 5 g KNO_3 l^{-1}) and incubated for 48 h. The positive for nitrate reduction was indicated by the appearance of distinct red colour after adding a drop of sulfanilic acid reagent and α-naphthylamine reagent.

3.4. Characterization of Actinomycete cultures

3.4.1. Morphological study

The morphological feature of actinomycetes was studied by following moist chamber glass bridge technique of Waksman and Henrici (1943). Actinomycete culture was inoculated in five ml of medium was used for identification and characterization of actinomycetes as per composition (Appendix I)

3.4.2. Petri dish moist chamber

A clean microscopic slide was placed over the ‘V’ shape glass rod in a Petri plate and the assembly was sterilized in a hot air oven. After cooling, sterile water was added to the dish to keep the chamber moist.

0.5ml of spore suspension was applied on the glass slide as a thin film by using a sterile fast flow pipette and incubated at 35ºC for 7 days. Glass slides were withdrawn and the morphological characters of the cultures viz., spore structure, aerial mycelium, branching habit were observed under microscope.

3.4.3. Cultural characterization of Actinomycetes

The actinomycete cultures were streaked on nutrient agar, yeast extract glucose agar, ken knight's agar, kuster's agar and potato dextrose agar medium and growth characters sporulation and pigment production were observed after incubation at 35^{o}C for 5 days.

3.4.4. Biochemical characterization of actinomycetes cultures

Nitrate reductase, MRVP, catalase test, urease test of wax degrading actinomycetes cultures were carried as described earlier.

3.5. Screening of efficient wax degrading bacteria and actinomycetes

The bacterial isolates and actinomycetes cultures were inoculated in mineral salt solution I with 0.5%, 1%, and 2% of paraffin wax, beeswax, coconut oil and n-hexadecane respectively. Wax degrading bacterial isolates and actinomycetes cultures were selected based upon their growth after incubation at 30^{o}C for 7 days.

3.6. Enumeration of wax degrading bacteria of enriched automobile waxy waste (Mc Kenna *et al*., 2002)

One gram of enriched automobile waxy waste samples were drawn at 0, 30, 60, 90, 120, 150, 180, 210 and 240th day. Each treatment samples was suspended in 100 ml of sterile water in 250 ml Erlenmeyer flask. Suspended sample of enriched automobile waxy waste were serially diluted up to 10^{-3} and one ml of aliquots were withdrawn and plated in mineral salt medium I (Akit *et al.*, 1981) with 0.5% beeswax. The wax degrading bacteria of enriched automobile waxy waste were enumerated after incubation at 28^{o}C for 7 days.

3.6.1. Enumeration of wax degrading microorganism by MPN techniques (Roper and Gupta, 2005)

One gram of enriched automobile waxy waste sample were drawn at 0, 30, 60, 90, 120, 150, 180, 210 and 240th day of each treatment was suspended in 0.1 % saline with 0.01 % tween 80 and serially diluted. One ml aliquots of 10^{-4}, 10^{-5}, 10^{-6} dilution were added to five replicated test tubes with 5 ml of sterile mineral salts solution I (Appendix I). One set of test tube was kept as control without inoculation and coconut oil (2%) was added to each test tube. The test tubes were incubated at 30^{o}C and growth of bacteria was observed on 5th day. The positive tubes were recorded based on emulsification of coconut oil. The MPN of wax degrading bacteria was determined by following most probable number method (Alexander, 1982).

3.7. Estimation of biosurfactant activity of wax degrading bacteria (Jain *et al.*, 1992)

The plate was coated with a thin film of coconut oil and air dried. A drop of wax degrading bacterial isolates such as *Bacillus* sp. WDB1, *Bacillus* sp. WDB2, *Bacillus* sp. WDB 3, *Pseudomonas* sp. WDB4, *Serratia* sp. WDB5, *Bacillus* sp. WDB6, *Bacillus* sp. WDB7, *Bacillus* sp. WDB8, *Pseudomonas* sp. WDB9, *Bacillus* sp. WDB10, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12, *Pseudomonas* sp. WDB13, *Bacillus* sp. WDB14, *Bacillus* sp. WDB 15, *Bacillus* sp. WDB16 and *Bacillus* sp. WDB 17 were placed separately on the glass plate by using a sterile disposable 1 ml glass syringe. The drops of bacterial isolates on the glass plate were observed carefully. The spread out area on the glass plate of bacterial isolates were measured. A drop of distilled water, placed similarly on the plate served as control. The spread out area was worked out carefully using millimeter graph sheet.

3.7.1. Estimation of biosurfactant activity of wax degrading actinomycetes cultures (Jain *et al.*, 1992)

Biosurfactant activity of actinomycetes cultures such as *Streptomycetes sp*. WDA1, *Streptomycetes sp*. WDA2, *Streptomycetes sp*. WDA3, *Streptomycetes sp*. WDA4, *Streptomycetes sp*. WDA5were estimated as described in 3.7.

3.8. Studies on growth pattern of wax degrading microorganism

3.8.1. Studies on growth pattern of wax degrading bacteria

3.8.1.1. Growth

The growth of the wax degrading bacterial isolates such as *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12 and *Bacillus* sp. WDB17 were inoculated into mineral salt solution with 1% n-hexadecane and incubated in a shaker for 15 days at 37^{o}C. The growth of bacterial isolates were estimated in a spectrophotometer at 540 nm on 1, 3, 5, 7, 9, 11, 13, 15th day.

3.8.1.2. Biomass

Biomass of wax degrading bacterial isolates such as *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12and *Bacillus* sp. WDB17 were determined by estimation of dry weight. Wax degrading bacterial isolates such as *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. *Bacillus* sp. WDB17 were inoculated into mineral salt solution I (Appendix I) with 1 % (v/v) n-hexadecane and incubated in an orbital shaking incubator with 150 rpm at 37°C for 15 days.

The bacterial pellets were obtained on 1, 3, 5, 7, 9, 11, 13, 15th day by centrifugation at 1500 rpm for 30 min and weighed after dried in a oven at 90^{o}C for 12 h.

3.8.1.3. Growth parameters of wax degrading bacteria

Wax degrading bacterial isolates such as *Bacillus* sp. 3, *Bacillus* sp. 5, *Bacillus* sp. 8, *Bacillus* sp. 9 and *Bacillus* sp. 12 were studied for their growth parameter like number of generation, generation time, growth rate (μ), growth ratio, growth yield (y), molar growth yield (Etoumi, 2007).

3.8.1.4. Measurement of emulsification activity

The emulsification activity of wax degrading bacterial isolates was estimated by following method kerosene test (Cooper and Goldenberg, 1982). Bacterial isolates such as *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12 and *Bacillus* sp. WDB17 were inoculated into the mineral salt solution I with 1 % (v/v) n-hexadecane and incubated in shaker with 150 rpm at 37 °C for 15 days. The

emulsification activity were estimated by with drawing 2 ml of bacterial supernatant such as *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12 and *Bacillus* sp. WDB17 on 1, 3, 5, 7, 9, 11, 13, 15th day and added with 2 ml of kerosene and vortexed at high speed for 2 min by using Vortex Top Mix. The emulsion activity was determined after 24 h by dividing the height of the emulsion layer by the mixture total height and multiplied by 100 (Banat, 1995).

3.8.2. Studies on growth pattern of wax degrading actinomycetes

3.8.2.1. Growth

The growth rate of wax degrading actinomycete cultures such as *Streptomyces* sp. WDA2, *Streptomyces* sp. WDA3 and *Streptomyces* sp. WDA4 were estimated as described earlier in 3.8.1.1.

3.8.2.2. Biomass

Biomass of wax degrading actinomycetes cultures such as *Streptomyces* sp. WDA2, *Streptomyces* sp. WDA3 and *Streptomyces* sp. WDA4 were estimated as described earlier in 3.8.1.2.

3.8.2.3. Growth parameters of wax degrading bacteria

Wax degrading actinomycetes cultures such as *Streptomyces* sp. WDA2, *Streptomyces* sp. WDA3 and *Streptomyces* sp. WDA4 were studied for growth parameter like number of generation, generation time, growth rate (μ), growth ratio, growth yield (y), molar growth yield as described earlier in 3.8.1.3.

3.8.2.3. Measurement of emulsification activity

The emulsification activity of wax degrading actinomycetes cultures such as *Streptomyces* sp. WDA2, *Streptomyces* sp. WDA3 and *Streptomyces* sp. WDA4 were estimated on 1, 3, 5, 7, 9, 11, 13, 15th day as described earlier in 3.8.1.4.

3.9. Antagonistic effect of wax degrading bacteria and actinomycetes (Dennis and Van doorn, 1988)

The wax degrading bacterial isolates of *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12, *Bacillus* sp. WDB17 and actinomycetes

cultures such as *Streptomyces* sp. WDA2, *Streptomyces* sp. WDA3 and *Streptomyces* sp. WDA4 were tested for antagonistic activity by following cross streak assay method.

3.10. Enzymes activity of wax degrading bacteria

3.10.1. Determination of amylase activity (Beisher, 1991)

The amylase activity of wax degrading bacterial isolates such as *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12 and *Bacillus* sp. WDB17 were estimated by following method of determination of liberated reducing sugar from starch.

Wax degrading bacterial isolates such as *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12 and *Bacillus* sp. WDB17 were inoculated into nutrient broth (Starch) and incubated at 35^{o}C for 15 days. The culture filtrate were with drawn on 3, 6, 9, 12 and 15th day by centrifugation at 13,000 rpm for 5 minutes and amylase activity was estimated. 0·2 ml of culture filtrate such as *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12 and *Bacillus* sp. WDB17 were incubated with 0.2 ml of starch (soluble) suspension (10 mg/ml in 0.1 M acetate buffer, pH 5·0) and 0·18 ml of acetate buffer at 40°C for 30 minutes and stopped by adding 0.4 ml of alkaline copper reagent. One ml of supernatant was drawn and reducing sugar was estimated. The standard was prepared with glucose and actual value of glucose was calculated and the release of one µ mol of glucose/ ml enzyme / min was expressed as one enzyme unit.

3.10.1.1. Estimation of reducing sugars (Nelson, 1944)

One ml of aliquots was added with one ml of freshly prepared alkaline copper tartarate reagent in a boiling test tube and placed in boiling water bath for 20 min. The tubes were then cooled and 1 ml arsenomolybdate reagent was added. The volume was made up to 25 ml and blue color developed was read at 495 nm. The unknown were calculated from a standard curve were prepared using glucose standard and expressed as glucose equivalents.

3.10.2. Determination of cellulase activity (Miller, 1959)

The cellulase activity of wax degrading bacterial isolates such as *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12 and *Bacillus* sp. WDB17 was determined by colorimetric method.

Wax degrading bacterial isolates such as *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12 and *Bacillus* sp. WDB17 were inoculated into nutrient broth (CMC) and incubated at 35^{o}C for 15 days. The culture filtrate was obtained on 3, 6, 9, 12 and 15th day by centrifugation at 13,000 rpm for 5 minutes and cellulase activity was estimated.

The culture filtrate 0.5 ml was incubated with 0.9 ml of CMC solution (1% w/v) in 50 mM sodium phosphate buffer (pH 7.0) and 0.25 ml distilled water at 40°C for 30 minutes. Blank were prepared in the same way and placed in ice instead of incubation. After incubation, the enzyme activity was stopped by adding 1.5 ml dinitro salicycylic acid reagent. Then the tubes were placed in boiling water both for 15 minutes, then cooled to room temperature and immediately measured at 575 nm. The standard was prepared with glucose and the actual value of glucose was calculated. One enzyme unit was expressed as 1 µ mol of glucose released / ml of enzyme/ min.

3.10.3. Determination of xylanase activity (Summer and Howell, 1935)

The xylanase activity of wax degrading bacterial isolates such as *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12, *Bacillus* sp. WDB17 were estimated by determination of liberated reducing sugar from xylan.

Wax degrading bacterial isolates such as *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12 and *Bacillus* sp. WDB17 were inoculated into nutrient broth (xylan) and incubated at 35^{o}C for 15 days. The culture filtrate were with drawn on 3, 6, 9, 12 and 15th day by centrifugation at 13,000 rpm for 5 minutes and xylanase activity was estimated.

The culture filtrate 0.1 ml of *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12 and *Bacillus* sp. WDB17 was incubated with 0.1 ml xylan and 0.2 ml of the acetate buffer at 40°C for 30 minutes and the reaction was stopped by

adding 0.5 ml alkaline copper reagents. The mixture was kept in a boiling water bath for 10 minutes, then cooled and incubated for 15 min with 0.2 ml arsenomolybdate reagent. The supernatant was diluted fivefold with water and measured in UV/VIS spectrophotometer at 495 nm. The standard was prepared with xylose and actual value of xylose was calculated. One enzyme unit was expressed as 1 µ mol of D-xylose/ released per ml of enzyme/ per minute.

3.10.4. Determination of lipase activity (Somkuti and Babel, 1968)

The lipase activity of wax degrading bacterial isolates such as *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12 and *Bacillus* sp. WDB17 were estimated.

Wax degrading bacterial isolates such as *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12 and *Bacillus* sp. WDB17 were inoculated into nutrient broth (coconut oil) at 35^{o}C for 15 days. The culture filtrate of bacterial isolates were with drawn on 3, 6, 9, 12 and 15th day by centrifugation at 13,000 rpm for 5 minutes and lipase activity was estimated.

The culture filtrate of 0.5 ml was added separately into 20 ml of neutralized coconut oil and 5 ml of phosphate buffer in a 500 ml beaker and pH was noted immediately. The pH drops was adjusted to initial value of pH. Every 10 min by the addition of 1N NaOH and continued for period of 30-60 minutes. The volume of alkali consumed was noted and the lipase activity was calculated from standard tributyrin. One enzyme unit was expressed as one milliequivalents of fatty acid produced / ml of enzyme/ min.

3.11. Enzymes activity of wax degrading actinomycetes

3.11.1. Determination of amylase activity

The wax degrading actinomycete cultures such as *Streptomyces* sp. WDA2, *Streptomyces* sp. WDA3 and *Streptomyces* sp. WDA4 were inoculated into basal medium and incubated at 35 ^{o}C for 15 days. The culture filtrate were obtain on 3, 6, 9, 12 and 15th day and amylase activity of actinomycetes cultures were estimated as described in 3.10.1.

3.11.2. Determination of cellulase activity

The wax degrading actinomycete cultures such as *Streptomyces* sp. WDA2, *Streptomyces* sp. WDA3 and *Streptomyces* sp. WDA4 were inoculated into basal medium and incubated at 35^{o} C for 15 days. The culture filtrate were obtain on 3, 6, 9, 12 and 15th day and cellulase activity of actinomycetes cultures were estimated as described in 3.10.2.

3.11.3. Determination of xylanase activity

The wax degrading actinomycete cultures such as *Streptomyces* sp. WDA2, *Streptomyces* sp. WDA3 and *Streptomyces* sp. WDA4 were inoculated into basal medium and incubated at 35^{o} C for 15 days. The culture filtrate were obtain on 3, 6, 9, 12 and 15th day and cellulase activity of actinomycetes cultures were estimated as described in 3.10.3.

3.11.4. Determination of lipase activity

The wax degrading actinomycete cultures such as *Streptomyces* sp. WDA2, *Streptomyces* sp. WDA3 and *Streptomyces* sp. WDA4 were inoculated into basal medium and incubated at 35^{o} C for 15 days. The culture filtrate were obtained on 3, 6, 9, 12 and 15th day and lipase activity of actinomycetes cultures were estimated as described in 3.10.4.

3.12. Estimation of beeswax degradation of wax degrading bacteria

Wax degrading bacterial isolates such as *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12 and *Bacillus* sp. WDB17 were inoculated in mineral salt solution I with beeswax and incubated for 15 days at 28^{o}C. The degradation of beeswax was determined by estimation of loss in weight of beeswax. The broth was centrifuged at 13,000 rpm for 5 minutes and beeswax with microbial pellets was taken out in a pre weighed container and fresh weight was noted and incubated in oven at 65^{o}C for 5 h. After incubation weight of pellets was taken and the actually weight of beeswax degraded by microorganisms were derived by subtracting the fresh weight of beeswax and microbial pellets with final weight of pellets and finally with weight of original beeswax added in medium.

3.12.1. Estimation of beeswax degradation of wax degrading actinomycetes

Actinomycete cultures such as *Streptomyces* sp. WDA2, *Streptomyces* sp. WDA3 and *Streptomyces* sp. WDA4 were inoculated in mineral salt solution I (Appendix I) with beeswax and incubated for 15 days at 28^{o}C and weight loss were estimated as described earlier.

3.13. Effect of wax degrading microorganism on sugarcane bagasse

Sugarcane Bagasse was collected from nearby juice shop and chopped into small pieces manually and amended with microbial consortium. Efficient wax degrading bacterial isolates and actinomycetes cultures such as *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12, *Bacillus* sp. WDB17 and actinomycetes culture *Streptomyces* sp. WDA2, *Streptomyces* sp. WDA3 and *Streptomyces* sp. WDA4 were grown in nutrient glucose broth for 3 days at 30^{o}C and 5 days at 30^{o}C respectively. Equal volume of each wax degrading bacterial isolates (10^{7}) and actinomycetes culture (10^{4}) were obtained and microbial consortium was prepared (v/w). Then the following treatments were given:

T_1: Sterilized Bagasse

T_2: Bagasse

T_3: Bagasse + 50 % Soil

T_4: Bagasse + 50 % Soil + 10 % Microbial consortium

T_5: Sterilized bagasse +10 % Micobial consoritum

T_6: Bagasse + 10 % Microbial consortium

The samples were drawn from sugarcane bagasse on 0, 15, 30, 45, 60, 75, 90 and 105th day and analyzed for microbial population, wax degrading bacteria and actinomycetes, total nitrogen content, phosphorous content, potassium content, organic carbon, pH and water repellency.

3.13.1. Microbial dynamics

The total aerobic bacteria, fungi, actinomycetes and wax degrading bacteria and actinomycetes population of sugarcane bagasse was estimated by following method of Allen (1953), Martin (1950), Allen (1953) and Akit *et al.* (1981) respectively.

3.13.2. Effect of wax degrading microbial inoculation on enzyme activity of bagasse

3.13.2.1. Estimation of amylase activity

Amylase activity of bagasse was estimated by following the method of Galstyan (1965). Five gram of bagasse was added with 1.5 ml of toluene and incubated for 15 minutes. Then 10 ml of distilled water was added and incubated with 5 ml of 2 % starch solution for 5 h at 37^{o}C. After incubation 15 ml of distilled water was added and mixed well. Then the contents were centrifuged at 5000 rpm. One ml of supernatant was drawn and reducing sugar was estimated by following the modified Somogyi method of Nelson (1944).

3.13.2.3. Estimation of cellulase activity

Cellulase activity of bagasse was estimated by following the method of Benefield (1971). One gram bagasse was added with 1 ml of phosphate buffer (pH 5). Two drops of toluene and then 1 ml of 1 per cent of carboxymethyl cellulose and incubated with 3 ml of distilled water for 24 h. After incubation 1 ml of the supernatant was taken and analyzed for reducing sugars as described earlier.

3.13.2.3. Estimation of xylanase activity

Xylanase activity of bagasse was estimated by following the method of Schinner and Von Mersi (1990). One gram of bagasse was added with 5 ml of 2 per cent xylan and 3 ml of 2 M acetate buffer and incubated at 37^{o}C for 24 h. After incubation 1 ml of the supernatant was drawn and analyzed for reducing sugars as described earlier.

3.13.2.4. Dehydrogenase activity (Casida *et al*., 1964)

5g of bagasse was added to 0.05 g of calcium carbonate in a 100 ml of beaker, one ml of 3% Triphenyl tetrazolium chloride (TTC) and 2.5 ml of distilled water was added. The content of beaker were mixed well with a glass rod and covered with aluminum foil and incubated at 37 ^{o} C for 24 h. The product tri phenyl formazan (TPF) was extracted by 94% methanol and with immediately filter paper. The final volume was made up to 100 ml with methanol and measured in a spectrophotometer at 460 nm standard was drawn using TPF in methanol. The dehydrogenase activity was expressed as µg of TFP formed g^{-1} dry soil 24 h^{-1}.

3.13.2.5. Assessment of water repellency of bagasse

Soil water repellency was determined by the Molarity of Ethanol Droplet (MED) method as described by King (1981). 40 µl of ethanol solution with concentration of 0.2M intervals in the range of 0-5 M were prepared and placed on bagasse surface with a Pasteur pipette and the water repellency of bagasse was estimated by calculating the entry time of ethanol in bagasse.

3.13.3. Analysis of bagasse

3.13.3.1. Total nitrogen

The total nitrogen content of bagasse was estimated by microkjeldahl method of Humphries (1956).

3.13.3.2. Total phosphorous

The total Phosphorous content of bagasse was estimated by following the method of Jackson (1973).

3.13.3.3. Total potassium

The total potassium content of bagasse was estimated by following the method of Jackson (1973).

3.13.3.4. Organic carbon

Organic carbon content of bagasse was estimated by following the method of Walkey and black as described by Piper (1950).

3.13.3.5. pH

The pH of bagasse was estimated by following method of Falcon *et al*. (1987).

3.14. Statistical analysis

The results of the experiments were subjected to statistical scrutiny as per the methods detailed by Panse and Sukhatme (1978). If the treatment differences are found significant in F test, critical differences were worked out at 5 percent probability

CHAPTER IV

EXPERIMENTAL RESULT

The present investigation was undertaken with a view to meet the overall objectives of effectively utilizing and degrading waxy rich agricultural waste in an ecofriendly manner. During this study, experiments were carried out for isolation and screening of wax degrading microorganism and their substrate use efficiency, enzymes activity were estimated. The results obtained in various experiments conducted during the present investigation are summarized below.

4.1. Isolation of wax degrading microorganisms

Wax degrading bacteria and actinomycetes were isolated from enriched automobile waxy waste in beeswax mineral salt medium, mineral salt medium I, mineral salt medium II, inorganic basal medium, modified mineral salt medium with 0.5, 1, 2 and 5per cent of beeswax and paraffin wax are presented in Table 1 and 2. Higher wax degrading bacterial isolates and actinomycetes culture were observed in waxy medium with 0.5 per cent concentration of wax (Plate 1). Wax degrading bacterial isolates were recorded maximum in mineral salt medium I and followed by beeswax mineral salt medium. The mineral salt medium I with beeswax was observed higher bacterial isolates than mineral salt medium I with paraffin wax. Waxy medium with low wax content of 0.5 per cent paraffin wax and beeswax recorded maximum bacterial isolates of 31 and 32 respectively. Least number of wax degrading bacterial isolates was observed in the waxy medium with 5per cent wax content.

Wax degrading actinomycetes cultures were obtained maximum in mineral salt medium I with 0.5 per cent paraffin wax followed by beeswax. Seventeen wax degrading bacterial isolates and five actinomycetes cultures were selected from mineral salt medium with 0.5 per cent beeswax and mineral salt medium with 1 per cent beeswax medium. The carbon source 1per cent beeswax recorded highest actinomycetes cultures than 0.5, 2 and 5 per cent in beeswax mineral salt medium I.

4.2. Characterization of wax degrading bacteria

4.2.1. Morphological characterization of wax degrading bacteria

The morphological character's of wax degrading bacterial isolates are presented in Table 3a and Plate 2. Wax degrading bacteria isolates such as WDB-1, WDB- 2, WDB- 6, WDB- 7, WDB- 8, WDB- 12, WDB- 13, WDB-14, WDB-15, WDB-16 and WDB-17 were shown rod shape, whereas WDB-3, WDB- 4, WDB- 9 and WDB- 10 was observed cocci shape. Wax degrading bacterial isolate WDB- 5 and WDB- 11 were observed long rod and small rod shape. The wax degrading bacterial isolates WDB-1, WDB- 2, WDB- 3, WDB- 6, WDB- 7, WDB- 8, WDB- 11, WDB- 12, WDB- 14, WDB-15, WDB-16 and WDB-17 were shown positive for gram staining and spore staining. The gram staining and spore staining were observed negative in wax degrading isolates WDB- 4, WDB- 5, WDB- 9, WDB- 10 and WDB- 13.

4.2.2. Colony characterization of wax degrading bacteria

The colony character's of wax degrading bacterial isolates are summarized in Table 3b and Plate 2. The wax degrading isolate WDB- 1, WDB- 4, WDB- 9 and WDB- 11 were formed irregular colonies. Circular and rhizoid colonies were observed in wax degrading bacterial isolates WDB- 3, WDB- 6, WDB- 7, WDB- 8, WDB- 10, WDB-12, WDB- 13, WDB- 14 and WDB- 5 respectively.

Margin of wax degrading bacterial colonies undulate and entire was observed in WDB-1, WDB-9, WDB- 16 and WDB- 3, WDB- 5, WDB- 6, WDB- 7, WDB- 8, WDB- 10, WDB- 12, WDB- 13, WDB- 17, whereas WDB-4, WDB-11, WDB-15 was observed crenate respectively. The wax degrading bacterial isolates WDB- 1, WDB- 9 and WDB- 3, WDB- 6, WDB- 7, WDB- 8, WDB- 10, WDB- 12, WDB- 13 and WDB- 14 were recorded smooth and transparent surface respectively.

Elevation of wax degrading bacterial colonies effuse, pulvinate, convex papillate was recorded in WDB- 1, WDB-3, WDB-4, WDB-6, WDB-9, WDB-14, WDB-15 and WDB-17. Wax degrading bacterial isolates WDB-1, WDB-4, WDB-12, WDB-13 and WDB-17 were recorded dirty white colonies. Wax degrading bacterial isolates WDB- 2,

WDB- 3, WDB- 6, WDB- 7, WDB- 9, WDB- 10, WDB- 11, WDB- 14, WDB- 15, WDB- 16 were recorded creamy white color whereas as the colony of wax degrading bacterial isolate WDB -5 was shown red color.

4.2.3. Biochemical characterization of wax degrading bacteria

Biochemical characterizations of wax degrading bacterial isolates are presented in Table 3c**.** Wax degrading bacterial isolates WDB-1, WDB- 2, WDB-3, WDB-4, WDB-6, WDB-7, WDB-8, WDB-10, WDB- 11, WDB-12 and WDB-15 were shown positive for starch

Wax degrading bacterial isolates WDB-1, WDB- 2, WDB-3, WDB-4, WDB-6, WDB-7, WDB-8, WDB-9, WDB-10, WDB- 11, WDB-12, WDB-13, WDB-14 and WDB-15 were observed cellulose degradation positive.

Citrate utilization test was recorded positive in wax degrading bacterial isolates WDB-1, WDB- 5, WDB-8, WDB-9, WDB-10, WDB- 11, WDB-12 and WDB-13. Wax degrading bacterial isolates WDB-3, WDB-5, WDB-6, WDB-7, WDB-10, WDB-11, WDB-10, WBD- 13, WDB-15, WDB- 16, WDB 17 were shown positive for indole test.

The methyl red and voges proskauer test was registered positive in wax degrading isolates WDB-1, WDB-2, WDB-4 WDB-6, WDB-7, WDB-9, WDB-10, WDB-12 WDB-13, WDB -14, WDB -17 and WDB-3 WDB- 5, WDB- 8 WDB-11, WDB-15, WDB-16 WDB- WDB17 respectively.

The gelatin hydrolysis test was shown positive in wax degrading bacterial isolate WDB-3, WDB-7 WDB-10, WDB-11, WDB-16 and WDB-17. The catalase activity was recorded positive in all wax degrading bacterial isolates. Wax degrading bacterial isolates WDB-5 was registered positive for nitrate reduction and hydrogen sulphate production test.

Wax degrading bacterial isolates such as WDB-1, WDB-2, WDB- 3, WDB- 4, WDB- 5, WDB- 6, WDB-7, 8, WDB- 9, WDB-10, WDB- 11, WDB-12, WDB-13, WDB- 14, WDB- 15, WDB- 16, WDB- 17 were tentatively identified as *Bacillus* sp. WDB1, *Bacillus* sp. WDB2, *Bacillus* sp. WDB 3, *Pseudomonas* sp. WDB4, *Serratia* sp. WDB5, *Bacillus* sp. WDB6, *Bacillus* sp. WDB7, *Bacillus* sp. WDB8, *Pseudomonas* sp. WDB9, *Bacillus* sp.

WDB10, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12, *Pseudomonas* sp. WDB13, *Bacillus* sp. WDB14, *Bacillus* sp. WDB 15, *Bacillus* sp. WDB16 and *Bacillus* sp. WDB 17 from the observation of morphological ,colony and biochemical characterization.

4.3. Characterization of wax degrading actinomycetes

The morphological, cultural and biochemical characters of wax degrading actinomycetes are summarized in Table 4 and Plate 3. Wax degrading actinomycetes WDA-1 was observed good growth in potato dextrose agar, yeast extract glucose agar and produces brown pinch colonies whereas WDA-2 produced dark brown pigmented colonies in nutrient agar and shown good growth in kuster agar medium. Wax degrading actinomycetes culture WDA-5was observed yellowish white powdery growth and brownish sporulation in nutrient agar and potato dextrose agar. The actinomycetes culture WDA-1, WDA-2, WDA-3, WDA-4, and WDA-5 showed positive for nitrate reduction, MRVP, catalase test and urease test. From the morphological and biochemical characterization of actinomycetes isolates WDA-1, WDA-2, WDA-3, WDA-4, WDA-5 were tentatively identified as *Streptomyces* sp. WDA1, *Streptomyces* sp. WDA 2, *Streptomyces* sp. WDA 3, *Streptomyces* sp. WDA 4 and *Streptomyces* sp. WDA 5.

4.4. Screening of efficient wax degrading bacteria and actinomycetes in waxy medium with different carbon sources

Screening of efficient wax degrading bacterial isolates and actinomycetes cultures in waxy medium with different carbon sources are summarized in Table 5 and Plate 4. The wax degrading bacterial isolates *Bacillus* sp. WDB3, *Bacillus* sp WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12 and *Bacillus* sp. WDB17 were observed good growth in 0.5, 1 and 2 per cent of paraffin wax, beeswax, n-hexadecane and coconut oil. WDB-3, WDB-7, WDB-17 are not able to utilize paraffin wax of 1per cent and 2 per cent respectively. Wax degrading bacterial isolates *Bacillus* sp. WDB12 was observed good growth in 1 per cent and 2 per cent of paraffin wax. The actinomycetes culture *Streptomyces* sp. WDA2, *Streptomyces* sp.WDA3 and *Streptomyces* sp.WDA4 were able to utilize all the carbon sources, *Streptomyces* sp.WDA2 were unable to utilize paraffin wax of 2per cent and exhibits lesser growth compared to other wax degrading actinomycete cultures *Streptomyces* sp.WDA3 and *Streptomyces* sp.WDA4.

4.5. Enumeration of wax degrading bacteria from enriched automobile waxy waste

The enumeration of wax degrading bacteria of enriched automobile waxy waste are presented in Table 6 and Fig.1. Wax degrading population was shown maximum in automobile waxy waste with 75 per cent cow dung and followed by automobile waxy waste with 75 per cent soil. Enrichment of automobile waxy waste with soil, FYM and cow dung showed increase in wax degrading bacterial population. Automobile waxy waste with 75 per cent soil recorded higher wax degrading bacterial population than automobile waxy waste enriched with 25 and 50 per cent of soil. Enrichment of automobile waxy waste with 75 per cent FYM was registered higher wax degrading bacterial population of 30 x 10^3 g^{-1}. The maximum wax degrading bacterial population of 40 x 10^3 g^{-1} was observed in automobile waxy waste with 75per cent cow dung on 240^{th} day. In general, the wax degrading bacterial population was enhanced during incubation. Wax degrading bacterial population was observed very poor in 100 per cent automobile waxy waste.

4.5.1. Enumeration of wax degrading bacteria from enriched automobile waxy waste by MPN technique

The enumeration of wax degrading bacteria of enriched automobile waxy waste by MPN technique are presented in Table 7 and Fig.1. Wax degrading bacterial population was shown higher in automobile waxy waste with 75 per cent cow dung and followed by the automobile waxy waste with 75 per cent soil. Enrichment of automobile waxy waste with FYM enhances wax degrading bacterial population and registered higher in automobile waxy waste with 75 per cent FYM than 25 and 50 per cent FYM. Automobile waxy waste amended with 75 per cent cow dung recorded higher wax degrading bacterial population than 25 and 50 per cent of cow dung. Wax degrading bacterial population maximum of 72 x 10^4 g^{-1} was observed in automobile waxy waste with 75 per cent cow dung on 240^{th} day. In general, the wax degrading bacterial population was enhanced during incubation.

4.6. Studies on biosurfactant activity of wax degrading bacteria and actinomycetes of enriched automobile waxy waste

Biosurfactant activity of wax degrading bacterial isolates and actinomycetes cultures in n-hexadecane are presented in Table 8. Wax degrading bacterial isolate

Bacillus sp. WDB12 recorded maximum biosurfactant activity of 152.14 mm^2 and followed by *Bacillus* sp. WDB11 of 119.93 mm^2, *Bacillus* sp. WDB3 of 94.39 mm^2, *Bacillus* sp. WDB17 of 92.00mm^2, *Bacillus* sp. WDB7 of 75.64 mm^2 on 15^{th} day. Wax degrading bacterial isolate produced higher biosurfactant activity than wax degrading actinomycetes culture. The wax degrading bacterial isolates and actinomycetes cultures were registered steady increase in biosurfactant activity during incubation. The actinomycetes culture *Streptomyces* sp. WDA3 produced higher biosurfactant activity of 95.64mm^2 than *Streptomyces* sp. WDA4. Statistical significant in biosusrfactant activity was observed in *Bacillus* sp. WDB17 on 15^{th} day.

4.7. Studies on growth of wax degrading bacteria and actinomycetes of enriched automobile waxy waste in n-hexadecane

The growth of wax degrading bacterial isolates and wax degrading actinomycetes cultures in n-hexadecane are summarized in Table 9 and Fig.2. The higher growth of wax degrading bacterial isolates *Bacillus* sp. WDB3, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12 were recorded on 9 and 11^{th} day. *Bacillus* sp. WDB7 and *Bacillus* sp. WDB17 wax degrading bacterial isolates was observed gradual increase in growth and observed maximum on 11^{th} day and declined further.

4.8. Studies on biomass of wax degrading bacteria and actinomycetes of enriched waxy waste in n-hexadecane

The biomass of wax degrading bacterial isolates and actinomycetes cultures in n-hexadecane are presented in Table 10 and Fig.2. The wax degrading bacterial isolate *Bacillus* sp. WDB11 produced maximum biomass of 0.28 mg in n- hexadecane and followed by *Bacillus* sp. WDB12 on 15^{th} day. The wax degrading bacterial isolates were observed slight increase in biomass production on 3^{rd} day in n-hexadecane. Wax degrading actinomycete cultures recorded gradual increase in biomass production in n-hexadecane and attained maximum on 15^{th} day. Wax degrading actinomycetes *Streptomyces*.sp WDA 3 were observed higher biomass than *Streptomyces* sp. WDA2. *Bacillus* sp. WDB12 was recorded higher biomass production than wax degrading actinomycetes *Streptomyces* sp. WDA3

4.9. Studies on Growth pattern of wax degrading bacteria and actinomycetes of enriched automobile waxy waste in n-hexadecane.

The number of generation, generation time, growth rate (μ), growth yield (y) and molar yield of wax degrading bacterial isolates and actinomycetes cultures are summarized in Table 11 and Fig. 2. Wax degrading bacterial isolate *Bacillus* sp. WDB12 recorded very low generation time and higher number of generation. Growth rate (μ) and growth ratio was recorded maximum in *Bacillus* sp. WDB12 of 9.61min and 16.61 respectively. Wax degrading bacterial isolate *Bacillus* sp. WDB11 shown maximum growth yield of 0.41. The actinomycetes culture *Streptomyces* sp. WDA2 was shown higher number of generation and low generation time than *Streptomyces* sp. WDA3 and *Streptomyces* sp. WDA4. Wax degrading actinomycetes cultures showed higher molar growth yield than wax degrading bacterial isolates. Actinomycetes cultures *Streptomyces* sp. WDA3 was recorded higher molar growth yield of 0.22 g/M than *Streptomyces* sp. WDA4.

4.10. Enumeration of wax degrading bacteria and actinomycetes of enriched automobile waxy waste in n-hexadecane

The enumeration of wax degrading bacteria and actinomycetes in n-hexadecane are presented in Table 12. The wax degrading bacterial population was higher in hexadecane inoculated with *Bacillus* sp. WDB12 than other wax degrading bacterial isolates on 9^{th} day. The slight increase in wax degrading bacterial population was recorded on 5^{th} day. Maximum wax degrading bacterial population was observed in n- hexadecane on 9 and 11^{th} day and declined further. The similar trend was observed in actinomycetes cultures. The n-hexadecane inoculated with wax degrading actinomycetes cultures *Streptomyces* sp. WDA1 exhibited higher wax degrading actinomycetes population than other actinomycetes cultures. Statistically significant was observed in wax degrading bacterial population and actinomycetes culture on 9^{th} day.

4.11. Studies on emulsification activity of wax degrading bacteria and actinomycetes of enriched automobile waxy waste in n-hexadecane

Emulsification activity of wax degrading bacterial isolates and actinomycetes cultures in n-hexadecane are summarized in Table 13. The wax degrading bacterial

isolates *Bacillus* sp.WDB12 was recorded maximum emulsification activity on 11th day and followed by *Bacillus* sp. WDB17 (Plate 5). Bacterial isolates *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11 recorded maximum emulsification activity on 13th day and markedly reduced further. The emulsification activity of wax degrading bacterial isolates *Bacillus* sp.WDB12 and *Bacillus* sp. WDB17 were increased to 59 and 56 per cent respectively on 11th day and wax degrading actinomycetes culture *Streptomyces* sp.WDA2 showed higher emulsification activity than *Streptomyces* sp. WDA3, *Streptomyces* sp. WDA4. The wax degrading actinomycetes culture shown highest emulsification activity in 5th day and slightly reduced further. In general the emulsification activity of actinomycete cultures was higher than bacterial isolate

4.12. Studies on *invitro* enzyme activity of wax degrading bacteria and actinomycetes

4.12.1. Studies on amylase activity of wax degrading bacteria and actinomycetes of enriched automobile waxy waste

The amylase activity of wax degrading bacterial isolates and actinomycetes cultures are presented in Table 14 and Fig.3. The wax degrading bacterial isolate *Bacillus* sp. WDB9 was recorded higher amylase activity of 3.97 unit ml^{-1} and followed by *Bacillus* sp.WDB11 of 3.78 unit/ml, *Bacillus* sp. WDB12 of 3.01unit ml^{-1} on 12th day and declined further. Marked variation in amylase activity was observed in wax degrading bacterial isolates. The amylase activity of wax degrading actinomycetes culture *Streptomyces* sp. WDA3 was shown 5.24 unit ml^{-1} on 12th day. Statistically significance in amylase activity was observed in bacterial isolates and actinomycetes on 12th day. In general, the amylase activity was observed higher in wax degrading actinomycetes culture than in wax degrading bacterial isolates.

4.12.2. Studies on cellulase activity of wax degrading bacteria and actinomycetes of enriched automobile waxy waste

The cellulase activity of wax degrading bacterial isolates and actinomycetes cultures are presented in Table 15 and Fig.3. The cellulase activity of wax degrading bacterial isolates *Bacillus* sp. WDA12 was recorded maximum of 3.89 unit ml^{-1} on 12th day. Marked variation in cellulase enzyme activity was observed in wax degrading bacterial isolates. The cellulase activity of wax degrading bacterial isolates was recorded

maximum on 12th day and reduced further. The actinomycetes culture *Streptomyces* sp. WDA2 was also recorded higher cellulase activity on 12th day. Statistically significance in cellulase activity was observed between wax degrading bacterial isolates and actinomycetes culture. The wax degrading actinomycetes culture *Streptomyces* sp. WDA2 was observed higher cellulase activity of 9.83 unit ml^{-1} than *Streptomyces* sp. WDA3

4.12.3. Studies on xylanase activity of wax degrading bacteria and actinomycetes of enriched automobile waxy waste

The xylanase activity of wax degrading bacterial isolates and actinomycete cultures are presented in Table 16 and Fig.3. The wax degrading bacterial isolate *Bacillus* sp. WDB7 was observed higher xylanase activity of 4.12 unit/ml than *Bacillus* sp. WDB11 of 3.99 unit ml^{-1} on 12th day. Significant increase in xylanase activity was recorded in wax degrading bacterial isolates. The xylanase activity of wax degrading actinomycetes cultures *Streptomyces* sp. WDA2, *Streptomyces* sp. WDA3 and *Streptomyces* sp. WDA4 were recorded 6.32, 6.34 and 6.19 unit/ml respectively on 12th day. In general, highest xylanase activity of wax degrading bacterial isolates was observed on 12th day and declined further. Significant increase in xylanase activity was observed in *Streptomyces* sp. WDA3, *Streptomyces* sp. WDA2 and bacterial isolates *Bacillus* sp. WDB7 and *Bacillus* sp. WDB11 on 12th day.

4.12.4. Studies on lipase activity of wax degrading bacteria and actinomycetes of enriched automobile waxy waste

The lipase activity of wax degrading bacterial isolates and actinomycete cultures are presented in Table 17 and Fig.3. The lipase activity of wax degrading bacterial isolates were recorded maximum on 12th day. Wax degrading bacterial isolates *Bacillus* sp. WDB12, *Bacillus* sp. WDB11, *Bacillus* sp. WDB7, *Bacillus* sp. WDB3 was shown lipase activity of 2.99, 2.32, 2.12 and 1.38 unit ml^{-1}. The wax degrading actinomycetes culture *Streptomyces* sp. 3 was recorded higher lipase activity of 3.07unit ml^{-1} on 12th day compared to *Streptomyces* sp. 2 and *Streptomyces* sp. 4. Significant increase in lipase activity was observed in bacterial isolates and wax degrading actinomycetes culture.

4.13. Effect of wax degrading bacteria and actinomycetes on degradation of beeswax

The effect of wax degrading bacterial isolates and actinomycetes cultures on degradation of beeswax are summarized in Table 18 and Plate 6. Wax degrading bacterial isolate *Bacillus* sp. WDB12 recorded higher degradation of beeswax of 0.05 mg and followed by *Bacillus* sp. WDB11 on 15^{th} day. Wax degrading bacterial isolates was shown slight reduction in beeswax content on 6^{th} and 9^{th} day. Wax degrading actinomycete cultures *Streptomyces* sp. WDA3 recorded maximum degradation of beeswax of 0.06 g on 15^{th} day and followed by *Streptomyces* sp. WDA2, *Streptomyces* sp. WDA4. In general, wax degrading actinomycete cultures utilized higher beeswax content than wax degrading bacterial isolates.

4.14. Studies on antagonistic activity of wax degrading bacteria and actinomycetes of enriched automobile waxy waste

The antagonistic activity of wax degrading bacterial isolates and wax degrading actinomycetes cultures are summarized in Table 19. The *Streptomyces* sp. WDA2 showed a inhibition zone of 0.5mm for *Bacillus* sp.WDB3, *Bacillus* sp.WDB17 and there is no inhibition zone was observed with other wax degrading bacterial isolates

4.15. Effect of wax degrading microbial consortium on microbial dynamics of sugarcane bagasse

4.15.1. Effect of wax degrading microbial consortium on total bacterial population of sugarcane bagasse

The effect of wax degrading microbial consortium on total bacterial population of sugarcane bagasse are presented in Table 20 and Fig.4.The total bacterial population in bagasse incorporated with soil and 10per cent microbial consortium was recorded highest population of 71 x 10^6 g^{-1} and followed by sterilized bagasse with 10 per cent microbial consortium. The increase in total bacterial population was observed in bagasse incorporated with soil and microbial consortium. Bagasse incorporated with soil and 10per cent microbial consortium recorded higher total bacterial population on 75^{th} day than on 105^{th} day. In general, bagasse incorporated with soil and 10per cent microbial consortium was observed increasing trend in total aerobic bacterial population up to 75^{th} day and declined further.

4.15.2. Effect of wax degrading microbial consortium on fungal population of sugarcane bagasse

The effect of wax degrading microbial consortium on fungal population in sugarcane bagasse are presented in Table 21 and Fig.4. The fungal population was recorded maximum in bagasse incorporated with soil and 10 per cent microbial consortium followed by the bagasse incorporated with soil. The fungal population was recorded very low of 15 x 10^4 g^{-1} sterilized bagasse on 75^{th} day. Significant increase in fungal population was observed in bagasse incorporated with soil.

4.15.3. Effect of wax degrading microbial consortium on actinomycetes population of sugarcane bagasse

The effect of wax degrading microbial consortium on actinomycetes population in sugarcane bagasse are presented in Table 22 and Fig.4. The bagasse incorporated with soil and 10 per cent microbial consortium was observed maximum actinomycetes population of 19 x $10^3 g^{-1}$ and followed by bagasse with 10per cent microbial consortium of 18 x 10^3 g^{-1}. The actinomycetes population was recorded higher in bagasse with soil and microbial consortium on 90^{th} day. The sterilized bagasse and bagasse were recorded very low actinomycetes population. The actinomycetes population was recorded higher in bagasse with 10 per cent microbial consortium than bagasse incorporated with soil. Significant difference was observed in actinomycetes population in bagasse incorporated with soil and microbial consortium on 90^{th} and 105^{th} day.

4.15.4. Effect of wax degrading microbial consortium on wax degrading bacterial population of sugarcane bagasse

The effect of wax degrading microbial consortium on wax degrading bacteria on sugarcane bagasse are presented in Table 23 and Fig.4. Sterilized bagasse with 10 per cent microbial consortium was observed maximum wax degrading bacterial population of 39 x $10^6 g^{-1}$ and followed by bagasse with 10 per cent microbial consortium. Wax degrading bacterial population was increased to 39 x 10^6 g^{-1} on 90^{th} day and declined further. The wax degrading bacterial population was higher in bagasse incorporated with soil than bagasse.

4.15.5. Effect of wax degrading microbial consortium on wax degrading actinomycetes population of sugarcane bagasse

The effect of wax degrading microbial consortium on wax degrading actinomycetes in sugarcane bagasse are presented in Table 24 and Fig.4. Wax degrading actinomycetes population was recorded highest of 15 x $10^3 g^{-1}$ in bagasse amended with 10 per cent microbial consortium on 90^{th} day and followed by sterilized bagasse with 10 per cent microbial consortium of 13 x 10^3 g^{-1}. Whereas sterilized bagasse recorded very low wax degrading actinomycetes population. Bagasse incorporated with soil and microbial consortium was observed increase in wax degrading actinomycetes population on 40^{th} day. Significant increase in wax degrading actinomycetes population was observed in bagasse with microbial consortium on 90^{th} day.

4.16. Effect of microbial consortium on enzyme activity of sugarcane bagasse

4.16.1. Effect of wax degrading microbial consortium on amylase activity of sugarcane bagasse

The effect of wax degrading microorganisms on amylase activity of sugarcane bagasse are summarized in Table 25 and Fig.5. The amylase activity was recorded maximum 3.08 unit ml^{-1} in bagasse incorporated with soil and 10per cent microbial consortium of and followed by bagasse with 50 per cent soil on 75^{th} day. The amylase activity in bagasse, sterilized bagasse and sterilized bagasse incorporated with 10 per cent microbial consortium was observed 1.30, 1.29, 1.18 and 1.58 unit ml^{-1} on 75^{th} day. Significant increase in amylase activity was observed in bagasse with soil, bagasse incorporated with soil and 10per cent microbial consortium on 75 and 90^{th} day.

4.16.2. Effect of wax degrading microbial consortium on cellulase activity of sugarcane bagasse

The effect of wax degrading microbial consortium on cellulase activity of sugarcane bagasse are presented in Table 26 and Fig.5. In general, the bagasse amended with 10 per cent microbial consortium recorded higher cellulase activity. Bagasse incorporated with soil and 10 per cent microbial consortium was recorded the maximum cellulase activity of 3.36 unit ml^{-1} and followed by bagasse incorporated with soil. The maximum cellulase

activity was observed in bagasse with 10per cent microbial consortium and sterilized bagasse with microbial consortium of 2.22 and 2.18 unit ml^{-1} respectively on 45^{th} day. Significant increase in cellulase activity was observed in bagasse incorporated with soil and 10 per cent microbial consortium on 45 and 60^{th} day.

4.16.3. Effect of wax degrading microbial consortium on xylanase activity of sugarcane bagasse

The effect of wax degrading microbial consortium on xylanase activity of sugarcane bagasse are presented in Table 27 and Fig.5. Sterilized bagasse with 10 per cent microbial consortium, sterilized bagasse and bagasse showed high xylanase activity of 2.61, 2.49 and 2.42 unit ml^{-1}. The xylanase activity was recorded maximum in sterilized bagasse with 10 per cent microbial consortium on 75^{th} day and slightly declined further. Significant increase in xylanase activity was observed in bagasse, sterilized bagasse and bagasse incorporated with soil and microbial consortium.

4.16.4. Effect of wax degrading microbial consortium on dehydrogenase activity of sugarcane bagasse

The effect of wax degrading microbial consortium on dehydrogenase activity of sugarcane bagasse are presented in Table 28 and Fig.5. Bagasse with soil and 10 per cent microbial consortium was observed maximum dehydrogenase activity and followed by bagasse incorporated with soil on 60^{th} day. Bagasse incorporated with soil and 10 per cent microbial consortium, bagasse with 10per cent microbial consortium was recorded higher dehydrogenase activity of 1.47 and 1.12 µg of TFP formed g^{-1} h^{-1} on 75^{th} day. Significant increase in dehydrogenase activity was noticed in bagasse with soil and 10 per cent microbial consortium and bagasse incorporated with 10 per cent microbial consortium on 60^{th} and 75^{th} day**.**

4.16.5. Effect of wax degrading microbial consortium on water repellency of sugarcane bagasse

The effect of wax degrading microbial consortium on water repellency of sugarcane bagasse are summarized in the Table 29 and Fig.5. Bagasse recorded slightly high water repellency than bagasse incorporated with soil. In general, water repellency in

bagasse is moderate and significant decrease was observed from 30th during decomposition (Plate 7). The maximum reduction in water repellency was observed in sterilized bagasse incorporated with 10 per cent microbial consortium of 0.8 MED on 105th day and followed by bagasse with microbial consortium. The water repellency was recorded maximum in bagasse of 2.2 MED on 15th day.

4.16.6. Effect of wax degrading microbial consortium on nitrogen, phosphorous and potassium content of sugarcane bagasse

The effect of wax degrading microbial consortium on nitrogen content, phosphorous content and potassium content of sugarcane bagasse are summarized in Table 30, 31, 32 and Fig.6. Bagasse incorporated with soil and 10 per cent microbial consortium was recorded higher nitrogen content of 0.54 per cent and followed by bagasse incorporated with soil on 45th day. Sterilized bagasse with 10 per cent microbial consortium, bagasse with 10per cent microbial consortium and bagasse was recorded nitrogen content of 0.4, 0.39 and 0.38 per cent respectively. The maximum higher nitrogen content was resulted in bagasse incorporated with soil and 10 per cent microbial consortium on 60th day which is followed by 75, 90 and 105th day. Significant increase in total nitrogen content was observed in bagasse incorporated with soil and 10 per cent microbial consortium and bagasse with 10per cent microbial consortium on 60th day.

The phosphorous content was recorded higher in bagasse incorporated with soil and 10 per cent microbial consortium on 60th day followed by bagasse with 10 per cent microbial consortium and low phosphorous content was recorded in sterilized bagasse.

The potassium content was recorded maximum of 0.3 per cent in bagasse incorporated with soil and followed by bagasse incorporated with soil and 10 per cent microbial consortium. The potassium content of sterilized bagasse with 10 per cent microbial consortium was shown 0.18 per cent. Significant increase in potassium content was observed in bagasse incorporated with soil and 10 per cent microbial consortium.

4.16.7. Effect of wax degrading microbial consortium on pH of sugarcane bagasse

The effect of wax degrading microbial consortium on changes in pH of sugarcane bagasse are summarized in Table 33. Bagasse incorporated with soil and bagasse with

soil and 10per cent microbial consortium were recorded the pH of 7.05 and 7.15 on 45th day. The change in pH from 7.13 to 7.78 in bagasse incorporated with soil and microbial consortium. The bagasse with 10 per cent microbial consortium, sterilized bagasse and bagasse recorded acidic pH ranges from 4.95 to 6.21. The change in pH from acidic to neutral was observed in bagasse on 90th day. Significant change in pH was observed in bagasse incorporated with soil and 10 per cent microbial consortium on 15th and 105th day.

4.16.8. Effect of wax degrading microbial consortium on organic carbon of sugarcane bagasse

The effect of wax degrading microbial consortium on organic carbon of sugarcane bagasse are summarized in Table 34. The organic carbon content of bagasse incorporated with soil and bagasse with soil and 10 per cent microbial consortium was recorded of 1.34 per cent and 1.40 per cent on 15th day. Significant reduction in organic carbon content was observed in bagasse incorporated with soil and microbial consortium during incubation. The higher significant reduction in organic carbon was observed in bagasse incorporated with microbial consortium than bagasse with soil. Significant change in organic carbon content was observed on 45th day.

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CHAPTER V

DISCUSSION

The growing food demand requires intensive and extensive cultivation of cropping to be done through out the year. The continuous intensive and extensive cultivation has exhausted soil nutrients and require frequent replenishment of nutrient that has been removed through cultivation. Exhausted soil nutrient could be replenished by natural resources, chemical sources and organic sources which include biomass, crop residues, farm yard manure, human waste and agro industrial wastes. It was estimated that 130 million tons of plant nutrients are available in the organic sources this could be utilized by exploring with suitable decomposer. The crop residues consists of cellulose, hemicellulose, lignin, pectin, cutin, fats and waxes and constituted about 68 per cent carbohydrates, 20 per cent lignin, 8 per cent proteins and 2 per cent fats and waxes.

Deposed wax as remnants of crop residues and agro industrial waste, degrades slowly due to complex chemical system and hydrophobicity. Plant waxes and their biodegraded products are major contributors of water repellency (Roberts and Carbon, 1972; Mc Ghie and Posner, 1980; Franco *et al.*, 2000*a*). Waxes are hydrolyzed by opportunistic group of microorganisms that facilitate the further succession of degradation by other group of microorganisms and enhance quicker degradation. Many of agricultural residues and municipal solid waste consist of cellulose, hemicellulose, lignin, pectin, cutin, oils, fats and waxes. These organic residues could be effectively composed by suitable microorganisms and obtained available form plant nutrients which will supplement nutrient requirement for plant growth and to obtain maximum yield. Wang and Ochoa (1972) reported mechanisms of wax utilization by microorganisms through pseudosolubilzation, adherence on waxes and adsorption by direct contact. Microbial surfactants are surface active compounds are glycolipids, lipoaminoacids, lipopetides, alkyl ether, alkano sulfano, olefin sulfonates, polyglycol ethers which is emulsify coconut oil and hexadecane. Husain *et al.* (1997) demonstrated the growth, biosurfactant and emulsifying activity in n- alkanes.

5.1. Isolation of wax degrading microorganisms

Waxes are n-alkanes, alkyl esters, fatty acids, fatty alcohols, fatty aldehydes, ketones, β-diketones with carbon atom of C_{21}- C_{33} and hydrolyses by microorganisms. Hankin and Kolathukudy (1969) isolated *Pseudomonas* species from soil of apple orchard and utilized apple wax, ursolic acid as sole carbon source. Stevenson (1970) also reported that the various plant waxes degrading microorganisms in soil.

The present study investigated the isolation of wax degrading bacterial isolates and actinomycetes cultures of enriched automobile waxy waste with soil, farmyard manure and cow dung in waxy medium beeswax mineral salt medium, mineral salt medium I, mineral salt medium II, inorganic basal medium and modified mineral salt medium with 0.5, 1, 2 and 5 per cent of beeswax and paraffin wax respectively. The wax content 0.5 per cent paraffin wax and beeswax were recorded higher bacterial isolates than 1, 2 and 5 per cent of paraffin wax and beeswax. The low concentration of 0.5 per cent paraffin wax and beeswax facilitated isolation of bacteria because bacteria are able to utilize through pseudosolubilization (Velankar *et al.,* 1975).

Wax degrading bacterial isolates were recorded maximum in mineral salt medium I with 0.5 per cent beeswax. Kawai *et al*. (1999) isolated microorganisms capable of degrading waxes especially actinomycetes from photodegradable polythene wax.

Roper (2004) obtained wax degrading bacterial isolates from soils of animal fats and oils, petroleum oils, sewage and composed animal manure with wool wax of 2 per cent as a sole carbon source. The results of present study clearly showed that bacterial isolates and actinomycetes culture of enriched automobile waxy waste might utilizes both paraffin wax and beeswax as a carbon source. The results are conformity with finding of Akit *et al*. (1981).

0.5 per cent beeswax exhibited higher bacterial isolates than paraffin wax due to chemical property of beeswax. Wax degrading actinomycetes culture isolation was facilitated by mineral salt medium I with 1per cent of beeswax and 0.5 per cent of paraffin wax as a sole carbon source. Wax degrading actinomycete cultures recorded higher in paraffin wax than beeswax. Five per cent beeswax and paraffin wax does not support the growth of wax degrading bacteria and actinomycetes due to low solubility. The present results are conformity with above findings.

5.2. Characterization of wax degrading bacteria and actinomycetes

Roper (2004) studied the cultural characteristic of wax degrading bacteria and identified. In this present study, morphological characteristic, growth characteristics and biochemical test of wax degrading bacterial isolates and actinomycetes culture were investigated. The members of *Bacillus* are gram positive, spore forming rods, the *Bacillus* colonies are irregular, filamentous and mycoids dirty white. The members of *Pseudomonas* are gram negative, cocci, non spore forming. The results clearly indicated that the wax degrading bacterial isolates contain *Bacillus* sp, *Serratia* sp and *Pseudomonas* sp. Etoumi (2007) identified the wax degrading bacteria like *Pseudomonas* species and filamentous type of *Actinomycetes* species according to Bergey's manual of systematic bacteriology on the basis of morphological feature and standard physiological tests.

The *Streptomyces* are exhibiting good growth, musty odur and brownish white growth, brown pigment, white mycelium, brown pigment production, yellowish white powdery growth in nutrient agar medium. The results clearly indicated that actinomycete cultures are *Streptomyces* species. The hydrocarbons n- alkane degrading eubacterial *Pseudomonas, Alcaligenes, Micrococcus, Nocardia, Cornebacteria, Arthtobacter, Bacillus, Streptomycetes, Rhodococcus* and *Proteus* were charactertised by Ridgway *et al*. (1990).

5.3. Screening of wax degrading bacteria and actinomycetes

In the present study, wax degrading seventeen bacterial isolates and five actinomycetes cultures were screened in paraffin wax, beeswax, coconut oil and n- hexadecane with 0.5, 1 and 2 per cent. The result clearly indicated that wax degrading bacteria and actinomycetes culture such as *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12, *Bacillus* sp. WDB17, *Streptomyces* sp. WDA.2, *Streptomyces* sp. WDA.3 and *Streptomyces* sp. WDA.4 grew well on paraffin wax, beeswax, coconut oil and n-hexadecane. Roper and Gupta (2005) have also suggested that coconut oil, n- hexadecane could be used as a carbon source for enumeration of wax degrading microorganisms.

5.4. Enumeration of wax degrading bacteria from enriched automobile waxy waste

The present study was investigated on enumeration of wax degrading bacteria in enriched automobile waxy waste with soil, FYM and cow dung in mineral salt medium I. Wax degrading bacterial population was recorded maximum in automobile waxy waste amended with 75 per cent cow dung compared to automobile waxy waste with other amendments on 240th day. The results clearly showed that automobile waxy waste with 75 per cent cow dung recorded highest wax degrading bacterial population because cow dung serves as microbial inoculum as well as initial organic source for establishment of microorganisms. The results clearly indicated that enrichment of automobile waxy waste enhances wax degrading bacteria. Roper and Gupta (2005) standardized the enumeration of wax degrading bacteria by standard plate count technique and with MPN technique. In present study, the wax degrading bacterial population attained maximum only on 240th day. This happened due to slow establishment of wax degrading bacteria in wax substrate.

In the present study beeswax was used as carbon source to enumerate wax degrading bacteria and it could not confirm the ability of isolates to perform the function of wax degradation particularly, if opportunistic groups of microorganisms were present that utilize the products of wax degradation (Mckenna *et al.*, 2002; Roper and Gupta, 2005). Roper (2004) reported that enumeration of wax degrading bacteria by using wool wax on the surface of agar plates. The MPN method proposed in this study relies on the expression of specific character that selects for the wax degrading microorganisms. Therefore this method is more relevant than plating technique because it combines the expression of function (wax degradation) with its ability of the organisms to grow. In MPN method also showed highest wax degrading bacterial population on 240th day in automobile waxy waste. However, the population size is ten folds higher in MPN technique method than plate count method. Because waxes of liquid based system has short chain fatty acids which is emulsified in short period.

Gupta and Roper (1994) supported the most probable number (MPN) method that offers the opportunity to measure the size of a particular microbial population by using a specific characteristic of wax degrading bacteria. On the other hand, MPN method used

liquid medium which ensured uniform distribution of the wax substrates. Hence, the number of wax degrading bacteria, determined by their ability to perform the wax degrading function, meant the measurement of population size were truly based on the capability to perform the function rather than on incidental growth on the media. In order to count potential wax degrading bacteria in liquid based system, it was necessary to choose carbon sources with components implicated as causative agents of hydrophobicity as like that of beeswax. Coconut oil contained some of the shorter chains known to be responsible for water repellency in soils and as liquid above 25^{o}C.

In MPN techniques coconut oil were used to measure the actual size of the wax degrading population. MPN counts statically recorded slightly higher than plate counts and it is clearly indicated that the MPN technique is one of the methods to use estimation of wax degrading bacterial population. The present results are conformity with above findings.

5.5. Studies on growth of wax degrading bacteria and actinomycetes in n- hexadecane

The present study investigated growth biomass, growth pattern of wax degrading bacterial isolates and actinomycetes cultures in 1 per cent n- hexadecane. Wax degrading bacterial isolates *Bacillus* sp. WDB11 and *Streptomyces* sp. WDA3 recorded higher growth, biomass and growth yield. The results clearly indicated that the highest biomass production of wax degrading bacterial isolates and actinomycetes culture were recorded on 15th day. Parra *et al.* (1989) isolated wax degrading bacterial isolates in soil enriched with crude oil and also reported establishment of bacterial growth in n- hexadecane. Cameora (1995) enumerated hydrocarbon utilizing soil bacteria in n- hexadecane. Wax degrading bacteria and actinomycetes were enumerated in n- hexadecane. Higher wax degrading bacteria and actinomycetes were recorded in n- hexadecane inoculated with *Bacillus* sp.WDB11 and *Streptomyces* sp. WDA2 on 9th day. The specific growth rates of the wax degrading bacterial isolate *Bacillus* sp. WDA12 grown rapidly found in mineral salt medium I with 1 per cent (v/v) n- hexadecane. The actinomycetes culture *Streptomyces* sp. WDA2 recorded higher biomass production of 0.23 g/25ml than other *Streptomyces* sp. Britton (1984) reported growth of hydrocarbon utilizing eubacteria *Bacillus, Pseudomonas, Streptomyces* in n- hexadecane.

5.6. Biosurfactant activity of wax degrading bacteria and actinomycetes in n- hexadecane

Biosurfactants are synthesized predominantly by hydrocarbon degrading microorganisms (Lang and Philip, 1998) and hydrolyze hydrocarbons. Cooper *et al.,* 1979) studied the production of monoglyceride biosurfactant and a more conventional polysaccharide bioemulsifier by *Bacillus cereus* IAF 346. The "drop collapse method" developed by Jain *et al*. (1992) appears handy and simple. Using this method a large number of bacteria can be screened in a short time for the biosurfactant activity.

In the present study, the results clearly indicated that wax degrading seventeen bacterial isolates and five actinomycetes exhibited biosurfactant activity. The biosurfactant activity had shown maximum in *Bacillus* sp. WDB12 of 152.14 mm^2 on 15^{th} day. However, wax degrading bacterial isolates observed higher biosurfactant activity than actinomycetes. Biosurfactant production is usually affected by incubation period and type of microorganisms used (Bicca *et al.,* 1999) Biosurfactants are produced by the bacteria and other contributing factor to the breakdown of paraffin. Persson *et al*. (1988) reported the production of a glycoprotein biosurfactant AP-6 by *Pseudomonas fluorescens* 378 under different growth conditions.

5.7. Emulsification activity of wax degrading bacteria and actinomycetes in n-hexadecane

Many microorganisms have been reported to produce solubilising agents such as biosurfactants and bioemulisiers when grown on hydrocarbons to facilitate uptake of organic substances (Zajic *et al.,* 1981). Banat (1995) reported that the emulsification activity and ability of the wax degrading microorganisms to immiscible hydrocarbon such as n-hexadecane. This phenomenon indicated the production of surface-active compounds by the microbial culture, which shown to provide substrate for further the metabolism and stimulated microbial growth. Wax degrading bacterial isolates and actinomycetes culture was studied for emulsification activity in n- hexadecane. The emulsifying activity was shown higher in wax degrading bacterial isolates *Bacillus* sp. WDB11 and actinomycetes culture *Streptomyces* sp. WDA2. The results clearly revealed that emulsifying activity recorded maximum on 11^{th} day and decline further. The production of biosurfactant and emulsifying activity of 60.6 percent in *Pseudomonas aeruginosa* was observed in kerosene (Desai and Banat, 1997)

Comparing these results with other biosurfactant production and emulsifying activity of microorganisms suggested that *Bacillus* sp. WDB12 may be a one of the good candidate for inhibiting paraffin deposition in petrochemical industries and allied sector.

In the present study, emulsification activity was evaluated at different stages of growth of wax degrading bacterial isolates and actinomycetes culture. The emulsification activity of the culture was high near to the stationary phase of growth in n-hexadecane substrate. This may be due to the production of biosurfactant which mainly occurs during this stage. The wax degrading bacterial and actinomycetes cultures exhibited their emulsification activity and recorded maximum on 11^{th} day. Roper and Gupta (2005) demonstrated emulsifying activity of *Achromobacter* and *Streptomyces* in coconut oil and n-hexadecane. The results are conformity with above findings.

5.8. Enzymes activity of wax degrading bacteria and actinomycetes

Microbial enzymes are extra cellular polypeptides. The enzyme activities such as amylase, cellulase, xylanase and lipase of these wax degrading bacteria and actinomycetes plays a vital role in degradation of waxy rich agricultural residues.

Pandey *et al.* (2000) mentioned *Bacillus subtilis, Bacillus stearothermophilus, Bacillus licheniformis* and *B. amyloliquefaciens* and *Streptomyces* sp. are known to be good producers of α-amylase and also demonstrated the amylase activity in submerged fermentation with starch polysaccharides.

In the present study, amylase activity of wax degrading bacteria isolates and actinomycetes cultures were assessed. Among the wax degrading bacterial isolates *Bacillus* sp. WDB11 recorded maximum amylase activity on 12^{th} day and reduced further. Actinomycetes culture *Streptomyces* sp. WDA3 recorded higher amylase activity than bacterial isolates.

Ekperigin (2007) reported that cellulase production in *Cellulomonas, Bacillus, Flavobacterium, Acinetobacter anitratus and Branhamella sp*. in solid state fermentation with cellulose. Gallagher *et al.* (1996) reported that the actinomycete *Micromonospora chalcae* produced an extracellular cellulase system when grown on cellulose-containing medium. Sodhi *et al.* (2005) reported that the thermostable cellulase was produced by *Streptomyces* sp T3-1 with maximum cellulase activity was attained on the fourth day.

The results of present study clearly indicated that marked variation in cellulase enzyme activity in wax degrading bacterial isolates. The cellulase activity of wax degrading bacterial isolates and actinomycetes cultures were steadily increasing to 12^{th} day. Actinomycetes culture recorded higher cellulase activity than bacterial isolates. The results of present study are in conformity with above findings.

Xylanases (1, 4-β-D-xylan xylanohydrolase) are glycosyl hydrolases that cleave the main chain of xylan of β -1, 4-linked xylose residues. The production of xylanase was confirmed in various microorganisms found in natural environments such as soil and in the rumen (Roberge *et al*., 1997). The present study investigated xylanase production of wax degrading bacterial isolates and actinomycetes cultures. Bacterial isolates were also found to produce significant amount of xylanase compared to actinomycetes culture. Among the actinomycetes cultures *Streptomyces* sp. WDA3 was producing higher xylanase activity. The results showed that xylanase activity of *Streptomyces* is higher than bacterial isolates. Battan *et al*. (2006) reported xylanase activity of alkaliphilic *Bacillus pumulis* in solid state fermentation

Among wax degrading bacterial isolates *Bacillus* sp. WDB12 showed higher lipase activity. *Streptomyces* sp. WDA3 was shown higher lipase activity than other bacterial isolates. Sugihara *et al*. (1991) reported that *Bacillus* sp. produces thermo stable alkaline lipase with corn oil, olive oil and coconut oil (1per cent) as carbon source.

Large *et al*. (1999) reported lipase activity in *Streptomyces lividans*, *Streptomyces clavuligerus*, *Streptomyces coelicolor*, and *Streptomyces rimosus*. Haba *et al*. (2000) reported that *Pseudomonas*, *Bacillus*, *Candida*, *Rhodococcus*, and *Staphylococcus* grew on the waste oils and produced lipolytic activity. Shah *et al*. (2007) reported that *Bacillus* sp. synthesize a variety of extracellular enzymes and the maximum synthesize occurs normal in the early stationary and late exponential phases.

5.9. Studies on growth of wax degrading microorganism on beeswax

The present study investigated the wax degrading bacteria such as *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB 12 *Bacillus* sp. WDB17 and actinomycetes culture *Streptomyces* sp. WDA2 and *Streptomyces* sp. WDA3 *Streptomyces* sp. WDA3 growth on 1 per cent beeswax, *Bacillus* sp. WDB 12 and

Streptomyces sp. WDA3 grown on beeswax becomes dirty brownish white and brownish black colored. This suggested that the bacteria were adhering to solid pieces of wax and grew on it on 7^{th} day because of pseudosolubilization, biosurfactant and emulsifying activity of wax degrading bacterial isolates and actinomycetes cultures. *Bacillus* sp. WDB9 and *Streptomyces* sp. WDA3 beeswax content settles down after 7 days of growth rather than floating earlier. 0.05 and 0.06 mg utilization of beeswax by bacterial strains and actinomycetes culture was observed. Marino (1998) observed that *Rhodococcus* IS01 and *Mycobacterium* OFS were grown with a piece of solid wax, the piece becomes pigmented with beige and orange respectively. The result clearly indicated that degradation of beeswax by wax degrading bacterial isolates *Bacillus* sp and actinomycetes culture *Streptomyces* sp.

Beeswax degradation by *Bacillus* and *Streptomyces* were demonstrated by growing in a medium with beeswax as sole carbon sources (Mckenna *et al.*, 2002). The results are conformity with above findings.

5.10. Antagonistic activity of wax degrading microorganisms

The biosurfactants could functions as antibiotics in which they have ability to bind hydrophobic molecules and form partition within membranes. Rhamnolipids and surfactin act as solubilization agent and solubilized the envelope of competing microorganisms. Biosurfactant such as lipopeptides obtained from *Bacillus licheniformis* exhibited antibiotic activity against a variety of yeast strains (Jenny *et al*., 1991).

The antagonistic activity between wax degrading bacterial isolates and actinomycetes cultures were assessed. The result clearly indicates that *Streptomyces* sp.WDA2 showed antagonistic activity against *Bacillus* sp.WDB3 and *Bacillus* sp. WDB12. The results are conformity with above findings.

5.11. Effect of wax degrading microbial consortium on microbial population of sugarcane bagasse

The present study investigated total aerobic bacteria, fungi, actinomycetes, wax degrading bacteria and actinomycetes population in bagasse and bagasse incorporated with soil and microbial consortium. The bagasse incorporated with soil and 10 per cent microbial

consortium recorded higher total aerobic bacteria, fungi, actinomycetes population on 75th day, whereas wax degrading bacterial and actinomycetes population was recorded higher in sterilized bagasse with 10 per cent microbial consortium. This might had happened due to inoculated wax degrading microbial consortium may be dominated and become zymogenous group of bacteria. The result clearly indicated that establishment of microbial population is very slow in bagasse because of chemical constituent of bagasse.

The above results are supported by Schmidt and Walter (1978) reported that bagasse consist micro flora of bacteria (74%), actinomycetes (6%), yeasts (13%), and fungi (7%). The yeasts dominate in early fermentation, followed by bacteria, and then by actinomycetes and fungi.

5.12. Effect of wax degrading microbial consortium on enzymes activity of sugarcane bagasse

Enzyme activity is considered as an important index for the soil biological activity in bagasse. Biological activity of sugarcane bagasse was monitored by the parameter like amount of microbial biomass (Friedel *et al*., 1996), which is usually supplemented by determining enzyme activity (Curci *et al*., 1997; Beyer *et al.,* 1992) or with recycling processes of carbon and nitrogen (Kandeler *et al*., 1999). The major contributors to enzyme pool in bagasse are mainly from microorganisms. Various microorganisms produce extracellular enzymes which are released in the surrounding bagasse to catalyze the depolymerization of polysaccharides. It has been recognized that various biochemical reaction are taking place to hydrolysis polysaccharides into simple organic and inorganic nutrients.

The present study amylase, cellulase, xylanase and dehydrogenase activity of bagasse and bagasse incorporated with soil and 10 per cent microbial consortium was assessed. The soil enzymes activity amylase, cellulase, xylanase and dehydrogenase were recoded higher in bagasse incorporated with soil and 10 per cent microbial consortium than other bagasse amendments because the microflora in soil and microbial consortium which added in bagasse might have contributed higher enzyme activity. The results clearly indicated that bagasse enzymes activity was steadily increasing upto 75th day which was directly proportional to the establishment of microorganism and wax degrading bacteria and actinomycetes in bagasse.

Dehydrogenase activity is one of the indicators of biological processes. Bagasse with 10 per cent microbial consortium registered higher enzyme activity of amylase, cellulase, xylanase and dehydrogenase activity than bagasse. This clearly indicated that inoculation of 10 per cent microbial consortium significantly enhances enzyme activity. Significant increase in enzyme activity was observed in bagasse with microbial consortium on 45^{th} day which support the degradation process and enhances physiochemical changes in bagasse amendments.

Karthikeyan *et al*. (2005) reported that xylanase activity of *Streptomyces violaceusniger* and found that reduction in the hemicellulose content of paper and sugarcane waste. Ramalingam (2007) reported that inoculation of cellulolytic bacteria, fungi and actinomycetes in soil amend with green manures enhanced cellulase, amylase, phosphatase and dehydrogenase activity.

The proliferation of microorganisms and break down of chemical compounds is very slow even in favourable condition due to lignocellulosic content of bagasse. The results are conformity with above findings.

5.13. Effect of wax degrading microbial consortium on water repellency of sugarcane bagasse

Water repellency is caused by hydrophobic substances on the surface of the soil and plant waxes. These biodegradative products are the main contributors to the phenomenon of repellency (Franco *et al.*, 2000*a*). The organic compounds of branched and unbranched C16-C36 fatty acids and their esters, alkanes, alcohols and sterols are causes for water repellency. Waxes also serve a diversity of other functions related to their water-repellent properties and their firm consistency. The present study investigated the water repellency in bagasse and bagasse incorporated with soil and 10 per cent microbial consortium. Water repellency of bagasse with soil and bagasse added with soil and 10 per cent microbial consortium was observed reduction after 45^{th} day of incubation. The results clearly shown that reduction in water repellency was occurred due to wax degrading microbial consortium. This might have happen due to wax degrading microbial consortium hydrolysis, waxes and other hydrophobic organic compound in bagasse.

5.14. Effect of wax degrading microbial consortium on NPK, organic carbon and pH of sugarcane bagasse

The incorporation of manure, green manure, crop residues in soil increases the major plant nutrient such as total nitrogen, phosphorous and potassium content in soil. It is estimated that 130 million tons of plant nutrients will be provided in available form by exploitation of agricultural residues by decomposition with suitable microorganisms. Total nitrogen content, phosphorous and potassium content of bagasse and bagasse incorporated with soil and microbial consortium was assessed. Bagasse incorporated with soil and 10 per cent microbial consortium was shown higher nitrogen content, phosphorous and potassium content than bagasse with other amendments. Because nitrogen, phosphorous and potassium content of bagasse was degraded rapidly by wax degrading microbial consortium. The results are clearly indicated that the addition of microbial consortium hastens the decomposition of bagasse and increase available nutrient content in bagasse. Dimalanta and Latiza (1990) reported decomposition of bagasse enriched with other substances or with certain cellulose degrading microorganisms. The bagasse with cellulolytic fungi *Trichoderma viridi* and *Aspergillus niger* increased available phosphorous content durning decomposition. The C: N ratio of bagasse reduced to 19:1 (Li-Xin *et al.,* 2002)

A higher reduction in organic carbon content was observed in bagasse inoculated with 10 per cent microbial consortium. There has been a steady and significant decrease in the C/N ratio as the decompostion proceeded in the bagasse incorporated with soil and microbial consortium. In present study, the results clearly indicated that steady reduction in organic carbon and changes in pH was from acidic to neutral was occurred due to inoculation of microbial consortium.

CHAPTER VI

SUMMARY

Waxes are n-alkanes, alkyl esters, fatty acids, fatty alcohols, fatty aldehydes , ketones, secondary fatty alcohols, β-Diketones , triterpenols and triterpenoid acids

Wax degrading bacterial isolates and actinomycetes cultures of enriched automobile waxy waste were isolated, characterized and screened, growth, biomass, biosurfactant and emulsifying activity of wax degrading bacterial isolates was increased in n- hexadecane. Rapid decomposition of bagasse was encounted with inoculation of wax degrading microbial consortium. The results of present investigation are summarized below.

- Wax degrading bacterial isolates were isolated in mineral salt medium I with 0.5 per cent of beeswax and paraffin wax. Wax degrading actinomycetes were isolated in mineral salt medium I with 1 per cent of beeswax and 0.5 per cent paraffin wax respectively.
- The wax content of 0.5 per cent of beeswax and paraffin wax was observed higher bacterial isolates and 2 per cent paraffin wax was observed very poor bacterial isolates. The wax degrading bacterial isolates were recorded maximum in mineral salt medium I with beeswax.
- Automobile waxy waste with 75 per cent cow dung recorded maximum population of 40×10^3 g^{-1} in beeswax was mineral salt medium I, whereas mineral salt solution I with coconut oil recorded highest wax degrading bacterial population of 72×10^4 g^{-1} of automobile waxy waste with 75 per cent cow dung in MPN method.
- Wax degrading bacterial isolates were tentatively identified as *Bacillus* sp. WDB1, *Bacillus* sp. WDB2, *Bacillus* sp. WDB3, *Pseudomonas* sp. WDB4, *Serratia* sp. WDB5, *Bacillus* sp. WDB6, *Bacillus* sp. WDB7, Bacillus sp. WDB8, *Pseudomonas* sp. WDB9, Bacillus sp. WDB10, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12, *Pseudomonas* sp WDB13, *Bacillus* sp. WDB14, *Bacillus* sp. WDB15, *Bacillus* sp. WDB16 and *Bacillus* sp. WDB17 based on the morphological, colony and biochemical characterization.

- Wax degrading actinomycetes cultures were tentatively identified as *Streptomyces* sp. WDA1, *Streptomyces* sp. WDA2, *Streptomyces* sp. WDA3, *Streptomyces* sp. WDA4 and *Streptomyces* sp. WDA5 based on the morphological and biochemical characterization.

- Efficient wax degrading bacterial isolates *Bacillus* sp. WDB3, *Bacillus* sp. WDB7, *Bacillus* sp. WDB11, *Bacillus* sp. WDB12 and *Bacillus* sp. WDB17 and three actinomycetes isolates *Streptomyces* sp. WDA2, *Streptomyces* sp. WDA3, *Streptomyces* sp. WDA4 were screened based on their growth in paraffin wax, beeswax, coconut oil and n- hexadecane.

- The inoculation of *Bacillus* sp. WDB12 in n- hexadecane was observed highest wax degradation bacterial population of 39 x 10^6 ml^{-1} on 9^{th} day, whereas wax degrading actinomycetes population was recorded maximum while n- hexadecane was inoculated with *Streptomyces* sp. WDA2.

- Wax degrading bacterial isolates was observed highest growth and biomass in n- hexadecane on 9^{th} day and 11^{th} day respectively. Maximum biomass of 0.28 mg/25 ml and 0.27 mg/25 ml were recorded in *Bacillus* sp. WDB11 and *Streptomyces* WDA2. Among bacterial isolates *Bacillus* sp. WDB11 was shown higher generation time, growth rate, growth ratio, growth yield and molar growth yield (g/M).

- Biosurfactant activity of wax degrading bacterial isolates and actinomycetes cultures were observed maximum on 15^{th} day, whereas emulsifying of wax degrading bacteria isolates and actinomycetes cultures were recoded highest in 11^{th} day and decline further. *Bacillus* sp. WDB12 recorded maximum biosurfactant activity of 152.14 mm^2 than wax degrading actinomycetes culture *Streptomyces* sp. WDA3 of 95.64 mm^2.

- Wax degrading actinomycetes culture *Streptomyces* sp. WDA3 recorded inhibition zone of 0.5mm for *Bacillus* sp. WDB3 and *Bacillus* sp. WDB17

- Higher amylase and cellulase activity was observed in wax degrading bacterial isolates and actinomycetes cultures was recorded higher amylase activity than

bacterial isolates and recorded maximum of 5.24 unit ml^{-1} in *Streptomyces* sp. WDA3 on 12^{th} day. Among the actinomycetes culture *Streptomyces* sp. WDA2 registered maximum cellulase activity of 9.83 unit ml^{-1}.

- The xylanase and lipase activity was observed higher in wax degrading bacterial isolates and actinomycetes culture. The bacterial isolates *Bacillus* sp. WDB 7 was observed maximum xylanase activity of 4.12 unit ml^{-1} on 12^{th} day. Wax degrading actinomycetes culture observed higher xylanase activity than bacterial isolates and recorded maximum of 6.62 unit ml^{-1} in *Streptomyces* sp. WDA2. *Bacillus* sp. WDB12 was registered maximum lipase activity of 2.99 unit ml^{-1}. Among actinomycetes culture *Streptomyces* sp. WDA3 was recorded maximum of 3.07 unit ml^{-1}.

- Wax degrading bacterial isolates and actinomycetes cultures exhibited beeswax degradation. Beeswax degradation was higher in wax degrading bacterial isolates on 15^{th} day. However, wax degradation was observed maximum of 0.06 mg in *Streptomyces* sp. WDA3 on 15^{th} day.

- The highest bacterial, fungal and actinomycetes population of 71 x $10^6 g^{-1}$, 36 x 10^4 g^{-1} and 19 x 10^3 g^{-1} was registered in bagasse incorporated with soil and 10 per cent microbial consortium on 75^{th} and 105^{th} day during decomposition.

- The wax degrading bacterial population recorded maximum of 39 x 10^6 g^{-1} in sterilized bagasse with 10 per cent microbial consortium. The maximum wax degrading actinomycetes population of 15 x 10^3 g^{-1} was observed in bagasse with 10 per cent microbial consortium on 90^{th} day.

- Bagasse incorporated with soil and microbial consortium increase enzymes activity of amylase, cellulase, xylanase and dehydrogenase. Highest amylase and cellulase activity of 3.08, 3.36 unit ml^{-1} were recorded in bagasse incorporated with soil and 10 per cent microbial consortium on 75^{th} day, whereas xylanase activity recorded maximum of 2.93 unit ml^{-1} in bagasse incorporated with 10 per cent microbial consortium on 60^{th} day.

- The inoculation of wax degrading microbial consortium in bagasse reduces water repellency. Sterilized bagasse amended with 10 per cent microbial consortium was observed low water repellency of 0.8 MED value on 105^{th} day and followed by bagasse incorporated with soil and 10 per cent microbial consortium.
- Nitrogen, phosphorous and potassium content in bagasse was increased during decomposition. Bagasse incorporated with soil and 10 per cent microbial consortium was shown higher nitrogen, phosphorous and potassium content of 0.56, 0.06 and 0.28 per cent and the reduction of organic carbon content and change in pH from acidic to neutral was recorded in bagasse with 10 per cent microbial consortium. The higher reduction in organic carbon content of 0.41 per cent was recorded in bagasse with 10 per cent microbial consortium.

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Table 1. Isolation of wax degrading microorganisms from enriched automobile waxy waste in beeswax incorporated mineral medium

Waxy Medium	Beeswax							
	Wax degrading bacteria				Wax degrading actinomycetes			
	0.5%	1%	2%	5%	0.5%	1%	2%	5%
Beeswax mineral salt medium	20	15	12	0	2	5	1	0
Mineral salt medium -I	32	25	20	0	3	4	2	0
Mineral salt medium- II	20	12	7	0	1	3	2	0
Inorganic basal medium	12	3	5	0	0	2	1	0
Modified mineral salt medium	0	0	1	0	0	0	0	0

Table 2. Isolation of wax degrading microorganisms of enriched automobile waxy waste in paraffin wax incorporated mineral medium

Waxy Medium	Paraffin wax							
	Wax degrading bacteria				Wax degrading actinomycetes			
	0.5%	1%	2%	5%	0.5%	1%	2%	5%
Beeswax mineral salt medium	20	16	11	0	6	4	1	0
Mineral salt medium -I	31	29	21	0	8	3	2	0
Mineral salt medium-II	15	15	14	1	3	2	0	0
Inorganic basal medium	20	13	12	0	1	0	1	0
Modified mineral salt medium	0	1	0	0	0	2	0	0

Table 8. Studies on biosurfactant activity of wax degrading bacteria and actinomycetes

Wax degrading bacteria and actinomycetes	Area of dispersal (mm^2)				
	Days				
	3	6	9	12	15
Bacillus sp. WDB1	03.27	03.32	05.60	06.48	07.42
Bacillus sp. WDB2	07.22	11.26	15.30	18.27	21.98
Bacillus sp. WDB3	62.05	73.62	86.23	90.24	94.39
Pseudomonas sp. WDB4	30.27	37.26	42.68	50.78	58.28
Serratia sp. WDB5	25.29	28.78	35.26	38.27	42.29
Bacillus sp. WDB6	03.30	03.32	04.75	06.28	07.42
Bacillus sp. WDB7	47.96	56.35	62.58	69.27	75.64
Bacillus sp. WDB8	18.76	22.78	30.69	35.32	38.76
Pseudomonas sp. WDB9	08.60	08.78	09.65	11.25	12.88
Bacillus sp. WDB10	20.58	23.87	25.26	30.29	38.76
Bacillus sp. WDB11	56.29	75.29	89.37	95.25	119.93
Bacillus sp. WDB12	60.89	75.29	102.01	117.26	152.14
Pseudomonas sp. WDB13	19.02	23.08	31.78	37.28	38.76
Bacillus sp. WDB14	06.29	07.19	08.93	11.10	11.94
Bacillus sp. WDB15	12.79	14.35	15.29	18.39	21.98
Bacillus sp. WDB16	43.89	50.27	54.23	60.79	62.24
Bacillus sp. WDB17	63.07	74.72	80.27	84.37	92.00
Streptomyces sp. WDA1	20.79	25.27	29.26	39.39	37.76
Streptomyces sp. WDA2	49.05	52.07	57.79	60.29	67.24
Streptomyces sp. WDA3	61.68	72.96	81.96	89.26	95.64
Streptomyces sp. WDA4	61.78	71.29	74.26	82.79	94.39
Streptomyces sp. WDA5	08.30	08.79	9.70	11.30	12.88
SEd	1.255	1.496	1.733	1.910	2.199
CD (0.05)	2.529	3.016	3.494	3.851	4.431

Table 9. Studies on growth of wax degrading bacteria in n- hexadecane

Wax degrading Bacteria	Growth at 560 nm							
	Days							
	1	3	5	7	9	11	13	15
Bacillus sp. WDB3	0.91	1.05	1.18	1.36	1.68	1.69	1.51	1.50
Bacillus sp. WBB7	0.91	1.05	1.10	1.56	1.79	1.95	1.61	1.50
Bacillus sp. WDB11	0.91	1.21	1.51	1.83	2.21	1.91	1.78	1.70
Bacillus sp. WDB12	0.91	1.10	1.21	1.83	1.97	1.47	1.38	1.26
Bacillus sp. WDB17	0.91	1.01	1.18	1.26	1.33	1.36	1.26	1.25
SEd	0.026	0.356	0.042	0.051	0.058	0.056	0.053	0.051
CD (0.05)	0.555	0.079	0.095	0.109	0.123	0.119	0.112	0.109

Table 14. Studies on amylase activity of wax degrading bacteria and actinomycetes

Wax degrading bacteria and actinomycetes	Amylase activity (U/ml)				
	Days				
	3	6	9	12	15
Bacillus sp. WDB3	0.27	0.86	1.23	1.58	1.27
Bacillus sp. WBB7	0.36	1.18	2.37	3.01	2.98
Bacillus sp. WDB11	0.51	1.26	2.18	3.78	2.91
Bacillus sp. WDB12	0.58	1.37	2.97	3.97	2.98
Bacillus sp. WDB17	0.46	0.87	1.26	1.87	1.56
Streptomyces sp. WDA 2	0.48	1.07	2.65	4.37	3.89
Streptomyces sp. WDA 3	0.64	1.18	3.18	5.24	4.48
Streptomyces sp. WDA 4	0.32	0.97	1.08	2.58	1.82

	SEd	CD(0.05)
Days	0.027	0.053
Microorganism	0.034	0.068
Days x Microorganisms	0.076	0.152

Table 15. Studies on cellulase activity of wax degrading bacteria and actinomycetes

Wax degrading bacteria and actinomycetes	Cellulase activity (U/ml)				
	Days				
	3	6	9	12	15
Bacillus sp. WDB3	1.26	2.42	2.68	2.86	1.98
Bacillus sp. WBB7	1.54	2.01	2.56	2.98	2.02
Bacillus sp. WDB11	1.26	2.27	3.67	3.87	2.08
Bacillus sp. WDB12	1.46	1.89	2.56	3.89	2.56
Bacillus sp. WDB17	1.30	2.37	2.56	2.68	1.97
Streptomyces sp. WDA 2	3.26	5.83	8.25	9.83	9.62
Streptomyces sp. WDA 3	3.26	4.68	6.64	8.62	8.41
Streptomyces sp. WDA 4	3.83	4.20	6.04	7.67	7.06

	SEd	CD(0.05)
Days	0.053	0.106
Microorganism	0.067	0.134
Days x Microorganisms	0.150	0.299

Table 16. Studies on xylanase activity of wax degrading bacteria and actinomycetes

Wax degrading bacteria and actinomycetes	Xylanase activity (U/ml)				
	Days				
	3	6	9	12	15
Bacillus sp. WDB3	1.27	1.97	2.28	3.97	2.82
Bacillus sp. WBB7	1.68	2.12	3.86	4.12	3.78
Bacillus sp. WDB11	1.72	2.29	3.67	3.99	3.87
Bacillus sp. WDB12	1.82	2.21	3.58	3.87	2.98
Bacillus sp. WDB17	1.32	1.82	2.26	3.58	2.67
Streptomyces sp. WDA 2	1.83	3.17	5.06	6.62	6.20
Streptomyces sp. WDA 3	1.96	3.66	4.85	6.34	6.07
Streptomyces sp. WDA 4	1.72	2.77	4.47	6.19	5.42

	SEd	CD(0.05)
Days	0.042	0.085
Microorganism	0.054	0.108
Days x Microorganisms	0.121	0.241

Table 17. Studies on lipase activity of wax degrading bacteria and actinomycetes

Wax degrading bacteria and actinomycetes	Lipase activity (U/ml)				
	Days				
	3	6	9	12	15
Bacillus sp. WDB3	0.52	0.83	1.26	1.38	1.07
Bacillus sp. WBB7	0.73	1.28	0.63	2.12	1.93
Bacillus sp. WDB11	0.67	1.45	1.83	2.32	2.18
Bacillus sp. WDB12	0.83	1.68	2.34	2.99	2.54
Bacillus sp. WDB17	0.48	0.98	1.32	1.83	1.76
Streptomyces sp. WDA 2	0.48	0.97	1.26	1.53	1.32
Streptomyces sp. WDA 3	0.63	1.23	2.86	3.07	2.91
Streptomyces sp. WDA 4	0.56	0.83	1.18	1.36	1.12

	SEd	CD(0.05)
Days	0.018	0.037
Microorganism	0.023	0.047
Days x Microorganisms	0.053	0.106

Table 18. Effect of wax degrading bacteria and actinomycetes on degradation of beeswax

Wax degrading bacteria and actinomycetes	Beeswax content (mg)				
	Days				
	3	6	9	12	15
Bacillus sp. WDB3	0.00	0.01	0.01	0.01	0.02
Bacillus sp. WBB7	0.00	0.02	0.02	0.03	0.03
Bacillus sp. WDB11	0.00	0.01	0.02	0.02	0.03
Bacillus sp. WDB12	0.00	0.02	0.03	0.05	0.05
Bacillus sp. WDB17	0.00	0.01	0.01	0.01	0.01
Streptomyces sp. WDA 2	0.00	0.02	0.02	0.03	0.04
Streptomyces sp. WDA 3	0.00	0.03	0.04	0.04	0.06
Streptomyces sp. WDA 4	0.00	0.02	0.02	0.03	0.03

Table 3a. Morphological characterization of wax degrading bacterial isolates of enriched automobile waxy waste

Wax degrading bacterial isolates	Morphological characters		
	Shape	Gram staining	Spore staining
WDB 1	Rods	+	+
WDB 2	Rods	+	+
WDB 3	Cocci	+	+
WDB 4	Cocci	-	-
WDB 5	Long rods	-	-
WDB 6	Rods	+	+
WDB 7	Rods	+	+
WDB 8	Rods	+	+
WDB 9	Cocci	-	-
WDB 10	Cooci	-	-
WDB 11	Short rods	+	+
WDB 12	Rods	+	+
WDB 13	Rods	-	-
WDB 14	Cooci	+	+
WDB 15	Rods	+	+
WDB 16	Rods	+	+
WDB 17	Rods	+	+

Table 19. Studies on antagonistic activity of wax degrading bacteria and actinomycetes

Wax degrading bacteria and actinomycetes	Inhibition zone (mm)				
	Bacillus sp. WDB3	*Bacillus* sp. WDB7	*Bacillus* sp. WDB11	*Bacillus* sp. WDB12	*Bacillus* sp. WDB17
Streptomyces sp. WDA 2	-	-	-	-	-
Streptomyces sp. WDA 3	0.5	-	-	-	0.5
Streptomyces sp. WDA 4	-	-	-	-	-

Table 5. Screening of efficient wax degrading bacterial isolates and actinomycetes cultures in waxy medium with different carbon sources

Wax degrading bacteria and actinomycetes	Carbon sources											
	Paraffin wax			Beeswax			Coconut oil			n-hexadecane		
	0.5%	1%	2%	0.5%	1%	2%	0.5%	1%	2%	0.5%	1%	2%
Bacillus sp. WDB1	0	0	0	1	0	0	2	1	1	0	0	0
Bacillus sp. WDB2	0	0	0	1	0	0	2	1	1	0	0	0
Bacillus sp. WDB3	2	0	0	2	2	1	2	2	2	2	2	2
Pseudomonas sp. WDB4	0	0	0	1	0	0	1	1	1	0	0	0
Serratia sp. WDB5	0	0	0	2	1	0	2	2	2	0	0	0
Bacillus sp. WDB6	0	2	0	0	0	0	1	0	0	0	0	0
Bacillus sp. WDB7	0	0	0	2	2	2	2	2	2	2	2	2
Bacillus sp. WDB8	0	0	0	1	0	0	1	0	0	0	0	0
Pseudomonas sp. WDB9	0	0	0	1	0	0	1	1	0	0	0	0
Bacillus sp. WDB10	0	0	0	1	0	0	1	0	0	0	0	0
Bacillus sp. WDB11	2	2	0	2	2	2	2	2	2	2	2	2
Bacillus sp. WDB12	2	2	2	2	2	2	2	2	2	2	2	2
Pseudomonas sp. WDB13	0	0	0	1	0	0	1	1	0	0	0	0
Bacillus sp. WDB14	0	0	0	0	0	0	1	0	0	0	0	0
Bacillus sp.WDB15	0	0	0	0	0	0	1	0	0	0	0	0
Bacillus sp. WDB16	0	0	0	1	0	0	1	0	0	0	0	0
Bacillus sp. WDB17	2	0	0	2	2	2	2	2	2	2	2	2
Streptomyces sp. WDA1	1	1	0	1	1	0	1	1	0	0	0	0
Streptomyces sp. WDA2	2	2	0	2	2	2	2	2	2	2	2	2
Streptomyces sp. WDA3	2	2	2	2	2	2	2	2	2	2	2	2
Streptomyces sp. WDA4	2	2	0	2	2	1	2	2	2	2	2	1
Streptomyces sp. WDA5	2	0	0	2	2	1	2	2	2	2	2	2

0-No Growth 1- Growth 2-High Growth

Table 4. Characterization of wax degrading actinomycetes cultures of enriched automobile waxy waste

Wax degrading actinomyce-tes cultures	Nutrient agar	Yeast extract glucose agar	Kenknights agar	Kuster agar	Potato dextrose agar	NR	MR (VP)	CT	UT	Tentative identifcation
WDA-1	Good growth musty odur, brownish white growth	Good growth & blackish brown tinch on the colonies	Sparse growth with little mycelium	Moderate growth with small colonies	Good growth with high mycelial growth	+	+(-)	+	+	*Streptomyces* sp. WDA1
WDA-2	Good growth dark brown pigment	Good growth brownish white pigment	Good growth exhibit high sporulation	Good growth with pinkish rose color on backside	Good growth whitish black mycelium	+	+(-)	+	+	*Streptomyces* sp. WDA 2
WDA-3	Moderate good growth, chalky white mycelium	Moderate growth with pigmented mycelium white and thin	Good growth brownish pigment	Moderate growth,no pigment production	Moderate growth,no pigment ,thin sporulation	+	+(-)	-	-	*Streptomyces* sp. WDA 3
WDA-4	Good growth brown pigment production	Good growth and dense packed colonies	Moderate growth small colony(pin head size)	Less growth round circular colonies	Good growth whitish brown mycelium	+	+(-)	+	+	*Streptomyces* sp. WDA 4
WDA-5	Good growth yellowish white powdery growth	Dense growth	Moderate growth with powdery	Moderate growth with high sporulation	Good growth with high brownish sporulation	+	+(-)	+	+	*Streptomyces* sp. WDA 5

NR –Nitrate Reductase, MR-VP- Methyl red Voges proskager, CT- catalase test, UR- urease test

Table 3c.Biochemical characterization of wax degrading bacterial isolates of enriched automobile waxy waste

Wax degrading Bacterial isolates	Biochemical characters										
	Starch hydrolysis	Catalase activity	Indole test	MR-VP	Cellulose utilization	Gelatin hydrolysis	Citrate utilization	Hydrogen sulphide production	Urease	Nitrate reduction	Tentative identification
WDA1	+	+	-	+ (-)	+	-	+	-	-	+	*Bacillus* sp. WDB1
WDA2	+	+	-	+ (-)	+	-	+	-	-	+	*Bacillus* sp. WDB2
WDA3	+	+	-	+ (-)	+	-	+	-	-	+	*Bacillus* sp. WDB3
WDA4	+	+	-	- (+)	+	-	-	-	-	-	*Pseudomonas* sp. WDB4
WDA5	+	+	-	- (+)	+	-	-	-	-	-	*Pseudomonas* sp. WDB5
WDA6	+	+	-	+ (-)	+	-	+	-	-	+	*Bacillus* sp. WDB6
WDA7	+	+	-	+ (-)	+	-	+	-	-	+	*Bacillus* sp. WDB7
WDA8	+	+	-	+ (-)	+	-	+	-	-	+	*Bacillus* sp. WDB8
WDA9	+	+	-	- (+)	+	-	-	-	-	-	*Pseudomonas* sp. WDB9
WDA10	+	+	-	+ (-)	+	-	+	-	-	+	*Bacillus* sp. WDB10
WDA11	+	+	-	+ (-)	+	-	+	-	-	+	*Bacillus* sp. WDB11
WDA12	+	+	-	+ (-)	+	-	+	-	-	+	*Bacillus* sp. WDB12
WDA13	+	+	-	- (+)	+	-	-	-	-	-	*Pseudomonas* sp. WDB13
WDA14	+	+	-	+ (-)	+	-	+	-	-	+	*Bacillus* sp. WDB14
WDA15	+	+	-	+ (-)	+	-	+	-	-	+	*Bacillus* sp. WDB15
WDA16	+	+	-	+ (-)	+	-	+	-	-	+	*Bacillus* sp. WDB16
WDA17	+	+	-	+ (-)	+	-	+	-	-	+	*Bacillus* sp. WDB17

Table 6. Enumeration of wax degrading bacteria from enriched automobile waxy waste (AWW)

Treatments	Wax degrading bacteria X 10^3 g^{-1} of EAWW*								
	Days								
	0	30	60	90	120	150	180	210	240
T_1: AWW	0	0	0	0	0	1	2	2	3
T_2: AWW +25% soil	0	0	0	2	7	7	8	8	8
T_3: AWW+50% soil	2	9	12	15	16	19	21	28	26
T_4: AWW+ 75% soil	1	8	6	15	15	20	29	33	36
T_5: AWW + 25% FYM	0	0	0	0	2	10	17	19	22
T_6: AWW + 50% FYM	5	7	6	12	19	21	26	28	30
T_7: AWW+75 % FYM	2	7	6	12	19	21	26	28	30
T_8: AWW +25 % cow dung	0	0	0	6	15	20	21	22	25
T_9: AWW +50 % cow dung	3	4	7	9	12	16	21	22	29
T_{10}: AWW+ 75 % cow dung	8	12	17	19	21	29	35	39	40
SEd	0.106	0.207	0.250	0.360	0.469	0.592	0.737	0.823	0.888
CD(0.05)	0.222	0.432	0.523	0.752	0.979	1.235	0.538	1.717	1.852

* EAWW: Enriched automobile waxy waste

Table 7. Enumeration of wax degrading bacteria from enriched automobile waxy waste (AWW) by MPN technique

Treatments	Wax degrading bacteria X 10^4 g^{-1} of EAWW*								
	Days								
	0	30	60	90	120	150	180	210	240
T_1: AWW	-	-	-	-	-	2	2	5	5
T_2: AWW +25% soil	2	5	5	6	9	9	9	14.6	14
T_3: AWW+50% soil	6	9	5	14	17	24	33	33	39
T_4: AWW+ 75% soil	6	17	30	34	45	40	47	47	54
T_5: AWW + 25% FYM	2	4	4	9	6	17	17	27	27
T_6: AWW + 50% FYM	9	9	12	12	21	28	39	45	48
T_7: AWW+75 % FYM	9	14	17	17	33	45	48	47	70
T_8: AWW +25 % cow dung	4	2	12	12	17	21	27	33	27
T_9: AWW +50 % cow dung	9	17	24	24	28	27	40	47	54
T_{10}: AWW+ 75 % cow dung	17	28	24	32	35	40	54	68	72
SEd	0.258	0.433	0.536	0.623	0.817	0.698	0.874	0.992	1.133
CD(0.05)	0.539	0.905	1.118	1.301	1.704	1.456	1.823	2.070	2.364

* EAWW: Enriched automobile waxy waste

Table 13. Studies on emulsification activity of wax degrading bacteria and actinomycetes in n- hexadecane

Days	Emulsification activity (%)							
	Wax degrading Bacteria					Wax degrading Actinomycetes		
	Bacillus sp. WDB3	*Bacillus* sp. WDB7	*Bacillus* sp. WDB11	*Bacillus* sp. WDB12	*Bacillus* sp. WDB17	*Streptomyces* sp. WDA2	*Streptomyces* sp.WDA3	*Streptomyces* sp. WDA4
1	30	30	36	43	40	46	49	46
3	30	37	43	53	45	57	50	51
5	32	36	48	55	54	52	51	50
7	30	36	47	46	53	48	50	52
9	30	32	35	40	38	43	43	43
11	31	36	39	59	56	53	51	49
13	32	36	46	55	45	58	55	54
15	30	34	38	47	41	49	47	46
SEd	0.001	1.121	1.349	1.620	1.517	1.645	1.597	1.579
CD (0.05)	0.002	2.376	2.861	3.434	3.216	3.488	3.886	3.349

Table 12. Enumeration of wax degrading bacteria and actinomycetes in n-hexadecane

Days	Wax degrading Bacteria (x 10^6 ml^{-1})					Wax degrading Actinomycetes (x 10^3 ml^{-1})		
	Bacillus sp. WDB3	*Bacillus* sp. WDB7	*Bacillus* sp. WDB11	*Bacillus* sp. WDB12	*Bacillus* sp. WDB17	*Streptomyces* sp. WDA2	*Streptomyces* sp.WDA3	*Streptomyces* sp. WDA4
1	6	8	9	8	5	3	2	2
3	13	16	19	15	9	9	8	6
5	14	17	27	26	15	13	13	16
7	17	20	29	36	16	23	17	19
9	19	26	33	39	21	27	20	22
11	25	29	30	29	21	23	17	20
13	28	27	28	16	21	21	19	18
15	19	23	25	18	17	19	12	17
SEd	0.612	0.708	0.811	0.831	0.540	0.616	0.478	0.535
CD (0.05)	1.298	1.502	1.768	1.763	1.146	1.305	1.014	1.135

Table 20. Effect of wax degrading microbial consortium on total bacterial population of sugarcane bagasse

Treatments	Total Bacterial population x 10^{6} g^{-1} of bagasse							
	Days							
	0	15	30	45	60	75	90	105
T_1 : Bagasse	10	17	20	28	29	36	42	50
T_2 : Sterilized Bagasse	09	19	29	39	40	49	51	56
T_3 : Bagasse + Soil	15	23	38	48	56	62	62	69
T_4 : Bagasse + Soil + 10 % Microbial consortium	18	28	36	50	62	71	66	65
T_5: Sterilized bagasse +10 % Microbial consoritum	10	21	39	40	50	66	62	68
T_6 : Bagasse + 10 % Microbial consortium	12	21	32	42	58	68	65	62

	SEd	CD(0.05)
Days	0.613	1.218
Treatments	0.540	1.053
Days x Treatments	1.500	2.978

Table 21. Effect of wax degrading microbial consortium on fungal population of sugarcane bagasse

Treatments	Fungal population x $10^{4}g^{-1}$ of bagasse							
	Days							
	0	15	30	45	60	75	90	105
T_1 : Bagasse	12	30	29	30	22	21	24	25
T_2 : Sterilized Bagasse	05	15	20	19	21	15	22	18
T_3 : Bagasse + Soil	08	12	17	18	21	30	31	32
T_4 : Bagasse + Soil + 10 % Microbial consortium	12	16	20	28	31	31	34	36
T_5: Sterilized bagasse +10 % Microbial consoritum	09	15	23	26	29	31	33	27
T_6 : Bagasse + 10 % Microbial consortium	12	29	30	32	23	25	26	29

	SEd	CD(0.05)
Days	0.330	0.660
Treatments	0.274	0.545
Days x Treatments	0.805	1.598

Table 22. Effect of wax degrading microbial consortium on actinomycetes population of sugarcane bagasse

Treatments	Actinomycetes population x $10^{3}g^{-1}$ of bagasse							
	Days							
	0	15	30	45	60	75	90	105
T_1 : Bagasse	03	06	11	10	09	10	13	12
T_2 : Sterilized Bagasse	02	04	08	11	11	09	10	12
T_3 : Bagasse + Soil	06	10	09	10	11	13	12	11
T_4 : Bagasse + Soil + 10 % Microbial consortium	10	14	12	17	16	18	19	19
T_5: Sterilized bagasse +10 % Microbial consoritum	05	08	10	12	15	10	11	12
T_6 : Bagasse + 10 % Microbial consortium	06	07	10	14	12	15	18	17

	SEd	CD(0.05)
Days	0.155	0.307
Treatments	0.134	0.266
Days x Treatments	0.379	0.753

Table 23. Effect of wax degrading microbial consortium on wax degrading bacterial population of sugarcane bagasse

Treatments	Wax degrading Bacterial population x 10^6 g^{-1} of bagasse							
	Days							
	0	15	30	45	60	75	90	105
T_1 : Bagasse	02	03	03	02	15	06	06	08
T_2 : Sterilized Bagasse	01	01	01	01	02	02	02	01
T_3 : Bagasse + Soil	02	04	7	08	11	13	14	14
T_4 : Bagasse + Soil + 10 % Microbial consortium	07	10	16	14	19	21	23	29
T_5: Sterilized bagasse +10 % Microbial consoritum	03	10	19	29	33	36	39	33
T_6 : Bagasse + 10 % Microbial consortium	04	12	16	19	30	33	35	32

	SEd	CD(0.05)
Days	0.238	0.472
Treatments	0.206	0.409
Days x Treatments	0.583	1.157

Table 24. Effect of wax degrading microbial consortium on wax degrading actinomycetes population of sugarcane bagasse

Treatments	Wax degrading actinomycetes population x 10^{3} g^{-1} of bagasse							
	Days							
	0	15	30	45	60	75	90	105
T_1 : Bagasse	01	02	04	07	06	04	03	01
T_2 : Sterilized Bagasse	01	01	01	02	04	02	01	02
T_3 : Bagasse + Soil	02	02	03	03	02	03	02	06
T_4 : Bagasse + Soil + 10 % Microbial consortium	03	02	06	08	12	06	07	08
T_5: Sterilized bagasse +10 % Microbial consoritum	04	09	08	07	09	11	13	10
T_6 : Bagasse + 10 % Microbial consortium	03	07	09	10	11	13	15	12

	SEd	CD(0.05)
Days	0.090	0.179
Treatments	0.078	0.155
Days x Treatments	0.221	0.439

Table 34. Effect of wax degrading microbial consortium on organic carbon of sugarcane bagasse

Treatments	Organic carbon (%)							
	Days							
	0	15	30	45	60	75	90	105
T_1 : Bagasse	0.74	0.72	0.69	0.65	0.63	0.62	0.63	0.60
T_2 : Sterilized Bagasse	0.72	0.67	0.61	0.53	0.49	0.49	0.43	0.47
T_3 : Bagasse + Soil	1.38	1.34	1.27	1.21	1.19	1.22	1.25	1.17
T_4 : Bagasse + Soil + 10 % Microbial consortium	1.42	1.40	1.35	1.30	1.29	1.24	1.24	1.25
T_5: Sterilized bagasse +10 % Microbial consoritum	0.74	0.68	0.63	0.58	0.50	0.45	0.44	0.41
T_6 : Bagasse + 10 % Microbial consortium	0.73	0.69	0.63	0.56	0.51	0.49	0.43	0.42

	SEd	CD(0.05)
Days	0.011	0.023
Treatments	0.010	0.020
Days x Treatments	0.028	0.057

Table 33. Effect of wax degrading microbial consortia on pH in sugarcane bagasse

Treatments	pH							
	Days							
	0	15	30	45	60	75	90	105
T_1 : Bagasse	5.15	5.12	5.14	5.75	5.78	5.91	5.89	6.05
T_2 : Sterilized Bagasse	5.15	5.39	5.67	5.58	5.91	6.21	6.19	6.24
T_3 : Bagasse + Soil	7.05	7.10	7.03	7.05	7.31	7.35	7.14	7.15
T_4 : Bagasse + Soil + 10 % Microbial consortium	7.15	7.20	7.11	7.13	7.72	7.78	7.63	7.65
T_5: Sterilized bagasse +10 % Microbial consoritum	5.15	5.10	5.18	5.05	5.98	5.93	6.03	6.05
T_6 : Bagasse + 10 % Microbial consortium	5.15	4.95	5.08	5.06	5.18	5.25	5.24	6.15

	SEd	CD (0.05)
Days	0.082	0.163
Treatment	0.071	0.141
Days x Treatment	0.201	0.400

Table 30. Effect of wax degrading microbial consortium on nitrogen content of sugarcane bagasse

Treatments	Nitrogen content (%)							
	Days							
	0	15	30	45	60	75	90	105
T_1 : Bagasse	0.33	0.33	0.36	0.35	0.37	0.35	0.38	0.32
T_2 : Sterilized Bagasse	0.32	0.33	0.35	0.34	0.38	0.37	0.36	0.38
T_3 : Bagasse + Soil	0.40	0.47	0.48	0.48	0.49	0.46	0.48	0.47
T_4 : Bagasse + Soil + 10 % Microbial consortium	0.42	0.48	0.53	0.54	0.56	0.49	0.50	0.51
T_5: Sterilized bagasse +10 % Microbial consoritum	0.33	0.35	0.37	0.38	0.40	0.41	0.41	0.4
T_6 : Bagasse + 10 % Microbial consortium	0.31	0.34	0.36	0.38	0.39	0.42	0.41	0.42

	SEd	CD (0.05)
Days	0.005	0.010
Treatment	0.004	0.009
Days x Treatment	0.013	0.026

Table 31. Effect of wax degrading microbial consortium on Phosphorous content of sugarcane bagasse

Treatments	Phosphorous content (%)							
	Days							
	0	15	30	45	60	75	90	105
T_1 : Bagasse	0.02	0.02	0.04	0.04	0.03	0.03	0.04	0.04
T_2 : Sterilized Bagasse	0.02	0.02	0.03	0.03	0.02	0.02	0.03	0.03
T_3 : Bagasse + Soil	0.02	0.03	0.03	0.02	0.04	0.04	0.03	0.03
T_4 : Bagasse + Soil + 10 % Microbial consortium	0.04	0.04	0.05	0.05	0.06	0.05	0.05	0.04
T_5: Sterilized bagasse +10 % Microbial consoritum	0.03	0.03	0.04	0.05	0.05	0.05	0.04	0.03
T_6 : Bagasse + 10 % Microbial consortium	0.04	0.05	0.05	0.05	0.06	0.05	0.04	0.04

	SEd	CD (0.05)
Days	0.005	0.010
Treatment	0.002	0.004
Days x Treatment	0.001	0.002

Table 32. Effect of wax degrading microbial consortium on potassium content of sugarcane bagasse

Treatments	Potassium content (%)							
	Days							
	0	15	30	45	60	75	90	105
T_1 : Bagasse	0.14	0.15	0.13	0.14	0.16	0.15	0.16	0.16
T_2 : Sterilized Bagasse	0.16	0.15	0.14	0.15	0.14	0.14	0.13	0.13
T_3 : Bagasse + Soil	0.24	0.23	0.26	0.27	0.26	0.30	0.29	0.29
T_4 : Bagasse + Soil + 10 % Microbial consortium	0.25	0.24	0.23	0.28	0.27	0.29	0.28	0.29
T_5: Sterilized bagasse +10 % Microbial consoritum	0.18	0.17	0.18	0.19	0.20	0.18	0.17	0.17
T_6 : Bagasse + 10 % Microbial consortium	0.19	0.18	0.17	0.18	0.16	0.19	0.18	0.18

	SEd	CD (0.05)
Days	0.002	0.005
Treatment	0.002	0.004
Days x Treatment	0.003	0.013

Table 25. Effect of wax degrading microbial consortium on amylase activity of sugarcane bagasse

Treatments	Amylase activity (U/ml)							
	Days							
	0	15	30	45	60	75	90	105
T_1 : Bagasse	0.48	0.73	0.82	0.93	1.10	1.30	1.29	1.24
T_2 : Sterilized Bagasse	0.54	0.63	0.67	0.83	1.12	1.29	1.25	1.20
T_3 : Bagasse + Soil	1.06	1.18	1.38	1.49	1.53	1.67	1.57	1.59
T_4 : Bagasse + Soil + 10 % Microbial consortium	1.54	1.98	2.64	2.81	3.06	3.08	2.88	2.82
T_5: Sterilized bagasse +10 % Microbial consoritum	0.69	0.73	0.92	1.36	1.67	1.58	1.50	1.49
T_6 : Bagasse + 10 % Microbial consortium	0.52	0.82	0.85	0.93	1.13	1.18	1.27	1.22

	SEd	CD (0.05)
Days	0.020	0.040
Treatments	0.017	0.034
Days x Treatments	0.049	0.098

Table 26. Effect of wax degrading microbial consortium on cellulase activity of sugarcane bagasse

Treatments	Cellulase activity (U/ml)							
	Days							
	0	15	30	45	60	75	90	105
T_1 : Bagasse	1.48	1.96	2.01	2.14	2.24	2.19	2.18	2.13
T_2 : Sterilized Bagasse	1.37	1.46	1.78	1.86	2.13	2.15	2.06	2.07
T_3 : Bagasse + Soil	1.87	1.97	2.12	2.43	2.56	2.48	2.41	2.30
T_4 : Bagasse + Soil + 10 % Microbial consortium	1.98	2.12	2.56	2.83	3.34	3.36	3.29	3.28
T_5: Sterilized bagasse +10 % Microbial consoritum	1.63	1.72	1.93	2.18	2.25	2.29	2.18	2.12
T_6 : Bagasse + 10 % Microbial consortium	1.70	1.92	2.10	2.22	2.15	2.19	2.13	2.09

	SEd	CD (0.05)
Days	0.029	0.058
Treatments	0.025	0.051
Days x Treatments	0.072	0.144

Table 27. Effect of wax degrading microbial consortium on xylanase activity of sugarcane bagasse

Treatments	Xylanase activity (U/ml)							
	Days							
	0	15	30	45	60	75	90	105
T_1 : Bagasse	2.01	2.10	2.26	2.38	2.63	2.71	2.68	2.65
T_2 : Sterilized Bagasse	1.97	2.08	2.13	2.56	2.78	2.91	2.87	2.68
T_3 : Bagasse + Soil	1.63	1.68	1.93	2.19	2.21	2.29	2.25	2.20
T_4 : Bagasse + Soil + 10 % Microbial consortium	1.79	1.92	2.09	2.21	2.46	2.50	2.39	2.37
T_5: Sterilized bagasse +10 % Microbial consoritum	2.26	2.30	2.52	2.78	2.93	2.82	2.73	2.58
T_6 : Bagasse + 10 % Microbial consortium	2.17	2.20	2.36	2.56	2.53	2.25	2.18	2.14

	SEd	CD (0.05)
Days	0.031	0.062
Treatments	0.027	0.054
Days x Treatments	0.077	0.153

Table 28. Effect of wax degrading microbial consortium on dehydrogenase activity of sugarcane bagasse

Treatments	Dehydrogenase activity (µg of TFP formed $g^{-1}h^{-1}$)							
	Days							
	0	15	30	45	60	75	90	105
T_1 : Bagasse	0.43	0.59	0.78	0.83	1.01	1.10	1.07	1.02
T_2 : Sterilized Bagasse	0.21	0.28	0.31	0.34	0.51	0.57	0.49	0.45
T_3 : Bagasse + Soil	0.61	0.83	1.13	1.15	1.20	1.25	1.20	1.18
T_4 : Bagasse + Soil + 10 % Microbial consortium	0.83	1.21	1.37	1.38	1.56	1.47	1.42	1.40
T_5: Sterilized bagasse +10 % Microbial consoritum	0.43	0.72	0.80	0.83	0.78	0.87	0.70	0.65
T_6 : Bagasse + 10 % Microbial consortium	0.60	0.81	0.92	1.02	1.06	1.12	1.08	1.05

	SEd	CD (0.05)
Days	0.012	0.025
Treatments	0.010	0.021
Days x Treatments	0.031	0.061

Table 29. Effect of wax degrading microbial consortium on water repellency of sugarcane bagasse

Treatments	Molarity ethanol droplet value (MED Value)							
	Days							
	0	15	30	45	60	75	90	105
T_1 : Bagasse	2.2	2.2	2.0	2.0	2.0	2.0	2.0	1.8
T_2 : Sterilized Bagasse	2.2	2.2	2.0	2.0	2.0	2.0	2.0	2.0
T_3 : Bagasse + Soil	2.0	2.0	1.8	1.6	1.4	1.4	1.2	1.2
T_4 : Bagasse + Soil + 10 % Microbial consortium	2.0	2.0	2.0	1.6	1.4	1.4	1.2	1.0
T_5: Sterilized bagasse +10 % Microbial consoritum	2.2	2.2	2.0	1.6	1.6	1.4	1.2	0.8
T_6 : Bagasse + 10 % Microbial consortium	2.2	2.2	2.0	2.0	1.6	1.6	1.2	1.0

	SEd	CD (0.05)
Days	0.022	0.450
Treatments	0.019	0.038
Days x Treatments	0.055	0.110

Table 10. Studies on the biomass of wax degrading bacteria and actinomycetes in n- hexadecane

Wax degrading bacteria and actinomycetes	Biomass (mg/25 ml)							
	Days							
	1	3	5	7	9	11	13	15
Bacillus sp. WDB3	0.0	0.01	0.01	0.06	0.15	0.16	0.18	0.15
Bacillus sp. WBB7	0.0	0.05	0.08	0.06	0.11	0.14	0.16	0.19
Bacillus sp. WDB11	0.0	0.03	0.03	0.09	0.14	0.20	0.28	0.28
Bacillus sp. WDB12	0.0	0.01	0.05	0.09	0.14	0.08	0.24	0.26
Bacillus sp. WDB17	0.0	0.02	0.03	0.05	0.10	0.11	0.12	0.15
Streptomyces sp. WDA 2	0.0	0.05	0.09	0.15	0.16	0.17	0.20	0.23
Streptomyces sp. WDA 3	0.0	0.07	0.12	0.13	0.18	0.24	0.25	0.26
Streptomyces sp. WDA 4	0.0	0.02	0.09	0.13	0.18	0.20	0.25	0.27
SEd	0.0	0.013	0.023	0.003	0.004	0.005	0.007	0.311
CD (0.05)	0.0	0.002	0.005	0.007	0.010	0.011	0.014	0.661

Table 11. Studies on growth pattern of wax degrading bacteria and actinomycetes in n- hexadecane

Wax degrading bacteria and actinomycetes	Growth Parameters					
	Number of generation	Generation time (h)	Growth rate (μ) (min)	Growth ratio	Growth yield (y/g)	Molar growth yield (g/M)
Bacillus sp. WDB3	1.68	21.41	2.4	4.66	0.20	0.11
Bacillus sp. WBB7	1.93	18.62	6	10.32	0.20	0.11
Bacillus sp. WDB11	2.00	18.0	3	5.55	0.41	0.21
Bacillus sp. WDB12	6.00	6.00	9.6	16.61	0.32	0.18
Bacillus sp. WDB17	1.79	20.13	2.4	4.97	0.18	0.10
Streptomyces sp. WDA 2	1.89	19.01	3	5.25	0.32	0.18
Streptomyces sp. WDA 3	1.02	31.04	1.8	3.22	0.41	0.22
Streptomyces sp. WDA 4	1.16	35.29	1.2	2.83	0.37	0.20
SEd	0.085	0.733	0.002	0.258	0.010	0.005
CD (0.05)	0.181	1.555	0.005	0.547	0.021	0.011

Table 3b.Colony characterization of wax degrading bacterial isolates of enriched automobile waxy waste

Wax degrading bacterial isolates	Colony characters				
	Form of colony	Margin	Elevation	Surface	Color
WDB 1	Irregular	Undulate	Effuse	Smooth	Dirty white
WDB 2	Filamentous	Crenate	Convex papillate	Coarse granular	Creamy white
WDB 3	Circular	Entire	Effuse	Transparent	Creamy white
WDB 4	Irregular	Crenate	Effuse	Opaque	Dirty white
WDB 5	Rhizoid	Entire	Low convex	Transparent	Red
WDB 6	Circular	Entire	Effuse	Transparent	Creamy white
WDB 7	Circular	Entire	Convex papillate	Transparent	Creamy white
WDB 8	Circular	Entire	Low convex	Transparent	Creamy white
WDB 9	irregular	Undulate	Effuse	Smooth	Creamy white
WDB 10	Circular	Entire	Raised	Transparent	Creamy white
WDB 11	Irregular	Crenate	Convex papillate	Opaque	Creamy white
WDB 12	Circular	Entire	Pulvinate	Transparent	Dirty white
WDB 13	Circular	Entire	Low convex	Transparent	Dirty white
WDB 14	Circular	Entire	Effuse	Transparent	Creamy white
WDB 15	mycoids	Crenate	Effuse	Filamentous	Creamy white
WDB 16	Circular	Undulate	Low convex	Transparent	Creamy white
WDB 17	Circular	Entire	Effuse	Opaque	Dirty white

APPENDIX I

1. Beeswax mineral salt medium (Mckenna *et al*., 2002)

KNO_3	-	2 g
K_2HPO_4	-	1 g
MgSO4	-	0.5 g
NaCl	-	0.5 g
$CaCO_3$	-	3 g
$FeSO_4$	-	0.01 g
Beeswax	-	20 g
Agar	-	20 g
Distilled water	-	1000ml

2. Mineral salt solution I (Akit *et al.*, 1981)

$NaNO_3$	-	2 g
K_2HPO_4	-	1 g
KH_2PO_4	-	0.5 g
KCl	-	0.1 g
$MgSO_4.7H_2O$	-	0.5 g
$CaCl_2$	-	0.01 g
NaEDTA	-	0.0015 g
$FeSO_4.H_20$	-	0.01 g
Beeswax	-	20 g
Agar	-	20 g
Distilled water	-	1000ml

3. Mineral salts solution II (Skinner *et al.*, 1952)

$CaCO_3$	-	0.33 g
$CaSO_4$	-	0.8 g
$MgSO_4.7H_2O$	-	0.7 g
K_2SO_4	-	0.025 g
K_2HPO_4	-	0.005 g
$NaHCO_3$	-	0.2 g
$FeCl_3$	-	Trace
Yeast Extract	-	0.005 g
Beeswax	-	20 g
Agar	-	20 g
Distilled water	-	1000ml

4. Inorganic Basal Medium (IBM) (Sorkoh *et al*., 1995)

$NaNO_3$	-	0.85 g
K_2HPO_4	-	0.56 g
Na_2HPO_4	-	0.86 g
K_2SO_4	-	0.17 g
$MgSO_4.7H_2O$	-	0.37 g
$CaCl.6H_2O$	-	0.0007 g
FE^{3++} EDTA	-	0.004 g
Trace Element	-	2.5ml
Distilled water	-	1000ml

Trace element solution

EDTA	-	1 g
$ZnSO_4.7H_2O$	-	2.08 g
$MnSO_4.4H_2O$	-	1.78 g
H_3BO_3	-	0.56 g
$CuSO_4.5H_2O$	-	1.0 g
$Na_2MO_6O_4.2H_2O$	-	0.39 g
KI	-	0.664 g
$FeSO_4.7H_2O$	-	0.4 g
$NiCl_2.6H_20$		0.004 g

5. Modified mineral salt medium (Sorkoh *et al*., 1995)

NH_4NO_3	-	4 g
KH_2PO_4	-	4 g
Na_2HPO_4	-	6 g
$MgSO_4.7H_2O$	-	0.2 g
$CaCl_2.2H_2O$	-	0.01 g
$FeSO_4.7H_2O$	-	0.01 g
Na_2EDTA	-	0.014 g
Agar	-	20 g
Distilled water	-	1000ml

6. Nutrient agar medium

Glucose	-	5.0g
Peptone	-	5.0g
Beef extract	-	3.0g
NaCl	-	5.0g
Distilled water	-	1000ml
Agar	-	15g
pH	-	7

7. Potato dextrose agar medium

Dextrose	20g
Potato	250g
Agar	15-20g
Distilled water	1000ml
pH	7

8. Martin' s Rose Bengal agar medium

Glucose	-	10g
Peptone	-	5.0g
KH_2PO_4	-	3.0g
$MgSO_4$	-	5.0g
Streptomycin Sulphate	-	0.03g
Rose Bengal	-	0.935g
Distilled water	-	1000ml
Agar	-	15g
pH	-	7

9. Kenknight's agar medium

Dextrose	-	1.0g
KH_2PO_4	-	1.0g
$NaNO_3$	-	0.1g
KCl	-	0.1g
Distilled water	-	1000ml
Agar	-	15g
pH	-	6.8

10. Basal salt medium for actinomycetes

Yeast extract	-	1.0g
KH_2SO_4	-	1.0g
NaCl	-	0.1g
KCl	-	0.1g
$MgCl_2.6H_2O$	-	0.7g
KH_2PO_4	-	1.0g
$FeCl_3.6H_2O$	-	0.05g
$CaCl_2$	-	0.1g
CMC	-	1.0g
Distilled water	-	1000ml
Agar	-	15g
pH	-	6.8

APPENDIX II

1. Voges Proskaeur test Baritts reagent

Solution A

α- Napthol	-	1.0 g
Ethanol	-	100 ml

Solution B

40 % potassium hydroxide

2. Urea broth

Peptone	-	1.0 g
Sodium chloride	-	5.0 g
Potassium Monohydrogen phosphate	-	2.0 g
Glucose	-	1.0 g
Phenol red (0.02% solution)	-	1ml
Urea (20 % aqueous solution)	-	100 ml
Distilled water	-	1000 ml

3. Reagents for nitrate reductase

Sulfanilic acid	-	0.8 g
5N acetic acid	-	150 ml

α – napthylamine reagent

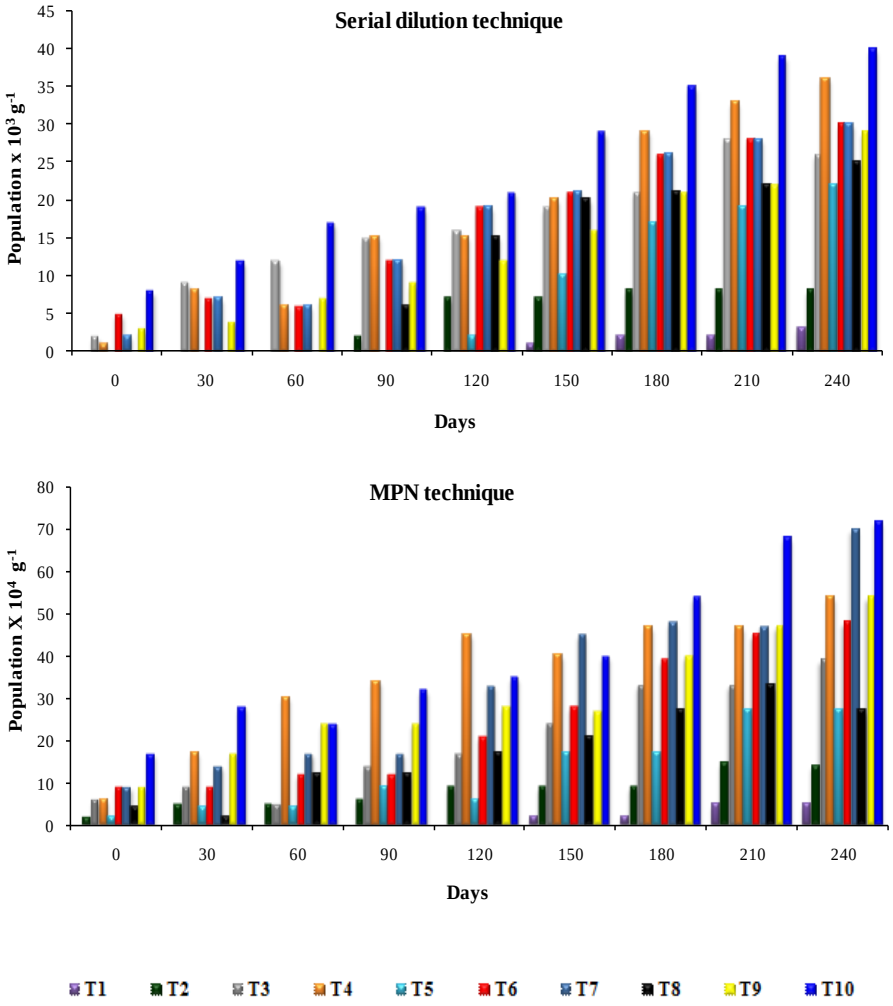
Dimethyl α – napthylamine	-	0.5 g
5 N Acetic acid	-	100 ml

4. DNS- reagent (Miller, 1972) for estimation of cellulase activity

Dinitro salicylic acid	-	10 g
Phenol	-	2 g
Na_2SO_3	-	0.5 g
NaOH	-	20 g
Potassium sodium tartarate	-	400 g

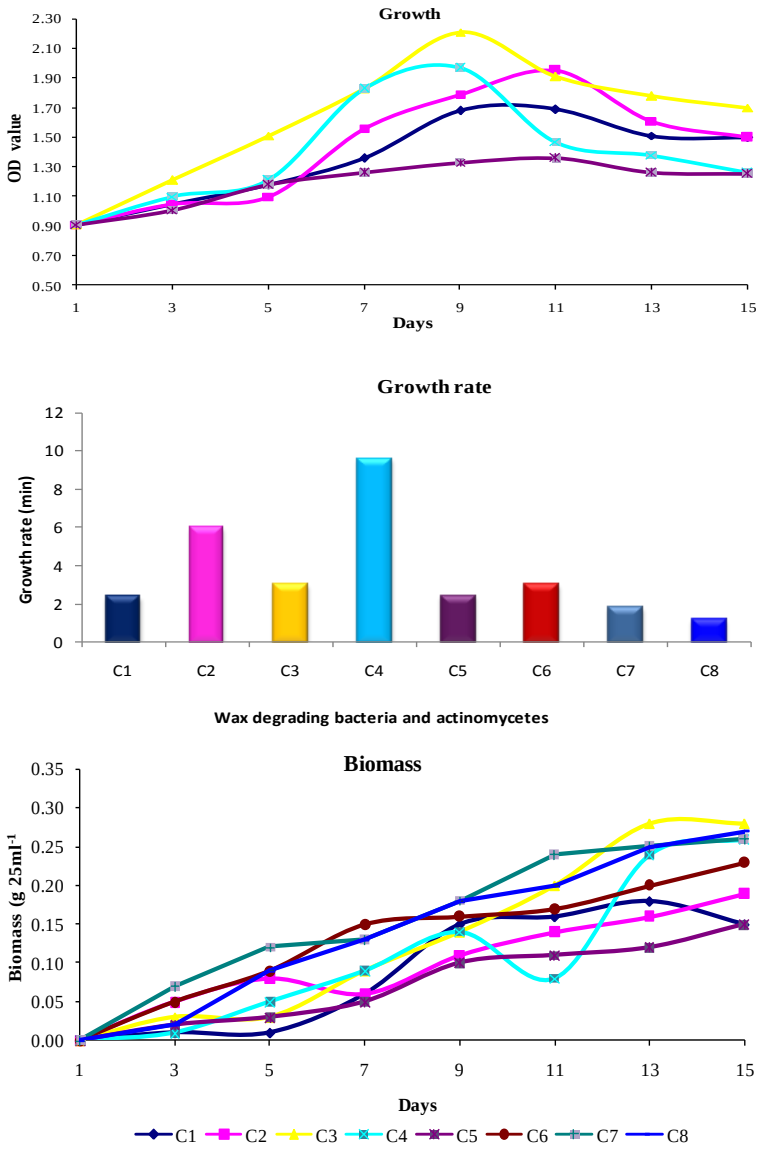
Components were dissolved in 1 litre of distilled water by stirring and heating at 50°C and stored in brown glass bottles at room temperature

Fig. 1. Enumeration of wax degrading bacteria from enriched automobile waxy waste (AWW)



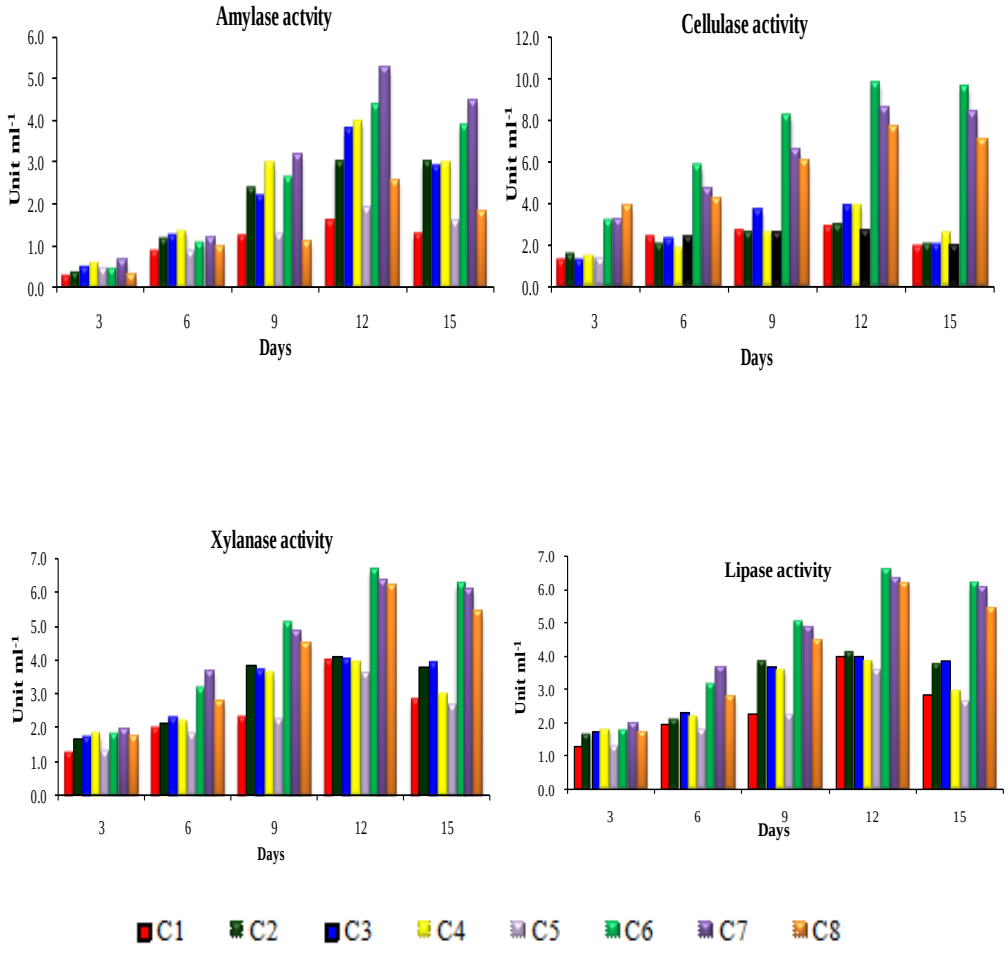
T_1 : Automobile waxy waste
T_2 : Automobile waxy waste + 25% soil
T_3 : Automobile waxy waste + 50% soil
T_4 : Automobile waxy waste + 75% soil
T_5 : Automobile waxy waste + 25% FYM
T_6 : Automobile waxy waste + 50% FYM
T_7 : Automobile waxy waste + 75% FYM
T_8 : Automobile waxy waste + 25% cow dung
T_9 : Automobile waxy waste + 50% cow dung
T_{10} : Automobile waxy waste + 75% cow dung

Fig.2. Growth pattern of wax degrading bacteria and actinomycetes in n-hexadecane



C1 - *Bacillus* sp. WDB3
C2 - *Bacillus* sp. WDB7
C3 - *Bacillus* sp. WDB11
C4 - *Bacillus* sp. WDB12
C5 - *Bacillus* sp. WDB17
C6 - *Streptomyces* sp. WDA2
C7 - *Streptomyces* sp. WDA3
C8 - *Streptomyces* sp. WDA4

Fig. 3. *Invitro* enzymes activity of wax degrading bacteria and actinomycetes



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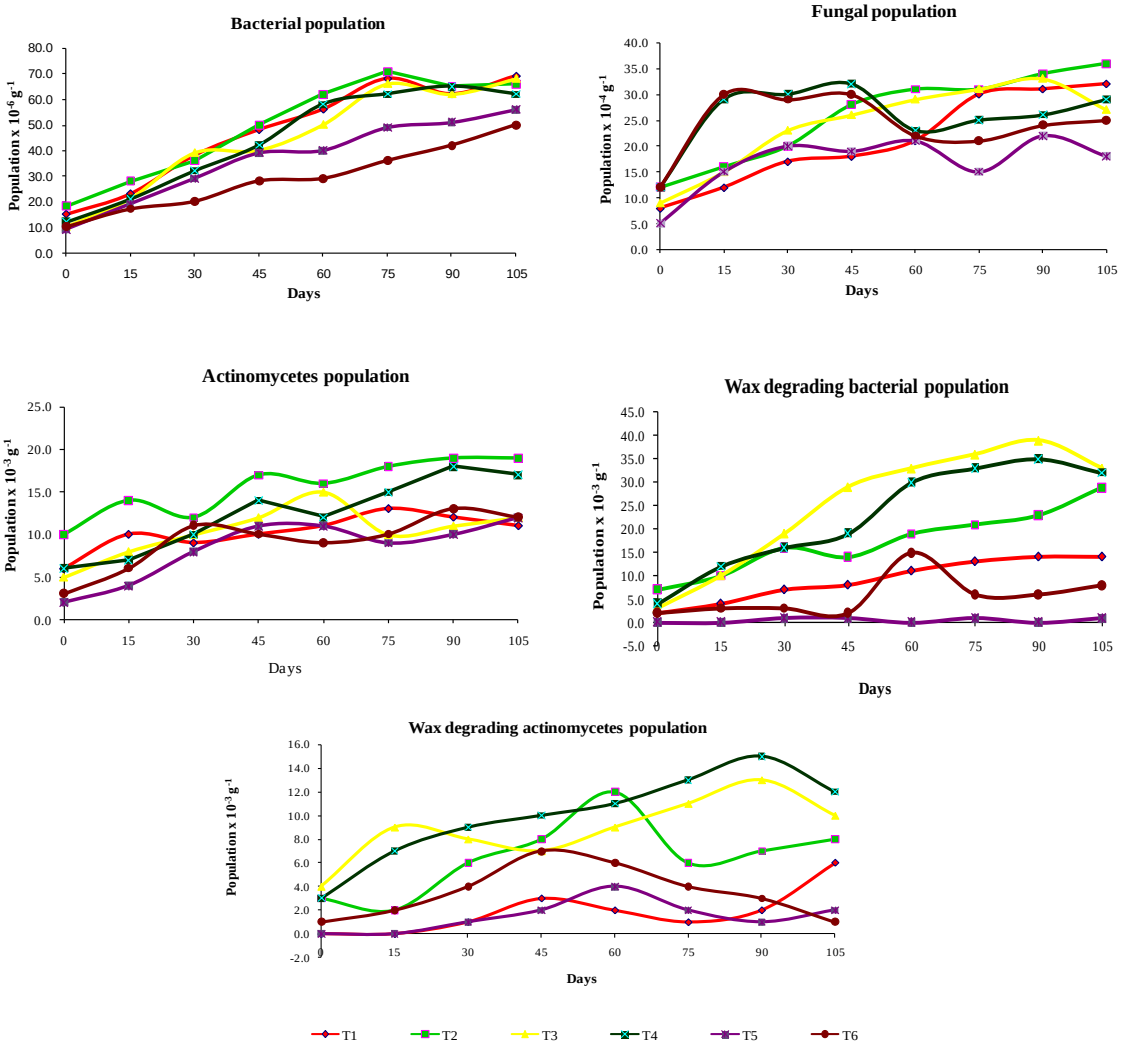
C4 - *Bacillus* sp. WDB12
C5 - *Bacillus* sp. WDB17

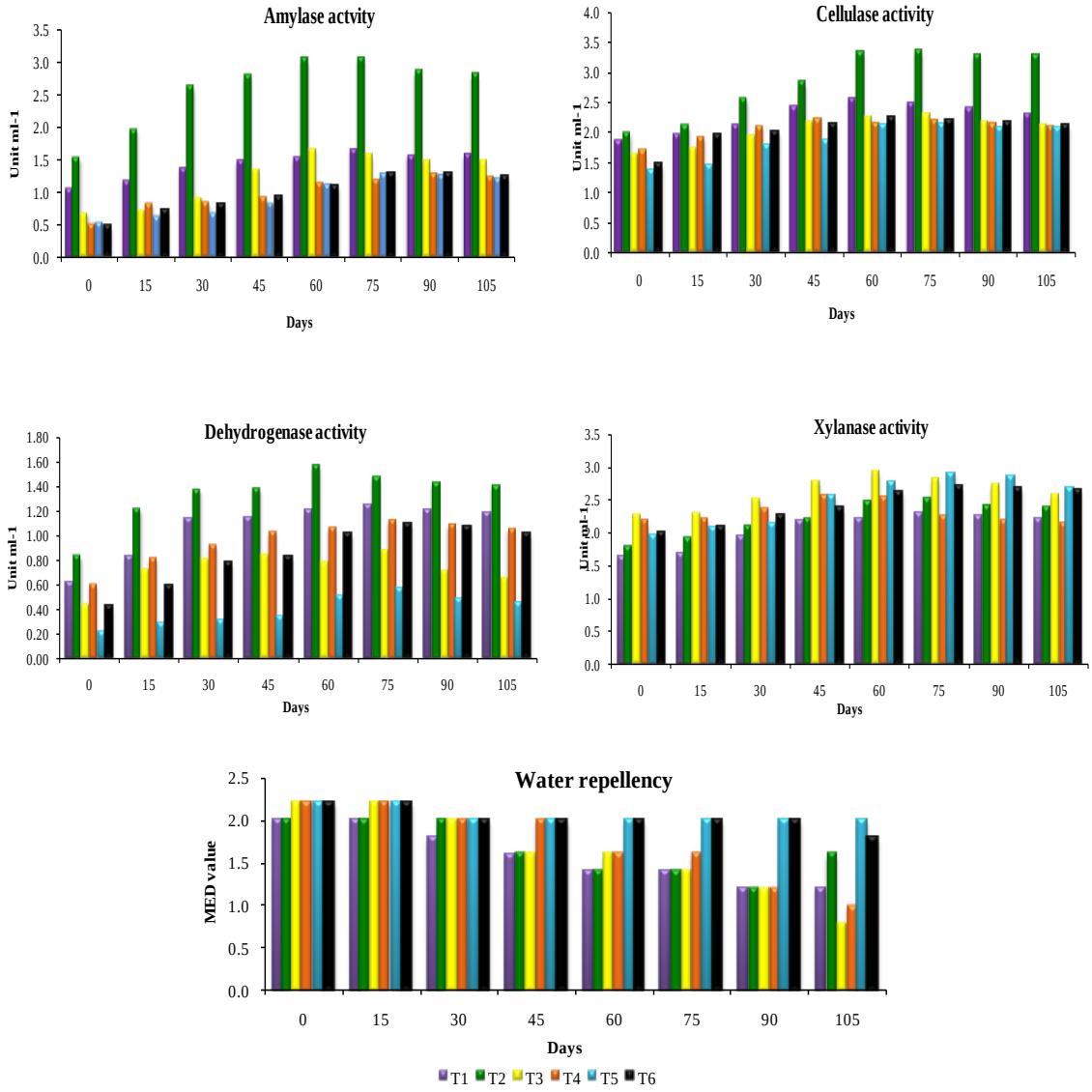
C6 - *Streptomyces* sp. WDA2
C7 - *Streptomyces* sp. WDA3
C8 - *Streptomyces* sp. WDA4

Fig. 4. Effect of wax degrading microbial consortium on microbial dynamics of sugarcane bagasse



T1 - Sterilized Bagasse
T2 - Bagasse
T3 - Bagasse + 50 % Soil
T4 - Bagasse + 50 % Soil + 10 % Microbial consortium
T5 - Sterilized bagasse +10 % Micobial consoritum
T6 - Bagasse + 10 % Microbial consortium

Fig. 5. Effect of wax degrading microbial consortium on enzymes activity of sugarcane bagasse



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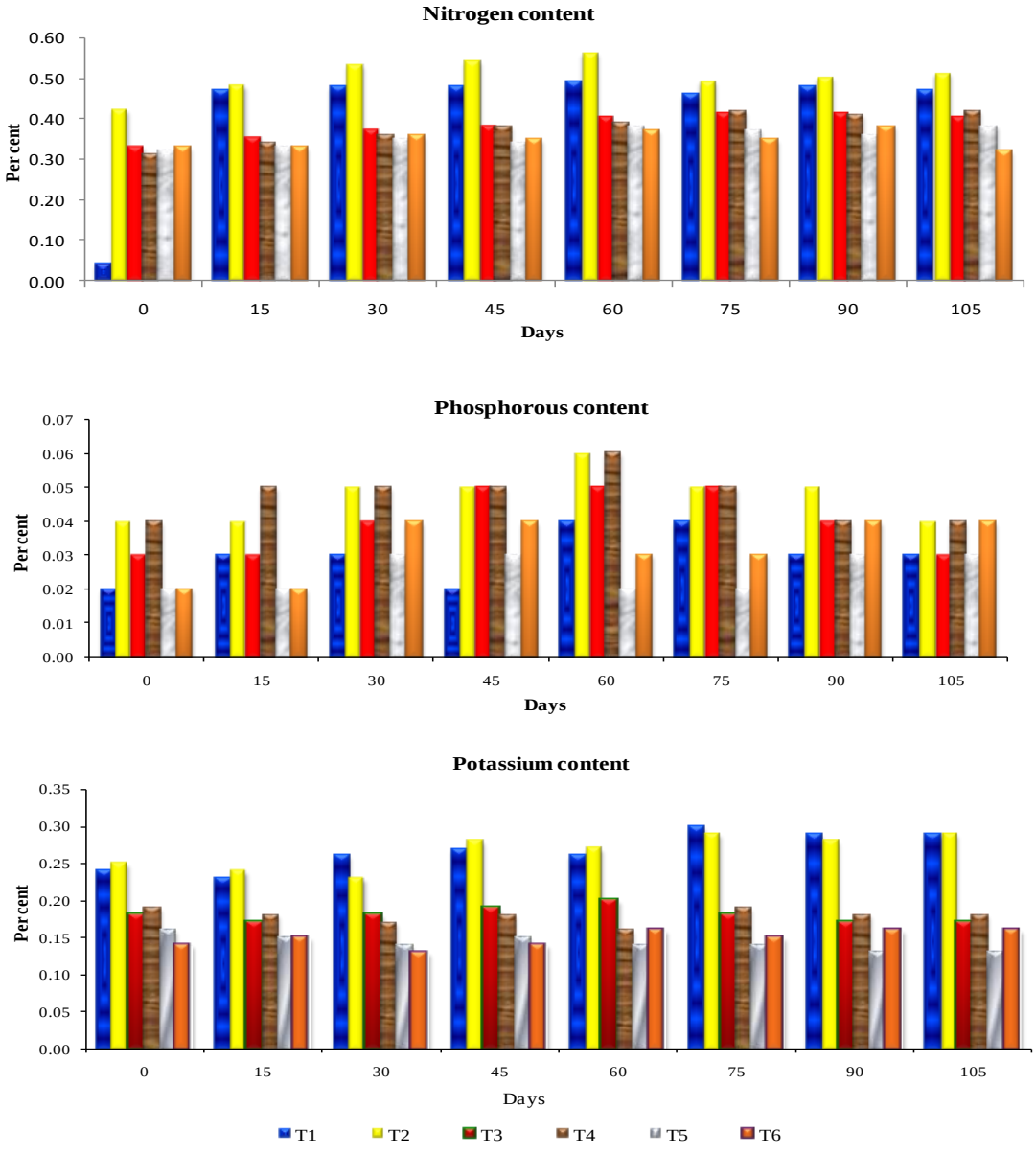
T4 - Bagasse + 50 % Soil + 10 % Microbial consortium
T5 - Sterilized bagasse +10 % Micobial consoritum
T6 - Bagasse + 10 % Microbial consortium

Fig. 6. Effect of wax degrading microbial consortium on nitrogen , phosphorous, potassium content of sugarcane bagasse



T1 - Sterilized Bagasse
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T4 - Bagasse + 50 % Soil + 10 % Microbial consortium
T5 - Sterilized bagasse +10 % Micobial consoritum
T6 - Bagasse + 10 % Microbial consortium