Chapter-1

General Introduction
1.1 Introduction to Biofuels

One of the most important challenges of the twenty-first century is to meet to the rising energy needs of the population across the globe. However, with ever increasing awareness on the impact of climate change; the search for a sustainable alternate energy source is presently the most important research question facing the scientific community. Conventional fossil fuels produce greenhouse gases and are considered as one of the major factors along with coal based power-plants for exacerbating the greenhouse effect leading to climate change. Any sustainable alternative to fossil fuels both for energy and transportation should be able to reduce the current levels of greenhouse gases according to existing international norms. The technology that is most widely adapted as a substitute to the fossil fuel for transportation purposes and which also has the potential to reduce the highest concentration of CO$_2$ is biofuel (Avci et al. 2013).

Biofuels have presently emerged as the most important alternative towards meeting both transportation and energy needs of the world. The manufacture and use of biofuels has increased exponentially in the recent past, from 18.2 billion liters in 2000 to 88.69 billion liters in 2014. 85% of all the biofuel being used in the world is bioethanol. The two major markets for biofuels in the form of bio-ethanol are USA and Brazil which account for more than 80% of the global biofuel production. The biggest advantage of biofuels is that they are renewable and can use any of the available plant based sources. Also, biofuels produce significantly less carbon emissions which in turn help to decrease climate change, providing a safer alternative for environmental protection (Gupta and Verma 2015).
1.2 Classification of Biofuels

Biofuels are liquid, solid, or gaseous fuels made from renewable biomass. Biofuels can be classified as primary or secondary biofuels. Primary biofuels are obtained from burning unprocessed biomass to generate heat or electricity. Examples of biomass used for primary biofuels include wood, chips, pellets and animal wastes. Secondary biofuels are obtained after a number of predefined processing steps with biomass to yield ethanol and biodiesel. Secondary biofuels have industrial applications and also can be used with conventional gasoline as a substitute. Secondary biofuels are further classified into first, second and third-generation, based on the substrate and the technology used for production. First-generation biofuels (FGB) are mostly bio-ethanol and biodiesel which use simple sugars as substrates obtained from crop plants. The processing steps include fermentation of sugars and trans-esterification of oils (Nigam and Singh 2011).

1.3 Benefits of Second Generation Biofuels

Second-generation biofuels (SGB) are produced after hydrolysis of lignocellulosic biomass to ethanol. Finally, third-generation biofuels use algae to produce biodiesel or bioethanol. Most of the bioethanol produced for transportation purposes across the globe have been generated using food crops and are thus first generation in nature. The main advantages in the production of biofuels are highlighted in the Figure 01. However, first generation biofuels drastically affect food crop production and output, as highly fertile land is used up for non-food grain purposes (Dalla Marta et al. 2014).
There have been many instances where prices of food crops have increased due to diversion of land for non-food purposes. One such instance is the drought of USA in 2013 when the price of maize per bushel increased to 6.89$ from 6.22$ in the previous years. Additionally, due to increased profit margins, large holding farmers tend to divert their farmland for non-food purposes. In USA 40% of corn produced currently is being used for bioethanol production. In order to reduce this dependence on fertile land and food crops for biofuel production, it is envisaged that biofuels be produced from non-food crops which are rich in cellulose, hemicellulose and lignin (Kim and Dale 2004).

Further, it has been reported that approximately 442 billion liters of second generation biofuel can be produced worldwide per year from available non-food crop residues. Therefore, lignocellulosic bio-refineries have been attracting increasing attention in many countries around the world, mainly in Brazil, the USA, Canada, Japan, India, China and Europe (Mussatto et al. 2010).
1.4 Sources of Biomass for the Production of Second Generation Biofuels

Second-generation biofuels are basically made from biomass rich in cellulose/lignocellulose (LC). Such biomass is abundant in the form of nonfood crops, its residues and specialized crop species called as “energy crops”. Some examples of LC/Cellulosic biomass include residues after agricultural processing like corn stalks, forest residues, and grasses specifically grown for energy (Figure 02). These products contain composite material consisting primarily of polysaccharides namely cellulose, hemicellulose and lignin bonded to each other in the plant cell wall. Cellulose is mostly present in the secondary plant cell walls along with hemicelluloses and lignin forming a crystalline complex which is resistant to hydrolysis. These polysaccharides have to be broken down to small mono or oligosaccharides which can be then converted to ethanol (Nigam and Singh, 2011).

![Biomass Sources Diagram]

**Figure 02. Types of biomass for second generation biofuels.**

Ideal Properties of cellulosic/LC biomass used for production of ethanol are as follows (1) they should be non-edible and should not affect food production. (2) Should be produced as specific energy crops which do not require highly productive land. (3) Should be produced in higher quantities per unit of land area when compared to FGB. Lignocellulose or specifically cellulose is the most abundant bio-material on the earth. Therefore, introduction of the ethanol
from the cellulose/LC offers the most sustainable alternative to either of fossil fuels of FGB (Nigam and Singh, 2011).

Biomass in the form of corn stover, sugarcane bagasse, cereal straw, rice stubble, dedicated perennial produces such as Miscanthus, poplar and willow Krzyzaniak et al. (2014) are among the prominent feedstocks for which bioethanol production technology after hydrolysis of cellulose/LC has been implemented in bio refineries across the globe. Among perennial species, short rotation woody crops (SRWC) like Miscanthus provide a stable return and there is a well-developed technology available for their culture. Miscanthus can produce 6.1 kilo-litre of (KL) ethanol given current conversion technologies, and 8.6 KL ethanol under advanced technologies (Arnoulta et al. 2015).

For each cubic meter of water used Miscanthus could reach a potential biofuel production limit of 1.8 lakh liters per annum. Experiments indicate that switching from maize to Miscanthus is much more competitive than switching to switchgrass in terms of the use efficiencies of water and land (Gao et al. 2014).

Miscanthus, a perennial grass, yields high amounts of biomass, requires fewer nutrients and is good at carbon sequestration. Miscanthus can grow to over 3 m tall and is able to produce 20 to 25 tons of dry matter per hectare (Brosse et al. 2009).

Miscanthus undergoes an annual cycle of senescence leading to low removal of nutrients during harvesting (Somerville 2010). It contains 40% cellulose and 20% hemicellulose, which is higher than most other warm season grasses (Table 01). As a C4 perennial plant characterized with high biomass yield and relatively low nitrogen and water requirement, Miscanthus
is considered to be one of the top candidates of second-generation energy crops. It has been estimated that the energy that can be obtained from LC/Cellulosic biomass is estimated to be 100 EJ (Energy joule)/year (Somerville 2010).

<table>
<thead>
<tr>
<th>Crop</th>
<th>Cellulose (%)</th>
<th>Hemicellulose (%)</th>
<th>Lignin (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn stover</td>
<td>35%</td>
<td>28%</td>
<td>10.4%</td>
<td>Karp and Shield, 2008</td>
</tr>
<tr>
<td>Miscanthus</td>
<td>57.6%</td>
<td>15.9%</td>
<td>10.5%</td>
<td>Karp and Shield, 2008</td>
</tr>
<tr>
<td>Poplar</td>
<td>40%</td>
<td>14%</td>
<td>20%</td>
<td>Karp and Shield, 2008</td>
</tr>
<tr>
<td>Sugarcane</td>
<td>24%</td>
<td>8%</td>
<td>7%</td>
<td>Karp and Shield, 2008</td>
</tr>
<tr>
<td>Sweet sorghum</td>
<td>26.3%</td>
<td>20%</td>
<td>7.1%</td>
<td>Rooney et al, 2007</td>
</tr>
<tr>
<td>Switchgrass</td>
<td>31.6%</td>
<td>36%</td>
<td>6.1%</td>
<td>Karp and Shield, 2008</td>
</tr>
<tr>
<td>Willow</td>
<td>55.9%</td>
<td>14%</td>
<td>19%</td>
<td>Karp and Shield, 2008</td>
</tr>
</tbody>
</table>

**Table 01. Content of cellulose, hemicellulose and lignin in various crops used as substrates for production of second generation biofuels.**

However, the cost of production of SGB in the form of ethanol is still quite high and thus is not economical enough for market penetration and acceptance as a substitute for fossil fuels. The primary reason for exceptionally high cost is the need for several steps before the ethanol is produced in usable form (Nigam and Singh 2011).

The steps in a bio-refinery include pre-treatment, enzymatic hydrolysis of pre-treated biomass to monosaccharides, ethanol fermentation of monosaccharide solution, distillation, rectification and dehydration of the obtained alcohol. Pre-treatment of the biomass and enzymatic hydrolysis of
the pre-treated biomass are the two processes which add to the cost of production of second generation biofuels due to the complexity and crystalline structure of the secondary plant cell wall of which cellulose, hemicellulose and lignin are the primary components (Nigam and Singh 2011).

1.5 Polysaccharides of the Plant Cell Wall

1.5.1 Cellulose

It is the main structural component in the plant cell wall. It is a linear homopolysaccharide consisting of anhydrous glucose units (500–15,000) that are linked by β-1, 4-glycosidic bonds, with cellobiose as the smallest repetitive unit. The β-1, 4 orientations of the glucosidic bonds results in the potential formation of intramolecular and intermolecular hydrogen bonds which makes cellulose highly crystalline, insoluble, and resistant to enzyme attack (Saini et al. 2015).

1.5.2 Hemicellulose

Hemicellulose is a small, branched polysaccharide made up of pentoses (D-xylose and L-arabinose), hexoses (D-manose, D-galactose, and D-glucose) and sugar acids making up a chain of 50–200 units. The composition of hemicellulose varies extensively within the different organs of an individual plant and also within the species of a particular genus. Softwood hemicelluloses largely consist of glucomannans, arabinogluconoxylans (xylans), arabinogalactans, xylglucans and other glucans, whereas hardwoods primarily consist of xylans and glucomannans. Hemicellulose provides linkage between lignin and cellulose. Hemicelluloses make up 20 to 35% of the total available biomass of the plants and thus affect the efficiency of conversion of biomass processing to ethanol (Holtzapple 1993).
1.5.3 Lignin
Lignin is a vital component of the secondary cell walls of plants. It provides structural support by filling the spaces between cellulose, hemicelluloses and pectin components. Lignin is highly heterogeneous and does not have a primary structure. It consists of multiple cross-linked phenylpropanoids, believed to be obtained from three methoxylated monolignol monomers: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Freudenberg and Nash 1968). The resulting molecule is hydrophobic, has an elevated carbon content and energy value which makes it very difficult to breakdown (Lebo et al. 2001 and Martone et al. 2006).

1.6 Pre-Treatment Methods for efficient Breakdown of Biomass
The production of ethanol from cellulose/LC feedstocks and energy grasses like Miscanthus as described above is severely restricted because of their complex crystalline structure which is extremely recalcitrant to hydrolysis. Therefore, the conversion of cellulose/LC to ethanol requires a very efficient pre-treatment method followed by hydrolysis of the pre-treated polysaccharides to mono or oligosaccharides. In order to achieve sustainable and environmentally friendly biomass deconstruction consisting of complex polysaccharides into fermentable monosaccharaides it is necessary to overcome the chemical and structural complexity of biomass through various pre-treatment methods (Martone et al. 2006).

1.6.1 Physical Pre-treatment Methods
Complexity of Lignocellulosic biomass can be reduced by reducing its size either by chipping, grinding, shearing, or milling, which cuts down the particle size and increases surface area of cellulose availability for enzymatic attack for
Some of the most prominent methods include Shammer mills or Wiley mills to produce molecules that can pass through 3 to 5mm diameter sieve. Other physical treatment methods include pyrolysis, gamma irradiation, microwave, infrared and sonication (Alvira et al. 2010).

1.6.2 Chemical Pre-treatment Methods

Chemical methods are extremely effective in breaking down of the polysaccharides but are not environmentally friendly and produce a number of toxic byproducts. Some of the methods used are ozone, acids, alkali, peroxide and organic solvents (Mosier et al. 2005).

1.6.3 Physico-Chemical Pre-treatment Methods

These methods are significantly new and are highly effective than physical or chemical methods of pretreatment alone. Some of the physicochemical methods used for pretreatment are ammonia fiber explosion (AFEX) Mosier et al. (2005), autohydrolysis (steam explosion), SO2 steam explosion Tooyserkani et al. (2013), acid and ionic liquids (Sorensen et al. 2008).

Other popular methods include organosolv, wet explosion liquid hot water (LHW) pretreatment Khullar et al. (2013) and wet oxidation (Sorensen et al. 2008). LHW has now emerged as the pre-treatment method of choice as it is compatible with biological treatment with enzymes and is less toxic. In addition, no extra chemicals are required, no special non-corrosive materials are required for reactor building, and fewer toxic degradation products are formed (Laser et al. 2002 and Li et al. 2010).

1.6.4 Biological Pre-treatment Methods

Biological treatment of feed stocks mostly involves enzymatic hydrolysis with enzymes such as cellulases, hemi-cellulases, lignin peroxidases, manganese
dependent peroxidases, polyphenol oxidases, laccases and quinosine-reducing enzymes. These enzymes are produced from a diversity of microbial sources and treatment with enzymes alone will not achieve sufficient saccharification required for economical yield of ethanol. In addition, the chosen pre-treatment method should be compatible with the enzymatic hydrolysis in order to avoid toxicity to the enzyme used which reduces the efficiency of saccharification of the polysaccharides. Further, various factors like substrate concentration, enzyme loading, temperature and time of saccharification affect enzymatic degradation process. Since, structure of lignin is highly heterogeneous, it is expected that a number of enzyme combinations should be used for breakdown of lignin from the biomass thus adding to the cost of the ethanol produced. Due to extreme variations in the polysaccharide compositions of the feedstock it is necessary to use different categories of enzymes individually and then create cocktails for hydrolysis of different types of biomass. Therefore, the most prudent approach would be to use either physical or physico-chemical method to reduce the complexity of the biomass and increase the cellulose exposure which could then be hydrolysed by a number of cellulosases to various monosaccharides for conversion to ethanol (Brown 2003).

1.7 Cellulose Degrading Enzymes

Cellulose-degrading enzymes can be grouped into three major groups: endoglucanases (EG), exoglucanases (cellbiohydrolases, CBH) and β-glucosidase (BGL) (Saini et al. 2015).
1.7.1 Endoglucanases

Endo-β-(1,4) β-glucanases (1,4-β-D-glucan-4-glucanohydrolases, EC 3.2.1.4) are usually referred to as endoglucanases. They are characterized by their random hydrolysis of β-(1,4)glucosidic linkages. The random cleavage by endoglucanase causes rapid reduction in chain length and therefore changes in viscosity relative to the release of reducing end groups occurs (Saini et al. 2015).

1.7.2 Exoglucanases

Exo-β-(1-4)-glucanase (1,4-β-D-glucanellbiohydrolases, EC 3.2.1.91) cleave cellobiose units from the non-reducing ends of cellulose particles. Exo-β-(1,4) -glucosidase (1,4-β-D-Glucan glucohydrolases, EC3.2.1.74) cleaves glucose units successively from the non-reducing end of the glucan (Saini et al. 2015).

1.7.3 Beta-glucosidases

β-glucosidase (or β-D-glucoside glucohydrolase, EC 3.2.1.21) hydrolyzes complex polymers to very short chain β-1,4-oligoglucosides up to cellohexose to form glucose. Most β-glucosidases are active on a range of β-dimers of glucose (Saini et al. 2015).

1.8 Need for Discovery of Efficient Cellulases

Combining cellulose hydrolysis of biomass with a suitable pre-treatment method also has number of advantages over the various pre-treatment methods used alone. These advantages include: a. mild process b. high yield c. maintenance costs are lesser when compared to acid or alkaline hydrolysis d. the whole process is compatible in any scalable bio-refinery. Although
substantial progress has been made in saccharification using cellulases, several challenges still remain (Pablo et al. 2013).

Full commercial-scale utilization is hindered by the high cost of these enzymes. Currently, high enzyme loadings of cellulases are needed because of their low specific activities compared to that of other polysaccharide-degrading enzymes. It has been estimated that bioethanol process would need approximately 11 million filter paper units (FPU) of cellulase to generate 84 gallons of ethanol or 15–25 kg cellulose per ton of biomass. Thus, high energy cost is the most important step that adds to the increasing economies of bioethanol production in a bio-refinery (Carroll and Somerville 2009).

Therefore, the first challenge in cellulosic hydrolysis is to produce an efficient enzyme for speedy and less expensive saccharification of cellulose. Because of the high price and limited structural capability of the currently available cellulases it is absolutely essential to look at discovery of enzymes from natural sources which mimic breakdown of cellulose. Multifaceted approaches to reduce costs of cellulases and/or improve the efficiency of enzyme cocktails have therefore received growing attention, and a number of approaches are in use, including streamlining bioprocess designs, development of cheaper feedstocks for enzyme production, improving and designing feedstock specific cellulase cocktails, and bioengineering microorganisms secreting cellulases and its associated enzymes (Carroll and Somerville 2009).

1.9 Microbial Sources of Cellulases

Cellulases are produced naturally by a variety of microbial species including bacteria and fungi. Many cellulases have been isolated and characterized and many more are yet to be identified. Availability of genome sequences of
Trichoderma reesei as reviewed by Martinez et al. (2008) and other organisms as cited by Rubin (2008) has increased the inventory of cellulases. Trichoderma reesei is the major fungi used for the production of cellulases for second generation bio-refineries. White-rot fungi also have the enzymatic machinery required to completely break down all plant cell wall components including cellulose, hemicelluloses and lignin. However, the use of white-rot fungi for industrial production of cellulases for biofuel production is severely restricted because of stringent culture and nutrient conditions required. Brown-rot fungi also possess the capacity to depolymerize cellulose through the action of highly reactive hydroxyl radicals but this mechanism cannot be exploited commercially to produce cellulase as the culture conditions are difficult to mimic (Pocas-Fonseca and Maranhac 2005).

Various fungal groups have been reported to possess the ability to produce cellulases. These include the basidiomycete Phanerochaete chrysosporium, the ascomycetes Neurospora crassa, Aspergillus species like nidulans, fumigatus, oryzae, niger, cluvatus, Fischeri, flavus and terreus. In addition to the normal microbiological methods used for identification of cellulase producing fungi, genomic approaches have been widely used in the recent past. Genomic approaches for identification of cellulases include curating available fungal genomes for cellulase genes (Martin et al. 2011).

Many fungal genomes have been sequenced and the most important fungi from which many cellulase related genes have been identified are Fusarium graminearum and Ustilago maydis. Even though in silico annotations of curated fungal genomes are able to produce information on putative lignocellulose-degrading enzymes, validation in the form of number of in vitro
assays are necessary in order to characterize, purify and over express these genes for commercial exploitation. Databases like CAZy give valuable information on extracellular hydrolytic and redox enzymes activities of various enzymes involved in lignocellulose degradation, curated genomic data on such enzymes of more than twenty different fungi are available in the CAZy database. Further, validation of the genomic information needs high end proteomic tools which further adds to the time and cost of the enzyme production (Cantare et al. 2009).

Therefore, in addition to genomic methods used for discovery of novel cellulases; it is essential that other biotechnological methods which combine microbiological assays for hydrolysis of biomass combined with sensitive analytical methods for quantification of sachharification products are essential. It is thought that the highly effective plant biomass degrading capacity is found in naturally endophytic fungi which could provide an important and interesting biotechnological source of microorganisms and enzymes for cellulose degradation (Leonid et al. 2011).

1.9.1 Endophytic Fungi as a Source of Cellulases

Generally, plants develope or carry symbiotic/mutualistic relationship with the endophytic fungi which posses a highly efficient enzyme arsenal that allows the fungi to rapidly deconstruct the plant biomass. Endophytic fungi are generally defined as “those species that live within the living tissues of plants, for some or all of their life cycle without causing any harm to their hosts.” Endophytic fungi have been isolated from almost every tissue of every plant sampled. They perform diverse roles ranging from symbionts to latent pathogens. Endophytes have been implicated in increasing environmental
stress tolerance, improving plant vigour and decreasing herbivore attack. Plants in natural ecosystems demonstrate increased colonization by endophytic fungi. Life style of the endophytes exhibit a vast diversity between necrotrophy and biotrophy, depending on the benefits conferred to their host and their colonization strategies (Schulz and Boyle 2005 and Weiss et al. 2011).

Since fungal endophytes interact biochemically with host tissues for the survival of the symbiotic relationship, their physiological adaptation and metabolic activity require a detailed investigation which might result in identification of novel biochemical intermediates with wide range of biotechnological applications. Plant-endophyte interactions are essentially physical in nature, leading to crossing of several host generated barriers ultimately establishing the symbiotic relationship. The “balanced antagonism” hypothesis which is the most well accepted theory on the life style of the endophytes as suggested by Schulz et al. (1999) and reviewed in detail by Schulz and Boyle (2005) suggests that a-symptomatic colonization is a balance of antagonism between the host and the endophyte.
Figure 03. Applications of endophytic fungi

Even though the association between plants and endophytic fungi is ecologically important the physiological characteristics of the interaction are not well understood. An important aspect of physiological studies involving endophytic fungi is the involvement of these microorganisms in the decomposition of plant material, especially the cell wall. Since, the fungi are already present in the plant tissues, they may be able to initiate the decomposition of the host tissue before it becomes dominated by saprophytic species. Therefore, the production of hydrolytic enzymes by endophytic species is not only important for the nutrition of the fungus during the endophytic stage but also to help in the competition for substrates during the saprophytic stage (Kumaresan and Suryanarayanan 2002)
Investigations on the Secretome of Endophytic Fungi and Hydrolysis of Minimally Treated Biomass Feedstocks

Figure 04. Balanced Antagonism Hypothesis for the survival of endophytes

(Schulz and Boyle 2005)

Endophytes display a vast diversity in the range of substrates that can be utilized. Studies have shown that endophytes are capable of metabolizing in vitro most substrates found in plants and produce enzymes including proteases, amylases, phenol oxidases, lipases, laccases, polyphenol oxidases, cellulases, mannanases, xylanases, and pectin lyase. It can be hypothesized that due to their ecologically unique lifestyle, endophytes might have evolved to possess the necessary enzymatic machinery to breakdown and utilize lignocellulosic biomass in situ (Lumyong et al. 2002 and Schulz and Boyle 2005).

1.10 Need for Discovery of Fungal Endophytes

Endophytic fungi produce many enzyme complexes in response to the various ecological stimuli which results in a biochemical and molecular regulation of such responses. These responses can be exploited under lab and industrial conditions for production of some these enzymes. Using controlled
fermentation conditions by altering the culture and process parameters like media type and composition, aeration, harvest points etc. the enzymes produced by fungal endophytes might be optimized. The optimisation could lead to a cost-effective, environmentally friendly, continuous and reproducible yield of enzymes compatible for commercial scale-up which is highly desirable in bioenergy applications (Diogo et al. 2013).

The quest for identifying novel enzyme sources from the endophytic fungi has resulted in the sampling of host vegetative tissues such as herbs, shrubs, tree species, and vines in unique places of ecological adaptations such as rainforests of the world. Such niches including Western Ghats of India harbour great species diversity, without human intervention. Efforts in this direction to sample plants located in the rainforests around the world with potential ethno-medicinal values have resulted in the isolation of fungal endophytes, unique to a particular plant species with distinct bioactivity. The exploration of the biotechnological potential of woody perineal plants of Western Ghats, as a source for the discovery of new or improved enzymes and microorganisms for second-generation biofuel production would offer several simultaneous benefits. It could result in the increased protection and preservation of these ecosystems, leading to the maintenance of their ecological and biotechnological potential. Thus, screening for cellulolytic enzymes among the endophytic community may lead to the potential breakthrough in cellulose conversion in the form of highly specific enzymes (Diogo et al. 2013).
1.11 Advantages of Extracellular Cellulases

Cellulases are often produced by microorganisms as a part of the cellulosome complex and location of such complexes are mostly sub-cellular. However, the sub-cellular location of such complexes is still not clear or has not been studied in most of the fungi. Fungal cellulases are usually preferred by the industry, because they are extracellular, adaptive in nature and usually secreted in large quantities during growth. Endophytic fungi due to its balanced antagonism approach are known to secrete a number of cellulases either as a complex or individually in order to establish a symbiotic relationship with the host. Based on the genomic data of various endophytic fungi it has been speculated that large number of proteins related to saprophytic life style are present. Many of the proteins are expected to be related to hydrolytic enzyme machinery like cellulases. However, very little work has been carried out on the efficiency of saccharification of either crude or purified secreted cellulases of endophytic fungi on lignocellulosic biomass (Alvarez et al. 2013).

1.12 Methods for Sensitive Quantification of Monosaccharides

Quantification of monosaccharides after pre-treatment and hydrolysis is the most important step in assessing the efficiency of the method being followed. In order to develop, refine, and validate complete processing process of bioethanol production; accurate analytical methods to determine the chemical composition of biomass samples before, during, and after conversion processing are essential. Such sensitive analytical methods can be used to determine feedstock compositions, mass balances and monosaccharide product yields from conversion processes. The methods include HPLC analysis of monosaccharides like glucose, xylose, arabinose etc... In addition
multivariate analysis and calibration models are also used for identifying appropriate biomass loading. Therefore, it is essential to establish a simple, sensitive and robust workflow for hydrolysis and quantification of monosaccharides for economical production of bioethanol (Alltech 2005).

1.13 Existing Gaps In Research and Hypothesis of the Investigation

Developing efficient, environmentally benign and affordable technologies to produce lignocellulosic biofuels will require major breakthroughs in biomass deconstruction. The most important aspect of lignocellulosic biofuel production is the need for novel, efficient, economical enzyme systems that are robust, efficient and is compatible with a range of biomass pre-treatment strategies. Therefore, discovery of such enzymes from microbial sources is the scientific challenge that many investigators are facing.

Naturally, microorganisms evolve and adapt in a way that communal capacity is developed in order to use available growth substrates. Thus, for the discovery of such microorganisms and enzymes capable of breaking down lignocellulosic biomass; corresponding habitats rich in such materials would seem to be the most productive sites (Singh et al. 2014). Examples include wood decaying regions of biodiversity rich regions like Western Ghats. The variations in temperatures, water availability and soil alkalinity of these environments and the balanced anatagnism of endophytic fungi in order to establish a mutually sustainable saprophytic lifestyle may lead to discovery of novel, lignocellulolytic/cellulosic enzyme systems capable of efficient, prolonged catalysis under biorefinery-approximating conditions (Singh et al. 2014).
In addition, if these enzymes are secreted then the cost of downstream processing will be drastically reduced and the use of such enzyme systems for hydrolysis and the corresponding monosaccharides produced could be easily quantified using sensitive analytical methods. Another important aspect of bioethanol production which involves the overall economy of production is the pre-treatment. As, discussed above number of pre-treatment methods are available. However, the best and the most compatible method would be to combine a physo-chemical method followed by enzymatic hydrolysis. The method of choice preceding enzymatic treatment should ideally be designed in such a way that it will not in any way affect the enzymatic activity. Therefore, it would be safe to state that only minimally treated biomass will lead to efficient hydrolysis of biomass (Singh et al. 2014).

The current gap in scientific literature shows that a systematic screening of endophytes from perinneal woody plants of Western Ghats has not been undertaken with specific reference to utilization of simple and complex lignocellulosic substrates. Further, utilization of such carbon sources as a sole substrate after a nutrient drain process is yet to be ascertained. Once, such an efficient endophyte has been identified, its ability to secrete a repertoire of cellulases and its quantification is yet to be conducted. However, such a systematic screening followed by pre-treatment of minimally treated biomass, hydrolysis with secreted cellulases in the crude fraction and analytical quantification of monosaccharides released by HPLC has not been carried out in a single study. Such a study will enable establishing an economical, novel and efficient workflow for biomass hydrolysis which can be easily scaled up in an integrated bio-refinery (Singh et al. 2014).
In order to conduct such a study we hypothesize that “endophytic fungi are capable of secreting a number of extracellular cellulases under tightly regulated conditions which can hydrolyse a variety of lignocellulosic substrates.” Further, we also hypothesize that these enzymes in their crude form is able to hydrolyze minimally treated biomass such as those present in energy crops like Miscanthus sinensis and the efficiency of their hydrolysis is not reduced by use of various pre-treatment methods, thus releasing sufficient quantities of monosaccharides which could then be used for bioethanol production.

Therefore, based on the hypothesis proposed above and the existing scientific gap discussed in the previous section, the following objectives have been planned for this investigation:
**Figure 05:** Graphical representation of the work flow for production of endophytic cellulases planned for the present study

**Objective 1:** Isolation, diversity analysis of endophytic fungi and their ability to use complex cellulosic substrates.

**Objective 2:** Cellulase activities in the secretome of endophytic fungi and its quantification.

**Objective 3:** Studies on saccharification of *Miscanthus sinensis* biomass by secretome of endophytic fungi.