Bioconversion of Cellulosic agricultural wastes

A
DISSERTATION

Submitted in partial fulfillment of the requirements
for the Award of the Degree of

Masters of Technology
(Environment Science & Technology)

Under the Guidance of
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Candidate's Declaration

I, hereby declare that the work presented in the dissertation entitled **"Bioconversion of cellulosic agro-wastes"** in partial fulfillment of the requirement for the award of the degree of Masters of Technology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is an authentic record of my own work during the period of ten months from August 2007 to May 2008, under the supervision of Dr. Dinesh Goyal, Associate Professor, Department of Biotechnology & Environmental Sciences, Thapar University. The report has not been submitted for the award of any other degree or certificate in this or any other university.

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This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

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This is to certify that the thesis entitled "**Bioconversion of cellulosic agro-wastes**" submitted by Somesh Ajnavi in partial fulfillment of the requirements for the award of Degree of Masters of Technology in Environment Science and Technology to Thapar University, Patiala, is a record of student's own work carried out by his under my supervision and guidance. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

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(Somesh Ajnavi)

Abstract

Recycling of agricultural wastes comprising of garden waste (grass cutting) and leaf litter was carried out by different fungi (*Aspergillus niger* FS1 and *Trichoderma reseei* MTCC-164) and using cow dung and DAP (Di Ammonium Phosphate @ 0.1%) as activators. The results indicated that organic carbon was decreased from 28.6 % to 14.5% and cellulose from 534.4 ppm to 115.1 ppm, with concomitant increase in available nitrogen content and reducing sugars from 74.6 ppm to 356.5 ppm and from 117.7 ppm to 428.4 ppm respectively over 90 days of incubation. In garden waste cellulose was decreased by 77% and in leaf litter (comprising mainly with *Bambusa vulgaris* leaves) by 70 % when the biomass was treated with fungal consortia with the addition of 0.1% DAP as activator. The results indicated that activator like DAP enhances the rate of decomposition when agricultural wastes are either treated with fungal consortia or cow dung.

In field conditions, the mixed agricultural waste was given hot water pretreatment at 80-90 ºC for 1 hour residence time to accelerate the degradation process over a period of 42 days. The results showed that organic carbon was decreased from 25.3% to 21.3% in hot water pretreated biomass. Available nitrogen in hot water pretreated biomass increased from 830 ppm to 1250 ppm, whereas in untreated biomass the increase was from 784 ppm to 1017 ppm. The cellulose degradation was also more pronounced in hot water pretreated biomass, it degraded to 57% from its initial concentration i.e., 365.1 ppm after 42 days of incubation. Reducing sugars also increased in hot water pretreated biomass as compared to the untreated one. The results indicated that the pretreatment had accelerated the degradation rate and led to an increase in nitrogen and reducing sugars as well as a decrease in organic carbon and cellulose.

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Chapter 1: Introduction

Growth of population, increasing urbanization, rising standards of living due to technological innovations have contributed to an increase both in the quantity and variety of solid wastes generated by industrial, mining, domestic and agricultural activities. Globally the estimated quantity of the wastes generation was 12 billion tones in a year 2002 of which 11 billion tones were industrial wastes and 1.6 billion tones were municipal solid waste. About 90 billion tones solid wastes are expected to be generated annually by the year 2025. Annually, Asia alone generates 4.4 billion tones of solid wastes and municipal solid waste comprises 790 million tones of which about 48 million tones are generated in India. By the year 2047, municipal solid waste generation in India, is expected to reach 300 million tones and land requirement for disposal of this waste would be 169.6 km^2.

Solid waste generation from organic sources include municipal and urban wastes, animal wastes, farming wastes, horticulture wastes, domestic refuses and agro-industrial wastes. India is one of the richest countries in agricultural resources. Agricultural wastes are the byproducts of various agricultural activities such as crop production, crop harvest, saw milling, agro-industrial processing and others. The major quantity of wastes generated from agricultural resources are sugarcane baggase, paddy and wheat straw and husk, wastes of vegetables, food products, jute fiber, groundnut shell, coconut husk and cotton stalk etc. The main objective of waste management system is to maximize economic benefits and at the same time protection of environment. However, it is envisaged that the total solid wastes from municipal, agricultural, non-hazardous and hazardous wastes generated from different industrial processes in India seem to be even higher than the reported data. Already accumulated solid waste and their increasing annual production are a major source of pollution. Due to the environmental degradation, energy consumption and financial constraints, various organizations in India and abroad, apart from the regulatory frame work of United States Environmental Protection Agency, has

recommended various quantitative guidelines for generation, treatment, transport, handling, disposal and recycling of non-hazardous and hazardous wastes.

In developing countries, there is a different approach to dealing with organic waste. In fact, the word 'waste' is often an inappropriate term for organic matter, which is often put to good use. The economies of most developing countries dictates that materials and resources must be used to their full potential, and this has propagated a culture of reuse, repair and recycling. In many developing countries there exists a whole sector of recyclers, scavengers and collectors, whose business is to salvage 'waste' material and reclaim it for further use.

But these problems have also provided a window of opportunity for them to find solutions, involving the community and private sector; involving innovative technologies and disposal methods and involving behavior changes and awareness raising. These issues have been amply demonstrated by good practices from many cities and towns around the world. Current global initiatives on recycling issues have brought forth a number of lessons, which we are quite familiar with. Recycling can be promoted by increasing the content of recycled and recyclable materials in the products, which in turn, can be stimulated by actions such as public procurement regulations, product standards/specifications and research/development on the uses of recycler. Regulations restricting the landfill or incineration promoting recycling, a mandatory requirement for recycling and providing requisite infrastructure promotes involvement in recycling schemes.

In the present study different agricultural wastes were examined for rapid degradation by fungal consortia (*Aspergillus niger* FS1 and *Trichoderma reseei* MTCC-164), cow dung and DAP alongwith pretreatment of agricultural waste by hot water. Parameters like pH, moisture content, organic carbon, available nitrogen, cellulose content, reducing sugars and lignin were determined to evaluate the rate of biodegradation process.

Chapter 2: Review of literature

1. Waste

Waste, rubbish, trash, garbage or junk is unwanted or undesired materials. "Waste" is the general term; though the other terms are used loosely as synonyms, they have more specific meanings: rubbish or trash are mixed household waste and including paper and packaging; food waste or garbage is kitchen and table waste and junk or scrap is metallic or industrial material. There are other categories of waste as well: sewage, ash, manure and plant materials from garden operations, including grass cuttings, fallen leaves and pruned branches (www.wikipedia.org).

Some components of waste can be recycled once recovered from the waste stream, e.g. plastic bottles, metals, glass or paper. The biodegradable component of the wastes (paper & food waste) can be composted or anaerobically digested to produce soil improvers and renewable fuels. If it is not dealt within a suitable manner, biodegradable waste can thus contribute to greenhouse gas emissions and by implication climate change. There are some definitions of wastes:

1.1 Waste according to Basel Convention

*" Wastes are substances or objects which are disposed or are intended to be disposed or are required to be disposed of by the provisions of national laws" (*www. wikipedia.org*).*

1.2 Waste according to United Nations Statistics Division (UNSD)

*"Wastes are the materials that are not prime products for which the generators has no further use in terms of his/her own purposes of production, transformation or consumption and of which he/she wants to dispose. Wastes may be generated during the extraction of raw materials, the processing of raw materials into intermediate and final products, the consumption of the final products and other human activities. Residues recycled or reused at the place of generation are excluding" (*www.zerowasteamerica.org*).*

1.3 Waste according to European Union

The EU defines waste as an object the holder discards, intends to discard or is required to discard is waste under the Waste Framework Directive (European Directive 75/442/EC as amended).

*" Once a substance or object has become waste, it will remain waste until it has been fully recovered and no longer poses a potential threat to the environment or to human health" (*www. wikipedia.org*).*

1.4 Waste according to United Kingdom

The UK's Environmental Protection Act, 1990, indicated waste includes any substance which contributes a scrap material, an effluent or other unwanted surplus arising from the application of any process or any substance or article which requires to be disposed of which has been broken, worn out, contaminated or otherwise spoiled; this is supplemented with anything which is discarded otherwise dealt with as if it were waste shall be presumed to be waste unless the contrary is proved. This definition was amended by the Waste Management Licensing Regulations, 1994 defining waste as:

*" Any substance or object which the producer or the person in possession of it, discards or intends or is required to discard but with exception of anything excluded from the scope of the Waste Directive" (*www. wikipedia.org*).*

2. Organic wastes

Organic waste is produced wherever there is human habitation. The main forms of organic waste are household food waste, agricultural waste, human and animal waste. In industrialized countries the amount of organic waste produced is increasing dramatically each year. Although many gardening enthusiasts compost some of their kitchen and garden waste, much of the household waste goes into landfill sites and is often the most hazardous waste. The organic waste component of landfill is broken down by micro-organisms to form a liquid 'leachate' which contains bacteria, rotting matter and maybe chemical contaminants from the landfill. This leachate can present a serious hazard if it reaches a watercourse or enters the water table. Digesting organic matter in landfills also generates methane, which is a harmful greenhouse gas, in large quantity. Human organic waste is usually pumped to a treatment plant

where it is treated, and then the effluent enters a watercourse, or it is deposited directly into the sea. Little effort is made to reclaim the valuable nutrient or energy content of this waste.

In developing countries, there is a different approach to dealing with organic waste. In fact, the word 'waste' is often an inappropriate term for organic matter, which is often put to good use. The economies of most developing countries dictates that materials and resources must be used to their full potential, and this has propagated a culture of reuse, repair and recycling. In many developing countries there exists a whole sector of recyclers, scavengers and collectors, whose business is to salvage 'waste' material and reclaim it for further use (www.epa.gov).

Table 1: Types and nature of solid wastes and their recycling and utilization potentials

S.No.	Types of solid wastes	Source details	Recycling and utilization in building application
1.	Agro waste (organic nature)	Baggase, Rice & Wheat straw and husk, Cotton stalk, Saw mill waste, Ground nut shell, Banana stalk and jute, sisal and vegetable residues.	Particle boards, insulation boards, wall panels, printing paper and corrugating medium, roofing sheets, fuel, binder, fibrous building panels, bricks, acid proof cement, etc.
2.	Industrial waste (inorganic nature)	Coal consumption residues, steel slag, bauxite red mud, construction debris	Cement, bricks, blocks, tiles, paint, aggregate, concrete, wood substitute products, ceramic products
3.	Mining/ Mineral waste	Coal washeries waste, mining overburden waste, tailing from iron, copper, zinc, gold industries	Bricks, tiles, lightweight aggregates, fuel

4.	Non-hazardous other process waste	Waste gypsum, lime sludge, lime stone waste, marble processing residues, broken glass and ceramics, kiln dust	Gypsum plaster, fibrous gypsum boards, bricks, blocks, cement clinker, super sulphate cement, hydraulic binder
5.	Hazardous waste	Metallurgical residues, galvanizing waste, tannery waste	Cement, bricks, tiles, ceramics and board

Source: www.epa.gov

3. Cellulosic agro-waste

Cellulosic biomass constitutes a huge and renewable resource that can be converted to compost and fuel feedstocks. More efficient means for conversion of agricultural and forest waste are sought so that useful biomass-derived products can not only compete with or eventually replace petroleum based products but also supplement and complement the use of petroleum based fuels as additives to promote more efficient burning and lower emissions. Using these cellulosic resources efficiently can thus reduce the disposal problems and pollution resulting from accumulation of these wastes (Edwin, 2001).

4. Cow dung

According to the Agriculture Ministry of India, about 5.06 million tonnes of dung (cow and buffalo) is daily produced. The Major portion of this cow dung that is, approx 85% is used as a fuel mostly for households in the form of solid and dry cow dung cake and only 3.7% of this cow dung is used as a fuel in the form of biogas. Biogas is very good alternate fuel, but in country like India where most of the areas are suffering from draught, it is impractical to set biogas plant everywhere as it requires heavy investment for installation, huge amount of water and large area for installing the plant.

Cow dung manure is a nitrogen rich material and has economic importance as fertilizer, feed supplement or as energy source. Cow dung manure has been collected

and used to supply nitrogen, phosphorous, potassium and calcium to the soil for plant production (Smith and Wheeler, 1979). Cow dung has relatively high carbon to nitrogen ratio.

5. Biocycling

Due to the development of civilization, vast amount of organic waste is being produced. Its return to the environment is highly desirable, as it is characterized by significant amount of macro- and micronutrients. However, some of the organic waste can not be used directly, and needs improvement in its physical and chemical properties. One way of organic waste utilization is composting with usage of the appropriate biopreparations. Biological methods of decomposition of solid waste and liquid waste are known all over the world. They are regarded, to high extent, as effective, relatively cheap and most environmentally friendly way for utilizing most of the organic waste, in which organic fraction is usually represented by proteins, fats and carbohydrates. However, one of the biggest problems in this kind of process is a proper choice of micro-organisms, in terms of both quantity and quality, which determines the utilization effects of the biopreparation as well as the speed of the process. The complete biodegradation of organic waste can not be assumed, as it may contain compounds that can not be decomposed, i.e. hemicellulose, but biodegradation of most proteins, fats and carbohydrates in organic waste, originating from for example households seems to be possible (Bujak and Targoński 1998). Organic manure obtained in this process is characterized by better properties in comparison with the initial waste and can be used in agriculture, if its chemical composition (content of heavy metals) and health and sanitary properties are unquestioned.

6. Microbes involved in Biocycling

6.1 Bacteria

Lignocellulose biodegradation by prokaryotes is essentially a slow process characterized by the lack of powerful lignocellulose degrading enzymes, especially lignin peroxidases. Grasses are more susceptible to actinomycete attack than wood

(Crawford *et al.,* 1981; McCarthy 1987). Together with bacteria, actinomycetes play a significant role in the humification processes associated with soils and composts (Trigo & Ball 1994). The enzymatic ability to cleave alkyl-aryl ether bonds enable bacteria to degrade oligomeric and monomeric aromatic compounds released during fungal lignin degradation (Vicuna *et al*., 1993; White *et al*., 1996).

Bacillus subtilis, *Bacillus macerans*, *Pseudomonas fluorescens*, *Pseudomonas fragi*, *Serratia liquefaciens*, *Acinetobacter junii*, *Acinetobacter lvoffii*, *Cytophaga* sp. (Bujak and Targoński, 1998) have the ability to degrade the lignocellulosic waste. Microbial degradation of organic matter was investigated using culture of heterotrophyc bacteria enriched from solid samples collected with waste heap. A mixture of bacteria containing *Pseudomonas fluorescen, Areomonas hydrophilia, Oligella* sp. *Bacillus megaterium, Streptomonas maltophilia, Stenotrophomonas maltophilia, Bacillus amyloliquefaciens, Bacillus circulans, Bacillus pumilus and Burkhordelia capacia* was used for the biodegradation experiments (Vicuna, 2000).

6.2 Fungi

Most fungi are capable cellulose degraders. However, their ability to facilitate rapid lignocellulose degradation attracted attention from scientists and entrepreneurs alike. White-rot fungi comprise powerful lignin degrading enzymes that enable them in nature to bridge the lignin barrier and, hence, overcome the rate-limiting step in the carbon cycle (Elder & Kelly 1994). *Phanerochaete chrysosporium* is the best studied. New information regarding the identities of the cellulose, hemicellulose or lignin degrading enzymes, their unique catalytic capabilities, the physiological conditions required for optimum secretion or activity etc. is constantly being added to an already impressive volume of work and varies between fungi and bacterial genera, species and even strains. Anaerobic fungi (*Piromyces* spp., *Neocalli-mastix* spp. and *Orpinomyces* spp.) form part of the rumen microflora. These fungi produce active polymer degrading enzymes, including cellulases and xylanases (Hodrova *et al.,* 1998). Their cellulases are among the most active reported to date and able to solubilise both amorphous and crystalline cellulose (Wubah *et al.,* 1993). These

fungi can be used in situations where process principles and design necessitate anaerobic conditions.

Several brown rot fungi, including *Gloeophyllum sepiarium* (Mansfield *et al.*, 1998), *Gloeophyllum trabeum* (Herr *et al.,* 1978a; Mansfield *et al*., 1998), *Polyporus schweinitzii* (Bailey *et al.,* 1969), *Serpula incrassata* (Kleman-Leyer & Kirk, 1994) and *Tyromyces palustris* (Ishihara & Shimizu, 1984), *Trichoderma koningii* and *Phanerochaete chrysosporium,* fungi of the genera *Neocallimastix*, *Piromonas* and *Sphaeromonas* (Leschine 1995; Tomme *et al.,* 1995) white rot fungi *P. chrysosporium*, *Pleurotus ostreatus* and *Trametes versicolor* (Kuhad *et al*., 1997; Leonowicz *et al*., 1984) are also capable to degrade the cellulosic biomass.

7. Cellulose hydrolysis

Hydrolysis of cellulose is carried out by cellulase enzymes which are highly specific (Bégiun and Aubert, 1994). The products of the hydrolysis are usually reducing sugars including glucose. Both bacteria and fungi can produce cellulases for the hydrolysis of cellulosic materials. These microorganisms can be aerobic and anaerobic, mesophilic or thermophilic (Bisaria, 1991). Fungi that have been reported to produce cellulases include *Sclerotium rolfsii, Penicillium chrysosporium* and species of *Trichoderma, Aspergillus, Schizophyllum* and *Penicillium*(Sternberg, 1976; Fan *et al.*,1987; Duff and Murray, 1996). Of all these fungal genera, *Trichoderma* has been most extensively studied for cellulase production (Sternberg, 1976).

There are a number of ancillary enzymes that attack hemicellulose such as glucuronidase, acetylesterase, xylanase, β-xylosidase, galactomannase and glucomannanase (Duff and Murray, 1996). The addition of β- glucosidases into the *Trichoderma reseei* cellulases system achieved better saccharification than the system without β-glucosidases (Excoffier *et al*., 1991; Xin *et al*., 1993).

8. Pretreatment of lignocellulosic materials

The effect of pretreatment of lignocellulosic materials has been recognized for a long time (McMillan, 1994). The purpose of the pretreatment is to remove lignin and

hemicellulose, reduce cellulose crystallinity and increase the porosity of the materials. Pretreatment must meet the following requirements:

1. Improve the formation of sugars or the ability to subsequently form sugars by enzymatic hydrolysis.
2. Avoid the degradation or loss of carbohydrate.
3. Avoid the formation of byproducts inhibitory to the subsequent hydrolysis and fermentation process.
4. Cost effective treatment.

Physical, physico-chemical, chemical and biological processes have been used for pretreatment of lignocellulosic materials.

8.1 Physical pretreatment

8.1.1 Mechanical comminution

Waste materials can be comminuted by a combination of chipping, grinding and milling to reduce cellulose crystallinity. The size of the material is usually 10-30 mm after chipping and 0.2-2 mm after milling and grinding. Vibratory ball milling has been found to be more effective in breaking down the cellulose crystallinity of sporuce and aspen chips and improving the digestibility of biomass of the biomass than ordinary ball milling (Millet *et al.,* 1976). The power requirement of the mechanical comminution of agricultural wastes depends on the final particle size and the waste biomass characteristics (Cadoche and López, 1989).

8.1.2 Pyrolysis

Pyrolysis has also been used for pretreatment of lignocellulosic materials. When the materials are treated at temperatures greater than 300 ºC, cellulose rapidly decomposes to produce gaseous products and residual char (Kilzer and Broido, 1965; Shafizadeh and Bradbury, 1979). The decomposition is much slower and less volatile products are formed at lower temperatures. Mild acid hydrolysis (1 N H_2SO_4, 97 ºC, 2.5 hr) of the residues from pyrolysis pretreatment has resulted in 80-85% conversion of cellulose to reducing sugars with more than 50% glucose (Fan *et al.,* 1987). The process can be enhanced with the presence of oxygen (Shafizadeh and

Bradbury, 1979). When zinc chloride or sodium carbonate is added as catalyst, the decomposition of pure cellulose can occur at a lower temperature (Shafizadeh and Lai, 1975).

8.2 Physico-chemical pretreatment

8.2.1 Steam explosion (Autohydrolysis)

Steam explosion is the most commonly used method for the pretreatment of lignocellulosic materials (McMillan, 1994). In this method, chipped biomass is treated with high-pressure saturated steam and then pressure is swiftly reduced, which makes the materials undergo an explosive decompression. Steam explosion is typically initiated at a temperature of 160-260 ºC (corresponding pressure 0.69-4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure. The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis. The factors that affect steam explosion pretreatment are residence time, temperature, chip size and moisture (Duff and Murray, 1996). Optimal hemicellulose solubilization and hydrolysis can be achieved by either high temperature and short residence time (270 ºC, 1 min) or lower temperature and longer residence time (190 ºC, 10 min)(Duff and Murray, 1996). Recent studies indicate that lower temperature and longer residence time are more favorable (Wright, 1998).

Addition of H_2SO_4 or CO_2 in steam explosion can effectively improve enzymatic hydrolysis decrease the production of inhibitory compounds and lead to more complete removal of hemicellulose (Morjanoof and Gray, 1987). The advantages of steam explosion pretreatment include the low energy requirement compared to mechanical comminution and no recycling or environmental costs. The environmental mechanical methods require 70 % more energy than steam explosion to achieve the same size reduction (Holtzapple *et al.,* 1989). Steam explosion is recognized as one of the most cost effective pretreatment process for hardwoods and agricultural residues, but it is less effective for softwoods (Clark and Mackie, 1987). Limitations of steam explosion include destruction of a portion xylan fraction, incomplete disruption of the lignin-carbohydrate matrix and generation of

compounds that may be inhibitory to microorganisms used in downstream processes (Mackie, 1985). Because of the formation of degradation products that are inhibitory to microbial growth, enzyme hydrolysis and fermentation, pretreated biomass needs to be washed by waster to remove the inhibitory materials along with water soluble hemicellulose (McMillan, 1994).

8.2.2 Ammonia fiber explosion (AFEX)

AFEX is another type of physico-chemical pretreatment in which lignocellulosic materials are exposed to liquid ammonia at high temperature and pressure for a period of time and then pressure is swiftly reduced. The concept of AFEX is similar to steam explosion. In a typical AFEX process, the dosage of liquid ammonia is 1-2 kg ammonia /kg dry biomass, temperature 90ºC, and residence time 30 minutes. AFEX pretreatment can significantly improve the saccharification rates of various herbaceous crops and grasses. It can be used for the pretreatment of many lignocellulosic materials including alfalfa, wheat straw, wheat chaff (Mes-Hartree *et al.,* 1988), barley straw, corn stover, rice straw (Vlasenko *et al.,* 1997), municipal solid waste, softwood newspaper (Holtzapple *et al.,* 1992a), coastal Bermuda grass, switch grass (Reshamwala *et al.,*1995), aspen chips (Tengerdy and Nagy, 1988) and bagasse (Holtzapple *et al.,* 1991). The AFEX pretreatment does not significantly solubilize hemicellulose compared to acid pretreatment and acid-catalyzed steam explosion (Mes-Hartree *et al.,* 1988; Vlasenko *et al.,* 1997). The composition of materials after AFEX pretreatment was essentially the same as the original materials. Over 90 % hydrolysis of cellulolse and hemicellulose has been obtained after AFEX pretreatment of Bermuda grass and bagasse (Holtzapple *et al.,* 1991).

To reduce the cost and protect to environment, ammonia must be recycled after the pretreatment. In an ammonia recovery process, a superheated ammonia vapor with the temperature up to 200ºC was used to vaporize and strip the residual ammonia in the pretreated biomass and the evaporated ammonia was then withdrawn from the system by a pressure controller for recovery (Holtzapple *et al.,* 1992b). The ammonia pretreatment does not produce inhibitors for the downstream biological processes, so water wash is not necessary (Dale *et al.,* 1984; Mes-Hartree *et al.,*

1988). AFEX pretreatment does not require small particle size for better efficiency (Holtzapple *et al.,* 1990).

8.2.3 CO_2 explosion

Similar to steam and ammonia explosion pretreatment, CO_2 explosion is also used for the pretreatment of ligocellulosic materials. It was hypothesized that CO_2 would form carbonic acid and increase the hydrolysis rate. Dale and Moreira (1982) used this method to pretreat alfalfa (4 kg CO_2/ kg fiber at the pressure of 5.62 MPa) and obtained 75 % of the theoretical glucose released during 24 h of enzymatic hydrolysis. The yields were relatively low compared to steam or ammonia explosion pretreatment, but high compared to the enzymatic hydrolysis without pretreatment. Zheng *et al.,* (1998) compared CO_2 explosion with steam and ammonia explosion for pretreatment of recycled paper mix, sugarcane bagasse and repulping waste of recycled paper and found that CO_2 explosion was more cost effective than ammonia explosion and did not cause the formation of inhibitory compounds and could occur in steam explosion.

8.3 Chemical pretreatment

8.3.1 Ozonolysis

Ozone can be used to degrade lignin and hemicellulose in many lignocellulosic materials such as wheat straw (Ben-Ghedalia and Miron, 1981), bagasse, green hay, peanut, pine (Neely, 1984), cotton straw (Ben-Ghedalia and Shefet, 1983) and popular sawdust (Vidal and Molinier, 1988). The degradation was essentially limited to lignin and hemicellulose was slightly attacked but cellulose was hardly affected. Ozonolysis pretreatment has the following advantages: (1) it effectively remove lignin; (2) it does not produce toxic residues for the downstream processes; and (3) the reactions are carried out at room temperature and pressure (Vidal and Molinier, 1988). However, a large amount of ozone is required, making the process expensive.

8.3.2 Acid hydrolysis

Concentrated acids such as H_2SO4 and HCl hav been used to treat lignocellulosic materials. Although they are powerful agents for cellulose hydrolysis, concentrated acids are toxic, corrosive and hazardous and require reactors that are resistant to corrosion. In addition, the concentrated acid must be recovered after hydrolysis to make the process economically feasible (Sivers and Zacchi, 1995).

Dilute acid hydrolysis has been successfully developed for pretreatment of lignicellulosic materials. The dilute sulphuric acid pretreatment can achieve high reaction rates and significantly improve cellulose hydrolysis (Esteghalalian *et al.,* 1997). At moderate temperature, direct saccharification suffered from low yields because of sugar decomposition. High temperature in dilute acid, treatment is favorable for cellulose hydrolysis (McMillan, 1994).

There are primarily two types of dilute acid pretreatment processes: high temperature (temperature >160ºC), continuous flow process for low solids loading (5-10%, weight of substrate/weight of reaction mixture) (Brenann *et al.,* 1986; Converse *et al.,* 1989) and low temperature (temperature <160ºC), batch process for high solids loading (10-40%)(Cahela *et al.,* 1983; Esteghlalian *et al.,* 1997). Although dilute acid pretreatment can significantly improve the cellulose hydrolysis, its cost is usually higher than some physico-chemical pretreatment processes such as steam explosion or AFEX. A neutralization of pH is necessary for the downstream enzymatic hydrolysis or fermentation process.

8.3.3 Alkaline hydrolysis

Some bases can also be used for pretreatment of lignocellulosic materials and the effect of alkaline pretreatment depends upon the lignin content of the materials (Fan *et al.,* 1987; McMillan, 1994). The mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds crosslinking xylan hemicellulosic and other components, for example, lignin and other hemicellulose. The porosity of the lignocellulosic materials increases with the removal of the crosslinks (Tarkow and Feist, 1969). Dilute NaOH treatment of lignocellulosic materials caused swelling, leading to an increase in internal surface area, a decrease in the degree of

polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates and disruption of the lignin structure (Fan *et al.,* 1987). The digestibility of NaOH treated hardwood increased from 14 to 55 % with the decrease of lignin content from 24-55% to 20%. However, no effect of dilute NaOH pretreatment was observed for softwoods with lignin content greater than 26% (Miller *et al.,* 1976). Dilute NaOH pretreatment was also effective for the hydrolysis of straws with relatively low lignin content of 10-18% (Bjerre *et al.,* 1996). Chosdu *et al.,*(1993) used the combination of irradiation and 2% NaOH for pretreatment of corn stalk, cassava bark and peanut husk.

8.3.4 Oxidative delignification

Lignin biodegradation could be catalyzed by the peroxidase enzyme with the presence of H_2O_2 (Azzam, 1989). The pretreatment of cane bagasse with hydrogen peroxide greatly enhanced its susceptibility to enzymatic hydrolysis. About 50% of lignin and most hemicellulose were solubilized by 2% H_2O_2 at 30ºC within 8 hr and 95% efficiency of glucose production from cellulose was achieved in the subsequent saccharification by cellulase at 45ºC for 24 hr (Azzam, 1989). Bijerre *et al.,* (1996) used wet oxidation and alkaline hydrolysis of wheat straw (20 g straw/l, 170ºC, 5-10 min), and achieved 85% conversion yield of cellulose to glucose.

8.3.5 Organosolv process

In the organosolv process, an organic or aqueous organic solvent mixture with inorganic acid catalysts (HCl or H_2SO_4) is used to break the internal lignin and hemicellulose bonds. The organic solvents used in the process include methanol, ethanol, acetone, ethylene, glycol, triethylene glycol and tetrahydrofurfuryl alcohol (Chum *et al.,* 1988; Thring *et al.,* 1990). Organic acids such as oxalic, acetylsalicylic and salicylic acid can also be used as catalysts in the organosolv process (Sarkanen 1980). At high temperatures >180ºC, the addition of catalyst was unnecessary for satisfactory delignification (Sarkanen, 1980; Aziz and Sarkanen, 1989). Usually, a high yield of xylose can be obtained with the addition of acid. Solvents used in the process need to be drained from the reactor, evaporated, condensed and recycled to

reduce the cost. Removal of solvents from the system is necessary because the solvents may be inhibitory to the growth of organisms, enzymatic hydrolysis and fermentation.

8.4 Biological pretreatment

In biological pretreatment processes, microorganisms such as brown, white and soft –rot fungi are used to degrade lignin and hemicellulose in waste materials (Schurz, 1978). Brown rots mainly attack cellulose, while white and soft rots attack both cellulose and lignin. White-rot fungi are the most effective basidiomycetes for biological pretreatment of lignocellulosic materials (Fan *et al.* 1987). In order to prevent the loss of cellulose, a cellulase less mutant of *Sporotrichum pulverulentum* was developed for the degradation of lignin in wood chips (Ander and Eriksson, 1977).

The white rot fungus *P. chrysoporium* produces lignin degrading enzymes, lignin peroxidase and manganese dependent peroxidases, during secondary metabolism in response to carbon to nitrogen limitation (Boominathan and Reddy, 1992). Both enzymes have been found in the extracellular filtrates of many white-rot fungi for the degradation of wood cell wall (Kirk and Farrell, 1987; Waldner *et al.,* 1988). Other enzymes including polyphenol oxidases, laccases, H_2O_2 producing enzymes and quinone-reducing enzymes can also degrade lignin (Blanchette, 1991). The advantages of biological pretreatment include low energy requirement and mild environmental conditions. However, the rate of hydrolysis in most biological pretreatment processes is very slow.

8.5 Enzymatic hydrolysis

Enzymatic hydrolysis of cellulose is carried out by celullase enzymes which are highly specific (Béguin and Aubert, 1994). The products of hydrolysis are usually reducing sugars including glucose. Utility cost of enzymatic hydrolysis is low as compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45-50 ºC) and does not have a corrosion problem (Duff and Murray, 1996). Both bacteria and fungi can produce

cellulases for the hydrolysis of the lignocellulosic materials. These microorganisms can be aerobic or anaerobic, mesophilic or thermophilic. Bacteria belonging to *Clostridium, Cellulomonas, Bacillus, Thermomonospora,* and *Streptomyces* etc. can produce cellulases (Bisaria, 1991). Because anaerobes have a very low growth rate and require anaerobic growth conditions, most research for commercial cellulase production has focused on fungi (Duff and Murray, 1996).

Fungi has been reported to produce cellulases include *Sclerotium rolfsii, P. chrysoporium* and species of *Tricoderma, Aspergillus* and *Penicilium* (Sternberg, 1976; Fan *et al.,* 1987; Duff and Murray, 1996). Of all these fungal genera, *Tricoderma* has been most extensively studied for cellulase production (Sternberg, 1976).

Cellulases are usually a mixture o several enzymes. At least three major groups of cellulases are involved in the hydrolysis process: (1) endoglucanase which attacks regions of low crystallinity in the cellulose fiber, creating free chain-ends; (2) exoglucanase or cellobiohydrolase which degrades the molecule further by removing cellobiose units from free chain ends; (3) β-glucosidase which hydrolyzes cellobiose to produce glucose (Coughlan and Ljungdahl, 1988). During the enzymatic hydrolysis, cellulose is degraded by the cellulases to reducing sugars that can be fermented by yeast or bacteria to ethanol.

9. Value added products of agricultural wastes

9.1 Compost

Composting is the nature's way of recycling. Composting biodegrades the organic waste i.e. food waste, manure, leaves, grass trimmings, paper, wood, feathers, crop residue etc., and turns it into a valuable organic fertilizer.

Composting is a natural biological process, carried out under controlled aerobic conditions. In this process, various microorganisms, including bacteria and fungi break down organic matter into simpler substances. The effectiveness of the composting process is dependent upon the environmental conditions present within the composting system i.e. oxygen, temperature, moisture, material disturbance, organic matter and the size and activity of microbial populations.

Composting is relatively simple to manage and can be carried out on a wide range of scales in almost any indoor and outdoor environment and in almost any geographical location. It has a potential to manage most of the organic matter in the waste stream including restaurant waste, leaves and yard wastes, farm wastes, animal manure, animal carcasses, paper products, sewage sludge, wood etc. and can be easily incorporated into any waste management plan (www.soil.ncsu.edu).

Since approximately 55-55% of the waste stream is organic matter, composting can play a significant role in diverting waste from landfills thereby conserving landfill space and reducing the production of leachate and methane gas. In addition, an effective composing program can produce a high quality soil amendment with a variety of end uses.

Composting is applied microbiology at its most complexes, involving the interaction of thousands upon thousands of different species of microorganisms in a highly complex ecosystem. The composting process kills weed seeds and suppresses human and plant pathogens that doesn't happen when leaves and other detritus rot down on their own. Once applied, compost "balances" the soil flora that is for each of the scores or more of disease organisms that can affect each species of plant at least12 to 15 different species of bio-control microorganisms need to be present, with the food and conditions they require, if the plant is to be healthy. Composting accomplishes that among other things (www.soil.ncsu.edu).

The essential elements required by composting microorganisms are carbon, nitrogen, oxygen, moisture. If any of these elements are lacking or if they are not provided in the proper proportion, the microorganisms will not flourish and will not provide adequate matter into stable compost that is odor and pathogen free and poor breeding substrate for flies and other insects. In addition, it will significantly reduce the volume and weight of organic waste as the composting process converts much of the biodegradable component to gaseous carbon dioxide (www.epa.gov).

9.2 Bioethanol

Cellulosic resources such as paper, cardboard, wood, agricultural residues and other fibrous plant material are in general very widespread and abundant. For example, forests comprise about 80% of the world's biomass. Ethanol can be produced from different kinds of raw materials. Cellulosic biomass can be supplied from a variety of resources at a low price. They can be classified in four groups based on type of resources: wood, municipal solid waste, waste paper and crop residue resources. Being abundant and outside the human food chain makes cellulosic materials relatively inexpensive inputs for ethanol production. Cellulosic wastes are comprised of lignin, hemicelluloses and cellulose and other are sometimes called lignicellulosic materials.

Based on average 52% cellulose and 21% hemicelluloses in wood, the maximum theoretical yield of ethanol can be calculated as 0.32 grams of ethanol per gram of wood. This calculation is based on full conversion of cellulose and hemicelluloses to sugars and conversion of sugars to ethanol at the theoretical yield of 0.51 g/g. Since pentose molecules comprise a high percentage of the available sugars, the availability to recover and ferment them into ethanol is important for the efficiency and economics of the process.

Specific feedstocks under consideration include:

- Agricultural residues (leftover material from crops, such as the stalks, leaves, etc.)
- Forestry waste (chips and sawdust from lumber mills, dead trees and tree branches)
- Municipal solid waste(household garbage and paper products)

Although the choice of feedstock for ethanol conversion is largely a cost issue, feedstock selection has also focused on environmental issues. Materials normally targeted for disposal include forest thinnings collected as part of an effort to improve forest health, MSW and certain agricultural wastes such as rice straw. Although forest residues are not large in volume, they represent an opportunity to decrease the fire hazard associated with the dead wood present in many national forests. Agricultural residues, in the long term, would be the source of biomass that could

support substantial growth of the ethanol industry. The cost of agricultural residues is not nearly as sensitive to supply as is the cost of forest residues (www1.eere.energy.gov).

Extensive research has been completed on conversion of cellulosic materials to ethanol in the last two decades (Dale *et al.,* 1984; Wright, 1998; Azzam, 1989; Cadoche and López , 1989; Reshamwala *et al.,* 1995; Bjerre *et al.,* 1996; Duff and Murray, 1996). Ethanol represents closed carbon dioxide cycle because after burning of ethanol, the released carbon dioxide is recycled back into plant material because plants use CO2 to synthesize cellulose during photosynthesis cycle (Wyman, 1999). Ethanol production process only uses energy from renewable energy sources; no net carbon dioxide is added to the atmosphere, making ethanol an environmentally beneficial energy source. In addition, the toxicity of the exhaust emissions from ethanol is lower than that of petroleum sources (Wyman and Hinman, 1990). Ethanol derived from biomass is the only liquid transportation fuel that does not contribute to the green house gas effect (Foody, 1988).

Chapter 3: Objectives

1. Characterization of different types of cellulosic agricultural wastes.
2. Standardization of biomass handling and pretreatment of agricultural wastes.
3. Biodegradation of mixed agricultural wastes.
4. Pilot plant bioconversion of agro-wastes.

Chapter 4: Materials and Methods

1. Agricultural wastes

Agricultural wastes from different plant/tree species i.e. *Tylophora indica, Hevea brasilienosis, Eucalyptus, Polyalthia longofolia, Tectona grandis, Mangifera indica, Bamboosa* vulgaris and grass cuttings, weeds and mixed waste were collected from the field of STEP, Thapar University, Patiala, were overnight oven dried at 105°C. After complete drying the waste material was crushed using a mixer grinder. Cow dung was collected from the dairy nearby Thapar University, Patiala and was air dried to the moisture content of 30%.

For pilot/field scale the mixed agricultural waste comprising of leaf litter (shown in Plate: 13) was collected from different places of Thapar Technology Campus in a very large quantity (around 15 quintals).

2. Fungal consortia

The fungi used in the study were *Aspergillus niger* (FS1) and *Trichoderma reseei* (MTCC 164). The mother cultures were obtained from the culture bank of Department of Biotechnology & Environmental Sciences, Thapar University. A agar disc of fungal mycelia was subcultured in Potato Dextrose Broth (PDB) for mass cultivation and incubated at 28 ± 2 ºC and 120 rpm for 48 hours. After 48 hours of growth, the inoculum was mixed with the waste.

3. Bioconversion of agricultural wastes

After the processing (chopping) of agricultural waste, cow dung, fungal consortia and DAP were mixed to the waste in different proportions (w/w) in a conical flask of 500 ml capacity in triplicates as follows:

1. Garden waste (GW)(25 gm) control;
2. Garden waste + cow dung (CD)(2:1);
3. Garden waste + cow dung (2:1) + DAP (0.1 gm);
4. Garden waste + fungal consortia (FC)(2.5:1);

5. Garden waste + fungal consortia (2.5:1)+ DAP (0.1 gm);
6. Leaf litter (LL)(25 gm) control;
7. Leaf litter(LL) + cow dung (CD)(2:1);
8. Leaf litter (LL) + cow dung (2:1) + DAP (0.1 gm);
9. Leaf litter (LL) + fungal consortia (FC)(2.5:1);
10. Leaf litter (LL) + fungal consortia (2.5:1) + DAP (0.1 gm).

The flasks were plugged with cotton, were kept at 30 ± 0.2 ºC in incubator. The samples were withdrawn from the flasks at 15 days interval from day 0 to day 90. The above setup was monitored on laboratory scale for 90 days, at pilot scale the experiment was set in two different pits with two different treatments as: Hot treated biomass with cow dung (1:1, w/w) in pit-1(18*12*2 ft^3)† and untreated biomass with cow dung (1:1, w/w) in pit-2 (18*10*2 ft^3)†. Hot treatment was given in tin drums of 100 liter capacity each. The waste was boiled in the drums in batches, each batch contained around 10 kg of waste and residence time provided to each batch was 1 hour at temp. 80-90 ºC. Then it was placed in the pit and cow dung was mixed to it in equal proportion (w/w). The moisture was required to be maintained daily because of the evaporation losses. The moisture was not same always because of different weather conditions so the quantity of water added to maintain moisture was not same also and it was maintained 40-60 % in each pit. Turning of the waste was provided once in every week to ensure aerobic condition and the same pattern (except boiling) was followed with untreated waste. At every 7 days interval the samples were withdrawn from the pits and for other parameters estimation in the laboratory. The data was generated for 42 days.

4. Analytical methods

Moisture was maintain at 50 %, estimated by gravimetric method and pH was measured potentiometrically in a supernatant liquid that was in equilibrium with waste sample suspension of a 1:10 waste to distilled water. A digital pH/mV meter with a combination electrode and an automatic compensator made by EUTECH Instruments was used for pH measurement. The carbon % was estimated by the

(† dimension of the pit as length * width *depth; all dimensions are in feet)

method as prescribed by Walkley and Black (1934), available nitrogen was estimated as per the protocol given by Jackson (1967), at an interval of 15 days upto 90 days. The concentration of cellulose and reducing sugars were estimated as per the protocol described by Updegraff (1969) and Ghose, (1987) respectively. Lignin was estimated as per method described in TAPPI Test method, (1989).

4.1 Estimation of pH (Potentiometry)

Method

1. Weighed 2g of air dried waste sample into a 100 ml beaker.
2. Added 20 ml of distilled water to it.
3. Thoroughly stirred it for 10 seconds using a glass rod.
4. Further stirred the suspension four five times during the next 30 minutes.
5. Allowed suspension to settle for 30 minutes.
6. In the meanwhile, rinsed the electrodes with distilled water and carefully wiped with filter paper.
7. Measured pH of the sample by immersing the combination electrode in supernatant solution.
8. Recorded pH value when the reading had stabilized.

4.2 Estimation of percentage organic carbon (Walkley and Black, 1934)

Materials

1. 1 N $K_2Cr_2O_7$ was prepared by dissolving 49.04 gm of Potassium dichromate in 1000 ml of distilled water.
2. 0.5 N Ferrous ammonium sulphate was prepared by adding 198 g salt in 1000 ml distilled water and also added 20 ml of concentrated sulphuric acid.
3. Diphenylamine indicator was prepared by adding 0.5 g of diphenylamine in a mixture of 20 ml distilled water and 100 ml concentrated sulphuric acid.
4. Concentrated sulphuric acid.
5. Orthophosphoric acid (85%) was prepared by mixing 15 ml distilled water to orthophosphoric acid.
6. Sodium fluoride.

Method

1. Took 0.1 g of dried waste sample in a 500 ml conical flask and added 10 ml of 1N $K_2Cr_2O_7$ to it. Swirled the flask for mixing the waste and reagent.
2. Added 20 ml of H_2SO_4 and allowed the flask to stand undisturbed for 30 minutes after which added 200 ml of distilled water.
3. To the mixture, added 10 ml of 85% Orthophosphoric acid, 0.5 g of NaF and I ml of diphenylamine indicator.
4. Ultimately titrated with 0.5 N ferrous ammonium sulphate till the end point was observed from violet blue to green.

Also a blank was taken without waste sample.

Calculation

Organic Carbon (%) = 10* (B-T) * 0.003 * 100/(B * weight of sample taken)

Where B = volume of ferrous ammonium sulfate consumed for blank titration

T = volume of ferrous ammonium sulfate consumed for sample titration

4.3 Estimation of Available Nitrogen (Jackson, 1967)

Materials

1. 0.32% Potassium per manganate was prepared by dissolving 3.2 g of $KMnO_4$ in distilled water and final volume made up to 1000 ml.
2. NaOH (2.5%) was prepared by dissolving 25 g of sodium hydroxide pellets in distilled water and volume made upto 1L.
3. Boric acid (2%) was prepared by dissolving 10 g of boric acid powder in warm distilled water by stirring and diluted to 500 ml.
4. Mixed indicator was prepared by dissolving 0.066 g of methyl red and 0.099 g of bromocresol green in 100 ml of ethyl alcohol. 10 ml of mixed indicator was added to 500 ml of 2% boric acid solution.
5. NaOH (0.1 N) was prepared by dissolving 4 g of sodium hydroxide pellets in distilled water and diluted to 1 L.

6. Sulphuric acid (0.02 N) was prepared by adding 0.56 ml of concentrated sulphuric acid to about 990 ml of distilled water.

Method

1. Weighed 1 g of dried waste sample and placed in 500 ml kjeldahl flask.
2. Moisten the sample with 2 ml of distilled water and added 20 ml of 0.32% $KMnO_4$ and also added 0.4 mm glass beads to prevent bumping.
3. Measured 20 ml of 2% boric acid containing mixed indicator in a 100 ml beaker and placed under the receiving tube. Dipped the receiver tube in boric acid.
4. Allowed tap water to run into the condenser.
5. Added 20 ml of 2.5% NaOH solution and quickly fitted the stopper in the alkali trap.
6. Switched on the heater on to continued distillation until 40 ml distillate was collected.
7. Removed the beaker containing distillate before switching off the heater to avoid back suction.
8. Titrated the distillate against 0.02 N H_2SO_4 taken in burette until pink colour appeared.

Calculation

Available N (ppm) = (X) * 0.00028 * 100 * 10000

Where X = volume of 0.02 N H_2SO_4 consumed for sample titration

4.4 Estimation of cellulose by Anthrone Assay (Updegraff, 1969)

Materials

1. Standard Stock Solution of Cellulose was prepared by dissolving 50 mg of pure cellulose in 10 ml of 67 % H_2SO_4 with gentle heat; it was further diluted to 500 ml to a final concentration of 100 µg cellulose/ml.
2. Acetic Nitric Reagent was prepared by adding 150 ml of 80 % acetic acid and 15 ml of concentrated HNO_3.
3. Sulphuric acid 67 % (v/v).

4. Anthrone reagent was prepared by dissolving 0.2 gm of anthrone in 100 ml cold H_2SO_4 and kept in ice bath.

Method

1. **Standard curve**
    a. Different dilutions of standard cellulose were prepared by mixing stock cellulose solution (1 mg/ml) and water in the test tube.
    b. The final volume in each of the test tube was 1 ml. The cellulose range was 10 to1000 µg/ml.
    c. This solution was kept in ice bath for 2 – 3 mins.
    d. Chilled 5 ml of anthrone solution was added to this.
    e. This solution was kept in ice bath for 5 min, further in boiling water bath for 15 mins and again in ice bath for 5 mins.
    f. Optical density was measured at 620 nm using spectrophotometer.
2. **Sample Analysis**
    a. 0.5 ml of diluted sample was taken.
    b. To this 0.5 ml of distilled water was added to make total volume of 1 ml.
    c. Same procedure was followed as for standard curve.

4.5 Estimation of reducing sugar by Dinitrosalicylic Acid (DNS) method (Ghose, 1987)

Materials

1. Citrate Buffer was prepared by dissolving 210 g of Citric acid monohydrate ($C_6H_8O_7.H_2O$) in 750 ml of distilled water. It was diluted to 0.05 M buffer and the desired pH was set using NaOH
2. DNS Reagent was prepared by mixing 10.6 g of 3, 5 - Dinitrosalicylic acid and 19.8 g NaOH in 1416 ml of distilled water. To this solution 306 g of Rochelle salt (Na – K tartarate), 8.3 g of Na metabisulfite and 7.6 ml of Phenol (melt at 50°C) were added.
3. Substrate: CarboxyMethyl Cellulose (1 %)

4. Blank consisted of 1 ml citrate buffer, 3 ml DNS Boiled for 5 min.
5. Enzyme blank consisted of 0.5 ml citrate buffer, 0.5 ml enzyme solution, 3 ml DNS boiled for 5 min.

Methods

1. **Standard curve**
    a. Different dilutions of glucose solution were prepared by mixing stock glucose solution (10 mg/ml) and Distilled water in the test tube.
    b. The range of glucose concentration was 2.0 to 6.7 mg/ml.
    c. To this 3 ml of DNS was added & mixed.
    d. This solution was boiled for exactly 5 min in a vigorously boiling water bath.
    e. Sample was diluted & OD was measured at 540 nm against the spectro zero.
2. **Sample Analysis**
    a. 0.5 ml enzyme was taken.
    b. To this 0.5 ml of substrate CMC was added.
    c. This solution was mixed well & incubated at 50ºC for 30 min.

Same procedure was followed as for the standard curve.

Sample digestion

0.5 g of dried sample were added to 10 ml of acetic nitric reagent and mouth of tubes were covered with marbles to avoid evaporation. Tubes were placed in boiling water bath for refluxing for 4-5 hours. Further tubes were centrifuged at 8000-10000 rpm for 10 minutes and supernatant was discarded. To the residue 10 ml of distilled water was added, again centrifuged as above and again supernatant was discarded. Same procedure was repeated for 2-3 times. 10 ml of 67% H_2SO_4 was added in two installments of 5 ml each by mixing well on vortex mixer. The reaction mixture was incubated at room temperature for 1 hour and diluted to 100 times for color measurement.

Enzyme preparation

The enzyme was extracted from fungi *A. niger* and *T. reseei* , for this the fungal cultural were inoculated on Potato Dextrose media and kept at 28 ± 2 ºC for 48

hours. For the suspension of fungal spores 0.1% Tween-80 solution was used. Spore count of the fungal suspensions was set to approximately 5 x 10^6 spores/ml using haemocytometer. 1 ml of fungal suspension was inoculated in 100 ml of Duff's medium and incubated at varying temperatures viz 20º, 30^o, 37^o and 45^o for 48 hrs with shaking at 120 rpm and enzymes were extracted by filtration (Ghose *et al., 1987).*

Duff's media (per liter solution, pH = 5.5)

Components	quantity
Potassium bisulfate (KH_2PO_4)	2 gm
Ammonium sulfate $(NH_4)_2SO_4$	1.4 gm
Urea	0.3 gm
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	0.3 gm
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	0.3 gm
Peptone	1 gm
Tween – 80	2 ml
Ferrous sulfate heptahydrate ($FeSO_4.7H_2O$)	5 mg
Magnus sulfate dihydrate ($MnSO_4.2H_2O$)	1.6 gm
Zinc sulfate heptahydrate ($ZnSO_4.7H_2O$)	1.4 gm
Cobaltous chloride hexahydrate ($CoCl_2.6H_2O$)	2 mg
Lactose	10 gm
Total volume made up to 1 liter with distilled water.	

4.6 Estimation of acid insoluble lignin (TAPPI test methods, 1989)

Materials

1. For the preparation of 72% sulphuric acid solution, 720 ml of concentrated sulphuric acid was carefully poured to 200 ml of distilled water and after cooling made the volume to 1000 ml.

Method

1. To the beaker containing 0.5 g of dried waste sample, 7.5 ml of cold (10-15º C) 72% H_2SO_4 was added. The acid was added gradually in small increments

while stirring and macerating the material with the glass rod. The beaker was kept in a bath at 20 ± 1° C during the dispersion of material.

2. After the sample was dispersed, the beaker was covered with watch glass and was kept in a water bath 20 ± 1° C for 2 hours. The material was stirred frequently during this time to ensure complete dissolution.
3. 150 to 200 ml of distilled water was added to a round bottom flask and the material was transferred from the beaker to the flask containing distilled water and was rinsed and diluted with water to 3% concentration of sulphuric acid to a total volume of 300 ml.
4. The solution was boiled for 4 hours, maintaining constant volume by using a reflux condenser.
5. The insoluble material was allowed to settle by keeping the flask in inclined position.
6. Without stirring up the precipitate, the supernatant solution was filtered through a Whatman filter paper 42.
7. The lignin was washed free of acid with hot water.
8. The filter paper having lignin, was dried in an oven at 105 ± 3° C to a constant weight. The filter paper was cooled in desiccators and weighed.

Calculation

Lignin (%) = A * 100/ W

Where A = weight of lignin

W = weight of sample

4.7 Estimation of ethanol

Materials

1. Potassium dichromate ($K_2Cr_2O_7$) solution was prepared by dissolving 33.76 gm of $K_2Cr_2O_7$ in 400 ml distilled water and 325 ml concentrated sulphuric acid, cooled and then made total volume to 1 liter with distilled water.
2. Glucose Yeast Extract medium was prepared by dissolving 10 gm of sucrose, 0.1 gm of Potassium bisulfate (KH_2PO_4), 0.5 gm of Ammonium sulfate

$(NH_4)_2SO_4$, 0.1 gm of yeast extract and 0.05 gm of Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) in some amount of distilled water and made final volume to 100 ml with distilled water. pH of the medium was maintained at 4.5 - 4.7.

Method

1. **Standard curve**
    a. Different dilutions of ethanol solution were prepared by mixing stock ethanol solution (5 %) and diluted to 10 ml with distilled water in the test tube.
    b. The range of ethanol percentage was 0.2 to 1 %.
    c. To this 2 ml of $K_2Cr_2O_7$ (1N) was added & mixed.
    d. This solution was boiled for exactly 10 min in a vigorously boiling water bath.
    e. Took the absorbance at 600 nm in spectrophotometer after 10 minutes.
2. **Growth of Yeast cells**
    a. Glucose Yeast Extract medium was prepared having 10% glucose.
    b. Inoculated 1 gm of Baker's yeast to this medium.
    c. Incubated it at 37 ºC for 48 hours with shaking at 120 rpm in shaker.
    d. After 48 hours, 1 ml of yeast suspension was taken and centrifuged the yeast cells at 10000 rpm for 10 minutes.
    e. Took the pellets and added 5 ml of sterile distilled water and again centrifuged at 10000 rpm for 10 minutes.
    f. Again discarded the supernatant and diluted these pellets to 5 ml with sterile distilled water.
3. **Sample preparation**
    a. Took 10 gm sample in 250 ml flasks and added 1 ml of starter yeast culture in each flask.
    b. Maintained the moisture around 60 % in all the flasks.
    c. All the flasks were incubated for 72 hours at 37 ºC with shaking at 120 rpm.

4. Sample analysis

a. Took 1 gm of sample in 500 ml round bottom flask.

b. Added 29 ml of distilled water.

c. Distillated each sample at 60 – 70 ℃ and collected the distillate in a 100 ml beaker containing 25 ml of $K_2Cr_2O_7$ solution.

d. Collected 25 ml of distillate for each sample and then incubated at 62.5 ℃ for 20 minutes in a water bath.

e. Cooled the solution and took 5 ml of each sample diluted with 5 ml of distilled water (1:1 dilution).

f. Took the optical density at 600 nm in spectrophotometer.

Chapter 5: Results and Discussions

pH

Analysis of grass cuttings (garden waste) and leaves of *Bamboosa vulgaris* indicated that pH was 8.3 in garden waste and 7.82 in leaf litter at day 0. Treatments comprising of fungal consortia, cow dung and DAP showed a gradual decrease in pH over a period of 90 days and it varied from 7.82 in garden waste (control), 7.98 in garden waste amended with cow dung and 8.08 in garden waste with fungal consortia; whereas it was 7.03, 7.35 and 7.41 in leaf litter (control), leaf litter with cow dung and in leaf litter amended with fungal consortia respectively.

pH in mixed agricultural waste (mixed leaf litter) in Hot water pretreated biomass and untreated biomass indicated that pH was 8.16 in hot water pretreated biomass and 8.21 in untreated biomass and it decreased to 8.04 in hot water pretreated biomass and 8.1 in untreated biomass over a period of 42 days.

Organic Carbon

Organic carbon estimation in different agricultural wastes comprising of different plant/tree species i.e., *Tylophora indica, Hevea brasilienosis, Eucalyptus, Polyalthia longofolia, Tectona grandis, Mangifera indica, Bamboosa* vulgaris and grass cuttings, weeds and mixed waste, showed that the maximum organic carbon was present in *Bamboosa vulgaris* i.e. 24.81% and minimum in grass cuttings i.e. 18.8 % (Table 2). Analysis of organic carbon in grass cuttings (garden waste) and leaves of *Bamboosa vulgaris* indicated that organic carbon was 22.8% in garden waste and 26.1% in leaf litter at day 0(Texture color shown in Plate: 1, 7). Treatments comprising of fungal consortia, cow dung and DAP showed a gradual decrease in organic carbon content over a period of 90 days. Organic carbon content was decreased to 15.8% from 22.8% in garden waste (control) whereas, 15.1% from 26.1% in leaf litter (control). The maximum decrease in organic carbon was observed between 75-90 days of incubation in all different treatments. After 90 days organic carbon was found to vary from 14.5 % to 16.1 % (Table 4), (Figure 1 & 2)

(Texture color shown Plate: 2-6). The results indicated that the organic carbon reduction rate was faster in different combinations of leaf litter but the maximum reduction was found in garden waste amended with fungal consortia and DAP.

Organic carbon in mixed agricultural waste (mixed leaf litter) pretreated with hot water and untreated biomass indicated that organic carbon was 25.3% in hot water pretreated biomass and 25.9% in untreated biomass (Table 5), (Figure (3) (Texture color shown in Plate: 13). Organic carbon was decreased up to 21.3% from 25.3% in hot treated biomass and to 22.5% from 25.9% in untreated biomass after 42 days of field incubation (Texture color shown in Plate: 14, 15). The results indicated that the hot water pretreated biomass gave better results in terms of decreasing organic carbon content as compared to untreated biomass over a period of 42 days.

The organic carbon was found to be decreasing with the progression of decomposition. As the decomposition progressed due to the losses of carbon mainly as carbon dioxide, the carbon content of the agricultural waste decreased with time (Goyal *et al.*, 2005).

Available Nitrogen

Available nitrogen estimation in different agricultural wastes comprising of different plant/tree species, showed that the minimum available nitrogen was present in *Bamboosa vulgaris* i.e. 147.46 ppm and maximum in grass cuttings i.e. 205.3 ppm (Table2) (Texture color shown in Plate: 1, 7).

Analysis of available nitrogen in garden waste (grass cutting) and leaves of *Bamboosa vulgaris*) indicated that available nitrogen was 1008 ppm in garden waste and 765.3 ppm in leaf litter at day 0. Treatments comprising of fungal consortia, cow dung and DAP showed a gradual increase in available nitrogen when studied over a period of 90 days. Available nitrogen was increased up to 1288 ppm from 1008 ppm in garden waste (control) whereas, 1222.7 ppm from 765.3 ppm in leaf litter (control). The maximum increase in available nitrogen was observed between 15-30 days of incubation in all different treatments. After 90 days available nitrogen was observed to vary from 1194.7 to 1773 ppm. The maximum available nitrogen was found in garden waste amended with fungal consortia and DAP (1773 ppm) and

minimum in leaf litter amended with cow dung (1194.7 ppm). Overall, the maximum increase in available nitrogen was observed as 75.8 % in garden waste and 90.2 % in leaf litter with due course of time (Table 6), (Figure 4 & 5). Thus the results indicated that the available nitrogen increased at a faster rate in different combinations of leaf litter but the maximum available nitrogen was found in garden waste amended with fungal consortia and DAP.

Available nitrogen in mixed agricultural waste (mixed leaf litter) pretreated with hot water and untreated biomass indicated that available nitrogen was 830.6 ppm in hot water pretreated biomass and 784 ppm in untreated biomass. Available nitrogen was found to increase up to 1250 ppm from 830.6 ppm in hot treated biomass and 1017.3 ppm from 784 ppm in untreated biomass after 42 days of field incubation (Table 7), (Figure 6). The results indicated that the hot water pretreated biomass gave better results in terms of increasing available nitrogen content as compared to untreated biomass over a period of 42 days.

The decrease in available nitrogen in early stages of experiment was due to losses of nitrogen in the form of ammonia which in turn depends upon the type of material and its C:N ratio (Reddy *et al.,* 1979; Sanchez-Monedero *et al.,* 2001).

Cellulose

Analysis of cellulose in garden waste (grass cuttings) and leaves of *Bamboosa vulgaris* indicated that cellulose was 534.4 ppm in garden waste and 399.7 ppm in leaf litter at day 0. Treatments comprising of fungal consortia, cow dung and DAP showed a gradual decrease in cellulose content when studied over a period of 90 days. Cellulose content was decreased to 118.4 ppm from 534.4 ppm for garden waste (control) whereas 117.1 ppm from 399.7 ppm in leaf litter (control). After 90 days cellulose was found to be varying from 113.7 ppm to 135.1 ppm in all different treatments of garden waste and leaf litter and the minimum cellulose was observed in garden waste with cow dung and DAP (113.7 ppm) and maximum in leaf litter with cow dung (135.1 ppm). Overall, the maximum reduction in cellulose was observed as 78.7% in garden waste and 71.2% in leaf litter with the due course of time (Table 8), (Figure 7 & 8). Results indicated that the cellulose reduction rate was faster in

different combinations of garden waste and the minimum cellulose was present in garden waste amended with cow dung and DAP after 90 days of incubation.

Cellulose in mixed agricultural waste (mixed leaf litter) with hot water pretreated biomass and untreated biomass indicated that cellulose was 365.06 ppm in hot water pretreated biomass and 421.7 ppm in untreated biomass and afterwards, it showed a gradual decrease in cellulose in both the treatments. Cellulose decreased up to 169.06 ppm from 365.06 ppm in hot treated biomass and 180.4 ppm from 421.7 ppm in untreated biomass after 35 days of field incubation. The maximum decrease in cellulose was observed in both the treatments between 7-14 days (Table 9), (Figure 9). The results indicated that in hot water pretreated biomass the decrease in cellulose content was higher as compared to untreated biomass over a period of 35 days.

Reducing sugars

Reducing sugars analyzed in garden waste (grass cutting) and leaves of *Bamboosa vulgaris*), indicated that reducing sugars were 249.3 ppm in garden waste and 230.4 ppm in leaf litter at day 0. Treatments comprising of fungal consortia, cow dung and DAP showed a gradual increase in reducing sugars when studied over a period of 90 days. Reducing sugars were increased up to 412.1 ppm from 249.3 ppm for garden waste (control) whereas 406.7 ppm from 230.4 ppm in leaf litter (control). After 90 days reducing sugars were found to be varying from 406.7 to 428.2 ppm in all different treatments of garden waste and leaf litter and the maximum reducing sugars were observed in garden waste with fungal consortia and DAP (428.2 ppm) and minimum in leaf litter (control) (406.7 ppm). Overall, the maximum increase in reducing sugars was observed as 71.7 % in garden waste and 79.6 % in leaf litter with the due course of time (Table 10), (Figure 10 & 11). Thus the result indicated that the reducing sugars increment rate was faster in different combinations of leaf litter but the maximum reducing sugars were present in garden waste amended with fungal consortia and DAP.

Reducing sugars in mixed agricultural waste (mixed leaf litter) with hot water pretreated biomass and untreated biomass indicated that reducing sugars were

238.64 ppm in hot water pretreated biomass and 230.6 ppm in untreated biomass. Reducing sugars increased up to 243.3 ppm from 238.64 ppm in hot water pretreated biomass and 235.3 ppm from 230.6 ppm in untreated biomass after 35 days of field incubation (Table 11), (Figure 12). The results indicated that the hot water pretreated biomass gave better results in terms of increasing reducing sugars content as compared to untreated biomass over a period of 35 days.

Various hydrolytic enzymes are believed to control the rate at which various substrates are degraded. Enzymes are the main mediators of various degradative processes (Tiquia *et al.*, 2002). These enzymes help to degrade the non-starch polysaccharides in substrate to reducing sugars. Thus with the decrease in the amount of cellulose, a corresponding increase in reducing sugars was obtained. *A. niger* and *T. reseei* were able to bring the highest percentage in cellulose degradation due to its vigorous growth and therefore ability to produce more celluloytic enzymes within the short period of time (Mckinley *et al.*, 1985; Tiquia *et al.,* 1996). Fungi have the ability to produce enzymes such as *Aspergillus niger*, *A. flavus* and *Trichoderma reseei* have been reported to be the main source of cellulase, amylase, hemicellulase, catalase and xylanase (Hamlyn, 1998).

Lignin

Lignin estimation in different agricultural wastes comprising of different plant/tree species i.e., *Tylophora indica, Hevea brasilienosis, Eucalyptus, Polyalthia longofolia, Tectona grandis, Mangifera indica, Bamboosa* vulgaris and grass cuttings, weeds and mixed waste, showed that the minimum lignin was present in *Tylophora indica* i.e. 2.7% and maximum in grass cuttings i.e. 13.9 % (Table 3).

Ethanol

Analysis of ethanol in garden waste (grass cutting) and leaves of *Bamboosa vulgaris*) with different treatments comprising of fungal consortia, cow dung and DAP, showed variation in ethanol percentage from 1.33 % to 1.4 % when studied after a period of 90 days (Table 12).

Ethanol estimation in mixed agricultural waste (mixed leaf litter) with hot water pretreated biomass and untreated biomass indicated that ethanol percentage was 0.83 % in hot water pretreated biomass and 0.77 % in untreated biomass after 35 days of field incubation and showed a gradual increase in ethanol percentage in both treatments after 49 days (Table 13).

The yeast *Saccharomyces cerevisiae* cannot utilize cellulosic materials, these materials must first undergo saccharification to glucose before ethanol production can take place. Various cellulase and β -glucosidase genes have been expressed in *S. cerevisiae* with the aim of direct ethanol production from cellulose (Rensburg V., *et al., 1998,* Henrissat B., *et al.,* 1985). The yield in terms of grams of ethanol produced per gram of cellulose utilized was 0.48 g/g, which corresponds to 93.3% of the theoretical yield. The highest ethanol concentration was around 16.5 g/kg after 48 h of fermentation in barley β-glucan, a linear polysaccharide composed of an average of 1,200 glucose residues, Since the ethanol concentration increased as the total sugar concentration decreased, production of ethanol occurred by simultaneous saccharification and fermentation of β –glucan (Yasuya, *et al.,* 2002).

Chapter 6: Conclusion

1. Agricultural wastes comprising of different plant/tree species (*Tylophora indica, Hevea brasilienosis, Eucalyptus, Polyalthia longofolia, Tectona grandis, Mangifera indica, Bamboosa* vulgaris & grass cuttings, weeds and mixed waste) were studied for organic carbon and available nitrogen content. The least organic carbon was observed in grass cuttings (18.8%) and maximum in *Bamboosa vulgaris* (24.81%) while available nitrogen was maximum in grass cuttings (205.3 ppm).

2. Over 90 days of incubation the biomass of grass cuttings degraded at much faster rate when treated with fungal consortia (*Aspergillus niger* FS1 and *Trichoderma reseei* MTCC- 164) and DAP.

3. Degradation of mixed leaf litter waste was observed on pilot scale with hot water pretreated biomass and untreated biomass for a period of 6 weeks. It was observed that hot water pretreated biomass gave batter results in terms of decrease in organic carbon and cellulose content and increase in available nitrogen and reducing sugars content as compared to untreated biomass.

4. The results indicated that activator like DAP enhances the rate of decomposition when agricultural wastes are either treated with fungal consortia or cow dung. Use of the fungi and DAP in the biodegradation of the agricultural wastes is the best option for rapid conversion of agricultural wastes and more suitable in biocycling.

Chapter 6: References

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http://www.edugreen.teri.res.in/explore/solwaste/types.htm

http://www.soil.ncsu.edu/publications/Soilfacts/AG-439-19/

http://www.epa.gov/compost/

Table 2: Organic Carbon (%) and Available Nitrogen (ppm) in different types of agro-wastes

Sample	% Org. C	Available N (ppm)
Tylophora indica	20.8	183.6
Hevea brasilienosis	24.4	149.5
Eucalyptus	19.9	198.2
Polyalthia longofolia	19.6	196.4
Techtona grandis	23.6	153.4
Mangifera indica	21.3	173.6
Grass cutting	18.8	205.3
Bamboosa vulgaris	24.8	147.5
Weeds	22.1	170.2
Mixed waste	24.3	151.1

Table 3: Lignin (%) in different type of agro-wastes

Sample	% lignin
Tylophora indica	2.7
Hevea brasilienosis	5.4
Eucalyptus	5.0
Polyalthia longofolia	4.8
Techtona grandis	4.0
Mangifera indica	5.0
Grass cutting	13.9
Bamboosa vulgaris	3.4
Weeds	12.4
Mixed waste	11.1

Table 4: Changes in Organic Carbon (%) at different time interval (Laboratory scale)

Treatment	0 day	15 days	30 days	45 days	60 days	75 days	90 days
GW + C	22.8±0.14	22.1±0.1	21.5±0.1	20.4±0.2	19.4±0.1	17.4±0.3	15.9±0.1
GW + CD		25.1±0.4	24.8±0.1	21.8±0.1	20.4±0.4	18.3±0.4	15.6±0.1
GW + CD + DAP		24.9±0.1	23.9±0.2	22.7±0.1	20.5±0.1	18.2±0.5	15.7±0.2
GW + FC		24.3±0.1	22.1±0.3	19.5±0.09	18.7±0.5	16.6±0.1	15.1±0.1
GW + FC +DAP		26.2±0.5	24.1±0.3	22.7±0.2	20.6±0.1	18.1±0.1	14.5±0.1
LL+ C	26.1±0.14	28.6±0.9	25.9±0.1	21.1±0.1	18.9±0.1	17.6±0.3	16.1±0.3
LL + CD		24.3±0.1	22.7±0.1	21.8±0.7	19.7±0.7	16.7±0.3	15.8±0.2
LL + CD +DAP		25.1±0.1	22.6±0.2	20.4±0.1	19.2±0.1	17.5±0.3	15.9±0.2
LL + FC		27.6±0.1	23.1±0.5	20.7±0.3	19.3±0.3	17.5±0.1	16.3±0.1
LL + FC + DAP		27.6±0.1	22.8±0.1	21.5±0.8	19.2±0.7	17.7±0.2	16.1±0.1

GW: Garden Waste; C: Control; CD: Cow Dung; FC: Fungal Consortia; DAP: Di Ammonium Phosphate; LL: Leaf Litter.
Temperature during dark incubation: 30 ºC; Moisture content: 50%

Table 5: Changes in Organic carbon (%) at different time interval (Pilot Scale)

Days	% Org. carbon in Hot water pretreated biomass	% Org. carbon in untreated biomass (Control)
0	25.3 ± 0.1	25.9 ± 0.1
7	26.3 ± 0.1	26.1 ± 0.3
14	26.1 ± 0.3	25.4 ± 0.2
21	22.9 ± 0.1	24.3 ± 0.1
28	22.7 ± 0.1	23.2 ± 0.1
35	22.2 ± 0.1	22.8 ± 0.1
42	21.3 ± 0.2	22.5 ± 0.5

Temperature: Field conditions; Moisture content: approx. 50%

Table 6: Changes in Available Nitrogen (ppm) at different time interval (Laboratory scale)

Treatment	0 day	15 days	30 days	45 days	60 days	75 days	90 days
GW + C	1008±16.1	541.3±9.3	718.6±9.3	905.3±18.6	924.0±16.1	1194.6±24.6	1288.0±16.1
GW + CD		522.6±9.3	840.0±0	1036.0±0	1222.7±18.1	1325.3±9.3	1521.3±9.3
GW + CD + DAP		662.6±9.3	681.3±18.6	774.6±24.6	1428.0±16.1	1577.3±9.3	1717.3±18.6
GW + FC		494.6±9.3	896.0±16.1	933.3±8.5	1222.7±18.6	1278.6±9.3	1652.0±16.1
GW + FC +DAP		429.3±9.3	933.3±18.6	989.3±4.9	1176±2.8	1456.0±3.2	1773.0±9.3
LL+ C	765.3±9.3	522.6±9.3	541.3±9.3	634.6±9.3	914.6±9.3	1008.0±16.1	1222.7±18.6
LL + CD		578.6±9.3	625.3±18.6	606.6±24.6	672.0±16.1	877.3±9.3	1194.7±18.6
LL + CD +DAP		448±16.1	569.3±9.3	681.3±18.8	961.3±9.3	1026.6±4.9	1325.3±9.3
LL + FC		718.6±18.6	774.6±9.3	849.3±3.7	1194.7±18.6	1400.0±3.2	1344.2±18.6
LL + FC + DAP		588.0±16.1	746.6±9.3	905.3±3.7	1045.3±18.6	1213.3±8.1	1456.0±9.3

GW: Garden Waste; C: Control; CD: Cow Dung; FC: Fungal Consortia; DAP: Di Ammonium Phosphate; LL: Leaf Litter.
Temperature during dark incubation: 30 ºC; Moisture content: 50%

Table 7: Changes in Available Nitrogen (ppm) at different time interval (Pilot Scale)

Days	Available Nitrogen (ppm) in Hot water pretreated biomass	Available Nitrogen (ppm) in untreated biomass (Control)
0	830.6 ± 24.6	784.0 ± 16.1
7	709.3 ± 18.6	681.3 ± 13.6
14	653.3 ± 18.6	634.6 ± 14.6
21	690.3 ± 9.3	681.3 ± 14.9
28	933.3 ± 24.6	830.6 ± 14.6
35	1082.6 ± 23.6	886.6 ± 13.6
42	1250.0 ± 24.6	1017.3 ± 14.6

Temperature: Field conditions; Moisture content: approx. 50%

Table 8: Changes in Cellulose (ppm) at different time interval (Laboratory scale)

Treatment	0 day	15 days	30 days	45 days	60 days	75 days	90 days
GW + C	534.4±6.4	417.1±8.9	277.1±4.8	151.7±3.3	146.4±2.0	134.4±2.3	118.4±1.1
GW + CD		405.7±6.9	199.7±7.4	177.1±2.4	131.1±1.7	123.7±2.4	119.7±1.7
GW + CD + DAP		433.7±9.3	249.1±6.3	128.4±3.4	118.4±0.2	117.7±1.7	113.7±0.6
GW + FC		463.7±6.3	227.1±7.8	172.4±3.1	152.4±3.1	145.1±3.5	132.4±1.1
GW + FC +DAP		451.7±7.6	225.7±3.5	147.1±5.9	135.7±2.4	130.4±3.1	124.4±1.1
LL+ C	399.7±9.2	366.4±5.3	279.1±4.3	273.1±5.2	121.1±3.5	120.4±2.3	117.1±1.3
LL + CD		301.1±3.5	235.1±5.8	214.4±5.2	163.1±2.6	150.4±2.3	135.1±2.4
LL + CD +DAP		368.4±8.3	278.4±7.1	147.7±5.9	141.1±2.9	139.1±2.9	120.4±3.1
LL + FC		354.4±8.3	151.1±9.9	125.1±2.4	121.7±2.9	119.1±1.7	115.1±1.7
LL + FC + DAP		395.1±4.4	253.1±7.6	195.7±4.1	144.4±3.4	135.7±2.9	120.4±1.1

GW: Garden Waste; C: Control; CD: Cow Dung; FC: Fungal Consortia; DAP: Di Ammonium Phosphate; LL: Leaf Litter.
Temperature during dark incubation: 30 ºC; Moisture content: 50%

Table 9: Changes in Cellulose (ppm) at different time interval (Pilot Scale)
Temperature: Field conditions; Moisture content: approx. 50%

Days	Cellulose conc.(ppm) in Hot water pretreated biomass	Cellulose conc.(ppm) in untreated biomass (Control)
0	365.1 ± 6.9	421.7 ± 10.9
7	352.4 ± 3.3	390.4 ± 11.3
14	270.4 ± 1.2	294.4 ± 4.1
21	247.1 ± 2.9	266.4 ± 7.2
28	215.1 ± 4.8	235.1 ± 4.8
35	169.1 ± 6.3	180.4 ± 6.1

Table 10: Changes in Reducing Sugars (ppm) at different time interval (Laboratory scale)

Treatment	0 day	15 days	30 days	45 days	60 days	75 days	90 days
GW + C	249.3±2.7	217.1±3.2	246.6±3.0	268.6±1.6	368.7±0.9	379.9±1.1	412.1±1.2
GW + CD		195.4±1.1	246.2±3.2	304.5±4.1	366.9±1.9	401.8±1.1	418.1±1.3
GW + CD + DAP		200.1±2.8	229.9±1.5	283.3±2.3	330.1±3.4	369.1±1.9	408.6±1.3
GW + FC		175.9±1.1	255.1±1.1	309.8±0.6	318.5±2.1	378.3±1.2	415.7±0.8
GW + FC +DAP		162.2±2.8	257.1±3.8	358.3±2.5	361.4±0.6	388.7±1.9	428.2±1.6
LL+ C	230.4±2.7	207.3±4.5	209.1±2.7	273.5±4.5	313.7±2.9	390.2±1.5	406.7±2.4
LL + CD		214.7±8.3	225.7±1.3	286.6±1.9	335.5±5.3	395.3±2.9	413.9±1.7
LL + CD +DAP		207.4±4.0	223.5±1.2	278.6±1.2	343.1±3.1	397.8±1.8	411.4±2.2
LL + FC		117.7±2.8	253.9±2.8	355.7±0.9	372.7±2.4	401.5±3.1	410.6±3.8
LL + FC + DAP		196.7±2.0	258.7±0.5	291.5±0.8	328.1±1.1	391.8±3.6	408.6±2.4

GW: Garden Waste; C: Control; CD: Cow Dung; FC: Fungal Consortia; DAP: Di Ammonium Phosphate; LL: Leaf Litter.
Temperature during dark incubation: 30 ºC ; Moisture content: 50%

Table 11: Changes in Reducing Sugars (ppm) at different time interval (Pilot Scale)

Days	Reducing sugars (ppm) in Hot water pretreated biomass	Reducing sugars (ppm) in untreated biomass (Control)
0	238.6 ± 2.7	230.6 ± 2.3
7	227.9 ± 2.2	226.2 ± 2.7
14	223.1 ± 1.6	225.8 ± 1.6
21	237.1 ± 2.3	229.5 ± 1.3
28	241.1 ± 1.7	231.4 ± 1.4
35	243.3 ± 1.5	235.3 ± 1.7

Temperature: Field conditions; Moisture content: approx. 50%

Table 12: Ethanol (%) in different treatments of agricultural wastes after 90 days (laboratory scale)

Treatment	Ethanol (%)
GW + C	1.35
GW + CD	1.37
GW + CD +DAP	1.34
GW + FC	1.36
GW + FC + DAP	1.40
LL + C	1.33
LL + CD	1.36
LL + CD +DAP	1.35
LL + FC	1.35
LL + FC + DAP	1.34

GW: Garden Waste; C: Control; CD: Cow Dung; FC: Fungal Consortia; DAP: Di Ammonium Phosphate; LL: Leaf Litter.
Temperature during dark incubation: 30 ºC; Moisture content: 50%
Fermentation temperature: 37 ºC; Fermentation period: 72 hours

Table 13: Changes in ethanol (%) at different time interval (Pilot Scale)

Days	Ethanol (%) in Hot water pretreated biomass	Ethanol (%) in untreated biomass (Control)
35	0.83	0.77
42	0.87	0.79
49	0.93	0.84

Fermentation temperature: 37 ºC
Fermentation period: 72 hour; Moisture content: approx. 50%

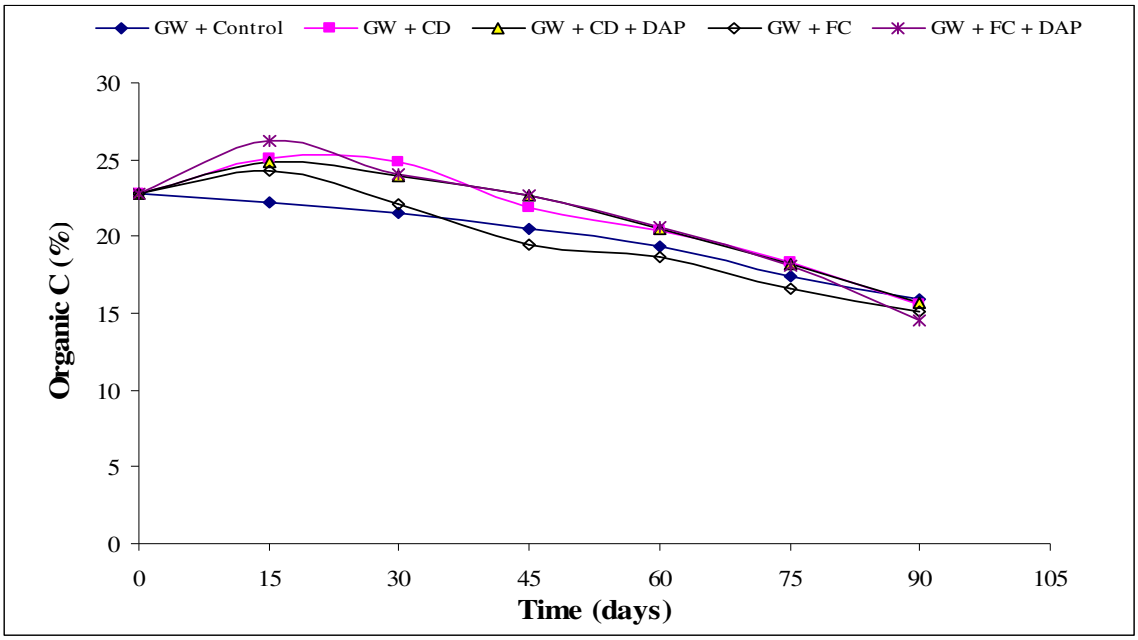

Figure 1: Organic carbon (%) in various treatments at different time intervals
GW: Garden Waste; CD: Cow Dung; FC: Fungal Consortia; DAP: Di Ammonium Phosphate
Temperature during dark incubation: 30°C; Moisture content: 50%

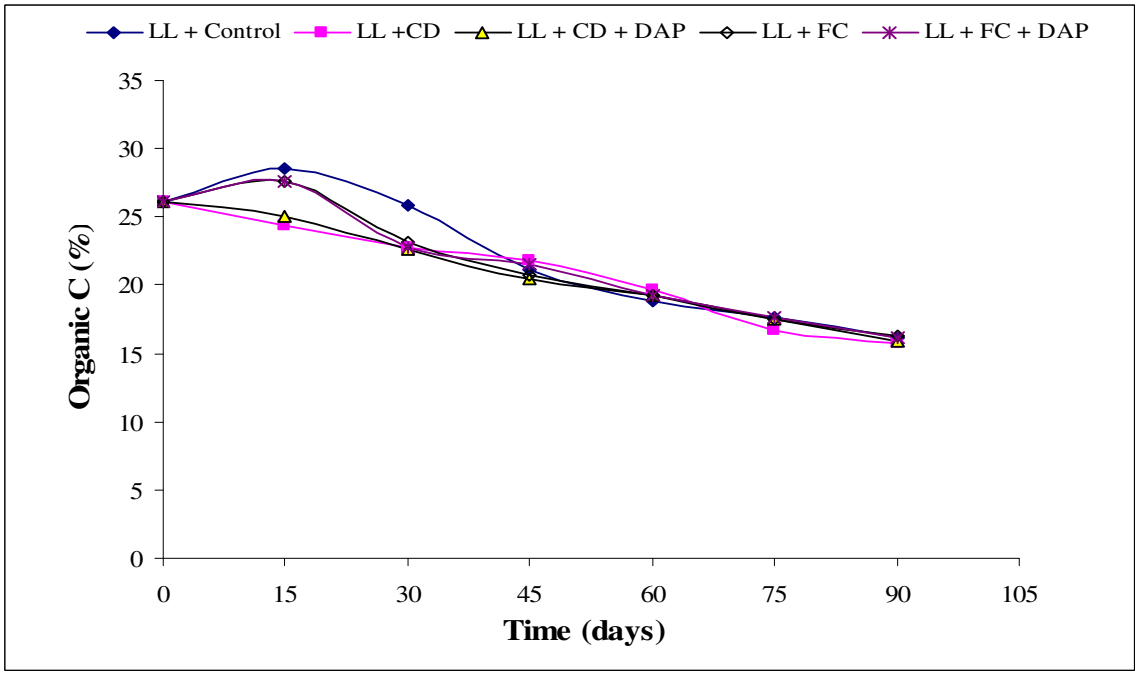

Figure 2: Organic carbon (%) in various treatments at different time intervals
LL: Leaf Litter; CD: Cow Dung; FC: Fungal Consortia; DAP: Di Ammonium Phosphate

Temperature during dark incubation: 30°C; Moisture content: 50%

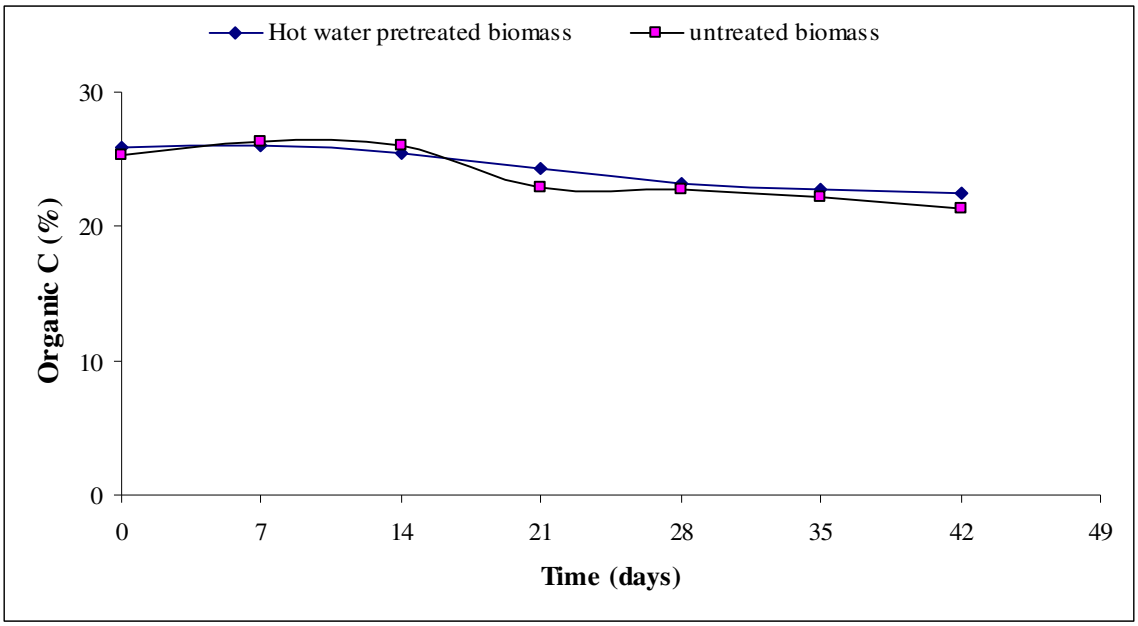

Figure 3: Organic carbon (%) in two different treatments at different time intervals
Temperature: Field conditions; Moisture content: 50%

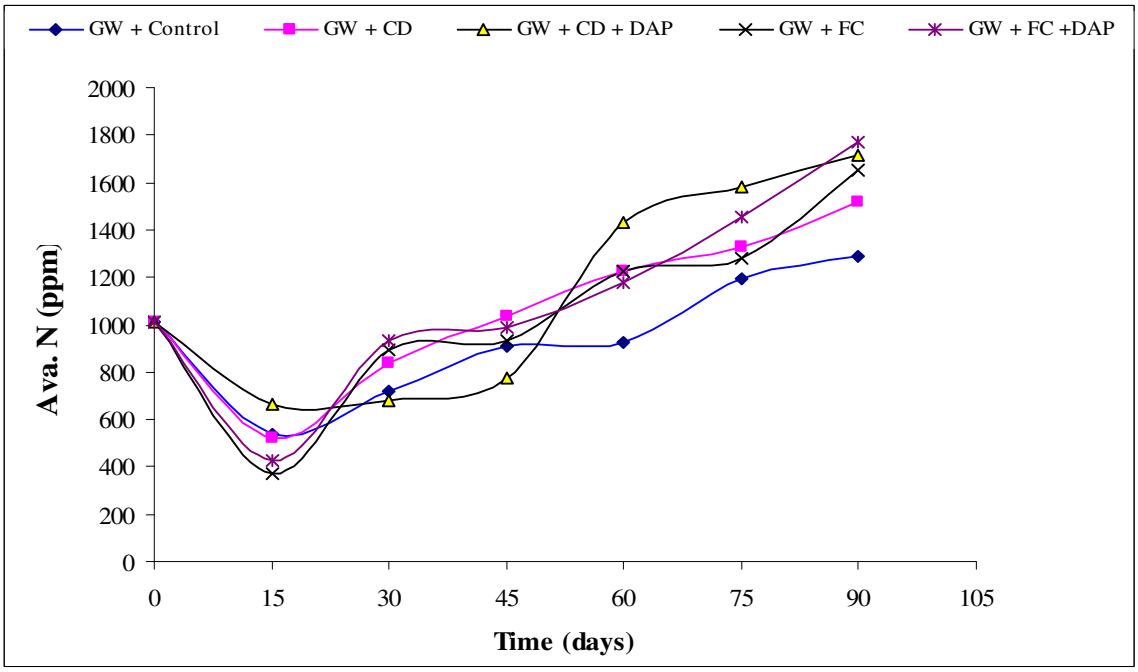

Figure 4: Available nitrogen (ppm) in various treatments at different time intervals
GW: Garden Waste; CD: Cow Dung; FC: Fungal Consortia; DAP: Di Ammonium Phosphate

Temperature during dark incubation: 30°C; Moisture content: 50%

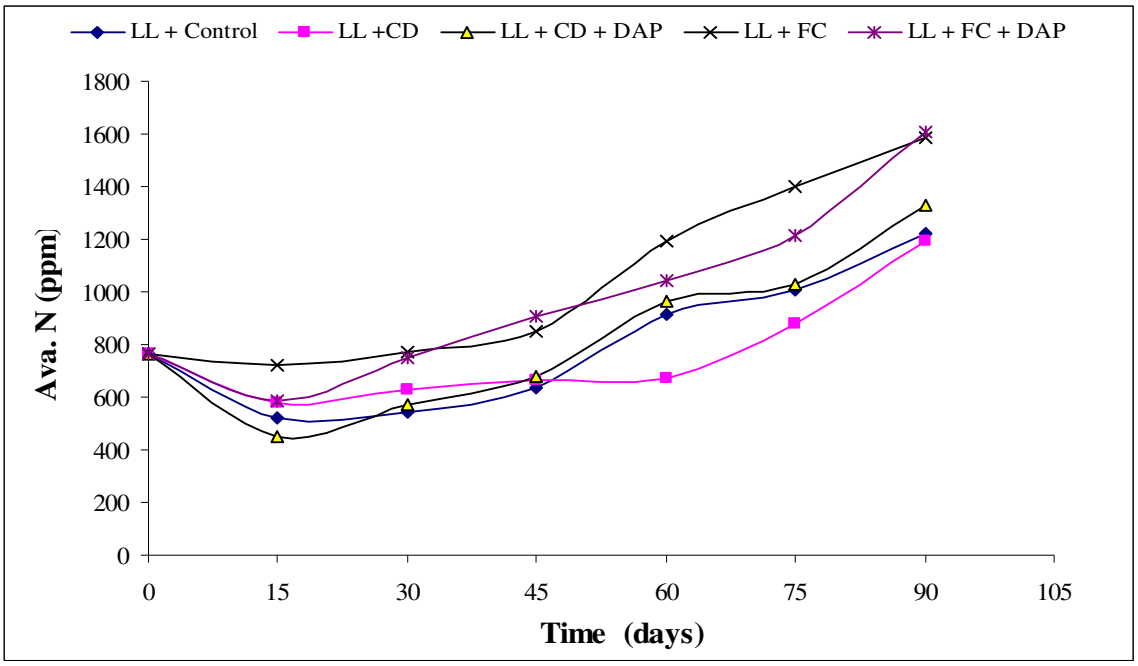

Figure 5: Available nitrogen (ppm) in various treatments at different time intervals
LL: Leaf Litter; CD: Cow Dung; FC: Fungal Consortia; DAP: Di Ammonium Phosphate
Temperature during dark incubation: 30°C; Moisture content: 50%

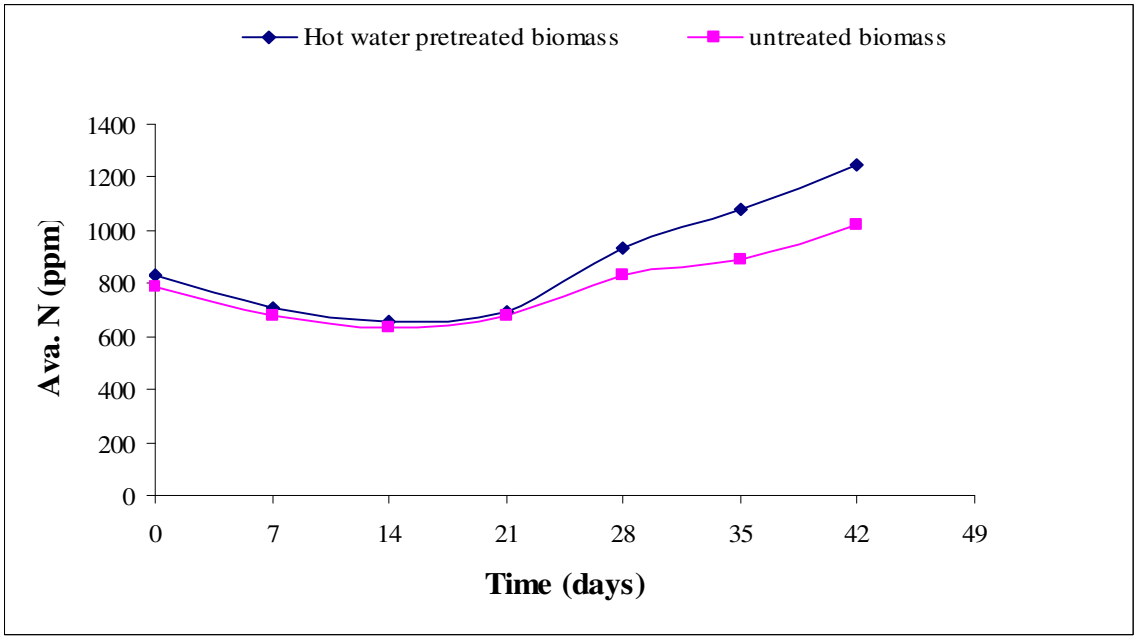

Figure 6: Available nitrogen (ppm) in two different treatments at different time intervals
Temperature: Field conditions; Moisture content: 50%

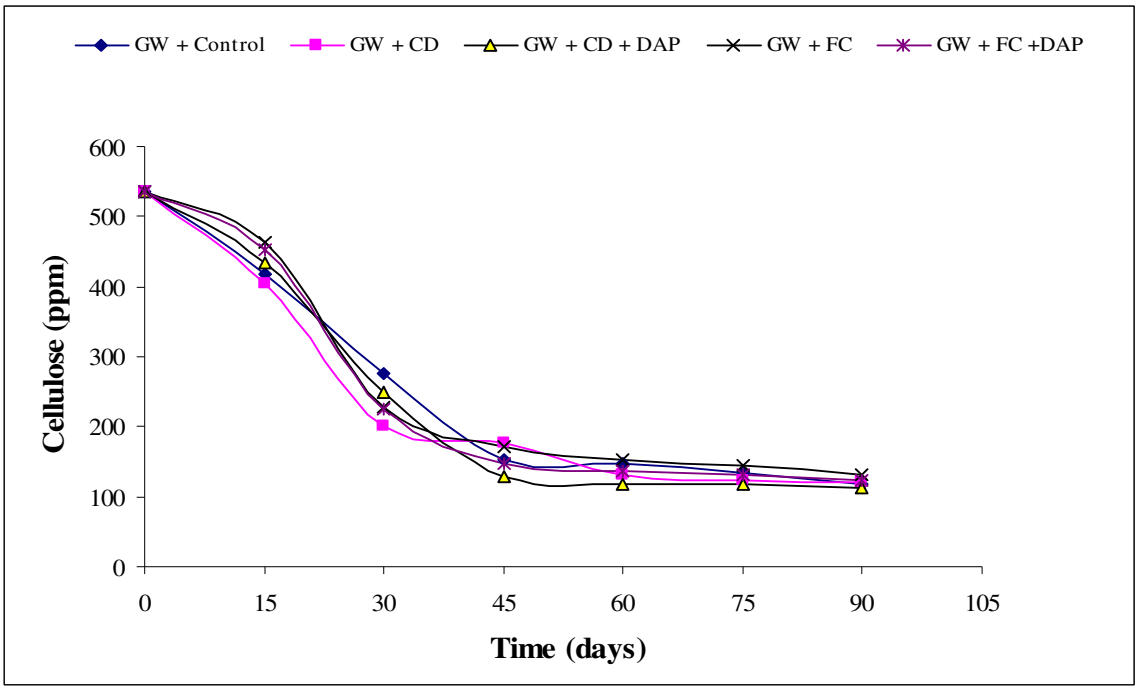

Figure 7: Cellulose (ppm) in various treatments at different time intervals
GW: Garden Waste; CD: Cow Dung; FC: Fungal Consortia; DAP: Di Ammonium Phosphate
Temperature during dark incubation: 30°C; Moisture content: 50%

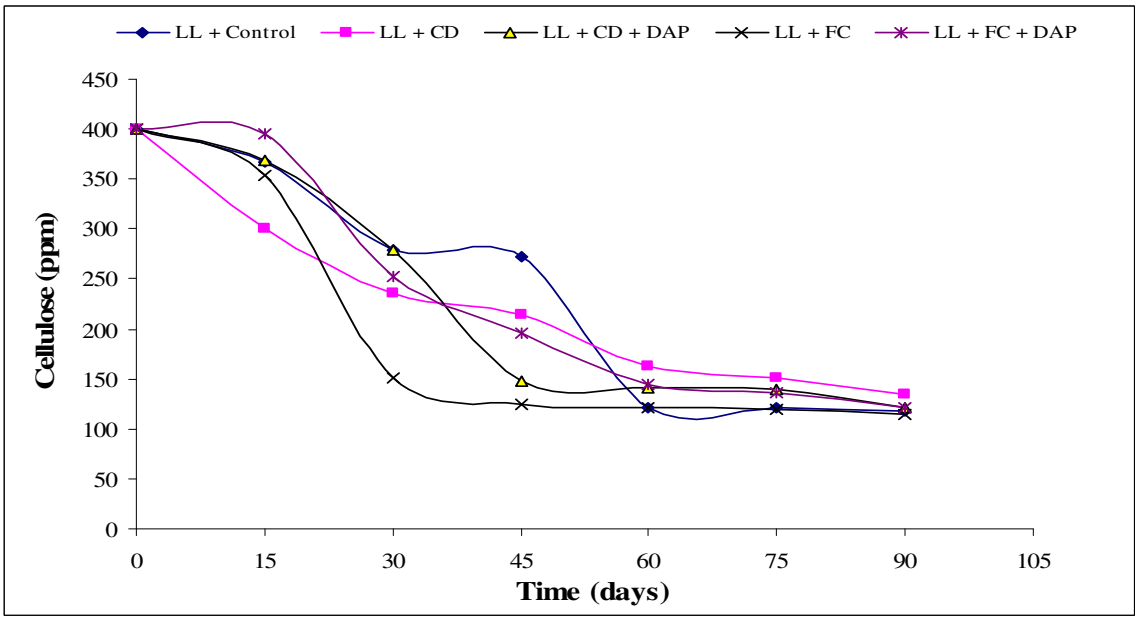

Figure 8: Cellulose (ppm) in various treatments at different time intervals
LL: Leaf Litter; CD: Cow Dung; FC: Fungal Consortia; DAP: Di Ammonium Phosphate

Temperature during dark incubation: 30°C; Moisture content: 50

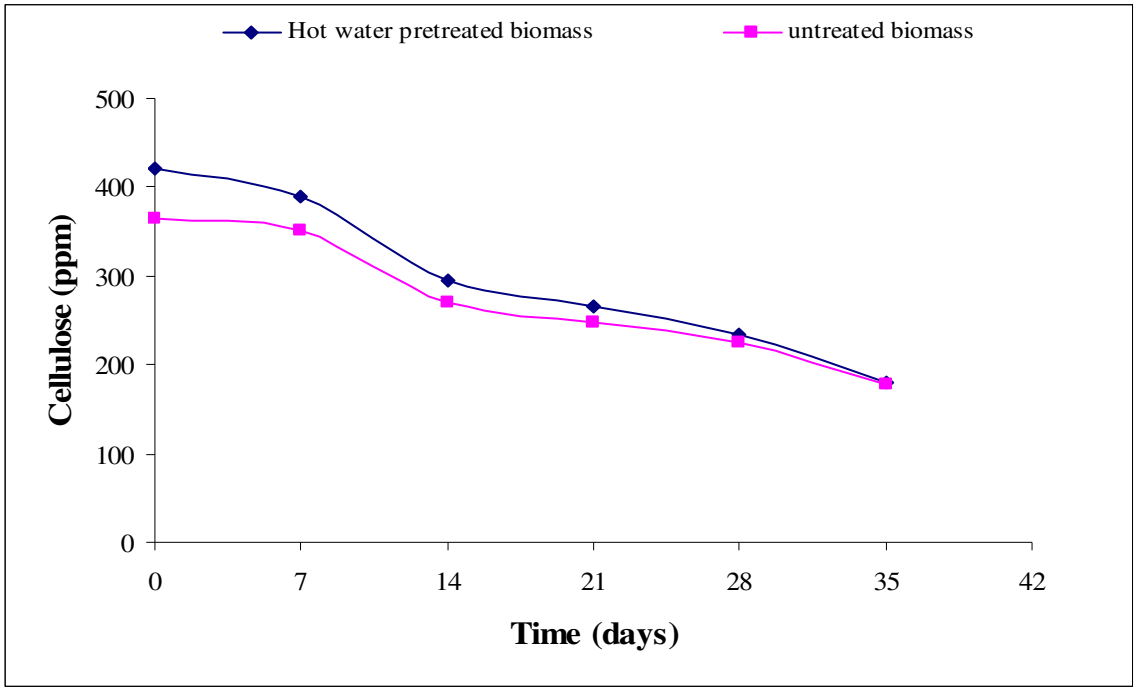

Figure 9: Cellulose (ppm) in two different treatments at different time intervals
Temperature: Field conditions; Moisture content: 50%

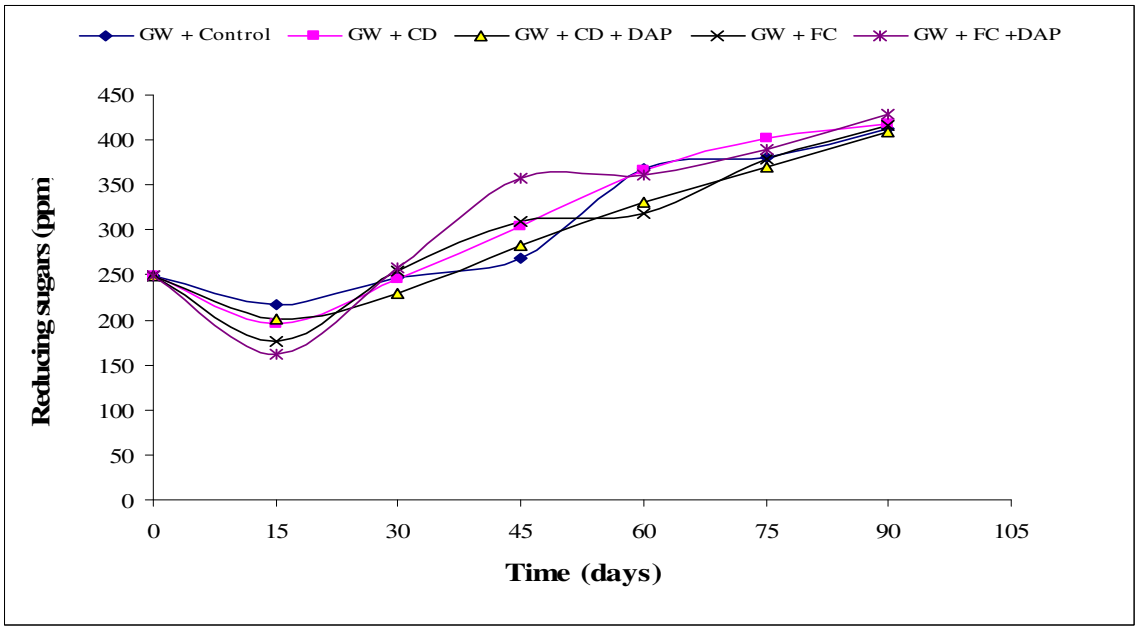

Figure 10: Reducing sugars (ppm) in various treatments at different time intervals
GW: Garden Waste; CD: Cow Dung; FC: Fungal Consortia; DAP: Di Ammonium Phosphate

Temperature during dark incubation: 30°C; Moisture content: 50%

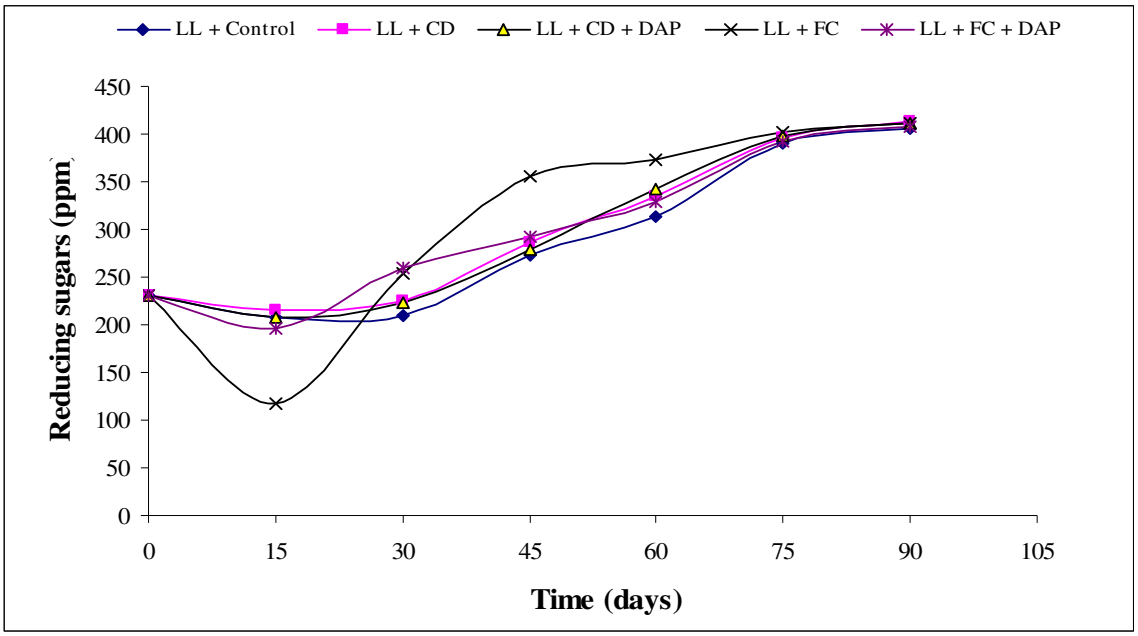

Figure 11: Reducing sugars (ppm) in various treatments at different time intervals
LL: Leaf Litter; CD: Cow Dung; FC: Fungal Consortia; DAP: Di Ammonium Phosphate
Temperature during dark incubation: 30°C; Moisture content: 50%

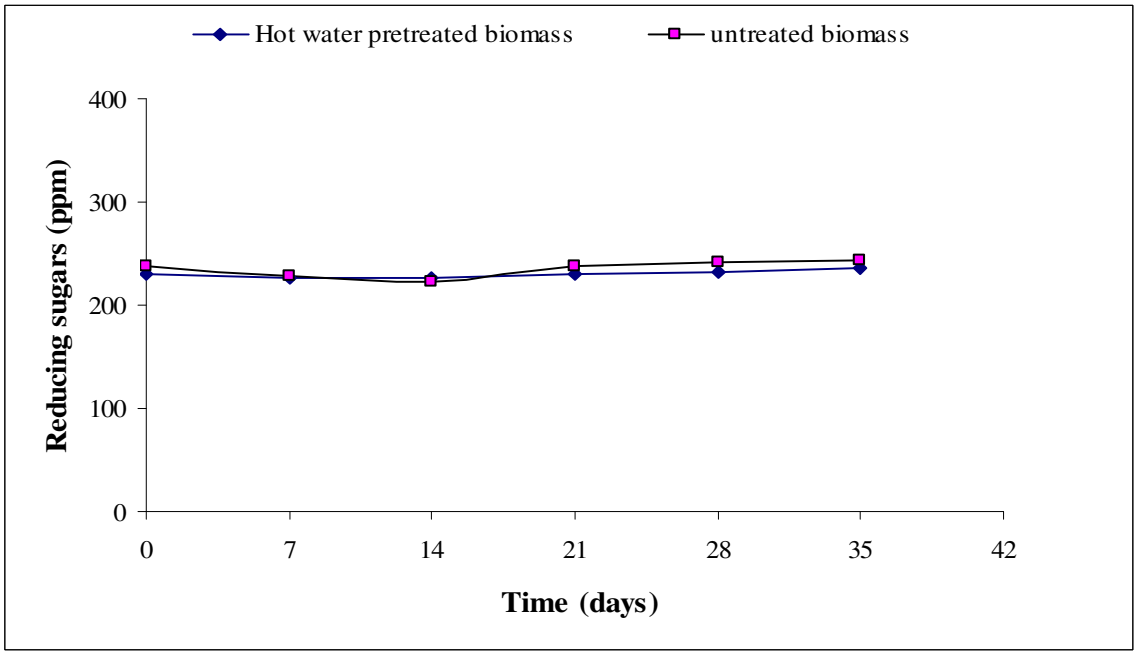

Figure 12: Reducing sugars (ppm) in two different treatments at different time intervals
Temperature: Field conditions; Moisture content: 50%

Plate 1: Garden waste (day 0)

Plate 2: Garden waste as control (day 90)

Plate 3: Garden waste with cow dung (day 90)

Plate 4: Garden waste with cow dung and DAP (day 90)

Plate 5: Garden waste with fungal consortia (day 90)

Plate 6: Garden waste with fungal consortia & DAP (day 90)

Plate 7: Leaf litter (day 0)

Plate 8: Leaf litter as control (day 90)

Plate 9: Leaf litter with cow dung (day 90)

Plate 10: Leaf litter with cow dung & DAP (day 90)

Plate 11: Leaf litter with fungal consortia (day 90)

Plate 12: Leaf litter with fungal consortia & DAP (day 90)

Plate 13: Mixed agricultural waste (day 0) (field set up)

Plate 14: Mixed agricultural waste (field set up) with hot treatment (day 42)

Plate 15: Mixed agricultural waste (field set up) without hot treatment (day 42)