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DEVELOPING SYNTHETIC BIOLOGY TOOLS FOR LIGNOCEL-
LULOSE DEGRADATION
Master of Science Thesis

ABSTRACT

Lignocellulose is mainly composed of cellulose, hemicelluloses and lignin. Lignocellulose is abundant in nature and hence an attractive low cost feedstock for bioprocesses such as biofuel production. Bioproduction initially requires separation and hydrolysis of polysaccharides content of lignocellulose. Lignocellulose is decomposed either by physiochemical means or by biodegradation. Biodegradation is accomplished mainly by fungi and bacteria in nature. The cellulases involved in cellulose biodegradation include endoglucanase, cellobiohydrolase or exoglucanase, and β-glucosidase. The hemicellulases comprise of endo-β-1,4-xylanase, β-xylosidase and the accessory enzyme which include α-L-arabinofuranosidases, α-D-glucuronidases and some esterases. The major ligninolytic enzymes include lignin peroxidases (LiPs), manganese peroxidases (MnPs) and laccases.

Unfortunately production of biofuels from lignocellulose is an expensive process. To increase the economic feasibility of the process, various aspects have been focused including the biological means of degradation. In the current project a photosensitizer protein, red fluorescent protein variant (KillerRed, KR) was engineered and tested for its capability to degrade cellulose. The KR protein was added with a cellulose binding peptide (CBP) at its N terminal. The engineered protein CBP-KR was expressed in *Escherichia coli* cells using conventional expression system and purified.

The binding property of purified CBP-KR was analyzed by simply incubating the protein solution with different cellulosic substrates and observing changes in fluorescence signals after incubations. The degradation capability of CBP-KR was tested by a modified Congo red assay. The CBP-KR exhibited clear binding to printing paper but degradation of carboxymethyl cellulose (CMC) could not be recorded by the modified Congo red assay.

PREFACE

The study was completed in the department of Chemistry and Bioengineering, Tampere University of Technology, Tampere, Finland.

First of all I thank to the almighty Allah (God) who gave me the opportunity to learn and complete this thesis. I also deeply thank my research supervisor Ville Santala for his training, motivation and kind attitude. I feel obligated to thank Professor Matti Karp and his group for their immense help and guidance during the research. Lastly I am thankful to my family especially my father Siddique Ahmad Munawar and my mother Amtul Basit for their immense prayers, support, care and love which I even felt from five thousand kilometer distance.

'If we knew what it was we were doing, it would not be called research, would it?' Albert Einstein

Tampere, 18 October 2014

MUSTAFA AHMAD MUNAWAR

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TERMS AND DEFINITIONS

Cellulases	Enzymes responsible of cellulose degradation
Cellulosome	A complex supramolecule integrating cellulases on a scaffoldin
Hemicellulases	Enzymes responsible of hemicellulose degradation
Ligninolytic Enzymes	Enzymes responsible of lignin degradation
Biocatalyst	Biocatalyst means biological catalyst like protein and enzyme but the whole cell biocatalyst means the whole cell which carry out chemical transformations.
SSF	Simultaneous Saccharification and Fermentation means combining the process of hydrolysis of cellulose and the process of fermentation of glucose.
KR	KillerRed is a red fluorescent protein which maximally absorbs light of 585nm and emits light of 610nm.
CBP	Cellulose Binding Peptide
CBD	Cellulose Binding Domain
His tag	Six or more histidine residues tagged to N or C terminal of a protein. Histidine has affinity to metal ions and this affinity facilitates purification of His tagged proteins.
IMAC	Immobilized Metal Ion Affinity chromatography is a method for purifying His tagged protein. Immobilized metal ions in the column reversibly bind His tagged protein and later the tagged protein is eluted.
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis is method for protein separation while the negatively charged SDS acts as detergent in protein electrophoresis
CMC	Carboxymethyl cellulose is a soluble derivative of cellulose
OD_{600}	Optical Density at 600nm represents absorbance value of a substance for the light of 600nm.

1. INTRODUCTION

Lignocellulose is a complex and heterogeneous structure of cellulose, hemicelluloses and lignin. Lignocellulose is present excessively in straws, hulls, stems, stalks, and woods. The proportion of cellulose, hemicellulose and lignin in lignocellulose varies among plants; cellulose ranges from 35 to 50%, hemicellulose 20-35% and lignin 10-25%. Cellulose, chemically represented as $(C_6H_{10}O_5)$n, is the major component of plant cell wall. Cellulose is a homo-polysaccharide of D- glucose monomers. The glucose monomers are linked by β-1,4-glucosidic bonds. Hemicelluloses are copolymer of different pentoses and hexoses, which are also present in plant cell wall. The sugar residues of hemicelluloses include D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose, D-glucuronic acid and 4-O-methyl-D-glucuronic acid. Compared to cellulose, hemicellulose is shorter and branched molecule. Lignin is a complex long polymer of phenyl propane units with average molecular weight of 8,000 to 11,000. The phenyl propane units include p-coumaryl alcohol (*p*-hydroxyphenyl propanol), coniferyl alcohol (guaiacyl propanol) and sinapyl alcohol (syringyl propanol). These units are linked by alkyl–aryl, alkyl–alkyl and aryl–aryl ether bonds. Lignin is bonded to both hemicellulose and cellulose in cell wall. Lignin provides support, impermeability and protection to plant cells from microbial attack and oxidative stress. The structure of lignocellulose is presented in Figure 1.
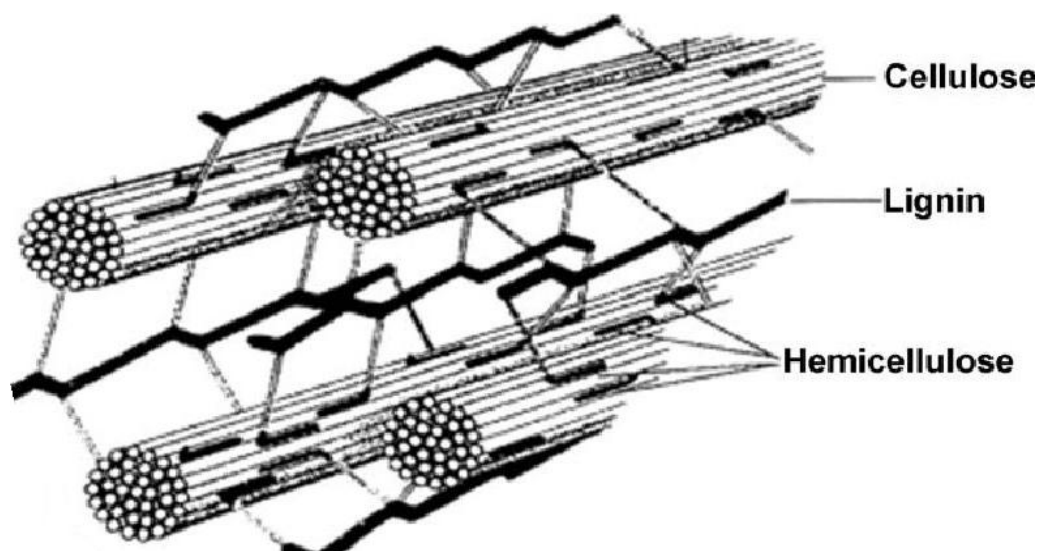
Figure 1: *Structure of lignocellulose. Lignin serves as linker between the cellulose and hemicelluloses polymers. (Yinghuai 2013.)*

Lignocellulose is the most abundant renewable biomass on earth as half of the mass produced by photosynthesis comprises of lignocellulose. Although lignocellulose is a low cost feedstock but among the lignocellulose produced from agriculture and forestry, only a small proportion is utilized. The lignocellulose and its components can be converted in different valuable products. Biofuels can be synthesized from cellulose and hemicellulose fractions while lignin is sometimes employed for heating the process of biofuel synthesis. Cellulose and hemicelluloses can be converted in alcohols (ethanol, n-butanol, isobutanol, 2-methyl-1-butanol or 3-methyl-1-butanol), esters, ethers and hydrocarbons biofuels. Interestingly fatty esters, fatty alcohols and waxes have also been biosynthesized from hemicelluloses (Steen et al. 2010). Among the biofuels, ethanol is produced in the highest quantity. Ethanol production of 84 billion liters was recorded for the year 2010 and the production is estimated to reach 125 billion liters in the year 2017 (Walker 2011). Ethanol is already being mixed in petrol up to 25% in different counties (E10 Petrol 2014). Currently alternative fuels have been focused seriously as fossil fuel is not environment friendly and its prices are ever increasing. (Nigam & Pandey 2009.) Moreover the ethanol being produced from food sources like sugar cane or corn endangers the food availability and prices. So there is a need to switch ethanol production from food source to lignocellulose but unfortunately conversion of lignocellulose into ethanol is an extensive and costly process. (Saha 2004.)

Among the cost contributors of cellulosic ethanol production, one contributor is recalcitrant nature of lignocellulose. A cost effective mean to degrade lignocelluloses will increase economic feasibility of the process. Recalcitrant lignocellulose can be degraded by either conventional physiochemical degradation methods (also known as pretreatment) or by biodegradation. (Pérez et al. 2002.) The common physical and chemical pretreatments include steam explosion, ammonia fiber explosion (AFEX), acid pretreatment, alkaline pretreatment and organosolv process while biodegradation of lignocellulose is processed by fungi and bacteria. Biodegradation need lesser energy and mild operational conditions compared to physiochemical treatments. Unfortunately biodegradation by microbes is time consuming as the rate of biological hydrolysis is quite slow (Sun & Cheng 2002). Biodegradation is overall an attractive approach as technologies based on microbes and their enzymes are more environment friendly (Hatakka 2005).

The current research thrived to develop an effective biological mean to degrade lignocellulose. A fluorescent and photosensitizer protein, killerRed was manipulated to develop its affinity for cellulose substrate. A cellulose binding peptide (CBP) was added at N terminal of killerRed to obtain Cellulose Binding Peptide-KillerRed (CBP-KR) protein. Then CBP-KR was analyzed for its affinity and degradation capabilities for cellulose. A control protein, His tagged KillerRed (His-KR) was also included in the experiments.

2. THEORETICAL BACKGROUND

Synthetic biology has been originated from the finding of mathematical sense in gene regulation and from the recombinant DNA technology. Recombinant DNA technology or genetic engineering focuses individual genes but synthetic biology targets systems. (Andrianantoandro et al. 2006.) Similar to synthetic biology, system biology also focuses biological system rather than individual genes or pathways. Although system biology and synthetic biology both target systems but the objectives of both disciplines are quite different. System biology analyzes interaction of components of systems and tries to acquire a quantitative understanding of the natural biological systems. Synthetic biology, on the other hand, engineers biological systems to generate novel artificial tasks. Synthetic biology is an engineering discipline and it utilizes the knowledge and tools generated from system biology. (Serrano 2007.) Synthetic biology has two approaches. One is about developing artificial behaviours from natural biology while other approach searches for interchangeable parts from natural biology, which can be reassembled for building artificial life. (Benner & Sismour 2005).

The concept of synthetic biology is parallel to computer engineering. The biomolecules like DNA, RNA, proteins and metabolites are analogous to material layer of resistors, transistors and capacitors in computers. The higher layer of devices such as logic gates is equivalent to biochemical reactions in synthetic biology. The device layer in biology controls flow of information and operates physical processes while in computers devices are responsible for computations. For engineering modules in synthetic biology, different biological devices are integrated into complex pathways. Connecting modules and integrating modules with host cell, generate new behavior from cells. Synthetic biology not only engineer individual cells but also target cell populations. Sophisticated coordination is achieved by involving multiple communicating cells similar to the computer networks. So similar to computer engineering, synthetic biology require bottom-up approach to obtain new behaviors.

Researchers encounter typical problems while engineering biological systems. The devices and modules used for biological engineering are not independent and require biological milieu to function. Researchers usually utilize host cell machinery to engineer devices or modules as biological devices requires cellular environment to function. Interestingly the host cells also get altered due to those engineered biological devices and nodules. Another problem is about the unpredictability of biological devices. Functioning of even simple devices in host cells cannot be fully predicted and hence complex system cannot be precise and reliable. The uncertainty in single cell engineering arises from limited knowledge of inherent cellular properties. The known contributors to the

unpredictability include effects from gene expression noise, mutation, cell death and altering extracellular environments. Moreover the interaction between devices and cellular context also cause perturbations. The issue of uncertainty is overcome by using cell population rather than single cell. The predictability and reliability of multiple cell systems is achieved by two strategies. Either big population of independent cells is employed or intercellular communication is exploited to achieve synchrony among individual cells. Intercellular communication can generate harmony in a heterogeneous cell populations and it is consider as a finest choice to draw complex but sophisticated tasks from multicellular systems. (Andrianantoandro et al. 2006.)

The era of applications in synthetic biology started with the success in engineering biological devices. The first biological devices, a genetic toggle switch and a repressilator (oscillator), were engineered in 2000. Gardner et al. engineered the first toggle switch which could be activated by either IPTG or with transient increase in temperature. In the switch, transcription of *lacI* was derived from the P_L promoter. The P_L promoter has origin from λ phage. The protein product form *lacI* was repressing the second promoter, Ptrc2 while the promoter Ptrc2 was deriving expression of *cI-ts* gene. The *cI-ts* genes produce temperature sensitive (ts) λ CI repressor protein which in return suppresses P_L promoter. The functioning of the circuit was observed by expression of an GFP associated promoter. The switch could be toggled in either direction depending upon the activator (IPTG or temperature rise) and the switch was able to retain existing state after removal of activating signals, which depict characteristic of memory. (Khalil & Collins 2010.) Gardner's switch is presented in Figure 2. Elowitz and Leibler engineered the first synthetic genetic oscillator by employing three transcriptional repressors (repressilator); the products from *lacI, tetR* and *cI* genes. LacI suppress *tetR* transcription, TetR inhibit expression of *cI* while CI restrain *lacI* expression in the final step of the cycle. Strong repressible hybrid promoters $P_L$lacO1 and $P_L$tetO1 were employed to reduce leakiness of transcription and destruction tags were added to repressors to reduce their life. The destruction tags were *ssrA* RNA sequence based carboxy-terminal tags which are recognized by proteases of *E. coli*. The genetic oscillator was a negative feedback cycle and loop was monitored by the second plasmid enclosing PLtetO1 fused to *gfp*. The plasmid construct and repression scheme of the loop have been presented in Figure 3. (Elowitz & Leibler 2000.)

Synthetic biology has applications in three fields; biosensing, therapeutics and manufacturing of biofuels, pharmaceuticals and new kind of biomaterials. Biosensing rely on cell's natural ability to sense and respond to diverse kind of environmental signals. Cells have a sensing module of tailored sensitive element and a transducer module to filter the signals and activate a cellular response. Biosensing have been engineered at levels of transcription, translation and post-translation. For transcriptional biosensing, promoters and transcription factors have been targeted commonly. The familiar environment-responsive promoters include promoters *lac*, *tet* and *ara* operons from *Escherichia coli*. Except promoters and transcription factors, RNA polymerase and genes itself can be engineered for biosensing. The environment-responsive promoters have not

only been engineered but sometimes these promoters have been embedded in engineered networks. Kobayashi et al. (2004) developed an *E. coli* biosensors by coupling an engineered gene circuit with an environment-responsive promoter and its transcription factor. The biosensing circuit in sensing whole cell was a toggle switch which got ON when deleterious signals had exceeded threshold. For translational biosensing, riboswitches, antisense RNAs and the self cleavage property of RNA are employed while post-translational biosensing target protein receptors. Hybrid approaches combining transcriptional, translational and post-translational levels of biosensing have provided more sophisticated whole cell sensors. (Khalil & Collins 2010.)
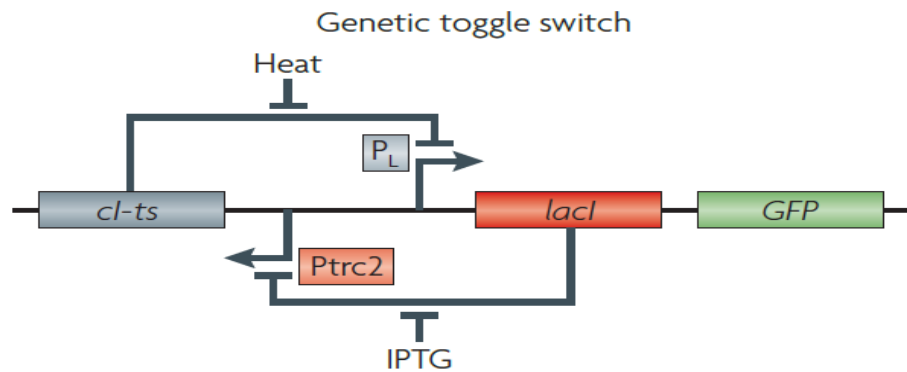
Figure 2: *Schematic diagram of Gardner group's toggle switch. The toggle switch employed P_L and Ptrc2 promoters and could be toggled in two directions with IPTG and temperature rise. (Khalil & Collins 2010.)*
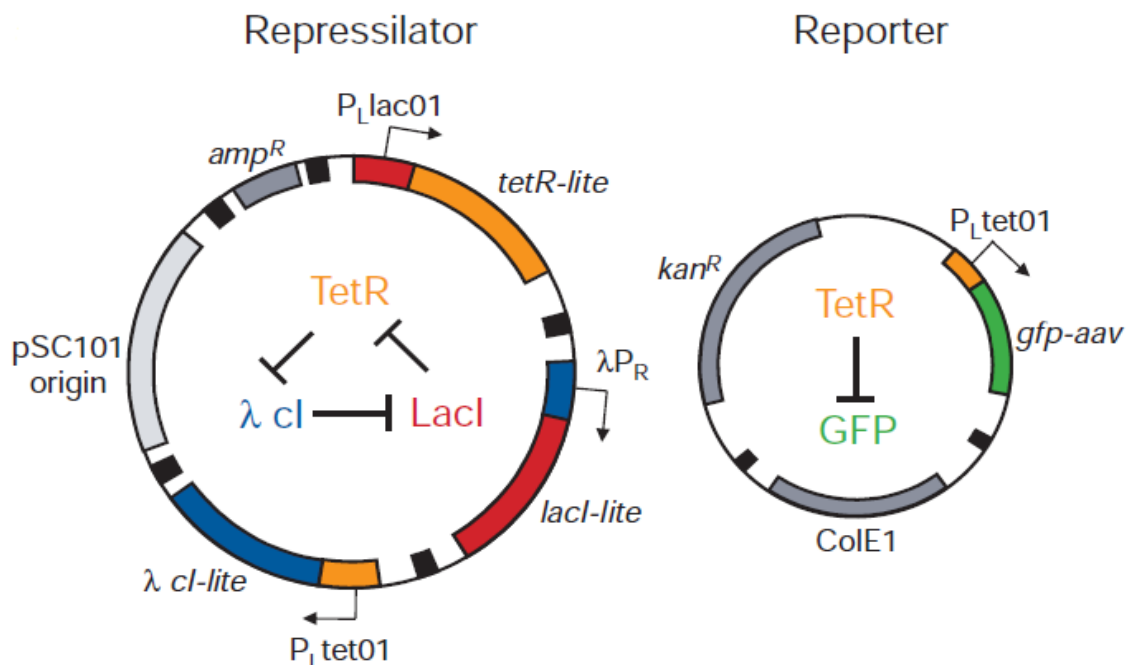
Figure 3: *The plasmid constructs used by Elowitz & Leibler to develop the first repressilator. In the repressilator cycle, one promoter causes expression of second promoter's repressor while GFP expression is also connected to genetic oscillator. (Elowitz & Leibler 2000).*

In therapeutics, synthetic biology has a distinct importance. Disease mechanisms can be understood by exploring pathological behaviours from reconstructed biological systems. Similarly viral genomes could be reconstructed to understand pathogenesis of viral infections. Moreover synthetic biology devices deployed in biological systems can identify drug-targets and with detection of faulty components in biological pathways, drugs could be discovered from engineered whole cell screening systems. Except understanding disease mechanisms, indentifying drug targets and discovering drugs, synthetic biology devices can be used for therapeutic treatment purposes, control drug delivery, gene therapy and metabolic therapy. (Khalil & Collins 2010.) Viruses have been engineered to target faulty components and pathogenic agents (Lu & Collins 2007; Lu & Collins 2009).

The most relevant application of synthetic biology to the current topic is about manufacturing of biofuels, pharmaceuticals and new kind of biomaterials. Cost effective production of biofuels, lowering cost of expensive drugs and synthesis of novel biomaterials are exciting applications of synthetic biology. These applications require selection of optimal biosynthetic pathway and host organism. Host organisms are selected on the base of presence of required biosynthetic activities. For instance, some microbes have innate ability to convert lignocellulose into ethanol or biobutanol. Such microbes have catabolic activity close to the desired one and are tolerant to toxic substances but usually not suitable for industrial use. To bring these organisms at commercial scale, they are either reengineered or their synthetic pathways are transplanted in industrial model organisms like *Escherichia coli* or *Saccharomyces cerevisiae.*

Among biofuels, ethanol is most widely produced but its microbial production is not cost effective yet. (Khalil & Collins 2010.) Among other alcohol biofuel alternatives, n-butanol is intensively investigated. n-Butanol has higher energy content compared to methanol and ethanol and it is also less miscible with water. On the other hand, n-butanol has two times higher viscosity than ethanol and five to ten times higher viscosity than gasoline. At industrial scale, n-butanol is produced from propylene and carbon monoxide. The reaction of propylene and carbon monoxide produce n-butyraldehyde and isobutyraldehyde and then n-butyraldehyde is reduced to n-butanol. (Wackett 2008.) Microbial synthesis of biobutanol has also been experimented. Butanol is naturally produced by *Clostridium acetobutylicum* from acetyl-CoA buts its native production is low and it is produced in mixture with acetone and ethanol. (Khalil & Collins 2010.) Atsumi et al. (2008a) engineered *Escherichia coli* to produce n-butanol. As wild-type strains of *E. coli* do not produce n-butanol, genes synthesizing acetoacetyl-CoA, 3-hydroxybutryl-CoA, crotonyl-CoA, butyryl-CoA, butyraldehyde, and n-butanol were introduced into the host *E. coli*. The poor expression of some genes was overcome by replacing those genes with related genes from other microorganisms and a maximum titer of 552 mg/l was obtained for n-butanol. In contrast to *Clostridium acetobutylicum,* *E.coli* did not produce acetone and absence of acetone production make the process more suitable. Some advance studies have raised the titer of n-butanol up to 1–2% (w/v)

but it should be further increased to compete with high ethanol titers generated by ethanologenic yeast (Wackett 2008).

Atsumi et al. (2008b) adapted a non fermentative approach for producing long chain alcohols (isobutanol, 2-methyl-1-butanol or 3-methyl-1-butanol) from *E.coli*. The 2-keto acids intermediates generated from amino acid biosynthesis pathways were synthetically shunted to butanols by introducing two enzymes. The enzymes were 2-keto-acid decarboxylases (KDCs) and alcohol dehydrogenases (ADHs). KDC converts 2-keto acids intermediates into aldehydes while ADH converts aldehydes into alcohols.

Eggeman and Verser (2006) employed a homoacetogenic bacterium which can consume both hexoses and pentoses concurrently to yield acetic acid. Eggeman and Verser presented a novel approach which was about esterifying acetic acid from fermentation broth with ethanol to form ethyl acetate. Ethyl acetate is less water soluble and hence can be separated easily. Upon hydrogenation, one molecule of ethyl acetate gives two molecules of ethanol and this way yield was improved significantly.

In alcohol fermentations, one of the major issues is about solvent toxicity to fermenting organism. A study has reported 251 genes of *S. cerevisiae* are involved in ethanol tolerance. Alcohol tolerance can be improved if the product is water insoluble and in solid state at fermentation temperatures. Synthetic biology has potential to solve the tolerance problem. (Wackett 2008.)

With alcohols researchers also focus on esters, ethers and hydrocarbons as alternative biofuels. Biosynthetic alternatives which can replace the components of common fossil fuels are presented in Table 1. Hydrocarbons are synthesized in the form of long waxy hydrocarbons in animals, plants, and microbes. Among bacteria, Bacillus produce isoprene. Withers et al. (2007) recognize the genes (*yhfR* and *nudF*) responsible for isoprene biosynthesis in Bacillus. Withers et al. introduced *nudF* and biosynthetic pathway of mevalonate-based isopentenyl pyrophosphate in *E. coli*. The engineering resulted in isopentenol production. Many biochemical mechanisms have been found to be involved in hydrocarbon synthesis. Alkyl carboxylic acids are converted in alkanes or alkenes by decarboxylation reaction. Similarly for biofuel production, fatty acids obtained from plants or animals can be converted to alkanes by high-temperature and catalytic route. Biological decarboxylations happen with activated substrates and the carbon going through decarboxylation is involved in resonance through a carbonyl carbon, a carbon to carbon double bond or an aromatic ring. *Rhodotorula minuta* have an alternative mean to convert alkyl carboxylic acids into alkanes. *R. minuta* converts branched chain carboxylic or amino acids into isobutene. The decarboxylation is proposed to proceed by isovalerate. (Wackett 2008.)

In addition to alkyl carboxylic acids, alkyl aldehydes have been reported to convert into alkanes by decarbonylation in a study (Dennis & Kolattukudy 1992). The enzyme responsible for decarbonylation was partially purified and proposed to possess a cobalt-porphyrin. Unfortunately follow up studies regarding alkyl aldehydes conversion into alkanes are missing. Moreover a novel enzymatic conversion of fatty alcohol into alkane was reported by Park (2005). Park reported *Vibrio furnissii* M1 membrane to

possess ability to reduce fatty acid into corresponding alkane via the related alcohol intermediate. Wackett et al. (2007) investigated the interesting finding of Park about alkane synthesis and reported absence of an alkane synthesis in *V. furnissii* M1. Wackett et al. employed methods of whole-genome sequencing and biochemical analysis for the investigation. Upon sequencing complete genomic DNA of *V. furnissii* M1 with 21-fold coverage, no gene for alkane synthesis or alkane degradation was found. Similarly gas chromatography-mass spectrometry did not detect *n*-alkanes from the extracts of *V. furnissii* M1 culture.

Table 1: *Alternative biofuels for replacing components of gasoline, diesel and jet fuel. (Lee et al. 2008)*

Fuel type	Major components	Biosynthetic alternatives
Gasoline	C_4–C_{12} hydrocarbons	Ethanol, *n*-butanol and *iso*-butanol
	Linear, branched, cyclic, aromatics	Short chain alcohols
	Anti-knock additives	Short chain alkanes
Diesel	C_9–C_{23} (average C_{16})	Biodiesel (fatty acid methyl esters/ FAMEs)
	Linear, branched, cyclic, aromatic	Fatty alcohols, alkanes
	Anti-freeze additives	Linear or cyclic isoprenoids
Jet fuel	C_8–C_{16} hydrocarbons	Alkanes
	Linear, branched, cyclic, aromatic	Biodiesel
	Anti-freeze additives	Linear or cyclic isoprenoids

Wackett also led a study in which prevalent bacterial head-to-head hydrocarbon biosynthesis was reported. The *oleABCD* genes responsible for head-to-head olefinic hydrocarbon synthesis were identified by sequence analysis through structural alignments and gene context. Moreover hydrocarbons were characterized by Gas chromatography–mass spectrometry (GC-MS) after extractions. Among the bacteria analyzed, maximum were producing a long-chain olefin containing nine double bonds; 3,6,9,12,15,19,22,25,28-hentriacontanonaene. *Xanthomonas campestris* was reported to

synthesize maximum number of olefins. *X. campestris* produces fifteen olefin products ranging from C28 to C31 with one to three double bonds. The study defined OleA within thiolase superfamily, OleB within α/β-hydrolase superfamily, OleC within AMP dependent ligase/ synthase superfamily and OleD within short-chain dehydrogenase superfamily. The study also proposed role of *oleA*. OleA was found responsible for nondecarboxylative thiolytic condensation of fatty acyl chains. The condensation produce β-ketoacyl intermediates and these intermediates spontaneously decarboxylate into ketones. (Sukovich et al. 2010.) Fatty acid derived biofuels have been discussed in details in the section 2.2.3.

In addition to engineering biosynthetic pathways, expressions of components are also optimized to get optimal metabolic flux and maximum product. Metabolic pathway flux is optimized either by evolutionary techniques or by rational design and in silico modeling approach. Sometimes both evolutionary strategies and rational design approach are combined to optimize pathway flux. (Khalil & Collins 2010.)

Novel functionalities can also be programmed in the engineered organisms. The novel functionality is generated by integrating basic circuits which can perform simple tasks like sensing and processing signals, logic operations and activating biological responses. The examples of novel functionality include a circuit which can sense bioreactor environment and move metabolism accordingly to achieve maximum yield of biofuel. Genetic toggle switches have been programmed by promoter engineering to behave as biological timers. Such timers were applied to the time-dependent flocculation of yeast cells which is frequently eccountered in fermentation process. The timer circuit facilitated separation of cells from alcohol produced in the process. Other novel functionalities presented include extraction of product from cells or purification of product. (Khalil & Collins 2010.)

A hybrid chemical and biological synthetic approach also has potential to generate those fuel molecules whose biological synthesis is not feasible. Hybrid biological/chemical processes are expected to bring success in producing cost effective renewable fuels. Many of the hybrid chemical and biological synthetic approaches have been adopted. Román-Leshkov et al. (2007) employed enzymes to break starch and to convert glucose into fructose (isomerisation). Fructose produced was added with acid and metal catalysts. The five oxygen atoms were removed by acid catalysis and 2,5-dimethylfuran (DMF) was produced. DMF possesses more energy content and has higher boiling point when compared with ethanol. DMF has an octane number of 119 and hence can be utilize as fuel in a spark ignition engine. The performance of DMF in engines, toxicity in environment, and possibility to produce DMF at large scale are still unanswered. This strategy of DMF production can also be applied to glucose content obtain from cellulose. The detailed reactions involved in conversion of starch into DMF have been presented in Figure 4.
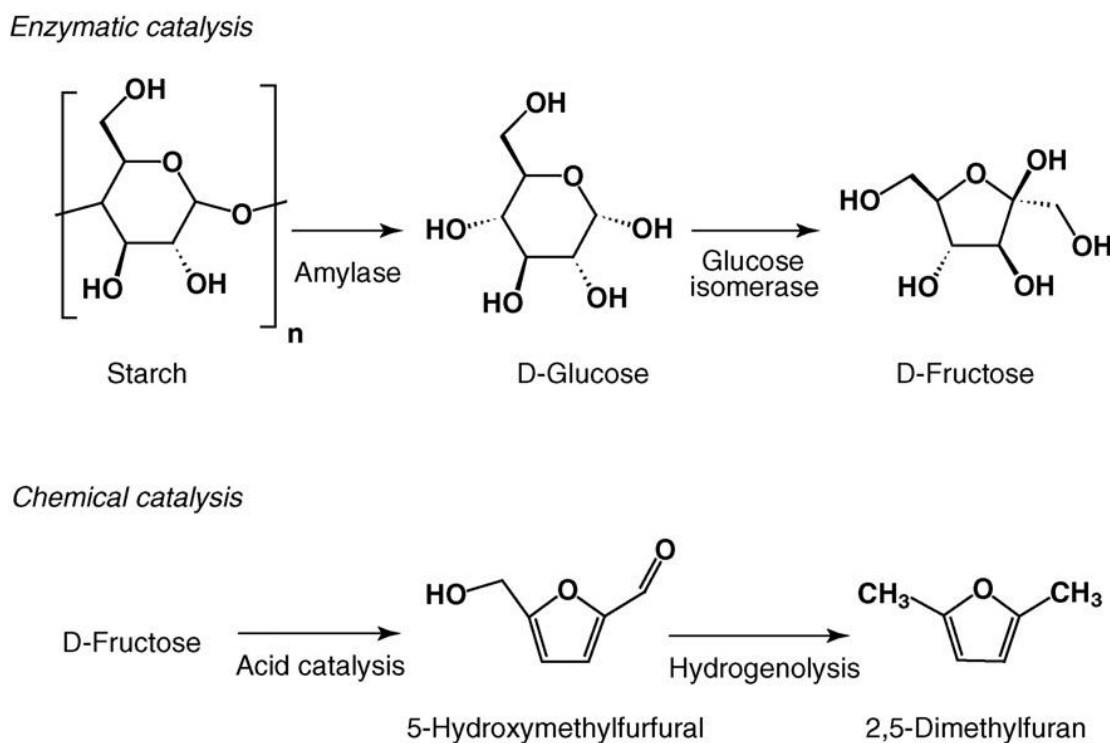
Figure 4: *Schematic diagram of hybrid chemical and biological synthetic approach adopted by Leshkov et al. (2007) to convert starch into 2,5-dimethylfuran (DMF) (Wackett 2008).*

Generation of new biofuels require extensive integration of biology, chemistry and other sciences. The information required for applying hybrid chemical and biological synthetic approaches are usually fragmented. A database has been initiated to break barriers and to provide the required data in an unbiased way. The database 'BioFuels Database' contains information about fuel molecules: alcohols, esters, ethers, and hydrocarbon. In short, all the efforts going on will end with a sustainable fuel which will be cheap, cost effective, with higher energy content, highly combustible, non explosive, stable for long term storage, in liquid form and easily transportable by pipes. (Wackett 2008.)

In the next sections of this theoretical background, biodegradations and commercially attractive bioconversions of cellulose, hemicelluloses and lignin have been presented. The lignocellulosic biomass present in nature is biodegraded by collaboration of several microbes as a part of earth carbon cycle. Haruta et al. (2002) mimicked the natural biodegradation of lignocellulose and obtained a microbial community which was capable of degrading 60% of rice straw at 50 °C in four days. The community of microbes comprised of aerobic and anaerobic bacteria and the community was found stable even after several subcultures or at extreme temperatures of 95 °C or –80 °C. In addition to biodegradations, the last section of this theoretical background summarizes the efforts for developing whole-cell biocatalyst which can produce ethanol from lignocellulose in a consolidated manner.
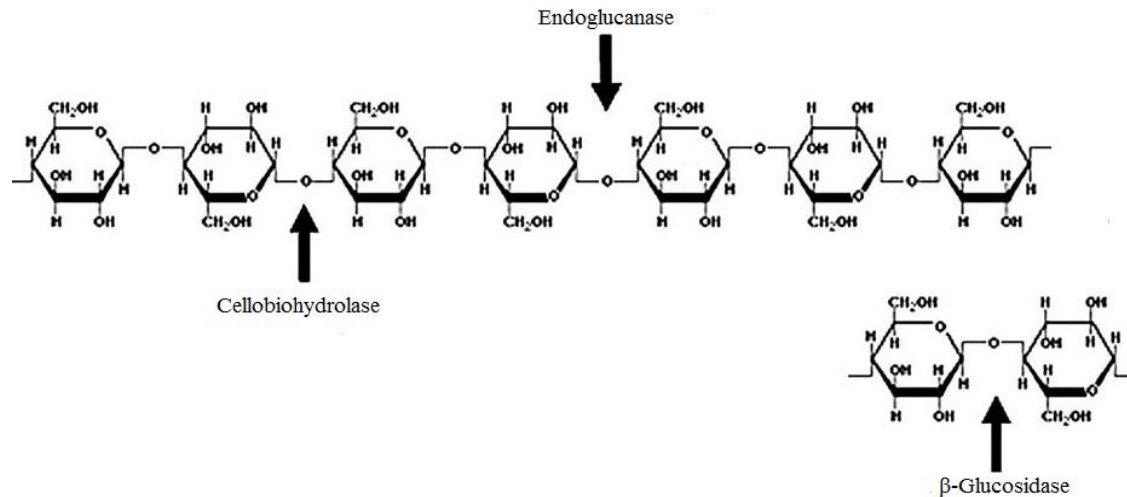
2.1. Biodegradation of Cellulose

Plant cell wall is mainly composed of cellulose and the cell wall contains cellulose in form of fibres (macrofibrils). Macrofibrils are collections of microfibrils while microfibrils are parallel polymers linked by hydrogen bonds. In a microfibril, β-1,4-glucosidic linkages straighten the cellulose polymer while hydroxides groups on both sides of glucose monomer facilitate hydrogen bonding. Cellulose exists in both crystalline and non-crystalline form. (Harmsen et al. 2010.) Properties of cellulose affect its microbial degradation. The properties influencing biodegradation include capillary structure of cellulose compared to size of cellulases, extent of crystallinity, the facets of the crystalline part of cellulose microfibrils and nature of cellulose binding lignin. (Kirk 1983.) Cellulose degrading organisms possess cellulases and cellulosomes.

2.1.1. Cellulases

Hydrolysis of cellulose requires combination of at least three enzymes; endoglucanase (EC 3.2.1.4), cellobiohydrolase or exoglucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). Endoglucanases randomly hydrolyze accessible β-1,4-glucosidic bonds between linked glucose residues and cellulose is broken down into smaller polymers. Cellobiohydrolases act on non-reducing chain ends of cellulose fibers and release cellobiose (glucose dimmers). Endoglucanases and exoglucanases act on solid substrates and produce soluble cellobiose or glucose. (Percival Zhang et al 2006; Saha 2004.) Moreover an endo-exo form of synergism was observed between cellobiohydrolases II and endoglucanases of *Trichoderma reesei* (Henrissat et al. 1985). Except synergism, adsorption of cellulases on insoluble cellulose substrate is an essential step before hydrolysis (Divne et al. 1994). The binding of cellulases with insoluble cellulose is facilitated by cellulose binding domains (CBDs). CBDs ensure localized and restricted hydrolysis of cellulose and prevent cellulases from being washed off from cellulose surface. (BEguin & Aubert 1994.) CBD is attached to the catalytic domain of cellulase via a peptide linker and CBD can be linked to either N or C terminal of the catalytic domain (Linder & Teeri 1996).

The soluble cellobiose produced by cellobiohydrolase is hydrolyzed by β-glucosidases. The actions of endoglucanase, cellobiohydrolase and β-glucosidase is shown in Figure 5. The rate-limiting steps of enzymatic cellulose degradation are the reaction involving endoglucanase and exoglucanase depolymerizations. (Percival Zhang et al 2006; Saha 2004.)

Figure 5: *The action of endoglucanase, cellobiohydrolase and β-glucosidase in cellulose degradation (Modified from Kumar et al. 2008).*

2.1.1.   Cellulosome

The first cellulosome was discovered by Lamed (1983) from *Clostridium thermocellum*. Cellulosomes is a supramolecular complex containing cellulases organized on a scaffoldin. The scaffoldin contains multiple cohesin domains and each cellulase attached to cellulosome possesses dockerin domain. The integration of cellulases in cellulosome is facilitated through cohesin-dockerin interactions. Moreover cellulosomal cellulases can be differentiated from individual or free cellulases by the presence of dockerin domain. (Bayer et al. 1998.)

CBDs are also attached to scaffoldin of cellulosome and interestingly some of the cellulases (catalytic domains) within a cellulosome may have their own CBD which is not attached to the scaffodin (Linder & Teeri 1996). A schematic representation of cellulosome is shown in Figure 6. Another cohesin-dockerin interaction system was also found in the cellulosome of *Clostridium thermocellum* which facilitate binding of scaffoldin to surface of cells (Bayer et al. 1998).
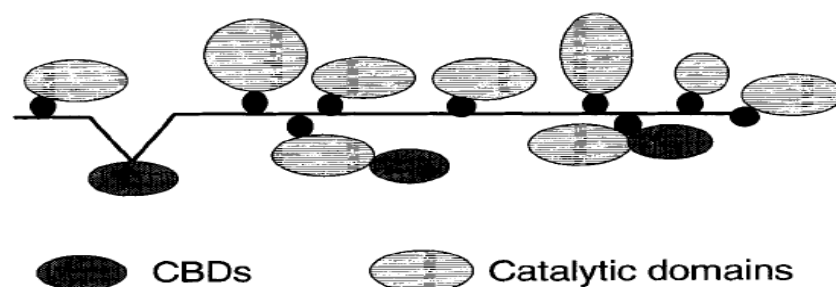
Figure 6: *Structure of cellulosome. The central scaffoldin is containing multiple CBDs and catalytic domains. CBDs are attached either to central scaffoldin or to catalytic domains. (Modified from Linder & Teeri 1996.)*

2.1.2.　Cellulose Binding Domains (CBDs)

A cellulose-binding domain (CBD) is present in individual cellulases acting on cellulose substrates and in the complex cellulosomes. CBDs have also been found in some hemicellulases and some domains similar to CBD are found in other enzymes like in Glucoamylases. (Linder & Teeri 1996; Carrard et al. 2000.) CBDs are classified into 10 families but most CBDs are placed in family I, II and III. Family I contains all CBDs from fungi and the well studied CBDs among the family I are from *Trichodermu reesei.* In the family II, CBDs from *Cellulomonas fimi,* and *Pseudomonas fluorescens* are well characterized. In family III, many CBDs are from *Clostridium thermocellum* and *Clostridium cellulovorans* which are cellulosome producing bacteria. (Tomme et al 1995a.)

CBD has a minute influence on hydrolysis of soluble substrates but CBD significantly enhances cellulase activity on insoluble substrates. Kruus et al. (1995) have showed that providing a CBD to a tiny artificial cellulosome resulted in better catalytic activity of cellulosome. CBDs are also found interchangeable to some extent. Tomme et al. (1995b) replaced the family II CBD from Cex with a fungal family I CBD. Cex is mixed function xylanase-exoglucanase and found in bacteria. The modified Cex was found with little bit lesser binding ability and hydrolytic activity for the bacterial microcrystalline cellulose. For soluble cellulose and xylan, the modified Cex showed improved activity. The length of the linker between catalytic domain and CBD also affects cellulase activity. From various research results, it has been suggested that CBD and catalytic domain work in coordination during hydrolysis of cellulose. Moreover a relatively longer linker length for CBD is supposed to be required for maximum cellulase activity. (Linder & Teeri 1996.)

Binding or adsorption of CBD on cellulose is sometimes considered tight but cellulases get adsorbed and desorbed successively which ensures progressive hydrolysis of cellulose and relocation of the cellulases on the cellulose surface. The enzyme relocation was confirmed by the fast lessening in degree of polymerization (DP) of substrate upon action of endoglucanases. (Kleman-Leyer et al., 1994, 1996.) Moreover according to Tomme (1998), CBDs from family I and IV attach cellulose reversibly while CBDs from family II and III generally bind irreversibly. Din et al. (1994) showed ability of some CBDs from family II to enter the discontinuities present on the surface of cellulose and break non covalent bonds between cellulose fibers. This way family II CBDs exposes new fibers for further hydrolysis.

As a biotechnological application, CBDs are utilized as affinity tags for purification or immobilization of proteins. For expression of CBD-fused proteins, commercial vectors are also available nowadays. (Novy et al. 1997).

2.1.3.   Sources of Cellulases

The common sources of cellulases include the aerobic fungi; *Trichoderma viride, Trichoderma reesei, Penicillium pinophilum, Sporotrichum pulverulenlum, Fusarium solani,Talaromyces emersonii and Trichoderma koningii* (Bhat & Bhat 1997). *Trichoderma reesei* has been reported to produce at least nine cellulases. Among the cellulases EGI, EGII, EGIII, EGIV and EGV are the endoglucanases, CBHI and CBHII are the exoglucanases and BGLI and BGLII are the β-glucosidases. (Kubicek & Penttilä. 1998.) Moreover an isolated fungi *Mucor circinelloides* strain has been report-ed to produce a complete cellulase enzyme system (Saha 2004).

Cellulases have also been found in many other bacteria and fungi including aerobes and anaerobes and mesophiles and thermophiles. Cellulases producing thermophilic aerobic fungi include *Sporotrichum thermophile, Thermoascus aurantiacus, Chaetomium thermophile and Humicola insolens*. Celluases are also produced by mesophilic anaerobic fungi which include *Neocallimastix frontalis, Piromonas communis and Sphaeromonas communis*. Anaerobic bacteria also produce cellulases and the species include *Acetivibrio cellulolyticus, Bacteroides cellulosolvens, Bacteroides succinogenes, Ruminococcus albus, Ruminococcus flavefaciens, and Clos-tridium thermocellum*. Among aerobic bacteria, *Cellulomonas* species, *Cellvibrio* spe-cies, *Microbispora bispora, and Thermomonospora* species are active cellulases pro-ducer. (Bhat & Bhat 1997.)

Cellulolytic rumen bacteria include *Clostridium cellobioparum, Butyrivibrio fibrisolvens, Bacteroides succinogenes, Eubacterium cellulosolvens, Ruminococcus flavefaciens* and *Ruminococcus albus* (Teather & Wood 1982). Actinomycetes, *Thermomonospora fusca* has also been reported to actively produce cellulases (Bhat & Bhat 1997).

Termites and the associated microbial symbionts are described as the most efficient decomposer of lignocelluloses in nature. A dual system of cellulose degradation has been proposed for lower termites. The dual system consists of termites' own cellulases and cellulases from its gut protists. Higher termites do not possess symbiotic protists and hence degrade cellulose by their own cellulases. (Ohkuma 2003.) Interestingly cellulase genes have also been found in arthropods like cockroaches and crayfish and from nematodes like *Globodera rastochiensis, Heterodera glycines* and *Meloidogyne incognita* (Watanabe & Tokuda 2001).

2.1.4.   Commercially Attractive Bioconversions of Cellulose

Commercial scale conversion of lignocellulose into fermentable sugars requires cut in the expenses of cellulases consumed in the process. Biotechnology companies are trying to reduce cellulases prices by increasing production volumes and utilizing cheaper sub-strates for enzymes production. Cellulases with higher stability and specific activity have also been focused. Genencor International and Novozymes Biotech companies

claimed to reduce cellulases cost for the process of cellulose-to-ethanol from US$5.40 to 20 cents per gallon of ethanol. (Moreira 2005; Saha 2004.)

Properties of cellulases have been improved by rational engineering and directed evolution. Rational engineering of cellulases is limited due to insufficient knowledge about the properties of cellulose substrates and the complex interactions between cellulases and cellulose substrates. Moreover the synergism and/or competition between cellulases components has not been unveiled completely. (Percival Zhang et al 2006.) Various cellulases have been improved by rational design and directed evolution. Among success stories of rational design, one is about improving an endocellulase, Cel5A of *Acidothermus cellulolyticus*. Baker et al. (2005) replaced amino acid Tyr245 of Cel5A with Gly and the mutated enzymes exhibited an increase of 1480% in K_i value for inhibition of Cel5A by cellobiose. Moreover the improved Cel5A released soluble sugars from cellulose at a rate 40% higher compared to the wild-type enzyme.

Among several achievements of the directed evolution or simply irrational design, one is about improving the thermostability of endoglucanase EngB. Endoglucanase EngB is a component of cellulosome of *Clostridium cellulovoran*. Genes of EngB were shuffled (recombination) with genes of non-cellulosomal cellulase EngD. A mutant was obtained possessing seven times higher thermostability compared to the EngB from *Clostridium cellulovoran.* (Murashima et al 2002.) Apart from manipulation of cellulases, thermostable and other novel cellulases are present in nature. *Candida peltata* had been reported to secrete a highly glucose-tolerant β-glucosidase (Saha & Bothast 1996).

Cellulases recycling reduce net enzyme requirement for cellulose hydrolysis. Recovery of cellulases from substrate is though limited, especially when the lignin content is high. Castanon and Wilke (1981) significantly improved hydrolytic activity cellulases and the recovery of enzymes by the addition of Tween 80 as surfactant to newspaper substrate.

New novel cellulases have been discovered which can directly break cellulose without extracting chains from crystalline matrix. The new cellulases are bacterial carbohydrate binding modules (CBM33) and fungal glycoside hydrolases 61 (GH61). CBM33 and GH61 have substrate-binding surfaces and break cellulose by an oxidative mechanism. The genes of CBM33 and GH61 have been found abundantly in the genome of microorganisms responsible for biomass conversion. The oxidative enzymes have provided a new model for future degradation of cellulose and other recalcitrant polysaccharides at commercial scales. (Horn et al. 2012)

Recently cellulases have been conjugated with nanoparticles. The cellulases have been adsorbed on polystyrene nanospheres (Blanchette et al. 2012) and silica nanoparticles (Lupoi & Smith 2011). The cellulases conjugated with nanoparticles have shown improved cellulolytic ability for crystalline cellulose but not for CMC. Conjugation of different enzymes with nanospheres has potential to improve economic feasibility of bioethanol production.

2.2.   Biodegradation of Hemicelluloses

Hemicellulose is a family of heterogeneous polysaccharides and hence biodegradation of hemicelluloses also requires heterogeneous enzymes. Hemicelluloses usually exist in non-crystalline form and include xylan, glucomannans and galactoglucomannans. Xylans are the principal hemicellulose of hardwood while glucomannans are the main hemicellulose of softwood.

Xylans are heteropolysaccharide molecules with homopolymeric backbone chains. The homopolymeric chains are composed 1→4-bonded β -D-xylopyranose units and the xylopyranose units can have O-2 or O-3 linked α-glucuronosyl residues, 4-methyl-α-glucuronosyl residues or one α-arabinofuranosyl residue depending on the nature of the source. (Saha 2004; Burton et al. 2010.) In the hardwood xylan, the homopolymeric backbone has substituents of 4-O-methyl-D-glucuronic acid or glucuronic acid. The substituents are linked to 10% of the xyloses in xylan backbone through α (1→2) bonds. Additionally 60% to 70% xyloses in the xylan of hardword are esterified by acetic acid. The chemical structure of a typical hardwood xylan is presented in Figure 7. In contrast to hardwood xylan, soft wood xylans are non-acetylated or rarely acetylated. Softwood xylans have side chains of uronic acids and one L-arabinofuranose attached to the back-bone through α (1→2) and/or α (1→3) glycosidic linkages. The xylans from graminaceous plants have α-arabinofuranosyl substituents esterified with ferulic acid and ρ-coumaric acid. (Jeffries 1994; Shallom & Shoham 2003; Burton et al. 2010.)


Xylan backbone of 1→4 linked β -D-xylopyranose units
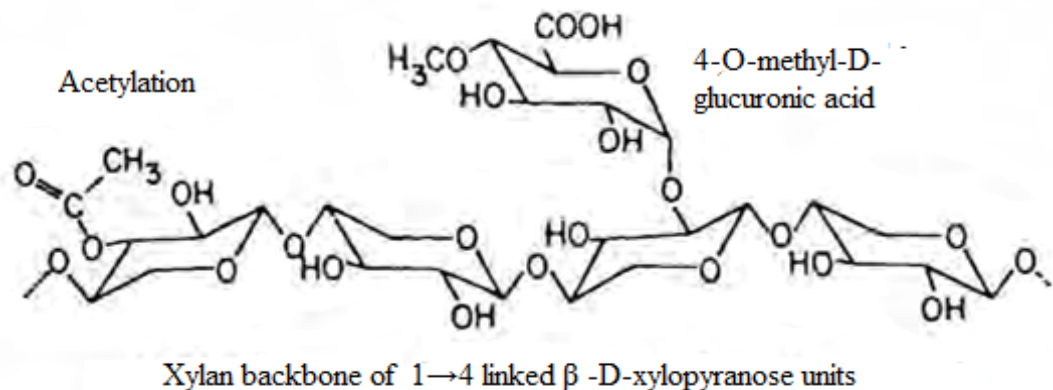
Figure 7: Chemical structure of hardwood xylan. The O-acetyl-4-O-methylglucuronoxylan has one acetylated xylose residue and one xylose residue linked to 4-O-methyl-D-glucuronic acid. (Modified from Kirk 1983.)

Glucomannans are composed of randomly β (1→4) linked mannose and glucose residues. Softwood also contains galactoglucomannans. Galactoglucomannans have similar backbone of β (1→4) linked mannose and glucose residues but there are additional galactose residues linked to backbone through α (1→6) bonds. These α (1→6) bonds of

galactoglucomannans are cleaved during alkaline extractions. Moreover the O-2 and O-3 of the mannose in galactoglucomannans can be found acetylated (Jeffries 1994; Shallom & Shoham 2003.) The structure of a typical galactoglucomannan is presented in Figure 8.

Arabinan and arabinogalactans are sometimes classified as hemicelluloses but their origin is either hairy parts of galacturonan or glycoproteins of cell wall. Arabinan has a backbone comprising of α-1,5-bonded L-arabinofuranosyl units and further linked to side chains of α-1,2- and a α-1,3-linked L-arabinofuranosides. The arabinogalactans have backbone of β-1,3-linked galactoses and substitutions of β -1,6-linked galactoses and α-1,3-linked L-arabinofuranosyl or arabinans sidechains. (Shallom & Shoham 2003.)
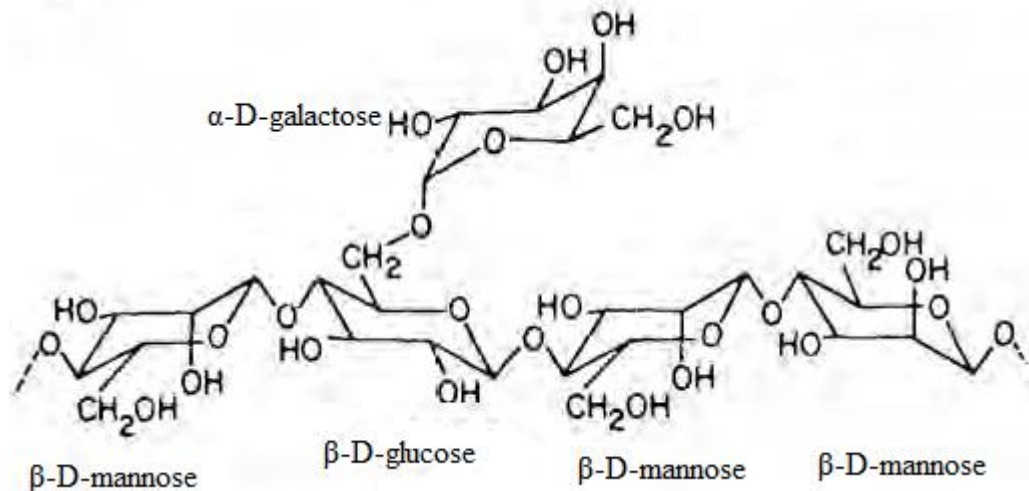

Figure 8: *Chemical structure of galactoglucomannan.The backbone consist of β (1→4) bonded mannose and glucose residues while a substituent galactose is attached through α (1→6) bond. (Modified from Kirk 1983.)*

2.2.1.   Hemicellulases

Hemicellulases are either glycoside hydrolases (GHs) or carbohydrate esterases (CEs). GHs hydrolyze glycosidic bonds while CEs hydrolyze ester linkages between sugar residues and acids like acetic acid, ferulic acid or ρ-coumaric acid. The homopolymeric backbone xylan is biodegraded by endo-β-1,4-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37).   The endo-β-1,4-xylanase hydrolyses β (1→4) bonds between xylose residues and convert xylan backbone into shorter xylooligomers. Then β-xylosidase, being a exo-type glycosidases, hydrolyses xylooligomers into xylose monomers. The side chains of xylans are removed by several accessory enzymes. Among the accessory enzyme, α-L-arabinofuranosidases (EC 3.2.1.55) cleaves terminal non-reducing α-arabinofuranose from arabinoxylans. α-D-Glucuronidases (EC 3.2.1.131) remove the side chains of the 4-O-methyl-D-glucuronic acid from the xylan backbone. The ester linkages are removed by hemicellulolytic esterases. Acetyl xylan esterases (EC

3.1.1.72) hydrolyzes acetyl esters from acetyl xylans. Ferulic acid esterase (EC 3.1.1.73) hydrolyzes ester bond between arabinose side chains and ferulic acid while ρ-coumaric acid esterase removes ester bond between arabinose side chains and ρ-coumaric acid. Figure 9 represents action of different hemicellulases on xylan. (Shallom & Shoham 2003; Saha 2003.)
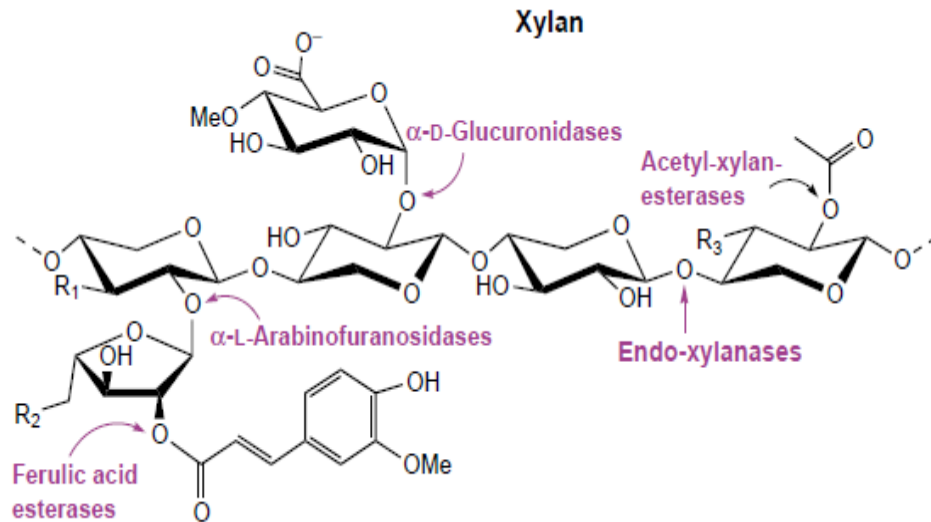
Figure 9: *Xylan degradation by different hemicellulases. The xylan backbone is cleaved by endo-xylanases while side chains of 4-O-methyl-D-glucuronic acid and α-arabinofuranose are removed by α-D-Glucuronidases and α-L-arabinofuranosidases respectively. Esterases remove ester linkages of acetic acid and ferulic acid. (Shallom & Shoham 2003.)*

Mannan-based hemicelluloses are first degraded into β-1,4-manno-oligomers by β-mannanases (EC 3.2.1.78) and then β-mannosidases (EC 3.2.1.25) hydrolyze β-1,4-manno-oligomers further into mannose units. Figure 10 represents hemicellulases responsible for degradation of Galactoglucomannan. For arabinan, α-L-arabinanases (EC 3.2.1.99) is responsible of separating arabinoses. (Shallom & Shoham 2003; Saha 2003.)

Bachmann & McCarthy (1991) reported synergism in the action of endo-xylanase, β-xylosidase, α-arabinofuranosidase and acetylxylan esterase from *Thermomonospora fusca*. Poutanen et al. (1989) also reported synergism between depolymerizing hemicellulases and side-group breaking hemicellulases. (Saha 2004.) Importantly many xylanases cannot hydrolyse the glycosidic bonds linking xylose in xylans. Such xylanases require removal of side chains by other accessory hemicellulases. (Lee& Forsberg 1987.) Similarly many accessory enzymes cannot remove side chains directly from xylans and their action is limited to xylooligosaccharides (Poutanen et al. 1991).
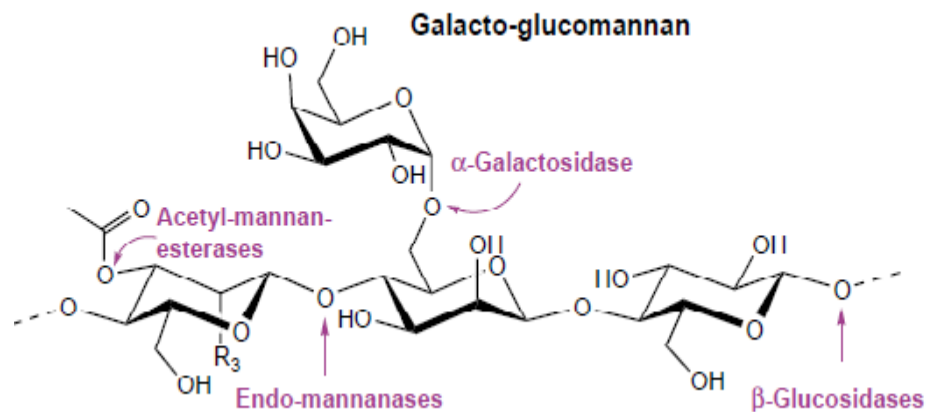
Figure 10: *Galacto-glucomannan degradation by different hemicellulases. The backbone is cleaved by endo-mannanases and β-glucosidase while the side chains of galactose are removed by α-galactosidase. Esters from acetic acids are removed by acetyl-mannan-esterases. (Shallom & Shoham 2003.)*

2.2.2.   Source of Hemicellulases

Several aerobic and anaerobic bacteria and fungi possess or secrete hemicellulases. Some microorganisms like *Penicillium capsulatum* and *Talaromyces emersonii* have been reported to have complete set of hemicellulases for xylan degradation (Filho et al. 1991). Moreover the cellulosomes from anaerobic Clostridia integrate hemicellulolytic enzymes with cellulolytic enzymes (Kosugi et al. 2002).

Xylanases are more common compared to β-xylosidases. Xylanase had already been isolated from terrestrial fungi *Aspergillus batatae, Irpex lacteus, Chaetomium globosum* more than fifty years ago (Dekker & Richards 1976). β-Xylosidases are mostly cell bound and larger than xylanases. β-Xylosidases are found in *Aureobasidium pullulans and Bacillus sterothermophilus.* Mannanases producing microbes include *Aeromonas hydrophila*, *Cellulomonas* sp., *Streptomyces olivochromogenus* and *Polyporous versicolor.* Acetyl xylan esterase had been recovered from many microbes including *Schizophylum commune*, *Aspergillus nige*, *Trichoderma reesei, Rhodotorula mucilaginosa* and *Fibrobacter succinogenes*. (Jeffries 1994.) Ferulic and ρ-coumaric acid esterases were reported to be present in *Streptomyces viridosporus* (Deobald and Crawford 1987). Some fungi secrete hemicellulases in addition to cellulases. Fungus *Neurospora crassa* was reported to secrete both cellulase and xylanase (Deshpande et al. 1986).

The Table 2 and Table 3 represent the occurrences of hemicellulases genes from some of the representative organisms and the provided data in the tables was originally acquired from Carbohydrate-Active Enzymes database. The online server of Carbohy-

drate-Active Enzymes database provides a comprehensive and updated classification of carbohydrate active enzymes (Carbohydrate-Active enZYmes Database 2014).

Hemicellulases are also present in rumen organism. The anaerobic cellulolytic bacteria *Ruminococcus flavefaciens* also degrades hemicelluloses (Dehority 1967). Interestingly *Butyrivibrio fibrisolvens* produce a bifunctional hemicelluase which possess α-L-arabinofuranosidase with the activity of β-xylosidase (Utt et al. 1991). Among rumen ciliate protozoa, protozoa from cellulolytic entodiniomorphid genera (*Polyplastron, Diploplastron, Eremoplastron, Epidinium, Ophryoscolex and Eudiplodiniumhave*) were reported to have higher hemicellulolytic activities (Williams & Coleman 1985). Hemicellulolytic activity is also present in bacteria and yeast of termite gut (Schäfer et al. 1996).

Table 2: *The occurrence of hemicellulase genes in some representative microbe. (Modified from Shallom & Shoham 2003).*

Microorganisms	Xylanase	Xylosidase	Arabinofuranosidase/ arabinanase
Bacillus subtilis 168	2	1	4
Clostridium acetobutylicum ATCC 824	3	4	2
Thermotoga maritima	2	–	1
Xanthomonas axonopodis pv. Citri str. 306	3	7	1
Cellvibrio japonicus	6	–	3
Geobacillus stearothermophilus T-6	2	2	2
Aspergillus niger	4	1	5
Trichoderma reesei	3	1	1

Table 3: *The occurrence of hemicellulase genes in some representative microbes (Modified from Shallom & Shoham 2003).*

Microorganisms	α-Glucuronidase	Mannanase	Mannosidase	Acetyl xylan esterase
Bacillus subtilis 168	–	–	–	–
Clostridium acetobutylicum ATCC 824	–	4	–	1
Thermotoga maritima	1	1	1	–
Xanthomonas axonopodis pv. Citri str. 306	1	–	–	–
Cellvibrio japonicus	1	1	–	–
Geobacillus stearothermophilus T-6	1	–	–	1
Aspergillus niger	–	–	1	1
Trichoderma reesei	1	1	–	1

2.2.3.   Commercially Attractive Bioconversions of Hemicelluloses

Hemicellulose fraction (liquid fraction) collected after pre-treatment of lignocellulosic biomass can be fermented into ethanol or converted into other valuable chemicals. Xylose in the liquid fraction of wood hydrolyzates can also be converted into xylitol by mean of chemical reductions. Xylitol is commonly used as a natural sweetener in food industry and it is also consumed by diabetics as an alternative of sweeteners. (Saha 2004.)

Corn fiber acquired as byproduct in corn wet milling facility contains about 35% hemicelluloses (Saha et al. 1998). Corn fiber xylan has high recalcitrance and digestion of the xylan cannot completed by commercially available hemicellulases. A dilute acid sulphuric acid pretreatment at 120°C with subsequent enzymatic saccharification has recovered 85-100% fermentable sugars from corn fiber. (Saha & Bothast 1999.) The xylan from corn fiber was utilized by two newly discovered fungal cultures *(Fusarium proliferatum* NRRL 26517, *F. verticillioides* NRRL Y-26518). The isolated endo-xylanases from those fungal cultures were not able to degrade corn fiber xylan and accessory enzymes were needed. (Saha 2004.)

The thermophilic fungi *Thermomyces lanuginosus* produce cellulase-free thermostable xylanase and that make the fungi an ideal source of xylanase at commercial scale (Singh 2003). Tsujibo et al. (1990) isolated three alkaline active endo-xylanases from culture of *Nocardiopsis dassonvillei* subsp. *alba* OPC-18. The xylanases active at alkaline pH have industrial applications such as enzymatic treatments of kraft pulps.

Many thermostable and commercially attractive hemicellulases have been discovered. Thermophilic actinomycete *Thermonospora* secrete a thermostable xylanase which was stable at 55 °C for 1 month or up to 80 °C for 10 minutes (Ristroph and Humphrey 1985). Another thermostable xylanase from a thermophilic bacillus was described by Grüninger and Fiechter (1986). The xylanase optimum activity was observed at 78°C and half life was recorded as 15 hours at 75°C. Themostable xylanases have been also reported from *Thermoascus aurantiacus* and *Talaromyces emersonii.* (Tan et al. 1987; Tuohy and Coughlan 1992). The fungus *Aureobasidium pullulans* secretes highly thermostable α-L-arabinofuranosidase which acts on arabinan and arabinoxylans. The novel α-L-arabinofuranosidase cleaves arabinan, removes branches from arabinan and releases arabinose from different arabinoxylans. (Saha & Bothast 1998.)

Co-culture of cellulolytic and hemicellulolytic organisms can improve degradation of cellulosic biomass. The degradation of orchard grass by cellulolytic *Bacteroides ruminicola* was improved slightly by adding *Fibrobacter succinogenes. F. succinogenes* is both cellulolytic and hemicellulolytic in nature but cannot utilize the degraded hemicelluloses. (Osborne & Dehority 1989.) Previously combination of hemicellulolytic *Bacteroides ruminicol* with different cellulolytic organisms was reported to significantly increase the cellulose consumption (Dehority & Scott. 1967).

Hemicellulose has been converted into fatty esters, fatty alcohols and waxes by an engineered *Escherichia coli*. Steen et al. (2010) integrated innate fatty acid synthetic ability of *E. coli* with new pathways. Through this synthetic biology approach, fatty acid metabolism was directed towards biodiesel production. Biodiesel is composed of fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs). Fatty acids derived from plant and animal oils are converted into biodiesel and other valuable chemicals like surfactants, solvents and lubricants. Chemically biodiesel is prepared by transesterification of oils while *E. coli* has also been engineered to utilize its endogenous ethanol to esterify the added exogenous fatty acids into biodiesel. Today diesel is

in high demand and its market growth rate is three times higher than that of gasoline. The renewable biodiesel has a consumption of two billion gallons per year and due to the increasing demand of biodiesel, the prices of food oils may raise. Therefore, there is a need to produce fatty acids from abundant and cost-effective renewable raw material like lignocelluose. Steen et al. (2010) engineered biodiesel generating *E. coli* cells which were fed on simple sugars. The strains were further engineered to express hemicellulases and hence biodiesel was produced from hemicelluloses fraction.

Steen and colleague scientists introduced cytoplasmic thioesterase in the host cell to hydrolyze acyl-ACPs (acyl carrier protein). Fatty acyl-ACP is the product of fatty acid synthesis in microbes and its accumulation inhibits biosynthesis of fatty acids. So a native thioesterase (*'tesA*, a leaderless version) was expressed in *E. coli* to increase production of fatty acids. Expression of TesA resulted in a yield of around 0.32 gram fatty acids per liter which was similar to previous studies. Steen et al. (2010) improved the biodiesel yield from host cell by expression of wax-ester synthase (*atfA*) gene, overexpression of first enzyme of β-oxidation, *fadD* and removal of second enzyme of β-oxidation, *fadE*. The engineered *E. coli* synthesized FAEEs by utilizing exogenous glucose and ethanol. A yield of 0.4 gram per liter was obtained in 48 hours for FAEEs with carbon ranging from C12 to C18. In addition to fatty esters, fatty alcohols are potential candidates of biofuels. Fatty alcohols are chemically synthesized by hydrogenation plant source FAMEs or from petrochemical precursors. Some microbes also possess fatty acyl-CoA reductases for synthesis of fatty alcohol. A fatty acyl-CoA reductase from *Acinetobacter calcoaceticus* BD413, *acr1*, was also introduced in the place of *atfA* in the FAEE synthesizing strain. The strain with *acr1* synthesized around 60 mg per liter of medium chain fatty alcohol.

To simplify and decrease cost of the process of microbial biodiesel production, Steen et al. engineered the FAEE producing strain to endogenously produce ethanol. Pyruvate decarboxylase gene, *pdc* and alcohol dehydrogenase gene, *adhB* from *Zymomonas mobilis* were added to the FAEE producing strain. Addition of *pdc* and *adhB* decrease the yield to 37 mg of FAEE per liter. The yield of FAEE was raised by overexpression of an acyl-CoA ligase (FAA2) from *Saccharomyces cerevisiae* and an acyl-CoA ligase from *E. coli* A1A. Additionally the yield of FAEE was further increased up to 427 mg per liter by expression of an extra copy of *atfA*. Steen et al. also overlaid a dodecane layer to reduce FAAE evaporation and this way the yield of 427 rose to 674 mg per liter. Hence the synthetic biology approach of joining synthesis of FAEE and ethanol resulted in a strain which can competently synthesize FAEE from glucose as sole feed. To utilize hemicelluloses content of lignocelluloses, Steen group introduced two hemicellulases in the FAEE producing strain. For the expressing hemicellulases, genes coding endoxylanase catalytic domain (Xyn10B) and xylanase (Xsa) from *Clostridium stercorarium* and *Bacteroides ovatus* respectively were employed. The hemicellulases were fused with native OsmY22 protein of *E. coli*. A schematic diagram which includes all the synthetic biology applied to build a biodiesel producing strain is presented in Figure 11. The biodiesel producing strain added with

hemicellulases synthesized 3.5 mg per liter FAEE with 0.2% glucose as sole carbon source. The strain produced three times higher yield of FAEE (11.6 mg per liter) when two percent xylan was added in the medium. The strain utilized both glucose and xylan without addition of any exogenous enzymes. So in future, expression of cellulases from this strain can enable co-utilization of cellulose and hemicelluloses.

As a summary, Steen's group applied synthetic biology approach and joined hydrolysis of fatty acyl-ACP with the reactivation of the fatty acid carboxylate group. The thioesterase facilitated overproduction of fatty acids while fatty acyl-CoA-synthase diverted fatty acid metabolism towards fatty acyl-CoA. Fatty acyl-CoA is the substrate required for synthesis of esters and alcohols. In addition to combining two pathways, the strain was provided capability to utilize hemicelluloses which is a promising success towards consolidated bioprocessing. Interestingly the yields of products are also competent to bring the engineered strain at commercial scales to produce cheaper biofuel molecules and other valuable chemicals.
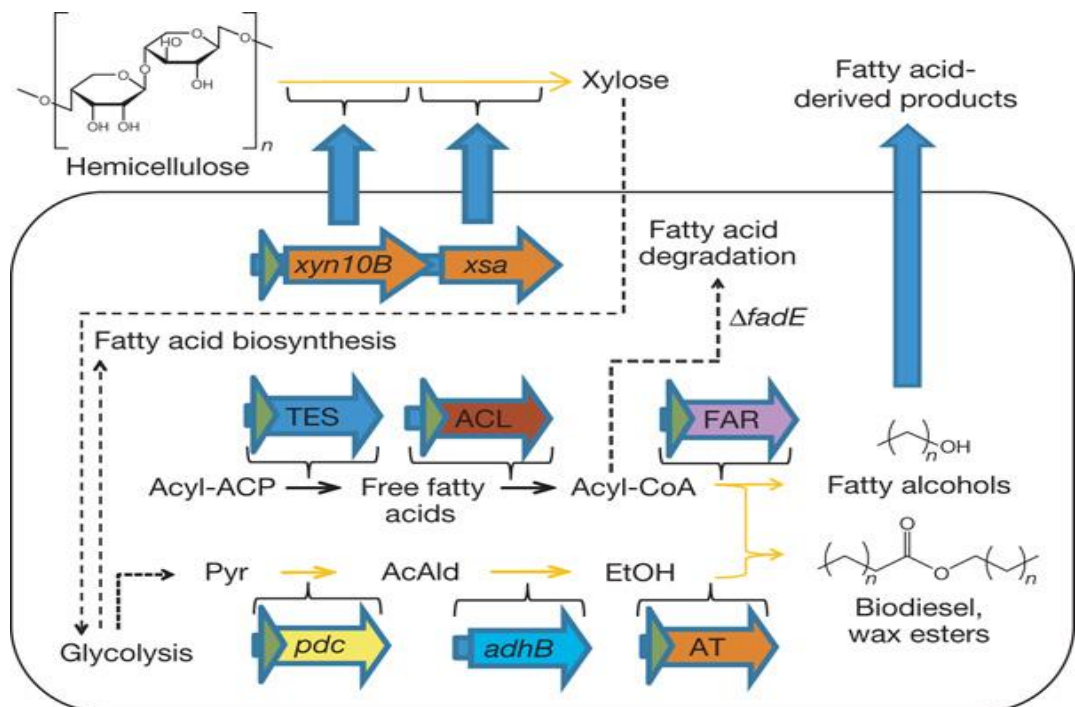
Figure 11: *Schematic representation of the engineering provided to E. coli for synthesizing fatty acid-derived compounds from hemicelluloses or glucose. Production of fatty acids and acyl-CoAs was raised by removing fadE and over expressing thioesterases (TES) and acyl-CoA ligases (ACL). Fatty acyl-CoA reductases (FAR) was over expressed to convert fatty acyl-CoAs into fatty alcohols while acyltransferase (AT) expression synthesized fatty esters. For ethanol synthesis, the genes pdc and adhB were added to the host strain. Finally hemicellulases encoding genes (xyn10B and xsa) were introduced for consumption of xylan. The orange lines highlight the non-native pathways while green triangles symbolize the lacUV5 promoter. The abbreviation of AcAld, EtOH and pyr stand for acetaldehyde, ethanol and pyruvate respectively.(Steen et al. 2010.)*

The biodiesel synthesizing strain of Steen was not stable due to the imbalances and toxicity of heterologous gene products. Metabolic imbalances in the synthetic pathways limit product yield. Metabolic imbalance is generated by high expression or low expression of gene(s) in a pathway. Low expressions become bottlenecks while high expressions result in wastage or burden of intermediates in pathways. To improve stability and the yield of the biodiesel synthesizing strain, Zhang et al. (2012) developed a dynamic sensor-regulator system (DSRS). For developing DSRS, fatty acid–sensing protein FadR and its associated regulator were selected as biosensor for fatty acyl-CoA. Fatty acyl-CoA is the key intermediate for biosynthesis, degradation and transport of fatty acids. FadR is a transcription factor which binds FadR-recognition DNA sequences and regulate expression of genes involved in fatty acid metabolism. FadR is a ligand-responsive transcription factor and its binding with DNA is antagonized mainly by fatty acyl-CoAs. Before constructing DSRS, Zhang et al. engineered two FA/acyl-CoA–regulated promoters with higher dynamic ranges. The promoters were based on lambda promoter and phage T7 promoter and these synthetic promoters were containing FadR-binding DNA sequence. The promoters coupled with red fluorescence protein (*rfp*) were tested for their functionality. Both promoters detected exogenously added fatty acids and endogenous synthesized fatty acids (fatty acid synthesizing strain). The design of FA/acyl-CoA–regulated promoter/ biosensor has been shown in Figure 12.
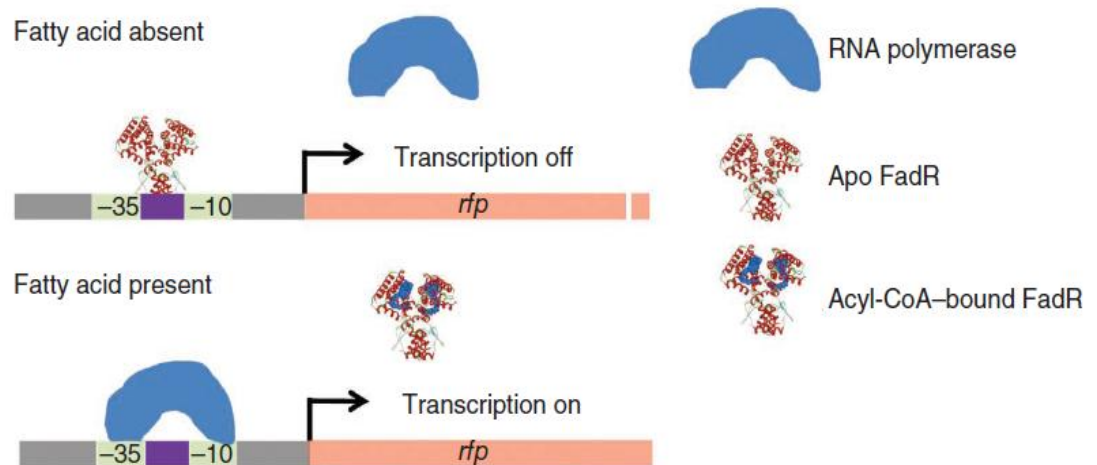
Figure 12: *Coupling expression of red fluorescence protein for testing FA/acyl-CoA–regulated promoter. In this biosensor, presence of fatty acid results in expression of rfp. Fatty acids are activated into acyl-CoAs and the acyl-CoAs bind to FadR. This binding in turn releases the promoter bound FadR and expression of rfp is initiated. In the absence of fatty acids, FadR binds to the synthetic promoter and inhibit polymerase to transcribe rfp. (Zhang et al. 2012.)*

After engineering promoters (sensors), the next task was about reducing the leaky expression of those FA/acyl-CoA–regulated promoters. For this purpose hybrid promoters, containing both FadR and LacI DNA recognition DNA sequences, were engineered. These hybrid promoters were able to sense both IPTG and fatty acyl-CoA. Zhang et al. defined the biodiesel synthesizing strain of Steen comprising of three modules. Module A produces fatty acids through native fatty acid synthesis pathway and heterologous thioesterase, TesA. Module B synthesizes ethanol while the final module, module C, synthesizes FAEEs from ethanol and fatty acids. Module C comprises of acyl-CoA synthase and wax-ester synthase.

In DSRS, biosensor-derived promoters were cloned upstream of modules B and C and hence modules B and C were regulated in relation to availability of fatty acid. In absence of fatty acid, synthesis of ethanol and acyl-CoA were repressed by FadR. In contrast excess of intracellular fatty acid resulted in surplus acyl-CoA and hence acyl-CoA hindered binding of FadR to those biosensor-derived promoters in module B and C. This way the free promoters were occupied by polymerases and more ethanol and fatty acyl-CoA were synthesized. Moreover higher expression of wax-ester synthase in module C resulted in higher yield of FAEE. The detailed functioning of DSRS is presented in Figure 13. The DSRS dynamically balanced the enzymes and improved the yield of FAEE three times compared to the strain of Steen. The biodiesel synthesizing strain provided with DSRS produced 1.5 g/l FAEE after three days of incubation.

Interestingly both Steen group and Zhang group were led by Keasling. Keasling is associated with LS9 INC (Life Sustain 9 Billion INC). LS9 is a biotechnology startup company which is applying synthetic biology for developing renewable biofuels. The company has gathered funding of 20 million dollars for its initial research and have sites in California, Florida, and southern Brazil. As described earlier, the company has developed microbes which can convert sugars into biodiesel. Moreover, as indicated by name, the LS9 plans to develop a sustainable fuel to fulfil demands of 9 billion humans residing on earth. (BIOFUEL 2014.)
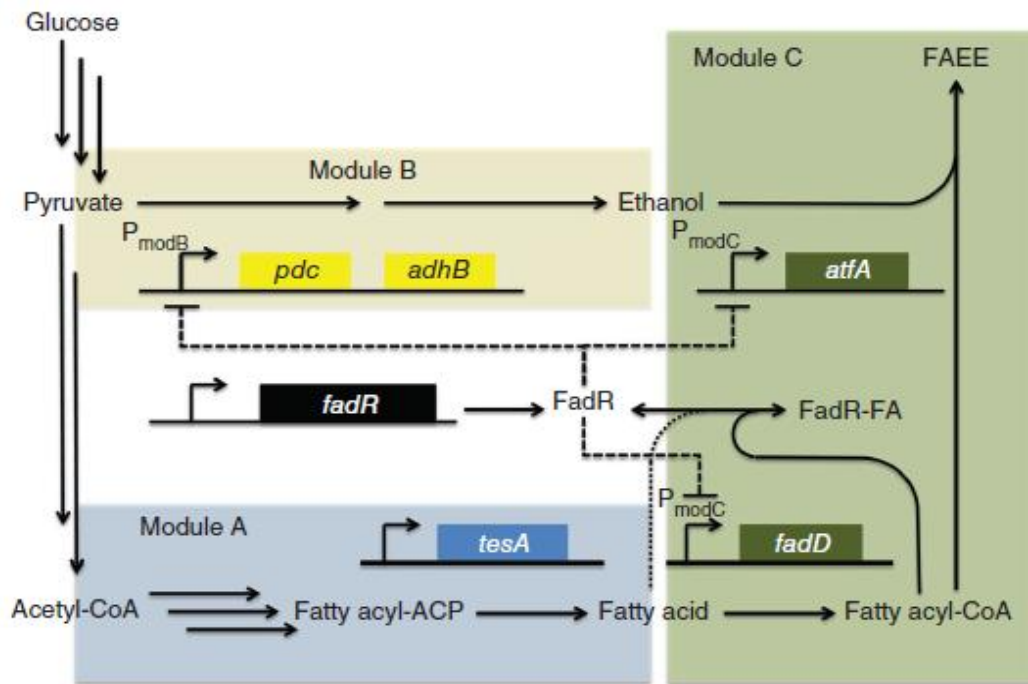
Figure 13: *DSRS regulating synthesis of FAEE. Module A is synthesizing fatty acids from native fatty acid synthase and the heterologous thioesterase gene (tesA) while module B is producing ethanol from the genes of pyruvate decarboxylase (pdc) and alcohol dehydrogenase (adhB). Module C synthesizes FAEE from the genes of acyl-CoA synthase (fadD) and wax-ester synthase (atfA). PmodB and PmodC represent the FadR-regulated promoters and the repressor gene fadR. When fatty acid concentration is low, FadR limit PmodB and PmodC. This results in limited synthesis of ethanol, acyl-CoA and FAEE from module B and C. In contrast when fatty acid concentration is high, acyl-CoAs binds FadR and release the bound FadR from FadR-regulated promoters. This result in simultaneously and enhanced synthesis of ethanol, acyl-CoA and FAEE. (Zhang et al. 2012.)*

2.3. Biodegradation of Lignin

Lignin is amorphous, insoluble in water and optically inactive. Lignin, like hemicellulose, also exhibits polydispersity. Lignin obtained from gymnosperm contains more than 90% coniferyl alcohol and rest mainly comprise of p-coumaryl alcohol units. In contrast to gymnosperm, lignin extracted from angiosperm contains coniferyl and sinapyl alcohol units with varying ratios. (Harmsen et al. 2010.) Lignin content of gymnosperm (softwood) is higher than lignin content of angiosperm (hardwood) (Martínez et al. 2010). In grasses, lignin is composed of all three lignin subunits; *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Jeffries 1994). The chemical structure of these subunits is presented in Figure 14.
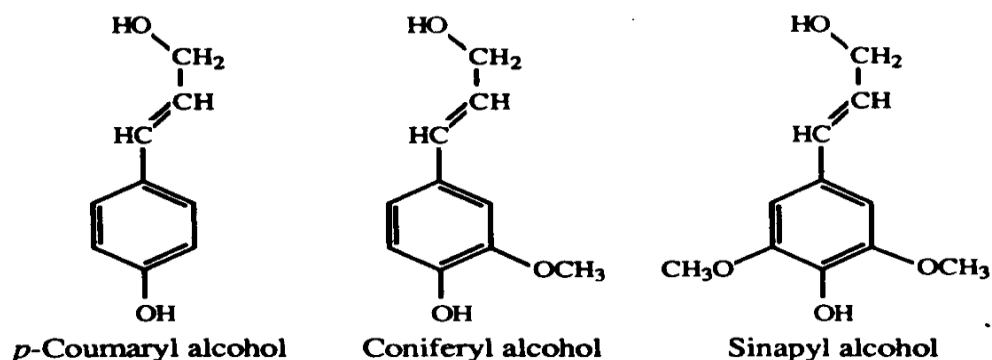
Figure 14: *The chemical structures of lignin subunits. The subunits include p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. (Jeffries 1994.)*

β-Aryl ether is the most abundant lignin substructure in softwoods and around 40% interphenylpropane bonds are formed by β-aryl ether (Higuchi 1985). β-Aryl ether has a free benzyl hydroxyl and a free phenolic hydroxyl in lignin structure and these free hydroxyl groups are thought to be responsible for lignin-carbohydrate bonds (Jeffries 1994). In lignin, the phenolic benzyl ether bonds hydrolyses in alkaline environment while nonphenolic benzyl ether bonds are alkali stable (Košíková et al. 1979). The structure of β-aryl ether is presented in Figure 15.

Guiacylglycerol-β-aryl ether
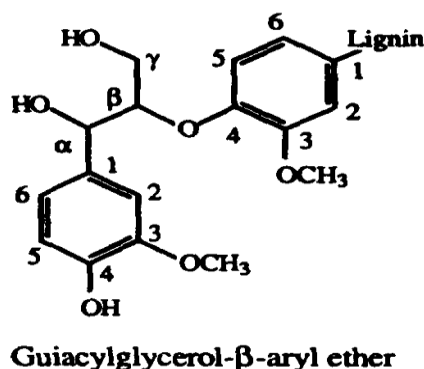
Figure 15: *The model of β-aryl ether presented by guaiacylglycerol-β-guaiacylether dimer. In the structure of lignin, β-aryl ether has one free benzyl hydroxyl group at α position and one free phenolic hydroxyl while the other hydroxyl groups are esterified. (Jeffries 1994.)*

The ligninolytic microorganisms undergo different environments while infiltrating softwood and hardwood. Softwood has tracheids and the cell wall completely covers cell lumen. So the ligninolytic microorganism crosses the the cell wall or pit membrane. Hardwood on the other hand contains vessels, fibers, parenchyma cells and some tracheids. The vessels comprised of open ended vessel constituents and hence fungal hyphae or bacteria can invade without crossing cell wall or pit membrane. In particular the wood decay has distinct types depending on the chemistry and morphology of the decay. Fungal decay is divided into three types; decay from white, soft, or brown rot. Similarly bacterial decay is classified as erosion, tunneling, and cavity formation. (Hatakka 2005.)

2.3.1.   Ligninolytic Enzymes

Lignin is a stereoirregular compound which requires oxidative enzymes rather than hydrolyzing enzymes. There are three major families of extracellular fungal enzymes responsible for lignin degradation; lignin peroxidases (LiPs, "ligninases", EC 1.11.1.14), manganese peroxidases (MnPs, "Mn-dependent peroxidases", EC 1.11.1.13) and laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2). Except of these three families, there are some accessory enzymes required for hydrogen peroxide production. The accessory enzymes include glyoxal oxidase (GLOX) and aryl alcohol oxidase (AAO) (EC 1.1.3.7). (Hatakka 2005.)

The first lignin peroxidase was discovered from *Phanerochaete chrysosporium* as an extracellular enzyme and the enzyme was depolymerizing methylated spruce lignin. The LiPs were found capable of degrading several lignin model compounds including the *β-aryl ether*. (Tien & Kirk 1983; Glenn et al. 1983.) After *Phanerochaete chrysosporium*, LiPs were also found from *Phlebia radiata*, *Trametes versicolor and* other white-rot fungi. LiPs have also been reported in ascomycete *Chrysonilia sitophila* (Ferrer et al. 1992). LiPs acquire single electron from the phenolic and nonphenolic rings in lignin and leave cationic phenoxy radicals. This results in partial depolymerization of methylated lignins. (Jeffries 1994.)

MnPs and LiPs are both extracellular and glycosylated haem proteins. The first MnP was also discovered from *P. chrysosporium* in 1985. Later MnPs were also reported from *P. radiata, P. tremellosa*, *T. versicolor* and some other white-rot organisms. MnPs convert Mn(II) into Mn(III) and hence Mn(III) oxidizes phenols into phenoxy radicals. (Jeffries 1994.)

P. chrysosporium do not produce laccase but laccases have been found in many white-rot fungi including *Phlebia* and *Trametes* spp. Laccases are non haem extracellular proteins. Laccases is a family of oxidases which contain four copper ions and these oxidases are also known as blue copper oxidases. Laccases oxidizes the phenolic subunits of lignin by one electron reaction into phenoxy radicals. (Jeffries 1994.)

2.3.2. Sources of Ligninolytic Enzymes

Wood-rotting basidiomycetous is classified into groups; white-rot and brown-rot fungi. Both white-rot and brown-rot fungi both degrade lignin by radicals, low pH and organic acid (Kirk 1975; Jin et al. 1990). White-rot fungi are more prevalent in hardwood compared to softwood (Gilbertson, 1980). Burlat et al. (1998) have reported white-rot fungi to target different location of plant tissue for lignin removal. *Ceriporiopsis subvermispora* and *Pleurotus eryngii* partially target middle lamella while *Phlebia radiata* target secondary cell walls of straw. Brown-rot fungi mainly degrade cellulose and hemicelluloses while lignin is also modified slightly (Eriksson et al. 1990). Among Brown-rot fungi, *Gloeophyllum trabeum* has been studied extensively (Hatakka 2005.)

Lignin biodegradation is most efficient with basidiomycetous white-rot fungi and related litter-decomposing fungi but other microbes also degrade the lignin present in soil and compost (Tuomela et al., 2000). The bacteria and fungi in the rumen also degrade lignin to some extent (Akin & Benner 1988).

Several bacteria have been reported capable of degrading lignin. *Streptomyces viridosporus* T7A was reported to secrete lignin peroxidase (Ramachandra et al. 1988) and soil bacteria *Nocardia* and *Rhodococcus* (Zimmermann 1990) were reported to degrade lignin in ^{14}C-labelled lignin assay. Ahmad et al. (2010) reported strains of *Pseudomonas putida* mt-2 and *Rhodococcus jostii* RHA1 to degrade lignin. Ahmad et al. designed two new lignin degradation assays for reporting the lignin degradation of the starins; fluorescently labeled lignin based assay and UV-visible assay employing chemically nitrated lignin. Among bacteria, *S. viridosporus* T7A secrete many peroxidases, several actinomycetes also produce extracellular peroxidases and bacterial laccases have also been reported. Lignin degradation capabilities of these bacteria were found much lesser compared to the white-rot fungus *Phanerochaete chrysosporium* but close to some other lignin digesting fungi. (Bugg et al. 2011.)

The lignin degradation capabilities of microbes residing the termite gut is unclear yet and conflicting reports have been published. Many researchers have confirmed the aromatic degradation capability of the bacteria residing termite gut. Chung et al. (1994) reported a gram positive bacterium, *Rhodococcus erythropolis* strain TA421, from termite gut which was possessing capability to degrade polychlorinated biphenyl. Moreover a metagenomic study has searched for genes of lignin degradation in microflora of the hindgut of a higher termite. The hindgut of termite *Nasutitermes* contains microbe with cellulose degradation genes but genes for lignin degradation could not be found. (Warnecke et al. 2007.) Although lignin degradation by termite gut flora is unclear but the extensive compartmentalization and extreme alkaline environment (pH even more than 12) in the gut of soil-feeding termites assist oxidation and solubilization of organic matter. (Ohkuma 2003.)

2.3.3. Role of Carbohydrates in Lignin Biodegradation

Wood-rotting fungi need supplementary carbohydrates for lignin degradation. Kirk et al. (1976) showed that lignin-decomposing fungi *Phanerochaete chrysosporium* and *Coriolus versicolor* could not grow on lignin as sole carbon source and additional growth substrates like cellulose or glucose were required. Moreover Jeffries et al. (1981) observed carbohydrates regulating the ligninolytic activity of *Phanerochaete chrysosporium*. In the carbohydrate-limited cultures of Jeffries, ligninolytic activity emerged when the provided carbohydrate was depleted. The quantity of carbohydrate supplied was directly influencing the degree of lignin degradation by *Phanerochaete chrysosporium*. Carbohydrate depletion was also associated with a reduction in mycelial dry weight.

Except carbohydrates as supplementary growth substrates, glycosides might also contribute in lignin biodegradation. Glycosides were first observed by Kondo and lmamura (1987) in media growing wood-rotting fungi. The media were vanillyl alcohol or veratryl alcohol (model lignin compounds) supplemented glucose or cellobiose containing media. Involvement of glycosides in the depolymerization of lignin had been suggested in some experiments. Kondo et al. (1990) employed two synthetic lignin polymers; dehydrogenative polymers (DHP) and DHP-glucoside. Both lignins were treated with lignin-oxidizing enzymes. The enzymes included horseradish peroxidase, laccase and lignin peroxidase. Upon enzyme treatment, DHP-glucoside lignin depolymerised while DHP became more polymerized. Kondo et al. (1990) also reported reduced repolymerization of lignin during action of laccase III from *Coriolus Versicolor.* Additionally veratryl alcohol and vanilyl alcohol were found harmful for *Coriolus versicolor* and *Tephrocybe palustris* while glycosides of veratryl alcohol and vanilyl alcohol were lesser toxic to the growth of *C. versicolor* and *T. palustris* growth *(*Kondo & Imamura 1989a.)

Polysaccharides were found better carbon source for lignin degradation compared to monosaccharides. Kondo and Imamura (1989b) degraded three lignin model compounds by *P. chrysosporium* and *Coriolus versicolor.*The model compounds were 4-*O*-ethylsyringylglycerol-b-syringyl ether, veratryl alcohol and veratraldehyde. Kondo and Imamura used media containing monosaccharides and polysaccharides as carbon source and observed faster lignin consumption in the media containing polysaccharides.

2.3.4. Commercially Attractive Bioconversions of Lignin

Among polymers from plant cell wall, lignin is the most recalcitrant polymer. Lignin cannot be degraded completely by the enzymes obtained from wood-rotting fungi. Evans and Palmer (1983) collected extracellular proteins from the wood chip culture of *Coriolus versicolor* and observed the effect of the extracellular proteins from *Coriolus versicolor on* isolated milled wood lignin. Evans and Palmer observed small lessening in molecular weight of the lignin molecules and increase in hydroxyl groups of the lig-

nin polymer. Similar results were found with extracellular proteins from *Phanerochaete chrysosporium* and *Phlebia radiate* in other experiments. So lignin degradation was found limited when using cell free enzyme system. (Jeffries 1994).

Conventional conversion of lignocellulose into fermentable sugars produces lignin as by-product. The lignin produced can be burn for heat or electricity generation. Lignin is also used in synthesis of other chemicals. (Saha 2004.)

White-rot fungi and their enzymes have many industrial applications. They are used for biomechanical pulping and pulp bleaching in pulp and paper industry. The biopulping process urges for selective lignin degraders (Eriksson et al. 1990) but unfortunately removal of lignin from wood is not completely selective and some concomitant removal of carbohydrates is always there (Jeffries 1994). Co-culturing two white rot fungi can enhance lignin biodegradation depending on the species of fungi and hence biopulping could be improved by co-cultivations. Chi et al (2007) has showed that co-cultivation of *Ceriporiopsis subvermispora* and *Pleurotus ostreatus* considerably enhanced wood decay compared to individual cultures of *C. subvermispora* and *P. ostreatus*.

Brown-rot fungi have been tested for its biotechnological applications. Agosin et al. (1989) performed solid-state fermentation to convert radiata pine *(Pinus radiata)* into cattle feed. The brown-rot fungi employed for fermentation were *Gloeophyllum trabeum, Lentinus lepideus*, and *Wolfiporia cocos*. Agosin et al. reported improvement in dry matter digestibility. Lignin has also been tested as wood adhesive for many times. Brown rotted lignin is rich in phenolic hydroxyl content and Jin et al. (1991) proposed brownrotted lignin as replacement of phenol in phenol-formaldehyde flakeboard resin. On the negative side, brown-rot fungi also infect and destroy woods in buildings. *Serpula lacrymans, Coniophora puteana, Meruliporia incrassata* and *Gloeophyllum trabeum* cause destruction of wood (Blanchette 1995).

2.4. Efforts for Developing a Multipurpose Yeast Biocatalyst

Conversion of lignocelluloses into bioethanol starts with initial degradation of lignocellulose by a physical or chemical pretreatment. The pretreatment of lignocellulose result in liquid and solid fraction. The liquid fraction contains pentoses and inhibitors while the solid fraction comprises of cellulose and lignin. Cellulose is hydrolyzed into fermentable sugars by cellulases and then sugars are fermented into ethanol. The liquid fraction is separated as the pentose fermenting organisms are slower in fermentation compared to hexose fermenting organism. Fermentation of pentoses and glucose can be combined (co-fermentation) if the hexose fermenting organism develops ability to utilize pentose efficiently. Hydrolysis and fermentation can also be combined (SSF, simultaneous saccharification and fermentation) but excessive sugars can also affect fermenting organism. The whole process can also be combined if an organism could be engineered which produces cellulases and co-ferment pentoses and hexoses. Such a process will be a consolidated bioprocessing. Figure 16 represents a schematic diagram of different possibilities of the process. (Cardona & Sa´nchez 2006.)

Consolidated bioprocessing is supposed to be cost effective but it needs development of an ideal whole-cell biocatalyst. The concept of whole-cell biocatalyst has a potential to reduce cost of bioethanol production as cellobiose and glucose, the inhibitors of cellulase and β-glucosidase, are immediately consumed after their production. Secondly the sterilization requirements are lesser and thirdly, only one reactor is needed for the whole process of cellulosic ethanol production. (Fujita et al. 2003.)

Nowadays some researchers focus to develop whole-cell biocatalyst by engineering yeast or bacteria. *Saccharomyces cerevisiae* being an outstanding fermentor has been engineered by most researchers. The engineerings have focused either developing ability in *Saccharomyces cerevisiae* to degrade cellulose (for SSF process) or improving pentose utilization of *Saccharomyces cerevisiae* (for co-fermentation).
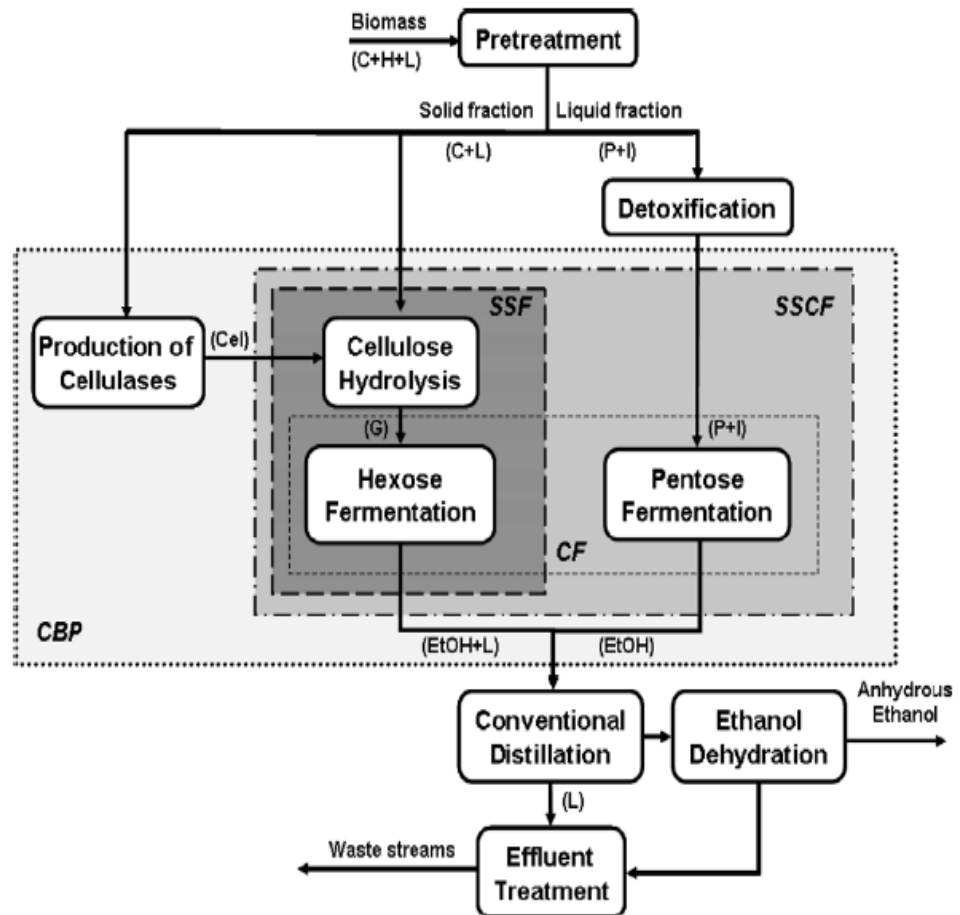
Figure 16*: Schematic diagram of the integration possibilities for the process of conversion of lignocellulose into ethanol. CF means co-fermentation, SSF is simultaneous saccharification and fermentation, SSCF represents simultaneous saccharification and co-fermentation and CBP means consolidated bioprocessing. The components of the process are also abbreviated; C for cellulose, H for hemicelluloses, L for lignin, Cel for cellulases, G for glucose, P for pentoses, I for inhibitors and EtOH for ethanol. (Cardona & Sa´nchez 2006.)*

2.4.1.   Biocatalyst for SSF Process

Fujita et al. (2003) were the first to co-display three kinds of cellulolytic enzymes on *Saccharomyces cerevisiae* surface. The three enzymes include β-glucosidase I from *Aspergillus aculeatus* and endoglucanase II, and cellobiohydrolase II from *Trichoderma reesei*. The engineered *Saccharomyces cerevisiae* was co-transformed with three expression plasmids (BG211, pEG23u31H6, and pFCBH2w3) and found to degrade amorphous phosphoric acid-swollen cellulose synergistically and sequentially. Ethanol yield of 0.45 in term of 'grams of ethanol produced per gram of sugar consumed' was obtained after 40 hours.

Tsai *et al.* (2009) brought concept of displaying miniscaffoldin (Scaf-ctf) on surface of *Saccharomyces cerevisiae* and then separate loading of cellulases on the scaffoldin.

The miniscaffoldin displayed was containing a CBD flanked by three different cohesin domains from *Clostridium thermocellum* (t), *Clostridium cellulolyticum* (c), and *Ruminococcus flavefaciens* (f). Cellulases were separately cloned in *Escherichia coli* and a lysate containing different cellulases was prepared to load the displayed miniscaffoldin on the surface of *S. cerevisiae*. Among cellulases, endoglucanase (CelA) was coupled with a dockerin domain from *C. thermocellum* (At), an exoglucanase (CelE) from *C. cellulolyticum* fused with its own dockerin domain (Ec), an endoglucanase (CelG) from *C. cellulolyticum* was joined with a dockerin domain from *R. flavefaciens* (Gf) and β-glucosidase (BglA) from *C. thermocellum* was also fused with dockerin from *R. flavefaciens*.

Assembling endoglucanase (At), an exoglucanase (Ec) and β-glucosidase (BglA) to the displayed miniscaffoldin resulted in better simultaneous saccharification and fermentation from phosphoric acid swollen cellulose simultaneously. Ethanol yield of 0.49 in term of 'gram ethanol per gram of sugar consumed' was reported. The scheme adopted for the assembly of minicellulosome on the surface of yeast has been shown in Figure 17. (Tsai *et al.* 2009.)
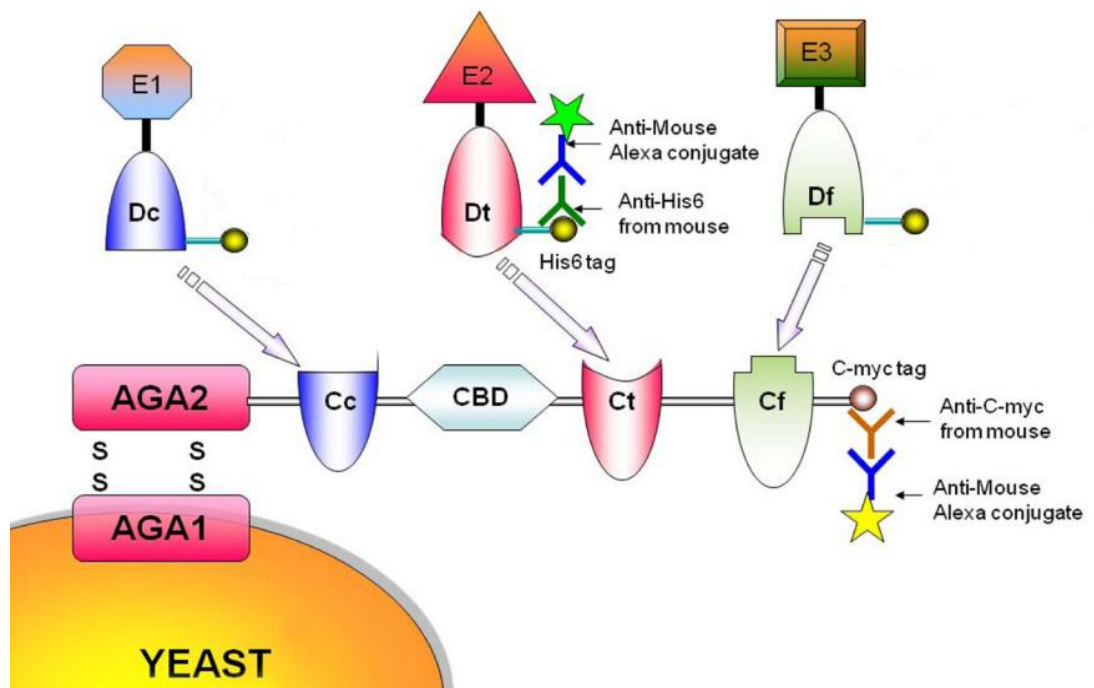

Figure 17: Assembly of minicellulosome has been shown. The miniscaffoldin (Scaf-ctf) is containing a CBD, cohesin domains from Clostridium thermocellum (t), Clostridium cellulolyticum (c), and Ruminococcus flavefaciens (f). (Tsai et al. 2009.)

Wen et al. (2009) displayed complete tri-functional minicellulosome on the surface of *Saccharomyces cerevisiae.* The minicellulosome was capable of self-assembly and comprised of a miniscaffoldin, and three cellulases. The minicellulosome was made of a cellulose-binding domain and three cohesion modules. The cellulases included

endoglucanase EGII from *T. reesei*, cellobiohydrolase CBHII from *T. reesei* and glucosidase BGL1 from *Aspergillus aculeatus*. The prepro signal peptide and alpha factor signal peptide were employed as secretion signal peptide for cellulases. The dockerin modules were from *C. thermocellum*. AGA2 protein was utilized to bind miniscaffoldin to yeast surface.

Wen et al. engineered seven strains of *S. cerevisiae* in total which were displaying either uni-, bi-, or trifunctional minicellulosomes. Figure 18 illustrates the strain with tri-functional minicellulosome, CipA3-EGII-CBHII-BGL1 and the strain with 3 kinds of unifunctional minicellulosomes, CipA1-EGII-CBHII-BGL1. The strain CipA3-EGII-CBHII-BGL1 was having 1.6 times higher activity compared to the CipA1-EGII-CBHII-BGL1. The enhance activity of trifunctional minicellulosomes explained the significance of the enzyme proximity synergy. The ethanol yield of CipA3-EGII-CBHII-BGL1 was 0.31g of ethanol per one gram of phosphoric acid-swollen cellulose.
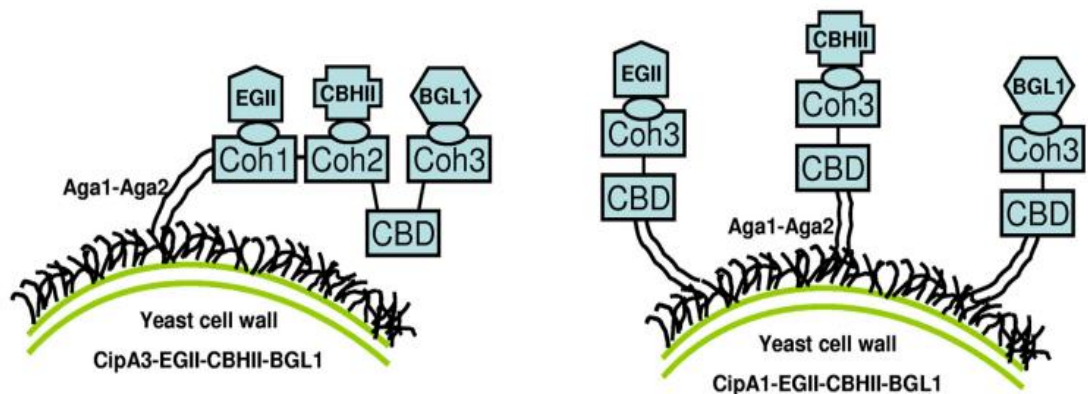

Figure 18: On the left, the strain CipA3-EGII-CBHII-BGL1 is displaying tri-functional minicellulosome, while on right side the strain CipA1-EGII-CBHII-BGL1 is displaying 3 kinds of unifunctional minicellulosomes. AGA2 protein fused with mini-CipA binds with yeast a-agglutinin mating adhesion receptor and cause display of minicellulosome on surface of yeast. (Wen et al. 2009.)

Tsai *et al*. (2010) introduced a concept of consortium rather than engineering a single yeast strain. Consortium concept is an attractive approach for the consolidation of bioethanol production process. Strain like Wen´s CipA3-EGII-CBHII-BGL1 was over burdened but a consortium distributes the burden of heterologous genes among yeast members. Additionally the previous concept of Tsai *et al*. (2009) about separate production of cellulases for loading the displayed scaffoldin also makes the process extensive and hence consolidation is not achieved. Moreover production ratios of cellulases cannot be efficiently regulated in single host and host consortium is always needed for fine tuning of cellulases ratios.

Tsai *et al*. (2010) used the previously described miniscaffoldin (Scaf-ctf) displaying *Saccharomyces cerevisiae* strain and made a consortium by adding three yeast strains which were secreting one of the three kinds of cellulases. For the three cellulases,

endoglucanase CelA from *C. thermocellum* was fused with *a* dockerin from *C. cellulolyticum*, an exoglucanase CelE from *C. cellulolyticum* was combined with its own dockerin and β-glucosidase BglI from *Thermoascus aurantiacus* was fused with dockerin from *R. flavefaciens.* The α-factor secretion peptide was used to facilitate secretion of cellulases. By adjusting different ratios of the four strains, a maximum ethanol yield of 0.475 in terms of 'gram of ethanol per gram of cellulose consumed' was obtained. An equal ratio of the four strains could only produce almost half of the ethanol obtained by the fine-tuned consortium. (Tsai *et al*. 2010.)

Kim *et al.* (2013) also develop a consortium but allowed a random assembly of cellulosome components as three cohesion domains of the same type were added to the scaffoldin, mini CipA. Among the four yeast EBY100 strains of consortium, one was displaying mini CipA and other three yeast strains were secreting cellulases. The cellulases included CelA (endoglucanase) from *C. thermocellum*, CBHII (exoglucanase) from *Trichoderma reesei* and BGLI (β -glucosidase) from *Aspergillus aculeatus*. CelA was fused with its native dockerin (DocA) while CBHII was expressed with dockerin from *C. thermocellum* CelS (DocS). BGLI was express without dockerin domain as its natural binding affinity to yeast surface. The four stains engineered by Kim *et al.* are shown in Figure 19. Twenty percent higher yield was obtained by an adjusted strains ratio compared to equal ratio of the four strains. The concept of random assembly could not significantly increase ethanol yield. An equal yield of ethanol was obtained by Kim's consortium compared to the consortium of Tsai. Importantly, all the engineered yeast strain or the consortiums developed could only digest cellulose partially and additional cellulases were always required for complete hydrolysis.
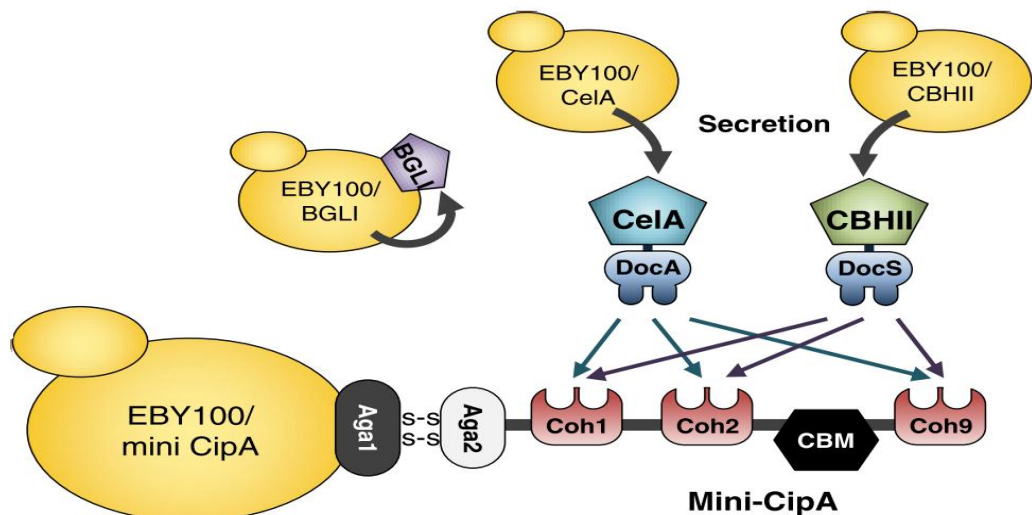
Figure 19: *Schematic representation of the research concept. One engineered strain of yeast is displaying mini CipA while other three strains are secreting one of the three kinds of cellulases: CelA, CBHII and BGLI. (Kim et al. 2013.)*

2.4.2.   Biocatalyst for Co-Fermentation

For increasing the economic feasibility of ethanol production from lignocellulose, there is a need to utilize hemicellulose fraction along with cellulose. Regarding xylose and arabinose utilization, bacteria have an isomerase pathway by which those pentoses are directed to the central metabolism. In xylose and arabinose utilizing fungi, reductase/dehydrogenase pathway is present to direct xylose and arabinose towards the central metabolism. Figure 20 represents the xylose and arabinose metabolism in bacteria and fungi. Although *S. cerevisiae* is the most suitable fermenting organism but it cannot utilize the pentose sugars xylose and arabinose. (Hahn-Hägerdal et al. 2006.) Different causes have been reported for the inability of *S. cerevisiae* to utilize xylose. The inefficient xylose uptake is one of them (Kotter and Ciriacy, 1993). A redox imbalance also occurs in the initial steps of xylose metabolism that hinder efficient utilization of xylose (Bruinenberg, 1986). Moreover the xylulokinase (XK) activity is not competent and pentose phosphate pathway (PPP) is also reported to be inefficient (Walfridsson et al., 1995). Several strategies have been adopted to improve pentose utilization abilities of *S*. cerevisiae.
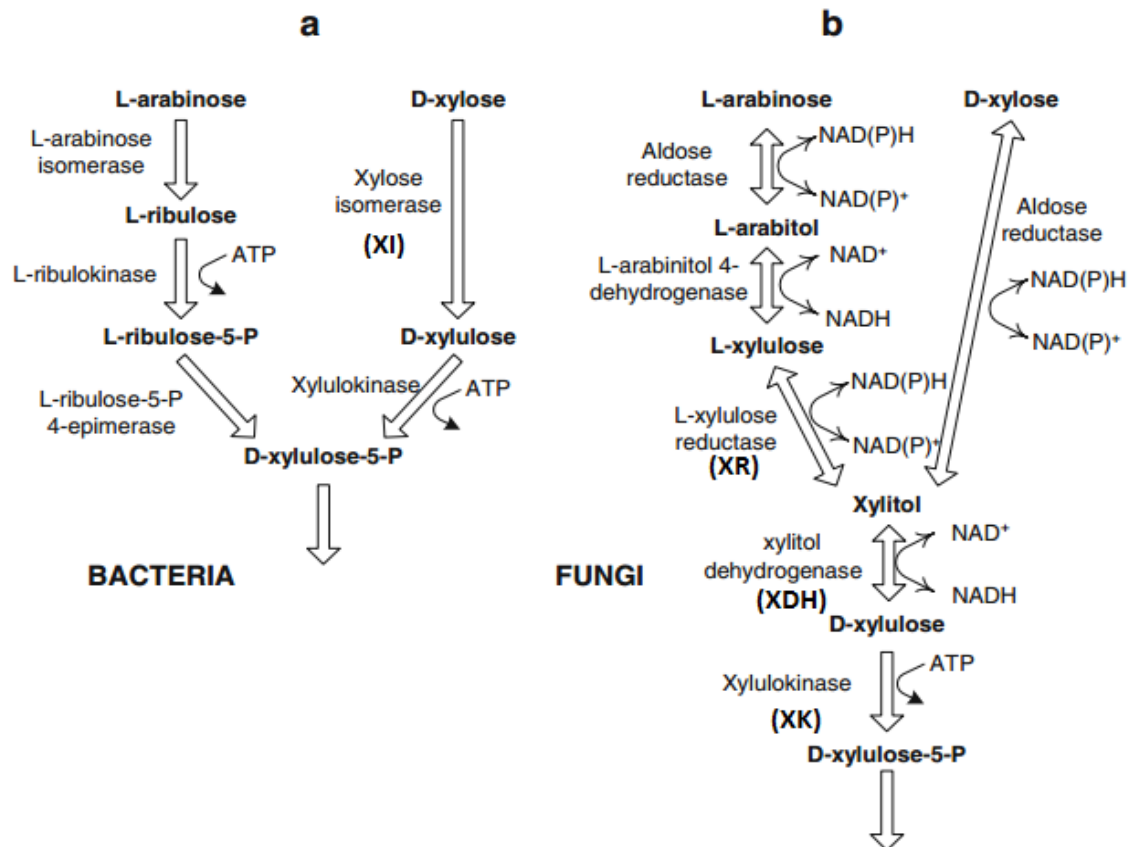
Figure 20*: Comparison of D-Xylose and L-arabinose utilization pathways between (a) bacteria and (b) fungi (Modified from Hahn-Hägerdal et al. 2006).*

Xylose utilization genes in *S. cerevisiae* are expressed at low levels (Hahn-Hägerdal et al. 2006) and hence xylulose reductase (XR), xylitol dehydrogenase (XDH) and xylulokinase (XK) have been over expressed by some researchers. Initially XYL1 and

XYL2 genes from *Pichia stipitis* were introduced in *S. cerevisiae* (Kötter and Ciriacy 1993; Tantirungkij et al. 1994). The genes XYL1 and XYL2 encode xylulose reductase (XR) and xylitol dehydrogenase (XDH). Kötter and Ciriacy achieved 0.07 g/g as a yield for ethanol and 0.46 g/g yield for xylitol while Tantirungkij et al. (1994) acquired an ethanol yield of 0.05 g/g. Few years after Tantirungkij, it was known that the xylulokinase (XK) from endogenous XKS1 gene should also over expressed with XR and XDH to get more xylulose-5-P and hence more ethanol from *S. cerevisiae* (Moniruzzaman et al. 1997; Ho et al. 1998; Eliasson et al. 2000; Toivari et al. 2001). By over expressing XK in addition XR and XDH, Moniruzzaman et al. achieved an ethanol yield of 0.34 g/g and a xylitol yield of 0.06 g/g. Similarly Ho et al. achieved an ethanol yield of 0.3 g/g and a xylitol yield of 0.08 g/g.

Walfridsson et al. (1997) overcame the redox imbalance (or accumulation of NADH from XDH reaction) caused by lower Km value of XR for NADPH compared to NADH. Walfridsson et al. adjusted the ratio of XR and XDH in *Saccharomyces cerevisiae*. The yeast vectors YEp24 with alcohol dehydrogenase 1 (ADH1) and phosphoglycerate kinase (PGK1) promoters were utilized to control expression of XYL1 and XYL2 genes. The endogenous genes coding transketolase and transaldolase were also over expressed to enhance utilization of xylose. The strain with XR: XDH ratio of 0.06 was found optimal. The optimal strain was not producing xylitol at all and lesser glycerol and acetic acid in comparison to the strains with the higher XR: XDH ratios

Xylose transport in *S. cerevisiae* has also been manipulated. Hamacher et al. (2002) proved genetically that the yeast hexose transport system is responsible for xylose transport. Hamacher et al. reported Hxt7and Gal2 as high affinity while Hxt4 and Hxt5 as low affinity transporters of xylose in *S. cerevisiae*. Hamacher et al. also employed *S. cerevisiae* PUA strains to test if over expression of the xylose transporting gene can result in better growth PUA strains. The *S. cerevisiae* PUA strains is a xylose utilizing and fast growing yeast strain but over expression of the transporting gene could not improve growth rate of the strain in either aerobic or anaerobic conditions. In contrast to Hamacher, Gardonyi et al. (2003) reported that a fast xylose utilization rate gets influenced by xylose uptake rate but xylose uptake rate don't affect a slow xylose utilization rate.

Inefficient Pentose Phosphate Pathway (PPP) contributes for incompetent xylose utilization by *S. cerevisiae*. Walfridsson et al. (1995) reported over expression of transaldolase (TAL) to cause better growth of *S. cerevisiae* on xylose. Selective pressure has also been utilized to adapt *S. cerevisiae* for xylose metabolism. Different strategies have been adapted to evolve a recombinant and native *S. cerevisiae* strain. For instance growing a recombinant *S. cerevisiae* strain on xylose as sole carbon source, then growing on xylose–glucose mixture and later again growing on xylose as sole carbon source. This strategy has evolved a strain capable of growing on 4.5% xylose and producing ethanol with a yield of 0.14 gram per gram. Native *S. cerevisiae* has also been evolved to grow on xylose by extensive natural selection and breeding. The improved strain,

MBG-2303, was able to grow on xylose as sole carbon source and the duplication time for MBG-2303 was less than 6 hours. The MBG-2303 was possessing higher xylose reductase and xylitol dehydrogenase activities and the strain was the first non-genetically modified *S. cerevisiae* that can utilize xylose. (Attfield & Bell 2006.)

The xylose utilization of bacteria which does not require cofactors has been introduced in *S. cerevisiae* by some researchers but the recombinant *S. cerevisiae* with XYLA gene from *Actinoplanes missouriensis, Bacillus subtilis, Clostridium thermosulfurogenes, Lactobacillus pentosus* and *Escherichia coli* have resulted into inactive XI or very minute XI activity. (Amore et al. 1989; Hallborn 1995; Ho et al. 1983; Moes et al. 1996; Sarthy et al. 1987.) In 1996, Walfridsson et al. became successful to express functional XYLA gene from *Thermus thermophiles* in *S. cerevisiae* (close phylogeny). The XYLA gene was inserted in the multicopy yeast expression vector pMA91, between phosphoglycerate kinase gene (PGK1) promoter and terminator. The resultant plasmids, pBXI, were then introduced in *S. cerevisiae*. The recombinant strain was able to grow on 3% xylose and to convert xylose into ethanol. Ethanol yield of 0.04 g ethanol/ g xylose and xylitol yield of 0.13 g xylitol/ g xylose were obtained. The low activity of XI was due to the need of higher temperature for optimum activity. Walfridsson et al. performed fermentation at 38 °C but they observed maximum thermophilic XI activity at 85°C. Later Kuyper et al. (2003) also expressed XylAgene from anaerobic cellulolytic fungus *Piromyces* sp. E2 into *Saccharomyces cerevisiae*. The transformed *S. cerevisiae* CEN.PK113-5D was observed to grow slowly on xylose and activity of XylA gene from the transformed *S. cerevisiae* was recorded as 1.1 U per mg protein. The slow growth of the transformed *S. cerevisiae* was a hindrance for Kuyper et al. to record xylose consumption and ethanol production.

For developing a co-fermenting yeast, there is a need of extensive metabolic improvement. No *S. cerevisiae* strain could be engineered which can co-ferment lignocellulose hydrolysate at industrial scale.

3. INTRODUCTION TO EXPERIMENTAL PART

Inspired from radical based pretreatment of lignocellulose, the current project aimed to develop a novel mean to degrade lignocellulose. The first step was about searching a biological source of radicals. So cytotoxic KillerRed (KR) was chosen for its ability to generate reactive oxygen species (ROS) upon illumination. After selecting KR, the next challenge was about developing affinity between lignocellulose and KR. Affinity was supposed to cause a focal action of ROS on lignocellulose and a better degradation of lignocellulose. To engineer affinity for cellulose, KR was added with a cellulose binding peptide (CBP) at its N terminal. The design of the approach has been shown in Figure 21.

The engineered CBP-KR was tested for its affinity by incubating it with cellulosic substrates (filter paper and printing paper) and then collecting supernatants. The changes in fluorescence signals from supernatants were recorded to investigate the affinity. Moreover eluent was also added to those cellulosic substrates to estimate the extent of binding between CBP-KR and cellulosic substrates. To evaluate degradation capability of the ROS generated by CBP-KR, the mixture of carboxymethyl cellulose (CMC) and CBP-KR were illuminated with green light. Then a modified Congo red assay was employed to estimate the extent of CMC degradation.

The model of CBP-KR protein bound to paper can be practical for the companies planning to use paper as raw material for production of fermentable sugars. FibreEtOH Project (EU Commission funded project) planned to convert paper fiber into bioethanol. The main player in the project was UPM- The Biofore Company while AB Enzymes GMBH, Skandinavisk Kimiinformation AB, Poyry Forest Industry Consulating OY, Sila & Tikanoja OYJ, ST1 Biofuels, Roal and Valtion Teknillinen Tutkimuskeskus (VTT) were also participating. FibreEtOH Project intended to collect papers fibers from solid waste and de-inked sludge from paper mills. Both sources of waste paper and sludge from paper mils have high content of hexoses and their supplies are massive. The FibreEtOH concept is attractive as pre-treatments and extensive fractionations are not required when directly using paper fibers as raw material. Moreover the low prices of waste could make the process more economic. FibreEtOH Project planned to start a demonstration plant with bioethanol production capacity of 20000 cubic meterss per year by using 250 000 tons waste of Helsinki metropolitan. The plant was also designed to ultilize the byproducts for electricity, biogas and heat production. (European Biofuels 2014.)
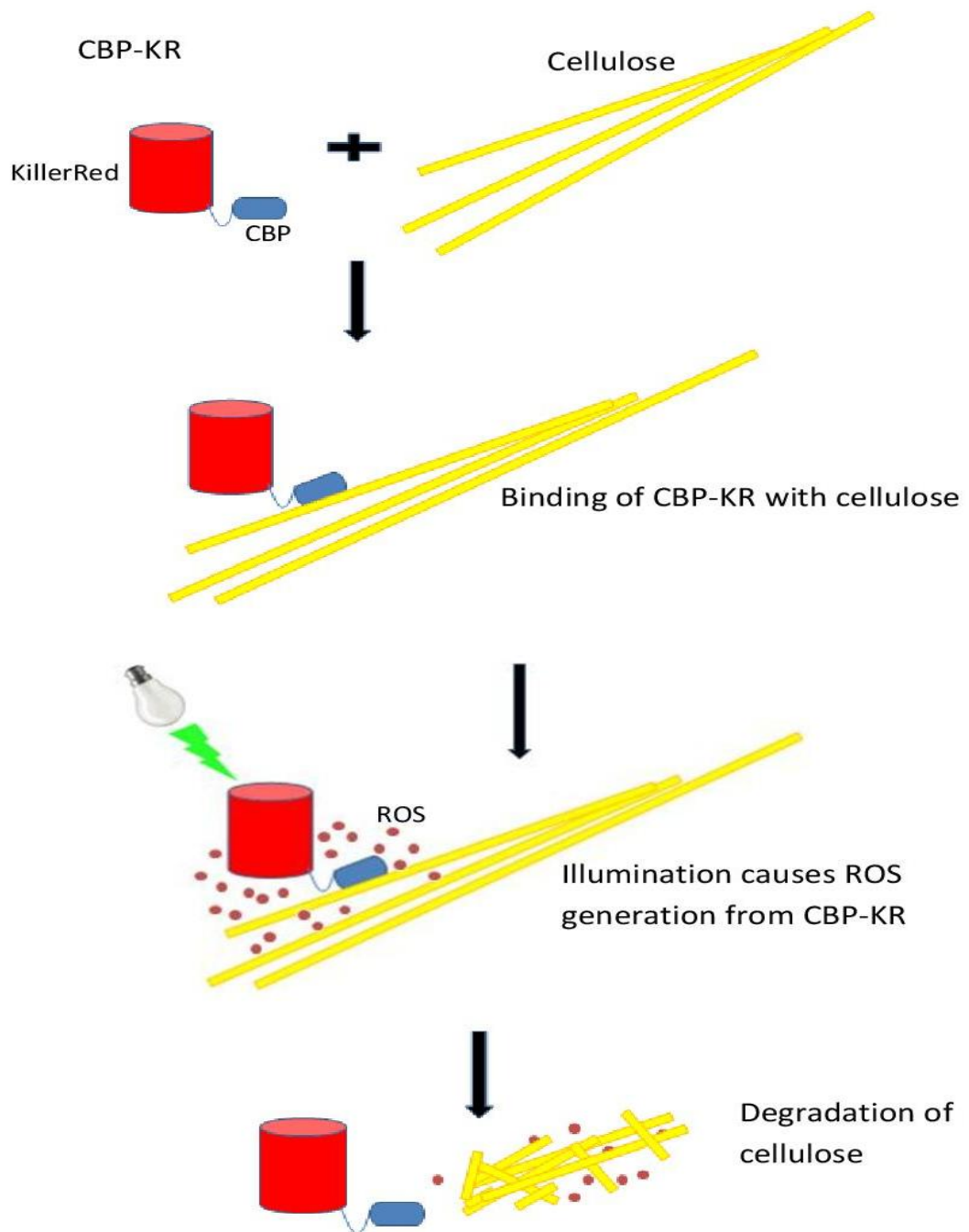
Figure 21: *Design of the current study. Incubation of CBP-KR with cellulose results in binding of CBP part to cellulose fibers. Then illumination of CBP-KR with green light generates ROS which in turn degrade cellulose fibers.*

4. RESEARCH METHODS AND MATERIALS

3.1. Construction of Expression Plasmids

The details of expression vector and genetic constructs of this study are shown in Table 4.

Table 4: Genetic constructs utilized and designed in this study

Plasmids	Source	Purpose
pET28	Invitrogen	Expression Vector
pET28-N-His-KR	This Study	Construct for expression of His-KillerRed
pET28-N-CBP-KR	This Study	Construct for expression of Cellulose Binding Peptide- KillerRed

4.1.1. Polymerase Chain Reaction (PCR) and PCR Product Purification

Two PCR tubes were employed; one for His-KR and second for CBP-KR. Both tubes were filled to obtain 1.5 mM of $MgCl_2$, 250 μM of each dNTP, primer 0.5 μM each and 1X buffer as final concentrations. Additionally ⸺100ng template, 2 Units of Phusion polymerase from FINNZYMES, Finland and MQ water to fill volumes of tubes up to 50 μl were added. The primes added in the tube labeled as His-KR were vs13_1 and ab135 while the primers vs13_3 and ab135 were added in the tube for CBP-KR. The primer vs13_3 was including the sequence of CBP. The CBP was adapted from the cellulose binding peptide CEL121 of Qi et al. (2008). The sequences of the primers and the amino acid sequence of CBP are presented in Appendix A.

In PCR tubes, phusion polymerase was added as the last component; just before thermocycling. Negative controls were also included in which all PCR components were added except DNA template.The thermocycler was programmed for 29 thermo cycles. One thermocycle of PCR was set as denaturation at 98°C for 10 seconds, annealing at 50°C for 10 seconds and elongation at 72°C for 20 seconds. The thermocycler was set to hold the reactions after 29 cycles by dropping temperature at 4°C for infinite time.

After completion of PCR reactions, the concentrations of PCR products were recorded by NanoDrop from Thermo Fisher Scientific and then 2 μl from each PCR prod-

ucts, negative controls and 100 bp DNA ladder from NEW ENGLAND BioLabs Inc. were run on electrophoresis gel. The details of agarose gel preparation are provided in Appendix B. Later the PCR products were purified by using GeneJET PCR Purification Kit from Thermo Scientific and concentrations of purified PCR products were recorded again by the NanoDrop.

4.1.2. Digestion of PCR Products

Two PCR tubes were used for the digestion of PCR products; one tube for His-KR and second tube for CBP-KR. The digestion tube reserved for His-KR was filled with 2 µl respective PCR product, 2 µl *NdeI*, 1 µl *SalI*, 3 µl of 10 X Buffer R and 22µl MQ water while the tube labelled as PCR digestion for CBP-KR was added with 2 µl respective PCR product, 1 µl *NcoI*, 1 µl *SalI*, 3 µl of 10 X Buffer R and 23 µl MQ water.

4.1.3. Cloning of Expression Vector

Plasmid pET28 was provided as an expression vector. The expression vector was amplified by molecular cloning. For the cloning, KRX competent cells were transformed with the expression vector, pET28. In transformation, an electroporation cuvette was pre-cooled on ice. Then 2 µl of the expression vector was added in 40 µl of KRX competent cells (previously prepared) and the whole mixture was transferred into the electroporation cuvette. The electroporation cuvette was filled carefully, avoiding any air bubble inside the cuvette. Moreover the vials and cuvette were kept on ice until electroporation. The electroporation was processed with Eco-1 program of MicroPulser^{TM} Electroporator from BIO-RAD, USA.

Immidiately after electroporation, 1 ml pre-warmed (at 37^{0}C) Luria Broth enriched with 0.4% glucose was added in the electroporation cuvette and the cuvette was incubated at 37^{0}C for 1 hour. Following incubation, 900 µl and 100 µl of the culture was inoculated on two Luria Agar media plates containing 50 µg/ ml kanamycin and 0.4% glucose. The plates were incubated overnight at 37^{0}C. The compositions of Luria Broth (LB) and Luria Agar (LA) medium are provided in Appendix B.

On the next day, two colonies were picked from the plates and two culture tubes containing 5 ml LB media with 50 µg/ ml kanamycin and 0.4% glucose were inoculated. The culture tubes were incubated for 12-16 hour at 37^{0}C with shaking of 200-250 rpm. After incubation, cells were harvested and vector pET28 was isolated by GeneJET Plasmid Miniprep Kit from Thermo Scientific. The concentrations of vector preparations were recorded by NanoDrop.

4.1.4. Digestion of Expression Vector

Two PCR tubes were employed for vector digestion; one tube for His-KR and second tube for CBP-KR. The tube reserved for the vector of His-KR was added with 10 µl plasmid preparation, 2 µl *Nde*I, 1 µl *Xho*I, 3 µl of 10 X Buffer R and 14 µl MQ water.

The tube labelled as vector digestion for CBP-KR was added with 10 µl plasmid preparation, 1 µl *NcoI*, 1 µl *XhoI*, 3 µl of 10 X Buffer R and 15 µl MQ water. Both tubes were incubated at 37^0C for 1 hour and then stored at 4^0C.

4.1.5. Electrophoresis and Extraction of Fragments

The digested PCRs and plasmids were loaded on 1 percent agarose gel while leaving empty wells as gaps between individual samples. The ladder MassRuller^{TM} DNA Ladder Mix (Ready to Use) from Fermentas was also added to one well. The gel was run for 1 hour.

After gel electrophoresis, the PCR fragments and vector backbones were precisely cut out from gel with a sharp clean blade and added to pre-weighted 1.5 ml tubes. Then the fragments were extracted from the gel by utilizing GeneJET^{TM} Gel Extraction Kit from Fermentas. The DNA concentrations after extraction were measured by the NanoDrop from Thermo Fisher Scientific.

4.1.6. Ligation Reaction

Ligation was the last step in preparation of pET28-N-His-KR and pET28-N-CBP-KR. For preparation of pET28-N-His-KR, 5.5 µl of the respective PCR fragment which was extracted from gel, 12 µl of the relevant extracted vector backbone, 2 µl of 10 X T4 Ligation Buffer and 0.5 µl T4 ligase were added in ligation tube. For preparing pET28-N-CBP-KR, the ligation tube was filled similarly with 4.5 µl of respective PCR fragment, 13 µl of particular vector backbone, 2 µl of 10 X T4 Ligation and 0.5 µl T4 ligase. Ligation controls were included in which the volumes of PCR fragments were replaced with MQ water while all other components of the ligation reactions were included.

4.2. Transforming *E. coli* with Expression Plamids

4.2.1. Competent Cell Preparation

KRX competent cells were grown in 2.5 ml of Super Broth (SB) enriched with 0.2 % glucose and containing 50 µg/ ml kanamycin. The culture was incubated at 37^0C with shaking of 300 rpm. Next day, the 2.5 ml culture was transferred in 250 ml SB medium enriched with 0.2 % glucose and containing 50 µg/ ml kanamycin. The culture was grown at 37^0C with shaking of 300 rpm to the OD_{600} (Optical Density at 600nm) of 0.7. The flask was chilled for 15 minutes on ice. Then KRX cells were harvested in a pre-cooled centrifuge by rotating culture at 1500g for 15 minutes at 4^0C. Then cells were carefully re-suspended in 100ml of ice cold 15% glycerol. The suspension was spin again at 1500g for 15 minutes at 4^0C and supernatant was removed. The cells were again re-suspended with 60 ml of ice cold 15% glycerol and cells were harvested by

spinning. Lastly the re-suspension was done with 30 ml of ice cold 15% glycerol. After harvesting KRX competent cells, the cells were re-suspended with smallest volume of 15% glycerol. The cells were divided in aliquots and 40 µl of competent cells were added in each aliquot. The aliquots were stored at -80 ^{0}C. Preparation of 15% glycerol and SB medium is provided in Appendix B.

4.2.2. Transformation and Clone Verification

For electroporation, 2 µl of ligation reaction was added in 40 µl of KRX and then the mixture was added to pre-cooled electroporation cuvette. The electroporation procedure has been mentioned previously. Four cuvettes were prepared for electroporation; 2 cuvettes with ligation reaction for pET28-N-His-KR and pET28-N-CBP-KR while 2 cuvettes with respective control ligations.

The cuvettes were filled with 1 ml pre-warmed (at 37^{0}C) Luria Broth enriched with 0.4% glucose immediately after electroporation and incubated at 37^{0}C for 1 hour. After incubation, from each cuvette 900 µl and 100 µl of the culture was inoculated to two Luria Agar (LA) media plates containing 50 µg/ ml kanamycin and 0.4% glucose. The eight LA plates were labelled as His-KR test 100, His-KR test 900, His-KR control 100, His-KR control 900, CBP-KR test 100, CBP-KR test 900, CBP-KR control 100 and CBP-KR control 900. After overnight incubation at 37^{0}C, numbers of colonies were counted and compared for all test and control plates.

From the plate His-KR test 100 and the plate CBP-KR test 100, six colonies (three from each plate) were picked and added to six tubes containing 9 ml SB medium supplement with 50 µg/ ml kanamycin and 0.4% glucose. The tubes containing colonies from the plate His-KR test 100 were labelled as H1, H2 and H3 while tubes with colonies from the plate CBP-KR test 100 were named as C1, C2 and C3 tubes. The tubes were incubated at 37^{0}C with shaking of 250 rpm till OD$_{600}$ of 0.8.

From the tubes, 4ml culture from each tube was transferred into new tubes and induced with 0.1% L-rhamnose as final concentration. The induced cultures were incubated overnight at 30^{0}C with shaking of 250 rpm. The preparation of L-rhamnose stock solution is provided in Appendix B.

From the remaining 5 ml culture in each tube, glycerol preparations and plasmid extracts for clone verification were obtained. Glycerol preparations were prepared by adding 800 µl culture in 200 µl of 80 % glycerol and then preparations were saved at -80 ^{0}C. The method for preparing of 80% glycerol is provided in Appendix B. Plasmids were isolated from tube H1, H2,H3, C1, C2 and C3 by GeneJET Plasmid Miniprep Kit from Thermo Scientific and concentrations of extracted plasmids were estimated by NanoDrop from Thermo Fisher Scientific.

For clone verification, digestion reactions were prepared for all the extracted plasmids. Digestion reactions were containing 12 µl plasmid, 1 µl *NcoI*, 2 µl of 10 X Buffer R and 5 µl MQ water. A tube as control digestion was included which was added with 12 µl of the pET28 plasmid, 1 µl *NcoI*, 1 µl *XhoI*, 2 µl of 10 X Buffer R and 4 µl MQ

water. All the tubes were incubated at 37^0C for 1 hour and later digested plasmid were stored at 4^0C. Latter digestion reactions, control digestion and GeneRuler[TM] DNA Ladder Mix from fermentas were run on 2 % agarose gel for 4 hours.

4.3. Expression and Purification of Proteins

4.3.1. Growing and Inducing the Verified clone

From the glycerol preparations of verified clones, H1 and C1, two LA plates were inoculated. The LA plates were enriched with 0.4 % glucose and containing 50 µg/ ml kanamycin. The inoculated plates were incubated overnight at 37^0C. Next day, single colonies from plates were picked and transferred in 5 ml SB medium containing 4 % glucose and 50 µg/ ml kanamycin. The tubes were labelled as pre-culture His-KR and pre-culture CBP-KR and incubated at $37\ ^0$C with 275 rpm shaking for overnight period. On the next day, OD_{600} were measured from both pre cultured tubes and 1 ml culture from each tube was transferred to 100 ml SB medium supplemented with 0.4 % glucose and 50 µg/ ml kanamycin. The flasks were labelled as His-KR and CBP-KR and incubated for 3 hours at $37\ ^0$C with 275 rpm shaking. After 3 hours, OD_{600} were estimated from both cultivations and the flasks were transferred to a shaker with temperature of $20\ ^0$C and shaking of 275 rpm. After one hour incubation at $20\ ^0$C, flasks were added with 0.1% L-rhamnose and 1mM IPTG as final concentrations. The incubation at $20\ ^0$C with shaking of 275 rpm was continued for overnight period. Next day, ODs_{600} were again estimated and then 90 ml of culture from each culivation was saved at $-80\ ^0$C for protein purification. The engineered proteins of this study are presented in Table 5.

Table 5: Details of the proteins engineered in this study

Engineered Protein	Description	Purification Method
His-KillerRed (His-KR)	KillerRed added with His tag at N terminal	Immobilized Metal Affinity Chromatography (IMAC)
Cellulose Binding Peptide-KillerRed (CBP-KR)	KillerRed added with Cellulose Binding Peptide at N terminal	Three-Phase Purification

4.3.2. Fluorescence Measurements

From the culture left in the cultivation of His-KR and CBP-KR, dilutions were prepared in a way that micro-wells were containing 10^7, 10^6 and 10^5 cells. As a control of fluorescence, the KRX competent cells previously transformed with pET28 were grown in 5

ml SB medium supplemented 4 % glucose and 50 μg/ ml kanamycin. Similar dilutions of 10^7, 10^6 and 10^5 were prepared for control. Fluorescence signals were recorded by Fluoroskan Ascent FL, from Thermo Labsystem. The excitation filter was set at 560 nm while emission was recorded at 590 nm.

4.3.3. Immobilized Metal Affinity Chromatography (IMAC)

The frozen culture from His-KR cultivation was thawed and cells were collected by centrifuging at 3000g for 20 minute at 4°C. The cells were resuspended in ice cold 1X binding buffer (3ml/gram wt). The stock 8X binding buffer was a solution with 4M NaCl, 160 mM Tris-HCl (pH 7.9) and 40 mM imidazole as final concentrations. Then lysozyme (1 mg/ ml) and 0.5 μl benzonase were added in the cell suspension. The suspension was left on ice for 30 minutes and then sonicated for two minutes. To avoid heating and a consequent damage to protein, sonication was completed with suitable breaks in the process. After sonication, the mixture was centrifuged at 10,000g for 30 minutes and lysate was separated. The lysate was filtered with 0.45 μm filter.

Ready to use column 'His GraviTrap' from GE Healthcare was used for IMAC. The storage buffer was removed from the column by cutting the lower outlet of the column. Then 1 ml 1X binding buffer was added to the 1 ml resin bed of His GraviTrap column and buffer was allowed to drain. Then lysate was loaded and the flow through was collected in a vial labeled as sample flow though. Then the column was drained with 3 ml 1X binding buffer and 3 ml 1X wash buffer and all the flow through collected was labeled as sample wash. The stock 8X wash buffer was 4M NaCl, 160 mM Tris-HCl (pH 7.9) and 390 mM imidazole. Then 3ml of 1X elution buffer was added to column and His-KR protein was collected in a clean vessel. A picture taken during elution phase is presented as Figure 22.


Figure 22: *Elution phase of Immobilized Metal Affinity Chromatography (IMAC). His-KR protein was collected in a vial placed under IMAC column*

The His-KR protein solution was stored at 4 ^{0}C in a vessel covered with aluminum foil. The stock 4X elution buffer was a solution with 2M NaCl, 80 mM Tris-HCl (pH 7.9) and 800 mM imidazole as final concentrations. Lastly the column was drained with 3 ml of 1X strip buffer and flow though was collected as a sample strip. The outlet of the column was capped and 1X strip was added in the column as storage buffer. The stock 4X strip buffer was 2M NaCl, 80 mM Tris-HCl (pH 7.9) and 4mM EDTA. The upper inlet of the column was also capped and the column was stored at 4 ^{0}C for its reutilization.

4.3.4. Three-Phase Purification

The frozen culture from CBP-KR cultivation was thawed and cells were suspended in 10 ml of buffer 1. Then 10 ml t-butanol was added in same falcon tube and vigorous vortexing was done. The mixture was centrifuged at 12,000g for 2 minutes to obtain separate layers. The red lower layer of CBP-KR obtained after centrifugation is shown in Figure 23A. The lower layer containing CBP-KR protein was carefully transferred to new falcon tube by pipetting and rest of the layers were discarded. Then 10 ml t-butanol was again added and vigorous vortexing was repeated. The mixture was centrifuged at 12,000g for 2 minutes and a thin red layer was obtained as shown in Figure 23B. The red layer containing CBP-KR was removed carefully and resuspended in 10ml of buffer 2. The insoluble material was settled down by centrifugation at 3200g for 10 minutes and supernatant was filtered by 0.45 μm filter. After filtration, CBP-KR protein solution was stored at 4 ^{0}C and covered with aluminum foil. Buffer 1 was 1.6 M $(NH_4)_2$ SO_4 and 10 mM Tris-HCl solution with pH adjusted to 8.0 while buffer 2 was 50 mM Tris-HCl and 0.1 M NaCl solution with pH adjusted to 7.5.
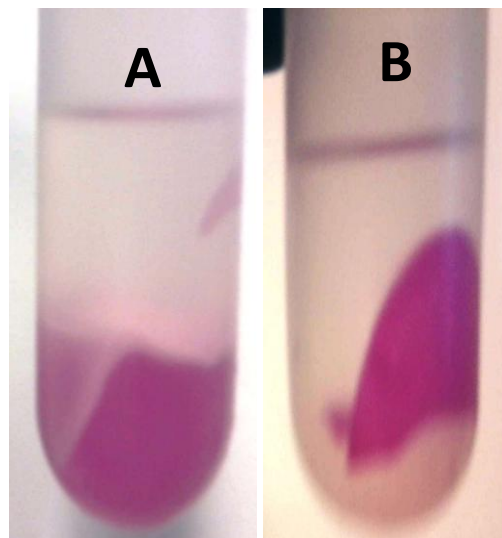
Figure 23*: Three-Phase Purification of CBP-KR. (A) After first centrifugation, a lower layer containing CBP-KR protein was obtained. (B) Second centrifugation resulted in a thin layer of CBP-KR.*

4.3.5. SDS-PAGE (Polyacrylamide Gel Electrophoresis)

The purified CBP-KR, His-KR and fractions collected during IMAC were run on SDS-PAGE. The samples and 5X Protein Loading Buffer from Fermentas were pre-mixed with 4: 1 ratio respectively and mixtures were heated at 95°C for 4 minutes. PageRuler^{TM} Plus Prestained Protein Ladder from Fermentas was also included as size standard for SDS-PAGE. The Mini-PROTEAN®Tetra Cell system from BIORAD was used for SDS-PAGE. The glass plates were cleaned and dried. Then Mini-PROTEAN casting stand and frame were assembled and the comb was placed between spacer plate and short plate. Then the short plate was marked 1cm below the comb. The comb was removed and resolving gel was prepared. Ten milliliter of 10 % resolving gel buffer was prepared by adding 4.1 ml MQ water, 3.3 ml of 30% Acrylamide/Bis, 2.5 ml gel buffer (1.5 M Tris-HCl, pH 8.8) and 100μl of 10% w/v SDS. Just before pouring the gel, 50 μl of 10% freshly prepared ammonium persulfate (APS) and 5 μl TEMED were added. After pouring the resolving gel upto the mark, the gel was immediately overlaid by ethanol. Upon solidification of gel, ethanol was poured off and MQ water was added to rinse gel surface. The gel top layer was dried by using filter and a comb was inserted. Then stacking gel was added and gel was allowed to solidify. Ten milliliter of 8 % stacking gel buffer was prepared by adding 4.7 ml MQ water, 2.7 ml of 30% Acrylamide/Bis, 2.5 ml gel buffer (0.5 M Tris-HCl, pH 6.8) and 100μl of 10% w/v SDS. Then just prior to pouring, 50 μl of 10% freshly prepared APS and 10 μl TEMED were added. The comb was removed after gel solidification and the assembly was filled with 1 X running buffer to almost the edge of outer short plate. Then samples were loaded and the electrode assembly was placed in Mini-PROTEAN Tetra Tank. The tank was filled with 1 X running buffer up to the mark and electrophoresis was run with 180 V for 45 minutes. The stock 10 X running buffer was containing 30.3 g Tris base and 144.1 g glycine per 1000ml MQ water and its pH was adjusted to 8.3. After electrophoresis, the gel was carefully removed and then stained with PageBlue Protein Staining Solution from Thermo Scientific for overnight period.

4.4. Analyzing Cellulose Degradation Capability of Purified Proteins

4.4.1. Analyzing Binding of Proteins to Cellulosic Substrates

To test affinity of CBP-KR protein to cellulosic substrates, His-KR was employed as a control. A 1/200 dilution was prepared from His-KR stock solution while 1/100 dilution was prepared for CBP-KR stock solution. Then 6 clean 1.5ml vials were labeled as His-Filter, His-Paper, His-Blank, CBP-Filter, CBP-Paper and CBP-Blank. A clean filter paper and a printing paper were cut in tiny pieces with a clean scissors. Then the vials His-Filter and CBP-Filter were added with 25 mg filter paper while the vials

His-Paper and CBP-Paper were filled with 25 mg printing paper. The vials His-Filter, His-Paper and His-blank were added with 1ml dilution of His-KR while the vials CBP-Filter, CBP-Paper and CBP-blank were filled with 1 ml dilution of CBP-KR. After incubation of 1 hour with rotation, 800 µl of the supernatant was removed from each tube. The fluorescence signals were recorded by adding 200 µl supernatant in duplicate in a clean microplate. The signals were recorded by Fluoroskan Ascent FL, from Thermo Labsystem and the excitation filter was kept at 560 nm while emission filter was set at 590 nm. Fluoresence signals were also recorded from the dilutions prepared from His-KR and CBP-KR stock solution and those signals were utilized as controls.

The second phase of the experiment was about elution of the bound CBP-KR and His-KR. All the six vials were added with 500 µl of 5M Guanidine-HCl and left for 30 minutes at room temperature. After 30 minutes, eluent was collected from each vial and the fluorescence signals were recorded as previously.

The experiment was repeated to confirm the results. In the repetition, the stocks of CBP-KR and His-KR were diluted to get an approximately equal fluorescence signals from both. An equal fluorescence was obtained by repeatedly measuring fluorescence signals during dilution process. Moreover only printing paper was incubated in equal concentrations (according to fluorescence signals) of CBP-KR and His-KR while filter paper vials and blank vials were excluded from the experiment.

4.4.2. Congo Red Assay

To analyze degradation capabilities of CBP-KR protein Congo red assay was optimized. The procedure of Congo red assay was modified from Haft et al 2011. As standards, 0.1 %, 0.2% and 0.4% solution of carboxymethyl cellulose (CMC) were prepared using 20 mM solution of 3-(N-morpholino)propanesulfonic acid (MOPS)–HCl as solvent. CMC dissolve slowly and hence CMC was left in MOPS–HCl for overnight period at room temperature.

From each standard CMC solutions, 500 µl was transferred into a 2ml vial. Then 100 µl of MOPS–HCl solution and 400 µl of 0.1M NaOH were added. Then 500 µl of 0.25 mg/ml aqueous solution of Congo red dye and 500 µl of 2M NaCl were added. Now the final concentrations in vials were 0.0625 mg/ ml of Congo red dye and 0.5 M NaCl. The vials were left for 30 minute incubation at room temperature with rotations. After incubation, 200 µl from each vial was transferred into a clear base microplate in duplicates and absorbance was recorded at 540 nm using Multiskan Ascent from Thermo Labsystem.

A test and a control vials were also included in the assay. For the assay, one in two dilution of CBP-KR protein solution was prepared. Then 1200 µl of 0.2 % CMC was added with 240 µl of the CBP-KR dilution. After proper mixing, 600 µl of the mixture was added in test vial and 600 µl in control vial. The test vial was induced by green light from the bulb of a fluorescent microscope for 1 hour. The control tube, on the other hand, was protected from light. The control and test vials were later added with

400 µl of 0.1M NaOH, 500 µl of 0.25 mg/ml Congo red dye solution and 500 µl of 2M NaCl and the vials were left for 30 minute incubation at room temperature with rotations. Later absorbance was recorded as described previously. A blank vial was also included to correct test and control absorbance values as KillerRed also absorb light at 540nm. The blank was containing 100 µl CBP-KR dilution and 1900 µl of the MOPS HCl solution.

5. RESULTS

The PCR product concentrations were 425 ng/ µl and 512 ng/ µl for His-KR and CBP-KR respectively. The concentration of PCR products after purification were 95.3 ng/ µl for tube His-KR and 101.1 ng/ µl for the tube labelled as CBP-KR. The Figure 24 represents the gel electrophoresis picture for PCR products verification. The size of PCR products were around 700bp.
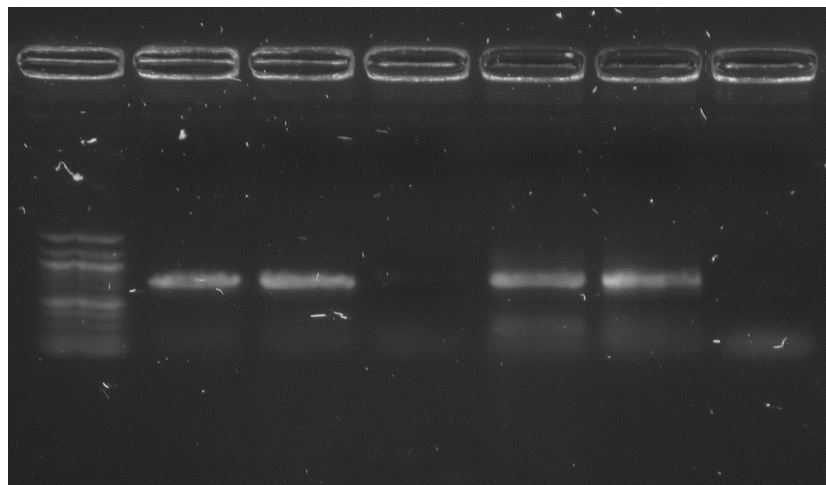
Figure 24: *Gel electrophoresis. From left to right first well was loaded with 100 bp DNA ladder from NEW ENGLAND BioLabs Inc, second and third with PCR products for His-KR while the fourth well was loaded with negative control for PCR of His-KR. Fifth and sixth well are PCR products for CBP-KR while last well is negative control for PCR of CBP-KR.*

The concentrations of the prepared expression vectors (pET28) were 73.8 ng/ µl and 80.0 ng/ µl. The Figure 25 shows gel electrophoresis picture of the digested PCRs and plasmids. The gaps between samples were left to facilitate cutting of fragments from the gel. The fifth well with digested vector from the tube His-KR is showing 3 bands. The third band or the heaviest band appeared because of partial digestion of the expression vector. The partial digestion was due to *NdeI* enzyme. The *NdeI* enzyme used lost its optimal activity, which was confirmed elsewhere. Among the three bands, middle band was extracted and utilized as vector backbone for the His-KR case.
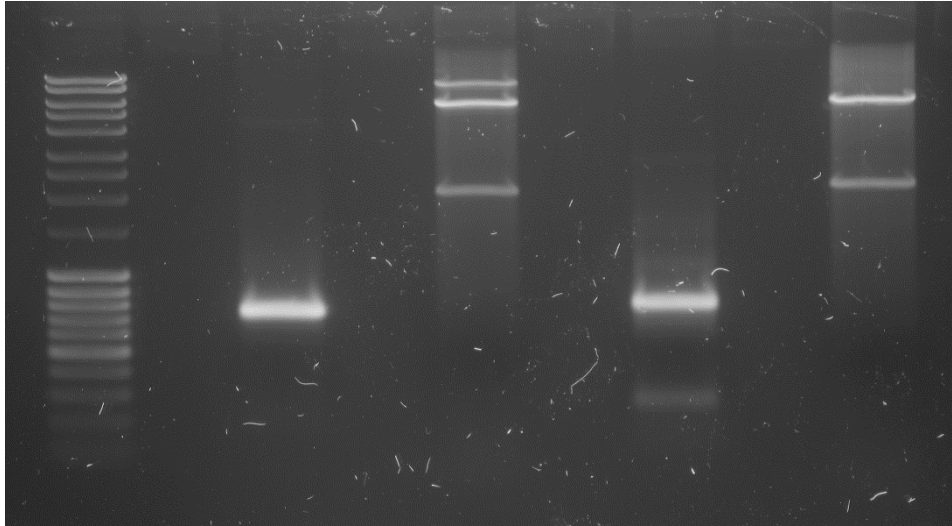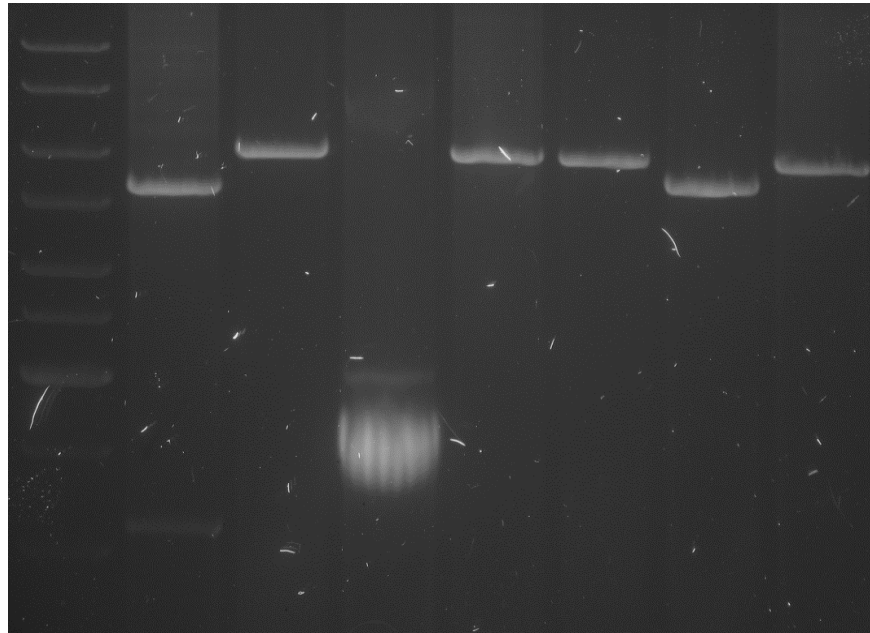
Figure 25: *Gel electrophoresis. From left to right first well was loaded with MassRuller*^{TM} *DNA Ladder, third well with digested and purified PCR product for His-KR and fifth well was containing digested expression vector from the tube His-KR. The seventh position shows the fragment from digestion of purified PCR product for CBP-KR while ninth position represents fragments from digestion of expression vectorby NcoI and XhoI (from digestion tube labeled as CBP-KR).*

The concentrations of PCR fragments after gel extraction were 14.5 ng/ μl and 25.4 ng/ μl for His-KR and CBP-KR respectively while concentrations of extracted vector backbones were recorded as 18.7 ng/ μl and 14.3 ng/ μl for His-KR and CBP-KR respectively.

The numbers of colonies on control ligation plates were around 20 percent of the number of colonies present on respective test ligation plates and hence ligation reaction was successful. The DNA concentration of plasmids isolated from tubes H1, H2, H3, C1, C2 and C3 for clone verification were 46.1 ng/ μl, 137.4 ng/ μl, 57.8 ng/ μl, 34.7 ng/ μl, 53.3 ng/ μl and 49.5 ng/ μl respectively. The clone verification gel is shown in Figure 26. The clone H1 and H3 were verified for having pET28-N-His-KR while C1 and C3 clones were containing pET28-N-CBP-KR.

The OD_{600} of pre-culture His-KR and pre-culture CBP-KR were 1.38 and 1.27 respectively. The optical densities of flask labelled as His-KR and CBP-KR were 0.7 and 0.54 after 3 hours incubation and 2.6 and 2.4 on the next day. The images of His-KR and CBP-KR culture flask have been presented as Figure 27. The fluorescence signals from dilutions of 10^7, 10^6 and 10^5 cells from His-KR cultivation, CBP-KR cultivation and control cultivation are presented in Figure 28. Cells from His-KR cultivation and CBP-KR cultivation were emitting almost equal fluorescence signals and the minor differences in fluorescence signals arrised from dilution prepapration. The pictures of purified CBP-KR and His-KR are shown in Figure 29. The image of SDS-PAGE gel has been shown in Figure 30. The molecular weight of KillerRed is 27 kDa and the bands of KillerRed can be seen around the red 25 kDa band of the ladder. The smears appear as

the samples were stored at 4 ^{0}C for almost 1 week prior to SDS-PAGE. The smears probably indicate partial degradation of proteins. Repetition of SDS-PAGE provided similar gel.

Figure 26: *Gel electrophoresis for clone verification. From left to right first well was loaded with GeneRuler^{TM} DNA Ladder Mix, second well was loaded with control diges- tion/ verification reaction while next wells were loaded with digestion reaction from H1, H2, H3, C1, C2 and C3.*
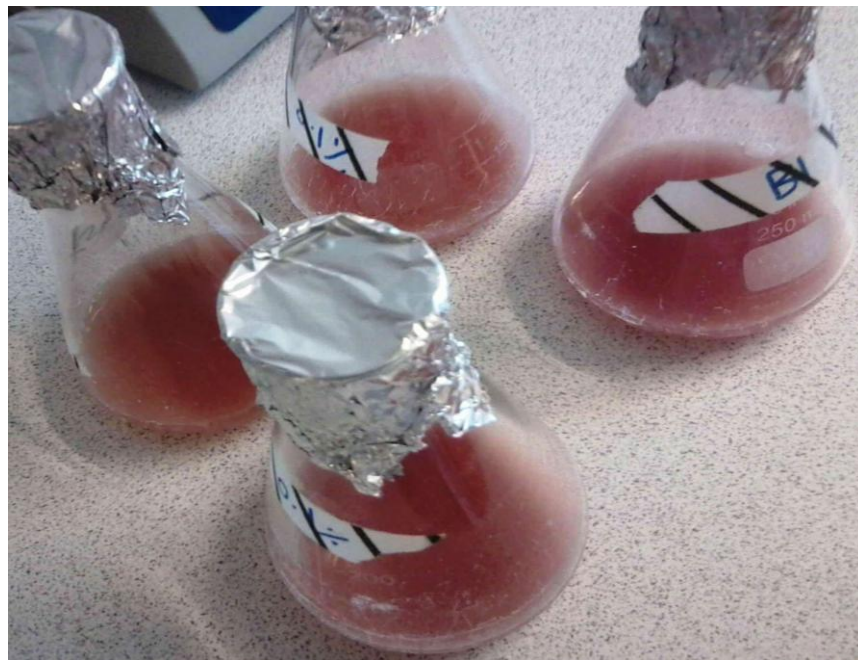
Figure 27: *Culture flasks with cell expressing KillerRed protein*
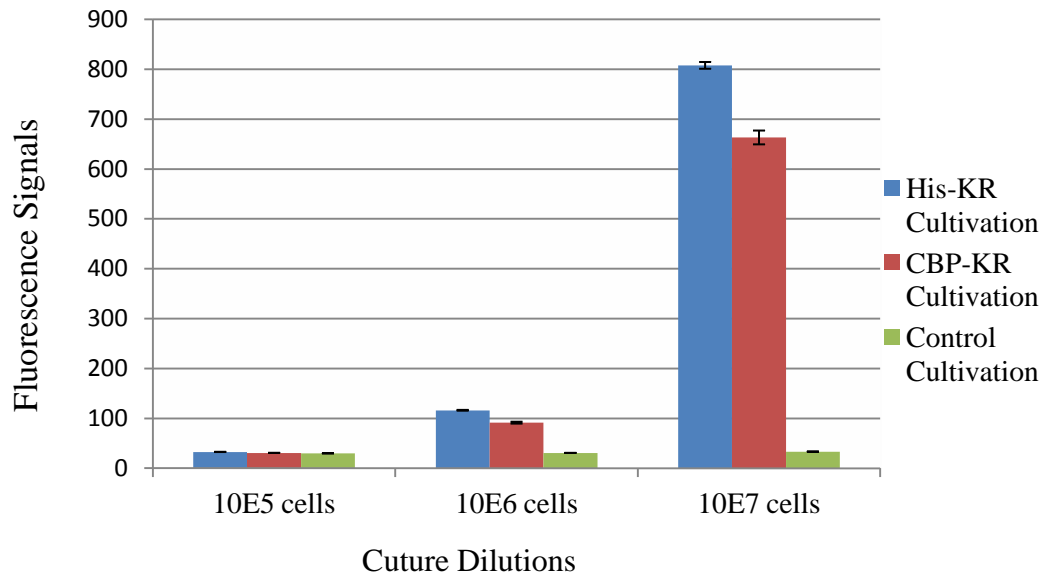
Figure 28: *Fluorescence signals recorded from equal dilution of cells from His-KR cultivation, CBP-KR cultivation and control cultivation. The control cultivation comprises of pET28 containing KRX competent cells. Fluorescence signals were recorded in duplicates and the error bar represents standard deviation for replicates.*
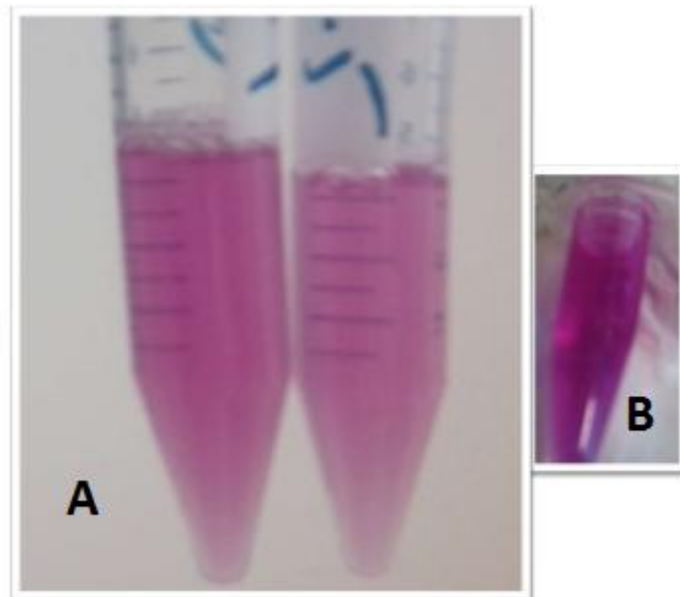
Figure 29: *(A) CBP-KR purified by Three-Phase Purification method and (B) His-KR isolated by Immobilized Metal Affinity Chromatography (IMAC).*
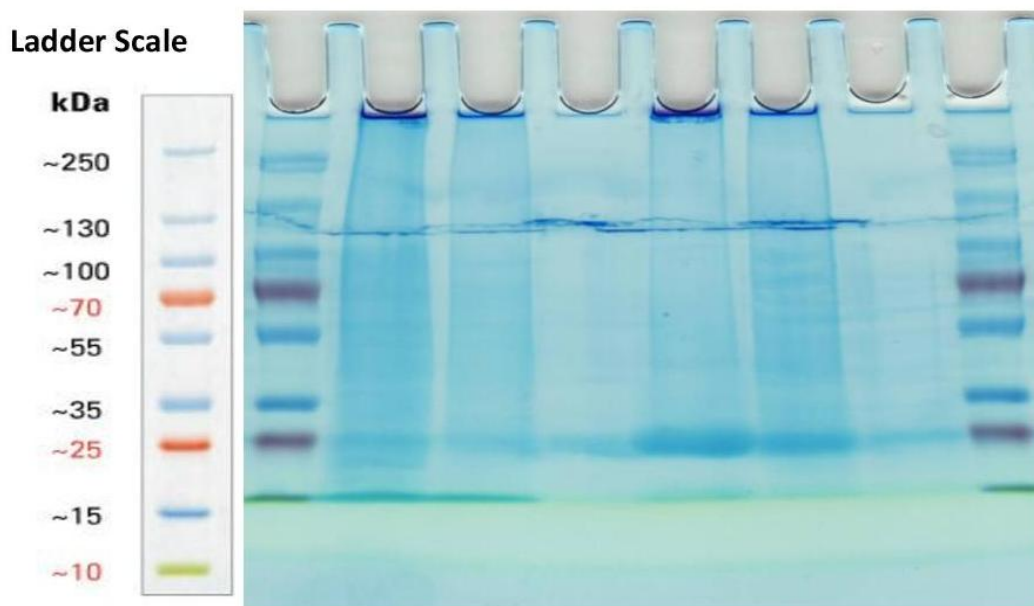
Figure 30: *Image of SDS-PAGE. From left to right first well was loaded with PageRuler^TM Plus Prestained Protein Ladder, second well with sample flow though, third with sample wash, fourth well with sample strip, fifth with purified His-KR, sixth well with stock CBP-KR solution, seventh with dilution of CBP-KR stock solution and last well was again filled with same ladder. The ladder scale on the left side represents SDS-PAGE gel for PageRuler^TM Plus Prestained Protein Ladder (Modified from ThermoSCIENTIFIC 2014).*

Before analyzing affinity of CBP-KR to cellulosic substrates, effect of Guanidine-HCl eluent on fluorescence signals of CBP-KR and His-KR was recorded. Five molar Guanidine-HCl eluent was found not affecting the fluorescence signals. A schematic diagram summarizing the affinity analysis is presented in Figure 31. The detailed results of affinity analysis are presented in Table 6 while key findings are plotted in Figure 32. The fluorescence signals from 1/200 dilution of His-KR stock solution were 460.4 and 462.8 while fluorescence signals from 1/100 of CBP-KR stock solution were recorded as 610.1 and 606.9. These signals were used as controls.

In the affinity analysis, a 43.5 % reduction in fluorescence signals was observed in supernatant collected from His-paper vial while a 38.6 % decrease was there in supernatant from CBP-paper vial after first incubation. The decrease can also be seen as a decrease of 201 fluorescence signals in the supernatant from His-paper vial while a reduction of 235 fluorescence signals in the supernatant from CBP-Paper vial. In elution phase, the elute collected from His-Paper vial after second incubation was emitting 32.3% of fluorescence signal compared to control His-KR dilution while elute from CBP-Paper was emitting 44.6 % signals compared to control CBP-KR dilution.
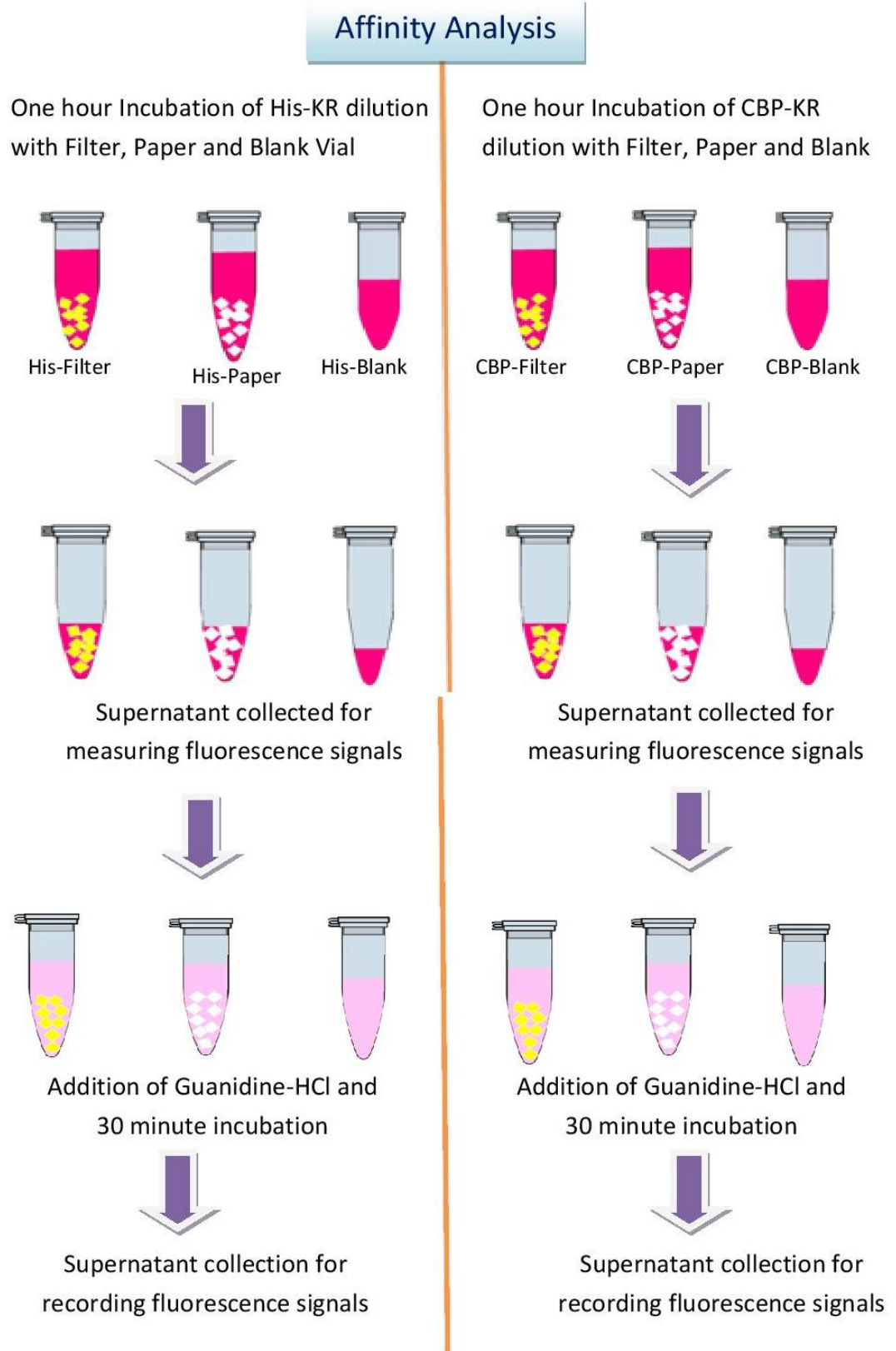
Figure 31: *Summary of the afinity analysis. Two phase analysis of CBP-KR affinity to cellulosic substrates (filter paper and printing paper). His-KR played a role of control in the experiment.*

Table 6: *Detailed results from the experiment analyzing affinity of CBP-KR to cellulosic substrates*

	Fluorescence Signals from Control			Fluorescence Signals from Test		
	His-Filter	His-Paper	His-Blank	CBP-Filter	CBP-Paper	CBP-Blank
Supernatant collected after first incubation	400.7, 406.7	260.0, 261.1	466.0, 469.7	568.0, 576.2	374.1, 373.1	610.2, 604.6
Supernatant collected after elution/ second incubation	126.7, 127.4	148.4, 149.4	125.0, 126.1	209.9, 210.3	269.1, 273.7	158.2, 158.4
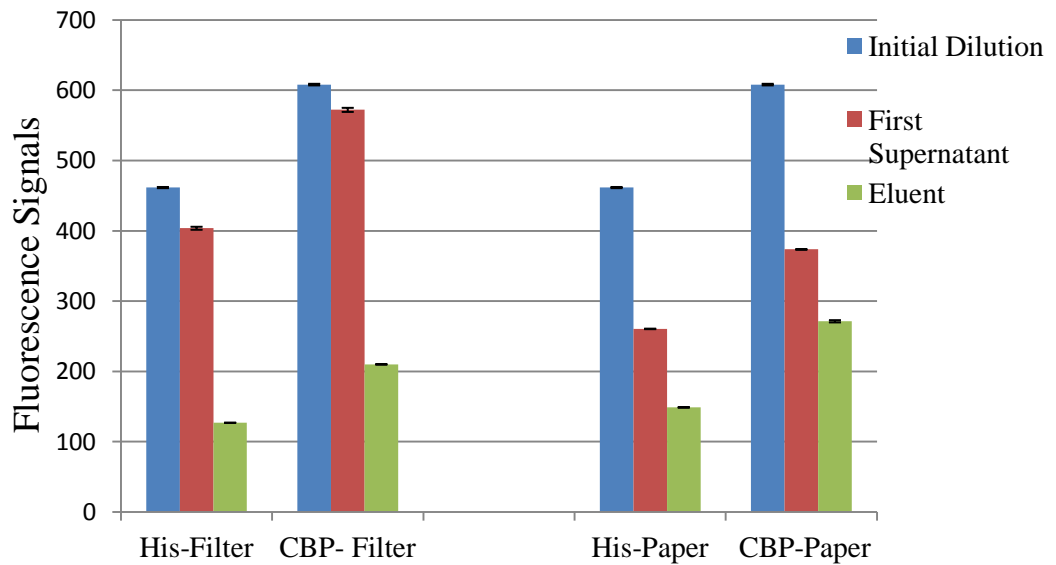
Figure 32: *Key results from the experiment analyzing affinity of CBP-KR to cellulosic substrate. The blue bars represent fluorescence signals from initial dilution which is considered as control dilution. The red bars show the fluorescence signals from the first supernatant collected after incubating cellulosic substrates and protein dilution. The green bars represent fluorescence signals from eluent collected in second phase of the experiment. Fluorescence signals were recorded in duplicates and the error bar corresponds to standard deviation for replicates.*

The results of the reanalysis of CBP-KR affinity to printing paper is shown in Figure 33. In the experiment His-KR dilution and CBP-KR dilution prepared were emitting approximately equal fluorescence signals. The signals from the dilution of His-KR were 334.8 and 342.6 while dilution of CBP-KR was emitting 346.8 and 344.8 fluorescence signals. These signals were used as controls. The results of the reanalysis of CBP-KR affinity to printing paper confirm binding of CBP-KR to printing paper. Supernatants collected after first incubation show a fluorescence signal decrease of 32.9% in the case of His-Paper vial while 60.7% signal reduction in the case of CBP-Paper. In elution phase, the elute collected from His-Paper vial after second incubation was emitting 38.2% of fluorescence signal compared to control His-KR dilution while elute from CBP-Paper was emitting 51.1 % signals compared to control CBP-KR dilution
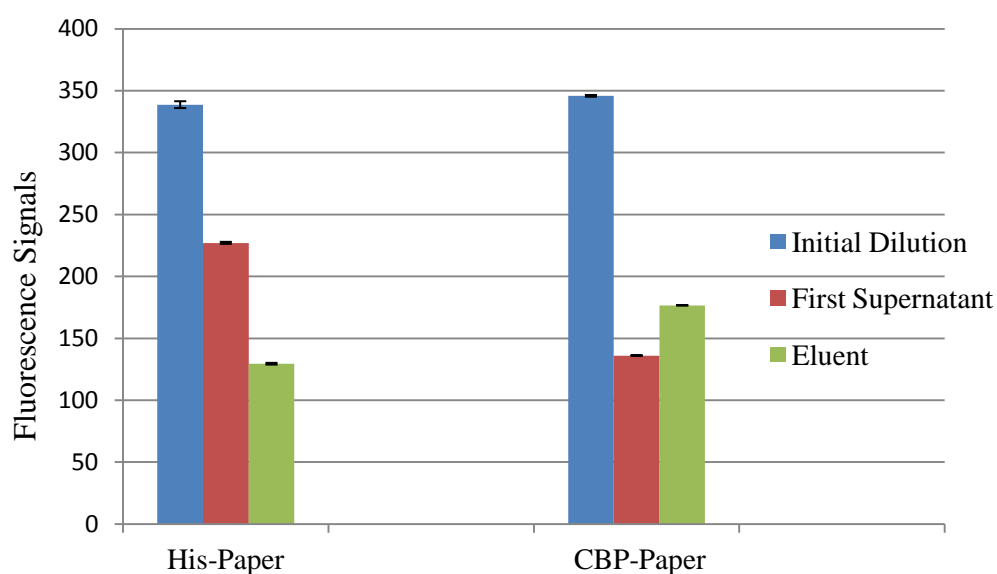
Figure 33: *Fluorescence signals recorded from the repetition of experiment analyzing affinity of CBP-KR to printing paper. The fluorescence signals from initial dilution are presented by blue bars while signal from first supernatant are presented by red bars. The final readings from eluent are shown in green bars similar to Figure 32. Fluorescence signals were recorded in duplicates and the error bar shows standard deviation for replicates.*

A schematic diagram summarizing the modified Congo red assay is presented in Figure 34 while the results of Congo red assay are presented in Figure 35. The average absorbance values from the Test vial and the Control vial (both added with 0.2% CMC) were equal and the value was 1.48 with a standard deviation of $\pm$ 0.02. Similarly the absorbance value of the 0.2% CMC Standard vial was 1.52 with a standard deviation of $\pm$ 0.01. An almost equal absorbance value from Test Vial and the 0.2% CMC Standard vial indicate absence of CMC degradation in the Test Vial.
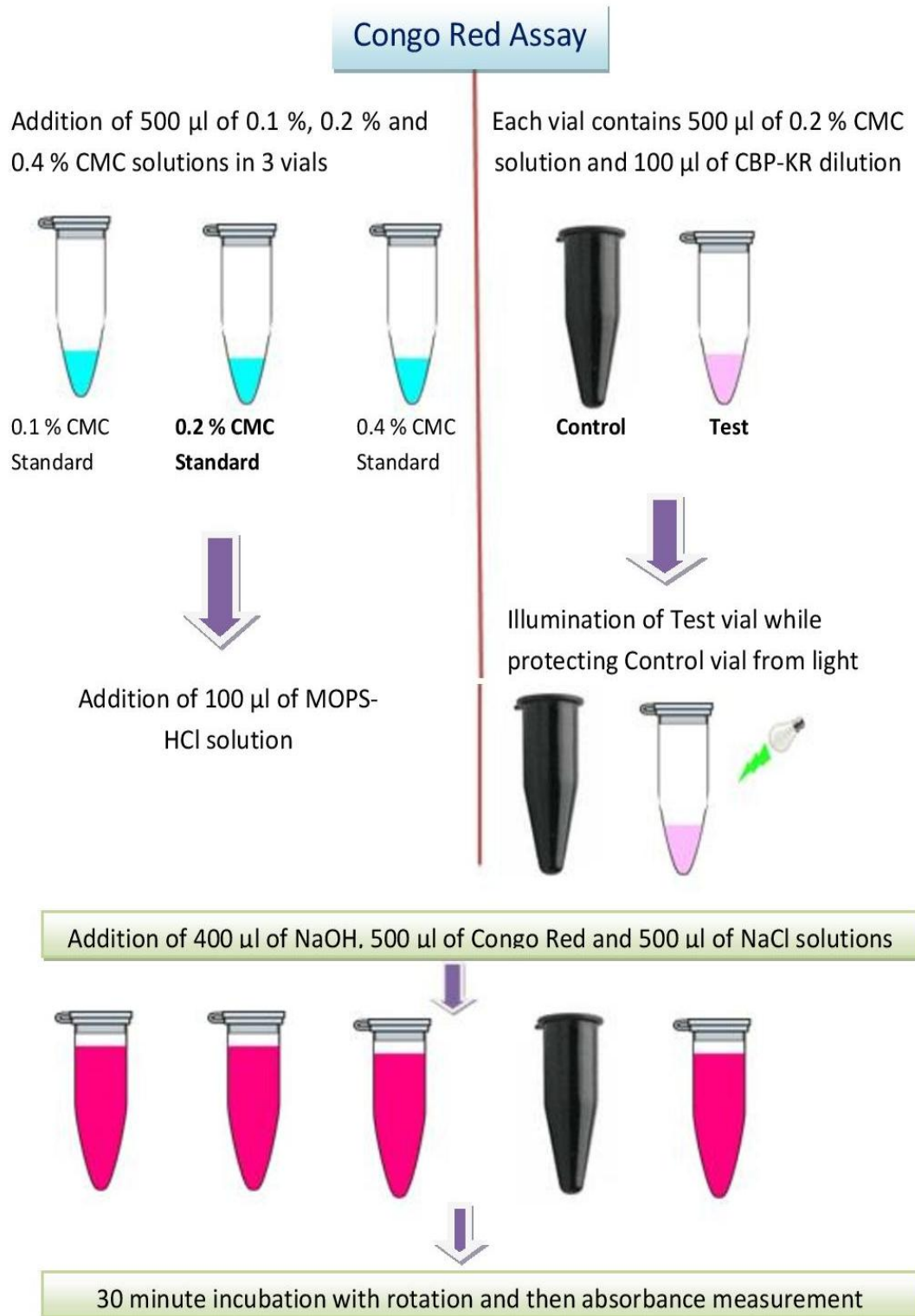
Figure 34: *Summary of the modified Congo red assay. After addition of corresponding CMC percent solution, Test and Control vials were added with CBP-KR while standard vials were added with MOPS-HCl. Then Test vial was illuminated and Control vial was protected from light. Lastly Congo red staining was completed and absorbance was recorded.*
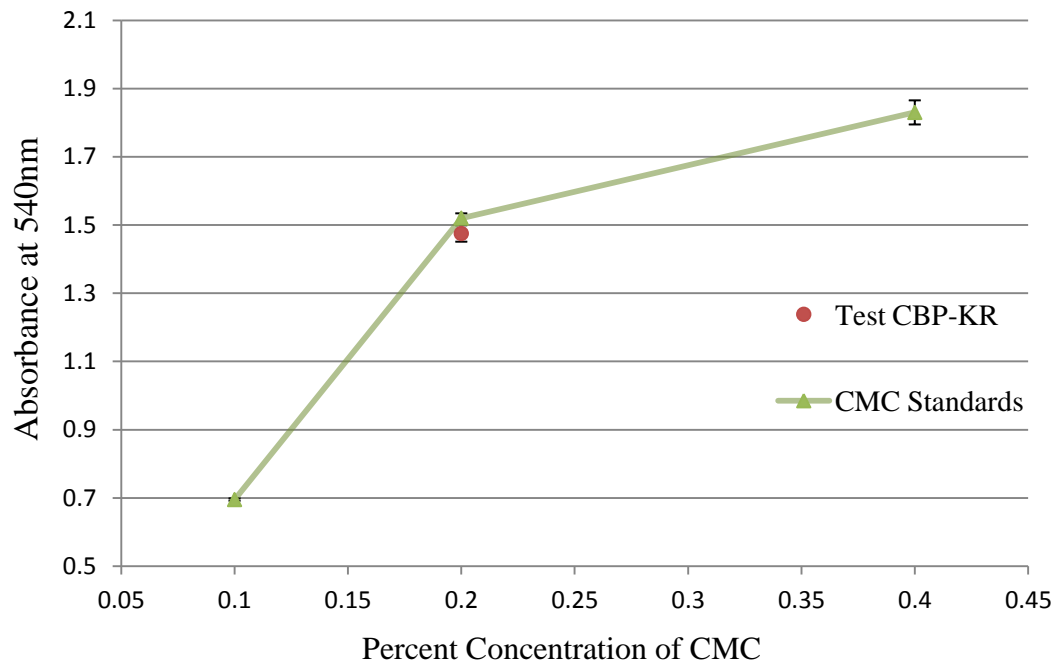
Figure 35: *Results of Congo Red Assay. The green line represents absorbance by CMC standard vials while red spot shows absorbance value of Test vial. The Test vial was added with 0.2% CMC and CBP-KR and it was induced with green light. Absorbance values were recorded in duplicates and the error bar demonstrates standard deviation for replicates.*

The Congo red assay was modified from Haft et al. (2011). Haft et al. utilized the absorbance shift property of Congo red dye, which is exhibited upon binding of the dye with soluble cellulose, and developed a simple, quick, quantitative assay to determine extent of cellulose degradation. The assay was named as Congo Red Analysis of Cellulose Concentration (CRACC) and was comprising of four steps. Initially cellulase and CMC were incubated. Secondly, the enzymatic digestion was blocked by addition NaOH. The third step was about incubating Congo red with NaCl. Lastly, change (decrease) in absorbance at 530 nm was recorded to estimate degradation of CMC.

In the current modified Congo red assay, enzyme was replaced with the engineered KillerRed protein, CBP-KR. The absorbance value of the 0.2% CMC Standard vial was set as standard for the Test vial while the Control vial was included to correct any possible interfering absorbance by CBP-KR. Although no obvious absorbance change (neither decrease nor increase) was observed in the Test vial, but some facts about Congo red assay were observed. Optimization of the assay revealed the importance of NaCl in the assay. A dynamic range of absorbance by Congo red dye (in relation to change in CMC concentration) was always requiring a final concentration of 0.5 M NaCl. Moreover NaOH was not required in our modified Congo red assay and Haft et al. used NaOH to block the activity of cellulases.

Prior to current experiments, histidine tag was added at C terminal of KillerRed. The pAK400 vector and XL1 Blue Competent Cell were employed but expression of the C terminal tagged KR was not successful due to some reasons. Moreover Cellulose fiber degradation by CBP-KR was scanned by routine microscopy but no gross breakage in fibers was observed.

6. DISCUSSION

Expression of CBP-KR and His-KR was although successful but testing affinity of CBP-KR to cellulosic substrate was somehow challenging. A simple approach of incubating CBP-KR with cellulosic substrate and then recording changes in fluorescence signals was adopted. The results from the first incubation experiment were not obvious. The percent decrease in fluorescence signals from the first supernatant collected from test vials (CBP-Filter, CBP-Paper and CBP-Blank) and control vials(His-Filter, His-Paper, His-Blank) were almost equal but higher fluorescence signals from the elute collected from CBP-Paper vial (vial initially incubating CBP-KR and printing paper) were recorded compared to other vials. Elute from CBP-Paper vial was giving 12.3 % higher fluorescence signals compared to His-Paper vial. This means higher affinity of CBP-KR to printing paper compared to affinity of His-KR to printing paper. To confirm the results of first incubation experiment, a second incubation experiment was performed. In the second experiment, the vials of CBP-Filter, His-Filter, CBP-Blank and His-Blank were excluded. The stocks of His-KR and CBP-KR were diluted step wise and dilutions of His-KR and CBP-KR with equal fluorescence signals were obtained. Equal fluorescence brought uniformity in the experiment and lead to final results showing clear and specific affinity of CBP-KR to printing paper.

Although a clear affinity of CBP-KR to paper was observed but reliability of results could be further improved by bringing uniformity in protein purification methods. So rather than isolating control protein His-KR with IMAC and test protein CBP-KR with three phase purification method, both test and control proteins could be purified by three phase method. Isolating both test and control proteins through three phase purification not only provides uniformity, but also simplify the methodology as need of His tagging the control protein will be omitted.

After affinity analysis, testing degradation ability of CBP-KillerRed was more challenging. Degradation of cellulose by CBP-KR might have happened in the current experiments but could not be detected by Congo red assay. Congo red assay is not suitable for analyzing minute cellulose degradations. The Congo red assay is considered as a sensitive assay for estimating degradation of CMC by cellulases but in the current research an alternative assay with higher sensitivity was required. Except sensitivity, an assay with lower detection limits can be more appropriate. Chromogen bicinchoninic acid (BCA) assay has lower detection limit. An improved version of BCA assay can even quantify sugars in range of 1 to 20 nmol concentrations. Unfortunately, BCA assay quantify all substances which can reduce copper and hence proteins also interfere the

assay. So presence of CBP-KR protein in our experiments also made BCA assay as an inappropriate option.

Besides BCA, an assay based on 3-Methyl-2-benzothiazolinonehydrazone (MBTH) has been reported for quantification of reducing sugars. MBTH assay does not rely on copper reduction and the assay is based on condensation of sugars and MBTH. So proteins and other reducing substances cannot interfere the assay. The MBTH assay is highly sensitive. For a 5 nmol concentration increase in sugars, an increase of around 0.2 in absorbance value at 620nm has been recorded. MBTH assay is linear for sugar concentrations up to 20 nmol and the linearity can be extended up to 100 nmol by dilution. (Anthon & Barrett 2002.) So MBTH assay can be an ideal choice for analysis of degaradtion capabilities of CBP-KR.

KillerRed is a fluorescent protein derived from a non-fluorescent chromoprotein, hydrozoan chromoprotein anm2CP. The excitation maxima for KillerRed is 585nm while emission maxima is 610nm. The extinction coefficient value of KillerRed at 585 nm is 45000 $M^{-1}cm^{-1}$. (Bulina et al. 2005.) KillerRed is a dimeric protein with an eleven stranded β-barrel structure, similar to GFP structure. In fluorescent proteins, β-barrel enclosing the chromophore limits the diffusion of oxygen and reactive oxygen species (ROS) from inside or outside the proteins. In contrast to other fluorescent proteins, KillerRed protein has a funnel shaped cavity under the chromophore. The cavity contains eight water molecules and serves as special pathway for movement of oxygen and ROS. (Carpentier et al. 2009.) The structure of KillerRed from X-ray crystallography has been shown in Figure 36.

KillerRed is the foremost genetically-encoded photosensitizer (Bulina et al. 2005). Being a photosentizer, KillerRed generates ROS upon light exposure and it possesses strong cytotoxicity (Carpentier et al. 2009). The current research analyzed the ROS generating property of KillerRed for its potential to degrade lignocelluloses as some of the pre-treatments of lignocellulose are already based on radicals. For instance pre-treatment of lignocellulose with hydrogen peroxide. Moreover the oxidative mechanism adapted by fungi for lignocellulose degradation is also ROS dependent. (Chaturvedi & Verma 2013.) Interestingly non catalytic proteins have been reported to enhance cellulases action. Swollenin protein from *Trichoderma reesei* is reported to disturb structure of cotton fibers without releasing reducing sugars. Swollenin has cellulose binding domain at N terminal. The gene sequence of swollenin is similar to plant expansins. Plant expansins break the hydrogen bonds present between polysaccharides of cell wall without hydrolysis. (Saloheimo et al. 2002.) Addition of bovine serum albumin (BSA) to the pre-treated corn stover has also been reported to improve cellulose degradation. BSA treatment prior to addition of cellulases increased glucose yield from 82% to 92%. BSA irreversibly binds to lignin and inhibits binding of cellulases to lignin substrates. (Yang & Wyman 2006.)

Except CBP, KillerRed can be added with cellulose binding domain (CBD). Addition of CBD would improve affinity of KillerRed to cellulose. For developing affinity of KillerRed to lignocellulose, lignin binding domain can also be appended to

KillerRed. Some lignin binding domains have been reported (Bianchetti et al. 2013 & Berlin et al. 2005) while new lignin binding domains can be identified by phage display method as adopted by Qi et al. (2008). Qi et al. utilized phage display libraries (Ph.D.-7 and Ph.D.-12 from New England Biolabs) for the biopanning process. In the simplest process from New England Biolabs, the library of phage-displayed peptides is incubated with target and after incubation the unbound phages are washed away. The bound fraction of phages are eluted and amplified. The amplified phages are again passed through additional 3-4 binding/amplification cycles and finally the enriched displayed phages are obtained and sequenced. In the New England Biolabs phage display system, M13 phages are engineered for pentavalent display of peptides and these clones peptides are fused to N-terminal of minor coat protein pIII.
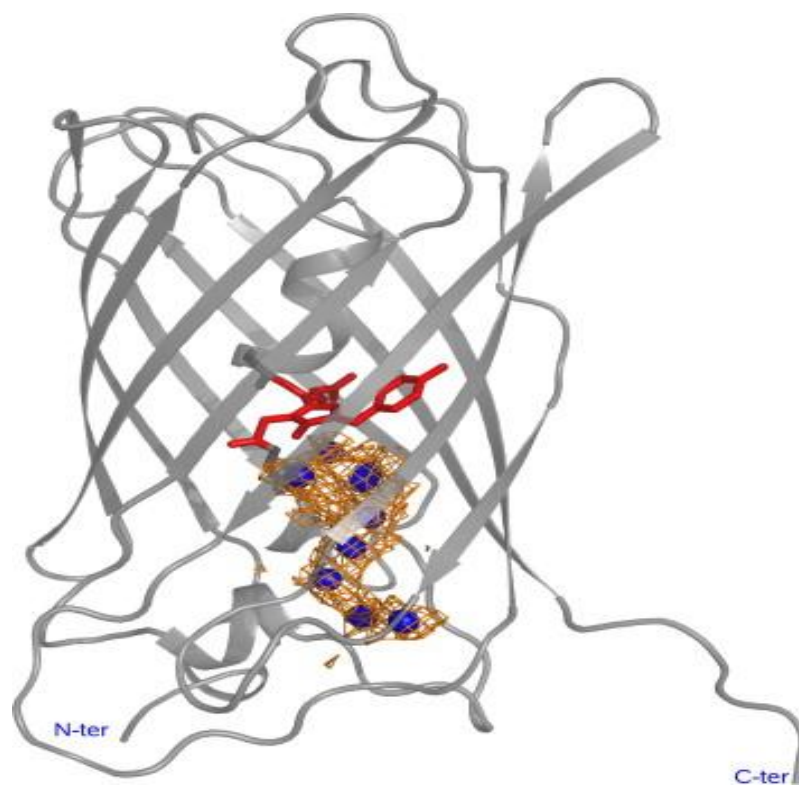
Figure 36: Structure of KillerRed monomer from X-ray crystallography. The backbone is presented with grey color, chromophore with red, cavity for ROS and oxygen transport is shown in orange color while water molecules inside the cavity are shown as blue spheres. (Carpentier et al. 2009.)

Fusion of KillerRed with CBD or lignin binding domain might not be successful due to the dimerization tendency KillerRed. Fortunately Takemoto et al. (2013) have resolved the dimerization problem by developing a monomeric variant of KillerRed. The monomeric variant posses 6 mutations (G3V/N145S/L160T/F162T/L172K/M204T) compared to KillerRed sequence. The variant was evolved by random mutagenesis and named as SuperNova. SuperNova fused with other proteins exhibited proper localiza-

tion and maintained ROS generating ability. So addition of CBP, CBD or lignin binding domain can also be tested with SuperNova.

Rather than analyzing capabilities of CBP-KR to degrade CMC, the focus should be ideally about examining degradation potential of CBP-KR for cellulose substrate. A consensus has been established that CBD has a little or no influence on hydrolysis of soluble substrates. Deduction of CBD from cellulase does not affect hydrolysis of soluble substrates but removal of CBD significantly decreases cellulase activity on insoluble substrates. (Linder & Teeri 1996.) Moreover Kruus et al. (1995) have showed improved degradation of crystalline cellulose after addition of a CBD to a cellulase catalytic domain. The catalytic domain was obtained from *Clostridium thermocellum* and a short scaffoldin was engineered which was containing a docking region and a CBD. Combining the scaffoldin with catalytic domain through the docking region enhanced the hydrolytic activity compared to sole catalytic domain.

The binding sites of CBP-KR on cellulose substrate and degree of cellulose degradation should be observed by electron microscope. Electron microscope based studies of lignocellulose degradation are already in practice. Akin & Benner (1988) utilized scanning electron microscopy to analyze degradation of bermudagrass leaf blades and whole cordgrass by ruminal bacteria and fungi. Scanning electron microscope differentiated three distinct levels of degradation based on the extent of fiber or component losses from tissue. Similarly Akin and Amos (1975) investigated pattern of degradation of forage grass via transmission and scanning electron microscopy. Akin and Amos additionally observed the ultrastructures involved in binding of large cocci to plant cells though electron microscopy. Degradation of sole cellulose has also been investigated by electron microscope. Chanzy and Henrissat (1985) observed degradation of *valonia* cellulose microcrystals by cellulases from fungus *Trichoderma reesei* through electron microscopy. Chanzy and Henrissat were also capable of differentiating exo/ endo actions of the cellulases employed. Moreover cooperative endo-exo actions of those cellulases were also observed by electron microscope. Except enzymatic degradation, chemical degradation of cellulose had also been studied. In 1953, Mukherjee and Woods employed electron microscopy to study breakdown of cellulose from ramie and cotton fibres by sulphuric acid. Interestingly electron microscopy is not only utilized for studying degradation of wood or lignocellulose but also used for studying wood structure and morphology.

7. CONCLUSION

Unfortunately the demands and prices of the environment unfriendly fossil fuels are ever increasing. To overcome human dependence on fossil fuel, there are worldwide efforts to develop an alternative renewable fuels which are cost effective and environmentally safe. For developing such alternative biofuels synthetic biology has potential. By using synthetic biology approaches, alcohols, esters, ethers, hydrocarbons, fatty esters, fatty alcohols and waxes biofuels have already been synthesized from carbohydrate content of lignocellulose. Lignocellulose is the most plentiful renewable biomass on earth as fifty percent of the mass produced by photosynthesis encompasses lignocellulose and among the lignocellulose waste generated from agriculture and forestry, only a minute proportion is utilized by humans. Lignocellulose being a non-food source and low cost feedstock has been focussed by many researchers for the synthesis of biofuels but its recalcitrant nature is the major barrier. Isolating components of lignocellulose is an extensive and costly process. Similarly enzymes utilized to convert carbohydrate content of lignocellulose into smaller sugar units are expensive. So considering these cost contributors, we applied synthetic biology approach to invent a system of cellulose degradation by ROS. We focused the synthetic biology approach of developing interchangeable parts and hence developed a cellulose binding and ROS generating protein, CBP-KR.

Upon testing CBP-KR, we found it binding with printing paper but we could not observe any extensive degradation of CMC by CBP-KR. So we found the ROS generating property of the cytotoxic KillerRed not competent enough to effectively break down cellulose. Importantly, the current research method does not completely exclude degradation potential of KillerRed and minor degradation of cellulose might have happened. There is a need to further investigate the extent of degradation capabilities of KillerRed by employing other sensitive means (like MBTH assay) and to reevaluate critically the degradation potential of ROS from excited KillerRed protein. Unfortunately the current study could not be further expanded due to constraints of time, finance and resources.

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APPENDIX A

Nucleotide Sequence of ab135
5´-TAATAGTCGACTTAATCCTCGTCGCTACCGATGG-3´

Nucleotide Sequence of vs13_1
5´-TAATACATATGGGTTCAGAGGGCGGC-3´

Nucleotide Sequence of vs13_3 (the primer including CBP sequence)
5´-TAATACCATGGCGACCCATAAAACCAGCACCCAGCGTCTGCTGGCAGC
AGGTTCAGGTCATATGGGTTCAGAGGGCGGCC-3´

Amino Acid Sequence of the CBP from Qi et al. 2008
THKTSTQRLLAA

APPENDIX B

Agarose Gel Preparation

One percent agarose gel was prepared by adding three gram agarose powder in 300ml of 1X TAE (Tris-Acetate-EDTA) buffer. The solution was boiled in a microwave and then cooled to 50°C to add 30 µl Syber Safe from Invitrogen, USA. After adding Syber Safe, the solution was mixed gently and poured in a template set with combs. After gel solidification, the combs were removed and the gel was transfer to electrophoretic tank containing 1X TAE. The buffer level was adjusted to have few millimeter layer of buffer on the top of the gel. Lastly the wells were loaded and electricity was applied.

LB Medium Preparation

In 1 liter Milli-Q water, 10 gram tryptone, 5 gram yeast extract and 5 gram NaCl were added. Then the medium was sterilized by autoclaving.

SB medium Preparation

In 1 liter Milli-Q water, 30 gram tryptone, 20 gram yeast extract and 10 gram MOPS were added. Then the medium was sterilized by autoclaving.

LA Plate Medium Preparation

For preparing LA plate medium, 10 gram tryptone, 5 gram yeast extract, 5 gram NaCl and 15 gram agar were added in 1 liter Milli-Q water. The medium was sterilized by autoclaving and cooled to 50°C to add antibiotic and glucose. The final concentration of glucose was kept at 0.2%.

Fifteen Percent Glycerol Preparation

Seventy five milliliter of glycerol was added in 425 ml of Milli-Q water. The solution was mixed and sterilized by autoclaving.

Eighty Percent Glycerol Preparation

Twenty milliliter of Milli-Q water was added in 80 ml glycerol. The solution was mixed and sterilized by autoclaving.

Ten Percent L-Rhamnose Stock Solution Preparation

One gram of L-Rhamnose was added in 10 ml of Milli-Q water and mixed well.