ENGINEERING *ESCHERICHIA COLI* FOR BIOFUEL PRODUCTION

Benchmarking with Butanol in Microbes

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The *Clostridium acetobutylicum* microorganism has a natural butanol fermentative pathway which can also be grafted into engineered *Escherichia coli*. In Figure 1, the red arrow from Acetyl-CoA is the "natural" pathway which is what *C. acetobutylicum* uses for 1-butanol production. The green arrow is the engineered pathway grafted in *E. coli* for 1-butanol production. Representative titers from engineered *E. coli* and native Clostridia for 1-propanol and 1-butanol production are presented in Table 1.

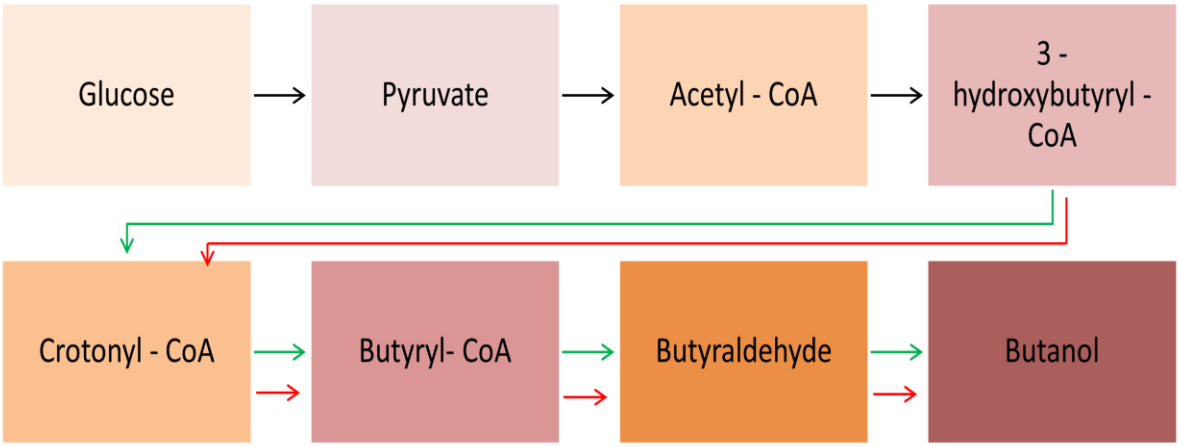

Figure 1 - Simplified 1-butanol fermentative pathway in natural *C. acetobutylicum* and engineered *E. coli*

Table 1 – 1-Propanol or 1-butanol production titers from engineered *E. coli* and native Clostridia

Engineered *E. coli* strains	Carbon source[a]	Aerobicity	1-Propanol[b]	1-Butanol[b]
E. coli CPC-PrOH3	Glycerol	Anaerobic	2.48	ND
E. coli CPC-PrOH3	Glucose	Anaerobic	0.47	ND
E. coli CPC-BtOH2†	Glycerol	Anaerobic	ND	ND
E. coli CPC-BtOH2	Glucose	Anaerobic	ND	1.17
Native clostridial strains	**Carbon source[a]**	**Aerobicity**	**1-Propanol[b]**	**1-Butanol[b]**
C. acetobutylicum ATCC 824†	Glycerol	Anaerobic	ND	ND
C. acetobutylicum ATCC 824	Glucose	Anaerobic	ND	11

Notes:

a – Batch cultivation using 30 g/L of respective carbon source

b – 1-propanol or 1-butanol titer in g/L at the end of batch cultivation

†Engineered butanogenic *E. coli* strain (CPC-BtOH2) and *C. acetobutylicum* cannot be cultivated using glycerol as the sole carbon source

The Global Energy Crisis of Engineering Biofuels

A great fraction of the world's energy requirements are presently met through the unrestricted use of carbonaceous fossil-derived fuels (primarily consisting of coal, oil, and gas) [1-3]. Nevertheless, the prognosticated demise of natural petroleum coupled with the mounting environmental and socioeconomic concerns associated with their use have led to investigations on the development of sustainable and environmentally friendly biofuel platforms. Biofuels are defined as fuels (either as a gas or liquid) produced by a renewable biological resource [1, 2, 4-6]. The biological resources (often termed, biomass) can be directly burned to obtain energy or it can serve as a feedstock in a bioprocess and converted into a liquid or gaseous energy carrier [2].

Biomanufacturing is one of the major activities for industrial biotechnology. Fundamentally, this involves the use of biological cells (in particular, microorganisms) with a specialized pathway. These cells are adopted as a biocatalytic system (sometimes referred to as cell factories) [1, 4-6]. There is even an open access scientific journal called Microbial Cell Factories (www.microbialcellfactories.com) to convert a low-value substrate feedstock into a high-value metabolite of interest. The organism can be a native producer of the target product or can be genetically manipulated to drive production of a novel product. Popular organisms for biomanufacturing of biofuels include Clostridial species, *Zymomonas mobilis*, *Saccromyces cerevisiae* and *Escherichia coli*. Among numerous microorganisms, the bacterium *E. coli* is considered the most popular and user-friendly organism for biomanufacturing [2, 7, 8]. This is primarily due to the well-characterized biological system and well-developed technologies for strain manipulation and bioprocessing.

However, like many other microorganisms, *E. coli* cannot produce every industrially useful metabolite. Recent technological advances in genetic engineering and metabolic engineering offer the possibility of genetically tailoring *E. coli* strains to produce non-native metabolites, an approach known as genetic/metabolic engineering biology [2].

Recently, the biological production of ethanol has become quite popular as a means to lessen the dependence on conventional fuels. The attractiveness of bioethanol as a transportation fuel stems from its high production efficiency, high octane rating (108) [1, 2, 4], and eco-friendly benefits. The corn-ethanol program in the United States and the sugarcane-ethanol program in Brazil have annual production of approximately 50 and 27 billion liters, respectively [2].

While bioethanol production platforms appear to be the most popular and successful in the biofuel market, as a fuel, ethanol possesses numerous unfavorable attributes such as low energy content, incompatibility with existing storage and distribution infrastructures, and hygroscopicity. As a result, 1-butanol, a high-energy four-carbon alcohol, is an attractive alternative to bioethanol as it possesses physicochemical properties that more closely resemble those of conventional gasoline [1, 2, 4-6]. It is safe to handle, less corrosive, less volatile, and environmentally friendly. 1-butanol can also be blended with gasoline or diesel fuel at any ratio. In addition, the pipeline systems for 1-butanol transport appear to be compatible with the existing one for ethanol distribution [1, 2, 4-8].

1-Butanol Production

The anaerobic microorganism *C. acetobutylicum* has a natural acetone-butanol-ethanol fermentative pathway. This particular organism, and its ability to produce potential fuels, was discovered in the early 20th century. In fact, the exploitation of clostridia for large-scale production of commodity chemicals represents one of the first worldwide industrial bioprocesses (known as the Weizmann process, see Figure 2). Prior to the dominance of the current petrochemical industry, large-scale production of acetone, and later 1-butanol, as solvents (referred to as AB fermentation) was carried out by several species of *Clostridium* during the World War 2 era [1, 2 4-6].

Nevertheless, due to volatility in the costs of molasses and maize feed stocks, coupled with the establishment of more economical petrochemical processes during the late-1950s, this led to the eventual downfall of fermentative AB production. The advancement of genetic engineering tools and strategies, however, make it possible to tailor *C. acetobutylicum* for biofuel production while overcoming many of the limitations faced previously [1, 4, 5]. Namely, issues such as high cost, tedious cultivation and lack of characterization of the organism no longer hinder the possibility of its use. Not only is this feasible in clostridial species, but it is also possible to graft many of the enzymes required for production into non-native butanol producing species, such as an *E. coli*, via rational metabolic engineering.

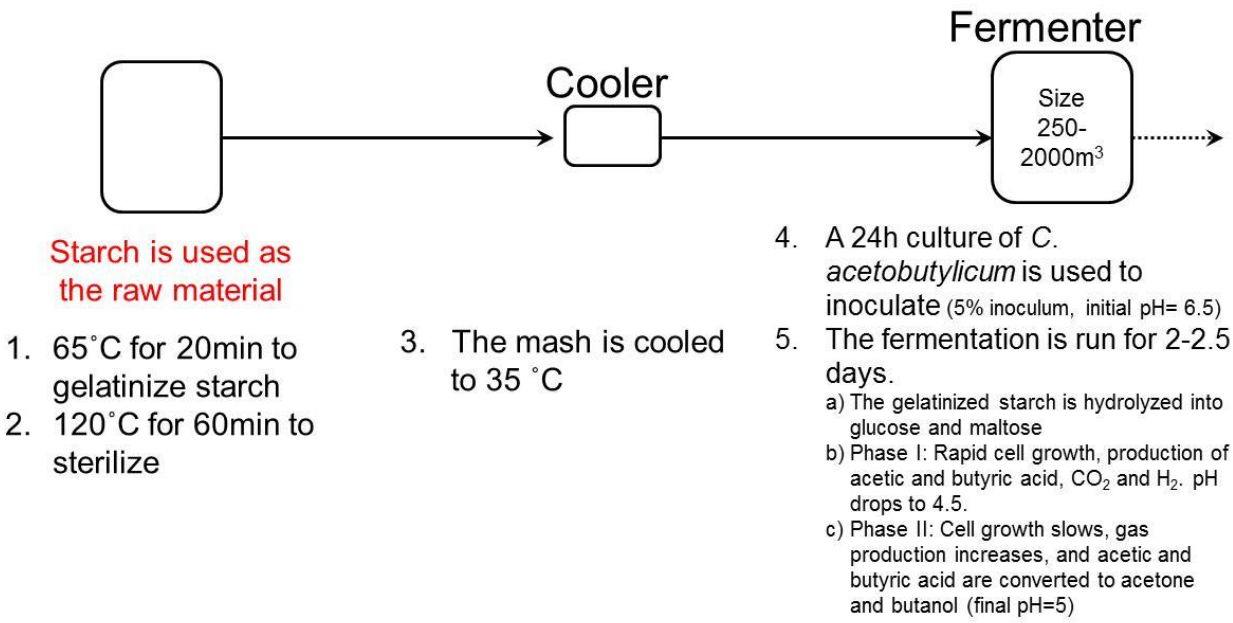

Figure 2 - Schematic representation of the Weizmann process. The process employs the bacterium *C. acetobutylicum* to ferment sugars (i.e. starch) into solvents ethanol, 1-butanol, and acetone.

Changing culture parameters to produce 1-butanol

Although *E. coli* is a facultative aerobe, it has to be cultivated under anaerobic conditions for the production of 1-butanol (this is because the enzymes required for this bioprocess are oxygen sensitive). To cultivate *E. coli* under anaerobic conditions, the cells must be first grown under aerobic conditions (to obtain biomass) and then transferred to an anaerobic medium. Given that *E. coli* is not an obligate anaerobe (such as *C. acetobutylicum*), it cannot sustain prolonged growth under anaerobic conditions unless supplemented with complex and enriched nutrients. See Appendix A for media considerations.

Problem Statement

Based on the above information:

1. Compare and contrast the media requirements for the two organisms. Explain why each component is needed.

2. Identify the benefits and drawbacks of each process used for the production of bio-butanol.

References

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Appendix A - Media Considerations

Table 2 – Anaerobic and aerobic media required to cultivate *E. coli* and *C. acetobutylicum*

***E. coli* media (engineered system):**	***C. acetobutylicum* media (natural system):**
Step 1: Grow *E. coli* aerobically in the following medium: *Per liter:* D-glucose*: 20 g $Na_2HPO_4 \cdot 7H_2O$: 12.8 g KH_2PO_4: 3 g NaCl: 0.5 g NH_4Cl: 1 g *(The pH of 7.0 is adjusted through the use of a phosphate buffer system)* And the following micronutrients: $MgSO_4$ 1 mM $CaCl_2$ 100 µM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ $3x10^{-9}$ M H_3BO_3 $4x10^{-7}$ M $CoCl_2 \cdot 6H_2O$ $3x10^{-8}$ M $CuSO_4 \cdot 5H_2O$ $1x10^{-8}$ M $MnCl_2 \cdot 4H_2O$ $8x10^{-8}$ M $ZnSO_4 \cdot 7H_2O$ $1x10^{-8}$ M $FeSO_4 \cdot 7H_2O$ $1x10^{-6}$ M **Step 2:** Once an optimal cell density has been obtained, the cells are harvested, washed and re-suspended in an anaerobic medium. The anaerobic medium is very similar to the media above, but it is also fortified with the following components: *Per liter:* Yeast extract: 5 g And the following micronutrients: Casamino acids: 2 g (Casmino acids are a mixture of amino acids and small peptides obtained from the hydrolysis of casein)	Given that *C. acetobutylicum* is an obligate anaerobe; it can be directly propagated in an anaerobic medium. **Step 1:** Grow *C. acetobutylicum* anaerobically in the following medium: *Per liter:* D-glucose*: 20 g Casein hydrolysate: 4 g K_2HPO_4: 5 g KH_2PO_4: 5 g *(The pH of 6.9 is adjusted through the use of a phosphate buffer system)* And the following micronutrients: $MgSO_4$ 1 mM $MnSO_4 \cdot 4H_2O$ 1 mM $FeSO_4 \cdot 7H_2O$ $1x10^{-6}$ M Vitamin BX 1 mM Vitamin B1 2 mM Vitamin H 1mM *Note: carbon sources, other than glucose can be used such as glycerol, xylose, or corn steep liquor, etc.