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REVIEW ARTICLE

STRATEGIES TO PRODUCE CLEAN FUELS FROM AGRICULTURAL AND ANIMAL WASTE...A REVIEW

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ABSTRACT

The concerns about energy and environment have encouraged governmental policies and scientific communities to develop novel approaches for the synthesis of biofuels. As economic growth is directly proportional to these scientific advancements, strategies for efficient utilization of readily available feedstock are grabbing extensive industrial attention. To accomplish these goals, metabolic engineering strategies are required to generate convenient host systems for successful production of biofuels. In this respect, this review aims at examining the potential bioresources and the manipulation of metabolic pathways for utilization of these resources for the production of biofuels. The review outlines the progress of metabolic engineering strategies for the production of pollution-free liquid transport fuels from alternative promising biomasses.

INTRODUCTION

An increase in emission rates of greenhouse gases has caused damage to global climate and atmosphere (Sagar & Kartha 2007). The worldwide release of CO_2 emitted from use of fossil fuels is approximately 7 Gt of carbon per year which is estimated to double by year 2050, that would elevate global warming by 2^0C, i.e., above the levels recorded in 1900 (Martinot *et al*. 2007; Liao 2016). The mounting sea levels, flagging of thermohaline movement and abolition of coral bank are one of the few examples that have raised the concerns about environment (O'Neill 2002). Along with these difficulties, a remarkable progression in the usage of petroleum over a century has also alarmed its increasing demand in the upcoming future. Theses unavoidable cause and effect relations has stimulated the scientific explorations and political acts to produce and support sustainable energy supplies within a targeted time span (Fargione *et al*. 2010.; Edenhofer *et al*. 2013). The compatibility and energy density of Biofuels has gained a lot of attention compared to their other bio renewable energy counterparts. Since, in the current scenario, biomass is the most economically favourable way to produce fuels, it has gained much of the prominence in the zone of biofuels (Lynd 1996).

Lignocellulose and lipid biomass are used as conventional feedstockstosynthesize fuels and chemicals. With the advancement of expertise, unusual resources such as waste proteins, syngas and CO_2 have been explored as renewable raw material to produce these compounds (Lan and Liao 2013). To utilize these kind of resources for fuel production, the host organism must first transform the feedstock to central metabolite, pyruvate and acetyl Coenzyme A (CoA) that are directed towards synthesis of fuels (Dien 2003).To uplift biofuel yields that could replace petroleum based fuels, synthetic biology approaches are required to engineer microorganism's metabolism (Jarboe *et al*. 2010). These constructions involve strategies to enhance consumption of resourcesthat lead to production of fuel molecules. It has been achieved either by overexpressing native pathways that are tinkered naturally over an evolutionary period or by introducing the resource utilizing pathways into the capable hosts to gain products of interest (Cintolesi *et al*. 2014).

The selection of approaches relies on the complexity of the metabolic networks and difficulties in the expression of desired proteins. The lack of well-developed genetic tools and complex physiology in non-traditional host organisms often limit the speed of strain development. Nevertheless, both strategies have demonstrated successful production of biofuel from non-conventional feedstock (Dellomonaco *et al*. 2010; Nevoigt and Nevoigt 2008).

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Pathways for biofuel production

Ethanol

Microorganisms produce ethanol by the process of fermentation using sugars derived from beetroots and bamboos, starches from maize and mueslis, and lignocellulosic materials ofmany plants. Ethanol has gained a place amongst gasoline-supplementing biofuels. However, the fermentation pathways of ethanol production also stimulate byproduct formation of lactic, formic, succinic and acetic acids (Saha & Cotta, 2012).

It is widely known that metabolic pathways that lead to production of ethanolin *Escherichia coli* opens with the transitional metabolite pyruvate. The enzyme, pyruvate formate lyase cleaves pyruvate into acetyl-CoA and formic acid (Fig. 1). Acetyl-CoA gets reduced in two steps to form ethanol by consuming two reducing equivalents. The intermediate acetaldehyde gets converted to alcohol with the help of enzyme alcohol dehydrogenase. In glycolysis, pyruvate is generated from one reducing equivalent using a 3-carbon entity, whereas, the production of ethanol requires consumption of two reducing equivalents from pyruvate (Fig. 1 A).

This process holds back fermentation course by means of formation of additional oxidized products to keep up a comprehensive redox equilibrium. This downside could be improved by devising metabolic engineering schemes to facilitate greater yields of alcohols from sugars.

versatile and flexible host systems. *E. coli* is one such facultative anaerobe which is able to metabolize an extensive range of sugars. Its lacks the genetic codon for pyruvate decarboxylase (PDC) gene (Hespell *et al*. 1996). In its place it expresses pyruvate formate lyase (PFL). PFL converts pyruvate to ethanol at an expense of two NADH molecules, leaving disproportion of NADH inside the cell (Fig 1 B).

The synthesis of organic compounds compensates this imbalance which creates NADH. Production of theses byproducts is responsible for lower yields of ethanol. This problem was rectified by incorporation of pfl genes inside a PET-operon comprising of genes encoding pyruvate decarboxylase and alcohol dehydrogenase inside the genome of *E. coli* (Fig. 1 B) (Ohta *et al*. 1991). When the mutations responsible for inhibition of succinate production were incorporated along with the integration of PET-operon, *E. coli* yielded ethanol as the sole fermentation product.

Though this kind of clever strategies enabled production of ethanol in higher yields, from an industrial point of view, this approach faced one difficulty. Host strains were incapable to grow in medias which were not rich in nutrients (Wang *et al*. 2013). The KO11 strain of *E. coli* was genetically modified for alcohol production by inserting complete ethanol pathway of *Zymomonas mobilis* encoding pdc, adhA, adhB genes, accompanied by removal of genes that enable synthesis of fermentation channels responsible for NADH synthesis (Ingram & Conway 1988) (Liu &Jarboe 2012).

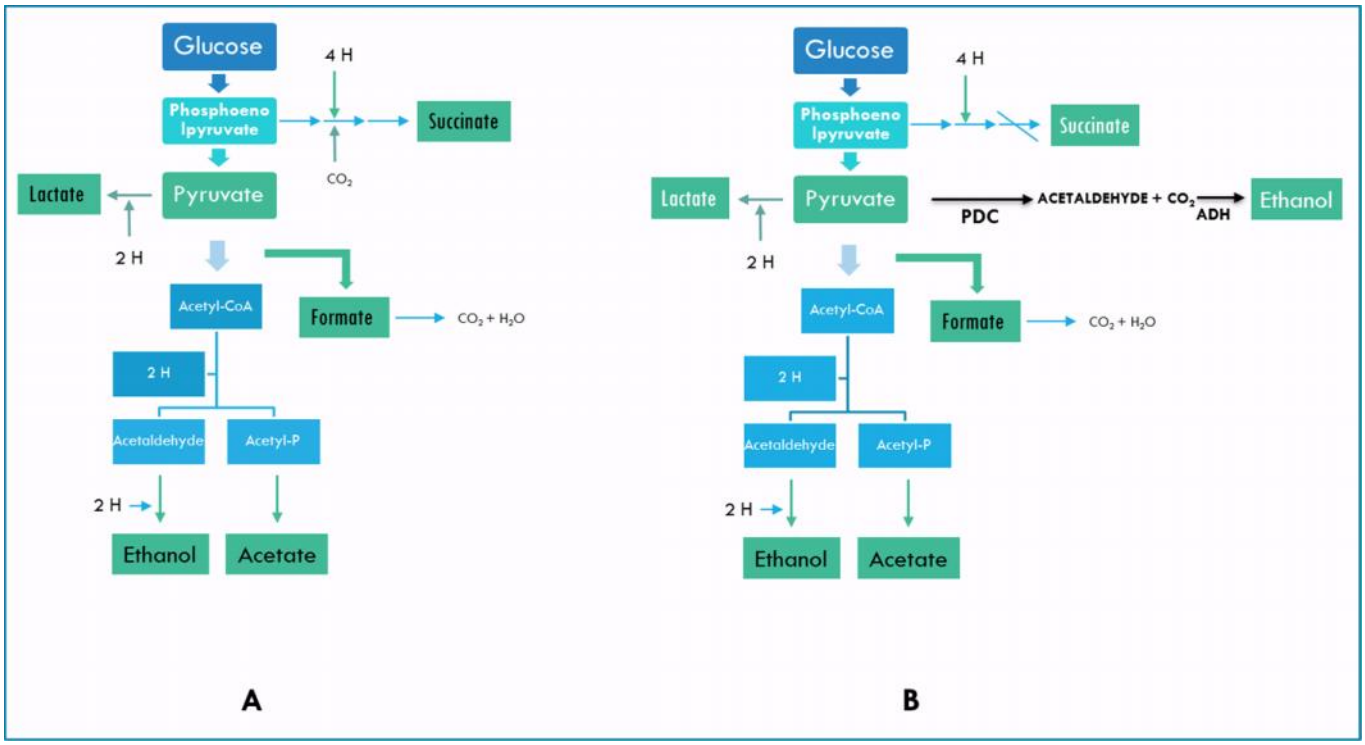

Fig 1 A Ethanol production in wild *E. coli* K-12
B Ethanol production in transformed strain of *E. coli* K-12 carrying pet operon

Historically, *Saccharomyces cerevisiae* has played a great role in development of biofuels (Nevoigt & Nevoigt 2008). But the cytotoxic effects caused by formation of fermented products has made scientists to explore metabolic networks of more

Higher chain alcohols

A range of alcohols were synthesized by manipulating routes of fermentative pathways. The next development in area of biofuels occurred in year 2008 when Liao *et al*. utilized

synthetic non fermentative pathways to synthesize higher chain alcohols which had greater energy density and closer structural compositions to petroleum based fuels (Atsumi *et al*. 2008).

2-Keto acids are common intermediates for amino acid biosynthesis as they are the immediate precursors of amino acids (Fig. 2).

networks. The overexpression of 2-ketoisovalerate decarboxylase (KivD) and alcohol dehydrogenase (AdhA) facilitate synthesis of isobutanol (Hazelwood *et al*. 2008).

In 2009, Atsumi *et al* reported the role of acetolactate synthase (alsS) isolated from *Bacillus subtilis* as a 2-Ketoisovalerate Decarboxylase for isobutanol biosynthesis in *E. coli*.

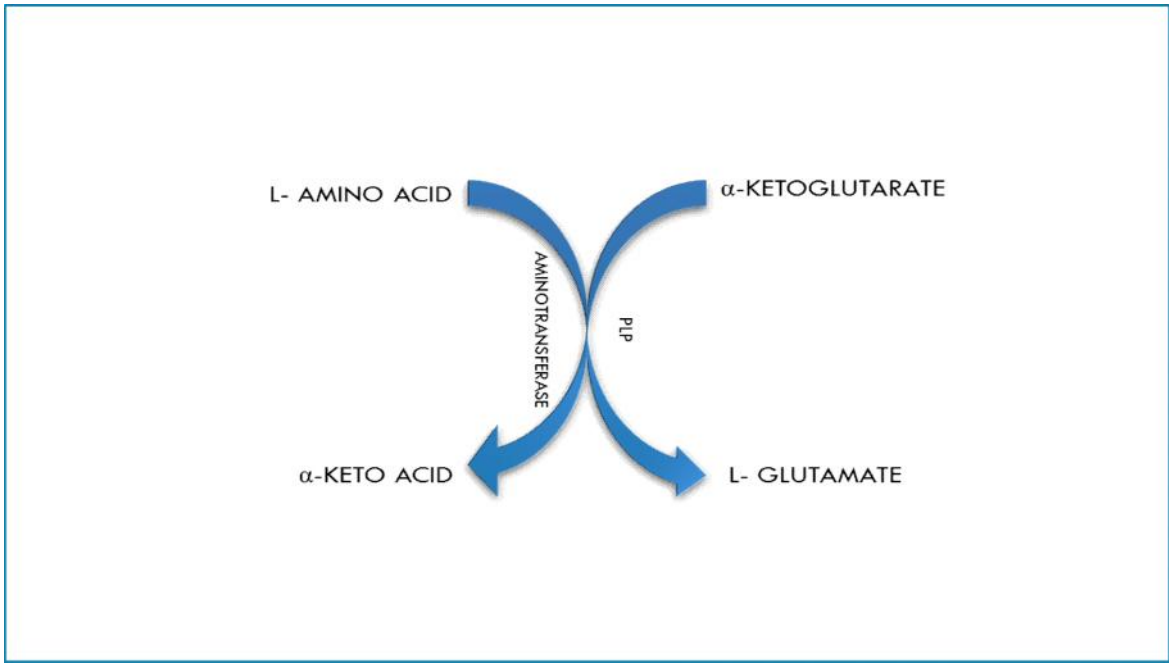

Fig 2 A reversible reaction of transamination

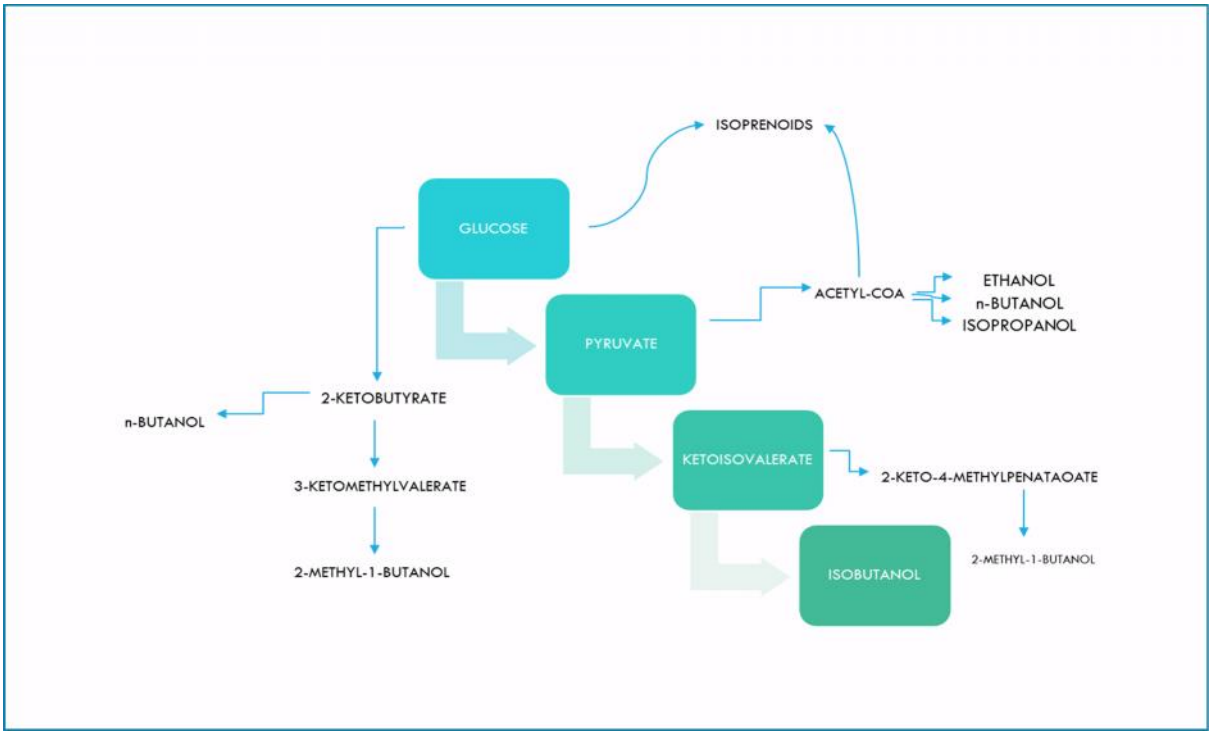

Fig 3 Metabolic pathways of higher chain alcohols

In particular, the precursor 2-keto acids of the aliphatic amino acids such as valine, isoleucine, and leucine can be diverted to synthesize higher alcohols (Fig. 3). The synthetic pathways designed for production of isobutanol are known to avert the carbon fluxes from branched chain amino acid metabolic

Its overexpression resulted in production of whooping 22 g/L of isobutanol (Atsumi *et al*. 2009). In the presence of norvaline, which is analog of valine, isobutanol productivity was improved (Lu 2016). The theoretical yield of isobutanol production

reached to its maxima, i.e., 100% yield, when second step of NADPH-dependent ketol-acid reductoisomerase (IlvC) was engineered to utilize NADH (Amara *et al*. 2011).

Isobutanol production has also been implemented in other heterotrophs such as *S. cerevisiae*. It utilized sugar to produce 0.14–0.18 g/L of isobutanol (Eden *et al*. 2001). Other heterotrophs such as *Corynebacterium glutamicum* and *B. subtilis* have also achieved production of isobutanol at concentrations 4.9–12.6 g/L and 2.62 g/L respectively (Smith *et al*. 2010). Along with isobutanol, C4 and C5 alcohols are also produced from 2-Keto-3-methyl- valerate and 2-keto-4-methylvalerate, respectively (Fig. 3 and 4). Synthesis of higher alcohols need longer 2-keto acid precursors (Fig. 4). To achieve these circumstances, a key enzyme known as 2-isopropylmalate synthase (IPMS; LeuA), in charge of elongation of 2-keto acid performs its part.

In case of recombinant organisms, their potential oxygen sensitivity demands a redox partner in the form of ferredoxin that causes poor expression of butyryl-CoA dehydrogenase electron transferring flavoprotein (Bcd/ Etf) complex. This failed expression was rectified by reducing crotonyl-CoA with the help of trans-2-enyol-CoA reductase (Ter). Expression of Ter is easy in *E. coli*. It makes use of NADH to reduce without requirement of additional ferredoxin partners (Liu *et al*. 2010; Na *et al*. 2010; Clomburg *et al*. 2012). This expression couples with glycolysis to generate NADH in the presence of glucose.This approach efficiently amplified yield of n-butanol in *E. coli*upto 1600 mg/L/d (Yan & Liao 2009).Metabolic networks for production of most of the alcohols follow decarboxylation at the completion of their pathways (Smith *et al*. 2010; Kang & Lee 2015). This steps forms a crucial reaction equilibrium near product formation where CO_2 is released in the solution. In native producers, this phase forms a driving force to produce n-butanol in higher titers.

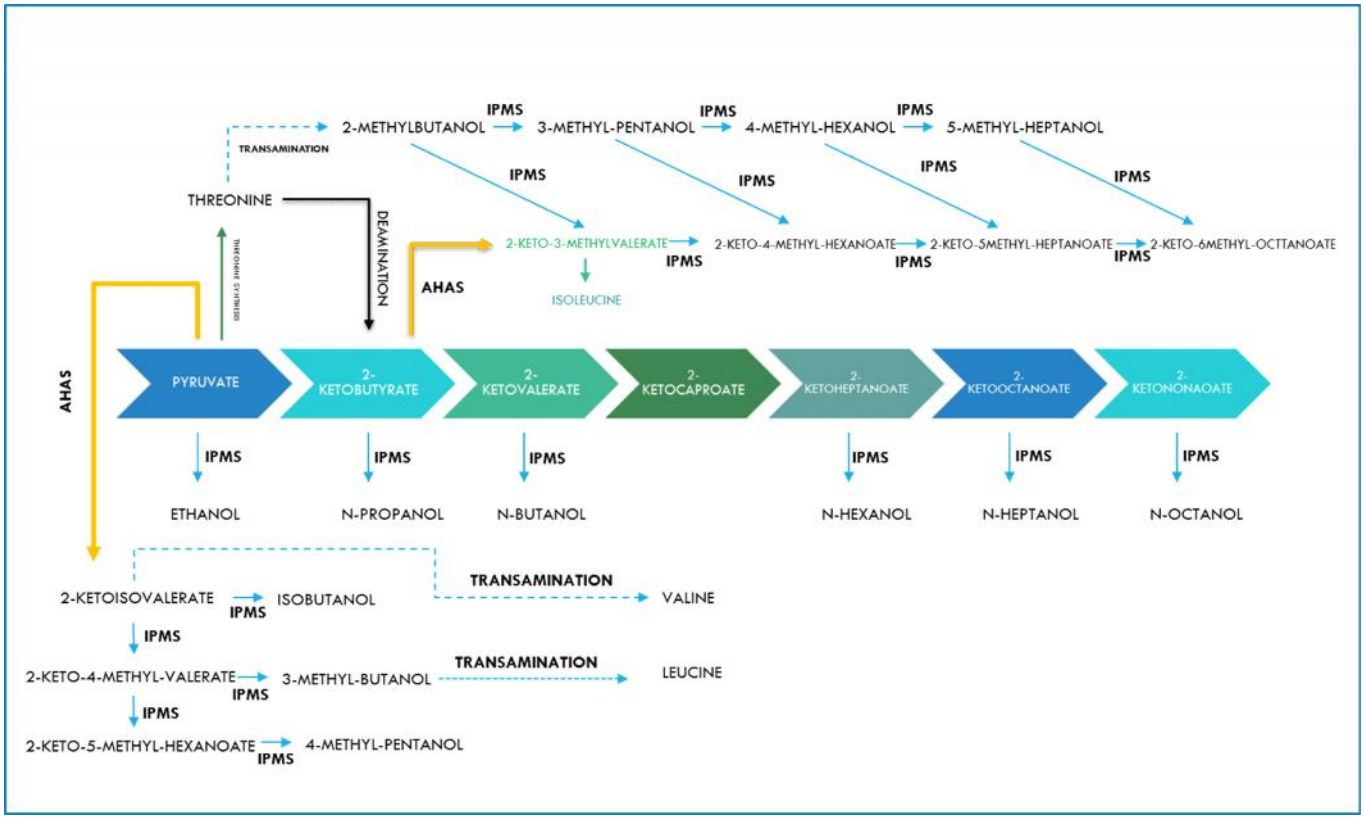

Fig 4 Production of higher chain alcohols via keto acid based metabolic pathways

Coenzyme A-dependent pathway

Clostridia produces n-butanol by Coenzyme A (CoA). The two molecules of acetyl-CoA get condensed to follow reduction and dehydration to form n-butanol. Although *Clostridia* is a natural producer of n-butanol production, its strict requirements for anaerobic conditions in fermentation procedures toughens the process of alcohol production (Kataoka *et al*. 2015). Therefore, *Clostridia's* pathway was overexpressed to other heterotrophs. These microorganisms like *E. coli*, *S. cerevisiae*, *Lactobacillus brevis*, *Pseudomonas putida* and *B. subtilis* were employed for butanol production using glycerol as a substrate, however, the titers of alcohol did not match with the original native's values (Lan & Liao 2013).

Thus, this concept was followed to engineer heterologous host systems. In 2011, Shen *et al* developed a strain that could synthetically accumulate precursor acetyl-CoA and cofactor NADH to delete mixed acid fermentation (Dldh, DadhE, and Dfrd) and acetate formation (Dpta) (Förster &Gescher 2014; Trinh 2012)

These reports conveyed importance of fluxes or driving forces for production of industrially important metabolites and therefore engineering strategies were designed to devise driving forces in the metabolic cell factories.

One such example includes overexpression of formate dehydrogenase (Fdh) to convert formate into NADH. It caused accrual of both acetyl-CoA and NADH inside *E.coli* resulting in

flux of metabolites to produce 15 g/L of n-butanol in fed batch fermentation under anaerobic condition (Shen *et al*. 2011).

These approaches of engineering host systems expanded by generating the constructs that had ability to extend carbon chain length of butyryl-CoA upon condensation with another acetyl-CoA. In *Ralstonia eutropha* this step is performed by 3-keto-thiolase (BktB) (Tseng & Prather 2012; Dekishima *et al*. 2011). On the basis of this knowledge, an *E. coli* strain was constructed to produce 469 mg/L of n-hexanol (Henne *et al*. 1999; Lan & Liao 2013; Lan & Liao 2012)

The isobutanol pathway was introduced into this strain. The biofuel production was enhanced by knocking out the genes which were responsible for polyhydroxybutyrate biosynthesis pathway (Zhang & Keasling 2012; Wells *et al*. 2011; Wlaschin *et al*. 2006). In the presence of formate, this manufactured strain of *R. eutropha* fashioned 846 mg/L of isobutanol and 570 mg/L of 3-methylbutanol within 150 hours (Lan & Liao 2013)

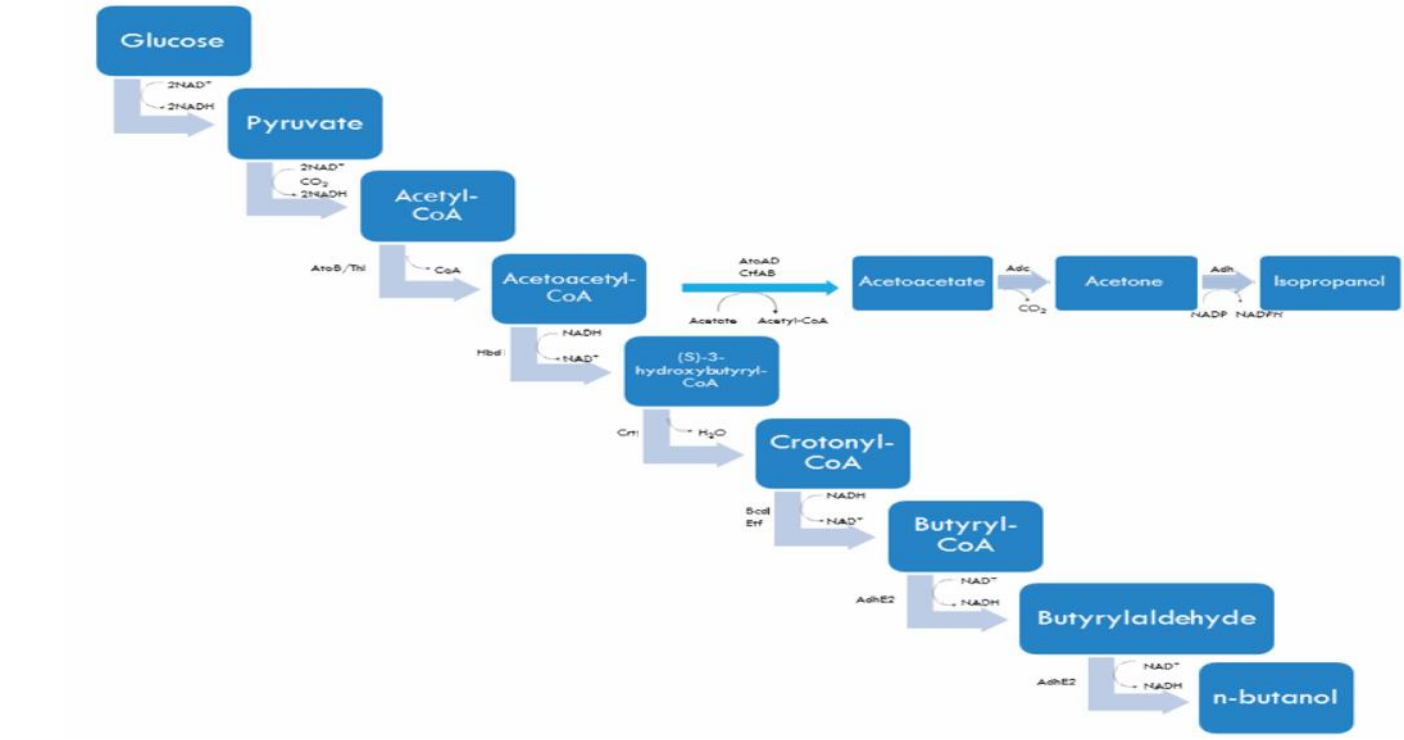

Fig 5 Coenzyme A based alcohol production

Photosynthesis for production of alcohol

Since, the CoA dependent pathway is mostly found in anaerobes, using *Cyanobacteria* for n-butanol production was extremely challenging. In 2011, Liao *et al* modified this pathway into *S. elongatus* PCC 7942 (Liao 2016). To drive the flux towards production of butanol in higher titers, the accumulation of NADH and acetyl-CoA was carried out using energy of ATP to favor thermodynamically unfavorable condensation of two acetyl-CoA (Lan & Liao 2012; Johnson & Egli 2014)

CO_2based alcohol production

While photosynthesis enables CO_2 fixation, production of biofuel by photoautotrophs demands photobioreactors that lifts the production cost and therefore is not economically favorable (Georgianna & Stephen 2012; Angermayr *et al*. 2012). This problem was solved by separating light reactions from the dark ones where CO_2 fixation and biofuel production takes place.
To use electricity for biofuel production, a chemoautotroph, *R. eutropha*, was used to fix CO_2 with formate as a basis of energy. It was engineered to harvest isobutanol and 3-methylbutanol in a combined process of electricity bound biofuel production (Zhu 2011; Kataoka *et al*. 2015).

Syngas, an energy source

The blend of CO and H_2 is known as Syngas. It is produced from both municipal waste as well as biomass and is recycled as an energy source for fixing CO_2 with the help of acetogens like *Clostridium carboxidivorans* and *Clostridium ljungdahlii* (Dellomonaco *et al*. 2010; Mcneely *et al*. 2010). *C. carboxidivorans* uses syngas to produce n-butanol, ethanol, butyrate and acetate (Hemme *et al*. 2011). *C. ljungdahlii* is known to produce ethanol from syngas. This strain was engineered to synthesize CoA-dependent pathway from *Clostridiumsps.*to produce 0.5 mM of n-butanol.

Protein as a feedstock

The nonfood Protein waste obtained from exoskeletons of animals and birds can be regarded as a potential biomass for production of biofuels. In order to convert proteins into fuel, their carbon skeletons need to be directed towards pyruvate. However utilization of amino acids from these protein rich sources creates a challenge when host cells utilize them for their own synthesis rather than making it available for production of biofuels. To solve this problem, a metabolic engineering strategy was developed by (Huo *et. al*., 2011) engineering

nitrogen flux. They generated *E. coli* strains by performing several rounds of chemical mutagenesis that deaminated protein hydrolysate. They created a metabolic driving force by removing the gdhA and glnA genes thatallow the reuptake of amino group and by introducing three exogenous transamination and deamination cycles. These metabolic conditions facilitated conversion of proteins to C4 and C5 alcohols at 56% of the theoretical yield. (Huo *et al*. 2011).

Concluding remarks

All the approaches discussed in this review have contributed significantly for production of biofuels. The basis of strategies centered on development of strains for efficient utilization of biomasses. It was achieved by using metabolic engineering techniques that either created fluxes of metabolites by deleting the competent pathways or introducing pathways that could produce alcohols of interest. Further, systems level approaches involving in silico modeling and omics technology could strengthen the yield and productivity of biofuels.

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