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# From the first drop to the first truckload: commercialization of microbial processes for renewable chemicals

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Fermentation of carbohydrate substrates by microorganisms represents an attractive route for the manufacture of industrial chemicals from renewable resources. The technology to manipulate metabolism of bacteria and yeast, including the introduction of heterologous chemical pathways, has accelerated research in this field. However, the public literature contains very few examples of strains achieving the production metrics required for commercialization. This article presents the challenges in reaching commercial titer, yield, and productivity targets, along with other necessary strain and process characteristics. It then reviews various methods in systems biology, synthetic biology, enzyme engineering, and fermentation engineering which can be applied to strain improvement, and presents a strategy for using these tools to overcome the major hurdles on the path to commercialization.

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## Introduction

The use of microbial fermentation to produce industrial chemicals from renewable resources has generated much interest lately, both in academia and industry. The rising cost of petroleum, concern over global $CO_2$ emissions, and a general desire for environmentally friendly processes render the market conditions ideal for introduction of sustainable chemicals. 1,3-Propanediol (PDO), polylactic acid (PLA), and poly-β-hydroxybutyrate (PHB) are examples of chemical precursors to novel polymeric materials which have recently been commercialized [1]. Other existing chemical intermediates are nearly commercial, including isobutanol, 1,4-butanediol (BDO), and isoprene [1]. However, the concept of carbohydrate fermentations to chemicals is not new. ABE (acetone–butanol–ethanol) fermentation using *Clostridium* species was the primary method for industrial butanol production from WWI through the 1960s, when it was eventually supplanted by petrochemical processes due to the abundance of cheap oil [2,3]. The ABE process suffered from a number of technical drawbacks. The organism has a complex life cycle that is intertwined with metabolic regulatory processes [4], and over time can undergo degeneration, or loss of solvent production [5]. However, the use of modern genetic tools and metabolic engineering approaches to solving problems such as these has created renewed interest in the ABE process. *Clostridium acetobutylicum* and *Clostridium beijerinckii* have been sequenced [6], and metabolic models have been utilized to help understand its complexities [7]. Although these species have been recalcitrant to directed genetic recombination and thus lag behind other industrial organisms in the development of tools for gene deletions and overexpressions, such methods have begun to emerge [8,9].

Like in the ABE example, many existing commercial processes are for the enhanced production of native metabolites such as amino acids and organic acids. Because no heterologous genes need to be introduced, such endeavors are of lower risk and have led to the establishment of 'platform chemicals' that can in theory be converted into useful compounds by subsequent chemical steps. One such chemical is succinic acid, a TCA cycle intermediate that is a natural fermentation product of many bacteria. Extensive literature exists reporting the successful engineering of *Escherichia coli* and other organisms to achieve near-theoretical yield of succinic acid [10]. On the basis of these successes, commercial processes have been announced by Myriant (http://www.myriant.com) using engineered *E. coli* [11], and by BioAmber (http://www.bio-amber.com/bioamber/en/products/succinic_acid) using a proprietary yeast strain that is acid tolerant, allowing direct production of the free acid rather than succinate salts [12].

Platform chemicals require subsequent downstream chemical steps to convert them to industrial chemicals currently used in industry; therefore, it is generally more economical to produce the target molecule directly by fermentation. Except in a few cases like *n*-butanol and lactic acid, intermediate chemicals important to the industry are not produced naturally from sugars. Thus the expression of heterologous genes or pathways, or even the introduction of entirely novel enzymatic conversions, is required. One of the most celebrated metabolic engineering success stories is the development and commercialization of an *E. coli* strain producing PDO by Genencor and DuPont [13]. Over a period of nearly a

decade, the PDO pathway, host metabolism, and fermentation process were optimized to achieve high PDO titer, yield, and productivity. PDO has been commercialized now for several years by DuPont and Tate & Lyle (http://www2.dupont.com/Sorona_Consumer/en_US), and used as a raw material for renewable carpet fiber. Other success stories in which commercially viable metrics have been announced include isobutanol from sugars via amino acid and 2-keto acid intermediates [14] by Gevo (http://www.gevo.com), adipic acid from renewable oils by Verdezyne [15], and BDO from sugars by Genomatica (http://www.genomatica.com). The latter is particularly noteworthy because it required introduction of two non-natural enzymatic conversion steps [16••].

## Considerations and challenges for commercialization of bioprocesses

There tends to be a large performance gap between early studies published in the open literature and the requirements for an economically attractive bioprocess for industrial chemical production. The most significant differences by far are in the key performance metrics of titer (g/L product), yield (g product per g substrate), and productivity (g/L h). Other considerations include the byproduct profile [17] and strain robustness [18]. In this section we address the challenges in achieving these metrics (Table 1), and in the next section present methods to overcome them.

For production of basic and intermediate chemicals with selling price near $1.00/lb or lower, the raw material cost of sugar represents a significant fraction of the value of the product even at near theoretical yield. Thus target yields generally need to be at least 80% of theoretical yield to even be considered for commercialization. In contrast, with a few notable exceptions cells do not direct a high percentage of carbon flux to these compounds, if any at all. Therefore, the metabolic engineer is faced with the challenge of redirecting a major portion of flux away from biomass production and natural fermentation products, and toward the product of interest. Limiting fluxes may be a result of poor expression, complex regulation at the gene or enzyme level, or unfavorable kinetics. Host central metabolism must also be engineered to remove byproduct pathways while maintaining redox and energy balance. Removal of common fermentation pathways under anaerobic or microaerobic conditions often results in excess NAD(P)H. A classic example is the anaerobic growth defect of an *E. coli ldhA pflB* double mutant due to excess NADH that cannot all be oxidized via flux through the reductive TCA cycle [19••]. Next to the sugar substrate, the most significant operating cost is in downstream processing. The higher the product titer in the fermentation broth, the easier and more efficient are the downstream separations. As a rule of thumb, 50 g/L is the minimum acceptable titer for any basic or intermediate chemical, and may be higher in many cases. These targets are often well above the tolerance limit of most of the microbes; for example, isobutanol is severely toxic to *E. coli* at 12.5 g/L [20•]. Volumetric productivity determines the rate at which the product can be formed in a given size fermenter, and thus dictates the overall fermentation volume needed for a given plant output. Fermenters are a significant portion of the plant capital investment, particularly for bacterial processes that require steam sterilization. Productivities below 2.0 g/L h are generally considered uncommercializable due to their high capital cost. A productivity of 3.5 g/L h has been reported for a

**Table 1**

**Summary of bioprocess performance metrics, impact on process economics, and major challenges in achieving them**

| Metric | Impact | Challenges | Tools and methods |
|---|---|---|---|
| Titer (g/L) | Downstream separations cost | Product toxicity | Adaptive evolution with sequencing and transcriptomics<br>Genome-wide methods (TRMR, SCALES, GTME) [26]<br>Efflux pumps |
| | | Enzyme inhibition | Protein engineering |
| Yield (g/g) | Substrate cost | Low pathway flux | Fermentation optimization<br>All approaches for productivity (below) |
| | | Byproducts | Metabolic modeling, fluxomics, transcriptomics<br>Fermentation optimization |
| | | Energetics | Metabolic modeling, transcriptomics, metabolomics<br>Fermentation optimization |
| | | NAD(P)H balance | Metabolic modeling, fluxomics, transcriptomics<br>Protein engineering<br>Fermentation optimization |
| Productivity (g/L h) | Bioreactor size | Bottleneck enzymes | Protein engineering<br>Protein scaffolding |
| | | Expression imbalance | Combinatorial expression testing<br>Promoter and RBS libraries<br>Transcriptomics<br>Proteomics |