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Research review paper

# Designing the perfect plant feedstock for biofuel production: Using the whole buffalo to diversify fuels and products

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## ABSTRACT

Petroleum-derived liquid fuels and commodities play a part in nearly every aspect of modern daily life. However, dependence on this one natural resource to maintain modern amenities has caused negative environmental and geopolitical ramifications. In an effort to replace petroleum, technologies to synthesize liquid fuels and other commodities from renewable biomass are being developed. Current technologies, however, only use a portion of plant biomass feedstocks for fuel and useful products. "Using the whole feedstock buffalo" or optimally using all portions and biochemicals present in renewable biomass will enhance the economic and environmental feasibility of biofuels and coproducts. To accomplish this optimization, greater understanding of the relationship between liquid fuel and bioproduct properties and plant chemistries is needed. Liquid fuel properties and how they relate to biochemistry and petrochemistry are discussed. Enhanced biofuel yields and high-value commodities from biomass are needed to sustainably replace petroleum-based products. Several metabolic engineering strategies are discussed. We will describe paths of possible fuel and product diversification using dedicated lignocellulosic biomass (e.g., switchgrass).

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*Abbreviations:* SSF, simultaneous saccharification and fermentation; FAME, fatty acid methyl ester; FAEE, fatty acid ethyl ester; PHA, polyhydroxyalkanoates; PHB, poly-3-hydroxybutyrate; TAL, tyrosine ammonia-lyase; HCT, hydroxycinnamoyl-CoA shikimate/quininate hydroxycinnamoyl transferase; DGAT, diacylglycerol acyltransferase; LEC, leafy cotyledon.

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## 1. Introduction

Modern, industrialized society relies on a single natural resource to provide a plethora of commodities and conveniences that would be hard to envision living without: petroleum. Petroleum not only provides liquid transportation fuels, but also provides the asphalt which literally paves the way for transportation. Petroleum provides heating fuels, plastics and other materials which have revolutionized everything from how we package and store food to modern medical

products (Thompson et al., 2009). Petroleum has in some way contributed to nearly every aspect of modern daily life, but the end of petroleum is in sight.

But for every positive benefit that petroleum has provided there seems to be a negative environmental ramification. The Deepwater Horizon oil spill released 4 million barrels of oil into the Gulf of Mexico which has had a series of ecological and economic impacts on the states and countries lining the Gulf (Camilli et al., 2010). Extensive use of asphalt have created a phenomenon known as 'urban heat islands' which increases energy consumption and can increase mortality rates in urban centers (Rizwan et al., 2008). Emissions from combustion engines have led to debate and growing concern over air quality and greenhouse effects. Plastics make up 10% of human wastes, do not readily degrade, and when they do they release toxic chemicals that have started to bioaccumulate across the globe (Thompson et al., 2009). There have also been negative geopolitical ramifications associated with petroleum production and consumption. Included in the list is war, internal friction within countries, economic and political instability, and increasing disparity between rich and poor countries. Taken together these considerations have led researchers to investigate a number of technologies to replace petroleum-derived commodities with renewable, ubiquitous, and more environmentally benign substitutes. Replacing petroleum commodities with an inexpensive, renewable resource that can be produced in any country in the world would lead to a second green revolution for human needs going beyond food (Mooney, 2009).

Bioplastics derived from natural polymers are renewable and biodegradable (Mooney, 2009; Suriyamongkol et al., 2007). Of specific interest, biofuel research has taken aim at replacing petroleum liquid fuels with chemicals derived from crop and forest residues, algae, and bioderived waste materials. There have been a number of policies and incentives directed at developing both ethanol (Hoekman, 2009; Martin, 2010) and biodiesel (Hoekman et al., 2009) into mature cost-effective technologies. However, current biofuels are not ideal liquid fuels when characteristics like fuel properties and compatibility with existing infrastructure are considered. These benefits and drawbacks will be further discussed in Section 2.1. Plant biotechnology and microbial biotechnology have been proven to be useful tools in improving biomass processing and biorefinery product yields (Hermann and Patel, 2007; Octave and Thomas, 2009). Biocatalyst reactions, or reactions driven by enzymes, have advantages over organic chemistry synthesis, e.g. the ability to produce complex molecules efficiently (Wohlgemuth, 2009). Although the use of biocatalysis of chemicals on large scale has been limited, biotechnology and bioprocessing have been applied extensively to biofuel production, which will be discussed in Section 3. The reasons why biofuels have become an attractive solution to replacing petroleum-derived liquid fuels has been addressed in a number of reviews, and as such is beyond the scope of this manuscript, e.g. Hoekman, 2009. However, it is important to briefly discuss how first generation (food crop-derived) and second generation (non-food crop-derived) biofuels developed to better understand next generation biofuels and bioproducts.

In 2007, the U.S. Energy Independence and Security Act set incentives and a goal of 144 billion liters of biofuels per year by 2022 (Martin, 2010). Industry and researchers turned to available technologies in an attempt to begin to displace petroleum fuels immediately. In the US, ethanol was first derived from fermented starch (usually maize grain); biodiesel was derived from alkyl esters of cooking or waste oils. Starch and plant oils are feedstocks easily accessible and have liquid fuel synthesis technologies already well developed (Octave and Thomas, 2009); in the case of ethanol fermentation, humans have been practicing it for millennia. But these technologies led to a now famous public outcry against using food sources to produce fuels. The outcry resulted from food prices that increased 4.0% in 2007 and 5.5% in 2008 compared to a 2.4% increase in 2006 and 2005 (Martin, 2010). In reality, the higher food prices were a result of several factors with corn-based ethanol production accounting for only about a fifth of the total food price

increase of 4.0 and 5.5% (Martin, 2010). Despite this, biofuel research shifted more heavily to non-food sources such as corn stover and dedicated biofuel crops such as poplar, switchgrass, and algae.

## 2. Replacing petroleum commodities: can we grow barrels of oil?

The ultimate goal of biofuels is to completely replace petroleum--derived liquid fuels, especially for the transportation sector. But biomass, like a barrel of oil, contains a diverse array of chemicals that could be used to create many different commodities in addition to liquid fuels. Indeed, fuel could be the essential loss leader in the emerging bioeconomy (Bozell, 2008). Petroleum itself is formed from organic matter such as marine algae and plants heated to specific temperatures in the Earth's crust on geologic time scales. The formation of petroleum occurs throughout the world and the chemicals that are formed differ based on different locations and different source rock (Speight, 1999). Petroleum is so chemically complex and variable between each deposit that it has been traditionally characterized by bulk properties like distillation ranges and total atomic percentage. In fact, it was not until recently that individual chemicals present in petroleum could be identified using high resolution mass spectrometry (Marshall and Rodgers, 2008). The chemical complexity of petroleum has led to the petroleum industry adopting a number of technologies, e.g. catalytic reforming, hydro-treating, etc., to separate, refine and alter chemical fractions for specific uses (Matishev, 1994).

By comparison, biomass is an immature feedstock compared with petroleum that needs to be converted and refined into chemicals useful for commodities. In the current biofuel industry, chemically complex plant biomass is separated, thermally cracked or degraded by enzymes, and then converted into products using chemical synthesis or biological conversion. The key is the efficient conversion of biomass into petroleum-like chemicals on a biological timescale (seconds to hours) rather than a geologic timescale (millennia). The main factor that distinguishes petroleum from biomass is the use of biotechnology to fundamentally alter enzymes present in biomass; essentially, biotechnology enables researchers to engineer and fine-tune barrels of renewable (biomass-derived) petroleum. To put the concept into petroleum terminology, biotechnology could be considered *in vivo* refining, and can occur in plant biomass, microbes used to ferment the biomass, or a combination of both. A significant amount of work has gone into altering fermentation products in microbes, and several comprehensive reviews are available (Lee et al., 2008; Liu and Khosla, 2010; Peralta-Yahya and Keasling, 2010). Currently suspension cells, micropropagated plantlets, and hairy root culture are the main mechanisms for industrial scale *in planta* production of high-value biochemicals, but each of these methods has major drawbacks that limit their wide commercial success (Weathers et al., 2010). Microbial fermentation of products has been thoroughly studied and has several advantages over *in planta* synthesis of metabolites such as rapid screening on culture chips, short life cycles, ease of engineering resulting from relatively simple metabolic pathways, and more sequence data available (Wohlgemuth, 2009). As dedicated biofuel crops become more commonplace, however, *in planta* synthesis of biochemical products offers several advantages such as simple extraction and separation to yield products, and direct (efficient) synthesis of hydrocarbons and high-value commodities using low--cost solar energy. Synthesis of biofuels and chemical commodities in traditional agricultural crops would allow countries without extensive infrastructure to produce modern commodities and could increase overall yields of biofuels by reducing loss of fixed carbon resulting from conversion. Cyanobacterial or algal production of biofuels might likely be the best combination of microbial and plant production systems, but there are still significant barriers to these technologies and their use on a sustainable industrial scale remains in the long-term (Wijffels and Barbosa, 2010).

With this in mind, our focus here is on plant feedstock metabolism and biotechnology strategies for producing the 'perfect' dedicated biofuel feedstock. We liken the goal of this bioenergy feedstock design process to the Native American paradigm of "using the whole buffalo." Prior to European settlers in America, North American plains people hunted buffalo (bison) for food, clothing, fuel, and many other needs in their daily lives. No part of the buffalo went to waste. We envisage, likewise, designer dedicated plant feedstocks that provide a plethora of high value fuels, bioproducts and materials. Biotechnology should be integral in designing this perfect feedstock; an ideal feedstock does not exist in nature (Gressel, 2008).

### 2.1. Plant-derived biofuels: two engines, two fuels, two crops?

There are many classes of biochemicals that are key targets to replace petroleum commodities. The complex hydrocarbon fraction of petroleum can be broken down into three general petrochemical fuel classes: paraffins (alkanes), naphthenes (cyclic alkanes), and aromatics with several subgroups such as isoparaffins (branched alkanes) and olefins (unsaturated alkanes) (Wallington et al., 2006). Ultimately, fuel and engine operability properties of liquid fuels result from a combination of engine type, chemical composition, environmental conditions, e.g. ambient temperature, and vehicle parameters, e.g. heavy load versus light load, which all vary in real world applications. However, experimentation with simplified surrogate fuel mixtures has led to some understanding of how chemical components affect quality of fuels. Petroleum-derived commodities, physicochemical properties, and the effect of chemical class constituent on those properties are illustrated in Table 1. Each petroleum chemical class present in a commodity yields different physicochemical properties for that commodity; essentially, there is no perfect chemical constituent that translates to a perfect liquid fuel. Market and engineering demands, such as a low cloud point property and a high cetane number in diesel fuel requires mixing chemical classes that counteract each other. For example, aromatics in diesel fuel will provide low cloud point properties but also a low cetane number, whereas paraffins will provide high cetane numbers but will also begin to solidify at high temperatures. Current liquid fuel demands and environmental regulations require catalytic cracking of heavier petroleum fractions, e.g. aromatics and naphthenes, to form smaller hydrocarbons (Dupain et al., 2003). Blending is a crucial process in petroleum fuel synthesis, because mixing different chemical classes

allows for the vast flexibility to meet market and environmental demands. Current biofuels, however, are conspicuously homogeneous in their chemistries, which is in great contrast to plant biochemistry where the typical vascular plant is composed of over 50,000 different chemicals (Hartmann, 2007). Ethanol or butanol for gasoline replacement are, obviously, single chemicals. Biodiesels derived from alkyl esters of either animal or plant oils contain more chemical diversity, but even then there are only 5–15 distinct chemicals based on the source material's composition of fatty acids. As there is a vast range of commodities derived from petroleum that could be replaced by biochemicals, in this review we will focus on the suitability of different biochemicals as liquid fuels.

Currently in plant-derived biofuels, biomass is either deconstructed and sugars are fermented to produce ethanol/butanol, or oils are collected from oilseed crops to produce biodiesel through alkyl esterification reactions. This separation of biofuel crops seems to stem from availability of first generation biofuels as well as a restriction in technologies to derive both gasoline and diesel replacements in the same crop. The overall suitability of biofuels as a replacement for petroleum-derived fuels will depend on a plethora of factors including fuel properties, combustion and operability properties, emissions, and fungibility or compatibility with existing infrastructure. All of these properties can be linked directly to the chemical composition of liquid fuels. Understanding how fuel chemistry influences fuel properties is still insufficient, and reports of biofuel effects in petroleum fuel blends are often difficult to compare due to inconsistent experimental designs, vastly different fuel chemistry between studies, and incomplete data sets (Lapuerta et al., 2008). A better understanding of petroleum fuel chemistry and how that relates to fuel properties will lead to a more intelligent design of biofuels (Pitz and Mueller, 2011). Fuel properties of any liquid fuel, whether petroleum or biomass derived, result from a combination of fuel chemistry and combustion engine type (Wallington et al., 2006). As such, we will discuss general gasoline chemistry for spark ignition engines and diesel fuel chemistry for compression ignition engines and how these two chemistries relate to fuel properties in more detail separately.

#### 2.1.1. Current production technologies toward biogasoline

Gasoline is used in spark ignition engines. In these engines, fuel is carburetor-distributed or injected into a combustion chamber and then ignited with a spark at the appropriate time. Gasoline, therefore, needs to have a high volatility to combust instantly in presence of a

**Table 1**  
Chemical class influence on petroleum distillates' fuel and physical properties and biochemical alternatives.

Petroleum Distillate (range °C)	Fuel Property	Paraffin	Iso-paraffin	Olefin	Naphthene	Aromatic	Oxygenates <sup>a</sup>	Saturated Fatty Acid Esters	References
Gasoline (30–200) C <sub>4</sub> –C <sub>12</sub> 50–60% paraffin	Octane number	-	+	±	-, ±	NR	+		(Pitz et al., 2007; Speight, 1999)
	Lower heating value (kJ L <sup>-1</sup> )	-	±	±	+	+	-		(Pitz et al., 2007; Speight, 1999)
	Volatility	+	+	±	±	±	-, ±		(Pitz et al., 2007; Speight, 1999)
Kerosene (140–320) C <sub>10</sub> –C <sub>16</sub>	Smoke emission	+	±	-	±	-	NR	NR	(Dagaut and Cathonnet, 2006; Speight, 1999)
	Cetane number	+	-, +	-	±	-	-	+	(Bacha et al., 2007; Benjumea and Agudelo, 2010; Li et al., 2005)
Diesel (126–258) C <sub>8</sub> –C <sub>18</sub>	Cloud point	-	+	±	+	+	+	-	(Bacha et al., 2007; Benjumea and Agudelo, 2010; Li et al., 2005)
	Lower heating value (kJ L <sup>-1</sup> )	-	NR	±	+	+	-	NR	(Bacha et al., 2007; Li et al., 2005)
	Fuel formulation	±	±	-	-	-	NR	NR	(Dagaut and Cathonnet, 2006; Speight, 1999)
Jet Fuel (126–287) C <sub>8</sub> –C <sub>16</sub> 60% paraffins	Freezing point <sup>b</sup>	±	+	±, +	+	+	+	-, ±	(Bacha et al., 2007; Dagaut and Cathonnet, 2006)

<sup>a</sup> oxygenates denote ethanol, butanol.

<sup>b</sup> fuel characteristics will be similar in diesel fuel distillates.

+ denotes a positive affect.

± denotes a negligible or mixed affect.

- denotes a negative affect.

NR – not reported.

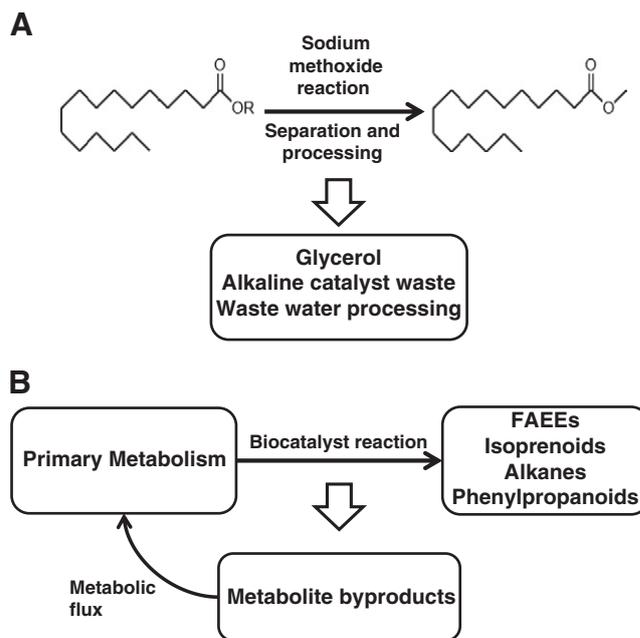
spark but not as volatile as to prematurely detonate or to be explosive in storage. This range makes predicting optimal chemical composition for biogasoline difficult as each chemical class can have chemicals inside or outside the volatility range depending on carbon number, chemical structure, or side groups (Table 1). Petroleum gasoline, therefore, distills at temperatures between 30 °C and 200 °C which contains the lower molecular weight paraffins, naphthenes, isoparaffins, olefins, and aromatics in crude oil (Speight, 1999, 2002). Gasoline distilled directly from petroleum has low octane rating, and as such, requires upgrading and blending with other refinery hydrocarbon streams (Pitz et al., 2007). Olefins, unsaturated alkanes, are not present in significant amounts in crude oil, but are refined and blended with gasoline fractions to meet market requirements for fuel and emission properties (Speight, 1999).

As there are a few biobased chemicals being investigated to be replaced by petroleum-derived gasoline, including ethanol, butanol, and hydrocarbons from thermochemical conversion, they will be referred to collectively as biogasoline. Thermochemical conversion of biomass, such as Fisher-Tropsch synthesis, will be discussed further in Section 2.1.3. Ethanol and butanol have different advantages and disadvantages as biofuels, and there is a debate centered on which is more suitable. Ethanol also has positive fuel characteristics when blended with gasoline such as reduced emissions of CO, CO<sub>2</sub>, and hydrocarbons (Demirbas, 2009a). However, ethanol has a lower energy density than gasoline or butanol which means that ethanol will carry a car a shorter distance liter for liter; reduced kilometers per liter have led to a lack of economic incentive for consumers to switch to using E85 fuel blends and flex-fuel cars (Martin, 2010). When compared to ethanol production, butanol has lower final concentrations (2% versus 15% for ethanol) and longer fermentation times which reduce its usefulness in meeting widespread demand for liquid fuels (Pfromm et al., 2010). Despite these restrictions, butanol has greater energy density and is more hydrophobic which means that it is more suitable as a drop-in replacement for gasoline and more compatible with existing infrastructure. The benefits and drawbacks of ethanol and butanol as fuels directly result from their oxygen content. Currently, both fuel chemicals are fermented from biomass whether it is starch or sugars derived from lignocellulosic feedstocks. Though lignocellulosic biofuels are not widespread currently, biotechnology improvements have led to better lignocellulosic feedstocks for ethanol production (Fu et al., 2011). These improvements will likely lead to lignocellulosic biofuels being industrially viable in the near future, and apply to all potential products derived from lignocellulosic sources.

### 2.1.2. Current production of biodiesel

Diesel fuel is used in compression ignition engines. In these engines, fuel is injected into a combustion chamber where it is compressed until it reaches a specific pressure which causes the fuel to heat and ignite producing mechanical work. High cetane diesel fuels will ignite quickly to produce the maximum amount of work or transferable power to the engine. Jet fuel is distilled from nearly the same petroleum fraction as diesel fuel with a few more restrictions such as a limit on the percentage of aromatics and the need for low temperature operability to -40 °C (Carlsson, 2009). Currently, diesel fuel demand is growing at 3.5% which is greater than gasoline, kerosene, or jet fuel (International Energy Agency, 2011).

There are two major diesel replacement technologies: the production of fatty acid alkyl esters (usually methyl esters or FAMES) and 'green diesel' or Fisher-Tropsch diesel, both of which are derived from extracted plant oils. Biodiesel has been primarily derived from four oilseed plants: soybean, oil palm, canola, and sunflower, although there are other crops being used in smaller amounts or being considered, e.g. *Camelina sativa*, cotton, and *Crambe abyssinica* (Carlsson, 2009). Plant-derived oils, which are primarily composed of acylglycerides, are too viscous to be used as fuel directly in engines without chemical structure modification or without



**Fig. 1.** A) Conventional chemical methyl esterification conversion of fatty acids for biodiesel production whether free or bound to glycerol. Methyl esterification reduces biodiesel viscosity while creating glycerol, alkaline catalyst waste, and waste water that needs to be processed. B) Conversion of metabolites with biocatalysts, bioconversion, *in planta* would produce biofuel chemicals that are extractable from plant biomass directly. The resulting metabolic byproducts such as glycerol would reenter plant metabolism for recycling and reduce processing waste.

heating to reduce viscosity. Synthesis of alkyl fatty acid esters requires an esterification reaction involving an alkyl alcohol, usually methanol, and a catalyst such as sodium hydroxide or a lipase biocatalyst (Demirbas, 2009b). The addition of a methyl ester group to a fatty acid does not radically alter the original fatty acid chemical structure, however separation from the glycerol backbone reduces viscosity and is the primary goal of this reaction (Fig. 1A). Ethanol for production of fatty acid ethyl esters (FAEEs) has gained some interest because it can be produced from biomass; although methanol is far more common for economic reasons (Demirbas, 2009b). This reaction produces glycerol as a byproduct in a 1:9 ratio and must be separated from the biodiesel product requiring processing and then disposal of alkaline or acid catalyst wastes (Du et al., 2008). Biotechnology research to improve biodiesels has largely focused on developing products from byproduct glycerol and altering the fatty acid profile of oilseed crops to modify biodiesel properties. Glycerol has been used in chemical conversion (Thompson et al., 2009) and biological conversion (García et al., 2008; Rahmat et al., 2010; Zhang and Memelink, 2009) to make new products which will be discussed in more detail in Section 3.1.2. However, direct biosynthesis of biofuel chemicals *in planta* would allow for byproducts to reenter the metabolic pathways and reduce waste catalyst and water processing (Fig. 1B). Altering the fatty acid composition of oilseeds has been suggested as a way to optimize biodiesel fuel properties (Agarwal, 2007; Knothe, 2009), but this approach will always limit biodiesel producers to the inherent properties of long-chain oxygenated alkanes and alkenes. In addition, annual food crops such as soybean, sunflower, and canola have unfavorable net energy output (Yuan et al., 2008). Increasing unsaturated fatty acids in biodiesel improves cold operability characteristics but increased hydrocarbon and NO<sub>x</sub> emissions, and lowered cetane rating (Benjumea and Agudelo, 2010). Butanol and ethanol have also been blended with diesel, biodiesel, and even raw canola oil to enhance fuel properties such as lowering viscosity and increasing cold temperature operability characteristics (Demirbas, 2009a; Laza and Berczky, 2011). However, there is still uncertainty

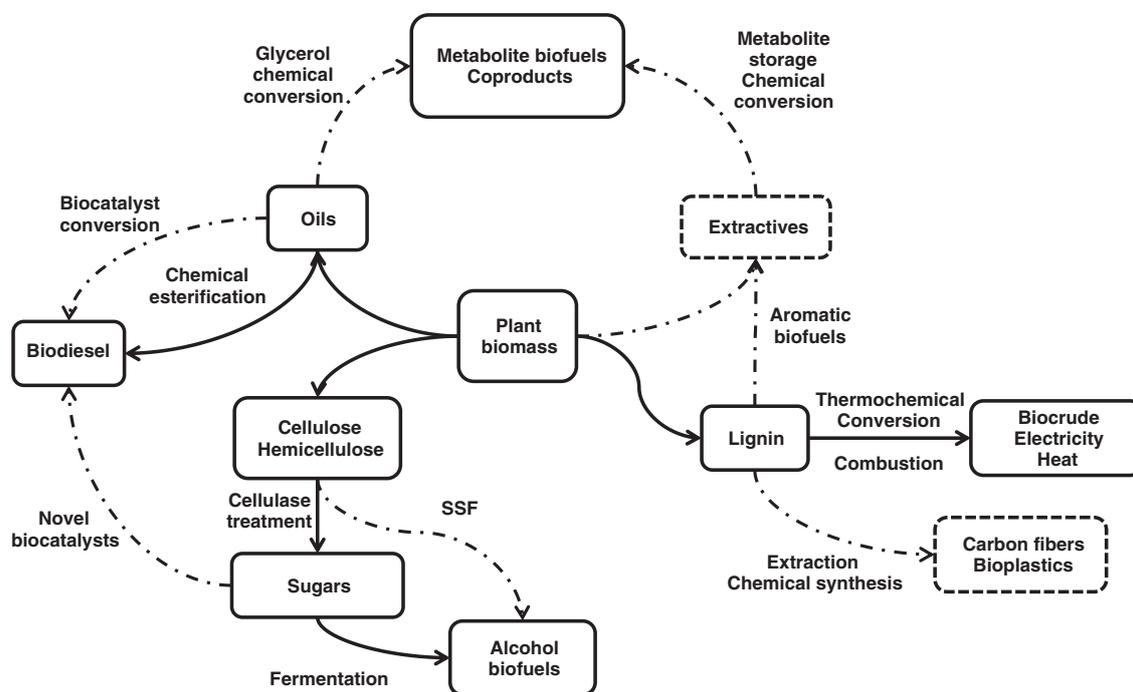
among reports regarding biodiesel fuel properties and emissions (Xue et al., 2011). This is best highlighted in a recent review by Xue et al. who created summary tables of biodiesel fuel properties and what percentage of reports showed an increase, similar, or decrease in the properties in relation to diesel fuel.

2.2. Coproduction of biogasoline, biodiesel, green chemicals, and high-value coproducts: towards growing green petroleum

To date thermochemical conversion of biomass is the only way to produce biogasoline, biodiesel, and chemical commodities from the same feedstock. Thermochemical conversion is more commonly used to process woody biomass that has higher lignin content than herbaceous crops because lignin increases biomass recalcitrance to degradation into fermentable sugar monomers for bioconversion. Each thermochemical conversion process breaks down and reforms biomass into small molecular building blocks to yield biosyngas or biocrude (Demirbas, 2009c). Biosyngas and biocrude can then be reformed or upgraded to produce drop-in fuels with fuel properties essentially identical to existing liquid fuels, e.g. high energy content through removal of oxygen. However, there are disadvantages to thermochemical conversion that reduce their economic and product efficiency which include the need for high temperature reactions, loss of energy from biomass to entropy, and catalyst fouling (Carroll and Somerville, 2009). Thermochemical conversion reaction conditions range from 450–950 °C depending on which thermochemical conversion process is being used (Demirbas, 2009c; Ong and Bhatia, 2010). Lower temperature conversion processes usually require catalysts which are eventually fouled by coke formation and require replacement (Kleinert and Barth, 2008). Additionally, thermochemical conversion favors construction of large reactors to make the process

economically viable but biomass has a diffuse distribution which favors construction of many smaller biorefineries to minimize transportation costs (Carroll and Somerville, 2009). Combining the quality of liquid fuels from thermochemical conversion with the product specificity, low energy inputs, and scalability of bioconversion will result in higher quality and economically viable renewable liquid fuels.

Lignocellulosic feedstock biomass has traditionally relied on pretreatment, which facilitates hydrolysis by making cellulose, hemicellulose and lignin polymers to supplemented enzymes which cleave the polymers into sugar monomers for fermentation (Fig. 2). The concept of consolidated bioprocessing, or simultaneous saccharification and fermentation (SSF), incorporates fermentation and pretreatment of biomass into one process which usually includes production of necessary conversion enzymes by fermentation microbes. This opens a sizable fraction of plant biomass for conversion to biofuels while simplifying the overall fermentation process and making bioconversion more economic. However, production of liquid fuels ranging from gasoline to diesel and jet fuel in a single fermentation vessel from a biomass source will take the next step in making consolidated bioprocessing truly consolidated. Coproduction of several biofuel chemicals has been discussed in other reports, and in itself, is not a novel concept. Hydrogen, methane, and ethanol coproduction from biomass in a biorefinery has been considered and even patented under the name 'Maxifuel Concept' (Ahring and Westermann, 2007). However, coproduction of biogasoline and biodiesel using biological conversion has rarely been considered. The first significant step in biogasoline and biodiesel coproduction from biomass was realized when a process to convert sugars into fatty acid esters using engineered *Escherichia coli* fermentation was coupled with hemicellulases (Steen et al., 2010). Further modification of plant feedstocks is needed – likely via genetic engineering. Biofuel



SSF: simultaneous saccharification and fermentation

**Fig. 2.** Current (solid lines) and future (dotted lines) conversion techniques for production of biofuels and coproducts from fractions of plant biomass. Cellulose and hemicellulose are currently converted to sugars through pretreatment and enzyme degradation which are then fermented to produce alcohol biofuels. Simultaneous saccharification and fermentation (SSF) techniques will allow for consolidated bioprocessing to reduce inefficiencies resulting from multistep processing. Biodiesel is generated from fatty acid chemical conversion. Lipases and other biocatalysts are being developed to enhance the esterification reaction, and conversion of glycerol into useful biofuel and coproduct chemicals. Novel biocatalysts have also been developed to produce biodiesel fatty acid ethyl esters (FAEEs) from sugars. Lignin is currently thermochemically converted into biocrude, syngas, or electricity/heat. Production of commodities such as carbon fibers and bioplastics are being developed from lignin fractions. Extractable hydrocarbon metabolites such as isoprenoids and alkanes are being considered for biofuels and coproducts. Monolignols would also be suitable as an aromatic biofuel fraction.

coproduction techniques and biotechnological engineering will lead to a generation of new biofuels where all chemicals present in biofuel crops are relevant for the production of biofuels and coproducts; a biofuel generation where the whole biofuel feedstock buffalo is used. While genetic engineering will likely be crucial for diversifying fuels and products, conventional breeding will play a role in diversifying adaptation and biomass yield.

### 3. Engineering plants to make the 'biofuel feedstock buffalo'

The first step in engineering the most suitable biofuel feedstock is the choice of the optimal crop for mass production of biofuels and bioproducts (Yuan et al., 2008). This can be a difficult choice to make because biomass will be produced around the world and each environment and climate will have varying requirements and adaptation for production. Several species have been considered as dedicated lignocellulosic crops. The major biomass feedstocks considered to be viable candidates in the United States are dedicated lignocellulosic feedstocks such as poplar (*Populus spp.*), switchgrass (*Panicum virgatum*), miscanthus (*Miscanthus x giganteus*), as well as microalgae. Oilseed crops are not considered here because it is not clear whether oilseeds will ever be dedicated biofuel crops, though there has been interest in *Camelina sativa* in recent years (Moser, 2010). Corn stover, the remnants of the corn plant after harvest, has been investigated as a major source of lignocellulosic biomass and represents agricultural wastes. Forest and agricultural residues will likely constitute a large portion of biomass supply for biofuel production (Perlack et al., 2005), but by their nature, will most likely not be engineered and as thus will not be discussed here. Perennial dedicated lignocellulosic feedstocks, e.g., switchgrass and miscanthus, have benefits compared to other potential crops that include requirement of less energy inputs for stand establishment, good nutrient- and water- use efficiency, and environmental benefits that include soil carbon deposition and ecosystem services (Carroll and Somerville, 2009). Certain tree species such as poplar and willow have been considered for perennial dedicated feedstocks, but they require large amounts of water which will ultimately limit their use (Allison et al., 2010). Algae might ultimately be the best feedstock for biofuel

production as it will not compete for arable land and has a large lipid fraction, but production engineering considerations and large capital outlay for production facilities will most likely put algae for biofuel production in the long term (Carlsson, 2009). In the southeastern United States, the dedicated lignocellulosic feedstock of choice will most likely be a perennial grass species such as switchgrass, miscanthus, or energy cane. Of these, switchgrass has received a lot of research attention as a dedicated biofuel crop. The BioEnergy Science Center selected switchgrass and poplar as primary research species, and companies such as Ceres and Metabolix have ongoing research projects that feature switchgrass. Recently, genetic modification of the lignin biosynthetic pathway in switchgrass has successfully produced plants that fermented up to 38% more ethanol than unmodified biomass (Fu et al., 2011). The first public field trials of transgenic switchgrass are also underway at the University of Tennessee, started in 2009, which will bring switchgrass a step closer to being a viable dedicated biofuel feedstock. With this in mind, we will focus on engineering approaches to engineering switchgrass and perennial grass feedstocks.

#### 3.1. Bale to barrel: strategies for engineering the perfect petroleum-replacement feedstock

The first way to improve biofuel production yields and economic viability is to convert all the chemicals present in biomass into useful liquid fuel chemicals or high-value commodities, and secondly to use all the biomass generated by the dedicated feedstock. Strategies for using and improving all portions of switchgrass biomass will be discussed further in Section 3.2. Efficient engineering of feedstocks and conversion of all biochemicals present requires in-depth knowledge of metabolites natively present in feedstocks. However, identifying all chemical constituents in biomass is difficult, and moreover, highly variable depending on season, biomass fraction, and extraction techniques employed (Yan et al., 2010; Yang and Ohlrogge, 2009). Biomass composition and metabolites present in major feedstocks are compiled in Table 2. Data were selected based on late season harvest for crops that have been considered for multiple harvests in a year, e.g. during senescence of switchgrass. As described

**Table 2**  
Lignocellulosic biofuel feedstock chemical composition in percent dry weight.

Biomass Fraction	Fraction Metabolite(s)	Biomass Composition of Lignocellulosic Feedstocks			
		<i>Panicum virgatum</i> Cv Alamo (% dry weight)	<i>Miscanthus x giganteus</i> (% dry weight)	<i>Zea mays</i> Stover (% dry weight)	<i>Populus</i> (% dry weight)
Cellulose		33.48 - 33.75	46.93 - 49.41	37.12 - 39.4	42.2 - 48.95
Hemicellulose		26.1 - 27.04	29.68 - 32.26	24.18	16.6 - 23.24
	Glucose	37.0	50.47	36.8	39.23
	Xylose	20.42, 28.8	21.68	22.2	13.07
	Arabinose	2.75, 3.7	2.78	5.5	0.89
	Galactose	0.92, 1.3	0.35	2.9	0.88
	Mannose	0.29	NR	NR	1.81
	Uronic acid	NR	NR	NR	4.31
Lignin		16.8 - 17.35, 22.7	11.97-13.24	23.1	21.4 - 29.1
Extractives		11.0 <sup>a</sup> , 15.50 <sup>a</sup> , 18.4 <sup>b</sup> , 10.2 <sup>c</sup>	1.13 <sup>d</sup> , 14.03	3.9 <sup>a</sup> , 5.61	2.4 <sup>c</sup> , 6.89
	Fatty acids	1.54 <sup>a</sup> , 5.5 <sup>d</sup>	3.93 - 4.53	NR	NR
	Sterols	1.0 <sup>a</sup>	2.75 - 9.49	NR	NR
	Trehalose	2.2 <sup>a</sup>	NR	NR	NR
Ash (inorganic elements)		5.76	3.2	10.06	2.03
References		(Carroll and Somerville, 2009; Sannigrahi et al., 2010; Yan et al., 2010)	(Allison et al., 2010; Le Ngoc Huyen et al., 2010; Villaverde et al., 2009)	(Carroll and Somerville, 2009; Hu et al., 2010; Sannigrahi et al., 2010)	(Carroll and Somerville, 2009; Sannigrahi et al., 2010)

<sup>a</sup> ethanol extraction.

<sup>b</sup> hot water.

<sup>c</sup> alcohol-benzene extraction.

<sup>d</sup> dichloromethane extraction.

<sup>e</sup> toluene extraction.

NR - not reported.

above in Section 2.2, technical advances in lignocellulosic ethanol production and consolidated bioprocessing have opened up the possibility of using entire aboveground biomass whole for production of biofuels. While much research has focused on the composition of cellulose and lignin present in switchgrass feedstocks, there has been little compositional analysis of the other portions of switchgrass biomass, namely the 'extractives' fraction.

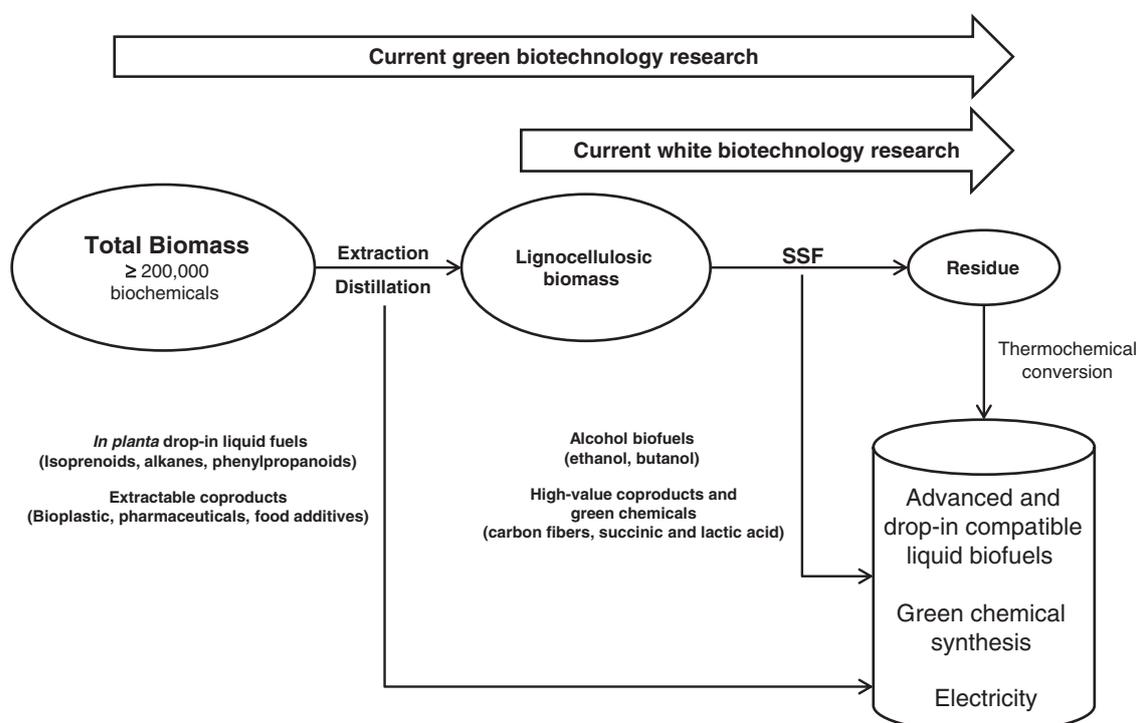
Switchgrass has a large extractives fraction that ranges overall from 11–17% of the biomass depending on cultivar, and 13.3–21.0% in different portions of the plant itself (Carroll and Somerville, 2009; Mann et al., 2009). However, the term 'extractives fraction' simply equates to a miscellaneous grouping used to describe the portion of biomass metabolites that is not lignocellulosic biomass and not inorganic components, i.e., ash. Remarkably, few studies on the chemical composition of the extractives fraction have been carried out in switchgrass. This could result from the highly variable nature of the extractive fraction. Switchgrass extractives percentages of dry biomass changes during storage, whether sheltered or outside (Wiselogel et al., 1996). The percentage of total dry biomass the extractives fraction of switchgrass also changes depending on the extraction procedure itself. One study has shown that a 95% ethanol extractives fraction included fatty acids, sterols, triglycerides, sugars, and other metabolites (Yan et al., 2010). However, this report only examined the composition of metabolites from one extraction method. These fractions likely include other secondary metabolites such as isoprenoids and phenylpropanoids, but no studies have been published on secondary metabolites present in switchgrass. Further investigation into existing metabolites and metabolic pathways in switchgrass will aid biofuel crop engineering efforts.

Ideally, dedicated plant biomass feedstocks would be processed for biofuel and coproduct production in three steps: 1) simple extraction or distillation to recover a liquid portion of biomass that would be

drop-in ready biofuels, 2) the resulting lignocellulosic fraction would be deconstructed and fermented to produce liquid fuels and chemicals for chemical synthesis precursors, 3) residual biomass would then be thermochemically converted to produce hydrocarbons for liquid fuels, coproducts, or heat for generation of electricity (Fig. 3). Drop-in ready biofuel chemicals in plant biomass that can be extracted or collected through simple distillation will allow for biofuel production in rural and non-industrialized areas. Additionally, converting more of the feedstock biomass to usable products, e.g., combustible metabolites for liquid fuels, that can be simply extracted will increase overall biofuel yields from biomass and increase biorefinery production efficiency while not requiring more infrastructure or investment. Bioconversion microbes are also subject to plant metabolite toxicity, and so extraction of biomass before fermentation would also remove potentially toxic metabolites from feedstocks. Engineering plant metabolism for the production of simple extraction drop-in ready biofuels will be discussed further in Section 3.1.1.

Modifying lignin content and structure in biomass feedstocks to reduce bioconversion recalcitrance has been the primary focus of green biotechnology. While lignin reduces the efficiency of biomass processing and subsequently fermentation, lignin has been used to produce high-value commodities and precursors for chemical synthesis. Significant reduction of lignin content could also lead to lodging, increased susceptibility to pathogens, and increased water loss. These considerations have led researchers to investigate ways to alter lignin monolignol composition rather than drastically decrease total lignin. Biotechnology approaches to increasing product yields from biomass with specific focus on biofuel chemicals will be discussed in Section 3.1.2.

Thermochemical conversion has been considered a competing technology to bioconversion, but in most biorefinery designs both



**Fig. 3.** Systematic processing of the ideal biofuel plant feedstock. Simple distillation or extraction of biomass would yield *in planta* biofuel chemicals and coproducts such as bioplastics (PHAs, PHBs), pharmaceuticals (artemisinin, taxol), and food and cosmetic additives (limonene, geraniol, citral). Biofuel feedstocks with this characteristic would allow for production of biofuels and biobased products in rural and areas without biorefinery capabilities, and help to offset costs associated with transportation of biomass to biorefineries. Lignocellulosic conversion would occur in areas with a biorefinery infrastructure. This segment of biofuel processing would allow for more complete conversion of biomass and produce a host of coproducts such as green chemical precursors that require either microbial fermentation or further processing to develop valuable coproducts. Examples include ethanol, butanol, carbon fibers, succinic acid, lactic acid, and valeric acid for biodiesel. The residue remainder of the biomass that cannot be bioconverted will be processed using thermochemical conversion to generate syngas, biocrude, and/or combusted to produce heat or electricity. This will reduce the volume of biomass that has to be converted at high temperatures, and reduce the chemical complexity of biocrude generated from fast pyrolysis.

technologies are included (Cherubini and Jungmeier, 2010; Lyko et al., 2009). Bioconversion and separation of products from feedstocks before thermochemical conversion allows for the production of high-value native coproducts and complex biochemical metabolites that are not feasible for chemical synthesis. Biocrude oil produced from fast pyrolysis is chemically diverse and needs to be catalytically upgraded (Yaman, 2004). Selectively removing large portions of biomass as extractable or fermentable biofuels and coproducts before thermochemical conversion could lead to biocrude with simpler chemistry and higher product specificity.

### 3.1.1.1. Plant metabolites for extractable biofuels

Production of switchgrass feedstocks with extractable portions of drop-in biofuels requires two key traits: 1) production of metabolites with suitable fuel properties for combustion in modern gasoline or diesel engines, and 2) storage of metabolites in high concentrations that will not be toxic to plant tissues. Plants produce a range of hydrocarbons that could be used as drop-in ready biofuels and coproducts (Table 3). Plants produce an incredible diversity of C<sub>10</sub>, C<sub>15</sub>, and C<sub>20</sub> isoprenoids, also called terpenoids, which are derived from precursors comprised of isoprene units. The chemical structures of monoterpenes (C<sub>10</sub>), sesquiterpenes (C<sub>15</sub>), and diterpenes (C<sub>20</sub>) are highly diverse and are primarily isoalkanes/enes, and cyclic alkanes/enes. Sesquiterpene synthesis has been shown to occur primarily in cytoplasm, whereas mono- and diterpene synthesis occurs primarily in plastids (Chen et al., 2011). The diverse array of terpenoid isoparaffins and naphthenes produced in plants is reminiscent of the gasoline and diesel fractions of petroleum (Table 1). Catalytic conversion of the monoterpene pinene yielded a biofuel that had similar net heat of combustion and density as jet fuel, but a higher freezing point (Harvey et al., 2009). A number of terpenoid or terpenoid derived metabolites may have potential as high-value extractable coproducts. Taxol and artemisinin are expensive drugs used in the treatment of cancer and malaria, respectively, which have moved to production through tissue culture or heterologous expression of plant genes in microbes (Kirby and Keasling, 2009). Many mono- and sesquiterpenes are volatile organoleptic compounds responsible for the taste and smell of fruits and flowers, and as such are commodities in the food and cosmetic industries.

Phenylpropanoids makeup one of the largest pools of plant metabolites and are involved in pathogen defense, ultraviolet light protection, and biosynthesis of lignin (Besseau et al., 2007). Lignin monomer chemical structures are directly comparable to aromatic compounds found in petroleum. Lignin has been widely considered, at best, a byproduct of biofuel production that should be burned or converted to liquid fuels by thermochemical conversion (Kleinert and Barth, 2008), and, at worst, a large fraction of plant biomass that interferes with biofuel production and, as such, is a candidate for decreased biosynthesis (Chen and Dixon, 2007; Fu et al., 2011). However, phenolic compounds show promise as precursors for bioplastics (Kleinert and Barth, 2008), carbon fibers (Baker et al., 2009), and even antioxidants in diesel fuel (Kleinert and Barth, 2008). Directing phenylpropanoid metabolites for storage in cellular compartments would create an aromatic biofuel fraction that would enhance properties in biogasoline such as lower (net) energy per volume, and cold flow properties in biodiesel (Table 1).

Identification and characterization of novel enzymes involved in unique reactions has been identified as an important line of research that will lead to the development of future biorefinery processes and industrial chemical synthesis (Wohlgemuth, 2009). For biofuels, production of short-chain alkanes from biomass could be the most important as they make up the largest chemical fraction of gasoline and diesel (Table 1). There are two known plants that produce short-chain alkanes: *Pinus jeffreyi* and *Pittosporum resiniferum*. *Pittosporum* spp. produce a range of n-alkanes including heptane, nonane, dodecane, and undecane (John et al., 2008). *P. jeffreyi* only synthesizes n-heptane in tissues and oleoresins; preliminary radiolabeled substrate feeding experiments suggested that n-heptane is formed from octanal precursors coming from fatty acid biosynthesis (Savage et al., 1996). However, no genes involved in either *Pittosporum* or *P. jeffreyi* alkane biosynthesis are known. Recently, identification and recombinant expression of cyanobacterial genes identified as an acyl-ACP reductase and an aldehyde decarbonylase led to tridecane, pentadecane, and heptadecane biosynthesis in *E. coli* (Schirmer et al., 2010). Further investigation into these unique biosynthetic pathways will lead to applications in biofuel property and combustion characteristic enhancement, and extractable drop-in fuels.

**Table 3**  
Biofuel chemicals and coproducts derived from biomass metabolites and conversion techniques used.

Metabolite Precursor	Chemicals Generated	Fuel Chemical Class	Products	Method	References
Glucose	Ethanol	Oxygenated alkane	Biogasoline Biodiesel	Microbial fermentation	(Agarwal, 2007)
	Valeric esters	Olefins	Biogasoline Biodiesel	Chemical conversion	(Bond et al., 2010; Lange et al., 2010)
	C <sub>8</sub> -C <sub>16</sub> alkenes	Oxygenated paraffin/olefin	Biodiesel	Microbial fermentation	(Steen et al., 2010)
	Fatty acid esters	For green chemical synthesis	Solvents, resins, antifreeze	Microbial fermentation	(Octave and Thomas, 2009)
	Lactic acid	For green chemical synthesis			
	Succinic acid	For green chemical synthesis	Bioplastics, paints, food additive	Microbial fermentation	(Lyko et al., 2009)
Xylose	Ethanol	Oxygenated alkane	Biogasoline Biodiesel	Microbial fermentation	
Monolignols	C8-C12 alkanes	paraffins and aromatics	Biogasoline Biodiesel	Thermochemical conversion	(Kleinert and Barth, 2008)
	Direct coproduct and biofuel	Aromatics	Biogasoline Biodiesel	Plant biomass	Proposed
Fatty acids	Fatty acid esters	Paraffin or olefin	biodiesel, lubricants, surfactants, food additives	Plant biomass Microbial fermentation	(Agarwal, 2007; Demirbas, 2009b)
	Short-chain alkanes	Paraffins	Biogasoline Biodiesel	Plant biomass Microbial fermentation	Proposed
	Glycerol	Oxygenated paraffin	Adhesives, polymers, plasticizers, ethanol, succinate, hydrogen, butanol, bioplastics	Microbial fermentation Chemical synthesis Catalytic cracking	(García et al., 2008; Zhang et al., 2010)
Trehalose	Direct coproduct	Direct coproduct	Food and pharmaceutical coproduct	Plant biomass	(Börnke and Broer, 2010)
Terpenoids (isoprenoids)	Direct coproduct and biofuel	paraffin, olefin, isoparaffin	Biogasoline, biodiesel, jet biofuel, pharmaceuticals, food additives	Plant biomass	Proposed, (Harvey et al., 2009; Lee et al., 2008)

Alkane and isoprenoid biofuels could also be enhanced through modifications such as additions of methyl groups to create isoparaffin-like biofuels which have higher octane values for biogasoline, and better cetane number and cloud points for biodiesel. Methyltransferases have been identified that add methyl groups to a wide range of metabolites including sterols derived from terpene metabolism (Zhou et al., 2008), fatty acids to make FAMES (Yang et al., 2006), and tocopherol (Bergmüller et al., 2003). Screening of methyltransferases with n-alkanes will be required to determine if any known enzymes will catalyze the formation of isoalkanes. Terpeneoid substrates have also been modified in bacteria using both native and plant-derived cytochrome P450 genes (Misawa, *In Press*). Terpeneoid engineering work has focused primarily on mono- and sesquiterpeneoid biosynthesis and modification, and as such should provide a fundamental basis for engineering terpenoids in plants and microbes for biofuel applications.

Ultimately, the effectiveness of extractable biofuels will depend on the extent to which metabolites can be synthesized and stored in large quantities in feedstock biomass. Investigation into increasing secondary metabolite concentrations in plants have yielded mixed results. Overexpression of substrate synthesis genes and localization of terpene synthases in non-native cell organelles have showed remarkable increases in specific terpeneoid products (Kirby and Keasling, 2009). Investigation into unique species could also shed insight onto mechanisms for increasing production of terpenoids in plants. Trees in the genus *Copaifera* produce a sesquiterpene-rich oleoresin when their trunks are tapped, and can produce anywhere from 0.46 to 1.8 L at a time (Medeiros and Vieira, 2008; Plowden, 2003). However, these researchers noted that production of oleoresin from these trees is unstable and varies with age of tree and environment. Investigation of *Copaifera* saplings grown in greenhouse conditions showed *in planta* sesquiterpene production varied with age and in tissues (Chen et al., 2009). The primary sesquiterpene detected in tissues and oleoresins was  $\beta$ -caryophyllene, a compound that is directly comparable to a bicyclic naphthene. Most interestingly, *Copaifera* oleoresins have been reportedly used directly in diesel engines for transportation and production of electricity in remote areas of the Amazon (Calvin, 1983; Da Costa et al., 2007). There are several challenges to terpeneoid metabolic engineering, namely: cross-talk between terpene synthases and other metabolic pathways that can lead to uncertain product synthesis, and a large diversity but low overall concentration of individual products. The terpeneoid biosynthesis pathway is highly complex, and a single terpene synthase can have multiple products. Cellular localization of terpene synthases can also lead to a change in their products, and has been suggested as a way that terpene biosynthesis has evolved from lower plants to flowering plants (Chen et al., 2011). However, terpene synthases can be engineered by altering amino acids present in the reaction pocket to influence product specificity (Köllner et al., 2006). Using a maize sesquiterpene synthase that natively had two major products, Köllner et al. were successful in creating amino acid mutations that could alter the enzyme activity to one specific major product or the other. Recombinant expression of terpene synthases that have been engineered for production of a single or a few select major products would enable biofuel production from this portion of plant metabolism. Expression of a *tyrosine ammonia-lyase* (TAL) from *Rhodobacter sphaeroides* in *A. thaliana* shunted more carbon into the phenylpropanoid by synthesizing p-coumaric acid from tyrosine (Nishiyama et al., 2010). Down regulation of the *hydroxycinnamoyl-CoA shikimate/quinic acid hydroxycinnamoyl transferase* (HCT) gene in *A. thaliana* resulted in increased accumulation of flavonoids and altered lignin profiles (Besseau et al., 2007). However, the accumulated flavonoids interfered with normal auxin transport in transgenic plants resulting in a dwarf phenotype.

Storage of biofuel metabolites *in planta* without toxicity to the cell is the second key step in engineering plant extractable biofuels. Modification of the phenylpropanoid biosynthetic pathway to reduce lignin resulted in dwarf *A. thaliana* growth from perturbation of auxin

transportation through cells (Besseau et al., 2007). *In planta* synthesis of polyhydroxyalkanoates (PHAs) polymers causes a measurable reduction in seed set and growth (Suriyamongkol et al., 2007). Storage of metabolites must be considered when engineering plants for specific applications. The vacuole in plant cells is usually the largest organelle, and stores a host of secondary metabolites generated by the cell during its life cycle. Therefore it is a perfect target for sequestration of novel biofuel metabolites. Vacuolar  $H^+$ -ATPase and vacuolar pyrophosphatase transporters are responsible for transport of a large fraction of metabolites into the vacuole (Roytrakul and Verpoorte, 2007). These vacuolar transporters and others are targets for engineering extractable biofuel metabolite accumulation, and will require further investigation in switchgrass and other feedstocks.

### 3.1.2. Enhancing production of biofuel chemicals from lignocellulosic biomass

Lignocellulosic fractions of switchgrass are currently being studied so that they can be modified to reduce recalcitrance to degradation into simple sugars. Several review papers addressing lignin biosynthesis and engineering strategies to modify lignin for enhanced biofuel and coproduct production have been written (Pauly and Keegstra, 2010; Simmons et al., 2010). Both the down-regulation of genes in the lignin biosynthesis pathway, and addition of novel monolignols, such as ferulic acid and coniferyl ferulate, to remodel lignin structure have been considered to enhance biofuel production from lignocellulosic feedstocks. Successful reports achieving reduced recalcitrance in switchgrass are just beginning to be published (Fu et al., 2011). Investigation of native switchgrass lignin biosynthesis genes have shed light on useful targets for down regulation, and perhaps more importantly, genes that are important in plant defense that should not be knocked down (Escamilla-Treviño et al., 2010). Interestingly, the down-regulation of lignin biosynthesis in *Medicago sativa* led to reduced growth and overexpression of drought tolerance genes and those encoding pathogen defense proteins (Gallego-Giraldo et al., 2011). Free monolignols and other phenylpropanoids may be present in higher concentrations in switchgrass biomass engineered for reduced lignin, and as such technologies to convert the aromatic chemicals or store them for extractable biofuels will need to be developed more fully.

Technology to synthesize useful biofuels and coproducts from sugars and metabolites present in switchgrass biomass is still rather new. Interest in producing better biofuel chemicals from biomass has led researchers to develop a myriad of microbial, chemical, and thermochemical conversion techniques (Table 3). Conversion of levulinic acid, derived from acid treatment of hexose sugars, to alkenes using catalysts for use as biogasoline and biodiesel has been demonstrated (Bond et al., 2010; Lange et al., 2010). Glycerol from biodiesel production has been studied extensively for conversion into useful chemicals. Increased fatty acid and acylglyceride composition in switchgrass would lead to a usable glycerol fraction that must be considered. Chemical and thermochemical conversion techniques have been most useful for converting glycerol into products. To date, these techniques have been used to convert glycerol into biofuels and many coproducts (García et al., 2008; Rahmat et al., 2010). However, fermentation of glycerol has produced succinate which can be used to generate biofuels or in green chemical synthesis (Zhang et al., 2010). Cellulose nanofibrils have been used to synthesize flexible, electrically conductive materials (Mattoso et al., 2009).

### 3.2. Improving unused portions of switchgrass biomass

Efficient utilization of all biomass of the dedicated feedstock will enhance biofuel yields and economic viability. However, data on the composition of other portions of switchgrass biomass, specifically seed composition, is scarce. One study found switchgrass seeds contained 62.9% dry weight carbohydrates, 7.4% fiber, 8.6% ash, 8.2% lipid, 12.9% protein (Christian and Lederle, 1984). Switchgrass seed yield has been

**Table 4**  
Calculated seed composition of switchgrass and yield per hectare of each component.

Fraction	Calculated content of <i>Panicum virgatum</i> seed (kg ha <sup>-1</sup> )	
	Cv Summer	Cv Sunburst
Carbohydrates	212.60	178.01
Fiber	25.01	20.94
Ash	29.07	24.34
Lipid	27.72	23.21
Protein	43.60	36.51

calculated in South Dakota at 338 and 283 kg ha<sup>-1</sup> for the cultivars Summer and Sunburst, respectively (Boe, 2007). Harvest of seed biomass in combination with leaf and stem biomass would add an additional source of high quality feedstocks such as starch (carbohydrates), protein for animal feed, and press extractable lipids for production of biofuels and/or coproducts (Table 4). Further investigation is needed to determine whether the production of biofuel chemicals from switchgrass seed biomass would outweigh the cost of harvest and processing. However knowledge from transgenic improved oilseed crop seed composition could be applied to switchgrass to increase the breadth of its utility. Overexpression of diacylglycerol acyltransferase (DGAT) in *Brassica napus* changed metabolic flux in the fatty acid biosynthetic pathway and increased overall seed oil accumulation (Weselake et al., 2008). Additionally, overexpression of a maize transcription factor involved in triglyceride biosynthesis increased seed oil content 46% but reduced seed starch content by 60% (Shen et al., 2010). Shen et al. (2010) also reported that expression of *ZmLEC1* increased oil concentrations in seeds, but delayed and decreased seed germination. A similar phenotype in switchgrass could be used as an interesting transgene containment phenotype. Expression of a fungal *DGAT2* gene increases oil in maize seed (Oakes et al., 2011), and coexpression of these genes or orthologs in switchgrass may be a viable strategy for enhanced seed quality for biofuel production. This strategy to enhance biofuel characteristics is not limited to seed biomass. Expression of *DGAT* and *LEC2* from *Arabidopsis thaliana* showed a two-fold increase of triglyceride content in *Nicotiana tabacum* leaf tissues (Andrianov et al., 2010).

After senescence, switchgrass leaves still have 10.6 µg mg<sup>-1</sup> fatty acids in extractable fractions (Yang and Ohlrogge, 2009). Increased biosynthesis and storage of fatty acids in leaf tissues could be achieved as discussed above for seed tissues. Additionally, direct synthesis of fatty acid ethyl esters (FAEEs) from glucose has been achieved in *E. coli* (Steen et al., 2010). Expression of a recombinant thioesterase for production of free fatty acids was coupled with expression of a recombinant pyruvate decarboxylase and alcohol dehydrogenase to produce FAEEs. Furthermore, hemicellulose excretion was engineered into the FAEE producing strains to liberate xylose from biomass which further enhanced FAEE production. Perhaps most interestingly, the FAEE composition could be controlled by expressing thioesterases with different substrate specificity. While this strategy may or may not be feasible to use directly in switchgrass to produce FAEEs, increasing fatty acid content in biomass would most likely increase the efficiency of FAEE production during fermentation using these engineered strains of *E. coli*.

#### 4. Conclusions

Current visions of biofuel production would utilize a portion of biomass, which might be unsustainable. Converting latent metabolites into valuable coproducts and biofuel chemicals will lead to not only a more robust biobased products industry, but reduced reliance on petroleum feedstocks for chemical synthesis and liquid fuels. Additionally, engineering production and storage of biofuel metabolites that are extractable from biomass using simple techniques such as distillation or cold pressing will enable liquid fuel production, and perhaps even isolation of coproducts in rural or undeveloped areas. Production of

biofuels in rural areas and farmlands of the United States will help to reduce costs associated with transportation of biomass to biorefineries, and lend more incentives to farmers to grow dedicated feedstock biomass. Any sustainable biorefinery concept will reach far beyond simple liquid fuels such as ethanol.

To create plant-extractable biofuels, we need a greater understanding of how biochemical structures combust in engines and we must be able to manipulate unique metabolite biosynthetic pathways, such as short-chain alkane biosynthesis. Additionally, genes and engineering strategies useful in transporting and storing large amounts of metabolites in plant organelles will be needed to avoid toxicity issues. Once these technologies are developed, they will then be applicable to any biomass feedstock for biofuel production being considered across the world. Use of biotechnology for optimization of biofuel feedstocks is critical in replacing petroleum as a natural resource. As such, strategies for transgene biocontainment and mitigation of gene flow and research to help inform and guide proper regulation of transgenic feedstocks are crucial in developing the biofuel industry's infrastructure (Kausch et al., 2010).

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