Biodegradation of leaf litter biomass by *Bacillus licheniformis*

A
DISSERTATION REPORT

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MASTERS OF SCIENCE
IN
BIOTECHNOLOGY

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Dated: 15/7/13

Place: Patiala

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the dissertation entitled **"Biodegradation of leaf litter biomass by *Bacillus licheniformis*"** in partial fulfillment of the requirements for the award of the degree of Masters of Science in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is an authentic record of my own work during the period of six months from Jan 2013 to July 2013, under the supervision of Dr. Dinesh Goyal, Professor, Department of Biotechnology & Environmental Sciences, Thapar University. The report has not been submitted for the award of any other degree or certificate in this or any other university.

Dated: 15/7/13.

Place: Patiala

Shalika Devi

Shalika Devi

CERTIFICATE

This is to certify that the thesis entitled **"Biodegradation of leaf litter biomass by *Bacillus licheniformis*"** submitted by Ms. Shalika Devi in partial fulfillment of the requirements for the award of degree of Masters of Science in Biotechnology to Thapar University, Patiala, is a record of student's own work carried out by her under her supervisor. The report has not been submitted for the award of any other degree or certificate in this or any other University or institute.

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Abbreviations

α	Alpha
ß	Beta
BSA	Bovine serum albumin
CMC	Carboxymethyl cellulose
CO_2	Carbon dioxide
°C	Degree Celsius
DNS	3, 5-dinitrosalicylic acid
et al	and others
$FeSO_4.7H_2O$	Ferrous sulphate heptahydrate
g	Gram
h	Hour
H_2O_2	Hydrogen peroxide
K_2HPO_4	Di potassium hydrogen phosphate
KOH	Potasium hydroxide
L	Liter
M	Molar
µg	Microgram
µl	Microliter
mg	Milligram
min	Minute
mm	Millimeter
mM	Millimolar
MM	Minimal Media
ml	Milliliter
M	Molar
$MgSO_4.5H_2O$	Magnessium sulphate pentahydrate
N	Normal
nm	Nanometer
NA	Nutrient Agar

NaOH	Sodium Hydroxide
NB	Nutrient Broth
NH_3	Ammonia
NaOH	Sodium hydroxide
NaCl	Sodium chloride
$(NH_2)_2CO$	Urea
OD	Optical Density
O_2	Oxygen
%	Percent
rpm	Rotation per minute
s	Second
U/ml	Units per ml
Viz.	As follows

ABSTRACT

Leaf litter biomass was collected, dried, processed and characterized for cellulose, hemicelluloses, lignin and reducing sugars and these were found to be 17.11 %, 4.55 %, 8.67 % and 5.02 % of biomass respectively. CMCase activity of *Bacillus licheniformis* T9 was 0.256 U/ml. Growth curve of bacteria was drawn and protein estimation was done simultaneously. Effect of pH and temperature on CMCase activity was also checked and optimum activity was at pH 7.0 and 50 °C. *Bacillus licheniformis* T9 was used for biodegradation of leaf litter biomass and cellulose was degraded gradually with formation of reducing sugars. Reducing sugar content decreased initially due to consumption by bacteria and increased at a later stage of growth. *Bacillus licheniformis* T9 was able to effectively utilize leaf litter biomass as sole carbon source.

Keywords: Lignocellulosic biomass, Bacteria and fungi, Cellulases, Biodegradation.

INTRODUCTION

Exponential rise of world's population and industrialization have jacked up demand for fossil fuels which are non-renewable (Uihlein & Schbek et al., 2009). Fossil fuels have a huge influence not only on the world's economy but on our lives too. Bioethanol is one of the most promising biofuel from renewable resources. Ethanol is at present the most widely used liquid biofuel for motor vehicles (Demirbas, 2005). Ethanol is a clean-burning fuel that makes no net contribution to global warming because the carbon dioxide produced by the combustion of ethanol is consumed by plant growth. Ethanol can be produced from inexpensive and abundant lignocellulosic biomass. Crops such as sugarcane and corn cannot be utilized for the production of bioethanol because of their primary value as a source of food. Therefore, lignocellulosic materials such as agricultural wastes or residues are of great interest in bioethanol production owing to their use as a promising substrate (Sarkar et al., 2012). It is important that we look for sources of lignocellulosic biomass from which ethanol can be produced at a reasonable cost with available resources. The lignocellulose is the most abundant renewable natural resource and substrate available for conversion into fuels.

On a worldwide basis, terrestrial plants produce 1.3×10^{10} metric tonnes (dry weight basis) of wood per year, which is equivalent to 7×10^{9} metric tonnes of coal or about two-thirds of the world's energy requirement. Available cellulosic feed stocks from agriculture and other sources are about 180 million tonnes per year (Lynd et al., 2003). The biomass include agricultural residues such as corn stover (leaves, stalks and cobs), wheat straw, paddy straw, sugarcane bagasse as well as forest products (hardwoods and softwoods) and potential energy crops like switch grass and *Miscanthus* (Gray, 2007).

India stands fourth in the world in ethanol production with a production of about 1.3 billion litres per annum from 105 ethanol plants. Brazil, which produces about 16.1 billion litres per year stands first, USA is next with a production level of 5.75 billion litres and China with 2.83 billion litres occupies third place (Thomas, 2003).

Bioethanol from celluloses holds great potential due to the wide availability and relatively low cost of cellulose material (Badger, 2002). The three main raw materials for ethanol production are sugars (from sugarcane, sugar beet, molasses and fruits), starch (from corn, cassava, potatoes and root crops) and cellulosics. Most of the materials in the first two categories come under food stuffs. This limits their use for ethanol production. So, the interest obviously relies on the abundant cellulosics (Lin and Tanaka, 2006).

Bioconversion of cellulose to sugars is a prerequisite for the subsequent production of bioenergy and can be readily fermented to fuel ethanol (Kumar et al., 2008). Cellulose biodegradation by cellulases and cellulosomes, produced by numerous microorganisms, represents a major carbon flow from fixed carbon sinks to atmospheric CO_2 (Berner, 2003).

In the present work leaf litter biomass was characterized for cellulose, hemicelluloses, lignin and sugars and processed biomass was subjected to biodegradation by cellulose degrading bacterial isolate under pure culture conditions.

REVIEW OF LITERATURE

A defining feature of terrestrial plants is the highly developed cell walls which is a dynamic structure. It is a complex composite of polysaccharides, aromatic compounds and proteins. In general, the three major constituents of secondary cell walls are cellulose, hemicelluloses and lignin; from which the generic term 'lignocellulose' is coined. Plant biomass is mainly composed of cellulose, hemicelluloses and lignin, along with smaller amounts of pectin, protein, extractives (soluble nonstructural materials such as nonstructural sugars, nitrogenous material, chlorophyll and waxes) and ash (Jorgensen et al., 2007).

Cellulose

The main framework of the cell wall is the cellulose fibrils. Being the most abundant polymer and entirely made up of fermentable glucose units, cellulose is the main target for bioconversion. The D-glucose units of cellulose are linked by ß-1, 4-glycosidic bonds into a linear structure with a high degree of polymerization. This structure results in the formation of intra- and intermolecular hydrogen bonds, creating para-crystalline cellulose fibrils (Klemm et al., 2005). Cellobiose is the repeat unit established through this linkage, and it constitutes cellulose chains. The long-chain cellulose polymers are linked together by hydrogen and vander Waals bonds, which cause the cellulose to be packed into microfibrils. Hemicelluloses and lignin cover the microfibrils. Fermentable D-glucose can be produced from cellulose through the action of either acid or enzymes breaking the ß-1, 4-glycosidic linkages. Cellulose in biomass is present in both crystalline and amorphous forms. Crystalline cellulose comprises the major proportion of cellulose, whereas a small percentage of unorganized cellulose chains form amorphous cellulose. Cellulose is more susceptible to enzymatic degradation in its amorphous form (Beguin et al., 1994).

Hemicellulose

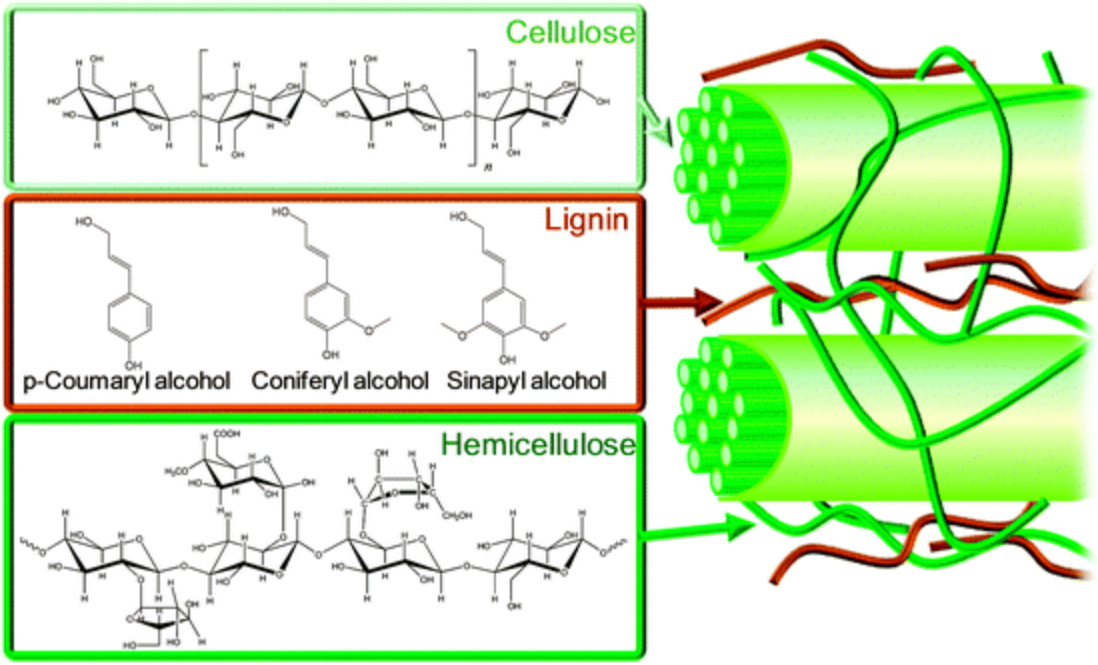
The main feature that differentiates hemicellulose from cellulose is that hemicellulose has branches with short lateral chains consisting of different sugars. These monosaccharides include pentoses (xylose, rhamnose and arabinose), hexoses (glucose, mannose and galactose), and uronic acids (4-O-methylglucuronic, D-glucuronic, and D-galactouronic acids). The backbone of hemicellulose is either a homopolymer or a heteropolymer with short branches linked by ß-1, 4-

glycosidic bonds and occasionally ß-1, 3-glycosidic bonds (Kuhad et al., 1997). The hemicellulose fraction of hardwoods and agricultural raw materials is rich in pentose sugars while softwood hemicellulose only contains minor fractions of the pentose sugar D-xylose (Hayn et al., 1993).

Lignin

Lignin is the principal aromatic component of wood. The lignin molecule is a polymer with a DP (degree of polymerization) of 450-550, formed by the free radical, oxidative condensation of the three monomers, coniferyl alcohol, sinapyl alcohol and coumaryl alcohol (Wayman and Parekh, 1990). It is present in the primary cell wall imparting structural support, impermeability, and resistance against microbial attack (Perez et al., 2002).

Figure 1. Structure of lignocellulosic biomass (Source: Alonso et al., 2012)



Biodegradation of Lignocellulosic Biomass

Lignocellulosic biomass consists of three types of polymers- cellulose, hemicelluloses and lignin that are strongly intermeshed and chemically bonded by non-covalent forces. A great variety of fungi and bacteria can fragment these macromolecules by using a variety of hydrolytic or oxidative enzymes. Most of the cellulolytic microorganisms belong to eubacteria and fungi, even though some of the anaerobic protozoa and slime moulds are also able to degrade cellulose.

Although cellulose is a homopolymer, a number of enzymes are needed to degrade it. These can be divided into the following three types: Endoglucanases (EC 3.2.1.4), which hydrolyse internal ß-1, 4-D-glucosidic linkages randomly in the cellulose chain; Cellobiohydrolases, also known as exoglucanases (EC 3.2.1.91), which progress along the cellulose line and cleave off cellobiose units from the ends; ß-glucosidases (EC 3.2.1.21), which hydrolyse cellobiose to glucose and also cleave off glucose units from cello-oligosaccharides. These three groups of enzymes work synergistically to degrade cellulose by creating new sites for each other and preventing product inhibition (Eriksson et al., 2002; Valjamae et al., 2003). Over the years, culturable, cellulase-producing bacteria have been isolated from a wide variety of sources such as composting heaps, decaying plant material, feces of ruminants, and hot springs (Doi, 2008). Bacteria has high potential in cellulase production as compared to fungi (Ariffin et al., 2008). Cellulose degradation is largely an aerobic process and the primary cellulolytic bacterial isolates are *Pseudomonas* sp. (Kitamura et al., 2002), *Bacillus subtilis* (El-Helow et al., 2002), *Bacillus licheniformis* (Malet et al., 1993), and *Bacillus brevis* (Louw et al., 1993). *Fibrobacter succinogenes* from the rumen is also an important cellulose degrader (Kobayashi et al., 2008).

In case of lignin, lignin peroxidases, manganese peroxidases (MnPs), and laccases are three types of enzyme families that are implicated in the lignin degradation (Glen et al., 1983; Tien and Kirk, 1983). Hemicelluloses are made up of a number of different monosaccharides so it needs various enzymes for degradation such as xylanases, mannanases, galactanases, acetyl xylan esterase etc.

Table 1. Microorganisms involved in lignocellulosic waste degradation

Microorganism	Target Substrate	Reference
Bacteria		
Bacillus sp.	Cellulose	Sun and Cheng, 2002; Rabinovich et al., 2002
Cellulomonas sp.	Cellulose	Sun and Cheng, 2002; Rabinovich et al., 2002
Thermonospora sp.	Cellulose	Sun and Cheng, 2002; Rabinovich et al., 2002
Clostridium sp.	Cellulose	Sun and Cheng, 2002; Rabinovich et al., 2002
Ruminococcus sp.	Cellulose	Sun and Cheng, 2002; Rabinovich et al., 2002
Acetovibrio sp.	Cellulose	Sun and Cheng, 2002; Rabinovich et al., 2002
Erwinia sp.	Cellulose	Sun and Cheng, 2002; Rabinovich et al., 2002
Streptomyces sp.	Cellulose	Sun and Cheng, 2002; Rabinovich et al., 2002
Microbispora sp.	Cellulose	Sun and Cheng, 2002; Rabinovich et al., 2002
Bacteriodes sp.	Cellulose	Sun and Cheng, 2002; Rabinovich et al., 2002
Clostridium cellulovorans	Cellulose	Arai et al., 2006
Fungi		
Brown-rot Fungi	Cellulose	Prasad et al., 2007
White-rot Fungi	Cellulose and lignin	Prasad et al., 2007
Trichoderma reesei	Cellulose	Arai et al., 2006
Aspergillus niger	Cellulose	Arai et al., 2006
Penicillium sp.	Cellulose	Fan et al., 1987; Duff and Murray, 1996; Sternberg, 1976
Phanerochaete sp.	Cellulose	Fan et al., 1987; Duff and Murray, 1996; Sternberg, 1976
Sclerotium rolfsii	Cellulose	Fan et al., 1987; Duff and Murray, 1996; Sternberg, 1976
Schizophyllum sp.	Cellulose	Fan et al., 1987; Duff and Murray, 1996; Sternberg, 1976
Phanerochaete chrysosporium	Lignin	Glenn et al., 1983; Tien & Kirk, 1983
Phlebia sp.	Lignin	Kirk and Farrell, 1987; Kirk and Shimada, 1985
Schizophylum commune	Hemicelluloses	MacKenzie and Bilous, 1988

Ethanol as fuel

Increasing cost and rising demand for energy worldwide from population growth and economic development have focused world attention on developing larger and more sustainable supplies of transportation fuel. The major energy demand is still supplied from conventional fossil fuels such as oil, coal and natural gas. Utilization of fossil fuels over the last century has drastically increased the level of green house gases in the earth's atmosphere (Ballesteros et al., 2006). One fuel that has the potential to match the convenient features of petroleum at a low price is ethanol produced from lignocellulosic biomass resources conveniently referred to as bioethanol. Lynd et al., (1991). Ethanol can be produced from inexpensive and abundant lignocellulosic biomass such as agricultural and forestry residues, wastepaper, a significant fraction of municipal solid waste, and woody and herbaceous energy crops grown as feed stocks for ethanol production. Lignocellulosic waste materials obtained from energy crops, wood and agricultural residues represent the most abundant global source of renewable biomass (Lin & Tanaka et al., 2006). However, even though lignocellulosic biomass provides a low-cost resource, its conversion into ethanol is difficult (Wyman et al., 1996). A promising approach is to break down the celluloses and hemicelluloses chains, which comprise two-thirds to three-quarters of the biomass, into their component sugars and then ferment those sugars into ethanol.

Conversion of Biomass to Fuel

Biological ethanol production, from renewable feed stocks, uses lower pressures, temperatures and acidities suitable for enzymes and fermentation organisms. In biological ethanol production, lignocellulosics are processed for bioethanol production through three major operations: pretreatment for delignification is necessary to liberate cellulose and hemicellulose before hydrolysis; hydrolysis of cellulose and hemicellulose to produce fermentable sugars including glucose, xylose, arabinose, galactose, mannose and fermentation of reducing sugars (Balat et al., 2008). Action of microorganisms and enzymes on biological sources can lead to the production of mostly ethanol. These agents carry out the fermentation of sugar, starch, hemicellulose or cellulose. Pretreatment is required to alter the size and structure of the biomass, as well as its chemical composition, so that the hydrolysis of the carbohydrate fraction to monomeric sugars can be achieved rapidly and with greater yields. The hydrolysis process can be significantly

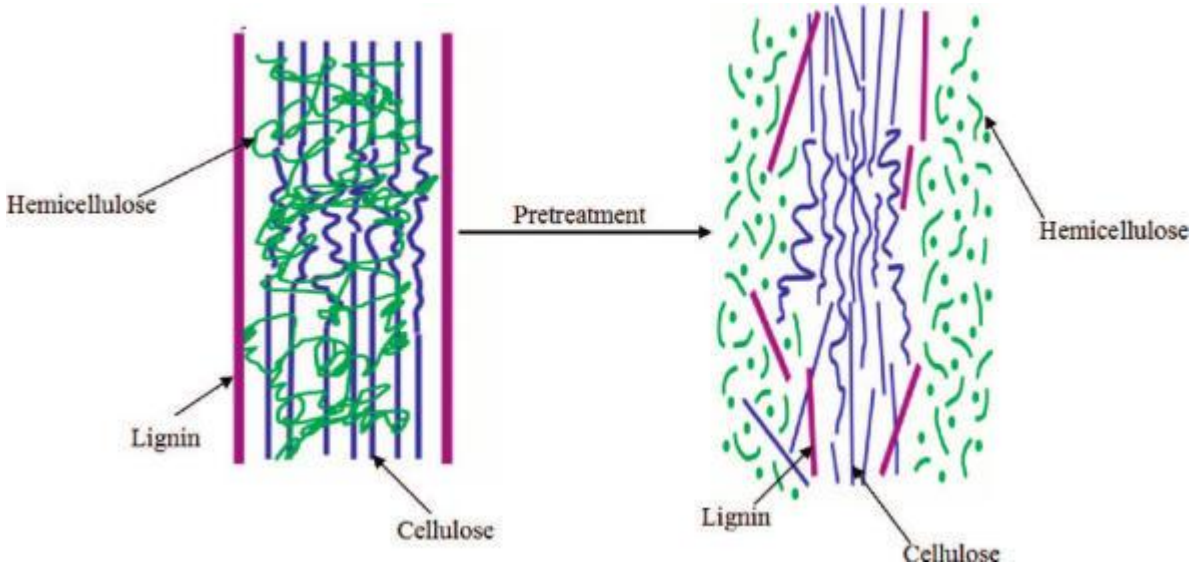
improved by removal of lignin and hemicellulose, reduction of cellulose crystallinity and increase of porosity through pretreatment processes (McMillan et al., 1994).

1. Pretreatment

The most important processing challenge in the production of biofuel is the pretreatment of biomass. Pretreatment methods refer to the solubilization and separation of one or more of these components of biomass. It makes the remaining solid biomass more accessible to further chemical or biological treatment (Demirbas, 2005). The lignocellulosic complex is made up of a matrix of cellulose and lignin bound by hemicelluloses chains. The pretreatment is done to break the matrix in order to reduce the degree of crystallinity of the cellulose and increase the fraction of amorphous cellulose, the most suitable form for enzymatic attack (Sanchez & Cardona, 2008). Pretreatment is undertaken to bring about a change in the macroscopic and microscopic size and structure of biomass as well as submicroscopic structure and chemical composition. It makes the lignocellulosic biomass susceptible to quick hydrolysis with increased yields of monomeric sugars (Mosier et al., 2005). Goals of an effective pretreatment process are (i) formation of sugars directly or subsequently by hydrolysis (ii) to avoid loss and/or degradation of sugars formed (iii) to limit formation of inhibitory products (iv) to reduce energy demands and (v) to minimize costs.

Physical, chemical, physicochemical and biological treatments are the four fundamental types of pretreatment techniques employed (Talebnia et al., 2010; Sanchez & Cardona, 2008; Prasad et al., 2007; Hu & Wen, 2008; Wana et al., 2011). In general a combination of these processes is used in the pretreatment step (Figure 2).

Figure 2. Schematic diagram of the role of pretreatment (Source: Hsu et al., 1980)



Biological Pretreatment

Degradation of the lignocellulosic complex to liberate cellulose can be brought about with the help of microorganisms like brown rot, white rot and soft rot fungi. Biological pretreatment renders the degradation of lignin and hemicelluloses (Sun and Cheng, 2002; Talebnia et al., 2010, Prasad et al., 2007) and white rot fungi seem to be the most effective microorganism. Brown rot attacks cellulose while white and soft rots attack both cellulose and lignin (Prasad et al., 2007). Lignin degradation by white-rot fungi occurs through the action of lignin-degrading enzymes such as peroxidases and laccase (Lee et al., 2007). White-rot fungi are the most effective for biological pretreatment of lignocellulosic materials (Fan et al., 1987). Hatakka et al. (1983) studied the pretreatment of wheat straw by 19 white-rot fungi and found that 35% of the straw was converted to reducing sugars by *Pleurotus ostreatus* in 5 weeks. Similar conversion was obtained in the pretreatment by *Phanerochaete sordid* (Ballesteros et al., 2006) and *Pycnoporus cinnabarinus* (Okano et al., 2005) in 4 weeks. To prevent the loss of cellulose, a cellulase-less mutant of *Sporotrichum pulverulentum* was developed for the degradation of lignin in wood chips (Ander & Eriksson, 1977). Akin et al., (1995) also reported the delignification of bermudagrass by white-rot fungi. The biodegradation of Bermuda grass stems was improved by 29-32% after 6 weeks using *Ceriporiopsis subvermispora* and by 63-77% using *Cyathus*

stercoreus. Lee et al. (2007) studied the effects of biological pretreatment on the Japanese red pine *Pinus densiflora*, after exposure to three white rot fungi: *Ceriporia lacerata*, *Stereum hirsutum*, and *Polyporus brumalis*. This method is safe and energy saving due to less mechanical support (Sun and Cheng, 2002; Talebnia et al., 2010). It needs no chemicals but low hydrolysis rates and low yields impede its implementation (Balat et al., 2008; Hamelinck et al., 2005). Biological pretreatment of bamboo culms with white rot fungi has been performed at a temperature of 25 °C (Zhang et al., 2007).

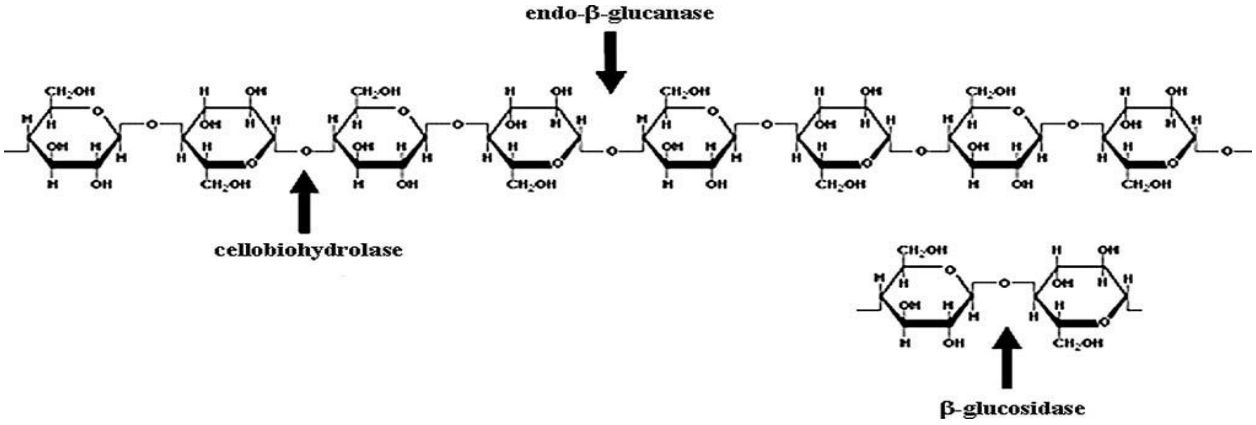
2. Enzymatic Hydrolysis

Saccharification is the critical step for bioethanol production where complex carbohydrates are converted to simple monomers. Compared to acid hydrolysis, enzymatic hydrolysis requires less energy and mild environment conditions (Ferreira et al., 2009). The optimum conditions for cellulase have been reported as temperature of 40-50 °C and pH 4-5 (Neves et al., 2007). Therefore, enzymatic hydrolysis is advantageous because of its low toxicity, low utility cost and low corrosion compared to acid or alkaline hydrolysis (Taherzadeh & Karimi, 2007; Sun & Cheng, 2002). Cellulose hydrolysis is catalyzed by a class of enzymes known as cellulases. These enzymes can be produced by fungi such as *Trichoderma reesei* and *Aspergillus niger* and/or bacteria such as *Clostridium cellulovorans* (Arai et al., 2006). These enzymes are highly substrate specific (Banerjee et al., 2010; Taherzadeh & Karimi, 2007). Cellulase and hemicellulase enzymes cleave the bonds of cellulose and hemicellulose respectively. Hemicellulolytic enzymes are more complex and are a mixture of at least eight enzymes such as endo-1, 4-ß-D-xylanases, exo-1, 4-ß-D xylocuronidases, α-L-arabinofuranosidases, endo-1, 4-ß-D mannanases, ß-mannosidases, acetyl xylan esterases, α-glucuronidases and α-galactosidases (Jorgensen et al., 2003). Cellulose is hydrolysed to glucose whereas hemicellulose gives rise to several pentoses and hexoses. Several species of *Clostridium, Cellulomonas, Thermonospora, Bacillus, Bacteriodes, Ruminococcus, Erwinia, Acetovibrio, Microbispora, Streptomyces* are able to produce cellulase enzyme. Many fungi such as *Trichoderma, Penicillium, Fusarium, Phanerochaete, Humicola, Schizophillum* sp. also have been reported for cellulase production (Sun & Cheng, 2002; Rabinovich et al., 2002).

Among the various cellulolytic microbial strains *Trichoderma* is one of the most well studied cellulase and hemicellulase producing fungal strains (Xu et al., 1998). *Trichoderma* is able to produce at least two cellobiohydrolases and five endoglucanases and three endoxylanases (Xu et al., 1998; Sandgren et al., 2001). On the other hand, Aspergillus is a very efficient ß-glucosidase producer (Taherzadeh & Karimi, 2007). *Trichoderma* cellulase supplemented with extra ß-glucosidase has been studied several times (Krishna et al., 2001; Itoh et al., 2003; Ortega et al., 2001).

Various factors influence yields of monomer sugars from lignocellulose. Temperature, pH and mixing rate are the main factors of enzymatic hydrolysis of lignocellulosic material (Taherzadeh & Karimi, 2007; Olsson et al., 1996). Belkacemi and Hamoudi (2003) studied enzymatic hydrolysis of corn stalk hemicelluloses at 30 °C and pH 5.0. Saccharification was 90% and sugar was released after 10 h.

Figure 3. Molecular structure of cellulose and site of action of endoglucanase, cellobiohydrolase and ß-glucosidase (Source: Kumar et al., 2008)



3. Fermentation using lignocellluloses as substrate

The saccharified biomass is used for fermentation by several microorganisms (Talebnia et al., 2010). Some native or wild type microorganisms used in the fermentation are *Saccharomyces cerevisiae, Escherichia coli, Zymomonas mobilis, Pachysolen tannophilus, Candida shehatae, Pichia stipitis, Candida brassicae, Mucor indicus* (Balat et al., 2008; Talebnia et al., 2010; Sanchez & Cardona, 2008; Bjerre et al., 1996; Sukumaran et al., 2010; Girio et al., 2010; Moniruzzaman, 1995; Nigam J N, 2001). Among all the best known yeast and bacteria employed in ethanol production from hexoses are *Saccharomyces cerevisiae* and *Zymomonas mobilis* respectively (Talebnia et al., 2010). Main disadvantage of the native strains of *Saccharomyces cerevisiae* and *Zymomonas mobilis* is their inability to utilize xylose, the main C5 sugar derived from hemicelluloses (Talebnia et al., 2010; Xu et al., 1998). Native organisms such as *Pichia* and *Candida* species can be used in place of *Saccharomyces cerevisiae* and they can utilize xylose but their ethanol production rate is at least fivefold lower than that observed with *Saccharomyces cerevisiae* (Xu et al., 1998). Genetic engineering has been employed to develop the various aspects of fermentation from higher yield to better and wide substrate utilization to increased recovery rates. A number of genetically modified microorganisms such as *Pichia stipitis* BCC15191 (Buaban et al., 2010), *Pichia stipitis* NRRLY-7124 (Moniruzzaman, 1995; Nigam J N, 2001), recombinant *Escherichia coli* KO11 (Takahashi et al., 2000), *Candida shehatae* NCL-3501 (Abbi et al., 1996), *Saccharomyces cerevisiae* ATCC 26603 (Moniruzzaman, 1995) have been developed. Strict anaerobic hemophilic bacteria such as *Clostridium* sp. and *Thermoanaerobacter* sp. have been proposed (Sanchez & Cardona, 2008; Talebnia et al., 2010).

MATERIALS AND METHODS

1. Sample collection and processing

Sample collection

Leaf litter biomass from different trees (Table 2) was collected from different places of Thapar University, Patiala, Punjab (India).

Table 2. Sources of leaf litter biomass

Sr. No.	Tree	
	Common Name	**Botanical Name**
1.	Jamun	*Syzygium cumini*
2.	Poplar	*Populus alba*
3.	Eucalyptus	*Eucalyptus tereticornis*
4.	Ashoka	*Saraca asoca*
5.	Mango	*Mangifera indica*
6.	Bamboo	Bambus indica

Processing

Leaf litter biomass was washed thoroughly to remove adhering debris and then dried. It was then grinded using mechanical blender and sieved to a mesh size of 2 mm. Equal amount of leaf litters of different tree species by weight were taken and mixed biomass samples were stored in air tight containers so as to avoid moisture and used for further characterization and degradation study.

2. Characterization of untreated leaf litter biomass

Untreated leaf litter biomass was characterized for cellulose, hemicelluloses, lignin and reducing sugars.

Estimation of cellulose in leaf litter biomass

Estimation of cellulose in untreated mixed leaf litter biomass was done by Anthrone assay (Updegraff, 1969).

Materials

a. Acetic/Nitric reagent: Mix 150 ml of 80% acetic acid and 15 ml of concentrated nitric acid.
b. Anthrone reagent: Dissolve 200 mg anthrone in 100 ml concentrated sulfuric acid.
c. Sulfuric acid (67%).
d. Stock solution of cellulose: 10 mg/ml.

Method

a. Acetic/Nitric reagent (5 ml) was added to 0.1 g of the sample in a test tube and was mixed by using a vortex mixer.
b. The test tubes were placed in a water-bath at 100 $^{\circ}$C for 30 min.
c. The contents were then cooled and centrifuged at 10,000 rpm for 15 min.
d. The supernatant was discarded and residue was washed with distilled water.
e. Ten ml of sulfuric acid (67%) was added to it and it was allowed to stand for 1 h.
f. The above solution was then diluted 100 times by using distilled water.
g. In 200 µl of this diluted solution, 2 ml of anthrone reagent was added.
h. The tubes were then heated in a boiling water-bath for 10 min.
i. The contents were cooled and OD was measured at 630 nm.
j. The standard graph was prepared by taking different concentrations of cellulose and cellulose in unknown sample was then calculated.

Table 3. Standard curve of cellulose

Cellulose (mg/ml)	**Stock (µl)**	**DW (µl)**	67% H_2SO_4 (10 ml) was added	Diluted 100 times and 200 µl was taken for the next step	Anthrone Reagent (2 ml)	Boiled for 10 min	OD was taken at 630 nm
0	0	1000					
2	200	800					
4	400	600					
6	600	400					
8	800	200					
10	1000	0					

Estimation of hemicelluloses in leaf litter biomass

Hemicelluloses in untreated leaf litter biomass were estimated by a method developed by Goerging and Van soest (1979).

Materials

a. Biomass samples
b. Di-sodium ethylene diamine tetraacetate
c. Sodium borate decahydrate
d. Sodium lauryl sulphate
e. 2-ethoxy ethanol
f. Di-sodium hydrogen phosphate
g. Decahydronaphthalene (Decalin)
h. Sodium sulphite
i. Sulphuric acid (1N)
j. Cetyl trimethyl ammonium bromide (CTAB)

Neutral Detergent Fibre (NDF)

Preparation of neutral detergent solution:

a. Distilled water (200 ml) was taken in a beaker and then disodium ethylenediamine tetraacetate (18.61 g) and sodium borate decahydrate (6.81 g) were added and dissolved by intermittent heating.
b. To this 100 ml of a solution containing 30 g of sodium lauryl sulphate and 10 ml of 2-ethoxy ethanol was added.
c. To this about 100 ml of a solution containing 4.5 g of disodium hydrogen phosphate was added.
d. Then the volume was made upto one litre and pH was adjusted to 7.0.

Method

a. Biomass sample (0.5 g) was taken in a refluxing flask. Cold neutral detergent solution (100 ml), 2 ml of decahydronaphthalene (Decalin) and 0.5 g of sodium sulphite were added.
b. The mixture was heated to boiling.
c. Then the heat was reduced to avoid foaming and refluxed for one hour.
d. After cooling, the sample was filtered through a previously weighed gooch crucible of G-1 grade under suction using a vacuum pump. The residue remained in the gooch crucible was washed with hot water repeatedly.
e. Finally the residue was given two washings of acetone. The crucible containing residue was dried at 100 $^{\circ}$C for 8 h in hot air oven.
f. Then it was cooled in a desiccator and the dry weight was recorded.

Acid Detergent Fibre (ADF)

Preparation of acid detergent solution:

In one litre of 1N sulphuric acid, 20 g of cetyl trimethyl ammonium bromide was dissolved.

Method

a. Biomass sample (0.5 g) was transferred to a refluxing flask.
b. To this 100 ml of acid detergent solution and 2 ml of decahydronaphthalene were added.
c. This mixture was heated to boiling and the heat was reduced to avoid foaming and refluxed for one hour.
d. After one hour of refluxing, the mixture was cooled and filtered through a previously weighed gooch crucible of G-1 grade under suction using a vacuum pump.
e. The sample in the crucible was washed with hot water to remove acid followed by two washings with acetone.
f. The crucibles were dried at 100 $^{\circ}$C for 8 h in hot air oven. After 8 h, the crucibles were cooled in a desiccator and dry weight was recorded.

Calculations:

$$\text{NDF (\%)} = \frac{Y-X}{W} * 100$$

Where,

Y: Weight of crucible + NDF.

X: Weight of empty crucible.

W: Weight of the sample.

$$\text{ADF (\%)} = \frac{Y-X}{W} * 100$$

Where,

Y: Weight of crucible + NDF.

X: Weight of empty crucible.

W: Weight of the sample

Hemicelluloses (%) = NDF (%) – ADF (%)

Estimation of lignin in leaf litter biomass

Untreated leaf litter was also characterized for its lignin content as per the method developed by Sluiter et al., 2012.

Materials

a. Sulfuric Acid (72% w/w)
b. Calcium Carbonate
c. Biomass samples

Procedure

a. An appropriate number of filtering crucibles were placed in the muffle furnace at 575 ± 25 °C for a minimum of four hours. These crucibles were directly placed in the desiccators after removing from the furnace and cooled down for a specific period of time.
b. Crucibles were weighed and weight was recorded.
c. Weighed 300 ± 10 mg of the biomass sample into tared test tube. This weight was recorded as W_1.
d. Run all the samples and standards in duplicate, at minimum.
e. Added 3 ± 0.01 ml of 72% H_2SO_4 to each test tube. Glass rod was used to mix it for about one minute, until the sample was thoroughly mixed.
f. The test tubes were then placed in water bath at 30 ± 3 ^{0}C for 60 ± 5 min. Using the stirring rod, each sample was stirred after every 5-10 min without removing them from water bath. Stirring is essential to ensure even acid to particle contact and uniform hydrolysis.
g. Upon completion of the 60 min hydrolysis, test tubes were removed from the water bath. Diluted the acid to 4% concentration by adding 84 ± 0.04 ml of distilled water.
h. Sample was mixed by inverting the tubes to eliminate phase separation between high and low acid concentrations.
i. Sealed samples and sugar recovery standards were autoclaved at 121 $^{\circ}$C for one h. After completion of the autoclave cycle, the hydrolysates were allowed to slowly cool to room temperature before removing the caps.
j. The samples were vaccum filtered and filtrate was used for further.
k. On a UV-Visible spectrophotometer, run a background of distilled water or 4% H_2SO_4.
l. The absorbance of hydrolysis liquor was measured at 205 nm.
m. The samples were diluted so as to fall between OD 0.7-1.0 if needed.
n. The blank should always be diluted in the same ratio.

Calculations:

Amount of lignin estimated (g/l) = $\frac{A}{b*a} * df$

Where,

A: Absorbance of the sample

b: Path-length

a: Absorptivity (110 l/g-cm)

df: Dilution Factor

Estimation of reducing sugars

Reducing sugars in the leaf litter biomass were estimated using DNS (3, 5-dintrosalicyclic acid) method as per Miller (1959).

Materials

a. Stock: 1 mg/ml glucose
b. DNS reagent (Appendix I)

Method

a. Different concentrations of glucose were prepared ranging from 0.2 – 1 mg/ml in Potassium Phosphate buffer pH - 7.0 (Appendix III) and volume was made to 1 ml using distilled water.
b. Then 3 ml of DNS reagent was added and placed in boiling water bath for 10 min.
c. The solution was cooled down to room temperature and absorbance was taken at 540 nm.
d. A standard graph was plotted using stock solution.
e. Leaf litter biomass (20 mg) was taken in a test tube containing 50 mM potassium buffer pH - 7.0 (Appendix III) and volume was made to 1 ml using distilled water.
f. Then, same procedure was followed as for standard curve.

Table 4. Standard curve of glucose for reducing sugars

Glucose (mg/ml)	Stock Volume (µl)	Distilled Water (µl)	Phosphate Buffer (µl)	DNS (ml)		
0	0	500	500	3	Boiled for 5 min	OD was taken at 540 nm
0.2	100	400	500	3		
0.4	200	300	500	3		
0.6	300	200	500	3		
0.8	400	100	500	3		
1.0	500	0	500	3		

3. Bacterial strain

***Bacillus licheniformis* T9**

A cellulose degrading bacterial strain (isolated and characterized in the same laboratory) was screened for CMCase activity and then it was used for degradation of untreated leaf litter biomass.

CMCase activity in cell free supernatant

Cellulase activity was determined by estimating the amount of reducing sugar using DNS (3, 5-Dinitrosalicyclic acid) method as per Miller (1959).

Materials

a. Stock: 2 mg/ml glucose.
b. DNS reagent (Appendix I).

Method

a. The overnight grown culture (OD = 1.0) of *Bacillus licheniformis* T9 (1%) was inoculated in flask containing media (Appendix II) supplemented with 0.5% CMC and incubated at 37°C under shaking condition (120 rpm).
b. After each 4 h of incubation up to 28 h, sample was withdrawn and centrifuged in cooling centrifuge (4 °C) at 10,000 rpm for 10 min.
c. Cell free supernatant (500 µl) was added in a test tube containing CMC (500 µl) dissolved in 50 mM potassium phosphate buffer pH 7.0 (Appendix III).
d. Test tubes were incubated in water bath at 50°C for 10 min.
e. Then 3 ml of DNS reagent (Appendix I) was added and placed in boiling water bath for 10 min.
f. Test tubes were cooled at room temperature and then OD was taken at 540 nm.
g. A standard graph was drawn and concentration of sugar was calculated.

$$\text{CMC} = \frac{0.185}{\text{Critical enzyme concentration}} \text{ U/ml}$$

Table 5. Standard curve of glucose for enzyme activity

Glucose (mg/ml)	Distilled water (µl)	Stock Volume (µl)	DNS (ml)	Boiled for 10 minutes	OD taken at 540 nm
0	1000	0	3		
0.4	800	200	3		
0.8	600	400	3		
1.2	400	600	3		
1.6	200	800	3		
2.0	0	1000	3		

Growth curve of *Bacillus licheniformis* T9

Growth curve of *Bacillus licheniformis* T9 was drawn by taking OD of the sample at 600 nm at an interval of every four hours viz. 0, 4, 8, 12, 16, 20, 24, 28 h.

Method

a. Flasks containing CMC media (Appenix II) were inoculated with 2% of *Bacillus licheniformis* T9.
b. These flasks were incubated at 37 °C under shaking conditions (120 rpm).
c. Sample was taken out from the flask at an interval of every four hours viz. 0, 4, 8, 12, 16, 20, 24, 28 h till 28 hours.
d. OD of the culture was taken at 600 nm.

Protein estimation of *Bacillus licheniformis* T9

Protein estimation of *Bacillus licheniformis* T9 was done using the method of Lowry et al. (1951). A standard curve of BSA fraction V was drawn and using the standard curve protein content of the samples taken at an interval of every four hours viz. 0, 4, 8, 12, 16, 20, 24, 28 h was estimated.

Materials

a. Stock of BSA: 1 mg/ml
b. Reagent A: 2 g of sodium carbonate + 0.4 g of sodium hydroxide in 100 ml distilled water
c. Reagent B1: 0.1 g of copper sulphate in 10 ml distilled water
d. Reagent B2: 0.2 g of sodium potassium tartarate in 10 ml distilled water
e. Solution C:
   1 ml of reagent B1
   1 ml of reagent B2
   100 ml of reagent A
   Mix in the above order.
f. Solution E: Folin-Ciocalteau reagent (2 N)
   (Diluted to 1 N for use by adding equal amount of reagent and water).

Method

a. Flasks containing CMC media (Appenix II) were inoculated with 2% of Bacillus licheniformis T9.
b. These flasks were incubated at 37 °C under shaking conditions (120 rpm).
c. Sample was taken out from the flask at an interval of every four hours viz. 0, 4, 8, 12, 16, 20, 24, 28 h.
d. Two ml of sample was taken and centrifuged in a cooling centrifuge at 10,000 rpm for 5 min.
e. From the supernatant solution, 10 µl of sample was taken and volume was made upto 200 µl with distilled water.
f. To the above sample, 1 ml of solution C was added and mixed well.
g. Allowed to stand for 10 min.
h. To the above mixture, 200 µl of solution E was added and mixed well.
i. The above solution was incubated at room temperature in the dark for 30 min.
j. OD was taken at 695 nm.
k. A standard curve of BSA was plotted and amount of protein in the sample was calculated.

Table 6. Standard curve of BSA fraction V

BSA (mg/ml)	**Stock Volume (µl)**	**DW (µl)**	**Solution C (ml)**	Allowed to stand for 10 min.	**Solution E (µl)**	Incubated at room temperature in dark for 30 min.	OD was taken at 695 nm
0	0	200	1		200		
0.1	20	180	1		200		
0.2	40	160	1		200		
0.4	80	120	1		200		
0.6	120	80	1		200		
0.8	160	40	1		200		
1	200	0	1		200		

Effect of pH on CMCase activity of *Bacillus licheniformis* T9

Effect of pH on CMCase activity of *Bacillus licheniformis* T9 was determined by using buffers of different pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 (Appendix III). The reaction mixture of various pH buffers were incubated for 30 min at 50°C. The enzyme assays were carried out by determining the amount of reducing sugars released as described above.

Table 7. Different buffers used to check the effect of pH on CMCase activity

pH	50 mM buffer
4.0	Sodium citrate
5.0	Sodium citrate
6.0	Sodium citrate
7.0	Potassium phosphate
8.0	Glycine-NaOH
9.0	Glycine-NaOH
10.0	Glycine-NaOH

Effect of temperature on CMCase activity of *Bacillus licheniformis* T9

The optimum temperature for CMCase activity of *Bacillus licheniformis* T9 was determined by assaying the enzyme at various temperatures ranging between 30°C to 80°C in reaction mixture containing 2% CMC dissolved in 50mM potassium phosphate buffer pH - 7.0 (Appendix III) with incubation time of 30 min and the amount of reducing sugar was determined as described above.

4. Biodegradation of Leaf Litter Biomass

Untreated leaf litter biomass (1%) was taken and degraded by *Bacillus licheniformis* T9 (2%). Degradation pattern was checked for a period of eight days using five different treatments by varying the carbon source and media components.

Procedure

a. A loopful culture of *Bacillus licheniformis* T9 was added in a test tube containing nutrient broth (5 ml).
b. Culture was incubated at 37 °C under shaking condition (120 rpm).
c. Then 2% of overnight grown culture (OD = 1.0) was transferred to a 100 ml nutrient broth.
d. The culture was again incubated at 37 °C under shaking condition (120 rpm).
e. Three different types of media viz. Minimal media (Appendix II), CMC media (Appendix II) and Nutrient broth (pH 7.0) were prepared.
f. Experimental setup was, using a final volume of 25 ml in 150 ml flask.
g. Five different treatments were given to the leaf litter biomass and experiment was done in 5 sets of such treatments.

Table 8. Degradation of leaf litter biomass in different treatments

S. No.	Treatment
1	Mixed LLB (1%) + Distilled water (Control)
2	Mixed LLB (1%) + Distilled water + T9
3	Mixed LLB (1%) + Minimal Media (Appendix II) + T9
4	Mixed LLB (1%) + Nutrient Broth + T9
5	Mixed LLB (1%) + CMC Media (Appendix II) + T9

MLLB – Mixed leaf litter biomass, T9 – Bacterial strain

Figure 4. Biodegradation of leaf litter biomass in five different treatments

h. Overnight grown bacterial culture (OD =1) was added (2 %) to all twenty flask and then incubated at 50 °C under shaking condition (120 rpm).

Reducing sugar estimation

Sugar release in the culture suspension was estimated using DNS method as described earlier.

a. Samples were withdrawn from each flask after a regular interval of 24 h till 8 days.
b. These samples were subjected to membrane filtration (0.45 µm) and then used estimated for sugar.

Cellulose estimation

a. After 48 h, out of five sets of experiment, one set is taken out from incubation and the whole culture along with suspended leaf litter biomasses were filtered using Whatman filter No. 1, separately.
b. The residual cellulose of leaf litter biomass was estimated using Anthrone assay described earlier.

RESULTS AND DISCUSSION

Leaf litter biomass was processed and sieved through a mesh size of 2 mm. Untreated mixed leaf litter biomass in powdered form was characterized for cellulose, hemicelluloses, lignin and reducing sugars. *Bacillus licheniformis* T9 was checked for CMCase activity. A growth curve of the strain was plotted and protein estimation was done at definite intervals. Effect of pH and temperature was seen on the same strain by varying the buffers and temperature of incubation. *Bacillus licheniformis* T9 was used for biodegradation of native leaf litter biomass and the concentration of cellulose and reducing sugars was checked before and after biodegradation.

1. Characterization of untreated leaf litter biomass

Cellulose content of untreated mixed leaf litter biomass was checked by the Anthrone assay and it was found to be 17.11 ± 0.22 mg/100 mg of biomass. Similarly, hemicelluloses content of biomass was checked by a method developed by Goering and Van Soest (1979) and were found to be 4.55 ± 0.49 g/100 g of biomass. Lignin was estimated by the method of Sluiter et al., 2012 and was found to be 8.67 ± 0.07 g/100 g of biomass.

Table 9. Standard curve of cellulose using different concentrations

Cellulose (mg/ml)	Stock (µl)	DW (µl)	67% H_2SO_4 (ml)	Anthrone Reagent (ml)	OD (630 nm)
0	0	1000	10	2	0
2	200	800	10	2	0.053
4	400	600	10	2	0.104
6	600	400	10	2	0.141
8	800	200	10	2	0.198
10	1000	0	10	2	0.229

Figure 5. Standard curve of cellulose

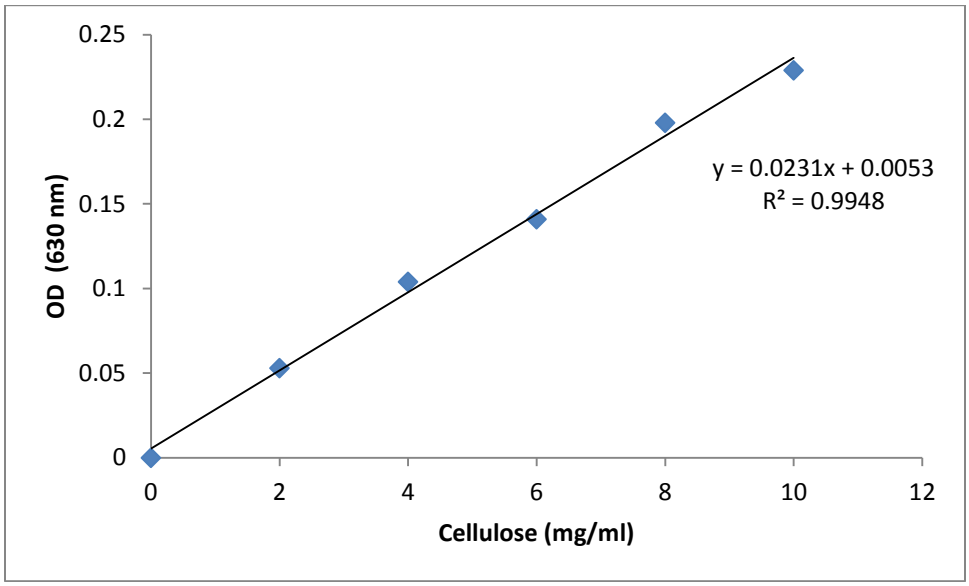


Table 10. Estimation of cellulose, hemicelluloses and lignin in untreated leaf litter biomass

Sample	Mixed Leaf Litter R 1	Mixed Leaf Litter R 2	Mean ± SD
Cellulose (mg/100 mg of biomass)	17.27	16.96	17.11 ± 0.22
Hemicelluloses %	4.2	4.9	4.55 ± 0.49
Lignin (g/100 g of biomass)	8.69	8.58	8.67 ±0.07

Estimation of Reducing Sugars

By the standard curve, reducing sugars were estimated in mixed leaf litter biomass and found to be 5.02 ± 0.19 mg/100 mg of biomass.

Table 11. Standard curve of glucose for reducing sugars

Stock Conc. (mg/ml)	Stock volume (µl)	DW (µl)	Pot. Phosphate Buffer (µl)	DNS (ml)	OD (540nm)
0	0	500	500	3	0
0.2	100	400	500	3	0.069
0.4	200	300	500	3	0.202
0.6	300	200	500	3	0.313
0.8	400	100	500	3	0.468
1	500	0	500	3	0.656

Figure 6. Standard curve of glucose for reducing sugars

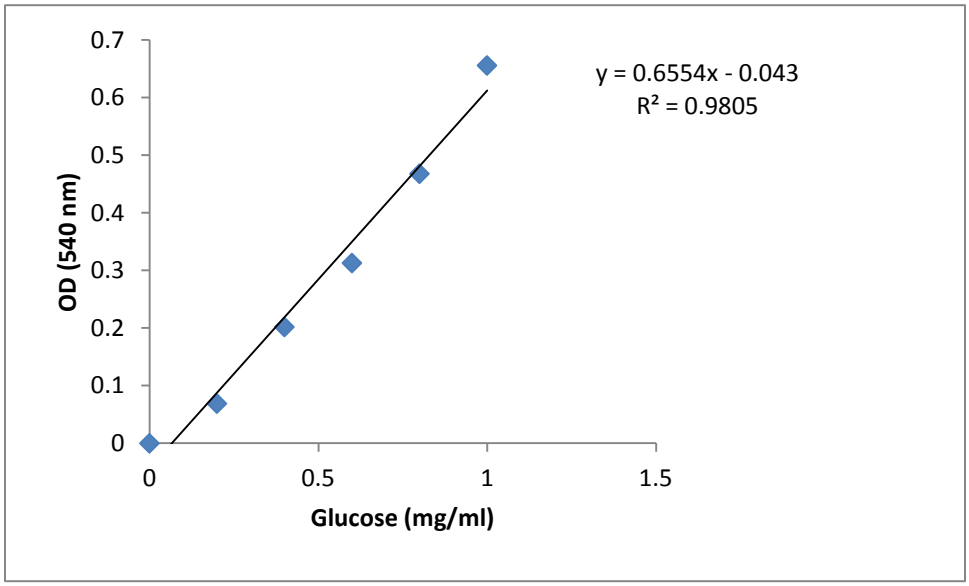


Table 12. Reducing sugars estimation in untreated leaf litter biomass

Sample	Amount (mg)	DW (µl)	Pot. Phosphate Buffer (µl)	DNS (ml)	OD (540 nm)	Amount of sugars in mg/100 mg of biomass
Blank	20	500	500	3	0	0
Mixed 1	20	500	500	3	0.632	5.16
Mixed 2	20	500	500	3	0.596	4.88

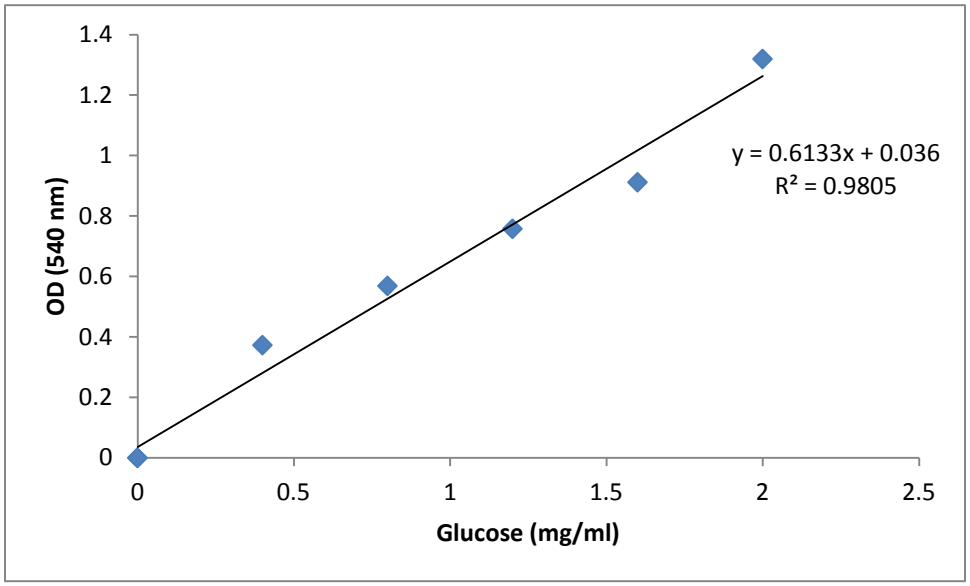
2. Cellulase activity in cell free supernatant of *Bacillus licheniformis* T9

A standard curve of glucose was drawn for checking cellulase activity in cell free supernatant of *Bacillus licheniformis* T9. Cellulase activity in cell free supernatant was 0.256 U/ml.

Table 13. Standard curve of glucose for CMCase activity

Glucose (mg/ml)	Stock Volume (µl)	DW (µl)	DNS (ml)	OD (540 nm)
0	0	1000	3	0
0.4	200	800	3	0.373
0.8	400	600	3	0.569
1.2	600	400	3	0.758
1.6	800	200	3	0.912
2.0	1000	0	3	1.32

Figure 7. Standard curve of glucose for enzyme activity



Enzyme Activity in *Bacillus licheniformis* T9 cell free supernatant is 0.256 U/ml.

Growth curve and protein estimation

Bacillus licheniformis T9 was inoculated in a media containing 0.5% CMC. The culture was incubated at 37 °C under shaking conditions (120 rpm). After an interval of every four hours viz. 4, 8, 12, 16, 20, 24, 28 h, absorbance of the culture was recorded and protein estimation was done by using standard curve of BSA fraction V made by Lowry Method as described earlier.

Table 14. Absorbance of *Bacillus licheniformis* T9 at different time of incubation

Time (h)	OD (600 nm)
0	0
4	0.21
8	0.353
12	0.461
16	**0.532**
20	0.5
24	0.49
28	0.45

Bacillus licheniformis had a log phase upto 16 h since there was an increase in absorbance at 600 nm and also the protein content. Thereafter, there was almost stationary phase (Table 14, Figure 9). Fang et al., (2013) also found the log phase of growth of *Bacillus licheniformis* between 6 and 18 h of incubation.

Table 15. Standard curve of BSA fraction V

BSA conc. (mg/ml)	BSA stock (µl)	Distilled Water (µl)	Solution C (ml)	Solution E (µl)	OD (695 nm)
0	0	200	1	200	0
0.1	20	180	1	200	0.23
0.2	40	160	1	200	0.446
0.4	80	120	1	200	0.796
0.6	120	80	1	200	0.98
0.8	160	40	1	200	1.194
1	200	0	1	200	1.479

Figure 8. Standard curve of BSA fraction V

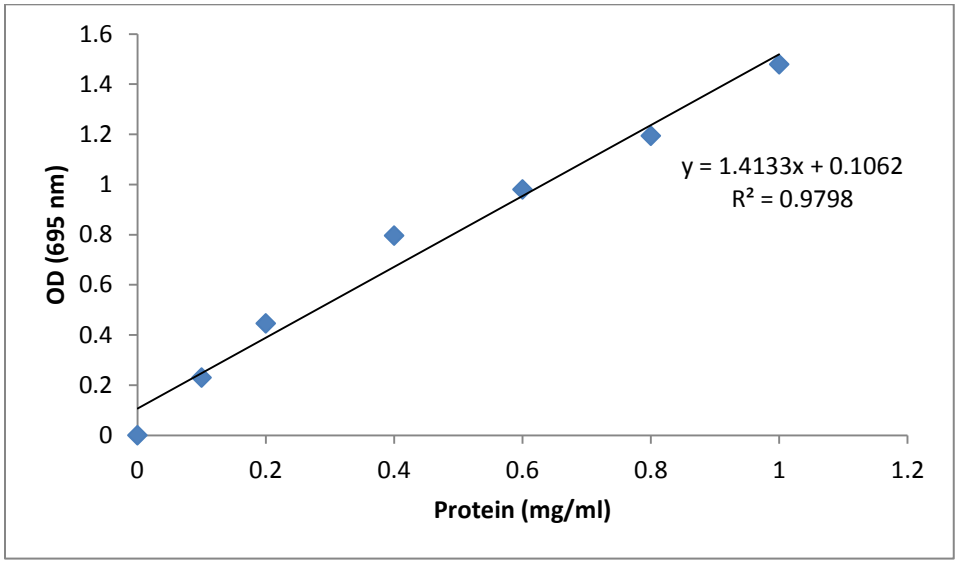
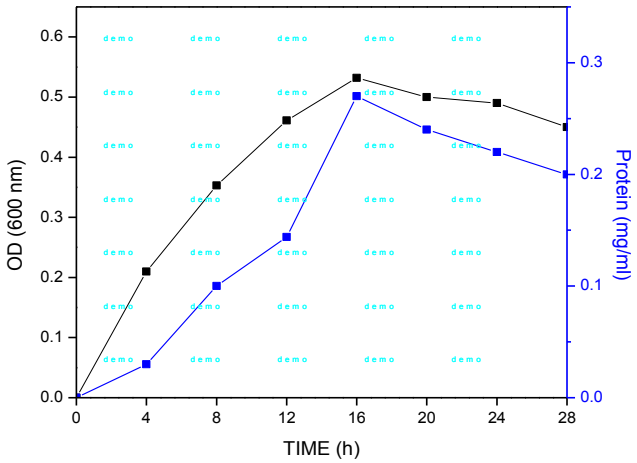


Figure 9. Growth curve and protein concentration of *Bacillus licheniformis* T9



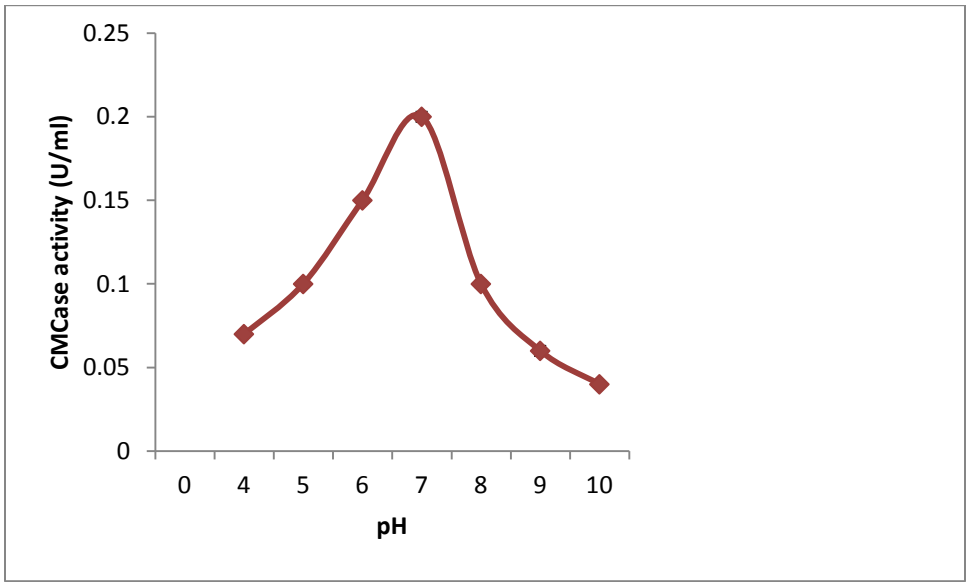
Effect of pH on CMCase activity of *Bacillus licheniformis* T9 in cell free supernatant

Enzyme activity in cell free supernatant of *Bacillus licheniformis* T9 was checked at different pH by using buffers of different pH viz. sodium citrate buffer (pH 4.0,5.0,6.0), potassium phosphate buffer (pH 7.0) and glycine-NaOH buffer (pH 8.0,9.0,10.0). The enzyme activity was found to be maximum at pH 7.0 (Table 16, Figure 10). Fagade and Bambigboye (2012) also found the maximum CMCase activity of *Bacillus licheniformis* at pH 7.0. Akhtar et al., 2012 also found the CMCase activity of *Bacillus* sp. at different pH and it was found optimum at pH 7.0.

Table 16. Effect of different pH on CMCase activity of *Bacillus licheniformis* T9

pH	OD (540 nm)	Enzyme Activity (U/ml)
4.0	0.285	0.07
5.0	0.368	0.1
6.0	0.52	0.15
7.0	**0.771**	**0.2**
8.0	0.336	0.1
9.0	0.239	0.06
10	0.15	0.04

Figure 10. CMCase activity of *Bacillus licheniformis* T9 at different pH



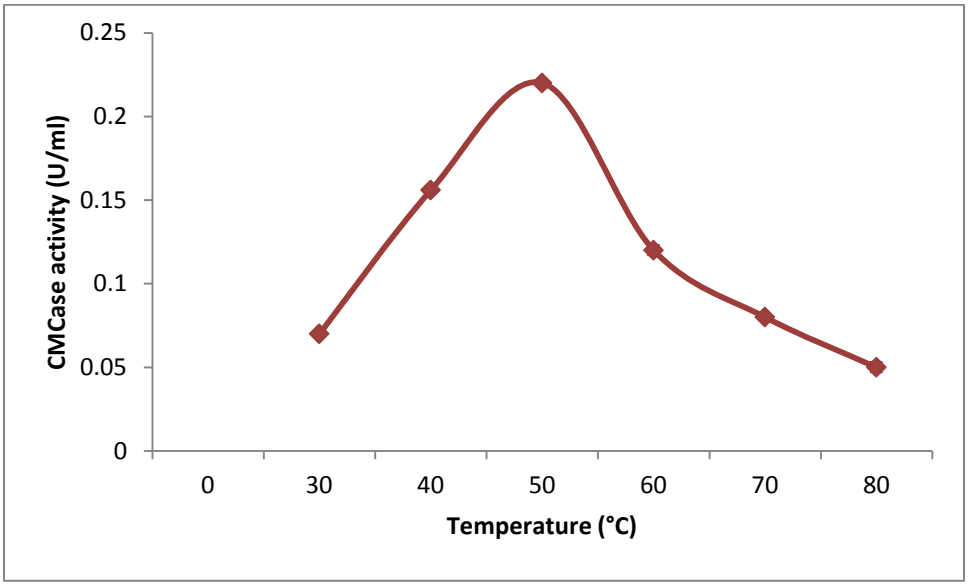
Effect of temperature on CMCase activity of *Bacillus licheniformis* T9 in cell free supernatant

Cellulase activity in cell free supernatant of *Bacillus licheniformis* T9 was checked at different incubation temperatures viz. 30°C, 40°C, 50°C,60°C,70°C,80°C. Temperature was observed to influence CMCase activity significantly. CMCase activity was found to be maximum at a temperature of 50 °C (Table 17, Figure 11). Fagade and Bambigboye (2012) found the optimum temperature for CMCase activity of *Bacillus licheniformis* to be 40 °C and value of CMCase activity to be 0.52 mg/ml. Akhtar et al., 2012 found the CMCase activity maximum at 50 °C.

Table 17. Effect of different temperatures on CMCase activity of *Bacillus licheniformis* T9

Temperature (°C)	OD (540 nm)	Enzyme Activity (U/ml)
30	0.272	0.07
40	0.551	0.156
50	**0.76**	**0.22**
60	0.437	0.12
70	0.306	0.08
80	0.2	0.05

Figure 11. CMCase activity of *Bacillus licheniformis* T9 at different temperatures



3. Biodegradation of Mixed Leaf Litter Biomass by *Bacillus licheniformis* T9

Biodegradation of leaf litter biomass after 8 days showed a decrease in cellulose content. Cellulose was 17.11% which was reduced to 14% forming reducing sugars 5-7%. Maximum reduction was observed in case of leaf litter biomass supplemented with CMC. Akhtar et al., (2012) also observed the degradation of leaf litter biomass by *Bacillus* sp. showing an 8% increase in reducing sugars and 7% decrease in cellulose.

Biodegradation of mixed leaf litter biomass was done by *Bacillus licheniformis* T9 and amount of cellulose and reducing sugars were estimated. Cellulose was degraded by bacteria and converted into sugars. It was maximally degraded in media containing 1% leaf litter biomass supplemented with 0.5% CMC. Nutrient broth and minimal media supplemented with 1% leaf litter biomass showed nearly same pattern in terms of cellulose degradation. Biodegradation of cellulose was also observed even in case of bacteria inoculated in distilled water containing only 1% leaf litter biomass (Table 18, Figure 12).

Reducing sugar was maximally increased in media containing 1% leaf litter biomass supplemented with 0.5% CMC. Bioconversion of leaf litter biomass into reducing sugars in minimal media and nutrient broth was nearly same. Whereas even without minimal media and nutrient media, there was bioconversion of leaf litter biomass by bacteria into reducing sugars as observed when bacteria was inoculated in distilled water containing only 1% leaf litter biomass (Table 18, Figure 13). The result shows that *Bacillus licheniformis* has more preference for CMC as compared to native processed leaf litter biomass. With degradation of cellulose there was increase in reducing sugars which was also maximum when CMC was used in medium. *Bacillus licheniformis* T9 was able to grow and effectively utilize leaf litter biomass as sole carbon source.

Table 18. Biodegradation of leaf litter biomass in different treatments

Days	Treatment	Cellulose (mg/100 mg)	Reducing Sugars (mg/100 mg)
DAY 0	MLLB+WATER	17.11	5.43
	MLLB+WATER+T9	17.31	5.64
	MLLB+MM+T9	17.01	5.21
	MLLB+NB+T9	17.09	5.23
	MLLB+CMC+T9	17.51	5.12
DAY 1	MLLB+WATER	-	5.45
	MLLB+WATER+T9		5.51
	MLLB+MM+T9		5.13
	MLLB+NB+T9		5.1
	MLLB+CMC+T9		4.97
DAY 2	MLLB+WATER	17	5.46
	MLLB+WATER+T9	16.84	5.46
	MLLB+MM+T9	16.88	5.06
	MLLB+NB+T9	16.76	4.95
	MLLB+CMC+T9	16.29	4.71
DAY 3	MLLB+WATER	-	5.5
	MLLB+WATER+T9		5.61
	MLLB+MM+T9		5.33
	MLLB+NB+T9		5.58
	MLLB+CMC+T9		5.59
DAY 4	MLLB+WATER	16.92	5.51
	MLLB+WATER+T9	16.12	5.87
	MLLB+MM+T9	16.12	5.93
	MLLB+NB+T9	16.01	5.93
	MLLB+CMC+T9	15.44	6.12
DAY 5	MLLB+WATER	-	5.52
	MLLB+WATER+T9		5.98
	MLLB+MM+T9		6.49
	MLLB+NB+T9		6.49
	MLLB+CMC+T9		6.86
DAY 6	MLLB+WATER	16.87	5.63
	MLLB+WATER+T9	15.86	6.1
	MLLB+MM+T9	15.52	6.72
	MLLB+NB+T9	15.43	6.79
	MLLB+CMC+T9	14.61	7.12
DAY 7	MLLB+WATER	-	5.65
	MLLB+WATER+T9		6.22
	MLLB+MM+T9		6.9
	MLLB+NB+T9		6.9
	MLLB+CMC+T9		7.43
DAY 8	MLLB+WATER	16.53	5.69
	MLLB+WATER+T9	15.65	6.4
	MLLB+MM+T9	15.04	7.18
	MLLB+NB+T9	15.11	7.18
	MLLB+CMC+T9	14.02	7.84

MLLB-Mixed leaf litter biomass, MM-Minimal media ,CMC-Carboxy methyl cellulose, NB-Nutrient broth,T9-Bacterial strain

Figure 13. Estimation of reducing sugars after biodegradation

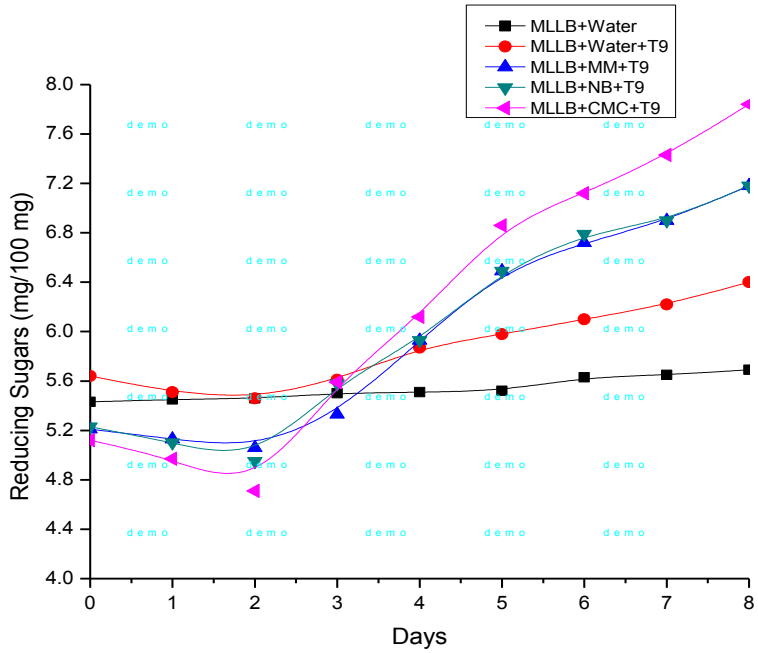
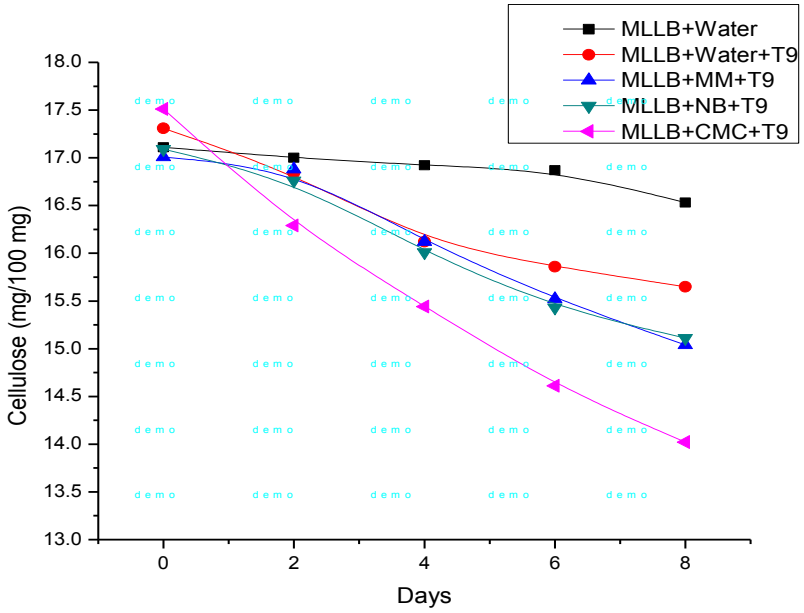


Figure 12. Estimation of cellulose after biodegradation



CONCLUSIONS

1. Native untreated lignocellulosic leaf litter biomass was checked for cellulose, hemicelluloses, lignin and reducing sugars and their percentage in biomass was 17.11%, 4.55 %, 8.67 % and 5.02 % respectively.
2. CMCase activity of *Bacillus licheniformis* T9 was 0.256 U/ml and optimum at pH 7.0 and 50°C.
3. Bacterial strain had a log phase upto 16 h of incubation, was then used for biodegradation of leaf litter biomass in different sets of treatments with either leaf biomass or CMC as sole carbon sources.
4. Cellulose in the leaf litter was slowly degraded which was estimated by Anthrone Assay. The graph clearly shows decreasing cellulose levels with increase in days of incubation. Maximum decrease was found with CMC as carbon source. The result shows that *Bacillus licheniformis* has more preference for CMC as compared to native processed leaf litter biomass.
5. With degradation of cellulose there was increase in reducing sugars which was also maximum when CMC was used in medium.
6. *Bacillus licheniformis* T9 was able to grow and effectively utilize leaf litter biomass as sole carbon source.

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Appendix-I

DNS Reagent Composition (per liter)

Ingredients	Amount
3, 5-dinitrosalicylic acid	10 g
NaOH Solution (0.5 N)	700 ml
Rochelle Salt (Sodium Potassium Tartarate)	300 g
Phenol	7.6 ml
Sodium Metabisulfite	7.6 ml

Appendix-II

Minimal Media Composition

Ingredients	%
Di-Potassium hydrogen phosphate	0.2
Magnesium sulphate pentahydrate	0.05
Ferrous sulphate heptahydrate	0.05

CMC Media Composition

Ingredients	%
Tryptone	0.5
Yeast Extract	0.5
Di-Potassium hydrogen phosphate	0.1
Magnesium sulphate pentahydrate	0.025
Ferrous sulphate heptahydrate	0.025
Carboxy Methyl Cellulose	0.5

Appendix-III

Sodium Citrate buffer (pH 4.0, 5.0 and 6.0)

Stock solutions: A - 0.1 M citric acid, B - 0.1 M sodium citrate

Use x ml of A+ y ml of B and dilute to 100 ml with distilled water.

A (ml)	B (ml)	pH
33.0	17.0	4.0
20.5	29.5	5.0
9.5	41.5	6.0

0.1 M Potassium phosphate buffer (pH 7.0)

Stock solutions: A - 1 M K_2HPO_4, B - 1 M KH_2PO_4

A (ml)	B (ml)	pH
61.5	38.5	7.0

Glycine-NaOH buffer (pH 8.0, 9.0 and 10.0)

Stock solutions: 0.2 M glycine, 0.2 M NaOH

Combine 25 ml of glycine stock with x ml of 0.2 M NaOH and dilute with distilled water to make 100 ml solution.

0.2 M NaOH (ml)	pH
2.0	8.0
6.0	9.0
19.3	10.0