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GENETIC ENGINEERING, A HOPE FOR SUSTAINABLE BIOFUEL PRODUCTION: REVIEW

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Abstract

The use of recently developed genetic engineering tools in combination with organisms that have the potential to produce precursors for the production of biodiesel, promises a sustainable and environment friendly energy source. Enhanced lipid production in wild type and/or genetically engineered organisms can offer sufficient raw material for industrial transesterification of plant-based triglycerides. Bio-diesel, produced with the help of genetically modified organisms, might be one of the best alternatives to fossil fuels and to mitigate various environmental hazards.
Key words: Biodiesel, Gene, Global Warming, CO_2-Emission, Lignin, Cellulose, Ethanol

Introduction

Petroleum is the major source of energy being used as fuel in kitchen, transportation, industry, etc. However, our fuel resources are running out due to an unsustainable consumption by an ever increasing world population. Indeed, a 50% growth in fuel demand by 2025 has been projected due to the rapid increase in energy consumption especially in rapid developing countries (Arthur et al., 2006). The indiscriminate extraction and use of limited reserves of petroleum has caused not only a reduction in fossil fuel reserves but also led to environmental deterioration. Depletion of fossil-fuel reserves combined with the increase in global temperature due to increased level of atmospheric carbondioxide (CO_2) are inevitable twin problems of burning fossil fuels. Large efforts in search of alternative and sustainable sources of energy to substitute or at least

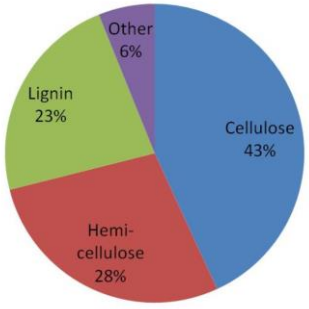

Fig 1: Key global biomass resources from agricultural residues, wood, and herbaceous energy crops (Modified Arthur et al 2006).

to supplement fossil fuel being made all over the world. The day-to-day increase in the demand for fuel is still being matched by petroleum production but being a limited resource and uncertainty in its availability in the future are major triggering factors for this massive global research in alternative energy sources. Bio-fuel specifically biodiesel derived from vegetable oils, holds great promises for the future because of its renewability, better quality of exhaust gas emission, biodegradability, photosynthetic origin of atmospheric carbon, non-toxicity and it is essentially free of sulfur & aromatics. Biodiesel is not only an unlimited alternative fuel for diesel engines but it also has the potential to reduce the level of pollutants and potential carcinogens (Sing and sing, 2010).

Carbohydrates, the first stable photosynthetic product in plants, are the major chemical constituents of the cells that can be fermented into ethanol (utilizable bio-energy) and carbon dioxide in anaerobic condition using appropriate organism (e.g. yeast). The cell wall of plants ontributes to the structural framework and strength of the plant and is primarily made up of carbohydrates. There are three main components to the plant cell wall: cellulose, hemicelluloses and lignin. The relative amount–of these components varies by species and cell type. For instance, xylem of trees has ~40-45% cellulose, 25-30% hemicelluloses, 20-25% lignin and the rest is ash, pectin, etc (Fig. 1). Cellulose and hemicellulose act as rebar and lignin as concrete of the cell wall. This combination of biopolymers is found in wood, gramineous plants and in the hard stems of some annual species and is known as lignocellulose. Solely glucose monomers are used for the synthesis of cellulose. Unlike cellulose, hemicellulose is composed of a combination of different sugars such as the five carbon long aldopentoses xylose and arabinose and the six carbon long aldohexoses mannose and galctose. However, the monomer of p-hydroxycinnamyl alcohol, dimethoxylated (syringyl, S), monomethoxylated (guaiacyl, G) and non-methoxylated (p-hydroxyphenyl, H) phenylpropanoid units are used for the oxidative coupling reactions during lignin synthesis.

That natural agrarian and forest residues represent an abundant cellulose and ultimately renewable source of energy as ethanol, the fermented product. The cellulosic ethanol not only replaces gasoline but also reduces green house gas emission into the environment thereby decreasing the rate of global warming. As a result, several governments are aiming to establish ethanol as a substitute of gasoline. For instance, the Energy Independence and Security Act of 2007 sets goals of 36 billion gallons of ethanol per year to be blended with gasoline by 2022 and 16 billion gallons/year must come from cellulosic sources by 2022. Nevertheless, the hydrolytic procedures needed to release fermentable carbohydrates from biomass polysaccharides, especially lignin, becomes an obstacle because of the associated high cost. Although, hydrolysis of cellulose and hemicelluloses is relatively cheap, the recalcitrant lignin associated with cellulose and hemicelluloses, becomes a barrier to commercialize cellulosic ethanol (Martinez et al., 2008).

Nature of lignin

Lignin is a heterogeneous polymer, synthesized by free radical coupling mechanism from phenolic cinnamyl alcohol residues. The monomers are interconnected through carbon-carbon, β-O-4-linked ether bonds that are highly resistant to chemical hydrolysis. The β-O-4-linked ether represents about half of the total lignin component, followed by phenylcoumarans, resinols, and various minor subunits (Fig 2, Fig 3).

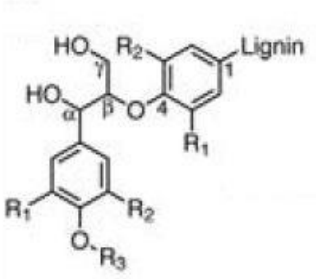

Fig 2: The principal β-O-4 structure of lignin.

Lignin is a racemic mixture of isomers of the β-O-4-linked monolignols. The number of isomers increases geometrically with increasing the number of subunits. The three dimensional surface of the lignin is complex and non-repeating (Hammel and Cullen, 2008).

Natural Degradation of Lignocellulose

After cellulose, lignin is the most abundant biopolymer on earth. None of the aromatic biopolymer or even aliphatic biopolymer can outperform it in resistance to oxidation and as a result it is extremely recalcitrant to degradation. This recalcitrant lignin in association with cellulose and himicellulose creates a barrier to penetration of any lignocellulolytic enzymes or any other solutions into the interior structure of the plant cell. Some naturally occurring fungi, (e.g. basidiomycetes ascomycetes, etc.) are able to break the covalent linkages between two moieties of monolignoles monomers or to mineralize lignin. These modifications increase the susceptibility of lignin towards degrading enzymes using either a single or a combination of extracellular ligninolytic enzymes (Dashtban et al., 2010).

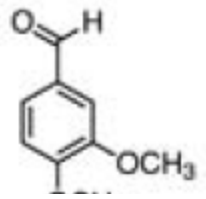

Fig 3 Veratryl alcohol, lignin metabolite.

In nature, more than 90% of all angiospermic wood is degraded by White-Rot fungi. Usually guaiacyl (G) units of lignin are more resistant to degradation as compared to syringyl (S) units. These enzymes include two broad classes of phenol oxidase (laccase) and heme-peroxidases. Heme-peroxidases are sub divided into lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP). These enzymes are rather large in size (e.g. molecular mass- laccase: 60–80 kDa, MnP: 30–62.5 kDa and LiP: 38–42 kDa) and therefore they need smaller but active enzyme accessories like hydrogen peroxide generating oxidases and non-enzyme species of free hydroxyl radicals (•OH) called mediator to enter the tightly packed lignin (Dashtban et al., 2010). Laccases is considered to operate in conjunction with diffusible redox mediators and iron-reducing systems use free Fe^{2+} to generate hydroxyl radicals (Hammel and Cullen, 2008).

All or some of these enzymes are randomly distributed among the fungal members and also found in some other saprophytes like bacteria, termites, etc. Laccase, manganese peroxidase (MnP), lignin peroxidase (LiP) and versatile peroxidase (VP) are in the front line of proteins

expressed during the fungal catabolism of lignin. Versatile peroxidase shares the structural–functional properties of LiP and MnP. Moreover, LiP can catalyze difficult chemical transformations. Because of this ability, this enzyme is the point of attraction of the scientific community not only as the potential candidate in bio-remedial waste treatment but also as an agricultural and environmental tool (Akbar et al., 2013).

Lignin peroxidases

Lignin peroxidases (LiPs) were first reported from *Phanerochaete chrysosporium* growing in medium under nitrogen scarcity. They are iron containing hemoproteins. The four heme tetrapyrol nitrogen and a histidine residue of this monomeric protein establish five covalent co-ordination bonds with Fe^{3+}. Upon two-electron oxidation by hydrogen peroxide, this enzyme forms a Fe(IV) containing intermediate (compound I) with the characteristic cation free radical on the tetrapyrrole ring (or on nearby amino acid) (Hammel and Cullen, 2008). One electron is then transferred from the donor substrate to Compound I, that yields a substrate-free radical and Compound II. Like as Compound I, Compound II iron also remains in the Fe (IV) state, but the tetrapyrrole becomes a free-radical. The second molecule of substrate gets oxidized by Compound II, yielding another substrate-free radical and the enzyme peroxidase enters the resting state. Unlike other peroxidases, lignin peroxidase can oxidize moderately activated aromatic rings by electron-donating substrates and is capable of abstracting an electron from aromatics that carry only two or three ether bonds and thus resemble the major non-phenolic structures of lignin (Hammel and Cullen 2008). LiPs become stronger oxidants as compared to classical peroxidases because the iron in the porphyrin ring is more electron-deficient and a tryptophan residue – trp171 is present in an exposed region on the enzyme surface enabling the enzyme to participate in long-range electron transfer from aromatic substrates that cannot make direct contact to the oxidized heme. This second characteristic plays a major role to enable LiPs to oxidize bulky lignin-related substrates directly (Hammel and Cullen, 2008).

Coding lignin peroxidase

LiP belongs to the class II of fungal secretory peroxidases. There are over 65 fungal lignin targeting protein coding genes that have been identified based on sequence homology comparison and were submitted to GeneBank as of 2014. They are characterized by 8-9 introns of 49-78 base pairs each and the mature proteins are composed of 343-345 amino acids. LiP exists in several different isoforms and the number of isoform depends on the species. Gaskell (1994) identified 10 genes dedicated for the synthesis of LiPs and designated them as lipA through lipJ from *Trametes versicolor*. Gene analysis of LiP proofs that different but homologous open reading frame are responsible for the synthesis of each isoforms. Every LiP protein consists of 343–345 amino acids with a 27–28 secretion signal peptide.

Manganese peroxidases (MnPs)

Although, lignin peroxidase is considered the central lignolytic enzyme of White Rot fungi, many species in this group actually lack lignin peroxidase and express alternative peroxidases.

Manganese peroxidases (MnPs) is one of these alternatives. Extensively researched and more widespread than LiPs, MnPs belong to a different group of secreted enzymes. They differ from LiPs by lacking the invariant tryptophan residue 171 that plays a central role for electron transfer to aromatic substrates in LiPs. Because of this lack of an activated tryptophan MnPs cannot oxidize non-phenolic lignin related structures directly. Unlike LiPs, MnPs have one iron propionate group in addition to several acidic amino acid residues which collectively form a manganese binding domain. The mechanism of catalysis is similar to LiPs. Instead of Fe^{3+}, Mn^{3+} plays the major role here associated with peroxidase. (Hammel and Cullen, 2008).

Coding MnP

Genomic analysis of *Phanerochaete chrysosporium* reveals that it encodes for five MnPs assigned as *mnp1* through *mnp5*. The genes mnp1, mnp2 and mnp3 are better studied and cDNAs have been isolated (Janusz et al., 2013). Whereas the two genes, *mnp4* and *mnp5* were recently reported and identified with the Basic Local Alignment Search Tool (BLAST) against data bases. In the number and position of introns, these five gene sequences are remarkably conserved. MnP proteins coded by those genes have 330–370 amino acids sequence and they contain 21-29 long amino acid sequence as a leader peptide as well as short sequences including N-terminal end. Met-Ala-phe and Ala-Ala-Pro are conserved sequences in the N-terminal end in each mnp genes and the typical MnP secretion signal peptides. Gene *mnp5* corresponds to the N-terminal amino acid sequence of MnP (Janusz et al., 2013).

Versatile peroxidase

Substitution of iron by Mn^{2+} in LiP or the insertion of a tryptophan residue analogue to LiP into MnP, by site directed mutagenesis, yield chimeric enzymes that shows activities similar to both enzymes LiP and MnP. However, recently, enzyme of this type have been found to occur naturally in various species and were termed versatile peroxidase (VP) (Hammel and Cullen 2008).

Biodiesel

The National Soy Diesel Development Board [presently National Bio Diesel Board (USA)] coined the term biodiesel in 1992 as a synonym for the long chain mono alkyl esters formed by transesterification of triglycerides (TGA) with short chain alcohols. These chemicals are also referred to as fatty acid methyl ester (FAME) because during the transesterification reaction usually methanol is used in presence of a catalyst such as NaOH or KOH.

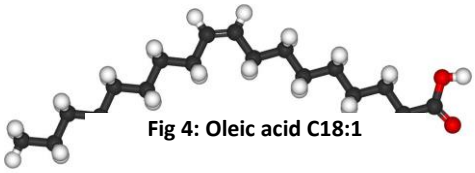

Fig 4: Oleic acid C18:1

The co-product of the biodiesel production process is glycerin. The TGAs are obtained from plant oils and/or animal fat (lipids). Naturally occurring fat or oil consists of 80-90% triglycerides (three fatty acids and one glycerol) and small amounts of mono- and diglycerides. These lipids (fats and oils) from different sources have different compositions (Fig 5) and physical and chemical properties because of variation in the length of the carbon chain and different numbers and positions of unsaturated bonds (Biodiesel Handling and Use Guide (4th edition) 2008).

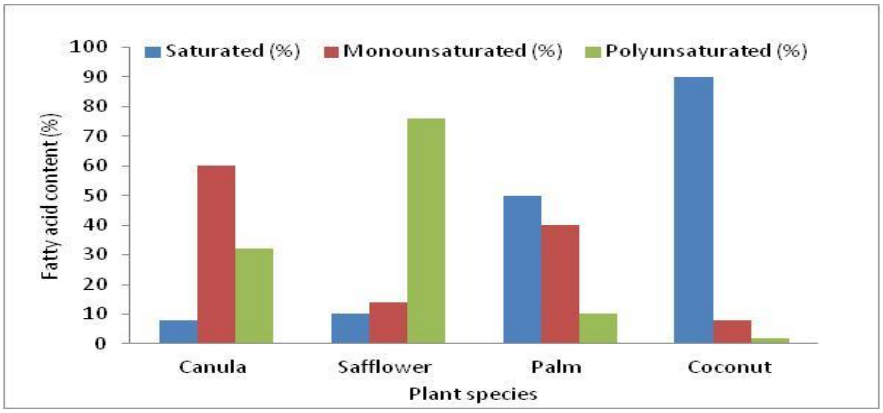

Fig 5: Composition (saturated fatty acid content) of various Biodiesel Feedstock (Source: Biodiesel Handling and Use Guide (4th edition) 2008

Vegetable (plant derived) oils are liquid at room temperature but are highly viscous, low volatile, and have poor cold-flow properties. However, animal fats are solid at room temperature since they contain higher levels of saturated fatty acids. Because of these properties the plant oils or animal fats need to be modified in order to be used as biodiesel before fueling engines. These lipids (fat/oils), either edible or non edible fatty oils, are used as the raw material for biodiesel production. They are obtained from linseed, coconut, soya bean, rapeseed, palm, sunflower, canola, or cotton seed, jatropa, and others. Soybean is the most common feedstock in USA, whereas rapeseed (canola) in Europe and palm oil in tropical countries are most common crops for biodiesel production. The source of biodiesel depends mainly on the crop availability according to the regional climate (Singh and Singh, 2010 and Vasudevan and Briggs, 2008).

Viscous vegetable oils with low volatility and poor cold flow properties of raw or recycled greases reduce-the life of diesel engines. This is due to accumulation of deposits in the engine, lube oil gelling, and other maintenance problems including ring sticking at high concentration. However, dilution of these vegetable oils with petroleum, after transesterification reaction, yields better result. Unlike vegetable oils, straight or branch chain or aromatic configuration of hydrocarbon present in petroleum diesel offers better ignition quality (Biodiesel Handling and Use Guide (4th Edition) 2008).

Transesterification

Transesterification is the process of releasing individual methylated fatty acid from triglycerides and methanol in presence of a catalyst. The catalyst may be an acid, base, or other biocatalyst but most often for industrial application, NaOH or KOH are used. Since the reaction is reversible, alcohol is used excessively to shift the equilibrium towards the glycerol + methyl

ester reaction side. In general, the alcohol is methanol but alcohols up to 8 carbon atoms are used for this reaction and hence it is also known as alcoholysis. The process is a consecutive but reversible reaction. In each step one mole of ester is produced. The triglycerides consequently get converted into diglycerides, monoglycerides and ultimately glycerol (Fjerbaek et al., 2009).
Alkaline transesterification is the most commonly used industrial method. However, for industrial use, the raw materials need to be pretreated with an acidic catalyst to reduce soap formation (saponification) during the reaction. Pretreatment is also necessary for separation of biodiesel and glycerol, the catalyst, and the alkaline wastewater. (Fjerbaek et al., 2009; Singh and Singh, 2010).

In acid catalyzed transesterification, carbocation, the target of the nucleophilic attack of the alcohol to form a tetrahedral intermediate, is produced due to protonation of the carbonyl group of the ester after acid treatment. The tetrahedral intermediate eliminates glycerol forming a new ester and regenerates the catalyst.

Biocatalyst (eg. lipase enzyme, derived from bacteria and fungi are the most commonly used enzymes) mediated transesterification is almost similar to the alkali catalyzed reaction but the ratio of catalyst and stirring time needs to be seriously considered. Lipase transesterification of TGA with an alcohol involves a two-step mechanism: hydrolysis of the ester bond and release of the alcohol moiety and esterification of the second substrate. Contrary to the alkaline transesterification, enzyme mediated reactions do not form soap and increase the efficiency of esterification of both free fatty acids and triglycerides (Fjerbaek et al., 2009; Singh and Singh, 2010). In addition, enzymes are potentially useful because the enzymes are especially compatible to the substrate, reusable, require fewer steps in the process (i.e. less energy), drastically reduced water consumption, and yield a higher quality glycerol. However, currently enzymes are not being used industrially because of their slow reaction rate, the high cost of enzymes themselves and the loss of their activity typically within 100 days of operation (Fjerbaek et al., 2009).

The relevance of genetic engineering

Any biological system is basically a pool of orderly orchestrated enzymatic reactions. In cellular metabolic activity, thousands of organic macromolecules are being synthesized and broken down simultaneously. Metabolic pathway engineering is a branch of genetic engineering that aims to either increase or decrease certain metabolic compounds or groups of compounds. A rate limiting enzyme of an enzymatic pathway can be over expressed to increase the production of target compounds in an organism or a pathway can be transferred to another organism. Also, genes can be 'knocked out' to decrease the production of unwanted compounds using various approaches, for example over-expression of antibodies against the enzyme of choice or by the use of RNAi technology aimed to reduce the corresponding mRNA of the unwanted protein (Verpoorte and Memelink, 2002). In general there are two approaches to increase the production of the desire compound. The first approach is to change the expression of regulatory genes that control structural genes downstream of multiple biosynthesis genes, and the second approach is changing the expression of specific genes to overcome specific rate -limiting steps in the target

pathway that results in decreased catabolism of target compounds combined with inhibition of competitive pathways (Verpoorte and Memelink, 2002).

The rising complications of the relentless use of fossil-fuels and the extreme scientific progress towards synthetic genomics and metabolic engineering in last few years, led to the development of genetically engineered organisms (GMOs) as promising strategy to produce 'green fuels'. The possible alternatives for petroleum, discovered so far are hydropower, geothermal power, wind power, nuclear power and bio-energy. Presently none of those technologies can entirely substitute fossil fuel solely or in combination, because of the requirement of large amounts of money and energy involved in these processes (Fjerbaek et al., 2009).

Ethanol, the first generation biofuel, is derived from anaerobic microbial digestion of sugars and competes spatiotemporally with food crops and food itself. Ethanol's low energy density, hygroscopic nature, and the competition with food crops are some limitation of 1st generation bio-fuels that triggered investigation into genetically modified organisms to produce alternative fuels comparable to petroleum. For the production of alcohols, alkyl esters, fatty acid and other biofuel products, bacteria, yeast, cyanobacteria and several algal members are considered as potential host organisms (Fjerbaek et al., 2009). In the second generation of bio-fuel, lignin is the major barrier to produce cellulosic ethanol. In the third generation of bio-fuel, cultivation, harvesting, extraction of potential bio-fuel from the host are the limiting factors in terms of energy and cost for commercial production. (I would like to denote bio-fuel produced from GMOs as the third generation bio-fuel).

Many organisms naturally produce compounds that can be considered advance biofuels. Among thousands of organisms, algae are mostly likely the best candidate to accumulate proportionally high amount of biofuel molecules. On the basis of lipid profile and productivity, the credit for large proportion of the ancient deposition of biological compounds that led to today's crude oil reserves can be attributed to diatoms, a member of microalgae (myxophyceae). Also, various scientific efforts demonstrated that many other microalgae have bio-fuel producing properties. For instance, The U.S. Department of Energy's Aquatic Species Program recommended approximately 3,000 different microalgae for their potential to produce biofuels (Radakovits, 2010).

To advance the potential of microalgae in biofuel production, significance advances have been achieved in microalgal genomics. For instance, various databases like nuclear, mitochondrial and chloroplast genome, and expressed sequence tag (ETS) have been established and several similar projects are ongoing. Tools for the knock-down and expression of transgenes have been developed for *Chlamydomonas reinhardtii* and being developed for diatoms and other algae like *Phaeodactylum tricornutum* and more than 30 other species that are of great interest for industrial applications. A number of direct and indirect gene transfer techniques have been adapted in algal cell transformation. *Agrobacterium tumefaciens* mediated gene transfer is a very common indirect method of gene transfer. Direct methods consist of physical and chemical methods like agitation in the presence of glass beads or silicon carbide whiskers, electroporation, biolistic microparticle and bombardment with a DNA fragment. Development of high throughput

artificial-micro-RNA (armiRNA) techniques are supposedly more specific and stable gene-knockdown strategies than traditional RNA interference (RNAi) approaches for *C. reinhardtii* (Radakovits, 2010; Dyera and Mullen, 2008).

Currently, the focus in technology is to develop engineered organisms which can secret bio-fuel compounds in their extracellular milieu or techniques to modify organisms genetically to make them to secrete (metabolic engineering) the product of interest continuously to reduce energy and costs (Dunlop, 2011). In addition, scientific effort is concentrated to achieve modified and increased accumulation or secretion of advance biofuel compounds like polysaccharides, lipids, hydrocarbons, alcohols, and other potential energy storage compounds in photosynthetic and heterotrophic organisms (yeast and bacteria) through genetic engineering. Numerous improvements have been reported including increased lipid and carbohydrate production, successful diversion of metabolic pathways resulting in production of fungible biofuels from GMOs (Radakovits, 2010). However, many advanced bio-fuel compounds are toxic to microorganisms and provide an obstacle at high concentrations. While engineering metabolic pathways, one need to consider this fact otherwise the genetic modification of the host cell for the production of those toxic compounds can be detrimental for survival of the organism. On the other hand, various organisms use different strategies to address cellular toxicity. For instance, efflux pumps, a group of membrane transporters that uses the protonmotive force to export the toxins to the extracellular environment, if synthesized within the cells, may be promising in reducing compound toxicity (Dunlop, 2011).

Bacterial transport proteins known as efflux pumps offer a direct mechanism for mitigation of biofuel toxicity and consist of three subunits, an outer membrane channel, a periplasmic linker and an inner membrane protein. Each of them is specialized for a specific function and they are coded by corresponding genes arranged together in one operon. The inner membrane protein recognizes the substrate and plays a role in proton exchange. In *E. coli* there are 43 such pumps, expressed heterologous. Among them some of them restore the growth of the *E. coli* strain in presence of toxic biofuel (Dunlop, 2011). Dunlop (2011) introduced efflux pumps as a new genetic engineering tool for improved yields in biofuel secretion by direct tests in a biofuel producing strain of *E. coli*.

Fjerbaek (2009) demonstrated secretion of biofuel to the extracellular environment in an *E. coli* test system using membrane embedded transporter proteins responsible for the efflux of lipids and drugs. These proteins belong to the ubiquitous ATP-binding cassette family and are of equally pharmacological interest. These proteins mediate the secretion of potential biofuel products and isoprenoid compounds that are synthesized within bacterial cells. Utilization has been reported to increase the transporter mediated isoprenoid compound production by 3-5 fold. The authors have proposed this system as “plug-and-play biofuel secreting system”. This system can be useful for the production of economically important chemicals/products using variety of organisms from Monera to Plantae.

Gene cloning

Genetic manipulation techniques have paved the way in search of cheap and efficient ways of lignin digestion. Scientists are using these molecular tools and techniques in two different aspects of research to achieve this goal.

1. Genetic manipulation of lignin

The understanding of the lignin synthesis pathway combined with genetic engineering tools, allowed for the genetic manipulation of lignin itself. That goal is to reduce recalcitrance of the biopolymer and to improve ethanol yield. For example, transgenic Switchgrass developed from a normal growth phenotype by down-regulation of the caffeic acid 3-O-methyltransferase (COMT) gene in the lignin pathway has reduced lignin content, and an altered lignin composition. These modifications improved forage quality, increased saccharification efficiency, and increased ethanol production yield compared to wild-type controls. Moreover, the transgenic plant materials requires less severe pretreatment and much lower cellulase dosages to obtain ethanol yields equivalent to yields in controls. These transgenic switchgrass lines and the approach are valuable for developing improved cultivars of biofuel crops (Fu et al., 2010). However, strongly reduced lignin amounts result in altered plant development, but more importantly modest reductions can lead to normal development (Vanholme et al., 2010).

2. Cloning and expression of lignases

Genetic manipulation of lignin causes alterations in plant development and may increases the susceptible to pathogens which ultimately causes an undesired loss in productivity. The alternative way that creates a win-win situation is the isolation, cloning and expression of the gene that encodes lignases in to a suitable host to produce large amounts of the protein for lignin degrading applications. For instance, the gene lipH2 that encodes the expression of lignin peroxidase from *Phanerochaete chrysosporiu* was cloned and modify to lack the peroxidase secretion signal. The lipH2 gene was expressed in yeast and inserted into the expression vector pPICZalpha. The produced enzyme showed maximum peroxidase activity of 15 U/L after 12 hours of induction (Wang and Wen, 2009). Similarly, two genes ylpA and ylpB, encoding YK-LiP1 and YK-LiP2 respectively from the white-rot fungus *Phanerochaete sordida* YK-624 were expressed using the promoter for glyceraldehyde-3-phosphate dehydrogenase cloned from *P. sordida* (PsGPD) in the expression vector pGPD-g-ylpA and transformed into a *P. sordida* an uracil auxotrophic mutant, UV-64. The physical and catalytic properties of the purified enzymes synthesized after transformation are similar to the enzymes isolated from wild type of source. The concentration and time of unset of expression varies according to the species and cultured medium (Sugiura et al., 2009).

Conclusion

Our complete reliance on non-renewable fossil fuel, the dangers of increase in green house gases and the accompanied climate change, the instability in price and an unsure supply of petroleum constantly triggers a quest for renewable and sustainable energy sources. Solar energy

is an unlimited source of energy. Various photoautotrophic organisms assimilate solar energy and store the energy in the form of chemical energy. Photosynthetic plants use atmospheric CO_2 and soil water to assimilate solar energy. Especially algae have appealing properties to serve as a potential bio-diesel resource for decades. Ethanol production from lignocellulosic biomass is a renewable, environment friendly alternative to fossil fuel and corn or sugarcane derived ethanol. However, the presence of lignin in plant cells is unfortunate and becomes a barrier for breakdown of the carbohydrate polymers cellulose and hemicellulose, to smaller and fermentable subunits in fuel production. Recent developments towards the understanding of the composition of lignin, the mechanism of its synthesis, ,and the regulation and degradation combined with genetic engineering tools have paved new ways and revealed new opportunities towards manipulation of lignin composition and development of novel resources for lignin digestion in ethanol production. Various genetic tools are promising as platform for the production of alternatives source of energy for example starch derived alcohols, alkanes, bio-hydrogen and bio-diesel. Unlike petroleum, bio-diesel also offers recycling of atmospheric carbon and reduces toxic gas emissions.

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References

Akbar, M.T., Habib, A.M., Chowdhury, D.U.S., Bhuiyan, I.K., Mostafa, K.MdG., Mondol, S. & Mosleh, IMHAI., 2013. An insight into the lignin peroxidase of Macrophomina phaseolina Bioinformation: *Biomedical Informatics*, 9(14): 730-735.

Biodiesel Handling and Use Guide (Fourth Edition) 2008: U.S. Department of Energy Office of Scientific and Technical Information http://www.osti.gov/bridge

Dashtban, M., Schraft, H., Syed, T.A. and Qin, W., 2010. Fungal biodegradation and enzymatic modification of lignin (Review). *International Journal of Biochemistry and Molecular Biology,* 1(1):36-50.

Dunlop, M.J., Dossani, Z.Y., Szmidt, H.L., Chu, H.C., Lee, T.S., Keasling, J.D., Hadi, M.Z. and Mukhopadhyay, A., 2011. Engineering microbial biofuel tolerance and export using efflux pumps. *Molecular System Biology* 7:487.

Dyera, J.M. and Mullen, R.T., 2008. Engineering plant oils as high-value industrial feedstocks for biorefining: the need for underpinning cell biology research (Review). *Physiologia Plantarum* 132, 11–22.

Fjerbaek, L., Christensen, K.V., Norddahl, B., 2009. A Review of the current state of biodiesel production using enzymatic transesterification. *Biotechnology and Bioengineering* 102 (5), 1298-1315.

Fua, C., Mielenz, J.R., Xiaoa, X., Gea, Y., Hamilton, C.Y., Jr, M.R., Chenc, F., Fostonc, M., Ragauskasc, A., Boutona, J., Dixonc, R.A., and Wanga, Z.Y. Genetic manipulation of

lignin reduces recalcitrance and improves ethanol production from switchgrass : www.pnas.org/cgi/doi/10.1073/pnas.1100310108 : 1-6.

Gaskell, J., Stewart, P., Kersten, P.J., Covert, S.F., Reiser, J. and Cullen, D., 1994. Establishment of genetic linkage by allele-specific polymerase chain reaction—application to the lignin peroxidase gene family of *Phanerochaete chrysosporium*. *Biotechnology* 12:1372-1375.

Hammel, K.E. and Cullen, D., 2008. Role of fungal peroxidases in biological ligninolysis. *Current Opinion in Plant Biology*, 11:349–355.

Hatakka, A. (In: Hofrichter, M., Steinbu¨chel, A. (eds))., 2001. Biodegradation of lignin. *Biopolymers, Lignin, humic substances and coal, Wiley-VCH, Weinheim* 1: 129–180.

Janusza, G., Kucharzyk, K.H., Pawlik, A., Staszczak, M. and Paszczynski, A.J., 2013. Fungal laccase, manganese peroxidase and lignin peroxidase: Gene expression and regulation (Review). *Enzyme and Microbial Technology* (52), 1– 12.

Ljungdahl, L.G., 2008. The cellulase/hemicellulase system of the anaerobic fungus Orpinomyces PC-2 and aspects of its applied use. *Annals of the New York Academy of Science* 1125, 308-321.

Martinez, D., Berka, R.M., Henrissat, B., Saloheimo, M., Arvas, M., Baker, S.E., Chapman, J., Chertkov, O., Coutinho, P.M., Cullen, D., Danchin, E.G.J., Grigoriev, I.V., Harris, P., Jackson, M., Kubicek, C.P., Han, C.S., Ho, I., Larrondo, L.F., Lopez, de L. A., Magnuson, J.K., Merino, S., Misra, M., Nelson, B., Putnam, N., Robbertse, B., Salamov, A.A., Schmoll, M., Terry, A., Thayer, N., Westerholm-Parvinen, A., Schoch, C.L., Yao, J., Barabote, R., Nelson, M.A., Detter, C., Bruce, D., Kuske, C.R., Xie, G., Richardson, P., Rokhsar, D.S., Lucas, S.M., Rubin, E.R., Dunn-Coleman, N., Ward, M. and Brettin, T.S., 2008. Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. Hypocrea jecorina). *Nature Biotechnology* 26,553–560.

Murphy, D.J., 1999. Production of novel oils in plants: *Current Opinion in Biotechnology* 10,175–180.

Pradhan, A., Shrestha, D.S., McAloon, A., Yee, W., Haas, M. and Duffield, J.A., 2011. Energy life-cycle assessment of soybean biodiesel revisited: *American Society of Agricultural and Biological Engineers* 54(3), 1031-1039.

Radakovits, R., Jinkerson, R.E., Darzins, A. and Posewitz, M.C., 2010. Genetic engineering of algae for enhanced biofuel production: *Eukaryotic cell* 9 (4), 486–501.

Ragauskas, A.J., Williams, C. K., Davison, B.H., Britovsek, G., Cairney, J., Eckert, V., Frederick, Jr W.J., Hallett, J.P., Leak, D.J., Liotta, C.L., Mielenz, J.R., Murphyn, R.,Templer, R. and Tschaplinski, T., 2006. The Path Forward for Biofuels and Biomaterials. *Science* 311(5760), 484–89.

Singh, S.P. and Singh, D., 2010. Biodiesel production through the use of different sources and characterization of oils and their esters as the substitute of diesel (review). *Renewable and Sustainable Energy Reviews* 14, 200–216.

Sugiura, T., Yamagishi, K., Kimura, T., Nishida, T., Kawagishi, H. and Hirai, H., 2009. Cloning and homologous expression of novel lignin peroxidase genes in the white-rot fungus

Phanerochaete sordida YK-624. *Bioscience, Biotechnology and Biochemistry* 73(8), 1793-1798

Vanholme, R., Demedts, B., Morreel, K., Ralph, J., and Boerjan, W., 2010. Lignin Biosynthesis and Structure. *Plant Physiology* 153, 895–905.

Vasudevan, P.T. and Briggs, M., 2008. Biodiesel production—current state of the art and challenges (Review). *Journal of Industrial Microbiology Biotechnology* 35, 421–430.

Verpoorte, R. and Memelink, J., 2002. Engineering secondary metabolite production in plants: *Current Opinion in Biotechnology* 13, 181–187.

Wang, W. and Wen, X., 2009. Expression of lignin peroxidase H2 from *Phanerochaete chrysosporium* by multi-copy recombinant Pichia strain. *Journal of Environmental Sciences* (China) 21(2), 218-222.

Weng, J.K., Li, X., Bonawitz, N.D. and Chapple, C., 2008. Emerging strategies of lignin engineering and degradation for cellulosic biofuel production. *Current Opinion in Biotechnology* 19, 166–172.

Yoon, J.J., Cha, C.J., Kim, Y.S., Son, D.W. and Kim, Y.K., 2007. The brown-rot basidiomycete *Fomitopsis palustris* has the endo-glucanases capable of degrading microcrystalline cellulose. *Journal of Microbiology and Biotechnology*, 17: 800-805.