Improving Butanol Fermentation To Enter the Advanced Biofuel Market

Bryan P. Tracy

Elcriton Inc., New Castle, Delaware, USA

ABSTRACT **1-Butanol is a large-volume, intermediate chemical with favorable physical and chemical properties for blending with or directly substituting for gasoline. The per-volume value of butanol, as a chemical, is sufficient for investing into the recommercialization of the classical acetone-butanol-ethanol (ABE) (E. M. Green, Curr. Opin. Biotechnol. 22:337–343, 2011) fermentation process. Furthermore, with modest improvements in three areas of the ABE process, operating costs can be sufficiently decreased to make butanol an economically viable advanced biofuel. The three areas of greatest interest are (i) maximizing yields of butanol on any particular substrate, (ii) expanding substrate utilization capabilities of the host microorganism, and (iii) reducing the energy consumption of the overall production process, in particular the separation and purification operations. In their study in the September/October 2012 issue of *mBio*, Jang et al. [mBio 3(5):e00314-12, 2012] describe a comprehensive study on driving glucose metabolism in *Clostridium acetobutylicum* to the production of butanol. Moreover, they execute a metabolic engineering strategy to achieve the highest yet reported yields of butanol on glucose.**

In efforts to develop renewable alternatives to petroleum-based chemicals and fuels, there is rejuvenated interest in clostridial acetone-butanol-ethanol (ABE) fermentation. ABE fermentation was first commercialized in the 1910s in the United Kingdom for the production of acetone, which was used as a solvent in the production of cordite, a smokeless ammunition propellant (1). The ABE process spread across the globe during the first and second World Wars and was further capitalized upon for the production of butanol (i.e., 1-butanol), which remains an important feedstock for the production of paints and coatings today. In the 1950s, however, the oxo process, which hydroformylates and hydrogenates propylene to butanol, displaced the ABE process as the most economically viable method of butanol production.

Fast-forward to today, when the ABE process is competitive with the oxo process for manufacturing butanol to be sold as a chemical (1). Butanol is also used as an alternative liquid transportation fuel and blending agent and has demonstrably better physical properties than ethanol (2). Moreover, it can be catalytically converted into jet fuels (3).

However, before butanol can stake a significant claim in the advanced biofuel markets, the economics of ABE manufacturing must improve. Three areas of greatest opportunity are (i) reaching theoretical maximum yields of butanol, (ii) expanding substrate utilization such that a diversity of feedstocks can be used, and (iii) minimizing energy consumption during separation and purification. Significant advances are being made toward these goals, as briefly described below.

Feedstock is typically the greatest operating expense of ABE fermentation. Thus, employing the cheapest feedstock and reaching the theoretical maximum conversion to butanol will result in the optimal ABE process. Papoutsakis previously calculated the theoretical maximum to be 0.939 mol butanol/1 mol glucose, which is based upon ABE fermentation equations (4). More recently, Fast and Papoutsakis calculated that this could be further improved to ~1.33 mol butanol/1 mol glucose by mixotrophic fermentation whereby glucose and CO_2 (evolved during fermentation) are fully utilized in the presence of sufficient H_2 (5). As reviewed previously (6), metabolic engineering studies in clostridia have improved yields to various degrees. However, a major limitation is the production of acetone and CO_2 during acid reassimilation, which Jang et al. investigated and minimized for butanol production in their study in *mBio* (7).

Jang et al. investigate the two butanol production pathways in *Clostridium acetobutylicum* ATCC 824 (i.e., cold and hot channel[s]) and propose a metabolic strategy to improve yields of butanol. The cold channel refers to the metabolic process of reassimilating organic acids, acetate and butyrate, into acetyl and butyryl coenzyme A (CoA), respectively, through the CoA transferase (CoAT) pathway. Acetyl-CoA is then reduced to ethanol or converted to butyryl-CoA through condensation and a series of hydrogenation reactions. Butyryl-CoA is then reduced to butanol. For every mole of reassimilated acid, 1 mol of acetoacetate is generated, which is then decarboxylated into acetone and CO_2, and detracts from butanol yields. The hot channel avoids acid reassimilation and refers only to the direct route of acetyl-CoA condensation to butyryl-CoA and subsequent reduction to butanol. Consequently, the hot channel avoids yield losses to acetone and CO_2.

Previous studies attempted to reduce the cold channel and force carbon flux through the hot channel. Tummala et al. (8) decreased cold-channel flux by separately downregulating the activity of the two CoA transferase subunits (CoAT A and B, coded, respectively, by the genes *ctfA* and *ctfB*) via antisense RNA (asRNA) expression from a multicopy plasmid. Acetone production was reduced, suggesting that the cold channel was impaired. However, acetate production increased and butanol production decreased, suggesting that the hot channel was unchanged or even impaired. Subsequently, they realized that the primary, bifunctional alcohol/aldehyde dehydrogenase (i.e., ADHE1, coded by *adhE1*) is translated from the same transcript as *ctfA*/*B*. Conse-

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Address correspondence to BryanTracy@elcriton.com.

quently, downregulating the activity of CoAT A and B also likely downregulated the activity of ADHE1. Tummala et al. performed another study (9) in which they concurrently overexpressed *adhE1* along with the *ctfB* asRNA from a multicopy plasmid. The resulting strain exhibited reduced acetone levels, wild-type (WT) ATCC 824 levels of butanol, and 23-fold-higher levels of ethanol. Since ADHE1 catalyzes the production of both ethanol and butanol, a follow-up study (10) attempted to reduce and increase the pool of acetyl- and butyryl-CoA by increasing the activity of the thiolase enzyme. Thiolase catalyzes the condensation of two acetyl-CoA molecules to acetoacetyl-CoA. Additionally, they varied the promoters used for overexpressing *adhE1* and the asRNA against *ctfB*. Neither approach was successful at optimizing the hot channel. Instead, record titers of ethanol (305 mM) were again witnessed.

A significant limitation in earlier studies was access to genetic tools. Five years ago, gene disruptions were difficult to perform. Fortunately, and as reviewed elsewhere (11), genetic tools have improved, and a recent article by Al-Hinai et al. (12) demonstrates the latest of these improvements. Accordingly, a more recent study (13) attempted to reduce the cold-channel flux by disrupting the acetoacetate decarboxylase gene, *adc*, in a mutant *C. acetobutylicum* strain designated 2018. With pH control, the *adc* mutant exhibited reduced acetone levels and 2018 levels of butanol, but it also exhibited high titers of acids, particularly acetate. Since butanol production requires sufficient reducing equivalents (e.g., NADH), they decided to supplement cultures with methyl viologen (MV) to increase the NADH throughput. As a result, acetone remained low, butanol levels remained constant, and acid production was reduced. However, acid levels were still not as low as they were in the 2018 cultures, and butanol yields maxed out at 70.8%.

Jang et al. present a more comprehensive and systematic analysis of deleting cold-channel pathways individually and in combination and overexpressing genes in hot-channel pathways (7). In accordance with previous investigations, they noticed higher titers and yields of acids when acid reassimilation was disrupted. Interestingly, the best approach for improving the ratio of hot-to cold-channel fluxes did not include modifying the acid reassimilation path. Rather, they determined that a particular combination of acid formation gene disruptions was optimal, which they combined with overexpression of a mutant *adhE1* that they proposed had increased affinity for NADPH. Finally they optimized continuous cell culture conditions to demonstrate the highest reported butanol yields to date at 0.76 mol butanol/1 mol glucose. Interestingly, they did not witness an increase in ethanol production with the overexpression of the mutant *adhE1*, as seen by two previous studies (9, 10). There is no report of the mutated *adhE1* having increased affinity for butyryl-CoA over acetyl-CoA, but these results suggest that this could be the case. Additionally, acid production was not fully eliminated, which is consistent with even the earliest acid formation gene disruption study in ATCC 824 (14). How acids are still formed is unclear, but Jang et al. provide some potential explanations. Overall, this study is an important step forward in improving butanol yields.

Returning to the issue of feedstock cost, another way to minimize that expense is commercializing strains that utilize a diversity of substrates. As colleagues and I recently reviewed (11), clostridia can directly utilize numerous monomeric sugars, complex carbohydrates (e.g., starch, hemicellulose, and cellulose), industrial wastes (e.g., cheese whey and glycerol), short-chain fatty acids (e.g., acetic, butyric and lactic acids), and gaseous feedstocks (e.g., CO and CO_2/H_2). Although ATCC 824 cannot ferment cellulose, it does contain a cellulosome (15) that appears to be nonfunctional due to a single inactive component, Cel48A (16). Assuming cellulosome function is reinstated, ATCC 824 would be an ideal platform for a consolidated bioprocess (CBP) for butanol production. CBP refers to combining enzyme production, hydrolysis, and fermentation into a single-unit operation, which has bioprocessing and economic advantages over separate unit operations (17). Lastly, there are acetogenic clostridia, such as *Clostridium carboxidivorans* (18), that perform chemoautotrophic growth on CO_2 and H_2 to form butanol. Other acetogens, such as *Clostridium ljungdahlii*, can be genetically engineered to produce butanol (19). Thus, there are opportunities to use syngas and industrial waste gases as cheap feedstocks. Additionally and as previously mentioned, CO_2 evolved during glucose fermentation can be fed to an acetogenic culture or used concurrently in the same culture to generate theoretical yields of 1.33 mol butanol/1 mol glucose (5).

Lastly, it was shown (20) that the energy required just for butanol separation from fermentation broth by distillation (the industry standard) can be multiples of the energy embodied in the actual butanol, which attenuates the argument for butanol as a fuel. The major issue is the low titers (i.e., concentrations) that are achievable in the fermentation broth due to butanol toxicity. Researchers have attempted to increase tolerance with limited success, as reviewed previously (21). Moreover, screening of known solvent-tolerant microorganisms revealed tolerance only up to 3% (i.e., 30 g/liter) (22). In order to reduce distillation energy consumption to one-third of the heat of combustion of butanol, broth concentrations would need to be greater than 4% (23). Consequently, ABE fermentation is a great candidate for alternative separation technologies, which as reviewed previously (23) include gas stripping, liquid-liquid extraction, adsorption, pervaporation, and combinations. Speaking from our development experience, we can achieve significant reductions in energy consumption. Furthermore, some technologies can be integrated with continuous or semicontinuous fermentation processes, which increases capacity of capital equipment. The question moving forward is whether such technologies are scalable and economically viable.

In conclusion, given continual research, development, and investment into the three areas mentioned above, butanol's entrance into the advanced biofuel market could be accelerated. Studies such as that by Jang et al. highlight that fact.

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