Role of *Escherichia coli* in Biofuel Production

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ABSTRACT: Increased energy consumption coupled with depleting petroleum reserves and increased greenhouse gas emissions have renewed our interest in generating fuels from renewable energy sources via microbial fermentation. Central to this problem is the choice of microorganism that catalyzes the production of fuels at high volumetric productivity and yield from cheap and abundantly available renewable energy sources. Microorganisms that are metabolically engineered to redirect renewable carbon sources into desired fuel products are contemplated as best choices to obtain high volumetric productivity and yield. Considering the availability of vast knowledge in genomic and metabolic fronts, *Escherichia coli* is regarded as a primary choice for the production of biofuels. Here, we reviewed the microbial production of liquid biofuels that have the potential to be used either alone or in combination with the present-day fuels. We specifically highlighted the metabolic engineering and synthetic biology approaches used to improve the production of biofuels from *E. coli* over the past few years. We also discussed the challenges that still exist for the biofuel production from *E. coli* and their possible solutions.

KEYWORDS: *Escherichia coli* (*E. coli*), biofuel, metabolic engineering, biodiesel, bioethanol, *n*-butanol, isobutanol, propanol, isopropanol, microbial production of fuels

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Introduction

Utilization of fossil fuels such as petroleum has increased tremendously in the twentieth century and the demand for its use is continuously increasing. This enhanced utilization of petroleum reserves has raised concerns such as depletion of reserves for future availability, unequal distribution of reserves, and global climate change due to increased greenhouse gas emissions. In order to reduce the dependency on fossil fuel reserves, significant attention has been paid in the recent past few decades to develop alternate renewable energy sources such as biofuels through cellular conversion of biomass into fuels.[1,2] Significant success has been achieved in the production of bioethanol in industrial scale as major biofuel alternatives to the traditional transportation fuels.[3–6] In 2007, the United States has produced 6.4 billion gallons of bioethanol.[7] Many studies have investigated the economics, utility and environmental benefits of bioethanol.[8–12] Bioethanol is currently in use in combination with gasoline at many places. Although bioethanol is often touted as a major alternative, it does present some limitations such as high vapor pressure, low energy density, and high hygroscopicity leading to corrosiveness.[13] Another biofuel, biobutanol, is also under investigation as a promising alternative to bioethanol because of its better energy density, low vapor pressure and less hygroscopicity over bioethanol.[13,14] Alternate biofuels such as biodiesel, propanol, and synthetic hydrocarbons are also under investigation.[15–20] Regardless of which one would become a major alternative fuel source, microbial conversion of biomass into biofuels is considered at present a major route of production.

Successful use of microorganisms for catalysis of biomass into biofuels depends on the organism's ability to produce biofuels in industrial scale at a faster rate and low cost. Recent calculations indicate that a titer of 100 g/L medium with a rate of 2 g/L per hour and a yield of 95% of the theoretical maximum yield is required.[21] Many microorganisms (often environmental isolates) possess native biochemical pathways that convert biomass into products that resemble biofuels. However, industrial scale overproduction of biofuels from these isolated microorganisms often need genetic modification and gene import to fine tune the multistep biochemical processes leading to biofuels, and are thus limited in their use due to the dearth of extensive knowledge on genetic regulation. In this regard, microbial organisms such as *Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae* are explored extensively for their potential to produce biofuels due to the presence of well-established tools for the genetic modification, well-studied growth metabolism, and their successful use in other industrial applications.[8,19,22] Especially, *E. coli* has the unique advantage of being the best-studied model organism in terms of gene regulation and expression, and also as an

organism with the largest molecular tools available for genetic engineering. *E. coli* strains can naturally utilize a variety of carbon sources (including sugars and sugar alcohols) under both aerobic and anaerobic conditions and is best suited for a variety of industrial products in addition to biofuels such as hormones, proteins, amino acids, and diverse high-volume chemicals including 1–3 propanediol and polyhydroxy butyrate.[19,23–25] Other organisms such as *Corynebacterium glutamicum* and *Closteridia* species are also successfully used in the production of various biofuels depending on the nature of the target material and the type of biofuel.[26,27]

Recent advances in metabolic engineering, systems biology, and synthetic biology[17,28–33] have played a major role in generating interest in the commercial production of biofuels from microorganisms including *E. coli*. These advances enabled us to improve natural pathways, to construct new biosynthetic pathways de novo for the optimal production of the desired biofuel products. In addition, the development of new sequencing technologies enabled the identification of the genetic variations, understanding the diversity, and characterization of the genetic makeup of organisms, which could play a role in generating new classes of biofuels.[34,35] All the biofuels derived from *E. coli* so far are derived from the modification of central carbon catabolism and the process includes the conversion of hexose/pentose sugar molecule into C2 molecules, and the further modification of C2 molecules.[19,22] Given the recent advances in technologies for the microbial production of biofuels, we highlight the metabolic engineering and systems biology approaches utilized in *E. coli* for making biofuels, and also discuss the problems that still exist and the possible solutions. Given the presence of enormous literature, we limit the review to the most promising biofuels such as bioethanol, biodiesel, *n*-butanol, isobutanol, *n*-propanol, and isopropanol.

Engineering *E. coli* to produce bioethanol. Currently, ethanol is dominating the biofuel industry and is commercially being produced as an alternate renewable fuel despite its limitations such as corrosiveness and low energy. The major source of ethanol production is lignocellulosic feed stock material (composed of lignin, hemicellulose, and cellulose) and is considered a cheaply available renewable energy source for ethanol production.[36] The hemicellulose component of lignocellulosic biomass hydrolyzes into hexose sugars (mannose, glucose, and galactose) and pentose sugars (xylose and arabinose), which are ultimately converted into ethanol through the fermentation process. Organisms such as *S. cerevisiae* and *Zymomonas mobilis* are currently used as front runners to produce ethanol through fermentation. However, these organisms cannot use pentose sugars and thus limit our ability to harness maximum productivity. In search of other alternatives, organisms such as *E. coli* and *Clostridia* sp are considered because of their ability to use both pentose and hexose sugars. Here, we focus on strategies that are being used to produce ethanol from *E. coli*.

The native *E. coli* is capable of producing ethanol through an endogenous process in which under anaerobic conditions one mole of glucose is metabolized into two moles of formate, two moles of acetate, and one mole of ethanol (Fig. 1A). The last step in the endogenous ethanol production process (Fig. 1A) involves the reduction of acetyl-coA into ethanol by AdhE.[37,38] The reduction reaction consumes two NADH molecules, while the initial glycolysis in order to convert glucose to puruvate produces only 1NADH (1NADH for each glyceraldehyde 3 phosphate

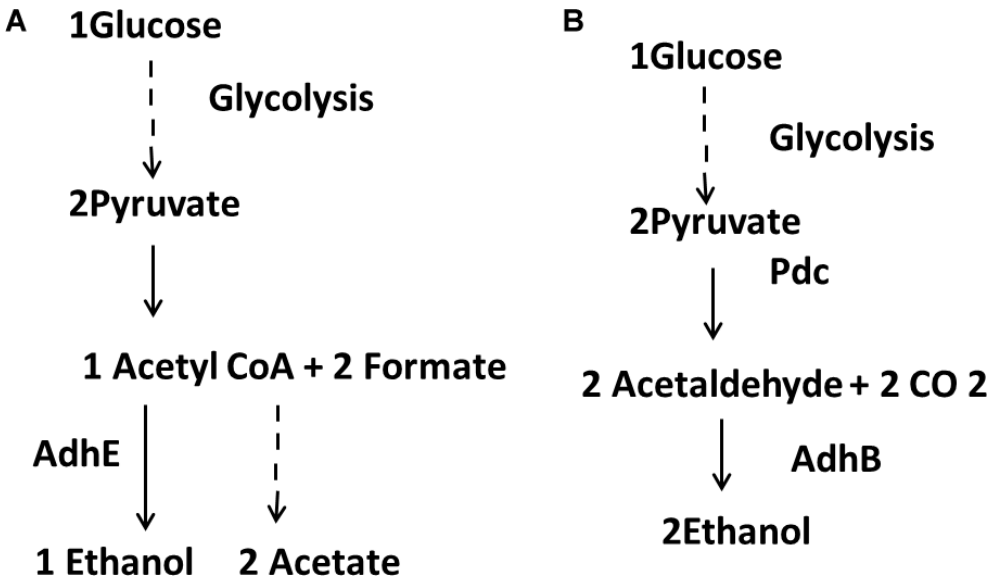

Figure 1. Strategies for the production of bioethanol from *E. coli*. (**A**) Endogeneous ethanol production pathway/Heterofermentative pathway for ethanol production in *E. coli*. (**B**) Metabolic engineering pathway for ethanol production in which endogenous *E. coli* ethanol production pathway was engineered by expressing *pdc* and *adhB* genes of *Zymomonas mobilis*. Broken arrows represent the pathways that involve multiple enzymes and steps.
Abbreviations: Pfl, pyruvate formate lyase; AdhE, alcohol dehydrogenase; Pdc, pyruvate decarboxylase; AdhB, alcohol dehydrogenase II.

to 1,3—Bisphosphoglycerate) leading to redox imbalance. To overcome the redox imbalance, the native *E. coli* balances the production of ethanol by oxidation of acetyl-coA into acetate, which requires no NADH. This native fermentation process leads to the sub-optimal level of production of ethanol, which is estimated to be 0.26 g ethanol/g of glucose, whereas the maximum possible theoretical yield is 0.51 g ethanol/g of glucose.[5]

To mitigate the problems existing in the endogenous ethanol production process, Ingram et al[3] have made successful attempts of genetic engineering in *E. coli* to produce high quantities of ethanol by inserting genes such as *pdc* and *adhB* from *Z. mobilis*. The *pdc* and *adhB* genes were expressed in operon from a plasmid under a constitutively expressed artificial *pet* (production of ethanol) promoter to produce pyruvate decarboxylase and alcohol dehydrogenase II, respectively. This heterologous fermentation pathway shown in Figure 1B produces 95% of the final products as ethanol without creating any redox imbalance (consumes only one NADH). In order to further stabilize the *E. coli* to continuously generate ethanol production, Ohta et al[39] constructed an *E. coli* ATCC 11303 strain KO3 through chromosomal integration of *pdc*, *adhB* genes along with a selective chloramphenicol resistance gene.

To further enhance ethanol production, an *frd* gene (encoding fumarate reductase) was deleted from a KO4 strain (isolate of KO3) leading to 95% reduction in succinic acid in the resulting KO11 strain.[39,40] Relative to the KO4 strain, this KO11 strain witnessed higher ethanol productivity (41.6 g/L ethanol over 72 h as opposed to 36 g/L of KO4) and theoretical yield (104% as opposed to 94% in KO4) in 8% xylose and equal productivity (52.8 g/L) and yield (~110%) in 10% glucose. KO11 strain successfully produced ethanol from various lignocellulosic hydrolysates at 10,000 L capacity.[4,40] Directed evolution of KO11 was carried out to enhance its ethanol-tolerance capabilities through alternate cycles of selection in liquid media (to increase ethanol tolerance) and solid media (to increase ethanol production) leading to the LYO1 strain.[41] A lactate-producing isolate of KO11, the SZ110 strain, was reengineered to delete all fermentative routes for NADH and insert complete ethanol-producing pathway genes *pdc*, *adhA*, and *adhB* into chromosomes. The generated LY160 strain has produced high ethanol (46 g/L) in minimal medium and with lower-grade carbon source xylose, thus leading an economical way to produce ethanol.[42]

Engineering *E. coli* to produce *n*-butanol (1-butanol). Higher-carbon alcohols, such as *n*-butanol and isobutanol, as fuels are much better than ethanol due to their less corrosiveness, high energy, high blending capability, and use in conventional combustion engines without modification.[14] *Clostridium* has a native butanol-production pathway (Fig. 2) and produces

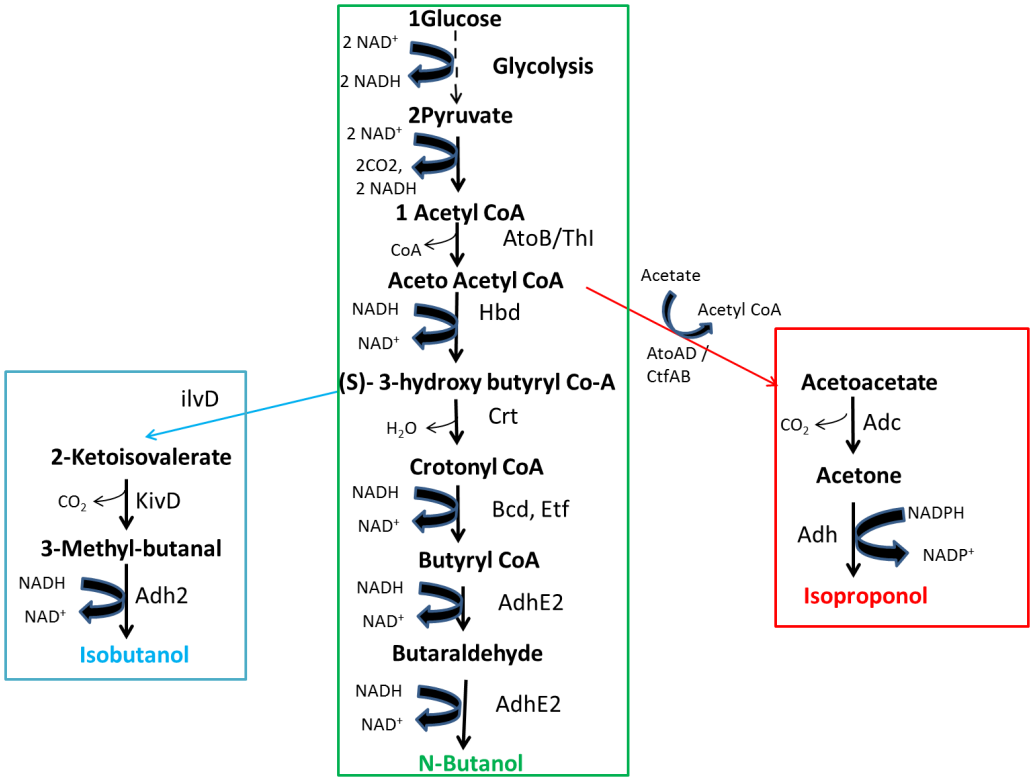

Figure 2. Metabolic pathways engineered to produce isobutanol (specific steps in the left box), *n*-butanol (middle box) and isoproponol (specific steps in the right box) from *E. coli*.

Abbreviations: ilvD, dihydroxy acid dehydratase; KivD, ketoacid decarboxylase; Adh2, alcohol dehydrogenase; AtoB/Thl, acetyl-CoA acetyltransferase; Hbd, 3-hydroxybutyryl-CoA dehydrogenase; Crt, crotonase; Bcd, butyryl-CoA dehydrogenase; Etf, electron transfer flavoprotein; AdhE2, aldehyde/alcoholDehydrogenase; AtoCD/CtfAB, acteoacetyl-CoA transferase; Adc, acetoacetate decarboxylase; Adh, alcohol dehydrogenase.

a carbon yield of 0.41 g butanol/g of glucose. However, it also produces several byproducts and causes NADH redox imbalance, thus lowering the productivity yield. Due to the nonavailability of genetic tools to modify *Clostridium* to remove byproducts, *E. coli* is considered an alternate choice. The native butanol synthesis pathway of *Clostridium* was introduced into *E. coli* by inserting *thl*, *hbd, crt, bcd, etfAB,* and *adhE2* genes. Introducing the synthetic isobutanol pathway into *E. coli* produced very low-level butanol (13 mg/L) under anaerobic conditions using glucose.[13] Further engineering by introducing *E. coli atoB* gene in place of the *thl* gene helped a threefold increase in the butanol production.[13] In order to reduce alternate carbon using pathways and butanol byproducts, additional changes were made by deleting *ldhA, adhE, frdBC, pta,* and *fnr* genes leading to the reduction in byproducts and increase in yield to 1.2 g/L (in 60 h in glucose medium) with a 15% theoretical yield of *Clostridium*.[43] More improvements to *E. coli* strain were done to lower the redox imbalance by manipulating pyruvate dehydrogenase (PDH) and formate dehydrogenase (FDH) to produce additional 2NADH in glycolysis. The resulting strain also showed an increase in production titer.[44,45]

Recently, in 2015, Saini et al[46] developed a potential production platform by developing and co-culturing a butyrate-producing strain and a butyrate conversion strain. The butyrate-producing strain was equipped with a pathway comprising *atoDA* and many heterologous genes for the synthesis of butyrate. The butyrate conversion strain was developed by removing undesirable genes, recruiting endogenous *atoDA* and *Clostridium adhE2*. By co-culturing the butyrate-producing strain and a butyrate conversion strain in M9 medium, butanol yield of 5.5 g/L with a theoretical yield of 69% was achieved. In 2016, another improved production platform was developed by the same group.[47] This platform involved multiple manipulations to drive acetyl-CoA conversion into butanol. These include enhancing acetyl CoA production, deleting genes whose expressions are needed to convert acetyl-CoA into ethanol and acetate, inhibition the conversion of acetyl-coA into carboxylic acid (TCA or Krebs cycle), and improving NADH production from glucose-6-phosphate through the pentose-6-phosphate pathway. The final strain has produced high NADH levels, *n*-butanol production of 6.1 g/L *n*-butanol with a yield of 0.31 g/g of glucose (76% of maximum theoretical yield).

Engineering *E. coli* to produce isobutanol. Isobutanol is an isomer of *n*-butanol and just like n-butanol it also possesses better fuel properties (less corrosiveness, high energy, and high blending capability) than ethanol. To engineer *E. coli* toward the production of isobutanol, two genes *kivD* and *adh2* were introduced from *lactobacillus lacti* and *S. cerevisiae*, respectively.[48] KivD converts ketoacids into methyl butanol and Adh2 converts methyl butanol into isobutanol (Fig. 2). The productivity of isobutanol in this *E. coli* strain was dependent on the level of ketoacids. For example, greater accumulation of ketoacids in the previously mentioned *E. coli* strain through the overexpression of *alsS* from *Bacillus subtilis* and *ilvCD* from endogenous *E. coli* resulted in greater accumulation of ketoacids and subsequently in the production of very high isobutanol (22 g/L isobutanol over 110 hours with 86% of the theoretical carbon yield). Owing to the successful production of 22 g/L isobutanol, it is emerging as an alternate biofuel along with ethanol and *n*-butanol.[48] However, *E. coli* is found to be nontolerant to isobutanol accumulation of over 8 g/L. To alleviate this problem, in 2014 Chong et al[49] developed an isobutanol-tolerant strain engineering the global regulator cAMP receptor protein (CRP) of *E. coli* through error-prone PCR. The resultant *E. coli* strain was capable of tolerating isobutanol levels of up to 12 g/L. Further improvements in isopropanol tolerable limits would promise a great hope for isobutanol as a fuel. Although the key intermediate steps differ, introduction of recombinant *kivD* and *adh2* facilitated the production of several other short chain alcohols from *E. coli*, including 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenylethanol, whose potential is yet to be explored fully and described elsewhere.[48]

Engineering *E. coli* to produce n-propanol and isopropanol. Propanol and its isomer isopropanol are higher chain alcohols and have similar fuel properties as *n*-butanol and isobutanol. In addition to being a potential biofuel, propanol also serves as an important solvent and chemical in many industrial applications. Here, we review the latest metabolic engineering developments used to produce these potential biofuels in *E. coli*.

In 2008, Atsumi et al[48] engineered native L-threonine pathway of *E. coli* to produce propanol. In this pathway, L-threonine is first converted into 2-ketobutyrate (using *llvA, tdc*) and the 2-ketobutyrate is further converted to 1-proponal (using *kdc* that encodes 2-ketoacid decarboxylase and *adh* that encodes alcohol dehydrogenase; Fig. 3). Later, the conversion bioprocess was further enhanced by evolving a heterologous citramalate pathway (Fig. 3).[50] In this bioprocess, *E. coli* was first engineered to express *cimA* gene of *Methanococcus jannaschii* to convert pyruvate to 2-ketobutyrate, bypassing threonine biosynthesis (shortest 2-ketobutyrate synthesis pathway). Later, *cimA* gene variants for enhanced growth were isolated by error-prone PCR. The best variant developed in this way has produced up to 3.5 g/L of propanol in 92 hours. Later, Choi et al in 2012[51] further engineered *E. coli* to improve production titer to 10.8 g/L medium (0.11 g/g of glucose) by deleting competing pathways, stress response genes, and releasing feedback inhibition of amino acid biosynthesis. In 2013, Shen and Liao[52] combined the citramalate pathway and threonine pathway to synergistically produce 1-propanol in *E. coli*. Using this synergistic method, they have shown a high 1-propanol yield (0.15 g/g of glucose) and the rate of production (0.12 g/L/h) than individual ones alone: the threonine pathway (0.09 g/g; 0.04 g/L/h) or the citramalate pathway (0.11 g/g; 0.04 g/L/h). In 2013, Srirangan et al[53] have shown

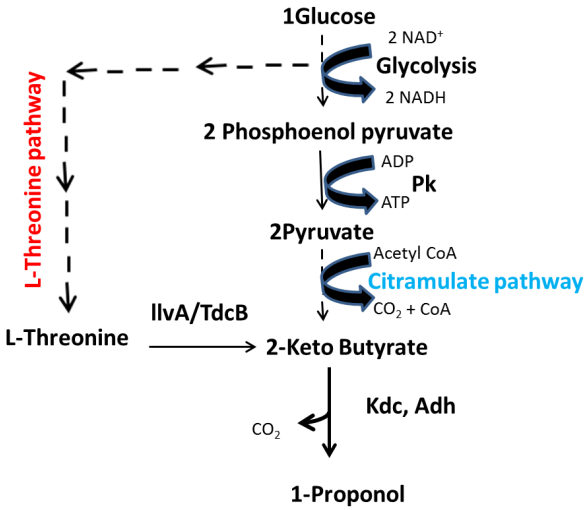

Figure 3. Metabolic pathways engineered to produce 1-proponol from *E. coli*. Broken arrows represent the pathways that involve multiple enzymes and steps.

Abbreviations: Pk, Pyruvate kinase; IlVA, L-Threonine dehydratase biosynthetic IlvA; TdcB, L-Threonine dehydratase catabolic TdcB; Kdc, 2-ketoacid decarboxylase; Adh, alcohol dehydrogenase.

1-propanol production from a totally different pathway using native sleeping beauty mutase (Sbm) operon, heterologous genes encoding bifunctional aldehyde/alcohol dehydrogenases and native succynyl CoA synthase gene. Although this novel route yielded a mere 150 mg/L of 1-propanol, it represented an alternate choice, which can be further explored to improve yield and productivity.

The synthetic pathway to produce isopropanol from the host *E. coli* was derived from *Clostridium acetobutylicum* (just like *n*-butanol). This involved engineering the *E. coli* strain by inserting genes *thl, ctfAB,* and *adc* encoding enzymes that produce acetone (Fig. 2). To further convert acetone into isopropanol, *adh* gene of *Clostridium beijerinckii* was expressed in *E. coli*. The resulting strain has produced isopropanol yield of up to 5 g/L, which is higher than the isopropanol yield from *Clostridium*. The strain also witnessed 44% of the theoretical maximum carbon yield (0.33 g isopropanol/g of glucose).[54]

Engineering *E. coli* to produce biodiesel. Biodiesel along with bioethanol constitutes almost 90% of the industrial production of biofuels. The main source of the current industrial biodiesel production is from triacylglyceride-rich vegetable oils such as rape seed oil. The production process involves a catalytic transesterification of vegetable oil with petro chemical–derived methanol. Given the raising public concerns of utilizing vast land area to produce vegetable oils for diesel rather than for food, alternative ways such as utilizing microalgae and bacteria are being explored. Although the use of *E. coli* to produce biodiesel is still in its infancy, we made an effort to put together the relevant available knowledge in this field.

Kalscheuer et al 2006[55] introduced the idea of transesterification of fatty acids with bioethanol (instead of currently petro chemically derived methanol) to produce fatty acid ethyl ester (FAEE) biodiesel (microdiesel). First, *E. coli* was engineered to produce bioethanol by introducing *Z. mobilis* genes *pdc* (encodes puruvate decarboxylase) and *adhB* (encodes alcohol dehydrogenase) as outlined in Figure 1B. To esterify ethanol with fatty acid–derived Acyl CoA, *E. coli* was engineered with gene *atfA* (encodes unspecific acyltransferase) from *Acinetobacter baylyi*. The process needed external addition of fatty acids, as the acyltransferase did not use the fatty acids produced in *E. coli* when grown on glucose. In 2011, Steen et al[56] developed an *E. coli* strain, A2A, which is capable of utilizing hemicellulose or glucose to produce fatty acids that can be used for biodiesel production. Using this strain, they observed the production of FAEE biodiesel with a yield of 9.4% of the theoretical maximum. In 2012, Zhang et al[57] developed a dynamic sensor-regulator system (DSRS) in A2A *E. coli* to improve the stability of the strain. They engineered *E. coli* with transcription factors that regulate the expression of genes involved in biodiesel production leading to increased titer to 1.5 g/L and increase in yield by threefold to 28% of the theoretical maximum. Attempts to make fatty acid methyl esters (FAME) diesel using *E. coli* by transesterification of fatty acids with methanol is also progressing rapidly. Although these are good starting points in using *E. coli* for biodiesel production, further improvements in yield and productivity are needed to practically replace petroleum-derived diesel.

Opportunities and challenges for the production of biofuels in *E. coli*. The success of using any microorganism for industrial production of fuels depends on its ability to quickly convert renewable raw material into fuel with high productivity at a low price without being toxic to the organism itself. Availability of genetic and molecular tools to engineer existing native pathways or to create a synthetic new pathway has made *E. coli* as the microorganism of best choice in order to produce biofuels from renewable energy sources. Although significant work has been done, some challenges still exist when the use of *E. coli* is considered a cost-efficient strategy for commercial production of bioethanol, higher chain alcohols, and biodiesel.

To date, the state-of-the-art bioethanol-producing *E. coli* strains showed titers in the range of 40–55 g/L and yield of ~100% theoretical maximum from various cellulosic and hemicellulosic feed sources and are similar to bioethanol produced through *S. cerevisiae*. However, cheap raw materials (cellulosic and hemicellulosic hydrolysates) used as a source contain toxic compounds such as organic acids, furan derivatives, and phenolic compounds that inhibit the growth of *E. coli* than *S. cerevisiae*. Improved pretreatment and genetic engineering approaches to improve tolerance might prove useful.[58–60]

In addition, the use of highly concentrated sugars would lead to osmotic stress, thereby decreasing the growth of *E. coli*; this can be tackled by adding osmolyte supplements that increase the overall production cost.[61,62]

Compared to ethanol, long-chain alcohols such as *n*-butanol, isobutanol, *n*-propanol, and isopropanol are fuel of best choice because they are less corrosive (low water solubility) and possess high energy and high blending capability. The physicochemical properties of these fuels are much similar to gasoline and can thus be transported with existing infrastructure and storage. However, further research is needed to improve yield and productivity of these fuels to commercialize to industrial scale. For example, the best existing *E. coli* strain for isobutanol production showed a titer of 12 g/L medium, while the same was 585.3 g/L from *C. acetobutylicum*.[63]

At present, nearly 100% of biodiesel production is nonmicrobial and involves transesterification of triglycerides in vegetable oils with methanol. Amid concerns of corrosiveness, less energy density and glycerol deposition during transesterification, biodiesel from microbial sources including *E. coli* can be considered an excellent choice replacement diesel. Moreover, diesel is suitable for microbial production due to low toxicity.[64] However, more research is needed to improve the productivity of diesel to such an extent that it can be viewed as an alternative to ingenious technology of biodiesel production from nonmicrobial sources.

Author Contributions

Conceived and designed the experiments: VK and VKRV. Analyzed the data: VK and VKRV. Wrote the first draft of the manuscript: VK and VKRV. Contributed to the writing of the manuscript: VK and VKRV. Agree with manuscript results and conclusions: VK and VKRV. Jointly developed the structure and arguments for the paper: VK and VKRV. Made critical revisions and approved final version: VK and VKRV. Both authors reviewed and approved of the final manuscript.

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