Chapter 1

Introduction and literature review

1.1. Non-renewable and renewable energy sources: Current status in India:

Recent concerns about future availability of conventional or non-renewable fuels, climate changes and increased energy demand have raised worldwide interest in exploring unconventional or alternative renewable sources of energy, such as wind, solar, hydropower, geothermal, biomass and nuclear. In a report on energy status published by Government of India (GOI), the amount of total coal (in billion tonnes), crude oil (in million tonnes) and natural gas (in billion m^3) is 308.8, 621.1 and 1227.23 respectively in March 2016, which was 306.6, 635.60 and 1521.90 in 2015 respectively. The figures suggest that, though there is minor increase in coal amount, the amount of other fossil fuels like crude oil and natural gas is decreasing remarkably and will be vanished within a century (*Energy Statistics 2017*, 2017). The report also counts the resourcefulness of unconventional renewable sources of energy, i.e., solar (62%) and wind (34%) while the remaining 4% are served by small hydro powers (1.76%), biomass power (1.56%), cogeneration of bagasse (0.44%) and from waste (0.24%). Thus, only 2.24% portion is the bioenergy which is achieved through use of biomass. Source wise and state wise estimated potential of renewable power ranked Gujarat on second position with 13.11% of potential energy sources, Rajasthan being the first with 13.95%. The details suggested that, though Gujarat produces highest renewable energy from wind power, the biomass to bioenergy conversion is moderate. There is only 1% increase in energy production from biomass in 2016 than 2015 (*Energy Statistics 2017*, 2017).

1.2. Biofuel:

The term "Biofuel" refers to the biomass and the refined products to be combusted for production of energy i.e., heat and light (Naik et al., 2010; Guo et al., 2015). Being in the largest amount on earth, the plant biomass and its refined products can serve as good source of biofuels and can be used to fulfil the major energy demand. Biofuel is a renewable source of energy and produced directly and indirectly from biomass derived from plant and animal wastes as opposed to fossil fuel such as petroleum, coal and natural gas. The non-renewable energy sources are grouped as solid (coal), liquid (crude oil) and gaseous (natural gas) fuels. Guo et al., (2015) have categorized plant biomass and their products in different categories of biofuels i.e., solid, liquid and gaseous biofuels as explained further in details.

1.2.1. Solid Biofuels:

This category includes firewood, wood chips, wood pellets and charcoal. These four mostly comprise of terrestrial plant biomass as a major energy source. Hard woods, i.e, dead branches and cut trunks of trees are commonly used as fire wood as a traditional source of energy since ages. Wood chips are the raw and small pieces made from the trunk and branches of hard woods using wood chippers. Whereas, the wood pellets are more processed than the wood chips. The saw dust of woody mass and small ground fragments of grasses, agrowastes leaf-litters etc., are compressed in the pelletizer under high pressure and temperature which shapes the pellet due to inherent properties of lignin components which glues it. Charcoal is the processed firewood turned in greyish black, porous solid with enriched carbon. It has more energy yielding ability than firewood.

In India, in majority rural areas the household requirements of energy is gained in form of bioenergy from the cogeneration of agricultural residues. India is the second largest producer of sugarcane in the world after Brazil. Sugarcane bagasse is the residual fibrous by-products discarded from sugar processing units after squeezing it for sugar juice. Biotechnological potentials of SCB agro residues has been described in details by several investigators for their utilization in biorefinery, pulp-paper industries, food industries etc., (Pandey et al., 2000; Rabelo et al., 2011; Chandel et al., 2012; Mishra et al., 2014; Rahmani et al., 2014). Heat and steam both are the products of cogeneration of bagasse biomass, which are traditionally consumed in sugar processing units to run boiler and turbine for electricity production (Mishra et al., 2014). Thus in small scale industries also cogeneration of biomass serves the energy production. But, in an ordinary or conventional furnace during the household burnings and cogeneration processes, the plant biomass is rarely combusted completely. This results in release of immense volatile organic compounds and carbon black particulates mixed with water vapor creating a smoke which is hazardous to human health and the environment. Besides this, the other products like CO, CH_3, and oxides of nitrogen and sulphur are generated which contributing to greenhouse effects. All these factors cumulatively have given rise to air pollution, health issues and several other environmental problems like global warming (Shepherd, 2017). Thus, the cogeneration of biomass is not an eco-friendly. To overcome this issue, Government of India (GOI) has recently invited proposals motivating research for process of development of agricultural waste management without burning them. In last one decade, GOI has developed a policy

called “National Bioenergy Mission” under which the efforts are made to complete major of the regular energy demands by means of bioenergy and other renewable energy sources instead of conventional ones. (*India Energy Outlook - World Energy Outlook Special Report 2015*, 2015).

1.2.2. Liquid Biofuels:

Liquid biofuels are the decent alternative solution to the problems created by solid biofuel cogeneration as well as the depletion in the conventional energy sources. Unlike the solid biofuels that involve majority of the terrestrial biomass, the liquid biofuels can be generated using aquatic biomass like algae. (Laurens and Wolfrum, 2011). Liquid biofuels category includes bioethanol, biodiesel, pyrolysis bio-oil, and drop-in biofuels. The bioethanol is the fermentation product of hexose and pentose sugars, the structural monomer units of plant cell wall polysaccharides. The process of bioethanol production involves two steps of saccharification and fermentation (Banerjee et al., 2010a; Maurya et al., 2015). Saccharification, the process of conversion of plant biomass structural polysaccharides to its constitutive monomer units, is the keystone step in the biofuel production as it controls the availability of the fermentable sugar for the subsequent fermentation step. Usually feed stocks used for biofuels varies in range from food-based products to recalcitrant plant-based biomass which decides the generation of biofuel-ethanol. Matured technologies producing the first-generation bioethanol were dependent on the food crops like sugarcane juice, corn starch which can easily yield fermentable sugars creating a food and animal feed clashes. This competition gave rise to need of sustainable feed stocks other than human food and animal feed, i.e., agrowaste biomass with recalcitrant properties (Zaldivar et al., 2001) (EERE, 2006; Sharif Hossain et al., 2008; Weng et al., 2008; Banerjee et al., 2010b; Knothe, 2010; Yue et al., 2014; Williams et al., 2016; Zabed et al., 2016; G. Kumar et al., 2017). The major bottleneck in the process of second generation bioethanol production lies in the saccharification of cellulosic components to the fermentable sugars step as explained in details ahead. In last two decades, numerous investigators have expressed their interests in studies on pretreatment processes of biomass to ease and improve its enzyme cocktail mediated saccharification process. The bioethanol can be blend with crude oil derived petrol or gasoline up to 20% which eases the combustion process and reduces the production of different oxides thereby benefiting the environment.

Third generation liquid biofuel is a biodiesel, which is derived from different biomass products like animal fats, vegetable oils or oil seed plants like coconut, canola, jatropha, palm, soybean, sunflower etc., and oils and lipids of algal cells like, *Dunaliella salina*, *Dunaliella teriolecta*, *Phaeodactylum tricornutum*, *Scenedesmus obliquus*, *Chlamydomonas reinhardtii* etc., This yellowish colored liquid is derived through the trans-esterification process of these lipids and can be used as a substitute for petrol and diesel. The research on biodiesel production also has bloomed in last two decades. Where, low cost-cultivation and harvesting of biomass along with oil extraction process are studied for the further improvement. Another type of liquid biofuel is a Pyrolysis bio-oil. As consequences of pyrolysis, i.e., heating of plant biomass 300–900 °C in absence of air, biochar (solid black residuals), bio-oil (the brown-vapor condensate) and syngas (the incondensable vapor) are generated. This bio-oil can be utilized as heating fuel and it is an important industrial feedstock material.

The natural structural polysaccharides are abundant in amount and can be found from potentially cheap agricultural waste (wheat straw, corn stalks, soybean residues, sugar cane bagasse, barley straw, sorghum straw and many others), forestry residues, municipal solid waste, wood chips, etc., (Wiselogel et al., 1996) and hence can be used for production of bioethanol from its cellulose. While fatty acids and lipids can be found as storage materials from algal biomass and can be used for production of biodiesel. It has been stated that biofuels hold the potential to deliver at least one-quarter of the world's energy needs if more and more agricultural, forestry and industrial biomass is used in a better way (Kopetz, 2013).

Countries like, USA, China, Canada, Japan, Brazil, etc., have established plants for production of second generation bioethanol and third generation biodiesel and have implemented their use in transportation fuels. India is now taking a huge leap as the major crude oil refinery companies like Bharat Petroleum Corporation Limited (BPCL), Indian Oil Corporation Limited (IOCL), Hindustan Petroleum Corporation Limited-Biofuel Limited (HBL), Reliance Industries Limited now have established their research and development (R&D) centres for improvement of biofuel production process. Sardar Patel Renewable Energy Research Institute (SPRERI), Vallabh Vidyanagar, Gujarat is a recognized facility by S. P. University, Nirma University and Junagadh Agricultural University for research in this field.

1.2.3. Gaseous biofuels:

This category includes the biogas and syngas. The biogas is a good alternative fuel to natural gas. It is synthesized by anaerobic microorganisms during the digestion of organic wastes under anaerobic conditions. Raw biogas consists of 60–65% of methane (CH_4), 30–35% of CO_2, and small percentages of water vapor, H_2, and H_2S. Once the CO_2 and H_2S are removed through purification, the biogas now can be said as biomethane and is a great substitute of natural gas. The methanogenic bacteria play a key role in production of CH_4 gas. Whereas, the syngas is a gaseous product obtained during biomass pyrolysis. Chemically syngas consists of 30–60% CO, 25–30% H_2, 5–15% CO_2, 0–5% CH_4, and smaller proportion of water vapor, H_2S, NH_3, and others, depending on the feedstock types and production conditions, the product is either directly used for burning or gas is purified and used as a source material for synthesizing transportation fuel, methanol, ethanol, methane, dimethyl ether, and other products.

Thus, currently, most of the transportation vehicles are dependent on the gasoline and/or diesel-based system. Hence, in this context, the liquid biofuels specifically bioethanol and biodiesel have gained major interests. Any crude plant biomass which is rich in polysaccharide components can be converted to simple sugars and than fermented to alcohol.

1.3. Plant cell wall and its' lignocellulosic components:

Plant biomass contains cellulose, hemicellulose, lignin, pectin and some amount of lipids and proteins. Out of which cellulose is a major component followed by hemicellulose and polyphenolic lignin. Their interactions generate a complex matrix of lignocellulose leading to the term "**Lignocellulosic Biomass"** (McKendry, 2002). Figure 1.1(A) provides the brief idea about the general arrangement of the plant cells. As can be seen from the image, middle lamella is the cementing layer of plant cell wall which joins two adjacent plant cells. It is made up of calcium pectate, a calcium salt of pectic polysaccharides. On luminal side of the middle lamella the plant cells synthesize their cell wall. Initially, only the primary cell wall is synthesized on the luminal side of the middle lamella. As shown in Figure 1.1(B) in some plants like dicotyledons, during secondary growth, the secondary layer of plant cell wall is being added towards the luminal side, inner to primary cell wall. The secondary cell wall is usually several fold thick than the primary cell wall. Thus, the lumen size decreases with growth of secondary cell wall.

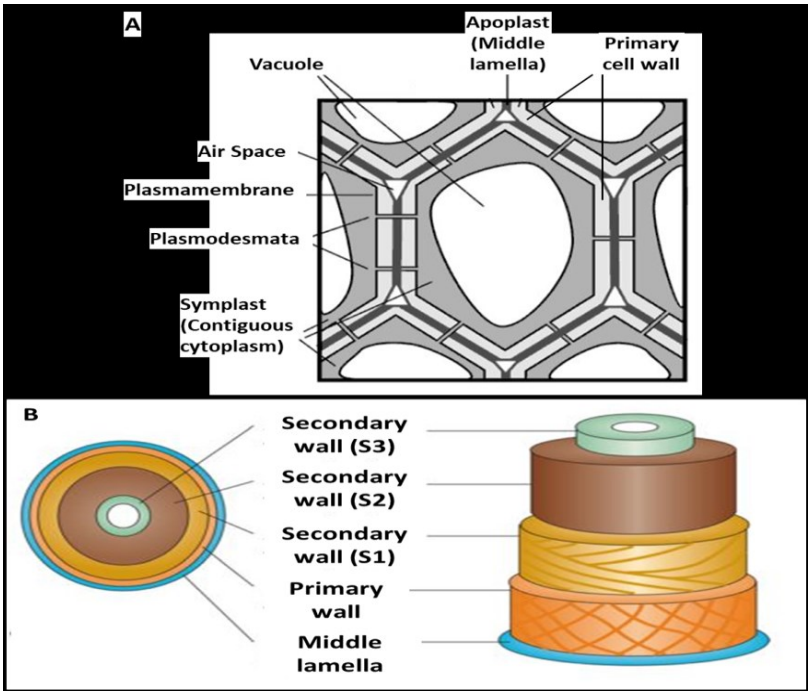

Figure 1.1. Schematics of arrangement of plant cells and cell wall layers:
(A) Schematic showing arrangement of plant cells (Image Source: Complex carbohydrate research centre, University of Georgia, (https://www.ccrc.uga.edu/~mao/intro/ouline.htm); **(B)** Schematic showing deposition layers of secondary cell wall in plant ell (adapted from, Mathews et al., 2015)

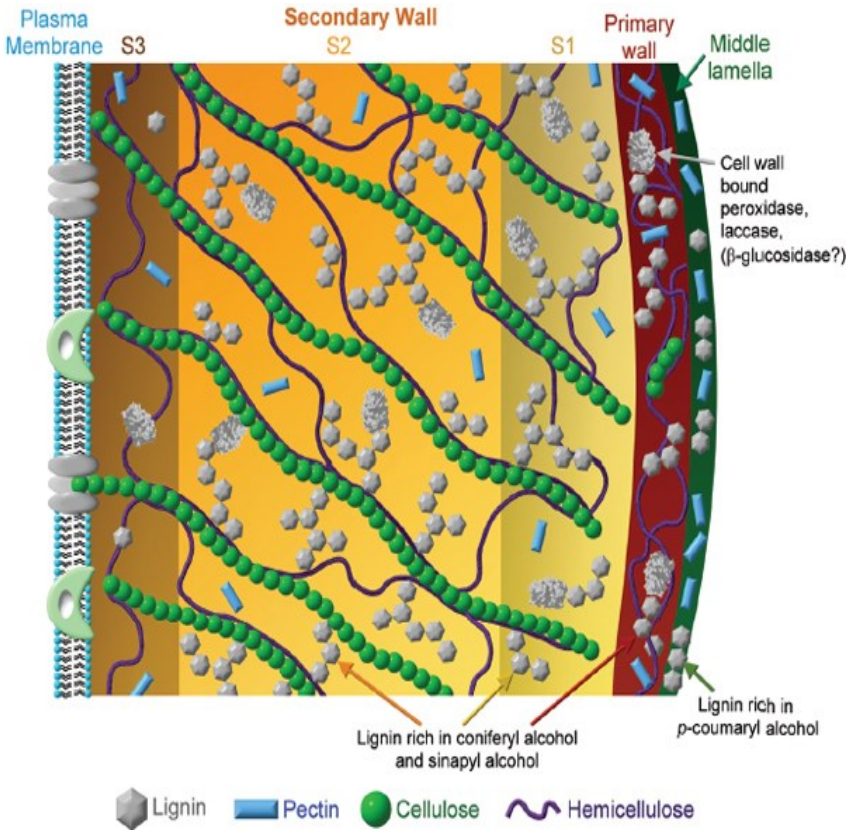

Figure 1.2. Schematic of distribution of plant cell wall components in cell wall:
Distribution of cellulose, hemicellulose, lignin and pectin in primary as well as secondary cell wall structure (adapted from, Achyuthan et al., 2010).

The primary cell wall is thin while the secondary cell wall is several fold thick. The cellulose, hemicellulose, lignin and pectin are distributed throughout the plant cell wall. The distribution becomes intense in the secondary layer. But as the growth increases, the secretion of secondary cell wall becomes more complex. The secondary layer contains more complex interactions between cellulose, hemicellulose, pectin and lignin components.

Following is the Table 1.1, depicting the amount of lignocellulosic components estimated and reported from different sources. It can be seen that the amount of these individual components from lignocellulosic matrix varies from plant to plant. Based on the genus, species, and growth stage, each of the plant ideally possesses a particular amount of these structural polysaccharide components. Yet drought, low or high environmental temperature, ultraviolet irradiation, mineral deficiency, mechanical wounding etc. are the parameters which affects the growth at different developmental stages of plants and alters the ratio of the cellulosic, hemicellulosic and lignin components (Achyuthan et al., 2010; Sawada et al., 2018).

Table 1.1. The contents of cellulose, hemicellulose, and lignin in common agricultural residues and wastes:

Lignocellulosic material	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwoods stems	40-55	24-40	18-25
Softwood stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30
Paper	85-99	0	0-15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seed hairs	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Waste papers from chemical pulp	60-70	10-20	5-10
Primary waste water solid	8-15	NA	24-29
Swine waste	6.0	28	NA
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0

(adapted from, Sun and Cheng, 2002)

1.4. Structural components of lignocellulose biomass:

Cellulose, hemicellulose, and pectin are the polysaccharide components while lignin is the polyphenolic component that forms the plant cell wall. Very fine and minute details about their structures and interactions have been studied by several investigators from which some relevant information is presented as below.

1.4.1. Lignin:

Figure 1.3 represents the monolignols and most abundant aromatic polymer in nature i.e., lignin. Lignin is a macromolecule with phenolic character, being the dehydration product of three monomeric alcohols, *trans-p*-coumaryl alcohol, *trans-p*-coniferyl alcohol and *trans-p*-sinapyl alcohol commonly known as monolignols derived from *p*-cinnamic acid (Figure 1.3A) (Abdelaziz et al., 2016).

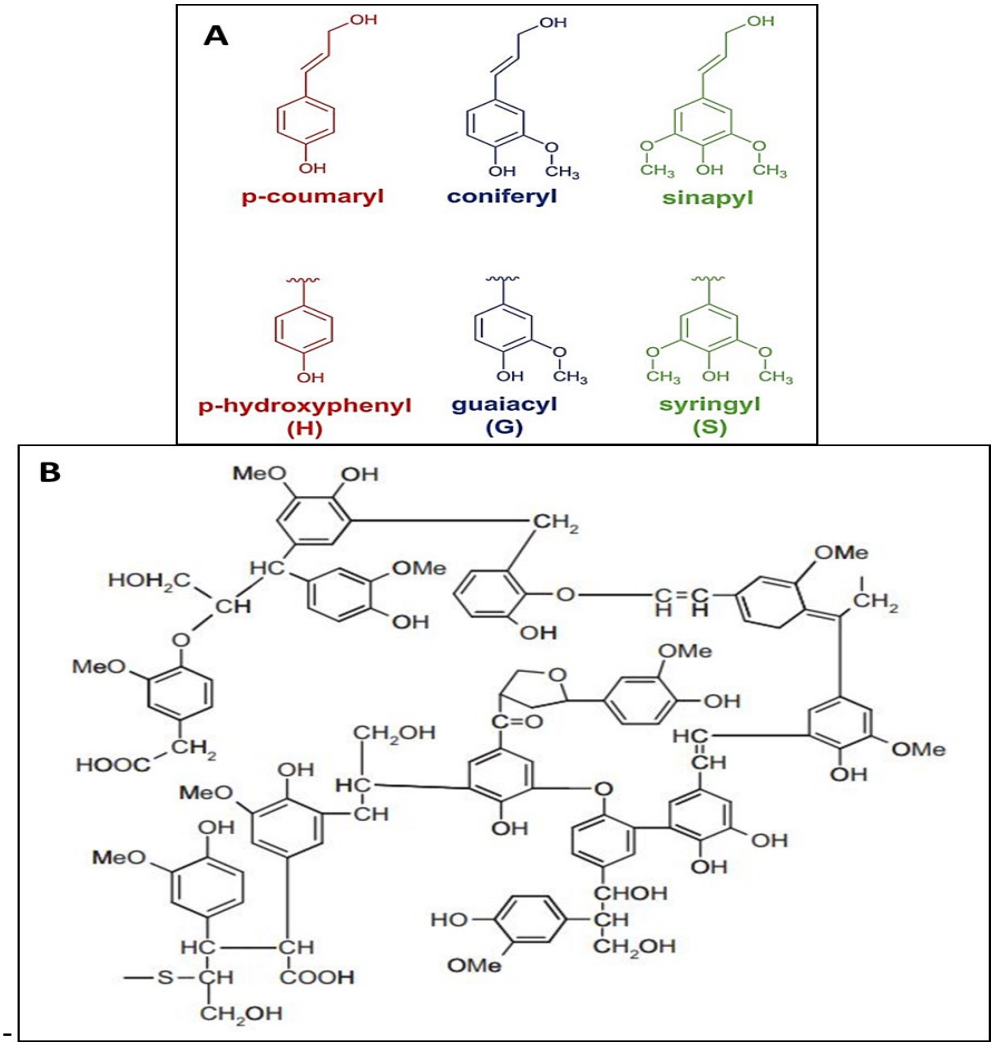

Figure 1.3. Schematic structure of monolignols and their polymer lignin:
(A) Structure of monolignols and their respective precursor forms: p-coumaryl alcohol as p-hydroxyphenyl (P); p-coniferyl alcohol as guaiacyl (G) and p-synapyl alcohol as syringyl (S) (Adapted from, Kai et al., 2016); **(B)** Structure of lignin polymer molecule. (Adapted from, Norgren and Edlund, 2014).

These monolignols are incorporated into lignin in the form of the phenylpropanoids *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), respectively (Figure 1.3 B). Gymnosperms have a lignin that consists almost entirely of G with small quantities of H. The monocotyledonous lignin is a mixture of all three types whereas, that of dicotyledonous angiosperms is more often than not a mixture of G and S (with very little H) (Boerjan et al., 2003; Maitan-Alfenas et al., 2015a).

1.4.2. Cellulose:

It is the most abundant polysaccharide compound in the world and is a linear, crystalline homopolymer made up of D-glucopyranose units ranging from 8,000 to 15,000 residues per chain that are linked by β-1,4 glycosidic bonds (Figure 1.4A). A dimer of two glucose units linked by β-1,4 bond is known as cellobiose (red box), which is a building block of cellulose (Zaldivar et al., 2001).

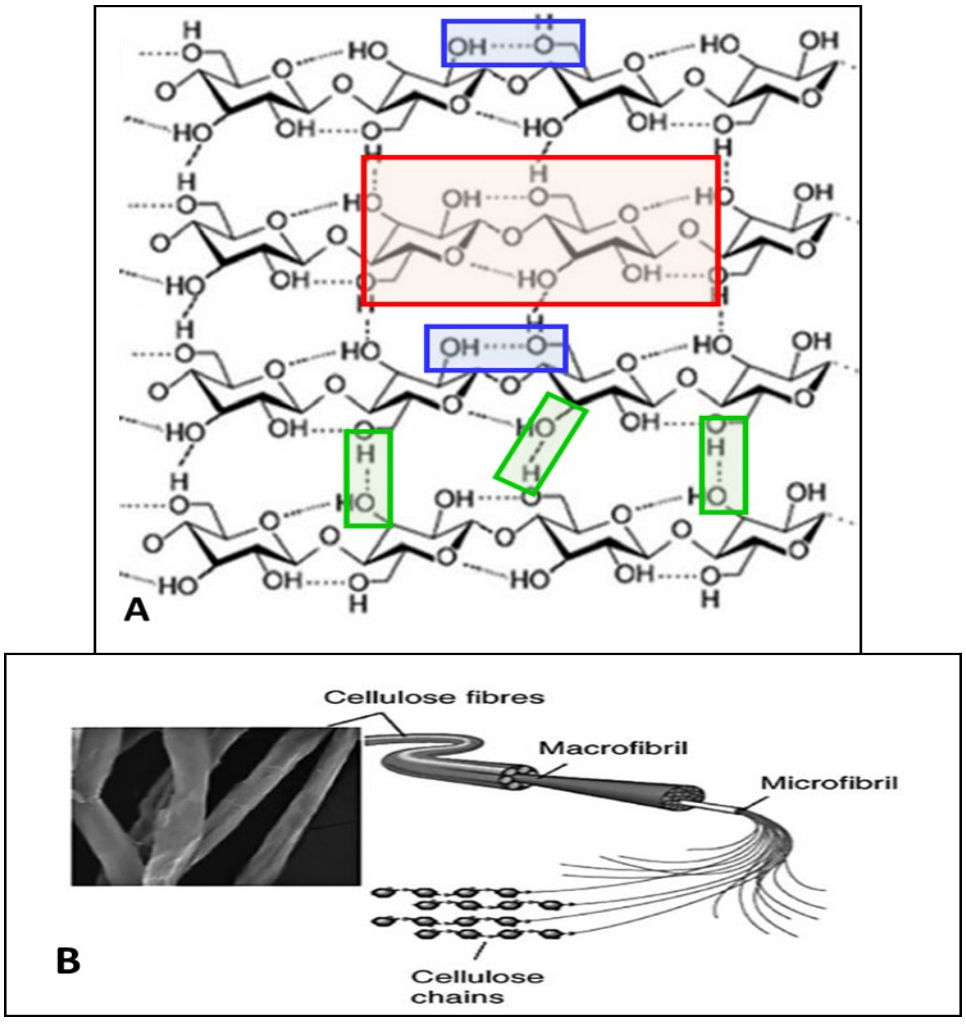

Figure 1.4. Molecular structure and microfibril arrangements in cellulose:
(A) Skeletal structure of cellulose molecule representing its constitutional units-cellobiose (red box), intrachain (blue box) and interchain (green box) hydrogen bonds (Adapted from, Zhang et al., 2015); **(B)** Microfibrill arrangements arrangement of cellulose (Yuan and Cheng, 2015).

Individual cellulose chains come close to each other and are arranged in such a manner that they form a crystalline array of molecules in micelle, having intrachain (blue box) and interchain (green box) type of hydrogen bonding. Figure 1.4B explains how these crystalline micelle forms the cellulose macrofibrils. The crystalline arrays of the cellulose molecules are organized in the micelles get associated with each other to form a cellulose micro-fibril. Several such microfibrils are arranged in a cluster to form a macrofibrils and interact with hemicellulose and lignin components (Karpe, 2015; Zhang et al., 2015).

1.4.3. Hemicelluloses:

They are branched heterogeneous polysaccharides of shorter lengths compared to cellulose and composed of pentose and hexose sugars such as glucose, xylose, mannose, galactose, rhamnose, arabinose, 4-*O*-methyl-glucuronic acid, galacturonic acid and glucuronic acid. Simplified structures of some of the hemicellulosic polymers are depicted in Figure 1.5. The backbone is made up of β-1,4 linkages. The two main hemicellulose types are xylans and mannans. Xylans are usually composed of partially acetylated poly β-1,4-D-linked xylose. Xyloglucan is a polymer having backbone of β-1,4-D-linked D-glucose which have several substitutions of xylose, xylose-galactose, xylose-arabinose, xylose-galactose-fucose etc. on the 6^{th} carbon of glycosyl residues. Depending on the sugar present on branch point of second carbon of xylose, presence of L-arabinose or 4-*O*-methylglucuronic acid will give rise to arabinoxylan or glucuronoxylan. Mannans are usually composed of partially acetylated poly β-1,4-D-linked mannose. Glucomannan is a heteropolymer of β-1,4-D-linked D-glucose and D-mannose. Similarly, galactoglucomannan is heteropolymer of D-galactose, D-glucose and D-mannose while arabinogalactan is heteropolymer of D-galactose and D-arabinose. These hemicelluloses, with arabinose substitutions or sometimes with acetylated regions play an important role in their association with lignin through ester bond formation e.g., feruloyl acid ester. Such interactions add on to the complexity of cell wall (de Souza, 2013; Smith et al., 2017).

1.4.4. Pectin:

Polymer of α-1,4-D-galacturonic acid is commonly known as polygalacturonic acid. The carboxyl groups of galacturonic acid are methyl esterified to varying extent. As the amount of methyl-esterified galacturonic acid residues increases in backbone,

the Degree of Esterification (DE) also increases and such pectic compounds are called pectins which are more complex to digest. Thus, pectins are copolymers of galacturonic acid and the methyl esterified galacturonic acid. As presented in figure 1.6, Certain galacturonic acid residues possess rhamnose side chains substitutions which increases the complexities from homogalacturonan to rhamnogalacturonan-I and rhamnogalacturonan-II. (Jayani et al., 2005).

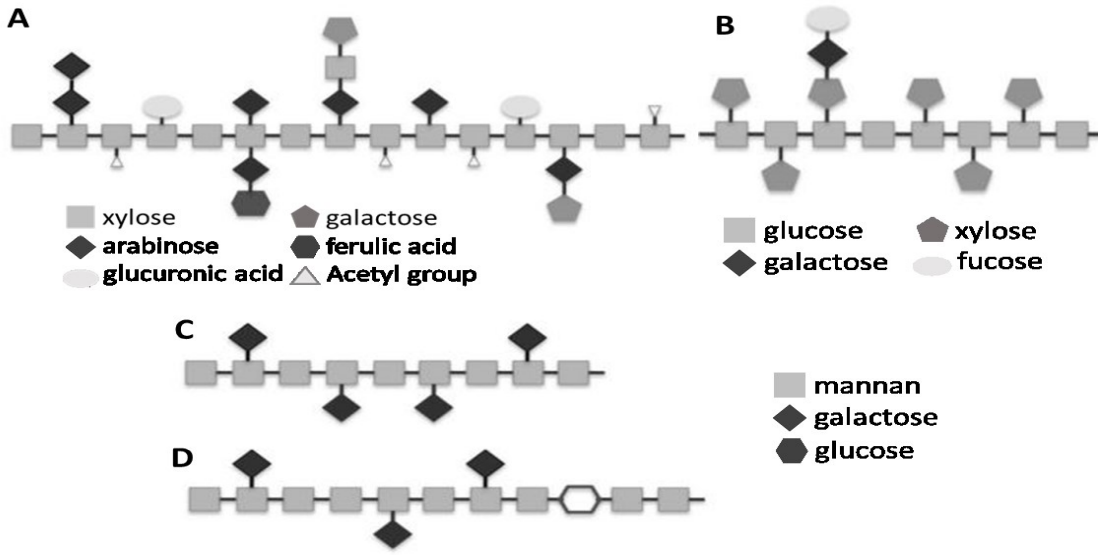

Figure 1.5. Schematic representation of different hemicelluloses:
(A) Xylan, **(B)** Xyloglucan, **(C)** Galactomannan and **(D)** Galacto-glucomannan. (adapted from de Souza, 2013)

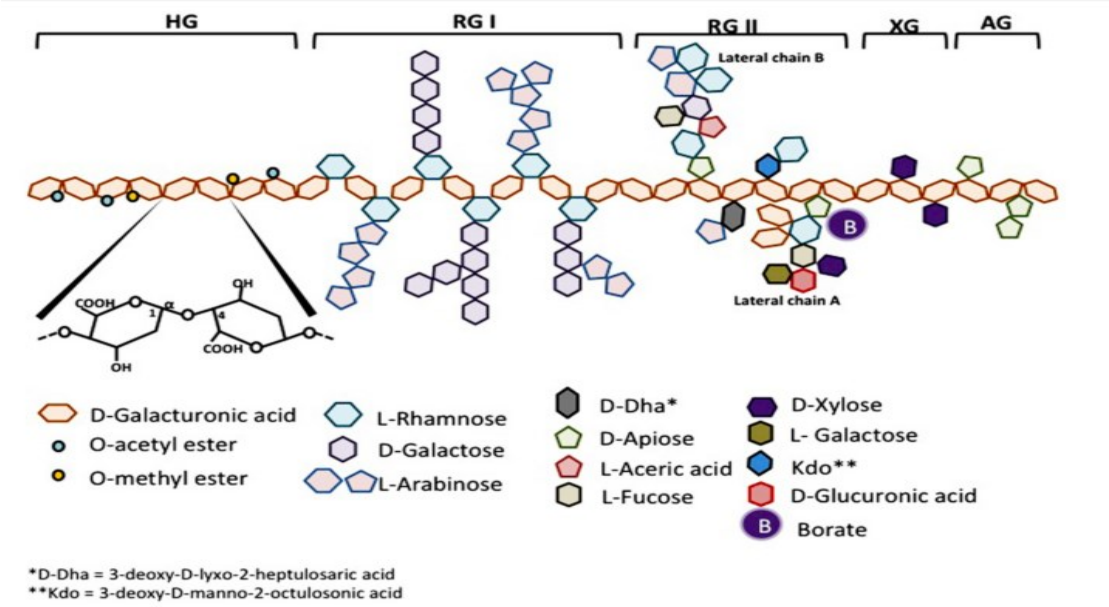

Figure 1.6. Schematic representation of pectin structure:
HG: Homogalacturonan, RG I: Rhamnogalacturonan I, RG: II Rhamnogalacturonan II, AG: Arabinogalacturonan, XG: Xyloglucan (Leclere et al., 2013).

The information about these polyphenolic and polysaccharide structural polymers points towards the complex nature of the cellulose-hemicellulose-lignin matrix. These acetylated ends most commonly form the ester bonds with the -OH group present in lignin and the ester linkages between hemicellulose and lignins.

1.5. Structural complexities of carbohydrates in plants:

Pectic middle lamella makes the plant cell wall elastic. The hemicelluloses with pectin, glycoproteins and lignin forms a matrix in which the cellulose micro-fibrils are embedded. Whenever the secondary wall layer is added up, it mainly consists of cellulose microfibrils arranged in layered structures with hemicellulose fibres and lignin. Wall matrix polymers (xyloglucan, pectin and glycoproteins) and lignin are covalently linked to one another. The binding of xyloglucan to cellulose microfibrils results in a non-covalently cross-linked cellulose-hemicellulose network. Fig 1.7 shows the cellulose fibrils embedded in a network of pectin, hemicellulose and lignin.

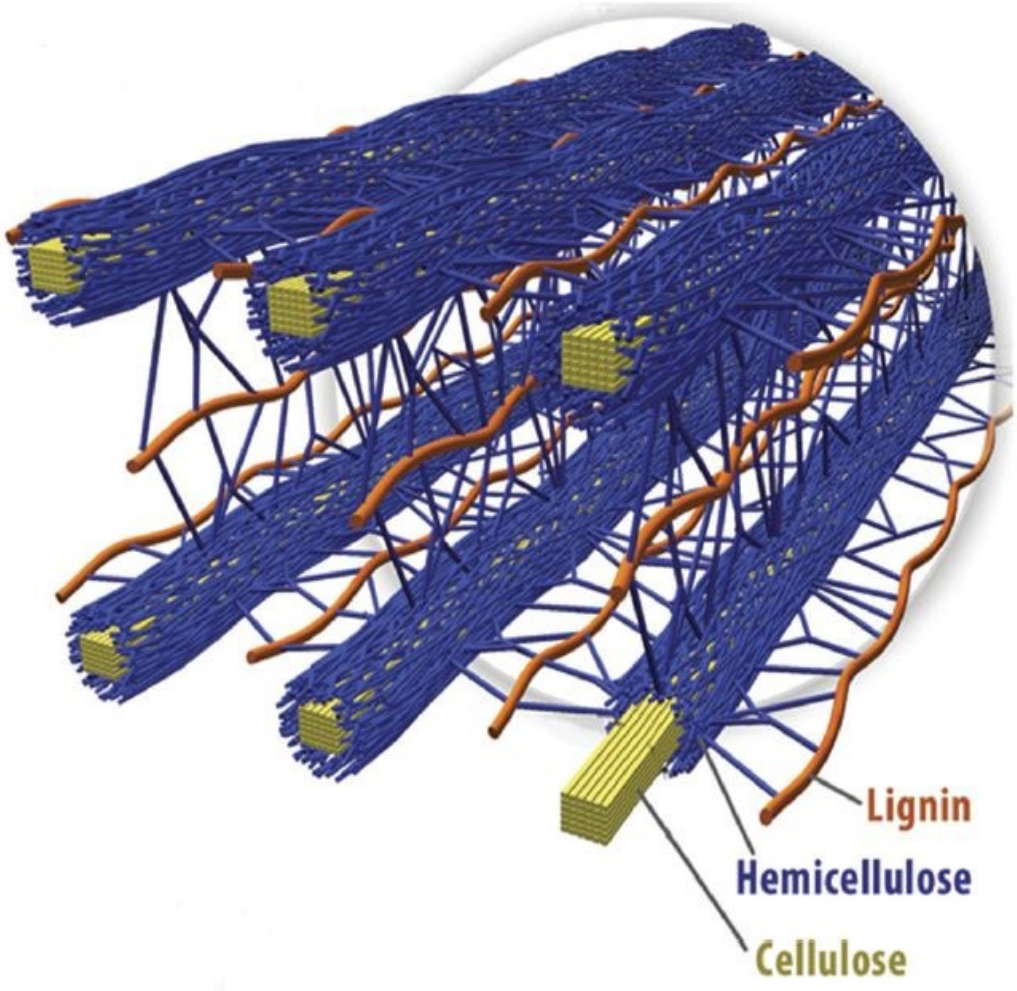

Figure 1.7. Spatial arrangement of cellulose, hemicellulose and lignin in the plant cell wall: Adapted from (Brandt et al., 2013).

Such complex cross-linking of this network is believed to result in the elimination of water from the wall and the formation of a hydrophobic composite that limits accessibility of hydrolytic enzymes and is a major contributor to the stiffness of walls (Smith, 2001; Achyuthan et al., 2010; Brandt et al., 2013) But hemicellulose, pectin and lignin like compounds in biomass exert significant restraints on cellulose hydrolysis, e.g., it binds enzyme components non-productively or it may restrict the access of cellulolytic enzymes by coating cellulose fibers. Cellulose crystallinity, porosity, etc. are also other important factors affecting enzymatic saccharification. So enzymatic hydrolysis finally depends on structural and spatial conformation of hemicellulose-cellulose-lignin and their composition. Lignin and hemicellulose removal, reduction of cellulose crystallinity and increase of porosity by pretreatment processes can significantly improve the enzymatic saccharification (Smith, 2001; Yoshida et al., 2008).

1.6. Sugarcane stem anatomy:

Being from *Poaceae* family, Sugarcane (*Saccharum officinarum)* contains the complex lignocellulosic cell wall matrix which comprises majorly cellulose and hemicellulose in structure along with all three polyphenolic lignins interacting with these polysaccharides. The amount of cellulose (glucan), hemicellulose (xylan and galactan) and lignin in sugarcane biomass has been reported as 41.4, 28.2, 1.3, and 23.6 respectively (Ferreira-Leitão et al., 2010). Different components of structure of sugarcane stem is visible in transverse section of its anatomy Figure 1.8. Several vascular bundles are present in the soft pith tissue region of stem. Bundle sheath comprises of two to several layers of sclerenchyma tissue cells which surrounds each vascular bundle. The vascular bundle mainly composed of xylem and phloem. The xylem vessels consist of two different type of cavity cells. The vascular bundles in sugarcane bagasse are collateral type where xylem vessels are arranged in the form of letter ‘Y’ or ‘V’ or ‘U’ as visible in Figure 1.8. Arms of the alphabets are formed by large metaxylem vessel elements while the rest is composed by protoxylem. The part of protoxylem disintegrates in later stages and the lysogenous or protoxylem cavity or lacuna is formed. As the phloem elements are situated in the grooves of Y or V or U-shaped xylem, the xylem encircles phloem on the three sides. Jacobsen et al., (1992) had explained these developmental changes in anatomy of sugarcane. Among, above mentioned tissues, protoxylem, metaxylem and sclerenchyma cells are the dead ones

which contains lignin depositions on the lumen side of the cell wall. The phloem tissues lack the lignin and majorly composed of cellulosic and hemicellulosic cell wall. The parenchymatous tissues in pith region contains major cellulosic and hemicellulosic fractions in the cell wall with minor amount of polyphenolic lignin and pectin content.

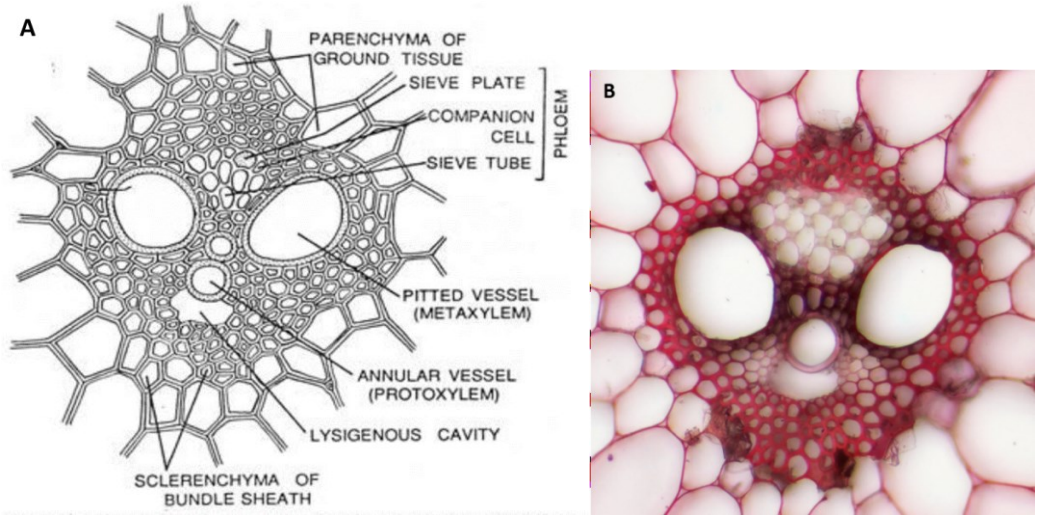

Figure 1.8. Anatomy of vascular bundle in sugarcane stem section:
(A) Schematic diagam showing image of a vascular bundle from sugarcan stem (http://www.biologydiscussion.com/stems-2/monocotyledonous-stems-regions-features-and-types-botany/20507) **(B)** Transverse section of sugarcane stem prepared in this work representing a single vascular bundle observed after safranin staining at 10X magnification under brightfield microscope.

1.7. Biomass saccharification by different enzymes:

Literature survey on biomass saccharification revealed that investigators have used chemical as well as enzymatic methods which can breakdown these structural polysaccharides to their constitutional monomers. Involvement of most chemical methods leads to synthesis of some toxic or inhibitory molecules which hinders the subsequent fermentation process. Hence, such methods require downstream processing increasing the cost of production. On the contrary the downstream processing is not required in case of saccharification and the hydrolysate can directly be used for fermentation. Several investigators have studied concurrent enzymatic saccharification and ethanol production by fermentation (Ko et al., 2009; Rattanachomsri et al., 2009; Oberoi et al., 2011; Sakamoto et al., 2012; Sandhu et al., 2012; Huang et al., 2015). Three major type of enzymes are required for lignocellulose saccharification are cellulase, xylanase and pectinase.

1.7.1. Cellulase:

Table 1.2 enlist the various cellulase enzymes along with EC classification, their substrate, action and end-products. The incompletely hydrolyzed end-products suggest

that the hydrolysis of cellulose to glucose is never complete with a single cellulase enzyme. Core cellulase group contains the following enzymes, Endo-glucanases which randomly cleaves beta,1-4, linkages in backbone to release triose, tetrose or larger oligosaccharides. Further cellobiohydrolases cleave these oligosaccharides to liberate cellobiose units. Cellobiase strictly hydrolyze cellobiose to beta-D-Glucose while exoglucanase cleaves terminal beta-1,4- bond to liberate D-Glucose gradually. Thus, all these enzymes are mandatory when core cellulase group is applied to achieve the saccharification.

Table 1.2. Cellulases, the reactions they catalyze and released end-products:

Type of Cellulases	**E.C. No.**	**Reaction catalyzed**	End-products
Endo-1,4-β-D-glucanase (Endoglucanase, CMCase, Avicelase)	3.2.1.4	Endohydrolysis of (1→4)-β-D-glucosidic linkages in cellulose	Oligosaccharides
Cellulose 1,4- β -D-Cellobiosidase (Cellobiohydrolase, reducing end)	3.2.1.176	Hydrolysis of (1→4)- β -D-glucosidic linkages in cellulose, releasing cellobiose from the reducing ends of the chains.	Cellobiose from reducing end
Cellulose 1,4- β -cellobiosidase (Cellobiohydrolase, non-reducing end)	3.2.1.91	Hydrolysis of (1→4)- β -D-glucosidic linkages in cellulose and cellotetraose, releasing cellobiose from the non-reducing ends of the chains	Cellobiose from non-reducing ends
Exo-1,4- β -D-glucosidase.	3.2.1.74	Hydrolysis of (1→4)-linkages in (1→4)- β -D-glucans, to remove successive glucose units; Acts on 1,4- β -D-glucans and related oligosaccharides; Cellobiose is hydrolyzed, but very slowly.	β -D- Glucose
β -glucosidase (Cellobiase)	3.2.1.21	Hydrolysis of terminal, non-reducing β -D-glucosyl residues with release of β -D-glucose.	β -D-glucose

1.7.2. Xylanase:

Table 1.3 enlists the several xylanases which act on complex xylan substrates. The breakdown of acetylated or lignin bound xylan requires esterases which free these xylan from ester bonds of acetyl groups or lignols. Such Xylans are now available for action of endoxylanases which release xylobiose or xylooligomers. Further β -xylosidase converts theses xylobiose to the β -D xylose residues subsequently. Thus, complete conversion of simple or complex xylan to xylose also requires action of more than one enzyme just like cellulose hydrolysis.

Table 1.3. Xylanases, the reactions they catalyze and released end-products:

Type of Xylanases	E.C. no.	Reaction catalyzed	End-products
Esterases (Mode of action: Hydrolysis)			
Acetyl xylan esterase	3.1.1.72	Deacetylation of xylan and xylo-oligosaccharides	Ethanol, Xylan
Ferulic acid esterase	3.1.1.73	Feruloyl-polysaccharide to ferulate and polysaccharide	Ferulic acid and Xylan
Glycosylases (Mode of action: Hydrolysis)			
Endo-1,4-β-xylanase	3.2.1.8	Random hydrolysis of (1,4)-β-D-xylosidic linkages in xylan	Oligosaccharides
Xylan 1,4- β -xylosidase. β-xylosidase. Exo-1,4- β-xylosidase. Xylobiase.	3.2.1.37	Hydrolysis of (1, 4)- β -D-xylans, to remove successive D-xylose residues from the non-reducing termini. Also hydrolyzes xylobiose.	D-Xylose
α-L-arabinofuranosidase	3.2.1.55	Hydrolysis of terminal non-reducing α-L- arabinofuranoside residues in α-L-arabinosides	L-arabinose and Xylan polymer
Endo-1,3-β-xylanase	3.2.1.32	Random hydrolysis of (1-3)-β-D-glycosidic linkages in (1,3)- β-D-xylans	Oligosaccharides
Xylan 1,3-β-xylosidase	3.2.1.72	Hydrolysis of successive xylose residues from the non-reducing termini of (1, 3)-β-D-xylan	D-Xylose
Xylan α-1,2-glucuronosidase	3.2.1.131	Hydrolysis of (1, 2)-α-D- (4-O-methyl) glucuronosyl links in the main chain of hardwood xylan	Xylan polymer and substituted D-glucuronic acids
Glucuronoarabinoxylan endo-1,4-β-xylanase	3.2.1.136	Endohydrolysis of (1, 4)-β-D-xylosyl links in some glucuronoarabinoxylans	Oligosaccharides
Oligosaccharide reducing-end xylanase	3.2.1.156	Hydrolysis of (1, 4)-β-D-xylose residues from the reducing end of oligosaccharides	D-Xylose

1.7.3. Pectinase:

Table 1.4 depicts the list of the pectinases which can convert the simple or highly esterified pectin to the monogalacturonic acid residues. Esterases do play an important role here. Pectin Methyl esterases or Pectin acetyl esterase hydrolyses the ester bonds and reduces the DE, liberating pectic acids. These pectic acids can directly hydrolysed by endo- or exo- polyygacturonases. Similarly, pectin with high DE can be hydrolysed directly with endo- or exo-polymethylgalacturonases. Apart from the hydrolases, the pectins can also be broken down by trans-elimination process carried out by lyase enzymes. Lyases perform non-hydrolytic breakdown of pectates or pectinates characterized by a trans-eliminative split of the pectic polymer. The lyases break the glycosidic linkages at C-4 and simultaneously eliminate H from C-5, producing a D-4:5 unsaturated product which might be unsaturated oligo-or di-saccharides. Thus, hydrolases or lyases together, can perform better saccharification of pectin and the

mechanism of these reactions are well explained by Jayani et al., (2005).

Table 1.4. Pectinases, the reaction they catalyze and released end-products:

Type of Pectinases	E.C. No.	Reaction catalyzed	Product
Esterases (Mode of action: Hydrolysis)			
Pectin acetyl esterases	3.1.1.6	Hydrolysis of ester bond of acetyl pectin	Pectic acid + Ethanol
Pectin methyl esterases	3.1.1.11	Hydrolysis of ester bond of methyl pectin	Pectic acid + Methanol
Glycosylases (Mode of action: Hydrolysis)			
Endopolygalacturonase	3.2.1.15	Random hydrolysis of (1->4)- α -D-galactosiduronic linkages in pectate and other galacturonans	Oligogalaturonates
Exopolygalacturonase	3.2.1.67	Terminally hydrolyze (1->4)- α -D-galacturonide	Monogalacturonates
Lyases (Mode of action trans-elimination)			
Endo Pectate Lyase	4.2.2.2	Eliminative cleavage of (1->4)- α -D-galacturonan	Unsaturated oligogalacturonates with4-deoxy- α -D-galact-4-enuronosyl groups at their non-reducing ends
Exo Pectate Lyase	4.2.2.9	Eliminative cleavage of 4-(4-deoxy- α -D-galact-4-enuronosyl)-D-galacturonate from the reducing end of pectate, i.e. de-esterified pectin	Unsaturated digalacturonates
Endo Pectin Lyase	4.2.2.10	Eliminative cleavage of (1->4)-α-D-galacturonan methyl ester	Unsaturated oligogalacturonates with 4-deoxy-6-O-methyl- α -D-galact-4-enuronosyl groups at their non-reducing ends

Thus, a survey of these enzymes suggests that the there is a potential of maximum possible saccharification of plant biomass when cocktails of such different enzymes are applied leading to improvement of biomass conversion to fermentable sugars for production of biofuels.

1.8. Microbial sources of hydrolases:

Most of the enzymes used for the saccharification belong to hydrolase group of enzymes. Many of such hydrolases have been found in bacteria and fungi isolated from variety of habitats. Forest and farm soil, where large amount of leaf litter and wood rotting usually occurs, can be considered as major sources for obtaining potent carbohydrate hydrolase producing bacterial and fungal isolates. Few invertebrate arthropods like phytophagous beetles and termites possess large number of gut symbionts, which secretes such hydrolases to support the plant digestion. Since plants

are food for herbivores majority of ruminant vertebrates, their rumen liquor and faecal material contain microorganisms, which can produce the target enzymes. So, microorganisms present in different plant material digesting or decaying niches, do possess a long list of corresponding cellulolytic, hemicellulolytic, amylolytic and lignolytic enzymes. Besides cloning and purifying these enzymes for application studies from the culturable organissms, the functional metagenomic methods are also used to procure and identify the enzymes from unculturable microorganisms which can play an important role in their niche for the plant polysaccharide degradations. Orgnisms and metagenome sources from which these enzymes have been obtained are reviewed and reported widely (Wojciechowicz et al., 1982; Duskova and Marounek, 2001; Dehority, 2002; Sá-Pereira et al., 2003; Ferrer et al., 2005; Zhu et al., 2011; Scully et al., 2013).

1.8.1. *Bacillus* spp. as source of polysaccharide degrading enzymes:

A number of reports about cellulase, xylanase and pectinase enzymes have been published till now which deals with the information about physicochemical characteristics like optimum temperature and pH, substrate specificities, enzyme kinetics, effect of modulators, microbial sources of the enzymes and potential applications of enzymes, etc. Study of literature regarding xylanase, pectinase and cellulase enzymes suggested that a large number of *Bacillus* isolates are capable of producing these enzymes (Subramaniyan and Prema, 2000; Sukumaran et al., 2005; Jayani et al., 2005; Kuhad et al., 2011; Juturu and Wu, 2012; Sharma et al., 2012, 2013; Goswami and Pathak, 2013; Mandal, 2015; Walia et al., 2017; Rebello et al., 2017) Table 1.5 enlists some of the *Bacillus* isolates reported to produce xylanase, pectinase or cellulase enzymes within the span of year 2017-18. Many reports as discussed above indicated that, *Bacillus* isolates produced these polysaccharide degrading enzymes form raw agrowaste biomass. All *Bacillus* spp. has been placed under biosafety level-1 except the *B. anthracis*, a causative agent of anthrax, is categorized under biosafety level-3. Biosafety level 1 (BSL-1) is allotted to those organisms or their products which do not cause disease in healthy humans, plants or animals. Thus, BSL-1 microorganisms are not suspected to contribute to human disease and are appropriate as well as safe to work.

Table 1.5. Literature survey of xylanase-pectinase and cellulase producing *Bacillus* spp. in span of 2017-18:

Enzyme	*Bacillus* species	References
Xylanase	*Bacillus oceanisediminis* SJ3	Boucherba et al., (2017)
	Bacillus sp.	Nkohla et al., (2017)
	Bacillus circulans	Shah et al., (2017)
	Bacilllus sp. BS5	Xu et al., (2017)
	Bacillus pumilus AJK	Kaur et al., (2017)
	Bacillus subtilis Lucky9	Chang et al., (2017)
	Bacillus subtilis 168	Wang et al., (2018)
	Bacillus altitudinis DNH8	Adhyaru et al., (2017)
	Bacillus licheniformis	Kumar et al., (2017)
Pectinase	*Bacillus sonorensis* MPTD1	Mohandas et al., (2018)
	Bacillus subtilis	Kuvvet et al., (2017)
	Bacillus pumilus	Kuvvet et al., (2017)
	Bacillus pumilus AJK	Kaur et al., (2017)
	Bacillus subtilis SAV-21	Kaur and Gupta, (2017)
	Bacillus sp. ZJ1407	Yu and Xu, (2018)
	Bacillus cereus	Zhao et al., (2018)
	Bacillus sp. TMF-1	Salim et al., (2017)
Cellulase	*Bacillus subtilis*	Ho et al., (2017)
	Bacillus sphaericus CE-3	Ekwealor et al., (2017)
	Bacillus cereus	Abada et al., (2018)
	Bacillus licheniformis	J. Wang et al., (2018)
	Bacillus subtilis BM1	Kumar et al., (2017)
	Bacillus amyloliquefaciens	Sun et al., (2017)
	Bacillus cereus KA3	Kalaiyarasi et al., (2017)
	Bacillus licheniformis 380	De Marco et al., (2017)

As discussed in earlier sections, the plants are rich source of cellulose, hemicellulose and also some amount of pectin. Microorgaisms that are able to produce the plant cell wall degrading enzymes, can break these polysaccharides down to mono- or oligomers and consume them for their growth. Hence the agrowaste biomass such as wheat husk, barley husk, rice bran, wheat bran, corn cobs etc., can serve as the best carbon source for the organisms (Nandini and Salimath, 2001; Das et al., 2008a; Choudhary et al., 2012; Kaur et al., 2017; Reginatto et al., 2017). Moreover, such biomass is easily available in bulk from the local agricultural fields and possess high potential to serve as inducer for production of the plant cell wall degrading enzymes. The polysaccharides present in biomass serves this purpose as they induce enzymes like cellulase, xylanase, pectinase, mannanase and several others. If two different biomass rich in two different components are amended together, they have a high probability to induce the enzymes which can degrade both the polysaccharide components from the provided biomass. Hence, the application of crude substrates for production of enzymes

has been studied many investigators.

1.9. Polysaccharide hydrolases and their synergism in lignocellulose saccharification:

Plant cell wall is composed of several polysaccharides like cellulose, xylan, mannan, glucomannan, glucuronomannan, xylomannan, glucoxylan, rhamnogalcturonan etc. Such plant biomass becomes a promising source material of green energy in terms of biofuels provided the polysaccharides from such biomass can be easily converted to fermentable reducing sugars which further can be fermented to bioalcohol. Two broad groups of enzymes are recognized that deconstruct biomass. Core enzymes' group comprising enzymes acting on cellulosic component of plants converting it to sugars such as different endo-glucanases, exo-glucanases cellobiohydrolases, and β-glucosidase etc. 'Accessory enzymes' is the second group that includes variety of hemicellulases (like xylanases, mannanase, pectinases etc.,) that break the physical interference of hydrophobic composite created by hemicelluloses that covers cellulose, and increase the availability of cellulose to core enzyme cellulase for saccharification (Berlin et al., 2007; Banerjee et al., 2010b, 2017). Investigators have used about 3 to 80 enzymes together belonged to these core or accessory groups for improvement of plant biomass saccharification (Banerjee et al., 2010a). In last few decades, efforts have been made to explore such enzymes for their industrial potential of saccharification.

As explained above, enzymatic hydrolysis of lignocellulosic materials needs synergistic action of a group of different functional enzymes. Synergism is defined as a system where the two components when are added together demonstrate the enhanced effectiveness greater than the sum of individual effect. Therefore, instead of a single hydrolase enzyme, synergistic application of an enzyme cocktail is always beneficial. Since each core and accessory group of enzymes can comprises several enzymes, the synergism can be studied with the cocktail of different enzymes of either the core group or the accessory group, as well as the cocktail of core and accessory group or enzymes. Among all these, the cocktail comprising of "core" and "accessory" enzyme components, is suggested to enhance the saccharification maximum (Banerjee et al., 2010a; Hu et al., 2011; Streshinskaya et al., 2012; Laothanachareon et al., 2015).

This synergism can be studied using either additive or substitutive approach. In additive approach, one enzyme component is kept constant and other enzymes are added. As a result, the total enzyme load in cocktail formulated in this manner increases.

While in substitutive approach, a part of one enzyme loading amount is replaced by similar amount of other enzyme(s) in the system. As a result total enzyme load in cocktail formulated in this manner remains constant. (Kostylev and Wilson, 2012). It has been reported that Celluclast, a commercial cellulase from Novozymes, Denmark, was not sufficient to complete saccharification, when used on pretreated lignocellulose substrates. Such deficiencies of cellulases can be compensated with supplementation of non-cellulosic accessory hydrolases having appropriate activities to core cellulase (Hu et al., 2011, Qing and Wyman 2011).

In literature there are several reports on application of commercial enzyme products for biomass saccharification improvement. Such industrial products include core cellulase groups e.g. Avicellase, Novozyme, Megazyme and Accellerase has been vastly studied and characterized for their commercialization with this aspect. Their supplementation with other commercial multifect xylanases and multifect pectinases in enzyme cocktails to remove the covering of hemicellulose and improve performance of cellulase has been studied and yet newer combinations of cocktails are required (Berlin et al., 2007; Delabona et al., 2013; Li et al., 2014b; Reyes-Sosa et al., 2017). These commercial products, their sources and its commercial provide companies are listed in following Table 1.6.

Table 1.6. Commercial enzymes available for biomass saccharification studies:

Enzymes	Source organism	Company/References
Celluclast 1.5L	*Trichoderma reesei*	Novozyme
Novozyme 188	*Aspergillus niger*	Novozyme
Cellic CTec3	--	Novozymes
Spezyme CP	*Bacillus licheniformis* (GM)	Genencor
Primafast	*Trichoderma reesei* (GM)	Genencor
Accellerase	*Trichoderma reesei* (GM)	Genencor
Multifect Pectinase	*Trichoderma reesei* (GM)	Genencor
Multifect Xylanase	*Trichoderma reesei* (GM)	Genencor

1.10. Biomass pretreatment methods:

Pretreatment of lignocellulose biomass is crucial for achieving effective hydrolysis of substrates as enzymatic hydrolysis of native lignocellulose. Although pretreatment is costly, the cost of not pre-treating is even larger. Sharma et al., (2017) has grouped different types of pretreatments in four major groups, i.e., Physical, Chemical, Physicochemical and Biological pretreatments. Table 1.7 depicts the list of different caagories of pretreatments with their pros- and cons (Sharma et al., 2017).

Table 1.7. Comparison of advantages and disadvantages of pretreatments:

Pretreatment	Advantages	Disadvantages
Physical pretreatment:		
Milling, grinding, chipping, irradiation, ultrasonic pretreatment	➔Useful to get desired particle size by increasing the surface area; ➔No chemical required; ➔Effective in reducing cellulose crystallinity; ➔Help enzymatic hydrolysis	➔High operating costs; ➔High chances of equipment depreciation; ➔Not suitable for lignin removal; ➔High energy requirement
Chemical pretreatment		
Liquid hot water	➔No catalyst and chemical involved; ➔Reduction of feedstock size by disrupting the lignocellulosic components- mainly the hemicellulose; ➔Hydrates the cellulose and make it more accessible to hydrolytic enzymes; ➔Also removes part of lignin and have high xylose recovery	➔High water and energy demand; ➔Multi-stage pretreatment at low temperature and long residence time is required to recover hemicellulose and its valuable sugars
Acid hydrolysis: HCl, H_2SO_4, HNO_3	➔A powerful agent for removal of hemicelluloses and lignin; ➔Concentration of acids has its significant role in pretreatment; ➔Dilute acids are more favored in pretreatment that affectively remove hemicellulose, maximize glucose yield and can alter the lignin structure, while strong acids can hydrolyze cellulose. ➔Some acids such as H_2SO_4 and HCl are cheap	➔Acids are corrosive and it is crucial to recycle in order to lower cost; ➔The formation of degradation products such as furfural; ➔5-hydroxymethylfurfural levulinic acids and formic acid formed from cellulose and hemicellulose together with organic acids from lignin degradation act as inhibitors, that affect the subsequent stages of enzymatic hydrolysis and fermentation; ➔Requires high temperature and specific reaction vessels which is costly
Organic solvent: Methanol, ethanol, ethylene glycol, acetone, oxalic acid, salicylic acid, etc.,	➔Help in removal of lignin and hemicellulose, improve retention and enzymatic digestibility of the cellulose	➔High cost of solvent and catalyst; ➔Greater chances of environmental impact; ➔Some are inflammable and causes fire and explosion
Alkaline hydrolysis: $Ca(OH)_2$, NaOH, NH_4OH	➔Important in removal of lignin from the biomass and exposed the polysaccharides, sometime also breaks the crystalline cellulose; ➔Increase surface area and makes the hydrolysis faster	➔High operational cost, formation of inhibitors; ➔Generally, not suitable for woody biomass; ➔It requires chemicals and generally has harsh conditions
Oxidative delignification: Ozone, wet oxidation, hydrogen peroxide, peracetic acid	➔Removes hemicellulose and lignin from biomass; ➔Improve retention and enzymatic digestibility of the cellulose; ➔Very low formation of enzyme-inhibiting compounds	➔High operational cost; ➔Acids formed in the process act as inhibitor in fermentation; ➔Parts of hemicellulose are lost
Physiochemical pretreatment:		
Explosion, Steam explosion, Ammonia fibre explosion, CO_2 explosion, SO_2explosion	➔Low chemicals and energy consumption; ➔Hemicellulose and lignin disruption; ➔Acids help to improve hydrolysis; ➔Increases the assessable surface area and enzymatic digestibility of the cellulose. ➔Suitable for industries	➔Degradation products may inhibit further processes; ➔Need high pressure; ➔Low yield but high energy consumption; ➔Chances of chemical hazard

Biological pretreatment:		
Bacteria, Fungi	➔Environment friendly process; ➔Low energy requirement; ➔Cost effective; sustainable; no chemical required; useful in hydrolysis of cellulose, hemicellulose and lignin	➔Slow process, partial hydrolysis of hemicellulose; ➔Chances of health hazard

Further, Mohapatra et al., (2017) had explained the combination of two different pretreatments from these group, viz., ultrasonication with alkali or acid pretreatment, acid and alkali pretreatment, alkali and microbial pretreatment and so. Liquid hot water pretreatment (Maurya et al., 2015), use of different ionic solvent like DMA (N,N-dimethylacetamide), DMSO (dimethylsulfoxide), [EMIM]Cl (1-ethyl-3-methylimidazolium chloride), [BMIM]Cl (1-butyl-3-methylimidazolium chloride) etc., are the other pretreatments which were used to enhance the saccharification efficiency of the biomass(Tadesse and Luque, 2011; da Costa Lopes et al., 2013).

The advantages and disadvantages of these pretreatments in terms of increased accessible surface area, de-crystallization of cellulose, removal of hemicellulose, removal or relocation or alteration in structure of lignin etc., have been reviewed in details by several investigators (Mosier et al., 2005; Taherzadeh and Karimi, 2008; Mohapatra et al., 2017; Sharma et al., 2017).

Kumar and Wyman, (2009) suggested that although decline in the rate of saccharification or conversion is majorly dependent on enzyme features and the chemical/physical environment, the pretreatment technology and the biomass substrates also affects the accessibility of pretreated biomass and its conversion or saccharification. Depending on the structural composition of the substrates and specific pretreatment, different effects may be observed on individual substrate that can contribute to alter the accessibility of biomass and improved hydrolysis. Van Dyk and Pletschke, (2012) had listed certain effects viz., removal of some / all of the lignin which causes increased porosity in the substrate; disruption of the lignin structure and its linkages with the rest of the biomass; redistribution of lignin; removal of hemicellulose that hampers access of cellulases to cellulose; disruption of the hemicellulose structure; reduction in crystallinity and degree of polymerization of the cellulose; reduction in the particle size of biomass, increase in porosity etc.

Perhaps the pretreatment becomes the single most crucial step having large impact on the succeeding steps of saccharification as it affects the biomass in such a

way that the biomass can easily be hydrolysed by various enzymes yielding the fermentable sugars for production of ethanol in high yield. And therefore, from the above mentioned benefits and effects of pretreatments, Galbe and Zacchi, (2012) had derived several limiting factors which can be used to determine the most suitable pretreatment of individual biomass. The pretreatment should have highest recovery of all carbohydrate with none or minor loss of pentoses or hexoses. It should impart highest digestibility to the cellulose in the successive steps during saccharification by enzymatic hydrolysis. The by-products like aromatic and aliphatic compounds as well as furfurals originated from the lignin or carbohydrates during the pretreatments can inhibit or even cease the fermentation, which will reflect in the operating costs of the process. It should produce no or minor amount of sugar and lignin degradation products which might be toxic for the fermentation process. The fermentation of the pretreatment liquid should not require any detoxification. The pretreatment should result in high solid concentration containing sugars, or high concentration of liberated sugars in the liquid fraction. The process ultimately should require a low energy demand to lower the operational and capital cost.

Table 1.8. % Saccharification or glucan and xylan conversion of different pretreated biomass by different enzyme preparation commercial enzyme preparations:

Biomass	Pretreatment	Enzyme combination	Saccharification	Remark
Switchgrass	1% NaOH	Celluclast 1.5L and Novozyme 188	~38%	Mohapatra et al., (2017)
Switch grass	NH_4OH	Cellulase and Cellobiase	~72%	Chang et al., (1997)
Napier grass	NH_4OH	Cellulase and Cellobiase	~60%	Yasuda et al., (2014)
Sugarcane bagasse	Microwave pretreatment	Commercial cellulase (Zytex)	~66%	Binod et al., (2012)
Red sage (*Lantana camara*)	Dilute acid pretreatement	Celluclast 1.5 L and Novozyme 188	77.7%	Kuhad et al., (2010)
Cotton stalks	NaOH pretreatment	Celluclast 1.5 L and Novozyme 188	~38%	Silverstein et al., (2007)
L. camara, *P. juliflora*, and Corn cob	Alkali pretreatment	Celluclast 1.5 L and Novozyme 188	50.8-55.0%	Gupta et al., (2011)
L. camara, *P. juliflora*, and Corn cob	Acid pretreatment	Celluclast 1.5 L and Novozyme 188	39.2-48.0%	Gupta et al., (2011)
L. camara, *P. juliflora*, and Corn cob	Chlorite pretreatment	Celluclast 1.5 L and Novozyme 188	86.4-91%	Gupta et al., (2011)
Wheat straw	Ionic Liquid	Celluclast 1.5 L and Novozyme 188	45.9%	da Costa Lopes et al., (2013)
Miscanthus giganteus, Poplar and wheat straw	Steam Explosion	Cellic CTec2®, (Novozyme)	71%, 51%, 82%	Auxenfans et al., (2017a)
Sugarcane Bagasse	H_2O_2	Celluclast 1.5 L, Novozyme 188,	~23.8% glucan conversion	Li et al., (2014b)

		Endo-1, 4-β-Xylanase		
Sugarcane Bagasse	H_2SO_4	Celluclast 1.5 L, Novozyme 188, Endo-1, 4-β-Xylanase	~30.7% glucan conversion	Li et al., (2014b)
Sugarcane Bagasse	Steam Explosion	Celluclast 1.5 L, Novozyme 188, Endo-1, 4-β-Xylanase	~67.7% glucan conversion	Li et al., (2014b)
Sugarcane Bagasse	Alkali treated	Crude extract containing cellulase, xylanase pectinase from *Chrysoporthe cubensis*	12.5% of glucan, 44% of xylan conversion	Maitan-Alfenas et al., (2015a)

These, and other reports as reviewed by Mohapatra et al., (2017) and Sharma et al., (2017) suggested that, though these pretreatment techniques are efficient in enhancement of biomass saccharification. The current available core cellulase system requires the assistance of accessory enzymes for optimum saccharification process. Based on these, the rationale and objectives for the work decided were as follows.

Rationale and Objectives

Rationale:

The process of plant biomass conversion to biofuel is a priority area of research in the hunt for renewable energy resources that can replace fossil fuels. Two fundamental steps involved in this process are breakdown of polysaccharides from plant biomass to fermentable sugars and fermentation of these sugars to alcohol. Due to the complexity of the plant cell wall matrix, the bottleneck to industrial scale production of biofuel from lignocellulosic material lies at the keystone step of saccharification, i.e., hydrolysis of this material into soluble fermentable sugars like glucose and xylose. The rate and conversion efficiency of biomass to fermentable sugars is attributed to three major parameters, i.e., composition of the biomass material, its pretreatment(s) and contribution of several polysaccharide degrading enzymes in the process.

The enzymes that directly act on the major plant cell wall polysaccharides like cellulose i.e. cellulases, are important "core" enzymes par taking in the breakdown of these polysaccharides into fermentable sugars. Yet the presence of hemicellulose, pectin and lignin crosslinking in matrix becomes the major limiting factors. A second group of enzymes that indirectly increase the accessibility of plant cellulose to the cellulases are also important in lignocellulosic breakdown. These enzymes, termed as "accessory enzymes", include ones that degrade other hemicellulose, pectin and lignin components and unmask the cellulose. The accessory enzymes like xylanases and pectinases in enzyme cocktails for plant biomass saccharification assume an important place since they increase the efficiency of the core enzymes.

With this background in mind, to overcome the limitation of substrate accessibility in saccharification, it was hypothesised that application of pretreatment prior to enzymatic saccharification should alter the lignin fraction and enhance the biomass accessibility to the saccharifying enzymes. Moreover, addition of ''accessory'' enzymes such as xylanase and pectinase to the "core" cellulase enzymes should also stimulate cellulose hydrolysis efficiently by hydrolysing non-cellulosic polysaccharide meshwork that coat cellulose fibres. The efficiency of such enzyme cocktail is dependent on synergism between the enzyme components of the cocktail. As explained in Chapter 1, Section 1.9, the synergism studies between core and accessory enzymes can be done with two methods. Accessory enzymes are added to constant amount of core enzyme in additive method, while a part of core enzyme is substituted with accessory enzyme to keep protein load constant in substitutive method.

The enzyme cocktail mediated agrowaste hydrolysis process will lead towards their bioconversion to simpler fermentable mono- or di-saccharides, which will reduce the cost effectiveness and provide the raw material for bio-alcohol synthesis. An efficient cost-effective enzyme cocktail for enzyme catalysed depolymerization of the agrowaste polysaccharides is needed to get fermentable sugars from agro waste products for ethanol producttion, and also contribute to reduce some amount of environmental pollution. The enzymes involved in biomass deconstruction are currently derived from fungi and there is a need to screen other microbes to obtain efficient enzymes that act synergistically with the cellulases.

Isolation of bacteria from variety of niches for the production of "accessory" enzymes like xylanase and pectinase to improve cellulose accessibility and use of pretreatment strategies to improve agrowaste digestibility was proposed here. This included synergism and saccharification studies with enzyme cocktails on the pretreated agro-waste biomass as the main focus. In this perspective the following objectives were decided.

Objectives:

- Isolation, screening and identification of bacterial cultures producing polysaccharide hydrolases (xylanase and pectinase) from different sources and synergism studies with polysaccharide hydrolases for saccharification of raw agrowaste biomass.
- Agrowaste biomass deconstruction by pretreatment and saccharification by cocktails containing commercial cellulase along with crude xylanase and/or pectinase from selected isolates.
- Physicochemical characterization of crude hydrolases and optimization of inducer substrate concentration in medium for concurrent production of accessory enzymes from selected isolates.