Optimization of Pretreatment Process for Bioconversion of Lignocellulosic Waste into Bioethanol

May 2008

Submitted in partial fulfillment of the Degree of Bachelor of Technology

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CERTIFICATE

This is to certify that the work entitled **"Optimization of Pretreatment Process for Bioconversion of Lignocellulosic Waste into Bioethanol"** submitted by Shipra Singh (041510) and Rimi Rastogi (041515) in partial fulfillment for the award of degree of Bachelor of Technology in Bioinformatics of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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ACKNOWLEDGEMENT

It is with great pleasure and respect that we take opportunity to express our sincere thanks and gratitude to all those persons who are always related with us and given us a spiritual excitement time to time.

It gives us immense pleasure to express our sincere gratitude to our head of the department **Dr. R S Chauhan** for his invaluable and benevolent guidance at each stage of this work.

We gratefully extend our sincere thanks to **Dr. Sudhir Syal**, our project guide, whose untiring willingness to help, counsel and encourage at every juncture of present project work. Without whom this work could not have been possible. We are thankful to Mrs. Hemant Sood and Mrs. Mamta Mishra for their kind cooperation and help, which enabled me to accomplish this work. We also express our sincere gratitude to all senior members, staff, and technicians of biotechnology laboratory of our college.

We are also thankful to all our dear friends & relatives whose affection and encouragement always makes us do things differently to lead the present and coming era.

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LIST OF ABBREVIATIONS

CO_2.....Carbondioxide

Nm.....Nanometers

w/v.....Weight by Volume

CMC.....Carboxy Methyl Cellulose

MW.....Mega Watt

MPa.....Mega Pascal

WHO.....World Health Organization

DNS.....Dinitrosalicylic Acid

FPA.....Filter Paper Assay

ABSTRACT

Environmentally sustainable energy sources are needed to cater the increasing energy demands of rapidly growing industries. Biomass is an attractive, cheap, abundant and renewable source that can contribute to a cleaner environment .Plants have contributed to drugs, paper, adhesives and fuel industries. Production of bioethanol from crops like sugarcane, maize, corn, wheat and sorghum is well established but using a cheaper, diverse and rapidly renewable substrate like lignocelluloses can contribute to a clean green solution. The utilization and processing of lignocellulosic biomass is complex, differing in many aspects from crop based ethanol production. There are mainly two processes involved in conversion: - hydrolysis of cellulose to produce reducing sugars and fermentation of sugars to ethanol. Bioconversion of lignocelluloses can contribute enormously to the production of organic chemicals in an eco friendly manner[1]. The main challenges in the current technologies are the low yield and high cost of hydrolysis process. Efforts are being made to improvise on the pretreatment process to significantly enhance the hydrolysis of cellulose. Use of robust enzymes / microorganisms is the solution to various inhibitors such as acetic acid, furfural, hydroxyl methyl furfural and gypsum and the lignin released during the hydrolysis. The development of a cost – effective bioprocess for utilization of lignocellulosic biomass will be a significant solution for energy crisis[2].

CHAPTER-I

INTRODUCTION

The technology of producing ethanol from agricultural products has been known for quite some time. However, production of ethanol from primary agricultural products is often not cost-effective because the value of the crops often exceeds the value of the ethanol produced. However, the ready availability of lignocellulosic waste from herbal drug industries provides added advantage of not only reducing the expenditure incurred for its disposal but also recycling of waste into valuable products of economic importance.

The project thus aims at developing technologies for recycling herbal waste. Bioconversion of lignocellulosic waste from agriculture - like herbal waste - for production of bioethanol instead of burning or disposal by other means could indeed be an effective method in reducing environment pollution[3]. Bioethanol is considered as the most promising and useful product from agricultural waste, as it is a high performance fuel used in internal combustion engines. Bioethanol, when produced from lignocellulosic materials, is also an important CO_2-neutral energy source that has a great potential of reducing the worldwide environmental impact caused by the fossil fuel used in the transportation sectors.

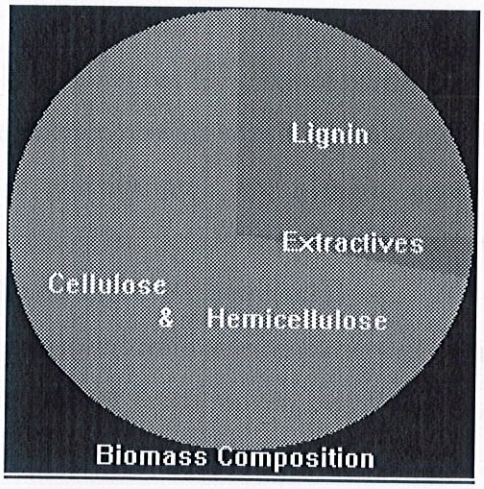
1.1. Why bioethanol?

Ethanol is a clean-burning, renewable and biodegradable fuel that could be produced from biomass resources in nearly every region of the country.

Bioethanol fuel is mainly produced by the sugar fermentation process, although it can also be manufactured by the chemical process of reacting ethylene with steam. Ethanol or ethyl alcohol (C_2H_5OH) is a clear colourless liquid; it is biodegradable, low in toxicity and causes little environmental pollution if spilt. Ethanol burns to produce carbon dioxide and water. Ethanol is a high octane fuel and has replaced lead as an octane enhancer in petrol. By blending ethanol with gasoline we can also oxygenate the fuel mixture so it burns more completely and reduces polluting emissions[4].

1.2. Biomass as potential substrate

Biomass is considered to be one of the key renewable resources of the future at both small- and large-scale levels. It already supplies 14 % of the world's primary energy consumption. But for three quarters of the world's population living in developing countries biomass is the most important source of energy. With increases in population and per capita demand, and depletion of fossil-fuel resources, the demand for biomass is expected to increase rapidly in developing countries. On average, biomass produces 38 % of the primary energy in developing countries. Biomass is likely to remain an important global source in developing countries well into the next century[5].



Graph.1. Biomass Composition[6].

The chemical composition of lignocellulosic biomass varies; the major components being cellulose (30-50%), followed by hemicellulose (20-35%) and lignin (10-25%)[7]. The carbohydrate fraction consists of many sugar molecules linked together in long chains or polymers. Two larger carbohydrate categories that have significant value are cellulose and hemi-cellulose. Nature uses the long cellulose polymers to build the fibers that give a plant its strength.

Biomass is called as a renewable resource since green plants are essentially solar collectors that capture and store sunlight in the form of chemical energy. Growing plants remove carbon dioxide from the atmosphere that is released back to the atmosphere when biomass fuels are used. Thus the overall concentration of atmospheric carbon dioxide

should not change, and global warming should not result. Cellulose is the single most important component of plant biomass[8] like starch; it is made of linked sugar components that may be easily fermented when separated from the cellulose polymer. The complex structure of cellulose makes separation difficult, but enzymatic means are being developed to do so. The biochemical conversion of biomass to ethanol currently involves three basic steps: **(1)** thermochemical treatments of raw lignocellulosic biomass to make the complex polymers more accessible to enzymatic breakdown; **(2)** production and application of special enzyme preparations (cellulases and hemicellulases) that hydrolyze plant cell-wall polysaccharides to a mixture of simple sugars; and **(3)** fermentation, mediated by bacteria or yeast, to convert these sugars to ethanol. Perfection of this technology will create a large potential for ethanol production using plant materials that are not human foods. The efficiency with which biomass may be converted to ethanol or other convenient liquid or gaseous fuels are a major concern. Conversion generally requires appreciable energy. The presence of lignin in the biomass lowers the biodegradability both of the cellulose and hemicellulose[9].

Cellulosic ethanol can be produced from a wide variety of cellulosic biomass feedstocks including agricultural plant wastes (corn stover, cereal straws, and sugarcane bagasse), plant wastes from industrial processes (sawdust, paper pulp) and energy crops grown specifically for fuel production, such as switchgrass[10].

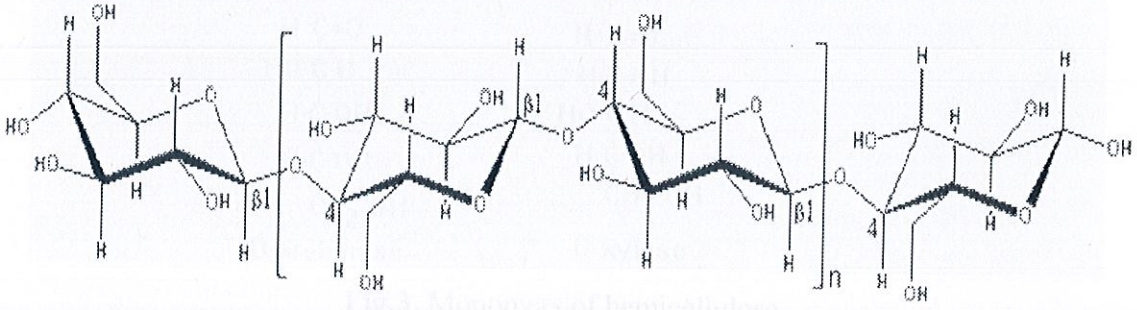


Fig.1. Structure of cellulose.

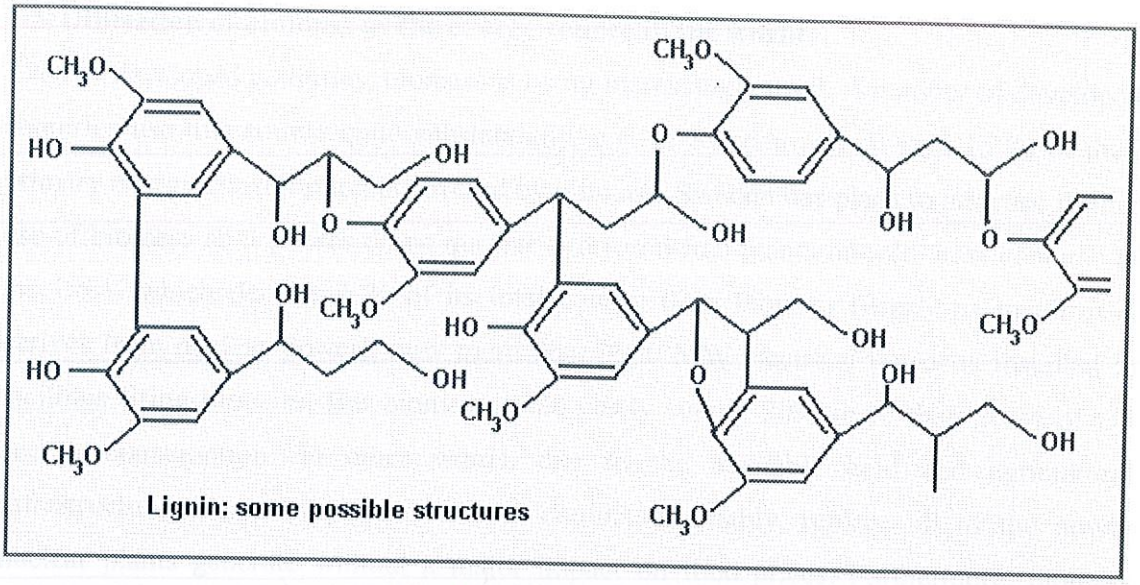


Fig.2. Structure of lignin.

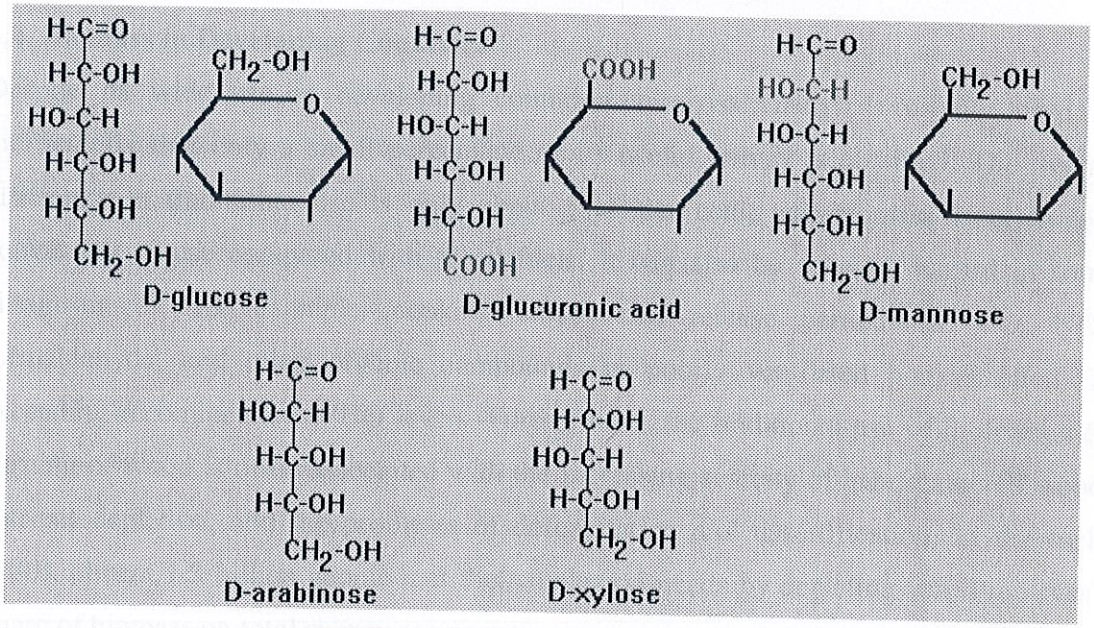


Fig.3. Monomers of hemicellulose.

1.3. Utilisation of biomass as the energy source in the world

Even in developed countries, biomass is being increasingly used. A number of developed countries use this source quite substantially, e.g. in Sweden and Austria 15 % of their primary energy consumption is covered by biomass. Sweden has plans to increase further use of biomass as it phases down nuclear and fossil-fuel plants into the next century. In the USA, which derives 4 % of its total energy from biomass (nearly as much as it derives from nuclear power), now more than 9000 MW electrical power is installed in facilities firing biomass. But biomass could easily supply 20% more than 20 % of US energy consumption. In other words, due to the available land and agricultural infrastructure this country has, biomass could, sustainably, replace all of the power nuclear plants generate without a major impact on food prices. Furthermore, biomass used to produce ethanol could reduce also oil imports up to 50%[5].

1.4. Biomass in Developing Countries

Despite its wide use in developing countries, biomass energy is usually used so inefficiently that only a small percentage of its useful energy is obtained. The overall efficiency in traditional use is only about 5-15 per cent, and biomass is often less convenient to use compared with fossil fuels. It can also be a health hazard in some circumstances, for example, cooking stoves can release particulates, CO, NOx formaldehyde, and other organic compounds in poorly ventilated homes, often far exceeding recommended WHO levels. Furthermore, the traditional use of biomass, i.e., burning of wood is often associated with the increasing scarcity of hand-gathered wood, nutrient depletion, and the problems of deforestation and desertification. In the early 1980s, almost 1.3 billion people met their fuelwood needs by depleting wood reserves[4].

Share of biomass on total energy consumption:

Nepal 95 %

Kenya 75 %

India 50 %

China 33 %

Brazil 25 %

Egypt 20 %

There is an enormous biomass potential that can be tapped by improving the utilization of existing resources and by increasing plant productivity. Bioenergy can be modernized through the application of advanced technology to convert raw biomass into modern, easy-to-use carriers (such as electricity, liquid or gaseous fuels, or processed solid fuels). Therefore, much more useful energy could be extracted from biomass than at present. This could bring very significant social and economic benefits to both rural and urban areas. The present lack of access to convenient sources limits the quality of life of millions of people throughout the world, particularly in rural areas of developing countries. Growing biomass is a rural, labour-intensive activity, and can, therefore, create jobs in rural areas and help stem rural-to-urban migration, whilst, at the same time, providing convenient carriers to help promote other rural industries[5].

1.5. Microorganisms producing cellulose-degrading enzymes

A large no of microorganisms including fungi, bacteria and actinomycetes have the ability to produce cellulose-degrading enzymes. But few of them produce all the necessary enzymes for degradation of crystalline cellulose. Fungi are the most studied microorganism with respect to degradation of cellulose and production of cellulolytic enzymes. Soft-rot fungi mainly degrade the polysaccharides. The best known of these producing a complete set of cellulases is ***Trichoderma viride***[11]. Other well known fungi causing soft-rot are ***Aspergillus niger, Chaetomium cellulolyticum, Neurospora crasa Pennicillium pinophilum***[12]. Brown rot fungi causing brown rot in plants degrade cellulose rapidly but the enzyme system seems to operate differently from those of soft-rot fungi and white-rot fungi[13].

Cellulotic enzyme systems of bacteria are not directly comparable to those of fungi. Bacteria often produce cellulases in small amounts and degradation of cellulose seems to take place by a cluster of multi-enzyme complexes, which are difficult to disrupt without the loss of total activity as well as of the individual components[14]. The most studied bacteria respect to cellulose systems are species of ***Clostridium, Cellulomonas, Bacillus and Pseudomonas***.

T. reesei has several advantages for industrial-scale production of homologous and heterologous cellulases and proteins in general. ***T. reesei*** is easy and inexpensive to

cultivate and it is currently grown in fermenters[15]. ***T. reesei*** has secretory machinery with protein modifications typical of eukaryotes[16]. ***T. reesei*** is considered to be a safe production organism, because it is non-pathogenic to healthy humans and does not produce mycotoxins or antibiotics under the conditions used for enzyme production[17]. ***T. reesei*** produces extracellular proteins and cellulases naturally in large quantities. Industrial ***T. reesei*** strains have been obtained by using classical mutagenesis techniques to enhance the release of extracellular proteins in general, to increase cellulose production levels.

1.6. Possible methods involved

The standard concept for producing ethanol from lignocellulosic biomass requires a biological/physical/chemical pretreatment. This is typically done using wet oxidation or steam explosion of macerated biomass to open the fibers and make them available for enzymatic attack. After the pretreatment, the hydrolysate is typically treated with microbial enzymes to release sugars that can be fermented to ethanol. The fermentation can be carried out either by a single microbial strain able to utilize all sugars in the hydrolysate or by a two-step concept using two different microorganisms, each optimized to convert a specific array of sugars. One of the significant obstacles in the bioethanol production process is the inhibitory effect of the lignin- and carbohydrate-derived compounds formed during pretreatment of the lignocellulose[1] when recirculation of process water is applied in production process, the inhibitory problems become even more critical. It is, therefore, important not only to investigate microorganisms that are capable of fermenting different sugars from lignocellulolytic waste under high substrate concentrations but it is also essential to recognize the need to identify and utilize microorganisms that can tolerate the inhibitory compounds in hydrolysates without expensive detoxification. The herbal waste from industries is a heterogenous mixture and, therefore, will require a blend of microbial strains for its decomposition and fermentation.

Table.1. Pretreatment Methods

METHOD	PROCESS	DISADVANTAGE
Steam Explosion	Chipped biomass is treated	Destruction of xylan

	with high pressure (0.69-4.83MPa) saturated steam at a temperature b/w 160-260°C and then the pressure is swiftly reduced which makes the materials to undergo an explosive decompression.	fraction. Incomplete disruption of the lignin-carbohydrate matrix. Generation of inhibitory compounds to microorganisms.
Acid Hydrolysis	Con. Acids and dilute acids like H_2SO_4 & HCL are used to treat the lignocellulosic materials.	Toxic, corrosive&hazardous. Requires special reactors which can withstand corrosion. Sugars are converted to inhibitory components. Acids must be recovered after hydrolysis Neutralization of pH in sugar solution is required.
Alkaline Hydrolysis	Dilute NaOH treatment of lignocellulosic material results in the Saponification of intermolecular ester bonds cross linking b/w lignin and cellulose	No good results and a slow process.
Oxidative delignification. OX-B treatment	Lignin is degraded by the peroxidase enzyme with the presence of H_2O_2. No energy input required. High cellulose digestibility	Price disadvantage.

Biological Pretreatment	Brown, white and soft rot fungi are used to degrade the lignin and hemicellulose.	Rate of hydrolysis is too slow and the yield is also too low.

1.7. Bioconversion

Enzymatic saccharification of lignocellulytic materials produces monosaccharrides, which subsequently can be fermented into a variety of products such as ethanol, other alcohols, organic acids, single cell protein, and lipids[12]. Bioethanol can be produced by using agricultural residues such as cereal straws and corn stalks and from industrial plant waste. It is an advanced new transport fuel with a unique combination of attributes including low life-cycle greenhouse gas emissions, a high level of sustainability, seemless integration into existing transport system and has the potential to have a large scale impact[18].

The key challenge in lignocellulosic biotechnology is to develop a robust enzyme production system that can produce adequate amounts of highly efficient cellulose to make biomass conversion more rapid and less expensive. Complete cellulolytic and hemicellulolytic enzyme systems are required to achieve maximum hydrolysis of complex substrates to yield monomeric sugars. Sugar obtained in saccharification processes can be further utilized for the production of protein-rich biomass.

OBJECTIVES OF THE PROJECT

- Isolation of cellulose degrading microorganisms from soil ecosystems of forest and decaying wood.
- Screening and comparative production of cellulose degrading microorganisms and their characteristics.
- Optimization and development of an efficient biological pretreatment system for delignification and bioethanol production from plant waste.
- Development of microbial consortia for enhanced bioethanol production from mixture of plant waste.
- To study the amount of sugars released after enzyme saccharification.
- *In situ* treatment of plant waste at laboratory scale.

CHAPTER-II

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Soil: The soil sample was collected from a nearby area where there was high density of wild plants and also from the vermi-composting unit.

2.1.2. Wood sample: Decaying wood sample was also collected from a site nearby where there were chances of finding potential microbes for degradation.

2.1.3. Samples from Palampur: Potential lignocellulytic fungi samples were obtained from Palampur.

2.1.4. Herbal waste procured from AyurVet Limited, Baddi: There were three different types of herbal plant waste **Dianoak, Streseroak, Superliad DS Premise** that were collected from the industry. It mainly consisted of dried plant parts like leaves, stem, twigs etc.

2.1.5. Chemicals: The chemicals used in the present study were obtained from S.D fine chemicals Limited, Merk Limited and Qualigens fine chemicals Limited. The various media used in the study namely Nutrient Broth, Nutrient Agar and Bushnell-Hass were manufactured by HiMedia Laboratories Pvt. Ltd. The commercially available cellulase enzyme was obtained from SIGMA.

2.1.6. Composition of media used for fungus is as under:

1) Carboxy Methyl Cellulose -10gm
2) Ammonium sulphate -0.5gm
3) L-Asparagine -0.5gm
4) Potassium dihydrogen phosphate -1gm

5) Potassium chloride	-0.5gm
6) Magnesium sulphate	-0.2gm
7) Calcium chloride	-0.1gm
8) Agar	-20gm

Note: The final pH of the medium is adjusted to 6.2. This media is basically used for the isolation and maintenance of cellulose-decomposing fungi.

2.2. METHODS

2.2.1. Selection of Site and Collection of Samples

Soil sample was taken from the area where soil was not disturbed and there was high density of wild plants. Soil samples were collected from their respective sites and taken to the lab for further processing. Soil samples were serially diluted and then spreaded on agar plates for different concentrations.

The wood sample was taken from a decaying trunk; the sample was spreaded, sprinkled and serially diluted on the agar plates respectively.

2.2.2. Characterization of the colonies obtained

Colonies were observed on the plates after incubating them for 24 hrs. Out of the various colonies that were obtained on the agar plates we took the colonies and inoculated them on cellulose agar plates, and only few of them were able to grow on it. Their colony characteristics were noted and gram nature was studied.

2.2.3. Liquid culture

The media mentioned above was used for the growth of potential fungal strains to obtain pure cultures for the strains.

2.2.4. Screening of Strains for Enzyme Production

- Clear zone experiment was carried out for various strains. The CMC plates were stained with congo red. Plates were destained with a 1 M solution of NaCl. Where

CMC hydrolysis occurred, a clear zone or yellowish halo was present around the fungal growth. If no degradation of CMC occurred, the plate remained red after staining with Congo red and destaining with 1 M NaCl.

- **Step 1**: Media plate inoculated with fungus.
- **Step 2**: Plates flooded with Congo Red Dye (0.1% w/v).
- **Step 3**: Plate destained with Sodium chloride (1M) solution after which clear zone was observed.

2.2.5. CMCase Activity

This assay is based on the ability of enzyme to hydrolyze CMC to reducing sugars. The same DNS Assay was conducted for the liquid cultures of the fungal strains after 5-7 days of inoculation.

2.2.6. Growth of fungal cultures

T.reesei strain was grown on 2.4% PDA at 28°C for 5-6 days.

2.2.7. Pretreatment Methods

2.2.7.1. Oxidative Delignification by OX-B Treatment

The biomass (5gm) was mixed with OX-B solutions at room temperature. The treatment was carried out for 40 minutes. After treatment the solids were recovered, washed with 100ml distilled water. The washed solid particles were then suspended for one hour in 0.6% NaOH at 25°C. The treated solids were again filtered washed with 100ml of distilled water and then analyzed for enzyme digestibility and weight loss[19]. In the original protocol the OX-B solution was made with six percent sodium hypochlorite and fifty percent hydrogen peroxide in 10:1 ratio but we made the OX-B solution with thirty percent hydrogen peroxide and four percent sodium hypochlorite in the original ratio.

2.2.7.2. Alkaline Hydrolysis

The biomass (5gm) was mixed with 1% NaOH solution (ratio 10:1) and kept for 2 hours at room temperature. After treatment the mixture was washed with distilled water and

autoclaved at 121°C for one hour. After autoclaving the mixture was again washed with distilled till the pH was 7.

2.2.7.3. Steam Hydrolysis

Grinded biomass is treated with high pressure (20-25 Psi) saturated steam at a temperature between 120-180°C for 2 hours and then the pressure is swiftly reduced which makes the materials to undergo an explosive decompression.

2.2.7.4. Ethanolysis

Herbal waste (15 gm) was taken and mixed with 60% ethanol (90 ml), here the liquor to wood ratio was 6:1 and was kept in a steel container at 140-200 °C for 2 hours. After heat treatment it was then cooled in ice water bath. Black liquor and waste were separated by filtering. Waste collected was washed twice with 50% ethanol (150 ml) and then was again washed with 300 ml distilled water and pulp fraction was obtained. The liquor collected after washing was freeze dried and soluble fraction was obtained.

2.2.7.5. Enzyme Hydrolysis

Grinded herbal waste (5 gm) was taken and mixed with 10 ml of distilled water. It was then sterilized at 121°C for 20 minutes. Discs of grown fungi were taken and the sterilized waste was inoculated. It was kept under stationary conditions at 28°C for 2-8 weeks. Slurry was made by addition of autoclaved distilled water and the sample was then analyzed for reducing sugars.

2.2.8. Determination of the weight loss

Weight loss was determined by weighing biomass before and after treatment either by directly weighing the biomass or after drying in an oven at 80°C to a constant weight.

2.2.9. Determination of lignin concentration

For each 0.3g of sample, 3ml of sulphuric acid (72%) is added and the mixture is swirled at room temperature. After 1 hour 50ml of distilled water is added and the sample is incubated at 120°C, in a dry oven for 1 hour. After cooling to room temperature, the

samples are separated in to liquid and solid fractions by filtration. From the solid fraction the lignin concentration is determined by measuring weight after drying at 80°C for 24 hours.

2.2.10. Enzyme saccharification and Fermentation

a). ***T.reesei*** was grown in the nutrient media and yeast inoculums were also prepared. 0.25 gm of ethanolysed pulp was taken and mixed with 2.5 ml of nutrient media and yeast inoculum each.2.5 ml of 0.05 M Sodium Citrate buffer (pH 5) was added and cellulose (10 FPU)was also mixed. The above mixture was then kept at 100 rpm for 96 hours at 37°C. The aliquots of the sample were collected after centrifugation at 4000 rpm for 10 minutes at 4 °C.

b). The pretreated pulp was inoculated with ***T.reesei*** and kept at 28°C for 7 days. Then saccharification was done by adding 0.1 M Citrate Buffer (pH 4.8) and kept at 150 rpm at 50°C for 2 days. Samples were withdrawn after intervals of 16 hours, centrifuged at 5000 rpm for 20 mins and the supernatant was analysed for reducing sugars[20].

2.2.11. DNS Assay[21]

DNS Reagent: Accurately weigh 10.6 gm of DNS into 2000 ml beaker. Add 19.8 gm of Sodium Hydroxide, 306 gm of Potassium Sodium Tartrate and 1000 ml of water. Heat the beaker and stir to dissolve.

Glucose standard: Take 1 ml sample each of different concentrations of glucose and make a standard curve.

Table.2. Glucose standard curve for DNS Assay.

Standard	Concentration (mg/ml)	Absorbance (540 nm)
S1	0.5	0.031
S2	1.0	0.110
S3	2.0	0.461
S4	4.0	1.052
S5	5.0	1.250
S6	10.0	2.434

Take 1 ml of sample and add 3 ml of DNS reagent into it. Stir well. Keep in boiling water bath for 5 minutes. Cool it to room temperature. Add 20 ml of water to dilute it and then take the absorbance at 540 nm.

CHAPTER-III

Results and Discussion

3.1. Selection of Site and Collection of Samples

Soil sample was collected from the vermicompost unit (Fig.5.a) of our college where plant waste, organic matter etc was being degraded by microflora and by exotic species of earthworm named as ***Eudrilas eugeniae***. The sample was taken to lab packed in aseptic bags for further processing. Wood sample was collected from a site nearby; the tree (Fig.5.b) seemed to be very old and was covered with microbial growth that were degrading it.

Fig.4. Site of collection of sample **a).** Vermicompost unit. **b).** Decaying wood.

The soil sample that was collected was serially diluted and we inoculated the PDA plates with different serial dilutions and got the following results after 3 days of inoculation.

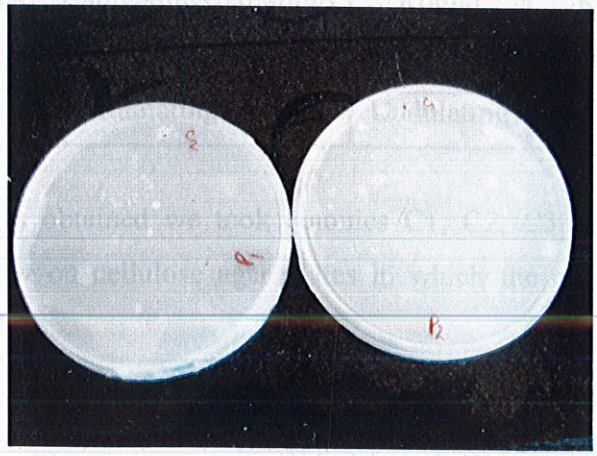


Fig.5. Bacterial growth on agar plates (sample C1 and C2)

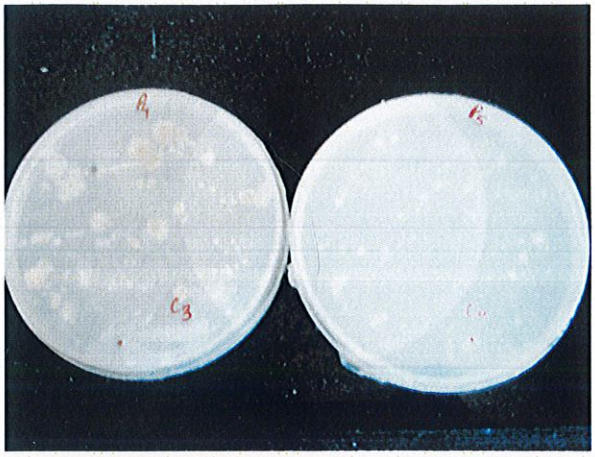


Fig.6. Bacterial growth on agar plates (sample C3 and C4)

3.2. Characterization: Different types of Bacterial Colonies obtained

Table.3. Different types of Bacterial Colonies obtained

	Whole Colony	Configuration	Edge	Elevation	Gram Nature
C1	**Irregular**	**Spreading**	**Undulating**	**Raised**	**+ve**
C2	**Irregular**	**Spreading**	**Wavy**	**Flat**	**-ve**
C3	**Irregular**	**Spreading with radiating margin**	**Wavy**	**Flat**	**-ve**
C4	**Irregular**	**Spreading**	**Smooth**	**Flat**	**+ve**
C5	Irregular	Spreading(radiating)	Round	Raised	+ve
C6	Merged	Irregular	wavy	concave	-ve
C7	Round	Radiating Margin	Undulating	Flat	-ve

Out of these colonies obtained we took colonies C1, C2, C3 and C4 randomly and allowed them to grow on cellulose agar plates in which the only carbon source was carboxymethyl cellulose.

After a growth of 5 days it was observed that the respective bacteria had grown and it implies that a part of cellulose that was present in the media was utilized.

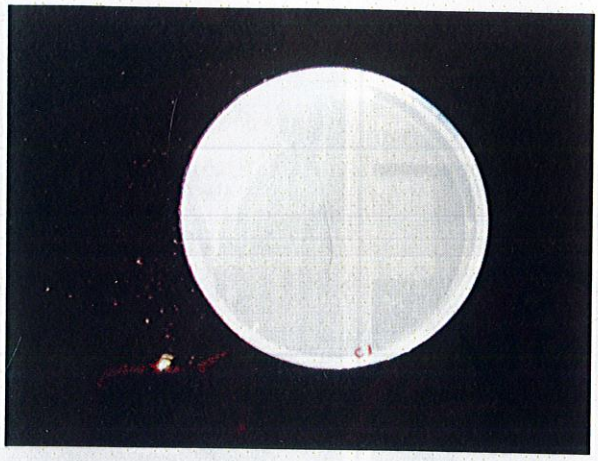


Fig.7. Bacteria sample C1 on cellulose agar plate.

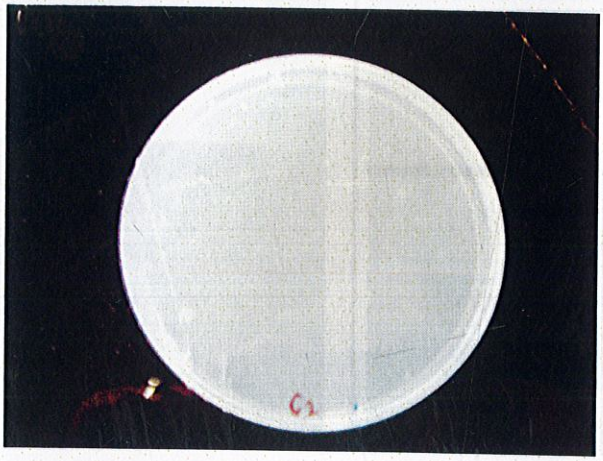


Fig.8. Bacteria sample C2 on cellulose agar plate.

3.3. Liquid culture

The media that was mentioned earlier in section 2.1.6 was taken and was inoculated with the potential cellulose degrading fungi that were obtained from **Chaudhary Sharvan Kumar HP Krishi Vishwavidyalay, Palampur**, just to check their growth in cellulose specific media. Fungi were grown in liquid culture so that large amount of cell mass could be obtained. The fungal strains that were used are: ***Peziza*** (3250), ***Hypholoma*** (3215), ***Auriwharia amicula*** (1580), ***Coriolus*** (3262), ***Pluerotus*** (3324), ***Coriolus*** (1678), Unidentified (3359), ***Hypholoma*** (3017), ***Mycopous sp.*** (1674),

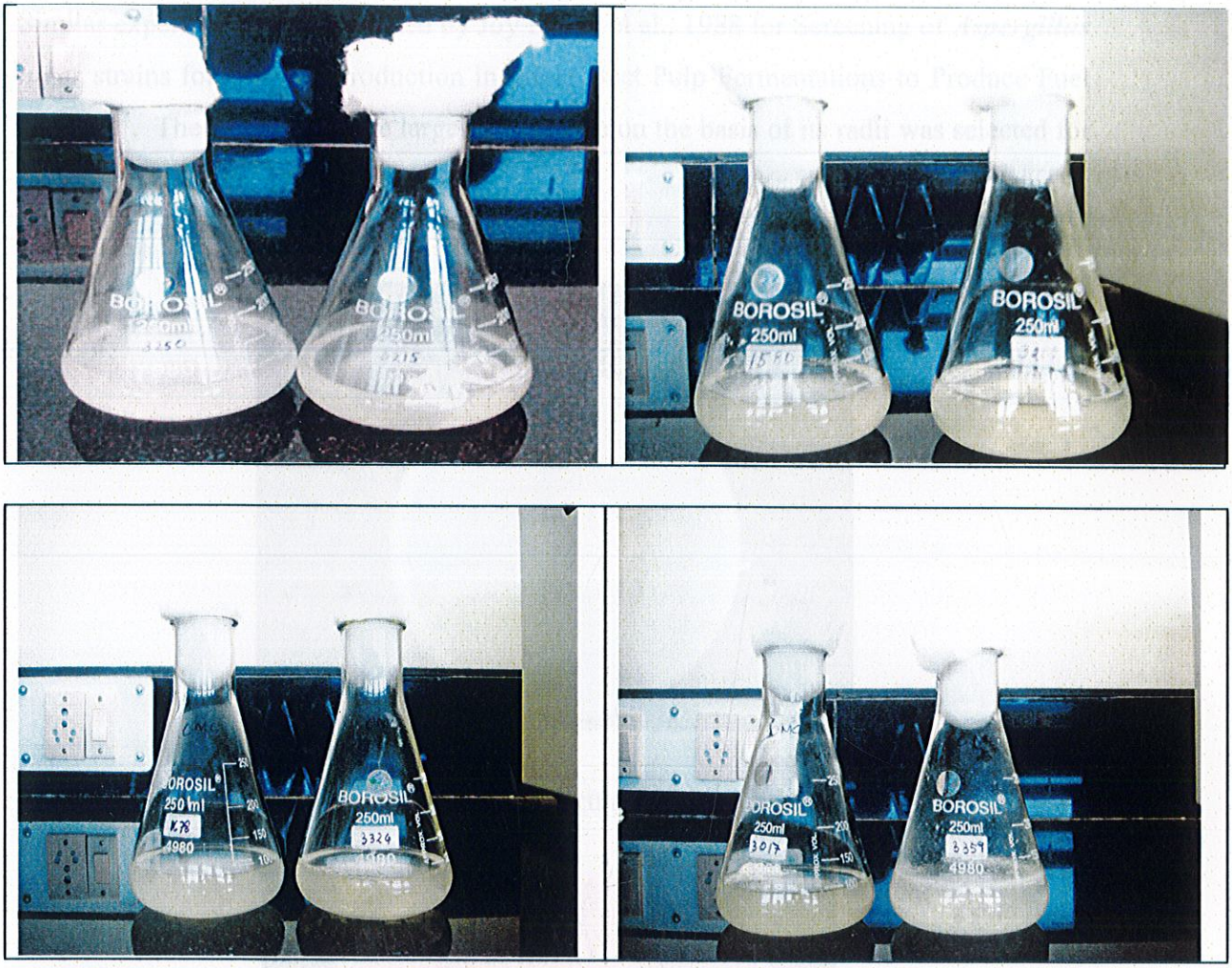


Fig.9. Liquid culture of microbes.

3.4. Screening of Strains for Enzyme Production

The experiment conducted helped us to study the enzymatic activity of the various cellulose degrading bacteria and fungus when grown specifically on plates containing CMC as the only carbon source. After 5 days of incubation at 22°C, CMC plates were stained with Congo red. After destaining with NaCl and distilled water, zones of clearance were visible around the fungal growth as a result of enzymatic activities. This showed that fungi utilized CMC present in the media for its growth and the places from where CMC was taken up gave clear zones after destaining. The clear zone formed shows the cellulose degrading activity of the various fungi/bacteria inoculated. We conducted this experiment on 9 strains of the fungi that we obtained and there were significant clear zones formed.

Similar experiment was conducted by Joy Doran et al., 1988 for Screening of ***Aspergillus niger*** strains for Enzyme Production in Sugar Beet Pulp Fermentations to Produce Fuel Ethanol[23]. The strain that gave largest clear zone on the basis of its radii was selected for ethanol production.

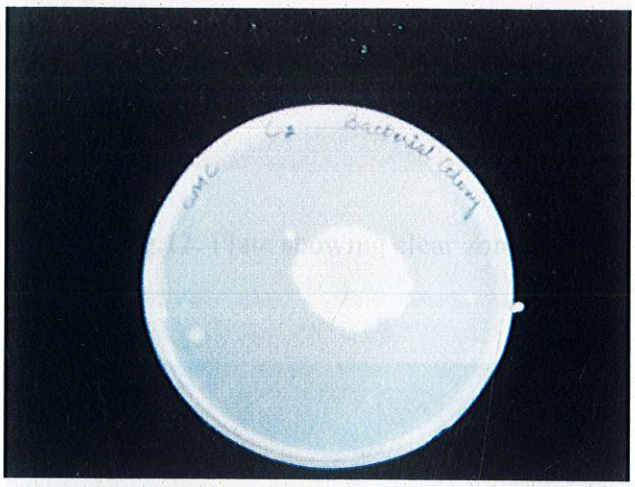


Fig.10. Media plate inoculated with fungus.

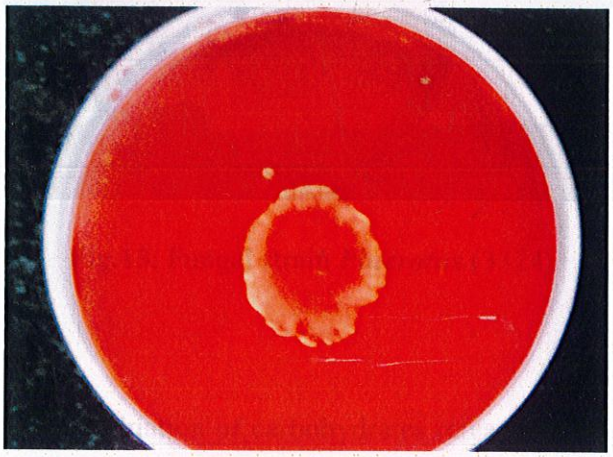


Fig.11. Plates flooded with Congo Red Dye (0.1% w/v).

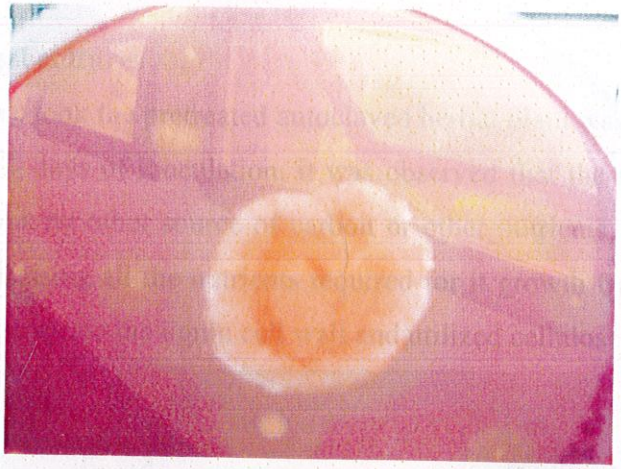


Fig.12. Plate showing clear zone.

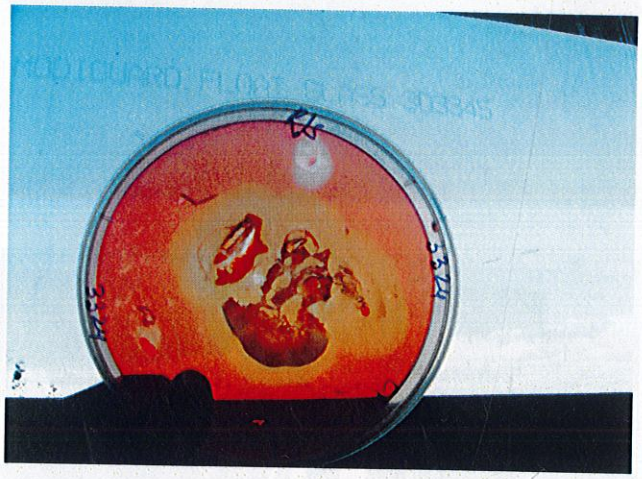


Fig.13. Fungal strain ***Pluerotus*** (3324).

3.5. CMCase Activity

DNS assay was done for evaluation of carbohydrates released after the microbes present in the solution utilized the carboxymethyl in the media. It is based on the ability of the enzyme to hydrolyze carboxymethyl cellulose to reducing sugars. The results showed that it was quite easy for bacteria and fungi to utilize the synthetic form of cellulose that is present in the media and degraded it with the help of cellulase enzyme that is released. Glucose liberated by C1 is 0.12mg/ml and that of C2 is 0.13mg/ml.

3.6. Growth of fungal cultures

In this experiment, we took the pretreated autoclaved herbal plant waste and inoculated it with ***T.reesei.*** After 7 days of inoculation, it was observed that the fungus grew on the waste. Since there was no other source of carbon or other nutrients, this shows that the fungus was very well using all the nutrients required for it growth from the waste itself. Thus, it was able to penetrate the lignin cell wall and utilized cellulose for its growth.

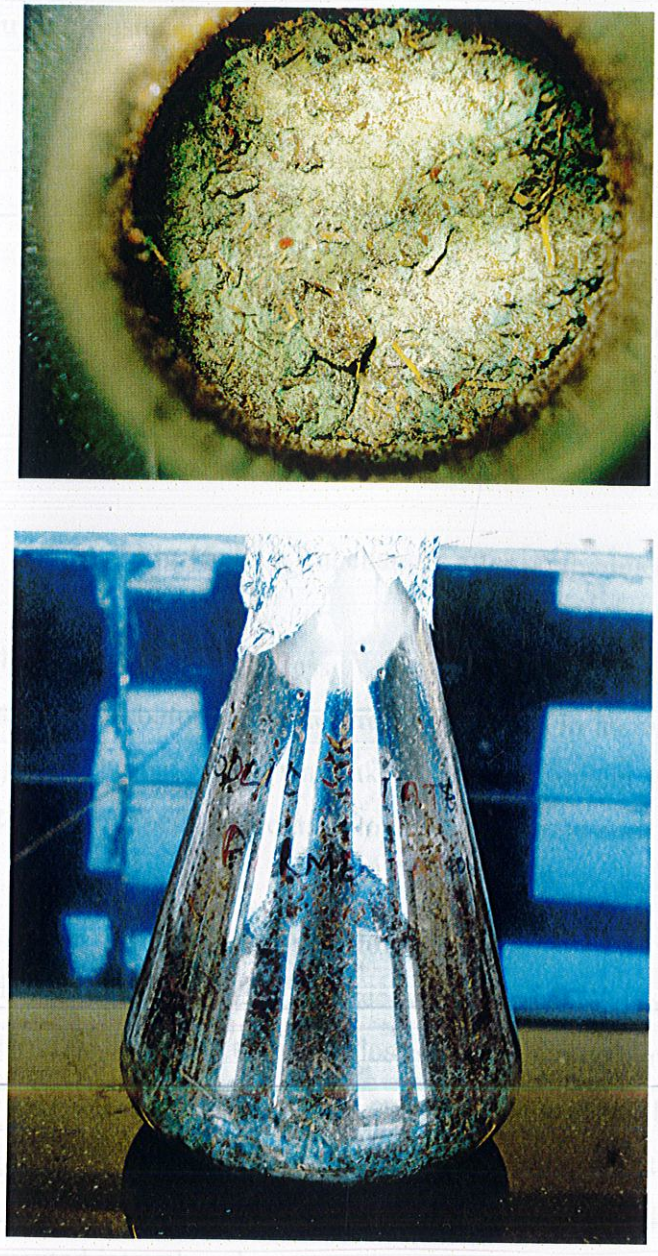


Fig.14. Fungus growing on pretreated herbal waste.

3.7. Pretreatment Methods

Since the herbal sludge to be used for ethanol production was purely of lignocellulosic nature they had to be pretreated. To enhance the degradation and removal of lignin from lignocellulosic materials, pretreatments are designed to open the structure of lignocellulosic biomass prior to enzyme hydrolysis to allow efficient production of C5 & C6 sugars. We applied different possible pretreatment techniques to study the degradation of lignocellulosic waste and the inoculated this pretreated waste with ***T.reesei***.

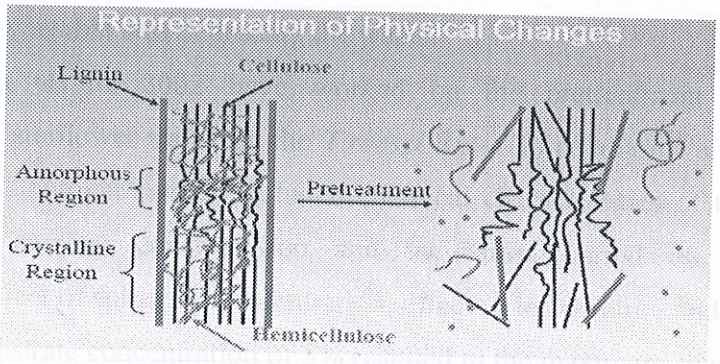


Fig.15. Physical changes after pretreatment.

3.8. Determination of the weight loss

Biomass was weighed before and after treatment, dried in an oven at 80°C to a constant weight. 2.5 gm of herbal waste was taken for pretreatment and 0.96g of waste was recovered after pretreatment. Percentage loss is 61.6.

3.9. Determination of lignin concentration

0.9g of sample was taken and 0.36g was recovered. Percentage loss is 60. This shows that after pretreatment of the waste there was loss in the lignin content and thus it told us that the pretreatment method was effective in breaking down of the lignocellulosic cell wall. After the breakdown of the lignocellulosic cell wall the crystalline cellulose will be exposed and enzyme saccharification leads to production of reducing sugars which can further be fermented into ethanol.

3.10. Enzyme saccharification and Fermentation

After inoculation and growth for 7 days, autoclaved distilled water was added and slurry was made and kept for 24 hours at 28°C at stationary conditions and then DNS and Filter Paper Assay were done. Since fungus had already grown well and penetrated well in the waste, means that it utilized cellulose exposed after cell wall degradation and thus it converted the complex sugar cellulose into its monomer that is glucose that can be easily detected by estimating the reducing sugar in the slurry. To estimate the reducing sugar formed we conducted DNS Assay and Filter Paper Assay was done to check the activity of the cellulase enzyme that was degrading the waste.

Sanjeev K.Sharma et al., 2002 have worked on the pretreatment and enzyme saccharafication of sunflower stalks by the ***T.reesei*** Rut-c 30 cellulase[20] and studied the various parameters. The parameters included chemical characteristics of the pretreated waste, effect of enzyme concentration and incubation period on the rate of saccharafication, effect of substrate concentration, effect of temperature and effect of pH. They found that substrate concentration of 5% resulted in the maximum saccharafication, most suitable temperature for enzymatic hydrolysis was found to be 50°C, and pH 5 was found to be the optimum for enzymatic saccharification.

We performed DNS Assay and Filter Paper Assay for the sample kept for 96 hours also.

Table.4. DNS Assay of samples kept for 24 hrs and 96 hours.

Sample	Absorbance (540nm)	Concentration (mg/ml)
Test 1(24hours)	0.250	0.80
Test 2(96 hours)	0.323	0.92

The sample that was kept for 24 hours liberated approximately 0.8 mg/ml glucose and the sample that was kept at incubation for 96 hours after pretreatment liberated 0.92 mg/ml reducing sugars which shows conversion of cellulose into simpler sugars by the action of cellulose enzyme on the herbal waste.

3.11. DNS Assay

In this experiment we observed the gradual increase in formation and liberation of reducing sugars for 48 hours. Reducing sugar liberation was estimated after 16, 32 and 48 hours and we found increase in the liberation of reducing sugars with time.

Table.5. DNS Assay for production of reducing sugars in 48 hours.

Incubation time	Test 1	Test 2	Test 3	Test 4
16 hours	0.491	0.452	0.397	0.361
32 hours	0.522	0.476	0.423	0.402
48 hours	0.588	0.495	0.471	0.451

Conclusion

Bioethanol is an attractive, sustainable energy source to fuel transportation. Based on the premise that fuel bioethanol can contribute to a cleaner environment and with the implementation of environmental protection laws in many countries, demand for this fuel is increasing. Efficient ethanol production processes and cheap substrates are needed. Current ethanol production processes using crops such as sugar cane and corn are well-established; however, utilization of a cheaper substrate such as lignocellulose could make bioethanol more competitive with fossil fuel. In the present study, by using enrichment culture technique we were able to isolate a large number of potential bacteria and fungi from soil and degrading wood which could be utilized for the bioconversion of lignocellulosic biomass into ethanol. We carried out various pretreatment methods to increase the accessible surface area of cellulose to enhance the conversion of cellulose to glucose. We used Oxidative Delignification, Enzymatic Hydrolysis, Alkali Treatment, Steam Hydrolysis and Ethanolysis for pretreating the lignocellulosic plant waste. Enzyme saccharification of pretreated biomass was carried out by culture filtrate of ***T.reesei.*** Commercially available cellulase enzyme was also used for saccharification.

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List of publications

1). Rimi Rastogi, Shipra Singh, Sudhir Syal, Woods to Wheels, CURRENT SCIENCE, VOL. 94, NO. 6, 25 MARCH 2008