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Strategies for production of butanol and butyl-butyrate through lipase-catalyzed esterification

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HIGHLIGHTS

- Butyl butyrate is produced by *Clostridium* sp. strain BOH3 in ABE fermentation.
- Indigenous lipases can be efficiently induced by olive oil or Bio-OSR.
- 6.3 g/L of butyl butyrate is obtained from an emulsified solution.
- Additional lipases and butyric acid increase the yield of butyl-butyrate.
- Up to 22.4 g/L of butyl butyrate can be obtained from fermentation with *in situ* product extraction.

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ABSTRACT

In this study, a fermentation process for production of butanol and butyl-butyrate by using *Clostridium* sp. strain BOH3 is developed. This strain is able to produce butyric acid and butanol when it ferments 60 g/L xylose. Meanwhile, it also excreted indigenous lipases (induced by olive oil) which naturally convert butyric acid and butanol into 1.2 g/L of butyl-butyrate. When Bio-OSR was used as both an inducer for lipase and extractant for butyl-butyrate, the butyl-butyrate concentration can reach 6.3 g/L. To further increase the yield, additional lipases and butyric acid are added to the fermentation system. Moreover, kerosene was used as an extractant to remove butyl-butyrate *in situ*. When all strategies are combined, 22.4 g/L butyl-butyrate can be produced in a fed-batch reactor spiked with 70 g/L xylose and 7.9 g/L butyric acid, which is 4.5-fold of that in a similar system (5 g/L) with hexadecane as the extractant.

1. Introduction

Esters derived from short-chain carboxylic acids and alcohols are a large group of flavor and fragrance compounds, which are widely used in food, beverage, cosmetic, perfumes, solvents and pharmaceutical industries (Horton and Bennett, 2006; Park et al., 2009). Among them, butyl butyrate is also considered as an aviation fuel constituent, which possesses good compatibility with kerosene with a melting point below −47 °C and a flash point above 38 °C, as well as miscibility with kerosene at low temperature. Meanwhile, it also shows high octane rating of 97.3, which is higher than the minimum rating (95) set out in the European Standard (EN) 228 (Rhodri et al., 2013).

Currently, most of the esters (R_1COOR_2) are produced through Fischer esterification method, in which an organic acid R_1COOH and an alcohol R_2OH react and are catalyzed by an inorganic catalyst (e.g. sulfuric acid) at high temperature (200–250 °C). However, the hazardous conditions caused by corrosive acid/base at the high temperature and increasing scarcity of fossil fuels – source of alcohol and carboxylic acid have limited large quantities production of these esters. Since esters are commonly found in living species, such as plants and microbes, the concept of a natural ester made by lipase and natural substrate components from renewable and sustainable feedstocks under mild reaction conditions is more attractive than those chemical routes (Horton and Bennett, 2006; Stergiou et al., 2013). As shown in literatures, lipases (triacylglycerol lipases E.C. 3.1.1.3) display catalytic activity towards a wide variety of alcohols and acids in ester synthesis (Stergiou et al., 2013). Ester synthesis catalyzed by lipases can be performed at room temperature, atmospheric pressure, and neutral pH, providing an energy-saving route for the production of esters.

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Enzyme-catalyzed esterification is also highly specific, which means less side products and waste (Rhodri et al., 2013). Therefore, the use of lipases for esterification synthesis alleviates the necessity of complex post-reaction separation processes and thus leads to lower overall operation costs.

The feasibility of enzymatic production of esters such as butyl butyrate by transgenic *Clostridium acetobutylicum* and *Escherichia coli* has been demonstrated, however, the final product concentration is very low (0.3 g/L) (Horton and Bennett, 2006). Recently, significant efforts have been made on the production of butyl-butyrate. A proof-of-principle experiment for the one-pot bio-ester production from glucose by using *C. acetobutylicum* led to 5 g/L butyl butyrate in the hexadecane phase (van den Berg et al., 2013). Although butyl-butyrate concentration was increased, however, the butyl-butyrate had to be extracted from the hexadecane after the fermentation and 5 g/L still cannot fulfill large-scale production. Therefore, new strategies and novel biocatalysts that can efficiently convert organic acids and alcohols into targeted esters are needed urgently. Solventogenic *Clostridium* sp. naturally produces organic acids and alcohols at two distinct phases, acidogenic and solventogenic phases, respectively, during acetone–butanol–ethanol (ABE) fermentation (Lee et al., 2008; Jones and Woods, 1986). The main metabolic products in these two phases (acids:butyric acid; alcohols:butanol) are the natural substrates which can be catalyzed by lipase for ester synthesis. Thus, the integration of ABE fermentation process and lipase-catalyzed esterification will not only alleviate butanol toxicity to the cells, but also be able to produce valuable esters for various applications.

Solventogenic *Clostridium* sp. strain BOH3 has shown great potential comparing with previously reported ABE-producing strains, such as its high butanol production, efficient utilization of xylose and simultaneous fermentation of glucose and xylose (Bramono et al., 2011; Xin et al., 2014). Moreover, bioprocessing strategies using strain BOH3 are also advantageous because of its capability of co-production of other value-added products, such as riboflavin (VB2). As shown previously, strain BOH3 produces both butyric acid and butanol as its main products in the biphasic fermentation processes. Butyric acid produced in the acidogenic phase could be assimilated by strain BOH3 to produce butanol in the solventogenic phase (Bramono et al., 2011; Xin et al., 2014). In this study, esterification of butyric acid and butanol to more valuable product – butyl butyrate is investigated. Accordingly, two efficient strategies were designed for butyl-butyrate production. First, butyl-butyrate production using indigenous lipases from *Clostridium* sp. strain BOH3 was explored. Second, a fed-batch fermentation study with an integration of ABE fermentation, additional lipases catalyzed esterification and simultaneous *in situ* extraction of butyl-butyrate using aviation fuels was conducted to obtain high butyl-butyrate concentration.

2. Methods

2.1. Growth medium and culture conditions

Bio-OSR was purchased from Alpha Biofuels & Keppel Offshore & Marine Technology Centre, Singapore. All the other chemicals were purchased from Sigma–Aldrich with a purity of >99%. *Clostridium* sp. strain BOH3 was used in this study (Bramono et al., 2011; Xin et al., 2014). Batch cultures were grown at 35 °C in a defined mineral salts medium containing (per liter of distilled water): KH_2PO_4, 0.75 g; K_2HPO_4, 0.75 g; CH_3COONH_4, 2 g; yeast extract, 5 g. In addition, 1 mL of trace element solution (Widdel and Hansen, 1992), 1 mL of Na_2SeO_3–Na_2WO_4 solution (Brysch et al., 1987) and 10 mg of resazurin were added to 1 L of the medium. After the medium was boiled and cooled down to room temperature under N_2, reductants Na_2S, L-cysteine, and DL-dithiothreitol were added to a final concentration of 0.2, 0.2, and 0.5 mM, respectively (Xin and He, 2013). Subsequently, 20 mM 2-(N-morpholino)ethanesulfonic acid (MES) and 30 mM sodium butyric were added to the medium and the initial pH was adjusted to 6.2. Then the medium was dispended to serum bottles, which were sealed with butyl stoppers, autoclaved for 20 min, and cooled down to room temperature. 70 g/L xylose was amended to the above medium before inoculation.

2.2. Shake bottle fermentation

Cultures for inoculation were grown in 50 mL mineral salts medium amended with xylose at 35 °C for ~20 h (late-exponential phase) unless otherwise stated. Inocula of 5 mL were added to 45 mL of the reduced mineral salts medium in 160 mL serum bottles. Furthermore, 0.2 g lipase from *Candida rugosa* and 25 mL O_2 free extractant (hexadecane, kerosene, or paraffin oil) or 10 mL oil-based materials (olive oil, glyceryl tributyrate or Bio-OSR) were added to the serum bottles, which were incubated in a shaker at a rotary rate of 130 rpm at 35 °C. The pH was adjusted to 5.2 using 2 M NaOH after 24 h. Experiments were carried out in duplicates.

2.3. Fed-batch fermentation with sodium butyrate feeding

Prior to inoculation, 25 mL (O_2 free) extractants (hexadecane, kerosene, paraffin oil) were added to 50 mL fermentation broth. After 48 h of cultivation, the initial sodium butyrate was consumed in the fermentation broth. Then, 3 M of concentrated sodium butyrate solution was added to the fermentation broth to raise the butyric acid concentration to 30 mM (2.6 g/L) at time points of 48 and 72 h.

2.4. Determination of partition coefficient

The partition coefficient of the expected fermentation products was determined as follows: a stock solution was prepared by dissolving 2.0 g butanol, 2.0 g butyl-butyrate, and 0.5 g butyric acid in 100-mL fermentation medium. Fifty milligram of extractant (hexadecane, kerosene, or paraffin oil) was mixed with the stock solution in a conical flask that was then sealed with a rubber stopper and incubated at 30 °C for 24 h in a constant temperature and humidity chamber. The concentrations of butanol, butyric acid and butyl-butyrate in the solvent and medium broth were then determined. The partition coefficient was calculated by the following formula: $K = C_E/C_B$, where C_E is the concentration of the analyst in the solvent and C_B is the concentration of the analyst in the medium broth.

2.5. Lipase assay

The activity of free lipase was determined spectrophotometrically using p-nitrophenyl palmitate (p-NPP) as a substrate according to the method of Gupa et al. with some modifications (Gupta et al., 2004). The reaction mixture containing 0.3 mL of 0.05 M phosphate buffer (pH 8.0), 0.1 mL of 0.8 mM p-NPP and 0.1 mL of lipase was incubated at 37 °C for 10 min. The reaction was then terminated by adding 1 mL ethanol. A control without addition of lipase was run simultaneously. Absorbance of the resulting yellow colored product was measured at 410 nm using a spectrophotometer. One International Unit (IU) of lipase activity was defined as the amount of enzyme catalyzing the release of 1 μmol of p-nitrophenol per min from p-NPP under the standard assay conditions.