BIOCONVERSION OF LIGNOCELLULOSIC SOLID WASTES OF A HERBAL PROCESSING INDUSTRY TO COMMERCIALLY VALUABLE PRODUCTS

By

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A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

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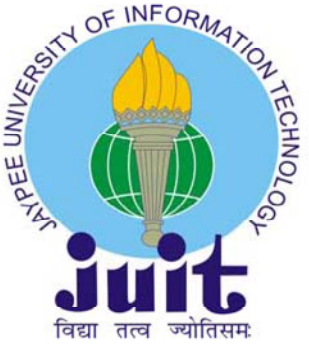

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CERTIFICATE

This is to certify that the thesis entitled **"Bioconversion of Lignocellulosic Solid Wastes of a Herbal Processing Industry to Commercially Valuable Products"** submitted by **Mamta Kumari** to the Jaypee University of Information Technology, Waknaghat in fulfillment of the requirement for the award of the degree of **Doctor of Philosophy in Biotechnology** is a record of bona fide research work carried out by her under our supervision and guidance and no part of this work has been submitted for any other degree or diploma.

Dr. Sudhir Kumar
Assistant Professor
(Guide)

Prof. R. S. Chauhan
Head of Department
(Co-guide)

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"Knowledge is in the end based on acknowledgement"

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Place:
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CHAPTER 1

INTRODUCTION

Growing urbanization and industrialization have led to the production of large quantities of household and industrial solid wastes and the problem of its handling is further augmented with shortage of landfills. Moreover strict environmental legislations and public consciousness have made land filling increasingly expensive and irresolute (Gupta et al., 2007). Present scenario represents it as one of the major concern of environmental problems. Management of solid wastes is a problem of increasing concern throughout the world. There is a need for cost effective and ecofriendly solid waste management technologies. Types of biomass such as agricultural residues, wood chips, municipal solid waste, food processing waste and industrial herbal waste etc qualify to be known as biodegradable solid waste having the potential to serve as low cost, abundant feed stocks. The problem of disposal of biodegradable waste generated after industrial operations was addressed by the Ayurvet Industry Ltd., situated in Baddi, distt. Solan (Himachal Pradesh). This Industry deals in herbal health care and nutritional products for a range of animal species in India as well as abroad especially in Africa, Europe, South-East Asia, Middle East and the Indian subcontinent. It is one of India's leading manufacturers of herbal veterinary medicine, feed supplements and animal feed. During the processing and manufacturing of veterinary products, 0.5 to 1.0 ton of medicinal plant waste is discarded daily. Although the waste is biodegradable but the industry is facing problem in disposing it off. Our study entails on finding some useful and viable alternatives to dispose off industrial herbal waste of a nearby industry so as to use a proportion of this waste biomass for organic farming and energy production.

Industrial herbal waste was heterogeneous in composition consisting of lignin, cellulose, hemicellulose and pectin due to presence of leftover parts of various medicinal plants. It was used as a substrate for the production of vermicompost which can be an appropriate, safe, hygienic and cost effective disposal of biodegradable waste rich in organic matter. It is the non-thermophilic biodegradation of organic material through the interaction between earthworms and microorganisms (Aracnon et al., 2004), whereby organic material residuals are fragmented rapidly into much finer particles by passing them through a grinding gizzard while maintaining nutrients (Ndegwa and Thompson 2001). Exotic species of earthworm *Eudrilus eugeniae* was used in the present study. Earthworms reduce the numbers of human pathogens, an effect obtained in traditional composting by increase in temperature, but vermicomposting is generally faster (Susan 1994; Ghatnekar et al., 1998). It is a finely-divided, peat-like material, with high porosity, aeration, drainage, water holding capacity and microbial activity, which make it an excellent soil conditioner (Sahni et al., 2008). Vermicompost contains plant-growth regulating materials, such as humic acids (Atiyeh et al., 2002) and plant growth regulators like auxins, gibberellins and cytokinins (Krishnamoorthy and Vajrabhiah 1986; Tomati et al., 1988, 1990), which are responsible for increased plant growth and yield of many crops (Atiyeh et al., 2002). In recent times organic farming has become the choice replacing chemical fertilizers. Though practice of vermicomposting is century old but now it's getting revived with ecological objectives such as waste management, soil detoxification, sustainable agriculture and demand of organic products free of chemical fertilizers (Suthar 2007, Pattnaik and Reddy 2010). Vermicomposting is an innovative component to stabilize the soil structure of an area could be a solution to decrease land degradation. During the composting process, microorganisms

decompose organic compounds, which consist of carbohydrates, sugar, proteins, fats, cellulose and lignin. Carbohydrates are more easily decomposed whereas lignin is more resistance to decomposition (Vinceslas –Akpa and Loquet 1997).

Lignocellulosic materials are cheap, abundant renewable resources and promising raw materials for ethanol production, yet, they are usually disposed or directly burned due to lack of effective utilization, thus often causing serious environmental pollution (Zhao and Xia 2009). Energy production from fossil fuels becomes more and more problematic since these resources are running short and burning of coal, oil and natural gas is connected with emissions of the green-house gases (Nikolic et al., 2009; Soni et al., 2010; Brijwani et al., 2010). For these reasons, production of renewable energy is promoted by national programs in many countries especially using the locally available abundant proportion of biomass for energy production. Long-term objectives of this policy are to ensure future energy supply and to reduce green-house gas emissions. However, achieving these objectives using discarded biomass as a substrate will be an added advantage focusing on the concept of "clean green solutions". The process can be accomplished under four steps: substrate selection, pretreatment, enzymatic saccharification and fermentation studies (Galbe and Zacchi 2002). Biomass composed of different percentage of lignin, cellulose, hemicellulose and pectin may act as an appropriate source for enzyme and bioethanol production. Certain pretreatment methods are needed to loosen the lignocellulosic matrix for making the complex polymers accessible for the enzymatic attack. With the enzymatic action complex polymers get converted into simple monomeric form of sugars so that can be further fermented to ethanol with the help of suitable fermenting microorganisms. The content of lignocellulosic biomass is rich and renewable, therefore the application of these biomass have extensive studies. In

order to reduce carbon dioxide resulted from burning lignocellulosic materials, acid hydrolysis or enzymatic hydrolysis are used during the production of monosaccharide or oligosaccharide from lignocellulose (Sukumaran et al., 2009). Cellulose, the major component of agro-residue, is degraded by enzymes known as cellulases that are able to hydrolyze the polymer to its monomer sugar glucose while pectinase are a heterogeneous group of enzymes catalyzing the degradation of pectic substances. Literature suggests that different substrates have been explored for the production of enzymes such as wheat bran, rice husk, sugarcane bagasse, rice straw and various other agro residues to make the process of enzyme production cost effective. Commercialization of process hindered mainly by the prohibitive cost of the enzyme preparations used for saccharification. Therefore, reduction in the cost of enzymes can be achieved only by concerted effort which addresses several aspects of enzyme production from the raw material used for production to microbial strain improvement (Soni et al., 2010). Use of cheaper raw materials and cost effective fermentation strategies like solid state fermentation can improve the economics of enzyme production (Sukumaran et al., 2009; Lynd et al., 2005) and these enzymes are industrially important too. Cellulases are complex enzyme that finds major application in textile (Gusakov et al., 2000; Belghith et al., 2001), detergents (Sukumaran et al., 2005), paper and pulp industry (Buchert et al., 1996) and food and feed industries (Acharaya et al., 2008). Similarly, pectinase also finds its commercial application in several conventional industrial processes, such as textile, paper and pulp, food and feed and plant processing sectors (Ranveer et al., 2005). *Trichoderma reesei* is a known producer of the enzymes and has been extensively studied (Kaur and Satyanarayana 2004; Alam et al., 2005; Liu and Yang 2007).

Biogas production from renewable resources or organic wastes is another energy generation method to fulfil energy demands by exploring and exploiting new sources of energy in an eco-friendly way (Weiland 2003; Yadvika et al., 2004). In rural areas of developing countries various cellulosic biomass (cattle dung, agricultural residues, etc.) are available in plenty which have a very good potential to cater to the energy demand, especially in the domestic sector (Yadvika et al., 2004). Biogas technology offers attractive route to utilize certain categories of biomass for meeting partial energy needs. In fact proper functioning of biogas system can provide multiple benefits to the users and the community resulting in resource conservation and environmental protection (Ofoefuele et al., 2010). Biogas is a product of anaerobic degradation of organic substrates, used for the treatment of industrial wastes and stabilization of sludges. It is a colourless, flammable gas produced via anaerobic digestion of animal, plant, human, industrial and municipal wastes amongst others, to give mainly methane (50-70%), carbon dioxide (20- 40%) and traces of other gases such as nitrogen, hydrogen, ammonia, hydrogen sulphide, water vapour etc. It is smokeless, hygienic and more convenient to use than other solid fuels (Ofoefuele et al., 2010; Maishanu et al., 1990). It is produced during anaerobic fermentation of organic substrates by specific microbial communities (Ohmiya et al., 2005). This process is biotechnologically exploited to produce biogas in commercial biogas plants (Angelidaki et al., 2003; Kleerebezem and van Loosdrecht 2007; Krober et al., 2009). It has been used as a source of fuel in countries like India, China, Sweden, Bangladesh etc. for lighting and cooking purposes. Work of Momoh and Ify (2008); Jain et al., (1981), Dunlop (1978) and Jash and Basu (1999) have studied biogas generation from animal and agricultural wastes.

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Objectives

a) Standardization of conditions for Vermicomposting of solid waste from herbal industry.

b) Bioefficacy of vermicompost on seasonal plants.

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CHAPTER 1

INTRODUCTION

Growing urbanization and industrialization have led to the production of large quantities of household and industrial solid wastes and the problem of its handling is further augmented with shortage of landfills. Moreover strict environmental legislations and public consciousness have made land filling increasingly expensive and irresolute (Gupta et al., 2007). Present scenario represents it as one of the major concern of environmental problems. Management of solid wastes is a problem of increasing concern throughout the world. There is a need for cost effective and ecofriendly solid waste management technologies. Types of biomass such as agricultural residues, wood chips, municipal solid waste, food processing waste and industrial herbal waste etc qualify to be known as biodegradable solid waste having the potential to serve as low cost, abundant feed stocks. The problem of disposal of biodegradable waste generated after industrial operations was addressed by the Ayurvet Industry Ltd., situated in Baddi, distt. Solan (Himachal Pradesh). This Industry deals in herbal health care and nutritional products for a range of animal species in India as well as abroad especially in Africa, Europe, South-East Asia, Middle East and the Indian subcontinent. It is one of India's leading manufacturers of herbal veterinary medicine, feed supplements and animal feed. During the processing and manufacturing of veterinary products, 0.5 to 1.0 ton of medicinal plant waste is discarded daily. Although the waste is biodegradable but the industry is facing problem in disposing it off. Our study entails on finding some useful and viable alternatives to dispose off industrial herbal waste of a nearby industry so as to use a proportion of this waste biomass for organic farming and energy production.

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Review of Literature

CHAPTER 2

REVIEW OF LITERATURE

2A.1 Problem of Disposing Solid Waste

Any material which is not needed by the owner, processor or producer is known as waste. With the dawn of a theoretic new century, we are knowingly and unknowingly contributing thousands of tonnes of solid waste per day which if not regulated or channelled properly might bury us within it, in the long run just to get composted, freely. With the advancement of human civilisation and its never-ending tertiary consumers, our planet is deliberately heading towards a fateful direction. The rapidly increasing population and high rate of industrialization has increased the problem of solid waste management. India has a large network of industries of varying capacity which produces different kinds of wastes such as domestic waste, e- waste, agricultural waste, food processing waste and biomedical waste etc. On an industrial scale, generally the waste is managed through destructive methods: land filling practices and incineration. But due to the prohibitive cost of the waste disposal; most of the industries like textile mills, dispose off their sludge in poorly designed sanitary landfills which can pollute surface or ground water causing public health hazards. The improper and indiscriminate disposal of industrial solids is posing a great challenge to India and other developing nations. Meanwhile the unavailability of land, stringent national waste disposal standards and public consciousness has made dumps and landfills expensive and impractical. Therefore, industries in India are under heavy pressure and are in search of cost effective sustainable technologies for the disposal of industrial wastes.

2A.2 Industrial Solid Waste as Source of Energy

A significant challenge confronting engineers and scientist in the developing countries is the search for appropriate solutions to the treatment and disposal of the domestic and industrial solids. Many industries generate biodegradable solid waste in bulk similar to discarded vegetable waste, kitchen waste of a locality. The solid waste generated from a locality or from a larger domain like in our study from an industry can be utilised in a healthier way depending on its source and the nature of the waste. Mostly industries give rise to tonnes of waste daily and to crossroad this unavoidable bulk we have to dispose it in safe, hygienic and ecofriendly manner. Proper disposal of these wastes by recycling proposed them as a promising source of energy to reclaim the valuable substance present in the waste for possible reuse. These biodegradable organic wastes can be converted into various value added products like vermicompost, biogas generation, and bioethanol and can also be used as a substrate for enzyme production.

2A.3 Vermicompost Production

Vermicomposting is the conversion of biodegradable waste into a high quality chemical free bio-fertilizer with the aid of earthworms. Zorba (2001, 2000) referred it as worm composting, in which redworms transform decaying organic matter into worm castings. It is all-together a natural system in which the earthworms play their major roles in degrading the organic portion of the waste, helping in managing the solid waste or vermistablization (Edwards 1988). Earthworms have played a key role by serving as versatile natural bioreactors to harness and destroy soil pathogens, thus converting organic wastes into valuable bio-fertilizers, enzymes, growth hormones and proteinaceous worm biomass. The worms do it by feeding voraciously on all

biodegradable refuse such as leaves, paper (non-aromatic), kitchen waste, vegetable refuse and industrial herbal waste. It then burrows deep into the soil, positioning its castings towards the surface of the soil thereby enriching the soil with a pre-digested, easy to assimilate bio-fertilizer which is rich with NPK. Certain types of earthworms ingest, digest, and excrete vermicompost with excellent nutrient content (Bhiday 1995). Ingestion ensures the sorting out of only organic matter while the digestion accelerates the maturing process.

2A.4 Process Mechanism

Although the product efficiency depends upon the nature of the substrate/organic waste but the mechanism of production remains the same. Major role is played by the earthworm, which convert the low value complex substrate into a beneficiary product. Excretion ensures the grading of the vermicompost as opposed to any inorganic matter, which may be existing in the waste and not concerned with the biological activity in the earthworm gut. During the composting process, microorganisms decompose organic compounds, which consist of carbohydrates, sugar, proteins, fats, cellulose and lignin. Carbohydrates are more easily decomposed whereas lignin is more resistance to decomposition. So, compositional analysis of the waste is an essential feature for process optimization. Aira et al., (2006) studied the cellulose decomposition during vermicomposting with *E. foetida*. Similarly, cellulose loss was reported by Vinveslas-Akpa and Loquet (1997) during vermicomposting of pruning waste with *E. foetida*.

2A.5 Role of Precomposting

Many factors affect the vermicomposting/composting process. As a result of microbial activity in vermibeds heat is liberated and, if contained within the

composting mass, the temperature rises. Temperature increases through the mesophilic phase into a thermophilic phase and then back in to the mesophilic phase. During the course of these transitions, the microbial population changes, thereby affecting the rate of organic matter decomposition. Gupta et al., (2007) reported that precomposting was essential to eliminate toxic gases potentially not favourable for earthworms and anaerobic conditions along with lowering the temperature within the vermibeds. Precomposting is digesting the organic matter with the microflora of the environment. It partially breaks down the material and increase the rate of vermicomposting. The organic matter gets fully colonized with microbes that lower the lag time of the process. It also helps in release of toxic gases with avoiding overheating within the system.

2A.6 Exotic, Epigeic Species of Earthworm

According to their feeding habits, earthworms are classified into detritivores and goephages (Ismail 1997). Geophagous worms feed deeper beneath the surface ingest large quantities of organically rich soil. These are generally called as humus feeders and comprise of endogeic earthworms. Detritivores feed near the soil surface. They feed mainly on the plant litter or dead roots and other plant debris in the soil. Detritivores further comprises of epigeic and the anecic forms. Epigeic are surface dwellers serving as efficient agents in fragmentation of organic matters on the soil surface. They ingest large amounts of undecomposed litter. These species produce ephemeral burrows into the mineral soil for short periods only. They are relatively exposed to climatic fluctuations and predator pressures, and tend to be small with rapid generation times. A common example is *Eisenia foetida* (redworm, manure worm) and *Eudrilus euginae* which is used in vermicomposting (Bouche 1977). Anecic earthworms build permanent burrows into the deep mineral layers of the soil

by dragging organic matter from the soil surface into their burrows for food while epigeic live on the soil surface without forming any permanent burrows along with feeding on decaying organic matter. Many workers like Garg and Kaushik (2005) ; Gupta et al., (2007); Nath et al., (2009) studied the vermicompost production using *E. foetida* with textile mill sludge mixed with poultry droppings, water hyacinth and mixture of different agroresidues respectively. While Gajalakshmi et al., (2002) reported the high rate vermicomposting of water hyacinth using *E.eugeniae*. Edwards (1988) explained that, earthworms ingest organic matter and egest to make it much finer after passing through the grinding gizzard. Edwards and Bates (1992) found that *Eisenia foetida* to be the best choice due to its wide temperature and moisture tolerance. The highest growth rate in *Eisenia foetida* is at 30^{o}C with 85% moisture. Different materials were mixed before processing for faster results and a better product. Worms were also found to have a limited tolerance to some chemicals. The most commonly used earthworms were *Eisenia foetida* and *Eudrilus eugeniae* and the best results obtained by using raised beds.

Earthworm occurs worldwide in most areas where water and temperatures are favourable. Across the range of habitats, earthworms display a wide array of morphological, physiological, and behavioural adaptations to environmental conditions. The abundance of earthworms across habitats is highly variable, depending on climatic and edaphic conditions, ecosystem type, and the degree to which the habitat has been altered. Under suitable conditions, carbon concentration in the substrate has shown to be highly correlated with earthworm population density and biomass (Edwards 1983; Hendrix et al., 1992). The distribution of earthworm is influenced by several factors such as aeration, temperature, moisture, pH, inorganic salts and the nature of organic matter (Govindan 1998).

2A.7 Factors Influencing Vermicompost Production

2A.7.1 Temperature

Temperature is an important factor in composting process. Considerable amounts of heat are released by aerobic decomposition. Since composting material has relatively good insulation properties, a sufficiently large composting mass will retain the heat of the exothermal-biological reaction and high temperatures will develop. High temperatures are essential for destruction of pathogenic organisms and undesirable weed seeds. Decomposition also proceeds much more rapidly in the thermophilic temperature range.

In normal practice composting begins at ambient temperature (mesophilic range) and progresses to and through a thermophilic phase, followed by a descent to the mesophilic level (Goluke 1977). In Vermicomposting, temperatures are generally kept below 35^{o}C (Riggle and Holmes 1994). Most worm species used in vermicomposting require moderate temperatures from. While tolerances and preferences vary from species to species, temperature requirements are generally pretty similar. The majority of vermicomposting worms can tolerate temperatures ranging from 10-40^{o}C but their activity decreases as temperatures move toward the extremes. Earthworms tolerate cold and moist conditions far better compared to hot and dry conditions (Slocum 2002, 2000).

2A.7.2 Moisture

Earthworm requires plenty of moisture for growth and survival, they need generally moisture at the range from (60 –75) %. Vermibed material should not be too wet else it may create an anaerobic condition which may drive the earthworms from the bed (Ronald and Donald 1977). It is very important to moisten the dry bedding material

before putting them in the bed, so that the overall moisture level is well balanced. Generally, the moisture level of organic wastes is maintained at about 65–70% during the vermicomposting period by periodically sprinkling with water (Tejada and Gonzalez 2009; Gupta et al., 2007; Garg and Kaushik 2005).

2A.7.3 pH

Although studies have suggested that worms perform best in neutral pH (Ronald and Donald 1977). It has been recorded by Edwards and Lofty (1976) that different species of earthworms have their own pH sensitivity and generally most of them can survive at the pH at a wide range (4.5 – 9). The alteration of pH in the bedding is due to the fragmentation of the organic matter under series of chemical reactions.

2A.7.4 Organic Carbon Breakdown

The rate of organic matter breakdown depends mainly on the type of litter/waste. Very soft plant and animal residues may be decomposed by the microflora but much of the organic matter; particularly the tougher plant leaves, stems and root or in other words lignocellulosic biomass does not break down without first being disintegrated by the earthworms (Aira et al., 2006; Vinveslas-Akpa and Loquet 1997). Earthworms have an important role in the initial process of the cycling of organic matter. These tiny creatures are responsible for translocation of the accumulated organic debris from the soil surface, the subsurface layers and during this process much of the organic materials are ingested, macerated and excreted. Earthworms also contribute several kinds of nutrients in the form of nitrogenous waste (Lakshmi and Vijaylakshmi 2000). There has been an extensive research done using earthworms to break down the various animal manures like pig, cattle solids, slurries, waste from the poultry farms etc. Edwards (1998) reported five earthworm species *(Dendrobaena veneta, Eudrilus*

eugeniae, Perionyx excavatus and *Pheretima hawayana* and *Eisenia foetida*) to be the most potential earthworms to breakdown the organic refuse. The earthworms are highly adaptable to different types of organic waste, provided the physical structure, pH and the salt concentration are not above the tolerance level (Seenappa et al., 1995). Although the optimal conditions of vermicomposting are well known but it varies with respect to type of substrates. Kaviraj and Sharma (2003) reported 45% loss of carbon during vermicomposting of municipality, or industrial wastes. Garg and Kaushik (2005) reported a loss of 30 - 42% in different feed mixtures of textile mill sludge spiked with poultry droppings by the end of vermicomposting period.

2A.7.5 Nitrogen Mineralization

Earthworms greatly increase the soil fertility, and part of this must be due to the increased amounts of mineralized nitrogen that they make available for the plant growth. There have been reports of increase in the amount of nitrogen in which the earthworms are reared (Edwards and Lofty 1976). This may be due to the decay of the bodies of dead earthworms. Since the body of the earthworm is rich in proteins. Govindan (1998) reported that earthworm body contains 65% protein, 14% fats, 14% carbohydrates and 3% ash. Similarly Ronald and Donald (1977) reported that 72% of the dry weight of an earthworm is protein and that the death of an earthworm will release up to 0.01 gram of nitrate in the soil. Earthworms consume large amount of plant organic matter that contains considerable quantities of nitrogen and much of this is returned to the soil in their excretions. Hand et al., (1988) and Suthar (2008) reported that nitrogen mineralization was greater in the presence of earthworms, and this mineral nitrogen was retained in nitrate form.

2A.7.6 C:N ratio

Plants root in general cannot assimilate the mineral nitrogen unless the C:N ratio is in the order of 20:1 or lower (Edwards and Lofty 1976) and it also indicates an advance degree of organic matter stabilization and mineralization during the process of decomposition (Senesi 1989; Suthar 2008; Senapati et al., 1980). Therefore the ratio of carbon to nitrogen is important for the proper growth of any plant. Daniel and Karmegam (1999) have done an experiment in vermicomposting of selected leaf litter and cow dung mixtures (1:1) and shown a substantial variation in the electrical conductivity, NPK, organic carbon and C/N ratio than compost (without earthworms). The C/N ratio also showed a remarkable reduction in the vermicompost (with earthworms) than the compost (without earthworms). Such type of reduction has been brought about by the respiratory activity and microflora present in the system (Daniel and Karmegam 1999).

2A.8 Effect of Vermicompost on Plant Growth

As feed passes through the earthworm gut, the material is mineralized and plant nutrients are made available. Presence of worms increases plant growth and N uptake as opposed to unfertilized soil. A higher plant growth was observed in the presence of worms cast (Edwards and Bates, 1992). Earthworm casts were found to increase nutrient uptake in Tomati et al., (1994), including nitrogen and several ions, particularly Mg and K. When used in horticulture, earthworm casts have a hormone-like effect. The biological effect of casts is linked to microbial metabolites that influence plant metabolism, growth and development (Tomati et al., 1997). N uptake increases in the presence of earthworms and is correlated ($r = 0.85$) with the increase in CO_2 production (Ruz – Jerez et al., 1992). Casts increase plant dry weight and N, P,

K and Mg uptake from the soil. The application of earthworm casts (0, 100, and 300 g per 3.5 kg soil) increased the dry weight of soybean by 40 to 70%. The nitrogen absorbed by the plants increased to 30 to 50%. Phosphorous and potassium in the plant were twice that of the control. The amount of organic matter, total nitrogen, phosphorous and potassium in the soil also increased along with available phosphorous and potassium in the soil (Lui et al., 1991). Studies conclude that vermicompost dramatically increases germination and growth in many plants. Adding only 5% of the vermicompost to commercial growing media (95%) significantly increased plant growth (Edwards 1983). Vermicompost not only influences the plant growth but it also inhibits/kills the bad microbes (fungi, bacteria, etc.) that are plant pathogens and in the process they also increase the good beneficial microbes. It has recently been discovered that in feeding, earthworms consume spores of mycorrhizae; a beneficial fungi that help roots take up nutrients. To conclude, vermicompost is considered a good solid waste management practice because it converts the waste into some beneficial product, increases plant growth and productivity with subsequent mineralization of plant nutrients, and therefore an increase in soil fertility and quality is achieved (Aracnon et al., 2006; Tejada and Gonzalez 2009).

2B.1 Pretreatment and *In Vitro* Enzyme Production

The industrial waste was a heterogeneous kind of waste and an excellent example of lignocellulosic biomass. The research work on the lignocellulosic biomass sources such as agricultural residues (straws, brans, hulls, stems, bagasse etc.), fruit and vegetable wastes, municipal solid wastes (paper, cardboard, yard trash, wood products etc.), waste from the paper and pulp industry and herbaceous energy crops have the potential to serve as low cost and abundant feedstocks for production of fuel ethanol. The major component of these materials is cellulose (35-50%), followed by

hemicellulose (20-35%) and lignin (10-25%) (Soni et al., 2010; Brijwani et al., 2010). Proteins, ash and oils in widely varying ratios make up the remaining fraction of lignocellulosic fraction of lignocellulosic biomass (Wyman 1994). The structure of these materials is very complex, and native biomass is resistant to enzymatic hydrolysis. The steps for the production of ethanol from lignocellulosic biomass involve:

i. Feedstock preparation
ii. Pretreatment
iii. Cellulase production
iv. Enzymatic saccharification
v. Ethanol fermentation

Although, each of the above steps has been extensively studied in various laboratories (Ehara and Saka 2002; Neves et al., 2007; Brijwani et al., 2010), yet the processing techniques required for ethanol production from lignocellulosic materials are presently extensive and costly. Comprehensive process development and optimization are still required to make the process economically viable. The success of the process depends on the development of environmentally friendly pretreatment procedures, highly effective enzyme systems for conversion of pretreated biomass to fermentable sugars and efficient microorganisms to convert sugars to ethanol.

2B.2 Pretreatment of Lignocellulosic Biomass

In lignocellulosics, cellulose a linear polymer of glucose is associated with hemicellulose and surrounded by lignin seal. Lignin seal around cellulose microfibrils and its limited covalent association with hemicellulose prevents enzymes and acid from accessing some regions of the cellulose polymers. Crystallinity of the cellulose due to potential formation of six hydrogen bonds because of β-1, 4 orientations of its glucosidic bonds further impedes hydrolysis (Weil et al., 1994). Pectin consists of a

complex set of polysaccharides that are present and particularly abundant in the non-woody parts of terrestrial plants. Pectin is present throughout primary cell walls but also in the middle lamella between plant cells where it helps to bind cells together. The characteristic structure of pectin is a linear chain of α-(1-4)-linked D-galactouronic acid. The pretreatment of lignocellulosics primarily employed to increase the accessible surface area of complex polymers like cellulose and pectin, and to enhance their conversion into monomeric form of sugars. This can be accomplished by removing the lignin seal, solubilising hemicellulose, disrupting cellulose crystallinity, and or increasing pore volume (Mandels et al., 1976; Lin et al., 1985; Weil et al., 1994). Several pretreatment techniques have been reported. These include physical methods such as ball milling, hammer milling, boiling, high pressure stream, wetting, etc. and chemical methods using sodium hydroxide, sulphuric acid, sulphur dioxide, alkaline peroxide, phosphoric acid, ammonia, organic solvents (Ethylenediamine), super critical carbon dioxide, etc. (Kosaric et al., 1980; Weil et al., 1994; Zhang et al., 1995).

2B.2.1 Physical Methods

2B.2.1.1 Grinding/ Milling

Milling of lignocellulosic material is a popular method for increasing the surface area for better digestibility. The changes in physical properties of cellulose like reduction in crystallinity, degree of polymerization and particle size and increase in water soluble fraction of the material can be achieved by milling (Kosaric et al., 1980).

Grinding of lignocellulosic material to a very small particle size makes it susceptible to enzymatic hydrolysis (Chahal 1982). Tewari et al., (1987b) studied the effect of grinding on the enzymatic hydrolysis of wheat straw, bagasse, corn cob and

groundnut shell. Mesh size of 40 was found to be the best except in sugarcane bagasse in which 20 mesh size gave the maximum enzymatic attack. Similarly, Sharma et al., (2002) also found mesh size 40 at its best for sunflower hulls and stalks. Alvo and Belkacemi (1997) used milling as the sole pretreatment for the enzymatic saccharification of timothy and alfalfa.

2B.2.2 Steam Explosion

It is usually performed at high temperature and pressure, followed by sudden depressurization. It increases the enzymatic accessibility of the lignocellulosic materials. The changes in the materials depend on the temperature, pressure and time of exposure to steam. The organic acids derived from acetylated polysaccharides hydrolyse the hemicellulose to soluble sugars. Secondary reactions which occur under more drastic conditions result in the formation of furfurals, hydroxymethyl furfurals and their precursors by dehydration of pentoses and hexoses (Casebier et al., 1969; Chahal 1982). The Stake and Iotech processes which are commercially operating in Canada involves the steam heating of wood biomass chips to approximately 180-200^{o}C for 5-30 min in a continuous operation and to higher temperatures (245^{o}C) for a shorter time (0.5-2 min) in batch mode, respectively (Wayman 1980).

Dekker and Wallis (1983) reported that auto hydrolysis of sunflower seed hulls at 200^{o}C for 5 min followed by explosive defibration solubilised >80% of the total hemicellulose and 85% of the pectic substances. Tewari et al., (1987b) reported the continuous improvement in the enzymatic hydrolysis of different lignocellulosic substrates with increase in duration of autoclaving at 15psi. The longer and higher temperature steam explosion treatments resulted in overall recovery of dry matter ranging from 96-46% overall and indicated that significant autohydrolysis and degradation of sugars can occur during pretreatment (Beltrame et al., 1992). These

explosion conditions produced a material highly susceptible to attack by hydrolytic enzymes, with great increase in pore volume.

2B.2.3 Chemical Methods

Chemical pretreatment of lignocellulosic materials using dilute acids, alkali, peroxides and organosolvents increase pore size, solubilise lignin and/or hemicellulose, and/or increase surface area (Lin et al., 1985; Baeza et al., 1991; Holtzapple et al., 1991; Thompson et al., 1991; Torget et al., 1991; Doran et al., 1994; Ishihara et al., 1995; Zhang et al., 1995 and Maekawa 1996).

2B.2.3.1 Sodium Hydroxide Pretreatment

It is probably the oldest and best known method of increasing digestibility of lignocellulosic biomass. The application of alkali results in disruption of lignin structure, hydration and swelling of the cellulose and decrease in cellulose crystallinity (Kosaric et al., 1980). A sodium hydroxide concentration of 1% and steam pressure of 1kg/cm^{2}for 1hr have been reported for delignification of water hyacinth (Motwani et al., 1993). Soto et al., (1994) treated the ground sunflower hulls with NaOH (0.5-3%) in an autoclave at 120^{o}C for 0.5, 1.0, and 1.5hr. Higher the NaOH conc. more was the delignification; however saccharification was little affected by alkali concentration. Sharma et al., (1996) reported that sodium hydroxide pretreated forest biomass is more susceptible to cellulase as compared to untreated one. Sharma et al., (2002) reported that 0.5% NaOH conc. at 1kg/cm^2 for 1.5hr was the best optimized condition of pretreatment.

2B.2.3.2 Sulphuric Acid Pretreatment

Acid hydrolysis of cellulosic biomass is commercially viable. Two acid catalyzed processes are known (i) dilute acid hydrolysis at elevated temperatures and pressures

(ii) concentrated acid hydrolysis at low temperature and ambient pressure. However, a weak acid hydrolysis is often combined with a weak acid prehydrolysis (Olsson and HahnHagerdal 1996). Song and Lee (1984) studied the kinetic parameters in acid hydrolysis of cellulose covering the conditions 170-190^{o}C, 4.4-12% H_2SO_4 and 1:16 solid to liquid ratio. They found that glucose yield increased with increase in reaction temperature and acid concentration. Singh et al., (1984a) obtained 78% and 74.5% saccharification with H_2SO_4 for NaOH delignified bagasse and rice husk while 58% and 49.5% saccharification was observed for untreated bagasse and rice husk respectively. Tewari et al., (1985) obtained effective saccharification when substrate was treated with 10% H_2SO_4 at 15psi for 15 min. While Kahlon and Chaudhary (1989) found 7.5% H_2SO_4 as optimum acid conc. to obtain maximum saccharification when sawdust was autoclaved for 45 min at 15psi.

2B.2.3.2.1 Concerns About Acid/Alkali Pretreatment Methods

Each method has its advantages and disadvantages, but the overriding factor in the long run must be low energy requirement and low pollution. Generally acid hydrolysis procedures give rise to a broad range of compounds in the resulting hydrolysate, some of which might negatively influence the subsequent steps in the process. Major problem is the presence of various toxic substances liberated from the structure of lignocellulosics during the hydrolysis process such as decomposition products of carbohydrates, lignin breakdown products, and extraneous materials from biomass. Among the identified toxins are furfurals, hydroxymethyl furfural (HMF), levulinic acid, acetic acid, formic acid and various phenolic compounds originating from lignin (Chung and Lee 1985; Wyman 1994; Larsson et al., 1999). In addition expensive corrosion proof equipment, high temperature and acid conc. are needed for hydrolysis resulting in high capital cost (Kosaric et al., 1980; Olsson and Hahn-

Hagerdal 1996). Enzymatic hydrolysis is not only energy sparing but also avoids the use of toxic and corrosive chemicals. Projected selling prices for ethanol produced from cellulose by acid hydrolysis are currently comparable to those for enzyme based processes. Enzymatic processes are yet to achieve technological maturity; however, research is likely to result in enzyme based processes that are significantly cheaper than acid based processes (Lynd et al., 1991; Saxena et al., 1992; Wyman 1994; Olsson and Hahn-Hagerdal 1996).

2B.3 Enzymatic Saccharification

Interest in the enzymatic hydrolysis of lignocellulosic wastes to get ethanol has increased as this involves milder conditions in comparison to acidic hydrolysis. Extensive studies on the physiological aspects, kinetics and economics of enzymatic saccharification have been reviewed (Wyman 1994; Szczodrak and Fiedurek 1996; Le Jee Won and Lee 1997; Bothast and Saha 1997). The rate and the extent of saccharification is affected by many factors such as source of enzyme, nature of the substrate, methods of pretreatment, enzyme and substrate concentration, product inhibition and enzyme stability (Lin et al., 1985; Saddler et al., 1986; Szczodrak 1988; Sukumaran et al., 2005; Vipan et al., 1994; Vidaurre et al., 1995; Jimenez et al., 1995).

2B.3.1Production of Enzymes

Even a cursory perusal of current scientific literature shows that hydrolysis of complex polymers like cellulose and pectin are the most extensively studied topics. Each and every aspect of enzyme production such as isolation and mutation of hydrolytic microorganisms, mode of fermentation, process optimization, genetics and regulation at molecular level, mode of action, process economics etc. Several workers

have reviewed the enzyme production and technology aspects (Wyman 1994; Bothast and Saha 1997; Sukumaran et al., 2005; Brijwani et al., 2010).

2B.3.1.1 Mechanism of Action

Complex enzyme system of cellulase for the conversion of cellulose to glucose has been characterized into 3 enzymes:-

i. Endo-1, 4 –β glucanase or carboxymethylcellulase (CMC) (EC3.2.1.4)

ii. Exo-1, 4- β glucanase or cellobiohydrolase (EC3.2.1.91)

iii. β glucosidase or cellobiase (EC3.2.1.21)

The cellulolytic enzymes with β glucosidase act sequentially and cooperatively to degrade crystalline cellulose to glucose. Endoglucanase act in a random fashion on the regions of low crystallinity of the cellulosic fibre, whereas Exoglucanase remove cellobiase units from the non reducing ends of the cellulose chains. Synergism between these two types of enzymes is attributed to the endo-exo form of cooperativity and has been studied extensively between cellulases in the degradation of cellulose by *Trichoderma reesei* (Henrissat et al., 1985). β glucosidase hydrolyse cellobiose and in some cases cello oligosaccharides to glucose. This type of enzyme is generally responsible for kinetic regulation of the whole cellulolytic process and is a rate limiting factor during enzymatic hydrolysis of cellulose as both endoglucanase and cellobiohydrolase activities are often inhibited by cellobiase (Coughlan 1985; Beguin 1990). Thus, β glucosidase not only produces glucose from cellobiose but also reduces cellobiase inhibition, allowing the cellulolytic enzymes to function more efficiently.

Pectinases are a family of enzymes acting on the polymers composed of pectin hydrolases, lyases and esterase with different specificities (Hemmink et al., 1996). The enzymes exhibit a β eliminative mechanism in the cleave of α -1, 4

galactouronosyl bonds of polygalactouronic acid, resulting in the formation of oligogalactouronates (Anurag et al., 2006).

2B.3.2 Solid State Fermentation (SSF) – A Low Cost Alternative

These enzymes can be produced using low cost enzyme production method i.e. solid state fermentation (SSF). It has gained interest as a cost effective technology, not only for enzyme production but also for the bioconversion of lignocellulosic biomass employing various hydrolytic enzymes (Pandey et al., 2000; Tengerdy 1998). Tengerdy (1998) indicated that there was about 10 fold reduction in the production cost in SSF than submerged fermentation (SmF). Various processes in the literature suggested the strategies to opt for hyper production of enzymes dealing with empirical optimization of process variables to improve productivity in a cost effective manner. In spite of several efforts by directed evolution, the cost of enzymes has remained high. Alternative strategies thought of in enzyme production include mainly SSF on lignocellulosic biomass particularly by using host/substrate specific microorganisms. There are several reports of using filamentous fungi in production of optimal enzyme complex for the degradation of host lignocellulose.

2B.3.2.1 Hydrolytic Microorganisms

Cellulases are widely distributed in bacteria (*Cellulomonas, Bacteroides, Cellvibrio, Cytophaga, Ruminococcus, Pseudomonas* etc), actinomycetes (*Streptomyces flavogriseus* and *Thermomonospora*), and Fungi (*Trichoderma reesei, Aspergillus oryzae, Penicillium funiculosum, Sporotrichum pulverulentum, Humicola grisea* and *Phanerochaete chrysosporium*) (Coughlan 1985; Klyolov 1986, Srinivasan and Seetalaxman 1988; Singh et al., 1995; Kaur and Satyanarayana 2004; Brijwani et al., 2010) while *Streptomyces* (Ladzama et al., 2007), *Penicillium chrysogenum* (Banu et

al., 2010), *Trichoderma harzianum* (Nabi et al., 2003) have also been investigated for the pectinases.

2B.3.3 Physiological Parameters for Enzyme Production

The nature of cellulosic substrate influences the induction of enzyme production (Acerbal et al., 1986; Kanotra and Mathur 1995). For cellulase production various substrates have been evaluated e.g. cellulose (Keskar 1992; Aiello et al., 1996; Giemba et al., 1999), wheat straw, wheat bran, barley husk, bamboo shoots, *Lantana camara*, Bermuda grass (Shamala and Sreekantiah 1986; Mehta et al., 1992; Steiner et al., 1994; Kuhad et al., 2010; Li et al., 2007). For pectinase production substrate like citrus peel (Nabi et al., 2003), orange bagasse, sugarcane bagasse and wheat bran (Martin et al., 2004), apple pulp, gram bran, dried amla (Kaur and Satyanarayana 2004) have been exploited.

Pretreatment of the lignocellulosic substrate is necessary for the maximum enzyme production. Pretreatment makes the substrate more easily accessible for microbial growth, because pretreatment facilitates the mycelium penetration or to provide some chemical constituents for growth and product formation (Pandey et al., 2001).

Moisture is one of the important parameters in SSF that influences the growth of organism and thereby enzyme production determining the success of the process (Sivaramkrishnan et al., 2006; Kaur and Satyanarayana 2004). As the moisture content of the medium changes during the fermentation as a result of evaporation and metabolic activities, adjusting the optimum moisture level of the substrate during SSF is therefore most important. 70% of the moisture content was found to be optimum using apple pomace, producing cellulase enzyme with *T. harzianum* (Sun et al., 2010) and using citrus peel as substrate for producing pectinase enzyme in a SSF system

(Nabi et al., 2003). Liu and Yang (2007) reported that 50% moisture content was optimum for producing cellulase enzyme using *T. koningii* in a SSF system with lignocellulosic vinegar industry waste as substrate.

Nystrom and Diluca (1977) carried out cellulase production at 29°C. While Duff et al., (1987) reported that optimum temperature for cellulase production by mixed culture of *T. reesei* Rut C30 and *A. phoenics* was 27°C. Other workers like Banu et al., (2010) found that production of pectinase enzyme from *Penicillium chrysogenum* in SSF system was maximum at 35°C. Similarly, Janas and Targonski (1995) studied the effect of temperature in the range of 26-38°C on the production of cellulase by four mutant strains of *T. reesei*. High temperature caused reduced secretion of cellulases.

On the other hand, different workers reported different time of production with their studies. Soni et al., (2010) obtained significant enzyme productivities after 96hr of incubation for cellulase production, while Alam et al., (2005) and Haq et al., (2006) reported 72hr as optimized time for maximum cellulase production.

Enzyme production is influenced by various factors and their interaction can affect the enzyme productivity (Tholudur et al., 1999). In *T. reesei*, a basal medium after Mandels and Reese (1957) has been most frequently used with or without modifications. Various carbon and nitrogen sources are known inducers. In most of the earlier studies; complex mineral salt solution was used as a moistening agent in the production media involving various lignocellulosic substrates such as grass, corn cob, spruce, wheat straw, sugarcane bagasse, wheat bran and rice hulls (Mekala et al., 2008; Sohail et al., 2009; Kovacs et al., 2009; Zhao et al., 2009).

In a comparative study, commercial enzymes are also used. Angadi and Theagarajan (1993) used commercial cellulose and crude cellulase obtained from *T.*

viride to hydrolyse saw dust. Maximum saccharification of 42.72% for the commercial enzyme and 47.40% for the crude enzyme was obtained after 48hr of incubation. Ramos et al., (1993) investigated the enzymatic hydrolysis of H_2O_2 pretreated *Eucalyptus* chips using commercial preparations. Complete saccharification of the substrate was obtained at a conc. of 6% (w/v) with an enzyme loading of 10 FPU/g of cellulose. Soto et al., (1994) studied the enzymatic saccharification of alkali treated sunflower hulls using a commercial cellulase supplemented with cellobiase in a cellobiase: cellulase activity ratio of 0.25. Maximum saccharification occurred after pretreating with 0.5% NaOH for 1.5hr at 121^{o}C using a solid liquid ratio of 0.1g/ml. The hydrolysis yield increased with enzyme concentration up to 50 FPU/g, higher conc. giving negligible further increase. 5-25 FPU/g range of enzymes is studied by other workers (Sharma et al., 2002; Grohmann and Baldwin 1992) as increase in hydrolytic efficiency has been reported within this range. Sriroth et al., (2000) took waste from cassava starch plant and reported the use of combination of two enzymes; cellulase and pectinase promoting higher amount of reducing sugar than that from single enzyme.

2B.4 Ethanol Fermentation

Efforts directed at ethanol production from biomass at industrial level have mixed result due to economic constraints and recalcitrant nature of biomass. The main problems encountered in the efficient conversion of the lignocellulosic hydrolysates to ethanol are twofold. Firstly, after pretreatment, the hydrolysate contains not only fermentable sugars but also a broad range of compounds having inhibitory effects on the microorganisms used for fermentation. The composition of these compounds depends upon the type of lignocellulosic material used and chemistry and nature of pretreatment process. Secondly, the hydrolysates contain not only hexoses but also

pentoses. Hexoses can easily be fermented by *Saccharomyces cerevisiae* with well known process techniques. The pentoses are more difficult to ferment. Efficient fermentation of pentoses is important for the overall economy of ethanol production from lignocellulosic materials (Olsson and Hahn-Hagerdal 1996; Moniruzzaman et al., 1997).

2B.4.1 Hexose Metabolism

The microorganisms most suitable for the production of ethanol from hexoses are yeasts of genera *Saccharomyces* and bacterium *Zymomonas mobilis*. *S.cerevisiae* is the most widely used organism providing high yields and productivities in addition to remarkable ethanol tolerance. However, *S.cerevisiae* is not able to utilize xylose for ethanol production whereas the isomer of xylose can be fermented (Deng and Ho 1990). The glucose is fermented to ethanol and CO_2 in almost an equimolar ratio through glycolytic pathway. *Z. mobilis* demonstrates high ethanol yield and tolerance, high fermentation selectivity and specific productivity, the ability of ferment sugars at low pH, and considerable tolerance to the inhibitors found in lignocellulosic hydrolysate.

2B.4.2 Pentose fraction

The pentose fraction in lignocellulosic hydrolysate consists mainly of xylose and arabinose, which can be converted to ethanol by bacteria, yeast and fungi. Various bacteria such as *Bacillus macerans, Bacteroides polypragmatus, Clostridium saccharolyticum, C. thermohydrosulfuricum, Zymomonas anaerobia, Thermoanaerobacter ethanolics* have been reported to ferment xylose to ethanol (Ng et al., 1981; Patel 1984; Asther and Khan 1985; Tolan and Finn 1987; Lacis and Lawford 1988). Generally bacteria have a broad host range but ethanol is seldom the

only product which is reflected in the low yields i.e. 0.16-0.39g/g (Olsson and Hahn-Hagerdal 1996). Large no. of fungi are able to ferment xylose to ethanol such as *Aeurobasidium pullulans, Fusarium avenaceum, F. culmorum, F. Solani, Monilia sp*, *Mucor*-105 and *Paecilomyces* (Gong et al., 1981; Suihko and Enari 1981; Wu et al., 1986). Fungi can utilize a broad range of substrates including not only monosaccharides, but also disaccharides, cellulose and xylan. With *F. oxysporum* VTT-D-80134, *Neurospora Crassa* NCIM870 and *Paecilomyces sp.* NFI ATCC 20766, yields of 0.50, 0.35 and 0.40 g/g respectively, have been achieved (Olsson and Hahn-Hagerdal 1996). Yeasts like *Candida, Calvispora, Kluveromyces, Pachysolen, Pichia, Schizosaccharomyces* have been evaluated to metabolize xylose (Maleszka and Schneider 1982; Gong et al., 1983; du Preez and vander Walt 1983; Slininger et al., 1985). *Candida shehatae*, *Pachysolen tannophilus* and *Pichia stipitis* are the most thoroughly investigated and most efficient occurring xylose fermenters among the yeasts. The yield for these microorganism are average to high (0.28-0.45g/g) and the productivities reasonable (0.02-0.99g/L/hr). In last decade, a major goal of research in biofuels has been to metabolically engineer microorganisms to ferment multiple sugars from biomass or agricultural wastes to final ethanol. Recombinant bacteria like *Erwinia chrysanthemi* has been used for the fermentation of xylose and arabinose to ethanol by Tolan and Finn (1987) with ethanol tolerance up to 2%. *Escherichia coli* strains, genetically engineered to contain the *pet* operon (*Z. mobilis* pyruvate decarboxylase and alcohol dehydrogenase B genes) produce high levels of ethanol. Strains carrying the *pet* operon in plasmid (*E. coli* B pLO 1297) or in chromosomal (*E. coli* K011) sites require antibiotics in the media to maintain genetic stability and high ethanol productivity (Ohta et al., 1990). Hexoses are preferred carbon source for recombinant *E. coli*. The problem of pentose sparing i.e. in complete fermentation has

been found when hexose dominate in the medium (Barbosa et al., 1992; Beall et al., 1992; Lawford and Rousseau 1993). Hexoses and pentoses can be utilized simultaneously by ethanologenic *E.coli*, but exercise mutual inhibition. Bera et al., (2010) reported more than 40% of ethanol production with genetically engineered *S.cerevisiae* 424A (LNH-ST). The genetically engineered *Scizosaccharomyces pombe* containing xylose isomerise gene showed a yield of 0.42g/g and a productivity of 0.19g/L/hr. However, the organism was dependent on nutrient supplementation and without yeast extract, malt extract and peptone the yield was only 0.15g/g.

2B.5 Production of Ethanol

The yeast *P.tannophilus* was found to be capable of converting xylose to ethanol. Batch cultures initially containing 50g/L xylose yielded 0.34g of ethanol per gram of sugar consumed (Slininger et al., 1982; Kurtzman 1983). Maleszka and Schneider (1982) reported that growing cultures of *P.tannophilus* concurrently consume and produce ethanol in the presence of substantial concentration of xylose. Tewari et al., (1985) carried out the fermentation of acid and enzymatic hydrolysate of saw dust by *S.cerevisiae var ellipsoideus*. The enzymatically hydrolysed samples supported better ethanol production on the basis of reducing sugars than the acid treated samples. For growth *P.tannophilus* require a source of thiamine and biotin such as yeast extract in the media. Yeast is tolerant to 250g/L xylose and 100g/L ethanol. Although *P.tannophilus* is capable of anaerobic fermentation, dependence of volumetric ethanol productivity on aeration conditions has been demonstrated in batch and continuous culture (Slininger et al., 1987). *S.cerevisiae var ellipsoideus* was found to be most efficient closely followed by *K. marxianus* in its ability to ferment enzymatically hydrolysed mash to ethanol. An incubation period of 24hr was found optimum for the production of ethanol by *S.cerevisiae* from acid and enzymatic hydrolysate of

agricultural residues (Tewari et al., 1987a). Laplace et al., (1991) investigated the combined fermentation of glucose and xylose to ethanol by separate or co-culture processes using *P. stipitis*, *C. shehatae*, *S. cerevisiae* and *Z. mobilis*. Laplace et al., (1992) studied the alcoholic fermentation of glucose and xylose derived from the cellulose and hemicellulose fractions of lignocellulose, respectively. Devi and Singh (1995) reported the fermentation of xylose-glucose (4:1) mixture to ethanol by *C. shehatae* NCIM 3501 with alcohol production rate of 0.71g/L/hr and ethanol yield of 0.35g/g. Inhibitory effects of lignocellulose acid degradation products (furfural, its hydroxymethyl derivative HMF, acetate, vanillin, hydroxybenzaldehyde and syrinaldehyde) on glucose fermentation by *S.cerevisiae* and *Z. mobilis* and on xylose fermentation by *P. stipitis* and *C. shehatae* were studied in batch culture. Vanillin at 1g/L strongly inhibited both growth and ethanol fermentation by xylose fermenting yeasts and *S.cerevisiae*. Larson et al., (1999) studied the effect of acetic acid, formic acid, levulinic acid, furfural and 5-hydroxymethylfurfural (5-HMF) (compounds generated during dilute acid hydrolysis of softwood) on fermentability of dilute acid hydrolysate of softwood by *S.cerevisiae*. Ethanol yield and volumetric productivity decreased with increasing concentrations of acetic acid, formic acid and levulinic acid. Furfural and 5-HMF decreased the volumetric productivity but did not influence the final yield of ethanol.

2C.1 Biogas Production

Another alternative for utilizing bulk of lignocellulosic biomass or industrial wastes can be as a feedstock in biodigester as a source of energy. Biogas, a clean and renewable form of energy could very well substitute (especially in the rural sector) for conventional sources of energy (fossil fuels, oil, etc.) which are causing ecological–environmental problems and at the same time depleting at a faster rate (Yadvika et al.,

2004). The production of inflammable gas in marshes and swamps has been reported from the beginning of ancient civilization of Egypt, China and Rome. Varma and Behera (2003) reported that methane is formed from simple carbon compounds by the action of microorganism.

2C.2 Substrate Selection

Laube and Martin (1981) reported that cellulose was the chief source of energy for methane producing bacteria. Many feedstock based on waste biomass are widely used as potential substrate for methane production (Cho et al., 1995; Chynoweth et al., 1993). Cow dung, urban organic wastes, algae, vegetable wastes, human excreta and waste animal flesh are used as common substrate for biogas generation (Kalia et al., 2000; Singh et al., 1995; Kang and Weiland 1993). It is noted that systematic studies on biomethanation by psychrophilic microflora are lacking (Kashyap et al., 2003). The European Union (EU) is striving for more renewable sources of energy in efforts to decrease their use of fossil fuels and meet the Kyoto Protocol (EU Commission of Energy 2005).

Generally digested sludge from a running biogas plant or a municipal digester, material from well-rotted manure pit, or cow dung slurry is used as seed. Addition of inoculum tends to improve both the gas yield and methane content in biogas. It is possible to increase gas yield and reduce retention period by addition of inoculum (Dangaggo et al., 1996; Kanwar and Guleri 1995; Kotsyurbenko et al., 1993).

2C.3 Factors Affecting Methane Production

2C.3.1 Pretreatment

Sometimes feedstocks require pretreatment to increase the methane yield in the anaerobic digestion process. Pretreatment breaks down the complex organic structure

into simpler molecules which are then more susceptible to microbial degradation. Dar and Tandon (1987) observed an improvement of 31–42% in microbial digestibility and an almost two fold increase in biogas when alkali treated (1% NaOH for 7 days) plant residues were used as a supplement to cattle dung. Patel et al., (1993) found that thermo-chemical pretreatment of water hyacinth improved biomethanation and the best results were obtained when water hyacinth was treated at pH 11.0 and at 121^{o}C. Ultrasonic pretreatment of waste activated sludge for 30 min resulted in a 64% increase in methane production (Wang et al., 1999).

2C.3.2 Particle Size

Indirectly particle size also affects the biogas production. Sharma et al., (1988) found that out of five particle sizes (0.088, 0.40, 1.0, 6.0 and 30.0 mm), maximum quantity of biogas was produced from raw materials of 0.088 and 0.40 mm particle size. Physical pretreatment such as grinding could significantly reduce the volume of digester required, without decreasing biogas production (Gollakota and Meher 1988; Moorhead and Nordstedt 1993; Yadvika et al., 2004).

2C.3.3 pH

pH is an important parameter affecting the growth of microbes during anaerobic fermentation. pH of the digester within a range of 6.8–7.2 was optimum at an optimum loading rate (Yadvika et al., 2004). Jain and Mattiasson (1998) found that above pH 5.0, the efficiency of CH_4 production was more than 75%. The two phase anaerobic reactor using cheese whey and dairy manure as substrate operated as a single-phase reactor when the pH was not controlled while when pH of whey was controlled in the methanogenic stage, it operated as a two-stage two-phase reactor (Ghaly 1996). The major problem related to drastic reduction in pH due to rapid

acidification of onion storage waste (OSW) was overcome by Sharma (2002) by mixing cattle dung with OSW in a suitable ratio so that medium is well buffered to take care of acid accumulation.

2C.3.4 Temperature

Temperature inside the digester has a major effect on the biogas production process. There are different temperature ranges during which anaerobic fermentation can be carried out: psychrophilic (<30^{o}C), mesophilic (30–40^{o}C) and thermophilic (50–60^{o}C). However, anaerobes are most active in the mesophilic and thermophilic temperature range (Mital 1996; Umetsu et al., 1992; Maurya et al., 1994; Takizawa et al., 1994; Desai and Madamwar 1994; Zennaki et al., 1996). The length of fermentation period is dependent on temperature. Angelidaki and Ahring (1994) observed that when the NH_3 load was high, temperature below 55^{o}C resulted in an increase of biogas yield and better process stability, with reduced VFA concentration. Garba (1996) observed that methanogens were very sensitive to sudden thermal changes. Nozhevnikova et al., (1999) proposed a two step anaerobic treatment of cattle dung i.e. (i) acidogenic fermentation at high temperature (55–82^{o}C), and (ii) separation of solid and liquid fractions and treating the liquid manure under low temperature conditions (5–20^{o}C). Long term adaptation of active psychrophilic microbial communities was found to be essential for efficient treatment of cattle dung at low temperature (Nozhevnikova et al., 1999; Meher et al., 1994). Some precautions taken during the installation of biogas plants and coating them with insulating materials also helps in keeping the temperature in the digester within the desired range (Molnar and Bartha 1989). In order to increase gas yield, it is preferred to construct biogas plants sun-facing and in a manner as to protect them from cold winds. Biogas plants should be covered with locally available crop residues for minimizing heat losses from the plants. A simple technique of charcoal coating of

ground around the digester had been found to improve gas production in KVIC biogas plant by 7–15% (Anand and Singh 1993).

2C.3.5 Co-digestion

Co-digestion is the simultaneous digestion of more than one type of waste in the same unit (Agunwamba 2001). Advantages include better digestibility, enhanced biogas production/methane yield arising from availability of additional nutrients, as well as a more efficient utilization of equipment and cost sharing (Agunwamba 2001; Mshandete and Parawira 2009; Parawira et al., 2004). Studies have shown that co-digestion of several substrates, for example, banana and plantain peels, spent grains and rice husk, pig waste and cassava peels, sewage and brewery sludge, among many others, have resulted in improved methane yield by as much as 60% compared to that obtained from single substrates (Ezekoye and Okeke 2006; Ilori et al., 2007; Adeyanju 2008; Babel et al., 2009). Results of co-digestion of food waste and dairy manure in a two-phase digestion system conducted at laboratory scale showed that the gas production rate (GPR) of co-digestion was enhanced by 0.8 - 5.5 times as compared to the digestion with dairy manure alone (El-Mashad et al., 2007).

2C.3.6 BOD and COD

Perez et al., (2007) examined the effect of organic loading rate (OLR) on the removal efficiency of COD and total organic carbon (TOC) in anaerobic thermophilic fluidized bed reactor (AFBR) in the treatment of cutting oil waste waters at different hydraulic retention time (HRT) conditions. Davidson and Brown (1952) reported 60 – 90% of BOD removal with anaerobic digestion of waste water from a distillery industry using different support materials such as charcoal, coconut coir and nylon fibres under varying hydraulic retention time and organic loading rate respectively. Hossain et al., (2009) reported the maximum reduction of BOD and COD are 76.82%

(w/w) and 81.65% (w/w) with maximum OLR of 39.513kg $CODm^{-3}$ hr^{-1} at optimum conditions.

2C.3.7 Total Solids and Volatile Solids

Total solids are used to determine the loading rate of anaerobic digester and give clues as to when maintenance is needed. Typically, total solids amount to less than 10% of the total volume. The volatile solids content can give an estimate on the amount of substrate that can potentially be turned into (Wilkie 2003). The study by El-Mashad et al., (2004) did not list the final results of TS or VS, but did indicate that TS was within the average range at 5%. The results of Karim et al., (2005) found a general trend that as the ratio of TS/VS increased, the amount of methane produced also increased. While Oleszkiewiz and Poggi-Varaldo (1997) reported that lowest ratio of TS/VS corresponded to the highest production of methane and the highest TS/VS gave the lowest methane production. Shivaraj and Seenayya (1994) reported that digesters fed with 8% TS of poultry waste gave better biogas yield than the higher TS levels. Budiyono et al., (2010), reported the results showing the best performance for biogas production in the digester with 7.4 and 9.2% of total solid i.e., gave biogas yield 184.09 and 186.28 mL gVS-1, respectively after 90 days observation.

Considering the wholesome of literature, it was clear that lignocellulosic biomass being a waste and a major cause of environmental pollution still holds the potential of producing various value added and commercially viable products if utilized appropriately. In different forms like straws, brans, residues from agricultural fields, localities, municipal wastes etc have already been explored, exploited and studied extensively. But this is the first report of using such kind of industrial herbal waste as substrate for vermicompost, crude enzymes and biogas production. Majorly it depends upon the composition of the waste which suggests it as an appropriate substrate for a particular process. Few objectives were framed to be investigated in a manner to

develop technologies for the bioconversion of industrial herbal waste into valuable end products.

CHAPTER 3

MATERIAL AND METHODS

SECTION A (Vermicompost)

3A.1 Materials

3A.1.1 Glassware

Borosil and Scott Duran glassware were used throughout the study, which were washed twice with detergent with final rinsing in distilled water. It was then dried in hot air oven at 70°C and used.

3A.1.2 Plasticware

Tarsons and Laxbro plasticware were used during the study. It was also washed twice with the detergent, rinsed with distilled water, dried in hot air oven at 70°C and used.

3A.1.3 Chemicals and Reagents

All the chemicals and reagents used in the study were of high purity obtained from Hi-Media, SD Fine Chemicals, Sigma, E-Merck, Qualigens, and SRL, India.

3A.1.4 Source and Composition of the Waste

Three different kinds of industrial herbal wastes (samples) provided by Ayurvet Industry Ltd., Baddi, Solan (H.P.). named as Stresroak, Diaroak and Superliv Premix were used as substrate. Different types of wastes were amalgamations of various remains of medicinally important herbs after industrial operation (Table 3A.1). All these wastes were dried and ground separately (mesh size 40 with particle size

0.42mm) and then stored at room temperature till further use. And fresh cow dung was procured from live stock from nearby village.

Table 3A.1: Composition of Three Different Types of Industrial Herbal Wastes

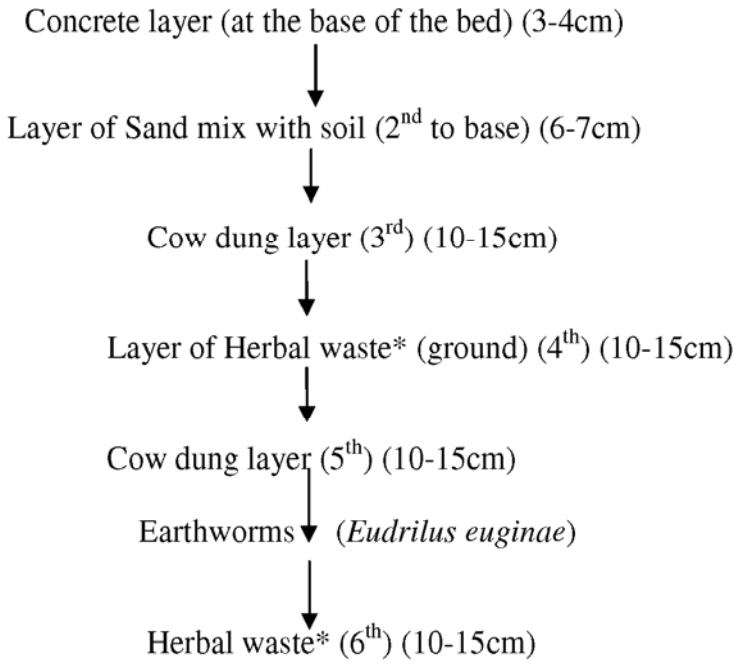
Stresroak (Left over of following plant parts)	%	**Superliv premix** (Left over of following plant p arts)	%	**Diaroak** (Left over of following plant parts)	%
Ashwagandha (roots)	30-33	Neem (leaves)	7	*Punica granatum* (fruit)	24
		Phyllanthus (fruit)	3	*Symplocus racemosa* (bark)	12
Tulsi (leaves)	33	*Terminalia chebula* (fruit)	6	*Andrographis paniculata* (stem)	10
Amla (fruit)	25	*Citrullus colocynthus* (fruit)	5		
Mangifera (bark) Asphaltum (bark)	5	*Phyllanthus niruri* (roots and leaves)	8	*Woodfordia fruticosa* (flowers)	14
		Achyranthes aspera (roots)	8	*Salmalia malabarica* (bark)	3.5
		Sida cordifolia	5	*Berberis aristata* (root)	13.5
		Eclipta alba	8	*Aeglemarmelos* (fruit)	22
		Tinospora cordifolia	1		
		Fumaria indica	3		
		Ichnocarpus frutescens	6		
		Andrographis paniculata	8		
		Solanum nigrum (fruit)	8		
		Borracharia diffusa	9		
		Aphanamixispolystachya	5		
		Convolvulus alsinoides	2		
		Tephrosia purpurea	3		

3A.2 Procurement and Maintenance of Vermiculture

Epigeic species of earthworms i.e. *Eudrilus eugeniae* used in the present study was procured from stock culture maintained at Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni –Solan (H.P.) for increasing and maintaining the population of earthworms. Master bed was made using cow dung as a major substrate for nurturing the population of this exotic species of earthworm.

3A. 3 Construction and Setting of Vermicompost Beds

1. The vermicompost was prepared in cemented area of 6×3×3 feet divided into 3 partitions for 3 different substrates for nurturing the population of exotic species of earthworm.
2. The floor of the bed was filled with stones and pieces of bricks.
3. Then different layers were set :-



*(Each time herbal waste replaced with respective substrate: Stresroak, Diaroak and Superliv Premix. 5kg of each substrate was taken in the vermibed.)

In another experiment vermibeds were made using mixture of herbal waste and cow dung (1:1) in comparison with only cow dung as substrate. Vermicompost beds of $50\times50\times15cm^3$ were prepared. One vermicompost bed was having 5kg of the cow dung where as other was having 1:1 ratio of cow dung and herbal waste with total substrate as 5kg. Both vermicompost beds were inoculated with 200 earthworms with an average initial weight of 38.96 grams. Further experiment was set to know the best ratio for the vermicompost made using combination of industrial herbal waste in a mixed blend with cow dung. Ten vermibeds ($50\times30\times12cm^3$) with 5 different combinations as S1 (5kg cow dung), S2 (3.75kg cow dung +1.25kg herbal waste), S3 (2.5kg herbal waste +2.5kg cow dung), S4 (1.25kg cow dung+3.75kg herbal waste) and S5 (5kg herbal waste) were made (Fig. 3A.1). Two sets of experiments were compared. Each of the five combinations was conducted with and without earthworms (as control).

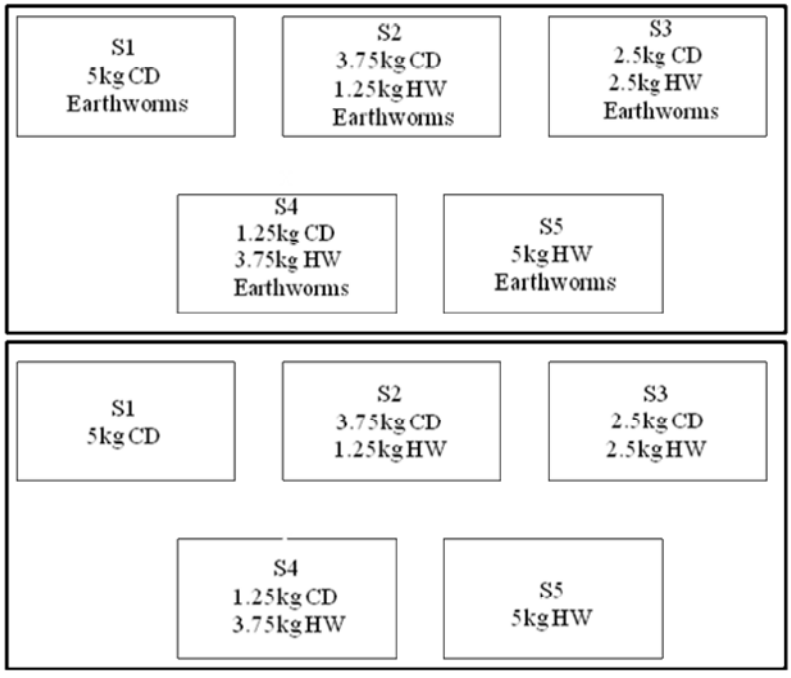


Fig. 3A. 1: Vermicompost beds with different ratios of cow dung and herbal waste with and without earthworms, CD- Cow dung; HW- Herbal waste.

4. Water was sprinkled and a wet gunny bag was placed on it to keep the environment wet and humid.
5. Sprinkling of water was done to maintain 70-80% moisture content.
6. A shed was constructed over the vermicompost beds which was essential to prevent entry of rainwater and direct sunshine (Fig 3A.2)

Fig 3A.2: Cemented vermibeds in the field of Jaypee University of Information Technology campus

7. Maturity was judged visually by observing the formation of granular structure of the compost at the surface of the beds.

3A.4 Harvesting of Vermicompost

Harvesting was done manually. A tarp/paper was placed on the ground in a well lit area, and then vermicompost was scooped out of the beds and placed on that paper. Since worms don't like light, they burrow down towards dark, at the end of the heap. Top layer of vermicompost was removed which was relatively worm free and placed in the container. Vermicompost was brown in colour, rich in humus, very light in

weight without any odor. In the end vermicompost was sieved to get rid of cocoons and other developmental stages of earthworms. Fig 3A.3 shows the sieving of vermicompost and Fig 3A.4 shows the population of earthworm from a vermibed with herbal waste as substrate.

Fig 3A.3: Sieving of vermicompost

Fig 3A.4: Earthworms (*E.eugeniae*) from a vermibed with herbal waste as a substrate

3A.5 Nutrient Analysis of the Vermicompost

3A.5.1 Determination of pH

pH of the vermicompost was determined using method of Mclean (1982). Elico - L1-127 was used to measure pH. Before measuring the pH, the meter was calibrated by using solutions of pH 7 and 4. The pH was determined using a double distilled water

suspension of vermicompost in ratio of 1:10 (w/v) that was agitated mechanically for 20 min and filtered through Whatman no. 1 filter paper.

3A.5.2 Determination of Total Organic Carbon

Total organic carbon content of the vermicompost was estimated using the method of Nelson and Sommers (1982). 0.05g of moisture free, oven dried sample was taken in a 250ml Erlenmeyer flask. Then 10ml of 1N potassium dichromate solution was added and the contents were mixed slowly followed by addition of 20ml of sulphuric acid. After complete mixing, the flask was kept at room temperature for half an hour. Then after incubation mixture was diluted to 200ml with distilled water. Further 10ml of 85% phosphoric acid, 0.2g sodium fluoride and 1ml diphenylamine indicator were added in sequence. Then solution was back titrated with 0.5N ferrous ammonium sulphate solution. Colour changes from initial dull green to turbid blue which further turn out to be brilliant green as end point.

Calculation:

$$\% \text{ TOC} = \frac{10\ (B\text{-}T) \times 0.003 \times 100}{B \times \text{weight of the sample}}$$

B- Volume used in Blank titration

T- Volume used in Test titration

1ml of 1N $K_2Cr_2O_7$ – 3mg or 0.003g organic carbon

3A.5.3 Determination of Total Kjeldahl Nitrogen (TKN)

It was determined after digesting the sample with conc. sulphuric acid (1:20 w/v) followed by distillation (Bremner and Malvaney 1982). 0.5g of finely ground oven dried sample (moisture free) was taken in Kjeldahl digestion flask. 20ml sulphuric acid was added carefully and mixed gently. Acid digestion was done by heating the mixture; slowly the temperature was increased up to 80^{o}C and heated till it becomes

white. Then it was removed and cooled at room temperature. It was further diluted to 100ml with distilled water. 5ml of aliquot was taken in micro Kjeldahl apparatus and 10ml of 40% NaOH was added slowly. Up to 50ml of distillate (ammonium ions along with water) was collected in 150ml Erlenmeyer flask containing 10ml of 4% working boric acid solution. Then colour was changed from wine red to blue. After the end point was achieved it was back titrated with 0.01N sulphuric acid and colour changed from blue to wine red at the end. Reading was noted. In a similar way, a blank sample was also run without adding aliquot.

Calculation

$$\% N = \frac{\text{Factor x volume of acid consumed x 100 x total volume made}}{\text{Weight of sample x volume taken}}$$

$$= \frac{0.00014 \times (S-B) \times 100 \times 100}{W \times 5}$$

Where W-weight of the sample (g)

Volume made = 100ml

Volume taken for distillation = 5ml

Amount of acid used for sample titration = S (ml)

Amount of acid used for Blank titration = B (ml)

Volume of acid consumed = S-B (ml)

1000 ml of 1N H_2SO_4 = 14g of nitrogen

1ml of 1N H_2SO_4= 14/1000

1ml of 0.01N H_2SO_4 (Factor) = 14 x 0.01/1000=0.00014 nitrogen

3A.5.4 Diacid digestion for Potassium (K) and Phosphorous (P) estimation

One gram of (oven dried) ground sample was taken in 100ml Erlenmeyer flask. Then 10ml of diacid mixture (conc. nitric acid and perchloric acid [9:4]) was added to it and the contents were mixed by swirling. Contents were placed on hot plate and heated gently until the solution became colourless. After cooling the flask; 20ml of distilled

water was added and final volume made up to 100ml and then it was filtered. This aliquot was further used for potassium and phosphorous estimation.

3A.5.4.1 Determination of Phosphorous

It was estimated by colorimetric method (Bansal and Kapoor 2000).

Vanado-molybdate reagent: 25g ammonium molybdate was dissolved in warm 400ml distilled water in a beaker. This was solution 'a'. Then 1.25g ammonium metavandate was added in 300ml boiling water. It was cooled and 250ml conc. nitric acid was added and again cooled at room temperature. This was solution 'b'. Solution 'a' was poured into 'b' and mixture was diluted to 1L and that was known as Vanado molybdate reagent.

Preparation of Standard Phosphorous (50ppm) Solution: KH_2PO_4 was dried at 40^{o}C and 0.2195g was dissolved in about 400ml distilled water contained in 1L volumetric flask. Then 25ml 7N conc. H_2SO_4 (17.7%) was added and finally volume made to 1L.

An aliquot of 5ml was taken from sample (diacid digested) in 25ml volumetric flask. Then 5ml of Vanado - molybdate solution was added and made the final volume to 25ml with distilled water. Similarly, samples with known concentration were taken from the standard solution and a blank was run along with. After 20 minutes of incubation time, optical density was measured at 470nm using UV/VIS Spectrophotometer.

3A.5.4.2 Determination of Total potassium (TK)

It was determined after digesting the sample in diacid mixture (conc. nitric acid and perchloric acid [9:4]) by flame photometer (Bansal and Kapoor 2000).

Preparation of Standard Potassium solution: 1.9069g of dried KCl was dissolved in distilled water and made the final volume to 1L. This solution contained 1000ppm of potassium. Further it was diluted 10 times to make solution of 100ppm.

An aliquot of 50ml was taken from sample (diacid digested). Similarly, samples with known concentration were taken from the standard solution (5, 10, 15, 20 and 40ppm) and a blank was run along with. And then the concentration of potassium was estimated using flame photometer.

3A.6 Plant Growth Experiment Studies

3A.6.1 Selection of Plants: Two seasonal plants – *Pisum sativum* (Pea) and *Tagetes erectus* (Marigold) were taken. Flower and Pod numbers per pot were counted each 10^{th} day after they appeared. Shoot and root were washed with tap water and got air dried and fresh weights were recorded. And for dry weights shoot, root and leaf material were placed in an oven (60°C) for overnight and dry weights were recorded. Experiment was done in triplicates.

3A.6.2 Determination of Chlorophyll 'a' and 'b' and Total Carotenoids

It was determined by spectophotometric methods in acetone solvent (Wellburn 1994).

1. 1g (wet weight) of minced fresh leaves was homogenized in 10ml of distilled water.
2. An aliquot 0.5ml was mixed with 4.5ml of 80% acetone.
3. The optical density was measured at 645, 663, 638 and 490nm using UV/VIS Spectrophotometer.

Calculation:

Carotene (g/L) = (OD 490) - (0.114) (OD 663) - (0.638) (OD 645)

Total chlorophyll (g/L) = (0.0202) (OD 645) + (0.00802) (OD 663)

Chlorophyll a (g/L) = (0.0127) (OD 663) + (0.00269) (OD 645)

Chlorophyll b (g/L) = (0.0229) (OD 645) + (0.00488) (OD 638)

One way ANOVA was used to analyze the statistical significance at $P < 0.05$ in all the experimental data on various parameters.

SECTION B (*In vitro* Enzyme Production and Fermentation Studies)

3B.1 Material

The industrial herbal waste used in the present study was given by Ayurvet Industry Ltd., Baddi, Solan (H.P.). The waste named as DIAROAK (refer to Table 3A.1) was dried, ground and stored at room temperature for further use.

3B.2 Chemical Analysis of the Substrate

3B.2.1 Determination of Cellulose Content

Cellulose content of the waste was determined by the method of Crampton and Maynard (1938). One gram of oven dried sample was taken in a 250ml Erlenmeyer flask. Then 25ml of acetic nitrate reagent (Acetic acid 650ml; Nitric acid 80ml; Distilled water 150ml) was added and the contents were boiled till the evolution of brown fumes. The residue was then transferred into a dried preweighed crucible and filtered using a vacuum pump. After the filtration, three washings each of water, alcohol and acetone were given. The crucible was then placed in oven (150^{o}C) for 1hr. After cooling in a dessicator, it was weighed. Finally, the crucible was kept in a

muffle furnace at 450°C for 1hr, cooled and weighed. Loss in weight was observed as the amount of cellulose present in the sample.

3B.2.2 Determination of Hemicellulose Content

Hemicellulose content of the waste was determined by estimating the percentage of NDF and ADF by the method of Georing and Vansoest (1970).

3B.2.2.1 Determination of NDF (neutral detergent fibre)

Reagent : NDS - Neutral detergent solution (SDS 30g/L , EDTA 18.61g/L, sodium borate decahydrate 6.81g/L, Disodium hydrogen phosphate 4.56g/L, 2-ethoxy ethanol 10ml)

Preparation of NDS: EDTA and sodium borate decahydrate were taken in a beaker containing 500ml of distilled water and dissolved by heating. Sodium lauryl sulphatae and 2- ethoxy ethanol were dissolved separately in boiling distilled water and then mixed with above solution. Disodium hydrogen phosphate was separately dissolved in boiling water and then added to above solution containing the other components. The pH was adjusted to 7.0 and volume was made to 1L with distilled water.

One gram of the dried sample was taken in a spoutless beaker. Then 100ml of neutral detergent solution, 2ml decaline and 0.5g sodium sulphite were added in sequence. The contents were boiled for 5 to 10 minutes and refluxed slowly for 1hr. The refluxed sample was got filtered by transferring it into the weighed crucible (A1) placed on the filtering apparatus. It was then rinsed with hot water, followed by acetone. The crucible was then dried at 105°C for 12hr and weighed (A2). The neutral detergent fibre content was calculated as:

NDF (%) = (A2 - A1/ W) × 100

W – Weight of the sample taken.

3B.2.2.2 Determination of ADF (acid detergent fibre)

Reagent: ADS- Acid detergent solution (Cetyl trimethyl ammonium bromide (CTAB) 20g in 1000ml of 1N sulphuric acid). One gram of the sample was taken in a 500ml spoutless beaker. 100ml acid detergent solution and 2ml decaline were then added to it. The contents in the beaker were boiled for 10 min and thereafter refluxed slowly for 1hr. The digested sample was filtered through a previously weighed crucible (A3) which was set on a filtering apparatus. The residue was washed first with hot water, then alcohol and finally with acetone. The crucible was then dried at 105^{o}C for 12hr, cooled in a dessicator and weighed (A4). The dried weight of the residue was recorded as ADF given by

ADF (%) = (A4 – A3/ W) ×100

Where W is the weight of the sample taken.

Hemicellulose content (%) in the herbal waste was ascertained as per the formula

Hemicellulose (%) = NDF (%) – ADF (%)

3B.2.3 Determination of ADL (acid detergent lignin) (Georing and Vansoest 1970)

The crucible containing ADF was kept on 500ml conical flask containing water. The contents of the crucible were covered with 20ml of 72% sulphuric acid and stirred with a glass rod to a smooth paste. The crucible was then refilled with acid and kept this on ice bucket carefully. After 3hr excess of acid was filtered off using a vacuum pump. The residue was then washed with hot water till it was acid free. The crucible was dried at 100^{o}C, cooled in a dessicator and weighed (A5). The crucible was then

placed in a muffle furnace at 500°C for 3hr cooled and weighed. (A6). The acid detergent lignin content was computed as

ADL (%) = (A5 –A6/W) x 100

Where W is the weight of the sample taken

The ADL content (%) worked out would be equivalent to lignin content (%).

3B.2.4 Pectin Estimation (Ranganna 1979)

50g of sample was taken in 1L beaker and 300ml 0.01N HCl was added to it. It was boiled for 30 min and filtered under suction. Then the residue was washed with hot water and the filtrate was collected. 100ml 0.05N HCl was added to it and again boiled for 20 min. Then it was filtered, washed with hot water and filtrate was collected. Further, 100ml 0.3N HCl was added to the residue and then boiling, filtering and washing with hot water was repeated. All the filtrates were pooled, cooled and final volume was made up to 500ml. 100ml of aliquot was taken in 1L beaker and 250ml water was added to it, acid was neutralized with 1N NaOH using phenolphthalein indicator. Further 10ml of 1N NaOH was added with constant stirring and it was allowed to stand overnight. Next day, 50ml 1N acetic acid was added along with 25ml 1N calcium chloride solution with stirring after 5 min. It was allowed to stand for 1hr. Then it was boiled for 1 – 2 min and filtered through pre-weighed Whatman No. 1 filter paper. Lastly, precipitate was washed with almost boiling water until the filtrate was free from chloride. Filtrate was tested with 1% $AgNO_3$ for presence of chloride. Then filter paper with calcium pectate was kept for drying overnight at 100°C. Next day, it was cooled in dessicator and weighed.

Calculation

The pectin content is expressed as % calcium pectate

$$\% \text{ calcium pectate} = \frac{\text{Wt. of calcium pectate} \times 500 \times 100}{\text{ml of filtrate taken} \times \text{Wt. of sample for estimation}}$$

Where 500 indicate the final dilution made

100- for percentage conversion

3B.3 Estimation of Reducing Sugars

Reducing sugars were estimated by the method of Miller (1959).

Preparation of DNS reagent

Composition	**(g/L)**
Sodium potassium tartaratc	300.0
Sodium sulphite	0.5
Phenol Crystals	2.0
Sodium hydroxide	19.8
Dinitrosalicylic acid	10.0
Distilled water	1L

1ml of sample was taken with 3ml of DNS reagent in a test tube. It was further heated in boiling water bath for 10 minutes. Then tubes were cooled to room temperature and optical density was measured at 540nm. A control was run simultaneously using 1ml of distilled water. Standard curve was prepared by taking 1mg/ml of glucose.

3B.4 Production of Cellulase and Pectinase Enzyme

3B.4.1 Procurement and Maintenance of Culture

The fungi used in the present study *viz Trichoderma reesei* procured from IMTECH-Institute of Microbial Technology, Chandigarh. The culture was grown on potato dextrose agar (PDA) plates and slants, pH- 4.8, maintained at 4^{o}C and subcultured fortnightly.

3B.4.2 Preparation of Inoculum

Fungal culture was grown on PDA slants at 30°C and the spores were harvested aseptically from 5 day old PDA slants. Then sterile Potato dextrose broth (PDB) supplemented with 0.2% Tween 20 (2ml) was added to the slant and shaken vigorously for preparing uniform suspension, which was used as inoculum. This suspension was further added to 50ml PDB supplemented with 0.2% Yeast extract incubated at 30°C for 72hr under shaking conditions (150rpm).

3B.4.3 Enzyme Production

Enzyme production by *T.reesei* was studied under solid state fermentation using Andreotti basal medium or Mandels medium (1976), pH 4.8-5.0; using industrial herbal waste and wheat bran as substrate. Fermentation was done at 28±2°C for 5 days.

Composition of the Basal/ Mandels medium (g/L)

$(NH_4)_2NO_3$	1.4
KH_2PO_4	2.0
$CaCl_2$	0.03
$MgSO_4$	0.03
$FeSO_4.7H_2O$	0.5
$MnSO_4.H_2O$	0.16
$ZnSO_4.H_2O$	0.14
Peptone	0.75
Tween-80	2%
Distilled water	1000ml

3B.4.4 Solid State Fermentation (SSF)

5g of the substrate (industrial herbal waste (HW), wheat bran (WB) and combination of both (HWB) in 1:1 ratio, respectively) was taken in different petri plates, was autoclaved at 121^{o}C at 15psi for half an hour. Distilled water and Mandels medium were used as moistening agents separately and 0.5ml of the inoculum with a spore count of 4x10^{6}/ml was inoculated. Plates were incubated at 28±2^{o}C for 5 days at stationary conditions.

3B.4.5 Optimization of Time of Enzyme Production in SSF

This was studied by comparing different enzyme yields at different time intervals from respective substrates such as industrial herbal waste, wheat bran and their combination in 1:1 ratio.

3B.4.6 Effect of substrate for the different enzyme productions in SSF

Three different materials were explored as substrate for the enzyme production, industrial herbal waste, wheat bran and their combination in 1:1 ratio for the maximum enzyme yield.

3B.4.7 Effect of moistening agent on enzyme yield in SSF

This was studied by altering the moistening agent in the production medium consisting of industrial herbal waste, wheat bran and their combination (1:1) using distilled water and Mandels medium (pH 4.8-5.0) in different sets of production medium.

3B.4.8 Effect of moisture percentage on enzyme yield in SSF

It was studied by altering the level of moisture content in the production medium consisting of wheat bran, herbal waste and their combination (1:1). At two different levels i.e. 62% and 81% (with addition of 1ml and 3ml moistening agent respectively) the relative humidity was varied and its effect on maximizing the enzyme yield was studied.

3B.5 Enzyme Harvesting

Whole plate was sacrificed and whole content was dipped in about 30 ml of extraction buffer [0.05M citrate buffer (pH 4.8) + 0.2% Tween 20] to extract the enzyme. Then it was filtered with muslin cloth and centrifuged at 8000 rpm for 15 min at 4^{o}C.

3B.6 Enzyme Assay

The crude enzyme filtrate was assayed for cellulase and Pectinase activity. The term cellulase includes filter paper activity, carboxymethyl cellulase and cellobiase activity.

3B.6.1 Filter Paper Activity

Filter paper activity of crude enzyme filtrate was assessed by the method of Mandels et al., (1976). The test tubes containing 0.8ml of 0.05M citrate buffer (pH 4.8), 0.2ml diluted enzyme filtrate (0.1ml filtrate + 9.9ml citrate buffer) and a rolled strip of (1x6cm) whatman no.1 filter paper strip were incubated at 50^{o}C for 1hr. Control was run simultaneously. Three ml of DNS was then added to stop the reaction. The tubes were heated in boiling water bath for 10 min. The absorbance was read at 540nm.

3B.6.2 Carboxymethyl Cellulase Activity

Carboxymethyl cellulase activity of crude enzyme was worked out as suggested by Mandels et al., *(*1976). The test tubes containing a mixture of 1% CMC solution (0.5ml) and diluted enzyme filtrate (0.5ml) were incubated at 50^{o}C for 30 min. Control with inactivated enzyme was run simultaneously. Three ml of DNS was then added to stop the reaction. The reducing sugars were then estimated by DNS method (Miller 1959).

3B.6.3 Cellobiase Activity

Cellobiase activity of the crude enzyme filtrate was determined by the method of Srivastava *et* al., (1987). The test tubes containing 0.5ml (0.1ml filtrate + 9.9ml citrate buffer) enzyme solution and 10mM cellobiose solution (0.5ml) were incubated for 10 min at 50^{o}C. The reaction was stopped by adding 3ml of DNS reagent and was then heated in a boiling water bath. The reducing sugars were then estimated by DNS method (Miller 1959).

3B.6.4 Pectinase Assay

Pectinase activity of the crude enzyme filtrate was determined by the method of Jayani et al., (2005). The test tubes containing a mixture of 1% pectin solution (0.8ml) and 0.2ml enzyme filtrate (0.1ml filtrate + 9.9ml citrate buffer) were incubated at 50^{o}C for 30 min. Control with inactivated enzyme was run simultaneously. Three ml of DNS was then added to stop the reaction. The reducing sugars were then estimated by DNS method (Miller 1959). The enzyme activities were expressed in terms of International unit (IU) equivalent to 1µmole of glucose liberated in one min. The enzyme yields were expressed as IU/g of dry substrate (IU/gds).

3B.7 Pretreatment of Substrate

3B.7.1 Physical Pretreatment

Diaroak waste was dried, ground and then sieved through a sieve with 40 mesh pore size.

3B.7.2 Pretreatment of Industrial Herbal Waste

Ground waste was subjected to various physicochemical pretreatments including steam hydrolysis, acid and alkali hydrolysis.

3B.7.2.1 Steam Explosion Pretreatment

It was performed at various temperatures at 121°C, 140°C and 160°C for 0.5hr, 1hr, 1.5hr and 2hr followed by sudden depressurization by fully opening the steam exhaust valve of autoclave. 20g waste was added into 200ml of distilled water in 500ml Erlenmeyer flask.

3B.7.2.2 Acid and Alkali Hydrolysis of Steam Exploded Pulp

Acid and alkali hydrolysis were performed to accelerate the process at160°C for different time intervals (for 0.5hr, 1hr, 1.5hr and 2hr). NaOH (alkali pretreatment) and conc. H_2SO_4 (acid pretreatment) at a concentration level of 0.5%, 1%, 1.5% and 2% w/v were used.

3B.7.3 HPLC Analysis

HPLC analysis was performed to characterize the sugars released after pretreatment steps. Shim pack CLC-NH_2 (M) 2281787892 column was used equipped with LC10AT HPLC pumps, refractive index detector (RID-10A), 20µl injection valve in a Shimadzu HPLC instrument. Acetonitrile: water (60:40) of HPLC grade was used as mobile phase at room temperature around 30°C with flow rate 0.8ml/min.

Concentration of standard sugar solutions of arabinose (1.5mg/ml), xylose (2.5mg/ml), fructose (5mg/ml), ribose (10mg/ml), galactose (10mg/ml), and glucose (20mg/ml) was taken and for dimeric sugars like sucrose concentration taken was 1mg/ml.

3B.8 Saccharification with Enzyme

The hydrolysates obtained after pretreatment were enzymatically saccharified with commercial and crude preparation of cellulase and pectinase enzymes.

3B.8.1 Enzyme Concentration v/s Saccharification

To study the effect of enzyme concentration, hydrolysate after pretreatment were inoculated with different doses of enzymes (5-25 FPU/g of the substrate). Saccharification was compared with both crude and commercial preparation of enzyme and was carried out at 50^{o}C under shaking conditions (150rpm). Samples were withdrawn after 24hr, centrifuged at 8000 rpm for 20 min and the supernatant was analyzed for reducing sugars by the method of Miller (1959).

3B.9 Fermentation Studies

3B.9.1 Cultures

The bioconversion of hydrolysates into ethanol was carried out using:

Organisms	Culture conditions	Source
Saccharomyces cerevisiae MTCC 475	Grown on YPD medium, 30^{o}C temperature, pH 5.0	MTCC, Institute of Microbial Technology, Chandigarh
Pachysolen tannophilus MTCC 1077	Grown on GYE medium, 30^{o}C temperature, pH 4.5	MTCC, Institute of Microbial Technology, Chandigarh

The cultures were maintained on YPDA slants at 4^{o}C.

3B.9.2 Inoculum preparation

3B.9.2.1 *Saccharomyces cerevisiae*

The inoculum was prepared in YPD [(g/L): Yeast extract 3; peptone 10; glucose 20; distilled water 1000ml] broth. A loopful of 24hr yeast culture was transferred to 100 ml broth in 250ml Erlenmeyer flask and incubated at 30±1^{o}C for 24 hr on rotary shaker (150rpm). A cell count was obtained through Plate Count Method.

3B.9.2.2 *Pachysolen tannophilus*

A loopful of 38hr *P.tannophilus* culture was aseptically inoculated into Erlenmeyer flask (250ml capacity) each containing 100ml of GYE [(g/L): Malt extract 3; yeast extract 3; peptone 5; glucose 10; agar 20, distilled water 1000ml] broth and incubated for 38 hr at 30±1^{o}C under shake flask conditions (150rpm). A cell count was obtained through Plate Count Method.

3B.9.2.3 Ethanol Fermentation

The enzymatic hydrolysates were inoculated with *S.cerevisiae* and *P.tannophilus*.

Yeast	**Inoculum level**
Saccharomyces cerevisiae	10%
Pachysolen tannophilus	10%
Mixed culture (*S. cerevisiae and P.tannophilus*)	5% of each

Fermentation was carried out in screw capped reagent bottles and incubated at 30^{o}C, stationary conditions.

3B.10 Ethanol Estimation

Ethanol in the fermentation broth was estimated by method of Caputi et al., (1968).

Reagent

Potassium dichromate solution: 33.768g of $K_2Cr_2O_7$ dissolved in 400ml of distilled water with 325ml of sulphuric acid and volume raised to 1L.

One ml of the fermented wash was taken in 100ml distillation flask containing 29ml of the distilled water. The distillate was collected in 50ml volumetric flask containing 25ml of potassium dichromate solution. About 20ml of distillate was collected in each sample. The flasks were kept in a water bath maintained at 62.5°C for 20 minutes. The flasks were cooled to room temperature and the volume raised to 50ml. Five ml of this was diluted with 5ml of distilled water for measuring the optical density at 600nm using a spectrophotometer. Standard solutions of ethanol were prepared. The standards contain 1, 2, 3, 4, 5 to 10 % ethanol (v/v).

Section C (Biogas Production)

A portable, easy to maintain and economical biogas digester was installed and fabricated. It consists of two plastic tanks: a digester of 1000L capacity (or fermentation tank) and a gas holder of 750L (Fig 3C.1, 3C.2). Construction of the biodigester involved the contribution of various PVC fittings. Construction of the biodigester involves the contribution of various PVC fittings named as follows:-

3C.1 Requirements

i. Black colored molded plastic tanks (1000L and 750 L) – Fig 3C.3

ii. 12.5 mm Galvanized iron elbow (1piece) – Fig 3C.4

iii. Barrel piece/ nipple 40mm – Fig 3C.5

iv. Ball valve (40mm) – Fig 3C.6

v. Rigid PVC pipe – 90 mm diameter (3m long) - Fig 3C.7

vi. 90mm T with cap (1piece) – Fig 3C.8

vii. 90mm PVC socket (1piece) – Fig 3C.9

viii. 63 mm rigid PVC pipe (1.5m) – Fig 3C.10

ix. PVC elbow 63mm– Fig 3C.11

x. PVC Union 63mm (1piece) – Fig 3C.12

xi. Gas cork – Fig 3C.13

xii. Nipple 12.5mm– Fig 3C.14

3C.2 Others

i. 12.5mm PVC socket (1piece)

ii. PVC adhesive solution

iii. M-Seal (epoxy resin and hardener)

iv. Barrel piece/ nipple 90mm, 63mm, 12.5mm

v. Thread

vi. Teflon tape

vii. Gas rubber pipe

viii. Biogas stove

3C.3 Schematic diagram of Biogas Plant (Biodigester) in JUIT Campus

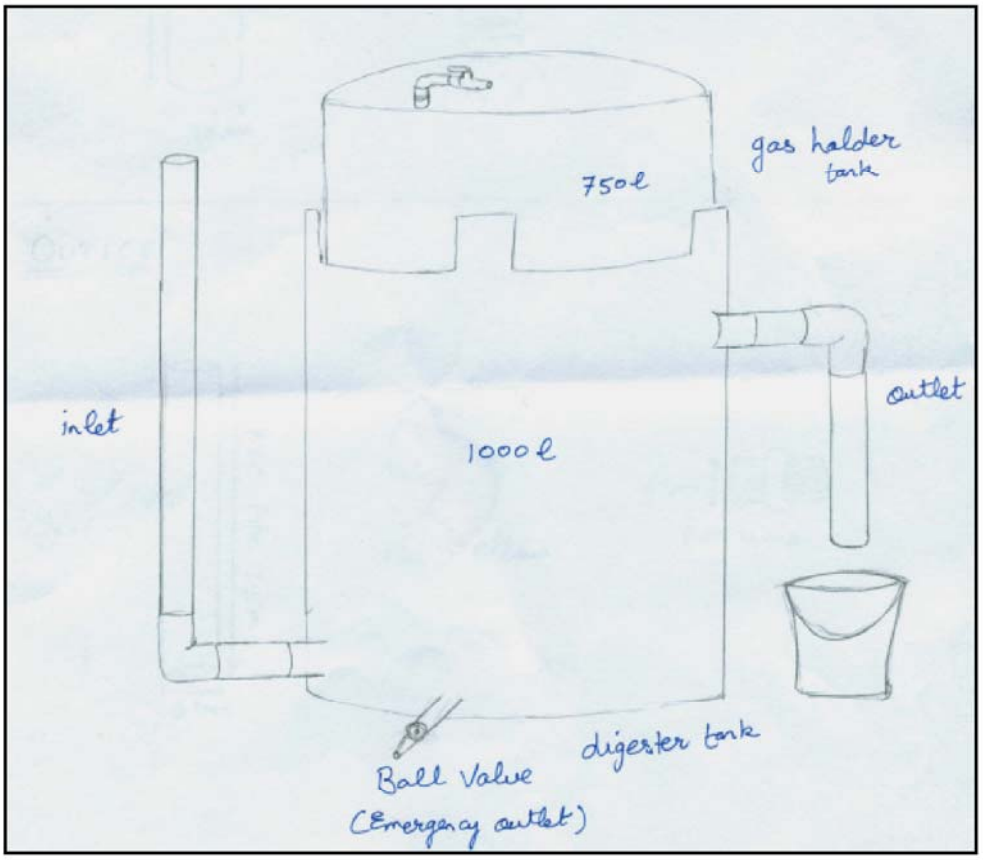


Fig. 3C.1 Schematic diagram of Biodigester

3C.4 Existing Biogas Plant in JUIT Campus

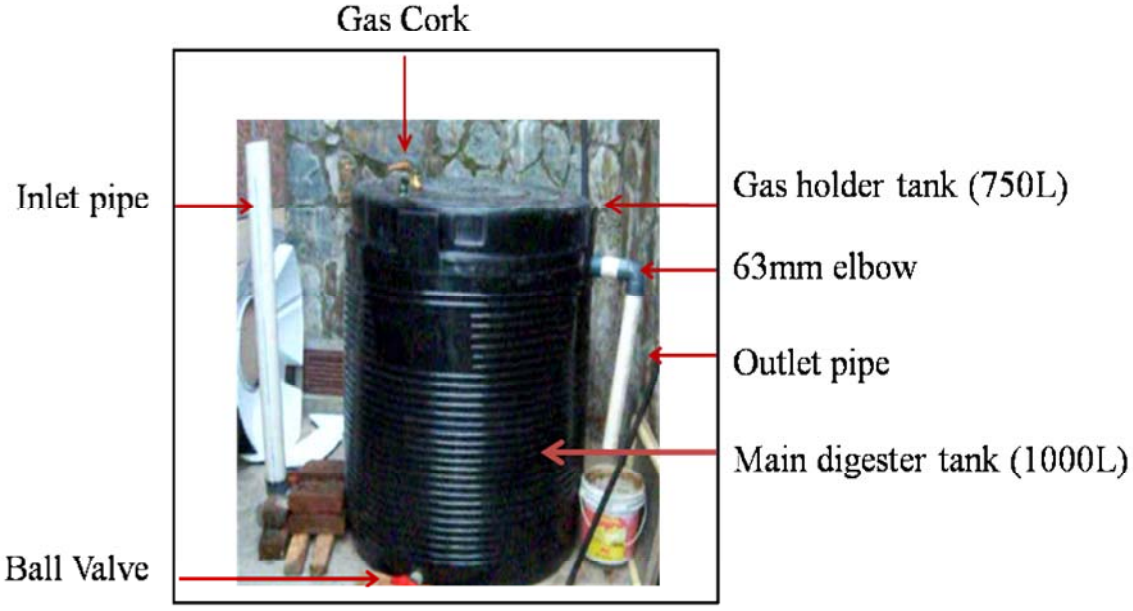


Fig. 3C.2 Biogas Plant Installed at JUIT

3C.5 Requirements

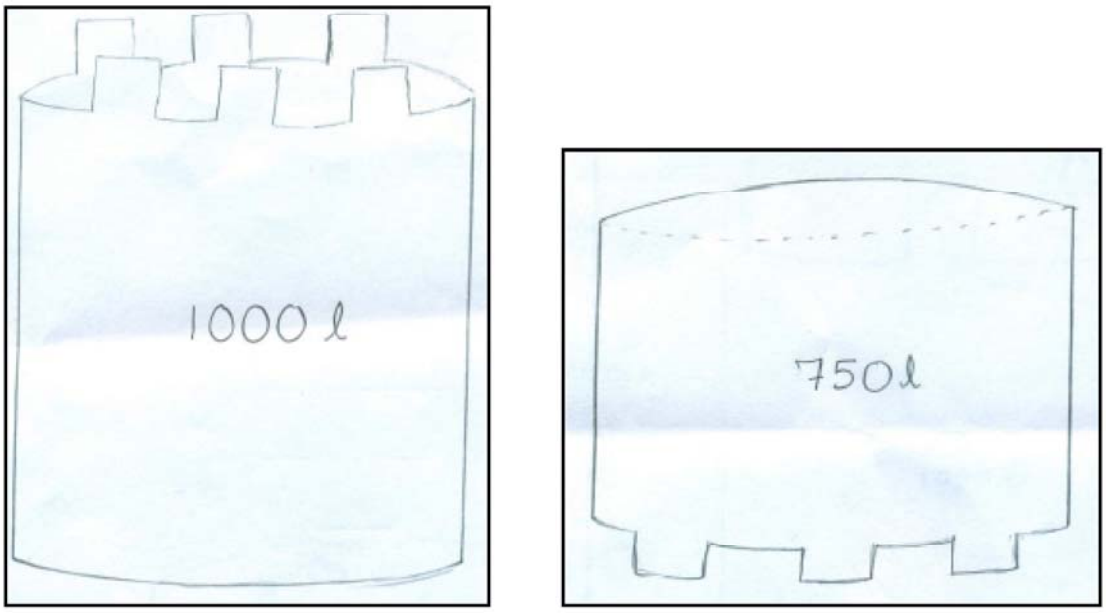


Fig 3C.3 Plastic tanks of 1000L and 750L

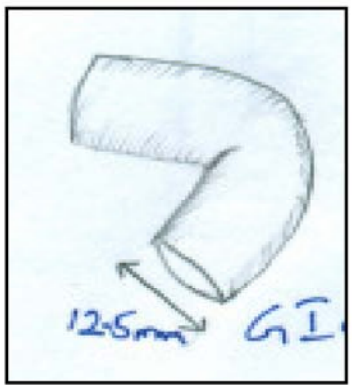


Fig 3C.4 GI elbow (12.5mm)

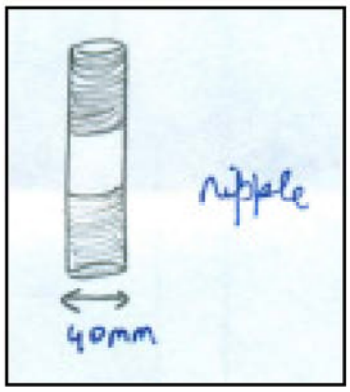


Fig 3C.5 Nipple (40mm)

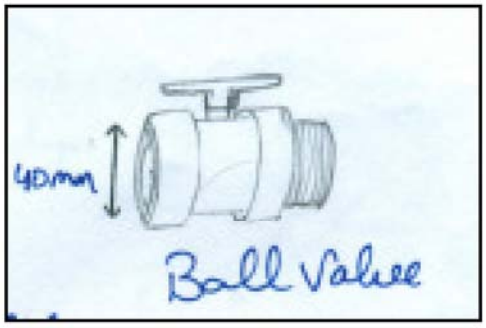


Fig 3C.6 Ball valve (40mm)

3C.5.1 INLET

Fig 3C.7 PVC Pipe (90mm)

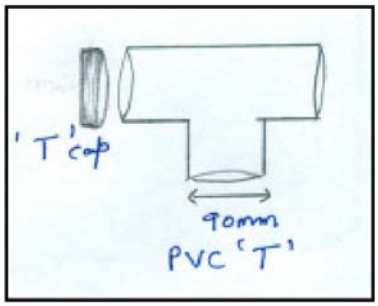


Fig 3C.8 T with cap (90mm)

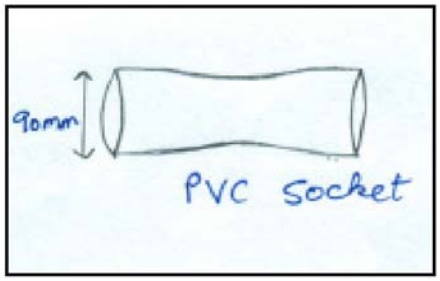


Fig 3C.9 PVC socket (90mm)

3C.5.2 OUTLET

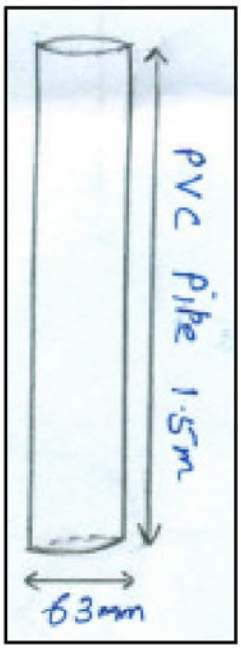


Fig. 3C.10 PVC pipe (63mm)

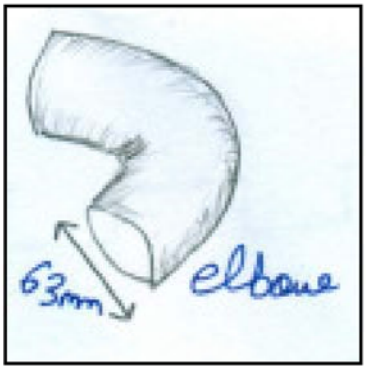


Fig. 3C.11 PVC elbow (63mm)

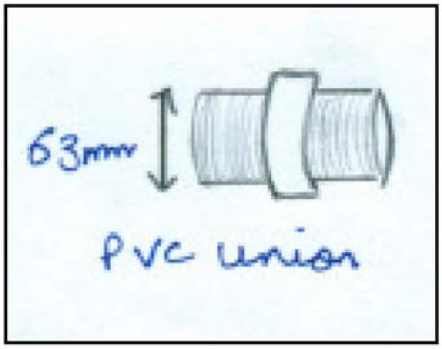


Fig. 3C.12 PVC Union (63mm)

Fig. 3C.13 Gas cork

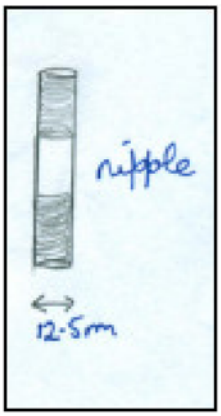


Fig. 3C.14 Nipple (12.5mm)

3C.6 Fabrication and Installation of Biogas Plant:

Two plastic tanks of 1000L and 750L were taken as main digester and gas holder tanks. Fabrication involved following steps:

a) 1000L tank was taken and was cut from the top, perforated near the base with 90mm preheated barrel piece or nipple, another perforation was done near bottom with 40mm barrel piece and at top with 63mm barrel piece. So the tank had one perforation near top and 2 near bottom. The socket of 90mm at the base perforation of 90mm was fixed with PVC adhesive solution and extra adhesive solution had been wiped with cloth and small piece of PVC pipe got fixed into it.

b) Then with PVC adhesive solution 90mm T (with cap) was fixed to it representing continuous linear horizontal pipe, then on to free end of T; 90mm PVC pipe had been attached with PVC adhesives and a funnel was added to its top as inlet for the feedstock /or the slurry. Then to inside of the tank a 60cm piece of 90mm pipe was attached to socket in such a way so that its free end projected into the tank up to its centre.

c) Approximately 45cm long PVC pipe of 63mm got fixed into the tank with 63mm union with PVC adhesives projected horizontally out of the tank with a 63mm fitted elbow joined with another PVC pipe and serve as the outlet for spent slurry.

d) Then 3rd hole which was perforated with the 40mm preheated barrel piece and barrel piece was left there, and 40mm ball valve was screwed to it and a mixture of epoxy resin and hardener (M seal) was applied to all over the joints wherever pipe was fitted to the tank. Bricks were placed inside the fermenter tank to create a support for the gas holder cap to rest on.

e) Gas holder of 750L capacity was taken and by inverting it, a hole was created at the base of 12.5mm with preheated 12.5mm barrel piece and to that barrel only, 12.5mm GI elbow was fitted. After it gets cooled gas cork screwed on to it and between every joint M seal was applied.

To start up we took cow dung slurry as a preinoculum with approximately 40kg of cow dung mixed with almost 40L of water by removing all extraneous matter and breaking all the lumps and then the main digester tank was filled with water. After that gas holder tank was placed in inverted position over the main digester tank with opened gas cork, and when gas holder tank sunk half way gas cork was closed and left as such for next 10 – 12hr for checking the leakage from joints. Then after gas cork was opened and tank was allowed to sink fully in fermenter tank. The gas production was indicated by the uplift of gas holder tank. Then after, Industrial herbal waste and rice slurry (mess waste) was used as feedstock.

3C.7 Physico-Chemical Analysis

3C.7.1 Determination of BOD (Sawyer et al., 1994)

pH of the slurry sample was adjusted to neutral using 1N HCl or NaOH solutions. The slurry samples were further filled in 6 BOD bottles without bubbling. 1 ml of allylthiourea (0.5%) was added to each bottle. Dissolved oxygen in three of the 6 BOD bottles were determined by titration method. Mean of three reading were taken (D1). The rest of 3 bottles were incubated at 27^{o}C in a BOD incubator for 3 days. Oxygen concentration in all the three incubated samples was estimated. And the mean of three readings were taken (D2).

Calculation:

BOD (mg/litre) = D1- D2

Where D1- Initial DO of the sample (mg/L)

D2- DO after 3 days incubation (mg/L)

3C.7.1.1 Dissolved Oxygen Estimation (DO)

Slurry sample was taken in 250ml glass bottles. 2ml each of manganous sulphate and alkaline iodide –azide solutions were added in succession right at the bottom of the bottle with separate pipettes and stopper was replaced. Bottle was shaken upside down at least six times. Precipitates were allowed to settle down. Then further 2ml of conc. sulphuric acid was added and the bottles were shaken to dissolve the precipitates. Then 50ml of sample was taken in flask and titrated with thiosulphate (0.025N) solution till the colour changed to pale straw. Afterwards 2 drops of starch solution was added to the flask which changed the colour from pale to blue. Then it was titrated with thiosulphate solution till the blue colour disappeared.

Calculation

$$\text{D.O. (mg/L)} = \frac{(8^* \times 1000 \times N)}{V} \times v$$

V- Volume of the sample taken

v- Volume of titrant used

N- Normality of the titrant

* 8 is the constant since 1ml of 0.025 sodium thiosulphate solution is equivalent to 0.2mg oxygen.

3C.7.2 Determination of COD (Sawyer et al., 1994)

50ml of slurry sample was taken from the biogas digester and 5ml of potassium dichromate solution (0.1N) was added and kept the flask at 100^{o}C in water bath for 1hr. Then samples were allowed to cool for 10 min. 5ml of potassium iodide solution (10%) was added to it followed by 10ml of 2M H_2SO_4 addition. Further the contents were titrated with 0.1M sodium thiosulphate until the pale yellow colour appeared. To

that 1ml of starch solution was added which turn it to blue. It was again titrated with 0.1M sodium thiosulphate until the blue colour disappeared completely.

Calculation

$$COD\ (mg/L) = \frac{8 \times C \times (B-A)}{S}$$

Where C – Concentration of titrant (mM/L)

A – Volume of titrant used for blank (ml)

B – Volume of titrant used for sample (ml)

S – Volume of sample taken (ml)

3C.7.3 Determination of Ash content (AOAC 1990)

0.5g of the oven dried sample was taken and kept in muffle furnace set at 500°C for 2 – 3hr. Note the change in weight as percentage of ash present.

3C.7.4 Determination of Fat content (Pearson 1976)

5g of the oven dried sample was taken in a cotton wool and that later into the thimble. Then it was further inserted into Soxhlet apparatus. About 100 - 120ml of the Petroleum ether was taken in the round bottom flask and heated at about 60 – 70°C. The heat source was so adjusted that solvent drips from the condenser into the sample chamber. The extraction was continued till the solvent changes its colour from transparent to light yellow. Thimble was removed from the extractor and the sample was transferred to a 100ml beaker. It was further placed in an oven at 102°C till a constant weight was reached. At the end it was cooled in a dessicator and the contents were weighed.

Calculation

% Crude Fat = (W1 – W2) x 100

Where W1– Initial weight of the sample

W2- Final weight of the sample

3C.7.5 Determination of Total Solids (TS) (Meynell 1976)

50 ml thoroughly mixed slurry sample from the biogas digester was taken in a preweighed china dish. Then the dish was placed on water bath at 95^{o}C for 3 – 4hr till it got completely dried. Further it was cooled in a dessicator and weighed. This process was repeated till we got a constant weight. Then final weight was recorded.

Calculation:

$$\text{Total solids (TS \%)} = \frac{(W1 - W2) \times 100}{A}$$

Where W1- Initial weight of sample with dish

W2- Final weight of sample with dish

A- Initial sample taken in ml

3C.7.6 Determination of Volatile Solids (VS) (Meynell 1976)

The residue produced by TS analysis was taken in a muffle furnace and kept at a temperature of 550^{o}C for 30 min. Then it was cooled in a dessicator and weighed.

Calculation:

$$\text{Volatile solids (VS \%)} = (W1 - W2) \times 100$$

Where W1- Initial weight of residue with dish after TS analysis

W2- Final weight of sample with dish

3D. Statistical Analysis

The results were presented as Mean $\pm$ SE (standard error of mean). One way/Two way analysis of variance (ANOVA) was used for multiple comparisons. Values of $P < 0.05$ were considered to be significant.

CHAPTER 5

SUMMARY AND CONCLUSION

Industrial herbal waste an excellent example of lignocellulosic biomass generated in bulk was explored as an alternate source of energy for its bioconversion into commercially viable products. It is a heterogeneous kind of waste composed of various medicinal plants, fruits and fruit parts. Although it was biodegradable but still industry was facing problem in its disposal. Considering the composition of the wastes, as a rich source of organic waste it was explored as a substrate for the vermicompost production and exotic species of the earthworm *Eudrilus eugeniae* was used. Although this technology is centuries old but it's getting revived as this vermicomposting technology is now replacing the chemical fertilizers as a part of organic farming. Vermicompost was prepared using industrial herbal waste and cow dung; and their nutrient profile was compared. Vermicompost made from industrial herbal waste was superior with respect to nutrient content in comparison to latter. Maximum amount of nitrogen fixed within the vermibeds having industrial herbal waste as substrate was 26.3g/kg. Thereafter, different parameters were checked and compared for the vermicompost made from blend of cow dung and industrial waste in 1:1 ratio (vermicompost I) in comparison with cow dung as substrate (vermicompost II). In the vermibed I having cow dung and industrial herbal waste blend (1:1), number of earthworms within the vermibeds were increased indicated that earthworms were capable of utilizing the substrate as a carbon source for its growth and reproduction. Simultaneously, total organic carbon got decreased from 430 to 180g/kg and nitrogen content increased from 12 to 26.3g/kg which decreased the C: N ratio upto 6.84 from 35.8 depicted a high rate of mineralization. Further effect of both the vermicomposts made from blended herbal waste and cow dung was checked on

two seasonal plants *Tagetes* (Marigold) and *Pisum sativum* (Pea). Results showed that vermicompost I significantly increased the growth and productivity of both the plants. Further experiments were done with variable substrate ratio to find out the best combination for the vermicompost production with a promising nutrient profile and to spot the role played by the earthworms in utilizing a different kind of waste. Results showed that vermicompost made from S4 set (3.75kg Herbal waste + 1.25kg Cow dung) came to be the best combination with the best nutrient profile fixing maximum of 6.5% nitrogen and C:N ratios was lower than 20:1. At the end technology has been transferred to the industry and vermicompost product made using industrial herbal waste as substrate has been launched in the local market. This gave an alternate method of managing the waste as a source of energy in a cost effective and ecofriendly manner.

On the other hand, herbal industrial waste was being checked for bioethanol production. As it was an example of lignocellulosic biomass, a recalcitrant type of waste so certain pretreatment methods were needed to loosen the complex lignocellulosic matrix to make them accessible for the enzymatic attack to convert the complex polymers into simple monomeric fermentable form of sugars. In the compositional analysis of the waste - lignin (23%), cellulose (25%), hemicellulose (11%) and pectin (11%) were found to be present in it. Different physico-chemical methods were applied as steam explosion (at different temperatures like 121^{o}C, 140^{o}C and 160^{o}C for 0.5hr, 1hr, 1.5hr and 2hr) and acid (with different % of H_2SO_4 0.5%, 1%, 1.5% and 2% for 0.5hr, 1hr, 1.5hr and 2hr at 160^{o}C) and alkali (with different % of NaOH 0.5%, 1%, 1.5% and 2% for 0.5hr, 1hr, 1.5hr and 2hr at 160^{o}C) hydrolysis of steam exploded pulp. Reducing sugar content was increased with respect to

affectivity of pretreatments. Maximum amount of reducing sugar released after steam explosion, acid and alkali hydrolysis of steam exploded pulp was 18.75mg/g, 145.9mg/g and 125.5mg/g respectively. Thereafter, enzymatic hydrolysis was done to convert the complex polymers into simple monomeric form of sugars. Commercial preparations of cellulase and pectinase enzymes were taken at the rate of 10 FPU/g of the substrate. After the enzymatic hydrolysis the sugar content was increased significantly with steam hydrolysis up to 81mg/g, with acid up to 157.5mg/g and with alkali up to 138.9mg/g. Further the hydrolysate with maximum reducing sugars was checked for its suitability for fermenting it into bioethanol using *S.cerevisiae* and *P.tannophilus*. But the ethanol conc. was marginally low. On similar lines, considering the composition of the industrial herbal waste, it was taken as substrate for *in vitro* enzyme production of two industrially important enzymes; cellulase and pectinase. In comparison wheat bran was taken as it was a traditionally known substrate in enzyme sector. A low cost production method SSF was chosen for the enzyme production. In the solid media of industrial herbal waste and wheat bran with 3ml of moistening agent (Mandels medium) at 30^{o}C after 72hr gave maximum enzyme productivities. After standardization of whole process our studies concluded that maximum endoglucanase 21.85 IU/gds, exoglucanase 90.8 IU/gds, β glucosidase 27.4 IU/gds and pectinase 0.4 IU/gds was obtained from herbal waste while from wheat bran maximum production was endoglucanase 25.09 IU/gds, exoglucanase 90.54 IU/gds, β glucosidase 32IU/gds and pectinase 0.3 IU/gds. The results depicted that enzyme productivities were comparable and herbal waste can be used as an alternate and additional substrate for the production of industrially important enzymes. Moreover, it has the potential to replace the existing ones from the enzyme

sector as it is produced in bulk, so abundant in amount, cost effective too and its not seasonal dependent. And the technology has been transferred to the industry.

The lignocellulosic waste had also been used as a feedstock in the biodigester for biogas generation. A very easy to maintain and portable kind of biogas plant was fabricated and installed in our campus consisting two plastic tanks one of 1000L as main digester tank and another 750L as gas holder tank. Rice waste from the mess and industrial herbal waste were used as substrates for the biogas generation in different ratios. Maximum of 0.440m3 gas was produced in the biodigester using industrial herbal waste as feedstock which is able to cook for approximately 2hr. Further the technology has been transferred to the Ayurvet Industry and a biogas plant with 2000L of gas holder tank and 2500L of main digester tank was installed over there and gas produced is used in their canteen for cooking purpose. In our campus another biogas plant has been installed with 2000L of gas holder and 3000L of main digester tank and biogas produced will be used for cooking purpose in workers mess. The technology has also been transferred to a school near Jabli (H.P.) also with the help of Rural Development Department, Himachal Pradesh. At the end we can enunciate that the industrial herbal waste has the potential to serve as a substrate for vermicompost and crude enzyme production along with biogas generation. The efforts to process industrial herbal waste into useful commercial products will be continuing in the R and D of the Ayurvet Industry and few more researchers of our laboratory will take up the problem of solid waste management; may be with different perspective. As a concluding remark we strongly feel that a kind of interactions between Industries and Research academics should be regular as it plays an imperative role in upgrading the research work from laboratories to the industrial level. Such kind of joint ventures poses new challenges for the researchers which can be helpful for the industry too.

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Publications

Publications

A. Patent Filed

1. Kumari, M., Kumar, S. Chuahan, R. S., Ravikanth, K. 2011. Patent Application No. CL/747/2011 filed in India on "Process for production of cellulase and pectinase from herbal industry residue".

B. Journals

1. Kumari, M., Kumar, S., Ravikanth, K., Chauhan, R. S., (2010). Bioconversion of herbal industry waste into vermicompost using an epigeic earthworm *Eudrilus eugeniae*. *Waste Management and Research* (doi: 10.1177/0734242X10384432). **ISSN: 0734-242X; Online ISSN: 1096-3669**.
2. Kumari, M., Kumar, S., Ravikanth., K., Chauhan, R. S., (2010). Pretreatment and saccharification of steam exploded waste of pharma industry. *International Journal of Pharma and Bio Science*. 1(2). 1-7. **ISSN 0975-6299.**
3. Kumari, M., Kumar, S., (2009). Biorefineries: India's future option for energy and chemical feedstock. *As. J. Energy Env.* 10 (3), 160-164. **ISSN 1513-4121**.
4. Kumari, M., Syal, S., (2009). Biofuels: concerns about substrate selection. *Current Science*. 96 (5): 630. **ISSN 0011-3891.**

C. International and National Conference Papers

1. Kumari, M., Kumar, S., Anaerobic digestion of lignocellulosic biomass for biogas production. Delhi International Renewable Energy Conference (DIREC) (October 27-29th 2010, Knowledge Park (II), Noida.
2. Kumar, S., Kumari, M., Ravikanth, K., (February 11-12, 2010). Herbal waste: A waste or a fortune. In 7th International Biofuels Conference held at Hotel Le-Meridian organized by Winrock International India, New Delhi, India.
3. Kumari, M., Syal, S., Ravikanth, K., Chauhan, R. S., (September 26-27, 2009). Bioconversion of Industrial Herbal Waste into Value Added Products. In a National Seminar on, "Biotechnology and Microbiology in Human Welfare: The Indian Scenario." Mody Institute of Technology and Science, Lakshmangarh, Sikar, Rajasthan.

4. Kumari, M., Syal, S., Chauhan, R. S., Ravikanth, K., (March 4-5, 2009). Bioconversion of herbal waste to bioethanol. 6th International biofuel conference in New Delhi.
5. Kumari, M., Syal, S., Dey, G., Chauhan, R.S., (September 6-10, 2008). Lignocellulosic biomass: A source of mandatory alternative bioenergy for future. Accepted for presentation at Field Research Laboratory of DRDO, at Leh.
6. Kumari, M. Syal, S., (March 13-14, 2008). Lignocellulosic Biomass: A clean green solution. In a national conference on Environmental degradation-Challenges and remedies. M.M. Engineering College, Ambala.

D. Print Media

1. News coverage of "Portable biogas plant developed" in The Tribune on December 06, 2009. (http://www.tribuneindia.com/2009/20091207/himachal.htm)

E. Conferences and Meeting

1. Kumar, S., Kumari, M., Verma, T., Kalia, T., Chauhan, R.S., Ravikanth, K., "Solid waste management vis a vis rural community empowerment: Indian perspective." in a meeting of Sigma Xi association in Rapid City, South Dakota, USA. (17 May, 2011)
2. Kumar, S., Kumari, M., Verma, T., Kalia, T., Chauhan, R.S., Ravikanth, K., Sani, R.K., "A green approach for industrial solid waste management and its bioremediation" to be presented in coming 6th annual Eastern South Dakota Water Conference, Brookings, SD, USA (October 13, 2011).

F. Manuscript under preparation

1. Anaerobic digestion of lignocellulosic industrial herbal waste for generation of biogas using a cost effective, portable and sustainable biogas plant, (Bioresource Technology).
2. Vermicomposting of different blends of industrial herbal waste using Eudrilus eugeniae (Waste Management and Research)