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Analysis of the Fatty Acid Biosynthetic Pathway for the Production of Fuels in Genetically Engineered Bacteria

On August 1, 2012, The National Petroleum Council (NPC) in approving its report, *Advancing Technology for America's Transportation Future*, also approved the making available of certain materials used in the study process, including detailed, specific subject matter papers prepared or used by the study's Task Groups and/or Subgroups. These Topic Papers were working documents that were part of the analyses that led to development of the summary results presented in the report's Executive Summary and Chapters.

These Topic Papers represent the views and conclusions of the authors. The National Petroleum Council has not endorsed or approved the statements and conclusions contained in these documents, but approved the publication of these materials as part of the study process.

The NPC believes that these papers will be of interest to the readers of the report and will help them better understand the results. These materials are being made available in the interest of transparency.

Analysis of the fatty acid biosynthetic pathway for the production of fuels in genetically engineered bacteria

Future Fuel Technologies, National Petroleum Council (NPC) Study

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Introduction:

About 80% of the world's energy is generated by burning fossil fuel and the demand for energy is projected to increase more than 30% by 2030 (Zhou and Li, 2010a). The increasing demand for energy, surging crude oil prices, environmental concerns and rapidly depleting fossil fuels necessitate sustainable and eco-friendly fuels. The renewable aspect of biofuel production relies upon converting and storing the "free" energy of the sunlight in carbon-carbon bonds of photosynthesizing organisms like plants and algae. This energy stored in carbon-carbon bonds can then be re-arranged by metabolic pathways present in living organisms or by catalytic methods developed in the petroleum refining industry to produce specific biofuels. Thus there exist a couple general biofuel production schemes including catalytic conversion of high-lipid content plants and algae into biodiesel or bio-conversion of plant-derived sugars (or sunlight) into various fuels like ethanol, diesels and jet fuels.

In the past, biofuel production has largely resulted from organisms that naturally produce high-levels of fuel-like chemicals, including ethanol and butanol. However, the nature of petroleum and the multitude of chemistries that are contained in gasoline, diesel and jet-fuel, which all contribute to the fuel's overall properties of combustion establishes a need to biosynthesize more chemistries that better mimic petroleum-derived fuels. Yet, metabolic pathways to these other chemistries do not naturally exist or have not been optimized to produce high-levels of product. Recently, progress in synthetic biology and metabolic engineering has enabled production of alternative "second-generation" biofuels like drop-in replacements or blends for gasoline, diesel and jet fuel (Antoni et al., 2007). Synthetic biology has enabled researchers to biosynthesize these fuels with superior properties compared to ethanol and butanol, resulting in higher energy density, heating value and compatibility with the existing transportation infrastructure including use in combustion, compression and jet engines and the

ability to transport in existing pipeline infrastructure. There are a limited number of metabolic pathways, which are being commercialized, that produce hydrocarbons relevant to fuel chemistry and these include derivations of the amino acid pathway to produce isobutanol (Gevo), the mevalonate pathway to produce farnesene (Amyris), the polyketide pathway to produce a variety of fuels (Lygos) and the fatty acid pathway to produce biodiesels (Solazyme, Solix, Joule, Sapphire, & LS9). These pathways all naturally exist in many different micro-organisms and can be genetically manipulated or transported into a "naïve" host to increase an organism's biosynthesis capacity for a specific fuel product. The biochemical discovery and understanding of these pathways has facilitated our ability to produce a suite of renewable hydrocarbons to be used as fuels.

In addition to different hydrocarbon-producing pathways there are different microbes such as bacteria, algae and yeast used for the production of fuels. Examples of industrially-relevant microbes with efforts from various companies include algae – Solazyme, Solix, Joule, Sapphire; yeast – Cargill, Gevo, Amyris; and bacteria– LS9. Generally, microbes are chosen based on a number of properties and can include industrial scalability, natural ability to produce a fuel of interest, tolerance to fuels, ability to grow on unique feedstocks, and ability to genetically modify them. Salient examples include the choice of yeast and E. coli for their proven industrial scalability and ease of performing genetic manipulations and algae for its ability to naturally accumulate high-levels of fatty acids and use sunlight as a "feedstock".

In this paper, we focus our analysis to two categories – the fatty acid pathway for hydrocarbon fuel biosynthesis and organism choice. First, we give a general overview of hydrocarbon biosynthesis pathways, discuss the fatty acid pathway biochemistry, and efforts to engineer the pathway, control chemistry, and achieve industrially-required production levels. We then follow with a discussion of host organisms being employed for fatty acid biofuel production, and conclude with a discussion of the various research institutions and the overall challenges they are facing.

Fatty acid biosynthesis for biofuel production

As discussed, there are a discrete number of biosynthetic pathways (amino acid, isoprenoid, and fatty acid pathways) that serve as the basis for the production of new fuels such as short-chain alcohols (ethanol, butanol, etc.), branched-chain alcohols (isobutanol, isopentanol,

etc.), and long-chain hydrocarbons (fatty acid esters, geranylgeraniol, etc.) (Fig1) those are common to many microbes. The fatty acid biosynthesis pathway yields a range of energy rich molecules suitable for use as biofuels and extensive research has been done to utilize this particular pathway, also reviewed elsewhere (Yu et al., 2011). Fatty acid biosynthesis is generally used by organisms to make their cell membranes and in microbes like *E.coli* is catalyzed by an enzyme system consisting of discrete proteins that all concertedly play a role in growing and fully reducing a linear hydrocarbon chain.

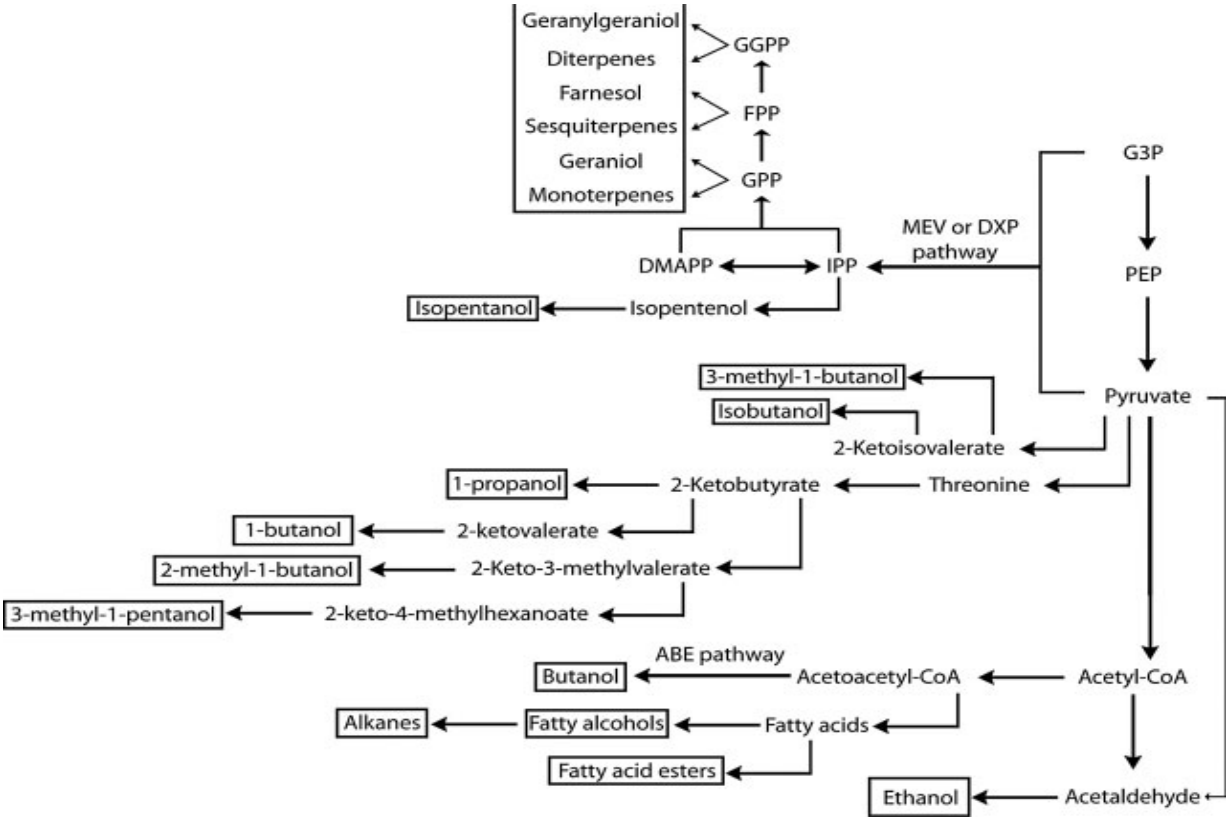

Fig 1. Biosynthetic pathways for biofuel production (Peralta-Yahya and Keasling, 2010)

In bacterial, fatty acid biosynthesis, acetyl-CoA carboxylase (ACC) is the enzyme responsible for the first committed step, catalyzing malonyl-CoA synthesis from acetyl-CoA and carbon dioxide. Malonyl-CoA is then transacylated to a fatty acyl carrier protein (acyl-ACP) by malonyl-CoA:ACP transacylase. Subsequently, acetyl-ACP and malonyl-ACP are condensed to

yield acetoacetyl-ACP in the presence of β-ketoacyl-ACP synthase with concomitant release of carbon dioxide. Then the β-ketoacyl substrate undergoes a cycle of reduction, dehydration and reduction resulting in the formation of the four-carbon fatty acyl-ACP (butyryl-ACP). This cycle of continues with two-carbon additions to the fatty acid chain provided by malonyl-ACP condensations and results in a 14 to 18 carbon fatty acyl-ACP final product (Fig 2). The fatty acid pathway in *E. coli* is tightly linked with biosynthesis of phospholipids and the pathway's final product is transferred to glycerol derivatives by glycerol-3-phosphate acyltransferase in order to build the cell membrane (Magnuson et al., 1993; Zhang et al., 2011). Fatty acid biosynthesis is energetically expensive for the cell and thus tightly regulated. Transcriptional and translational regulation balances the presence of pathway enzymes, while flux regulation prevents further, unnecessary biosynthesis of fatty acids under nutrient limiting conditions.

Efforts to engineer native pathways for the production of fatty acid biofuels have exploited the mechanisms of transcriptional, translational and flux regulation. Proven, successful strategies that result in high-level production of fatty acids are outlined in Fig 2.

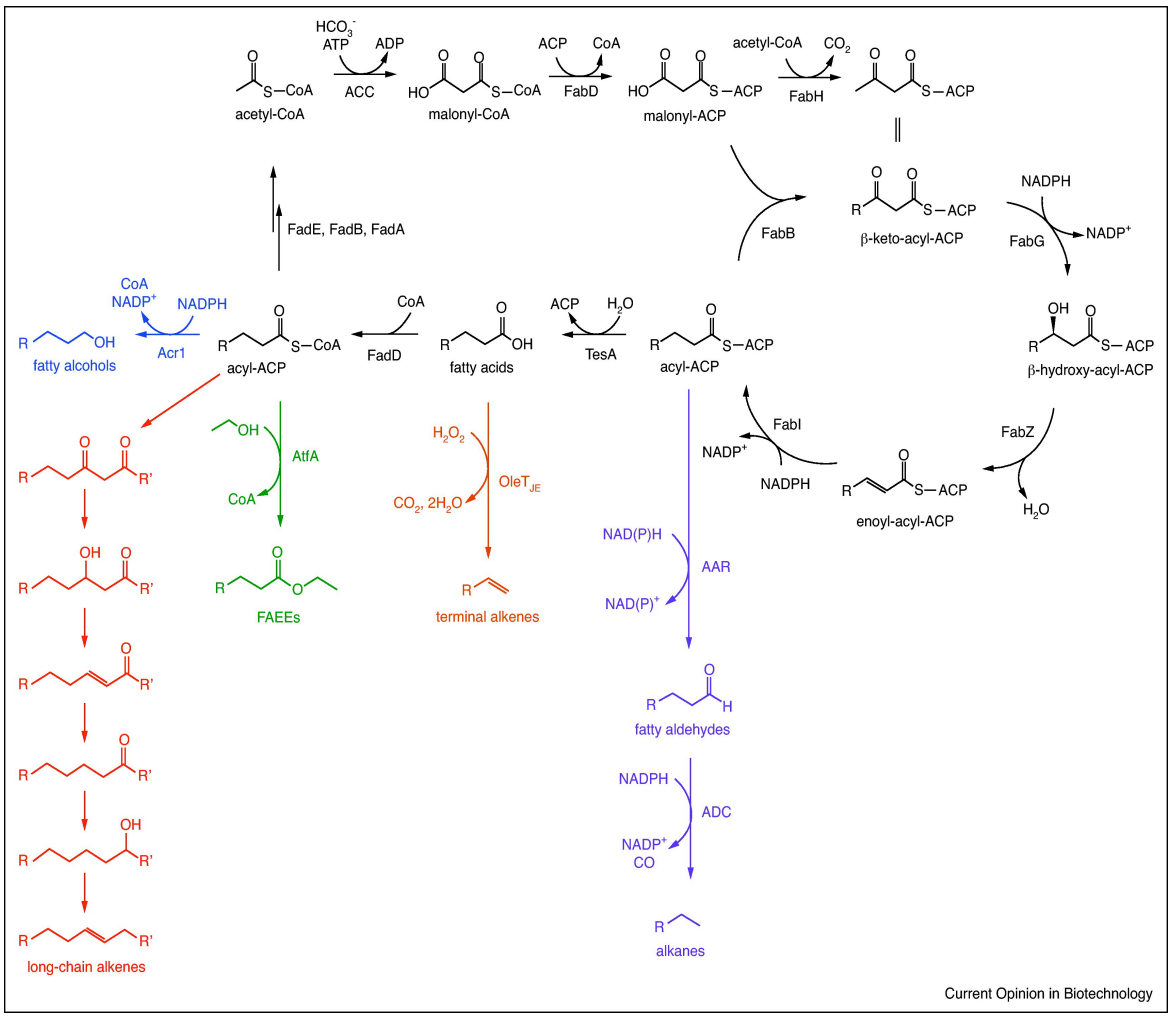

Fig 2. Pathways for the production of fatty acid-based biofuels.

Native E. coli fatty acid pathway is colored black. The proposed pathway for long-chain alkene biosynthesis is colored red. Engineered pathways for the production of other derivatives are in different colors (Zhang et al., 2011).

Host organism selection

Selection of a fitting host organism plays a key role in the productivity and yield of the biofuel production process. As the mechanisms associated with fuel production is host specific finding a microorganism with desirable properties is the pre-requisite for any metabolic effort in fuel production. The host organism should be amenable to genetic manipulation, efficient growth rate, ability to degrade lignocellulosic material, ferment various substrates at high rates with high

yields, tolerance to high temperature, pH, product toxicity and end product etc. The following are the most commonly used microorganisms for biofuel production.

E.coli and Yeast:

Though numerous host strains could be employed for fatty acid fuel production it is desirable to take advantage of the existing metabolic capability in a genetically tractable host like *Escherichia coli (E.coli*) to achieve maximal productivity (g/L/h), titer (g/L), and yield (g-substrate / g-fuel product) (Connor and Liao, 2009). Because of the tractability of microbial hosts like *E. coli*, one can use a variety of genetic and metabolic engineering technologies to rapidly modify metabolic pathways to increase fatty acid production and ultimately achieve industrially-relevant production metrics that approach price-parity with petroleum fuels. Among bacteria, *E.coli* is a well-studied organism and it has been used to produce valuable chemicals and biofuels. Although it can be altered genetically to produce a wide variety of fuels such as ethanol, isopropanol, hydrogen and biodiesel (Liu et al., 2010a), it has proven its ability to scale only in the case of 1,3 propanediol production (Katz, 2007). Though, the natural level of lipid stored in *E. coli* cell is low it has several advantageous traits such as, rapid growth rate, ability to grow in an anaerobic environment, efficient utilization of various biomass as feedstock for biofuel production and high fatty acid synthesis rate (0.2g/L/h/g dry cell mass) (Connor and Atsumi, 2010; Handke et al., 2011). As described earlier the heterotrophic bacteria (*E. coli*) can ferment glucose and other lignocellulose derived sugars as an energy substrate to produce different types of fuels. However, the recalcitrant nature of cellulosic biomass requires separation of polysaccharides from lignin and subsequent depolymerization of the polysaccharides by enzyme (Lu, 2010).

On the other hand, natural ethanologenic organisms such as *Saccharomyces cerevisiae (S.cerevisiae)* and *Zymomonas mobilis (Z.mobilis)* have the ability to produce ethanol from sugars and resulting in a theoretical maximum yield of 98%. However, these organisms lack the ability to ferment complex sugars like pentoses. To overcome this problem, research efforts have focused introducing pentose-metabolizing pathways into *S.cerevisiae* and *Z.mobilis* or engineering microorganisms to secrete cellulases and hemicellulases to depolymerize the complex sugars into sugars and subsequent conversion into fuels.

***Light-utilizing organisms: Photobacteria**:*

The use of photosynthetic organisms offers an alternate approach for the production of fuel compounds, in which they capture light energy and subsequently convert that into high energy organic compounds using water as the final donor. Many photosynthetic organisms such as algaes and bacteria have been used for the production of valuable metabolites including fuels, chemicals and organic acids etc. It is reported that employing photosynthetic bacteria for fuel and chemical production can overcome the energy intensive and costly biomass recovery (Liu et al., 2011), Lu, 2010; Tan et al., 2011). Among the photosynthetic bacteria *Cyanobacterium* offers unique advantages due to the following reasons.

Cyanobacteria have high potential for biofuel production due to their photosynthetic capability, rapid growth rate, efficient solar energy conversion, amenable to effective genetic manipulation, tolerance to high CO_2 content and ability to thrive in marginal environments (Lu, 2010; Zhou and Li, 2010b). To date, various strains of cyanobacteria are genetically engineered to produce energy rich compounds such as ethanol, isobutyraldehyde and other fatty alcohols. Fatty acid based biofuel production is carried out by genetic modification of cyanobacteria to overproduce free fatty acids (FFA)(Liu et al., 2010b) and fatty alcohols and hydrocarbons (Tan et al., 2011).

Though cyanobacteria are efficient in tapping the solar energy to generate reducing equivalents from water and fixing CO_2 into fuel (Liu et al., 2010b; Lu, 2010) the primary concern is contamination, toxicity of the host and development of sustainable production processes, etc.

Current maturity of the technology:

Current research on fatty acids to biofuels is advancing rapidly with new innovations being reported on a daily basis. Significant improvement and success in metabolic engineering and advances in synthetic biology tools to manipulate cellular metabolism for increased fatty acid production make this pathway more efficient for fuel development. Apart from academic institutes, private companies (like LS9) are actively involved in fatty acid based fuel using *E.coli* (Elshahed, 2010). Although there is a rapid progress in fatty acid derived fuel, improvements are

Table 1: Illustrates cost of fatty acid biofuels.

	Ethanol	Butanol	Isopentanol	Isopentenol	**Fatty acid**
Experimentally achieved yield (%)	98	15	33	1	**20**
Theoretical minimum cost per gallon ($/gal)	2.10	2.70	3.40	2.30	**3.25**
Current cost per gallon($/gal)	2.14	18.00	10.30	230.00	**16.15**

(Theoretical maximum cost per gallon was calculated using theoretical maximum product yield/gram of glucose and assuming a feedstock cost of $0.065/lb glucose and 40% of the total fuel cost represented by raw material. Whereas, the current cost/gallon was calculated using theoretical maximum cost per gallon/ highest experimental yield (Handke et al., 2011)).

still necessary to be cost-effective at industrial scales (Table 1). For example, the reported total fatty acid yields in genetically engineered *E.coli* are 2.5g/L and 4.5g/L with a conversion efficiency of 4.8% and 6% respectively representing approximately 20% of the maximum theoretical yield (Lu et al., 2008; Tiangang Liu et al., 2010). In another study the theoretical yield was 14% in shake flasks with glucose as a carbon source (2% glucose). It is also reported that the same group achieved near theoretical yield with 6g/L (personal communication). Thus, by combining genetic, metabolic and synthetic biology tools, improvements can be made in fine tuning the gene expression of this pathway for increased fuel production.

Recently, in an effort to overcome the energy related issues, the U.S. Department of Energy (DOE) has established bioenergy research centers and awarded funding to various institutes including: Oak Ridge National Laboratory, Lawrence Berkeley National Laboratory, Great Lakes Bioenergy Research Center, Arizona State University (ASU) (cyanobacterial biofuel research) and University of Minnesota (hydrocarbon fuel from *Shewanella* bacteria). The DOE also launches initiatives with companies that are involved in identifying and developing

breakthroughs in biofuels and other advanced energy technologies. The notable companies that have been awarded funding include Joule Unlimited and LS9 (Kagan, 2010; LS9, 2011). Detailed descriptions of these research projects are available at ARPA-E (Advanced Research Projects Agency – Energy).

Challenges:

Research efforts are focused on reducing the cost and increasing the production efficiency of the process. The major problems in the production process include feedstock cost, and their conversion efficiency into fuel, low productivity, and titers. Because of the significant cost for feedstock the host organism for fuel production should perform at high yields with any given substrate. This can be achieved by combining consolidated bioprocessing (CBP) and functional pathway expression in suitable host organisms. To increase the productivity and yield optimizing metabolic pathway is necessary. This could be achieved by combining metabolic engineering and synthetic biology tools to diagnose problems like toxicity that in turn can increase the yield(Dunlop et al., 2011). However, to make the fatty acid derived fuel economically viable further research is warranted in the following areas.

- Identification and employment of multiple and robust host strains are requisite to utilize different biomass at higher rates and produce a wide variety of biofuels. Host organisms should be either highly efficient native producer that naturally accumulates fuel precursors or non- native strains that can be converted into oleaginous organisms by engineering their metabolism.

- The major cost driving factor in microbe derived fuels is the feedstock and it accounts for roughly 2/3-3/4 of the total production costs (Rude and Schirmer, 2009). Hence the necessity to identify and utilize alternate carbon sources and develop highly efficient host organisms to metabolize these alternate carbon sources is warranted.

- Integration of various metabolic, synthetic, and bio-molecular techniques are required for a better understanding of microbe metabolism and carbon flux within the system. This

would aid in further development for pathway optimization for biofuel processes (Peralta-Yahya and Keasling, 2010).

- Higher production cost and the need to improve scalability necessitates the development of efficient methods to scale up biofuel production at the industrial level (Table 1). Further, there is a need to understand costs and technology associated with extracting the fatty acid fuels from fermentation broths.

- Overexpression of certain genes (e.g., *fadD*) that are responsible for increasing the fatty acid yield may result in antagonistic effect (Liu et al, 2010). Development of effective strategies to prevent such effect is critical for more efficient and increased fuel production.

Key Findings:

Fatty acid derived fuels and chemicals have diversified industrial uses resulting in active research within the biofuel industry. Described below are a few significant findings that would facilitate efficient and cost effective ways to engineer the microorganism for increased fuel production from a broad range of substrate.

1. New Technologies

Engineering microorganisms for fuel and chemicals production is a complex and challenging task. However, the recent developments in genomics, metabolic engineering, synthetic and system biology tools for advanced fuels has facilitated designing of new genetic constructs, identification of rate limiting factors in metabolic pathways, improvement of biocatalyst and fine tuning of other parameters. This enables higher production of novel fuels and other products. For example, the company LS9 employed synthetic biology tools to genetically alter its patent pending bacteria to convert fatty acid intermediates into "drop in" diesel fuel (http://www.ls9.com/technology/). Advances in the "omics" sciences and metabolic engineering techniques have contributed to a better understanding of cellular changes, the metabolic flux within the cells and engineering of proteins and biochemical pathways of cells to produce desired compounds such as alcohols, alkanes and alkenes in an efficient way. For

example, cell surface engineering technology in which functional proteins can be displayed on the cell surface provide molecular information about the localization mechanism of proteins. This would help to construct various biocatalyst for industrial use (Sakuragi et al., 2011). In another study, the metabolic capacity of fuel production pathways (mevalonate pathway) was increased while limiting protein expression to improve the productivity and yield by synthetic biology tools (Connor and Atsumi, 2010). It is also possible to regulate and control the genes that encode metabolic pathway by using specific promoters that in turn maximizes yield and titers (Keasling, 2010).

2. Feedstock

Many biomass feedstocks such as food and non-food crops, cellulosic materials and waste residues from different sources can be used as energy source for biofuel production. To make the cost of microbial fuel competitive with petroleum based products, the primary goal should be the production of these compounds directly from lignocellulosic biomass. ecause of its renewable nature and abundance, cellulosic biomass has the potential to be used as feedstock. However, the composition and complicated chemical structure of lignocellulosic biomass makes further processing difficult. Current processes include different pretreatments and enzymes to liberate sugar for further processing. An alternative processing technique called consolidated bioprocessing (CBP), is being developed, in which cellulase production, hydrolysis and fermentation are accomplished in a single step using genetically altered microorganism. The modified microbe possesses enzymes that hydrolyzes the pretreated biomass and obviates the need to add the enzymes, thus lowering the production cost. In a recent work, fatty acid ethyl esters (FAEE) were obtained from hemicellulose using a genetically engineered *E.coli* that expresses the gene for the production of the endoxylanse catalytic domain from C*lostridium Stercorarium* and xylanase from *Bacteroides Ovatus* (Steen et al, 2010,(Bokinsky et al., 2011)). Though the process needs significant improvement it provides a foundation for the production of fatty acid fuel from polysaccharides.

3.Overproduction of FA

The primary step in producing biofuel from fatty acid pathway is to increase the production of fatty acids using various genetic modifications. These modifications include overexpression of the rate limiting enzyme ACC that increases the supply of malonyl-CoA, deletion of *fadD* gene to prevent fatty acid degradation, overexpression of endogenous thioesterase enzyme to prevent feedback inhibition and expression of plant thioesterase to increase the yield of fatty acids. Two endogenous thioesterase enzymes (TesA and TesB) present in *E. coli* were overexpressed to release the fatty acid chain from acyl carrier protein and increase the accumulation of free fatty acid. Table 2 lists the research in genetically engineered cyanobacteria and *E.coli* for biofuel production.

Future Outlook:

The fatty acid biosynthetic pathway for the generation of fatty acid derived biofuels appears to be a promising technology in the biofuel industry. Fatty acid and their derivatives are valuable molecules for fuel and other industrial uses if the challenges can be overcome. Many academic research programs are actively involved in fatty acid production for biodiesel and other fuel derivatives. Researchers are now focused on producing new fuel-generating microorganisms and characterization of genes for efficient and high yield production of desired fuel molecules using various advanced tools to make these fuels cost competitive with petroleum products. Multiple approaches are currently employed by certain start-up companies that are focused on commercialization of biodiesel from microorganisms (Joule, LS9). Moreover, the government support through policy initiatives and funding has dramatically increased the level of scientific research in this field. If new research continues to maximize the yield of fatty acids, then the fatty acid pathway has the potential to serve as an important fuel platform in the future.

Table 2. Players and Research:

No.	Name	Institution	Research
1.	Rebecca M. Lennen	University of Wisconsin-Madison	Production of medium and short chain fatty acid in genetically engineered *E.coli* strain (RL08) that express plant bay thioesterase (BTE). The resulting fatty acid (C12) was further decarboxylated to produce alkanes.
2.	Xuefeng Lu	Stanford University	Different strains of *E.coli* were genetically modified to overproduce fatty acid. The modification includes deletion of *fadD* gene, overexpression of *Cinnamomum camphorum* acyl-ACP thioesterase and expression of an endogenous gene of *E.coli.* Glycerol was used as the carbon source and the strain accumulated 2.5g/l fatty acid.
	Tiangang Liu		*E.coli* strain XL100/pMSD8/pTL58 was modified to overexpress the genes encoding the endogenous ACC, endogenous thioesterase (TesA) and a plant thioesterase from *Cinnamomum camphorum*. Increased fatty acid production of 4.5g/l was obtained.
4.	Eric Steen	Joint Bioenergy Institute (JBEI), CA	Fatty acid overproduction in *E.coli* was achieved by overexpression of *fadD* and thioesterase (tesA) and deletion of *fadE* gene. They also demonstrated the possibility of FAEE production directly from hemicellulose.

Fatty acid production using photosynthetic bacteria			
5.	Xinyao Liu	Arizona State University	Genetically engineered cyanobacterium strain *Synechocytis* sp. PCC6803 by manipulating fatty acid metabolic pathway and deleting S layer for the increased production of fatty acids (C10-C18).The production efficiency was 133±12mg/l of culture. **S layer**: Is a cell surface protein layer that provides protection to the cyanobacterial cells and prevent the secretion of free fatty acids.
6.	Xiaoming Tan	Qingdao Institute of Bioenergy and Bioprocess Technology	Genetically altered cyanobacterium *Synechocystis* strains to produce fatty alcohols by expressing fatty acyl-CoA reductase (FAR) gene and obtained yield of 9.73± 2.73µg $OD^{-1}L^{-1}$

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