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Research review paper

Recent advances and state-of-the-art strategies in strain and process engineering for biobutanol production by *Clostridium acetobutylicum*

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ABSTRACT

Butanol as an advanced biofuel has gained great attention due to its environmental benefits and superior properties compared to ethanol. However, the cost of biobutanol production *via* conventional acetone-butanol-ethanol (ABE) fermentation by *Clostridium acetobutylicum* is not economically competitive, which has hampered its industrial application. The strain performance and downstream process greatly impact the economics of biobutanol production. Although various engineered strains with carefully orchestrated metabolic and sporulation-specific pathways have been developed, none of them is ideal for industrial biobutanol production. For further strain improvement, it is necessary to develop advanced genome editing tools and a deep understanding of cellular functioning of genes in metabolic and regulatory pathways. Processes with integrated product recovery can increase fermentation productivity by continuously removing inhibitory products while generating butanol (ABE) in a concentrated solution. In this review, we provide an overview of recent advances in *C. acetobutylicum* strain engineering and process development focusing on *in situ* product recovery. With deep understanding of systematic cellular bioinformatics, the exploration of state-of-the-art genome editing tools such as CRISPR-Cas for targeted gene knock-out and knock-in would play a vital role in *Clostridium* cell engineering for biobutanol production. Developing advanced hybrid separation processes for *in situ* butanol recovery, which will be discussed with a detailed comparison of advantages and disadvantages of various recovery techniques, is also imperative to the economical development of biobutanol.

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1. Introduction

Global environment concern and incessant fluctuations of crude oil price led to a resurgence of interest in biofuels production derived from renewable resources by microbial fermentation (Xue et al., 2013a). Compared to bioethanol, biobutanol is undoubtedly superior as fuel or fuel additive due to its more similar properties to gasoline and better compatibility with gasoline and current infrastructure. In addition, butanol produced by microbial fermentation is also used as an artificial flavorant in many food and beverage industries, as well as an extractant for various manufactured chemicals and pharmaceuticals. For the sake of human health and safety, butanol used in human-related field is mandatorily required from microbial fermentation in many developed countries, which supports the development of biobutanol production. Butanol is largely used as an industrial intermediate, particularly for the manufacture of butyl acetate and other industrial chemicals. Currently, it is mainly industrially produced *via* petrochemical synthesis (Oxo process), in which propylene is hydroformylated to butyraldehyde, and then hydrogenated to produce n-butanol.

ABE production all over the world has undergone ups and downs in the past decade. In China, over a dozen plants were built for ABE production in 2009, with annual production capacity of >200000 tons (Ni and Sun, 2009; Jiang et al., 2015). Many plants and projects were launched to produce butanol, acetone and ethanol along with increasing price trend of crude oil, but have been retrofitted or shut down in the past four years due to the rapid drop of crude oil price. Currently, butanol from petrochemical synthesis is more economically competitive than that from microbial fermentation. Although butanol price responds strongly to the international oil price, biobutanol will have its own market place sooner or later. Furthermore, it is still receiving increasing attention as a renewable alternative transportation fuel on the global level. New technologies for cell and process engineering will accelerate the industrial development of biobutanol production in the future.

Solventogenic clostridia are well-studied strains due to their specific ability in alcohols (butanol and ethanol) and acetone biosynthesis. *Clostridium acetobutylicum* used in ABE fermentation regained lots of interests in academia and industry in recent years. Even though enormous efforts have been made on strain and process engineering, butanol concentration in fermentation broth is difficult to exceed 2% (w/v) in conventional batch fermentation. Therefore, compared to ethanol fermentation with ~15% (v/v) ethanol in final fermentation broth, intensive energy consumption for butanol recovery from the diluted broth is the main challenge faced for butanol fermentation. For the revival of biobutanol production, the butanol-producing capabilities of the strains and the corresponding downstream process engineering are key factors. In addition, the use of carbon sources derived from low-cost raw feedstocks such as agricultural residues and industrial wastes is also important, which requires butanol-producing strains to efficiently utilize various sugars such as xylose, fructose and arabinose together with glucose (Wang et al., 2014). Despite many decades of research, only a limited number of genes responsible for central carbon metabolism and sporulation related genes had been engineered through various methods including single cross-over recombination, group II intron retargeting methodology *etc.* (Sillers et al., 2008; Tummala et al., 2003; Heap et al., 2007). Furthermore, the performances of all these metabolic engineered strains are not satisfactory, even not as good as that achieved by the famous mutants *Clostridium acetobutylicum* JB200 from long-term adaption and *Clostridium beijerinckii* BA101 from chemical mutagenesis (Annous and Blaschek, 1991; Xue et al., 2012). These facts, showing less effectiveness of rational metabolic engineering compared to traditional random mutagenesis and screening, suggested hampered strain development due to limited genetic tools and understanding of molecular mechanism. With the advance of genome editing technology and system biology, strain development for ABE fermentation is expected to make a significant breakthrough in the future. In addition, *in situ* product recovery techniques effectively reduce the production cost by continuous removal and purification of inhibitory products during fermentation, which will also promote the development of biobutanol production.

Recent synthetic biology efforts have successfully introduced butanol-producing genes into various non-native producing microorganisms including *E. coli*, *cyanobacteria*, *S. cerevisiae*, *C. tyrobutyricum etc.* (Lan and Liao, 2012a, 2012b; Krivoruchko et al., 2013; Yu et al., 2011), and which were extensively reviewed elsewhere (Jin et al., 2014; Zheng et al., 2015; Huang et al., 2010; Van Rossum et al., 2016). Although these studies have demonstrated the potential and feasibility of applying these not-native hosts for butanol production, the butanol titers and productivities achieved are very low, some of which are disappointingly at least one order of magnitude lower than that achieved by the native butanol-producing strains *Clostridium acetobutylicum* JB200 or *Clostridium beijerinckii* BA101. Furthermore, *C. acetobutylicum* is a typical strain for butanol production, and thus, the genetic manipulation and process engineering strategies developed based on *C. acetobutylicum* can also be implemented in other species of *Clostridium* and non-native producing strains.

Here, we review the problems and advances in butanol production by *C. acetobutylicum*, including mechanism and regulation of sugar uptake, metabolic engineering and genome editing for strain improvement, and integrated recovery technologies including conventional single integrated techniques and advanced hybrid recovery strategies. Based on the summarized work of the past decades, this review highlighted the important role of genome editing and impercipient metabolic regulation in *C. acetobutylicum*, which could contribute a lot for strain development. In addition, integration of fermentation and recovery aiming to improve the efficiency of biobutanol production was discussed. We hope this review could facilitate the development and expansion of strain and process engineering for microbial butanol production, and the final revival of biobutanol production in the future.

2. Mechanism and regulation of sugar uptake

Since the use of cheap feedstocks in ABE fermentation is necessary for reducing the production cost, the industrial strain should be adapted to use a broad range of carbohydrates from various wastes or cheap materials (Gu et al., 2014). Manipulation of associated sugars uptake and transport systems could enable clostridia to utilize various substrates for solventogenesis. Sugar transport process could be realized by various mechanisms including H^+-symport, Na^+-symport, ABC system and PEP-dependent phosphotransferase system (PTS) (Mitchell, 2016). Among them, the PTS mechanism has received more attention, in view of its importance in uptake of sugars and sugar derivatives. PTS, typically containing enzyme I (EI), enzyme II (EII) and a histidine-containing protein (HPr) plays an important role in sugar transport in a variety of bacteria. There are 13 complete PTS systems and one orphan IIA domain in *C. acetobutylicum*, mainly responsible for the uptake of glucose, fructose, mannitol, mannose, sorbose and galactose, disaccharides and glucoside. Encoding genes and functions of these PTSs have been well summarized in recent reviews (Mitchell, 2015; Gu et al., 2014).