Bioethanol Production from Lignocellulosic Biomass using Immobilized Cellulolytic Enzymes

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Miles to go before I sleep…

(Robert Frost)

-To my Parents, My Brother and Friends

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Karthik Periyasamy

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List of Symbols and Abbrevations

APTES	-	(3-Aminopropyl) triethoxysilane
AOX	-	Absorbable Organic Halogen
AFEX	-	Ammonia Fiber Explosion Method
ARP	-	Ammonia Recycle Percolation
APS	-	Ammonium Per Sulphate
AFM	-	Atomic Force Microscopy
ATR-FTIR	-	Attenuated Total Reflection-Fourier Transform Infrared spectroscopy
β-ME	-	β-mercaptoethanol
BSA	-	Bovine Serum albumin
CMC	-	Carboxymethyl Cellulose
Cm	-	Centimeter
COD	-	Chemical Oxygen Demand
Combi-CLEAs	-	Combined Cross-Linked Enzyme Aggregates
CBB	-	Coomassie Brilliant Blue
CLEs	-	Cross-Linked Enzyme
CLEAs	-	Cross-linked Enzyme Aggregates
CLECs	-	Cross-Linked Enzyme Crystals
CrI	-	Crystalline Index
DTG	-	Derivative Thermogravimetry
DCM	-	Dichloromethane
DEAE	-	Diethylaminoethyl
DME	-	Dimethoxyethane
DMSO	-	Dimethyl sulfoxide
DNS	-	3, 5-Dinitrosalicylic Acid
DTT	-	Dithiothreitol

DLS	-	Dynamic Light Scattering
EDTA	-	Ethylene diamine tetra acetic acid
$FeCl_3.6H_2O$	-	Iron(III) Chloride heptahydrate
$FeCl_2.4H_2O$	-	Iron(II) Chloride tetrahydrate
GRAS	-	Generally Regarded As Safe
G	-	Gram
HCl	-	Hydrochloric acid
H_2SO_4	-	Sulphuric acid
HPLC	-	High Pressure Liquid Chromatography
H	-	Hour
HIC	-	Hydrophobic Interaction Chromatography
ISN-CLEAs	-	Bifunctionalized Magnetic Cross-Linked Enzyme Aggregates
IU	-	International Unit
IAA	-	Iodoacetamide
IOMNPs	-	Iron Oxide Magnetic Nanoparticles
K	-	Kelvin
kDa	-	Kilo Daltons
L	-	Litre
V_{max}	-	Maximum velocity
K_m	-	Michaelis Menten Constant
µg	-	Microgram
µl	-	Microlitre
µm	-	Micrometer
µM	-	Micromolar
µmol	-	Micromole
m^3	-	Cubic meter
Mg	-	Milligram
Ml	-	Milliliter
Mm	-	Millimeter

mM	-	Millimolar
MNPs	-	Magnetic Nanoparticles
Min	-	Minute
M	-	Molar
NaOH	-	Sodium Hydroxide
TEMED	-	N,N,N,N'-Tetra Methyl Ethylene Diamine
Nm	-	Nanometer
Nmol	-	Nanomole
N	-	Normality
OD	-	Optical Density
PAGE	-	Polyacrylamide Gel Electrophoresis
PEG	-	Polyethylene Glycol
PDA	-	Potato Dextrose Agar
Rpm	-	Revolution Per Min
SEM	-	Scanning Electron Microscope
Sec	-	Seconds
SAA	-	Soaking in Aqueous Ammonia
SDS	-	Sodium Dodecyl Sulphate
SSF	-	Solid-State Fermentation
SmF	-	Submerged Fermentation
SCB	-	Sugarcane Bagasse
TEOS	-	Tetraethyl Orthosilicate
TGA	-	Thermogravimetric Analysis
TG	-	Thermogravimetry
TPP	-	Three Phase Partitioning
Tris	-	Tris-[Tris-(hydroxy methyl) Amino Methane]
U	-	Unit
U/mg	-	Unit per milligram
U/ml	-	Unit per millilitre
V	-	Volt

v/v	-	Volume per volume
w/v	-	Weight per volume
XDR	-	X-Ray Diffraction
YNBA	-	Yeast Nitrogen Base with Amino Acids
€	-	Euro
%	-	Percentage
ºC	-	Degree Celsius
A	-	Alpha
B	-	Beta

CHAPTER 1

INTRODUCTION

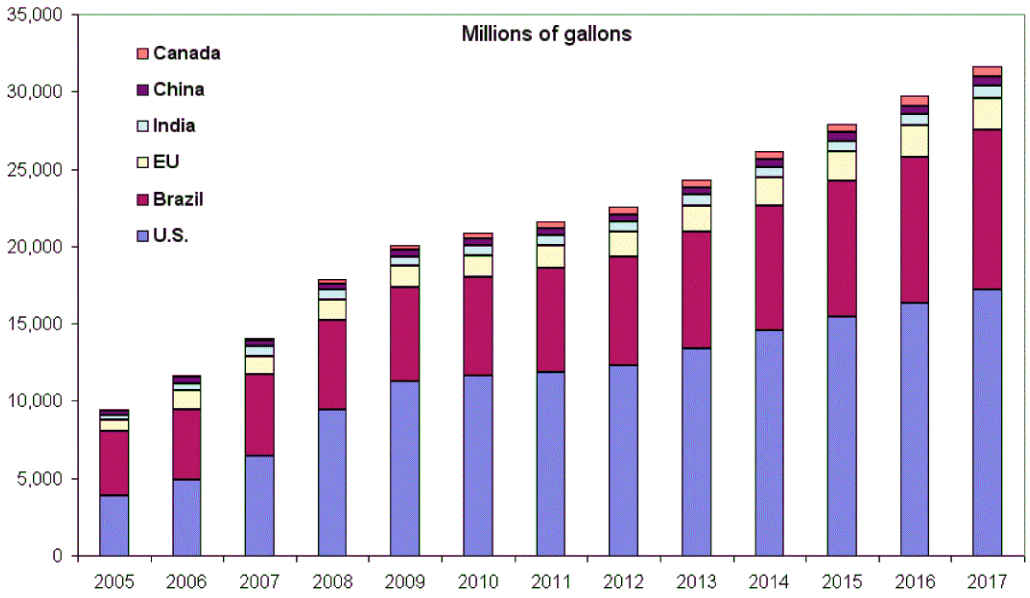
1.1 Background

Environmental concerns connected with climate change and global warming are of eminent importance for human sustenance and thus has attracted the attention of scientists worldwide. The rising greenhouse gas levels, depletion of oil supply and the increase in energy demands have necessitated the search for an alternative clean and sustainable fuel resource in order to balance the demand supply chain and to improve the quality of life. Currently, fossil fuel resources such as coal and natural gas contribute majorly towards the generation of power in the nonrenewable sector. In addition to this, a number of biofuels such as bioethanol, biodiesel, biomethane, biohydrogen and bio-butanol supplement the energy demand by reducing the usage of petroleum-based fuels. Biofuels are advantageous with certain limitations and hence a bio-refinery can prove to reduce the dependency on petroleum-based fuel in a more sustainable manner Bioethanol has established itself as the world's No. 1 biofuel and the bioethanol market has continued to grow rapidly in recent years. The oil demand is expected to increase to 57% from 2002 to 2030. In 2015, the world production of bioethanol rose by 2.7% year over year to 115.1 million m^3. The fuel sector accounted for over eighty percent (97.1 million m^3) of it. World leader in the production of bioethanol is the USA with 57.5 million m^3, followed by Brazil with 30.0 million m^3 (Crop energies, 2016).

Bioenergy is the largest source of renewable energy today as it supplies basic energy in the form of heat, electricity, and fuel for transport. However, globally, it accounts only up to 10% for electricity production and 3% as transportation fuel. Part of the difficulty in promoting the use of bioenergy lies in its complex supply chain, which is dependent on a variety of economic sectors. Moreover, these projects require relatively more careful consideration in terms of sustainability issues and appropriate regulatory frameworks than other low-carbon technologies (IEA, 2016).

Energy consumption is increasing at 6.5 percent per year whereas petroleum conservation is depleting day by day. India's consumption of crude oil amounts to 3.1% while production is only about 1% of global crude oil production. Government organizations and a number of private companies are involved in production and distribution of biofuel in India. The leaders in biofuel processing in India are D1 Oil Plc, Reliance Industries Ltd, Godrej Agrovet, Emami Group, Aatmiya Biofuels Pvt Ltd., Gujarat Oelo Chem Limited (GOCL), Jain Irrigation System Ltd., Nova BioFuels Pvt. Ltd., Sagar Jatropha Oil Extractions Private Limited etc., (Swain, 2014).

India is set to expand its biofuels market over the next few years in an effort to strengthen its energy security. Blending of 10% ethanol with gasoline or 5% biodiesel with a regular diesel could boost the market from 65 billion rupees to 500 billion rupees (€6.7 billion).



(Source: FAPRI 2008 U.S and World agricultural outlook).

Figure 1.1 World Fuel ethanol production from 2005 to 2017

1.2 Bioethanol

Bioethanol has a considerable potential to replace oil to a certain degree as it is a liquid fuel that can be easily integrated into the existing infrastructure for fuel distribution. There are three major challenges that researchers need to encounter in bioethanol production. Firstly, ensuring the large-scale availability of cellulosic feedstocks whenever required. Secondly, the conversion of cellulose to ethanol in a cost-effective manner in order to make it more competitive relative to cornstarch and gasoline based ethanol. Thirdly, altering the vehicle infrastructure to utilize renewable fuel such as cellulosic fuel mandated by the Renewable Fuel Standard.

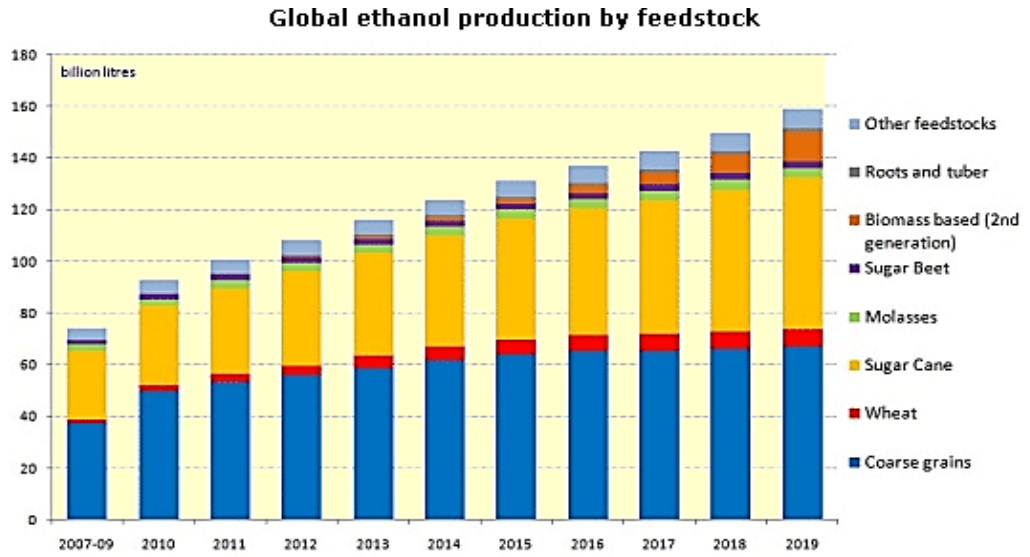
The major production pathways for bioethanol are defined based on the nature of the conversion process which includes the gasification pathway and the carbohydrate pathway. The carbohydrate pathway is further divided into the lignocellulosic pathway, sucrose (glucose) pathway and starch pathway (Keshwani et al. 2010). Conventionally, first generation bioethanol production utilizes starch and sucrose as raw materials. The production from lignocellulosic raw materials are categorized as the second generation which utilizes cheaper and non-food feedstocks like sugarcane bagasse or municipal solid waste making it more competitive with fossil fuels. Production of bioethanol from lignocelluloses requires a more complex upstream process compared to first generation ethanol production (Kootstra et al. 2009).

Lignocellulosic bioethanol production has become an active area of research over recent years because of a number of environmental and economic benefits. The depletion of the oil supply, its fluctuating price, and the negative impingement of fossil fuels on the environment predominantly greenhouse-gas emissions necessitate the need for alternative fuel resources. The challenge with utilizing lignocellulosic feedstock is overcoming the recalcitrant nature of cellulosic biomass which this study also aims to address. Enzymatic hydrolysis of cellulose is a recommended approach for obtaining fermentable sugars for various reasons, and in particular because the results of economic evaluations are in favour of enzymatic hydrolysis, when it is compared with chemical hydrolysis. (Michel D, Bouchra B M, 2016).

1.2.1 Bioethanol Production from Different Sources

1.2.1.1 Crops as Raw Materials

Ethanol can be produced directly from the free sugar-containing juices of some crops, converting sucrose or monosaccharides, especially, glucose, into ethanol by fermentation with microorganisms. A variety of feedstocks such as sucrose-containing sugar crops (e.g. sugarcane or sugar beet) and starchy cereals (e.g. wheat, corn, and barley) are the good sources of sugar-rich substances for bioethanol production. To produce ethanol from starch it is necessary to break down its carbohydrate chain for obtaining glucose syrup, which can be converted into ethanol by yeasts. This type of feedstock is the most utilized for ethanol production in North America and Europe (Cardona and Sánchez, 2007). Pretreatment of juices from sugar-containing plants has mainly involved the removal of impurities and disinfection. The sugar solution was fermented by yeast *Saccharomyces cerevisiae* to produce ethanol and it was purified by distillation. In terms of the design of ethanol production processes from sucrose-containing materials, the availability and transport costs of the feedstock continue to play an important role. On the other hand, this raw material base will not be fully sustainable due to the fact that wide planting of corn for ethanol production will compete for the finite arable land and will lead to food crisis (Binod et al. 2010; Zacchi, 2006). In contrast, lower value lignocellulosic materials from forestry, agricultural residues, as well as municipal and industrial wastes, was suggested as the promising alternative feedstock for bioethanol production. Intensive research and developments in the last decades on lignocellulosic materials will most likely make them important feedstock for ethanol production in the future. The primary feedstock for ethanol in the U.S and worldwide has been coarse grains (i.e., corn), however, the production of ethanol from these feeds is expected to plateau in 2015. The increase in ethanol production in the next 10 years is expected to be from sugar-based ethanol (cane, beets). It is expected that second generation biofuel production (from cellulosic feeds) will increase after 2015. Figure 1.2 shows graphically how the feedstocks compared.



(Source: http://www.agri-outlook.org/)

Figure 1.2 Global ethanol production by feedstock, projected until 2019.

Sugarcane is one of the most abundant crops in the world. it is a versatile plant grown for sugar production, and its major by-product is bagasse. Bagasse is the residue obtained after sugarcane is milled for juice extraction. In sugarcane production, the bagasse retrieved from crops is roughly 27–28 dry weight % of plant biomass. It is a highly heterogeneous material that consists roughly of 20–30% lignin, and 40–45% cellulose and 30–35% hemicellulose with limited amounts of extractives and ash. Its composition makes it a promising feedstock for second-generation biofuel production (Cardona, C. A et al. 2010).

1.2.2 Bioethanol from Lignocellulose

The term “lignocellulosic” covers a range of plant molecules/biomass containing cellulose, with varying amounts of lignin, chain length, and degrees of polymerization. Lignocellulosic materials have the potential for use as a feedstock for advanced diesel and drop-in biofuels (via thermochemical conversion) and for the production of cellulosic ethanol (via biochemical conversion). Lignocellulosic biopolymers extracted from wood and plants, typically cellulose, hemicelluloses, and lignin have been well recognized as the most abundant and potential alternative resources toward the production of bioethanol and other value-added products (Kumar et al. 2008; Rubin, 2008). Lignocellulosic biomasses like agricultural residues, wood, and wood wastes are mainly composed of cellulose (45–56 %) which is a homopolymer of glucose, hemicelluloses (10–25%) is a more complex

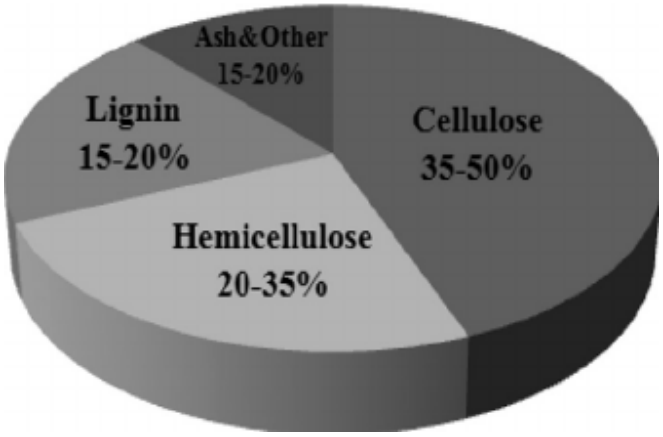
heteropolymer containing primarily xylose but also significant amounts of arabinose, mannose, glucose, galactose and lignin (18–30%) (Guerriero et al. 2015; Kang et al. 2004; Menon and Rao, 2012) with trace amounts of other components, like acetyl groups, minerals and phenolic substituents. The composition of these constituents may vary from one plant species to another. Cell wall polysaccharides of lignocellulosic biomass are composed of crystalline cellulose fibrils surrounded by a matrix of non-crystalline hemicelluloses, which are a group of heteropolysaccharides that bind with pectin to cellulose, forming a network of cross-linked fibers (Ramos, 2003).

The major component of lignocellulosic biomass is cellulose. Cellulose is a linear homopolysaccharide that consists of glucose (D-glucopyranose) units linked together by β-(1-4) glycosidic bonds (β-D-glucan). The repeating unit of the cellulose is the disaccharide cellobiose. The hydrophilic/hydrophobic nature of cellulose is based on its structural anisotropy. Cellulose chains are arranged in a parallel manner and are organized in sheets stabilized by interchain OH–O hydrogen bonds, whereas the stacking of sheets is stabilized by both van der Waals (vdW) dispersion forces and weak CH–O hydrogen bonds. Cellulose has a strong affinity to itself and materials containing hydroxyls, especially water. Based on the preponderance of hydroxyl functional groups, cellulose polymer is very reactive with water. Water molecular smallness promotes the reaction with the cellulose chains and immediately formed hydrogen bonds. Besides that, vdW dispersion forces play an important role between these two reactive entities. They stabilize the cellulose structure according to the considerable cohesive energy in the cellulose network. Hydrogen bonding, electrostatic interactions, and vdW dispersion forces play an important role in determining the cellulose crystal structure during the cellulose-water interactions.(Ha et al. 1998; Somerville et al. 2012).

Hemicelluloses are branched heteropolysaccharide with a low degree of polymerization and consists of different monosaccharide units such as glucose, mannose, galactose, arabinose and xylose. They are short-branched (~50-200) and amorphous which make them partially soluble in water (Demirbas, 2009). Hemicelluloses are able to bind with cellulose by multiple hydrogen bonds and bind with lignin by covalent bonds. They are not crystalline and are more accessible for hydrolysis than cellulose. Hemicelluloses are rod-shaped with branches and side chains folded back to the main chain by means of hydrogen bonding.

After cellulose, lignin is the second most abundant renewable carbon source available. Lignin is the cross-linked phenolic heteropolymers mainly derived from three hydroxycinnamoyl alcohol monomers i.e. *p*-coumaryl, coniferyl, and sinapyl alcohols. They are primarily formed by the free-radical polymerization of *p*-hydroxy cinnamyl alcohol units with varying methoxyl content. They are relatively hydrophobic and aromatic in nature. Depending on the degree of methoxylation, the aromatic group is *p*-hydroxy benzyl (derived from *p*-coumaryl alcohol), guaiacyl (derived from coniferyl alcohol) or syringyl (derived from sinapyl alcohol) (Ramos, 2003). Lignin not only gives strength to the plant tissue because of its rigidity but also affects the transport of water, nutrients and metabolites.

Lignocellulosic biomass appears to be an attractive feedstock for three main reasons. First, a renewable resource could be sustainably developed in the future. Second, it appears to have formidable positive environmental properties resulting in no net release of carbon dioxide and very low sulfur content. Third, it appears to have significant economic potential provided that fossil fuel prices increase (Demirbas, 2008). Besides, lignocellulosic materials do not have a negative effect on the human food supply chain by eliminating the food in favor of bioethanol production (Alvira et al. 2010).

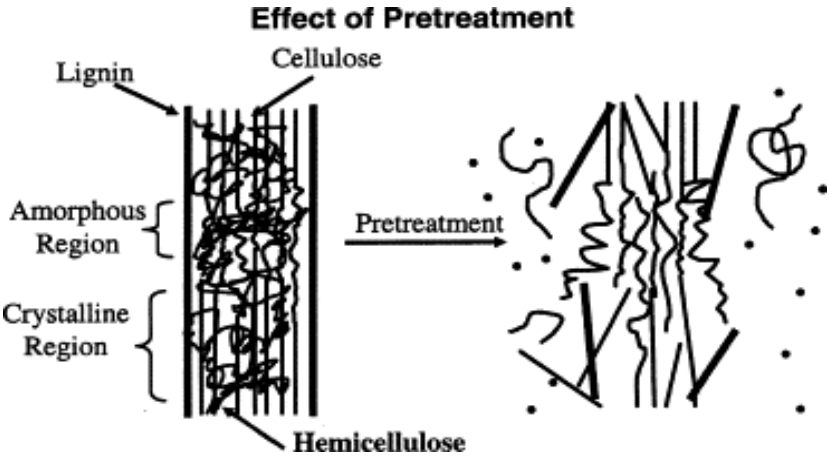


(Source: (Haghighi Mood et al. 2013)**.**

Figure 1.3 General composition of lignocellulosic biomass feedstock

Lignocellulosic biomasses are not easily broken into their composite sugar molecules, as they are resistant to enzymatic attack due to their impermeable structure. Three steps are required for the processing of lignocellulosic biomass to ethanol i.e. pretreatment, hydrolysis, and fermentation. Various steps, such as breaking the complex lignocellulose structure, enzyme

loading, interference of inhibitors during hydrolysis and fermentation, are all necessary components of the bioconversion process. Pretreatment is considered as one of the most expensive processing steps in the conversion of cellulose to fermentable sugars. It also has a great potential for improvement of efficiency and lowering of cost through research and development (Alvira et al. 2010; Taherzadeh and Karimi, 2008).



(Source: Hsu et al. 1980)

Figure 1.4 Schematic presentation of effects of pretreatment on lignocellulosic biomass

1.3 Pretreatment of Lignocellulosic Biomass

Pretreatment is an important step required to break down the lignin and hemicelluloses structure and disrupt the crystalline and increase the porosity of cellulose so that the enzymes or acids can have easy access and hydrolyze the cellulose. An effective pretreatment should improve the conversion of sugars. It should avoid the degradation or loss of carbohydrates and the formation of byproducts, which inhibit the hydrolysis as well as the fermentation process. It should be cost-effective and should minimize the energy demand. The cost barriers for biomass utilization can be overcome by developing an effective pretreatment method with optimal operating conditions. Limited formation of inhibitors, preserving cellulose and hemicellulose, reducing the usage of chemical and water, less demand for energy in the biochemical process are the key issues for a cost-effective pretreatment. Pretreatment method for lignocellulosic biomass can be a physical, physicochemical, chemical or a biological process.

1.3.1 Physical Pretreatment

Mechanical comminution: In this technique, biomass can be comminuted by several methods such as grinding, shredding, chipping, milling, extrusion, irradiation, etc. The main goal of chipping is to reduce heat and limit the mass transfer. Milling and grinding effectively reduce the size and crystallinity of the biomass. Studies have shown that chipping reduces biomass size to 10-30 mm while milling and grinding reduce to 0.2-2 mm (Sun and Cheng, 2002). However, it was found that reduction of biomass size beyond 0.4 mm has no substantial effect on rate and yield of hydrolysis (Chang et al. 1997). Several milling methods such as two-roll milling, hammer milling, colloid milling, and vibratory milling were used to improve the digestibility of the lignocellulosic materials. When compared to ordinary milling technique, vibratory ball milling was found to be more effective in breaking down the cellulose crystallinity and improved digestibility of biomass. Physical pretreatment does not cause toxic by-products during the treatment process (Brodeur et al. 2011). However, this method has its own disadvantages in terms of energy consumption. The energy requirements for mechanical comminution depends on the desired final particle size and the characteristics of the lignocellulosic material. Due to the high demand of energy, this method is economically not feasible (Zhu et al. 2010). The inability of removing the lignin and limiting the access to cellulose by the enzymes is another disadvantage (Berlin et al. 2006). It was observed that optimizing the conditions of pretreatment process and enzyme concentrations had a synergetic effect on the overall yield of the reducing sugars.

1.3.2 Chemical Pretreatment

a) **Acid:** In this method, the biomass is dried and milled. It is then presoaked in water and submerged in an acidic solution for a period of time under specific temperature. The pretreated biomass is further filtered in order to separate the liquor from the unhydrolyzed solid substrate. Further, the filtrate undergoes washing i.e. to remove acids and extract sugars or undergoes neutralization before saccharification. The purpose of acid pretreatment is to solubilize the hemicellulosic fraction of the biomass to increase the accessibility of cellulose to enzymes. Both concentrated and dilute acid

can be used for this method but utilization of concentrated acid is not favorable because they tend to form inhibiting compounds during the ethanol production.

The main advantage of using a dilute acid is the fast reaction rate which enables continuous processing of the biomass. Depending on the conditions of the pretreatment, the hydrolysis of the sugars could take from a few minutes to hours. It also has the advantage of solubilizing hemicelluloses as well as converting solubilized hemicelluloses to fermentable sugars. It results in increased recovery of sugars, increases porosity and improves enzymatic digestibility. Inorganic and organic acids (e.g. maleic acid, fumaric acid) are used for dilute acid pretreatment. Two types of acid pretreatment methods are widely employed on the industrial scale. Pretreatment at high temperature (above 180°C) for short duration (1-5min) and at low temperature (below 120°C) for long duration (30-90 min). After acid pretreatment, hydrolysis of biomass with enzymes could be avoided as acid itself hydrolyses the biomass into fermentable sugars. However, before fermentation of the sugars, extensive washing is required to remove the acid. Diluted sulphuric acid (H_2SO_4) is commonly used for the pretreatment of Switchgrass (*Panicum virgatum L.*) reed canarygrass (*Phalaris arundinacea L.*) (Digman et al. 2010) and corn stover (Avci et al. 2013). Rapid hydrolysis rates and high glucose yield during saccharification at a higher temperature. A major drawback of using dilute acid is its requirement of special corrosion-resistant reactors which are usually expensive and not feasible when compared to other pretreatment methods (Sharma and Kumar, 2013). Acid pretreatment also has some drawbacks due to its corrosive nature and toxicity. It has attracted less attention due to the formation of fermentation inhibitors like furfural and hydroxymethyl furfural, phenolic acids and aldehydes (Chen and Sharma-shivappa, 2007; Saha et al. 2005). Though diluted H_2SO_4 is relatively cheap and effective hydrolysis of lignocellulose, phosphoric acid (H_3PO_4) gives a milder effect and is more environment-friendly (Bensah & Mensah, 2013).

b) **Alkaline:** Alkaline pretreatment has gained more attention because it has a number of desirable features. The process involves soaking the biomass in the alkaline solution and mixing it at the required temperature for certain period of time. The effect of alkaline treatment depends on the lignin content in the biomass. Comparing to acid pretreatment, alkaline pretreatment is carried out at lower temperature and pressure and it also causes low sugar degradation (Kumar et al. 2009). They are used for removing

lignin by degrading the ester and glycosidic side chains and thereby increasing the digestibility of cellulose and partial solvation of hemicelluloses. In addition, it also removes acetyl and the various uronic acid substitutions on hemicellulose. It involves the use of mainly non-polluting and non-corrosive chemicals such as ammonia, sodium, potassium and calcium hydroxides. Lime is the least expensive available alkali as quick lime (CaO) or slaked lime ($Ca(OH)_2$). These chemicals provide an alternative low-cost lignin removal at higher pH values (Yang and Wyman, 2008). It also prevents condensation of lignin, resulting in high solubility of lignin for low lignin content biomass. It also removes the acetyl groups from hemicellulose thereby reducing enzyme steric hindrance. Lime has been used for effective pretreatment of switchgrass (Sun & Cheng, 2002), wheat grass (Chang et al. 1998) and poplar wood (Chang et al. 1998).

Sodium hydroxide (NaOH) has also been extensively studied in the pretreatment of biomass. It degrades the lignin by the cleavage of ether and ester bonds and the swelling of cellulose. Acetyl and various uronic acid substitutions on hemicelluloses are also removed, thereby increasing the enzymes accessibility to the cellulose and with low loss of polysaccharides. NaOH pretreatment is categorized into two processes: low concentration and high concentration. NaOH pretreatment is more effective on agricultural wastes, herbaceous crops, and hardwood (Chen et al. 2013) and treatment of spruce at low temperature (Zhao et al. 2008). The drawback in alkaline pretreatment is the conversion of alkali to unrecoverable salts into the biomass that may require further treatment. Highly recalcitrant biomass is not easily pretreated by the alkali method but requires severe pretreatment conditions. This condition leads to the loss of hemicellulose and formation of inhibitors (Bensah & Mensah 2013). NaOH pretreatment utilizes lower temperature and pressure. However, a large amount of heat is released during the dissolution of solid NaOH in water, which may be a threat to the environment. Before enzymatic saccharification, a neutralization step is required for the removal of lignin and inhibitors.

1.3.3 Physicochemical Treatment

a) Steam explosion method: This process has been used for the pretreatment of different biomass feedstocks by using both chemical and physical techniques in order to break the structure of the lignocellulosic material. This method increases the surface area of

the enzyme activity, which improves enzyme hydrolysis rate. The biomass is subjected to high temperature (160-260°C) and pressure for a short period of time (10 minutes) and then suddenly depressurized, which rapidly disrupts the structure of the biomass. This process offers several attractive features such as it is able to generate complete sugar recovery, utilization of low capital investment and low environmental impacts and less hazardous chemicals and conditions (Avellar & Glasser 1998). The efficiency of steam pretreatment can be effectively enhanced in the presence or absence of catalysts such as H_2SO_4, CO_2 or SO_2 (Biermann et al. 1984.). Without any catalyst, the process is catalyzed through autohydrolysis. A Steam explosion in the presence of acid catalyst has been found to be the most successful in improving enzymatic hydrolysis and to reduce the formation of inhibitory compounds. The addition of an acid catalyst has been also described to increase cellulose digestibility and improve hydrolysis of hemicellulose (Sun and Cheng, 2002). Steam explosion method has been widely used for the pretreatment of a wide range of raw materials such as wheat straw (Ballesteros et al. 2006), olive tree wood (Cara et al. 2006) and poplar wood (Oliva et al. 2003).

Tengborg et al. suggested two-step pretreatment technique to increase the sugar recovery (Tengborg et al. 1998). In the initial step, hemicellulose fraction was pretreated at low temperature to solubilize and later the cellulose fraction was pretreated at a higher temperature (above 210°C). This method has attractive advantages such as higher ethanol yield, better utilization of raw materials and less requirement of enzymes during the hydrolysis process. However, an economic evaluation is required to determine the effectiveness of an additional steam explosion (Söderström et al. 2002).

There are few disadvantages for steam explosion such as the formation of inhibitory products during downstream processing, high energy consumption, and possessing lowest energy efficiency (Liu et al. 2010). Another disadvantage is the partial degradation of hemicelluloses and generation of toxic compounds, which could affect the hydrolysis and fermentation process (Oliva et al. 2003). Acetic acid and uronic acid released from hemicellulose and formic and levulinic acids resulting from sugar degradation contribute to acidification and can inhibit downstream biochemical processes (Jönsson & Martín 2016).

b) **Ammonia Pretreatment:** Biomass is treated with aqueous ammonia at high temperature, which disrupts lignin and removes hemicellulose, while cellulose is de-

crystallized. There are different types of ammonia pretreatment: i) Ammonia Fiber Explosion-Method (AFEX), ii) Ammonia Recycle Percolation (ARP), and iii) Soaking in Aqueous Ammonia (SAA). In AFEX, biomass is exposed to liquid ammonia (in 1:1 ratio) at high temperature (90-100°C, 30-60min) with the pressure of 10-20 atmosphere, followed by immediate depressurization of the system. The desired temperature was held for 5 min, the valve is opened which explosively releases the pressure leading to evaporation of ammonia and drop in the system temperature (Alizadeh et al. 2005). This method causes swelling and phase change in cellulose crystallinity of biomass leading to increasing the reactivity of the leftover carbohydrates after pretreatment. The lignin structure is modified thereby increasing the digestibility and water holding capacity. If parameters of AFEX such ammonia loading, temperature, water loading and blowdown pressure, time and number of treatments are optimized then, 90% of cellulose and hemicellulose could be converted to fermentable sugars (Holtzapple et al. 1991). When compared to other pretreatment methods, AFEX treatment does not produce inhibitors and significantly cost effective due to the absence of additional steps such as recovery and reuse of large quantities of water, detoxification etc., which is highly desirable for downstream processing. The biomass recovered using this process is dry as water is not used. The biomass is stable for long periods and no degradation of cellulose and hemicelluloses was observed. The main advantage of the process is that nearly all the used ammonia can be recovered and reused. AFEX treatment has been used for various raw materials such as corn stover (Alizadeh et al. 2005), corn fiber (Moniruzzaman et al. 1815) and coastal bermudagrass (Holtzapple et al. 1991).

In Ammonia Recycle Percolation treatment, the biomass is pretreated with aqueous ammonia (5-15%) in a flow-through column reactor. The liquid flows at high temperature (140-210°C) through the reactor column, which is packed with biomass with the reaction time of 90min and 5 ml/min percolation (Kim et al. 2003; Sun & Cheng 2002). The solid fraction, rich in cellulose and hemicellulose, is separated from the liquid after the reaction. The ammonia is separated and recycled in this method. The main advantage of ARP is the ability to remove a large amount of lignin (75-85%), it solubilizes hemicelluloses but cellulose remains unaffected with limited production of inhibitors (Alvira et al. 2010). It results in a short-chained cellulosic material with high glucan content. The reactions occurring in the ARP are influenced by factors such as ammonia concentrations, temperature, reaction time, and the amount of liquid

throughput. The main drawback for ARP is high-energy costs and liquid loadings. ARP has been used for the pretreatment of herbaceous biomass like corn cobs/stover mixture (CCSM) and switchgrass (Iyer et al. 1996), corn stover (Tae & Lee 2005) and hybrid poplar milled from the whole tree (Yoon et al. 1995).

In Soaking in Aqueous Ammonia pretreatment method, the biomass is soaked in 15-30% dilute aqueous ammonia solution at a lower temperature (30–75°C). It effectively removes the lignin in the raw material by minimizing the interaction with hemicellulose resulting in the increase of surface area and pore size. This has mild reaction conditions when compared to AFEX and ARP pretreatment. The method has experimented on different biomass such as corn stover (Kim & Lee 2007) and barley hull (Kim et al. 2008).

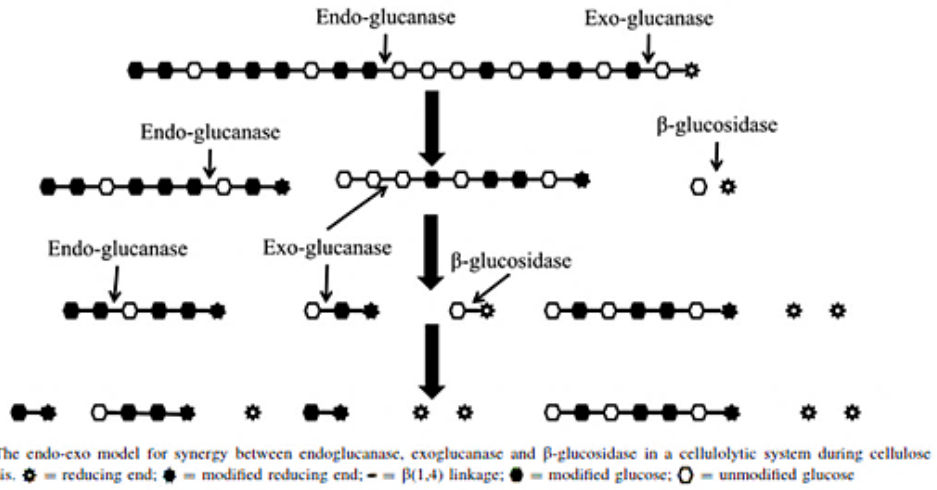
1.3.4 Biological Pretreatment

Biological treatment compared to conventional methods such as chemical and physical pretreatments is considered as an efficient, low-energy process and is environmental friendly. This method primarily uses microorganisms like white-, brown and soft-rot fungi or bacteria, which are capable of producing biomass-degrading enzymes. Fungi have distinct degradation characteristics on lignocellulosic biomass. It is a safe and environment-friendly method that does not require high energy or expensive equipment for lignin removal from the biomass despite extensive lignin degradation (Shrivastava 2008). This method has the higher yield of desired products when compared to other methods. Brown rots mainly degrade cellulose, whereas white and soft rots degrade both cellulose and lignin. The degradation occurs through the action of lignin degrading enzymes such as peroxidases and laccase (Galbe & Zacchi 2007). Lignin degradation by white- rot fungi occurs due to the presence of lignin degrading enzymes such as peroxidases and laccases with highest efficiency of pretreatment (Kumar et al. 2009). White-rot fungi *Phanerochaete chrysosporium* is known to give the highest efficiency pretreatment due to its high growth rate and lignin degradation capabilities. Simultaneous pretreatment and saccharification of rice husk by *P.chrysosporium* were studied. During the treatment, highest reducing sugar was obtained on the 18^{th} day. Fifty-one fungal isolates, which belong to white rot *basidiomycete Punctularia* sp. TUFC20056 and an unidentified *basidiomycete* TUFC20057 showed 50% lignin removal. White rot fungus *Irpex*

lacteus was reported to give promising effect for biological pretreatment of corn stalks. During the treatment, it can produce various oxidative and extracellular hydrolytic enzymes. Several white-rot fungi such as *Ceriporia lacerata, Cyathus stercolerus, Ceriporiopsis subvermispora, Pycnoporus cinnarbarinus* and *Pleurotus ostreaus* have been studied on different lignocellulosic biomass showing high delignification efficiency (Kumar et al. 2009).

1.4 Hydrolysis of Lignocellulosic Biomass

Hydrolysis breaks down the hydrogen bonds in the lignocellulosic materials into their sugar components: pentoses and hexoses, which are fermented into bioethanol. Commonly used hydrolysis methods are chemical hydrolysis (dilute and concentrated acid hydrolysis) and enzymatic hydrolysis. In the chemical hydrolysis, the pretreatment and the hydrolysis may be carried out in a single step. In this method, the degradation of glucose into HMF and other non-desired products is unavoidable. Currently, enzymatic hydrolysis of cellulose is carried out using cellulolytic enzymes with better results for subsequent fermentation because no degradation products of glucose are formed. Enzymatic hydrolysis is cost-effective when compared to acid or alkaline hydrolysis because of its mild conditions (pH 4.8 and temperature 40-50°C). Both bacteria and fungi can produce cellulases for the hydrolysis of lignocellulosic materials. Because most bacteria are anaerobes with low growth rate, most research works are focused on fungi for commercial production of cellulose. Fungi such as *Sclerotium rolfsii, P. chrysosporium* and species of *Trichoderma, Aspergillus, Schizophyllum* and *Penicillium* have been reported to produce cellulose.



The endo-exo model for synergy between endoglucanase, exoglucanase and β-glucosidase in a cellulolytic system during cellulose hydrolysis. ✪ = reducing end; ● = modified reducing end; ━ = β(1,4) linkage; ● = modified glucose; ○ = unmodified glucose

Figure 1.5 Schematic representation of mechanism of cellulase enzyme on cellulose (Saini et al. 2015)

1.5 Production of Cellulolytic Enzymes by *Trichoderma* Sp.

Trichoderma sp. is opportunistic avirulent plant symbionts fungi, abundant in nature, frequently found in all types of soil, manure and decaying plant tissues. Their dominance in soil may be attributed to their diverse metabolic capability and diverse heterotrophic interactions including decomposition of woody and herbaceous material, parasitism, and even opportunistic endophytes. They colonize readily in the presence of high levels of plant roots which are reflected both by their efficient utilization of the substrate as well as their secretion capacity for antibiotic metabolites and enzymes. Some strains are highly rhizosphere competent, i.e., able to colonize and grow on roots as they develop. In addition, they attack, parasitize and otherwise gain nutrition from other fungi. Till date, the majority of enzymes from fungi are being developed and tested for lignocellulose degradation.

Trichoderma sp. is able to degrade domestic waste relatively quickly without emitting bad odors (Elad 2000; Harman et al. 2004). Filamentous fungi, particularly *Trichoderma* sp. are good producers of lignocellulolytic enzymes from the industrial point of view due to extracellular release of the enzymes, higher yield when compared to yeast and bacteria, non-pathogenic and can be easily cultivated (Haltrich et al. 1997). These versatile fungi are commercially used in the various field such as food and textile, biocontrol agents, plant growth promotion, pulp and paper industry etc.

Trichoderma sp. is considered to be a good producer of cell wall degrading enzymes such as cellulase, chitinase, β-glucanase and xylanase in solid-state fermentation (SSF) which is gaining popularity for enzyme production as it is very simple and economical (Sun et al. 2010). Development of an economic process for the bulk production of enzyme cocktail comprising Cellulase, Xylanase, and β-1,3-glucanase through Submerged Fermentation (SmF) is hindered because of the high costs of media ingredients, chemicals, accumulation of secondary metabolites, operation and labor cost, etc. To overcome these bottlenecks, Solid State Fermentation (SSF) is an attractive process to produce a mixture of enzymes economically, with low capital investment and low operating cost due to the utilization of agro-industrial wastes for the production of enzymes in concentrated solutions with higher yield compared to SmF (Pandey et al. 2000). Several methods have been reported on the purification of enzymes such as ammonium sulfate precipitation, ion exchange or affinity chromatography, gel permeation, aqueous two-phase partitioning, hydrophobic interaction chromatography (HIC) and preparative electrophoresis (Sandrim et al. 2005). Most of the

techniques involve a number of steps, and the scale-up of these methods is difficult and also expensive. In this regard, three-phase partitioning is a simple and often used one-step procedure, for effective separation and purification of enzymes and proteins. This emerging method of purification has been reported for exo-polygalacturonase (Dogan & Tari 2008), beta-galactosidase (Choonia & Lele 2013), proteases (Chaiwut et al. 2010), laccase (Vinoth Kumar et al. 2012) invertase (Talekar et al. 2012) and xylanase (Periyasamy et al. 2016a)

Converting lignocellulosic biomass into fuels and chemicals along the standard biochemical route entails a physicochemical pre-treatment of the biomass, followed by enzymatic hydrolysis of the polysaccharide components cellulose and hemicellulose into monomeric sugars. These sugars can then be further fermented into ethanol or other desired compounds. Hence, with the aid of *Trichoderma* sp. economically reasonable production of second generation biofuels from waste products is on the way.

Xylanases (1,4-P-D-xylan xylanohydrolase; EC 3.2.1.8) are hemicellulases that hydrolyze xylan, which is a major constituent of the hemicellulose complex. It hydrolyzes xylan by cleaving the β-1,4-glycosidic linkages randomly to releases xylose and xylobiose. This enzyme is extensively used in food processing, chemical and pulp industries such as biopulping, biobleaching, clarifying and liquefying fruit and vegetable juices. In paper and pulp industries use of xylanase causes decrease in consumption of chlorine, Absorbable Organic Halogen (AOX), Chemical Oxygen Demand (COD) and improves thereby the quality of wastewater.

Cellulases are a multi-enzyme system that hydrolyzes cellulose. The enzymatic hydrolysis of cellulose to glucose requires the synergistic action of cellobiohydrolase or exoglucanase (E.C.3.2.1.91), endoglucanase or carboxymethyl cellulase (E.C. 3.2.1.4) and cellobiase or β-glucosidase (E.C.3.2.1.21) (Yin et al. 2010).

β-1,3-glucanases consist of two enzymes namely exo-glucanase (EC 3.2.1.58) and endoglucanase (EC 3.2.1.39) that act on (1, 3) and (1, 6) position of β-D-glucan to release glucose (Giese et al. 2012).

Insoluble enzyme aggregates engendered by extensive chemical cross-linking of enzyme dissolved in a solution have been recommended as an alternative approach for obtaining stable and reusable enzyme preparations. High enzyme loadings, thermos-stability and

operational stability of lignocellulolytic enzymes are major bottlenecks for the commercial processing of pre-treated lignocellulose biomass. Enzyme immobilization offers the lucrative prospect of improving thermostability and operational stability.

1.6 Enzyme Immobilization

Biocatalyst has many benefits for developing green and sustainable processes for the chemical industry. Biocatalyst, in their native form, has wide application in biochemical, biomedical, biotechnological, and food industrial fields mainly because of its mild reaction conditions, high activities and stereo-, regio-, and chemo-selectivities. Enzymes catalyze a chemical reaction by increasing the rate of the reaction without themselves being during the reaction. However, the use of enzymes in the industrial application is usually hampered by the lack of operational and storage stability, recovery, and reuse of the enzyme. Many techniques are available to overcome these drawbacks and improve the features of the enzymes and, immobilization is one of the technique. Immobilized Biocatalysts have recently gained much attention among many biotechnologists. Immobilization is a process in enzymes are physically confined or localized in a certain defined region of space with retention of their catalytic activities, and by using it repeatedly and continuously. Generally, the solid support system stabilizes the structure of the enzymes and thus, maintaining their activities. It consists of two essential components: the non-catalytic structural component (carrier), which is designed to aid separation and reuse of the catalyst and facilitates control of the process, and the catalytic functional component (enzyme), which is designed to convert the substrates of interest into the desired products (Cao et al. 2003). The performance of the immobilized enzyme depends on the characteristics of the matrix used as a support. Usually, inert polymers and inorganic materials are used as carrier matrices for the binding of the enzymes for immobilization. The carrier matrices should be inert, physically strong and stable, cost effective ; it should be easy to regenerate and should also reduce product inhibition. The physical characteristics of the matrices such as mean particle diameter, swelling behavior, mechanical strength and compression behavior are some of the major important features required for the performance of the immobilized systems and will determine the type of reactor employed under technical conditions. Nonporous supports have diffusional limitations with a low loading capacity and therefore porous supports are generally preferred because of the high surface area allows a higher enzyme loading and the immobilized enzyme receives greater protection from the environment. The hydrophilic character of the enzyme on

immobilization is one of the most important factors determining its level of activity. The enzymes can remain attached to the support via interactions ranging from reversible physical adsorption and ionic linkages to covalent bonds.

1.6.1 Advantages of Immobilization

There are several advantages of immobilized enzymes when compared to the free enzymes. Immobilized enzymes can be used multiple times thereby reducing the production cost with efficient recycling. They are usually more stable compared to the free enzymes and prevents degradation and deactivation of the enzymes. The products are free from contamination with the enzymes and are useful in the development of a multi-enzyme reaction system. The reaction with the immobilized enzyme can be immediately stopped by separating it from the reaction solution while with free enzymes it cannot be retrieved from the reaction mixture. The availability of a robust immobilized enzyme saves cost not only for the process development but also for its production.

1.6.2 Disadvantages of Immobilization

With all the advantages of the immobilized enzyme, there are also certain drawbacks in this method. It requires expensive experimental set up as well as additional time. Certain immobilization methods cause a problem with respect to the diffusion of the substrate on the enzyme. The enzyme may be less active as they are not freely available to the substrate for reaction. When they are mixed in a reaction solution with substrates, they might cause contamination and sometimes can't be recovered once the reaction is over. The immobilized enzymes are wasted and their reusability becomes a mandatory factor. So, numerous efforts have been made to the development of insoluble immobilized enzymes with functional efficiency and enhanced reusability as alternatives.

1.6.3 Types of Immobilization

There are several methods for immobilization based on the matrix or support and the types of bond involved. They are categorized as:

A. Physical method of immobilization.
   - Adsorption or carrier binding method.
   - Entrapment method.

- Encapsulation method.

B. Chemical method of immobilization.

- Covalent binding – support or cross-linking method.

1.6.3.1 Adsorption or Carrier Binding Method

Adsorption is one of the easiest and traditional methods of reversible enzyme immobilization where the enzymes are adsorbed to the external surface of the support/carrier materials through weak non-specific forces such as van der Waals, hydrophobic interactions and hydrogen bonds. Under mild conditions such as a change in pH, ionic strength or substrate concentration, the reversible immobilized enzymes can be removed from the support material because the bonds are so weak. This method is highly attractive because it is economically feasible and the support can be regenerated and reload with fresh enzymes when the enzymatic activity starts deteriorating. (Mohamad et al. 2015). The carrier used for immobilization in adsorption method can be divided into organic and inorganic carriers. The organic carriers used are chitosan, chitin, alginate, and cellulose, also the synthetic compounds, mainly polymers, while the inorganic carriers include hydroxyapatite, silicas, and titania. These matrices can be easily chemically altered to match requirements for a given enzyme and its application.

The immobilization of the enzymes on the solid carriers can be obtained using a wide variety of chemical and physical methods (Cao, 2005; R. a Sheldon, 2007). Physical absorption on an inert carrier requires soaking the enzyme solution with the carrier material and incubate for the absorption to occur. This method can be achieved chemically via direct covalent bonding between the enzyme solution and the electrode surface. However, these weak non-specific interactions do not alter the native structure of the enzyme, which prevents the active site of the enzyme from displacing thus retaining its activity (Hwang and Gu, 2013). The principles of protein-ligand interaction in chromatography are used in enzyme immobilization through ionic bonding between the enzyme and the support system. This is a very simple and reversible method but difficult to find conditions where the enzymes remain fully active and strongly bound to the support material. However, there are certain disadvantages in this method such as steric hindrance near the active center of the enzyme near or the electric charge of the enzyme is disturbed which reduces the activity of the enzyme. Another physical method for immobilization of enzymes is affinity binding in which the affinity between the enzyme and the carrier is very important. Affinity binding principle employs the selectivity

between complementary biomolecules in enzyme immobilization. The binding between enzyme and carrier occurs in two ways: i) an affinity ligand for the target enzyme is pre-coupled with the support ii) the enzyme is conjugated to an entity that develops an affinity towards the support. Further, the affinity binding was modified as bioaffinity layering that increases enzyme-binding capacity exponentially and reusability through several interactions such as van der Waals forces, hydrogen bonding and coulombic (Datta et al. 2012; Mohamad et al. 2015). Hydrophobic interactions are also employed to adhere the enzyme and the support material. This interaction is produced during immobilization when an enzyme molecule displaces a substantial amount of water molecules both from the support and its own surface (Hanefeld et al. 2009).

1.6.3.2 Entrapment Method

Entrapment is an irreversible method of enzyme immobilization where the enzymes or cells are trapped inside a porous/polymer matrix allowing diffusion of the substrate and the products. It is also defined as the physical restriction of an enzyme within a defined space or network. The main advantage of using this method is that they are easy, fast, and cheap and usually requires mild conditions. There are various ways for entrapping enzymes such as in a silica sol–gel, or an organic polymer network (gel lattice) or a microcapsule or a membrane device such as a hollow fiber. Entrapment is carried out by mixing the biocatalyst into a monomer solution, followed by polymerization initiated by a change in temperature or by a chemical reaction. Polymers like polyacrylamide, collagen, cellulose acetate, calcium alginate or carrageenan etc. are used as matrices for the entrapment method.

Entrapment method is classified into two lattice and microcapsules types. In the lattice type entrapment, the enzymes are entrapped within the interstitial space of a cross-linked water insoluble polymer. Synthetic polymers such as polyacrylamide, polyvinyl alcohol and natural polymers (starch) are utilized for immobilizing the enzyme. Calcium alginate is widely used matrices for entrapment method. It depends on the formation of crossed-linked mediated by calcium ions.

Alginate–gelatin– calcium hybrid carriers have been used for efficient encapsulation that prevents enzyme leakage and increased operational stability of the enzyme (Shen et al. 2011). Avnir et al described the successful immobilization of the enzyme in silica sol-gels prepared by hydrolytic polymerization of tetraethoxysilane (Avnir et al. 1994). polyethylene glycol, polyvinyl alcohol,

and albumin can be used as additives that can have a stabilizing effect on sol-gel entrapped enzymes. Microencapsulation involves the formation of spherical particles (microcapsule) in which the enzymes are confined inside a semi-permeable membrane. The effectiveness of the immobilization depends on the stability of the enzymes. Cellulose nitrate and nylon-based membranes are used for this method. Liposomes, due to their capability to support water-soluble enzymes within their aqueous medium are employed for encapsulation method (Walde and Ichikawa, 2001).

The limitation of this method is that the enzymes may leach out into the surrounding medium and the substrate cannot diffuse deep into the gel matrix. Binding to or encapsulation in a carrier inevitably leads to decrease in enzymatic activity and, consequently, leading to lower productivities due to a large proportion of a non-catalytic mass (R. A. Sheldon, 2011). In recent years, there is an increased interest in carrier-free immobilized enzymes, such as Cross-Linked Enzyme Crystals (CLECs), Cross-Linked Dissolved Enzymes (CLEs) and Cross-Linked Enzyme Aggregates (CLEAs). This method has major advantages, such as high stability highly concentrated enzyme activity in the catalyst, and economically feasible as a carrier molecule is not required. Their high resistance under severe conditions of pH, temperature and, particularly, in organic solvents as compared with their free enzymes and conventional carrier-bound immobilized enzymes has made them an attractive method.

1.7.3.3 Cross-Linking Method

Beginning of the 1960s, research in the field of solid phase protein chemistry led to the evolution of cross-linking of enzymes (R. a Sheldon, 2007). It is a method of irreversible enzyme immobilization which does not require a support system to prevent the loss of enzyme into the substrate solution (Mohamad et al. 2015; Wang et al. 2008). Immobilization by cross-linking of enzyme molecules affords carrier-free immobilized enzymes with high productivities and the resulting immobilized biocatalyst contains a high proportion of active enzyme. Cross-linking is a method of formation of intermolecular cross-linkages via the covalent bond between the enzyme molecules by means of bi- or multifunctional reagents producing aggregates in the presence or absence of support material. However, several drawbacks are there in the CLEAs by this method, such as poor reproducibility, low activity retention, low mechanical stability, and handling difficulties due to the gelatinous nature of CLEs (R. a. Sheldon, 2007). The use of CLECs was introduced in the early 1990s by Altus Biologics, where the enzymes are cross-linked in their crystalline state (St. Clair and Navia,

1992). Initial studies conducted with CLECs of thermolysin and subsequently proved to be applicable to a broad number of enzymes. The CLECs proved to be more stable to organic solvents; denaturation by heat, proteolysis than the corresponding soluble or lyophilized enzyme, high catalyst and volumetric productivities makes them ideal for industrial biocatalysis. However, CLECs have an inherent drawback: enzymes need to be crystallized, which is a difficult method and demands high purity enzyme, leading to their high costs. This led to the evolution of a new method of immobilized enzymes; cross-linked enzyme aggregates (CLEAs) (Cao et al. 2000). This idea was first introduced with penicillin G amidase by Sheldon (Cao et al. 2000). CLEAs are very efficient as biocatalysts as they are not expensive and there is no requirement of high purity of the enzyme. The synthesis is easy and exhibits competent stability and performance for selected applications and the CLEAs can readily be reused. The CLEAs method essentially consists of two processes, i.e. purification, and immobilization, into a single procedure causing minimum damage to the three-dimensional tertiary structure of the enzyme.

1.7 Preparation of Cross-Linked Enzyme Aggregates

Immobilization of enzyme using the CLEAs technique involves two steps: i) precipitation of soluble enzyme and ii) cross-linking of the aggregated enzymes. Initially, soluble protein molecules are aggregated by non-denaturing methods. Generally used precipitant are ammonium sulfate, polyethylene glycol (PEG 6000, PEG 8000), or *tert*-butyl alcohol. The aggregated proteins are cross-linked to each other by a cross-linking reagent and primary amino acid residues on the external surface of the enzyme leading to the formation of aggregates insoluble, stable and catalytically active. The cross-linking of these aggregated enzymes makes the enzyme permanently insoluble while retaining their catalytic activity as well as the structure of the enzymes. The best precipitant and cross-linker may differ from one another because of the difference in their structural and biochemical properties of enzymes. Apart from the properties of enzymes, factors such as the method of precipitation process, the concentration of cross-linking agent, and temperature and pH conditions play an important role in the formation of CLEAs (Cao et al. 2000; Sangeetha & Emilia Abraham, 2008; R. a Sheldon, 2007).

In this technique, the principal method of enzyme immobilization involves the initial precipitation of the enzymatic aggregates by the addition of salt, organic solvents, nonionic

polymers to the aqueous solutions of proteins, which are held together by non-covalent bonding without the disturbance to their tertiary structure and prevents denaturation.

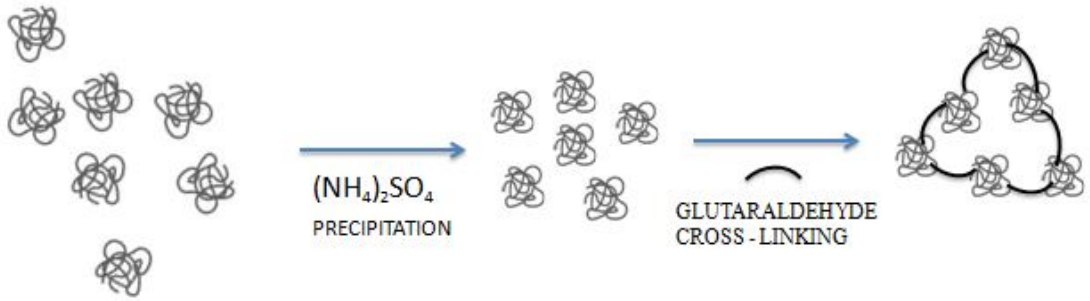


Figure 1.6 Enzyme immobilization using the CLEAs technique

Cross-linking gives a more stable structure and negates the possible loss of the aggregates even after removing the precipitating agent. The CLEAs establishes covalent bonds between the enzymatic protein molecule and the cross-linking reagent making them permanently insoluble (R. a Sheldon, 2007). Another important factor in the preparation of CLEAs is the cross-linking ratio i.e. the mass ratio between cross-linking agent and the enzyme molecule. Low concentrations of the cross-linking agent cause insufficient cross-linking resulting in unstable and fragile CLEAs causing leaching of the enzymes in the surrounding environment. While enormous concentration causes excessive cross-linking resulting in complete loss of enzymes flexibility thereby inhibiting the catalytic activity. Wilson et al. found that excess concentration of cross-linking agent inhibits the performance of the CLEAs of penicillin acylase for the production of cephalexin (Wilson et al. 2009). As every enzyme has a unique surface structure, comprising varying numbers of lysine residues, the optimum cross-linking ratio has to be determined for each enzyme for obtaining the desirable immobilization. Enzymes with few or no accessible lysine residues, insufficient cross-linking occur leading to CLEAs that are unstable and the enzyme leaches out in the aqueous media. This problem has been overcome by a novel methodology in which the CLEAs were prepared by co-aggregation of the Glutaryl Acylase (GAC) with an aminated polymer polyethyleneimine (López-Gallego et al. 2005). When the high concentration of cross-linking agent affects the activity of the enzyme or low concentration of enzyme in the protein aggregates, the addition of Bovine Serum Albumin (BSA) as a proteic feeder facilitates the formation of cross-linked enzyme aggregates (CLEAs) (Shah et al. 2006). Several enzymes such as aminoacylase (Dong et al. 2010), lipase, penicillin G amidase (Shah et al. 2006) have been successfully

immobilized as CLEAs with the recovery of high activity and specific activity directly from the crude fermentation broth.

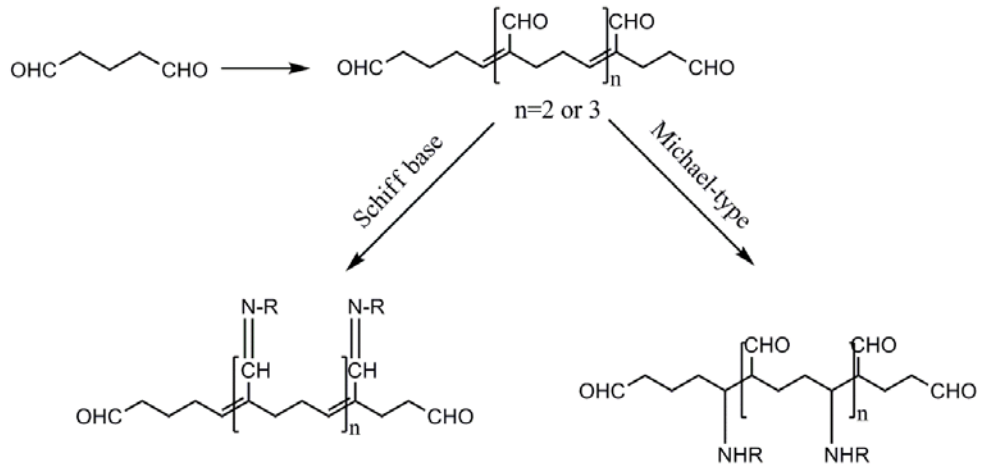
CLEAs has several benefits such as they are easy to prepare from crude enzyme extracts, and the costs of the expensive carrier are avoided. They generally show enhanced storage and operational stability towards denaturation by heat, organic solvents, autoproteolysis and prevents leaching in aqueous media. They are easy to recover and recycle with high productivity. CLEAs has the advantage of co-immobilizing two or more enzymes that are competent of catalyzing multiple biotransformations, independently or in sequence as catalytic cascade processes in a solid catalyst, all immobilized enzymes might have loss of activity due to diffusional limitations which are prevented in CLEAs as they are highly porous materials when used in biocatalytic transformations (Sheldon 2011).

Glutaraldehyde is a bifunctional, 5-carbondialdehyde compound, clear and colorless to pale straw-colored, pungent oily liquid that is soluble in all proportions in water, alcohol, and organic solvents. It is essentially available as acidic aqueous solutions (pH 3.0–4.0), in varying concentration from less than 2% to 70% (w/v). Among the commercially available cross-linking agents, glutaraldehyde (pentanediol) has gained the widest attention because of its commercial availability and high reactivity and low cost. They react rapidly with the primary amine group at neutral pH due to the presence of numerous reactive residues leading to various possible reaction mechanism (Migneault et al. 2004).

Glutaraldehyde easily reacts with several functional groups of proteins such as α-amino groups, thiol, phenol and imidazole but primary amino groups are the most reactive moieties (Habeeb et al. 1968). Due to the polarity of the amine groups, lysine residues are located on the protein surface exposed to the aqueous medium. At least 13 different structural forms can be represented depending on medium conditions such as temperature, pH, concentration, etc. Glutaraldehyde exists in monomeric and predominantly in a cyclic hemiacetal form in dilute solution and in the pH range of 3.0-8.0.

The monomeric form of glutaraldehyde's crosslinking reaction with the ε-amine groups of the lysine residues; involves the nucleophilic attack of the amino acid group on the aldehyde groups to yield a non-conjugated Schiff base, which is unstable under acidic conditions (Goldstein, 1976). At alkaline pH, crosslinking polymerize the monomeric glutaraldehyde. Crosslinking with polymeric glutaraldehyde occurs via two reactions: (I) Schiff base

formation: reaction between aldehyde groups of glutaraldehyde and the ε-amino groups of proteins (Monsan et al. 1976). Under acidic conditions, Schiff bases are unstable and tend to break down to regenerate the aldehyde and amine and (II) the reaction involved in the conjugate addition of another ε-amino group to the double bond to the α,β- unsaturated aldehyde groups of polymeric glutaraldehyde (Michael-type 1,4 addition) (Richards and Knowles, 1968) (Figure 1.8). The cross-linking mode is also dependent on the pH condition of the mixture (Wine, 2007).



(Source:Migneault et al. 2004)

Figure 1.7 Mechanism of cross-linking with glutaraldehyde

Enzymes such as nitrilases show less or no retention of activity when glutaraldehyde is used as a cross-linker. This could be due to the deactivation caused by the reaction of the cross-linker with amino acid residues that are crucial for the activity of the enzyme. This drawback could be avoided by using bulky poly aldehydes, obtained by periodate oxidation of dextrans as the cross-linkers (Mateo et al. 2004) and irreversible amine linkages were formed by reduction of the Schiff's base moieties with sodium borohydride.

1.8 Combined Cross-Linked Enzyme Aggregates (Combi-CLEAs)

CLEAs have been successfully applied in many biocatalytic processes including lignocellulosic bioconversions (Bhattacharya and Shrivastava, 2013; R. a. Sheldon, 2011; Talekar et al. 2013a). Improving the enzyme synergy of lignocellulolytic enzymes through CLEAs can be accentuated to a new level by simultaneously capturing multiple enzyme activities in a single CLEAs (Cao et al. 2003; Dalal et al. 2007; Sheldon et al. 2005). Combi-

CLEAs are a novel prospect for immobilization of a mixture of enzymes. Development of combi-CLEAs is based on the well-established carrier free enzyme immobilization technique of cross-linked enzyme aggregates (CLEAs). It involves multipoint attachment through intermolecular cross-linking between enzyme molecules and offers a number of potential benefits because there is no support, there is no dilution of catalytic activity by non-catalytic mass (Cao et al. 2003; Habulin et al. 2011). In general, combi-CLEAs are prepared by co-precipitating two or more soluble enzymes by agents such as inorganic salts or organic solvents to form aggregates without denaturation and subsequently linking the aggregates by using bifunctional agent such as suitable dialdehydes, usually glutaraldehyde (Dalal et al. 2007). Co-immobilized enzymes are more advantageous than conventional methods. They high specific enzyme activity increased space-time yields and catalyst productivity and low production cost. Moreover, co-immobilization of enzymes using combi-CLEAs does not require purified enzymes. Combi-CLEAs have been mostly used for attaining two sequential steps for a single biotransformation process. The co-immobilized enzymes multi-step enzymatic process can be combined into a one-pot, enzymatic cascade process without the need of separation of intermediates. The enhanced storage stability and reusability can significantly reduce the cost of enzymes and thus makes the industrial application economically feasible (Wu et al. 2015; Xiaoling Wu, 2015). There are several reports on co-immobilization of two or three soluble enzymes in a single CLEAs (Jung et al. 2013; Talekar et al. 2013b; Zhang et al. 2014).

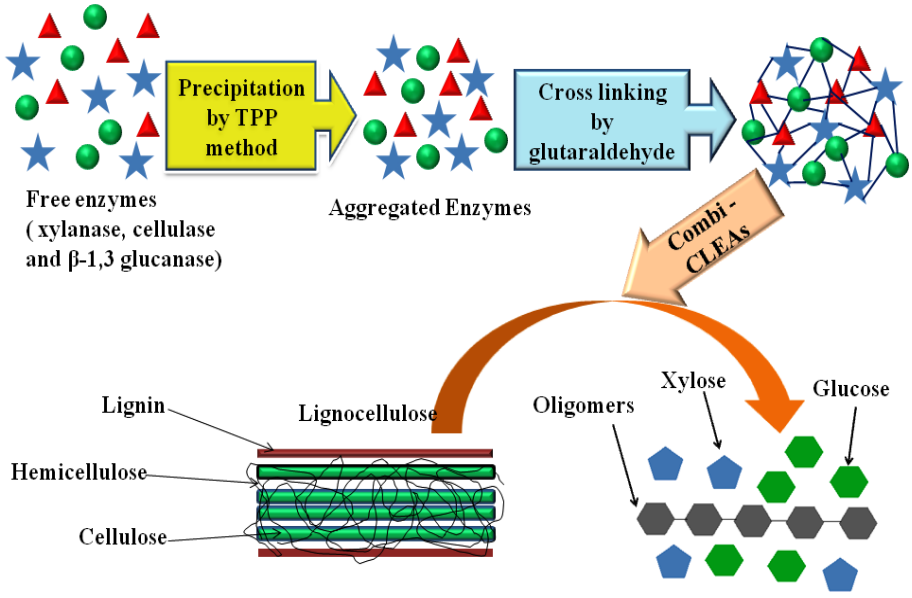


Figure 1.8 Schematic representation of combi-CLEAs preparation

1.9 Magnetic CLEAs

The magnetic-CLEAs methodology has been successfully employed for immobilization of a variety of enzymes. The most important reason for immobilizing enzymes is the easy recovery of the enzyme from the reaction medium and subsequent reuse of the biocatalyst. The major drawback of carrier free cross-linked enzyme aggregate is not easily recoverable from the reaction medium by conventional separation methods such as filtration and centrifugation (Montoro-García et al. 2010). To overcome these problems, covalent linking of enzymes onto the surface of Iron Oxide Magnetic Nanoparticles (IOMNPs) have attracted utmost attention because of simple preparation, allow for high specific enzyme activity and enhanced stability whereas maintaining maximum catalytic efficiency over several reaction cycles and also it consists of three functional parts which includes a magnetic core, an outer coating to shield the magnetic core and functionalized surface coating. IOMNPs are attracted to a magnetic field, but they retained residual magnetism in the absence of magnetic field. Hence the solution containing suspended IOMNPs can be recovered from a reaction mixture using an external magnetic field. It serves as a highly suitable catalyst support aiding enzyme immobilization and magnetic recovery of the catalyst. Cross-linking of a large number of different enzymes may be coated on the surface of IOMNPs due to their size and large surface area. This will also help in cut down the additional purification to remove the catalyst from the reaction mixture. Recently magnetic nanoparticles have attracted much attention as an alternative support material for enzyme immobilization (Dyal et al. 2003; Lu et al. 2007). If the size of the support material is reduced to the nanometer scale, high surface areas can be obtained, thus increasing the enzyme loading per unit mass of support. The nanoparticles where enzymes are attached when compared to porous carrier materials are subjected to minimum diffusion limitation. Because of the magnetic nanoparticles' superparamagnetic properties, the immobilized enzyme could be easily controlled and recycled by the application of a magnetic field (Hu et al. 2009). The application for biomolecules immobilization on the solid phase magnetic surface is able to achieve a rapidly easy separation and recovery from the reaction medium in an external magnetic field. In order to increase the loading amount of the biomolecules on the magnetic particles and improve the stability of immobilized biomolecules, the preparation of surface-functionalized magnetic particles with water soluble, biocompatible and reactive groups is much desired.

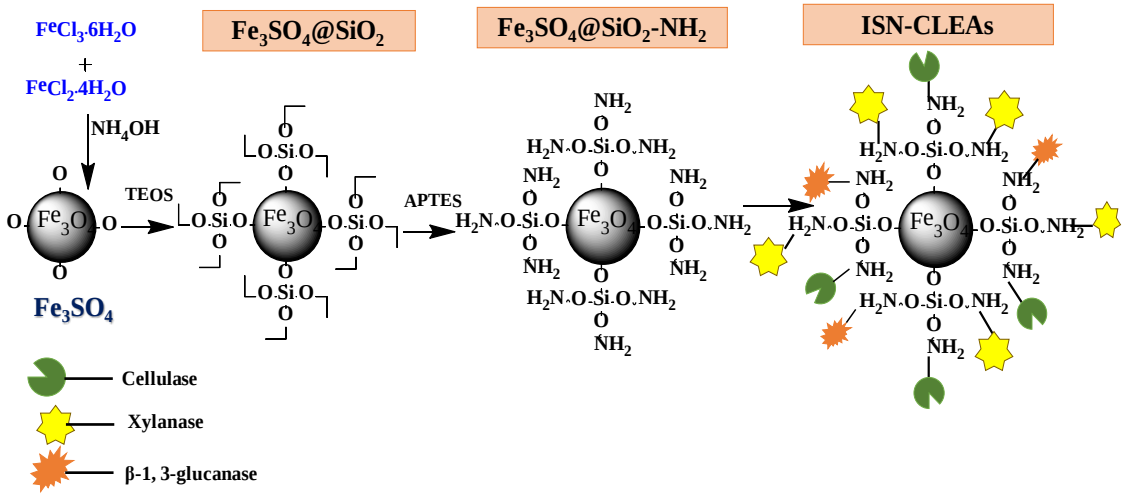


Figure 1.9 Schematic representation of ISN-CLEAs preparation

1.10 Bioethanol Fermentation

Conversion of lignocellulosic hydrolysate to bioethanol involves great challenges such as producing yeast or other microorganisms, which are high yield and tolerance to high ethanol concentration, the need of converting pentose and hexose sugars to ethanol; and resistance to inhibitors present in hydrolysates. Microorganism plays an important role in meeting these challenges during the fermentation process. Microorganisms such as yeast (e.g. Saccharomyces and Pichia species), bacteria (e.g. *Escherichia coli* and *Zymomonas*) and fungi (e.g. *Rizhopus*, *Mucor* and *Rhizomucor*) have been employed and are even genetically modified to achieve these goals.

Efficient production of bioethanol from lignocellulose hydrolysates needs robust glucose and xylose-fermenting and inhibitor resistance microorganisms. Although a number of particular research in lignocellulosic ethanol production has been carried out, there is still a need for robust microbes which can ferment both xylose and glucose effectively to make the process economical. Ko et al. (2016) reported that utilization of dilute acid-pretreated hydrolysates of rice straw and hardwood, co-fermentation of glucose and xylose by engineered isomerase-based xylose-utilizing strain such as Saccharomyces cerevisiae SXA-R2P-E produced 50 g/L of ethanol with a yield of 0.43 g ethanol/g sugars at 72 hours. Novy et al. 2014 investigated with a mutated strain of S. cerevisiae IBB10B05, they used enzymatic hydrolyzates obtained from wheat straw (15% dry mass) pretreated by steam explosion and it contained glucose and xylose in a mass ratio of 2. S. cerevisiae IBB10B05 capable of co-fermenting glucose and xylose to produce ethanol in 2 days. Lau and Dale, 2009 used corn stover as feedstocks and

pretreated using Ammonia Fiber Expansion (AFEX) method. *S. cerevisiae* 424A(LNH-ST) yield 191.5 g ethanol/untreated corn stover with the concentration of 40g/L ethanol. Demeke et al. 2013 studied on the mutated strain of *S. cerevisiae* GS1.11-26, which was capable of fermenting the hydrolysates containing pentose and hexose obtained from a mixture of Arundo donax (giant reed), spruce and a wheat straw/hay biomass. The maximum ethanol yield was 5.8% (v/v). It has been reported that genetically engineered *S. cerevisiae* with bacterial xylose isomerase can utilize xylose rapidly to produce ethanol under strict anaerobic condition (Parreiras et al. 2014). Another study has been conducted on the effect of inhibitory compounds in bioethanol production and reported that recombinant strain of *S. cerevisiae* (LNH-ST 424A) has the potential to ferment xylose and glucose in the presence of inhibitors such as furfural and acetic acid in hydrolysate after pretreatment process (Lu et al. 2009). According to Sedlak and Ho, 2004, Saccharomyces 424A(LNH-ST) strain containing the cloned xylose-metabolizing genes constructed into the yeast chromosome can efficiently ferment glucose and xylose to ethanol.

1.11 Scope of the Present Work

The present research work deals with the production, characterization, and immobilization of lignocellulolytic enzymes and its application in bioethanol production. The fungal strain of *Trichoderma citrinoviride* AUKAR04 was isolated on the basis of 5.8S ribosomal RNA sequencing. It produces lignocellulolytic enzyme such as xylanase, cellulase and β-1,3-glucanase under solid-State fermentation (SSF) and was partially purified by three-phase partitioning (TPP) method. Further, the enzymes were characterized to check the operational stability such as pH, temperature, metal ions and kinetic parameters. The free form of the enzymes was immobilized using the carrier-free crosslinking method to increase the performance of the enzymes, increase reusability and to prevent degradation and deactivation of the enzymes. Furthermore, the enzymes were immobilized on a magnetic support for better recovery and to increase the reusability and operational stability. Depolymerization of cellulosic biomass by free and immobilized enzymes was carried out and the resulting hydrolysate containing monomers were analyzed.

The scope of the present study is mentioned below:

- Screening of *Trichoderma citrinoviride* AUKAR04 for the production, partial purification and characterization of the lignocellulolytic enzyme by solid-state fermentation.

- To partially purify xylanase, cellulase and β-1,3-glucanase using TPP method by optimizing the effect of pH on crude ammonium sulfate saturation to the t-butanol ratio. Determination of molecular weight of the partially purified enzymes by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis.

- To characterize the effect of pH, temperature, metal ions and kinetic parameters on the activity of xylanase, cellulase and β-1,3-glucanase.

- To prepare and characterize Combined Cross-Linked Enzyme Aggregates (combi-CLEAs) and bi-functionalized magnetic Cross-Linked Enzyme Aggregates (ISN-CLEAs) with xylanase, cellulase and β-1,3-glucanase for the depolymerization of sugarcane bagasse.

- To optimize the effect of precipitant, glutaraldehyde concentration and crosslinking time to obtain the maximum activity recovery of xylanase, cellulase and β-1,3-glucanase in combi-CLEAs and ISN-CLEAs.

- To synthesize and functionalize Fe_3O_4 Magnetic Nanoparticles (IOMNPs).

- To study the surface morphology of combi-CLEAs and ISN-CLEAs by Environmental Scanning Electron Microscope (SEM).

- To analyse thermal stability, storage stability and reusability of free enzymes, combi-CLEAs and ISN-CLEAs.

- To prepare and characterize the sugarcane bagasse biomass and to study the ammonia pretreatment of SCB for ethanol production.

- To analyse the chemical and structural changes in the raw, ammonia pretreated and enzyme treated SCB biomass by using Attenuated Total Reflection-Fourier Transform Infrared spectroscopy (ATR-FTIR), Thermogravimetric analysis (TGA) and X-Ray Diffraction (XRD).

- To study the efficiency of enzymatic hydrolysis of SCB by free enzymes, combi-CLEAs and ISN-CLEAs and to analyze the sugar content by HPLC.
- To investigate the fermentation of SCB hydrolysate containing glucose and xylose by mono-culture fermentation by *Saccharomyces cerevisiae* LGP2Y1 and co-culture fermentation by using *C. utilis* ATCC 22023 and *S. cerevisiae* LGP2Y1 for the production of bioethanol.

CHAPTER 2

MATERIALS AND METHODS

2.1 Isolation of Xylanase-Cellulase Producing Strain

1 gram of soil sample mixed with 100 ml sterile distilled water was spread onto potato dextrose agar (PDA) plate containing beech wood xylan (0.5% w/v). These plates were incubated at 30°C for 48-64 hours. The fungal colonies from the plates were transferred onto the fresh xylan agar plate and CMC agar plates for screening of xylanase and cellulase producing colonies, respectively, which were again incubated at 30°C for 48 hours, and colonies developed on xylan agar and CMC agar were assayed for xylanase and Cellulase production, respectively, by Congo red (0.1%) for 15 min and then washed with 1M NaCl. The colonies showing a zone of clearance around them were picked up and maintained on potato dextrose agar slants at 4°C in a refrigerator. The prominent isolate was identified on the basis of morphological, cultural and 5.8S rRNA partial sequencing and the sequence was submitted in NCBI (http://www.ncbi.nlm.nih.gov/nuccore/KF698728). The strain was cultivated in potato dextrose agar (PDA) at 28ºC for 72 hours and subcultured at monthly intervals.

2.2 Growth media for the production of Xylanase, Cellulase and β-1,3-glucanase production

Trichoderma sp. was cultivated in the growth media containing the following ingredients (Table 2.1). The initial pH was adjusted to 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 by using 0.1N HCl and 0.1M NaOH. 50 ml of the above-mentioned seed media were inoculated with loopful of 48-72 hours old *Trichoderma* sp. spores and incubated at 30ºC for 36 hours with agitation of 180 rpm. These mycelial cells were used as a source of inoculum.

Table 2.1 composition of the growth media

Sl.No	Ingredients	Concentration (g/l)
1	wheat bran(fine)	25
2	lactose	10
3	soya flour	20
4	Corn Steep Liquor	20
5	KH_2PO_4	1
6	NaCl	0.1
7	Tween 20	0.1

2.3 Cultivation of *Trichoderma* sp. in Solid-State Fermentation (SSF)

The cultivation of isolated *Trichoderma* sp. in SSF system was carried out in a shallow aluminum tray of 30 cm x 23 cm x 6 cm. 500 g of wheat bran was moistened with 500 ml of 50 mM sodium acetate buffer (pH 5.0) autoclaved at 121°C for 45 min. After cooling the tray at room temperature, it was inoculated with 300 ml of growth medium with 40-50% of packed mycelium volume. The inoculum and the wheat bran were mixed well by using a sterile spatula in order to ensure a uniform distribution. The trays were incubated at 30ºC for 72 hours. The moisture content in this study was 56%. Experiments were carried out in triplicate, and the obtained results were reported as the mean of the triplicate experiments.

2.4 Extraction of Enzyme Cocktails (Xylanase, Cellulase and β-1,3-Glucanase)

5 g of the fermented solid substrate from each tray was transferred into a 250 ml Erlenmeyer flask and mixed with 40 ml of 0.1% (v/v) Tween-80 in 50 mM sodium acetate buffer (pH-5.0). The samples were then mixed using a rotary shaker (150 rpm) at 25°C for 1 hour. The suspension was centrifuged, and the supernatant were passed through Whatman No. 1 filter paper and the clear cell-free filtrate was used as the enzyme source.

2.5 Assay Methods

Xylanase activity was routinely measured in a reaction mixture (1.0 ml) containing 0.5 ml of 1% (w/v), beech wood xylan (Sigma, #X4252-25G) in 50 mM sodium acetate buffer (pH 5.0) and 0.5 ml of each enzyme solution. The substrate and enzyme solution were pre-incubated separately at 50°C for 5 min, and then the reaction was started by mixing the enzyme with the substrate. After 10 min incubation, the reaction was stopped by the addition of 1 ml of 3, 5-dinitrosalicylic acid (DNS) reagent. The reaction terminated immediately was used as a control. The absorbance of the reducing sugar was measured at 540 nm. The standard graph was prepared using 1 to 5 µmol ml^{-1} xylose in 50 mM sodium acetate buffer. One unit (U) of xylanase activity is defined as a number of enzymes required to release 1 µmol of xylose per minute in the reaction mixture under the specified assay conditions.

Cellulase activity was determined by measuring the amount of glucose released from CMC-Na by modified DNS method (Periyasamy K et al. 2017) with glucose as the standard. The reaction mixture contained 0.5 ml of 1% CMC-Na (Sigma #C5678) in 50 mM citrate buffer (pH 5.0) and 0.5 ml of enzyme solution and was incubated at 50ºC for 10 min. After incubation, the control and the samples were terminated by the addition of 1 ml DNS then kept in the boiling-water bath for 10 min and made it cool down at room temperature. The absorbance of the reaction solutions was measured at 540 nm. One unit (U) of Cellulase activity is defined as a number of enzymes that liberates 1µmol of glucose equivalents per minute under the assay conditions.

β-1,3-glucanase activity was measured by mixing 200 µl of culture filtrates with 200 µL of 50 mM sodium acetate buffer (pH 5.0), containing 0.8% β-glucan from barley (Sigma, #G6513) (Jia et al. 2012). The enzyme assay was carried out at 50ºC for 10 min. The reducing sugar liberated was quantified by DNS method (Miller, 1959). One unit (U) of activity is the amount of enzyme required to release 1µmol of reducing sugar per minute under the above conditions. All assays were performed in triplicates.

2.6 Partial Purification of Enzyme Cocktails by Three-Phase Partitioning (TPP)

2.6.1 Effect of Crude Extract to t-Butanol Ratio on Partitioning of Xylanase, Cellulase and β-1,3-Glucanase

The crude extract was saturated with 55% (w/v) ammonium sulfate and the t-butanol concentration was varied based on volume per volume in the range of 1:0.5, 1:0.75, 1:1, 1:1.25 and 1:1.5 with constant stirring at 25ºC for 1 hour. The mixtures were allowed to stand for 30 min at 25ºC and then subjected to centrifugation at 4000 rpm for 10 min to facilitate separation into three distinct phases (upper organic phase, middle precipitate and lower aqueous phase). The two phases were carefully separated from each other, and the middle precipitate was collected and dissolved in the sodium acetate buffer (10 mM; pH 5.0), and dialyzed against distilled water for 15 hours at 4ºC. The water for the dialysis was changed twice. The ratio which achieved the maximum recovery of enzymes was chosen for further purification procedures.

2.6.2 Effect of pH of Crude Ammonium Sulfate Saturation to t-Butanol Ratio on Partitioning of Xylanase, Cellulase and β-1,3-Glucanase

The pH of ammonium sulfate (55%) saturation was varied between pH 3.0 to 7.0 with 0.1 N HCl and 0.1 M NaOH respectively. After that, the t-butanol (1:0.5) was added and the mixtures were incubated at 25ºC for 1 hour under constant stirring and set aside to stand for 30 min. The mixtures were centrifuged (4000 rpm for 10 min) to facilitate the separation of phases then the interfacial precipitates were collected, dissolved in sodium acetate buffer (pH 5.0; 10 mM) and dialyzed against distilled water overnight at 4ºC.

2.6.3 SDS-PAGE Profile of Xylanase, Cellulase and β-1,3-Glucanase

The molecular weight of xylanase, Cellulase and β-1, 3-glucanase was determined by SDS-PAGE as described by Laemmli. A discontinuous system made of 5% stacking gel and 12% separating gel was used. The molecular weight of the enzyme was estimated using the low molecular weight calibration kit containing the standard protein marker: Phosphorylase b (97.0 kDa), Albumin (67 kDa), Ovalbumin (45.0 kDa), Carbonic amylase (30 kDa), Trypsin inhibitor (20.1 kDa) and α-Lactalbumin (14.4 kDa). To the TPP purified enzymes, an equal

volume of sample solubilizing buffer (10% glycerol, 0.05% bromophenol blue, 5% β-mercaptoethanol, 2% SDS and 0.25M Tris-HCl buffer; pH 6.8) was added and boiled at 100ºC for 2 min. Then the samples were loaded in SDS-PAGE (Acrylamide concentration in stacking gel and resolving gel was 5% and 12%, respectively). Electrophoresis was carried out at 25ºC with 100 volts for 2 hours.

2.7 Characterization of TPP Purified Xylanase, Cellulase and β-1,3-Glucanase

2.7.1 Effect of pH on the Activity of Xylanase, Cellulase and β-1,3- Glucanase

In order to determine the pH optima for the activity of TPP purified xylanase, Cellulase and β-1, 3-glucanase; the enzyme solution was incubated in different buffers (50 mM) ranging between 3.0 - 11.0 (Sodium acetate buffer, pH 3.0-6.5; Phosphate buffer, pH 6.5-7.5; Tris buffer pH 7.5-9.0; Glycine-NaOH, pH 9.0-10.0 and Carbonate buffer, pH 9.0-11.0) at 30ºC. The experiments were performed in triplicates.

2.7.2 Effect of Temperature on the Activity of Xylanase, Cellulase and β-1,3-Glucanase

The experiment was carried out to investigate the effect of different incubation temperature (30−70°C) on the activity of the enzyme. The TPP purified enzyme solution was incubated at different temperatures for 10min. The relative activities (%) were expressed as the ratio of the activity obtained at a certain temperature of each enzyme, to the maximum activity at the given temperature range.

2.7.3 Effect of Metal Ions on the Activity of free Xylanase, Cellulase and β-1,3-Glucanase

The effect of metal ions on the activity of TPP purified xylanase, Cellulase and β-1,3-glucanase was analyzed by incubation of the enzyme solution in the presence of particular metal ion solution (10mM) at 30°C for 30min. The residual activities (%) of the appropriate enzyme were analyzed by mentioned standard assay methods.

2.7.4 Determination of Kinetic Parameters of free Xylanase, Cellulase and β-1,3-Glucanase

The kinetic parameters K_m and V_{max} of xylanase, Cellulase and β-1,3-glucanase were determined by using various concentrations (2 to 20 mg/ml) of beech wood xylan, CMC-Na and β-glucan from barley, respectively at pH 5.0 and 50ºC (Bajaj et al. 2011). The experimental data were fitted in Lineweaver-Burk plot by using HYPER32 enzyme kinetics software.

2.8 Immobilization of the Enzymes

Enzyme Immobilization is a highly effective and advantageous method used to protect the enzyme from denaturation and increase the operational stability and reusability of enzymes in harsh conditions.

In the present work, two types of immobilization techniques were carried out: Combined Cross-Linked Enzyme Aggregates (combi-CLEAs) and bi-functionalized magnetic Cross-Linked Enzyme Aggregates (ISN-CLEAs).

2.8.1 Precipitation of Enzymes

Free xylanase (800 IU/ml), cellulase (120 IU/ml) and β-1,3-glucanase (550 IU/ml) were precipitated by ammonium sulfate, n-propanol, acetone, ethanol and Three-Phase Partitioning (TPP) method. TPP method was carried out by the following methodology: Initially, ammonium sulfate was added to a final concentration of 55% of saturation in 25 ml of crude enzyme solution containing xylanase, cellulase and β-1,3-glucanase. Then, t-butanol was added at a volume ratio of 1:0.75 (t-butanol: 55% ammonium sulfate saturated crude enzyme solution). The mixture was incubated at 25ºC for 1 hour under constant stirring (100 rpm). Then stirring was stopped after 30 min and the solution was left at rest. The mixture was centrifuged (4000 rpm for 10 min) to facilitate phase separation. Then, the interfacial aggregates were collected, redissolved in 10 ml of 10 mM sodium acetate buffer (pH 5.0) and dialyzed against distilled water overnight at 4ºC. This enzyme solution was used for further immobilization studies.

2.8.2 Preparation of Combi-CLEAs

Combi-CLEAs containing xylanase, cellulase and β-1,3-glucanase were prepared by successive aggregation and cross-linking of the enzymes. The latter operation includes the drop-wise addition of various concentrations of glutaraldehyde (20–140 mM) to the mixture and incubated at 30°C under agitation (220 rpm) for different time intervals (1.5–8.5 hours). The suspension was centrifuged at 10,000 rpm for 10 min. The supernatant was discarded, and the pellet was washed until there was no trace of enzyme activity in the supernatant. Finally, the aggregate was stored in 50 mM sodium acetate buffer (pH 5.0) at 4°C. The percentage activity recovery of xylanase, cellulase and β-1,3-glucanase in combi-CLEAs was determined by the following Equation (2.1).

$$\text{Activity recovery } (\%) = \frac{\text{Total activity of each enzyme in combi-CLEAs (IU)}}{\text{Total initial activity of each free enzymes (IU)}} \times 100 \quad (2.1)$$

2.8.3 Preparation of ISN-CLEAs

2.8.3.1 Synthesis and Functionalization of Fe_3O_4 Magnetic Nanoparticles (IOMNPs)

There are various chemical methods have been reported for the synthesis of IOMNPs. Modified co-precipitation method was adopted for the preparation of IOMNPs. In this method, 0.4 M of $FeCl_3.6H_2O$ (3.9 g) and 0.2 M of $FeCl_2.4H_2O$ (2.4 g) were dissolved in 60 mL of deoxygenated Millipore water and heated up to 80°C, then ammonium hydroxide solution (NH_4OH-25%) was added dropwise until the appearance of visible precipitates under constant stirring rate of 500 rpm for 30 min. The solution was left undisturbed for 1 hour for the settling of magnetic nanoparticles at the bottom. Finally, the resulting IOMNPs was separated by magnetic decantation and washed thrice with ultrapure water (MilliQ, Millipore co.,) and freeze dried at -80°C and 0.014 mbar for 3days.

2.8.3.2 Bifunctionalization of IOMNPs

Two types of functionalization have been carried out to obtain silica anchored-amine functionalized IOMNPs. First, anchoring of silica (SiO_2) group onto Fe_3O_4 NPs was carried out by salinization method. Briefly, 400 mg of Fe_3O_4 NPs were suspended in 5ml of ethanol-

deionized water (3:1), which was heated to 45°C and sonicated in a sweeping mode for 20 min. To this solution, 500 µl of TEOS and 400 µl of Triethanolamine were added and the mixture was kept in sonication for 30 min. Finally, the resulting silica anchored nanoparticles were separated by magnetic decantation and washed thrice with deionized water and ethanol and then freeze dried by using lyophilizer.

Second, amine functionalization on silica anchored IOMNPs was done by dispersing 200 mg of Fe_3O_4/SiO_2 NPs in a solution mixture of 5 ml deionized water and 5 ml of methanol, this solution was sonicated for 30 min. To this solution, 500 µl of APTES and 250 µl of triethanolamine were added and sonicated for 30 min. The resultant silica anchored-amine functionalized nanoparticles were collected by magnetic separation and washed 5 times with methanol and water and then freeze-dried in the lyophilizer.

2.8.3.3 Immobilization of Cellulase, Xylanase and β-1,3-Glucanase into Silica Anchored-Amine Functionalized IOMNPs

The cross-linking of cellulase, xylanase and β-1,3-glucanase onto silica anchored-amine functionalized IOMNPs was prepared as follows, 500 mg of $Fe_3O_4@SiO_2$-NH_2 NPs were dispersed in 10 ml of enzymes solution which contains cellulase (150 U mg^{-1}), xylanase (800 U mg^{-1}) and β-1,3-glucanase (500 U mg^{-1}) in 50 mM sodium acetate buffer (pH-5.0). Subsequently, glutaraldehyde solution with various concentrations (20-140 mM) was added dropwise into the mixture to crosslink the enzymes onto the magnetic nanoparticles. The mixture was incubated at 30°C under agitation (150 rpm) for different time intervals (2.0 - 12.0 hours). After crosslinking, the resulting immobilized cellulase, xylanase and β-1,3-glucanase onto $Fe_3O_4@SiO_2$-NH_2 NPs was separated by external magnetic field and washed thrice with 50 mM sodium acetate buffer (pH-5.0) and stored in the same buffer at 4°C for subsequent use. The activity recovery (%) of cellulase, xylanase and β-1,3-glucanase in the $Fe_3O_4@SiO_2$-NH_2 NPs -cross-linked enzyme aggregates (ISN-CLEAs) were determined by the following equation (Periyasamy et al. 2016b).

$$\text{Activity recovery (\%)} = \frac{\text{Total activity of each enzyme in ISN-CLEAs (IU)}}{\text{Total initial activity of each free enzymes (IU)}} \times 100 \quad (2.2)$$

The binding efficiency of cellulase, xylanase and β-1,3-glucanase onto ISN-CLEAs was calculated by the Equation (2.3)

$$\text{Binding efficiency} = \frac{E_i - E_f}{m} \times v \qquad (2.3)$$

Where, E_i and E_f are the initial and final concentration of enzymes (mg/ml), respectively, m is the mass of $Fe_3O_4@SiO_2$-NH_2 NPs (mg) and v is the volume of solution (mL).

2.9 Characterization of the Immobilized Enzymes

2.9.1 Structural Characterization of Combi-CLEAs and ISN-CLEAs.

The surface morphology of combi-CLEAs was analyzed by Scanning Electron Microscope (SEM). Samples were dried under vacuum and then placed on a carbon tape over a microscope slide to coat with gold particles using a sputter coater.

The surface morphology of ISN-CLEAs was examined on Environmental Scanning Electron Microscope (ESEM-FEI Quanta 200) and Atomic Force Microscopy (Veeco-icon ScanAsyst, USA). The FTIR spectra (4000 to 400 cm-1) were observed in ATR-FTIR (Perkin-Elmer Spectrum 65, USA). Dynamic light scattering (VASCOTM Particle Size Analyzer) was used to measure the size distribution of the magnetic nanoparticles.

2.9.2 Thermal stability of Free enzymes, Combi-CLEAs and ISN-CLEAs.

The thermal stability of free enzymes, combi-CLEAs and ISN-CLEAs were investigated by incubating them in 50 mM sodium acetate buffer (pH 5.0) without substrate at 30°C, 40°C, 50°C, 60°C and 70°C. Aliquots were withdrawn at every 2 hours interval for a total time of 8 hours. Supernatant was collected by centrifugation, and the immobilized biocatalyst was assayed to determine xylanase, cellulase and β- 1,3-glucanase activities under standard assay method. The residual activity of each enzyme at each temperature was compared with the activity at an initial time (0 min) taken as 100%.

2.9.3 Storage Stability of Free enzymes, Combi-CLEAs and ISN-CLEAs.

Storage stability of the free enzymes, combi-CLEAs and ISN-CLEAs were analyzed by incubating them in 50 mM sodium acetate buffer and the activity of each enzyme was measured at intervals of 1, 3, 5, 7, 9 and 11 weeks.

2.9.4 Reusability Studies of Combi-CLEAs and ISN-CLEAs

The reusability of immobilized enzymes is one of the crucial factors in industrial applications. The reusability of enzymes in combi-CLEAs and ISN-CLEAs were studied under standard assay conditions. After each cycle of a hydrolysis reaction, combi-CLEAs were separated by centrifugation and washed twice with 50 mM sodium acetate buffer (pH 5.0) and then re-suspended in the fresh substrate. On the other hand, ISN-CLEAs was separated by an external magnet and washed twice with the same buffer and then re-suspended in the fresh substrate. The activities acquired in the first cycle for the immobilized enzyme was taken as the control and correspond to 100% activity.

2.10 Preparation and Characterization of Biomass

Sugarcane Bagasse (SCB) is mainly of two polysaccharides such as cellulose, hemicelluloses, and polyphenolic macromolecule (lignin). Cellulose (30-35%) is a more abundant polysaccharide segment consisting of a linear chain of several hundred to over ten thousand of β-(1→4) linked D-glucose units that form crystalline regions and resultantly increases recalcitrance to the hydrolytic process. Hemicelluloses (25-30%) is the second predominant component and it made of heteropolysaccharides mainly xylan with β-D-xylose units. It can be more easily depolymerized than cellulose. Lignin is the third fractions with the complex structure made by the polymerization of aromatic alcohols that linked with the hemicelluloses, covers the cellulose matrix, giving resistance to enzymatic and chemical degradation.

SCB was locally harvested and was provided by E.I.D-PARRY (I) LTD, Tiruchirappalli, India. The biomass was finely chopped to 2-4 mm size and stored in a container at -20°C until further processing. The analysis of the lignin and sugar content profile of the SCB was made by classical hydrolysis using sulfuric acid, as follows: about 0.3 g of SCB (as dry matter) was mixed with 3 ml of 72% (w/w) sulfuric acid at room temperature; the mixture

was stirred at the beginning with a glass rod, and then occasionally during a total time of one hour; then 84 ml of de- ionized water was added to the mixture and autoclaved at 121°C for 1 hour under saturated water pressure. After cooling, the mixture was filtered on a sintered glass crucible of fine porosity (porosity index of 4). The filtrate was properly diluted (120 times for glucose and 40 times for the other sugars) for elemental sugar content analysis by High-Performance Anion-Exchange Chromatography and the dry lignin residue on the crucible was weighed after washing and oven-drying at 105°C during 24 hours.

2.11 Pretreatment of Biomass

In this present work, dilute ammonia pretreatment was carried out to break the complex structure of sugarcane bagasse, this pretreatment often been useful to improve the biomass digestibility and easy enzyme accessibility to cellulose. Pretreatment (delignification) consisted in mixing 10 g of SCB (dry basis) in 100 ml of 10% (w/v) liquid ammonia in a closed glass bottle. This reaction mixture was autoclaved at 140°C for 120 min. After this cooking step, the SCB was washed with distilled water till it attained a neutral pH. The biomass was then pressed and air- dried at room temperature, then ground in a Forplex hammer mill (Figure. 2.1) to obtain a biomass powder of mill metric size (0.5 to 1mm). it was further dried at 70°C to obtain constant weight and stored at 4°C (Periyasamy K et al. 2018).

Figure 2.1 Forplex hammer mill

2.12 CHEMICAL AND STRUCTURAL ANALYSIS OF SCB

2.12.1 Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

The structural and chemical changes of untreated SCB, liquid ammonia pretreated SCB and combi-CLEAs treated SCB were characterized by ATR-FTIR analysis (samples used were solid state dry powders placed on the sample holder of the spectrometer). All samples were dried at 80°C in an oven prior to analysis. The spectra were recorded by the FTIR spectrometer (Perkin-Elmer Spectrum 65) in the absorbance band mode in the range of 4000 cm^{-1} to 600 cm^{-1} with a resolution of 4 cm^{-1} and 40 scans per sample.

2.12.2 Thermogravimetric Analysis (TGA)

Thermal analysis techniques such as Thermogravimetric (TG) and derivative Thermogravimetric (DTG) were used to study the thermal decomposition behavior of biomass in different conditions. The thermal degradation of untreated SCB, liquid ammonia pretreated SCB and combi-CLEAs treated SCB were monitored by Thermogravimetric analysis (TGA) by using Thermogravimetric analyser-STA6000 (Perkin-Elmer Instruments, England). About 7-10 mg of samples were taken for the analysis and dynamic TG scans were recorded in a temperature range from 30°C to 600°C at a heating rate of 10°C/min. nitrogen atmosphere was supplied at the flow rate of 20 ml/min.

2.12.3 X-ray Diffraction Analysis (XRD)

The structural analysis of untreated SCB, liquid ammonia pretreated SCB and combi-CLEAs treated SCB were evaluated by XRD (X'Pert PRO MPD®, PAN analytical, Netherlands). The operating conditions of the refractometer were: copper Kα radiation (λ = 1.5418°A), 2θ (Bragg angle) between 2° and 56°, step size 0.067°, counting time 90 s. Each sample was measured once at the intervals of 0.2 s. The crystalline index (CrI) was determined based on the formula as follows (Segal et al. 1959).

$$\mathrm{CrI}(\%) = \left(\frac{I_{Total} - I_{am}}{I_{Total}}\right) \times 100 \qquad (2.4)$$

Where, I_{Total} is the scattered intensity at the main peak, whereas I_{am} is the scattered intensity of amorphous portion of cellulose

2.13 Enzymatic hydrolysis and Sugar Analysis

After the pretreatment of biomass, to enhance the accessibility of cellulose fibers, a mixture of enzymes with cellulolytic activity is used to hydrolyze the cellulose and other polysaccharides into fermentable sugars. Glucose and xylose yields were calculated as follows.

$$\text{Glucose yield (\%)} = \left(\frac{\text{Glucose (g)} \times 0.9}{\text{Cellulose content SCB(g)}}\right) \times 100 \qquad (2.5)$$

$$\text{Xylose yield (\%)} = \left(\frac{\text{Xylose (g)} \times 0.88}{\text{Hemicelluloses content SCB(g)}}\right) \times 100 \qquad (2.6)$$

where,

0.9 is a conversion factor for cellulose to equivalent glucose

0.8 is a conversion factor for hemicelluloses to equivalent xylose

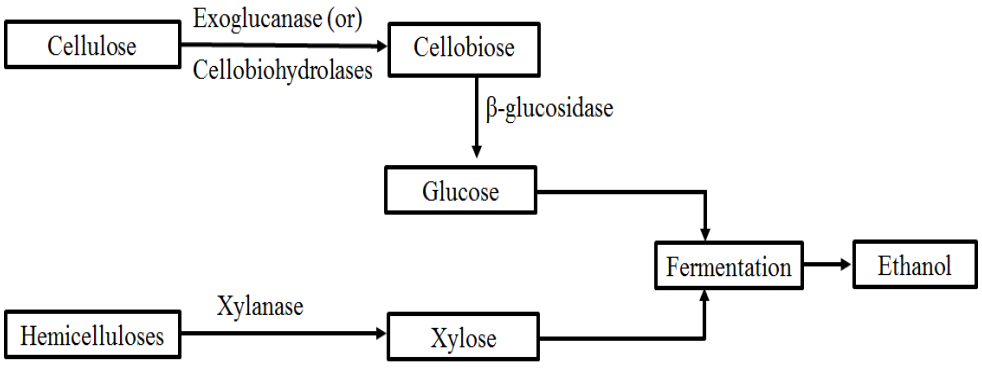


Figure 2.2 Schematic representation of enzymatic reaction on substrate

2.13.1 Enzymatic Hydrolysis of Sugarcane Bagasse by Free enzyme, Combi-CLEAs and ISN-CLEAs

Sugarcane bagasse (SCB) contained about 55% cellulose, 25% hemicelluloses and 20% lignin. Enzymatic saccharification of SCB was carried out by adding the free enzyme, combi-CLEAs and ISN-CLEAs separately in 250 ml shake flasks containing 4 g of pretreated SCB pulp in 100 ml of 50 mM sodium acetate buffer (pH 5.0). The reactions were carried out at 50°C on a thermostatic rotary shaker at 180 rpm for 60 hours. During the reaction time, aliquots were withdrawn at 4, 12, 24, 36, 48 and 60 hours. After the enzymatic hydrolysis, free enzyme containing hydrolysate was boiled at 80°C for 5 min to deactivate the free enzymes, combi-CLEAs containing hydrolysate was centrifuged and removed the aggregates and ISN-CLEAs was separated from hydrolysates by an external magnet. Finally, all the three hydrolysates were centrifuged to remove the unhydrolysed SCB.

2.13.2 Sugar Analysis

Monomeric sugar content was analyzed by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD, Dionex ICS 5000) equipped with a CarboPac PA 10 (250 × 4 mm, Dionex) column situated after a guard column (50 × 4 mm, Dionex). Columns and detectors were in a compartment regulated at 25°C. KOH was used as an eluent at a flow rate of 1 ml min^{-1} and the concentration of KOH was 2 mM during 30 min (analysis time) followed by 100 mM for 15 min (column washing and regeneration). Samples were diluted appropriately, and the pH was adjusted between 8 and 9 and then filtered through 0.45 mm syringe filters prior to injection. 25 ml of sample was injected into the column. Samples were measured against standards consisting of arabinose, galactose, glucose, xylose and mannose.

2.14 Fermentation

Bioethanol can be produced from feedstocks like biomass and organic waste matter by fermentation. A lot of research has been performed on the lignocellulosic sugar derived from renewable feedstocks. Despite the research on the production of cellulosic ethanol for more than two decades, it still lags to attract the industrial sector and continues to be in the R&D stage. The particular reason for this is, the wild strain of yeast *Saccharomyces cerevisiae* does not convert most sugars into ethanol due to the lack of biochemical activities and metabolic catabolic repression. Besides, fermentation of pentose sugars from the hemicelluloses

component of lignocellulosic biomass is still challenging. Conversion of xylose to ethanol is a crucial step to maximize the ethanol production economically. However, native *S. cerevisiae* cannot efficiently ferment xylose, as most strains have either lost or downregulated the activities of xylose catabolic enzymes and lack of specific xylose transporter. Though, the continuous developments in the field of the metabolic aspects of second-generation bioethanol production, still there are many divergences that hamper the use of metabolic engineering strategies to their full potential. Natural selection process such as the classical approach of evolutionary adaption of microbes remain an effective approach to improve the microorganism's performance.

Theoretically, 1 mol of glucose would yield 2 moles of ethanol as depicted in the following stoichiometric equation

$$1C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2\,CO_2$$

A similar equation can be written for xylose also,

$$1C_5H_{10}O_5 \rightarrow 1.67\,C_2H_5OH + 1.67\,CO_2$$

However, some amount of sugar is used for the growth and biomass development and therefore, the experimentally obtained yields can never correspond to the theoretical result. Furthermore, ethanol yield can also be affected by the metabolic pathway of the microorganism. The bioethanol yield is calculated as follows

$$\text{Ethanol yield}(\%) = \frac{\text{Ethanol concentration}\left(\frac{g}{l}\right)}{\text{Initial amount of sugar}\left(\frac{g}{l}\right) \times 0.511} \times 100 \qquad (2.7)$$

where sugar corresponds to glucose and xylose, and 0.511 is the conversion factor for glucose, and xylose to ethanol

2.14.1 Microorganisms and Growth media

In this study, two types of microorganisms were used to ferment the SCB hydrolysates containing glucose and xylose. Stress-tolerant, glucose fermenting yeast strain of *Saccharomyces cerevisiae* LGP2Y1 was obtained from Laboratoire Génie des Procédés Papetiers (LGP2) - Pagora - Université Grenoble Alpes, Grenoble, France. Xylose-

fermenting strain of *Candida utilis* ATCC 22023 was obtained from National Chemical Laboratory (NCL), Pune, India.

S. cerevisiae LGP2Y1 strain was maintained on Yeast Extract Peptone Dextrose (YEPD) agar plates containing yeast extract (10 g/l), peptone (20 g/l), glucose (20 g/l) and agar-agar (20 g/l). Similarly, the strain of *C. utilis* ATCC 22023 was maintained on Yeast Extract Peptone Xylose (YEPX) agar plates which contain yeast extract (10 g/l), peptone (20 g/l), xylose (10 g/l) and agar (20 g/l). These culture plates were stored at 4°C in a refrigerator.

2.14.2 Preparation of Inoculum

The preculture of *S. cerevisiae* LGP2Y1 was developed by inoculating single colony of LGP2Y1 strain in 10ml of media containing (g/l): yeast extract-10; peptone-20 and glucose-20 (pH-5.0±0.1). Similarly, the of *C. utilis* ATCC 22023 strain was cultivated in 10ml media containing (g/l): yeast extract-10; peptone-20 and xylose-20 and the pH was 5.0±0.1. These cultures were grown in thermostatic orbital shaker with the agitation of 150rpm for 16 hours at 30°C. These cells were harvested separately by using a refrigerated centrifuge (Sigma®, UK) at 5500 rpm for 6 min, re-suspended in 0.9% (w/v) NaCl and it was used as the inoculum for the ethanol production medium. The size of the inoculum was 5 g/l (dry weight) in both glucose and xylose fermentation. The dry weight of the cell was analyzed by UV-Vis Spectrophotometer (Shimadzu) at 600 nm. Samples were diluted in such a way to assure the absorbance is less than 0.5. In this range, the calibration curve was linear with a slope of 0.6 g dry weight/unit absorbance.

2.14.3 Fermentation Media

Yeast Nitrogen Base with Amino Acids (Y1250-250G- Sigma-Aldrich) medium (YNBA) supplemented with SCB hydrolysate was used for ethanol production and its compositions are mentioned below.

Table 2.2 Composition of Yeast Nitrogen Base with Amino Acids medium

Sl.No	Ingredients	Concentration
1	**Nitrogen Source (g/l)**	
	Ammonium sulfate	5.0
2	**Amino Acids (mg/l)**	
	L-histidine	10
	DL-methionine	20
	DL-tryptophan	20
3	**Vitamins (µg/l)**	
	Biotin	2
	Calcium pantothenate	400
	Folic acid	2
	Inositol	2000
	Nicotinic acid	400
	p-Aminobenzoic acid	200
	Pyridoxine HCl	400
	Riboflavin	200
	Thiamine HCl	400
4	**Trace Elements (µg/l)**	
	Boric acid	1
	Copper sulfate	1
	Potassium iodide	1
	Ferric chloride	1
	Magnesium sulfate	1
	Sodium molybdate	1
	Zinc sulfate	1
5	**Salts (g/l)**	
	Potassium phosphate monobasic	1.0
	Magnesium sulfate	0.5
	Sodium chloride	0.1
	Calcium chloride	0.1

Initially, 10x concentrated stock solution of the media was prepared by adding 6.7 g of yeast extract nitrogen base medium power in 100 ml of sterile distilled water. The solution was stirred for 5 min to ensure complete solubilization and it was filter sterilized and stored at 2-5°C. Finally, 1x working solution (YNBA, pH-5.0) medium was prepared by adding 10 ml of concentrated stock to 90ml of hydrolysate. SCB hydrolysate (contains glucose and xylose) was always autoclaved (120°C for 15 min) separately and mixed with 1x filter sterilized YNBA medium in order to avoid caramelization and other complexation reactions.

2.14.4 Ethanol fermentation by Mono and Co-culture

Two different ethanol fermentations were carried out : 90 ml autoclaved SCB hydrolysate was taken along with 10 ml of 10x sterile YNBA medium with the total volume of 100ml. Further it was divided into two cotton plugged Erlenmeyer conical flasks (50 ml each). In one flask *S. cerevisiae* LGP2Y1 inoculum was added at zero hour which was assumed as CF1 fermentation. In another flask only *C. utilis* ATCC 22023 was inoculated at zero hour and *S. cerevisiae* LGP2Y1 was inoculated at 10 hours which was consider as CF2 fermentation. The flasks were incubated at 30°C with the agitation of 80 to 100 rpm for the first 12 hours and then in static mode till the end of fermentation i.e. 48 hours. Aliquots of 500µl were withdrawn at 0, 8, 12, 24, 36 and 48 hours.

2.14.5 Quantification of Ethanol

The produced ethanol, acetic acid, formic acid, furfural and 5-hydroxymethyl furfural (HMF) was estimated by Ultra High-Performance Liquid Chromatography (Nexera-UHPLC 660) equipped with Refractive Index Detector (RID 20A, Shimadzu). Agilent Technologies pump and sample changer, a pre-column (3 mm × 5 mm, Varian) and a ligand exchange column (Agilent, Hi-Plex H 7.7 mm ✕ 300 mm, Varian) placed in a chamber thermostatically operated at 65°C with the flow rate of 0.6 ml/min. The detector used is an ERC 1715A refractometer. The refractive index is measured at 35°C. The eluent consists of 5 mmol /l sulfuric acid and the sample injection volume is 10 µl.

CHAPTER 3

RESULTS AND DISCUSSION

The present work focusses on Production, partial purification and characterization of the free xylanase, cellulase, and β-1, 3-glucanase by solid state fermentation. To increase the operational stability of the free enzymes, it was immobilized by using two methods : Carrier-free Combined Cross-Linked Enzyme Aggregates (combi-CLEAs) and Iron Oxide Magnetic Nanoparticles Cross-Linked Enzyme Aggregates (ISN-CLEAs). These immobilized enzymes were used to hydrolyze the pretreated sugarcane bagasse for bioethanol production.

3.1 Isolation of Xylanase-Cellulase Producing Strain

About 19 fungal isolates were screened from the soil sample, the prominent higher zone of clearance producing isolate was confirmed as *Trichoderma citrinoviride* with the partial 5.8S rRNA sequencing having a length of 607 bp nucleotide. The sequence was deposited in GenBank (Accession no. KF698728) and the blast search has shown 99% homology with the *T. citrinoviride* strain H09-105, 18S ribosomal RNA partial gene sequence, thus it is designated as *Trichoderma citrinoviride* strain AUKAR04. The phylogenetic relationships of this isolate was constructed using neighbor-joining method (Saitou, N & Nei M., 1987) by using a software (Mega 6.0) and the result is shown in Figure. 3.1.

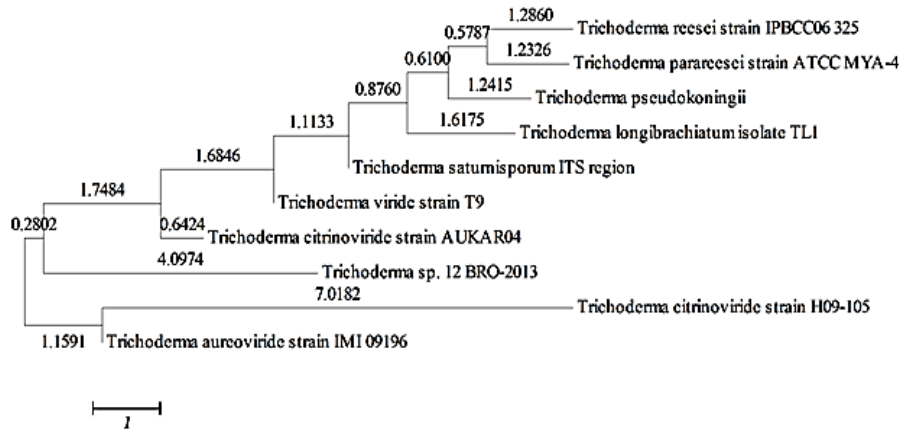


Figure 3.1 Phylogenetic tree of *Trichoderma citrinoviride* strain AUKAR04 using neighbor-joining method (MEGA 6.0) based on the 5.8S rRNA sequence

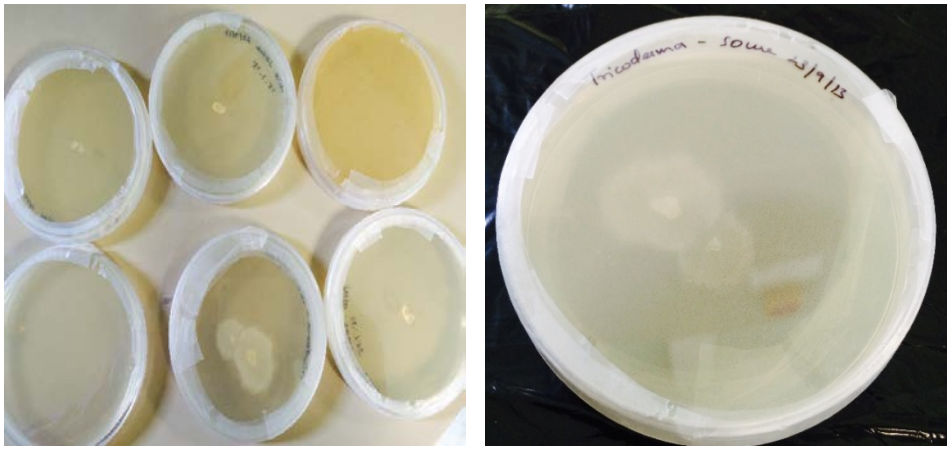


Figure 3.1. a *Trichoderma citrinoviride* strain AUKAR04

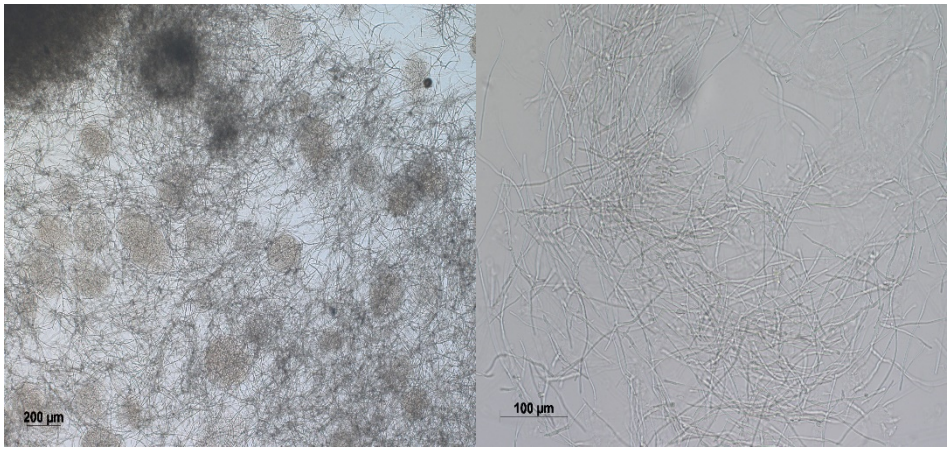


Figure 3.1. b. Optical Microscopy image of filamentous mycelia of *T. Citrinoviride* AUKAR04

3.2 Production of Xylanase, Cellulase and β-1, 3-Glucanase in SSF

Enzyme production from filamentous fungus in solid-state fermentation is also influenced by initial pH of seed media, growth and morphology. A filamentous fungus has the tendency to adhere to solid substrate surfaces, and the regulation of fungal physiology has not yet been thoroughly studied, especially when concerned with enzyme production (Ferreira et al. 2009). In SSF, the production of enzymes is not only based on the parameters of temperature, moisture, the size of the matrix, inoculum size, etc. but also correlated with the composition and initial pH of the seed media (Kang, 2004). In order to evaluate the effect of initial pH of the seed media corresponding with the fungal morphology on the production of the enzyme. the spore of *T. citrinoviride* strain AUKAR04 was inoculated in seed media with different pH ranging from 3.0 to 8.0. It was observed that the highest production of xylanase (55,000 IU/gds), Cellulase (385 IU/gds) and β-1, 3-glucanase (695 IU/gds) were obtained in SSF with

the seed media which was developed in initial pH 5.0±0.1 after 72 hours incubation at 30±1ºC and the results are shown in Table 3.1.

Table 3.1 Effect of initial pH of seed media and the relationship with xylanase, Cellulase, and β-1, 3-glucanase production

Initial pH of the Growth media	Activity (U/gds*)		
	Xylanase	Cellulase	β-1,3-glucanase
3±0.1	9047±15	204±3	250±4
4±0.1	40188±16	287±2	587±3
5±0.1	55000 ±20	385 ±5	695 ±5
6±0.1	40547±19	375±4	510±4
7±0.1	30487±20	150±3	277±3
8±0.1	19147±18	89±3	187±2

*Units per gram dry substrate (wheat bran)

3.3 Partial Purification of Xylanase, Cellulase and B-1,3-Glucanase by Three-Phase Partitioning

Protein purification by TPP is influenced by many factors such as ammonium sulfate concentration, t-butanol ratio, temperature and pH. An attempt using single-step partial purification of xylanase, Cellulase and β-1, 3-glucanase was made by TPP method. The effect of t-butanol ratio to crude ammonium sulfate saturation (55%) was varied. In this regard, 1 :0.5 ratio gave the highest xylanase yield (67.1%) with 2.4-fold purification, Cellulase yields (79.4%) with 2.8-fold purification and β-1,3-glucanase yields (45.0%) with 1.6-fold purification at the temperature of 25ºC. From this result, the 1:0.5 ratio of crude extracts to t-butanol was selected for investigating the importance of pH in TPP system. Protein concentration by salting out depends on the sulfate concentration and pH-dependent net charge of the proteins. The macromolecular contraction and conformational shrinkage are promoted by electrostatic forces and binding of sulfate ions to cationic protein molecules. Protein molecules have a tendency to precipitate near their *pI* (isoelectric point) (Özer et al. 2010). Below its *pI*, proteins are positively charged and quantitatively precipitated out by TPP (Ketnawa et al. 2014). As a rule of thumb, the crude enzyme precipitate was adjusted to different pH. Among the various pH (3.0-7.0) of TPP system, the maximum recovery of xylanase (99.8%) with 5.7 fold, Cellulase (96.5%) with 5.5 fold and β-1, 3-glucanase (98.4%) with 5.6 fold purification was obtained in interface with the pH of 5.0 and 1:0.5 (crude extract to t-butanol) at 25ºC and the results are presented in Figure 3.2 and Table.3.2.

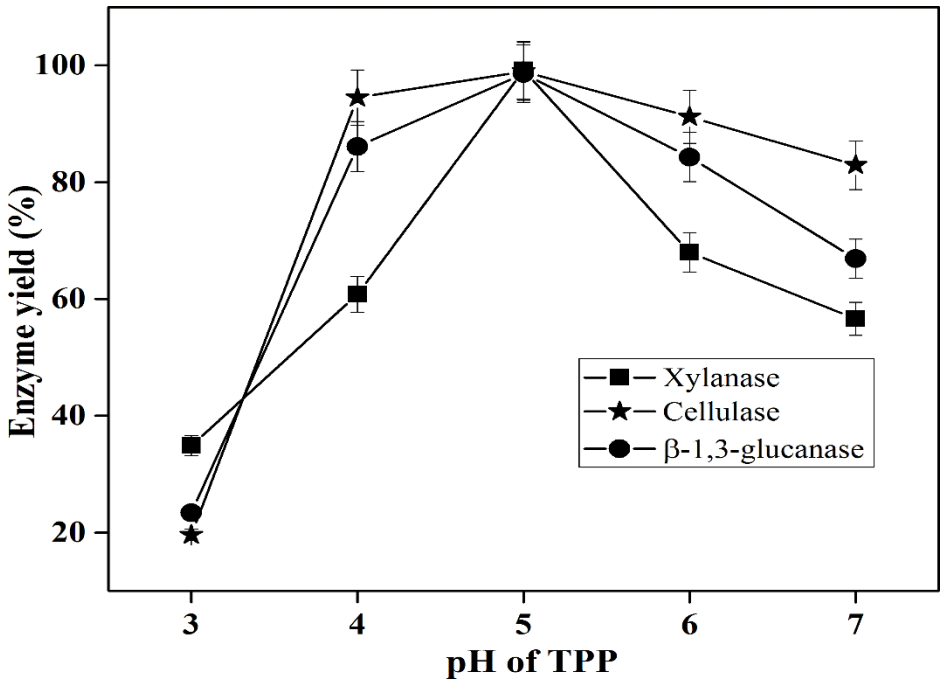


Figure 3.2. Effect of pH of crude ammonium sulfate saturation to t-butanol ratio on the partitioning of xylanase, cellulase and β-1, 3-glucanase. The crude enzyme precipitate was adjusted to different pH from 3.0 to 7.0 with the ratio of crude ammonium sulfate saturation to t-butanol was 1:0.5.

Table 3.2 Purification profile of xylanase, Cellulase and β-1,3-glucanase

Steps	Total protein (mg)	Total activity (U/ml)		Specific activity (U/mg)	Yield (%)	Purification Fold
Crude extract	9.2	Xylanase	35014	2202	100	1
		Cellulase	201	12	100	1
		β-1,3-glucanase	513	32	100	1
TPP purified enzyme (pH not adjusted)	4.5	Xylanase	23494	5221	67.1	2.4
		Cellulase	159	35	79.4	2.8
		β-1,3-glucanase	230	51	45.0	1.6
TPP purified enzyme (pH adjusted to 5.0)	1.6	Xylanase	34944	12480	99.8	5.7
		Cellulase	194	69	96.5	5.5
		β-1,3-glucanase	504	180	98.4	5.6

3.4 SDS-PAGE profile of Xylanase, Cellulase and β-1, 3-glucanase

SDS-PAGE analysis of the TPP purified enzymes showed that the molecular weight of Xylanase (~29.8 kDa) was comparable with those reported by different organisms like *Bacillus* sp. and fungal genus is in the range of 22-45 kDa (Saha, 2002; Tseng et al. 2002). The molecular weight of Cellulase (~58 kDa) (Iqbal, 2011) and β-1, 3-glucanase (~27 kDa) (da Silva Aires et al. 2012). The results are represented in Figure 3.3.

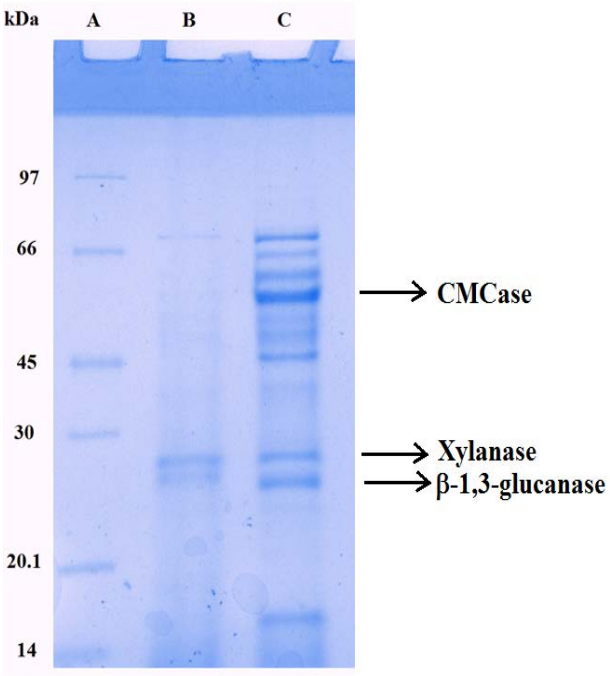


Figure 3.3 SDS-PAGE analysis of TPP purified enzyme cocktail [lane A: molecular weight marker (14-97 kDa) (20µg), lane B: TPP bottom phase and lane C: TPP purified enzyme cocktail (middle phase)]

3.5 Characterization of Free Xylanase, Cellulase and B-1, 3-Glucanase

3.5.1 Effect of pH on the Activity of Xylanase, Cellulase and β-1, 3-glucanase

The effect of pH for the TPP purified xylanase, Cellulase and β-1, 3-glucanase was investigated in the range of pH 3.0 to 10.0 and the results are shown in Figure 3.4. The xylanase activity towards the beech wood xylan was maximum at pH 5.0 (Chen et al. 2009), and at least 85% of the maximum activity was found from pH 4.0 to pH 6.0. Cellulase hydrolyzed CMC in the pH range of 3.0 - 10.0, and exhibited 100% activity at pH 5.0 and

6.0. The β-1, 3-glucanase attained maximal activity in the pH range of 4.0-6.0, and the highest activity found at pH 6.0. Substantial activities of the appropriate enzymes were also exhibited on either side of this optimum point, which indicated that xylanase, Cellulase and β-1,3-glucanase has the characteristically broad range of pH activity. An increase or decrease in the pH changes the ion concentration in the solution. These ions alter the structure of the enzymes and at times the substrate, either due to formation of additional bonds or breakage of already existing bonds. Ultimately, the chemical makeup of the enzyme and substrate are changed. Also, the active site of the enzyme is changed, after which the substrate can no longer identify the enzyme.

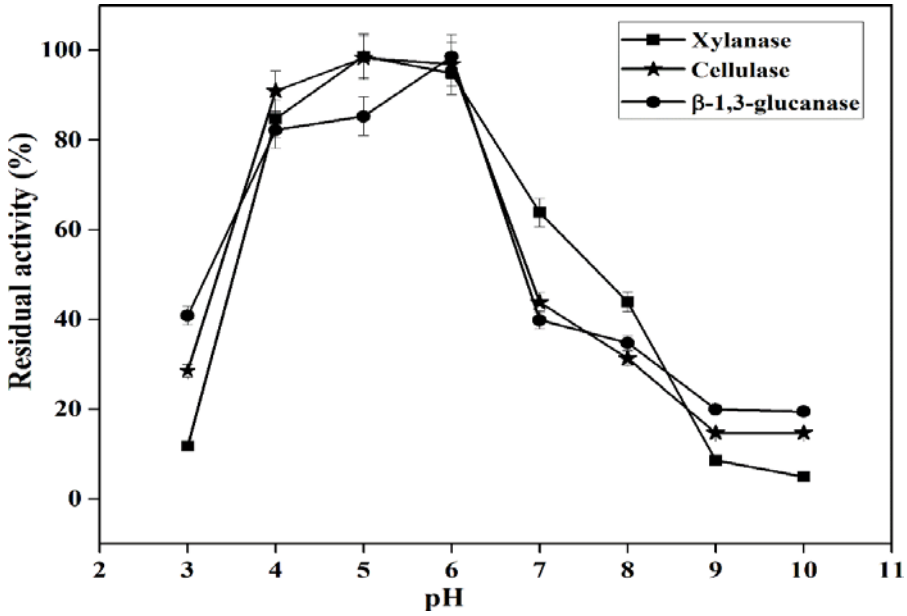


Figure 3.4 Effect of pH on the activity of Xylanase, Cellulase and β-1,3-glucanase

3.5.2 Effect of Temperature on the Activity of Xylanase, Cellulase and β-1, 3-Glucanase

Temperature is one of the crucial parameters to characterize the enzyme. The temperature optima of TPP purified xylanase, Cellulase and β-1,3-glucanase were analyzed. The optimum temperature for xylanase and β-1,3-glucanase were found to be 50ºC. The optimum temperature of xylanase from *Aspergillus fumigatus* MA28 exhibited at 50ºC (Bajaj et al. 2011), temperature optima of β-1,3-glucanase from *A. fumigates* was at 55ºC and 65ºC (Fontaine et al. 1997), whereas the maximum Cellulase activity was observed at 60ºC. Sadhu

et al. (2013) reported that the optimum temperature of Cellulase was at 50ºC. However, all the three enzymes showed 80% activity from 40ºC to 60ºC which significantly declined at 90ºC.The results are shown in Figure 3.5. The reason for the decrease in enzyme activity due to either denature of enzyme or as the temperature rises, reacting molecule has more and more kinetic energy, this increase the chances of successful collision and so the rate increases.

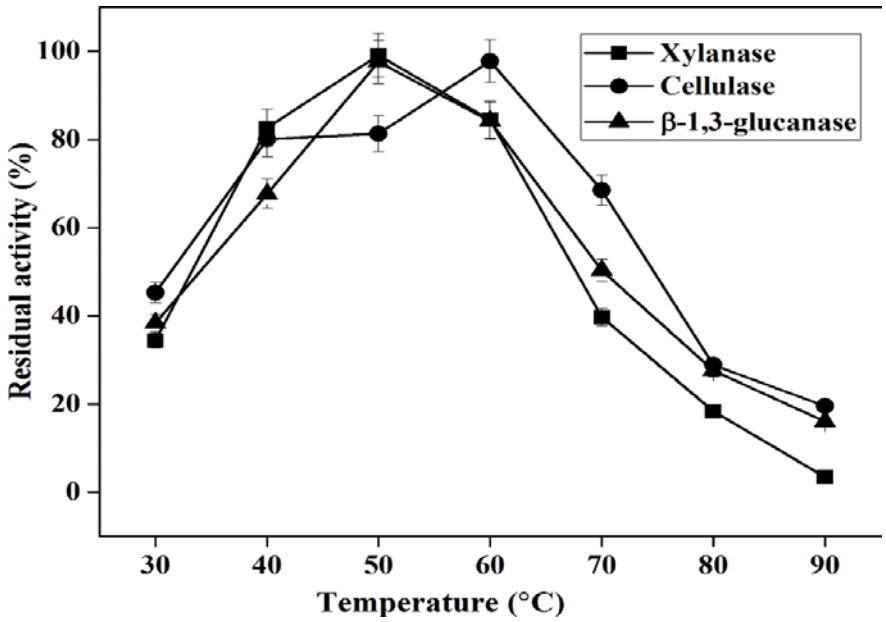


Figure 3.5 Effect of temperature on the activity of xylanase, Cellulase and β-1, 3-glucanase

3.5.3 Effect of Metal Ions and Metal chelator on the Activity of xylanase, Cellulase and β-1, 3-Glucanase

The effect of different metal ions and the metal chelator on the activity of TPP purified xylanase, Cellulase and β-1, 3-glucanase were measured at a final concentration of 10 mM, with the results shown in Table 3.3. Xylanase activity was very much enhanced by Mg^{2+}, Mn^{2+} and Zn^{2+}, whereas Cu^{2+} and Fe^{3+} inhibited activity up to 60%. Cellulase activity was inhibited by Cu^{2+} and significantly increased up to 111.1% and 154.5% by Mg^{2+} and Mn^{2+} respectively. Similarly, Yin et al.(2010) reported that Cellulase from *Bacillus subtilis* YJ1 was greatly activated by Mg^{2+} and Mn^{2+}. β-1,3-glucanase activity was strongly inhibited by Cu^{2+} and Zn^{2+} and raised up to 104% and 141% by Mg^{2+} and Mn^{2+} respectively. Complete inhibition of all the three enzymes activity was observed in the presence of Hg^{2+}, due to the

inhibitor metal has a similar shape to the usual substrate for the enzyme, and competes with it for the active site. However, once it is attached to the active site, nothing happens to it. It doesn't react - essentially, it just gets in the way. Sandrim et al. (2005) reported similar effects of Hg^{2+} on the activity xylanase I from *A. caespitosus.* On the other hand, the addition of EDTA had no remarkable effect on the activity of all the three enzymes, indicates that metal ions were not present in the active sites of the respective enzymes (Hauli et al. 2013). Enzymes can catalyze a reaction by the use of metals. Metals often facilitate the catalytic process in different ways. The metals can either assist in the catalytic reaction, activate the enzyme to begin the catalysis or they can inhibit reactions in solution. Metals activate the enzyme by changing its shape but are not actually involved in the catalytic reaction. Also, metals can promote binding of the enzyme and substrate by acting as a bridge to increase the binding energy and orient them correctly to make the reaction possible

Table 3.3 Effect of various metal ions and metal chelator on the activity of xylanase, Cellulase and β-1, 3-glucanase

Metal ions	Concentration (mM)	Relative activity (%)		
		Xylanase	Cellulase	β-1,3-glucanase
Control	(none)	100.0	100.0	100.0
Ca^{2+}	10	105.4	103.1	101.0
Cu^{2+}	10	46.2	56.7	33.0
EDTA	10	93.9	88.9	94.0
Fe^{3+}	10	41.6	88.2	106.0
Hg^{2+}	10	ND	ND	ND
K^{+}	10	97.7	97.5	93.0
Mg^{2+}	10	108.5	111.1	104.0
Mn^{2+}	10	126.9	154.5	141.0
Na^{+}	10	96.9	92.6	94.0
Zn^{2+}	10	112.7	82.7	33.0

3.5.4 Determination of Kinetic Parameters of Xylanase, Cellulase and β-1, 3-Glucanase

The kinetic parameters such as K_m and V_{max} of TPP partial purified xylanase, Cellulase and β-1,3-glucanase were calculated from Lineweaver-Burk double reciprocal plots (Figure 3.6) at 50ºC under appropriate enzyme standard assay methods, as mentioned in the experimental section. Xylanase had the lower K_m value of 0.713 mg ml^{-1} and highest V_{max} value of 24.28 mmol min^{-1} ml^{-1}. Similarly, the K_m value of Cellulase and β-1, 3-glucanase was found to be

0.811 mg ml^{-1}, and 1.31 mg ml^{-1}, respectively. The V_{max} values of Cellulase and β-1, 3-glucanase was found to be 630.9 µmol $min^{-1}ml^{-1}$ and 329.6 µmol $min^{-1}ml^{-1}$, respectively. In literature, different ranges of K_m and V_{max} for different fungal and bacterial species have been reported. According to Sandrim et al. (2005), K_m and V_{max} values of xylanase from *A. caespitosus* were 2.5 mg ml^{-1} and 1679 U,11mg^{-1} protein, respectively. Iqbal. (2011) reported that the K_m and V_{max} values of Cellulase from *T. viride* were 68 µmol and 148 U ml^{-1} respectively. K_m value of β-1, 3-glucanase was 0.9 mg/ml, and V_{max} of 0.11 U from *B. subtilis* NSRS 89-24 was reported by Leelasuphakul et al. (2006).

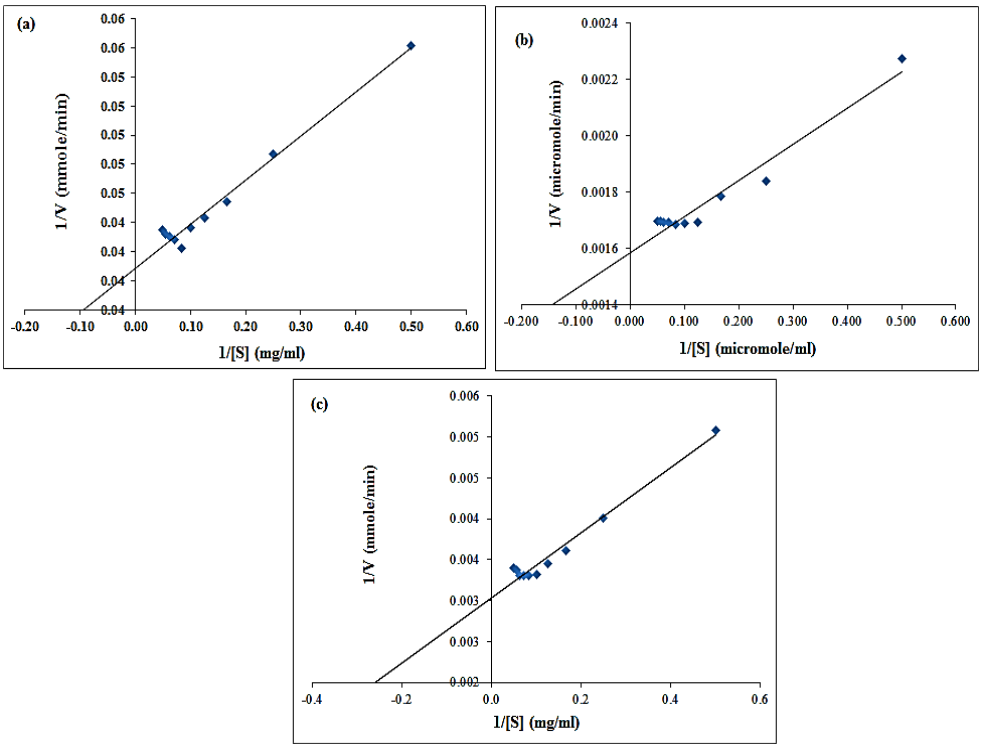


Figure 3.6 Lineweaver-Burk double reciprocal plots for the determination of K_m and V_{max} value of TPP partially purified xylanase (a), Cellulase (b) and β-1, 3-glucanase (c) from *T. citrinoviride* strain AUKAR04.

3.6 Immobilization of the Enzymes by COMBI-CLEAs

Combined Cross-Linked Enzyme Aggregates (combi-CLEAs) are a versatile immobilization method to improve the reusability and stability of the enzyme. It combines both purification and immobilization in a single process. In this investigation, we have studied the significance of various parameters such as the effect of precipitant on the recovery of the enzymes, cross-linker concentration (glutaraldehyde) and crosslinking time.

3.6.1 Effect of the Precipitant on the Recovery of Cellulase, Xylanase and β-1,3-Glucanase

During combi-CLEAs preparation, the precipitation efficiency and maximum recovery of enzyme activity depend on the nature of the precipitant used, and hence, it is necessary to select the best precipitant (R. a. Sheldon, 2011). In this study, five different types of precipitants were used to determine their effect on the enzyme activity recovery prior to the cross-linking step. Figure 3.7 shows that the combination of ammonium sulfate and t-butanol (TPP method) was found to be the best precipitant for obtaining maximum enzyme activity recovery (99%) of cellulase, xylanase and β-1,3-glucanase. TPP method exhibits the significant advantage of precipitating all proteins in their native structure (Dennison & Lovrien 1997).

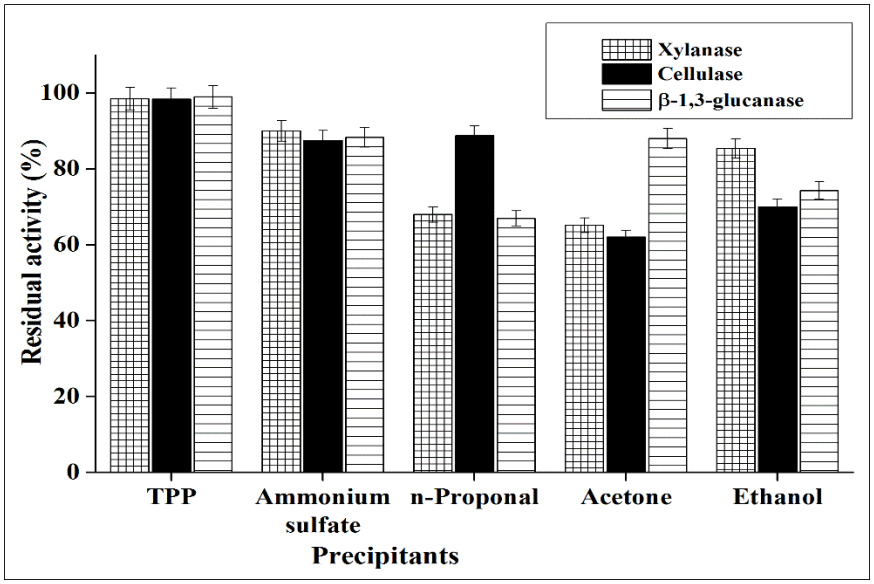


Figure 3.7 Effect of different precipitants on the residual activity of xylanase, cellulase and β-1,3 glucanase. The 100% activity recovery corresponds to 800 U/mg aggregate for xylanase, 120 U/mg aggregate for cellulase and 550 U/mg aggregate for β-1,3-glucanase. All the experiments were done in triplicate and the error bar shows the error percentage in each set of reading.

3.6.2 Effect of Glutaraldehyde Concentration on Combi-CLEAs Preparation

The concentration of glutaraldehyde is one of the critical parameters in the preparation and recovery of active enzymes in the combi-CLEAs process. A lower concentration of cross-linker leads to insufficient cross-linking and further leaching out of enzymes during the

hydrolytic process on the substrate (Talekar et al. 2013a). Conversely, a higher concentration of glutaraldehyde results in the loss of enzyme activity due to excessive cross-linking between active sites (Majumder et al. 2008). Figure 3.8 depicts the influence of glutaraldehyde concentration on the activity and recovery of cellulase, xylanase and β-1,3-glucanase in combi-CLEAs. The results indicate that the highest activity recovery in combi-CLEAs was attained by using 100 mM glutaraldehyde concentration: 98.1±2% for xylanase, 98.3 ±2% for cellulase and 98.8 ±2% for β-1,3-glucanase. Correspondingly, there was no significant activity of cellulase, xylanase and β-1,3-glucanase detected in the supernatant. The efficiency of reducing sugar release by the combi-CLEAs was checked against the appropriate substrates as described in the enzyme assay section. In this regard, the maximum amount of reducing sugar (24.5 mg/ml) was obtained by combi-CLEAs generated with the concentration of 100 mM glutaraldehyde (Figure 3.8).

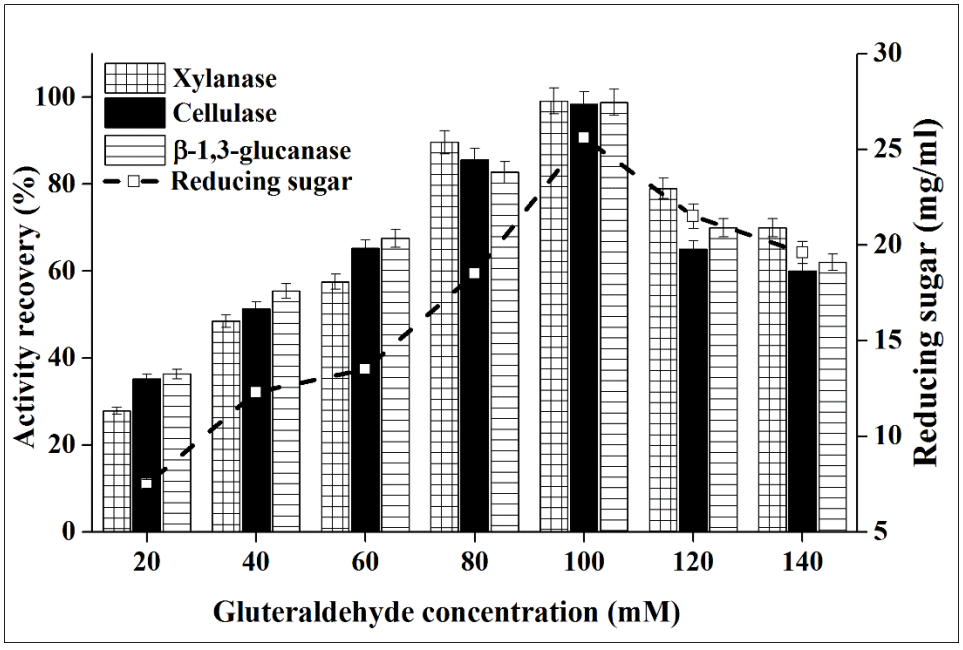


Figure 3.8 Effect of glutaraldehyde concentration on the activity recovery of xylanase, cellulase and β-1, 3-glucanase with the cross-linking time of 7.5 hours at 28°C. The 100% activity recovery corresponds to 800 U/mg aggregate for xylanase, 120 U/mg aggregate for cellulase and 550 U/mg aggregate for β-1,3-glucanase. All the experiments were done in triplicate and the error bar shows the percentage error in each set of reading.

3.6.3 Effect of Cross-Linking Time on Combi-CLEAs

For the optimization of the cross-linking step, the effect of cross-linking time on the activity recovery of cellulase, xylanase and β-1,3-glucanase in the resulting combi-CLEAs was studied. Based on the previous results, the amount of glutaraldehyde concentration was fixed at 100 mM. Results in Figure 3.9 show that an increased activity recovery is observed when the cross-linking time was increased up to 7.5 hours at 30ºC. This was checked for each of the three enzymes, and no enzyme activities remained in the supernatant at a time of 7.5 hours. Samples collected before that time exhibited some enzyme activity in the supernatant, due to incomplete cross-linking.

In the samples collected after 7.5 hours, we found a decreased activity of each enzyme in combi-CLEAs, but still no enzyme activity in the supernatant. This can be explained by the fact that prolonged cross-linking time hampers enzyme flexibility, due to more intensive cross-linking. Similar results were previously reported (Kim et al. 2013; Nguyen & Yang, 2014; Torabizadeh et al. 2014).

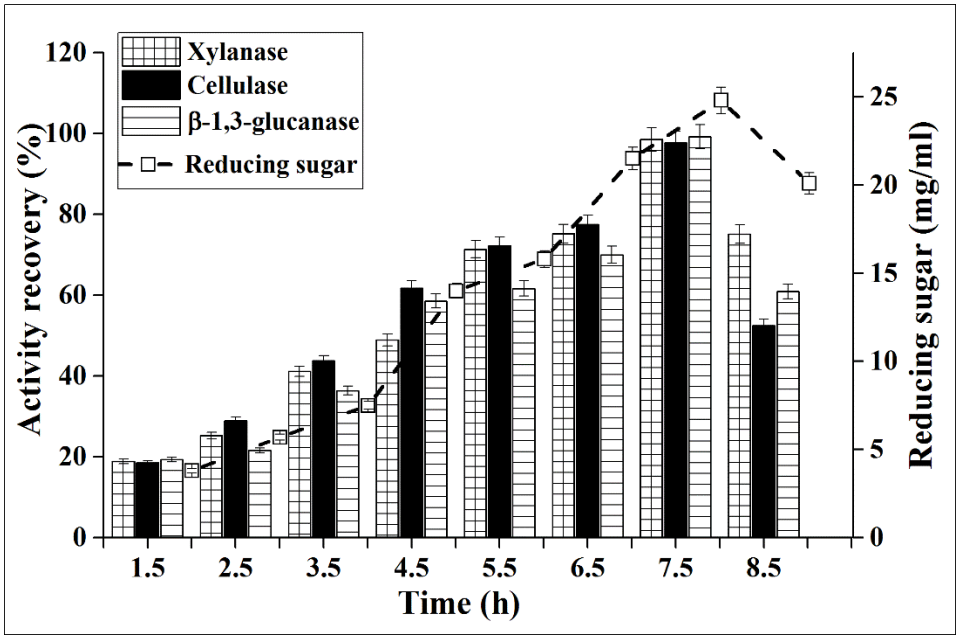


Figure 3.9 **Effect of cross linking time on combi-CLEAs preparation with 100 mM glutaraldehyde concentration.** The 100% activity recovery corresponds to 800 U/mg aggregate for xylanase, 120 U/mg aggregate for cellulase and 550 U/mg aggregate for β-1,3-glucanase. The percentage activity recovery of each enzyme in combi-CLEAs was calculated by taking the initial activity as 100%. All the experiments were done in triplicate and the error bar shows the percentage error in each set of reading.

3.7 Characterization of COMBI-CLEAs

3.7.1 SEM of Combi-CLEAs

The morphology of combi-CLEAs was characterized by SEM. Schoevaart et al. 2004, reported that CLEAs have either a spherical aggregate form (Type 1) or a less-structured appearance (Type 2). SEM of the combi-CLEAs (Figure 3.10a) before the first cycle illustrated that it had coarse-grained appearance ; more structured than type 2, but does not have the ball-like appearance of Type 1 either. Similar structure of CLEAs in the presence of bovine serum albumin was also reported for lipase(Shah et al. 2006). Figure 3.10b has a somewhat ball-type structure with few cavities (marked in redline) which might be due to the leaching out of enzymes.

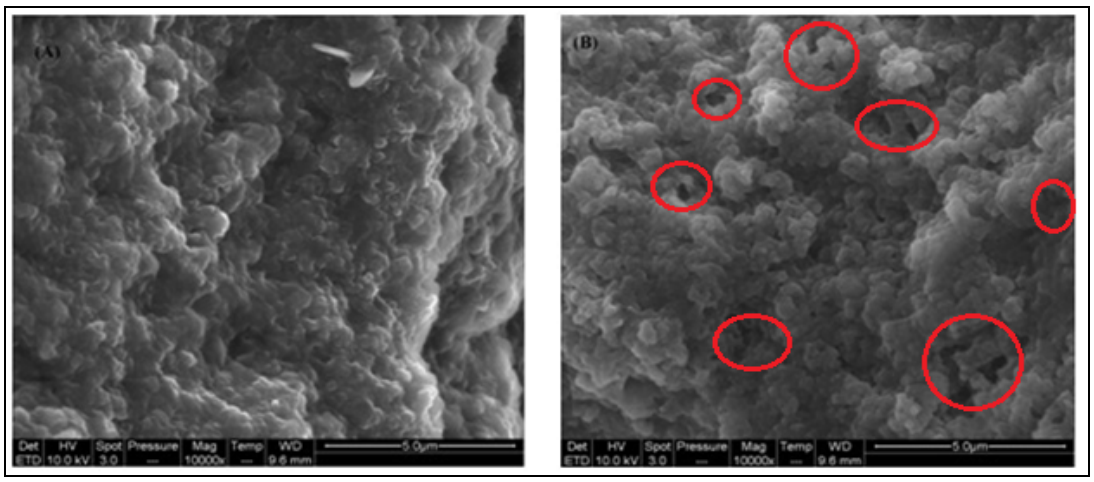


Figure 3.10 (A) SEM of the combi-CLEAs before first cycle ; (B) after tenth cycle

3.7.2 Thermal Stability of Free enzyme and Combi-CLEAs

The thermal stability of free enzymes (xylanase, cellulase and β-1,3-glucanase) and combi-CLEAs was analyzed by incubating them without substrate as specified in section 2.9.2. Residual activities are given in Figure 3.11. All the three enzymes in combi-CLEAs exhibited higher thermal stability than that of free enzymes : Xylanase, cellulase and β-1,3-glucanase in combi-CLEAs retained about 95% of their initial activity after 2 hours incubation at 50°C, while retention by free enzymes was 20-30% lower. At 70°C, combi-CLEAs retained more than 70% of their original activities, while about 60% was lost by the free enzymes. The increased thermal stability of enzymes in combi-CLEAs might be due the covalent cross-linking

between the enzyme aggregates (Wang et al. 2011). Investigations in the literature for different enzymes at different temperatures (30°C to 75°C) for various incubation times gave similar results as the herein presented ones (Abraham et al. 2014; Jiang et al. 2014; Talekar et al. 2012). Prolonged incubation to 4 hours and subsequently 8 hours led to the significant reduction in the residual activities.

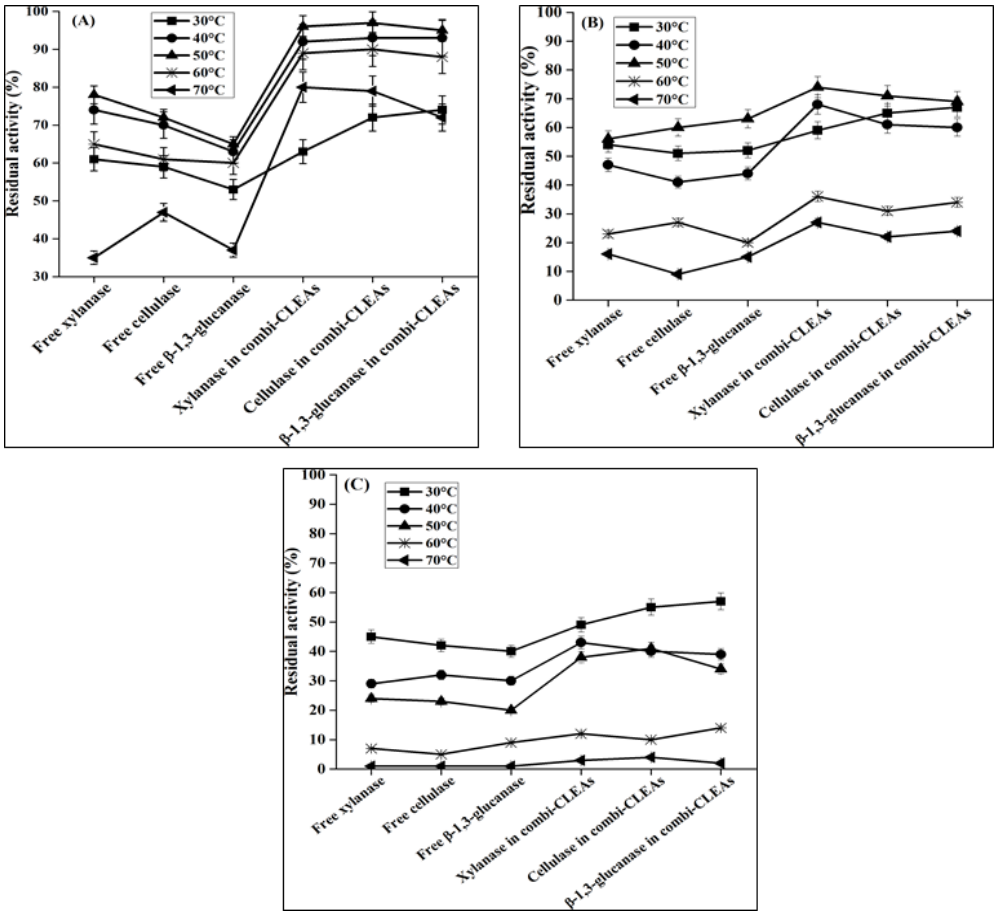


Figure 3.11 Thermal stability of xylanase, cellulase and β-1,3-glucanase in combi-CLEAs and free enzymes at 30°C, 40°C, 50°C, 60°C and 70°C after 2 hours (A), 4 hours (B) and 8 hours (C) of incubation

3.7.3 Storage Stability of Free enzyme and Combi-CLEAs

For storage study, combi-CLEAs and free enzymes were stored in 50 mM sodium acetate buffer (pH 5.0) at 4°C for a period of 11 weeks. Xylanase, cellulase and β-1,3-glucanase activities were checked at intervals of 1, 3, 5, 7, 9 and 11 weeks. As shown in Figure 3.12, free xylanase, cellulase and β-1,3-glucanase retained only 69%, 65% and 63.4% of its initial activities respectively, while combi-CLEAs retained more than 95% of their initial activities

after 11 weeks of incubation. Clearly, the combi-CLEAs have an extended storage stability compared to that of free enzymes.

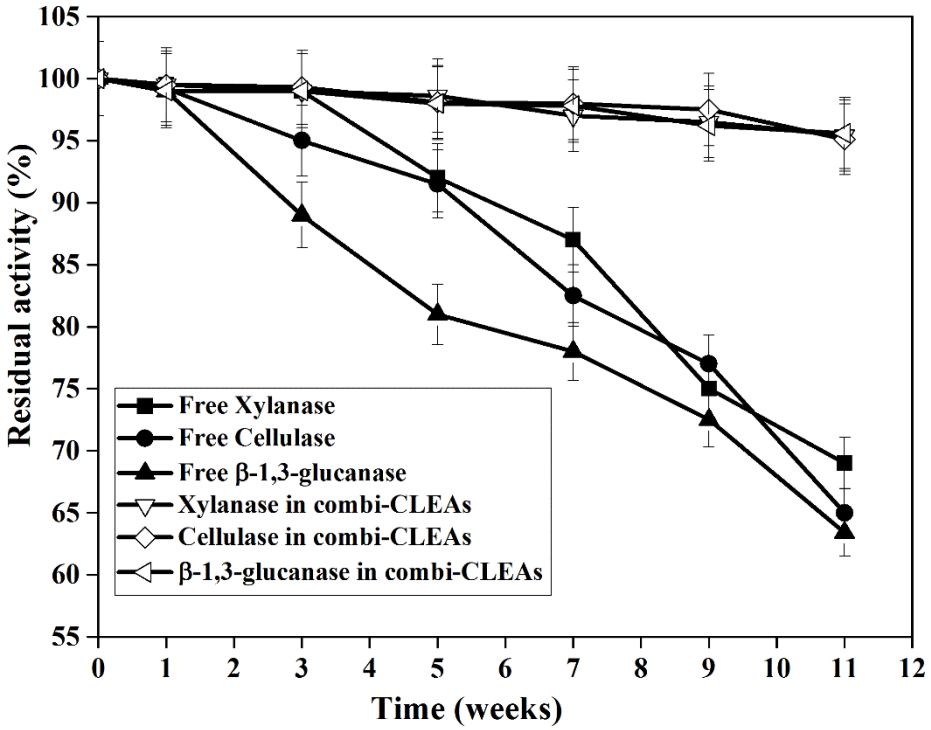


Figure 3.12 Storage stability of free xylanase, cellulase and β-1, 3-glucanase and combi-CLEAs at 4°C in 50 mM sodium acetate buffer (pH 5.0). The experiments were made in triplicate and the error bar stands for the percentage error in all set of experiments.

3.7.4 Reusability of Xylanase, Cellulase and β-1,3-Glucanase in Combi-CLEAs

The reusability of xylanase, cellulase and β-1,3-glucanase in combi-CLEAs was studied up to 10 cycles in batch operation mode at pH 5.0 at 50°C with beechwood xylan, carboxymethyl cellulose (CMC) and β-D-glucan respectively. After each cycle of reaction, combi-CLEAs was separated by centrifugation at 5000 g and washed in 50 mM sodium acetate buffer and then re-suspended in a fresh reaction mixture. As shown in Figure 3.13, the activities of xylanase, cellulase and β-1,3-glucanase in combi-CLEAs retained more than 98% up to five cycles, but started to decrease after the sixth cycle (90%), and subsequently to 50% after the ninth cycle. Some enzyme contamination during the hydrolysis step would take place and be responsible of the loss of activity after a certain number of hydrolysis cycles. After reaching a certain level of contamination, reusability might decrease (Lyu et al. 2014), possibly due to enzyme leaching from combi-CLEAs during the centrifugation step which exerts a high shear strength on the combi-CLEAs aggregates (Wang et al. 2011).

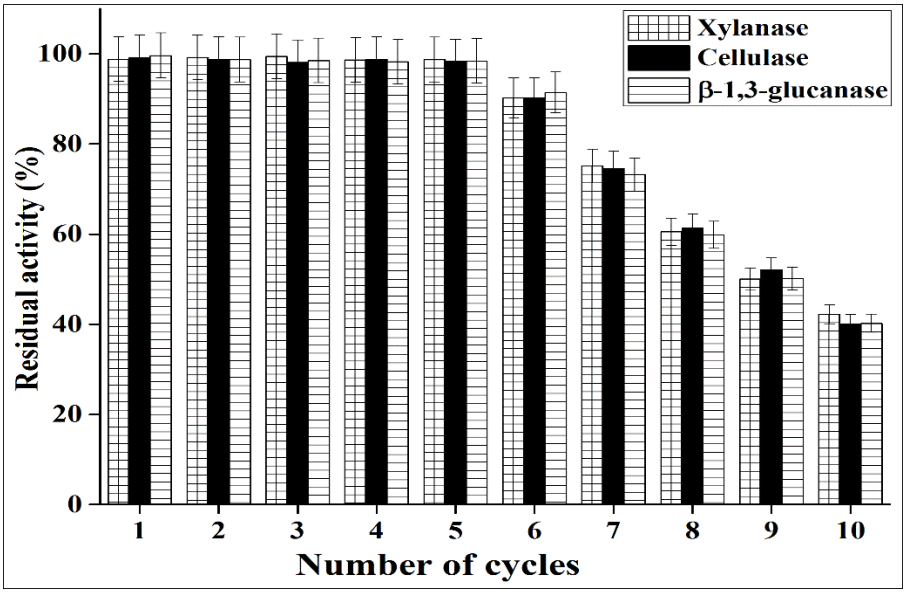


Figure 3.13 Reusability of xylanase, cellulase and β-1,3-Glucanase in combi-CLEAs.

Limitation in CLEAs Technology

There are certain limitations in CLEAs technology that are limiting its further applications such as the separation of CLEAs after the enzyme-substrate reaction is done by centrifugation or filtration, which leads to the formation of clump due to low compression resistance, which can further hamper the mass transport of substrate and decrease overall activities of the enzyme. Moreover, the enzymes with low lysine (amino acid) content might not form effective CLEAs, due to inadequate crosslinking of enzymes and hence it may lead to enzyme leaching into the reaction medium.

3.8 Immobilization of the enzymes on Functionalized Magnetic Nanoparticles

To overcome the problems in CLEAs technology, covalent linking of enzymes onto the surface of functionalized Iron Oxide Magnetic Nanoparticles (IOMNPs) were employed in this study. It is noteworthy that, the magnetic nanoparticles itself easily aggregates by its magnetic dipolar attraction, which limits their applications. Therefore, it is necessary to do surface modification/ functionalization, which helps to separate the magnetic-CLEAs from the reaction mixture.

3.8.1 Effect of Glutaraldehyde Concentration on ISN-CLEAs Preparation

It is very important to optimize the basic parameters such as glutaraldehyde concentration and crosslinking time in order to achieve the maximum cross-linking of xylanase, cellulase and β-1,3-glucanase on functionalized magnetic nanoparticles, Glutaraldehyde used as a crosslinker, helps to bind the enzymes on nanoparticles via $–NH_2$ terminal groups. As the results show in Figure 3.14, the maximum activity recovery of xylanase (97.8%), cellulase (97.5%) and β-1,3-glucanase (96.3%) in ISN-CLEAs was obtained at 135 mM glutaraldehyde concentration. Further increasing the concentration of glutaraldehyde up to 150 mM, did not make any notable difference in the enzyme recovery. At the same time, after crosslinking with less than 120 mM glutaraldehyde concentration, unbound enzyme activities were detected in the supernatant indicating insufficient crosslinking with the MNPs. From the results, it was clearly understood that amino functionalization of nanoparticles helps in binding maximum enzyme molecule on its surface. Besides, the resultant ISN-CLEAs became magnetic, it was easily separated by an external magnet.

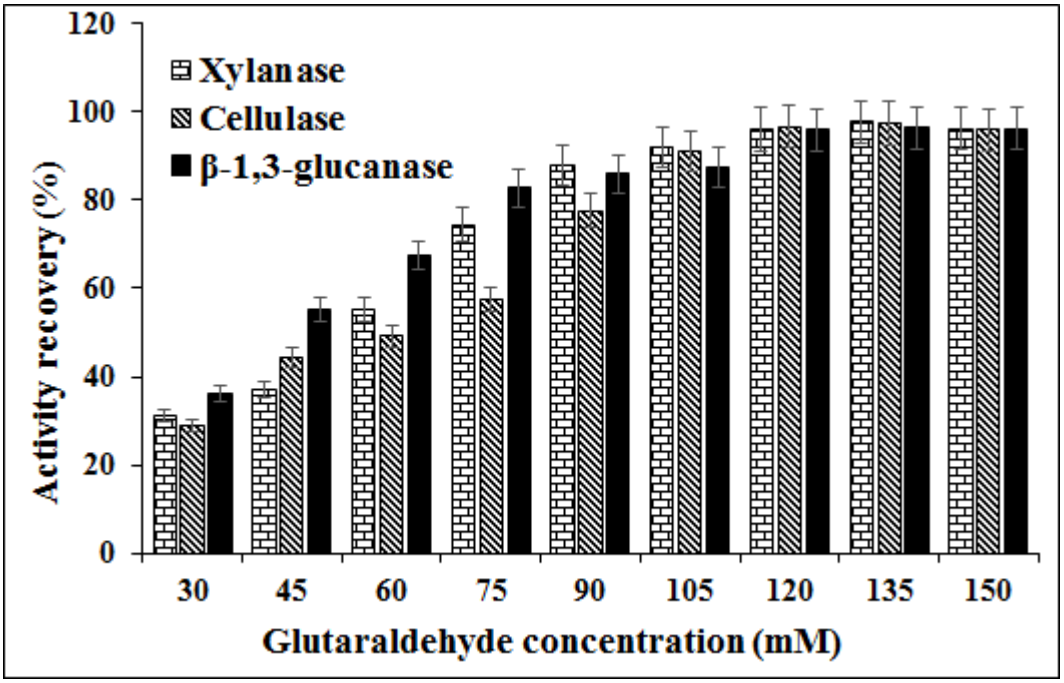


Figure 3.14 Activity recovery of xylanase, cellulase and β-1,3-glucanase on to magnetic nanoparticles with crosslinking time of 10 h at 30 °C. The 100% corresponds to 150 U/mg for cellulase, 800 U/mg for xylanase and 500 U/mg for β-1,3-glucanase. All the analysis was carried out in triplicate and the error bar indicates the percentage error in each set of determinations.

3.8.2 Effect of Cross-Linking Time on ISN-CLEAs Preparation

Crosslinking time is also a major factor for the maximum activity recovery of all the enzymes in ISN-CLEAs. Based on the previous results, the amount of glutaraldehyde was fixed at 120 mM. Figure.2 depicts the effect of crosslinking time on the recovery of xylanase, cellulase and β-1,3-glucanase. From the results, it was observed that increased activity recovery of xylanase (98.1%), cellulase (98.3%) and β-1,3-glucanase (97.3%) attained with crosslinking time of 10 h, further increasing the crosslinking time up to 12 h did not make noticeable changes in the activity recovery of all the three enzymes, but extended crosslinking time up to 14 h decreases the enzyme activities resulting in a loss of enzymes' flexibility due to intensive crosslinking times. Currently, crosslinking time less than 10 h led to low activity yield of enzymes due to insufficient crosslinking time.

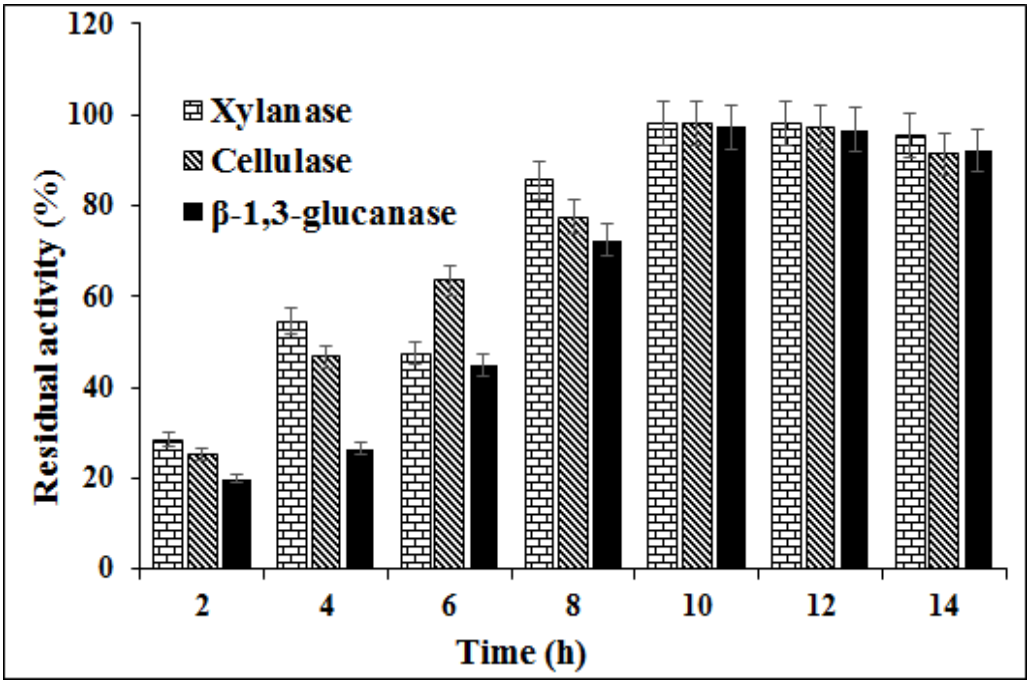


Figure 3.15 Effect of cross linking time on ISN-CLEAs preparation with 135 mM glutaraldehyde concentration. The 100% activity recovery corresponds to 800 U/mg aggregate for xylanase, 150 U/mg aggregate for cellulase and 500 U/mg aggregate for β-1,3-glucanase.

3.9 Characterization of ISN-CLEAs

3.9.1 Structural Analysis by Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra of Fe_3O_4, Fe_3O_4@SiO_2-NH_2 and ISN-CLEAs are illustrated in Figure 3.16. The peak at 591 cm^{-1} corresponds to Fe-O bond in Fe_3O_4 NPs(Reza et al. 2010), which shows the

successful synthesis of magnetic nanoparticles and depicted in Figure. 3.16 A. The silica group was attached to the Fe_3O_4 NPs by Fe-O-Si bonds and this was confirmed by the presence of SiO_2 stretching in the range from 691, 1024 and 1120 cm^{-1} (Figure. 3.16 B) (Reddy et al. 2017; Reza et al. 2010; Saikia and Parthasarathy, 2010). The characteristic peaks at 1317, 1482 and 1557 cm^{-1} attributed to the bending or vibration of the $-NH_2$ groups thus confirming the functionalization with amino groups onto the silica anchored MNPs. The decrease in the intensity of the band at 1024 cm^{-1} was observed in Figure.3.16 C which suggested that silica groups functionalized with amino groups. The peaks in the range of 1400 to 1600 cm^{-1} indicate the symmetric stretching of COO^-, C=O and C-O groups. The stretching pattern near 1557 and 1405 cm^{-1} also represents the binding of carboxyl group in the enzymes and amine groups of the MNPs. The observations of FTIR spectra led to conclusion that the enzymes were successfully cross-linked onto the bi-functionalized magnetic nanoparticles.

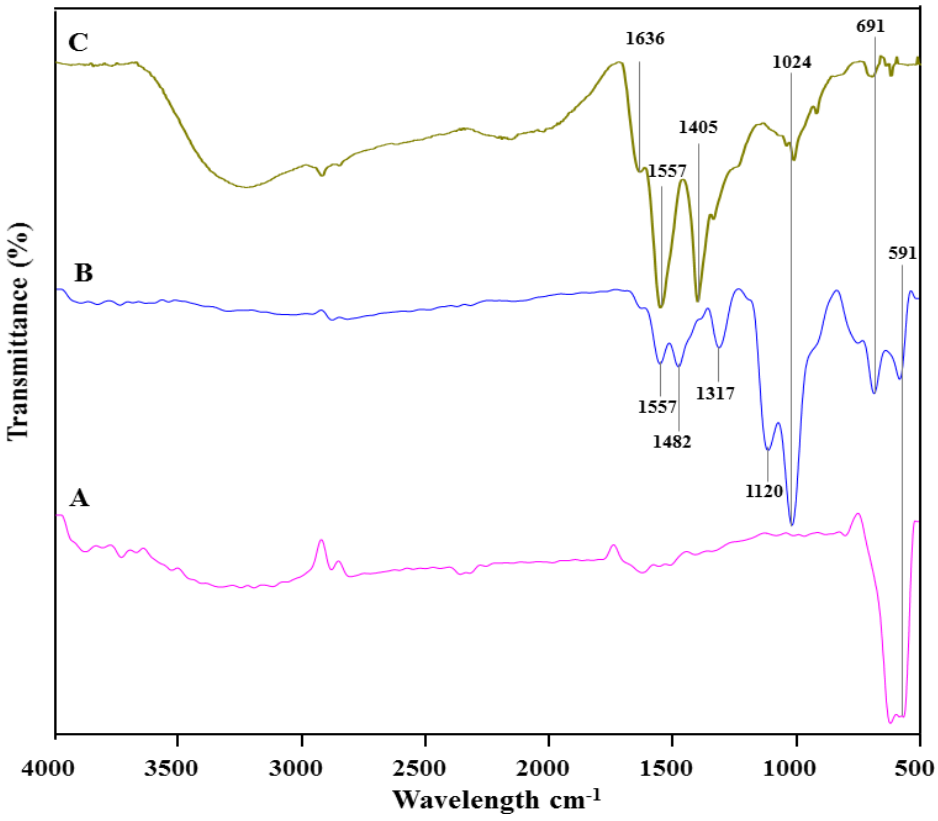


Figure 3.16 FTIR spectra of (A) Fe_3O_4 (B) Fe_3O_4@SiO_2-NH_2 and (C) ISN-CLEAs

3.9.2 Surface characterization of ISN-CLEAs by AFM and SEM

The morphology of the Fe_3O_4 NPs, Fe_3O_4@SiO_2-NH_2 NPs and ISN-CLEAs were examined by Atomic Force Microscopy (AFM) and Scanning Electron Microscope (SEM). The images in Figure.3.17 clearly depicts that the morphology of bi-functionalized NPs was significantly modified after immobilization with xylanase, cellulase and β-1,3-glucanase. The nanoparticles are the spherical shape with the average size of 0.4 to 0.5µm and the ISN-CLEAs were seen with large accumulated layers due to the binding of enzyme molecules on the bi-functionalized MNPs. To additionally characterize the variations in the surface topography of Fe_3O_4 NPs, Fe_3O_4@SiO_2-NH_2 and ISN-CLEAs, three-dimensional AFM images (Figure 3.17.B, D and F) were used to explain the strong visual impact of the structural variations(Cao et al. 2016). It is worth mentioning that the surface of the ISN-CLEAs exhibited gaps which showed that there was sufficient crosslinking between the enzymes and nanoparticles. The SEM images (Figure 3.18) of Fe_3O_4@SiO_2-NH_2 and ISN-CLEAs were the spherical structure of aggregates and the enzymes were bound together into balls onto the functionalized MNPs with the average diameter of about 0.12 µm. These observations led to the conclusion that the ball structure might pave way for the movement of the substrate to inner enzymes.

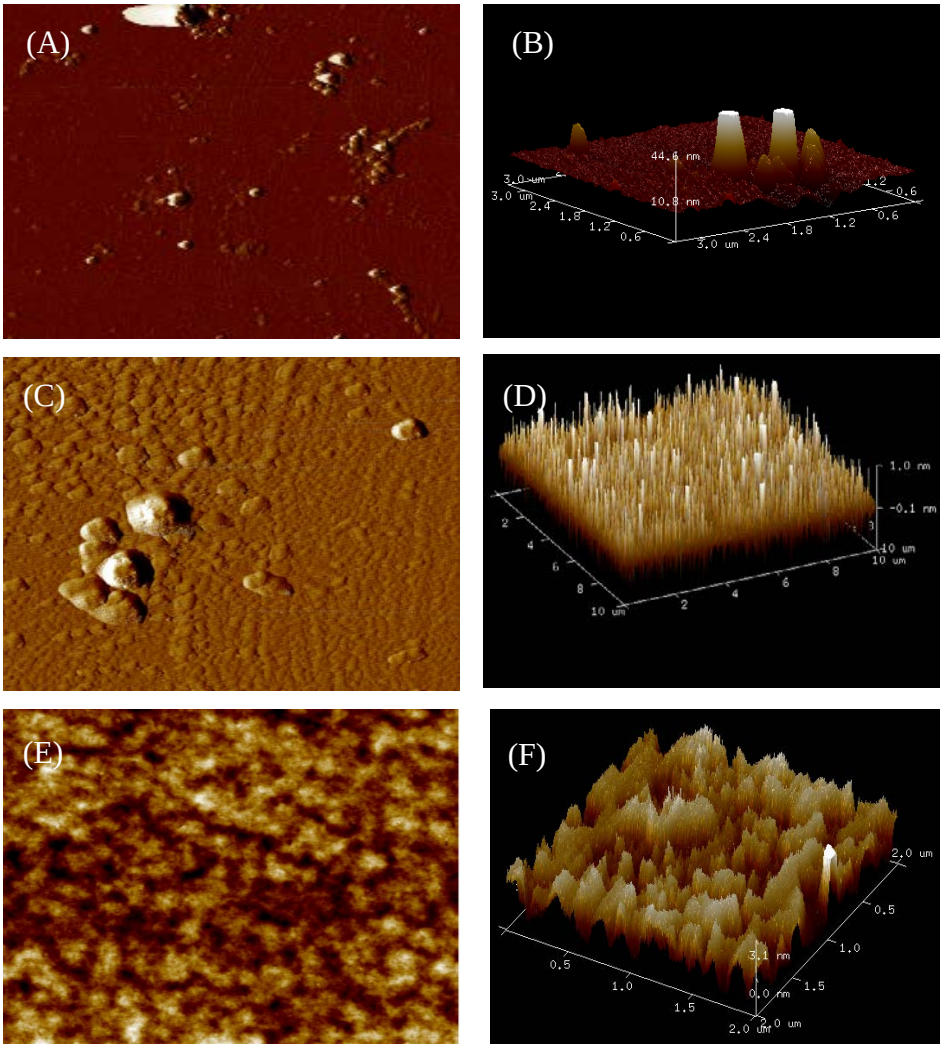


Figure 3.17 Topography of (A, B) Fe_3O_4 (C, D) Fe_3O_4@SiO_2-NH_2 and (E, F) ISN-CLEAs

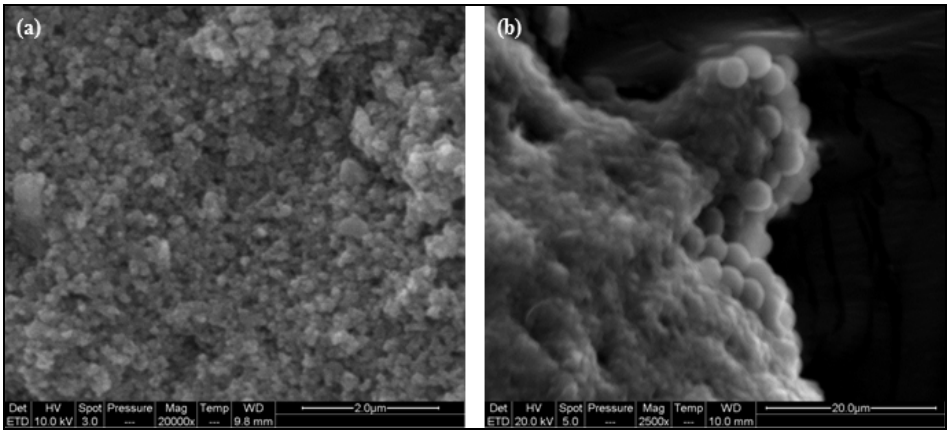


Figure 3.18. SEM of (a) Fe_3O_4@SiO_2-NH_2 and (b) ISN-CLEAs

3.9.3 Particle Size Analysis by Dynamic Light Scattering (DLS)

The hydrodynamic diameter of the Fe_3O_4, Fe_3O_4@SiO_2-NH_2 and ISN-CLEAs were determined by DLS method. The average diameter of the Fe_3O_4 NPs was 82.2 nm (Figure 3.19a.) after functionalized with -NH_2 and SiO_2 group, the size increased to 86.4 nm (Figure 3.19b.). On the other hand, the average diameter of the ISN-CLEAs was 1148.9 nm and it is shown in Figure 3.19c. This reveals that the immobilization of enzymes on the surface of magnetic nanoparticles.

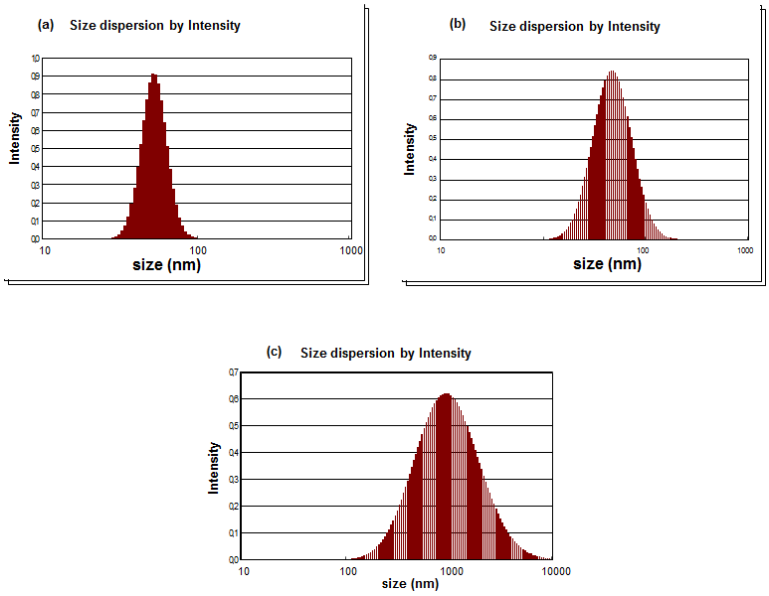


Figure 3.19 DLS of Fe_3O_4, Fe_3O_4@SiO_2-NH_2 and ISN-CLEAs

3.9.4 Thermal Stability of ISN-CLEAs

The thermal stability of free enzyme and combi-CLEAs were explained in section 3.6.2. In view of ISN-CLEAs, it was determined by incubating it without substrate as depicted in section 2.9.2. The results for thermal stability of ISN-CLEAs at 30°C, 40°C, 50°C, 60°C and 70°C with respect to different time is shown in figure 3.20. All the three enzymes in ISN-CLEAs showed slightly higher thermal stability than that of combi-CLEAs. At 50°C, ISN-CLEAs retained about 97% activity for at least 2 hours of incubation time. As observed ISN-CLEAs retained more than 75% of their initial activities at 70°C for 2 hours of incubation. Extended incubation to 4 hours and subsequently 8 hours led to the notable reduction in the residual activities of all the three enzymes in ISN-CLEAs. These observations led to the conclusion that amino-functionalized magnetic nanoparticles covalently linked with xylanase, cellulase and β-1,3-glucanase (ISN-CLEAs) provides more effective conformational stabilization to its secondary structure through a series of hydrogen bonds and hydrophobic

interaction leading to increased thermal stability as compared to the free enzymes. Besides, the smaller size of the MNPs may allow enzyme molecule to expand over its surface with the better exposure of the active site channel (Cao et al. 2016; Jafari Khorshidi et al. 2016; Mahmoud et al. 2013).

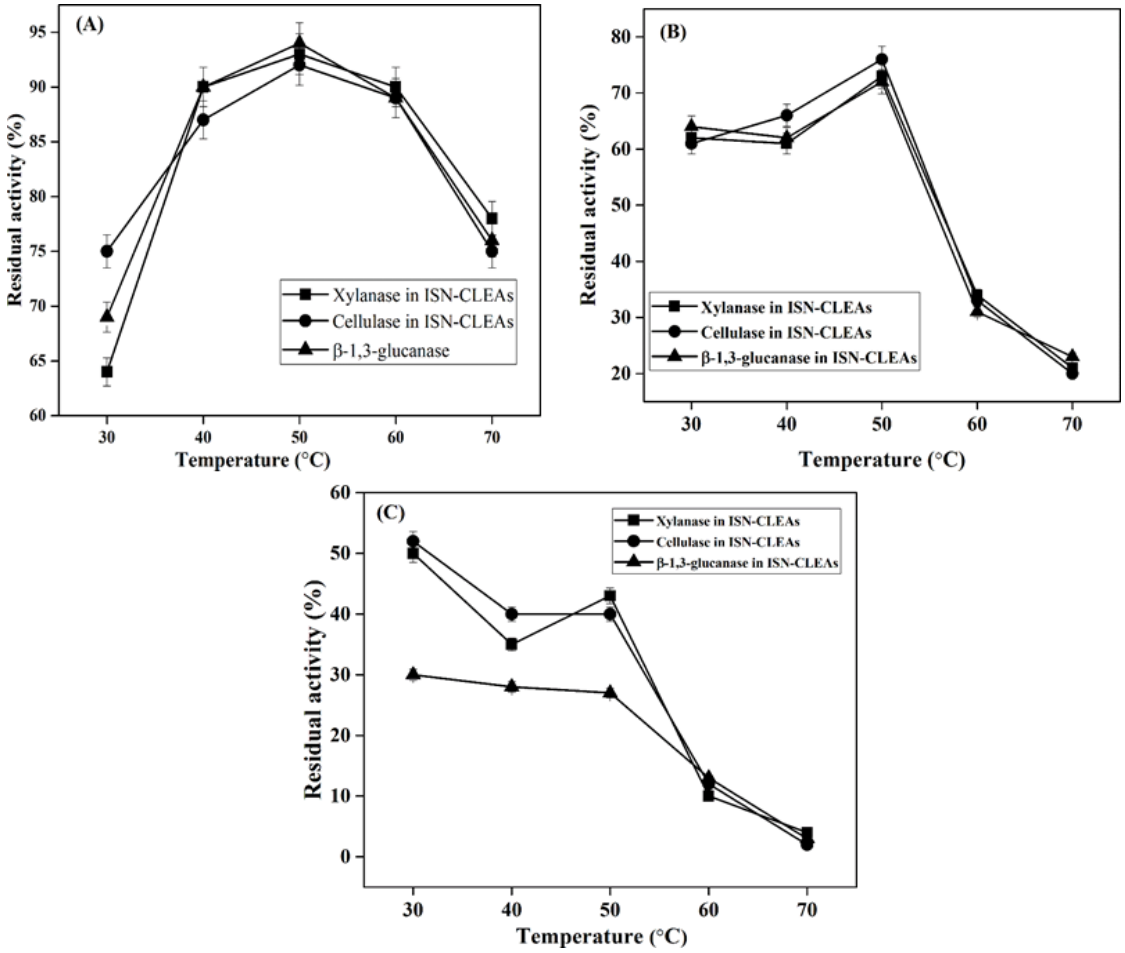


Figure 3.20 Thermal stability ISN-CLEAs at 30°C, 40°C, 50°C, 60°C and 70°C after 2 hours (A), 4 hours (B) and 8 hours (C) of incubation

3.9.5 Storage stability of ISN-CLEAs

For storage study, ISN-CLEAs was stored in 50 mM sodium acetate buffer (pH 5.0) at 4°C for a period of 11 weeks. Xylanase, cellulase and β-1,3-glucanase activities in ISN-CLEAs was checked at intervals of 1, 3, 5, 7, 9 and 11 weeks. The storage stability of free xylanase, cellulase and β-1,3-glucanase and combi-CLEAs were explained in section 3.6.3. As shown in Figure 3.21, ISN-CLEAs retained more than 97% of their initial activities after 11 weeks

of incubation. Which has slightly increased storage stability than combi-CLEAs and extended storage stability than that of free enzymes.

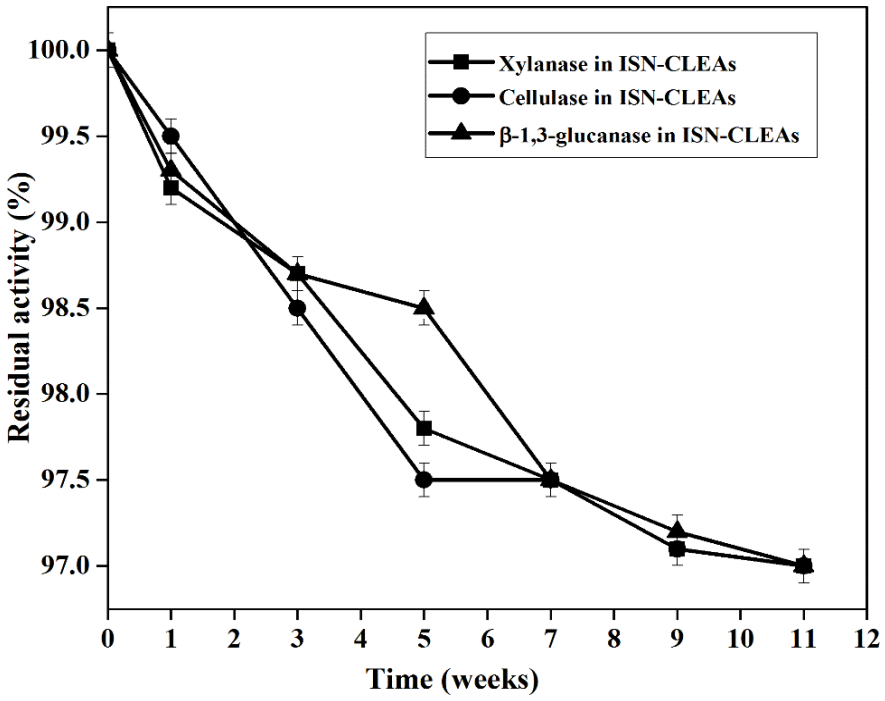


Figure 3.21 Storage stability ISN-CLEAs at 4°C in 50 mM sodium acetate buffer (pH 5.0).

3.9.6 Reusability of Xylanase, Cellulase and β-1,3-Glucanase in ISN-CLEAs

The reusability of xylanase, cellulase and β-1,3-glucanase is a key factor for industrial applications for an economic reason. The recyclability of xylanase, cellulase and β-1,3-glucanase in ISN-CLEAs were examined up to 10 cycles with beechwood xylan, carboxymethyl cellulose and β-D-glucan as substrate respectively, at pH 5.0 and temperature 50°C. After each cycle of the hydrolysis reaction, ISN-CLEAs was recovered by an external magnetic separation and washed with 50 mM sodium acetate buffer (pH-5.0) and then re-suspended in a fresh reaction mixture. As shown in Figure 3.22, the activities of xylanase, cellulase and β-1,3-glucanase in ISN-CLEAs retained more than 97% up to sixth cycles, but began to decrease after the sixth cycle (80%), and subsequently to about 60% after the ninth cycle. ISN-CLEAs retained the better residual activity of all the three enzymes than combi-CLEAs and easy to recover from the reaction mixture. The decrease in the enzymes' activity after a certain number of hydrolysis cycles in the ISN-CLEAs could be due to the gradual

leakage of enzymes or some kind of contaminations which inhibits the activity of all the three enzymes equally and loss of particles during magnetic separation, and/or enzyme denaturation (Mahmoud et al. 2013).

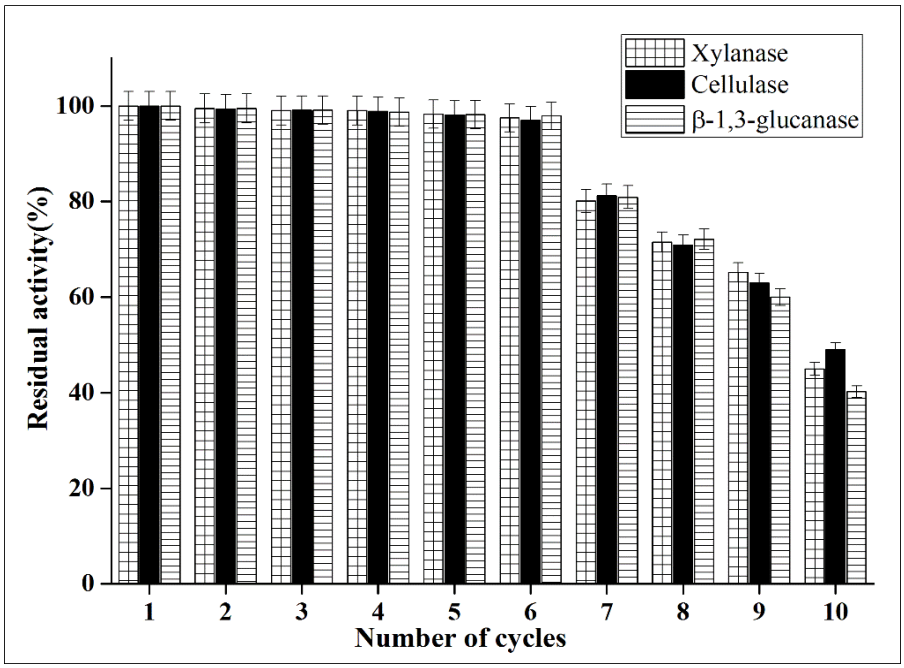


Figure 3.22 Reusability of xylanase, cellulase and β-1,3-glucanase in ISN-CLEAs

3.10 Characterization of Biomass

As mentioned in section 2.10, The composition of SCB raw material was analyzed by classical hydrolysis method using sulfuric acid and the sugar content was measured by HPLC. It contains about 43% of glucan, 27% of xylan, 4% of arabinan and 26% of lignin and other components such as ass and proteins. Similar results were also obtained in some literature by Aguilar et al. (2002); Aita et al. (2011).

3.11 Pretreatment of Biomass

Liquid ammonia pretreatment was adopted to loosen the structure of SCB biomass by partial removal of lignin and increase the enzymes' accessibility to cellulose and hemicelluloses. This pretreatment of SCB was carried out with 10% of liquid ammonia at 140°C for 120 minutes resulted in removal of about 40% of lignin and retained about 95% of glucan, 65% of xylan and 40% of arabinan in the raw material (Table 3.4) with the solid yields of about 70%, which indicated that some amounts of hemicellulose and lignin were removed during the

pretreatment process. Besides, fermentation inhibitors such as HMF, furfural and acetic acid were not generated during the pretreatment and it was confirmed my HPLC analysis.

Table 3.4 SCB composition expressed percentage (%) of dry biomass.

Sl. No	Biomass component	Individual component (g/100g dry biomass)		Yield (%)
		Untreated SCB	Ammonia treated SCB*	
1	Glucan	42.5±2.1	40.5±2.02	95.2
2	Xylan	27.3±1.4	17.9±0.89	65.4
3	Arabinan	3.8±0.2	1.5±0.08	40.6
4	Lignin	26.4±1.3	10.3±0.51	38.9**
5	Solid remaining	100.00	70.10	

*Liquid ammonia pretreatment (10%) performed at 140°C for 120 min with the solid to liquid ratio of 1:10.

**Corresponds to percentage (%) removal of lignin.

3.12 Chemical and Structural Analysis of SCB

3.12.1 Attenuated Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

The FTIR spectra of the untreated SCB, the ammonia-pretreated SCB pulp and ISN-CLEAs treated SCB pulp are shown in Figure 3.23. The band at 3338 cm^{-1} is ascribed to the stretching of –OH groups. There is no significant variation there, showing the large abundance of polysaccharides in the pulps. Peaks between 1640 cm^{-1} and 1700 cm^{-1} are generally ascribed to C=O stretching in carbonyl functions, which is typical of the presence of aldehydes, ketones, carboxylic acids and esters (Tsai et al. 2006; Wang et al. 2010). The peak at 1740 cm^{-1} refers to the acetyl group in hemicelluloses, which appears only in the untreated SCB. Absence of this peak in the ammonia-cooked SCB and in the ISN-CLEAs treated SCB pulp implies that ammonia cooking in alkaline medium removes all esters. Variations of the bands in the region between 1500 cm^{-1} and 1100 cm^{-1}, typical of aromatic C=C stretching (Sun et al. 2004) (1430 cm^{-1}) and various C-C and C-O linkages in lignin tend to indicate that delignification takes place not only during ammonia-cooking, but also during

the enzyme treatment of the SCB pulp. The aromatic (C-O) stretching peak in lignin at about 1250 cm^{-1} decreased significantly after ammonia-cooking of SCB. The peak in the region of 890 cm^{-1} indicates the presence of C-O-C vibration of β-glycosidic linkage in hemicelluloses and cellulose (Sun et al. 2004). It became weaker in ISN-CLEAs treated SCB but not absent. This allows to conclude that hemicelluloses and cellulose are partially de-polymerized by the synergetic action of xylanase, cellulase and β-1,3-glucanase in ISN-CLEAs.

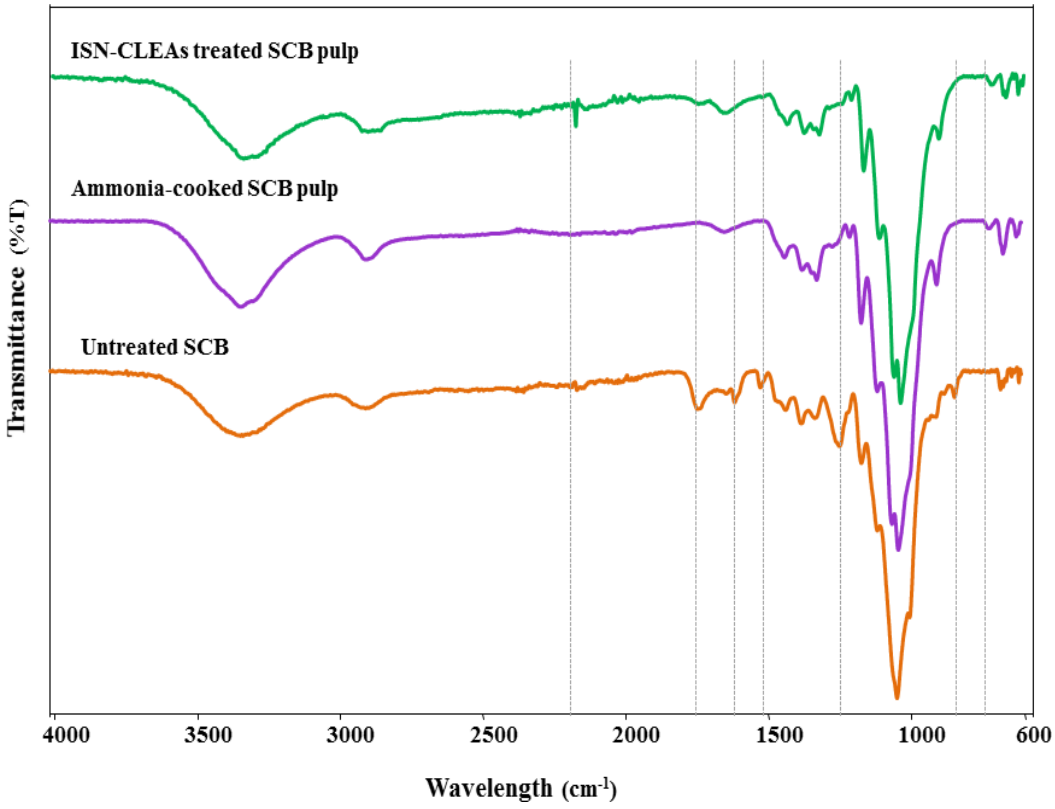


Figure 3.23 FTIR spectra of untreated, liquid-ammonia pretreated and ISN-CLEAs treated SCB

3.12.2 Thermogravimetric Analysis (TGA)

Thermogravimetric analysis was used to study the thermal properties of untreated, ammonia pretreated and ISN-CLEAs treated. The thermograms (TGA) and DTG curves are shown in Figure 3.24 (a and b) as weight loss and derivative of the thermogravimetric as a function of temperature respectively. The initial weight loss can be seen between 80-120ºC that is due to the moisture present in the SCB. Interestingly, the thermal stability of the ammonia pretreated and enzymatically treated samples shown remarkable improvement. In the untreated SCB, progressive degradation of lignin and hemicelluloses is well shown, by the progressive weight loss of the sample, since about 200°C, before the final step of cellulose degradation at

the highest temperature of about 340°C. At this final temperature, the content of organic matter remains rather high (about 30%), probably due to highly resistant high molar mass cellulosic chains in the highly crystallized part of the original untreated sample. Thus, after ammonia cooking, it is interesting to note that the sample highly stable until 300°C, the temperature at which cellulose starts to degrade. Most of the degradation continued until 350°C, higher for the more resistant chains i.e. highly ordered, long and crystalline. Such a small range of temperature at which thermal degradation occurs (between 340 and 350°C), and the low value of residual matter (20% by weight) after cellulose degradation shows that thermal degradation is a homogeneous process, meaning that the sample is made of ordered and homogeneous matter with homogeneous molar mass and homogeneous level of crystallinity.

After the enzyme treatment, it can be noticed that the thermal degradation starts at first sight at similar temperature and weight loss (but, after careful observation, little earlier (lower T and weight loss) and more progressively than in the untreated and ammonia-cooked sample. This is probably due to the slight increase of amorphous cellulose and disorder in the lower chains portions, and possibly, the slight reduction of their molar mass. Then, the main part of the cellulosic sample degrades similarly as the ammonia-pretreated SCB, but at the end, after about 50% degradation, thermal resistance increases significantly. After enzymatic treatment, such an increase in thermal resistance for a portion of about 50% by weight of the sample in the remaining undissolved part of the sample, could be due to a modification of ultrastructural or molecular nature: complexation between protein and cellulosic matter, or induced cross-linking due to the enzyme action, or possibly, the remainder is made of very resistant, highly crystallized cellulose chains, like in microcrystalline cellulose obtained by acid hydrolysis, in which all the amorphous chains were eliminated.

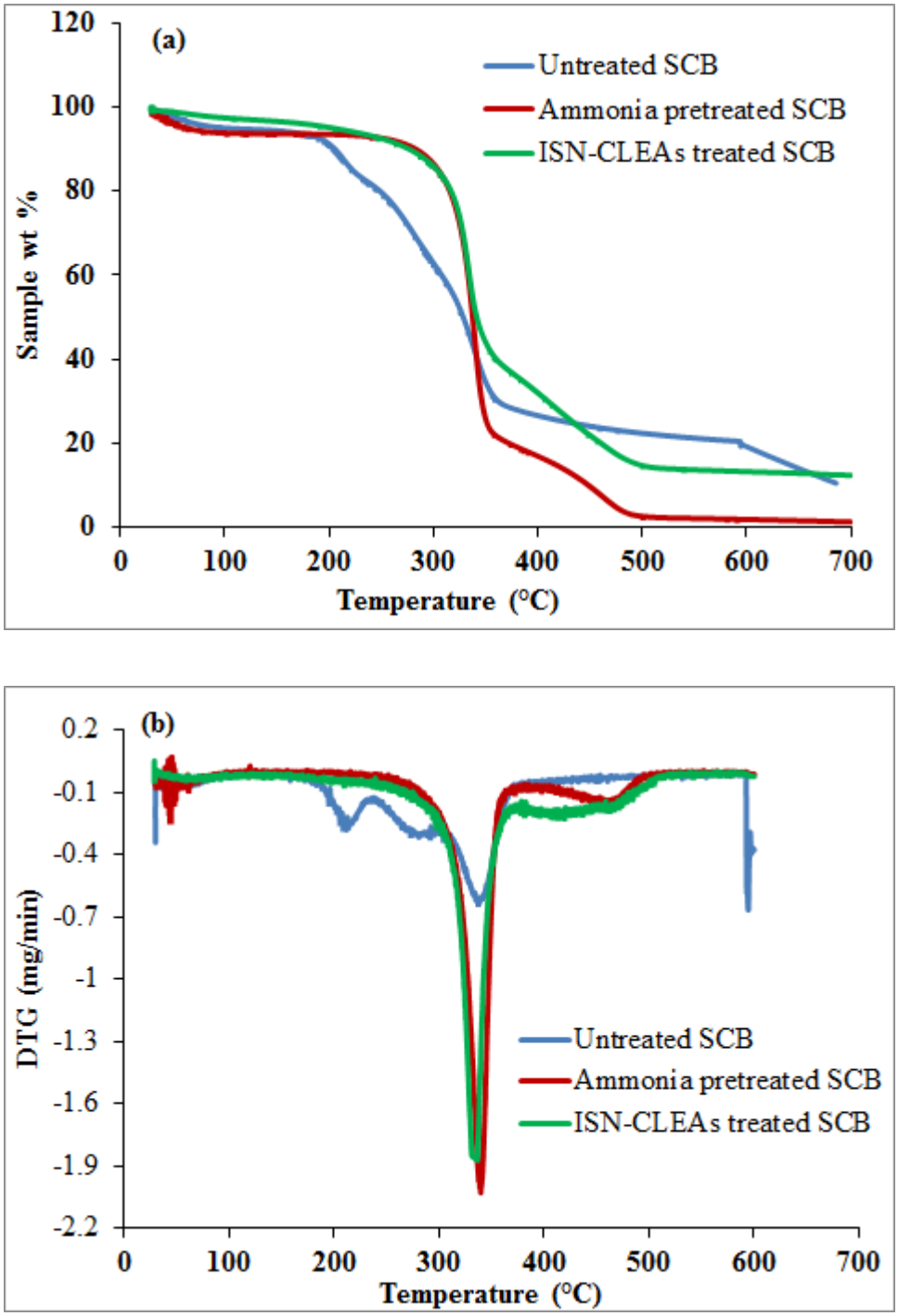


Figure 3.24 TGA (a) DTG (b) curves of the untreated, Ammonia pretreated and ISN-CLEAs treated SCB

3.12.3 X-ray Diffraction Analysis (XRD)

XRD was carried out to evaluate the crystallinity degree of untreated, ammonia pretreated and enzyme treated SCB. The crystallinity index is strongly influenced by the composition of biomass. The crystalline content in cellulose is used to estimate the recalcitrance of the biomass to enzymatic hydrolysis (Maeda et al. 2011). The results are shown in Figure 3.25 that the untreated SCB has the lowest crystallinity index (27.9±1%) because it has a higher content of lignin and hemicelluloses.

Interestingly, the ammonia-treated SCB shows a very significant increase in the crystallinity of cellulose (57.9 ±1%.). The curve fits well that of pure cellulose I, i.e. the pure cellulose originally in the raw sugarcane bagasse. Thus, it attributes that pretreatment has eliminated much of the amorphous polymers, like lignin and hemicelluloses, and probably also a lot of other small molecules like oligomers and soluble extractives. Indeed, in the alkaline cooking medium, a lot of small molecules are submitted to deacetylation and become soluble. Solubility is also enhanced by the high cooking temperature, the partial organic/aqueous and polar/non-polar character of the ammonia-water medium. During the ammonia pretreatment, removal of amorphous substances, and also possibly, partial rearrangement or recrystallization of cellulose molecular chains by solvent elimination, the crystallinity of the remained cellulose becomes quite high. This leads to a much significant increase of the heat resistance to degradation, as shown in the TGA analysis (Figure 3.24 a & b).

Compared to the ammonia pretreated SCB, the crystallinity index of the ISN-CLEAs treated SCB increased to 61.3±1%. Then it is realistic to think that the enzyme treatment leaves a greater part of highly crystalline cellulose after removing all the amorphous part of the sample, more or less similarly as a treatment of cellulose by deep acid hydrolysis. The additional peak at 34.4° observed after enzymatic (xylanase, cellulase and β-1,3-glucanase) treatment, the diffractogram was irregular which might be due to the conversion of the hemicelluloses (xylan) into xylose or the rearrangement of fibrils.

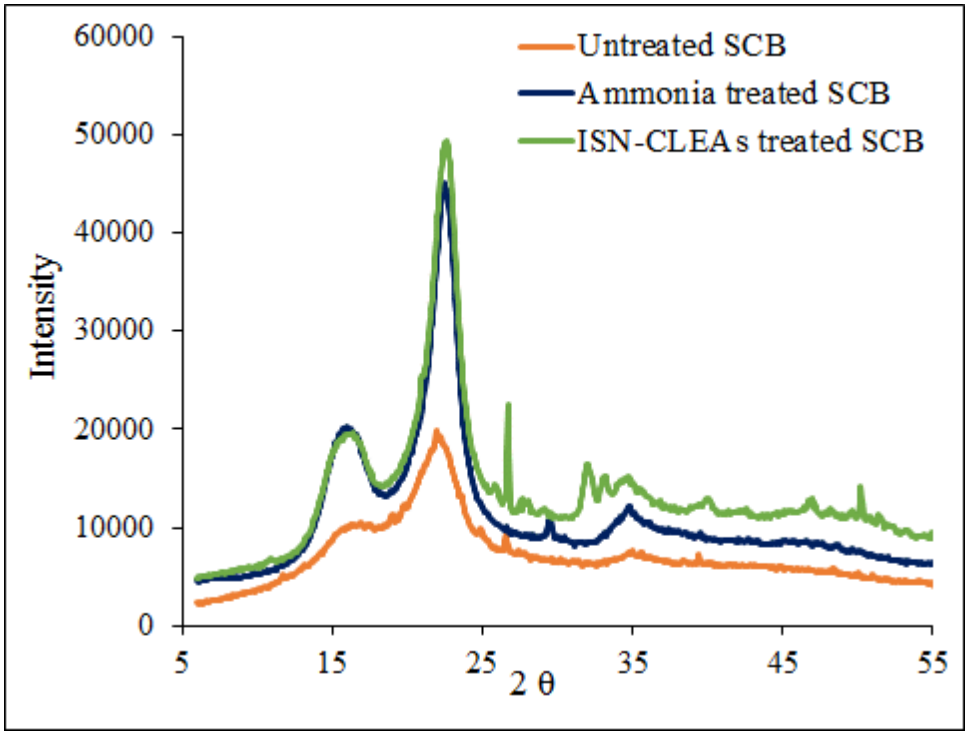


Figure 3.25 XRD patterns of the untreated, Ammonia pretreated and ISN-CLEAs treated SCB

3.13 Enzymatic Hydrolysis of SCB by Free enzyme, Combi-CLEAs and ISN-CLEAS

In this study, the enzyme cocktail such as xylanase (800 U/mg), cellulase (120 U/mg) and β-1,3-glucanase (550 U/mg) produced from *T. citrinoviride* AUKAR04 were used in each case i.e. the free enzyme, combi-CLEAs and ISN-CLEAs. Enzymatic hydrolysis was carried out on ammonia-pretreated SCB pulp as a substrate (detailed explanation in section 2.13.1). The liquid phase hydrolysate containing monomeric sugars were analyzed by HPLC. SCB hydrolyzed by free enzymes released a maximum amount of glucose (30.77 g/l), xylose (9.39 g/l) and arabinose (0.74 g/l) after 24 h of incubation at 50°C (Figure 3.26), prolonged incubation up to 48 hours did not make remarkable changes in the sugar yield.

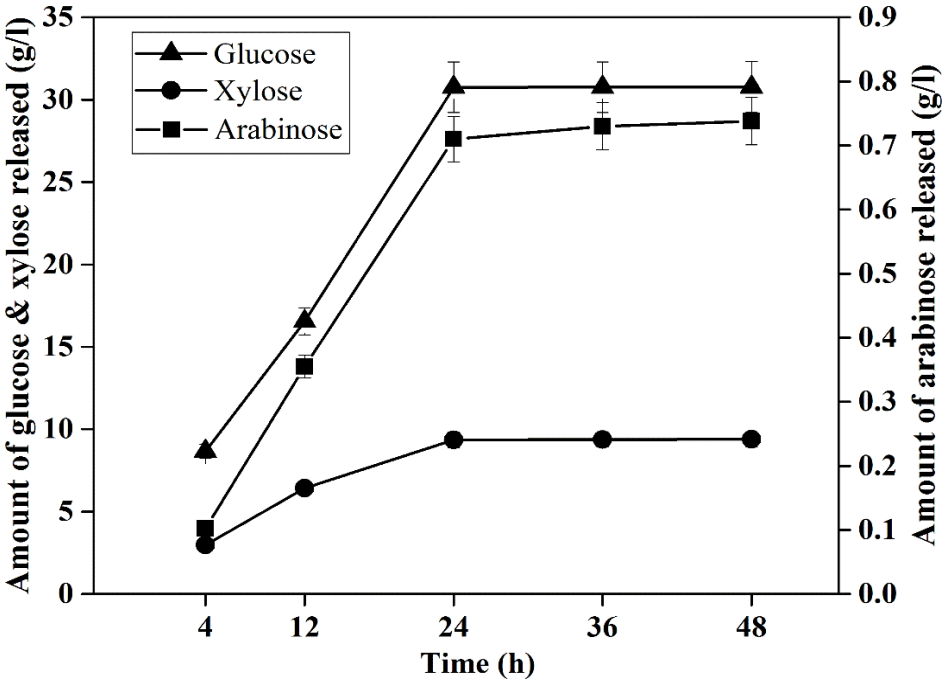


Figure 3.26 Yield of Arabinose, Glucose and Xylose by Free enzymes on the hydrolysis of pretreated SCB.

The reaction of combi-CLEAs on SCB pulp liberated a maximum amount of glucose (33.36 g/l), xylose (12.51 g/l) and arabinose (0.78 g/l) after 48 hours of incubation at 50°C (Figure 3.27), prolonged incubation for 60 hours did not lead to any remarkable improvement in the sugar yield. These results provided a vivid depiction that the cross-linked enzyme aggregates would take a longer time to hydrolyze the solid substrate might be due to the limited exposure of enzymes' active site to the substrate and restricting the internal mass transport of the substrate. In order to check the reusability of the combi-CLEAs on the solid substrate (SCB) after the first cycle of hydrolysis (after 48 hours), the reaction mixture was centrifuged at 7000rpm for 10 min. As a result, combi-CLEAs and the unhydrolyzed substrate were recovered and added to the fresh substrate (4%) and it was incubated at the same conditions like the first cycle, it hydrolyses the fresh substrate and released a maximum of about 2.64 g/l of glucose and 1.18 g/l of xylose in 8 hours.

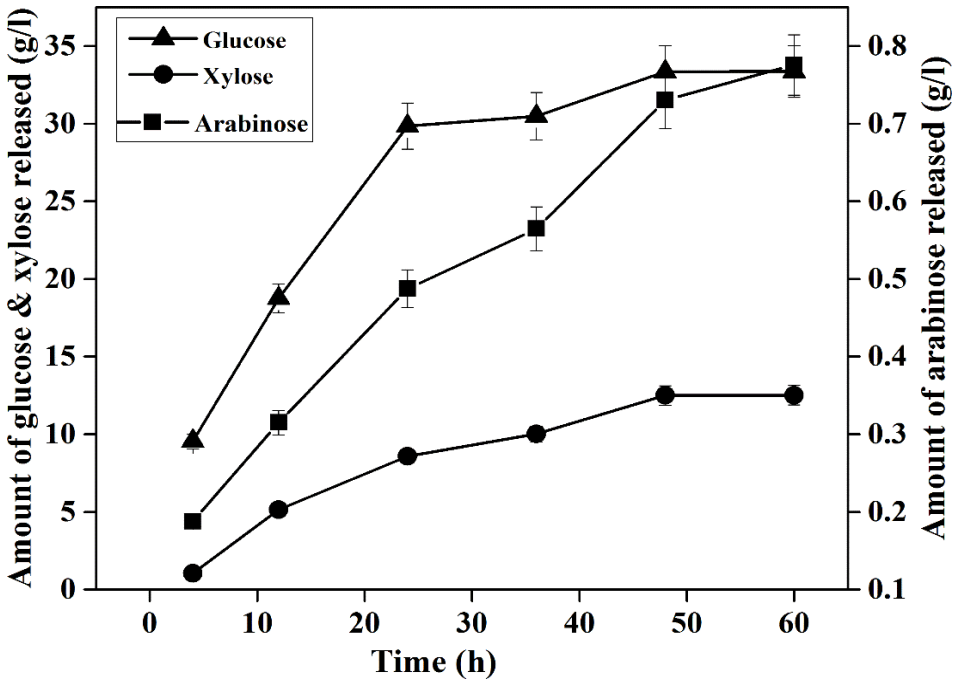


Figure 3.27 Yield of Arabinose, Glucose and Xylose by Combi-CLEAs on the hydrolysis of pretreated SCB

Subsequently, ISN-CLEAs hydrolyses the pretreated SCB with the incubation time of 48 h and yielded the maximum of 34.72 g/l of glucose, xylose (13.4 g/l) and 0.95 g/l of arabinose, the result depicts in Figure 3.28 further incubation up to 60 hours, no noticeable sugar yield in the reaction mixture. After the hydrolysis, ISN-CLEAs was separated by an external magnet and added to the fresh substrate (4%) and incubated at 50°C, it produced a maximum of glucose (6.26 g/l), xylose (2.22 g/l) and arabinose (0.11 g/l) after 8 hours of incubation. Having observed these results, it was concluded that ISN-CLEAs in the second cycle of hydrolysis yielded two times greater sugar conversion than that of combi-CLEAs. It can also be noticed that increasing agitation could increase the maximum sugar yield while decreasing the time to reach it, which is probably linked to the increased accessibility of the substrate to the enzymes.

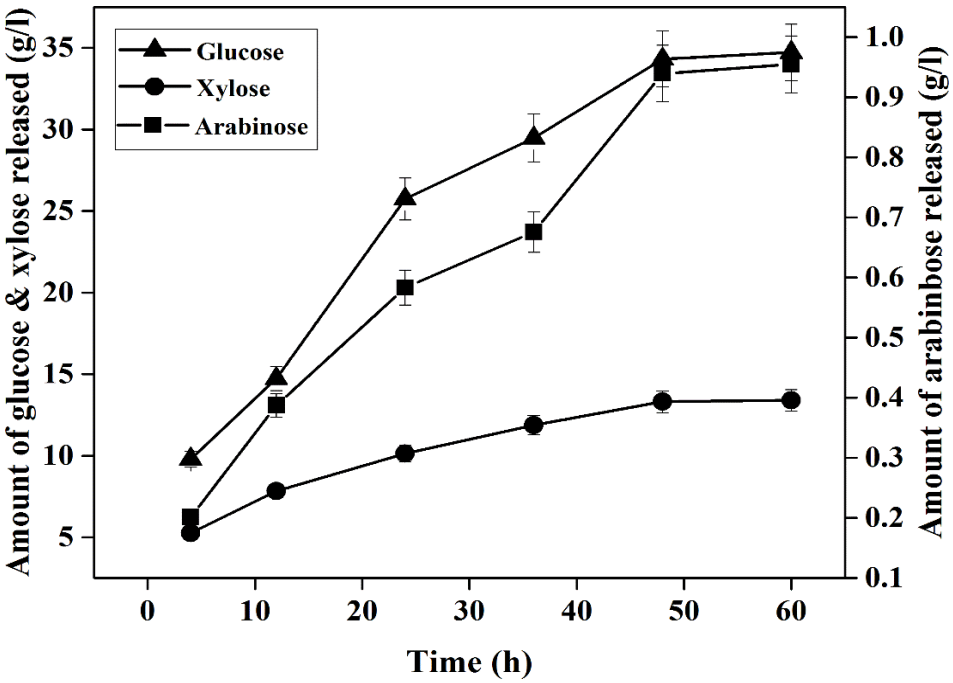


Figure 3.28 Yield of Arabinose, Glucose and Xylose by ISN-CLEAs on the hydrolysis of pretreated SCB

The percentage yield of glucose, xylose and arabinose from the SCB pulp after enzyme treatment are given in Table 3.5. Together with these observations, the result shows clearly that the immobilized enzyme treatments allow very high recovery yields of about 87% for glucose and 74% for xylose in the case of ISN-CLEAs treatment

Table 3.5 Sugar recovery yield (%) by free enzyme, combi-CLEAs and ISN-CLEAs applied on the SCB pulp

Form of Enzymes	Yield of recovered glucose, xylose and arabinose (% on initial substrate content) *		
	Glucose (%)	Xylose (%)	Arabinose (%)
Free enzymes	77±2.3	52±1.6	49±1.5
Combi-CLEAs	83±2.5	69±2.1	52±1.6
ISN-CLEAs	87±2.5	74±2.2	64±1.9

*Yields were calculated as % amount of sugar in the enzyme hydrolysate on amount of sugar in the substrate. Substrate analysis was made by sulfuric acid hydrolysis.

Finally, Monomers such as glucose and xylose are the two main fractions from SCB hydrolysate and arabinose fraction is substantial. After enzymatic hydrolysis, the hydrolysate was concentrated about 3-fold by incubating the solution at 75°C for 24 hours in order to increase the sugar concentration for ethanol production. Finally, the hydrolysate contains about 107.6 (g/l) of glucose and 40.7 (g/l) of xylose from the initial concentration of about (34.7 g/l) of glucose and 13.4 (g/l) of xylose.

3.14 Fermentation

3.14.1 Monoculture Ethanol Fermentation using *S. cerevisiae* LGP2Y1

S. cerevisiae LGP2Y1 is the most promising yeast that efficiently converts glucose to ethanol but unable to metabolize pentose present in the SCB hydrolysate. Co-culture of *S. cerevisiae* with another microbe which converts pentose is a possible way to overcome this problem (Qian et al. 2006). In this study, we have used *C. utilis* ATCC 22023 along with *S. cerevisiae* LGP2Y1 to convert xylose and glucose to ethanol, respectively. Yeast inoculation was initiated with the concentrated SCB hydrolysate obtained directly from enzymatic saccharification which contains 107.6 g/l of glucose and 41.5 g/l of xylose. Monoculture fermentation using *S. cerevisiae* LGP2Y1 utilized SCB hydrolysate containing 103.8±1.0 g/l of glucose and produced 41.95±0.5 g/l, with the yield of 0.4 g/g in fermentation time of 36 hours. Figure 3.29 clearly shows that, S. *cerevisiae* LGP2Y1 did not consume xylose even after the complete depletion of glucose and, the ethanol production was slightly declined after 36 hours.

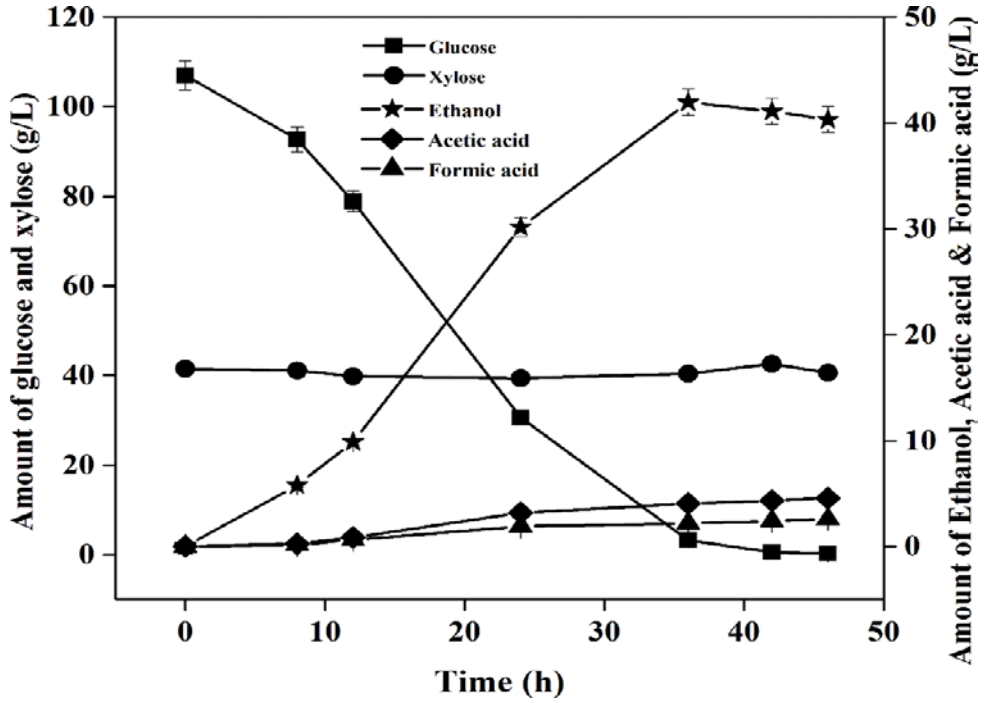


Figure 3.29 Monoculture ethanol production using *S. cerevisiae* LGP2Y1

3.14.2 Co-culture Ethanol Fermentation using *S. cerevisiae* LGP2Y1 and *Candida utilis* ATCC 22023

Co-culture of ethanol production using *Candida utilis* ATCC 22023 along with *S. cerevisiae* LGP2Y1 was carried out in batch fermentation. *C. utilis* ATCC 22023 was inoculated at zeroth hour and started consuming the maximum amount of xylose of about 31 g/l within 12 hours of incubation and produced 25 g/l of ethanol as shown in Figure 3.30. Then the fermentation was continued with *S. cerevisiae* LGP2Y1, which was inoculated after 10 hours. The fermentation was carried out for 48 hours. *C. utilis* ATCC 22023 and *S. cerevisiae* LGP2Y1 utilized 38.6±1.0 of xylose and 105.8±1.0 g/l of glucose, respectively and produced 64.8 g/l, with the ethanol yield on total sugar of 0.45 g/g. The overall percentage of experimental yield in co-culture ethanol fermentation was about 87.7% of theoretical yield whereas the monoculture yield of about 79%.

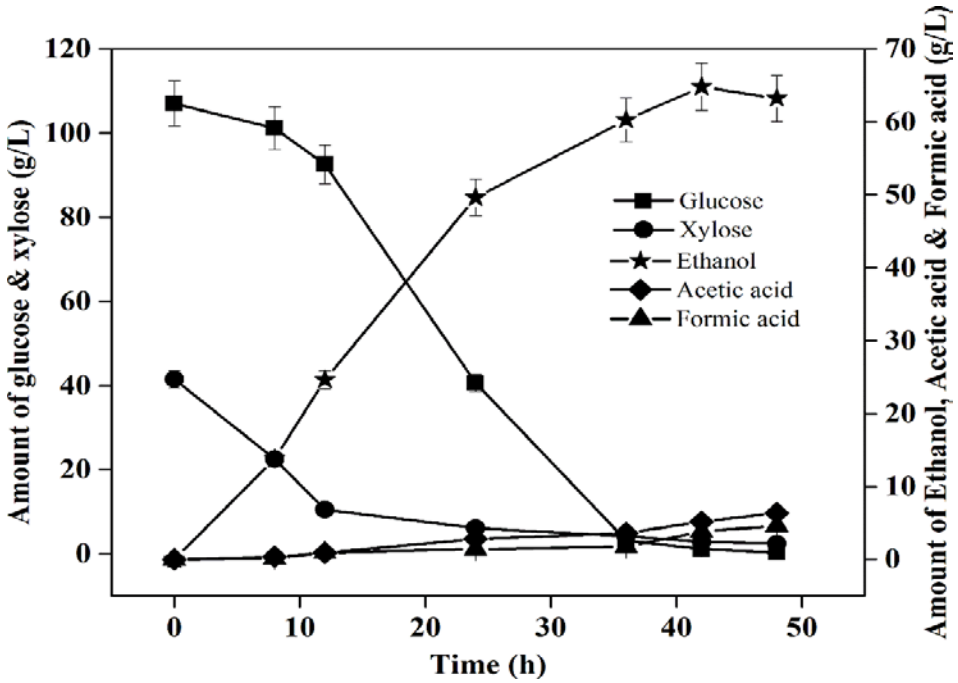


Figure 3.30 Co-culture ethanol production using *S. cerevisiae* LGP2Y1 and *C. utilis* ATCC 22023

CHAPTER 4

HIGHLIGHTS AND CONCLUSION

- In the present work, the fungal strain *Trichoderma citrinoviride* with the partial 5.8S rRNA sequencing having a length of 607 bp nucleotide. It is characterized as *Trichoderma citrinoviride* strain AUKAR04. The maximum production of xylanase (55,000 IU/gds), Cellulase (385 IU/gds) and β-1, 3-glucanase (695 IU/gds) were obtained in SSF with the seed media developed in initial pH 5.0±0.1 after 72 hours incubation at 30±1ºC.

- The enzymes were partially purified in a single step by Three-Phase Partitioning (TPP) method. 1:0.5 ratio of ammonium sulfate and t-butanol gave the highest xylanase yield (99.8%) with 5.7-fold purification, Cellulase yields (96.5%) with 5.5-fold purification and β-1,3-glucanase yields (98.4%) with 5.6-fold purification at the temperature of 25ºC. SDS-PAGE analysis showed the xylanase had molecular weight of ~29.8 kDa, cellulase ~58 kDa and β-1, 3-glucanase ~27 kDa.

- The partially purified enzymes were characterized. The enzymes showed good activity at acidic pH, where xylanase retained about 85% of the relative activity from pH 4.0-6.0. Cellulase exhibited 100% activity at pH 5.0 and pH 6.0. β-1, 3-glucanase showed maximum activity at pH 6.0 and retains about 80% of relative activity at pH 4.0 and pH 5.0.

- The three partially purified enzymes showed 80% activity from 40ºC to 60ºC. The optimum temperature for xylanase and β-1,3-glucanase were found to be 50ºC. The maximum cellulase activity was observed at 60ºC. Further incubation of the enzymes at 80ºC and 90ºC inhibits the activity of enzymes by 80%.

- All the three enzymes showed different behavior towards the metal ions. Mg^{2+}, Mn^{2+} and Zn^{2+} enhanced xylanase activity by 108.5%, 126.9% and 112.7% respectively but Cu^{2+} and Fe^{3+} inhibited up to 60% of xylanase activity. Mg^{2+} and Mn^{2+} enhanced cellulase activity by 111.1% and 154.5%

respectively and β-1,3-glucanase activity by 104% and 141%. Cellulase activity was inhibited by Cu^{2+} while β-1,3-glucanase activity was inhibited by Cu^{2+} and Zn^{2+}. The presence of Hg^{2+} caused complete inhibition of all the three enzymes.

- Kinetic parameters of TPP partial purified enzymes were calculated. V_{max} value of 24.28 mmol/min/ml, 630.9 µmol/ min/ml and 329.6 µmol/min/ml, respectively for xylanase, cellulase and β-1,3-glucanase. K_m value of 0.713 mg/ml/, 0.811 mg/ml, and 1.31 mg/ml respectively for xylanase, cellulase and β-1,3-glucanase.

- The three partially purified enzymes were cross-linked to improve the operational resistance in harsh conditions. The best CLEAs were obtained when the combination of ammonium sulfate and t-butanol was used as precipitant with maximum enzyme activity recovery (99%) of cellulase, xylanase and β-1,3-glucanase.

- 100mM glutaraldehyde gave the maximum activity recovery in combi-CLEAs with 99.1±3% for xylanase, 98.3±3% for cellulase and 98.8±3% for β-1,3-glucanase. The combi-CLEAs showed increased in activity recovery when the cross-linkng time was 7.5 hours at 30°C.

- Structural analysis of the combi-CLEAs showed a coarse-grained appearance (type 1) and are more structured. After the tenth cycle, a ball-type structure with few cavities appear on the surface of the cross-linked aggregates that can be due to the leaching out of the enzymes.

- Combi-CLEAs retained about 95% of its initial activity of xylanase, cellulase and β-1,3-glucanase after 2 hours incubation at 50°C while free enzymes lose about 30%. At 70°C, combi-CLEAs retained 70% while free enzymes lose about 60% of the activity. Incubation for 4 and 8 hours significantly reduced the residual activities of combi-CLEAs as well as free enzymes.

- Free xylanase, cellulase and β-1,3-glucanase retained only 69%, 65% and 63.4% of its initial activities respectively, while combi-CLEAs retained

more than 95% of their initial activities after 11 weeks of incubation at 4°C in 50 mM sodium acetate buffer (pH 5.0).

- Combi-CLEAs was reused for 10 cycles in batch operation mode at pH 5.0 at 50°C. The activities of xylanase, cellulase and β-1,3-glucanase in combi-CLEAs retained more than 98% up to five cycles, while decreased to 90% in sixth cycle and 50% after the ninth cycle.

- Limitations of CLEAs technology such as forming clump and not easily recoverable from the reaction medium were overcome by the immobilization of the enzymes on functionalized magnetic nanoparticles.

- 135mM glutaraldehyde concentration and cross-linking time of 10 hours gave the highest enzyme recovery in the case of ISN-CLEAs.

- FTIR analysis showed a corresponding peak of Fe-O bond in Fe_3O_4 NPs at 591cm^{-1} indicating successful synthesis of magnetic nanoparticles.

- Surface morphology of ISN-CLEAs was analyze by AFM and SEM. AFM images clearly showed that the morphology of bi-functionalized NPs was significantly modified after immobilization with xylanase, cellulase and β-1,3-glucanase and appears as spherical shape with the average size of 0.4 to 0.5µm. SEM images showed that the enzymes were bound together into balls onto the functionalized MNPs.

- DLS was used to measure the hydrodynamic diameter of the Fe_3O_4, Fe_3O_4@SiO_2-NH_2 and ISN-CLEAs with average diameter of 82.2 nm, 86.4 nm and 1148.9 nm respectively.

- The enzymes in ISN-CLEAs was slightly thermostable when compared to that of combi-CLEAs. ISN-CLEAs retained about 97% activity after 2 hours of incubation at 50°C and 75% of their initial activities at 70°C.

- ISN-CLEAs was stored for 11 weeks at 4°C in 50 mM sodium acetate buffer (pH 5.0) and it retained 97% of the original activities after 11 weeks.

- The enzymes in ISN-CLEAs were reused for 10 cycles and it retained about 97% of the original activities till the sixth cycles and reuduces to 60% by the ninth cycle.

- The composition of SCB raw material was analyzed and showed that it contains about 43% of glucan, 27% of xylan, 4% of arabinan, 26% of lignin and traces of ass and proteins.

- SCB was treated with 10% liquid ammonia at 140°C for 120 minutes removing 40% of lignin and retained 95% of glucan, 65% of xylan and 40% of arabinan.

- Chemical and structural analysis of SCB was done by ATR-FTIR, TGA and XRD. FTIR result showed a successful pretreatment of the SCB raw material. It showed that hemicelluloses and cellulose are partially de-polymerized by the action of xylanase, cellulase and β-1,3-glucanase in ISN-CLEAs.

- TGA studies showed that the thermal stability of the ammonia pretreated and enzymatically treated samples have improved remarkably.

- XRD results showed that the crystallinity index of the ISN-CLEAs treated SCB increased to 61.3±1% when compared to the ammonia-treated SCB.

- Maximum amount of glucose (30.77 g/l), xylose (9.39 g/l) and arabinose (0.74 g/l) was released when SCB was hydrolyzed by free enzymes at 50°C after 24 hours of incubation. Amount of glucose (33.36 g/l), xylose (12.51 g/l) and arabinose (0.78 g/l) was released with combi-CLEAs while ISN-CLEAs yielded the maximum of 34.72 g/l of glucose, xylose (13.4 g/l) and 0.95 g/l of arabinose after 48 hours of incubation at 50°C.

- Monoculture fermentation of using *Saccharomyces cerevisiae* LGP2Y1 utilized SCB hydrolysate containing 103.8±1.0 g/l of glucose and produced 41.95±0.4 g/l of ethanol in 36 hours of fermentation with the yield of 0.4 g/g. Co-culture of ethanol using *Candida utilis* ATCC 22023 along with *S. cerevisiae* LGP2Y1 utilized SCB hydrolysate containing 107.6 g/l of glucose and 41.5 g/l of xylose and produced 64.8 g/l of ethanol after 42

hours of incubation with the yield of 0.45 g/g. The overall percentage of experimental yield in co-culture ethanol fermentation was about 87.7% of the theoretical yield whereas the monoculture yield of about 79% of the theoretical yield.

4.1 Conclusion

The dissertation shows that the outstanding feature of *Trichoderma citrinoviride* strain AUKAR04 produces a beneficial amount of extracellular enzymes like xylanase, cellulase and β-1,3-glucanase in SSF with wheat bran as sole carbon and nitrogen sources, and the non-chromatographic, cost effective single-step purification processes by TPP are the major advantages in the industrial sector. The industries can reduce the import cost of the enzymes by producing in solid-state fermentation in large scale and make the whole process at a competitive cost.

This research study also provides experimental evidence of the efficiency of carrier free combined aggregated enzymes (combi- CLEAs) and formulations that include highly active xylanase, cellulase and β-1,3-glucanase. It was shown that combi-CLEAs could surpass the activity of isolated enzymes in terms of thermal stability and preservation during storage. There are certain limitations in combi-CLEAs technology that are limiting its further applications such as the separation of combi-CLEAs by centrifugation or filtration after the enzyme-substrate reaction leads to the formation of clump due to low compression resistance, which can further hamper the mass transport of substrate and decrease overall activities of the enzyme.

This study also provides a simple solution to recover and recycle the cellulolytic enzymes through the synthesis of Iron silica magnetic nanoparticles and crosslinking with cellulase, xylanase and β-1,3-glucanase (ISN-CLEAs) appears to be a promising tool for the pragmatic commercialization of lignocellulolytic enzymes with improved thermal stability, enhanced operational stability and reusability. Also, it evidently proves the effective synergy of the enzyme cocktail used in this study on lignocellulosic substrates breakdown into fermentable sugars for bioethanol production.

Monoculture Ethanol Fermentation using *S. cerevisiae* LGP2Y1 and co-culture Ethanol Fermentation using *S. cerevisiae LGP2Y1* and *Candida utilis* ATCC 22023 was carried out in order to ferment the total sugars i.e. glucose (C6) and xylose (C5) from the sugarcane bagasse

hydrolysate. The overall percentage of metabolic ethanol yield was achieved in co-culture fermentation was about 87.7% of theritical yield whereas the monoculture yield of about 79% of theritical yield.

4.2 Future Perspectives of Bioethanol

Lignocellulosic ethanol is a second generation biofuel relative to ethanol obtained via fermentation of sugars sourced from lignocellulosic biomass that have almost no food applications, cost-effective production of fermentable sugars from lignocellulosic biomass remains a challenge. As lignocellulose is difficult to hydrolyze into its constituent sugars, the biomass needs to be extensively pretreated to first remove the lignin and then depolymerize to fermentable sugars. A number of pretreatments have been developed and continue to be improved. Nevertheless, pretreatment processes contribute nearly 20% to the final cost of the lignocellulosic ethanol. A common pretreatment process does not exist, as the amenability of lignocellulosic feedstock to a given pretreatment varies with the source of the feedstock.

The future of bioethanol is very much intertwined with genetic and metabolic engineering. Capabilities of the lignocellulolytic enzymes for hydrolyzing cellulose can be enhanced and their cost of production can be reduced by genetic engineering the microorganisms. Ethanol from lignocellulosic biomass holds the greatest promise but remains relatively expensive. Other sources of ethanol, for example, microalgae, require extensive research for possible commercialization.

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LIST OF PUBLICATIONS

1. **Periyasamy Karthik**, Santhalembi Laishram, Mortha Gérard, Aurousseau Marc, Boyer Agnès & Subramanian Sivanesan 2018, 'Bioconversion of Lignocellulosic Biomass to Fermentable Sugars by Immobilized Magnetic Cellulolytic Enzyme Cocktails' Langmuir, DOI: 10.1021/acs.langmuir.8b00976.

2. **Periyasamy Karthik**, Santhalembi Laishram, Mortha Gérard, Aurousseau Marc, Guillet Agnès, Dallerac David & Subramanian Sivanesan 2017, 'Production, Partial Purification and Characterization of Enzyme Cocktail from *Trichoderma citrinoviride* AUKAR04 Through Solid-State Fermentation', Arabian Journal for Science and Engineering, vol. 42, no. 1, pp. 53-63.

3. **Periyasamy Karthik**, Santhalembi Laishram, Mortha Gérard, Aurousseau Marc & Subramanian Sivanesan 2016, 'Carrier-free co-immobilization of xylanase, cellulase and β-1, 3-glucanase as combined cross-linked enzyme aggregates (combi-CLEAs) for one-pot saccharification of sugarcane bagasse', RSC Advances, vol. 6, no. 39, pp.32849-32857.

ABSTRACT

The main objective of the current study is to produce bioethanol from Sugarcane bagasse by using free and immobilized cellulolytic enzymes. This study explored the highest production of cellulolytic enzymes from *Trichoderma citrinoviride* AUKAR04 and the enzymes were immobilized by two different techniques with extended reusability and storage stability. Further, the immobilized enzymes were used to hydrolyze the liquid ammonia-pretreated SCB for the bioethanol production.

The production of cellulolytic enzymes from *T. citrinoviride* AUKAR04 was investigated in solid-state fermentation. The highest production of xylanase (55,000 IU/gds), cellulase (385 IU/gds) and β-1, 3-glucanase (695 IU/gds) were obtained with the seed media was maintained at pH 5.0±0.1 after 72 hours incubation at 30±1ºC. The enzymes were partially purified by Three-Phase Partitioning (TPP) method with the highest yield of xylanase (67.1%), cellulase (79.4%) and β-1,3-glucanase (45.0%) at the temperature of 25ºC. The optimum temperature of xylanase, cellulase and β-1, 3-glucanase was 50°C. The residual activity of all the three enzymes showed 80% of activity when incubated from 40-60°C but further incubation at 90°C significantly inhibits the enzyme activity. Effect of the different metal ion on the activities of xylanase, cellulase and β-1,3-glucanase were evaluated. The kinetic parameters (K_m and V_{max}) of the free enzymes were studied at 50°C with the K_m value of 0.713 mg/ml, 0.811 mg/ml, and 1.31 mg/ml, respectively for xylanase, cellulase and β-1, 3-glucanase. The V_{max} values were 24.28 mmol/min/ml, 630.9 µmol/min/ml and 329.6 µmol/min/ml, respectively for xylanase, cellulase and β-1, 3-glucanase.

Industrial application of the free enzymes is usually hindered because of the instability for an extended period at elevated temperature, pH and organic solvents. Such drawbacks can be overcome by immobilization of the enzymes, which is easier to recover and recycle and enhanced the operational stability. Two types of immobilization techniques were studied: Combined Cross-Linked Enzyme Aggregates (combi-CLEAs) and Bifunctionalized Magnetic Cross-Linked Enzyme Aggregates (ISN-CLEAs). For combi-CLEAs, 100mM of glutaraldehyde concentration gave the maximum activity recovery in combi-CLEAs with the cross-linking time of 7.5 hours. Structural characterization of the combi-CLEAs shows a coarse-grained structure before the first cycle while after the tenth cycle the surface had few cavities, which might be due to the leaching out of the enzymes. Combi-CLEAs showed

higher thermal stability when compared to the native enzymes by retaining about 95% of its residual activity after 2 hours of incubation at 50°C. At 70°C, combi-CLEAs retained more than 70% original activity while free enzymes lose about 60% of the activities. Incubation for longer hours significantly reduces the residual activities. Storage stability study reveal that combi-CLEAs has an extended storage stability by retaining about 95% of its initial activities after 11 weeks of incubation at 4°C in 50 mM sodium acetate buffer (pH 5.0) while free xylanase, cellulase and β-1,3-glucanase retained only 69%, 65% and 63.4% of its initial activities respectively. Reusability of the combi-CLEAs was studied up to 10^{th} cycle where it retained about 98% till the 5^{th} cycle while it reduces to 90% by the 6^{th} cycle and by 50% in the 9^{th} cycle.

CLEAs technology has some disadvantages such as difficulties in recovery from the enzyme-substrate reaction and considered too soft for industrial applications. To overcome these problems, in our study, we prepared and characterized the covalently bound enzymes on the surface of functionalized Iron Oxide Magnetic Nanoparticles (IOMNPs). The resulting biocatalyst combines the relevant catalytic properties of CLEAs (thermal stability and reusability) and the magnetic property, and thus the final product ISN-CLEAs (Iron Silica Nanoparticles-Cross Linked Enzyme Aggregates) is a robust catalyst, which is more stable than the free enzyme, easily recoverable from the reaction medium and reusable for new reaction cycles. 135mM glutaraldehyde gave the maximum activity recovery of xylanase (97.8%), cellulase (97.5%) and β-1,3-glucanase (96.3%) in ISN-CLEAs with the cross-linking time of 10 hours. SEM analysis shows that nanoparticles appeared as a spherical shape. The particle size of Fe_3O_4, Fe_3O_4@SiO_2-NH_2 and ISN-CLEAs was analyzed by Dynamic Light Scattering (DLS). The three enzymes in ISN-CLEAs showed better thermal stability when compared to free enzymes and combi-CLEAs retaining about 97% of the initial activity at 50°C and 75% activity at 70°C after 2 hours of incubation. Storage stability study was done for 11 weeks at 4°C in 50 mM sodium acetate buffer (pH 5.0) and the enzymes in ISN-CLEAs retained more than 97% of its original activity after 11 weeks of incubation. ISN-CLEAs was reused for 10 times and it retained about 97% activity till the 6^{th} cycle while it reduces by 40% in the 9^{th} cycle.

Sugarcane Bagasse (SCB) was pretreated using 10% liquid ammonia at 140°C for 120 min, which removes about 40% of lignin and retained about 95% of glucan, 65% of xylan and 40% of arabinan. Chemical and structural analysis of ISN-CLEAs were done by Attenuated

Total Reflection-Fourier Transform Infrared Spectroscopy (ATR-FTIR), Thermal Gravimetric Analysis (TGA) and X-Ray Diffraction (XRD). SCB was enzymatically hydrolyzed by using the free enzymes, combi-CLEAs, and ISN-CLEAs. The result clearly shows that the treatment of SCB with immobilized enzymes gave high recovery yield of about 87% for glucose and 74% for xylose.

Mono-culture fermentation using *Saccharomyces cerevisiae* LGP2Y1 utilized SCB hydrolysate containing 103.8±1.0 g/l of glucose and produced 41.95±0.4 g/l of ethanol in 36 hours of fermentation with the yield of 0.4 g/g. Co-culture of ethanol using *Candida utilis* ATCC 22023 along *with S. cerevisiae* LGP2Y1 utilized SCB hydrolysate containing 144.5±1.5 g/l (glucose and xylose) and produced 64.8 g/l of ethanol after 42 hours of incubation with the yield of 0.45 g/g. The overall experimental yield of co-culture ethanol fermentation was about 87.7% of theoretical yield whereas the monoculture yield of about 79% of theoretical yield.

RESUME EN FRANCAIS

L'objectif principal de la présente étude est la production de bioéthanol par hydrolyse enzymatique de bagasse de canne à sucre (Sugar Cane Bagasse : SCB) utilisée comme substrat végétal, en mettant en oeuvre des enzymes cellulolytiques libres et immobilisées. L'étude a porté sur la méthode permettant de maximiser la production d'enzymes cellulolytiques à partir de *Trichoderma citrinoviride* AUKAR04 : culture sur le substrat, puis séparation des enzymes du substrat en vue de leur récupération et réutilisation. Les enzymes ont été immobilisées par deux techniques différentes favorisant leur réutilisation et une bonne stabilité en cas de stockage prolongé. Les enzymes immobilisées ont été utilisées pour hydrolyser la bagasse de canne à sucre à l'état de suspension en milieu aqueux, après prétraitement de celle-ci à l'ammoniac, dans l'objectif de produire du bioéthanol par fermentation ultérieure des sucres contenus dans les hydrolysats enzymatiques par des levures.

La production d'enzymes cellulolytiques à partir de *T. citrinoviride* AUKAR04 a été obtenue par fermentation à l'état solide. Les productions les plus élevées de xylanase (55 000 UI/gss[1]), cellulase (385 UI/gss[1]) et β-1,3-glucanase (695 UI/gss[1]) ont été obtenues avec le milieu de germination maintenu à pH 5,0 ± 0,1 après 72 heures d'incubation à 30 ± 1 ºC. Les enzymes ont été partiellement purifiées par la méthode TPP (Tri-Phase Partitioning) permettant d'atteindre le rendement le plus élevé en xylanase (67,1 %), en cellulase (79,4 %), et en β-1,3-glucanase (45,0 %), à la température de 25 ºC. La température optimale pour la xylanase, la cellulase et la β -1,3-glucanase était de 50 °C. L'activité enzymatique résiduelle des trois enzymes atteint 80% lors d'incubation à 40-60 °C. Cependant, une incubation ultérieure à 90°C inhibe significativement leur activité. Les effets de différents cations métalliques sur les activités des trois enzymes ont été évalués. Les paramètres cinétiques (K_m et V_{max}) des enzymes libres ont été mesurés à 50 °C, et les valeurs suivantes pour K_m ont été obtenues : 0,713 mg/ml pour la xylanase, 0,811 mg/ml pour la cellulase et 1,31 mg/ml pour la β-1,3-glucanase. Dans les mêmes conditions, les valeurs de V_{max} étaient de 24,28 mmol/min/ml pour la xylanase et de 630,9 et 329,6 micro-mol/min/ml pour la cellulase et la β -1,3-glucanase, respectivement.

[1]*UI/gss : Unité Internationale/gramme de substrat sec*

L'application industrielle des enzymes libres est habituellement limitée en raison de leur instabilité lorsqu'elles sont soumises pendant une période prolongée à des températures élevées, des variations de pH et des solvants organiques. Ces inconvénients peuvent être surmontés par l'immobilisation des enzymes, qui facilite leur récupération et leur recyclage, ce qui améliore la stabilité opérationnelle. Deux techniques d'immobilisation ont été étudiées : la formation d'agrégats enzymatiques réticulés combinés (Combined-Cross Linked Enzyme Aggregates : combi-CLEAs) et la formation d'agrégats enzymatiques réticulés magnétiques bifonctionnalisés (Iron Silica Nanoparticles-Cross Linked Enzyme Aggregates : ISN-CLEAs).

Pour les combi-CLEAs, une concentration de 100 mM en glutaraldéhyde a permis d'obtenir un recouvrement maximal d'activité, pour un temps de réticulation de 7,5 h. La caractérisation structurelle des combi-CLEAs montre une structure d'agrégats compacts à grains grossiers après leur formation (avant le premier cycle d'utilisation), tandis qu'après le dixième cycle d'utilisation, la surface des agrégats présente des cavités ce qui pourrait être dû à la lixiviation des enzymes.

Les combi-CLEAs montrent une stabilité thermique renforcée par rapport aux enzymes libres en phase liquide : environ 95% de l'activité résiduelle est maintenue après 2 h d'incubation à 50 °C. À 70 °C, les combi-CLEAs conservent plus de 70 % de leur activité initiale alors que les enzymes libres perdent environ 60 % d'activité. L'incubation pendant un temps plus long augmente considérablement la perte d'activité. Concernant la stabilité durant une période de stockage, les combi-CLEAs démontrent une stabilité élevée puisqu'elles conservent environ 95 % de leur activité initiale après 11 semaines d'incubation à 4 °C dans un tampon d'acétate de sodium à 50 mM (pH 5,0), alors que les enzymes libres (xylanase, cellulase et β-1,3-glucanase) ne conservent que 69 %, 65 % et 63,4 % de leur activité initiale respectivement. La réutilisation des combi-CLEAs a été étudiée jusqu'à 10 cycles : environ 98 % d'activité sont conservés jusqu'au 5ème cycle, puis l'activité est réduite à 90 % au 6ème cycle et à 50 % au 9ème cycle.

La technologie CLEAs présente cependant certains inconvénients, tels que la difficulté de récupération des enzymes après réaction enzyme-substrat, et elle est considérée comme trop complexe pour des applications industrielles. Pour remédier à ces problèmes, nous avons préparé et caractérisé des enzymes liées de manière covalente à la surface de nanoparticules magnétiques d'oxyde de fer fonctionnalisées (Iron Oxide Magnetic Nanoparticles : IOMNPs).

Le biocatalyseur ainsi formé allie à la fois les propriétés catalytiques pertinentes des CLEAs (stabilité thermique et possibilité de réutilisation) et une propriété magnétique facilitant la récupération. Le produit final ISN-CLEAs (Iron Silica Nanoparticles–Cross Linked Enzyme Aggregates) est donc un catalyseur robuste, plus stable que l'enzyme libre, facilement récupérable à partir du milieu réactionnel et réutilisable pour de nouveaux cycles de réaction. Ainsi, une concentration de 135 mM en glutaraldéhyde a permis une récupération d'activité quasi maximale de la xylanase (97,8 %), de la cellulase (97,5 %) et de la β-1,3-glucanase (96,3 %) dans les ISN-CLEAs, avec un temps de réticulation de 10 h. L'analyse par microscopie électronique à balayage (MEB) montre que des nanoparticules apparaissent sous forme de sphères. La taille des nanoparticules de Fe_3O_4 et Fe_3O_4@SiO_2-NH_2 ainsi que celles de ISN-CLEAs a été analysée par diffusion de lumière dynamique (DLS : Dynamic Light Scattering). Les trois enzymes contenues dans les ISN-CLEAs présentent une meilleure stabilité thermique, par comparaison aux enzymes libres et aux combi-CLEAs, en conservant environ 97 % d'activité initiale à 50 °C et 75 % d'activité initiale à 70 °C après 2 h d'incubation. La stabilité au stockage a été étudiée pendant 11 semaines d'incubation à 4 °C dans un tampon d'acétate de sodium de concentration 50 mM (pH 5,0). Les enzymes des ISN-CLEAs ont conservé plus de 97 % de leur activité initiale après la période d'incubation. Les ISN-CLEAs ont pu être réutilisées 10 fois en conservant environ 97 % d'activité jusqu'au $6^{ème}$ cycle et 60 % au $9^{ème}$ cycle.

La bagasse de canne à sucre (SCB), utilisée comme substrat, a subi un prétraitement préalable à base de 10 % d'ammoniac liquide à 140 °C pendant 120 min. Le prétraitement élimine environ 40 % de la lignine et retient 95 % de glucane, 65 % de xylane et 40 d'arabinane (pourcentages comptabilisés par rapport aux quantités initiales contenues dans le substrat). La caractérisation chimique et structurale des ISN-CLEAs a été effectuée par spectroscopie infrarouge à transformée de Fourier en mode ATR, par analyse thermogravimétrique et par analyse spectroscopique par diffraction des rayons X. La SCB prétraitée a été hydrolysée par voie enzymatique en utilisant les enzymes libres, les combi-CLEAs et les ISN-CLEAs. Les résultats obtenus montrent clairement que le traitement du substrat par des enzymes immobilisées donne un rendement de récupération élevé, d'environ 87 % pour le glucose et de 74 % pour le xylose.

Dans le but de produire du bioéthanol à partir des hydrolysats enzymatiques du substrat prétraité, la fermentation en monoculture utilisant la levure *Saccharomyces cerevisiae*

LGP2Y1, appliquée à un hydrolysat enzymatique contenant 103,8 ± 1,0 g/l de glucose, a produit 41,95 ± 0,4 g/l d'éthanol en 36 h de fermentation, soit un rendement d’environ 0,4 g d’éthanol par g de glucose. La co-fermentation utilisant simultanément *Candida utilis* ATCC 22023 et *S. cerevisiae* LGP2Y1 a produit 64,8 g/l d'éthanol à partir d’un hydrolysat enzymatique contenant 144,5 ± 1,5 g/l de mélange (glucose + xylose), après 42 h d'incubation, soit un rendement de 0,45 g d’éthanol par g de sucre. Ainsi, en co-culture fermentaire, le rendement expérimental de transformation du sucre en éthanol atteint 87,7 % du rendement théorique, alors qu’il est d'environ 79 % en monoculture.

Abstract: The overall objective of the study was to produce bioethanol from lignocellulosic biomass by using free and immobilized xylanase, cellulase and β-1, 3-glucanase. Specifically, this study was focused on the isolation of *Trichoderma citrinoviride* strain AUKAR04 able to produce xylanase (55,000 IU/gds), cellulase (385 IU/gds) and β-1, 3-glucanase (695 IU/gds) in solid state fermentation. Then the free enzymes were biochemically characterized depend on effect of pH, temperature and metal ion concentration and corresponding kinetic parameters were determined. Then the enzymes were subjected to two types of immobilization using carrier-free co-immobilization (combi-CLEAs) method or bifunctionalized magnetic nanoparticles (ISN-CLEAs) with higher thermal stability, extended reusability and good storage stability. Liquid ammonia pretreatment removed 40% of lignin from the biomass and retained 95% of glucan, 65% of xylan and 41% of arabinan in sugarcane bagasse (SCB). SCB was enzymatically hydrolyzed and converted to 87 % glucose from cellulose and 74% of xylose, 64% of arabinose from the hemicelluloses which is remarkably higher than the activity of the free enzymes. Chemical and structural analysis of SCB was done by ATR-FTIR, TGA and XRD. FTIR result showed a successful pretreatment of the SCB raw material. It showed that hemicelluloses and cellulose are partially depolymerized by the action of xylanase, cellulase and β-1,3-glucanase in ISN-CLEAs. TGA studies showed that the thermal stability of the ammonia pretreated and enzymatically treated samples have improved remarkably. XRD results showed that the crystallinity index of the ISN-CLEAs treated SCB increased to 61.3±1% when compared to the ammonia-treated SCB. Mono-culture fermentation using *Saccharomyces cerevisiae* LGP2Y1 utilized SCB hydrolysate containing 103.8 g/L of glucose and produced 42 g/L ethanol in 36 h of fermentation. The overall metabolic yield achieved was about 79% of theoretical yield. Co-culture fermentation using *Saccharomyces cerevisiae* LGP2Y1 and *Candida utilis* ATCC 22023 utilized SCB hydrolysate containing 107.6 g/L of glucose and 41.5 g/L xylose and produced 65 g/L ethanol in 42 h of fermentation. The overall metabolic yield in co-culture fermentation achieved was about 88 % of the theoretical yield.

Key words: Xylanase, Cellulase, β-1, 3-glucanase, Immobilization, Sugarcane bagasse, Pretreatment, co-culture and co-fermentation

Résumé : L'objectif global de cette étude était de produire du bioéthanol à partir de biomasse lignocellulosique en utilisant des enzymes libres ou immobilisées de type xylanase, cellulase et β-1,3-glucanase. L'isolement de la souche AUKAR04 de *Trichoderma citrinoviride* a permis de produire par fermentation solide ces trois enzymes à un taux de 55 000, 385 et 695 UI/gss[2] respectivement. L'activité biochimique des enzymes libres a été caractérisée en faisant varier différents paramètres : pH, température et concentration en cations métalliques, et les paramètres cinétiques correspondants ont été identifiés. Par la suite, les enzymes ont été immobilisées en phase solide, soit sous forme d'agrégats sans support de type (combi-CLEAs), soit par association avec des nanoparticules magnétiques bi-fonctionnalisées (ISN-CLEAs). Les enzymes ont ainsi montré de meilleures performances en termes de stabilité thermique, d'aptitude à une réutilisation (plusieurs cycles) et de stabilité après un temps de conservation prolongé. Le substrat végétal utilisé (SCB : bagasse de canne à sucre) a été prétraité chimiquement par cuisson à l'ammoniac, permettant d'éliminer 40% de la lignine initiale tout en préservant 95% de glucane, 65% de xylane et 41% d'arabinane. L'hydrolyse enzymatique du substrat prétraité a permis une conversion de la cellulose en 87% de glucose, et une conversion des hémicelluloses (arabinoxylanes) en 74% de xylose et 64% d'arabinose, chiffres notoirement supérieurs à l'activité des enzymes libres. L'analyse chimique et structurale du substrat a été faite par spectrométrie ATR-FTIR et DRX, et par analyse TGA. L'étude FTIR a prouvé l'efficacité du traitement enzymatique en montrant que les hémicelluloses et la cellulose subissent une dépolymérisation partielle par l'action simultanée des trois enzymes immobilisées dans les ISN-CLEA. L'étude TGA a montré que la stabilité thermique des échantillons prétraités à l'ammoniac puis traités par des enzymes est notoirement améliorée. L'analyse DRX a montré que l'indice de cristallinité du substrat prétraité à l'ammoniac puis traité par l'ISN-CLEA a augmenté de 61,3 ± 1%, par rapport au substrat avant traitement enzymatique. La fermentation par la levure *Saccharomyces cerevisiae* LGP2Y1 utilisée en monoculture, à partir d'un hydrolysat enzymatique contenant 103,8 g/L de glucose, a produit 42 g/L d'éthanol en 36 h de fermentation. Le rendement métabolique global atteint ainsi environ 79% du rendement théorique. La fermentation en co-culture avec *Saccharomyces cerevisiae* LGP2Y1 et *Candida utilis* ATCC 22023 d'un hydrolysat à 107,6 g/L de glucose et 41,5 g/L de xylose a produit 65g /L d'éthanol en 42 h de fermentation. Ainsi, en co-culture fermentaire, le rendement métabolique global atteint environ 88 % du rendement théorique.

Mots clé : Xylanase, Cellulase, β-1, 3-glucanase, Immobilisation, Bagasse de canne à sucre, Prétraitment, co-culture et co-fermentation

[2] *UI/gss : Unité Internationale/gramme de substrat sec*