Pretreatment of lignocelluloses biomass and its conversion to ethanol

A

Dissertation submitted

In partial fulfillment of the requirements for the award of the degree of

MASTER OF SCIENCE

IN

MICROBIOLOGY

By

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JULY, 2014

ACKNOWLEDGEMENT

I owe my profound gratitude and sincere regards to my supervisor Dr.Dinesh Goyal, Professor, Department of Biotechnology, Thapar University, who have been a source of inspiration and guiding light for me throughout my dissertation. His careful corrections during regular meetings and discussions always ensured a more thoughtful approach towards problem solving. I am very grateful to him for teaching me things that I would have never found in any textbooks.

I thank Prof Dr. Dinesh Goyal, Head of the Department, for providing the necessary infrastructure and facilities to carry out my work.

I would like to thank all my lab research scholars Mr. Nadeem Akhtar, Ms.Rajinder kaur, Ms.Mahiti Gupta, Mr.Vinit Meshram, Ms. Neha kapoor, Mrs. Taranpreet kaur and M.tech friend Ms. Kanika Gupta, for their constant support and guidance and their valuable suggestions during my course work.

I would like to thank my classmates with whom I share numerous memorable moments which made my M.Sc wonderful and really enjoyable. My heartfelt thanks to Sanjoli, Shilpi, Jaspreet, Pallak, Jyotika and Mani without whom I could not have imagined life at Thapar University. I would also like to thank my all other lab mates and staff members of STEP especially Mr. Pawan for their constant support and encouragement, who never failed to give me a boost, and made my life better in every possible way.

My parents and my sister Dr. Jasmeet kaur deserve special mention for their inseparable support, love and care. I cannot thank them enough for being the source of strength and inspiration all these years.

Lastly, I thank, almighty for giving me the patience to move in the right direction.

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CANDIDATE'S DECLARATION

I hereby declare that the work being presented in the thesis entitled "**Pretreatment of lignocellulosic biomass and its conversion to ethanol"** in the partial fulfillment of requirements for the award of degree of Masters of Science in Microbiology, Department of Biotechnology, Thapar University, Patiala is my own laboratory work during the period of January 2014 to June 2014, under the supervision of Dr. Dinesh Goyal, Head, Professor, Department of Biotechnology (DBT), Thapar University, Patiala.

Date: 18/July/14
Place: Patiala

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CERTIFICATE

This is to certify that the thesis entitled "**Assessment of Pretreatment of lignocellulosic biomass and its conversion to ethanol**" being submitted by Ms Manmeet kaur (Roll no. 301205006) in the partial fulfillment requirements for the award of degree of Masters of Science in Microbiology, The report has not been submitted for the reward of any other degree or certificate in this or any other university or institute.

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Abbreviations

α	Alpha
β	Beta
BSA	Bovine serum albumin
CMC	Carboxymethyl cellulose
CO_2	Carbon dioxide
°C	Degree celsius
DNS	3.5-dinitrosalicylic aci
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	Potassium hydroxide
L	Liter
µl	Microliter
mg	Milligram
min	Minute
mm	Millimeter
mM	Millimolar
$MgSO_4.7H_2O$	Magnesium sulphate pentahydrate
N	Normal
nm	Nanometer
NS	Nutrient solution
MLLB	Mixed leaf litter biomass

ABSTRACT

Mixed leaf litter biomass comprising of *Ashoka, Jamun, Mango, Poplar, Bamboo, Eucalyptus* was collected, dried, processed and characterized for organic carbon, cellulose, hemicelluloses, and total Kjeldahl Nitrogen content and these were found to be 22.5, 17.11, 4.55 and 11% respectively. Reducing sugar content was checked by 3,5- dinitro salicylic acid method and ethanol estimation was done by Di-n butyl phthalate (DBP) method. Maximum amount of sugar 8.68 mg/g of biomass and 4 g/l ethanol were observed in case of acid pretreated biomass samples. Cellulose and hemicellulose were degraded during pretreatment resulting in 32.2% increase in the sugar and 12.6% ethanol production after fermentation with *Saccharomyces cerevisiae* and *Candida shehate*.

Keywords: Lignocellulosic biomass, Cellulose, Leaf litter biomass, Biodegradation.

Chapter 1: Introduction

Bioethanol is one of the most promising fuel from renewable sources. It is used as liquid biofuel for motor vehicles (Demirbas, 2005). Ethanol is considered as cleaning fuel, with no impact on global warming. It can be produced from the inexpensive and abundant lignocelluolsic biomass. Lignocellulose is the most abundant renewable natural resource and substrate available for conversion into fuel. Various crops like sugarcane and corn are preferred for bioethanol production whereas most of the lignocellulosic materials can be used as agricultural wastes for bioethanol production.

Lignocellulosic are building blocks of all plants and are ubiquitous to most regions of our plants. A variety of microorgaanisms and mechanism are involved in the complete biodegradation of

lignocellulose in natural environments ranging from soil and rumen ecosystems to termite hindgut.

Terrestrial plants produces large amount of wood per year, various available agriculture and cellulosic feed stocks are about 180 million tones per year (Lynd et al., 2003). Biomass sources include agricultural residues such as leaves, stalks and cobs, wheat straw, paddy straw, sugarcane bagasse as well as forest products such as hardwoods and softwoods and potential energy crops like switch grass and *Miscanthus* (Gray, 2007).

Raw materials used for ethanol production are sugars (from sugarcane, sugarbeet, molasses and fruits), starch (from corn, cassava, potatoes, root crops) and cellulosics. Food stuffs materials limit their use for ethanol production. So the interest obviously relies on the abundant cellulosics (Lin and Tanaka, 2006).

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 the next with a production level of 5.75 billion litres (Thomas, 2003).

Bioconversion of cellulose to sugars is a prerequisitie for the subsequent production of bioenergy and can be readily fermented to fuel ethanol (Kumar et al., 2008). Biodegradation of cellulose by cellulases and, produced by numerous micro-organisms, represents a major carbon flow from fixed carbon sinks to carbon dioxide atmosphere (Berner, 2003).

Cellulose is often crystalline in the native stage and is surrounded by a mixture of amorphous cellulose, hemicelluloses, and lignin. Because of its structural rigidity, crystalline cellulose is resistant to action of individual cellulases. The commonly described mode of action for cellulosics is exo-and endo-cleavage. Using this classification system, cellobiohydrolases were classified as exo-acting based group, endoglucanases were classified as endo-acting cellulases.

Celluloses degradation occurs in three general steps (Bisaria et al., 1989). Firstly, the long chain polymer is degraded into random lengths of 4 to 6 glucose units by an endoglucanase, which is often reported to the environment outside the cell. Secondly, an exoglucanase cleaves the shortened chains into dimers. In the final step there is cleavage of dimer into beta glucosidase, commonly located in the cell.

Basic step in producing ethanol from cellulose biomass consist of an initial treatment (for example dilute acid, alkaline, steam explosion) to render cellulose more accessible to the step of enzymatic hydrolysis, which breaks down to polysaccharides to produce fermentable sugar. Glucose obtained by the fermentation leads to the production of ethanol by use of *Saccharomyces cerevisiae* which gives high ethanol yield from glucose.

In the present project work, characterization of mixed leaf litter biomass was done and different parameters such as pH and temperature were optimized for ethanol production.

Chapter 2: Review of Literature

Biomass of plant is mainly composed of cellulose, hemicelluloses, lignin along with small amounts of pectin, protein, extractives such as soluble non structural materials and nitrogenous materials (Jorgensen et al., 2007). Main feature of terrestrial plants is the highly developed cell wall, which is complex structure of polysaccharides, aromatic compounds and proteins.

Cellulose

Cellulose fibril is the major component of cellulose cell wall. It is homopolysacchride composed entirely of D-glucose units linked together by β-1,4-glycosidic bonds into a linear structure with higher degree of polymerization, which results into intra-and intermolecular hydrogen bonds resulting in the formation of cellulose microfibrils. Cellobiose is the repeat unit established through this linkage, which constitutes cellulose chains. Hydrogen and vanderwaals bonds are linked with long chain cellulose polymers, which cause the cellulose to be packed in microfibrils, which include hemicellulose and cellulose. The structure of cellulose with its hydrogen bond makes it insoluble in most solvents and is partly responsible for the resistance of cellulose against microbial degradation (Jorgensen, 2003).

Hemicellulose

Hemicellulose consists of short lateral chains of different sugars which differentiate hemicellulose from cellulose. It includes pentoses (xylose, rhamnose and arabinose), hexoses (glucose, mannose and galactose) and uronic acids (D-glucuronic and D-galactouronic acids). Its backbone comprises of homopolymer or hetropolymer with short branches linked by β-1,4-glycosidic bonds and β-1,3-glycosidic bond (Kuhad et al., 1997). Hardwood hemicellulose consist of agricultural raw materials rich in pentose sugars, while softwood hemicellulose only contain minor fractions of pentose sugar D-xylose (Hayn et al., 1993).

Lignin

Lignin is complex polymer of aromatic alcohols known as monolignosis. It is commonly derived from wood, and is an integral part of the secondary cell walls of plants. As a biopolymer, lignin is unusual because of its heterogeneity and is lack of a defined primary structure. Lignin molecule is a polymer with a DP (degree of polymerization) of 450-550, formed by the free radical, oxidative condensation of three monomers, coniferyl alcohol, sinapylalcohol and coumaryl alcohol (Wayman and Parekh, 1990). Its cell wall imparting structural support, impermeability, and resistance against microbial attack (Perez et al., 2002).

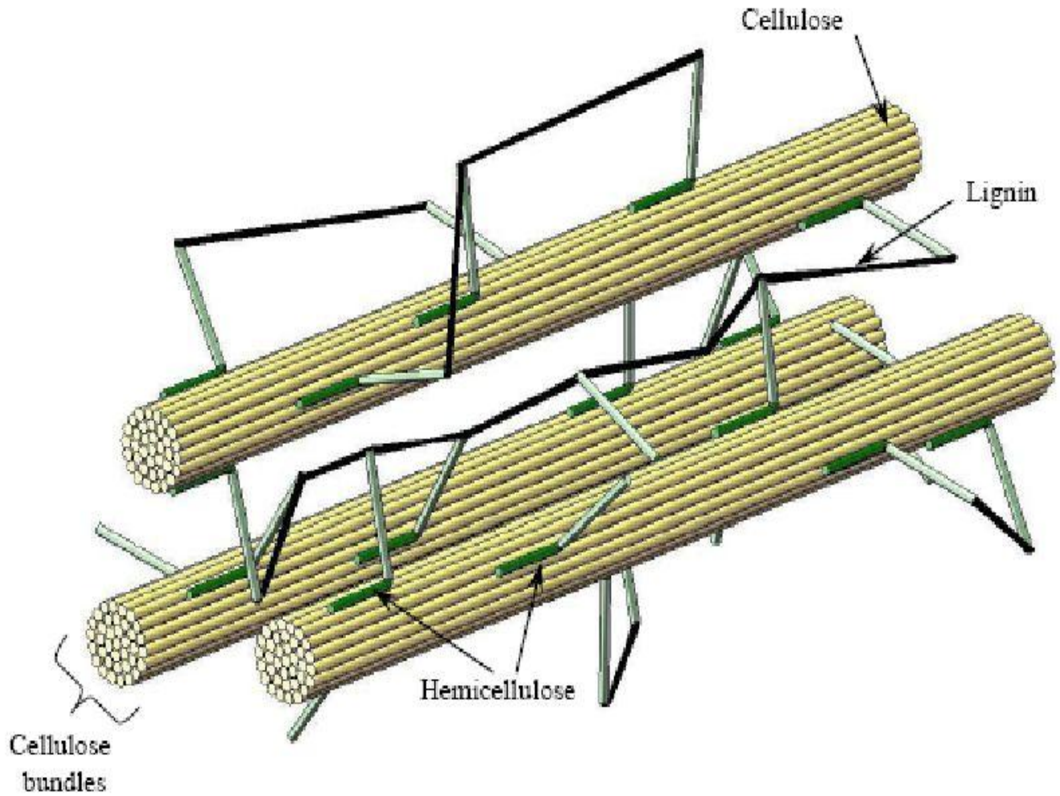

Figure 1 Structure of lignocellulosic biomass (Source: Murphy and McCarthy, 2005; Shaw, 2008)

Lignocellulosic Biomass Degrading Enzymes

Cellulose, hemicellulose and lignin are strongly intermeshed and chemically bonded by non-covalent forces. A variety of fungi and bacteria like cellulolytic micro-organisms belong to eubacteria and fungi, even some of the anaerobic protozoa and slime moulds are also able to degrade cellulose.

Cellulase is a family of three group of enzymes which are generally generally carried out by cellobiohydrolases, exoglucanases EC (3.2.1.91), endoglucanase EC (3.2.1.4), β-glucosidase EC (3 2.1.21) (Kuhad et al., 1997). The endoglucanase randomly attacks the internal O-glycosidic bonds, resulting in glucan chains of different lengths, exoglucanases acts on the ends of cellulose chains and release β-cellobiose as the end product and β-glucosidase acts specifically on the β-cellobiosedissachrides and produce glucose. These three group 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). Some organisms like *Trichoderma* sp. produces all three types of cellulases and efficiently degrades cellulose by their synergistic effect (Okada et al., 1995). Generally Fungi have low potential in cellulose production as compared to Bacteria (Ariffin et al., 2008). Cellulose degradation is generally carried out by the three primary cellulolytic bacterial *Pseudomonas* sp. (Kitamura et al., 2002), *Bacillus subtilis* (Helow et al., 2002), *Bacillus liceniformis* (Malet et al., 1993), and *Bacillus brevis* (Louw et al., 1993).

Lignin degradation is carried out by three group of enzymes; lignin peroxidases, manganese peroxidases, lacasses (Glen et al., 1983; Tien and Kirk, 1983). Hemicelluloses are made up of number of different monnossachrides so its needs various enzymes for degradation such as xylanases, mannanases, galactanases, acetyl xylan esterase etc.

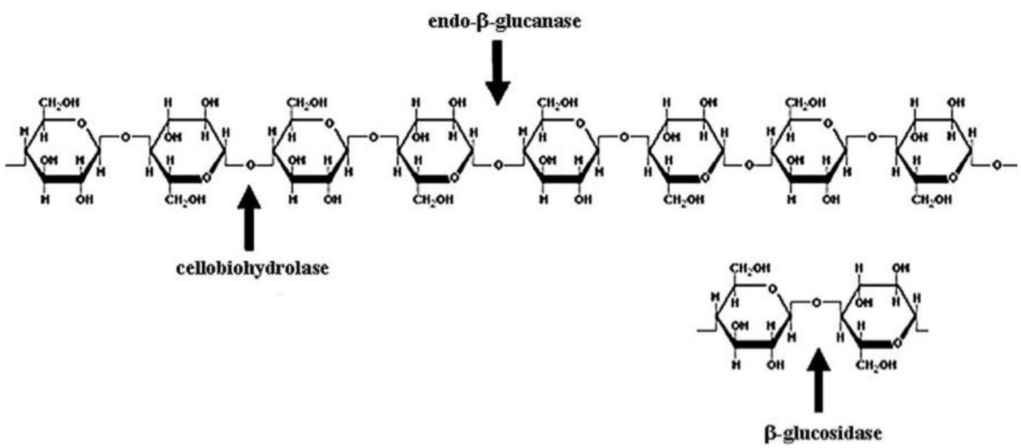

Figure 2 Molecular structure of cellulose and site of action endoglucanase, cellobiohydrolase, and β-glucosidase (Kumar et al., 2008)

Cellulosic substrates

Many different varieties of substrates are used are used as c carbon source for the growth of microrganisms; CMC, filterpaper, Cellobiose etc. CMC is often used as sodium salt, sodium carboxy methyl cellulose, these are cellulose derivative with carboxymethyl group bound to the hydroxyl group of glucopyranose monomers. Functional property of CMC depends on the cellulose.

Filter paper is a semi-permeable paper barrier placed perpendicular to flow of liquid.It comes in various porosities and grades depending on the applications it is meant for.It is used to rid a liquid of solid particles.

Cellobiose is disaccharide consist of two glucose molecules has eight free alcohol groups, one acetal linkage, another one is hemiacetal linkage, which give rise to the strong inter-and intramolecular hydrogen bonds. It is obtained by the enzymatic or acidic hydrolysis of cellulose and cellulose rich materials such as cotton, paper.

Table 1 Micro-organisms involved in bioconversion of lignocellulosic biomass

Bacteria	**Substrate**	**Refrence**
Bacillus sp.	Cellulose	Sun and Cheng, 2002; Rabinovich et al., 2002
Cellulomonas sp.	Cellulose	Sun and Cheng, 2002; Rabinovich et al., 2002
Clostridium sp.	Cellulose	Sun and Cheng, 2002; Rabinovich et al., 2002
Streptomyces sp.	Cellulose	Sun and Cheng, 2002; Rabinovich et al., 2002
Bacteriodes sp.	Cellulose	Sun and Cheng, 2002; Rabinovich et al., 2002
Microbispora sp.	Cellulose	Sun and Cheng, 2002; Rabinovich et al., 2002
Pseudomonas fluorescens	CMC	Bakare et al., 2005
Fungi	**Substrate**	**Refrence**
Trichoderma reesei	Cellulose	Arai et al., 2006
Aspergillus sp.	Cellulose	Arai et al., 2006
Pencillium sp.	Cellulose	Fan et al., 1987; Duff and Murray, 1996; Sternberg, 1976
Phanerochaete chrysosporium	Lignin	Glenn et al., 1983; Tien and Kirk, 1983
Phanerochaete sp.	Cellulose	Fan et al., 1987; Duff and Murray, 1996; Sternberg, 1976
Brown-rot fungi	Cellulose and Lignin	Prasad et al., 2007
Sclerotium rolfsii	Cellulose	Fan et al., 1987; Duff and Murray, 1996; Sternberg, 1976

Biomass Conversion to fuel

In biological ethanol production, lignocellulosics are processed for bioethanol production through three major operations; Pretreatment for delignification, hydrolysis of cellulose and hemicelluloses, feremention of sugars by bacteria and yeast. Micro-organisms and enzymes on biological sources can lead to the production of ethanol. Due to action of these micro-organisms fermentation of sugar, starch, hemicelluloses or cellulose takes place. Pretreatment is generally required to alter the size and structure of biomass as well as its chemical composition.

Hydrolysis plays very important role for the removal of lignin and hemicelluloses, reduction of cellulose crystallinity and increase of porosity through pretreatment processes (McMillan et al., 1994).

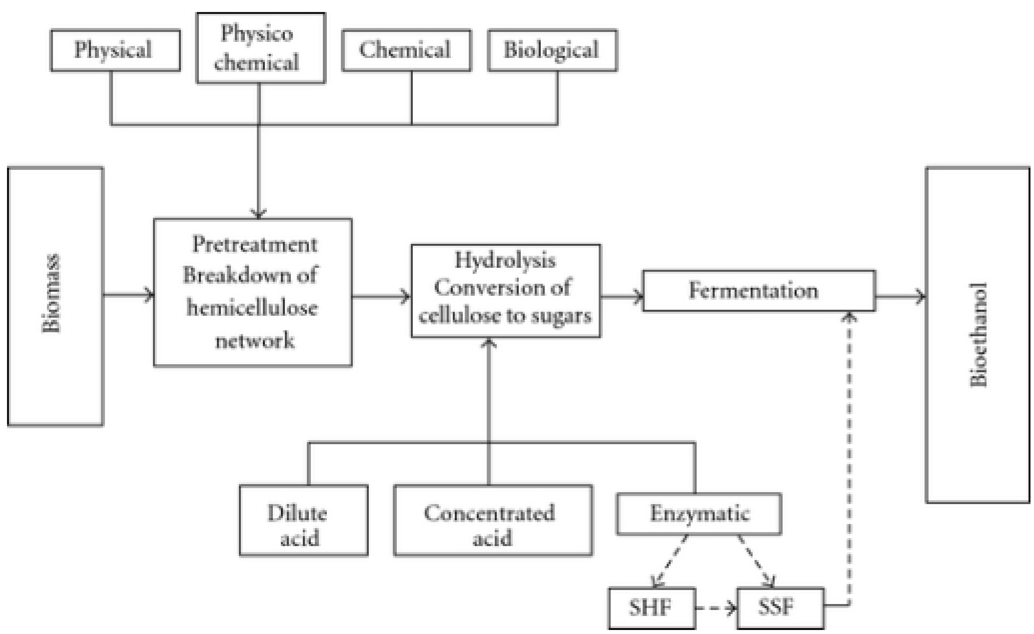

Figure 3 Simplified diagram of cellulosic ethanol production

1. Pretreatment

Pretreatment is usually employed since the biodegradation of untreated lignocellulose has low extent of conversion. This method refers to solubilization and separation of one or more of these components of biomas, which makes the remaining solid biomass more accessible to further chemical or biological treatment (Demirbas, 2005). It is done to break the matrix in order to reduce the degree of crystallinity of the cellulose and increase the fraction of amorphous cellulose (Sanchez and Cardona, 2008). It makes the lignocellulosic biomass susceptible to quick hydrolysis with increased yields of monomeric sugars (Mosier et al., 2005). Objectives of pretreatment are a) formation of sugars directly or hydrolysis b) degradation of sugars formed c) to limit formation of inhibitory products d) to reduce energy demands e) to minimize cost.

After pretreatment ,cellulose components can be converted to constituents monosaccharides by acid hydrolysis or enzymatic hydrolysis is found to require a shorter time than enzymatic sacchrification and pretreatment become unnecessary, however, neutralization and waste disposal increase the cost of production (Schell, Farmer, Newman and McMillan, 2003).

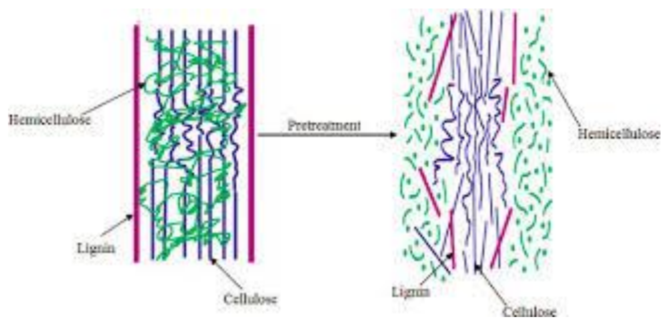

Figure 4 Diagram of role of pretreatment (Mosier et al., 2005)

Pretreatment techniques used were- physical, physicochemical, biological treatments. In general combination of these processes is used in the pretreatment step (Talebnia et al., 2010); (Sanchez and Cardona, 2008); (Prasad et al., 2007); (Hu and Wen, 2008 et al., 2011).

Physical Pretreatment

Physical pretreatment does not use chemical agents, and typically includes uncatalyzed steam explosion, liquid hot water treatment, and high energy radiation. The former two pretreatment methods are common than the later. (Sun and Cheng et al., 2002)

Chemical Pretreatment

Chemical pretreatment has been extensively used in paper industry for the production of lignocellulosic materials to produce high quality paper products. It plays very important role in improving biodegradability of cellulose removing lignin or cellulose and to a lesser degree decreasing the degree of polymerization and crystallainity of cellulose. Common pretreatment techniques include: catalyzed steam explosion, acid, alkaline, ammonia fibre explosion, Ionic liquid pretreatments (Bobleter et al., 1994).

Biological Pretreatment

Biological pretreatment employs wood degrading micro-organisms including white, brown,soft-rot fungi and bacteria. Brown rot attacks cellulose while white-rot fungi occur through the action of lignin-degrading enzymes such as peroxidase and laccase (Lee et al., 2007) whereas most effective for biological pretreatment of lignocellulosic materials are white-rot fungi (Fan et al., 1987). Hatakka et al., in 1983 studied the pretreatment of wheat straw by 19 white-rot fungi and found that 35% of staw was converted to reducing sugars by *Pleurotuso streatis* in five weeks.To prevent the loss of cellulose, cellulose-less mutant of *Sporotrichum. Pullverulentum* was developed for the degradation of lignin in wood-chips (Ander and Eriksson, 1997).

In 2007, Lee et al., studied the effects of biological pretreatment on the Japnese red pine *Pinus densiflora*, exposed to fungi; *Ceriporialacerate, Stereumhirsutum, Polyporusbrumalis.* This method is energy saving due to less mechanical support (Sun and Cheng, 2002; Talebnia et al., 2010).This process does not need any chemicals but low hydrolysis rates and low yields impede its implementation (Balat et al., 2008; Hamelinck et al., 2005). At temperature of 25 biological pretreatment of bamboo culms with white rot fungi has been performed (Zhang et al., 2007).

2. Hydrolysis

For bioethanol production sacchrfication is the critical step where complex carbohydrates are converted to simple monomers. In case of acid and enzymatic hydrolysis it requires less energy and mild environment conditions (Ferreira et al., 2009). Enzymatic hydrolysis is advantageous because of its low oxicity, low utility cost, low corrosion compared to acid or alkaline hydrolysis (Taherzadeh and Kamiri, 2007); Sun and Cheng, 2002). Optimum conditions for cellulose have been reported at temperature of 40-50˚C and pH 4-5 (Neves et al., 2007). Various enzymes are responsible for cellulose hydrolysis known as cellulases. These enzymes can be produced by fungi such as *Trichoderma reesei* and *Aspergillus niger* and bacteria such as *Clostridium cellulovorans* (Arai et al., 2006). These enzymes are highly substrate and specific (Banerje et al., 2010; Taherzadeh and Kamiri, 2007). Mainly cellulases and hemicellulases cleave the bonds respectively. In case of hemicelluloses, enzyme are more complex and are a mixture of at least eight different enzymes such as endo-1,4-β-D-xylanases,exo-1,4-β-Dxylocuronidase, α-L-arabino furanosidases, endo-,1,4-β-Dmannoses, acetylxylan esterases, α-glucouronidases and galacatosidases (Jorgensen et al., 2003).

Several species;-

Clostridium, Cellulomonas, Thermonospora, Bacillus, Bacteriodes, Ruminococcus, Erwinia, Acetovibrio, Microbiospora is able to produce cellulose enzyme. For cellulase production many fungi have been reported such as *Trichoderma, Pencillium, Fusarium, Phanerochaete, Schizophillum* sp. Among various cellulolytic microbial strains *Trichoderma* is able to produce at least two cellobiohydrolases and five endoglucanases and three endoxylanases (Xu et al., 1998; Sandgren et al., 2001). *Aspergillus* is a very efficient β-glucosidase producer (Taherzadeh and Kamiri, 2007).

Various factors influence yields of monomer sugars from lignocelluloses. Temperature, pH and mixing rate are the main factors of enzymatic hydrolysis of lignocellulosic material (Taherzadeh and Kamini, 2007; Olsson et al., 1996).

Acid hydrolysis

Acid pretreatment method was derived from the concentrated acid hydrolysis such as sulphuric acid (36N) and HCl hydrolysis, which had been a major technology for hydrolyzing lignocellulosic biomass for fermentable sugar production (Goldstein et al., 1983). Dilute acid pretreatment has been applied to a wide range of feed stocks, including softwood, hardwood, herbaceous crops, agricultural residues, wastepaper, and municipal solid waste. It performed well on most biomass materials.

Enzymatic Hydrolysis

In enzymatic hydrolysis, cellulose chains can be broken into glucose molecules by cellulase enzymes. This process uses several enzymes at various stages of this conversion. Using a similar enzymatic system, lignocellulosic materials can be enzymatically hydrolyzed at a relatively mild condition (50°C and pH 5), thus enabling effective cellulose breakdown without the formation of byproducts that would otherwise inhibit enzyme activity (Kim et al., 2006).

3. Fermentation

Traditionally, baker's yeast (*Saccharomyces cerevisiae*) has long been used in the brewery industry to produce ethanol from hexoses (six-carbon sugars). Due to the complex nature of carbohydrates present, significant amount of xylose and arabinose (five-carbon sugars derived from the hemicellulose portion of the lignocellulose) is also present in the hydrolysate. For example, in the hydrolysate of corn stover, approximately 30% of the total fermentable sugars is xylose. As a result, the ability of the fermenting microorganisms to use the whole range of sugars available from the hydrolysate is vital to increase the economic competitiveness of cellulosic ethanol and potentially bio based proteins.

The saccharified biomass is used for fermentation by several micro-organisms (Talebnia et al., 2010). Some wild type microorganisms used in the fermentation are *Saccharomyces cerevisiae*, *E.coli*, *Zymomonas*, *Mucorindicus* (Balat et al., 2010; Girio et al., 2010). Among all the bacteria and yeast employed for ethanol production *Saccharomyces cervisiae*, *Zymomonas mobilis* are the best.

Main disadvantage of the native strains 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 *Saccharomyce 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).

A number of genetically modified micro-organisms such as *Pichia stipitis,* BCC1591 (Buban et al., 2010), *Pichia stipitis* NRRLY7124 (Moniruzzaman et al., 1995) *Candida shehate* NCL-3501 (Abbi et al., 1996) have been developed. Some strict anaerobic and hemophilic have been reported like *Clostridium* sp. (Sanchez and Cardona, 2008); Talebnia et al., 2010).

Combined Hydrolysis and Fermentation

Some species of bacteria have been found capable of direct conversion of a cellulose substrate into ethanol. One example is *Clostridium thermocellum*, which uses a complex cellulosome to break down cellulose and synthesize ethanol. However, *C. thermocellum* also produces other products during cellulose metabolism, including acetate and lactate, in addition to ethanol, lowering the efficiency of the process.

Advantages of using this organism;

1. Higher growth rates
2. Higher metabolic activities
3. Thermophilic organism
4. Increased enzyme instability

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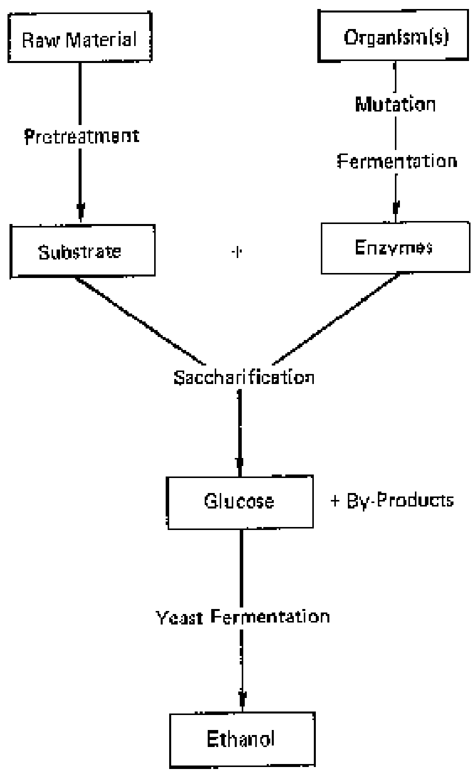

Figure 5 Schematic diagram of microbial fermentation

Chapter 3: Materials and Methods

1. Sample collection and Processing

Mixed leaf litter biomass from different trees such as: Ashoka, jamun, Mango, Poplar, Bamboo, Eucalyptus was Collected from Different places of Thapar University, Patiala Punjab (India).

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 only.

Table 2 Sources of leaf litter biomass

Sr.No.	Common Name	Family	Botanical name
1.	Ashoka	Annonaceae	*Polyathia longifolia*
2.	Jamun	Myrtaceae	*Syzygium cumini*
3.	Mango	Anacardiaceae	*Mangifera indica*
4.	Poplar	Saliacaceae	*Populus deltrides*
5.	Bamboo	Poaceae	*Dendrocalamus indica*
6.	Eucalyptus obliqua	Myrtaceae	*Eucalyptus oblique*

2. Characterization of Leaf Litter Biomass

Estimation of pH

pH was determined as per the method given by Jackson (1967) in a mixed leaf litter biomass water suspension of 1:2 ratio. Mixed leaf litter biomass (1g) was placed in a 100ml beaker and 50ml of distilled water was added and the sample was stirred for 20 min. and kept undisturbed for 30 min. pH was measured using a pH meter after calibration buffers of pH 4.0, 7.0 and 9.2.

Electrical Conductivity

Electrical conductivity was measured in μScm^{-1} as per the method given by Jackson (1967). Mixed leaf litter biomass (1g) was placed in 100 ml beaker and 50 ml of distilled water was added. The Mixed leaf litter biomass–water mixture was allowed to undisturbed for 30 minutes until settled completely. The conductivity meter was calibrated by 0.01 M potassium chloride.

Estimation of Organic Carbon Content by Titration Method (Walkley and Black, 1934)

Reagents:

1. 1N Potassium dichromate solution
2. Conc. sulphuric Acid
3. Orthophosphoric Acid
4. 0.5 N Ferrous Ammonium Sulphate Solution (FAS)
5. Diphenylamine (DPA)
6. Sodium fluoride

Procedure:

1. Took 0.5 g of Mixed leaf flitter biomass sample in a flask and added 10 ml of potassium dichromate (1N)
1. After adding potassium dichromate, added 20 ml of concentrated sulphuric acid to the sample.
2. Kept the sample in a shaker for 30 min. for complete digestion and diluted the sample with 200 ml distilled water.
3. In the next step, added 10 ml conc. orthophosphoric acid and 0.5g sodium fluoride to the sample.
4. In the last step added 1ml of diphenylamine and titrated it with ferrous ammonium sulphate (0.5N).
5. Took the three concordant reading, till colour changes from violet to greeny colour.

Calculations: 10 (B-T) $\times$ 0.003 $\times$ 10/B wt.of sample.

Where B = blank and T = test

Estimation of Cellulose (Updegraff, 1969)

Reagents:

1. Acetic/Nitric reagent
2. Anthrone reagent
3. 67% sulphuric acid-50 ml

Procedure:

1. Added 3 ml Nitric Reagent to a known amount (1 g) of sample in a test tube and mix in a vortexer.
2. Placed tube in a water bath at 100°C for 15-20 min. and cooled, then centrifuged contents for 15-20 min.
3. In the next step, discard supernatant and washed residue with distilled water.
4. Added 10 ml of 67% sulphuric acid and allowed it to stand for 1hr and dilute 1ml of above solution to 100 ml.
5. To 1ml of this diluted solution added 10 ml of anthrone reagent and mixed well.
7. In the last step, cooled the contents and measure absorbance at 630 nm.

Estimation of hemicelluloses (Goering and Soest, 1979)

Reagents:

1. Neutral Detergent Solution
2. Decahydronaphthalene
3. Sodium Sulphite
4. Acetone

Preparation of Reagents:

Neutral detergent Factor (NDF)

1. 18.61 g of disodium ethylene diamine tetracetate was weighed, and 6.81g of sodium borate decahydrate was transferred to beaker.
2. Dissolved about 200 ml distilled water by heating and to this added solution (50 ml) containing 30 g of sodium lauryl sulphate and 10 ml of 20 ethoxy ethanol.
3. In the next step, this added 4.5 g of disodium hydrogen phosphate.
4. Then the volume was made upto 1 L and pH was adjusted to 7.

Procedure:

1. 0.5 g of biomass sample was taken in a refluxing flask, 100 ml of cold detergent solution and 2 ml of decahydronaphthalene and 0.5 g of sodium sulphite were added.
2. The mixture was heated to boiling and then the heat was avoid foaming and refluxed for 1 hr.
3. After cooling, the sample was filtered through a previously weighed gooch crucible of G-I grade under suction using a vaccum pump.The residue remained in gooch crucible was washed with hot water repeatedly.
4. Finally the residue was given two washings of acetone.The crucible containing residue was dried at 100 C for 8 hr in hot air oven.
5. Then it was cooled in a dessicator and the dry weight was recorded.

Acid Detergent Factor (ADF)

Preparation of ADF

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

Procedure:

1. 0.5 g of biomass sample was transferred to a refluxing flask, to this 100 ml of acid detergent solution and decahydronaphthalene were added.
2. This mixture was heated to boiling and the heat was reduced to avoid foaming and refluxed for 1 hr.
3. After refluxing for 1 hr, the mixture was cooled and filtered through a previously weighed gooch crucible of G-I grade under suction using a suction pump.
4. The sample in the crucible was washed with hot water to remove acid followed by two washings with acetone.
5. The crucibles were dried at 100°C for 8 hr in hot air oven, after 8 hr the crucibles were cooled in a dessicator and dry weight was recorded.

Calculations:

$$NDF\ (\%) = y\text{-}x/\ z$$

Where y = weight of crucible +NDF

x = weight of empty crucible

z = weight of sample

$$ADF\ (\%) = y\text{-}x/w\times100$$

Where y = weight of crucible +NDF

x = weight of empty crucible

w = weight of sample

Estimation of Lignin (Sluiter et al., 2012)

Reagents:

1. Sulphuric acid (72% w/w)
2. Calcium Carbonate
3. Biomass samples

Procedure:

1. 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 dessicators after removing from the furnance and cooled down for a specific period of time.
2. Crucibles were weighed and weight was recorded.
3. Weighed 300 ± 10 mg of the biomass sample into tared test tube.This weight was recorded as $W_{1.}$
4. Run all samples and standards in duplicate at minimum.
5. Added 3 ± 0.01 ml of 72 % sulphuric acid to each test tubes. Glass rod was used to mix it for about one min, until the sample was thoroughly mixed.
6. The test tubes were then placed in water bath at 30˚C for 1 h. Using the stirring rod, each sample was stirred after every 5-10 min without removing them from water bath.
7. Upon completion of the 60 min hydrolysis, test tubes were removed from the water bath. The acid was diluted to 4% by adding 84 ± 0.04 ml of distilled water.
8. Sample was mixed by inverting the tubes to eliminate phase separation between high and low acid concentrations.

9. The samples were vacuum filtered and filtrate was used for further.
10. In the last step, OD of samples was taken at 205 nm.

Calculations: Amount of lignin estimated (g/l) = $A/b \times a \times df$

Where, A = absorbance of sample

b = Path length

a = Absorpitivity

df = Dilution factor

Estimation total Kjeldahl nitrogen (Jackson, 1967)

Reagents:

1. Concentrated H_2SO_4
2. 0.02 N H_2SO_4
3. Sulphuric-Salicylic acid: 1g salicylic acid mixed with 30 ml sulphuric acid
4. Sodium thiosulphate
5. 4 % boric acid
6. Mixed indicator. 0.066 g of methyl red and 0.099 g of bromocresol green dissolved in 100 ml of ethyl alcohol.
7. 50% NaOH
8. Digestion mixture: 10 g HgO, 5 g $CuSO_4$ and 100 g K_2SO_4 (2:1:20).

Procedure

1. Mixed leaf litter biomass sample of 0.1 g was mixed thoroughly with sulphuric-salicylic acid and, to this 5 g of sodium thiosulphate was added. Heating was carried out for 5 min followed by cooling and addition of 10 g digestion mixture. The contents were mixed well in a Kjeldahl flask.
2. The flask was kept in the digestion chamber at 100°C for 2 hr.
3. The colour change was monitored from dark brown to greenish white after that the contents were cooled and 300 ml distilled water was added.
4. 20 ml of the digested sample, 15 to 20 ml NaOH and glass beads were added to the distillation flasks through the open end of the condenser attachment and stopped. Water flow was maintained through the condenser.

5. The distillate was collected through a receiver tube in a beaker containing 15 ml boric acid and 2 drops of mixed indicator till the end-point colour changes from pink to green.
6. The distillate was titrated against 0.02 N sulphuric acid until the end point colour changed from green to pink.

Calculations

Total N % = 10 (B-T)$\times$ 0.03 $\times 100/$B$\times$ $wt. of\ sample$

Where T is the titre value for sample and B is for blank

2. Biodegradation of Mixed Leaf Litter Biomass

Untreated and Pretreated Mixed Leaf Litter biomass was taken. Degradation pattern was checked for sugar release in the culture suspension and for estimation of ethanol for a period of ten days using three different treatments by varying the carbon source and media component. This same experiment was conducted at pH 4.5 and 5.5, and at temperature of 25˚C and 35°C.

Inoculum preparation

Yeast extract peptone dextrose YPD broth media was prepared by adding yeast extract of 10 g, peptone 20 g and dextrose 20 g to 1 litre solution. Inoculum of selected fungi was prepared by *Saccharomyces.cerevisiae* and *Candida shehate* was mixed with yeast peptone dextrose broth. A loopful culture of both strains was mixed to YPD broth. Culture was incubated at 28˚C and 37˚C under shaking conditions for two days.

Preparation of Nutrient Solution Media

Nutrient solution media was prepared by adding adding media components: $MgSO_{4}.7H_2O$ of 10 g,$(NH_4)_2.SO_4$ of 20 g and Yeast Extract of 20 g to 1 L solution. 1ml of inoculum prepared culture was trransfered to 50 ml of each twenty flask containing both untreated and pretreated sample.

Experiment Design: Three different treatments was give to mixed leaf litter biomass was done in three sets of such experiment

Table 3. Biodegradation of Mixed Leaf litter Biomass

Sr.No.	Treatments
1	Mixed LLB + NS Media(control)
2	Mixed LLB + *Saccharomyces.cerevisiae*
3	Mixed LLB + *Candida. Shehate*
4	Mixed LLB + *Sacharomyces.cerevisiae* + *Candida.shehate*

Where, Mixed LLB = mixed leaf litter biomass

Estimation of Reducing sugar by 3, 5 dinitrosalicylic (DNS) acid method (Miller, 1959)

Reagents:

1. Sodium potassium tartrate
2. 3,5 – dinitro sakicylic acid solution
3. Stock solution (3 mg/ml)

Procedure:

1. Untreated and pretreated mixed leaf litter samples were withdrawn from each flask after a regular interval of 24 h till 10 days.
2. Samples were centrifuged at 10,000 rpm for 15 min and supernatant was discarded.
3. In the next step 3 ml of DNS Reagent was added to all the test tubes containing untreated and pretreated samples.
4. Plugged the test tubes with cotton and kept in water bath at 80˚C for 5 min.
5. Then allowed the test tubes to cool down at room temperature before taking the reading.
6. After cooling the samples, take the absorbance at 540 nm.
7. The same experiment was conducted for both the samples at pH 4.5 and 5.5 and temperature 25˚C and 35˚C.

Ethanol Estimation by DBP Method (Seo et al., 2009)

Reagents:

1. Ethanol
2. Potassium dichromate:10 g in 100 ml of 5 M Sulphuric acid
3. Di-n butyl phthalate (DBP)

Procedure:

1. Untreated and pretreated mixed leaf litter biomass samples were withdrawn from each flask after a regular interval of 24 h till 10 days.
2. Samples were centrifuged at 10,000 rpm for 15 min. and supernatant was discarded.
3. Different concentrations of ethanol were prepared ranging from 2 to 10% and volume was raised to 2 ml.
4. In the next step, 2 ml DBP was added to all the tubes and the mixture was shaked vigorously for 30 min.
5. After shaking the lower phase (1.5 ml) was transferred to a new microtube.
6. Then 1.5 ml of potassium dichromate was added to the microtube.
7. Again the samples were kept on the shaker for 30 min.
8. In the last step, after shaking OD of lower phase was taken at 595 nm.
9. The same experiment was conducted at pH 5.5 and 4.5 and at temperature of 25˚C and 35°C.

Chapter 4: Results and Discussion

Characterization of Leaf litter Biomass

Mixed leaf litter biomass was processed and sieved through mesh size of 2 mm. Untreated mixed leaf litter biomass in powdered form was characterized for pH, cellulose, hemicellulose, Lignin and reducing sugar.

Table 4: Physico-chemical characteristics of Mixed leaf litter biomass

S.no	Parameters	MLLB*
1	pH	6.45
2	EC	3.71mS/cm
3	Organic carbon	22.5%

MLLB* = Mixed leaf litter biomass

pH of mixed leaf litter biomass was checked by pH meter and in sample of mixed leaf litter biomass it was found to be 6.45. EC of mixed leaf litter biomass was checked by conductivity meter was found to be 3.71 mS/cm. Gaind and Lata, 2010 reported pH 8.3-8.6 of paddy straw and EC was reported 4.12 and 4.23 mS/cm in paddy straw and poultry manure. Similarly mixed leaf litter biomass samples were characterized for the estimation of organic carbon. Organic carbon content of mixed leaf litter biomass was determined by (Walkley and Black, 1934), it was found to be 22.5%. Gaind and Lata, 2010 reported total organic carbon content 39.2% in case of paddy straw substrate and 14.84% in case of poultry manure substrate.

Table 5: Estimation of Celluloses, hemicelluloses, lignin in mixed leaf litter biomass

S.no.	Substrate	Concentration (Mean ± SD)
1	Cellulose	17.11±0.22 mg/g
2	Hemicellulose	8.40 ± 0.56 %
3	Lignin	8.67 ± 0.07 mg/g

Cellulose content of mixed leaf litter biomass was checked by anthrone assay and it was found to be 17.11 ± 0.02 mg/100mg of biomass. Gaind, and Lata, 2010 reported 39.2 mg/g in case of paddy straw substrate. Hemicellulose content of biomass was checked by a method developed by Goering and Soest (1979). Goldstein, 1981 and Demibras, 1998 reported the 5-20 % of hemicellulose content in case of cotton, flax etc. Lignin was estimated by the method of Sluiter et al., 2012 and was found to be 8.67 ±0.05. Goldstein, (1981) and Demibras, (1998) reported 3-5 % of lignin content in corn stalk samples.

Table 6: OD at 630 nm for Standard curve of cellulose

S.NO.	**Conc. of cellulose (mg/ml)**	**Stock volume (μl)**	**Vol.of distilled water (µl)**	**67(%) sulphuric acid (ml)**	**Anthrone reagent (ml)**	**Incubation**	**OD (630 nm)**
1	0.2	200	800	10	3	Boiled the	0.686
2	0.2	400	600	10	3	samples	1.645
3	0.6	600	400	10	3	for 10 min.	2.495
4	0.8	800	200	10	3		3.47
5	1	1000	0	10	3		3.404

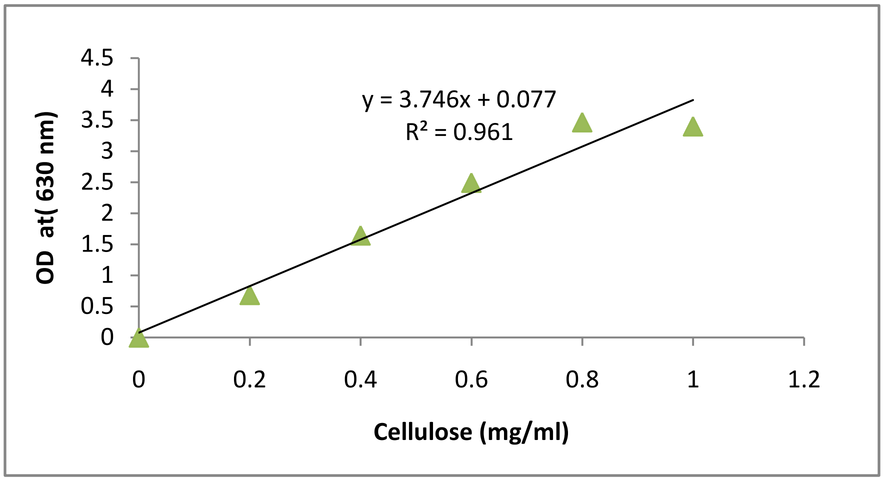

Figure 6: Standard curve for cellulose

Table 9: Estimation of Total Kjeldahl Nitrogen in leaf litter from different tree and mixture of all leaf litter biomass (MLLB)

S.No.	Samples	% age of TKN (Mean ± SD)
1	Jamun	16.5 ± 2.12
2	Eucalyptus	3.25 ± 1.06
3	Rice straw	6.25 ± 1.06
4	Mango	18.5 ± 2.12
5	Ashoka	15.5 ± 1.41
6	Bamboo	22 ± 1.76
7	MLLB	11± 1.41

MLLB: mixed leaf litter biomass

Total kjeldahl nitrogen content of untreated mixed leaf litter biomass samples and others samples such as jamun, mango, eucalyptus, ashoka, bamboo, rice straw samples were checked by digesting the samples with sulphuric acid, followed by distillation (Jackson, 1973). It was found that TKN in case of mixed leaf litter biomass samples was 11 % whereas TKN found to be maximum 22% in case of bamboo sample as compared to others leaf samples. Gaind and Lata reported the 13% TKN by using the paddy straw as major substrate.

2. Biodegradation of untreated and acid pretreated mixed leaf litter biomass

Biodegradation of untreated and acid pretreated MLLB was done by *Saacharomyces cerevisiae* and *Candida shehate* and their co-inoculation. It was checked by estimating sugar concentration and ethanol concentration at different time interval.

Table 10: Sugar estimation of the samples at 540 nm

Sr. No.	Conc. of dextrose (mg/ml)	Volume of distilled water (µl)	Volume of stock solution (µl)	Volume of DNS reagent added (ml)	OD. at 540 (nm)
1	0.2	200	800	3	0.686
2	0.4	400	600	3	1.645
3	0.6	600	400	3	2.495
4	0.8	800	200	3	3.470
5	1	0	1000	3	3.474

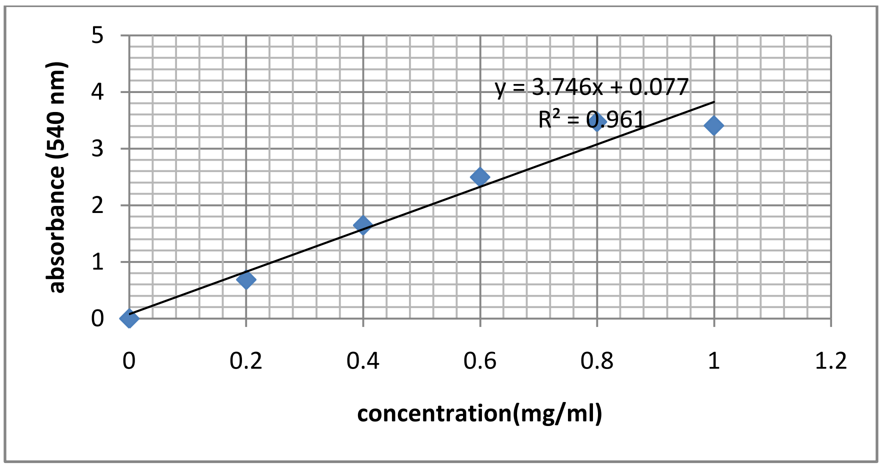

Figure 10: Standard curve for sugar estimation (mg/g)

Table 11: Standard curve for Ethanol estimation at 595 nm

S.NO.	Conc. of ethanol (g/l)	Stock volume (μl)	Volume of distilled water (ul)	DBP reagent (ml)	OD (630 nm)
1	0	0	2000	2	0.000
2	0.2	200	1980	2	0.312
3	0.4	400	1960	2	0.516
4	0.6	600	1940	2	0.655
5	0.8	800	1920	2	0.886
6	1	1000	1000	2	1.252

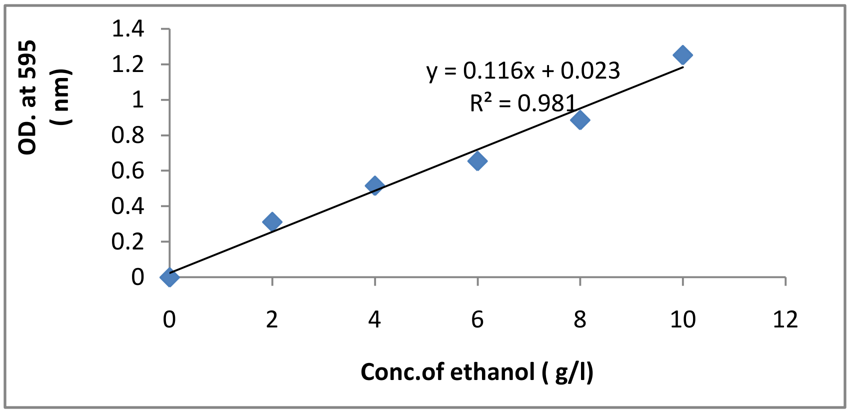

Figure 11: Standard curve for ethanol

Table 12; Sugar estimation for untreated samples

Days	**Cultures**		
	S.c.	**C.s**	**S.c + C.s.**
0	0.076 ± 0.02	0.067 ± 0.01	0.540 ± 0.02
1	1.633 ± 0.03	4.160 ± 0.02	4.100 ± 0.03
2	4.404 ± 0.02	4.489 ± 0.02	5.880 ± 0.01
3	2.460 ± 0.02	3.460 ± 0.01	5.00 ± 0.02
4	0.923 ± 0.02	1.783 ± 0.02	2.850 ± 0.02
5	1.090 ± 0.01	1.789 ± 0.01	1.912 ± 0.02
6	0.756 ± 0.02	1.290 ± 0.01	1.459 ± 0.02
7	0.213 ± 0.02	1.080 ± 0.02	1.090 ± 0.02
8	0.260 ± 0.03	1.200 ± 0.02	1.323 ± 0.02
9	0.196 ± 0.02	0.716 ±0.01	1.356±0.03
10	1.230 ± 0.01	1.486 ± 0.02	1.120 ± 0.02

S.c; *Saccharomyces cerevisiae*; C.s; *Candida shehate*

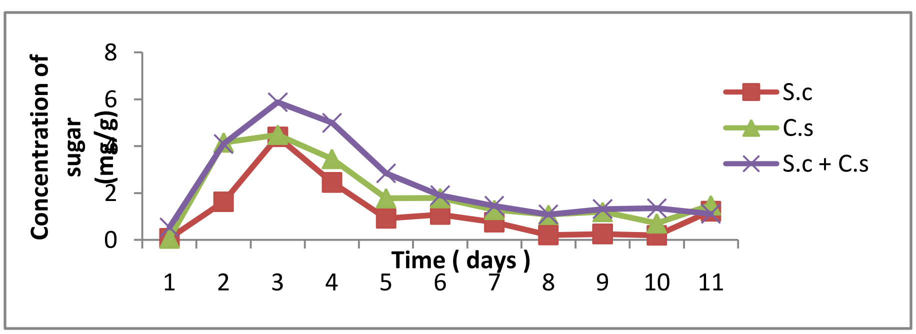

Figure 12: Sugar concentration for untreated samples (mg/g)

Untreated mixed leaf litter biomass samples were checked for sugar estimation (Table 12; Figure 12). There was increase in sugar in untreated biomass sample. Maximum sugar was observed on third day from the *Candida shehate* organism with maximum value of 5.88mg/g. A high concentration of reducing sugar released at the beginning of fermentation was due to presence of co-substrate and also the degradation of substrate occurred due to the presence of fungi. From the three sets of fungi, *Candida shehate* was found to be most suitable strains for sugar production using mixed leaf litter biomass as major substrate. Khan et al., 2007 reported the 2.58g/l obtained on the fourth day from the organism *Phanerochaete chrysosporium.*

Table 13: Ethanol estimation for untreated samples (g/l)

Days	**Cultures**		
	S.c.	**C.s**	**S.c + C.s.**
0	1.750 ± 0.03	1.773 ± 0.03	1.820±0.02
1	2.680 ± 0.02	2.563 ± 0.01	2.590±0.03
2	2.390±0.02	2.476±0.03	2.503±0.01
3	2.380±0.02	2.45±0.02	2.50±0.02
4	2.35±0.03	2.43±0.02	2.536±0.02
5	2.446±0.03	2.48±0.04	2.555±0.02
6	1.8±0.04	1.89±0.02	1.99±0.02
7	1.4±0.02	1.5±0.04	1.16±0.02
8	0.95±0.03	1±0.01	1.09±0.01
9	1.33±0.03	1±0.02	1.14±0.02
10	1.146±0.03	1.186±0.02	1.159±0.03

S.c; *Saccharomyces cerevisiae*; C.s; *Candida shehate*

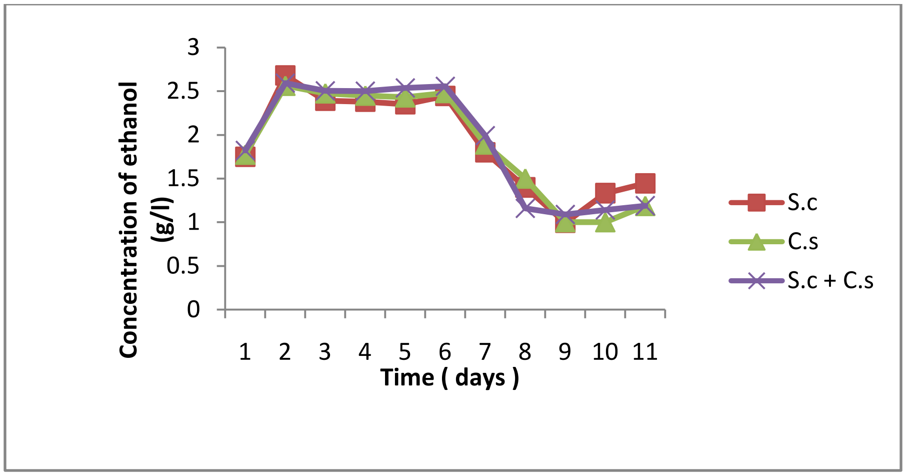

Figure 13: Ethanol concentration of untreated samples (g/l)

Ethanol estimation was done for the three treatments (Table 13; Figure 13), it was shown that in mixed leaf litter biomass samples high concentration of ethanol 1.99g/l was observed on sixth day treated with *Saccharomyces cerevisiae* and *Candida shehate*. From all the above three treatments both *S.cervisiae* and *C. shehate* were found to be most suitable for ethanol production using mixed leaf litter biomass as major substrate. In the production of ethanol, the concentration of reducing sugar released decreased with the fermentation time, indicating the degradation of substrates occurred and decreased at the end of fermentation. Due to the reduction of sugar there is maximum production of ethanol, they get metabolized during later stages. Alam et al., 2007 reported 10.1% ethanol on the sixth day, the highest reading was reported by a combination of *P. chrysosporium*, *T. harzianum*, *S. cerevisiae* using rice straw as major substrate.

Table 14: Sugar estimation for acid pretreated samples

Days	Cultures		
	S.c.	**C.s**	**S.c + C.s.**
0	0.086 ± 0.01	0.063 ± 0.02	0.686 ± 0.01
1	0.183 ± 0.01	0.073 ± 0.02	3.103 ± 0.01
2	2.160 ± 0.02	7.760 ± 0.03	3.726 ± 0.03
3	8.336 ± 0.01	8.680 ± 0.01	8.526 ± 0.02
4	4.830 ± 0.02	7.726 ± 0.02	5.070 ± 0.02
5	1.540 ± 0.02	1.583 ± 0.02	1.616 ± 0.02
6	0.490 ± 0.01	0.946 ± 0.03	0.480 ± 0.05
7	2.143 ± 0.03	3.280 ± 0.01	1.973 ± 0.02
8	1.676 ± 0.02	3.000 ± 0.02	1.653 ± 0.03
9	0.780 ± 0.02	2.373 ± 0.03	1.780 ± 0.02
10	0.783 ± 0.02	2.393 ± 0.05	1.826 ± 0.02

S.c; *Saccharomyces cerevisiae*; C.s; *Candida shehate*

Pretreated samples

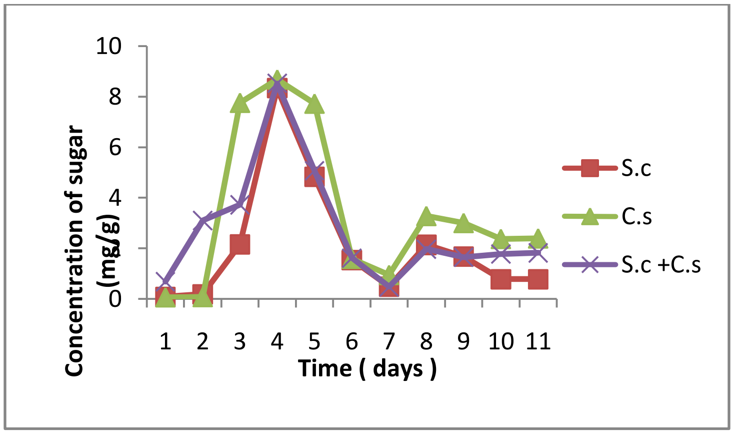

Figure 14 Sugar concentration for pretreated samples (mg/g)

Mixed leaf litter biomass was subjected to acid pretreatment and estimated for release of sugar (Table 14; Figure 14). Maximum concentration of 8.25mg/g sugar was observed on third day from the organism *Saccharomyces cerevisiae* and *Candida shehate.* Due to the acid pretreatment, reaction rate becomes faster, in the first step of reaction, cellulosic materials converted to sugars and in the second step, sugars converted to ethanol. When the biomass samples were subjected to the acid pretreatment there is degradation of cellulose and hemicelluloses and they get exposed, so there is maximum production of sugars in this case as compared to untreated samples. Alam et al., in 2007 reported the highest concentration of sugars 3.2 g/l on second day using rice straw as the major substrate from the organism *S.cerevisiae.*

Table 15 Ethanol estimation for pretreated samples

Days	**Cultures**		
	S.c.	**C.s**	**S.c + C.s.**
0	1.650 ± 0.02	1.470 ± 0.04	1.480 ± 0.02
1	2.566 ± 0.02	2.606 ± 0.02	2.693 ± 0.02
2	2.866 ± 0.02	2.893 ± 0.03	2.990 ± 0.03
3	2.586 ± 0.01	2.663 ± 0.02	2.880 ± 0.03
4	2.713 ± 0.03	2.683 ± 0.03	2.646 ± 0.02
5	2.853 ± 0.03	1.503 ± 0.01	2.896 ± 0.03
6	0.490 ± 0.01	0.946 ± 0.03	0.480 ± 0.05
7	2.143 ± 0.03	3.280 ± 0.01	1.973 ± 0.02
8	1.676 ± 0.02	3.000 ± 0.02	1.653 ± 0.03
9	0.780 ± 0.02	2.373 ± 0.03	1.780 ± 0.02
10	0.783 ± 0.02	2.393 ± 0.05	1.826 ± 0.02

S.c; *Saccharomyces cerevisiae*; C.s; *Candida shehate*

Pretreated samples

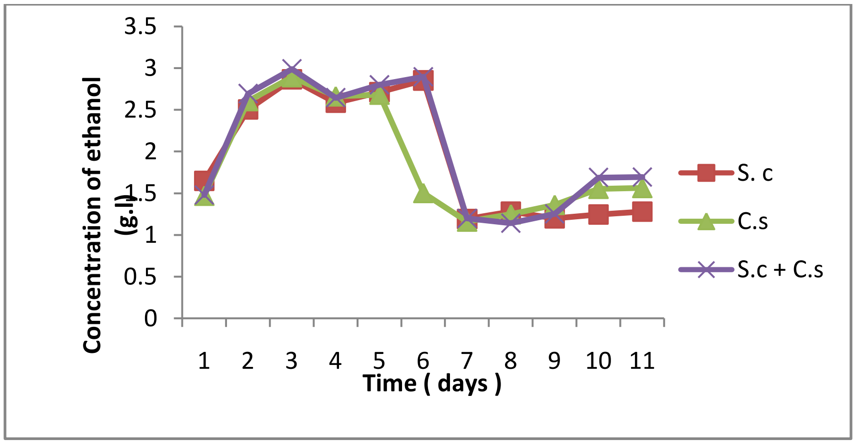

Figure 15 Ethanol estimation for pretreated samples (g/l)

Ethanol estimation was done in three microbial treatments of biomass (Table 15; Figure 15). It was found that in mixed leaf litter biomass samples high concentration of ethanol 1.99g/l was observed on sixth day from the organism *Saccharomyces cerevisiae* and *Candida shehate.* From all the above three strains both *S.cervisiae* and *C. shehate* were found to be most suitable for ethanol production using mixed leaf litter biomass as major substrate. In the production of ethanol, the concentration of reducing sugar released decreased with the fermentation time, indicating the degradation of substrates occured and decreased at the end of fermentation. Due to the reduction of sugar there is maximum production of ethanol, they get metabolized during later stages. Alam et al., 2007 reported 10.1% ethanol on the sixth day, the highest reading was reported by a combination of *P. chrysosporium*, *T. harzianum*, *S. cerevisiae* using rice straw as major substrate.

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2. Effect of pH on untreated and acid pretreated samples for sugar and ethanol estimation

In second set experiment, biodegradation of mixed leaf litter biomass for both untreated and acid pretreated samples were carried out different pH 4.5 (Table 16; Fig. 16) and 5.5 (Table 17; Fig. 17). Both the samples were estimated for the sugar and ethanol.

Table 16 Sugar estimation for untreated samples and acid pretreated samples at pH 4.5

Day	Untreated samples			Pretreated samples		
	S.c	**C.s**	**S.c.+ C.s.**	**S.c**	**C.s**	**S.c.+ C.s.**
0	0.166 ± 0.02	0.157 ± 0.03	0.125 ± 0.00	0.556 ± 0.02	0.760 ± 0.02	0.483 ± 0.02
1	2.103 ± 0.02	2.563 ± 0.02	1.666 ± 0.01	2.670 ± 0.03	2.430 ± 0.01	2.590 ± 0.01
2	0.956 ± 0.01	0.930 ± 0.02	0.950 ± 0.01	1.400 ± 0.02	1.345 ± 0.02	1.530 ± 0.03
3	0.780 ± 0.01	0.700 ± 0.01	0.686 ± 0.01	0.576 ± 0.02	0.380 ± 0.03	0.526 ± 0.04
4	0.513 ± 0.03	0.673 ± 0.01	0.556 ± 0.02	0.900 ± 0.01	0.890 ± 0.02	0.880 ± 0.02
5	0.763 ± 0.03	0.980 ± 0.01	0.880 ± 0.02	1.163 ± 0.02	1.250 ± 0.02	0.913 ± 0.02

S.c; *Saccharomyces cerevisiae*; C.s; *Candida shehate*

Untreated samples

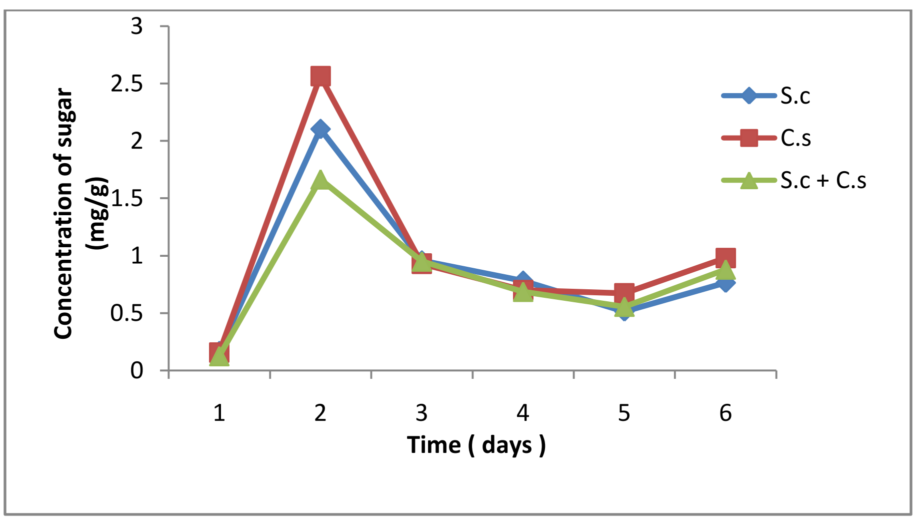

Figure 16 Sugar concentration for untreated samples (mg/g)

Untreated samples of mixed leaf litter biomass samples were taken and amount of sugar released was checked at pH 4.5 (Table 16; Figure; 16). It was observed that maximum sugar 0.96 mg/g was released on second day when treated with *Saccharomyces cerevisiae*. Although pH 4.5 was found to be optimum for the production of sugar, slight decrease in the sugar yield at lower pH was favourable for simultaneous saccharification and fermentation (SSF). It is known that pH optimum for the yeast growth was 5.0 and 5.5, but during fermentation pH decreases even below 4.0. The results showed that changes in the pH conditions cannot bring about great effect on the fermentation and thus there was maximum production of sugar released at pH 4.5. Srivastava et., al 2014 reported the 4.21 mg/g sugar using rice husk as major substrate.

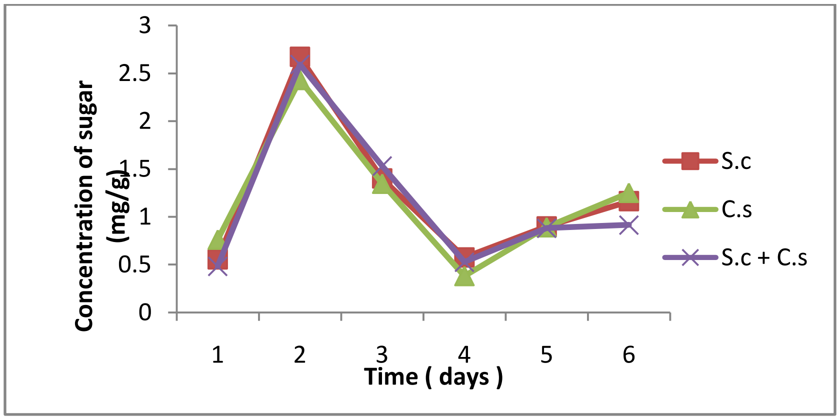

Figure 17 Sugar concentration for acid pretreated samples (mg/g)

Mixed leaf litter biomass samples were subjected to acid pretreatment. Maximum sugar content 2.59 mg/g (Table 17; Fig. 17) was observed on second day using *Saccharomyces cerevisiae* and *Candida shehate.* In case of pretreated samples, there is production of hemicellulose-derived sugars such as xylose and xylooligomers and associated degradation products such as furfural which leads to increase in the production of sugar and cellulose degradation products also.

Ethanol estimation at pH 4.5 for untreated and acid pretreated samples

Table 18 Ethanol estimation for Untreated Samples

Day	**Untreated samples**			**Pretreated samples**		
	S.c	**C.s**	**S.c.+ C.s.**	**S.c**	**C.s**	**S.c.+ C.s.**
0	0.690 ± 0.02	1.106 ± 0.02	0.700 ± 0.02	0.953 ± 0.02	0.986 ± 0.03	0.948 ± 0.01
1	1.660 ± 0.02	1.836 ± 0.03	1.373 ± 0.01	1.086 ± 0.01	1.256 ± 0.05	1.300 ± 0.01
2	1.790 ± 0.01	1.803 ± 0.02	1.486 ± 0.01	1.846 ± 0.02	2.070 ± 0.02	2.076 ± 0.01
3	1.560 ± 002	1.673 ± 0.02	1.450 ± 0.02	2.353 ± 0.02	2.480 ± 0.03	2.583 ± 0.01
4	2.480 ± 0.01	2.786 ± 0.04	2.623 ± 0.03	3.663 ± 0.01	3.643 ± 0.01	3.756 ± 0.03
5	3.150 ± 0.01	3.134 ± 0.03	3.200 ± 0.02	3.500 ± 0.01	3.800 ± 0.02	3.743 ± 0.02

S.c; *Saccharomyces cerevisiae*; C.s; *Candida shehate*

Untreated samples

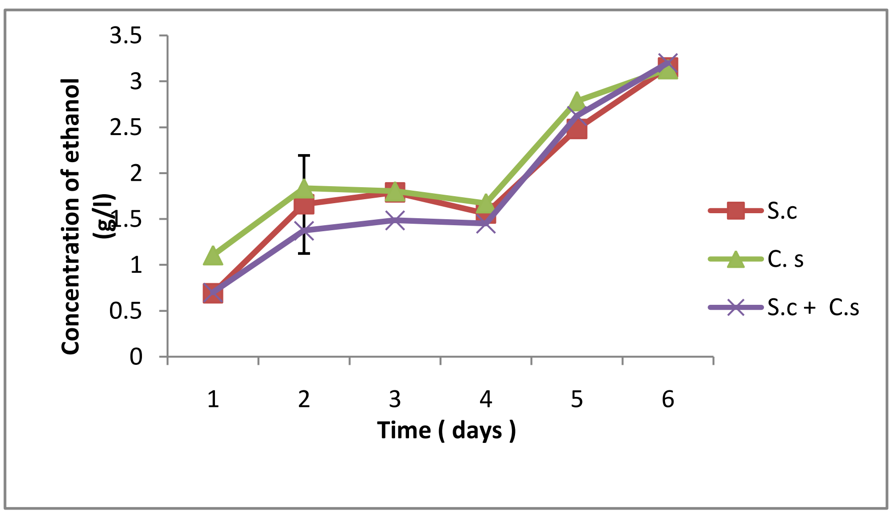

Figure 18 Ethanol concentration for untreated samples (g/l)

Untreated mixed leaf litter biomass samples were subjected to microbial treatment and ethanol was estimated at pH 4.5 (Table 18; Figure 18). Maximum amount of ethanol 3.20 g/l was produced on fifth day from the organism *Saccharomyces cerevisiae* and *Candida shehate* these were best among all the above strains for the production of ethanol using mixed leaf litter biomass as major substrate at pH 4.5, both the organisms has drawn considerable attention as an appropriate host for the production of lignin-degrading enzymes or direct application in lignocelluloses biomass conversion and there was increase in the ethanol production on the addition of both these pretreated samples.

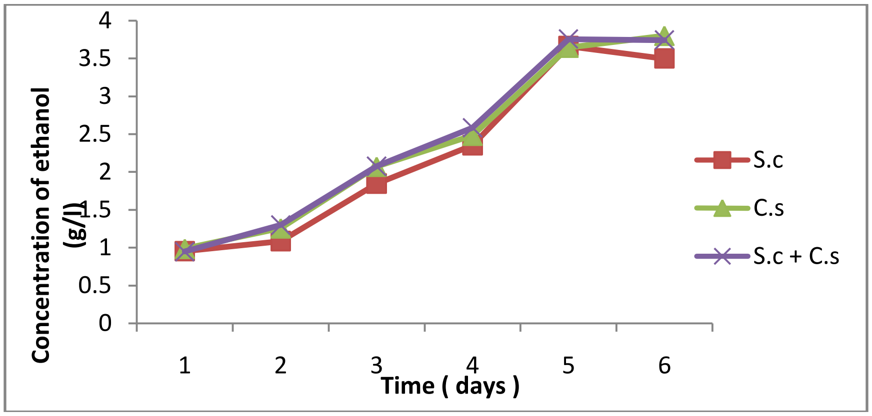

Figure 19 Ethanol concentration for pretreated samples (g/l)

Mixed leaf litter biomass samples were subjected to acid pretreatment and amount of ethanol produced were estimated (Table 19; Figure19). Maximum ethanol 3.75 g/l was produced on fith day from the organism *Candida shehate*. Due to the acid pretreatment there was easily degradation of cellulose and hemicelluloses takes place. Best strain for the production of ethanol was found to be *C.shehate* among all above two strains. Krishna et al., 1999 reported the concentration of ethanol in the range of 2.5-3.0 %.

Sugar estimation at pH 5.5 for untreated and acid pretreated samples

Table 20 Sugar estimation for untreated and acid pretreated samples

Day	Untreated samples			Pretreated samples		
	S.c	**C.s**	**S.c.+ C.s.**	**S.c**	**C.s**	**S.c.+ C.s.**
0	0.176 ± 0.02	0.160 ± 0.02	0.133 ± 0.03	0.400 ± 0.01	0.583 ± 0.02	0.670 ± 0.02
1	1.866 ± 0.03	1.760 ± 0.02	2.136 ± 0.01	2.436 ± 0.02	2.363 ± 0.02	2.436 ± 0.01
2	1.345 ± 0.02	1.286 ± 0.01	1.107 ± 0.03	2.483 ± 0.02	2.523 ± 0.05	2.486 ± 0.01
3	1.110 ± 0.02	0.980 ± 0.04	1.090± 0.04	1.670 ± 0.02	1.800 ± 0.02	1.666 ± 0.01
4	1.990 ± 0.01	1.560 ± 0.02	2.380± 0.02	1.545 ± 0.02	1.645 ± 0.01	1.743 ± 0.03
5	1.970 ± 0.02	1.590 ± 0.01	2.340 ± 0.02	3.186 ± 0.01	3.500 ± 0.02	3.400 ± 002

S.c; *Saccharomyces cerevisiae*; C.s; *Candida shehate*

Untreated samples

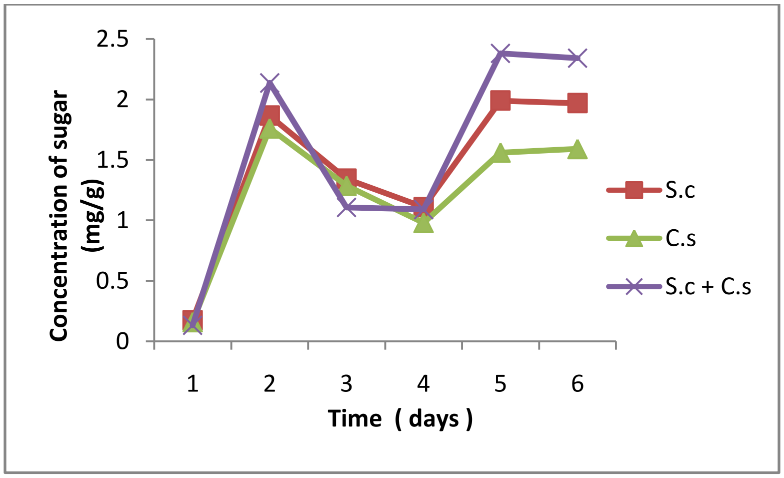

Figure 20 Sugar concentration for untreated samples (mg/g)

Untreated leaf litter biomass samples were microbially treated and sugar was estimated (Table 20; Figure 20). Maximum sugar content 1.9 mg/g observed on second day from the organism *Saccharomyces cerevisiae.* In case of untreated samples there is maximum increase in the sugar content due to degradation of cellulose and hemicelluloses takes place and they get exposed. There is increase in the cellulose extract. It was observed that at pH 5.5 there was maximum formation of sugars takes place as compared to the pH 4.5. From the above data it was observed that *S.cerevisiae* was found to be the best suitable strain for the release of sugar content.

Pretreated samples

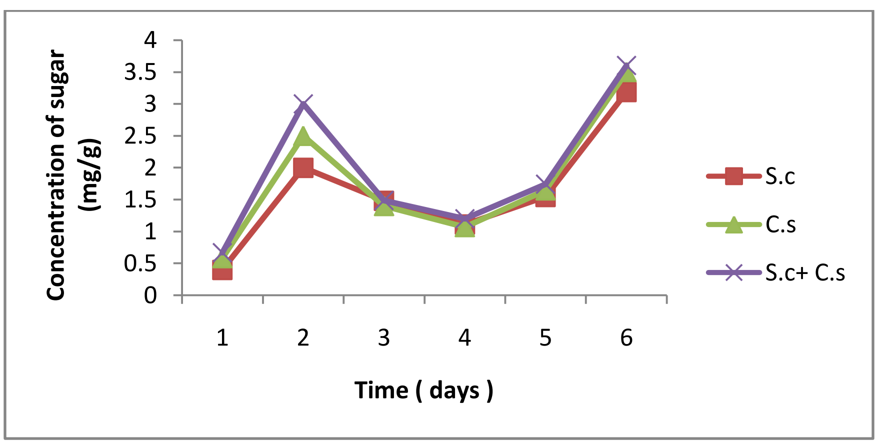

Figure 21 Sugar concentration for acid pretreated samples (mg/g)

Mixed leaf litter biomass samples were taken and subjected to acid pretreatment. From (Table 21; Figure 21), we concluded that maximum amount of sugar content 3.60 mg/g was formed on second day using *Saccharomyces cerevisiae* and *Candida shehate*. In case of pretreated samples sugar content increases as compared to untreated samples. In mixed leaf litter biomass samples after pretreatment due to degradation of cellulose and hemicelluloses, sugar content increases. The samples during fermentation typically with the use of microorganism converted the fermentable sugars in higher amounts.

Ethanol estimation at pH 5.5 for untreated and acid pretreated samples

Table 22 Ethanol estimation for untreated samples

Day	Untreated samples			Pretreated samples		
	S.c	**C.s**	**S.c.+ C.s.**	**S.c**	**C.s**	**S.c.+ C.s.**
0	0.790 ± 0.01	1.023 ± 0.04	1.103 ± 0.04	0.856 ± 0.03	0.976 ± 0.01	0.946 ± 0.04
1	1.900 ± 0.02	2.080 ± 0.01	1.956 ± 0.03	2.070 ± 0.02	1.956 ± 0.03	2.320 ± 0.02
2	2.440 ± 0.04	2.540 ± 0.04	2.533 ± 0.02	2.463 ± 0.02	2.503 ± 0.02	2.603 ± 0.03
3	2.660 ± 0.03	2.760 ± 0.02	2.803 ± 0.02	2.450 ± 0.02	2.479 ± 0.03	2.593 ± 0.01
4	3.550 ± 0.02	3.650 ± 0.05	2.666 ± 0.01	3.450 ± 0.02	3.780 ± 0.02	3.990 ± 0.03
5	3.256 ± 0.02	3.550 ± 0.02	3.850 ± 0.01	2.230 ± 0.03	2.206 ± 0.02	2.109 ± 0.02

S.c; *Saccharomyces cerevisiae*; C.s; *Candida shehate*

Untreated samples

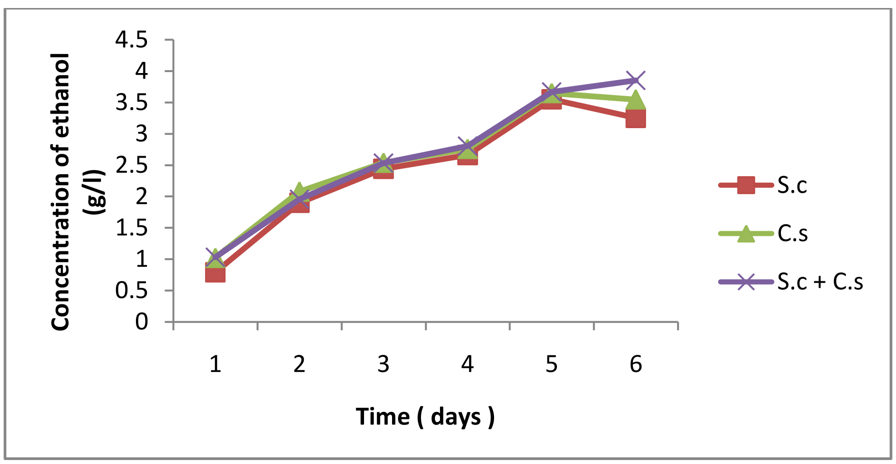

Figure 22 Ethanol concentration for untreated samples (g/l)

Untreated mixed leaf litter biomass samples were microbially treated and ethanol production was estimated (Table 22 and Figure 22). Maximum concentration of ethanol 3.55 g/l was observed on fifth day from the organism *Candida shehate.* In case of untreated samples at pH 5.5 during initial stages there was maximum production of sugars takes places, with the decrease in fermentation time sugar content decreases, so at that time of fermentation there is maximum production of ethanol takes place. From the above data we observed that *C.shehate* was found to be the best strain among all above strains, maximum production of ethanol takes place due to the presence of this organisms.

Pretreated samples

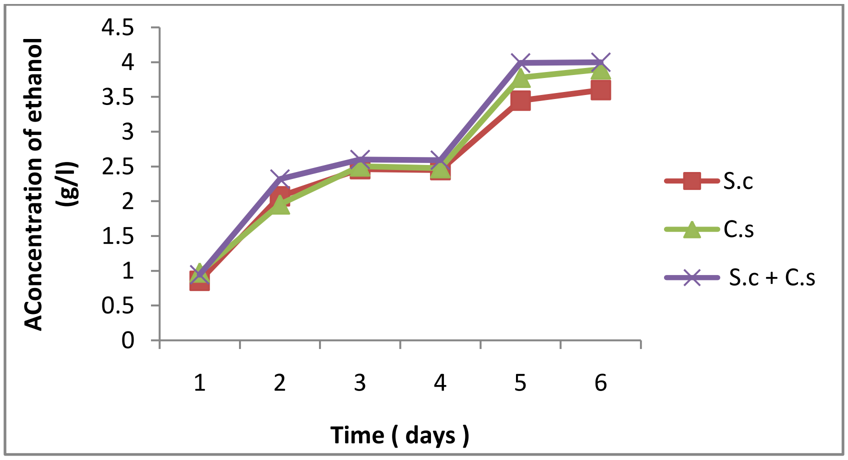

Figure 23 Ethanol concentration for pretreated samples (g/l)

Mixed leaf litter biomass samples were subjected to acid pretreatment thereafter it was microbially treated and production of ethanol was estimated (Table23; Figure 23). Maximum concentration of ethanol 4 g/l was produced from the organism *Saccharomyces cerevisiae* on fifth day. When the mixed leaf litter biomass samples were subjected to acid pretreatment maximum production of ethanol takes place due to the degradation of celluloses and hemicelluloses. In case of acid pretreatment with the increase in the pH maximum production of ethanol takes Place

3. Sugar estimation at temperature 25 ˚C for untreated and acid pretreated samples

At temperature 25˚C for untreated samples

Table 24 Sugar estimation for untreated samples

Day	Untreated samples			Pretreated samples		
	S.c	**C.s**	**S.c.+ C.s.**	**S.c**	**C.s**	**S.c.+ C.s.**
0	0.856 ± 0.03	0.976 ± 0.01	0.946 ± 0.04	0.393 ± 0.02	0.626 ± 0.03	2.403 ± 0.02
1	2.070 ± 0.02	1.956 ± 0.03	2.320 ± 0.02	6.500 ± 0.02	6.880 ± 0.02	7.093 ± 0.02
2	2.463 ± 0.02	2.503 ± 0.02	2.603 ± 0.03	4.250 ± 0.03	4.490 ± 0.01	4.340 ± 0.04
3	3.450 ± 0.02	2.230 ± 0.03	2.593±0.01	3.450 ± 0.02	3.666 ± 0.02	3.583 ± 0.07
4	3.450 ± 0.02	3.780 ± 0.02	3.990 ± 0.03	3.790 ± 0.03	3.773 ± 0.03	3.900 ± 0.02
5	2.230 ± 0.03	2.206 ± 0.02	2.109 ± 0.02	3.156 ± 0.03	3.580 ± 0.02	3.923 ± 0.03

S.c; *Saccharomyces cerevisiae*; C.s; *Candida shehate*

Untreated samples

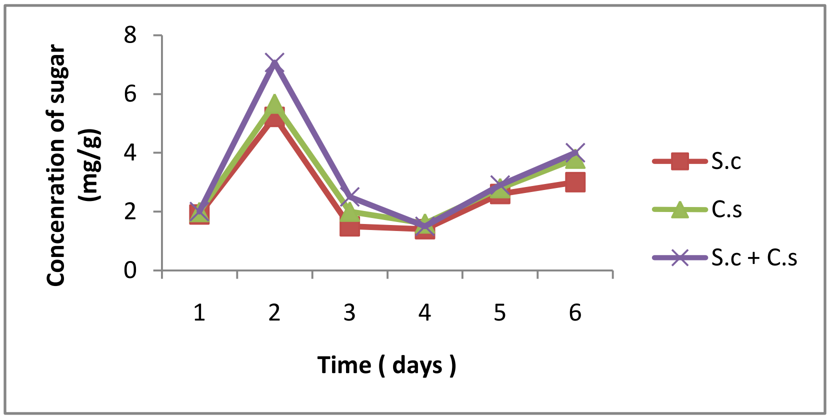

Figure 24 Sugar concentration for untreated samples (mg/g)

Mixed untreated leaf litter biomass samples were subjected to acid pretreatment.and there after it was microbially treated and production of sugar was estimated.(Table 24;Figure 24), we concluded that maximum content of sugar 7.07 mg/g was observed in second day from the organism *Saccharomyces cerevisiae and Candida shehate*. At temp.25°C. the increase in sugar concentration was linear upto 12-18 h, reaching maximum at 24-48h and remaining almost unchanged thereafter. Both the organism *S. cervisiae* and *C. shehate* were found to be the best among all the above strains.

Pretreated samples

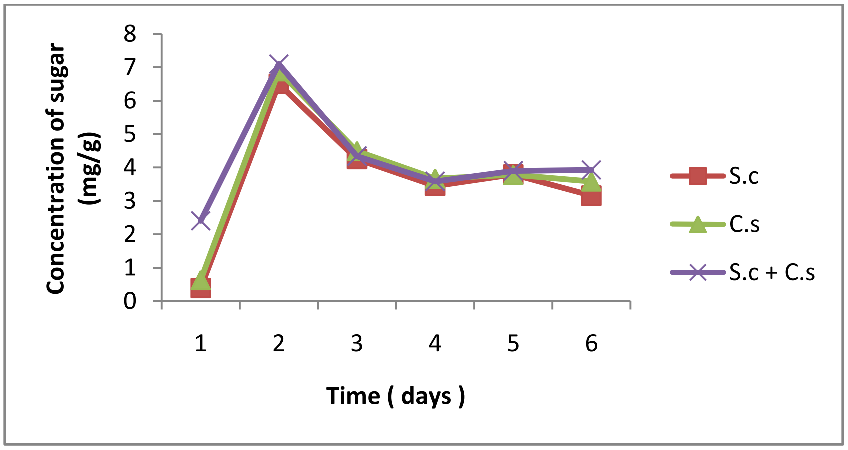

Figure 25 Sugar concentration for pretreated samples (mg/g)

Mixed leaf litter biomass samples were subjected to acid pretreatment and thereafter nicrobially treated and estimated for production of sugar .(Table 25 ; Figure 25) it was found that in case of acid pretreated samples it was found that maximum sugar 7.09 mg/g formed on second day from the organism *Saccharomyces cerevisiae* and *Candida shehate.* Due to degradation of cellulose and hemicelluloses there was increase in the production of sugar content.

Ethanol estimation at temperature 25˚C for untreated and acid pretreated samples

Table 26 Ethanol estimation

Day	Untreated samples			Pretreated samples		
	S.c	**C.s**	**S.c.+ C.s.**	**S.c**	**C.s**	**S.c.+ C.s.**
0	1.000 ± 0.03	1.330 ± 0.02	1.120 ± 0.02	0.990 ± 0.03	1.200 ± 0.03	1.300 ± 0.02
1	2.400 ± 0.01	2.570 ± 0.02	2.693 ± 0.02	2.516 ± 0.03	2.666 ± 0.01	2.500 ± 0.02
2	2.550 ± 0.04	2.506 ± 0.02	2.540 ± 0.03	2.600 ± 0.03	2.730 ± 0.03	2.750 ± 0.02
3	1.666 ± 0.02	1.669 ± 0.02	1.770 ± 0.03	2.450 ± 0.03	2.583 ± 0.02	2.560 ± 0.02
4	1.000 ± 0.03	1.200 ± 0.03	1.300 ± 0.02	1.853 ± 0.04	1.990 ± 0.01	2.000 ± 0.03
5	1.866 ± 0.02	1.846 ± 0.04	1.787 ± 0.02	1.900 ± 0.03	1.863 ± 0.02	1.900 ± 0.02

S.c; *Saccharomyces cerevisiae*; C.s; *Candida shehate*

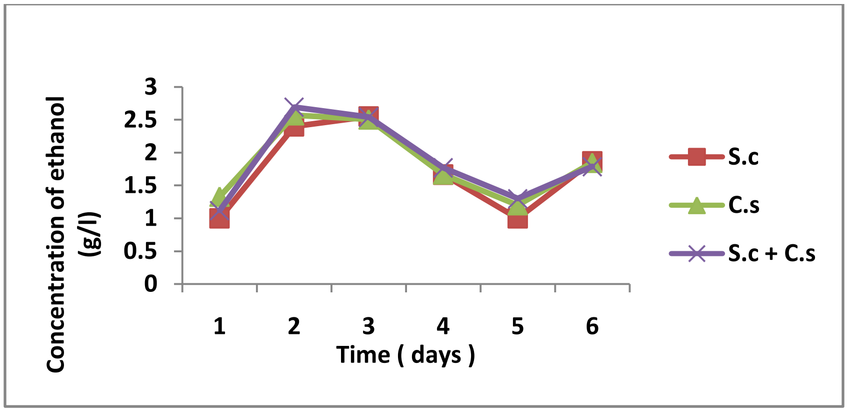

Figure 26 Ethanol concentration for untreated samples (g/l)

Mixed leaf litter biomass samples were checked for ethanol estimation (Table 26 ; Figure 26). Maximum value of ethanol was obtained at 2.57mg/g from the organism *Candida shehate* on second day. During initial stages of fermentation maximum production of ethanol takes place at the later stages, during initial stages maximum amount of sugars were released, as the fermentation time increases the amount of ethanol produced also increases. From the above data, we concluded that *C. shehate* was found to be the best strain for increase in the production of ethanol

Pretreated samples

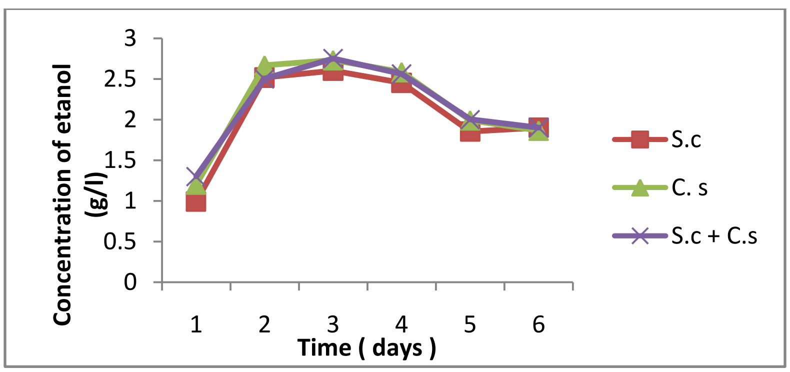

Figure 27 Ethanol concentration for pretreated samples (g/l)

Mixed leaf litter biomass samples were subjected to the acid pretreatment and thereafter microbially treated for production of ethanol (Table 27; Figure 27) Maximum ethanol concentration 2.73 g/l was observed on third day from the organism *Saccharomyces cerevisiae and Candida shehate*. In case of pretreated samples always there is production of ethanol takes place, a high yield of ethanol was observed because the biomass samples were acid pretreated. Due to pretreatment degradation of cellulose and hemicelluloses takes place which then used to convert the fermentable sugars into ethanol

Sugar estimation at temperature 35 ˚C for untreated and pretreated samples

Table 28 Sugar estimation for untreated samples

Day	Untreated samples			Pretreated samples		
	S.c	**C.s**	**S.c.+ C.s.**	**S.c**	**C.s**	**S.c.+ C.s.**
0	0.976 ± 0.03	2.493 ± 0.02	2.893 ± 0.02	0.395 ± 0.02	0.636 ± 0.03	2.393 ± 0.02
1	6.883 ± 0.02	6.786 ± 0.03	7.256 ± 0.01	6.700 ± 0.01	6.880 ± 0.02	8.550 ± 0.03
2	5.940 ± 0.03	5.966 ± 0.03	6.456 ± 0.03	3.253 ± 0.03	4.000 ± 0.03	5.000 ± 0.02
3	6.850 ± 0.02	6.413 ± 0.03	6.030 ± 0.03	3.450 ± 0.02	3.200 ± 0.02	3.400 ± 0.01
4	6.683 ± 0.02	5.973 ± 0.02	5.730 ± 0.05	3.786 ± 0.03	3.776 ± 0.03	2.986 ± 0.01
5	5.773 ± 0.02	5.290 ± 0.02	5.220 ± 0.02	5.153 ± 0.02	5.573 ± 0.01	5.600 ± 0.01

S.c; *Saccharomyces cerevisiae*; C.s; *Candida shehate*

Untreated samples

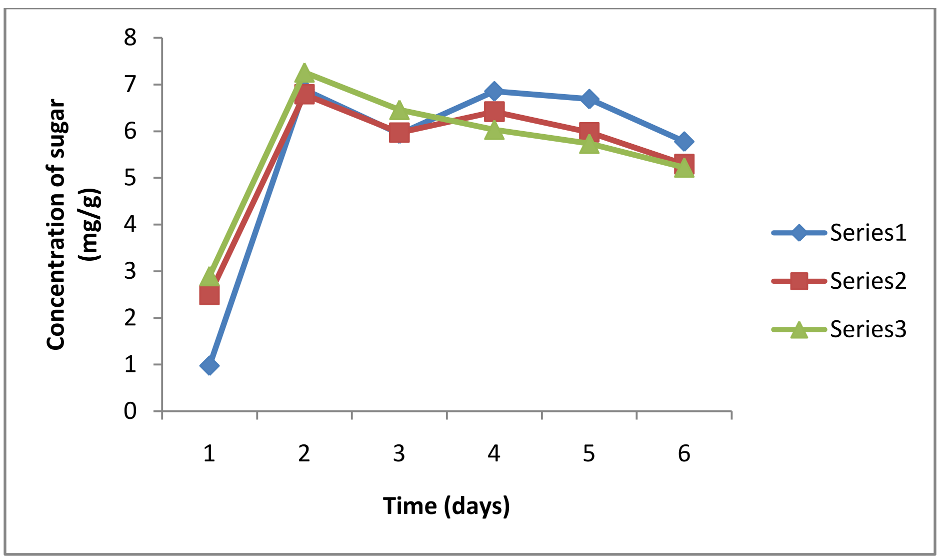

Figure 28 Sugar concentration for untreated samples (mg/g)

Mixed untreated leaf litter biomass samples were taken and checked for the sugar.(Table 28 ; Figure 28). Maximum sugar content 7.25 mg/g was observed on second day from the organism *Saccharomyces cerevisiae and Candida shehate.* With the increase in the temperature sugar content also increases during initial stages, as the fermentation time and temperature increases the sugar content increases. From the above data *S.cervi* was found to be best for the maximum production of sugars using mixed leaf litter biomass samples as the major substrate

Pretreated samples

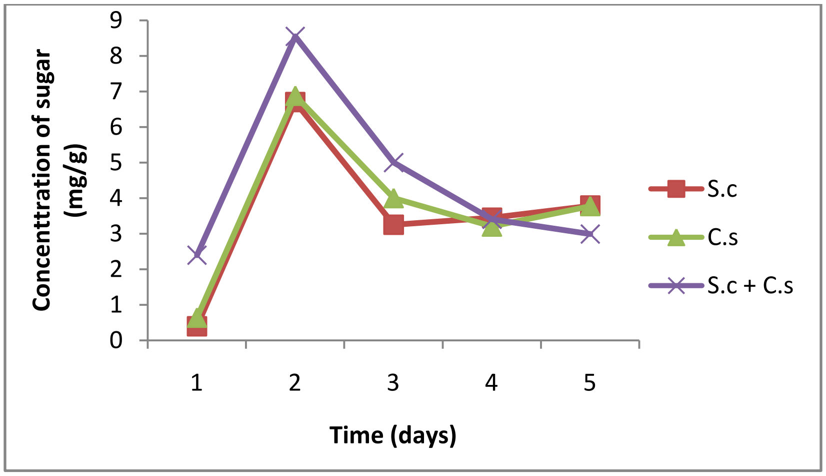

Figure 29 Sugar concentration for pretreated samples (mg/g)

Mixed leaf litter biomass samples were taken and subjected to acid pretreatment and thereafter microbially treated for production of sugar (Table 29 ; Figure 29).Maximum amount of sugar 8.55 mg/g was produced on second day from the organism *Saccharomyces cerevisiae* and *Candida shehate*. Maximum amount of sugar released during the fermentation was observed during the initial stages. When the samples were acid pretreated degradation of cellulose and hemicelluloses takes place in case of pretreated as compared to untreated samples. With increase in the temperature maximum production of sugars takes place.

Ethanol estimation at temperature 35˚C for untreated and pretreated samples

Table 30. Ethanol estimation for untreated samples

Day	Untreated samples			Pretreated samples		
	S.c	**C.s**	**S.c.+ C.s.**	**S.c**	**C.s**	**S.c.+ C.s.**
0	0.883 ± 0.03	0.620 ± 0.02	0.530 ± 0.02	1.500 ± 0.03	1.600 ± 0.01	1.800 ± 0.04
1	1.090 ± 0.05	1.110 ± 0.02	1.070 ± 0.02	2.400 ± 0.02	2.673 ± 0.01	2.653 ± 0.03
2	1.933 ± 0.03	1.826 ± 0.03	1.786 ± 0.05	2.580 ± 0.02	2.773 ± 0.03	2.630 ± 0.02
3	2.700 ± 0.03	2.496 ± 0.02	2.397 ± 0.03	2.643 ± 0.03	2.706 ± 0.04	2.670 ± 0.02
4	2.200 ± 0.03	2.300 ± 0.01	2.440 ± 0.02	1.200 ± 0.02	1.400 ± 0.03	1.100 ± 0.02
5	2.000 ± 0.04	2.100 ± 0.03	2.200 ± 0.02	1.953 ± 0.04	2.000 ± 0.02	1.890 ± 0.01

S.c; *Saccharomyces cerevisiae*; C.s; *Candida shehate*

Untreated samples

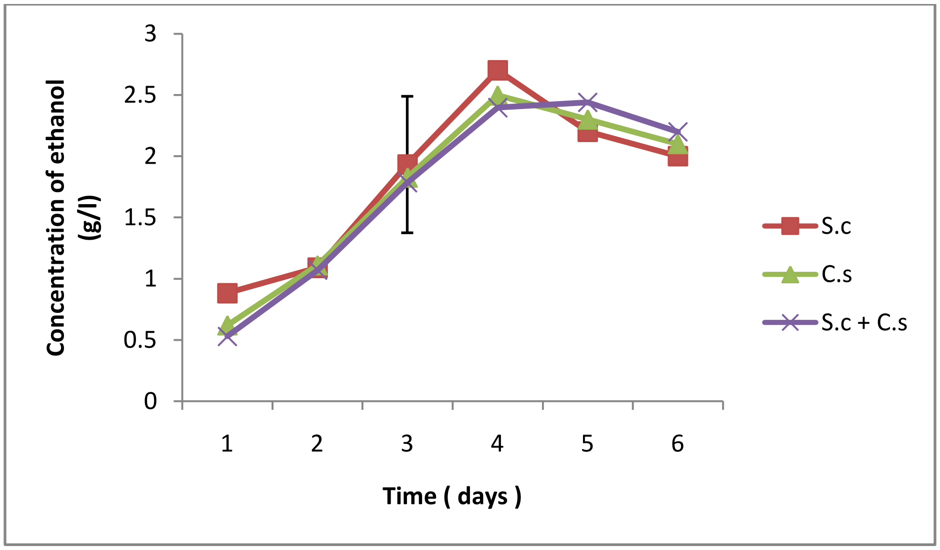

Figure 31 Ethanol concentration for untreated samples (g/l)

Untreated mixed leaf litter biomass samples were taken and checked for the sugar content (Table 31; Figure 31) .Maximum concentration of ethanol 2.7 g/l was observed in fourth day from the organism *Saccharomyces cerevisiae* and *Candida shehate*. With the increase in the temperature, sugar content also increases during initial stages of fermentation in case of untreated samples, during the end of fermentation time sugar content decreases and maximum production of ethanol takes places during later stages.

Pretreated samples

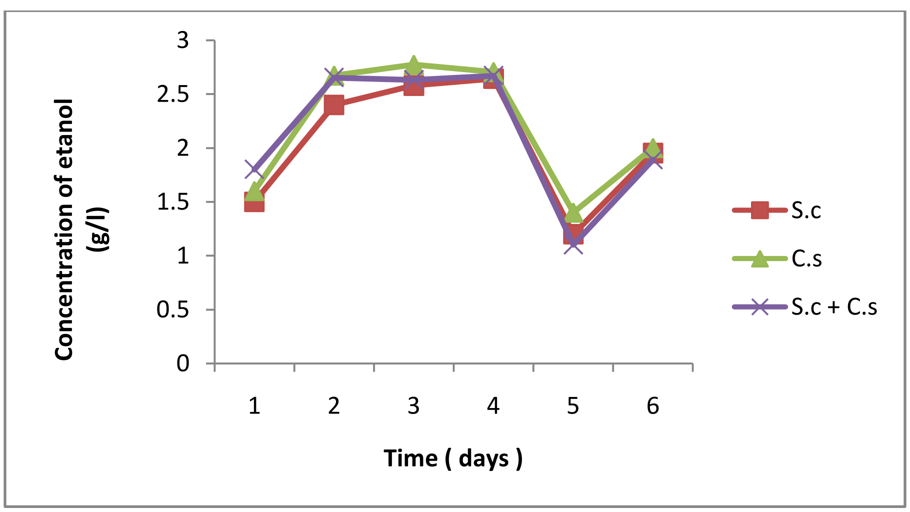

Figure 32 Ethanol concentration for pretreated samples (g/l)

Mixed leaf litter biomass samples were subjected to acid pretreatment and thereafter microbially treated for production of ethanol (Table 32 ; Figure 32). Maximum production of ethanol 2.67 g/l was observed on fourth day after fermentation with *Saccharomyces cerevisiae* and *Candida shehate.* In case of pretreated samples, highest amount of ethanol produced during later stages of fermentation. With the increase in the temperature maximum production of ethanol takes place in case of pretreated biomass.

Summary

1. Native untreated mixed lignocellulosic leaf litter biomass was checked for pH and EC, Organic carbon, TKN, Cellulose, Hemicellulose, lignin and reducing sugars pH was found to be 6.57 and EC 3.71 mS/cm and the percentage of biomass was 22.2% for organic carbon, 17.11 % for cellulose, 8.40 % for hemicellulose, 8.67% in case of lignin.
2. Biodegradation of untreated and pretreated mixed leaf litter biomass was carried out for ten days and amount of sugar present was checked by DNS method and ethanol was checked by DBP method. Due to degradation of cellulose and hemicelluloses, sugar content was increased 8.68 mg/g during initial days and amount of ethanol was 4 g/l produced.
3. Untreated and pretreated mixed leaf litter biomass samples were also checked at different pH conditions such as at pH 4.5 and 5.5 for the release of sugar and amount of ethanol produced. It was found maximum in pretreated samples, where sugar content was 2.59 mg/g and ethanol 3.75 g/l. At pH 5.5, 2.38 mg/g of sugar and 3.85 g/l of ethanol was formed.
4. Mixed leaf litter biomass samples both untreated and pretreated was also checked at different temperatures such as 25 and 35 ˚C. At temperature 25˚C, 7.09 mg/g of sugar and 2.75 g/l of ethanol was observed and at 35 ˚C 6.88 mg/g of sugar and 2.70 g/l of ethanol was observed.

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