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Engineering Microbial Fermentations: Acetone/Butanol/Ethanol to Fungible Fuels

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By

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Professor Douglas S. Clark, Co-Chair
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Abstract

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The batch fermentation of simple sugars to ethanol has thus far been the most successful process to displace transportation fuel consumption worldwide. However, the physical properities of ethanol limit its application in most of the world beyond 10-15 vol% blends with conventional gasoline. Additionally, ethanol is too volatile to be blended at an appreciable level with jet and diesel fuels. Alternative technologies are necessary to produce compounds that can be blended in all forms of transportation fuel and to higher levels. The biological production of longer chain oxygenates or hydrocarbons has been purposed to address this challenge. However, the increased toxicity of these molecules has limited their usefulness to-date. Alternatively, chemocatalytic routes to produce these fuel molecules do not suffer from toxicity issues but the highly functional saccharide-based feedstocks result in undesirable by-product formation and yield losses.

We propose that by effectively combining these biological and chemocatalytic approaches efficient production of all classes of transportation fuels can be achieved. *Clostridium acetobutylicum* is a well-studied industrial bacterium capable of fermenting a wide-variety of saccharides into a mixture of acetone, butanol, and ethanol (ABE). These mixed products contain functionalities that are amenable to C-C bond formation through transition-metal catalysis. Thus enabling the production of a suite of long-chain oxygenates from a variety of sugar substrates. This dissertation describes three major topics associated with the integration biological and chemocatalytic processes for fuel production: (1) the initial discovery and evaluation of such a combined process, (2) tailoring the fermentation products and catalyst to favor diesel production, and (3) engineering tunable ethanol and acetone co-production in *Escherichia coli*. Additionally, a thorough and reproducible protocol describing both biological and chemocatalytic procedures are provided.

Acetone, a product of the acetone-butanol-ethanol (ABE) fermentation, harbors a nucleophilic α-carbon, which is amenable to C-C bond formation with the electrophilic alcohols (ethanol and n-butanol) produced in ABE fermentation. This functionality enables the formation of higher molecular weight hydrocarbons similar to those found in current gasoline, jet, and diesel fuels. Using a palladium-catalyzed alkylation reaction efficient conversion of ABE fermentation products into ketones, ranging from 2-pentanone to 6-undecanone was achieved. Tuning of the reaction conditions permits production of either predominately gasoline or jet and diesel precursors. Glyceryl tributyrate was identified as a suitable extractant for selective *in situ* removal of both acetone and alcohols. Enabling simple integration of ABE fermentation and water sensitive chemical catalysis, while reducing the energy demand of the overall process. Additionally, extractive fermentation with glyceryl tributyrate removed several lignocellulosic-derived pretreatment inhibitors completely restoring solvent production titers.

Tailoring both the biological fermentation and chemocatalytic reaction conditions predictably increased the production of sustainable diesel blendstocks from lignocellulosic sugar. More specifically, engineering the metabolism of *C. acetobutylicum* to produce isopropanol-butanol-ethanol (IBE) as opposed to ABE by expression of the secondary alcohol dehydrogenase (*sadh*) from *C. beijerinckii* strain B593. Coupled with the optimization of a more water-resistant alkylation catalyst, hydrotalcite-supported copper(II) or palladium (0). Furthermore, *in silco* predictions of extraction efficiency using COSMO-RS were employed to identify oleyl alcohol as a superior extractant for *in situ* removal of IBE.

To achieve highly controllable acetone and ethanol production both metabolic and fermentation engineering approaches were employed. Strains capable of producing acetone without carboxylic acid reassimilation through a newly described pathway were developed. Furthermore, a more oxidized sugar-acid (gluconic acid) was used to drive production of acetone through redox balancing of NADH, predicatively reducing the molar ethanol:acetone ratio. Increases in the molar ethanol:acetone ratio were also described by the co-expression of either the aldehyde/alcohol dehydrogenase (*adhE*) from *E. coli* MG1655 or pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*) from *Z. mobilis*. Acetone titers reached wild-type *C. acetobutylicum* levels by controlling the fermentation sparge rate and pH in a bioreactor. Finally, catalytic strategies to upgrade the co-produced ethanol and acetone at both low and high molar ratios are described.

By leveraging the advantages of both biological fermentation and chemocatalytic reactions of fermentation products with well-defined functionality we are able to efficiently convert a variety of saccharides into long-chain oxygenates. These long-chain oxygenates can be blended with all forms of transportation fuels. Additionally, tuning the microorganism's metabolism, fermentation conditions, catalysis reaction, or all of the above can optimize the production for a specific class of fuel. The integration of mixed-product fermentation with chemical catalysis is thus a novel and potentially enabling route for the economical conversion of biomass into liquid transportation fuels.

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I am grateful for the constant guidance and support provided by my research advisors Professor Douglas S. Clark and Professor Harvey W. Blanch. Their imaginative approach to scientific research and discovery made the work described in this thesis possible. Professor F. Dean Toste collaborated on several of the projects presented in this work and provided much needed catalysis expertise. Furthermore, Professor Toste established an environment of creativity and openness, allowing our studies of chemocatalysis and biological fermentation to integrate seamlessly.

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Over the past 5 years the members of Chemical Engineering department at UC Berkeley have acted as my second family. Providing the necessary guidance, support, and compassion for me to overcome my academic and personal endeavors. The diversity of cultures, backgrounds and expertise enabled me to grow not only as a scientist/researcher but also as a human being. I would like to thank all of the incoming class of 2009, especially Matt Pavolvich, Yongkwan Kim, Tom Dursch, Josh Howe, and Eric Sacia for enriching my life and giving me the strength to complete this degree. I would also like to thank all members of the Clark lab past and present for the stimulating group meetings, entertaining ski trips, and memorable discussions.

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3. INTEGRATION OF CHEMICAL CATALYSIS WITH EXTRACTIVE FERMENTATION TO PRODUCE FUELS

4. TAILORING THE FERMENTATION PRODUCTS AND CATALYST TOWARDS BIODIESEL PRODUCTION

1. Background

1.1 Fermentation of sugars to fuels

The fermentation of sugars to ethanol by yeast or bacteria has been exploited by our society for thousands of years (Cavalieri et al. 2003). Recently, a significant proportion of the ethanol produced through this fermentative process is blended with gasoline as a means to reduce the CO_2 emissions associated with transportation fuels. This process is very efficient achieving yields in excess of 90% of theoretical and titers in excess of 100 g L^{-1} (Bai, Anderson, and Moo-young 2008). However, several of the physical properties of ethanol make it unsuitable for completely replacing all transportation fuels that are currently derived from petroleum sources. More specifically, ethanol is too volatile to be blended in jet and diesel fuels, requires blender pumps for fuels containing 15% or higher ethanol, and is hydroscopic causing engine corrosion problems when high blends (i.e. E85) are used in non-flex fuel vehicles (Hansen, Zhang, and Lyne 2010; Tuchscheerer et al. 2012). To address this challenge researchers have tried to identify additional processes that convert sugar to compounds that are more suitable for replacing petroleum (Blommel et al.; Peralta-Yahya et al. 2011; Machado et al. 2012). Several of these processes are highlighted in Figure 1-1. These compounds are commonly referred to as advanced biofuels and have been reviewed extensively (Chheda, Huber, and Dumesic 2007; Wen, Bond-watts, and Chang 2013; Blanch 2012). This study focuses on developing such a process that can efficiently convert sugars into blendstocks appropriate for all transportation fuels.

The ideal properties for the three major US based fuels (gasoline, jet, and diesel) can be found in Table 1-1. Currently, these properties are achieved by fractioning and blending different components found in petroleum. These blends contain little to no oxygen and are primarily composed of linear, branched, and cyclic hydrocarbons. Major considerations for these fuels include engine combustion properties like auto-ignition temperature and cetane or octane number, volatility for pump and storage operations, and freezing, cloud point and energy density for jet applications. The blended nature of these products enables the refinery to tune the composition of the fuel to achieve all of the specified properties. It is highly unlikely that the physical properties of single compound could meet all of the standards required for the various transportation fuels.

Table 1-1: Physical properities of transporation fuels and ethanol [a]

	Gasoline	Jet A	Diesel No.2	Ethanol
Chemical structure	$C_4 – C_{12}$	$C_8 – C_{16}$	$C_8 – C_{25}$	C_2H_5OH
Molecular weight	100-105	≈170	≈200	46.07
Flash point	- 45°F	100°F	165°F	55°F
Auto ignition temperature	495°F	473°F	600°F	793°F
Boiling temperature	80-437°F	302-554°F	370-650°F	172°F
Energy density MJ L^{-1}	34.2	35.3	36.1	24
Freezing point	-40°F	-40°F	-40-30°F	-173.2°F

[a] Values taken from U.S. Department of Energy, Office of Energy Efficiency and Renewable Energy, Alternative Fuels Data Center

1.2 Catalytic routes to fuels

Research using chemocatalytic routes to convert sugar to fuels have been very successful in reproducing fuel blends that exhibit properties similar to those from petroleum (Gürbüz, Kunkes, and Dumesic 2010; Xing et al. 2010; Corma et al. 2011). Though several approaches have been described two of the most promising processes are aqueous phase reforming and furan based fuels. Huber et al. (2004) first described the use of aqueous phase reforming with a Pt or Pd catalyst on an acid SiAl support for the production of gasoline range alkanes from sugar derived polyols. This work obtained high yields and could be operated continuously for several days without significant loss of catalytic activity. Subsequent work using aldol condensation and ketonization reactions on these smaller gasoline range components to form C-C bonds produced longer chain diesel products(Kunkes et al. 2008). Furans, specifically furfural, are readily produced by either dilute or concentrated acid treatment of biomass-derived sugars. Using these reduced products for the synthesis of fuel molecules has been described using a variety of different chemical reactions. Of particular interest is the etherification of hydroxy methyl furfural (HMF) with biologically derived ethanol and n-butanol over a variety of acid catalysts (Balakrishnan,

Sacia, and Bell 2012). These partially oxygenated furan-based molecules showed favorable properties for diesel applications with low freezing points and high cetane numbers.

However, all of these catalytic routes start with a clean sugar or furan stream and are only tested on a single species at a time. The blend of products produced by these catalytic approaches also requires hydrogen treating to remove excess oxygen from the fuel. The source of this hydrogen is typically not considered during the experiment; except for aqueous phase reforming, which naturally generates hydrogen from biomass. Currently, the most likely source of this hydrogen would come from steam reforming of natural gas. Additionally, carbohydrates and the intermediate species generated during these catalytic processes have multiple functionalities that are catalytically active making the production of undesirable by-products a challenge that must be continually addressed.

1.3 Advanced Biofuels

Until recently, the only major biological approach to mimic transportation fuel blends was the trans-esterification of fatty acids with methanol or ethanol to fatty acid methyl/ethyl esters (FAMEs or FAEEs). However, the sustainability and scalability of this process has been questioned (S. P. Singh and Singh 2010), especially given the potential impacts to food markets. A new biological process that directly converts sugar into blends of FAEEs by performing the esterification reaction *in vivo* was recently described in the model organisms *E. coli* and *S. cervisae*. However, after multiple rounds of optimization the titers still remain below 1 g L^{-1} and yields below 10% of theoretical.(Steen et al. 2010) Another promising strategy relies on the engineering the beta-oxidation cycle to function in the reverse direction. This method was first described by Dellomonaco et al.(2011) and with appropriate tuning of the termination enzymes could produce approximately 7 g L^{-1} free fatty acids for diesel applications or 300 mg L^{-1} of C_6-C_{10} primary alcohols for gasoline and jet applications. However, the oxygen that remains in these advanced biofuels has deleterious effects on the desired fuel properties, specifically freeze point and energy density. This limits the amount of biofuel that can be blended with conventional petroleum based fuels. Schirmer et al. (2010) described a biological route to produce C_{13}-C_{17} alkanes and alkenes from sugar. This strategy utilizes the cells fatty acid biosynthesis pathways and after extensive engineering was able to achieve titers in excess of 300 mg L^{-1} from 30 g L^{-1} glucose. The hydrophobic nature of the product makes it recovery from the fermentation broth very simple, requiring the oil layer be decanted.

Metabolic engineering, protein engineering and synthetic biology enabled these new processes, but they still require a significant amount of engineering and optimization to achieve yields, titers and productivities that are necessary for an industrial process. All of these advanced biofuel processes rely on driving carbon metabolism to the anabolic intermediate acetyl-CoA and/or malonyl-CoA for their synthesis. Much like the chemo-catalytic routes there are several different enzymatic reactions that can be exploited to produce a variety of different products for fuel applications. However, since most of these processes are anabolic they typically require excessive ATP and therefore require aerobic fermentation conditions. This limits the productivity of the fermentation because of oxygen mass transfer limitations into the culture broth.

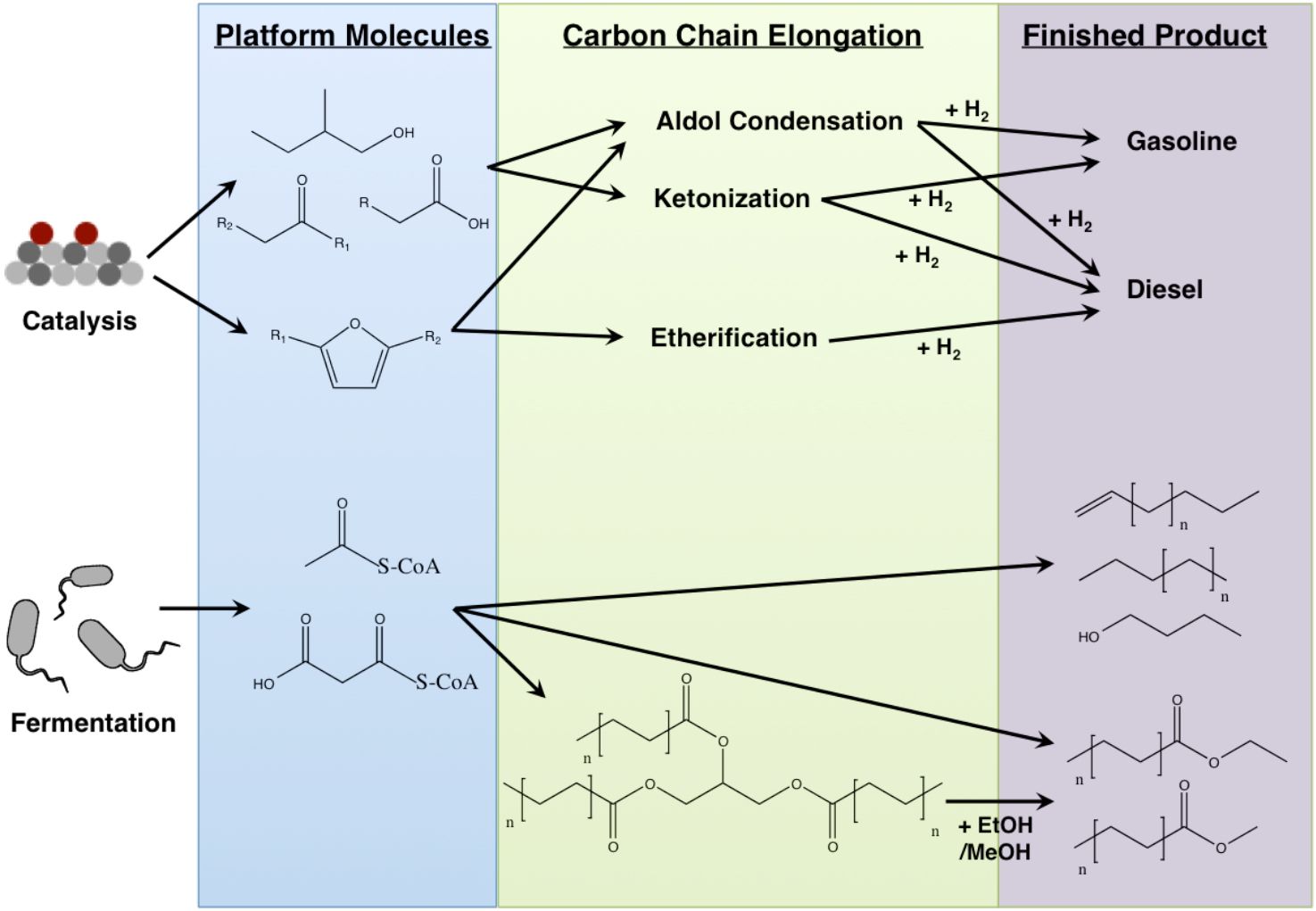


Figure 1-1: Established processes for the production of transportation fuel blends from sugar sources.

1.4 ABE Fermentation

Another advanced biofuel that has gained renewed interest is the fermentation of sugar to produce 1-butanol, a colorless four-carbon primary alcohol. Butanol is considered a superior fuel additive to ethanol in many regards: it has a higher energy density (29.2 MJ L^{-1}) and lower distillation costs, with less hydroscopic and corrosive properties (S. Y. Lee et al. 2008). Additionally, industrial fermentation of butanol from sugars has been carried out since the 1920's(Jones and Woods 1986). There are several species of bacteria from the genus *Clostridium* that naturally produce butanol. Acetone and ethanol are also usually co-produced in these organisms and this mixed solvent fermentation is commonly referred to as acetone-butanol-ethanol or ABE fermentation. ABE fermentation was carried out on the industrial scale from 1915-1960s. Originally the desired product was the acetone for the production of cordite (a smokeless munitions powder) during the First World War. After the war there was little need for acetone but the demand for butanol skyrocketed as a solvent for lacquers. It was during this period of history that ABE fermentation ranked second only to ethanol fermentation in scale and importance (Jones and Woods 1986). However, by the late 1950s routes to produce solvents from petroleum were made cost competitive with fermentation. Additionally, the major feedstock at the time molasses spiked in price because of animal feed demand. These two factors lead to the end of industrial ABE fermentation throughout most of the world. However, in the past 10 years China has recommissioned over 300,000 MT/year of butanol capacity with plans to increase capacity to over 1,000,000 MT/year (Green Biologics Data).

1.4.1 Solventogenic *Clostridium*

Several of the solvent producing or solventogenic *Clostridium's* genomes are sequenced, but most of the characterization and engineering to-date is on *Clostridium acetobutylicum* ATCC824. This gram-positive, endospore-forming, anaerobic bacterium is capable of fermenting starchy substrates in addition to a variety of mono- and disaccharides, including but not limited to: glucose, xylose, cellobiose, arabinose, and sucrose. *C. acetobutylicum* ATCC 824 undergoes a biphasic metabolism to produce solvents, as highlighted in Figure 1-2. In the first phase of the metabolism sugars are catabolized to acetic and butyric acids. This enables the cells to generate excess ATP via acetate and butyrate kinase mediated reactions to support anaerobic cell growth. To balance the cellular redox potential of the anaerobic acid fermentation *C. acetobutylicum* produces a significant amount of hydrogen during this rapid growth phase.

This initial growth phase is commonly referred to as the acidogenic phase of fermentation because of the high concentrations of acids that are produced.

High acid concentrations and decreased pH help to trigger the onset of the solventogenic phase, during which the cell reassimilates the acids produced during the acidogenic phase and converts them to acetone, butanol and ethanol. Though experiments done with a butyrate deficient strain of *C. acetobutylicum* by Papoutsakis and colleagues (Desai et al. 1999) suggest that butyryl-phosphate accumulation is actually the molecule responsible for the onset of solventogenesis. In solventogenic *Clostridium* the production of acetone is directly dependent on acid production and reassimilation. More specifically, acetoacetate, the precursor of acetone, is produced by the transfer of coenzyme A from acetoacetyl-CoA to the reassimilated acetate or butyrate via acetate and butyrate co-transferases(Wiesenborn, Rudolph, and Papoutsakis 1989). Acetoacetate is then decarboxylated by acetoacetate decarboxylase to acetone (Petersen and Bennett 1990). Additional sugar that is consumed during this phase of the fermtentaion is directly converted to ABE. The stoichometric ratios and ATP generation for the various acids, solvents, and gases produced during ABE fermentation are listed in eq. 1-1 thru 1-12. Wild-type *C. acetobutylicum* typically produce butanol, acetone, and ethanol in a 6:3:1 mass ratio, respectively, to a final titer of 20 g L^{-1} total solvents. Butanol the most toxic of the three solvents produced typically reaches a final titer of 12-13 g L^{-1} in optimized fermentations.

1 glucose => 2 acetate + 2 CO_2 + 4 H_2 + 4 ATP (1-1)

1 glucose => 1 butyrate + 2 CO_2 + 2 H_2 + 3 ATP (1-2)

1 glucose => 2 lactate + 2 ATP (1-3)

1 glucose => 1 acetate + 1 ethanol + 2 CO_2 + 2 H_2 + 3 ATP (1-4)

1 glucose => 2 ethanol + 2 CO_2 + 2 ATP (1-5)

1 glucose => 1 acetone + 3 CO_2 + 4 H_2 + 2 ATP (1-6)

1 glucose => 1 butanol + 2 CO_2 + 2 ATP (1-7)

1 glucose => 0.5 acetone + 0.5 butanol + 2.5 CO_2 + 2 H_2 + 2 ATP (1-8)

1 glucose => 0.6 butanol + 0.3 acetone + 0.2 ethanol + 2.3 CO_2 + 1.2 H_2 + 2 ATP (1-9)

1 glucose + 1 acetate => 1 acetone + 1 ethanol + 3 CO_2 + 2 H_2 + 2 ATP (1-10)

1 glucose + 1 acetate => 1 acetone + 0.5 butanol + 3 CO_2 + 2 H_2 + 2 ATP (1-11)

1 glucose + 1 butyrate => 1 acetone + 1 butanol + 3 CO_2 + 2 H_2 + 2 ATP (1-12)

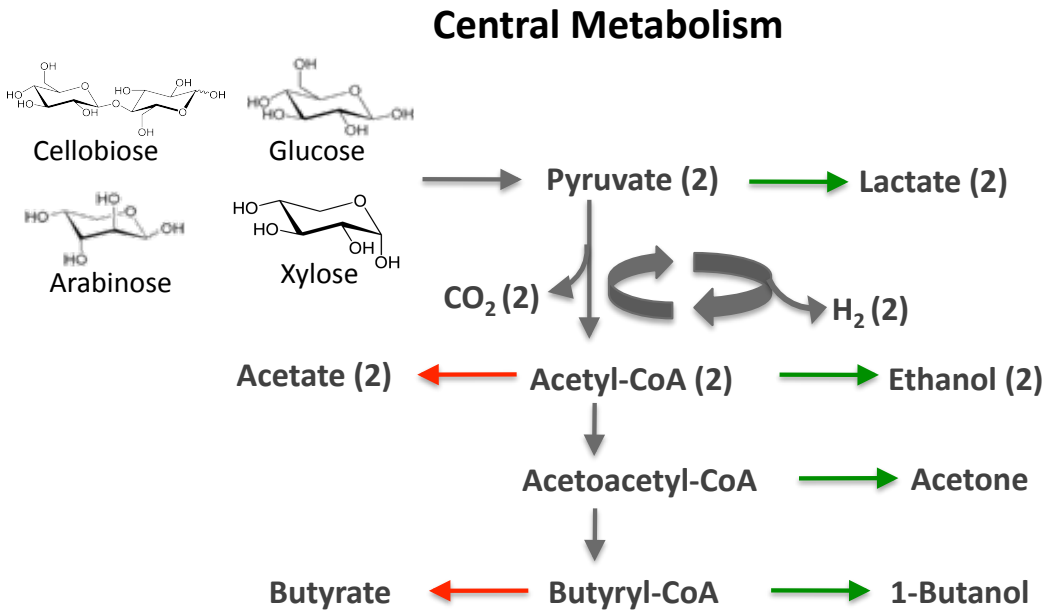


Figure 1-1: Proposed Central Metabolism of *Clostridium acetobutylicum*. Mono- and disaccharides are metabolized initially to pyruvate. Red arrows pointed left indicate acidogenic phase metabolic pathways, while green arrows pointed right indicate solventogenic phase metabolic pathways

1.4.2 Genetic and Metabolic Engineering of *C. acetobutylicum*

There have been several examples of mutagenesis and adaptation experiments on *C. acetobutylicum* to improve solvent production and yields. However, the use of rational genetic engineering approaches in *C. acetobutylicum* were severely hindered because of a native restriction endonuclease *CAC824I* (L. Mermelstein et al. 1992) that recognizes the sequence 5'-GCNGC-3'. Construction of a methylating plasmid pAN1, which contained a methyl transferase from the *Bacillus subitilis* prophage Φ3T, and co-transformation with a variety of shuttle vectors made it much easier to perform basic gene expression and knockout studies in *C. acetobutylicum* (L. D. Mermelstein and Papoutsakis 1993).

Several studies have been carried out on most of the central metabolism genes in *C. acetobutylicum* enabling the engineering of solvent production. Of particular interest is that the elimination or knockout of several genes responsible

for acid production enables *C. acetobutylicum* to produce higher titers of 1-butanol than are achieved by wild-type cultures. Additionally, Jang et al. (2012) reported the highest 1-butanol titer (18.9 g L^{-1}) in *C. acetobutylicum* to-date after combining knockouts of the acid production genes *pta* and *buk* with over-expression of an alcohol/aldehyde dehydrogenase variant. Thus indicating that the 12-13 g L^{-1} butanol titer limit seen in wild-type *C. acetobutylicum* is not simply a function of the solvents toxicity. Brown et al. (Brown et al. 2011) observed a similar finding in *Clostridium thermocellum* whereby an increase in ethanol tolerance was primarily attributed to a mutated alcohol dehydrogenase. Though studies have also shown that over-expression of native and heterologous heat shock proteins (HSPs) also improve butanol titers (Tomas et al. 2003). It is proposed that their chaperone function helps to mitigate the cellular stress associated with high solvent titers, enabling the organism to produce more solvents (Tomas, Beamish, and Papoutsakis 2006). These findings highlight the limit to our understanding of complex phonotypical traits like solvent stress and tolerance.

1.4.2 ABE fermentation with *In situ* product removal

It has been proposed through process economics modelling that increasing the butanol titer to 19 g L^{-1} would reduce distillation costs by almost 50% (Eleftherios T Papoutsakis 2008). This is significant because on the industrial scale the distillation step of the process is the most energy intensive unit operation. An alternative approach to conventional distillation of the fermentation broth is the use of an *in situ* product removal technology. Whereby the toxic solvents are removed from the fermentation broth enabling longer fermentation times and higher volumetric productivities. Several of these technologies have been previously described and reviewed. Figure 1-3 shows the most commonly employed *in situ* product removal techniques for ABE fermentation: gas stripping, pervaporation, and liquid-liquid extraction.

The volatile nature of the solvents in ABE fermentation makes gas stripping a viable process for the removal and enrichment of these toxic compounds. To remove the solvents large quantities (in excess of 1.5 vvm) of gas are sparged through the reactor or a separate stripping column. The volatiles and water are then condensed out of the gas before it is recycled back. Anaerobic conditions must be maintained during the fermentation so N_2 and/or recycled H_2 and CO_2 gas from the cellular metabolism of sugar are used. Ennis et al. (1986) first described the integration of a gas stripping operation and solventogenic *clostridia* fermentation. Ezeji et al. (2003)maintained a gas stripping ABE fermentation with *C. beijerinckii strain* BA101 for over 200 hours with high solvent productivity (1.16 g L^{-1} hr^{-1}) and yield (0.47 g g^{-1}). Average

selectivities (α), as defined in eq. 1-13, for butanol 16.16, acetone 9.71, and ethanol 7.81 highlights the considerable amount of water that must still be removed from these enriched condensates. Additionally, the high volatility of acetone makes complete condensation a very energy intensive process.

$$\alpha = \frac{\left[\frac{y}{(1-y)}\right]}{\left[\frac{x}{(1-x)}\right]}$$ where y and x are weight fractions in condensate and broth fractions, respectively (1-13)

Pervaporation is a membrane based separation process used to selectively remove compounds based on a physical property of the membrane and a gas-phase driving force (i.e. vacuum or sweep gas). In most ABE fermentations with pervaporation the solvents are selectively removed with a hydrophobic solid membrane leaving the sugars, nutrients, and cells in the reactor. Butyric acid has been observed to cross these membranes if the concentration in the fermentation broth is high (Nasibuddin Qureshi, Maddox, and Friedlt 1992). Polydimethylsiloxane (PDMS) based membranes are commonly employed in ABE pervaporation experiments and show no deleterious effects on the culture. However, ionic liquid (Izák et al. 2008), liquid (oleyl alcohol on polypropylene) (Matsumura et al. 1988), polytetrafluoroethylene (Vrana et al. 1993), and poly(ether-block-amide) with carbon nanotubes (Yen, Chen, and Yang 2012) have also been tested to improve ABE fermentation parameters. With the exception of the liquid membrane (oleyl alcohol on polypropylene) a large amount of water also crosses these membranes and must be removed by additional unit operations. Additionally, the fluxes of these membranes still remain quite low 7-784 g m^{-2} hr^{-1} (J. Li et al. 2014) and any time the membrane surface is in direct contact with the cell culture requires fouling considerations for continuous operation.

Unlike pervaporation, liquid-liquid extraction for *in situ* product removal has to-date been limited by the use of only non-inhibitory or biocompatible extractants. Organic solvents with a high affinity for the metabolite of interest are usually employed because of their insolubility in the fermentation broth. The metabolite is enriched by distillation or back extraction, regenerating the extractant. Ishii et al. (1985) first described the use of liquid-liquid extraction for *in situ* removal of ABE from the fermentation culture. Of the 29 compounds screened oleyl alcohol (*cis*-9-octadecan-1-ol) and C-20 guerbet alcohol were deemed the most suitable.

Later studies found dibutyl phthalate (Wayman and Parekh 1987) and poly(propylene glycol) 1200 (Barton and Daugulis 1992) were non-toxic extractants with high butanol distribution coefficients of 2.4 and 4.8, respectively. Roffler et al. (1987) demonstrated that fed-batch extractive fermentation whereby a concentrated feed of 339 g L^{-1} glucose and 67 g L^{-1} yeast extract enabled a butanol volumetric productivity of 1.5 g L^{-1} hr^{-1} and total solvent volumetric productivity of 2.3 g L^{-1} hr^{-1}. A key difference between extractive fermentation and the other *in situ* product removal methodologies described previously is the low water fraction that is removed by the water immiscible extractants. This makes the downstream purification of ABE less energy intensive.

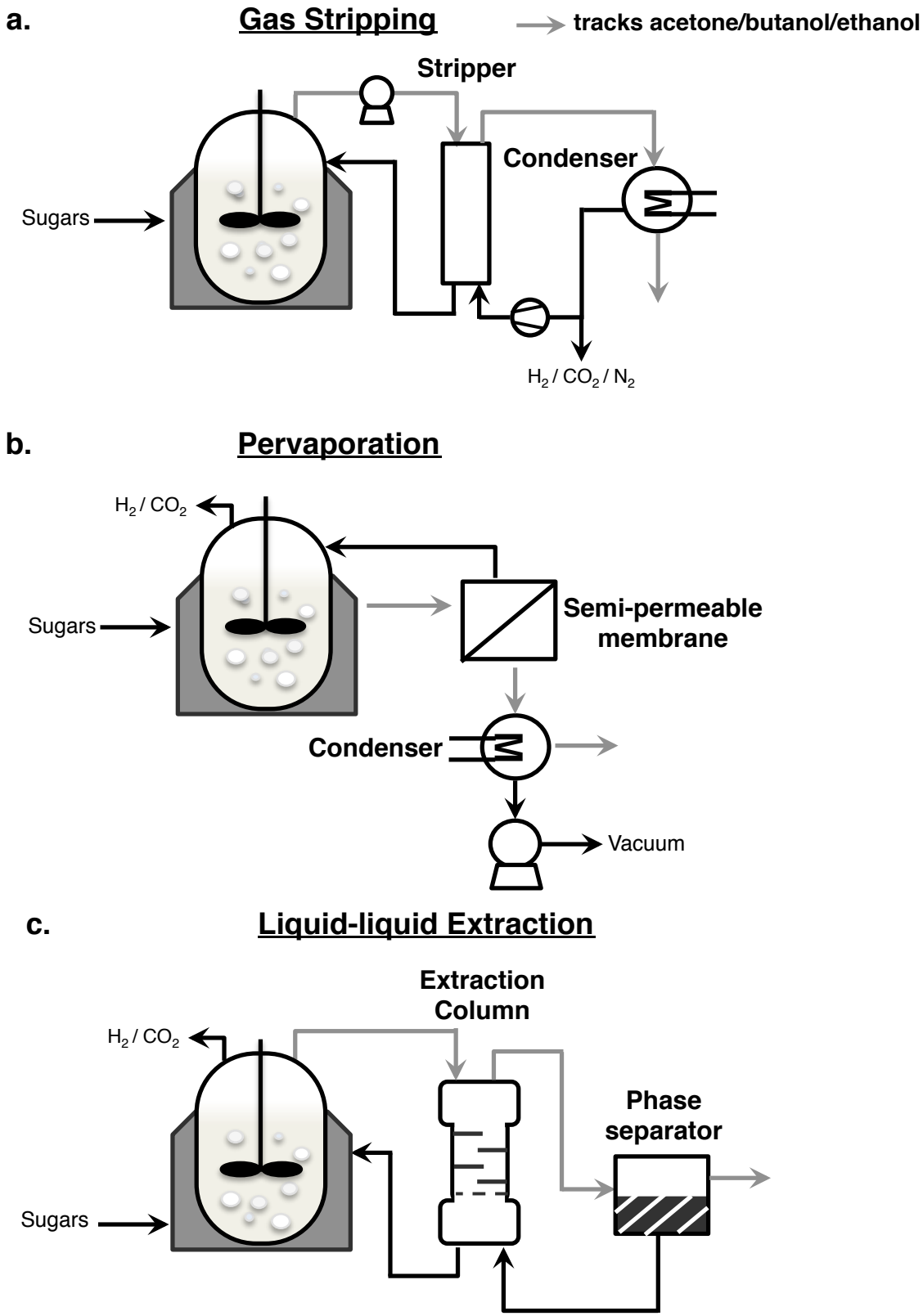


Figure 1-2: Methods for *in situ* product removal in ABE fermentation (a) gas stripping, (b) pervaporation, (c) liquid-liquid extraction.

2. OPTIMIZING FERMENTATION CONDITIONS FOR SOLVENT TITER AND YIELD

2.1 Introduction:

The commercial production of biofuels necessitates a highly efficient and low-cost process to compete with petroleum. Operating expenses for a conventional biorefinery are dominated by the feedstock and energy costs (L R Lynd et al. 2002). This is especially true in mixed product fermentations, specifically ABE fermentation, where the production unwanted carboxylic acids could severely reduce solvent yields (J. Y. Lee et al. 2009). Production of the carboxylic acids acetate and butyrate in *C. acetobutylicum* occurs predominately during the initial growth phase of the fermentation (S. Y. Lee et al. 2008). Under the appropriate pH conditions these acids are re-assimilated by acetyl-CoA transferase (*ctfa* and *ctfb*) and converted to acetone, butanol, and ethanol during the solventogenic phase of fermentation. However, *C. acetobutylicum* can also produce the carboxylic acid lactate, which is not re-assimilated by acetyl-coA transferases during the solventogenic phase of fermentation (Freier and Gottschalk 1987). Therefore it is highly desirable to minimize the final titers of these carboxylic acids, specifically lactate, while directing a majority of the catabolic carbon flux towards acetone, butanol, and ethanol.

Batch fermentation of the sugar substrate is the single largest unit operation for most conventional biofuel processes. 1-million gallon bioreactors have been used for biofuel fermentations making fermentation a significant percentage of the biorefineries total capital cost (McAloon et al. 2000). Increasing the volumetric productivity of the fermentation operation enables smaller or fewer bioreactors to be used, thus reducing capital costs. Volumetric productivity ($g_{ABE}\ L^{-1}\ hr^{-1}$) is a function of both the biocatalyst loading ($g_{dry\text{-}cell\text{-}weight}\ L^{-1}$) and the specific productivity of the cell ($g_{ABE}\ g_{dry\text{-}cell\text{-}weight}^{-1}\ hr^{-1}$). The biphasic nature of ABE fermentation enables increases in ABE specific productivity in *C. acetobutylicum* by simply decreasing the acidogenic phase of the fermentation.

Though the mechanism that regulates and initiates the switch from the acidogenic to solventogenic phase of fermentation has not been resolved (Sullivan and Bennett 2006; Latonia M Harris et al. 2000), several studies have identified pH control of culture as a major factor to solvent production (Bahl et al. 1982; Terracciano and Kashket 1986; Monot, Engasser, and Petitdemange 1984; Gottwald and Gottsehalk 1985). An initial study was conducted to investigate this pH effect on solvent titer, yield and productivity by varying both the initial pH of the culture and the set-point pH, for which the culture would be held at with concentrated base. A carbon balance of the fermentations was conducted to identify changes in metabolism associated with the various pH conditions.

2.2 Methods and Materials:

2.2.1 Growth:

C. acetobutylicum ATCC824 was routinely grown out of anaerobic -80°C glycerol freezer stocks in clostridium growth medium (65 g L^{-1} glucose, 5 g L^{-1} yeast extract, 2 g L^{-1} ammonium acetate, 1 g L^{-1} NaCl, 0.75 g L^{-1} K_2HPO_4, 0.75 KH_2PO_4, 0.5 g L^{-1} cysteine-HCL monohydrate, 0.1 g L^{-1} $MgSO_4$ heptahydrate, 0.01 g L^{-1} $FeSO_4$ heptahydrate, 0.01 g L^{-1} $MnSO_4$ monohydrate) at 37°C (Tomas et al. 2003) in anaerobic hungate tubes overnight. These precultures were then subcultured at 1:10 dilution in fresh clostridium growth medium (CGM) and incubated to mid-exponential growth phase before inoculating the fermentation. All shake flask experiments were conducted in an anaerobic chamber equipped with a culture incubator.

2.2.2 Batch Fermentation:

3-L Bioengineering fermentors were used to more accurately control the culture pH. The fermentors were made anaerobic by continuously sparging N_2 at 100 mL min^{-1} STP. Optimization of the fermentation's pH parameters was carried out in batch-mode with a 2L working volume. The initial pH was defined as the fermentor's pH prior to inoculation of the cells. Additionally, set point pH was defined as the pH the culture decreased to before control was initiated. The culture's cell density was measured using OD_{600} and correlated to dry cell weight (DCW) measurements from corresponding lyophilized cell pellet samples.

2.2.3 Analytical Methods

Concentrations of glucose and major metabolites were analyzed on a Shimadzu HPLC system using an Aminex HPX-87H column and equipped with both refractive index and UV/Vis detectors. 20 uL samples were injected onto the column at 35°C with a mobile phase flow rate of 0.7 mL min^{-1}. Acetone and butyric acid co-eluted from the column, requiring UV measurements at 255 nm and 210 nm respectively to accurately measure concentration.

2.2.4 Carbon Balance Calculation:

The carbon balance of the fermentation was determined from the carbon-molar (C-mole) contributions of biomass, lactate, ethanol, butanol, acetone, acetate, butyrate, acetoin, and CO_2 production. CO_2 concentrations were estimated based on the theoretical stoichiometry of ethanol, butanol, acetone, acetate, and butyrate production. We assumed that for every mole of ethanol or acetate formed, 1.0 C-mole of CO_2 is produced. Additionally, it was assumed

that for every mole of butanol, acetone, or butyrate formed, 2.0 C-moles of CO_2 are produced. The carbon content of the biomass was assumed to be 0.5 g of carbon per gram of DCW (Trinh et al. 2010).

2.3 Results and Discussion:

2.3.1 Optimizing Initial and Set Point pH

Initial fermentations were carried out to optimize pH conditions for maximum solvent production. It has been previously reported that solvent production is affected by the pH parameters of the fermentation (Bahl et al. 1982; Terracciano and Kashket 1986). To further investigate this phenomenon both the initial media pH and the set point pH were varied, see Figure 2-1. Decreasing the initial fermentation pH from 6.5 to 5.5 resulted in a 55% increase in butanol titer from 7.76 g L^{-1} to 12.04 g L^{-1} and a 48% increase in butanol volumetric productivity from 0.21 to 0.31 g L^{-1} hr^{-1}. However, only a 3% (56.7 g L^{-1} to 58.7 g L^{-1}) increase in glucose metabolism was observed between the two fermentations. Both fermentations had a set point pH of 5.0. Holding the initial pH constant and decreasing the set point pH from 5.0 to 4.8 resulted in an additional 10% increase in butanol titer to 13.25 g L^{-1} and a 165% increase in maximum butanol volumetric productivity to 0.82 g L^{-1} hr^{-1}. The dramatic increase in butanol titer associated with these optimizations in pH conditions could not be attributed to a significant increase in sugar consumption. Figure 2-2 shows that decreasing the initial fermentation pH from 6.5 to 5.5 resulted in a 9% decrease in lactate titer from 13.9 g L^{-1}. Similarly, decreasing the cultures set point pH from 5.0 to 4.8 decreased lactate titer an additional 25% to 9.5 g L^{-1}.

2.3.2 Evaluating fermentation variability via carbon balance

To better understand the observed increases in butanol titer and decreases in lactate titer, a carbon balance of the cell's major metabolites was constructed. Samples from the fermentations were spun down and lyophilized to correlate dry cell weight (DCW) to optical density at 600 nm (OD_{600}). Figure 2-3 shows a correlation of 0.2175 g DCW OD_{600}^{-1}, which is similar to values calculated for other rod-shaped bacterium. In all fermentations tested over 85% of the carbon in the glucose consumed was accounted for by dry cell weight, major metabolites and CO_2 respiration. The carbon balance plotted in Figure 2-4 shows that increased butanol titer associated with decreasing both initial and set point pH values is a direct result of the decreased lactate production during the fermentation. Decreasing the initial fermentation pH from 6.5 to 5.5 resulted in a 6.2% decrease in the C-mole of produced lactate and a 6.1% increase in the C-mole of produced butanol. Decreasing the fermentation set point pH from 5.0 to

4.8 further decreased the C-mole to produce lactate by an additional 3.9% and increased C-mole to produce butanol by 4.4%.

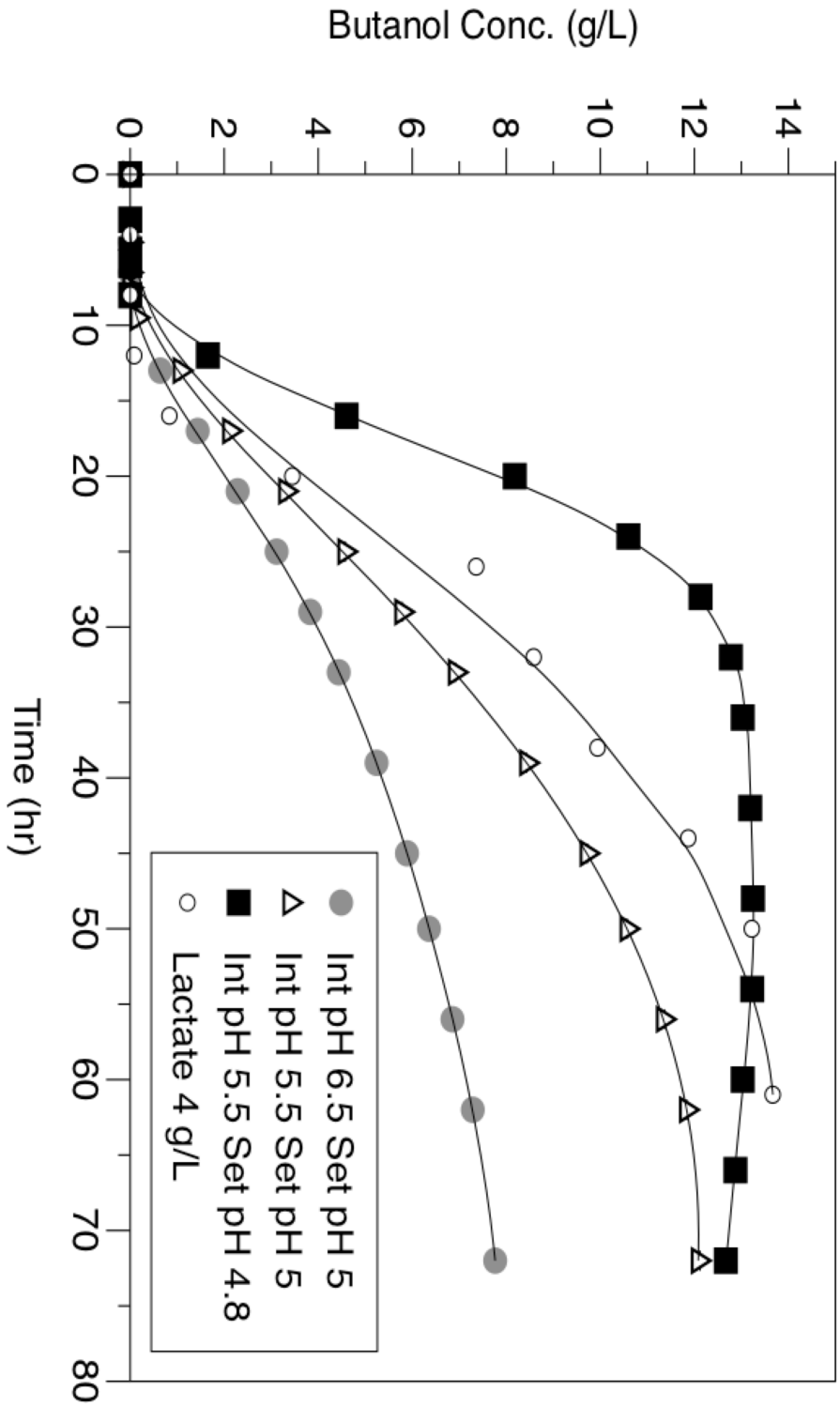


Figure 2-1: pH and external lactate effect on butanol production, lines drawn to guide the eye.

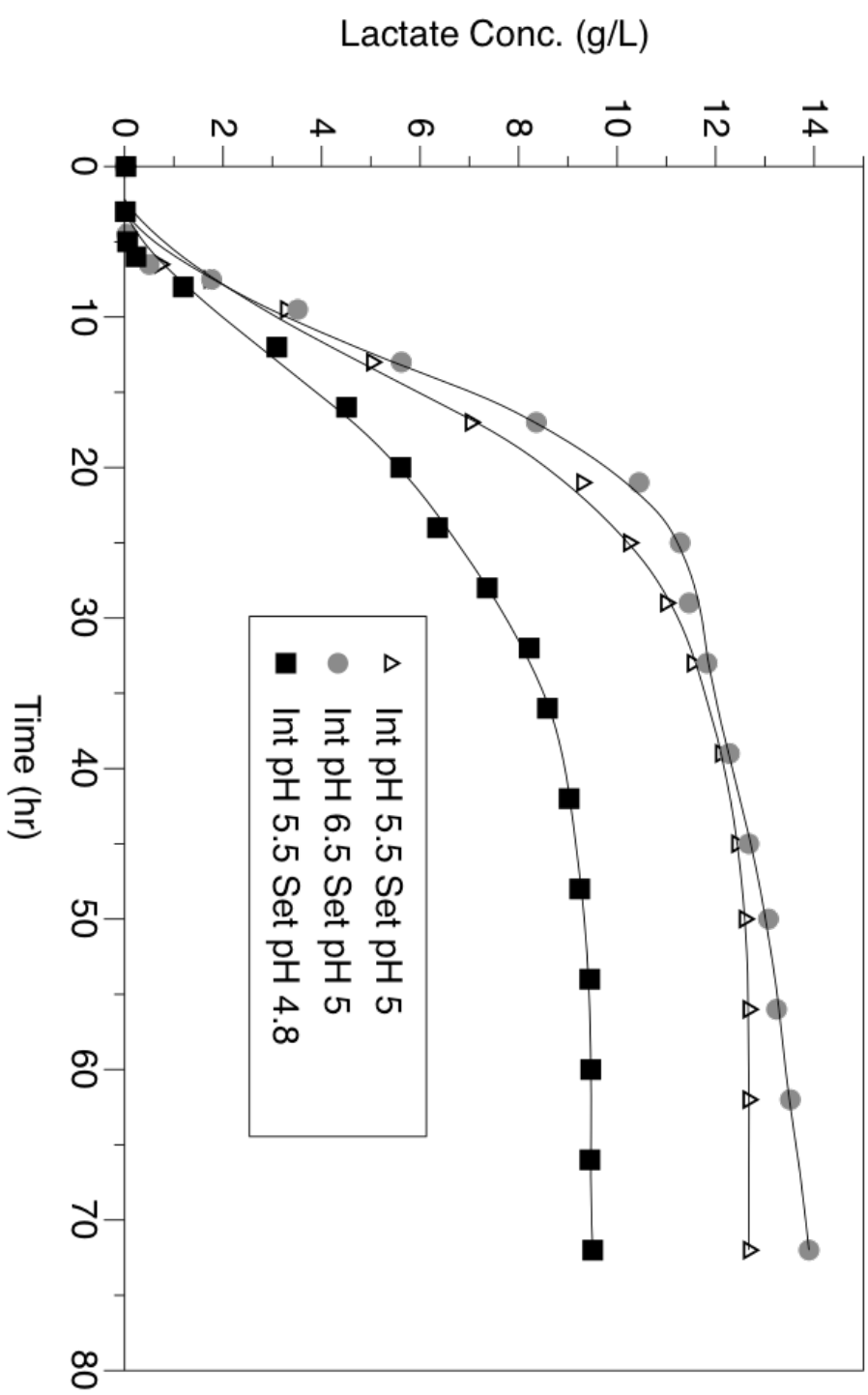


Figure 2-2: pH effect on lactate production, lines drawn to guide the eye.

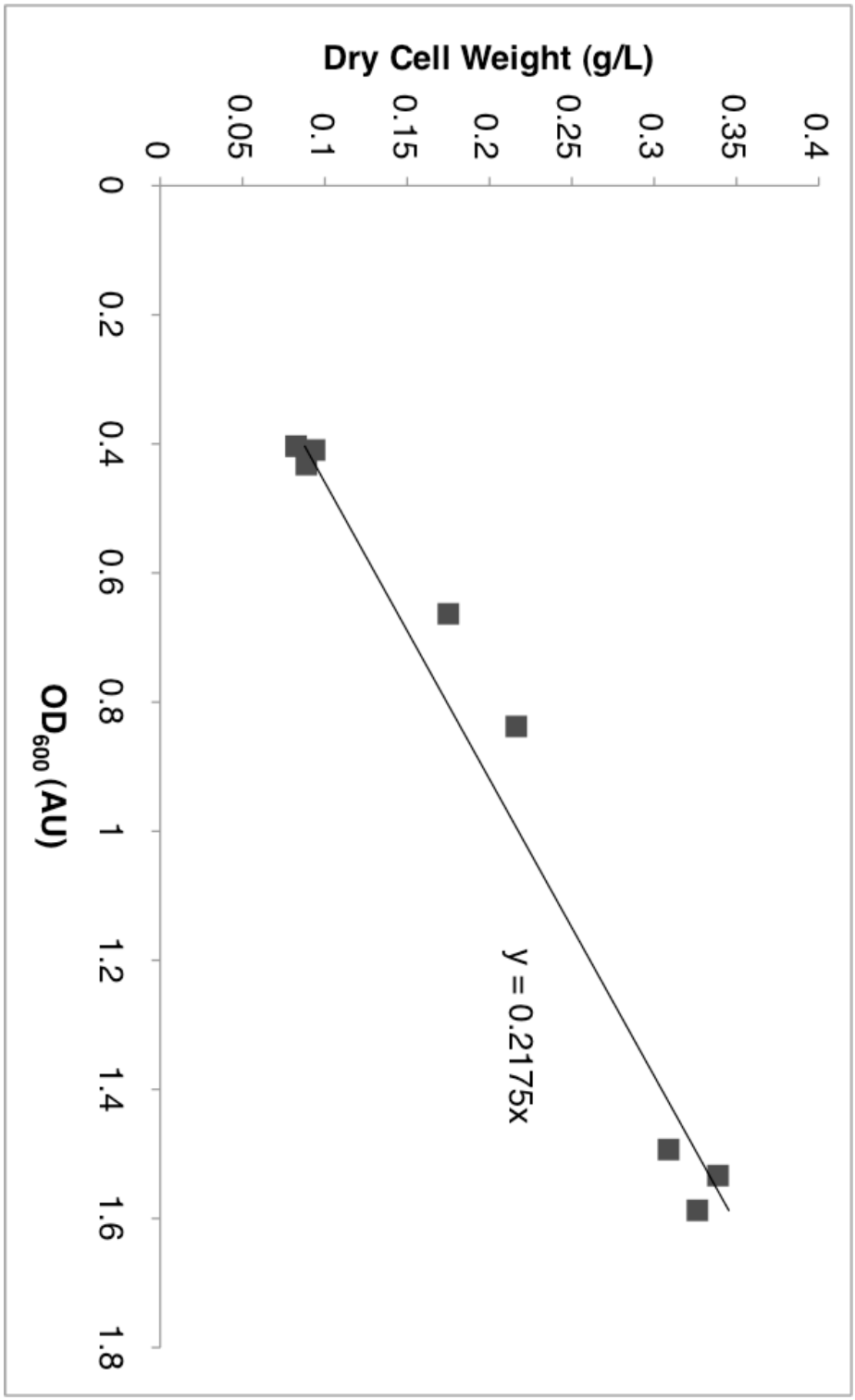


Figure 2-3: Correlation between Dry Cell Weight (DCW) and OD_{600} for *C. acetobutylicum* cultures during exponential growth.

Furthermore, the carbon balance analysis shows that metabolic pull towards lactate production decreases metabolic flux to acetone, butanol and ethanol.

Lactate, acetone, butanol, and ethanol all use pyruvate as an intermediate in their metabolic synthesis. The enzyme responsible for lactate production in *C. acetobutylicum*, lactate dehydrogenase, was previously shown to be very pH sensitive (Freier and Gottschalk 1987) with optimum activity at pH 5.8 and no activity below pH 5.0. *Clostridium acetobutylicum* does not maintain a constant cytosolic pH but instead maintains pH gradient across it's cellular membrane (Huang, Gibbins, and Forsberg 1985). It is also known that lactate dehydrogenases in the solventogenic *Clostridium acetobutylicum* strain P262 are also responsible for lactate catabolism and that this activity is improved when the culture is supplemented with additional lactate (Diez-Gonzalez, Russell, and Hunter 1995). Therefore to decrease the carbon flux towards lactate, 4 g/L of lactate was added to the fermentation media prior to inoculation, while the initial pH and set point pH remained at 5.5 and 4.8, respectively. The C-mole used to produce lactate was significantly decreased to 4.1% as compared to 14.5% without added lactate. Additionally, the final butanol titer was increased another 3.2% to 13.67 g/L. However, both total solvent yield and maximum butanol volumetric productivity decreased from 53.7% to 50% and 0.82 g L^{-1} hr^{-1} to 0.65 g L^{-1} hr^{-1}. These lactate results are in agreement with a similar study on *Clostridium saccharoperbutylacetonium* N1-4 (Oshiro et al. 2010). This study showed that the addition of 5g/L of lactate to the media increased butanol titer (Oshiro et al. 2010) through an unknown mechanism.

2.4 Conclusion:

The success of any biofuel technology requires an efficient fermentation process to first be established. This is particularly challenging in the ABE fermentation where a mixture of end products is desired. One of the major regulators of solvent production in solventogenic *Clostridium* is the culture pH. In this study it was determined that an initial pH of 5.5 and set point pH of 4.8 was ideal for maximum solvent titer, yield and productivity. Carbon balance analysis of the fermentations revealed that lactate production was pulling carbon from solvent production and that lactate production sensitive to the culture pH conditions. With the addition of 4 g/L of lactate to the media butanol titers increased slightly while total solvent yields and maximum butanol volumetric productivities decreased. Moving forward all wild-type *C. acetobutylicum* fermentations will have an initial pH of 5.5, a set point pH of 4.8, and not supplemented with lactate.

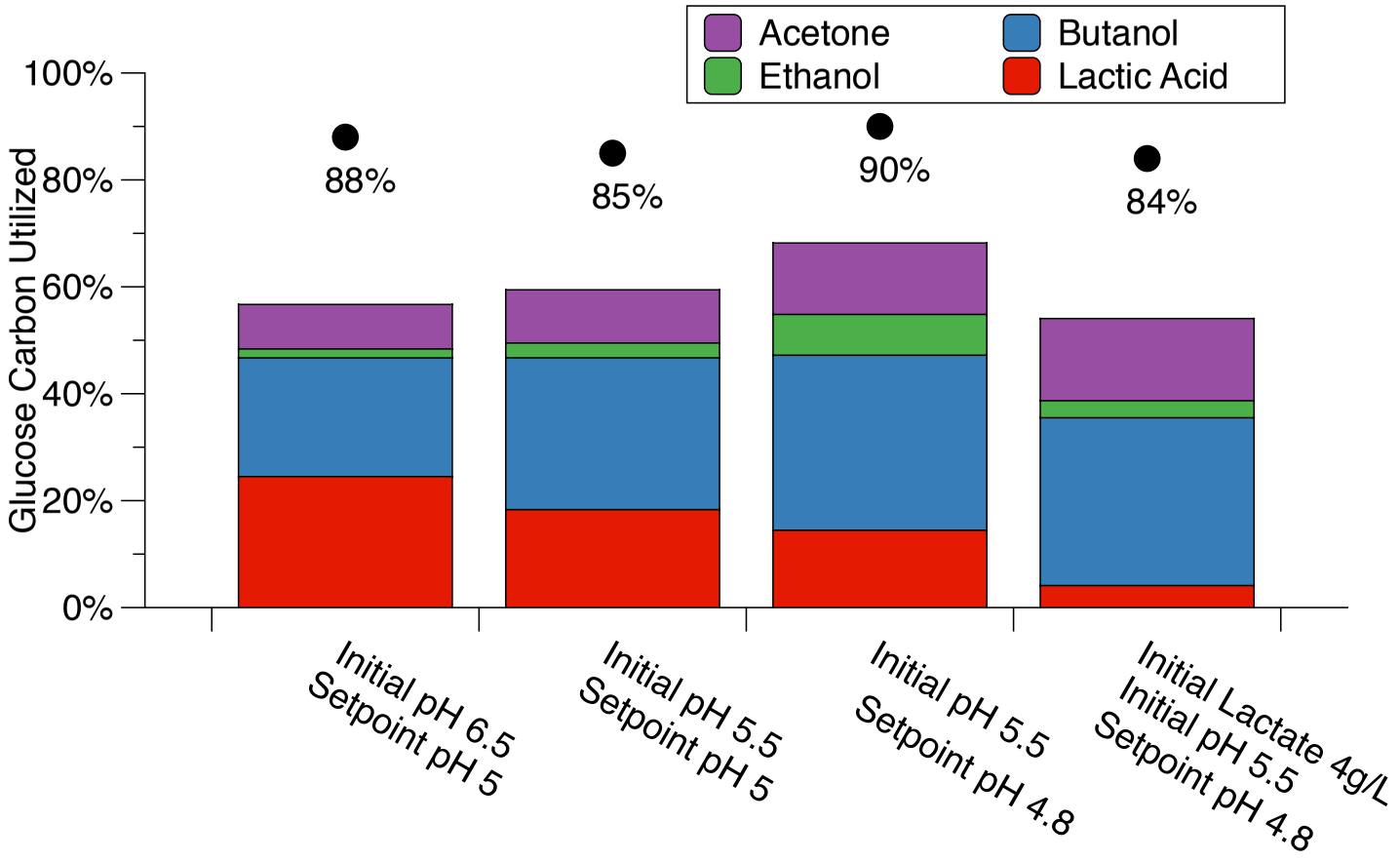


Figure 2-4: Carbon balance of major end products (from bottom to top: lactate, butanol, ethanol, and acetone). • total carbon balance of fermentation including contributions from biomass, acetate, butyrate, acetoin, biomass and CO_2 respiration.

INTEGRATION OF CHEMICAL CATALYSIS WITH EXTRACTIVE FERMENTATION TO PRODUCE FUELS

Work found in this chapter was previous published in the article “Integration of chemical catalysis and extractive fermentation to produce fuels”, Nature 2012. Catalysis experiments were conducted by Prof. Pazhamalai Anbarasan and Dr. Sanil Sreekumar.

3.1 Introduction

Natural biological routes to produce alcohols (ethanol and n-butanol) from carbohydrates have been known for more than 100 years, and these compounds have been produced in fermentations at high titres (~100 and 15 g l^{-1}, respectively) and at yields near their theoretical maxima. These low-molecular-mass compounds are primarily suitable as additives or in certain situations (for example, E100 flex fuel vehicles) as alternatives to petrol. Advances in metabolic engineering have enabled the biological production of several higher-molecular-mass jet and diesel fuel compounds from carbohydrates, but until now these processes have suffered from low titres and yields (Steen et al. 2010; Wang et al. 2011; Peralta-Yahya et al. 2011).

Here we propose a chemical route to convert fermentation products from a variety of renewable carbohydrate sources into hydrocarbons that can be used for petrol, jet fuel and diesel. Because solventogenic fermentation products have lower carbon numbers than are appropriate for these fuels, coupling chemistry can be used to produce molecules that are larger than these natural fermentation products, ideally achieved by exploiting the functionalities inherent in the starting materials (Blommel et al.; Corma et al. 2011; Huber et al. 2005). The acetone, n-butanol and ethanol (ABE) mixture produced by *Clostridium acetobutylicum* in a 2.3:3.7:1 molar ratio (3:6:1 mass ratio) provides such a system.
C. acetobutylicum is able to produce ABE from a variety of sugars and carboxylic acids (Ren et al. 2010), providing the flexibility needed to accommodate regionally specific feedstocks. The ABE products harbour both the nucleophilic a-carbons of the acetone and the electrophilic a-carbon of the alcohols. These paired functionalities enable us to construct higher alkanes from two-carbon, three-carbon and four-carbon precursors by the alkylation of acetone with the electrophilic alcohols. As shown in Figure 3-1, alkylation under suitable conditions results in C_5–C_{11} or longer-chain ketones, which may be deoxygenated to paraffins. These paraffins, from pentane to undecane, are components of petrol, diesel and jet fuel.

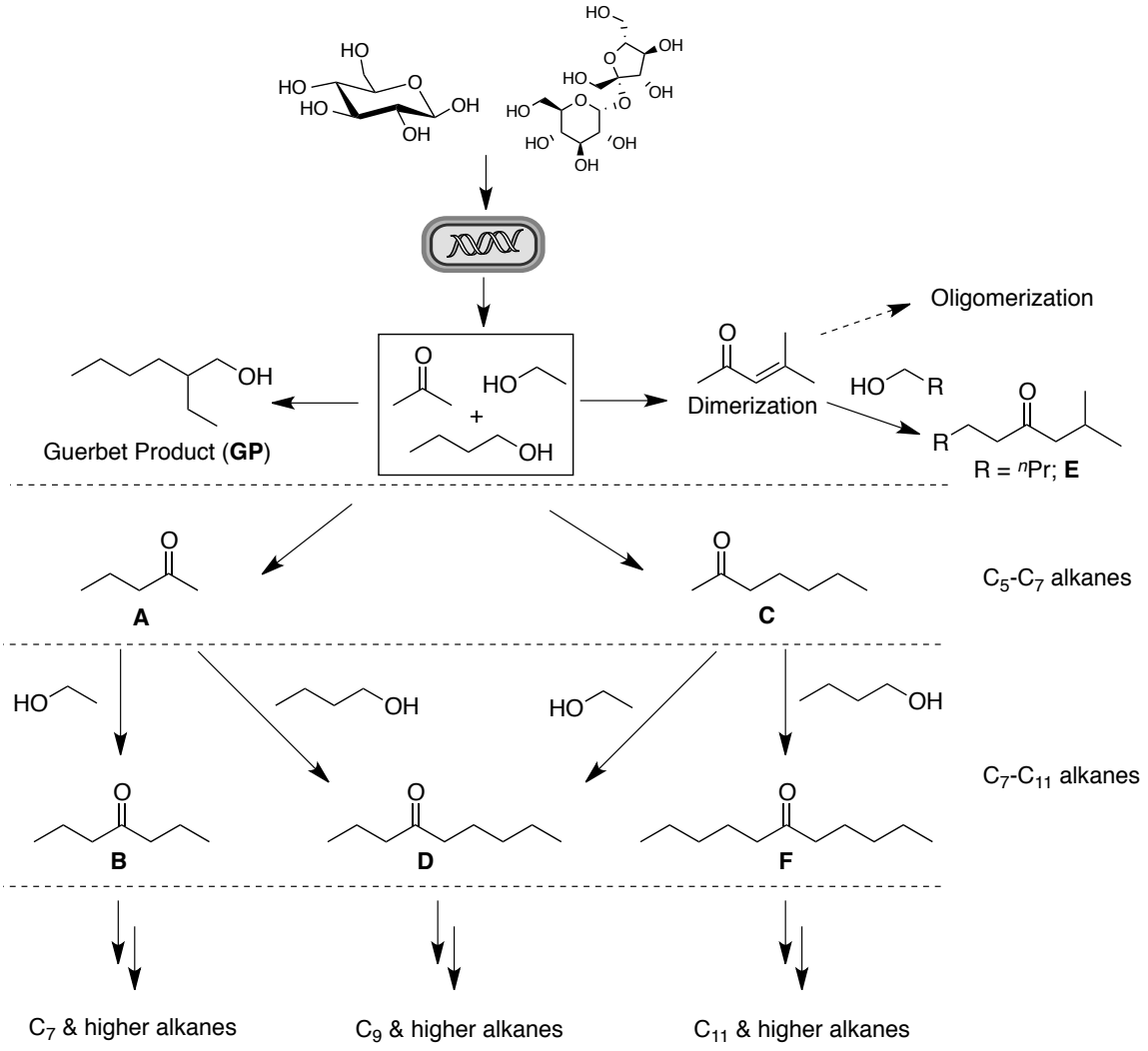


Figure 3-1: A general approach to the transition-metal-catalysed production of biofuels from the ABE fermentation mixture.

3.2 Methods and Materials

3.2.1 Reagents

All the metal sources were purchased from either Sigma Aldrich or Strem Chemicals and used as received. Chemicals were obtained from Sigma Aldrich and used without further purification.

3.2.2 Reaction analysis

All the reactions were analyzed by gas chromatography using dodecane as internal standard. Gas chromatography analysis was performed on a Varian CP-3800 instrument with a FID detector and VF5ms column (5% phenyl and 95% methylpolysiloxane) using helium as carrier gas.

3.2.3 Alkylation reaction

In a 12 mL Q-Tube (pressure tube) 5% palladium on carbon (containing 50% of water, 42 mg, 0.01 mmol), potassium phosphate tribasic (954 mg, 4.5 mmol) and magnetic stir bar were placed. To the tube, 1.5 mL of toluene was added followed by acetone (0.17 mL, 2.3 mmol), ethanol (60 μL, 1 mmol) and butanol (0.34 mL, 3.7 mmol) were also introduced. Next the tube was closed and kept at 145 °C in the pre-heated metal block. The reaction mixture was stirred for 20 h at the same temperature and then cooled to room temperature. Subsequently, dodecane (internal standard) was added and diluted with ethyl acetate. GC analysis of the reaction mixture yielded the ratio of products.

3.2.4 Extraction w/ glyceryl tributyrate

Simulated clostridia fermentation media was prepared with the following components glucose ($20gl^{-1}$), yeast extract ($5gl^{-1}$), ammonium acetate ($2gl^{-1}$), butyric acid ($2gl^{-1}$), acetoin ($3gl^{-1}$), ethanol ($20gl^{-1}$), acetone ($20gl^{-1}$), 1-butanol ($20gl^{-1}$), and lactic acid ($10gl^{-1}$). 5-mL of simulated fermentation media was combined with 5mL of glyceryl tributyrate and mixed for 5 minutes by inversion. The mixtures were then spun down at 5300 RPMs for 5 minutes and the extractant phase removed for GC analysis. Distribution coefficients were calculated based on equation 3-1.

$$K_{D_i} = \frac{\text{Kg of compound i in the extractant phase}}{\text{Kg of compound i in the aqeous phase}} \qquad (3\text{-}1)$$

ABE extraction experiments were run in quadruplicate. To measure hydrolysate inhibitor distribution coefficients, *Miscanthus giganteus* from the University of Illinois, Urbana-Champaign was first ground and placed through a 4mm size sieve. 5% w:w of *M. giganteus* was mixed with 1% H_2SO_4 in sealed Teflon tubes and reacted under the following conditions (30 minutes at 30°C, 6 minute ramp to

180°C, 2 minutes at 180°C). The liquid hydrolysate was pH adjusted to 5.0 using concentrated KOH. 3-mL of hydrolysate was combined with 3mL of glyceryl tributyrate and thoroughly mixed for 5 minutes by inversion. The mixtures were then centrifuged for 5 minutes at 5300 rpms and the aqueous phase removed. Inhibitors remaining in the aqueous phase were measured by first extracting into ethyl acetate, followed by drying with Na_2SO_4. The dried solution was then incubated with bis(trimethylsilyl)trifluoracetamide at 70°C for 30 minutes. Inhibitor concentrations were analyzed by GC/MS with isopropylphenol as an internal standard.

3.2.5 Extractive Fermentation

Clostridium acetobutylicum ATCC824 was grown in clostridial growth medium (CGM) as previously described in Section 2.2.1, with an initial glucose concentration of 65 g L^{-1} . Fed-batch fermentations were conducted in 3-L bioreactors (Bioengineering AG, Switzerland) with a 2L working volume. Additional glucose and yeast extract were added intermittently to the culture using a concentrated solution of 450 and 50 gl^{-1} respectively. Cultures were grown at 37°C anaerobically by sparging 100 mL/min of N_2 gas until solvent production was initiated. The culture pH was adjusted to 5.5 prior to inoculation with 1M phosphoric acid. After inoculation the bioreactor pH was controlled at pH ≥ 4.8 with 5M KOH. Sugars and major metabolites (glucose, sucrose, lactate, acetate, butyrate, acetoin, ethanol, acetone, and 1-butanol) were measured in the aqueous phase using an Agilent (Santa Clara, CA) HPLC system equipped with refractive index and UV/Vis detectors. A Bio-Rad (Hercules, CA) Aminex HPX-87H ion exchange column with a Cation H guard column at 30°C was used with a mobile phase of 0.05 mM sulfuric acid flowing at 0.7 mL min^{-1}. Acetone, 1-butanol and ethanol concentrations in the extractant phase were measured by GC/FID with a Varian (Palo Alto, CA) Factor four column VF-5ms. Extractive fermentation with sucrose was carried out in 100-mL shake flasks with 25mL of clostridia growth media inoculated with 2mLs of OD_{600} (0.6-1.0) cells. Cultures were grown at 37°C in an anaerobic chamber and pH adjusted to 4.8 using 1M KOH during the first 12 hours of growth. After 16 hours 25mL of glyceryl tributyrate was added to the culture.

3.2.6 One-pot alkylation and hydrogenation

5% palladium on carbon (0.01 mmol), K_3PO_4 (4.5 mmol, 96 mol%), and a magnetic stir bar were added to a high-pressure reaction vessel (HEL parallel synthesizer). To the vessel, 1.5 mL of toluene was added. Then, acetone (2.3 mmol), ethanol (1 mmol), and butanol (3.7 mmol) were also added to the vessel. The reaction vessel was sealed, and heated to 145°C and stirred at the same temperature for 20 hours. The reaction mixture was cooled to ~30 °C and 5% platinum on carbon (0.02 mmol) was added, and the reaction vessel was pressurized with H_2 gas (150 psi). The reaction mixture was stirred at 110 °C for

14 hours, and then cooled to room temperature. GC analysis of the final reaction mixture, using dodecane as internal standard, yielded the ratio of alcohol products.

3.3 Results and Discussion

3.3.1 Catalyst screening and reaction conditions optimization

Using a synthetic ABE mixture of pure acetone, n-butanol and ethanol, we investigated the double alkylation of acetone to obtain heptan-4-one (**B** in Figure 3-1) (alkylation with ethanol), nonan-4-one (**D**) (alkylation with one molecule each of ethanol and butanol) and undecan-6-one (**F**) (alkylation with butanol) under transition-metal-catalysed conditions (Figure 3-1) (Guillena, Ramón, and Yus 2007; Hamid, Slatford, and Williams 2007). Two possible side reactions in the transition-metal-catalysed alkylation of acetone with primary alcohols are self-condensation of the alcohol through the corresponding aldehyde (Guerbet reaction) (Carlini et al. 2004) and oligomerization of acetone into diacetone alcohol, mesityl oxide, cyclohexenones and other products (Salvapati, Ramanamurty, and Janardanarao 1989). In particular, self-condensation of acetone affects the overall efficiency of the desired process by lowering the ratio of ketone to alcohol in the reaction mixture. Similarly, if too much butanol reacts through the Guerbet pathway then not all of the acetone will be consumed; however, the Guerbet reaction does form higher branched alcohols that could themselves be valuable fuel precursors or be combined with acetone to form up to C_{19} ketones (Figure 3-2). The transition metal and base catalysts should therefore be chosen to minimize these competing processes. As previously observed[15], we found that solid potassium phosphate tribasic (K_3PO_4) was the optimal base for the transition-metal-catalysed alkylation of acetone (Table 3-1). Using solid K_3PO_4 as a heterogeneous base, we observed only minor amounts of products from acetone condensation (**E**) and no Guerbet products.

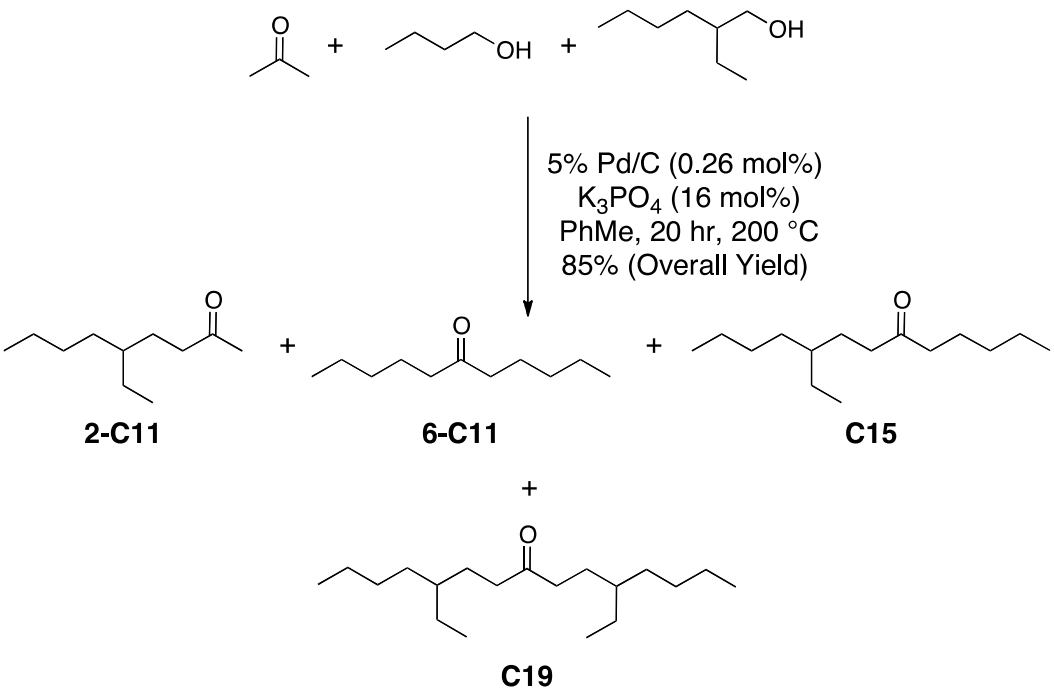


Figure 3-2: In a 12 mL Q-tube, 5 wt.% palladium on carbon (0.011 g, 0.0026 mmol, water *ca.* 50%), potassium phosphate tribasic (0.034 g, 0.16 mmol) and a magnetic stir bar were placed. To the reaction mixture, acetone (0.058 g, 1 mmol), 2-ethyl-1-hexanol (1.30 g, 10 mmol), butanol (0.22 g, 3 mmol) and 1 mL toluene were sequentially added. The Q-tube was sealed and the reaction mixture was stirred for 20 hours at 200 °C in a pre-heated metal block. The reaction mixture was cooled to room temperature and dodecane (internal standard) was added. The reaction mixture was diluted with tetrahydrofuran and the GC analysis of the reaction mixture was carried out (85% overall yield, **2-C11**: 4%, **6-C11**: 22%, **C15**: 37%, **C19**: 22%).

Our tests of different transition-metal catalysts (Ir, Ru, Rh, Pt, Pd and Ni) with K_3PO_4 in toluene for the alkylation reaction revealed that palladium was superior to the other metals (Table 3-2) (Kwon et al. 2005). Further, trials of various palladium precursors showed a marked effect of the precursor on the outcome of the reaction (Table 3-3). Although most of the palladium precursors examined catalysed the alkylation of acetone, Pd/C was significantly more active than the other palladium precursors at 110 °C in toluene.

Table 3-1: Effect of base species on product distribution and overall yield. A-F describe identified products: 2-pentanone (A), 4-hepatanone (B), 2-hepatanone (C), 4-nonanone (D), 2-methyl-4-nonanone (E), 6-undecanone (F). [a] Yields based on Acetone. Reaction conditions: Acetone (2.3 mmol), Ethanol, (1 mmol), Butanol (3.7 mmol), 5% Pd/C (0.01 mmol), Base (3 mmol), Toluene (1.5 mL), 145 °C, 20 h.

Base	Yield (%)[a]						
	A	**B**	**C**	**D**	**E**	**F**	Total
K_3PO_4	5	2	19	16	2	29	73
KOH	4	0	14	1	3	2	24
$Ba(OH)_2.8H_2O$	3	0	13	0	0	0	16
K_2CO_3	5	0	11	0	0	0	16
KOAc	0	0	0	0	0	0	0
KH_2PO_4	0	0	0	0	0	0	0
Na_2HPO_4	0	0	0	0	0	0	0
Pyridine	0	0	0	0	0	0	0
Et_3N	0	0	7	0	0	0	7

Table 3-2: Effect of metal species and precursors on product distribution and overall yield. A-F describe identified products: 2-pentanone (A), 4-hepatanone (B), 2-hepatanone (C), 4-nonanone (D), 2-methyl-4-nonanone (E), 6-undecanone (F). [a] Yields based on Acetone. Reaction conditions: Acetone (2.3 mmol), Ethanol, (1 mmol), Butanol (3.7 mmol), Metal precursors (0.01 mmol), K_3PO_4 (4.5 mmol), Toluene (1.5 mL), 145 °C, 20 h.

Metal	Yield (%)						
	A	**B**	**C**	**D**	**E**	**F**	Total
$[Ir(COD)Cl]_2$	0	0	0	0	0	0	0
$RuCl_2(COD)$	0	0	0	1	1	3	5
$PtCl_2(COD)$	0	1	1	3	1	9	15
$[Rh(COD)Cl]_2$	0	1	0	3	4	4	12
Ni/Silica-Alumina	0	0	0	0	0	0	0
Ru/C	0	0	0	0	0	0	0
Rh/C	0	0	0	1	1	1	3
Pt/C	0	1	1	7	1	14	24
Pd/C	3	2	10	21	3	39	78

Table 3-3: Effect of palladium source on product distribution and overall yield. A-F describe identified products: 2-pentanone (A), 4-hepatanone (B), 2-hepatanone (C), 4-nonanone (D), 2-methyl-4-nonanone (E), 6-undecanone (F). [a] Yields based on Acetone. Reaction conditions: Acetone (2.3 mmol), Ethanol, (1 mmol), Butanol (3.7 mmol), Pd-source (0.01 mmol), K_3PO_4 (3 mmol), Toluene (1.5 mL), 110 °C, 20 h.

Pd-Source	Yield (%)						
	A	**B**	**C**	**D**	**E**	**F**	Total
$Pd(OAc)_2$	2	2	5	6	4	6	25
$PdCl_2$	1	0	0	0	0	0	1
$Pd_2(dba)_3$	3	2	12	14	4	23	58
$Pd(OH)_2/C$	7	3	18	15	4	19	66
Pd/C	10	1	31	13	3	21	79
$Pd/CaCO_3$	0	0	7	4	7	13	31
Pd/Alumina	3	2	8	12	6	15	46
$Pd/BaCO_3$	2	0	2	1	1	1	7
Pd-Polyethylenimines on Silca	2	1	4	4	5	4	20
$PdCl_2(CH_3CN)_2$	0	0	1	1	1	1	4

3.3.2 Tuning product distribution

Increasing the reaction temperature to 145 °C resulted in a minor improvement in the selectivity for double alkylated products, but the overall efficiency of the reaction declined with increasing temperature (Figure 3-3a). Increasing the amount of base led to a marked improvement in both the selectivity for double alkylation and the overall yield. With 1.28 molar equivalents of K_3PO_4 with respect to total alcohols (ethanol and butanol) at 145 °C, an overall 86% molar yield, based on acetone as the limiting reagent, of identified ketone products was obtained (Figure 3-3b). We envisaged that tuning the reaction conditions would afford selective monoalkylation of acetone to obtain petrol-range hydrocarbon chains. Consequently, alkylation was examined at various temperatures with only 0.32 molar equivalents of K_3PO_4. Under these conditions, pentan-2-one and heptan-2-one became the major products, with C_5–C_7 species accounting for nearly 50% of the overall yield, with respect to acetone as the limiting reagent. The best result was obtained at 145 °C with the production of pentan-2-one and heptan-2-one in 11% and 38% yield, respectively, along with 17% of double-alkylated products.

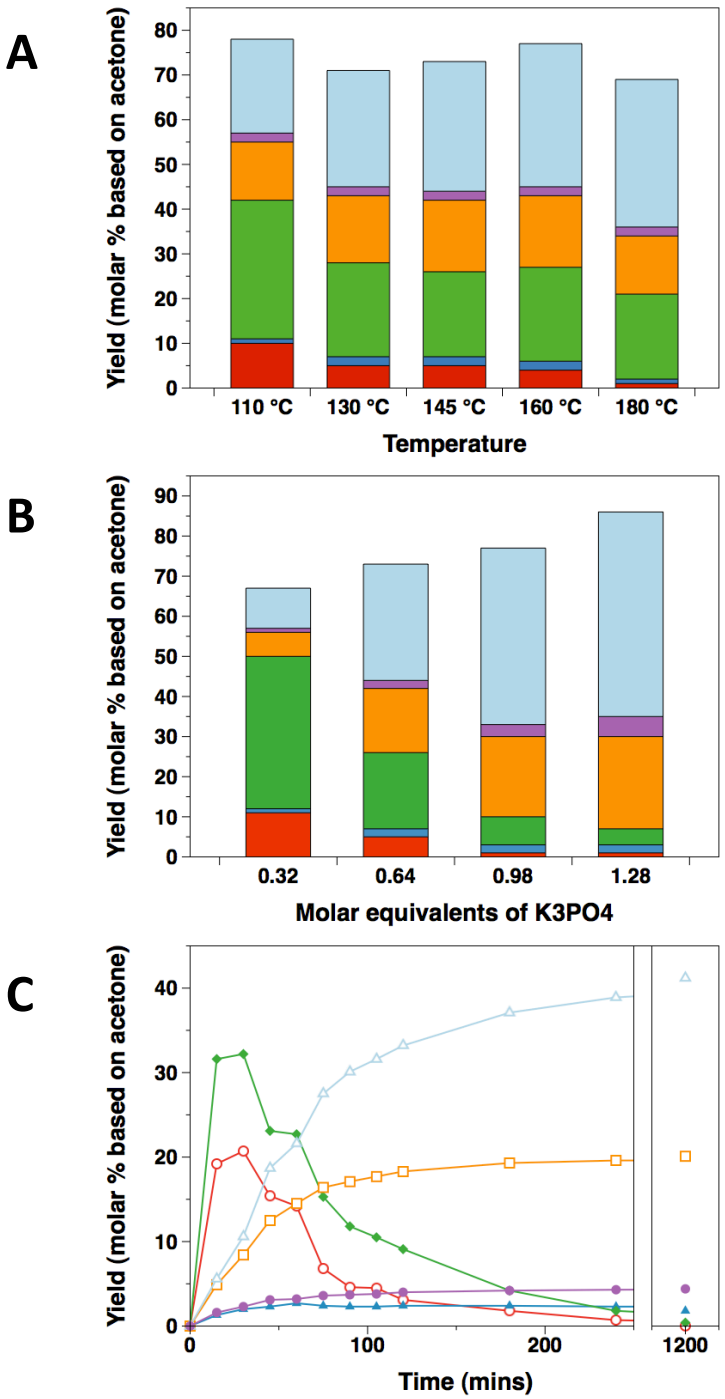


Figure 3-3 | Product distribution in Pd-catalysed alkylation as a function of reaction parameters and time course. **a**, Effects of temperature on product yields and distribution. Products listed in order from bottom to top (Red, pentan-2-one; dark blue, heptan-4-one; green, heptan-2-one; orange, nonan-4-one; purple, 2-methylnonan-4-one; light blue, undecan-6-one). Yields based on acetone. Reaction conditions: acetone (2.3 mmol), ethanol (1 mmol), butanol (3.7 mmol), 5% Pd/C (0.01 mmol), K_3PO_4 (3 mmol), toluene (1.5 ml), temperature (*x* axis), 20 h. **b**, Effect of K_3PO_4 loading on product yields. K_3PO_4 loading is represented as molar equivalents with respect to total alcohol loading (ethanol + butanol). Reaction conditions were identical to the temperature studies with the exception of K_3PO_4 loading (*x* equivalents) and T = 145 °C. **c**, Time course of product distributions during the palladium-catalysed alkylation reaction, lines drawn to guide the eye. Colors as in **b** (open circle, pentan-2-one; closed triangle, heptan-4-one; closed diamond, heptan-2-one; open square, nonan-4-one; closed circle, 2-methylnonan-4-one; open triangle, undecan-6-one. Reaction conditions: acetone (4.6 mmol), ethanol (2 mmol), butanol (7.4 mmol), 5% Pd/C (0.02 mmol), K_3PO_4 (9 mmol), toluene (3 ml), 145 °C.

3.3.3 Understanding the reaction mechanism

We explored the mechanism of the reaction by following the reaction progress. As shown in Figure 3-3c, monoalkylation of acetone with butanol and ethanol occurred quickly within the first 1.0–1.5 h of the reaction to produce pentan-2-one and heptan-2-one. These species underwent further reaction to form double-alkylated products. No aldehydes were observed during the reaction, suggesting that the aldehyde intermediates were present in very low concentrations and reacted rapidly with acetone and other ketones. Hence, the formation of Guerbet products was minimized, and acetone alkylation predominated.

On the basis of our experiments and previous studies of the alkylation of N-nucleophiles and C-nucleophiles (Guillena, Ramón, and Yus 2007; Guillena, J Ramón, and Yus 2010; Dobereiner and Crabtree 2010), we propose a mechanism for the double alkylation of acetone with ethanol and butanol (Figure 3-4). The alcohols (butanol and ethanol) are dehydrogenated by the palladium catalyst, generating the reactive aldehydes and hydrogen. At this stage the aldehydes undergo either a self-aldol reaction to form the Guerbet product precursor, or an aldol reaction with acetone. Under the conditions employed here, condensation with acetone seems to be favoured, because acetone is present at much higher concentrations than the transient aldehydes. Subsequent dehydration of the aldol product under the reaction conditions furnishes a,b-unsaturated ketones that undergo palladium-catalysed hydrogenation with the hydrogen generated in the first step. Completing the same cycle with monoalkylated products affords the expected double alkylation products. The relative ease of attack by the unsubstituted versus the substituted a-carbon of the monoalkylated products leads primarily to unbranched products. This combination of kinetic controls on the alkylation reaction therefore enables the conversion of a mixture of renewable metabolites into a well-defined range of ketone products.

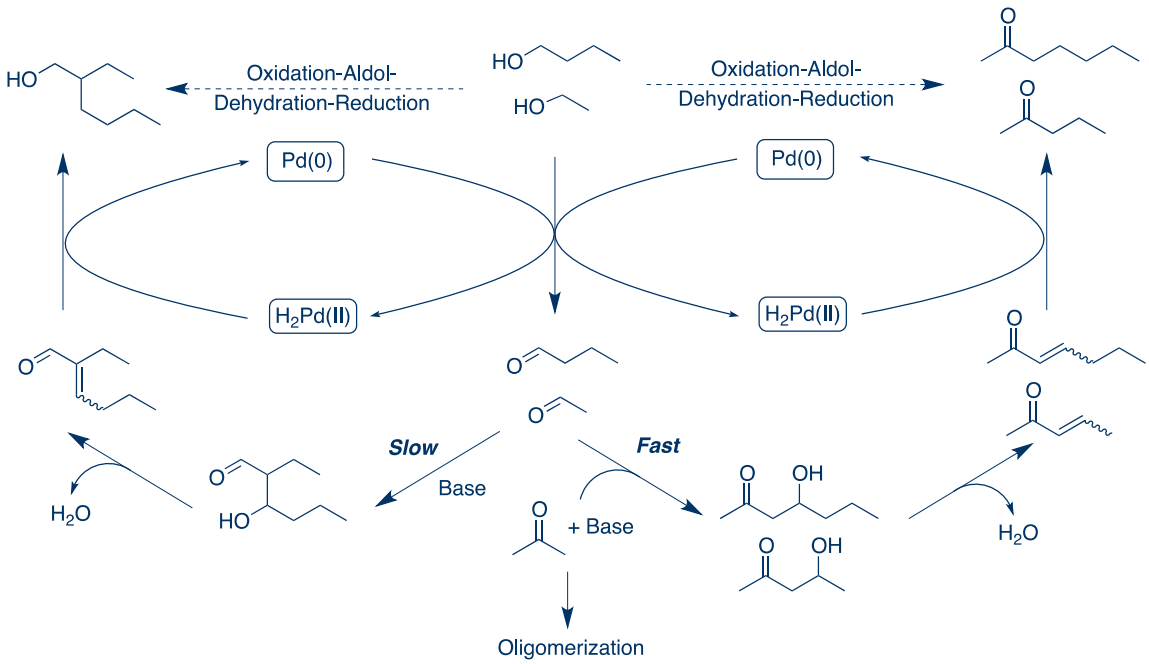


Figure 3-4 | Possible mechanism for the palladium-catalysed alkylation of acetone.

3.3.4 Catalyst recyclability and water inhibition

One important aspect of any catalytic process is the lifetime of the catalyst, which we investigated by adding additional starting material after the reaction had been allowed to proceed to near completion. In a typical experiment using Pd/C and 0.96 molar equivalents of K_3PO_4 at 145 °C, we added an additional ABE mixture equivalent to the initial charge at 10 and 20 h after the beginning of the reaction and measured the overall yield at 10, 20 and 30 h. In this case, the overall yield based on the total acetone feed for each time point was only slightly decreased: 80% at 10 h, 72% at 20 h, and 61% at 30 h, demonstrating that the catalysts remained active and continued to convert each new amount of starting material. The catalyst also retained activity in the absence of toluene or undiluted conditions, achieving catalytic turnover numbers of more than 3,000 (moles of product per mole of palladium; Table 3-4). Although wet organic solvents could be used for the alkylation reaction, no reaction was observed in water. The addition of water was found to slow the overall reaction, resulting in lower double alkylation and overall yields (Figure 3-5).

Table 3-4: Alkylation reaction under toluene free or neat conditions. [a] GC weight yield; [b] Turn Over Number based on alcohols. Reaction conditions: Acetone (4.6 mmol), Butanol (7.4 mmol), Ethanol, (2 mmol), 5% Pd/C (X mol%l), K_3PO_4 (molar equivalents to alcohols), Temp (°C), 20 h;

Pd/C (X mol%)	Base (molar equiv.)	Temp (°C)	Yield (%)[a]			TON[b]	Solvent
			C_5-C_{11}	C_{11}+	Total		
0.025	0.32	145	27	15	42	1574	Neat[f]
		180	36	19	55	2072	
0.0125	0.32	145	22	19	41	3074	
		180	26	17	43	3220	
0.0125	0.16	145	17	11	28	2080	
		180	31	14	45	3386	

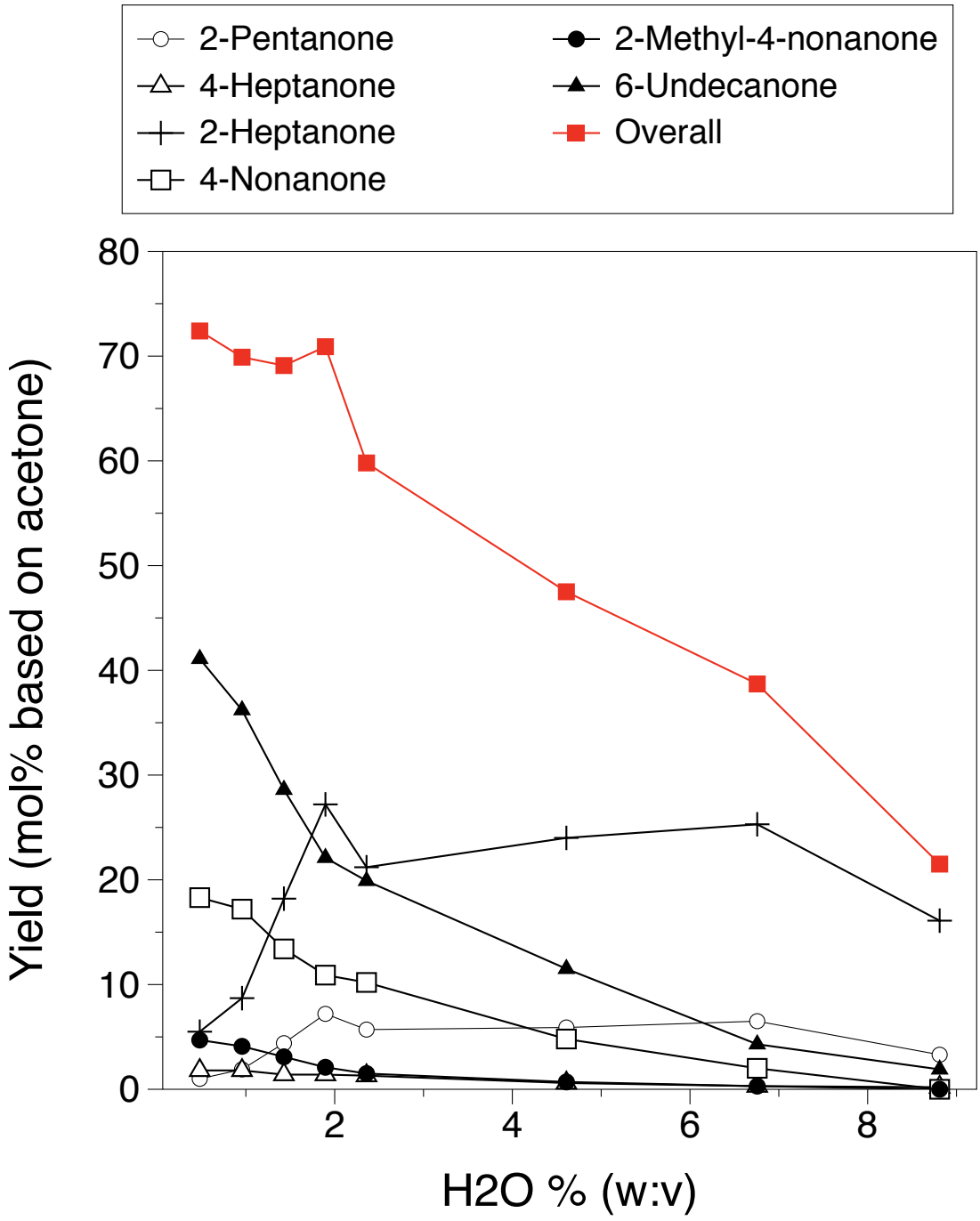


Figure 3-5: Effect of water addition on alkylation reaction. Yield based on Acetone. Reaction conditions: Acetone (2.3 mmol), Ethanol, (1 mmol), Butanol (3.7 mmol), Pd-source (0.01 mmol), K_3PO_4 (3 mmol), Toluene (1.5 mL), H_2O (wt%), 145°C, 20 h.

3.3.5 Extractant identification

To overcome the sensitivity of the catalysts to water, it was of interest to investigate highly selective water-immiscible extractants to remove acetone, n-butanol and ethanol *in situ*. *In situ* removal of the inhibitory product n-butanol during fermentation has been shown to increase solvent titres and yields, decrease distillation costs significantly, and decrease water use and reactor sizes (Wayman and Parekh 1987; Roffler, Blanch, and Wilke 1987b; Roffler, Blanch, and Wilke 1987a; Roffler, Blanch, and Wilke 1988). However, the extractants typically employed are only capable of efficiently removing n-butanol from the aqueous phase, leaving most of the acetone produced in the aqueous phase. Glyceryl tributyrate was identified as a water-immiscible solvent capable of efficiently recovering both acetone (K_d = 1.0) and n-butanol (K_d = 2.6) from aqueous solution (Jeon and Lee 1987). Ethanol, however, remains preferentially in the aqueous phase (K_d = 0.2), as shown in Table 3-5. Glyceryl tripropinate was also tested as a suitable extractant for ABE fermentation. The distribution coefficients for acetone (K_d = 1.1), butanol (K_d = 2.4), and ethanol (K_d = 0.25) were very similar to Glyceryl tributyrate.

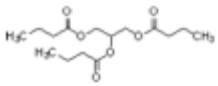
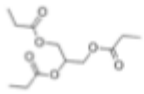
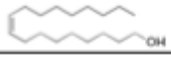
Extractant Structure	Name	Extracts Acids	Acetone K_D (Kg/Kg)	Butanol K_D (Kg/Kg)	Ethanol K_D (Kg/Kg)
	Glyceryl Tributyrate	No	1.0 ± 0.04	2.6 ± 0.04	0.19 ± 0.02
	Glyceryl Tripropionate	Yes	1.1 ± 0.04	2.4 ± 0.05	0.25 ± 0.02
	Oleyl Alcohol	No	0.35*	3.6*	0.25*

Table 3-5: Distribution coefficients (K_D) of acetone, butanol, ethanol for glyceryl tributyrate, glyceryl tripropinoate, and oleyl alcohol with water. Extraction of acids (butyric and acetic) could be detected on the GC but not accurately quantified. (*) values taken from (Roffler, Blanch, and Wilke 1987b).

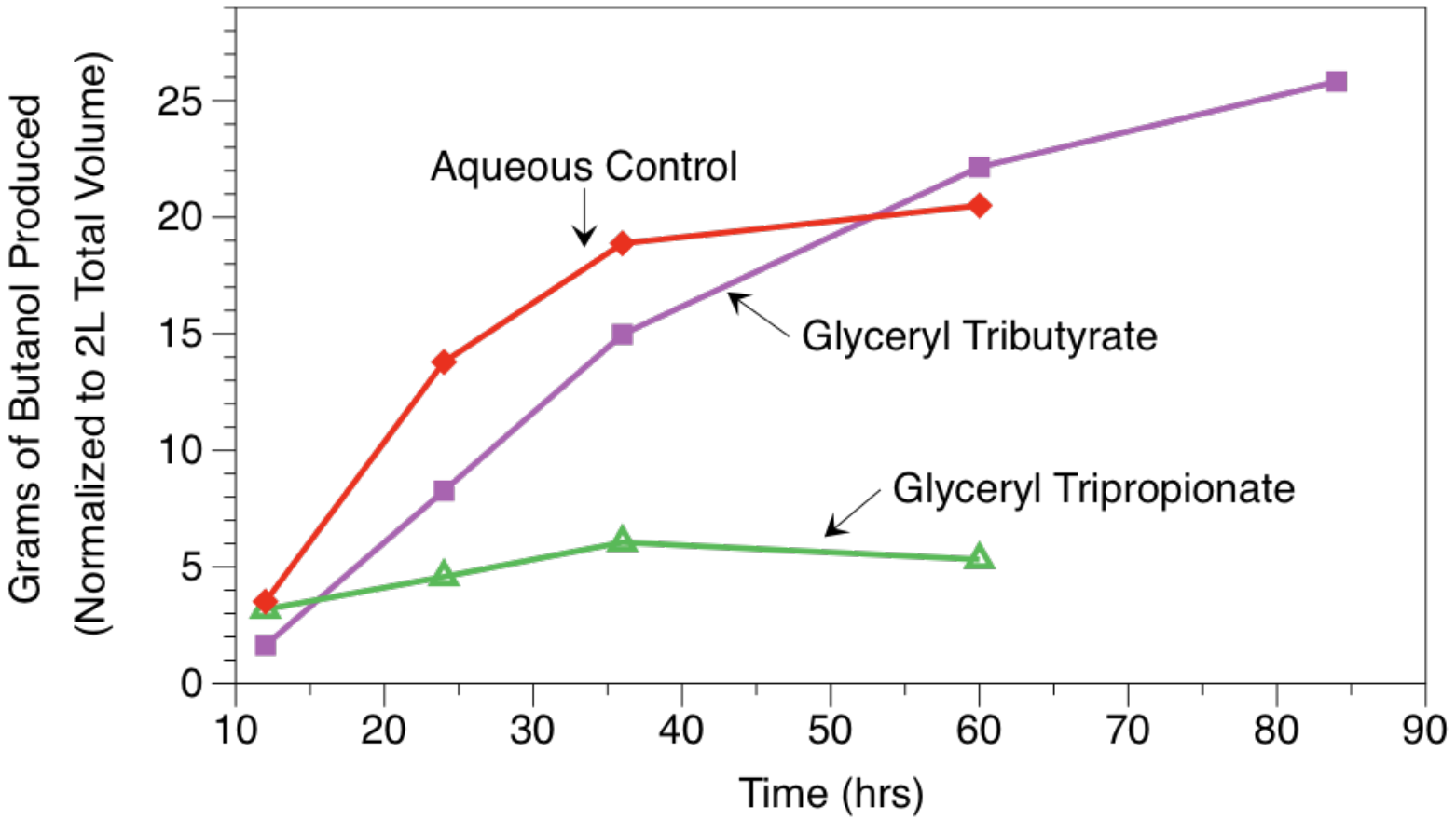


Figure 3-6: Comparison of butanol production when either glyceryl tripropinate (green triangles) or glyceryl tributyrate (purple squares) are utilized for *in situ* solvent removal. Fed-batch shake flask fermentations at 37 °C with a 1:1 extractant:media volume ratio. Extractant added 24 hours after culture inoculation. Aqeuous control (red diamonds) was operated in simple batch mode.

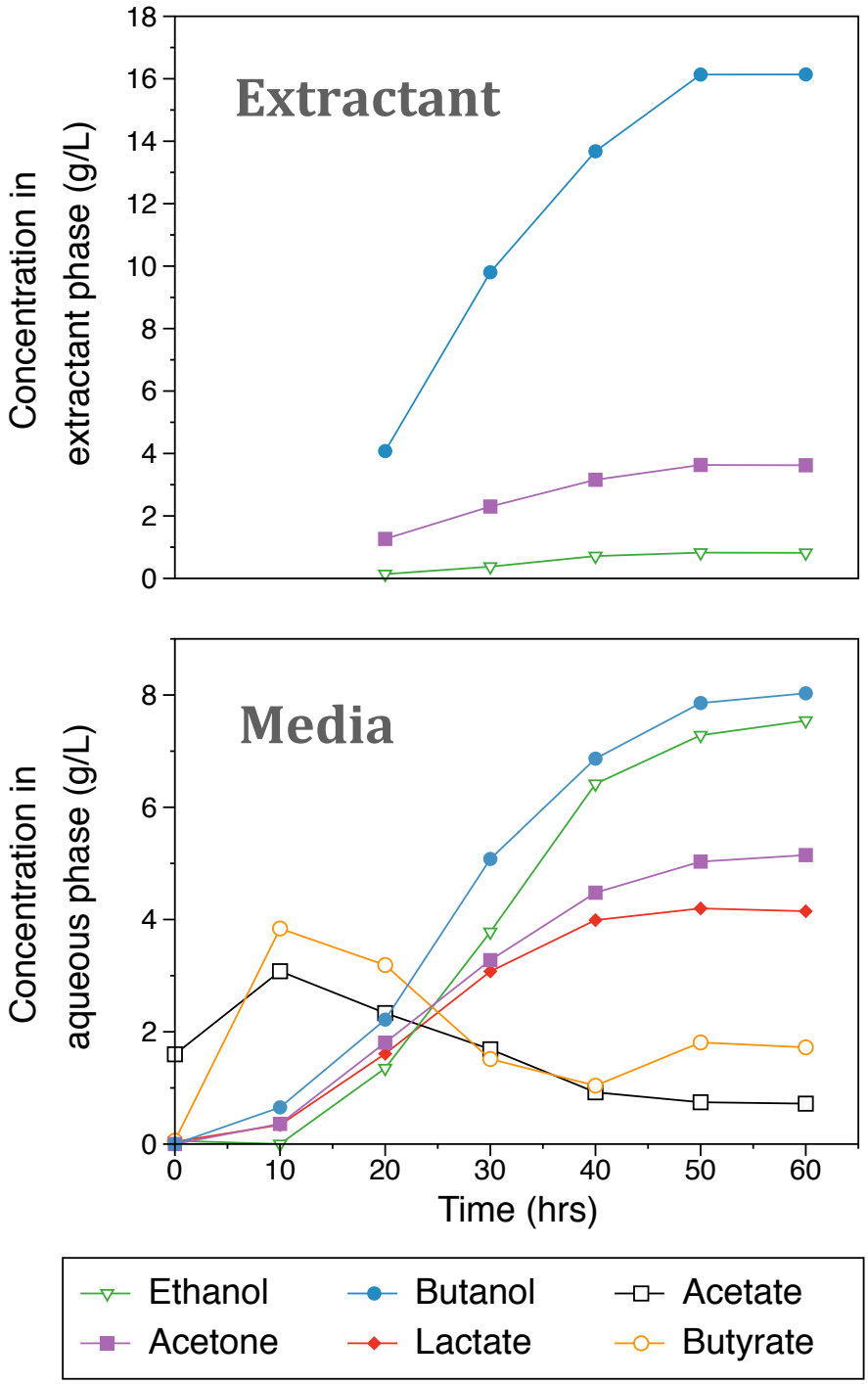


Figure 3-7: Time course of product formation in fed-batch extractive fermentation w/ glyceryl tributyrate extractant. Initial glucose concentration 65 gL^{-1}

3.3.5 Extractive fermentation

Studies of growth inhibition and cell viability with the use of up to 1:1 volume ratios of either of these glyceryl tri-esters to media showed no toxicity to *C. acetobutylicum*. However, as Figure 3-6 shows, solvent production was inhibited 41% compared to the aqueous phase control when glyceryl tripropionate was used as the extractant. This is in contrast to glyceryl tributyrate that showed no inhibitory effect towards solvent production in *C. acetobutylicum*. A 60-h 2-l fermentation of *C. acetobutylicum* on glucose with a 1:1 volume ratio of medium and glyceryl tributyrate produced 40.8 g of solvents with 16.4 g of n-butanol, 3.7 g of acetone and 0.8 g of ethanol, partitioning into the extractant phase (Figure 3-7). These solvents were produced from 105 g of glucose and 1.6 g of acetate, achieving an overall ABE weight yield of 90% of the theoretical maximum.

In addition, glyceryl tributyrate efficiently removed several of the inhibitors of biofuel fermentation (for example furfural, *p*-coumaric acid and ferulic acid) found in acid-pretreated lignocellulosic biomass, Table 3-6. The combined inhibition of these top 10 pretreatment compounds reduced solvent production in wild-type *C. acetobutylicum* batch fermentation by approximately 50% as shown in Figure 3-7. It should be noted that the concentration of these pretreatment inhibitors was based on a 10% biomass loading, which provides the minimum 60 g L^{-1} total sugar concentration for efficient solvent production. Diluting these inhibitor concentrations to 60% of their original values eliminated this reduction in solvent titers. This affords the simultaneous removal of residual inhibitors and the desired product during biofuel fermentation, a key advantage over other recovery technologies (Vane 2008). Addition of glyceryl tributyrate in a 1:1 ratio to the top 10 pretreatment inhibitor media 30 minutes prior to inoculation enabled similar solvent titers and volumetric productivities to the previously tested uninhibited extractive fermentation, see Figure 3-8. After 72 hours 27.5 g L^{-1} of butanol, 5,6 g L^{-1} of acetone, and 6 g L^{-1} ethanol were produced. The lower than expected acetone titer is likely a product of the fermentation being conducted in a shake flask without vapor condensation of the headspace.

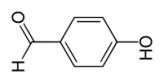
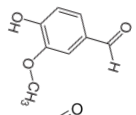
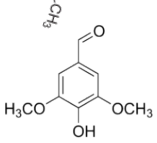
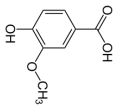
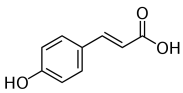
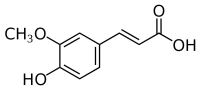
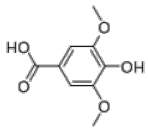
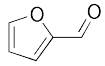
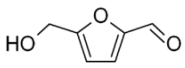
Inhibitor structure	Compound name	Initial Conc. (mg/L)	K_D in tributyrin
	**4-hydroxy-benzaldehyde**	**15.8**	**4.9**
	**vanillin**	**30.8**	**7.6**
	**syringaldehyde**	**18.9**	**5.3**
	**vanillic acid**	**14.5**	**1.3**
	**p-coumaric acid**	**35.5**	**3.8**
	**ferulic acid**	**41.8**	**4.2**
	**Syringic acid**	**8.8**	**0.7**
	**furfural**	**1926.1**	**6.5**
	**Hydroxymethyl furfural**	**205.1**	**0.5**

Table 3-6: Extraction of top 10 lignocellulosic hydrolysate inhibitors (by mass) produced during acid pretreatment of *Miscanthus giganteus* in glyceryl tributyrate. Concentration of inhibitors based on a 10-wt% biomass solids loading and 1-wt% sulphuric acid.

Figure 3-8: Effect of Top 10 pretreatment inhibitors on solvent production in wild-type *C. acetobutylicum*. Results average of biological triplicate. Line is drawn to guide the eye.

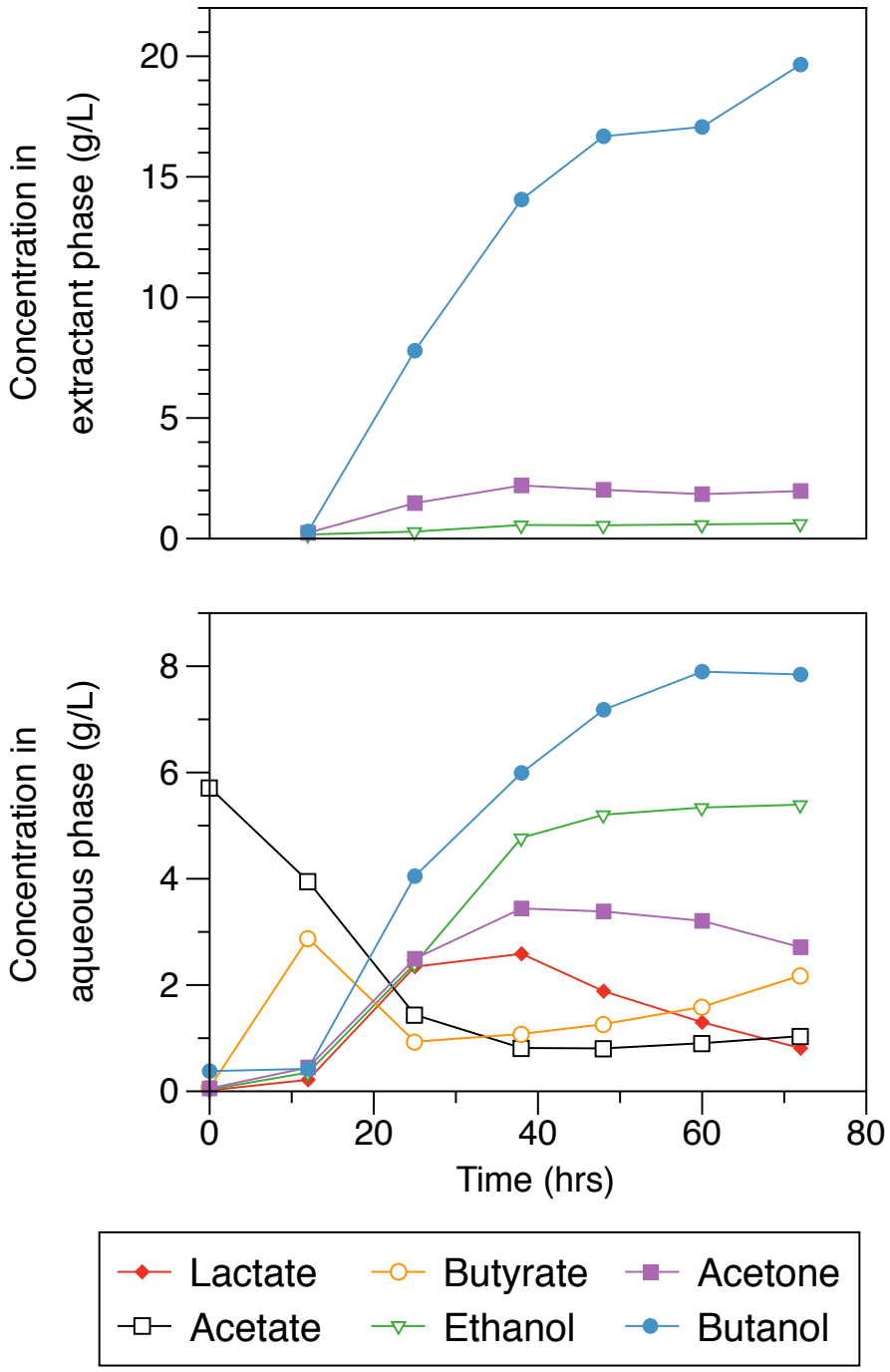


Figure 3-9: Time course of fed-batch extractive fermentation on glucose with Top10 lignocellulosic hydrolysate inhibitors. Initial glucose concentration 60 gL^{-1}. Lines drawn to guide the eye.

To demonstrate the feedstock flexibility of this process sucrose, the main carbohydrate of sugar cane, was used as the primary carbon source in an extractive fermentation. Though final solvent titers were similar to previously described fermentations with glucose the productivity was significantly reduced, see Figure 3-10. In a 1:1 volume ratio shake flask experiments with sucrose, after 150 hours 26.9 g/L of butanol, 6.6 g/L of acetone and 5.1 g/L of ethanol were produced for a total solvent titer of 38.6 g/L. *Clostridium acetobutylicum* ATCC824 was traditionally used for the fermentation of starchy substrates because of the strain's high α-amylase activity. Adaptation of *C. acetobutylicum* and isolation of new strains for the efficient fermentation of molasses has been previously described (Durre, 1998).

3.3.7 Catalytic Upgrading of Fermentation Products

The *in situ* removal of ABE from the aqueous phase by a high-boiling-point extractant such as glyceryl tributyrate decreases energy requirements for product distillation (Kraemer et al. 2011) and allows integration of the biological and catalytic processes. Furthermore, any high-boiling impurities present in the extractant (for example furfural, *p*-coumaric acid or ferulic acid) would remain in the distilled extractant upstream of the catalyst, thus having no effect on catalyst performance. Solvents distilled from the extractant phase of the 2-l fermentation were as reactive as the pure chemicals, achieving an overall molar yield of 93% and 97% conversion of acetone (Figure 3-11). Assuming complete recovery of ABE products from both the extractant and aqueous phases, ~20 g of C_7–C_{15} products would be formed, which represents ~38% of the carbon contained in the glucose feed (Table 3-7). The combined selectivity of the fermentation and the alkylation results in this particularly high-yielding transformation of carbohydrates into fuel ketones. In comparison, a previous study (Gürbüz, Kunkes, and Dumesic 2010) used similar aldol reactions to convert the alcohols and ketones in an aqueous-phase reformate into C_7 and higher species with a carbon yield of 42% from the intermediate oil feed and an overall carbon yield of 21% from the initial sorbitol feed. The lower yield of higher ketones resulted from the non-selective production of oxygenates that could not be upgraded in the aldol reaction. The 38% carbon yield in the present work does not account for the 15.1 g of lower-molecular-mass (C_4–C_6) petrol precursors also produced, which would then raise the overall carbon yield to ~58%. Further deoxygenation (Figure 3-12) of the products obtained from alkylation of the ABE mixture by using standard hydrotreating chemistry (Gürbüz, Kunkes, and Dumesic 2010; Kunkes et al. 2008; Xing et al. 2010) would yield alkanes compatible with refinery infrastructure and suitable for blending with petrol, diesel and jet fuels.

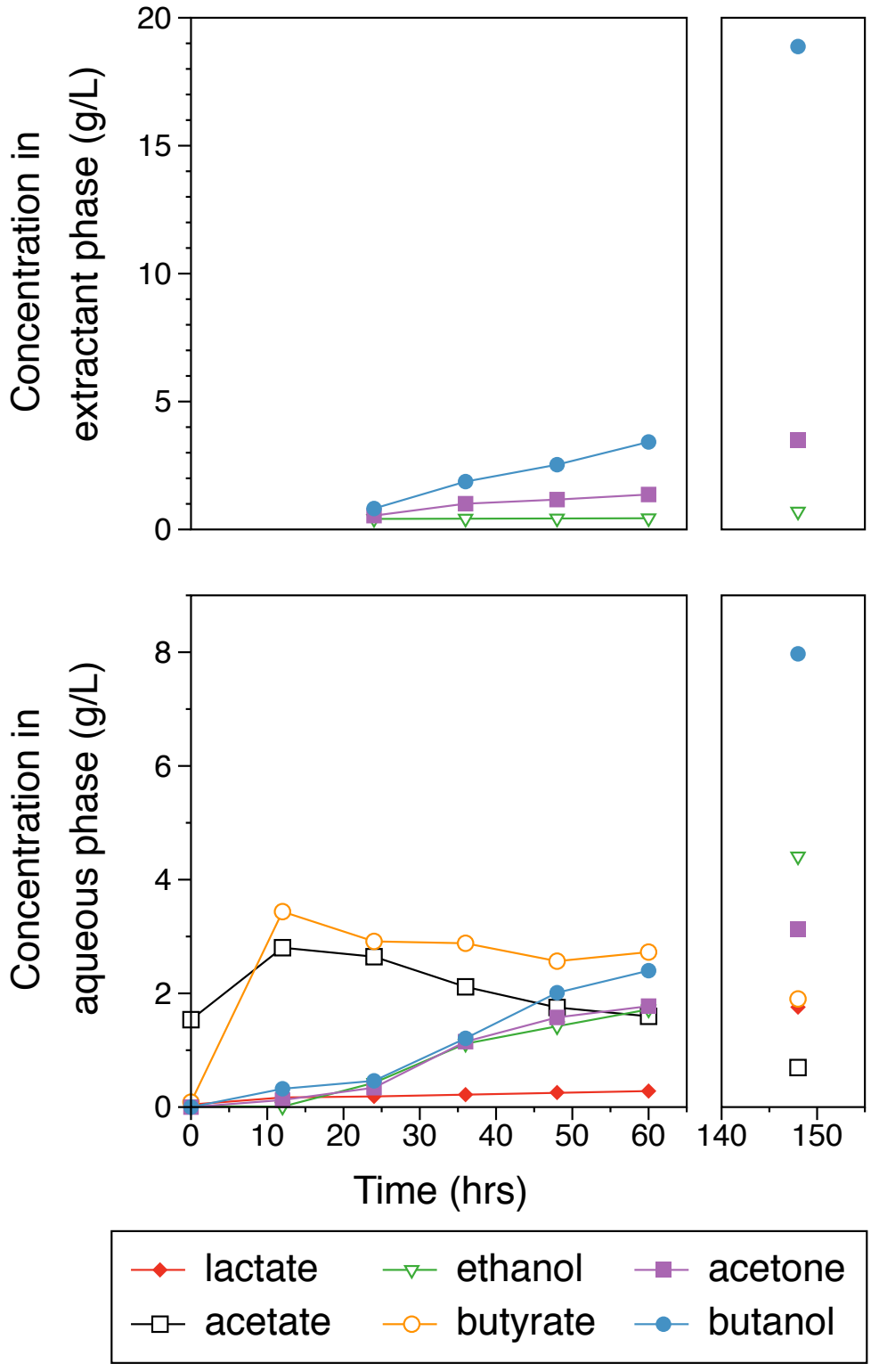


Figure 3-10: Time course of fed-batch extractive fermentation w/ sucrose. Initial sucrose concentration 60 gL^{-1}. Lines drawn to guide the eye.

Table 3-7: Alkylation reaction for ABE produced during extractive fermentation, ABE molar ratios were set based on the fermentation results. All reaction masses were determined by GC-FID. * Mass determined by average FID response factor. [a] Total Mass values based on fermentative production and total recovery of 153.5 mmol Acetone, 333.8 mmol Butanol, and 158.7 mmol Ethanol. Reaction conditions: Acetone (1.6 mmol), Butanol (3.7 mmol), Ethanol (1.7 mmol), 5% Pd/C (0.01 mmol), K_3PO_4 (6.91 mmol), Toluene (2.5 mL), 145°C, 20h.

Alkylated Product C_7+	**Reaction Mass (mg)**	**Total Mass[a] (g)**
4-Heptanone	2.8	0.2
2-Heptanone	7.6	0.7
4-Nonaone	48.5	4.2
2-Methyl-4-nonaone	0.8	0.1
6-undecanone	89.9	7.7
Higher MW products*	22.2	1.9
Alcohols and other products*	61.7	5.3
Overall	233.5	20.0

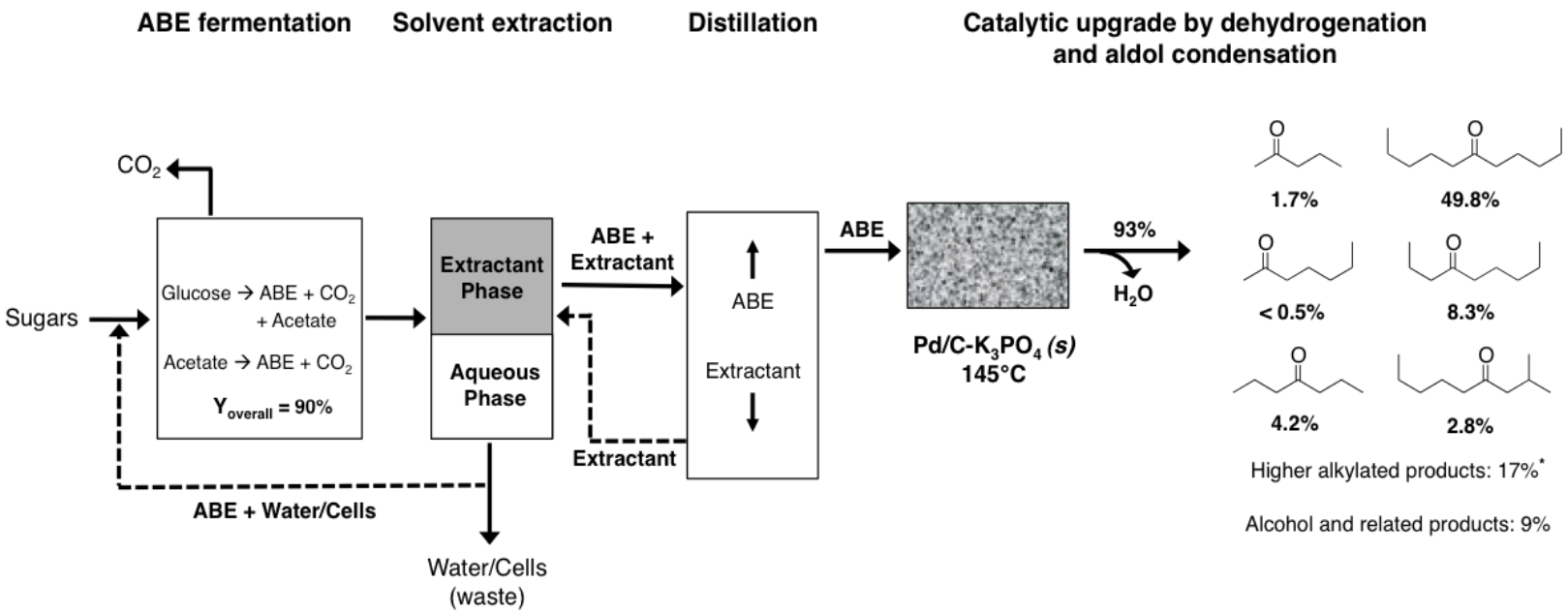


Figure 3-11: Block flow diagram for integration of ABE fermentation with chemical catalysis. Dashed lines represent proposed recycle streams for continuous operation. The results correspond to a 2-L 60-hr fed-batch extractive fermentation with glyceryl tributyrate to produce ABE from glucose. Alkylation reactions were performed on distilled solvents from the extractant phase after drying over molecular sieves; they were performed for 20hr in toluene at 145°C with 1.28 molar equivalents of K_3PO_4. Yields are based on acetone. Astericks, higher alkylated products and alcohol and related product yields were approximated by FID response factor and were assumed to have incorporated a single equivalent of acetone.

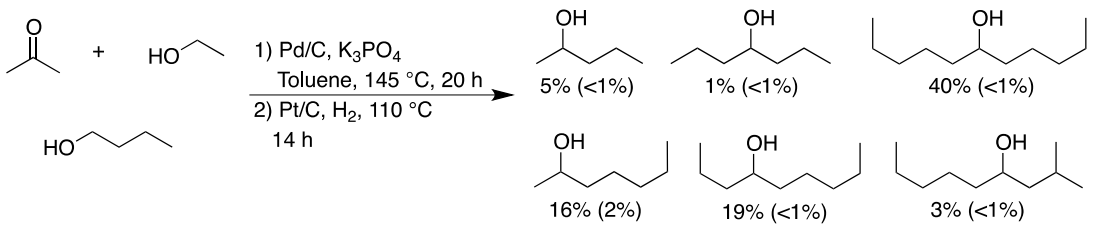


Figure 3-12: One-pot alkylation and hydrogenation reaction

3.4 Conclusions

Using this controlled alkylation of acetone, n-butanol and ethanol, we developed a high-yield method for transforming readily accessible fermentation products from a variety of carbohydrates into precursors for petrol, diesel and jet fuels. By catalytically upgrading low-carbon-number fermentation products we are able to exploit highly efficient metabolic pathways and achieve near theoretical yields (Dugar and Stephanopoulos 2011). Combined with the near theoretical yields attained during the alkylation reaction these higher-molecular-mass fuel precursors can be produced at relatively high titre. The tunability of this reaction to produce predominantly petrol or jet and diesel blend stocks is a significant advantage over other methods, and aligns well with current refining processes. Although further improvements will be required for commercial implementation, the results demonstrate that *in situ* extraction of the products from the ABE fermentation coupled with catalytic conversion of these products can provide hydrocarbon fuel blend stocks at high yields from biomass. The integration of extractive fermentation with chemical catalysis is thus a novel and potentially enabling route for the economical conversion of biomass into liquid transportation fuels.

4. TAILORING THE FERMENTATION PRODUCTS AND CATALYST TOWARDS BIODIESEL PRODUCTION

Work found in this chapter was previous published in the article “Chemocatalytic Upgrading of Tailored Fermentation Products Towards Biodiesel”, Chem. Sus. Chem. 2014. Catalysis experiments were conducted by Dr. Sanil Sreekumar. Characterization of the catalysts via TEM imaging and EXFAS was performed by Dr. Elad Gross.

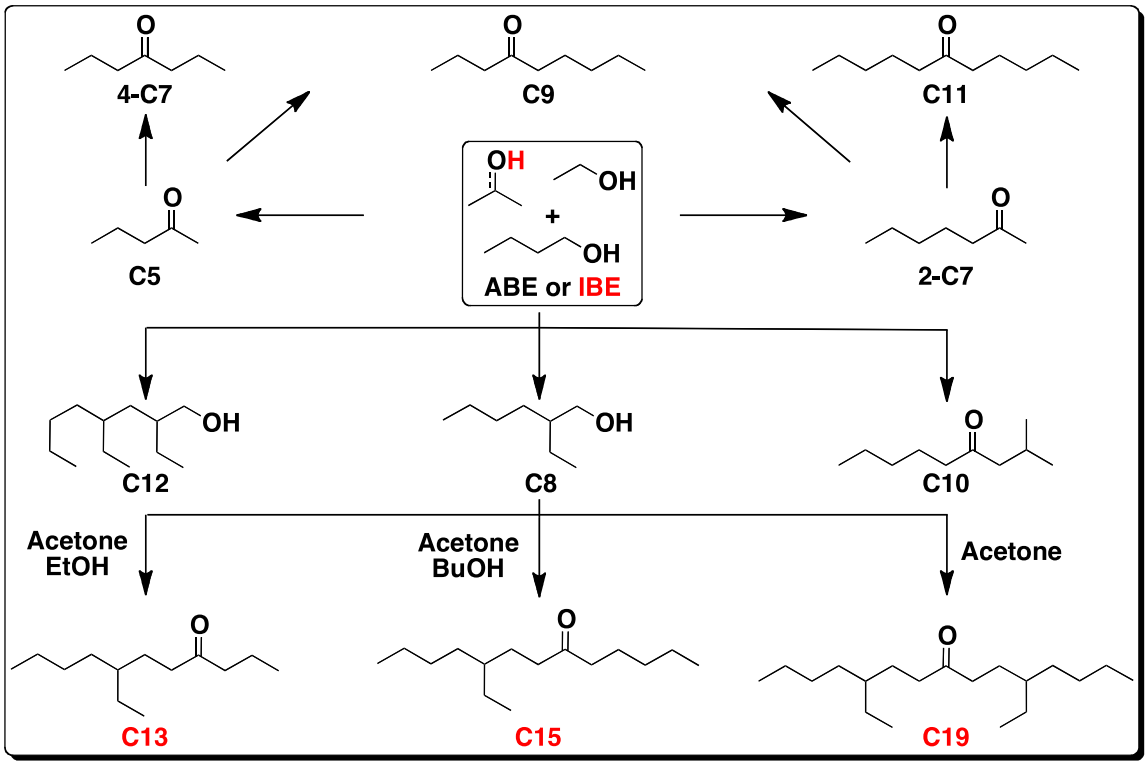
4.1 Introduction

Efficient routes for the synthesis of bio-based liquid fuels have gained considerable attention both commercially and scientifically for the sustainable production of renewable fuels for transportation (Corma et al. 2011; Xing et al. 2010; Kunkes et al. 2008; Gürbüz, Kunkes, and Dumesic 2010; Huber et al. 2005). Nonetheless, the high oxygen content in the biomass impedes its direct conversion to fuel molecules. The processes involved in converting lignocellulosic biomass to higher molecular weight compounds can be broadly classified as (i) thermochemical (e.g., gasification of biomass to produce syngas; aqueous phase reforming of sugars and hydrolysis of biomass to produce sugars)[6] or (ii) biological (e.g., fermentative and non-fermentative pathways to produce small chain alcohols) (Atsumi, Hanai, and Liao 2008; Dekishima et al. 2011; Machado et al. 2012). There are advantages and disadvantages associated with the aforementioned routes. Because sugars obtained from the biomass are highly functionalized molecules, their direct chemical conversion to higher molecular weight compounds represents a challenging task. Although lignocellulosic sugars can be converted by microorganisms such as *E. coli* and *Saccharomyces cerevisiae* to smaller chain alcohols for blending with gasoline (Atsumi, Hanai, and Liao 2008; Dekishima et al. 2011; Machado et al. 2012), production of longer chain alcohols for jet and diesel blendstocks suffers from low titers and yields due to their inherent toxicity to the microbial hosts (Green 2011; Lin and Tanaka 2006).

The initial discovery of a chemical catalysis route to generate aliphatic ketones (C5-C19) that are components of gasoline, jet, and diesel fuel from the acetone-butanol-ethanol (ABE) produced from a variety of sugar feedstocks by a Clostridial fermentation provided a strong foundation for additional scientific discovery and optimization (Anbarasan et al. 2012). In this process, the major pathway involves combining acetone with up to two molecules of alcohol to produce aliphatic ketones up to C11 (Scheme 4-1). A major goal of this integrated biofuels process is to make blendstock that are appropriate for high blend ratios in conventional petroleum fuels. However, C11 and lower carbon compounds only constitute a minor fraction (< 10 wt%) of conventional diesel fuel (see Figure 4-1, from Cheveron diesel fuel technical review 2007).

Hence, it would be highly desirable to develop a process wherein high concentration of C12+ (carbon length > C11) compounds could be formed in a single operation. This can be achieved by increasing the chain length of the alcohols used in the alkylation reaction (Anbarasan et al. 2012; Kwon et al. 2005). Biological production of longer-chained alcohols has been demonstrated (Dekishima et al. 2011) but the efficacy of the pathway and toxicity of the products has limited titers to below 1 g L^{-1}. Additionally, co-production of acetone and these longer-chain alcohols has never been demonstrated. An alternative approach was first described by Carlini et al. 2004 to produce the longer-chain alcohol, 2-ethyl-hexanol, catalytically from 1-butanol via the guerbet reaction. However, when acetone is present in the reaction mixture it competitively inhibits the formation of 2-ethyl-hexanol until all of the acetone has been alkylated with the 1-butanol. Changing the fermentation mixture of acetone, butanol, and ethanol too a mixture of alcohols (isopropanol, butanol, and ethanol) would reduce this competitive inhibition.

Herein, we demonstrate that a significant increase in the production of diesel range components (C12+) can be achieved by integrating improvements in the chemical catalysis with a tailored biomass-derived fermentation mixture of isopropanol-butanol-ethanol (IBE).



Scheme 4-1: Synthetic Strategy to Upgrade ABE/IBE to Biofuels. C5-C11: Gasoline and Jet, C13-C19: Diesel

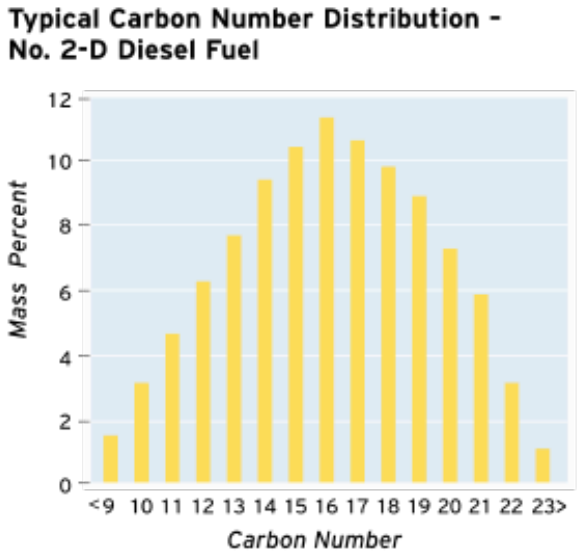


Figure 4-1: Carbon number distribution by mass percent of No. 2-D Diesel fuel. Data presented in Cheveron "Diesel Fuels Technical Reivew", 2007.

4.2 Methods and Materials:

4.2.1 Reagents

All the metal sources were purchased from either Sigma Aldrich or Strem Chemicals and used as received. Chemicals were obtained from Sigma Aldrich and used without further purification.

4.2.2 Catalyst Preparation

0.5 wt% Palladium-Hydrotalcite

A 250 mL round bottom flask was charged with commercially available hydrotalcite (5 g) palladium chloride (0.96 mmol, 0.17 g) and potassium chloride (0.25 g). Water (130 mL) was added and the resulting suspension was stirred at room temperature for 1 hour. The reaction mixture was then lowered in a preheated oil bath and was stirred at 100 °C under hydrogen atmosphere overnight. The reaction mixture was filtered and the solid was washed with copious amounts of water. The grey solid was dried under *vacuo* at 120 °C for 4 hours.

3.2 wt% Copper-Hydrotalcite

A 250 mL round bottom flask was charged with commercially available hydrotalcite (5 g) and copper acetate (11 mmol, 2 g). Water (130 mL) was added and the resulting suspension was stirred at 60 °C for 14 hours. The reaction mixture was filtered and the solid was washed with copious amounts of water. The solid was dried under *vacuo* at 120 °C for 4 hours. The copper loading on hydrotalcite was determined using ICP analysis.

4.2.3 Reaction analysis

All the reactions were analyzed by gas chromatography using dodecane as internal standard. Gas chromatography analysis was performed on a Varian CP-3800 instrument with a FID detector and VF-5 MS column (5% phenyl and 95% methylpolysiloxane) using helium as the carrier gas.

4.2.4 Representative procedure for ABE Alkylation reaction

In a 12 mL Q-tube containing a stir bar, 3.2 wt% Cu-HT (0.8 mol%) or 0.5 wt% Pd-HT (0.3 mol%) was charged. To the reaction mixture, acetone (0.058 g, 1 mmol), ethanol (0.046 g, 1 mmol) and butanol (2 mL were sequentially added. The Q-tube was sealed and the reaction mixture was stirred for 24 hours at 240 °C in a pre-heated metal block. The reaction mixture was cooled to room temperature and dodecane (internal standard) was added. The reaction mixture was diluted with tetrahydrofuran and the GC analysis of the reaction mixture was carried out.

4.2.5 Water Inhibitory Experiments

Water inhibitory study was carried out by adding different weight percentages of water with respect to the total weight of ABE employed in the reaction.

4.2.6 IBE Reaction Progress Study

The time course study was performed in a 4560 Mini Parr reactor. Samples at different time intervals were dispensed using an attached sample collection vessel and was analyzed using gas chromatography. Acetone and isopropanol concentration was determined using calibrated internal standard (dodecane) in the GC-MS.

4.2.7 Growth:

Clostridium acetobutylicum was routinely stored and grown out of anaerobic -80C glycerol stocks. Cultures were grown in CGM: glucose (6%), yeast extract (5.0 gl^{-1}), ammonium acetate (2.0 gl^{-1}), NaCl (1.0 gl^{-1}), K_2HPO_4 (0.75 gl^{-1}), KH_2PO_4

(0.75 gl^{-1}), cysteine-HCl (0.5 gl^{-1}), $MgSO_4 \cdot 7H_2O$ (0.4 gl^{-1}), $FeSO_4 \cdot 7H_2O$ (0.04 gl^{-1}), $MnSO_4 \cdot H_2O$ (0.02 gl^{-1}). Recombinant strains of *C. acetobutylicum* were supplemented with 80 mgl^{-1} erythromycin. Recombinant *E. coli* strains were grown in Luria broth medium supplemented with the appropriate antibioitics carbenicillin (100 mgl^{-1}) and chloramphenicol (35 mgl^{-1}).

4.2.8 DNA manipulation:

All strains and plasmids used in this study are listed in Table 4-1. *E. coli* Top 10 was used to propagate all plasmids before expression in *C. acetobutylicum* ATCC824. Methylation of all plasmids prior to transformation in ATCC824 was carried out with the pAN1 plasmid as previously described (L. D. Mermelstein and Papoutsakis 1993).

Table 4-1: Strains and plasmids used in this study

Name	Relevant features	Reference/Source
Strains		
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*q*ZΔM15* Tn*10* (Tetr)]	Stratagene
C. acetobutylicum	Wild-type	ATCC
Plasmids		
pIMP1	Ap^r MLS^r repL, ColE1 origin	Papoutsakis[1]
pAN1	Cm^r, φ3T I gene, p15A origin	Papoutsakis[1]
pACE	pIMP1 derivative with *adc-ctfAB* (*adc* promoter) insertion	This study
pSADH	*sadh* (*adc* promoter and terminator)	GeneArt
pSACE	pACE derivative with *sadh* (*adc* promoter and terminator) insertion	This study

The pIMP1 Clostridium/E. coli shuttle vector, generously provided by the Papoutsakis group, was used to construct all of the strains for this study. The construction of a synthetic acetone operon containing *C. acetobutylicum adc* (acetoacetate decarboxylase)*, ctfa* (acetyl-CoA transferase)*, ctfb* (butyryl-CoA transferase) genes was achieved as previously described (L. D. Mermelstein et al. 1993). DNA fragments to construct this operon were PCR amplified from genomic DNA. Primers used and the restriction sites inserted in the operon can be found in Table 4-2. This operon was expressed under control of the native *C. acetobutylicum* adc promoter.

The sequence for the *sadh* gene was identified from *C. beijerinkcii* strain B593 and synthesized by GeneArt® under the control of the *C. acetobutylicum* ATCC824 *adc* promoter and terminator. The synthesized SADH construct was cloned upstream of the synthetic acetone operon by restriction digest with *SfoI/EcoRI* followed by ligation to produce pSACE. The final construct sequence was verified before transformation into ATCC824.

Table 4-2: DNA primers used in this study

Name (Restriction site)	**Sequence**	**Target gene(s)**
ctfab fwd (BamHI)	AAAAAAGGATCCTTAAAAGGAGGGATTAA AATGAACTCTAAAATAATTAGATTT	*ctfa/b*
ace rev (SalI)	AAAAAGTCGACCTAAACAGCCATGGGTC TAAGTTCATTGGATATG	*ctfa/b*
adc rev (BamHI)	AAAAAAAGGATCCTATTTACTTAAGATAA TCATATATAACTTCAGCTCTAGGCAATAT	*Adc*
ace fwd (EcoRI)	AAAAAAGAATTCATAAAAACACCTCCACA TAAGTTTATATAAATC	*Adc*

4.2.9 DNA Transformation:

Transformation of plasmids in chemically-competent *E. coli* Top10 was performed via heat shock (42 °C) followed by preculturing in SOC media (20 g L^{-1} tyrptone, 5 g L^{-1} yeast extract, 0.5 g L^{-1} NaCl, 0.186 g L^{-1} KCl, 1 g L^{-1} $MgCl_2$, 20 mM glucose) at 37 °C for 1 hour before plating on selectable LB agar plates (100

μg mL^{-1} carbacillin and/or 30 μg mL^{-1} chloramphenicol). Transformation of *C. acetobutylicum* was performed by electroporation with a BioRad gene pulser in an anaerobic glove bag. Prior to transformation, an -80 °C glycerol stock of *C. acetobutylicum* was grown out overnight in CGM at 37 °C to and OD_{600} of 1.0 – 2.0. 5 mL of this culture was inoculated into 100 mL of 2xYTG media (16 g L^{-1} tryptone, 10 g L^{-1} yeast extract, 5 g L^{-1} glucose, 4 g L^{-1} NaCl, pH adjusted to 5.2) and grown in an anaerobic incubator at 37 °C until the OD_{600} reached 1.0. The culture was then pelleted via centrifugation at 4000x g for 10 minutes. Cells were immediately transferred back into the anaerobic chamber and the media decanted off. The cells were then resuspended in 30 mL of ice-cold anaerobic electroporation buffer (5 mM NaH_2PO_4, 270 mM sucrose, pH adjusted to 7.4). Resuspended cells were pelleted at 4000x g for 10 minutes, transferred back to the anaerobic chamber and liquid decanted. The cells were again resuspended in 30 mL of electroporation buffer, followed by centrifugation and removal of the liquid via decanting. Finally the cells were resuspended with 2 mL of electroporation buffer and kept on ice prior to electroporation. 500 uL of electrocompetent cells were loaded into a 4mm gap cuvette with 2 μg of methylated plasmid DNA. Electroporation parameters were as follows: voltage = 2.0 kV, capacitance = 25 μF, resistance = ∞ Ω, electroporation cuvette gap = 4mm. 1 mL of warm 2xYTG media was added to the cells immediately following electroporation. The resuspended cells were precultured in 10 mL of 2xYTG at 37 °C for 4 hours in an anaerobic incubator. The culture was then pelleted at 4000x g for 10 minutes. The cell pellet was resuspended in 1 mL of fresh 2xYTG and 200 μL alloquots spread onto anaerobic 2xYTG agar plates (15 g L^{-1} agar, pH 5.8) supplemented with 40 μg mL^{-1} erythromycin. Plates were anaerobically incubated for 48 hours at 37 °C. Several colonies were picked and grown in liquid CGM media supplemented with 100 μg mL^{-1} erythromycin. Colony PCR was performed on exponentially growing cells to confirm successful transformation of plasmid.

4.2.10 Batch Fermentation:

All fermentations of *C. acetobutylicum* strains were conducted in 3-L bioreactors (Bioengineering AG, Switzerland) with a 2L working volume. Cultures were grown at 37 °C anaerobically by sparging 100 mL/min of N_2 gas until solvent production was initiated. The culture pH was adjusted to 6.0 prior to inoculation. Fermentors were innoculated with 100 mL of preculture into 1.5L of CGM supplemented with 40 ug/mL clathromycin. The bioreactor pH was controlled with 5M KOH at pH ≥ 5.0. Sugars and major metabolites (glucose, lactate, acetate, butyrate, acetoin, ethanol, acetone, isopropanol and 1-butanol) were measured in the aqueous phase using an Agilent (Santa Clara, CA) HPLC system equipped with refractive index and UV/Vis detectors. A Bio-Rad (Hercules, CA) Aminex HPX-87H ion exchange column with a Cation H guard

column at 60°C was used with a mobile phase of 0.05 mM sulfuric acid flowing at 0.7 mL min^{-1}.

4.2.11 Liquid-Liquid Extraction of IBE:

A synthetic mixture was prepared with ethanol (5gl^{-1}), isopropanol (5gl^{-1}), and 1-butanol (10gl^{-1}). 5-mL of IBE media was combined with 5mL of glyceryl tributyrate or oleyl alcohol and mixed for 5 minutes by inversion. The mixtures were then spun down at 5300 RPMs for 5 minutes and the extractant phase removed for GC analysis. Distribution coefficients were calculated as previous described in Section 3.2.4, eq. 3-1.

ABE extraction experiments were run in triplicate.

4.2.12 Extractive Fermentation:

Extractive fermentations were operated in fed-batch mode with intermittent addition of a concentrated media solution containing 500 gl^{-1} glucose and 50 gl^{-1} yeast extract. 500 mL of N_2 degassed oleyl alcohol was added to the 1L fermentation broth 16 hours after inoculation. Isopropanol, 1-butanol, and ethanol concentrations were measured in the extractant phase by GC/FID.

4.3 Results and Discussion

4.3.1 Catalyst screening and optimization

In order to access greater quantities of diesel range hydrocarbons, we envisioned an alternative pathway in which the alcohols would undergo condensation (Guerbet reaction) prior to combining with acetone. However, this strategy was severely impeded by the preference for acetone alkylation over the Guerbet reaction pathway with the previously reported Pd/C-K_3PO_4 (Anbarasan et al. 2012). This limitation prompted us to investigate the ABE condensation reaction over hydrotalcite-supported metal catalysts, whose recyclability has been demonstrated in oxidation and condensation reactions (Debecker, Gaigneaux, and Busca 2009; Likhar et al. 2009; Mitsudome et al. 2008; Motokura et al. 2004). Table 4-3 summarizes the optimization studies on the ABE alkylation reaction using transition metal impregnated on the hydrotalcite support. Treatment of ABE mixture (2.3 : 3.7 : 1) in toluene with catalytic Ru-HT in the absence of external base at 200°C provided the desired aliphatic ketones in 20% yield (entry 1). We envisioned that replacing ruthenium with palladium(0) would increase the efficiency of the ABE alkylation reaction based on our previous studies (Anbarasan et al. 2012). Although switching to Pd-HT resulted in modest improvements (entries 1 *vs* 2), replacing toluene with butanol as the solvent increased the overall yield of aliphatic ketones to 58% (entry 3). However,

complete conversion of acetone was observed at 240°C, which also mirrored the excellent yield for aliphatic ketones (entry 4). It is highly desirable to replace palladium with an inexpensive and abundant transition metal from an economic standpoint. To this end, substituting palladium with Cu(II)-HT in the ABE alkylation reaction provided a mixture of aliphatic ketones along with the corresponding mixture of reduced alcohols in 92% overall yield (entry 5). The alkylation reaction also produced butyl butyrate (4%) as a by-product. Hydrogen for the reduction of aliphatic ketones is presumably formed by the dehydrogenation of hemiacetal generated from the *in situ* formed butyraldehyde and excess butanol.

4.3.2 Catalyst Recycling Experiments

The conversion of monoalkylated ketones (**C5** and **C7**) to the dialkylated ketones and the recyclability of catalyst in the alkylation reaction necessitate a catalyst that is fairly tolerant to the water formed in the reaction. A water inhibition study on Cu-HT revealed that the alkylation reaction could tolerate up to 0.5 wt% water with respect to the total weight of ABE, which was significantly higher than the previously employed Pd/C-K_3PO_4 mixture (Anbarasan et al. 2012). Further increasing the water content slowed the ABE condensation reaction and resulted in a decreased yield of dialkylated ketones and a lower overall yield (Figure 4-2). Additionally, the recyclability of both Cu-HT and Pd-HT were investigated by adding fresh ABE to the dried catalyst obtained by centrifugation and phase separation of the supernatant. In general, both Pd-HT and Cu-HT were recycled three times without significant loss of catalytic activity (see Table 4-4 and Table 4-5).

4.3.3 Catalyst Characterization

Catalyst characterization was carried in order to evaluate the activity of Cu-HT in the ABE alkylation reaction. The Cu-HT was prepared by adding hydrotalcite ($Mg_6Al_2(OH)_{16}CO_3{\cdot}4H_2O$) to an aqueous solution of copper(II) acetate at 60 °C. High-resolution Transmission Electron Microscope (HR-TEM) images of Cu-HT catalyst indicated the formation of Cu clusters 2±1 nm in diameter loaded on 150±50 nm HT support (Figure 4-3). The clusters were analyzed by extended x-ray absorption fine structure (EXAFS) and x-ray photoelectron spectroscopy (XPS). The x-ray absorption measurements indicated that CuO is the main species in the heterogeneous Cu-HT catalyst (Figure 4-3). *In situ* EXAFS measurements showed that CuO was reduced to Cu(0) at 250 °C under continuous flow of the ABE mixture, prior to the formation of products (Figure 4-4). These results indicate that the metallic copper nanoparticles are the active catalytic species involved in the dehydrogenation of alcohols to produce aldehydes, which undergoes aldol condensation with acetone.

Table 4-3: Optimization of ABE Reaction Using Metal/Hydrotalcite Catalyst

Entry[a]	Catalyst (mol%)	Temp (°C)	C5 (%)	4-C7 (%)	2-C7 (%)	C9 (%)	C10 (%)	C11 (%)	Alcohols[c] (%)	Yield (%)
1	Ru-HT (0.30)[b]	200	0.4	2.2	12.9	0.6	0.2	3.4	–	20
2	Pd-HT (0.15)[b]	“	0.1	1.3	17.6	0.6	0	11.9	–	32
3	Pd-HT (0.15)	“	0	0	39.6	1.8	0	16.8	–	58
4	Pd-HT (0.30)	240	12.1	4.4	38.2	7.3	1.6	29.5	2.0	95
5	Cu-HT (0.80)	240	1.5	4.4	31.4	2.4	0.1	28.4	23.8	92

Reaction Conditions: [a]Acetone (2.3 mmol), Butanol (2 mL), Ethanol (1 mmol), 24 hours, catalyst loading is represented as the molar percentages with respect to total alcohol loading (ethanol+1-butanol). Yield based on acetone determined by calibrated internal standard (*n*-dodecane). [b]Acetone (2.3 mmol), Butanol (3.7 mmol), Ethanol (1 mmol), Toluene (1 mL). [c]Alcohols yield (%) for Entry 4: **2-C7-OH**: 2%, Entry 5: **2-C7-OH:** 13.4, **4-C7-OH:** 9.7, **C9-OH:** 0.4 and **C11-OH:** 0.3.

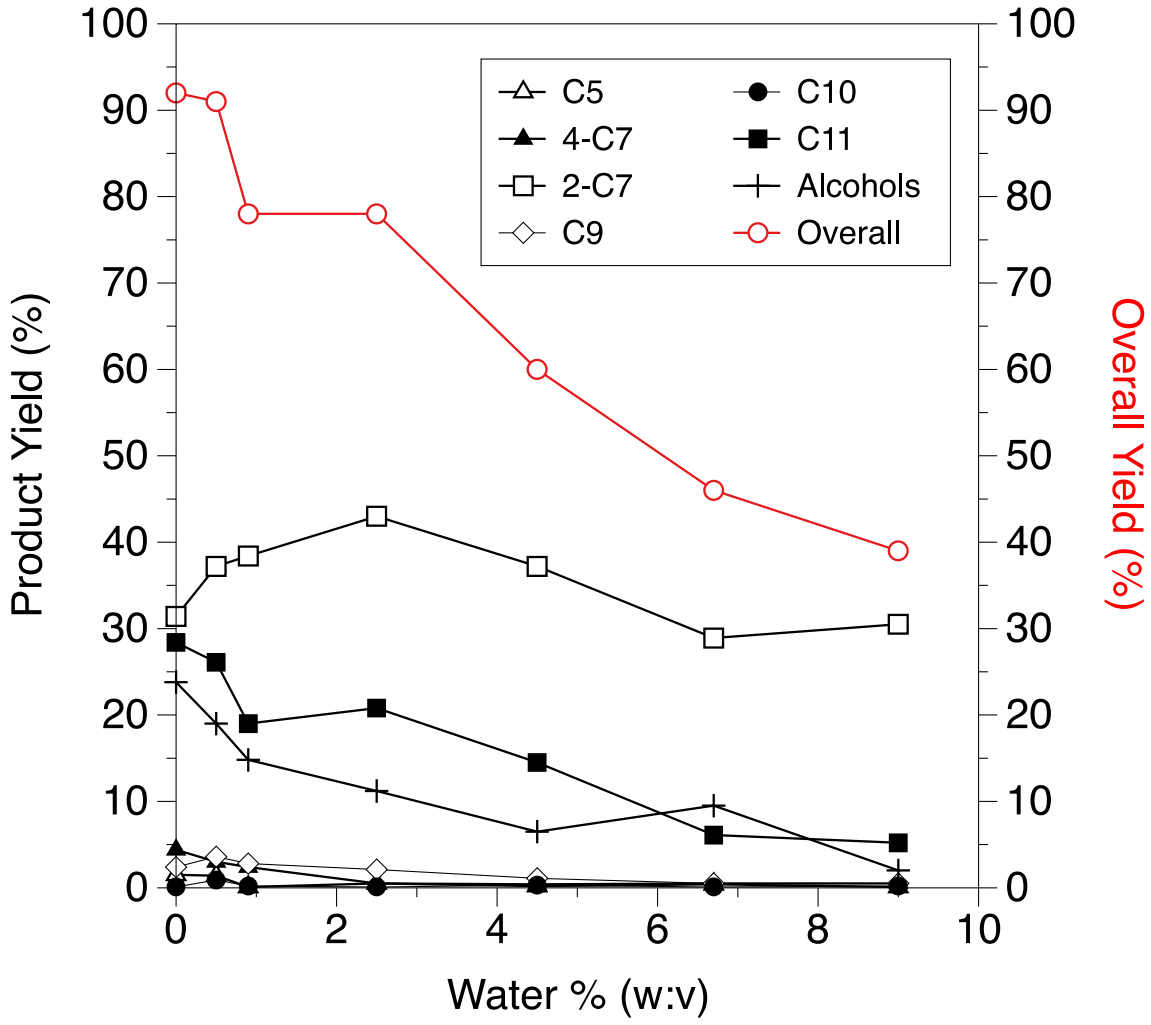


Figure 4-2: Effect of water addition on alkylation reaction. Yield based on Acetone. Reaction conditions: Acetone (2.3 mmol), Ethanol, (1 mmol), Butanol (2 mL), 3.2 wt% Cu-HT (0.8 mol%), H_2O (wt%), 240 °C, 24 h.

Table 4-4: Recycling experiments using 0.5 wt% Pd-HT (0.15 mol%). Reaction conditions: Acetone (2.3 mmol), Ethanol, (1 mmol), Butanol (2 mL), 240 °C, 24 h. Yields based on acetone.

Entry	C5 (%)	4-C7 (%)	2-C7 (%)	C9 (%)	C10 (%)	C11 (%)	Yield (%)
Cycle 1	0.1	36.4	3.2	2.5	0.1	25.3	68
Cycle 2	0.2	40.6	2.6	1.4	0.1	15	60
Cycle 3	0.1	18.2	8.8	2.9	0	36.8	67

Table 4-5: Recycling experiments using 3.2 wt% Cu-HT (0.8 mol%). Reaction conditions: Acetone (2.3 mmol), Ethanol, (1 mmol), Butanol (2 mL), 240 °C, 24 h. Yields based on acetone.

Entry	C5 (%)	4-C7 (%)	2-C7 (%)	C9 (%)	C10 (%)	C11 (%)	Alcohols (%)	Yield (%)
Cycle 1	1.5	4.4	31.4	2.4	0.1	28.4	23.8	92
Cycle 2	0.1	5.8	31.9	3.1	0.5	30.0	14.4	86
Cycle 3	0.2	6.3	33.6	2.9	0.5	28.2	12.7	84

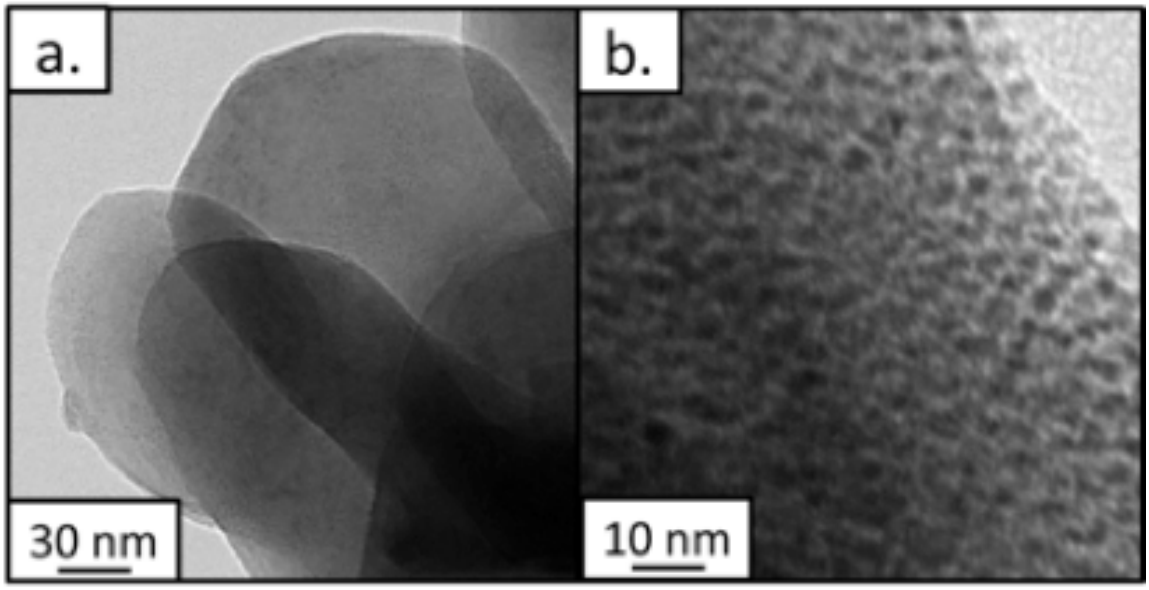


Figure 4-3: (a) HR-TEM images of 3.2 wt% Cu-HT. (b) Higher magnification image of Cu-HT catalyst reveals the size and distribution of the Cu nanoparticles.

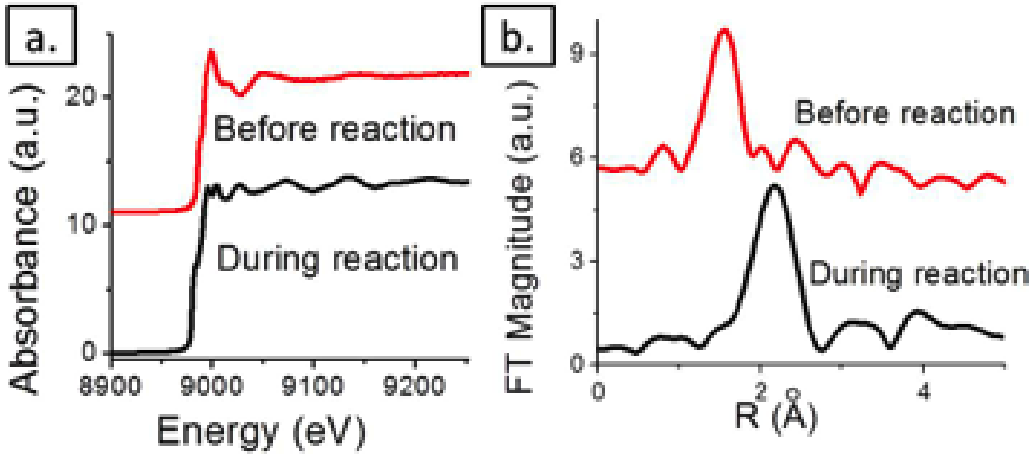


Figure 4-4: (a) EXAFS (b) and Fourier Transform of EXAFS data of Cu-HT catalyst before (red) and during (black) the catalytic reaction. The shift in the peak position of the FT-EXAFS spectra indicates the reduction of Cu oxide to Cu under reaction conditions.

4.3.4 Alkylation of IBE vs ABE

While we had been unable to achieve a significant enhancement in the production of **C12+** compounds with catalysts tuning alone, the improved efficiency of the catalytic process allowed us to consider the possibility of *in situ* formation of acetone by dehydrogenation of isopropanol. We envisioned that the slow production of acetone would potentially permit an increase in the rate of dimerization of butanol to 2-ethyl hexanal (**C8**) and effectively increase the selectivity toward diesel range components in the product mixture (Figure 4-5). As predicted, under the optimal condition for the IBE alkylation reaction, **C5**-**C11** aliphatic ketones were produced in 61% overall yield along with 25 wt% of **C12+** compounds (mainly **C13** and **C15**) in the product mixture, a two-fold increase compared to the ABE reaction under identical conditions (Table 4-6, entries 1 *vs* 2). Additionally, the time course study for the IBE condensation reaction showed that the concentration of acetone was lower than isopropanol during the entire course of the reaction (see Figure 4-6 and Table 4-7). It is worthy to note that the decrease in the rate of aldol reaction between *in situ* formed acetone and linear aldehydes led to a greater selectivity toward **C12+** compounds over minor constituents of diesel fuel (**C9-C11**).

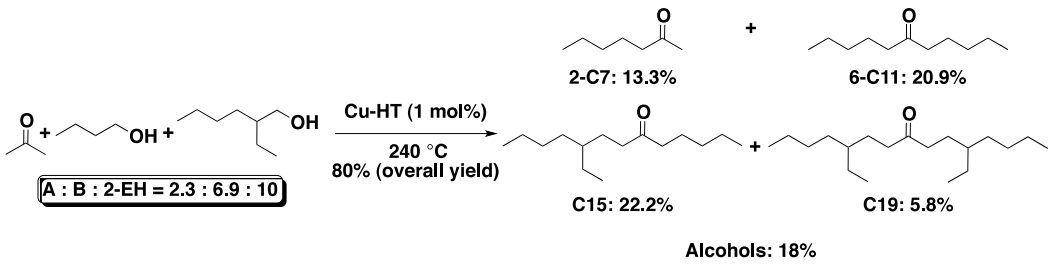
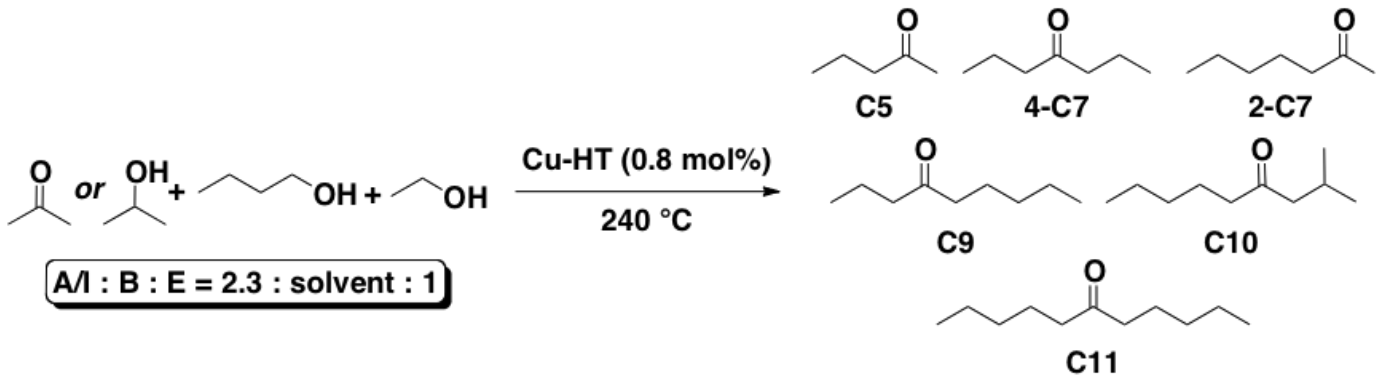


Figure 4-5: In a 12 mL Q-tube containing a stir bar, 3.2 wt% Cu-HT (0.350 g) was charged. To the reaction mixture, acetone (0.134 g, 2.3 mmol), 2-ethyl-1-hexanol (1.3 g, 10 mmol), butanol (0.51 g, 6.9 mmol) were sequentially added. The Q-tube was sealed and the reaction mixture was stirred for 24 hours at 240 °C in a pre-heated metal block. The reaction mixture was cooled to room temperature and dodecane (internal standard) was added. The reaction mixture was diluted with tetrahydrofuran and the GC analysis of the reaction mixture was carried out. Yields based on acetone.

Table 4-6: ABE Versus IBE Alkylation (See Scheme below).



Entry	Reaction	C5 (%)	4-C7 (%)	2-C7 (%)	C9 (%)	C10 (%)	C11 (%)	Alcohols[b] (%)	Yield (%)	C12+[c] (wt%)
1	ABE	1.9	1.0	23.5	4.2	0.4	35.5	9.1	73	14
2	IBE	0	1.9	24.0	1.9	0	21.8	11.1	61	25

[a]Reaction Conditions for ABE/IBE: Acetone/isopropanol (2.3 mmol), Butanol (1 mL), Ethanol (1 mmol), 0.8 mol% Cu-HT, 240 °C, 14 hours. Yield based on acetone determined by calibrated internal standard (*n*-dodecane). [b]Alcohols yield (%) for Entry 1: **2-C7-OH:** 3.5, **4-C7-OH:** 4.0, and **C11-OH:** 1.6. Entry 2: **2-C7-OH:** 2.9, **4-C7-OH:** 6.9, and **C11-OH:** 1.3. [c]Predominatly isomers of **C13** and **C15**

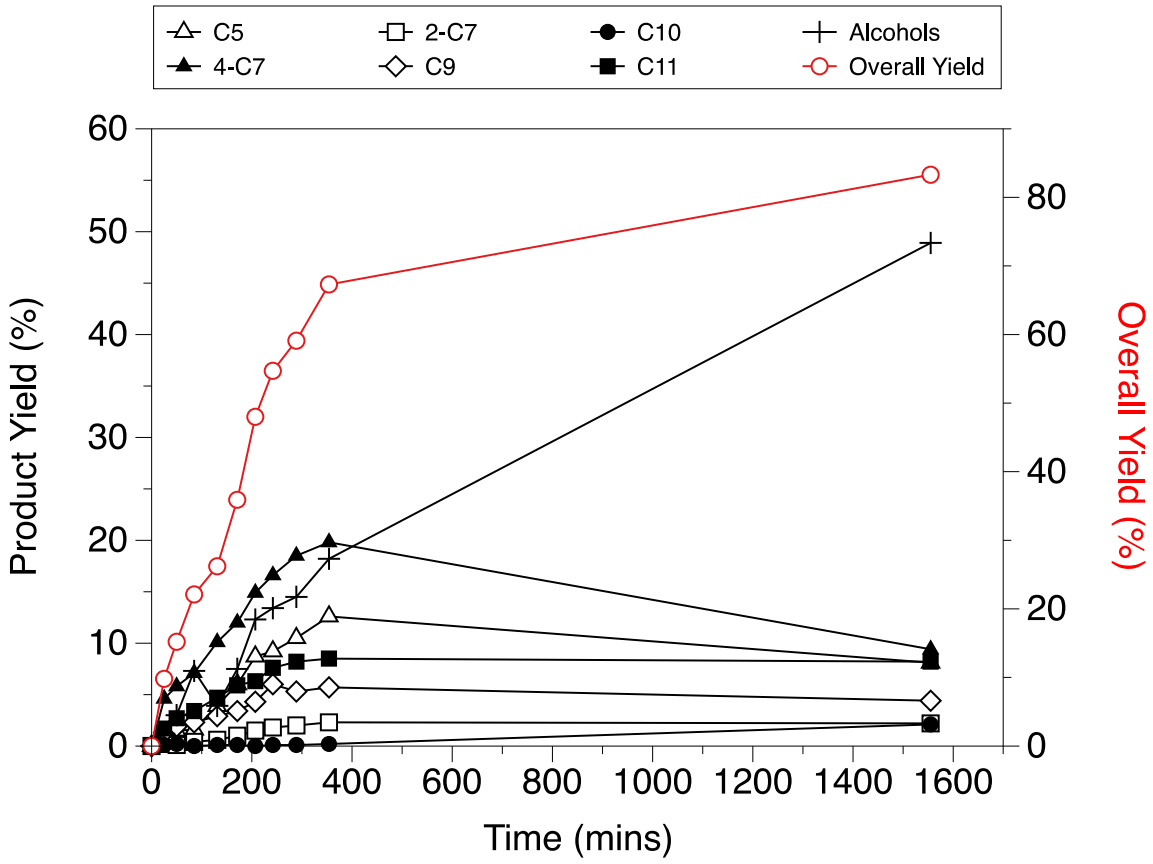


Figure 4-6: Time course study for the IBE reaction. Reaction conditions: Isopropanol (11.5 mmol), Ethanol, (5 mmol), Butanol (10 mL), 3.2 wt% Cu-HT (0.8 mol%), 275 °C, 24 h. Yield based on Isopropanol.

Table 4-7: Ratio of Isopropanol (IPA) and acetone during the IBE reaction progress

Entry	Time (mins)	IPA : Acetone
1	25	4.0 : 1
2	50	3.0 : 1
3	131	2.4 : 1
4	171	2.4 : 1
5	207	1.9 : 1
6	1555	1.6 : 1

4.3.5 Engineering IBE production in *C. acetobutylicum*

Given the favorable outcome in the chemocatalytic process of IBE, we explored the possibility of establishing a sustainable route to produce solvents for the IBE alkylation reaction from lignocellulosic sugars *via* Clostridial fermentation. It was recently reported that *C. acetobutylicum* would produce IBE by expression of the secondary alcohol dehydrogenase (SADH) from *Clostridium beijerinckii* strain B593 (J. Lee et al. 2012). To ensure sufficient metabolic flux through the isopropanol pathway a synthetic acetone pathway operon was inserted into the shuttle vector pIMP1 under control of the *adc* promoter (pACE) and expressed in *C. acetobutylicum*. L. D. Mermelstein et al. 1993 demonstrated a set-point pH optimum of ≥ 5.5, for both acetone and total solvent production, compared to set-point pH values of ≥ 4.5 and ≥ 6.5. However, in Section 2.3.1 it was demonstrated that changing the set-point pH by only 0.2 had an effect on total solvent production. To further resolve the pH optimum of the pACE strain Figures 4-7a,b show the effects of varying the bioreactor pH control between 5.5 and 5.0. Higher solvent titers are achieved when the set-point pH is raised from 5.0 to 5.5, 18.6 and 19.9 g L^{-1} respectively. However this minimal increase in solvent titer comes at the expense of significantly increased acid production and therefore reduced solvent yield. For these reasons controlling the fermentation at pH ≥ 5.0 was deemed superior. Fermenting the pACE strain at pH ≥ 5.0 increased acetone production only slightly to 5.2 g L^{-1}, but had a significantly effect on acid titers throughout the fermentation. Titers for both acetic and butyric acid remained below 2 g L^{-1} over the course of the 74-hour fermentation. This is contrast with typical wild-type *C. acetobutylicum* fermentation that undergoes a bi-phasic metabolism whereby acetate and butyrate concentrations initially reach titers of 4-6 g L^{-1} during the first 12 hours of the fermentation and then reduce until sugar catabolism ceases.

Construction of an isopropanol pathway was achieved by combining an artificially synthesized SADH operon controlled under the acetodecarboxylase (*adc*) promoter and terminator upstream of the modified acetone operon (pACE) resulting in the pSACE plasmid. A pH optimization study with the pSACE strain, set-point values of 4.8, 5.0, and 5.5 confirmed a set-point pH of 5.0 was ideal for IBE production and yield, see Figure 4-8. A 1.5-L batch fermentation of the SACE strain produced 21.5 g/L of alcohols (6.2 g/L isopropanol, 3.8 g/L ethanol, and 11.5 g/L butanol) from 54 g/L of glucose in 35 hours, see Figure 4-9.

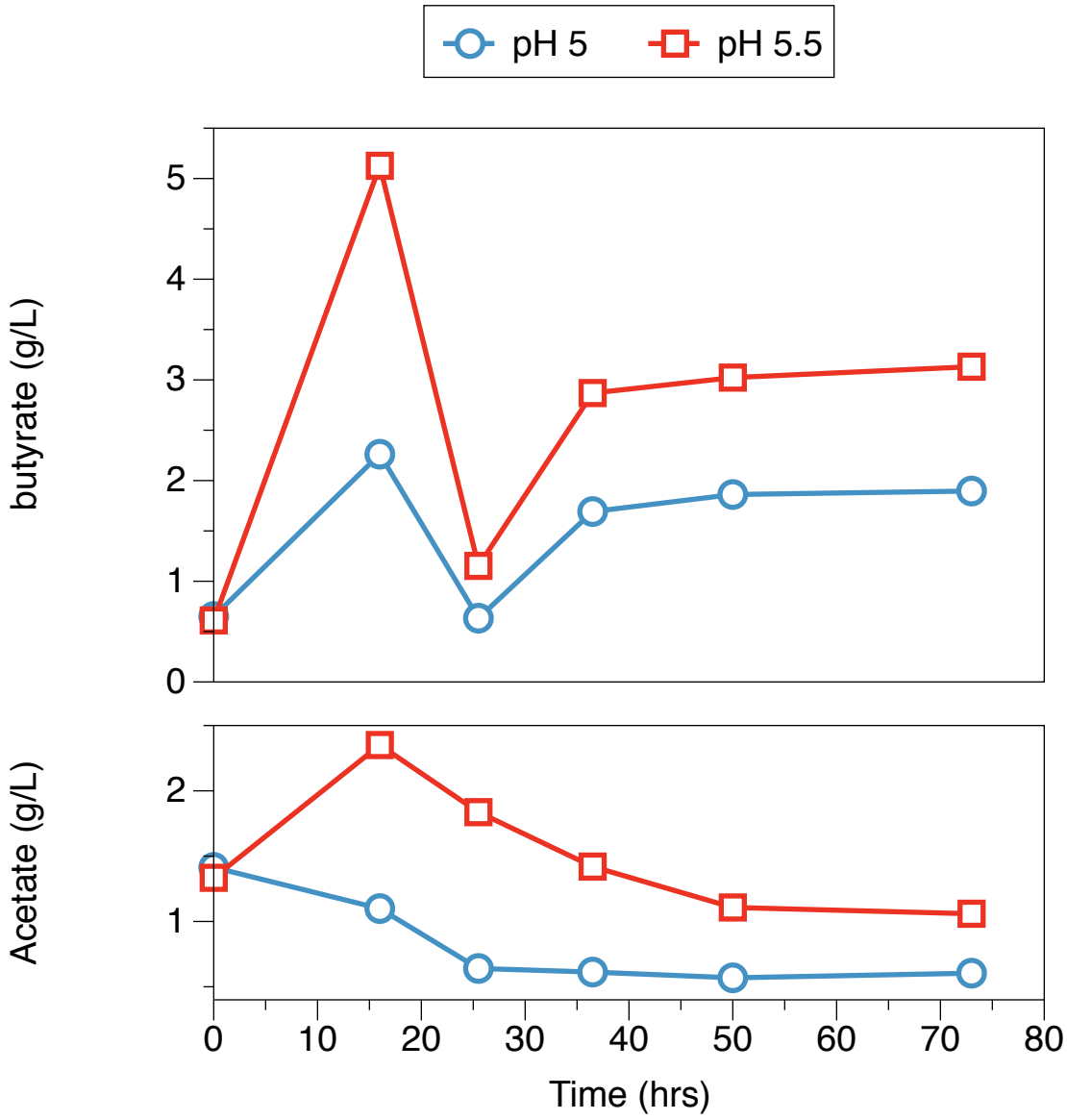


Figure 4-7a: Effect of set-point pH on acid production and reassimilation in the pACE *C. acetobutylicum* strain.

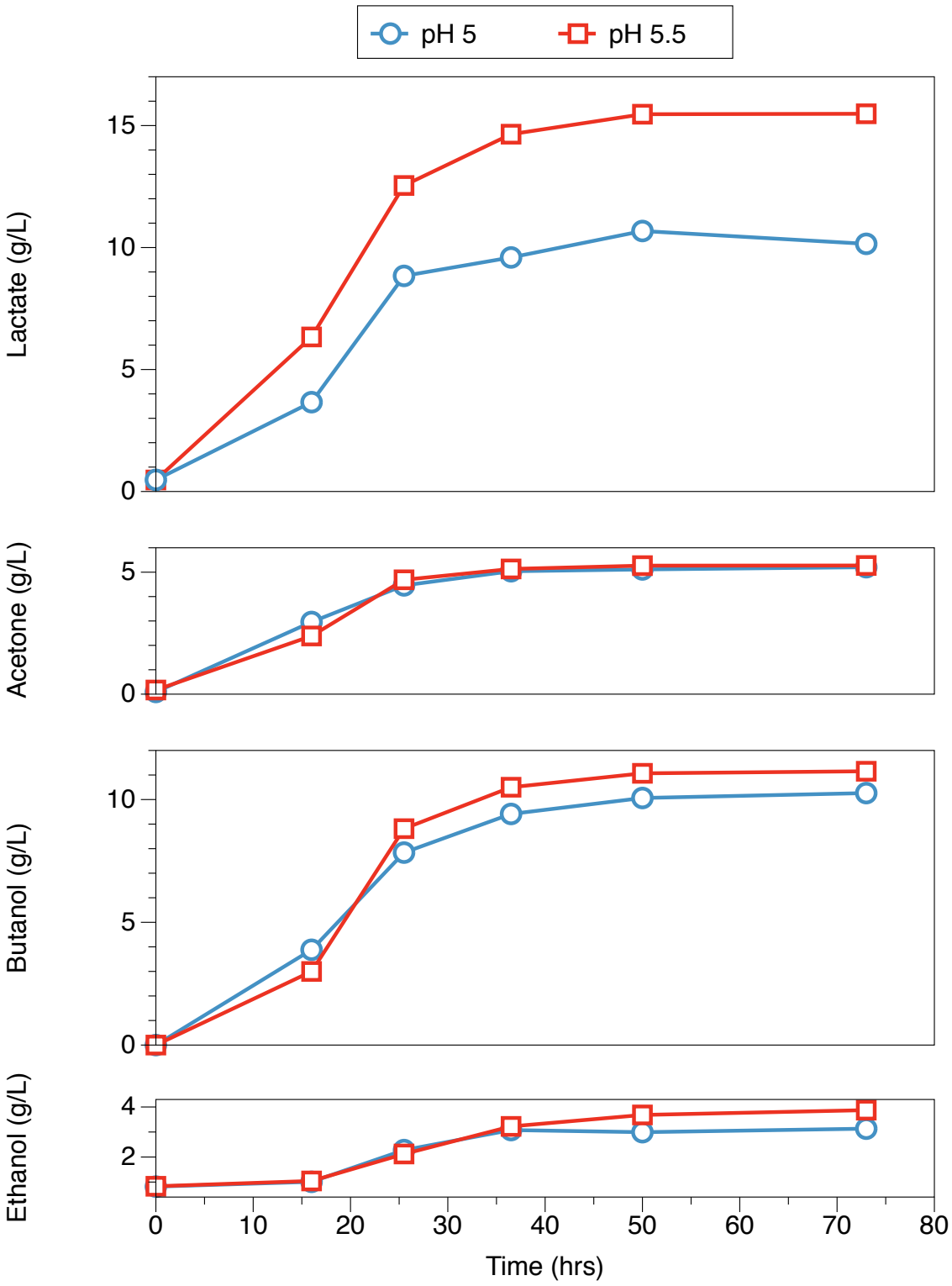


Figure 4-7b: Effect of set-point pH on solvent and lactic acid production in the pACE *C. acetobutylicum* strain.

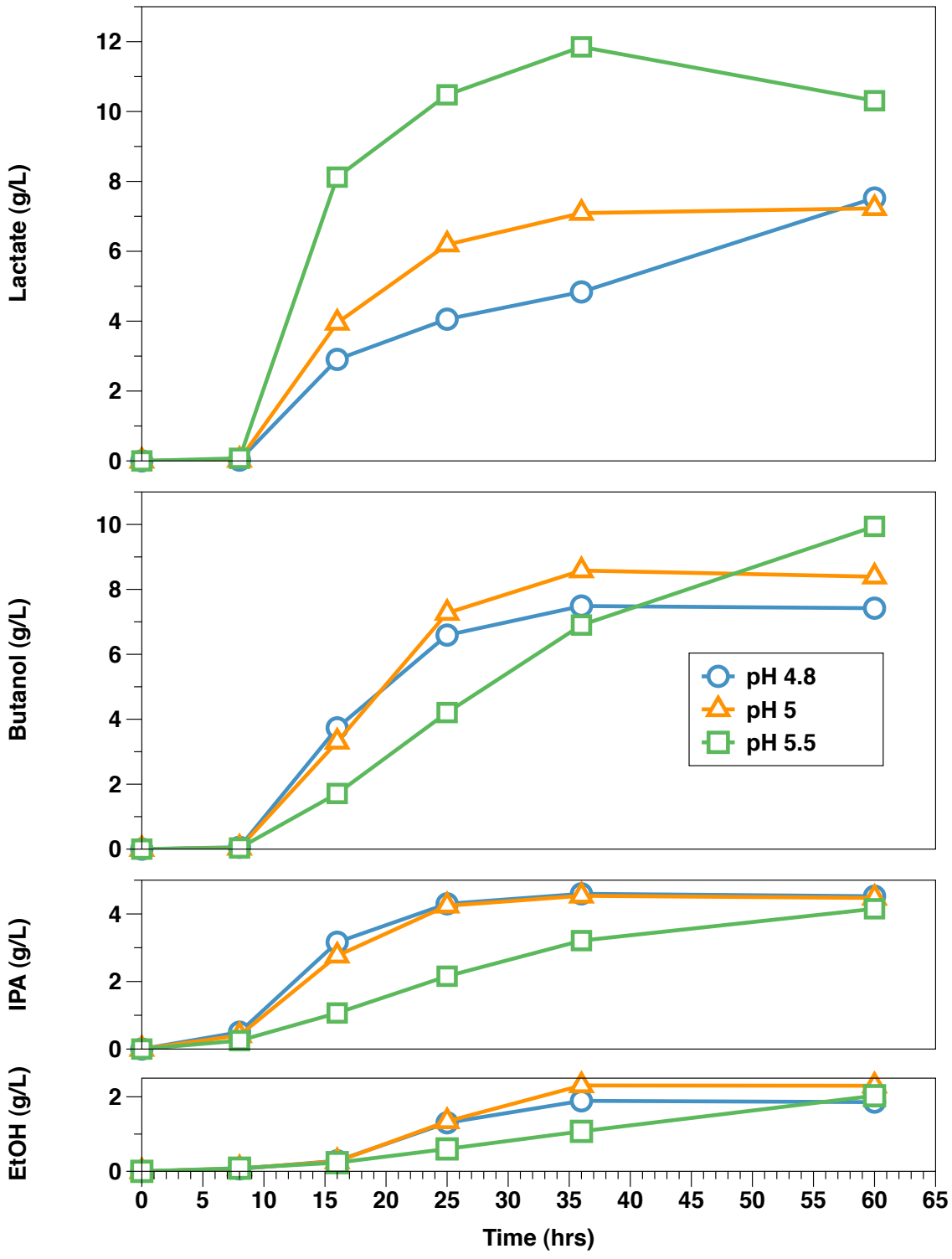


Figure 4-8: pH optimization for Isopropanol, Butatnol, Ethanol production. 1L batch fermentation, starting pH was 6 in all cases and set point pH was varied.

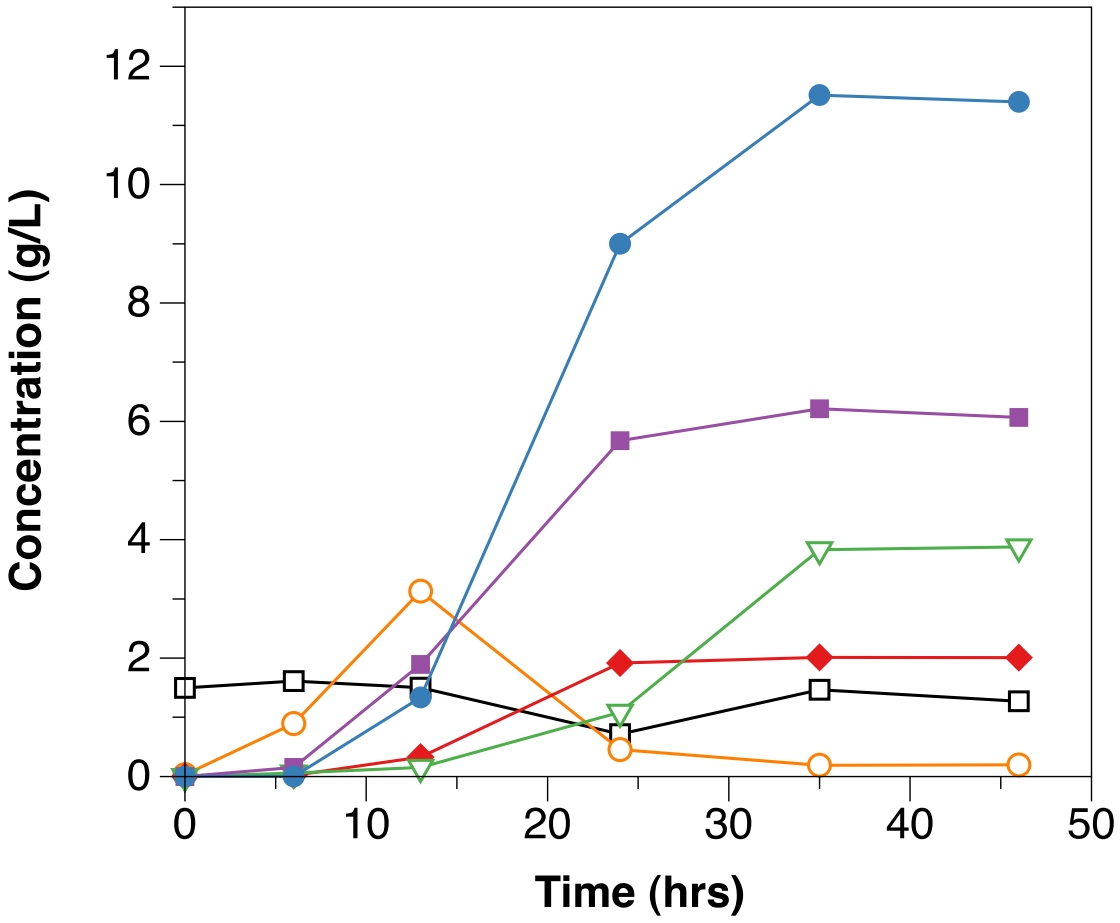


Figure 4-9: 46hr 1.5-L batch fermentation of *C. acetobutylicum* strain SACE on glucose. Major metabolites measured by HPLC: acetate (black open square), butyrate (orange open circle), lactate (red diamond), isopropanol (purple square), 1-butanol (blue circle), ethanol (green triangle).

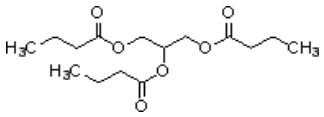
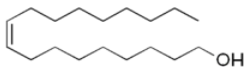
4.3.6 Predicatively Identifying an IBE Extractant

To integrate the biological IBE fermentation with the chemocatalytic approach, a selective, non-toxic, water-immiscible extractant was needed. Previous work found glyceryl tributyrate to be an excellent extractant for ABE fermentation (Anbarasan et al. 2012); however, preliminary testing showed poor removal of isopropanol from an aqueous environment. Since no data on extraction of isopropanol in aqueous environments existed and to avoid *ad hoc* testing of several potential extractants we took a thermodynamic modeling approach to select and efficient extractant. Klamt 1995 first described the conductor-like screening model for real solvents as a simple approach to predict thermodynamic properties. This approach has since been modified and optimized for various solvents and phase equilibra, including the calculation of infinite-dilution activity coefficients. Infinite-dilution activity coefficients describe the interactions between a solute and bulk solvent with values close to zero indicating a thermodynamically more favorable solvation as opposed to highly positive values. Comparing the infinite-dilution activity coefficients of various solvents on the same solute enables a rapid means to rank order solvents for their extraction potential of that particular solute. To test the validity of this methodology we employed COSMO-RS to calculate infinite-dilution activity coefficients for acetone and ethanol in either glyceryl tributyrate or oleyl alcohol (Table 4-8). In both case COSMO-RS correctly predicted the superior extractant by the calculated infinite-dilution activity coefficient. COSMO-RS was then used to calculate the infinite-dilution activity coefficients for isopropanol and predicted oleyl alcohol to be the superior extractant compared to glyceryl tributyrate. Liquid-liquid equilibrium experiments with isopropanol-butanol-ethanol mixtures in water confirmed the COSMO-RS results with a measured isopropanol distribution coefficient (K_D) of 0.8 (Table 4-9).

Table 4-8: Infinite dilution modeling of acetone and isopropanol in oleyl alcohol and tributyrin (glyceryl tributyrate) using COSMO-RS. Gamma infinity values, which correlate to distribution coefficients, were used to rank order extractants from lowest to highest.

Solute	γ_∞ in Oleyl	γ_∞ in Tributyrin
Acetone	**1.14**	**0.71**
Isopropanol	**1.48**	**2.71**
Ethanol	**1.18**	**3.61**

Table 4-9: Experimentally calculated distribution coefficients ($gl^{-1}_{extractant}/gl^{-1}_{media}$) of glyceryl tributyrate and oleyl alcohol from synthetic mixtures of ABE and IBE by HPLC and GC-FID.

Extractant Structure	Name	IPA K_D	Butanol K_D	Ethanol K_D
	Glyceryl Tributyrate	0.3	2.6	0.19
	Oleyl Alcohol	0.8	3.5	0.25

Furthermore, Cosmo-RS [1] was used to calculate infinite-dilution activity coefficients for all previously reported ABE fermentation extractants (Barton and Daugulis 1992; Roffler, Blanch, and Wilke 1987a; Jeon and Lee 1987; Wayman and Parekh 1987)available in the compound database. Using this approach it was determined that olyel alcohol would be the best extractant on the basis of isopropanol removal.

4.3.7 Extractive IBE Fermentation

A 90-hour 1.5-L fed-batch extractive fermentation of the SACE strain with a 1:2 volume ratio of medium and oleyl alcohol produced 39.6 g of alcohols, 5.6 g isopropanol, 17.9 g butanol, and 16.1 g ethanol (Figure 4-10). These alcohols were produced from 117 g of glucose achieving an IBE yield of 0.34 $g/g_{glucose}$ or 78% of the theoretical maximum. Significant ethanol production was observed after isopropanol production ceased approximately 25 hours into the fermentation. Alcohols distilled from the extractant phase of the fermentation were as reactive in the alkylation reaction as the previously tested synthetic mixture of IBE with a yield of 68%, see Table 4-10. Distilled IBE from the extractant phase of the fermentation mixture contained 20 wt% water, which was estimated by Karl Fisher titration. The high water content of the reactant pool diminished production of the double-alkylated products in the IBE reaction. Reducing the water content in the reaction mixture below the inhibitor level can be easily achieved with standard industrial distillation equipment.

[1] F. Eckert, A. Klamt, COSMOtherm, Version C2.1, Release 01.10, COSMOlogic GmbH & Co. KG, Leverkusen, Germany, **2009**.

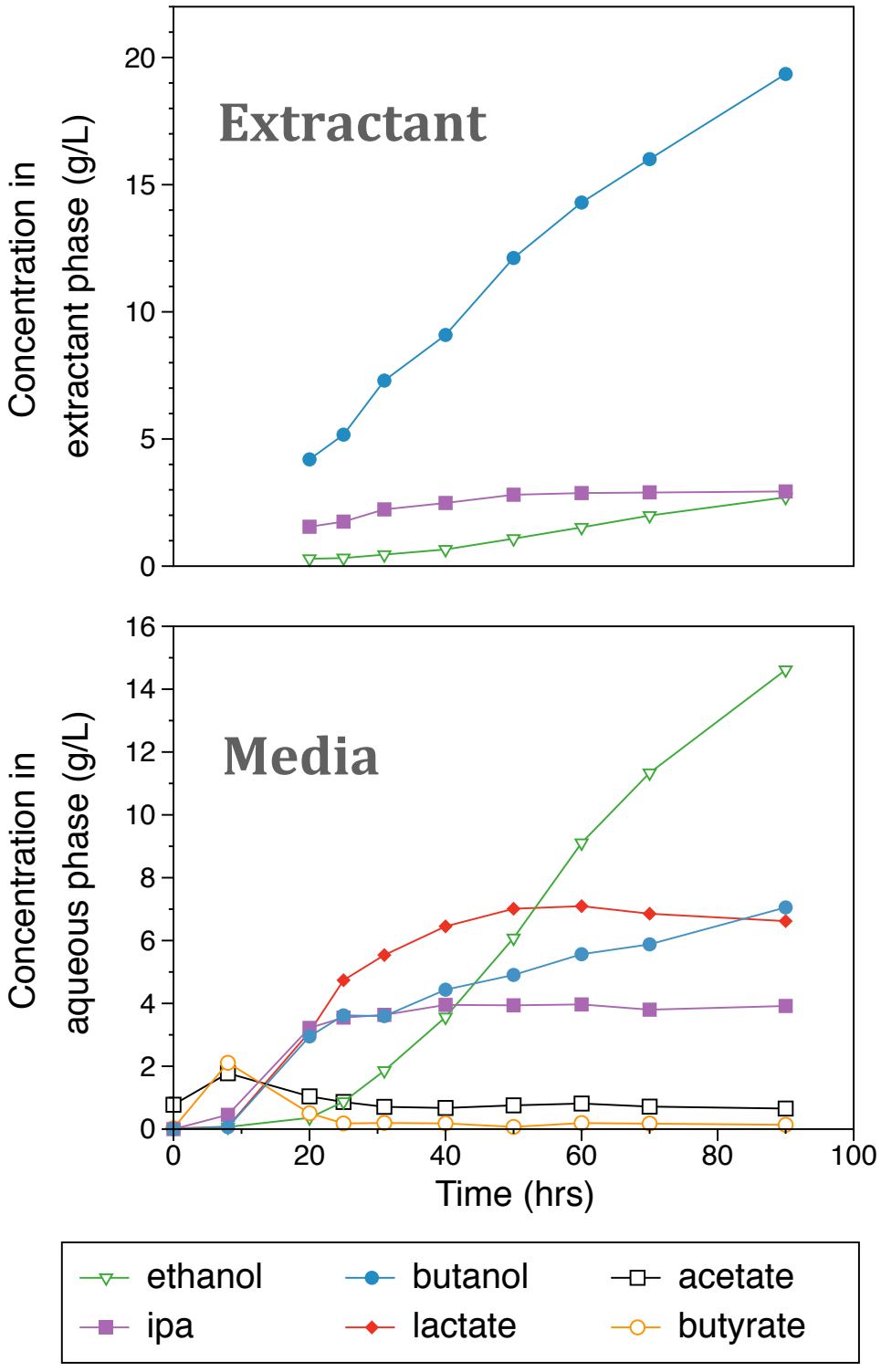


Figure 4-10: 1.5 L extractive fermentation w/oleyl alcohol. Metabolites measured: acetate (black open square), butyrate (orange open circle), lactate (red diamond), isopropanol (purple square), 1-butanol (blue circle), ethanol (green triangle).

Table 4-10: Alkylation reaction for IBE produced during extractive fermentation (contains 20 wt% water estimated by Karl Fisher titration). Reaction conditions: isopropanol (1.73 mmol, 0.104 g), Ethanol, (3.5 mmol, 0.161 g), Butanol (0.8 mL), 240 °C, 24 h. Yields based on isopropanol.

Entry	C5 (%)	4-C7 (%)	2-C7 (%)	C9 (%)	C10 (%)	C11 (%)	Alcohols[a] (%)	Yield (%)
1	2.0	5.9	13.1	2.9	0	9.4	34.4	68

[a]Alcohols yield (%) for Entry 1: **2-C7-OH:** 31.8, **4-C7-OH:** 1.2, **C9-OH:** 0.8 and **C11-OH:** 0.6.

4.4 Conclusion

In conclusion, we have leveraged advantages from both biological and thermochemical processes for the efficient conversion of sugar feedstocks to biodiesel. In the process, we have identified improved recyclable heterogeneous catalysts for the condensation of acetone and alcohols and demonstrated that an extractive IBE fermentation could be integrated with these catalysts. Thus, a significant increase in the production of desirable **C12+** components was achieved by tailoring *both* the fermentation and the heterogeneous catalyst. More importantly, these results demonstrate the benefits of a comprehensive approach to achieving synergy between chemical and biological processes (Turner and Reilly 2013).

5. PROTOCOL FOR THE PRODUCTION AND CONVERSION OF ABE MIXTURE TO HIGH-VALUE BIOFUELS

This protocol delineates a methodology to combine solventogenic *Clostidrial* fermentation and chemical catalysis *via* extractive fermentation for the production of biofuel blendstocks. Extractive fermentation of *Clostridium acetobutylicum* is operated in fed-batch mode with a concentrated feed solution for 60 hours, producing in excess of 40 g solvents (acetone, butanol and ethanol) between the two phases of the bioreactor. Following distillation of the extractant phase the acetone, butanol and ethanol are upgraded over a palladium-hydrotalcite catalyst. This reaction is generally carried out in batch with a high pressure Q-tube for 20 hours at 250 °C. Following this protocol one can produce up to a gram of high-value biofuel precursors. All required equipment, reagents and safety considerations are provided in the protocol below.

5.1 Introduction:

Acetone-butanol-ethanol (ABE) fermentation of sugar using solventogenic strains of *Clostridia* was a well-known industrial process and operational during early and mid 20th century for the production of solvents. Originally the desired product of the fermentation was the acetone for the production of cordite, a smokeless munitions powder, during the First World War (Durre 1998). After the war there was little need for acetone but the demand for butanol skyrocketed as a solvent for lacquers and by 1945 nearly 65% of all butanol was produced by ABE fermentation (Durre 2008). It was during this period of history that ABE fermentation ranked second only to ethanol fermentation in scale and importance (Jones and Woods 1986). However, by the late 1950s routes to produce solvents from petroleum were made cost competitive with fermentation (Green 2011). Additionally, the major feedstock at the time molasses spiked in price because of animal feed demand. These two factors lead to the end of industrial ABE fermentation throughout most of the world.

The growing demand for renewable transportation fuels to mitigate green house gas emissions is being enabled by both the development of new technologies (Huber, Cortright, and Dumesic 2004; Dellomonaco et al. 2011; Atsumi, Hanai, and Liao 2008) and the improvement of once commercially established processes (Harvey W Blanch 2012; Tracy et al. 2011). For this reason ABE fermentation has seen renewed attention for the production of butanol, which has wide applications in the energy and chemical industries. While China is leading the recommercialization of ABE fermentation with over 210,000 MT of butanol capacity (Ni and Sun 2009), plans for different stages of preparation and scale-up in Brazil, USA, UK and France are also underway.

However, for ABE to be a commercially viable process high butanol yields and titers are requisite; this is generally achieved by engineering the strains of *Clostridia*. Recently, with the aid of genetic, metabolic and protein engineering several groups have demonstrated strategies to selectively improve butanol production in solventogenic *Clostridia* (Y.-sin Jang, Lee, Lee, Park, Im, Eom, Lee, Song, et al. 2012; Latonia M Harris et al. 2000; Bormann et al. 2014). However, even with these modest improvements, the ABE process remains economically viable for the specialty chemical market alone. The physical properties of acetone, butanol, and ethanol do not align with traditional jet and diesel fuels, specifically energy density and volatility. Additionally, both ethanol and butanol form azeotropes with water, thus requiring multiple distillation columns and a significant amount of energy to purify each solvent separately.

To circumvent the need for these separation steps and expand the portfolio of available biofuel processes, we recently described a catalytic strategy to upgrade all three of the solvents produced during ABE fermentation to long-chain ketones, which after hydrodeoxygenation produces hydrocarbons that are components of gasoline, jet and diesel fuel (Anbarasan et al. 2012). We adopted a two-step approach wherein sugars were first catabolized to acetone, butanol and ethanol using *Clostridium acetobutylicum*. The overall solvent productivity and titer of this fermentation was significantly improved by employing *in situ* solvent removal with the water-immiscible non-toxic extractant, glyceryl tributyrate. The mixture fermentation products were then catalytically upgraded over Pd/C-K3PO4 to hydrocarbon ketones in excellent overall yield. Subsequently, recyclability of the catalyst was improved by employing multifunctional hydrotalcite, which played the role of the support for the metal in the dehydrogenation of alcohols and also served as the base in the condensation reaction (Sreekumar et al. 2014). By combining the fields of catalysis and fermentation this protocol is fundamentally different from all previously described routes to produce biofuels.

The protocol described in here allows experimenters to readily produce blends of ketones from glucose or sucrose *via* an extractive fermentation coupled with a heterogeneous catalytic reaction, see Figure 5-1. Both steps of the protocol can be carried out independently or combined by simple distillation of the extractant phase. Variations to the fermentation organisms end products or product ratios can be made by genetic or metabolic engineering strategies. However, changes to the catalysis reaction conditions maybe necessary to ensure high conversions and selectivity for the desired end-product. Following this protocol, the biological fermentation and chemical catalysis can be carried out on kilogram and gram scale respectively. This reaction scale produces sufficient blendstock precursors for fuel testing.

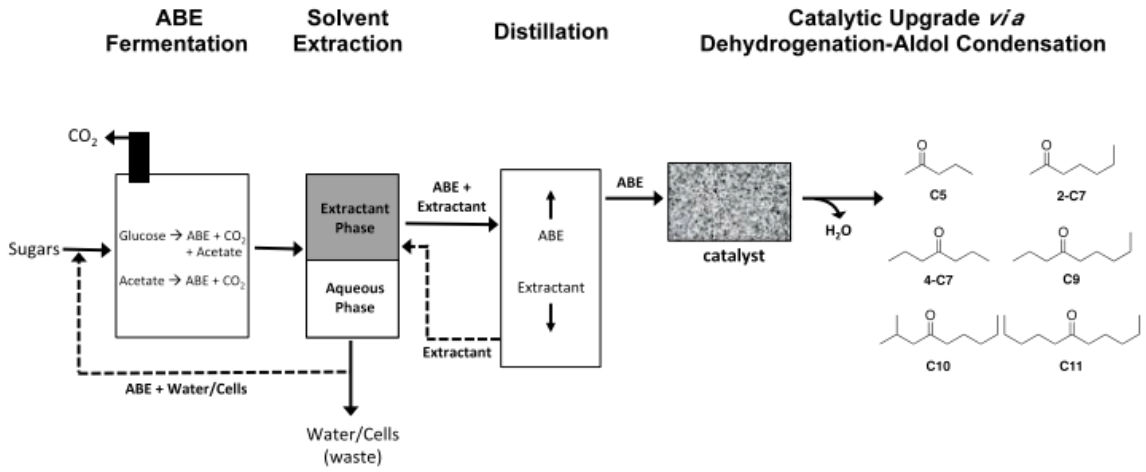


Figure 5-1: Schematic of ABE fermentation to biofuel blendstocks process.

5.2 Materials and Equipment:

Caution! All Chemicals in this protocol should be handled with care using standard safety equipment (eye protection, gloves, lab coat, ventilated fume hood, etc.)

- D-(+)-Glucose (Sigma Aldrich, cat. no. G8270)
- Bacto-Yeast Extract (BD, cat. no. 212750)
- Ammonium acetate (Sigma Aldrich, cat. no. 25006)
- NaCl (Fisher Scientific, cat. no. BP358)
- KH_2PO_4 (Fisher Scientific, cat. no. P285)
- K_2HPO_4 (Fisher Scientific, cat. no. BP363)
- KOH pellets (Sigma Aldrich, cat. no. 221473)
- Cysteine HCl□H_2O (Amresco, cat. no. 0206)
- $MgSO_4$□$7H_2O$ (Fisher Scientific, cat. no. M63)
- $MnSO_4$□H_2O (Acros Organics, cat. no. 423915000)
- $FeSO_4$□$7H_2O$ (Spectrum Chemicals, cat. no. F1060)
- Antifoam 204 (Sigma Aldrich, cat. no. A8311)
- Tributyrin 98% (Acros Organics, cat. no. 15088010)
- Phosphoric acid 85% (Sigma Aldrich, ca. no. 345245)
- Ethyl alcohol 200 proof (Sigma Aldrich, ca. no. 459844)
- Sulfuric acid solution 5M (Sigma Aldrich, ca. no. 35347)
- Ethyl acetate, anhydrous 99.8% (Sigma Aldrich, ca. no. 270989)

Synthesis:

- Palladium(II) nitrate dihydrate (Sigma Aldrich, cat. no. 76070)
- Copper(II) nitrate hemi(pentahydrate) (Sigma Aldrich, cat. no. 12837)
- Hydrotalcite, synthetic (Sigma Aldrich, cat. no. 652288)
- Acetone ≥99.9% (Sigma Aldrich, cat. no. 650501)
- 1-Butanol ≥99.7% (Sigma Aldrich, cat. no. 34867)
- Ethanol ≥99.5% (Sigma Aldrich, cat. no. 459844)
- Dodecane ≥99% (Sigma Aldrich, cat. no. 297879)
- Tetrahydrofuran, ≥99.9%, inhibitor-free (Sigma Aldrich, cat. no. 401757)
-

Equipment:

- Bioreactor: Any capable of controlling agitation, pH, and temperature; sparging sterile nitrogen gas and chilling off-gas back to the bioreactor. Ex. 3.5-L (2.5-L working volume) R'ALF bioreactor (Bioengineering AG, Switzerland)
  Bioreactor components:
- pH probe 405-DPAS-SC-K8S (Mettler Toledo)
- InPro6900 Dissolved Oxygen (DO) probe (Mettler Toledo)
- Temperature probe (Mettler Toledo)
- 0.22 µm filters (autoclavable) for nitrogen input and exhaust gases
- Cooling condenser
- Media sampling line with luer-lock fitting and cap
- Water bath (optional) – needed if bioreactor system does not compensate
- Extractant sampling line with luer-lock fitting and cap – end kept 2" above the final media volume line
- Concentrated media addition line with luer-lock fitting and cap
- Base feed line with luer-lock fitting and cap
- Silicone tubing – tubing of two sizes needed: one with an ID of 1.6 mm and OD of 4.8 mm and the other with ID of 4.8 mm and OD of 7.9 mm
- Pumpable base reservoir bottle
- Peristaltic pump and pump head – capable of pumping tubing with an 7.9 mm OD at a volumetric flow rate in excess of 50 mL min^{-1}
- Thermo RTE 7 115V/60Hz chiller with a Digital Plus controller – Chiller bath with ethylene glycol capable of holding at 2-4°C.

- Agilent HPLC with refractive index (RID) and UV/Vis detectors
  - Aminex HPX-87H ion exchange column

- Varian GC with flame-ionization detector (FID)
  - Factor four column VF-5ms (Varian, cat. no. CP8944)
- Cuvette spectrophotometer - Any can be used so long as it can measure absorbance at 600 nm.

- Benchtop centrifuge – capable of at least 5000 rcfs

Synthesis:

- Q-Tube - 12 mL (Q-labtech)
- Q-Parallel Synthesizer (Q-labtech)
- 20 mm blue butyl rubber septum stoppers (Bellco glass, cat. no. 2048-11800A)
- Teflon-coated magnetic stir bar
- Large safety shield (Scienceware, cat. no. S51223)
- Magnetic hotplate stirrer
- Porcelain mortar and pestle (100 mm diameter; 100 mL capacity)
- Weighing balance
- Weighing paper
- Weighing boats
- Medium frit filter funnel, lower vacuum assembly (Chem glass; 60 mL, 24/40 joint)
- Erlenmeyer filtering flask (Chem glass; 250 ml)
- Graduated cylinder (Vitlab; 250 ml)
- Yellow Teflon caps
- Disposable syringes (1 ml, 5 ml, 10 ml and 20 ml)
- Disposable needles (18, 20 and 22 gauge; 2, 4 and 6-inches long)
- Metal spatula
- Pasteur pipettes
- Rotary evaporator
- Vacuum pump
- Vacuum manifold with vacuum line
- Rubber vacuum tubes
- Reflux condenser
- Varian GC with flame-ionization detector (FID)
- Factor four column VF-5ms (Varian, cat. no. CP8944)
- Centrifuge – capable of at least 5000 rcfs

5.3 Reagent Setup:

Innoculum preparation:

- Hungate tube – capable of being autoclaved sealed and maintained anaerobic.
- Anaerobic jar – autoclavable sealed and capable of holding 100 mL of liquid
- 20 mm blue butyl stoppers – impervious to oxygen and needs to fit hungate tube and anaerobic jar
- 20 mm Aluminum crimp seals – capable of keeping butyl stopper in place during sealed autoclave

- Seal crimper – for 20 mm aluminum seals
- Syringes – 1 mL and 5 mL (sterile)
- Needles – 22G1 sterile
- Incubator capable of maintaining a temperature of 37°C ± 2°C

Clostridia growth media:

- Metals solution – 100 mL of 5gL^{-1} $MgSO_4 \cdot 7H_2O$, 0.5 gL^{-1} $MnSO_4 \cdot H_2O$, 0.5 gL^{-1} $FeSO_4 \cdot 7H_2O$. Sterile filter through a 0.22 µm filter.
- 110 mL of 70 gL^{-1} glucose, 5 gL^{-1} yeast extract, 2 gL^{-1} ammonium acetate, 1 gL^{-1} NaCl, 0.75 gL^{-1} KH_2PO_4, 0.75 gL^{-1} K_2HPO_4, 0.5 gL^{-1} cysteine $HCl \cdot H_2O$, 220 µL of metals solution. Sparge with N_2 for 30 minutes, then dispense 10 mL into the hungate tube and 100 mL into the anaerobic serum bottle. Seal tube and bottle with butyl stopper and crimp-on aluminum cap. Sterilize with autoclave for 20 minutes on liquid cycle. **Caution** Use standard precautions when handling pressurized glassware after autoclaving the sealed hungate tubes and serum bottles.

Note – glucose can be substituted with sucrose as the primary carbon source but this leads too much longer fermentation times before strong ABE production is observed.

70% ethanol: Add 350 mL of 200 proof ethyl alcohol to 150 mL of sterile water. This solution is used for sterilizing luer-lock connections and caps on the bioreactor and can be prepared and stored at room temperature up to 10 days prior to fermentation.

Bioreactor growth media: 200 mL of 350 gL^{-1} glucose, 25 gL^{-1} yeast extract, 10 gL^{-1} ammonium acetate, 5 gL^{-1} NaCl, 4.25 gL^{-1} KH_2PO_4, 4.25 gL^{-1} K_2HPO_4, 2.5 gL^{-1} cysteine $HCl \cdot H_2O$, 2 mL of metals solution, 100 µL Antifoam 204. Filter sterilize the solution through a 0.22 µm filter and pour into a sterile reservoir bottle. **Critical** Use the same sugar species in the bioreactor growth media as prepared for the hungate tube and anaerobic serum bottle.

Concentrated Clostridia media: 500 mL of 450 gL^{-1} glucose and 50 gL^{-1} yeast extract. Heat solution in 65°C water bath to completely dissolve solids. Sterile filter with a 0.22 µm filter. Prepare day of fermentation. **Critical** estimated time to completely dissolve the sugar and yeast extract is 2 hours at 65°C. At room temperature complete dissolution can take up to 8 hours.

Acid and Base solutions: Prepare 200 mL of 5M KOH by carefully dissolving 56.1 g of KOH pellets in 120 mL of de-ionized water. After pellets have completely dissolved adjust final volume to 200 mL with de-ionized water. **Caution** Solution will become very hot as KOH dissolves. Prepare solution in a chemical hood and wait for solution to return to room temperature before handling. Place solution in a pumpable reservoir bottle with a 0.22 um filtered

vent line. Prepare 10 mL of 20 wt% phosphoric acid in water. **Caution** Use standard precautions when handling concentrated acid.

HPLC standards: accurately weigh out 4.00 g sugar (glucose or sucrose), 1.00 g glacial acetic acid, 1.26 g sodium butyrate (1 g eq. butyric acid), 1.26 g sodium lactate (1 g eq. lactic acid), 1.50 g acetone, 1.50 g 200 proof ethyl alcohol, 1.50 g n-butanol into 80 mL of sterile water. Adjust final volume to 100 mL in a volumetric flask. Dilute sample 2x, 4x, 10x, 20x, and 50x with de-ionized water.

GC standards: accurately weigh out 2.0 g acetone, 2.0 g 200 proof ethyl alcohol, 4.0 g n-butanol into 80 mL of tributyrin. Adjust final volume to 100 mL in a volumetric flask. Dilute sample 2x, 4x, 10x, 20x, and 50x with tributyrin.

5.4 Equipment Setup:

Fermentor setup: (estimated time is 2 hours, plus 6 hours incubation time) depicted in Figure 5-2 and Figure 5-3.

1. Immerse pH probe in pH 7.0-solution for at least 20 minutes or until the probe output signal stabilizes. Calibrate the probe to pH 7.0. Rinse the probe with water and repeat in pH 4.0-solution. **Critical** pH calibration is sensitive to temperature if your bioreactor system does not compensate for temperature effects then keep the pH 7.0 and 4.0-solutions in a water bath held at 37°C while performing the pH calibration.
2. Thoroughly rinse the bioreactor vessel, ports, and lines with de-ionized water to remove any residual material from pervious experiments.
3. Add 750 mL of DI water to the bioreactor; insert the calibrated pH probe, DO probe and temperature probe. Cap all sampling, feed, acid and base ports and place 0.22 μm filters on the N_2 supply and cooling condenser exhaust. Cover filters with aluminum foil to prevent the filter from flooding with water. Autoclave the bioreactor for 30 minutes on liquid cycle. **Critical** the DO probe requires 6 hours to polarize post autoclaving.
4. Once bioreactor has cooled add 200 mL growth media and initiate 200 mL min^{-1} N_2 sparging to make the bioreactor anaerobic. Set the bioreactor temperature to 37°C ± 0.2°C and agitation to 200 rpm. Allow N_2 sparging for 3 hours before bioreactor is considered anaerobic. Prime the pump line connecting the 5M KOH base reservoir and fermentor.

HPLC analysis of fermentation broth: This HPLC method allows for quantification of sugar (glucose or sucrose), acetic acid, butyric acid, lactic acid, acetone, ethanol, and butanol. Calibration curves for these compounds should be prepared periodically.

- Agilent HPLC with refractive index (RID) and UV/Vis detectors
    - Aminex HPX-87H ion exchange column

- Mobile phase: 0.01 N H_2SO_4 in de-ionized water
- Flow rate: 0.7 mL min^{-1}
- Temp: 35°C
- Injection volume: 20 uL (10 uL for initial time point)
- Acetone measured with UV/Vis at 265 nm
- Butyric acid measured with UV/Vis at 190 nm
- All other compounds measured with RID

GC analysis of extractant phase: This GC method allows for the quantification of acetone, ethanol, and butanol. Calibration curves for these compounds should be prepared periodically.

- Varian GC with flame-ionization detector (FID)
  - Factor four column VF-5ms (Varian, cat. no. CP8944)
  - Wash fluid for injector: ethyl acetate
  - Injector temp: 260°C
  - Oven temp profile: 35°C hold for 3 minutes, ramp to 150°C at 10°C min^{-1}, ramp to 300°C at 20°C min^{-1}, 300°C hold for 7 minutes
  - Detector temp: 280°C
  - Detector Makeup flow: 25 mL min^{-1}
  - Detector H_2 flow: 30 mL min^{-1}
  - Air flow: 300 mL min^{-1}
  - Injection volume: 1 uL
  - Split ratio: 60

Bacteria inoculation and preculture:

1. 0.5 mL of frozen anaerobic glycerol stock of *Clostridium acetobutylicum* ATCC 824 is thawed and injected aspetically with a sterile 1 mL syringe and 22G1 needle into a hungate tube containing 10 mL of anaerobic clostridia growth media (CGM). **Caution** Use appropriate safety precautions when handling needles.
2. The culture is incubated at 37°C for 12-20 hrs to an OD_{600} of 1-2.0.
3. Using a sterile 5 mL syringe and 22G1 needle aspectically transfer 4 mL of the overnight culture to 100 mL of fresh CGM in an anaerobic jar. **Caution** Use appropriate safety precautions when handling needles.
4. The 100 mL preculture is incubated at 37°C for 4-6 hours to an OD_{600} of 1-2.0. To measure the OD_{600} on a cuvette spectrophotometer place the instrument in absorbance mode, set the absorbance wavelength to 600 nm and blank with a cuvette containing 1mL of de-ionized water. In a separate cuvette add 1 mL of the preculture and measure the absorbance value. **Critical** If the absorbance at 600 nm reads >0.5 dilute with de-ionized water. Calculate actual OD_{600} by multiplying (absorbance value) x (dilution factor). Doubling time for ATCC 824 at 37°C is approximately 1 hour

5.5 Procedure:

Batch phase Fermentation: Timing 30 mins incubation time 18 hours

1. Adjust the bioreactor pH to 5.8 prior to inoculating cells with a 20 wt% phosphoric acid solution (approximately 1-3 mL). Use a 5 mL syringe through the acid port of the bioreactor.
2. With a 60 mL syringe and 18G1 needle aseptically transfer 50 mL of culture into the 950mL anaerobic bioreactor. Starting OD_{600} of 0.05 to 0.1 should be measured.

   Caution Use appropriate safety precautions when handling needles.

3. Allow culture pH to decrease from 5.8 to 5.0 over the first 4-6 hours of fermentation. Then pH control the bioreactor ≥ 5.0 using 5M KOH
4. After inoculating the cells draw two 2-mL samples from the bioreactor culture. Aliquot 1 mL of each sample into a 1.5mL cuvette
5. In absorbance mode blank a cuvette spectrophotometer with de-ionized water at the 600 nm wavelength
6. Measure the samples absorbance (optical density) at 600 nm, dilute if necessary with DI water.
7. Filter the remaining sample volume with a 1 mL syringe through a 0.22 µm syringe filter into an HPLC vial and cap. High cell densities may make filtration difficult. **Critical** Centrifuging the sample for 3 minutes at 7,000-13,000 rpm will pellet the cells making filtration of the supernatant easier.
8. Repeat sampling periodically through out the fermentation (every 4-8 hours)

Addition of extractant: Timing 30 mins

Extractant is typically added after the cells begin to produce solvents. This typically takes about 15-20 hours and can be observed by a gradual increase in the culture pH.

9. Sparge N_2 gas through 1L of tributyrin for 1 hour prior to addition to the bioreactor to ensure that the extractant is anaerobic.
10. Decrease the bioreactor agitation to 50 rpm
11. Aseptically attached one end of 4.8 mm ID silcone tube line to the bioreactor using a luer-lock connection.
12. Feed the tubing through a peristaltic pump and immerse the other end into the anaerobic tributyrin
13. Quickly pump (>50 mL min^{-1}) the entire 1L of anaerobic tributyrin into the bioreactor
14. Aseptically disconnect the extractant line from the bioreactor, cap the luer-lock connection, and turn off N_2 sparging in the bioreactor.

Extractant sampling: Timing 30 mins, repeated every 8 hours for 40 hours

15. Draw three 1.5-mL samples from the extractant phase of the bioreactor through the extractant sampling line
16. Aliquot samples into 1.7 mL micro centrifuge tubes
17. Spin tubes at 10,000 rpm for 3 minutes to phase separate cells and water from the extractant sample
18. Collect and filter only the extractant phase of the spun down sample with a 1 mL syringe through a 0.22 μm filter into a GC vial and cap.
19. Repeat sampling periodically through out the fermentation (every 8 hours)

Critical Samples should be analyzed immediately on GC or stored at -20°C to prevent loss of volatiles.

Bioreactor feeding: Timing 30 mins incubation time 40 hours

24 hours after inoculation the bioreactor's glucose concentration should be monitored and adjusted every 4-8 hours.

20. Use a 5 mL syringe (sterile) to remove 1 mL of culture and place in a microcentrifuge tube
21. Centrifuge sample at 7,000 - 10,000 rpm to pellet cells
22. Dilute 100 μL of the sample supernatant into 900 μL of DI water
23. Measure glucose concentration on a YSI Biochemistry Analyzer
24. If the glucose concentration is below 25 gL^{-1} add enough concentrated clostridia media to raise the concentration above 35 gL^{-1}
25. Continue monitoring and feeding the reactor until the cells stop consuming glucose or the OD_{600} of the fermentation broth drops dramatically (2-4 units in 4-6 hours).

Note If accurate continuous feeding is possible with your bioreactor system, flow concentrated media at an initial rate of 6 mL hr^{-1} once the glucose concentration falls below 40 gL^{-1}. Every 6-8 hours measure the glucose concentration as described previously and adjust the media flow rate to maintain glucose concentrations between 15 gL^{-1} and 30 gL^{-1}.

Harvesting extractant for distillation: Timing 30 mins

Once the fermentation is complete the extractant phase is collected for distillation. **Critical** It is very important that little to no cells or excess water is in the harvested extractant.

26. 1 hour prior to harvesting the extractant turn off agitation in the bioreactor to ensure a clean extractant:broth interface.
27. Immerse one end of a 4.8 mm silcone tubing line into the extractant phase. Make sure the line remains 1" above the extractant:broth interface.
28. Feed the tubing through a peristaltic pump and place the other end into a 1L collection bottle.
29. Pump out the tributyrin making sure not to collect any fermentation broth or cells.
30. Distill extractant shortly after collection or store at -20°C

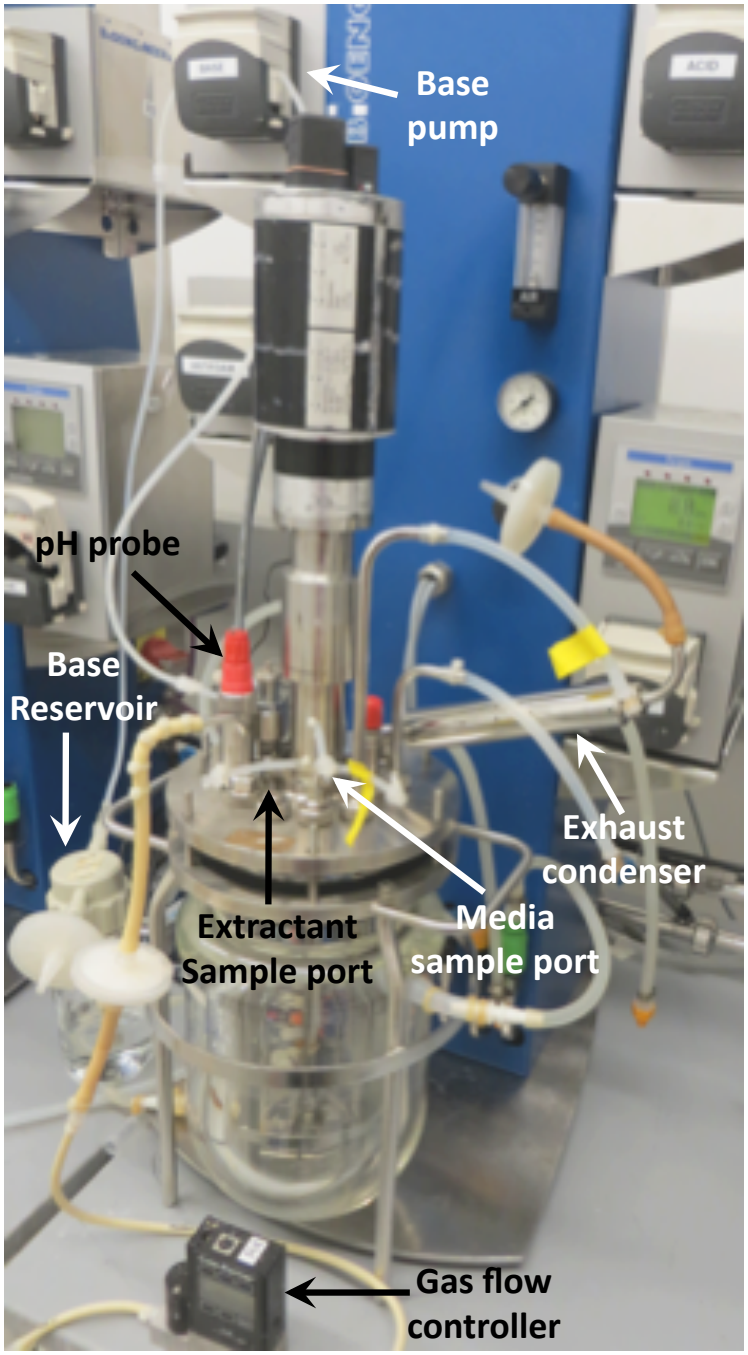


Figure 5-2: Assembled extractive fermentation bioreactor.

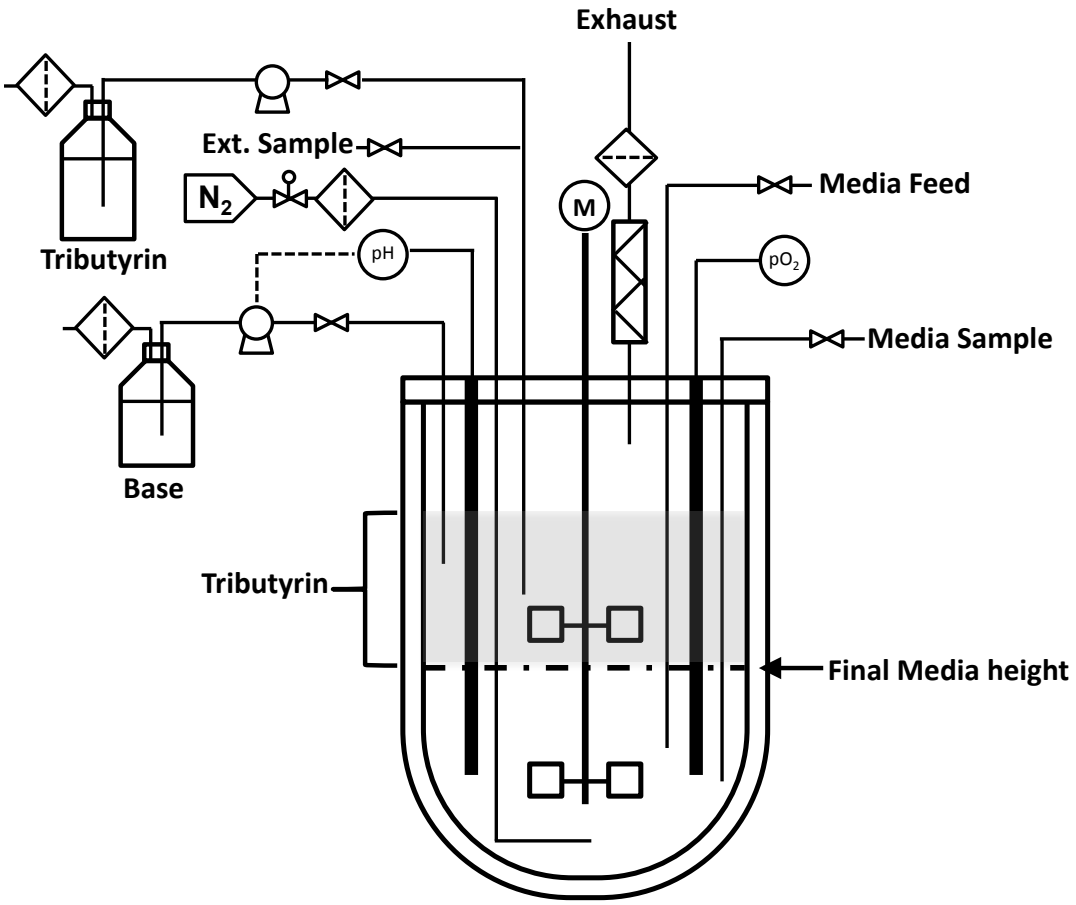


Figure 5-3: Bioreactor setup for extractive fermentation **without** continuous concentrated media feeding.

ABE distillation from extractant phase:

31. Place 100 mL of extractant phase from the ABE fermentation in a 250 mL round bottom flask. Assemble distillation apparatus as shown in Figure 5-4.
32. With the round bottom flask immersed in a silicone oil heating bath slowly increase the temperature to 150 °C and collect the distillate in the receiver flask. Repeat steps 1 and 2 with the remaining extractant phase.
33. Place all of the distillate into a 50 mL round bottom flask and attach to the distillation apparatus.
34. Begin slowly heating the distillation apparatus using the silicone heating bath to 80 °C collecting the distillate in a clean receiver flask. Critical do not allow the distillation temperature rise above 80 °C.

35. After liquid stops accumulating in the receiver flask remove, seal, and store at -20 °C. Install a new clean receiver flask to capture the butanol.
36. With the new receiver flask installed begin raising the distillation apparatus temperature to 150 °C. Hold the apparatus temperature at 150 °C until liquid stops accumulating in the receiver flask, at least 30 minutes.
37. Remove the receiver flask and carefully decant off the butanol-rich upper phase into the previously collected 80 °C distillate solution.
38. Dry this mixture over 2 g of activated 4Å molecular sieves for 1 hour
39. Remove the liquid phase and measure the water content using Karl Fisher titration. Critical – the water content must be below 0.5 wt% prior to running the alkylation reaction. Repeat drying over 4Å molecular sieves if necessary.
40. If not performing alkylation reaction immediately store solvent mixture in a sealed flask at –20 °C.

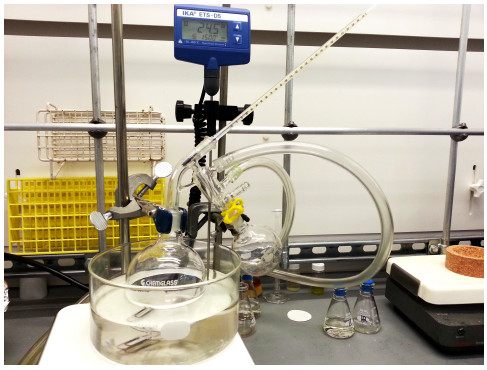


Figure 5-4: Distillation apparatus components and assembled setup.

ABE synthesis
Preparation of 2.4 wt% 3 : 1 Palladium/Hydrotalcite (Pd/HT)
Timing: 13hrs

41. Weigh 0.40 g of palladium(II) nitrate dihydrate (1.50 mmol) into a beaker and dissolve into 4 mL of deionized water.
42. Weigh 8 g of calcined hydrotalcite (calcination condition: 550 °C, 3 hours, 2 °C/min, cooled to room temperature) into a mortar.
43. By means of a syringe, add the palladium solution to the calcined hydrotacite in 0.2mL aliquots while grinding with a pestle, until all of the solution has been absorbed. Add an additional 10 mL water in 0.2mL aliquots while grinding so a thin paste is achieved.

44. Place the mortar in a drying oven at 100 °C for 3 hours.
45. After drying, finely grind the obtained solid using a pestle and transfer to a muffle furnace for calcination.
46. Calcine the catalyst under air at 550 °C for 5 hours using a 2 °C/min ramp followed by cooling to room temperature.
47. Transferred the solid to a calcination boat and place into a tubular furnace for reduction. Heat the sample to 550 °C for 2 hours using a 2 °C/min ramp under hydrogen (flow rate: 100 mL/min) followed by cooling to room temperature under an inert gas (helium flow rate: 100 mL/min).

Caution! When cooling, turn on helium before turning off hydrogen flow, otherwise fire can occur!
Critical Step After reduction of catalyst, store in air tight sample vial. Catalyst was found to slowly deactivate if left exposed to air for extended periods.

Catalytic ABE reaction setup
Timing 21hrs (1hr setup and cool down, 20hr reaction)

48. Place a dry Q-tube, equipped with a stir bar, in a test tube stand (Figure 5-5).
49. Weigh 0.35 g of 2 wt% Pd-HT and place it in the Q-tube.
50. Weigh 0.092 g of ethanol (2 mmol) and place it in the Q-tube.
51. Weigh 0.267 g of acetone (4.6 mmol) and place it in the Q-tube.
52. Add 0.548 g of 1-butanol (7.4 mmol) to the Q-tube.
53. Add a known amount of internal standard (dodecane) to the Q-tube.
54. Seal the Q-tube with the butyl rubber septum followed by the Q-tube sleeve fitted with the metal pressure adapter 1240.
55. Place the Q-tube in a preheated parallel synthesizer at 240 °C and stir it vigorously for 20 hours.
56. Remove the Q-tube from the parallel synthesizer and allow it to cool for 30 minutes.
57. Using a catch bottle fitted with needle, release the pressure in the Q-tube by piercing the butyl rubber septum.

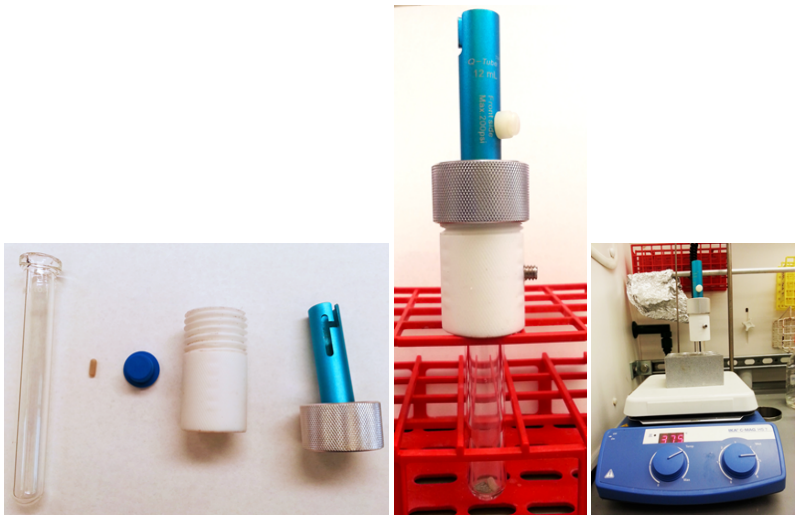


Figure 5-5: Assembly of Q-tube reactor and parallel synthesizer

Caution! Qtubes can build significant pressure during the reaction and can rupture. In addition to standard PPE, wear thick leather gloves and use a blast shield while piercing septa.

58. Dilute the Q-tube with tetrahydrofuran and mix the reaction mixture by means of a spatula.
59. Centrifuge the reaction mixture for three minutes to force catalyst to bottom.
60. Transfer the clear supernatant to a GC vial and dilute it with tetrahydrofuran.

Timing:

Reagent preparation: 2 hours (plus 8 hours incubation time)
Bioreactor assembly: 2 hours (plus 6 hours incubation time)
Steps 1-8, batch phase preparation: 30 mins (plus 18 hours incubation time)
Steps 9-14, addition of extractant: 30 mins
Steps 15-19, extractant phase sampling: 30 mins (repeated every 8 hours for 40 hours)
Steps 20-53, bioreactor feeding: 30 mins (plus 40 hour incubation time)
Steps 26-30, harvesting extractant phase: 30 mins
Steps 31-40, distillation of extractant phase: 6 hours (plus 4 hours incubation time)
Steps 41-47, catalyst preparation: 20 mins (plus 13 hours incubation time)
Steps 48-60, catalytic ABE reaction: 30 mins (plus 20 hours incubation time)

Step	Problem	Possible reason	Solution
3	Cells won't grow in bioreactor	Not anaerobic	Verify that only N_2 is being sparged into the vessel and check for leaks in the N_2 line
3	Culture pH continues to decrease below 5.0	Base pump failure or pump control sensitivity	Look for cracks in the base line and replace tubing. Adjust integral parameter of pump control
26	Extractant layer full of culture broth	Gas sparging is still on	Turn gas sparging off and reduce agitation rate
43	Hydrotalcite forms large clumps while adding metal solution	Solution is being added too quickly	Add smaller aliquots of liquid and grind more thoroughly after each addition
47	Water is forming in glass tube during reduction	Small amount of spilt catalyst is coating downstream end of reduction tube	Thoroughly clean glass tube between each use and always insert boats from upstream end of tube
55	Reaction mixture is not stirring in Q-tube	Catalyst has formed unsuspended clumps and stir bar has become stuck	Gently tap the tube against a hard surface
56	Q tube is stuck in parallel synthesizer block	Glass has expanded under heating	Blow lab air on Q tube while pulling gently to remove

- **Determination of Yield**

- Determine the experimental weight of the ketones by injecting an aliquot of the material obtained in Step X (~2 µl of a 1 mg ml^{-1} in 90:10 hexane/2-propanol) into the GC equipped with a factor four column VF-5ms after calibrating the retention time of the ketones with the authentic sample. Monitor the peaks with a FID detector.

5.6 Anticipated Results:

When *Clostridium acetobutylicum* ATCC824 was grown out and fermented with glyceryl tributyrate at a 1:1 volume ratio as described in the above protocol 24.8 g of butanol, 8.9 g acetone, and 7.3 g of ethanol were produced. Whereby 16.4 g of butanol, 3.7 g of acetone, and 0.8 g of ethanol partitioned into the glyceryl tributyrate phase. Production of these 40.8 g of solvents required the consumption of 105 g of glucose. Solvent production ceased 60 hours after inoculation of the culture into the bioreactor.

When the ABE alkylation reaction is carried out with acetone (4.6 mmol), 1-butanol (7.4 mmol) and ethanol (2 mmol) employing Pd-HT (0.350 g) in q-tube as described in the above protocol, mixture of hydrocarbon ketone in the following composition is obtained.

Yield (%)
2-Pentanone: 2.6
2-Heptanone: 9.4
2-Heptanone: 10.4
4-Nonone: 11.4
6-Undecanone: 23.6
2-Pentanol: 3.0
2-Heptanol: 5.1
4-Heptanol: 13.5
6-Undecanol: 6.6
Total: 86%

6. ENGINEERING CO-PRODUCTION OF ACETONE AND ETHANOL IN *E. COLI* FOR GASOLINE AND JET APPLICATIONS

6.1 Introduction:

The increasing demand for petroleum fuels coupled with the deterious effects of carbon dioxide emissions has led to major research in biotechnological alternatives over the last few years. Ethanol production from simple sugars has thus far been the most successful process to displace gasoline consumption worldwide. However ethanol production in 2010 was 23.0 billion gallons accounting for only 6.8% of the world's gasoline consumption (EIA data). The United States leads all nations in ethanol production at 13.3 billion gallons per year but has seen significant resistance recently to further increase production above the EPA suggested 10% blend-wall. Other technologies are trying to address the blend-wall problem by producing longer-chain biofuels (Atsumi, Higashide, and Liao 2009; Machado et al. 2012; Y.-sin Jang, Lee, Lee, Park, Im, Eom, Lee, Lee, et al. 2012). However, several of these longer-chain biofuel molecules are highly toxic to the fermenting organism severely limiting the final titer achieved during fermentation.

Acetone, also known as dimethyl ketone, is a colorless liquid used primarily as a solvent in the synthesis of polymers. Acetone is not a good fuel molecule because of its high volatility and polymer swelling susceptibility but is only mildly toxic to most organisms with tolerances in excess of 60 g L^{-1} reported (D. T. Jones and Woods 1986). The α-carbons on acetone are nucleophilic and can form carbon-carbon bonds with primary alcohols via an alkylation reaction under basic conditions with an appropriate transition-metal catalyst (Anbarasan et al. 2012). Therefore the co-production of acetone and ethanol combined with a chemical catalysis alkylation reaction enables the production of C_5 and C_7 mono-ketones as precursors for gasoline blendstocks. Bormann et al. 2014 demonstrated tuning the ratio of fermentation products for catalytic upgrade has a major impact on the distribution of long-chain fuel precursors. A high ethanol:acetone ratio (in excess of 6:1) would produce an improved ethanol blend enriched in C_5 and C_7 oxygenates. Alternatively controlling the ethanol:acetone ratio near 1-2:1 enables complete conversions of both solvents to mono-ketones. These mono-ketones produced from this initial alkylation reaction can be further upgraded to a highly branched C_{10} and C_{14} or cyclic C_{15} mono-ketones via self-condensation to dimers and trimers, respectively (unpublished data, Figure 6-1). This high degree of branching and the longer-chain length of these products make them well suited as a jet fuel.

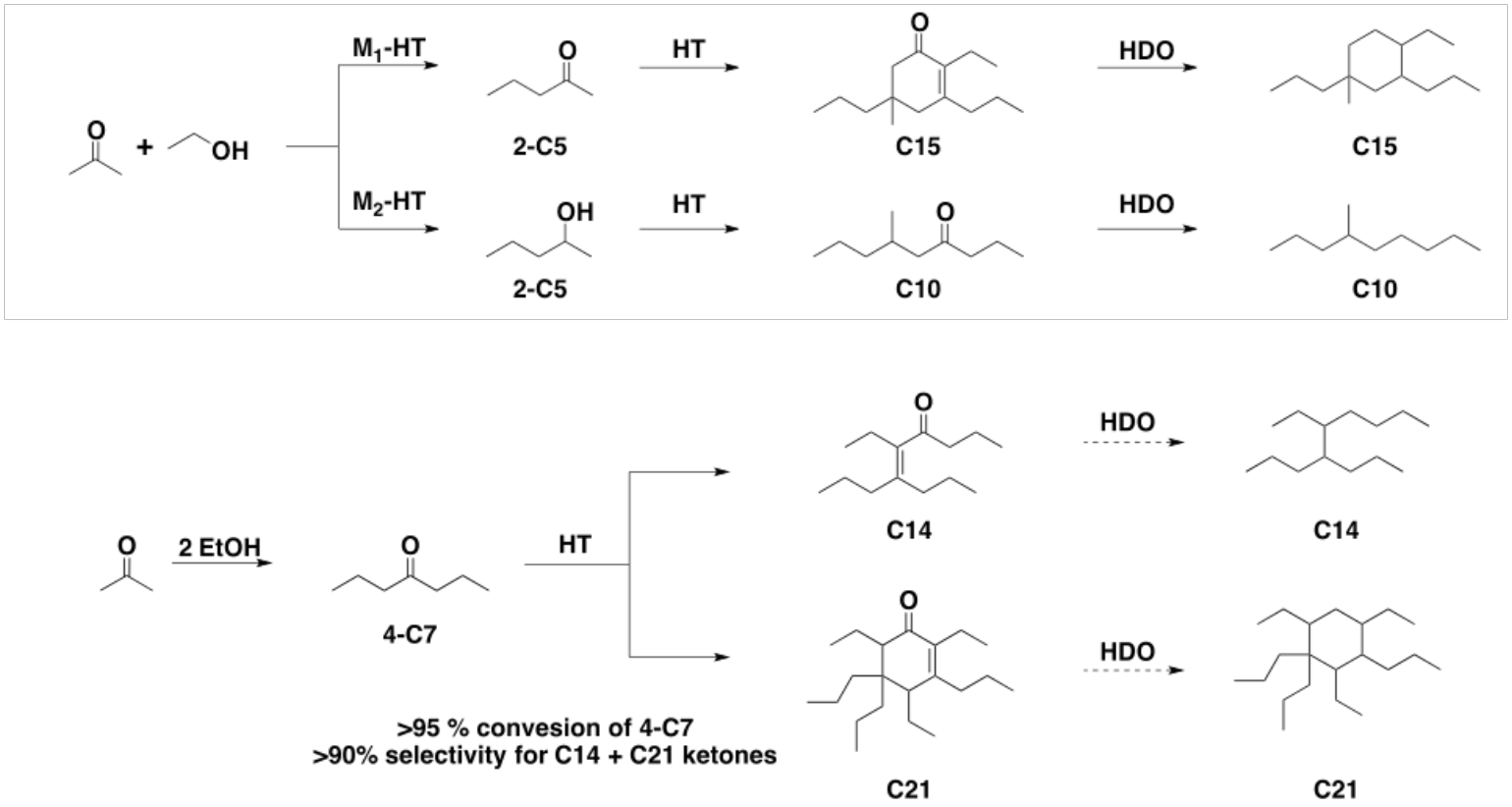


Figure 6-1: Catalytic alkylation and seld-condensation reactions to upgrade ethanol and acetone to linear, branched, and cyclic mono-ketones. Followed by hydrodeoxygenation reactions (HDO) to convert ketone products to pure hydrocarbons. HT, hydrotalcite (MgAlO) catalyst and M_x-HT, hydrotalcite supported transition metal catalyst.

Biological production of acetone was demonstrated on the industrial scale with *Clostridium acetobutylicum* from the 1920-1950s (Tracy et al. 2011). The metabolic pathway for producing acetone was successfully transferred to *E. coli* and resulted in comparable titers to wild-type *C. acetobutylicum* (Bermejo, Welker, and Eleftherios 1998). However, the pathway for acetone production in *C. acetobutylicum* requires the presence of acetate or butyrate in their un-dissociated form. Whereby a co-transferase (*ctfa* or *ctfb*) transfers a Co-A moiety from acetoacetyl-CoA to the un-dissociated acid producing acetyl- or butyryl-CoA and acetoacetate. Acetoacetate is then decarboxylated to acetone by acetoacetate decarboxylase (*adc*). May et al. 2013 described a modified acetone production pathway using a thioesterase from *Haemophilus influenzae* (*ybgC*) that did not require acetate or butyrate reassimilation and increased the acetone titer to 7.1 g L^{-1}. However, the thioesterase activity on acetyl-CoA coupled with the aerobic environment of the fermentation limited ethanol production to ≤ 10 mM. This low ethanol:acetone ratio is incompatible with the chemical catalysis upgrading, which requires a minimum molar ratio of 1:1. To address this challenge we identified an alternative acetone production pathway that did not require acid reassimilation and allowed for sufficient ethanol co-production by expressing a hydroxymethylgutyrl-CoA (HMG-CoA) synthase and HMG-CoA lyase as depicited in Figure 6-2.

HMG-CoA synthases are responsible for mevalonate production and have been investigated extensively for the microbial synthesis of isopreniods. While HMG-CoA lyases found in higher eukaryotes are responsible for final enzymatic step of ketoneogensis and leucine catabolism in the liver. Isolates from prokaryotes are implicated in mevalonate catabolism and maintain activity under oxidative conditions by lacking residue Cys^{323} (J. Pie et al. 2007). Because acetone production from glucose is not balanced with respect to reducing equivalents, fermentations in model organisms are carried out under aerobic conditions. Therefore only HMG-CoA lyase isolates from previously described prokaryotes were tested in this study.

In this study we describe the identification of an alternative acid-independent acetone production pathway in *E. coli* using both an HMG-CoA synthase and HMG-CoA lyase in a single inducible-operon with the *C. acetobutylicum* genes *thl* and *adc*. Several sources of the synthase and lyase enzymes are tested and suitable candidates are combined with a second inducible operon containing *E. coli* MG1655 alcohol/aldehyde dehydroganse *adhE* to improve the co-production of ethanol. Optimization of the fermentation conditions affords significant improvements in overall solvent production and control of the ethanol:acetone ratio. Control over the solvent ratio enables catalytic upgrading to either gasoline or kerosene precursors in high yields.

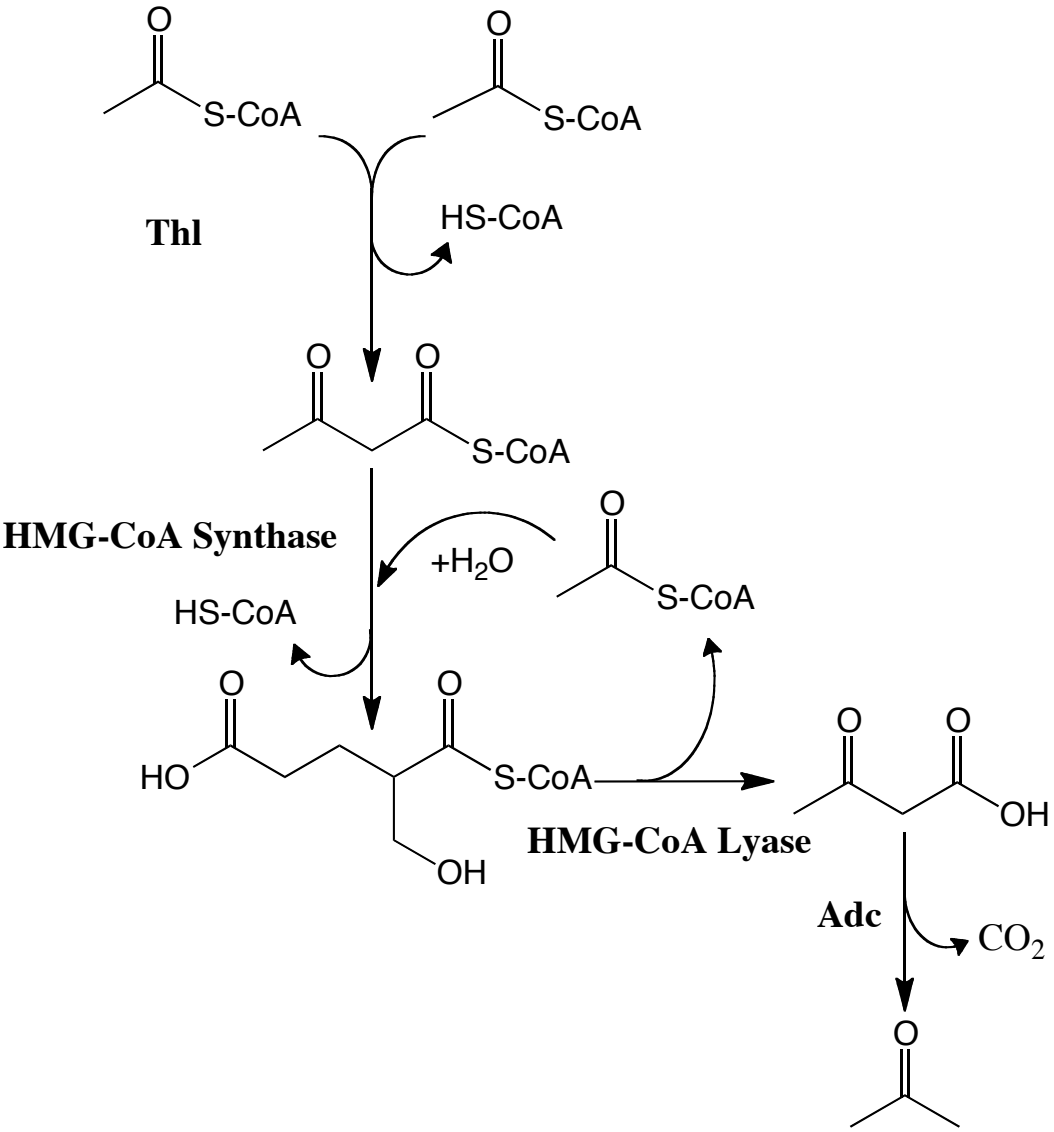


Figure 6-2: Proposed alternative acetone production pathway.

6.2 Material & Methods:

6.2.1 Chemicals, microorganisms, and plasmids

All chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) and Spectrum Chemicals. Enzymes were purchased from New England Biolabs (Ipswich, MA) and used according to the manufacturer's instructions. Strains used in this work and their sources are described in Table 6-1. Primers used and plasmids created in this work are described in the Table 6-2 and Table 6-3, respectively.

6.2.2 Strain maintenance

All *E. coli* strains were first streaked onto Luria Broth (LB) agar plates supplemented with the appropriate antibiotic (60 ug mL^{-1} Kanamycin and/or 100 ug mL^{-1} Carbacillin) if necessary. Single colonies were picked and inoculated into liquid LB media overnight at 37 °C before sub-culturing into fresh liquid LB media. Strains were stored in 15 wt% glycerol at -80 °C.

Table 6-1: Strains used in this study.

Strain	Description	Source
E. coli BL21 (DE3)	F– ompT hsdSB(rB– mB–) gal dcm (λDE3)	Invitrogen
E. coli MG1655 (DE3)	K-12;λ–; F– (λDE3)	Trinh et al. 2012
E. coli XL1B	F′ proAB lacIqZΔM15 Tn10 (Tetr)	Stratagene
E. coli BW25113	K-12; λ–; F– lacIq rrnBT14 ΔlacZWJ16 hsdR514 ΔaraBADAH33 ΔrhaBADLD78	Datsenko et al. 2000
E. coli BFA7 (DE3)	BW25113, λDE3 Δzwf Δmdh ΔfrdA Δndh Δpta ΔpoxB ΔldhA::Kan	Trinh et al. 2012
E. coli ATCC 11303	harboring pLOI297	ATCC
C. acetobutylicum ATCC 824	wild-type	ATCC

6.2.3 Assembly of expression plasmids

All plasmid used in this study were constructed using isothermal DNA assembly as previously described (Gibson et al. 2009). The genes *C. acetobutylicum thl, adc* and *E. coli* strain MG1655 *adhe* were amplified from genomic DNA. Pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*) from *Z. mobilis* was amplified from the pLOI297 vector. Hydroxymethylgutyrl-CoA (HMG-CoA) synthase and lyase genes: *E. faecalis EF1363 S. aureous mvaS, L. acidophilis LAC30SC_03150, P. mevalonii mvaB,* and *B. subtilis yngG* were synthesized with codon optimiation for both *E. coli* and *C. acetobutylicum* through geneArt, see sequences in Section 6.5. The HMG-CoA lyase gene from *A. baumannii AB57_1597* was also synthesized by geneArt but codon optimization could not be performed. All four of the genes for the recombinant acetone pathway were

first PCR amplified with appropriate primers (3-14 and 16) and then cloned into a pET21b vector fragment (primers 1 and 2) as a single operon (HMG-CoA synthase-HMG-CoA lyase-*adc*-thl). Plasmids harboring only three of the recombinant acetone pathway genes (p7ΔSYN), p7ΔLYS, p7ΔADC, p7ΔTHL) were constructed from the parent plasmid p7EFPM using universal vector primers (19 and 20) and the appropiate gene specific deletion primers (21-26). Ethanol production plasmids (pK7AT and pK7PA) were constructed from a pET24b vector fragment (primers 1 and 2). Genes for the pK7AT plasmid were amplified with the primers 15-18, while genes in pK7PA used primers 27-30. All PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) prior to the isothermal DNA assembly reaction.

Table 6-2: Primers used in this study. Primers contain both complimentary overlapping regions for Gibson assembly and priming regions for amplification.

Primer #	Name	Sequence
1	pET vector fwd	ctaagatccggctgctaacaaagc
2	pET vector rev	catatgtatatctccttcttaaagttaaacaaaa
3	EF gene fwd	tgtttaactttaagaaggagatatacatatgacaataggaatagataaaatatcattttt
4	EF gene rev	ttcctccttaataacaaaattgatagcttaatttctataacttctaactgtattatttat
5	LA gene fwd	ttttgtttaactttaagaaggagatatacatatgcaagttggaatagataaaatagg
6	LA gene rev	atgttcctccttaataacaaaattgatagcttatttttctttatattgtctaacatg
7	SA gene fwd	ttgtttaactttaagaaggagatatacatatgacaataggaatagataaaataaactttt
8	SA gene rev	atgttcctccttaataacaaaattgatagcttattcaggtctatgatattctctaa
9	HMG lyase gene fwd	gctatcaattttgttattaaggaggaacatatcatatg
10	HMG lyase gene rev	cctttaacataaaagtcaccttcctcgggcagtttaggcgggtttaaac
11	Adc gene fwd	gcccgaggaaggtgacttttatgttaaagg
12	Adc gene rev	cctcctttacttaagataatcatatataacttcagctc
13	Adc gene pET rev	cgggctttgttagcagccggatcttacttaagataatcatatataacttcagctctagg
14	Thl gene fwd	gagctgaagttatatatgattatcttaagtaaaggaggttagttagaatgaaagaag
15	Thl gene Adhe fwd	ccgcctaaactgcccgaggaggttagttagaatgaaagaag
16	Thl gene rev	cgggctttgttagcagccggatcttagcacttttctagcaatattgc
17	Adhe gene fwd	gtttaactttaagaaggagatatacatatggctgttactaatgtcgctg
18	Adhe gene rev	cgggcagtttaggcggttaagcggatttttcgctttttc
19	pET vector neg fwd	cgaggcagctgcggtaaagctcatcag
20	pET vector neg rev	ctgatgagctttaccgcagctgcctcg
21	Syn_neg fwd	ggagatataatgcaagcagttaaagtttttgaagttgg
22	Syn_neg rev	ctttaactgcttgcattatatctccttcttaaagttaaacaaaattatttc
23	Lys_neg fwd	gctatcaaaggaaggtgacttttatgttaaagg
24	Lys_neg rev	aaaagtcaccttcctttgatagcttaatttctataacttctaactg
25	Adc_neg fwd	gcccgaggaggttagttagaatgaaagaag
26	Adc_neg rev	ctaactaacctcctcgggcagtttaggcgggtttaaac
27	Pdc gene fwd	gtttaactttaagaaggagatatacatatgagttatactgtcggtacctatttag
28	Pdc gene rev	atgttcctccttaataacaaaattgatagcctagaggagcttgttaacaggc
29	AdhB gene fwd	atcaattttgttattaaggaggaacatatcatatggcttcttcaacttttatattcc
30	AdhB gene rev	cgggctttgttagcagccggatcttagaaagcgctcaggaagag

Table 6-3: Plasmids used in this study.

Name	Description	Source
pET 21b	f1 *ori,* Amp^r	Novagen
pET 24b	f1 *ori,* Kan^r	Novagen
p7EFPM	pET21b, pT7 *E. faecalis HMG-CoA SYN + P. mevalonii HMG-CoA LYS + C. acetobutylicum ADC + C. acetobutylicum THL tT7*	This study
p7LAPM	pET21b, pT7 L*. acidophilus HMG-CoA SYN + P. mevalonii HMG-CoA LYS + C. acetobutylicum ADC + C. acetobutylicum THL tT7*	This study
p7SAPM	pET21b, pT7 *S. aureus HMG-CoA SYN + P. mevalonii HMG-CoA LYS + C. acetobutylicum ADC + C. acetobutylicum THL tT7*	This study
p7EFAB	pET21b, pT7 *E. faecalis HMG-CoA SYN + A. baumanii HMG-CoA LYS + C. acetobutylicum ADC + C. acetobutylicum THL tT7*	This study
p7LAAB	pET21b, pT7 *L. acidophilus HMG-CoA SYN + A. baumanii HMG-CoA LYS + C. acetobutylicum ADC + C. acetobutylicum THL tT7*	This study
p7SABS	pET21b, pT7 *S. aureus HMG-CoA SYN + B. subtilis HMG-CoA LYS + C. acetobutylicum ADC + C. acetobutylicum THL tT7*	This study
pK7AT	pET24b, pT7 *E. coli strain MG1655 ADHE + C. acetobutylicum THL tT7*	This study
p7negSYN	pET21b, pT7 *P. mevalonii HMG-CoA LYS + C. acetobutylicum ADC + C. acetobutylicum THL tT7*	This study
p7negLYS	pET21b, pT7 *E. faecalis HMG-CoA SYN + C. acetobutylicum ADC + C. acetobutylicum THL tT7*	This study
p7negADC	pET21b, pT7 *E. faecalis HMG-CoA SYN + P. mevalonii HMG-CoA LYS + C. acetobutylicum THL tT7*	This study
p7negTHL	pET21b, pT7 *E. faecalis HMG-CoA SYN + P. mevalonii HMG-CoA LYS + C. acetobutylicum ADC tT7*	This study
pK7PA	pET24b, pT7 *Z. mobilis PDC + Z. mobilis ADHB tT7*	This study

6.2.4 DNA isolation and manipulation

All plasmids were propagated in *E. coli* XL1B (Stratagene, Carlsbad, CA) and purified using the QIAprep Spin Miniprep Kit (Qiagen). *E. coli* XL1B and BL21

(DE3) (Invitrogen, Carlsbad, CA) were transformed using the standard heat shock protocol. Transformations in *E. coli* BFA7 and MG1655 were performed by electroporation as previously described.

6.2.5 Fermentation

All fermentation experiments were carried out at 37 °C. Precultures were grown to an OD_{600} reached 1.0 before innoculating Terrific broth (24 g $L^{=1}$ yeast extract, 12 g L^{-1} tryptone, 4 mL L^{-1} glycerol, 17 mM KH_2PO_4, and 72 mM K_2HPO_4) at a 1:20 dilution. Initial 5-mL fermentation experiments were carried out with 14-mL round bottom tubes. Oxygen was provided to the culture by shaking at 250 RPM. 1mM of IPTG was added to the culture after the OD_{600} reached 0.6 - 1.0. Concentrated sugar and sugar acid feeding was initiated 1 hour after induction. 1M NH_4OH was used to stabilize the culture pH for all sugar acid fermentations.
Fermentations were scaled-up to a pH and oxygen-controlled 750-mL DasGIP bioreactor (Ependorff,) operated in fed-batch. Seed cultures (30 mL Terrific broth, 100 µg mL^{-1} carbacillin and 60 µg mL^{-1} kanamycin) were inoculated with 2 mL of preculture and grown to an OD_{600} of 2.0. 30 mL of the seed culture was inoculated into 720 mL of Terrific broth supplemented with 100 µg mL^{-1} carbacillin and 60 µg mL^{-1} kanamycin. Oxygen feed to the bioreactor was controlled by the sparge rate between 0.5 – 10 sL hr^{-1} while agitation was fixed at 500 rpm. The bioreactor pH was controlled at ≥ 6.0 with a 28% NH_4OH solution. 1mM of IPTG was used to induce acetone production after the culture OD_{600} reached 1.0. Concentrated feeding (400 g L^{-1} glucose or gluconic acid) was initiated after IPTG induction.

6.2.6 Analytical methods

Major metabolite concentrations for glucose, gluconic acid, acetate, succinate, formate, pyruvate, ethanol, acetone, and lactate were determined using a Shimadzu Prominence HPLC System (Shimadzu, Kyoto, Japan) equipped with both UV/Vis and refractive index detectors. Samples were separated with a Bio-Rad (Hercules, CA) Aminex HPX-87H ion exchange column and a Cation H guard column with 5 mM sulfuric acid as the mobile phase and a flow rate of 0.7 mL min^{-1} at 35°C.

6.3 Results and Discussion:

6.3.1 Acetone production in *E. coli* strains via the alternative pathway

To initially evaluate whether this alternative acetone pathway would express in *E. coli* plasmids (p7EFPM and p7LAPM) containing the HMG-CoA synthase from either *E. faecalis* or *L. acidophilis,* the HMG-CoA lyase from *P. mevalonii* and the *thl* and *adc* genes from *C. acetobuytlicum* were transformed into *E. coli* strain BL21(DE3). Expression of the pathway was induced by 1mM of IPTG and anaylzed by SDS-PAGE 18 hours after induction, see Figure 6-3. Expression of the HMG-CoA synthase, HMG-CoA lyase, and thiolase genes could be clearly detected compared to the negative control, while expression of the acetoacetate decarboxylase could not. However, 5-mL shake flask fermentations of BL21 p7EFPM and BL21 p7LAPM produced 190 mg L^{-1} and 120 mg L^{-1} of acetone, respectively from 6 g L^{-1} glucose as shown in Table 6-4. Table 6-5 shows that no acetone production was detected in uninduced cultures. Plasmids p7EFPM and p7LAPM were then transformed into *E. coli* strains MG1655 (DE3) and BFA7 (DE3). Fermentation of the BFA7 strain showed similar acetone titers as the previously tested BL21 strain whereby BFA7 p7EFPM and p7LAPM both produced 140 mg L^{-1} of acetone. However, both variants of the MG1655 strain produced only 30 mg L^{-1} of acetone, with most of the carbon flux going to acetate production, see Table 6-4. May et al. 2013 described similar strain variability with respect to acetone production, citing genetic background of the strain as a crucial factor for acetone production. Since the p7EFPM strain achieved the higher acetone titer further characterization of the alternative acetone pathway was performed using this variant.

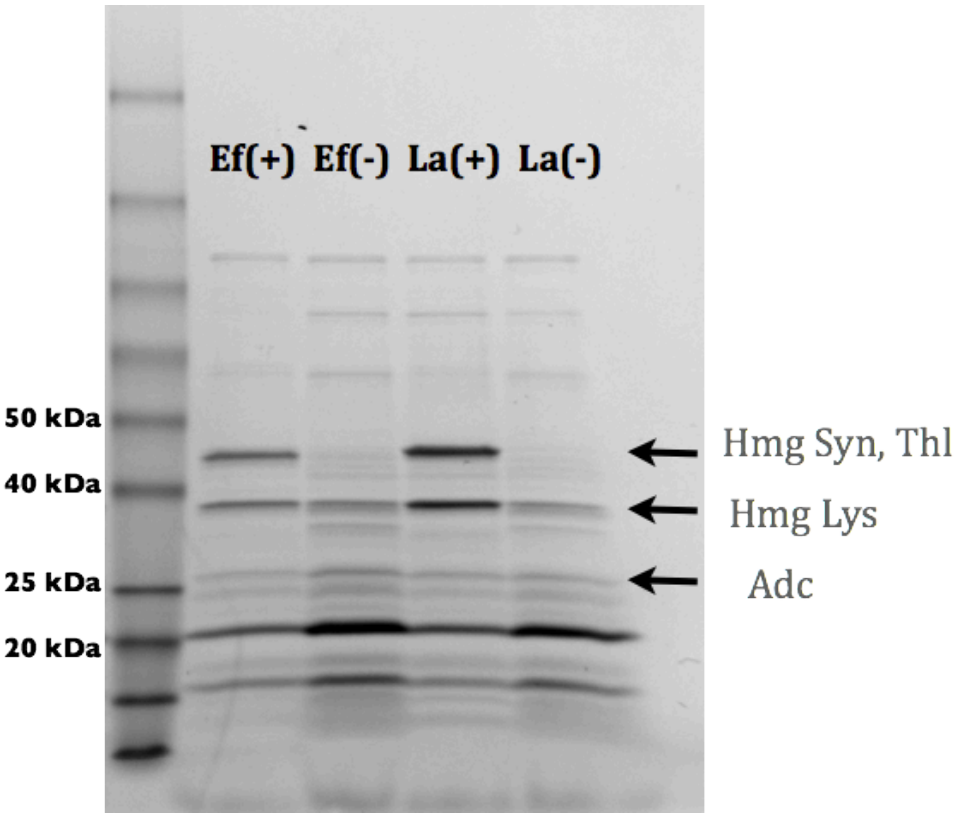


Figure 6-3: SDS-PAGE gel of *E. coli* strain BL21 (DE3) lysates harboring either the p7EFPM (Ef) or p7LAPM (La) plasmid. (+) indicates a culture induction of 1mM IPTG, while (-) denotes no induction of IPTG. Lysates were prepared 18 hours after induction of IPTG.

Table 6-4: Comparison of acetone production for variants p7EFPM and p7LAPM in various *E. coli* strains. Fermentation results from 5-mL shake flask experiments with 1mM IPTG induction and addition of 6 g L^{-1} glucose.

Variant	*E. coli* Strain	Acetate (mg L^{-1})	Ethanol (mg L^{-1})	Acetone (mg L^{-1})
p7EFPM	BL21	80	510	190
	BF7	70	620	140
	MG1655	2,540	310	30
p7LAPM	BL21	120	570	120
	BFA7	220	570	140
	MG1655	2,470	300	30

Table 6-5: Induction effects on major metabolite production in *E. coli* BL21 variants p7EFPM and p7LAPM.

Variant	Induced	Acetate	Ethanol	Acetone
p7EFPM	+	80	510	190
	-	420	190	0
p7LAPM	+	120	570	120
	-	400	180	0

6.3.2 Verifying the essential role of each gene in the pathway by single gene deletions

To verify that the observed variability in acetone production was not a function of a native enzyme and that all enzymes in the pathway are necessary for acetone production, plasmids with single gene deletions were constructed from p7EFPM see Table 6-3. These single gene deletion plasmids were transformed into strain BL21 and evaluated for acetone production in 5-mL shake flask fermentations. No acetone production was detected in the thiolase (p7ΔTHL), HMG-CoA synthase (p7ΔSYN) and HMG-CoA lyase (p7ΔLYS) deletion variants. Figure 6-4 shows that acetone production was however detected in the acetoacetate decarboxylase (p7ΔADC) deletion variant. Interestingly when the p7ΔADC variant was transformed into the BFA7 strain no acetone production was detected. No viable enzymes to explain this result could be identified from a homology blast search of the *C. acetobutylicum* acetoacetate decarboxylase gene in strain BL21. It is known that acetoacetate is naturally unstable under acid conditions and readily degrades to acetone. Therefore, this effect could be explained by differences in intercellular pH between the two strains.

5-mL shake flask fed-batch fermentations were examined to evaluate whether the deletion of the *adc* gene in the strain BL21 had any effect on final acetone titers. Figure 6-5A shows that there is no statistical difference in acetone production when grown on glucose between variants p7EFPM (0.85 g L^{-1}) and p7ΔADC (0.96 g L^{-1}) expressed in strain BL21, p-value = 0.52. Strain BFA7 p7EFPM had a lower acetone titer of 0.63 g L^{-1} and higher ethanol titer of 3.0 g L^{-1} compared to the BL21 variants. The BFA7 strain has knockouts for several of the competing metabolic pathways to ethanol and acetone production (e.g. acetate and lactate). A carbon balance analysis of the two strains of *E. coli* demonstrates that these knockouts in BFA7 enable more efficient production of the desired solvents with solvent yields on glucose of 0.65 of theoretical for the BFA7 strain as opposed to 0.51 in both variants of the BL21 strain, Figure 6-5B.

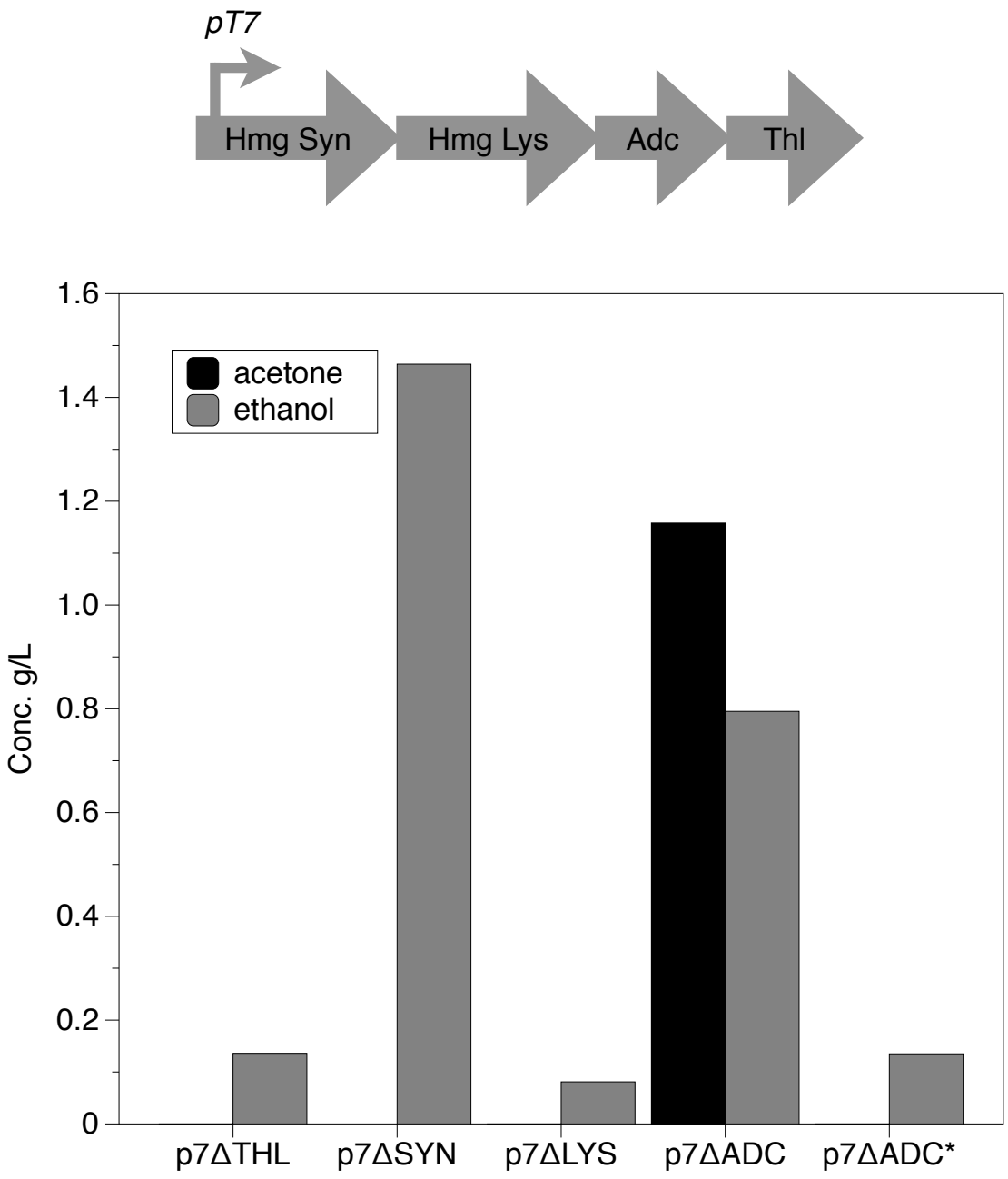


Figure 6-4: Single gene deletions of the alternative acetone pathway. Solvent concentrations for the single gene deletion variants fermented on 6 g L^{-1} glucose. Samples taken at least 24 hours after 1 mM IPTG induction. Δadc*, acetoacetate decarboxylase gene deletion plasmid expressed in *E. coli* strain BFA7.

6.3.3 Controlling the ethanol:acetone ratio

The ideal molar ratios of ethanol:acetone for the catalytic production of an enhanced gasoline blend and a jet fuel blendstock are quite different. To align with these ratios we envisioned engineering both the fermentation conditions and central metabolism of *E. coli*. By changing the substrate from glucose to the oxidize sugar acid gluconic acid acetone production in strain BFA7 p7EFPM was increased by almost 50% to 0.94 g L^{-1} while ethanol production decreased to 2.5 g L^{-1}. This effect is explained by the difference in reducing equivalent requirements for the production of acetone and ethanol, see Figure 6-6. The catabolism of gluconic acid generates 2 moles of pyruvate but only 1 mole of NADH and as opposed to the 2 moles generated from glucose. Additionally, each mole of pyruvate converted to ethanol requires either 1 or 2 moles of NADH depending on the metabolic pathway used, while acetone requires no NADH and consumes 2 moles of pyruvate. Therefore, in the more metabolically constrained BFA7 strain the cell must decrease the ratio of ethanol to acetone produced when the more oxidized gluconic acid is fermented. This effect is highlighted in Figure 6-5C whereby the ethanol:acetone ratio of BFA7 p7EFPM reduces from 6.0 to 3.4 by changing the carbon source from glucose to gluconic acid. Switching from glucose to gluconic acid in the BL21 strains had no effect on the ethanol:acetone ratio. This is because the BL21 strain is still capable producing acetate, which similar acetone requires no NADH for its production. Figure 6-7 shows that acetate production in the BL21 variants increased by ≥ 70% when the carbon source was changed from glucose to gluconic acid.

6.3.4 Evaluating different HMGS and HMGL variants

After all the strain and fermentation optimization acetone titers for variant p7EFPM increased by 5-fold but still remained below 1 g L^{-1}. To address this issue we evaluated acetone production with several additional HMG-CoA synthases and lysases (p7EFAB, p7LAAB, p7SAPM, p7SABS) in strains BL21 and BFA7, see Table 6-6. In all variants tested strain BL21 produced higher acetone titers. Of the new variants tested p7EFAB and p7LAAB produced the highest acetone titers of 0.54 and 0.77 g L^{-1} respectively.

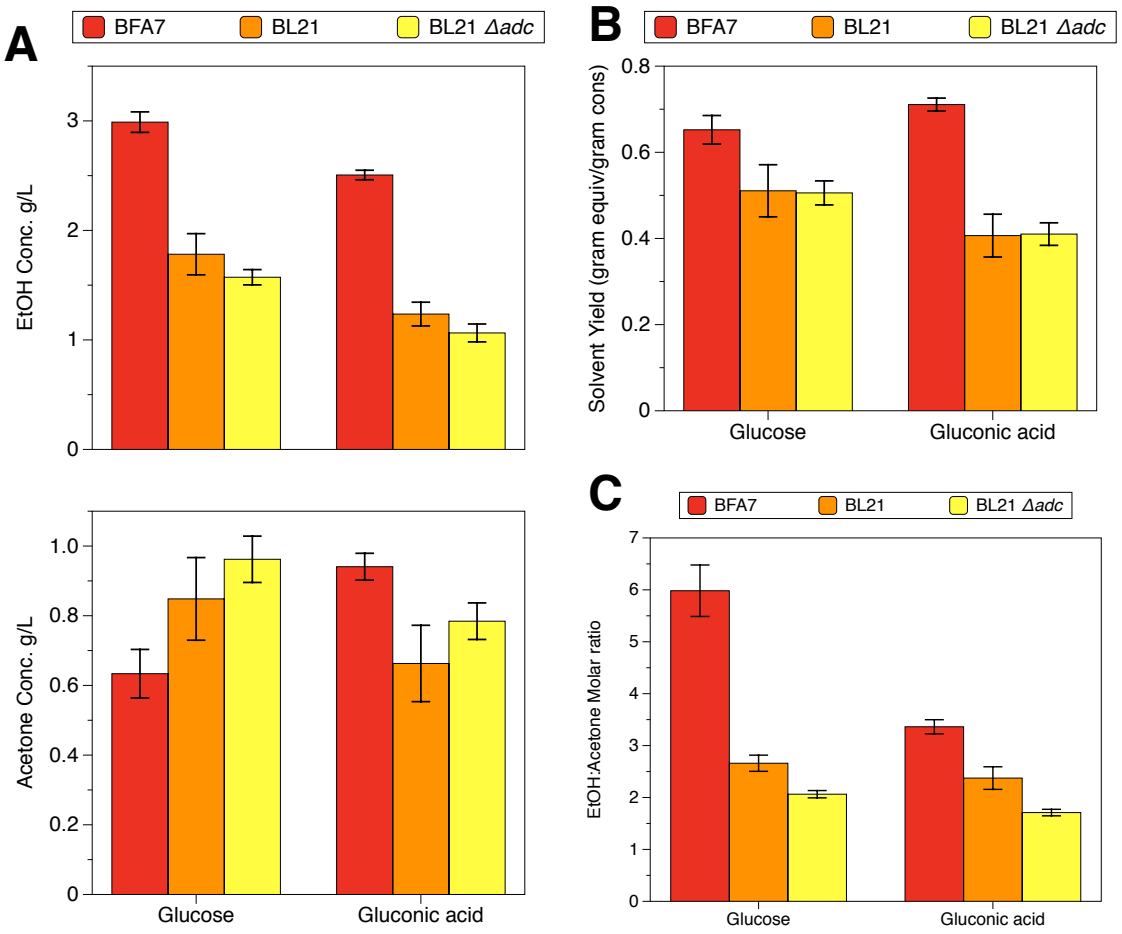


Figure 6-5: Fermentation results with glucose and gluconic acid as the major carbon source. Bars left to right for each condition are BFA7 (p7EFPM), BL21 (p7EFPM), BL21 (p7 ΔADC). (**A**) Final solvent titers, (**B**) solvent yields with respect to theoretical sugar consumption, (**C**) ethanol to acetone molar ratios. Values are the average of biological triplicates.

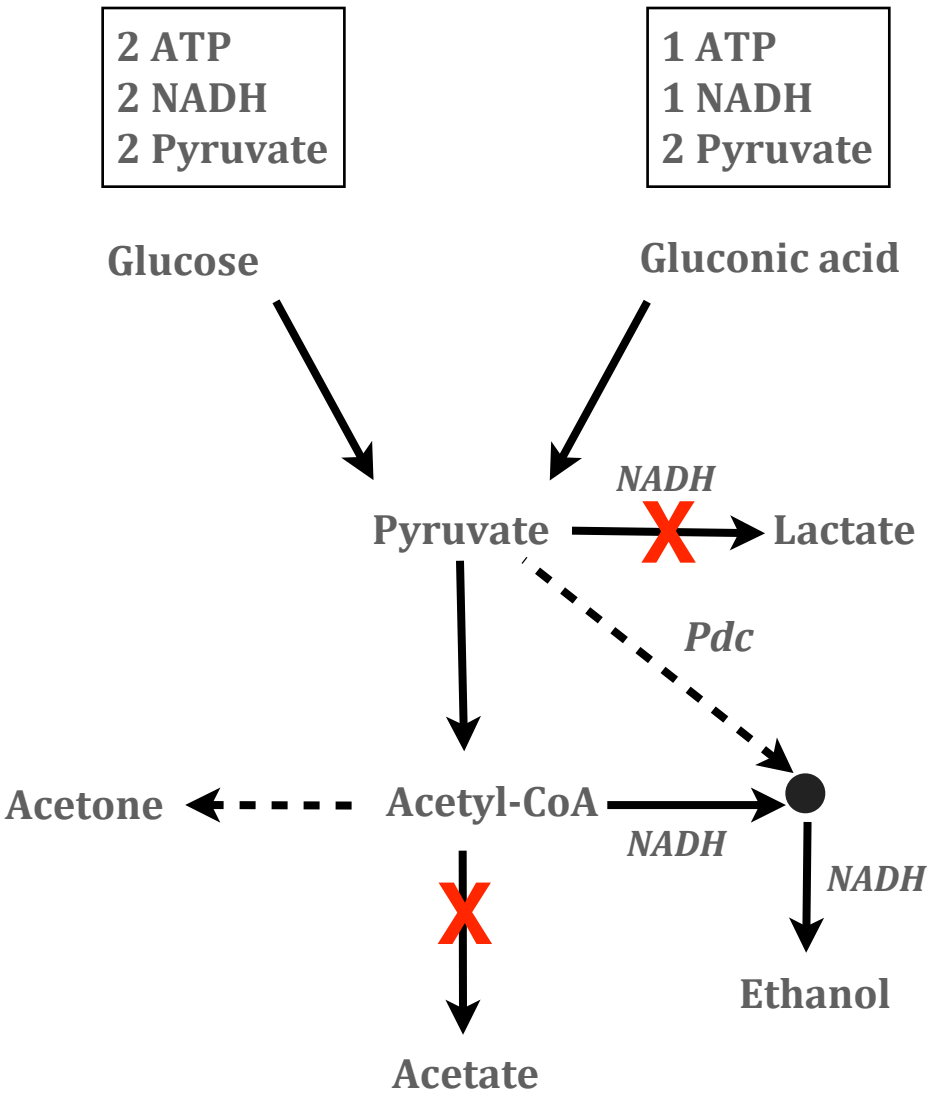


Figure 6-6: *E. coli* metabolic pathways for glucose and gluconic acid catabolism. Dashed lines represent non-native pathways in *E. coli*. Red "X" denotes knockouts found in *E. coli* strain BFA7. NADH requirements for all of the metabolic steps are depicted.

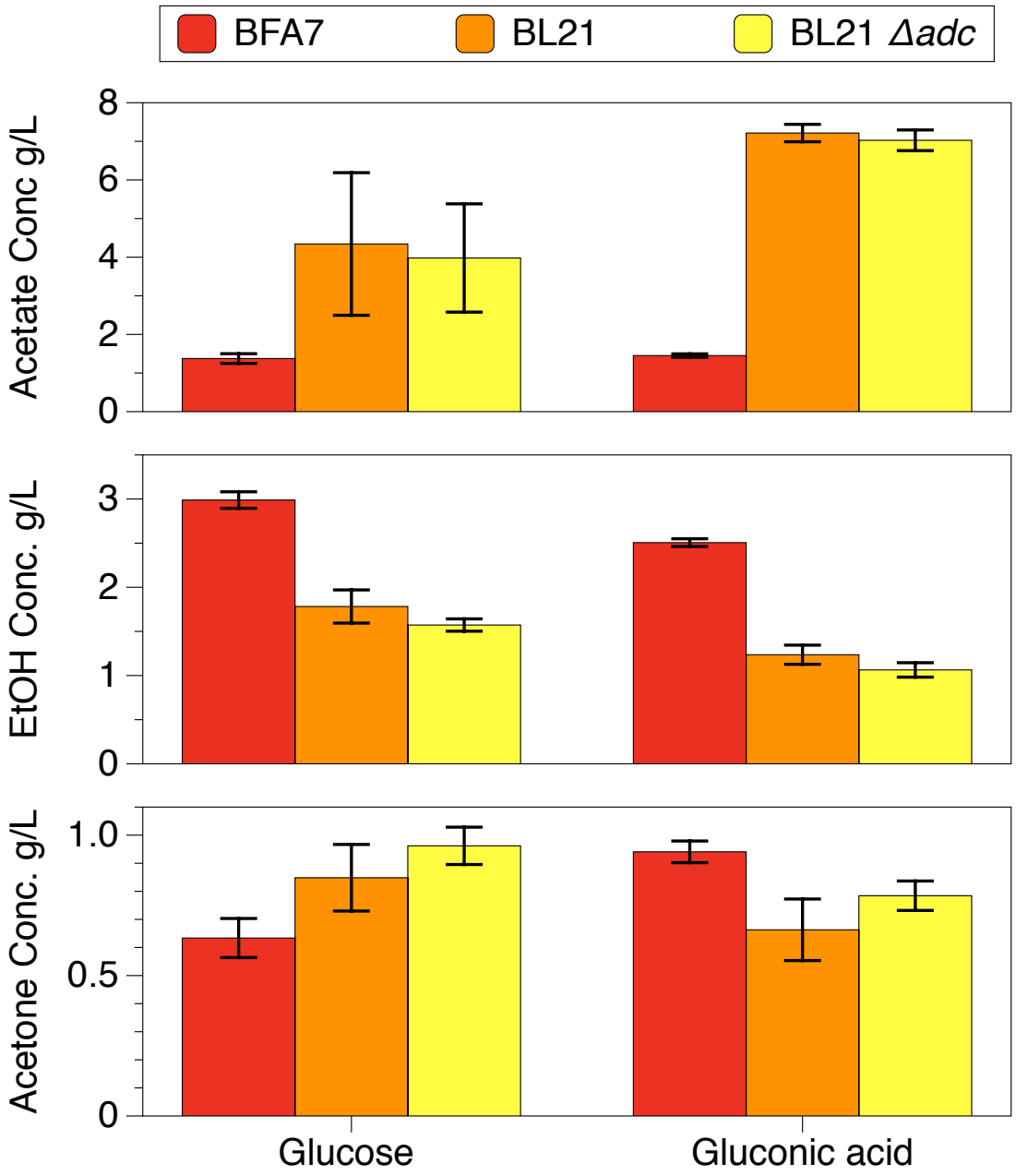


Figure 6-7: Major metabolite concentrations (acetone, ethanol, and acetate) from fermentations on glucose and gluconic acid as the major carbon source. Bars left to right for each condition are BFA7 (p7EFPM), BL21 (p7EFPM), BL21 (p7 ΔADC).

Table 6-6: Comparison of acetate and solvent production titers for new HMGS and HMGL variants in 5 mL shake flask fermentations. Values are the average of biological triplicates.

Variant	*E. coli* Strain	Acetate ($g\ L^{-1}$)	Ethanol ($g\ L^{-1}$)	Acetone ($g\ L^{-1}$)
p7EFAB	BL21	5.37 ± 0.30	1.7 ± 0.05	0.54 ± 0.07
	BFA7	0.73 ± 0.01	2.11 ± 0.02	0.08 ± 0.003
p7LAAB	BL21	2.94 ±0.04	1.77 ± 0.04	0.77 ± 0.03
	BFA7	0.617 ± 0.03	1.91 ± 0.06	0.14 ± 0.003
p7SAPM	BL21	5.687 ± 0.73	1.76 ± 0.04	0.41 ± 0.03
	BFA7	1.45 ± 0.03	3.15 ± 0.08	0.07 ± 0.02
p7SABS	BL21	8.52 ± 0.47	1.60 ± 0.06	0.27 ± 0.01
	BFA7	0.64 ± 0.01	2.01 ± 0.03	0.06 ± 0.001
p7EFPM	BL21	1.38 ± 0.13	1.78 ± 0.19	0.85 ± 0.12
	BFA7	4.34 ± 1.85	2.99 ± 0.09	0.63 ± 0.07

To improve the ethanol production in BL21 strains the alcohol/aldehyde dehydrogenase (*adhE*) gene from *E. coli* MG1655 was cloned into a pET24b plasmid (pK7AT) and co-transformed with the best acetone pathway variants (p7EFPM, p7EFAB, and p7LAAB). 5-mL shake flask fermentations of these BL21 variants were conducted in fed-batch mode with glucose or gluconic acid as the carbon source see Table 6-7. Ethanol production on glucose increased by 126%, 167%, and 111% for variants p7EFPM/pK7AT, p7EFAB/pK7AT, and p7LAAB/pK7AT compared to the strains not harboring the alcohol/aldehyde dehydrogenase plasmid. Ethanol production was further increased 4-fold to 15 g L^{-1} when plasmid pK7PA harboring the pyruvate decarboxylase and alcohol dehydrogenase from *Z. mobilis* was co-expressed with p7LAAB, Table 6-7. Acetone production in this strain was moderate at 1.8 g L^{-1} yielding a ethanol:acetone molar ratio of 10:1. Variant p7LAAB/pK7AT produced the highest acetone titer of 3.05 g L^{-1} when fermented on glucose, over a 3-fold improvement from variant p7EFPM.

Table 6-7: Product concentrations for improved ethanol variants in *E. coli* strain BL21. 5-mL shake flask fed-batch fermentations with glucose or gluconic acid as the major carbon source. Values are the average of biological triplicates.

Variant	Carbon Source	Acetate ($g\ L^{-1}$)	Ethanol ($g\ L^{-1}$)	Acetone ($g\ L^{-1}$)
p7EFAB /pK7AT	glucose	3.02 ± 1.100	4.54 ± 0.42	1.06 ± 0.27
	gluconic acid	6.75 ± 0.64	2.39 ± 0.11	0.78 ± 0.20
p7LAAB /pK7AT	glucose	3.32 ± 0.01	3.74 ± 0.33	3.05 ± 0.32
	gluconic acid	4.69 ± 1.43	2.53 ± 0.18	2.03 ± 0.27
p7EFPM /pK7AT	glucose	2.55 ± 0.50	4.03 ± 0.23	0.87 ± 0.13
	gluconic acid	3.13 ± 0.11	1.89 ± 0.02	0.49 ± 0.01
p7LAPM /pK7AT	glucose	4.68 ± 0.08	3.76 ± 0.22	0.58 ± 0.07
	gluconic acid	4.79 ± 0.23	2.00 ± 0.04	0.38 ± 0.01
p7LAAB /pK7PA	glucose	2.81 ± 0.78	15.13 ± 0.41	1.86 ± 0.07
	gluconic acid	4,25 ± 0.12	5.72 ± 0.46	0.81 ± 0.09

6.3.5 Optimizing fermentation for acetone and ethanol co-production

To further optimize culture conditions for higher solvent titers 750 mL fermentations were controlled in 1L working volume DasGIP (Eppendorff Hamburg, Germany) bioreactors. Initial fermentation studies showed that acetone production in *E. coli* could not be achieved under strictly anaerobic conditions (Figure 6-8A). This has also been previously demonstrated when expressing the native *C. acetobutylicum* and synthetic acetone pathways. When the air sparge-rate in the bioreactor was set to 10 $sL\ hr^{-1}$ making the environment fully aerobic, acetone was the only solvent produced (Figure 6-8B). Decreasing the sparge-rate to 1 $sL\ hr^{-1}$ kept the fermentation microaerobic enabling co-production of both acetone and ethanol (Figure 6-8C). Controlling the sparge-rate to ensure microaerobic conditions in the bioreactor a fed-batch fermentation of BL21 p7LAAB/pK7AT on glucose produced 3.90 $g\ L^{-1}$ acetone and 4.50 $g\ L^{-1}$ ethanol (Figure 6-9A). Switching the carbon source to gluconic acid resulted in 5.1 $g\ L^{-1}$ acetone and 2.76 $g\ L^{-1}$ ethanol production, reducing the ethanol to acetone molar ratio to 0.7:1 (Figure 6-9B).

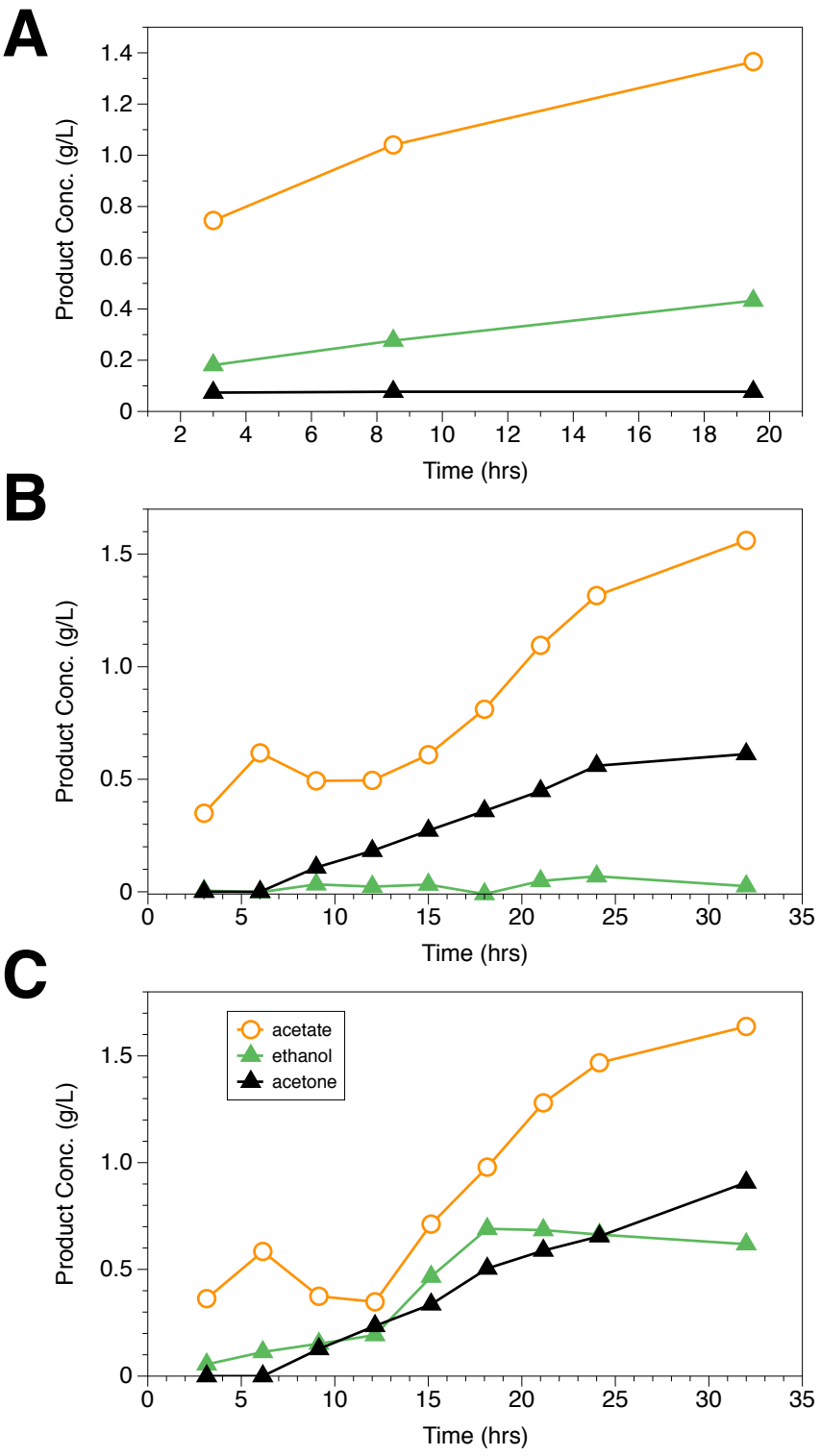


Figure 6-8: Air sparge rate effect on solvent and acetic acid production in *E. coli* BL21 p7EFPM. **(A)** anaerobic conditions in an oxygen free glove bag. **(B)** aerobic conditions, air sparge rate = 10 sL hr^{-1}. **(C)** micro-aerobic conditions, air sparge rate = 1 sL hr^{-1}.

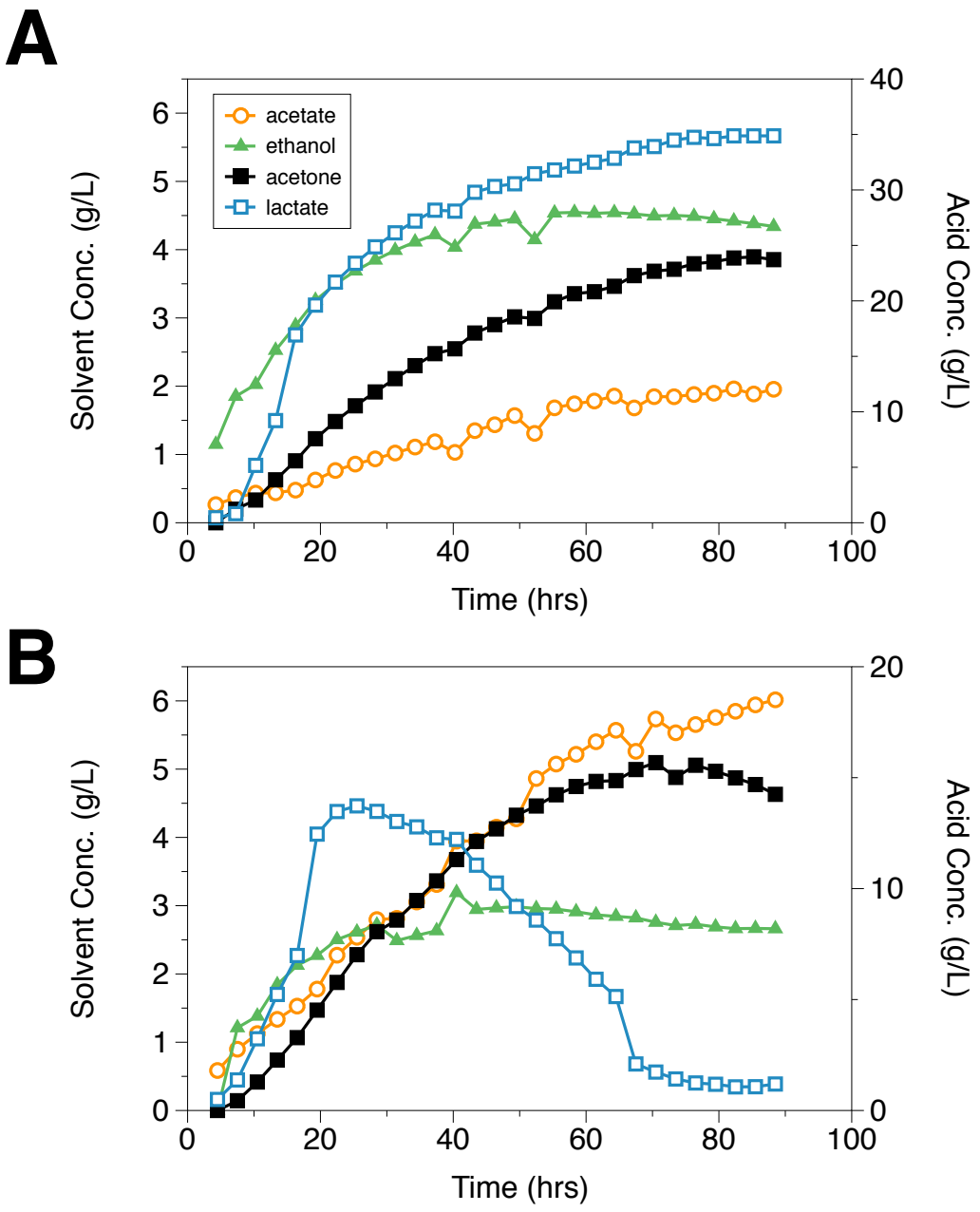


Figure 6-9: Time course of major metabolites in 750-mL pH controlled fed-batch fermentations of *E. coli* BL21 variant p7LAAB/pK7AT. Cultures fed with concentrated (40 wt%) glucose **(A)** and gluconic acid **(B)**. Air sparge rate controlled at 1-2 sL hr^{-1} and pH controlled ≥ 6.0 with 28 wt% ammonium hydroxide.

6.4 Conclusions

The co-production of acetone and ethanol with subsequent catalytic upgrading to C_5 to C_{15} oxygenates has major implications for the ethanol industry to address gasoline and jet blend limitations. Here we demonstrate an acid-independent pathway for the production of acetone through the mevalonate precursor hydroxymethylglutyrl-CoA. Acetone production was detected in all strains of *E. coli* tested however strain background contributed significantly to final titers and acetoacetate decarboxylation activity. Acetone production was predictably increased with respect to ethanol production when the primary carbon source was changed from glucose to gluconic acid in the metaboiically constrained BFA7 strain. Gluconic acid can be readily produced from glucose with glucose oxidase. This enabled control of the ethanol:acetone ratio, which is critical to align with the chemical catalysis. Several variants of the HMG-CoA synthase and HMG-CoA lyase were tested for acetone production, the HMGS from *L. acidophilus* and HMGL from *A. baumanii* produced the highest titer. Ethanol titers and ethanol:acetone ratios could be increased by the co-expression of either the aldehyde/alcohol dehydrogenase from *E. coli* MG1655 or pyruvate decarboxylase and alcohol dehydrogenase from *Z. mobilis*. After optimization of air sparge rates in pH controlled fed-batch fermentations acetone titers were improved to *C. acetobutylicum* wild-type titers. The combinations of these metabolic, strain, and fermentation engineering strategies improved acetone titers by over 35-fold and enabled ethanol:acetone molar ratios to range from 0.7:1 to 10:1.

6.5 Synthesized gene sequences

L.a. HMG-CoA Synthase synthesized with C. acetobutylicum and E. coli codon optimization:

ATGCAAGTTGGAATAGATAAAATAGGATTTTTTACACCTAATAAATATGTTGA
TATGGTTGATTTAGCACATGCAAGAAATCAAGATCCTAATAAATTTTTAATAG
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TTGAAGGACAAGATGAAGAAACAACAAAAAAACTTGAAAATAGTTTTGAAGC
AGCAAAACAATTAAGTAGAAGAGTTGGAAATGTTTATACAGCAAGTTTATATC
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GTTACAGATTATGAAAATGAAATAGATCAAGCAAAAGATATGGCATTATTAGA
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TAGAAGATCCTGAAGATGGACAAGAATTAGATAGTGATGATGAAAAAGGTAC
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E.f. HMG-CoA Synthase synthesized with C. acetobutylicum and E. coli codon optimization:

ATGACAATAGGAATAGATAAAATATCATTTTTTGTTCCTCCATATTATATAGAT
ATGACAGCATTAGCTGAAGCAAGAAATGTTGATCCTGGAAAATTTCATATAG
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CATTAGCATTTCATATACCTTATACAAAAATGGGAAAAAAAGCATTATTAGCA
AAAATAAGTGATCAAACAGAAGCAGAACAAGAAAGAATATTAGCAAGATATG
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TCAAATAGGATTATTTAGTTATGGAAGTGGTGCAGTAGCAGAATTTTTTACAG
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AGAAACATTAGATACAGATATAGATCAAACATTAGAAGATGAATTAAAATATA
GTATAAGTGCAATAAATAATACAGTTAGAAGTTATAGAAATTAA

S.a. HMG-CoA Synthase synthesized with C. acetobutylicum and E. coli codon optimization:

ATGACAATAGGAATAGATAAAATAAACTTTTATGTTCCTAAATATTATGTTGAT
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GAATAA

P.m. HMG-CoA Lyase with flanking regions synthesized with C. acetobutylicum and E. coli codon optimization:

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CAGCATTAGCAGGACATTTTCATGATACATGGGGAATGGCAATAGCAAATGT
TCATGCAGCTTTAGCACAAGGTGTTAGAACATTTGATAGTAGTGTTGCAGGA
CTTGGAGGATGTCCATATAGTCCTGGTGCAAGTGGAAATGTTGCAACTGAAG
ATTTACTTTATTTACTTCATGGATTAGGATATAGTACAGGTGTTGATCTTGAA
GCAGTAGCTCAAGTTGGAGTTAGAATAAGTGCACAACTTGGAACAGCAAATA
GAAGTAGAGCAGGATTAGCATTAGCTGCAAGAAGTGCAAGAGAACATTAAGT
TTAAACCCGCCTAAACTGCCCG

B.s. HMG-CoA Lyase with flanking regions synthesized with C. acetobutylicum and E. coli codon optimization:

GCTATCAATTTTGTTATTAAGGAGGAACATATCATATGCCTTATCCTAAAAAA
GTTACAATAAAAGAAGTTGGACCTAGAGATGGATTACAAAATGAACCTGTTT
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AGGACTTGGAGGATGTCCATATGCACCTGGAAGTAGTGGAAATGCAGCTAC
TGAAGATATAGTTTATATGTTAGAACAAATGGATATAAAAACAAATGTTAAATT
AGAAAAATTACTTAGTGCAGCAAAATGGATAGAAGAAAAAATGGGAAAACCT
TTACCTAGTAGAAATCTTCAAGTTTTTAAAAGTAGTTAAGTTTAAACCCGCCT
AAACTGCCCG

A.b. HMG-CoA Lyase with flanking regions synthesized without codon optimization:

GCTATCAATTTTGTTATTAAGGAGGAACATATCATATGTCTGAATTTGTTAAAA
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TAATTATGCAAATGCATATTGGCAAACAAAATGTGCATAAGTTTAAACCCGCC
TAAACTGCCCG

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