

**Simultaneous Fermentation and Esterification for Butyl Butyrate Production in Biphase
Medium with *Clostridium tyrobutyricum***

by

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Abstract

Butyl butyrate (BB) is a valuable chemical that can be used as flavor, fragrance, extractant, etc. in various industries. Meanwhile, BB can also be used as a fuel source with excellent compatibility as gasoline, aviation kerosene and diesel components. The conventional industrial production of BB is highly energy-consuming and generates various environmental pollutants. Recently, there have been tremendous interests in producing BB from renewable resources through biological routes. In this study, based on the fermentation using the hyper-butyrate producing strain *Clostridium tyrobutyricum* ATCC 25755, efficient BB production through *in situ* esterification was achieved by supplementation of lipase and butanol into the fermentation. Three commercially available lipases were assessed and the one from *Candida sp.* (recombinant, expressed in *Aspergillus niger*) was identified with highest catalytic activity for BB production. Various conditions that might affect BB production in the fermentation have been further evaluated, including the extractant type, enzyme loading, agitation, pH, and butanol supplementation strategy. Under the optimized conditions (5.0 g L⁻¹ of enzyme loading, pH at 5.5, butanol kept at 10.0 g/L), 34.7 g L⁻¹ BB was obtained with complete consumption of 50 g L⁻¹ glucose as the starting substrate. To our best knowledge, the BB production achieved in this study is the highest among the ever reported from the batch fermentation process. Our results demonstrated an excellent biological platform for renewable BB production from low-value carbon sources.

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List of Abbreviations

ABE	acetone-butanol-ethanol
ack	acetate kinase
adc	acetoacetate decarboxylase
adhE	acetaldehyde dehydrogenase
aq	Aqueous
BA	butyric acid
BB	butyl butyrate
bcd	butyryl-CoA dehydrogenase
bk	butyrate kinase
BOH	n-butanol
CoAT	CoA transferase
crt	crotonase
EMP	Embden-Meyerhoff pathway
GC	Gas chromatography
hbs	hydroxybutyryl-CoA dehydrogenase
HPLC	High performance liquid chromatography
LCA	Lipase immobilized on acrylic resin from <i>Candida antarctica</i>
LCR	Lipase from <i>Candida rugosa</i>
LCS	Novozymes Lipozyme® CALB
pta	phosphotransacetylase

ptb phosphate butyryltransferase
RCM reinforced clostridial medium
thl thiolase

Chapter 1 Introduction

1.1 Background

Fatty acid esters derived from alcohols and carboxylic acids are a large group of value-added chemicals usually with flavors and fruity fragrances, which are essential in food, cosmetic, and pharmaceutical industries (Horton and Bennett, 2006). Particularly, butyl butyrate (abbreviated as BB) is known to have a unique fruity smell like pineapple and banana, thus widely been used as food and fragrance additive to enhance the flavor. BB is also used as a solvent in plastic, texture and fiber industries, as well as important extractant in the processing of petroleum products (Horton and Bennett, 2006). Moreover, BB can be used as a valuable fuel source; it has a high octane rating of 97.3 with excellent compatibility and properties as gasoline, aviation kerosene and diesel components (Jenkins et al., 2013).

The conventional industrial production of BB, as for most other esters, undergoes the Fischer esterification process, which involves high temperature (200-250 °C) and needs concentrated sulfuric acid as catalyst. The reaction consumes a lot of energy and generates significant amount of hazardous wastes, further increasing downstream processing and treatment cost (Xin et al., 2016). An alternative approach for ester production would be to employ lipase, one of the most commonly existing enzymes in nature, to catalyze the reaction with renewable substrates under mild reaction conditions (Stergiou et al., 2013). This reaction can be performed at relatively low temperature, atmospheric pressure, and mild pH conditions. It is energy-saving and

environmentally friendly. Enzymatic reaction is also usually highly specific and efficient. Therefore, less byproduct or waste will be generated, reducing the downstream processing cost.

Previously, low-molecular-weight esters have been produced in genetically engineered *Escherichia coli* strains (Rodriguez et al., 2014). Although the production for some of the acetate esters (like isobutyl acetate) can reach a decent level, the produced final titer of BB particularly was rather low, probably due to the unavailability of intrinsic substrates (butyric acid (abbreviated as BA)/ butyryl-CoA and butanol (abbreviated as BOH)) for BB production (Rodriguez et al., 2014). From this sense, the solventogenic clostridia for biobutanol production through Acetone-Butanol-Ethanol (ABE) fermentation would be an excellent platform for BB production, because BA and BOH are co-produced during the fermentation. Indeed, van den Berg et al. reported that through *in situ* esterification and extraction of the BB into the non-aqueous extractant layer, 5 g L⁻¹ BB in the hexadecane phase was obtained in the fermentation using *Clostridium acetobutylicum* with glucose as carbon source (van den Berg et al., 2013). Another more recent study reported that, with strategic optimization and induction of indigenous lipase activity, 22.4 g L⁻¹ of BB was obtained in the extractive fed-batch fermentation using *Clostridium sp.* strain BOH3 (Xin et al., 2016). Moreover, 3.32 g L⁻¹ BB was obtained in the extractant layer when a *C. beijerinckii spo0A* negative mutant was employed for BB production with a similar procedure using lipase-catalyzed esterification and extractive fermentation (Seo et al., 2016). In all these attempts, solventogenic clostridia were employed for the fermentation; during biobutanol production, there are other ineluctable byproducts (including acetone, ethanol) competing for carbon sources for BA/BOH production, leading to low production of BA, BOH and final BB. *C. tyrobutyricum* (type strain ATCC 25755, also known as DSM 2637, KCTC 5387, JCM 11008, VPI 5392, NCIB 10635) is well known for its hyper-BA production (Wu and Yang, 2003). Recently it has been

completely sequenced and submitted to GenBank under the name of KCTC 5387 (Lee et al., 2016), providing deeper insight of the metabolic pathway and a solid platform for future metabolic engineering. With *C. tyrobutyricum*, a BA yield of 0.45 g g⁻¹-glucose can be achieved with negligible acetate production, corresponding to an extremely high BA selectivity of 91% (Wu and Yang, 2003). It has also been reported that *C. tyrobutyricum* has high BOH tolerance which holds greater than 80% and 60% relative growth rate (comparing to the control) at 1.0% and 1.5% (v/v) BOH, respectively (Yu et al., 2011). Therefore, we hypothesized that if BOH and lipase were supplemented to the fermentation with *C. tyrobutyricum*, high level of BB production can be achieved (Fig. 1). In addition, BB has a much higher partition coefficient in the appropriate organic extractant than either BA or BOH (van den Berg et al., 2013). Hence, BB is much easier to recover through extraction than either BA or BOH, and thus can significantly reduce the product recovery cost. On the other hand, BB production (along with an integrated extraction process) will decrease the end products' toxicity and thus be expected to achieve high productivity and titer. Therefore, the objective of this study was to attain efficient BB production through *in situ* esterification and extractive fermentation with the hyper-BA producing *C. tyrobutyricum*. We first evaluated various parameters for this bioprocess, and finally achieved high BB production under the optimized conditions. Further, we calculated the equilibrium constant of the reaction for BB production based on the biphasic reaction system, and successfully used the constant to simulate the BB production as we obtained from the experiment.

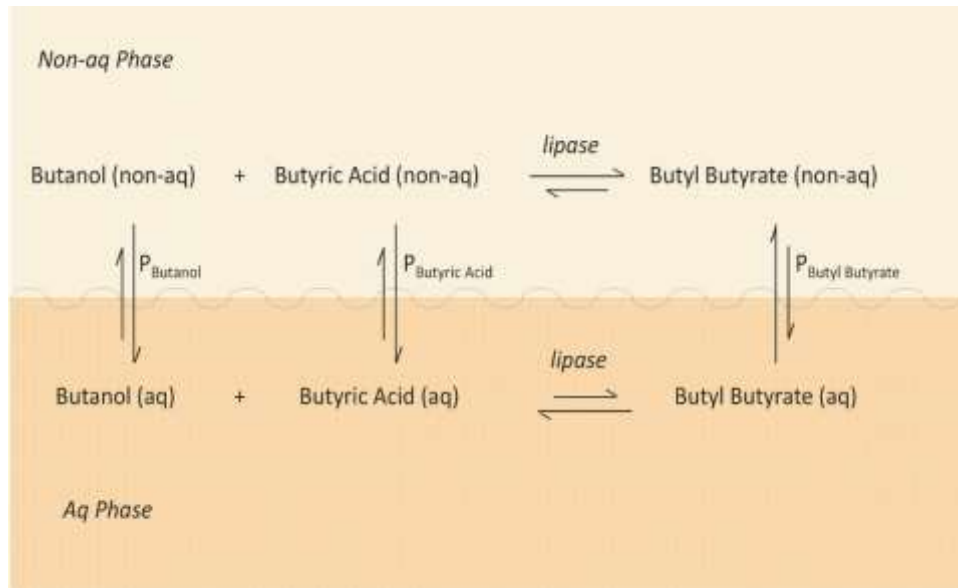


Figure 1. Partition and reaction scheme for lipase catalyzed esterification of butyric acid and butanol to produce butyl butyrate in biphasic medium. The relative tendency of reversible reaction and partition was indicated by the length of the arrow (the longer, the higher tendency), where in water (Aq Phase) the reaction tends to lysis and in organic solvent (Non-aq Phase) it tends to esterification direction.

1.2 Research Objectives

The first objective of this study is to evaluate fermentation and esterification conditions for production of BB with *C. tyrobutyricum*, including the extractant type, lipase type, enzyme loading, agitation, pH, and butanol supplementation strategy.

The second objective is to fine tune and optimize BB production with all the factors combined, to achieve a higher production yield and titer.

1.3 Significances

The enzyme catalyzed esterification is superior to chemical synthesis in many aspects. Firstly it is energy saving because it can be carried out in lower temperature and atmospheric pressure. Secondly it is a non-hazardous process compared to utilizing concentrated sulfuric acid as catalyst. Thirdly the down-stream processing is efficient since the product is almost completely extracted into the extractant layer.

The production of bio-based products involves extensive processing, such as fermentation and enzyme catalysis, thus the titer and yield of the desired product has to be high enough to be cost effective. By optimizing the reaction and fermentation conditions, the cost of producing BB through simultaneous esterification and extractive fermentation can be lowered.

This approach of optimizing in situ esterification and fermentation for BB production in this study is systematic and unique, that can be applied to similar enzyme catalyzed reactions for others' reference. Taking together, the results from this study demonstrated a promising biological platform for renewable BB production from low-value carbon sources.

Chapter 2 Literature Review

2.1 Clostridial Fermentation

Clostridia are Gram positive bacteria, typically rod-shaped, spore-forming and strict anaerobes (Patakova et al., 2011). They have a long history of being employed in several biotechnological processes, for instance, *C. acetobutylicum* in the conversion of renewable biomass for acetone/butanol production, *C. perfringens* for production of potent toxins such as enterotoxin and *C. botulinum* and *C. tetani* for neurotoxins, and *C. histolyticum* and *C. oncolyticum* for producing agents for cancer therapy (Pyne et al., 2014). In addition of *C. acetobutylicum*, other clostridia that are known to produce butanol as major fermentation endproduct include *C. aurantibutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, *C. saccharoperbutylacetonicum*, and *C. tetanomorphum* (Jones and Woods, 1986). It should be mentioned that *C. beijerinckii* NCIMB 8052, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* were originally designated as *C. acetobutylicum* (Poehlein et al., 2017). There are several other *Clostridium* species are known as hyper-butyrate producers, including *C. butyricum* (Zigová et al., 1999) and *C. tyrobutyricum* (Wu and Yang, 2003).

Clostridia can utilize a wide range of substrates from monosaccharides to polysaccharides, including di-, oligo-, like glucose, fructose, xylose, arabinose, lactose, saccharose, starch, pectin, inulin and others (Tangney et al., 1998). Among the various feedstocks used for biobutanol production, starchy substrates like corn and potatoes were used at first, and later blackstrap molasses became preferable. Nowadays, a lot of researchers aim to use lignocellulose, waste streams and gases, and synthesis gas in order to avoid competing with current food supply, as well as pollution reduction (Zhang et al., 2016).

2.2 ABE Fermentation

ABE fermentation is an anaerobic fermentation process, which mainly uses solvent-producing clostridia to produce acetone, butanol and ethanol from amylaceous or saccharous feedstocks (Jones and Woods, 1986). The first report of butanol production through fermentation was by Pasteur in 1862. At the beginning of 20th century, butanol fermentation was actively studied in England for rubber synthesis (Ewanick and Bura, 2010). After the break-out of World War I in 1914, the focus of ABE fermentation was changed to acetone production for cordite manufacture as acetone was required as the solvent. And butanol was produced meanwhile but mostly stored as a useless by-product. After the war, the acetone was no longer needed. However, butanol was in a big demand as a suitable solvent for quick-drying lacquers. In order to compete with newly developed industry for acetone and butanol synthesis from petroleum, new strains were attempted to isolate for fermentation of higher concentrations of starch and availability of cheap molasses, grain mash and soybean (Jones and Woods, 1986). When World War II started, acetone was in high level demand. As a result, continuous fermentation of acetone and butanol were reported and a large variety of substrates, including maize, wheat, rye, corn cob hydrolysates, were utilized for ABE fermentation (Wang, 2012). After World War II, acetone and butanol production declined rapidly. Until 1960s, in order to compete with the petro-chemically produced solvents and avoid the shortage of cheap molasses, solvent production through ABE fermentation continued to increase. In recent years, the research on butanol production through biological fermentation processes have been renewed since crude oil price increased and environmental problems associated with the fossil fuel are well noticed (Tashiro et al., 2013).

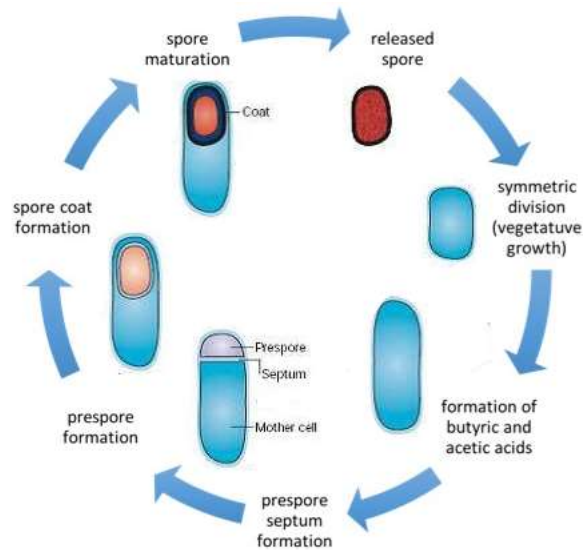


Figure 2. Life cycle of solventogenic clostridia (Patakova et al., 2011)

For the ABE fermentation, microorganisms can convert glucose to pyruvate through Embden-Meyerhoff pathway (EMP, or glycolysis) (Green, 2011). Then the fermentation can be divided into two distinct phases, namely acidogenesis and solventogenesis (Dürre, 2011, 2008; Jones and Woods, 1986). The first one couples with growth of cells and production of butyric and acetic acids as main products, while the second one is started with medium acidification by initiation of sporulation and metabolic switch, thereupon part of formed acids together with sugar carbon source are metabolized to ethanol, butanol and acetone. Among this whole process, the pH first decreases to lower levels with acids production and then increases when the cells enter stationary state with endospore forming. The life cycle of solventogenic clostridia is illustrated in Figure 2 (Patakova et al., 2011), while the simplified metabolic pathways of ABE fermentation are illustrated in Figure 3 (Ramey, 2004).

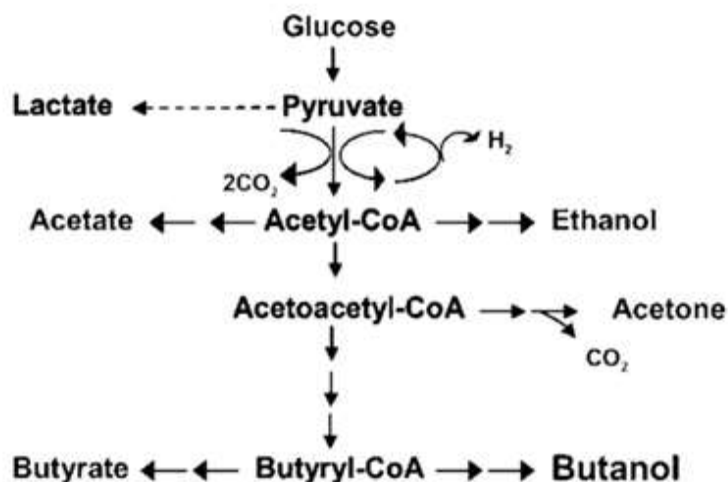


Figure 3. Metabolic pathway scheme of ABE fermentation (Ramey, 2004)

Solventogenic clostridia strains share the same metabolic pathways. During the acidogenic phase, acetate is created through acetyl-CoA, which is first phosphorylated to acetyl phosphate by phosphotransacetylase (pta) and then converted to acetate by acetate kinase (ack). However, butyrate formation is a relatively complicated process involving several steps. First, two molecules of acetyl-CoA are transformed into acetoacetyl-CoA through THL (acetyl-CoA acetyltransferase) (Stim-Herndon et al., 1995). Second, butyryl-CoA is formed from acetyl-CoA following a metabolic pathway with the assistance of four enzymes: thiolase (thl), 3-hydroxybutyryl-CoA dehydrogenase (hbd), crotonase (crt) and butyryl-CoA dehydrogenase (bcd). Butyryl-CoA is thereafter converted to butyryl phosphate by phosphotransbutyrylase (phosphate butyryltransferase, ptb) and butyrate kinase (bk) to generate butyrate (Figure 3) (Sillers et al., 2009). As the acid accumulates, pH drops to the lowest point during fermentation, which leads to the switch of acidogenesis phase to solventogenesis phase. The switch from acidogenesis to solventogenesis is usually associated with the induction of solvent formation gene transcription and decreased cell growth and motility. During the solventogenesis phase,

which is stationary, the formation of acids decreases, and acetone and butanol become the dominant products (with a small fraction of ethanol) (Awang et al., 1988). In the solventogenesis phase, two molecules of acetyl-CoA are transformed into acetoacetyl-CoA and acetoacetate through CoA transferase (CoAT) (Jones and Woods, 1986). Then acetone is obtained through acetoacetate decarboxylase (adc). Then acetyl-CoA goes through two pathways. In one route, it is converted to ethanol by acetaldehyde dehydrogenase (adhE) and ethanol dehydrogenase; in others, it is converted to butyryl-CoA through the butyryl-CoA formation pathway.

In the context of the development of a viable ABE production process, a number of disadvantages of this traditional process have been extensively reviewed and summarized in Table 1. In general, there is a need for substrate security, improved fermentation performance, sustainable process operations for solvent recovery, reasonable by-product utilization and so on (Dürre, 2011; Green, 2011).

Table I. The challenges and solutions for ABE fermentation

Challenges	Solutions
Expensive substrate cost and competition with nutrition	Cheaper feedstocks such as lignocellulosic hydrolysates and syngas, engineering of respective production strains
Low solvent yield	Targeted strain construction with high butanol yield
Formation of numerous by-products	Develop strains with higher butanol selectivity with elimination by-products production
Solvent recovery	Low energy methods such as liquid-liquid extraction and gas stripping

2.3 Fatty Acid Production with *Clostridium tyrobutyricum*

Clostridium tyrobutyricum is a Gram positive, rod-shaped and spore-forming strictly anaerobic bacterium growing at pH 6.0 and 37 °C (Song, Ventura, Lee, & Jahng, 2011). This strain is known to produce CO₂, hydrogen, acetic and butyric acids as main products. *Clostridium tyrobutyricum* (type strain ATCC 25755, also known as DSM 2637, KCTC 5387, JCM 11008, VPI 5392, NCIB 10635) is well known for its hyper butyric acid production and purity as well as its simple medium requirements for cell growth (Michelsavin, Marchal, & Vandecasteele, 1990a, 1990b, 1990c; Wu & Yang, 2003).

Recently, the genome of *C. tyrobutyricum* has been completely sequenced and submitted to GenBank under the strain name of KCTC 5387 (Lee, Jang, Han, Kim, & Lee, 2016). It was revealed from the genome that this microorganism does not use phosphotransbutyrylase and butyrate kinase for butyric acid production; instead, it uses a CoA transferase to mediate the butyric acid production from butyryl-CoA by reassimilation of acetic acid. This apparently is very different from the butyric acid production pathways in other well-known solventogenic clostridia strains.

2.4 Butyl Butyrate

Butyl butyrate (BB) is an ester that can be formed by the condensation of butyric acid and n-butanol. It is a clear, colorless liquid that is insoluble in water, but miscible with ethanol and diethyl ether. It is a valuable chemical that can be used in various industrial processes. BB has been widely used as food and fragrance additive to produce and enhance flavor with a unique fruity smell much like pineapple and banana. BB can also be used as the solvent in plastic, texture

and fiber industries, as well as extractant in petroleum products processing (Berger, 2009; Park et al., 2009; Rajendran et al., 2009; Rodriguez et al., 2014). Meanwhile, BB can also be used as a valuable fuel source since it has a high octane rating of 97.3 with excellent compatibility with Jet A-1 aviation kerosene and properties as gasoline, aviation kerosene and biodiesel enrichment components. (Chuck and Donnelly, 2014; Li et al., 2010).

2.5 Esterification

2.5.1 Fischer Esterification

Currently, Fischer esterification method is the most common way to produce most of the esters (R_1COOR_2), in which an organic acid R_1COOH and an alcohol R_2OH react and are catalyzed by an inorganic catalyst at high temperature. A generic mechanism for an acid Fischer esterification is shown in Figure 4. Commonly used catalysts for a Fischer esterification include sulfuric acid, tosylic acid, and Lewis acids such as scandium(III) triflate (Horton & Bennett, 2006). The reaction is often carried out without a solvent (particularly when a large reagent excess of alcohol is used) or in a non-polar solvent (e.g. toluene). The primary disadvantages are its thermodynamic reversibility, relatively slow reaction rates, and the hazardous conditions caused by corrosive acid/base at the high temperature.

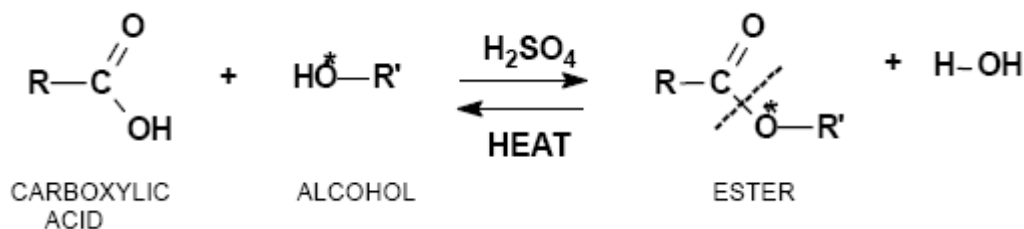


Figure 4. Fischer esterification mechanism

2.5.2 Enzyme Catalyzed Esterification and BB Production

Since esters can be commonly and easily found in living species, such as plants and microbes, an alternative approach for ester production would be to employ lipase, one of the most commonly existing enzymes in nature, to catalyze the esterification reaction with renewable substrates under mild reaction conditions. This is more attractive than those chemical routes for various reasons. This reaction can be performed at relatively low temperature, atmospheric pressure, and mild pH conditions. It is energy saving and environment friendly. Enzymatic reaction is also usually highly specific and efficient. Therefore, less byproduct or waste will be generated, reducing the downstream processing cost.

Previously, low-molecular-weight esters have been produced in genetically engineered *Escherichia coli* strains. Although the production for some of the acetate esters (like isobutyl acetate) can reach a decent level, the produced final titer of BB particularly was rather low, probably due to the unavailability of intrinsic substrates BA and BOH) for BB production (Jenkins et al., 2013). From this sense, the solventogenic clostridia for biobutanol production through acetone–butanol–ethanol (ABE) fermentation would be an excellent platform for BB production, because BA and BOH are co-produced during the fermentation. Recently significant efforts have been made on the production of BB. van den Berg et al. reported that through in situ esterification and extraction of the BB into the non-aqueous extractant layer, 5 g L⁻¹ BB was obtained in the fermentation using *C. acetobutylicum* (van den Berg et al., 2013). Another more recent study reported that, with strategic optimization and induction of indigenous lipase activity, 22.4 g L⁻¹ of BB was obtained in the extractive fed-batch fermentation using *Clostridium sp.* strain BOH3 (Xin et al., 2016). Moreover, 3.32 g L⁻¹ BB was obtained in the extractant layer

when a *C. beijerinckii* spo0A negative mutant was employed for BB production with a similar procedure using lipase-catalyzed esterification. However, during all these reports for BB production by integrating the esterification and ABE fermentation, the final BB yield is generally low. Because there are other ineluctable byproducts (including acetone, ethanol) in ABE fermentation competing for carbon sources for BA/BOH production, leading to low production of BA, BOH and final BB.

C. tyrobutyricum (type strain) is well known for its hyper-BA production (Wu and Yang, 2003). With *C. tyrobutyricum*, a BA yield of 0.45 g g⁻¹-glucose can be achieved with negligible acetate production, corresponding to an extremely high BA selectivity of 91% (Wu and Yang, 2003). It has also been reported that *C. tyrobutyricum* has high BOH tolerance which holds greater than 80% and 60% relative growth rate (comparing to the control) at 1.0% and 1.5% (v/v) BOH, respectively (Yu et al., 2011). Therefore, *C. tyrobutyricum* can serve as an excellent platform for BB production by supplementation of BOH and lipase for in situ esterification during the fermentation.

Chapter 3 Materials and Methods

3.1 Materials

The fermenting strain used in this study, *Clostridium tyrobutyricum* ATCC 25755, was acquired from American Type Culture Collection (ATCC, Manassas, VA, USA). The lyophilized culture was recovered in reinforced clostridial medium (RCM) and stored at -80 °C as glycerol stock. Lipase enzymes used in this study include Lipase from *Candida sp.* (recombinant, expressed in *Aspergillus niger*, in aqueous solution; Novozymes Lipozyme® CALB, minimum 5000 LU/g of liquid; abbreviated as LCS), Lipase from *Candida rugosa* (as solid powder, minimum 700 U/mg of solid; abbreviated as LCR), Lipase immobilized on acrylic resin from *Candida antarctica* (recombinant, expressed in *Aspergillus niger*, Novozymes 435, minimum 5000 U/g; abbreviated as LCA). LCS was kindly gifted by Novozymes (Franklinton, NC, USA), and LCR and LCA were purchased from Sigma-Aldrich (St. Louis, MO, USA). All enzymes were stored at 4 °C until use.

All chemicals used as GC and HPLC standards were purchased from Sigma-Aldrich and of analytical grades, including n-hexadecane, BOH, BA, BB, glucose, acetic acid (abbreviated as AA). Chemicals used for fermentation medium were purchased from VWR (Radnor, PA, USA), including RCM, glucose, yeast extract, tryptone, (NH₄)₂SO₄, K₂HPO₄, MgSO₄• 7H₂O, FeSO₄• 7H₂O and L-cysteine.

3.2 Fermentation Experiments

Unless otherwise stated, the fermentation medium was composed of (per liter of distilled water): 50 g glucose; 5 g yeast extract; 5 g tryptone; 3 g (NH₄)₂SO₄; 1.5 g K₂HPO₄; 0.6 g MgSO₄•

7H₂O; 0.03 g FeSO₄• 7H₂O and 1 g L-cysteine. The fermentation medium (except for the K₂HPO₄) along with the extractant (when appropriate) was sterilized by autoclaving at 121 °C and 15 psi for 30 min. To prevent precipitation by reacting with other chemicals under high temperature, K₂HPO₄ was autoclaved separately as 2x stock solution and then added into the main fermentation broth. The glycerol stock of *C. tyrobutyricum* was inoculated into RCM (at a 2% v/v ratio) and incubated at 35 °C for 12 h in an anaerobic chamber under N₂:CO₂:H₂ (volume ratio of 85:10:5) atmosphere.

For the small scale fermentation with 250 mL serum bottles, 5 mL of the actively growing seed culture (OD₆₀₀ is ~1.5) was inoculated into each bottle containing 50 mL fermentation medium along with 25 mL of organic extractant. Before inoculation, the mixture of medium and the extractant was placed in anaerobic chamber for an overnight to remove oxygen and then autoclaved. Afterwards, the bottles were put back into the anaerobic chamber for the inoculation. The fermentation was carried out in a shaker incubator set at 35 °C and 150 rpm (unless otherwise indicated). Gas generated during the fermentation was released every 12 h by piercing the rubber septa with syringe needles. All shaking bottle fermentations were carried out in triplicates. For the fermentation with bioreactors, the actively growing seed culture was inoculated at a 10% inoculum level to the fermentation medium in BioFlo 115 benchtop bioreactors (New Brunswick Scientific Co., Enfield, CT) with a 1.5 L working volume. Unless otherwise specified, hexadecane was added as the extractant, and the volume ratio of aqueous phase to organic phase was 2:1 in the fermentation (that is, 1.0 L fermentation broth and 0.5 L extractant). Oxygen-free nitrogen was flushed through the broth to initiate anaerobiosis before the inoculation and kept flushing after inoculation until the culture initiated its own gas production.

3.3 Determination of Partition Coefficient

For determining the partition coefficient of BA (or BOH) in hexadecane, we conducted following tests at fermentation temperature of 35 °C because partition coefficient is very sensitive to temperature (Dearden and Bresnen, 1988). 50 mL of DI water containing various levels (1-20 g L⁻¹) of BA (or BOH) was prepared in a 250 mL serum bottles. Then 25 mL of hexadecane was added into the solution and vigorously agitated at 35 °C overnight after which the mass transfer equilibrium was reached. The aqueous phase was sampled for HPLC measurement before hexadecane addition and after hexadecane addition (when mass transfer equilibrium was reached). After initial sampling, one additional sample was taken after 3 h of extended agitation. Generally, no change in the concentration of BA (or BOH) was observed within the additional sample, which confirmed that the mass transfer equilibrium could be reached after the overnight agitation. The concentration of BA (or BOH) in the hexadecane phase was calculated based on the amount of BA (or BOH) decreased in aqueous phase, and the corresponding partition coefficient of BA (or BOH) in the hexadecane/aqueous biphasic system was calculated as the ratio of the BA (or BOH) concentration in the hexadecane to that of the same compound in the aqueous phase. Similar experiment was conducted to determine the partition coefficient of BB. The mixed system was shaken vigorously at 35 °C overnight, and the mass transfer equilibrium was confirmed by additional sampling. The extractant phase was sampled for GC measurement, and the BB concentration in aqueous phase was calculated based on the difference of total loaded BB and BB detected in the hexadecane phase. Finally, the partition coefficient of BB in the hexadecane/aqueous biphasic system was determined.

3.4 Lipase Selection and Enzyme Loading Determination

Three commercially available lipases (LCS, LCR, LCA) were evaluated for their efficiency for catalyzing the BB production. The reaction was carried out in 250 mL serum bottles in an incubation shaker at 35 °C and 150 rpm. 50 mL of DI water and 25 mL of hexadecane (along with one of the three lipases) was added into the bottle, to mimic a volume ratio of 2:1 for the aqueous phase to the organic phase in the real extractive fermentation. For a fair comparison, the added lipase was set to 10,000 U for each. BA and BOH (0.23 M for each, to mimic the values in the real extractive fermentation) mixture was added and the reaction was started. Both aqueous phase and organic phase samples were taken and analyzed throughout the reaction.

Once the lipase with the best performance was identified, similar experiments were carried out to determine the appropriate enzyme loading, except that only the identified lipase (at a range of 0-15 g L⁻¹) was used.

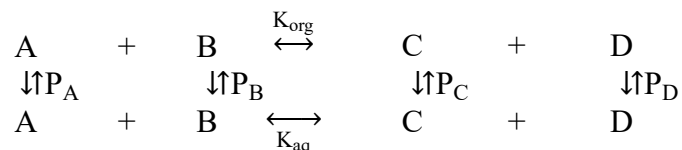
3.5 Analytical Methods

Cell culture growth was monitored by following the optical density in the fermentation broth at 600 nm (OD₆₀₀) using a cell density meter (Ultrospec 10, Biochrom Ltd, Cambridge, England). An HPLC (Agilent Technologies 1260 series) equipped with an automatic sampler/injector and a refractive index (RI) detector with an HPX-87H column (Bio-Rad, Hercules, CA, USA) was used to analyze all the compounds in the aqueous phase (including glucose, AA, BA, ethanol, BOH, and BB). 5 mM H₂SO₄ was used as the mobile phase with a flow rate of 0.6 mL min⁻¹ at 25 °C. A GC (Agilent Technologies 6890N) equipped with an HP-5 column (60m × 0.25 mm, 0.25 μm film thickness) was used to analyze the BB in the hexadecane phase. Helium at 8 kPa (20 ml mm⁻¹ total flow) was used as the carrier gas. The initial temperature of the oven was set

at 70 °C for 4 min, followed with a ramp of 10 °C min⁻¹, to reach the final temperature of 225 °C which was held for 3 min. The detector was kept at 225 °C. A split ratio of 5:1 was employed. Unless otherwise indicated, the reported BB concentration is the determined concentration in the extractant phase, while the BOH, BA and AA concentrations are the ones measured in the aqueous phase. For BA and BOH in pH controlled fermentations, concentration was adjusted by dilution factor due to the volume change caused by addition of base solution.

3.6 Determination of Equilibrium Constant of Lipase Catalyzed Esterification in Biphasic System

For the enzyme catalyzed esterification reaction studied here, we tried to determine an equilibrium constant to simulate the experiment result and predict the final BB production. It has been shown that in a biphasic medium, the apparent equilibrium constant changes with the volume ratio of organic and water phase (Martinek et al., 1981). For example,

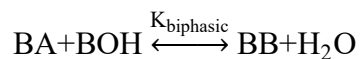


Where A, B are the reactants and C, D are the products of a reversible reaction in biphasic medium. The apparent equilibrium of this reaction can be calculated as

$$K_{biphasic} = K_{aq} \frac{(1 + \alpha \cdot P_C)(1 + \alpha \cdot P_D)}{(1 + \alpha \cdot P_A)(1 + \alpha \cdot P_B)}$$

In the above equation, $K_{biphasic}$ is the apparent equilibrium constant, in accordance with the principle of active mass (Martinek et al., 1981). K_{aq} is the equilibrium constant in water, P_A , P_B , P_C and P_D are the partition coefficient of the corresponding compound, and α is the volume ratio of the two phases.

A special case of this type of reaction scheme is that water is one of the products, in our case,



Equilibrium constant of a bimolecular reaction with one product as water in a biphasic system can be calculated as:

$$[\text{BB}]_{\text{total}} = K_{\text{biphasic}} \frac{[\text{BA}]_{\text{total}} [\text{BOH}]_{\text{total}}}{[\text{H}_2\text{O}]_{\text{total}}}$$

The total concentration of each compound is calculated by dividing total moles of that molecule in the system by total volume of the system (that is, the total volume of both phases) at equilibrium.

Chapter 4 Results and Discussion

The fermentation was carried out in serum bottles on a shaker incubator to investigate parameters including the extractant effect, BOH tolerance, agitation effect, partition coefficient and enzyme kinetics. The study of the effect of pH and the final optimized extractive fermentation for BB production was carried out in bioreactors.

4.1 Effect of Different Extractants on Cell Growth

Several organic solvents such as kerosene, hexadecane, olive oil and Alpha Bio-OSR have been reported to have low toxicity to the cell growth and thus can be used to perform effective *in situ* extraction for BB; among them kerosene and hexadecane were the most commonly used (Xin et al., 2016). Therefore, in this study, we first evaluated kerosene and hexadecane for their toxicity on the cell growth of *C. tyrobutyricum*.

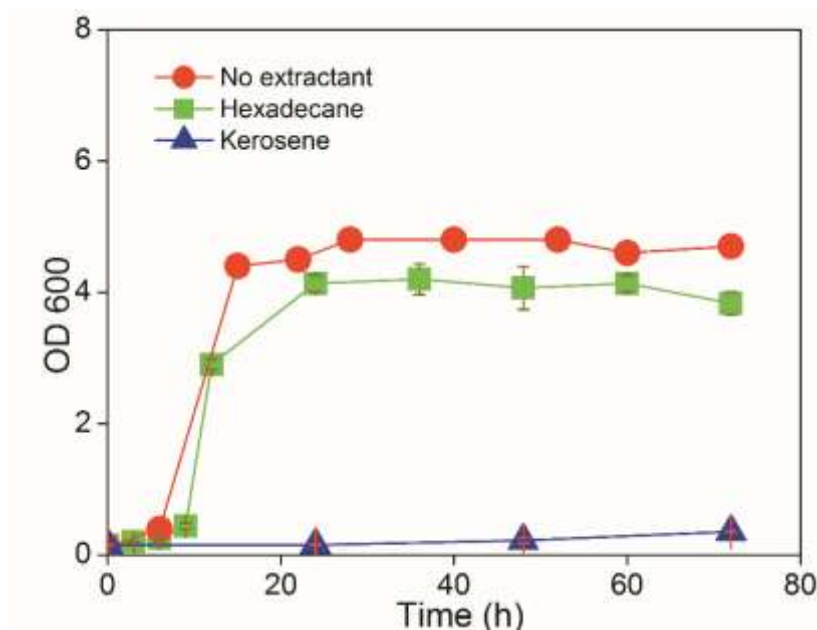


Figure 5. The effect of different extractants on the cell growth of *Clostridium tyrobutyricum*.

Our results indicated that the fermentation with kerosene as extractant showed no cell growth at all after incubation for 72 h, while hexadecane did not show noticeable inhibition on the cell growth under similar cultivation conditions (Fig. 5). This might be due to the fact that kerosene is a mixture comprising of a group of shorter carbon chain molecules (10-16 carbons) compared to hexadecane (16 carbons); as reported by Gill and Ratledge, the toxicity of hydrocarbons increases while chain length decreases, due to hydrophilicity and solubility in water (Gill and Ratledge, 1972). Another possible reason is that the purity of kerosene is lower compared to that of hexadecane. There might be other residual chemicals inhibiting the vital metabolism of cells.

Although hexadecane had no significant inhibition on cell growth in the early stage, cells in all the fermentation with hexadecane stopped growing at around 12-24 h of the fermentation, leaving a large amount of glucose unconsumed. The fermentation was carried out in serum bottles without pH control. Therefore, the pH value dropped rapidly with BA accumulation throughout the fermentation, which might lead to the cell death and the fermentation cease. We measured the final pH of the fermentation broth and confirmed the pH was < 4.0 . It was impractical to control pH in small bottles, and thus the effect of pH on the fermentation was further investigated in bioreactors as described in a later section. Here, these small scale fermentation tests demonstrated that, particularly for extractive fermentation with *C. tyrobutyricum*, hexadecane is a good candidate with low toxicity. Further, since other researchers already demonstrated that hexadecane is an efficient extractant for BB production (van den Berg et al., 2013; Xin et al., 2016a; Seo et al., 2016), we decided to use hexadecane for extractive fermentation in the following steps in this study.

4.2 Partition Coefficient Determination

BA, BOH and BB have very different hydrophilicity, which can largely determine the efficiency for BB production with *in situ* extraction. We decided to determine the partition coefficient of all three chemicals in the hexadecane/aqueous biphasic system. As illustrated in Table II, the partition coefficient of the ester is $\sim 10^4$ times higher than that of its substrates, indicating BB is much more hydrophobic compared to BOH and BA. Therefore, the selective *in situ* extraction by hexadecane for the fermentation is expected to be very efficient to drive the reaction towards BB production. van den Berg et al. reported very similar partition coefficient for BB in hexadecane-water biphasic system, but for BOH and BA, their results were very different from ours here (their partition coefficient for BA is 4.8 times higher, and that for BOH is 10.2 times higher) (van den Berg et al., 2013). There are two possible factors contributing to such difference in measuring the partition coefficient: temperature and BA loading. The temperature can significantly affect the solubility of the substrate in different media. Here, we conducted our measurement at 35 °C in order to closely simulate the actual fermentation condition. However, the temperature that was used for the partition coefficient measurement by van den Berg et al. was not reported (van den Berg et al., 2013). On the other hand, specifically for BA, the loading quantity can influence the partitioning drastically. Because BA has both dissociated and undissociated forms in the aqueous phase, and only the undissociated BA molecules can be involved in the partitioning. We measured BA partition coefficient at relatively low BA concentrations ($1\text{-}20\text{ g L}^{-1}$) in this study, simulating the extractive fermentation condition for *in situ* BB production. Under such conditions, BA would be more likely in the dissociated form and thus the determined partition coefficient was relatively low.

Table II. Partition coefficients in the hexadecane/aqueous system.

Butyric acid	Butanol	Butyl butyrate	References
0.13 ± 0.06	0.44 ± 0.04	340 ± 103	(van den Berg et al., 2013)
0.027 ± 0.03	0.043 ± 0.02	390 ± 47	This study

4.3 BOH Tolerance of *C. tyrobutyricum*

Since we would add exogenous BOH to the fermentation broth for BB production, we further evaluated the effect of BOH supplementation on the fermentation with *C. tyrobutyricum* and thus determined an appropriate strategy for BOH addition. It has been previously reported that *C. tyrobutyricum* has a good butanol tolerance (Yu et al., 2011). We here set out to test the BOH tolerance of *C. tyrobutyricum* in the biphasic setup for BB production. Various levels of BOH were added into the fermentation before the inoculation. As observed in Fig. 6, 5 g L⁻¹ BOH had little inhibition on the cell growth comparing to the control. The cell growth reached OD₆₀₀ of 13.6 at 48 h, which is comparable to the maximum OD of 13.8 at 36 h for the control. The further increase of BOH supplementation decreased the initial cell growth rate and extended the cell growth lag phase. At 10 g L⁻¹ BOH, the maximum OD of the cell growth reached 7.5 at 60 h. At 15 g L⁻¹ BOH, the cell started to grow after 24 h, and reached the peak level of OD₆₀₀ 3.0 after 60 h. Based on these results, apparently, if we do not want to bring about significant inhibition on the cell growth, the BOH supplementation for BB production should be not higher than 5 g L⁻¹. However, considering the continuous consumption of BOH in the *in situ* BB production process, higher BOH supplementation (like 10 g L⁻¹) might also be feasible. Therefore, in the following

extractive fermentation, we used this as a general guideline for the BOH supplementation for BB production.

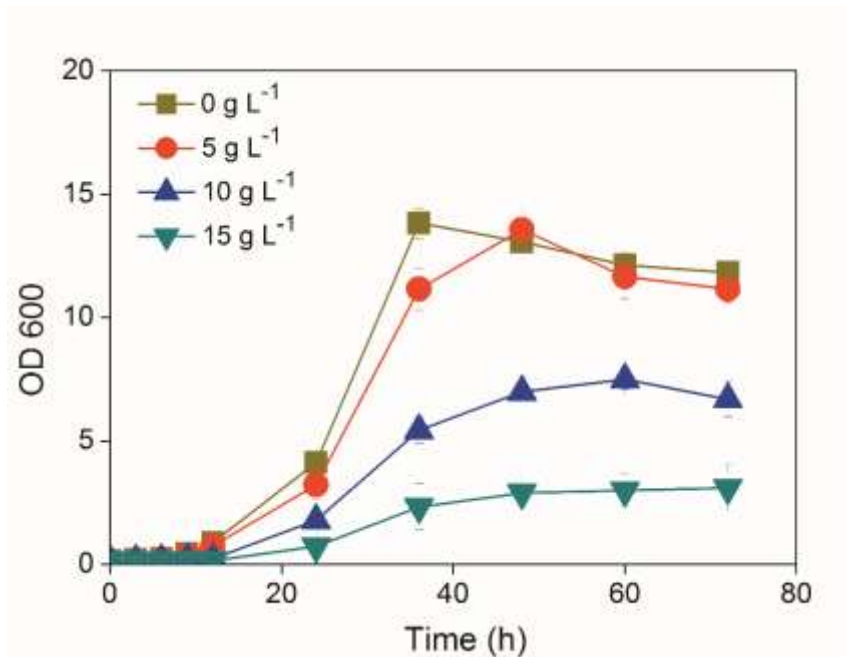


Figure 6. The effect of butanol supplementation on the cell growth of *Clostridium tyrobutyricum* in the extractive fermentation.

4.4 Lipase Selection and Enzyme Loading Determination

We further evaluated three different lipases including LCS, LCR, LCA that were reported by other researchers for BB production (van den Berg et al., 2013; Seo et al., 2016; Xin et al., 2016), for their effectiveness for *in situ* esterification for BB production. As shown in Fig. 7, the reaction with LCS reached equilibrium in < 20 h and generated 22.0 g L⁻¹ BB, corresponding to about 67.2% of the theoretical maximum yield based on the added substrates. The reaction with LCR reached a maximum BB of 18.6 g L⁻¹ within 24 h, while the reaction with LCA only generated

7.5 g L⁻¹ BB by the end of sampling period (72 h). Based on these results, we selected LCS to catalyze the BB production in the following steps.

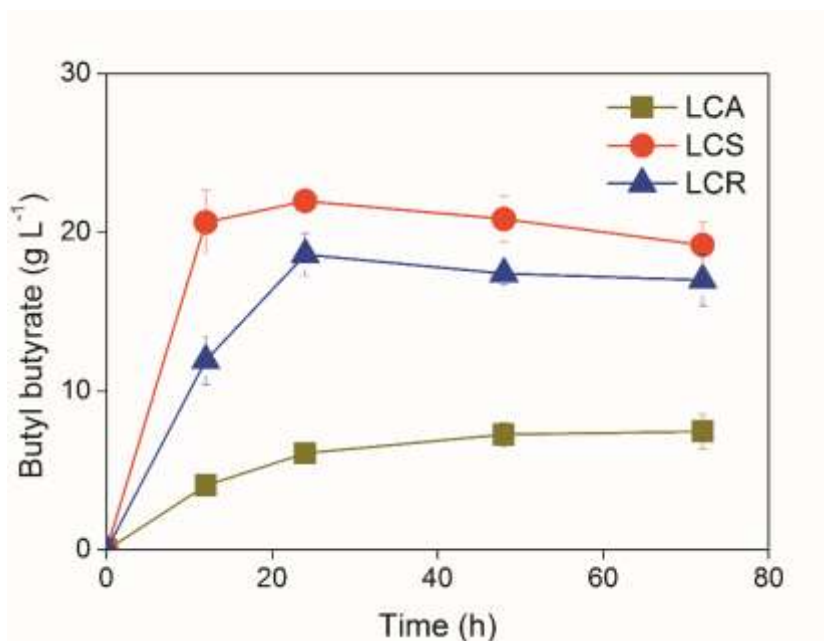


Figure 7. Butyl butyrate production through esterification catalyzed with three different lipases. LCR: Lipase from *Candida rugosa*, as solid power; LCS: Lipase from *Candida sp.*, recombinant, expressed in *Aspergillus niger*, in aqueous solution, also named as Novozymes Lipozyme® CALB; LCA: Lipase immobilized on acrylic resin from *Candida antarctica*, recombinant, expressed in *Aspergillus niger*, also named as Novozymes 435.

So far, the ever-reported highest BB production of 22.4 g L⁻¹ was achieved in a fermentation through the catalysis of esterification with LCR (Xin et al., 2016). In our study, LCR showed comparable performance as in the report, but was not as good as LCS under the same condition. On the other hand, LCA is the lipase from *Candida antarctica* immobilized on acrylic resin (expressed in *Aspergillus niger*, also known as Novozyme 435). It has been extensively studied in a variety of esterification reactions achieving high catalytic activity and ester production (Duan

et al., 2010; Yang et al., 2014). Generally, the immobilization of enzyme has various advantages, such as making the enzyme more stable and less likely to denature (and thus with better catalytic activity), increasing the resistance of the enzyme to condition variations (Mateo et al., 2007). However, in this study, the immobilized LCA demonstrated lowest esterification activity for BB production. This could be because the mass transfer of substrates to the non-immobilized enzyme is much more efficient than that to the immobilized enzyme in the resin. Similarly, Zhu and Zhang reported that non-immobilized enzymes demonstrated much higher catalysis activity than the immobilized ones in a closed biobattery system (Zhu and Zhang, 2015). However, one notable advantage for the immobilization of enzyme is that the immobilized enzyme can be easily separated and reused. For example, with immobilization, LCA has been reported to have robust catalysis capability even after one hundred cycles of washing and reusing (Duan et al., 2010). Therefore, although the non-immobilized LCS was selected in this study for high efficient BB production in the batch fermentation, enzyme immobilization (with various immobilization strategies) should be tested and evaluated in the future for further enhanced and economic BB production.

In the next step, the effect of enzyme loading of LCS on esterification for BB production was investigated. As shown in Fig. 8, the catalysis efficiency for BB production increased with the increase of enzyme loading. However, when the enzyme loading $> 5.0 \text{ g L}^{-1}$, the efficiency did not further increase significantly with the increase of enzyme loading. Therefore, 5.0 g L^{-1} LCS was used in the following fermentation for *in situ* BB production.

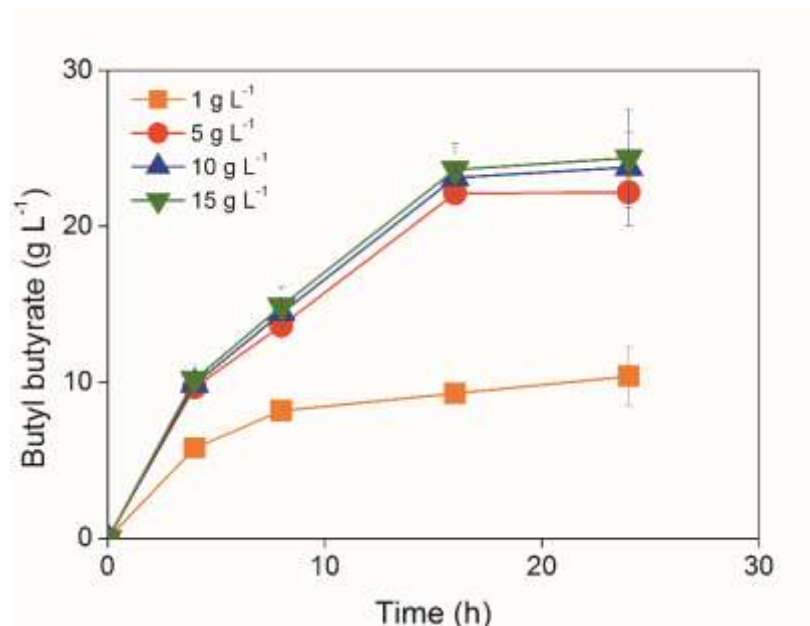


Figure 8. The effect of enzyme loading of LCS (the lipase from *Candida sp.*, recombinant, expressed in *Aspergillus niger*) on butyl butyrate production.

4.5 Effect of Agitation

Generally, high level of agitation is expected to enhance the mixing and thus improve the reaction efficiency. On the other hand, vigorous agitation might exert mechanic shearing stress on the cell and thus negatively influence cell growth and even break cells. Therefore, in this study, the effect of agitation ranging from 100-300 rpm on both enzymatic reaction and fermentation was investigated to optimize conditions for the extractive fermentation for BB production. For the enzymatic reaction, the experiment was carried out in shaking bottles with the same set-up as the experiment for determining the enzyme loading, except that 5 g L⁻¹ LCS was used for all reactions and various agitation rates were applied for the reaction. It was observed that the agitation rate had no significant impact on the esterification reaction rate within the range of

agitation studied (data not shown). Then the study of the effect of agitation on the cell growth was carried out in the bioreactor with the same set-up as for the extractive fermentation for BB production except that various agitation rates were applied. Fig. 9 illustrated the cell growth kinetics under different conditions. From the fermentation, the optimal cell growth rate was achieved at 150 rpm. Therefore, 150 rpm was selected for the following fermentation.

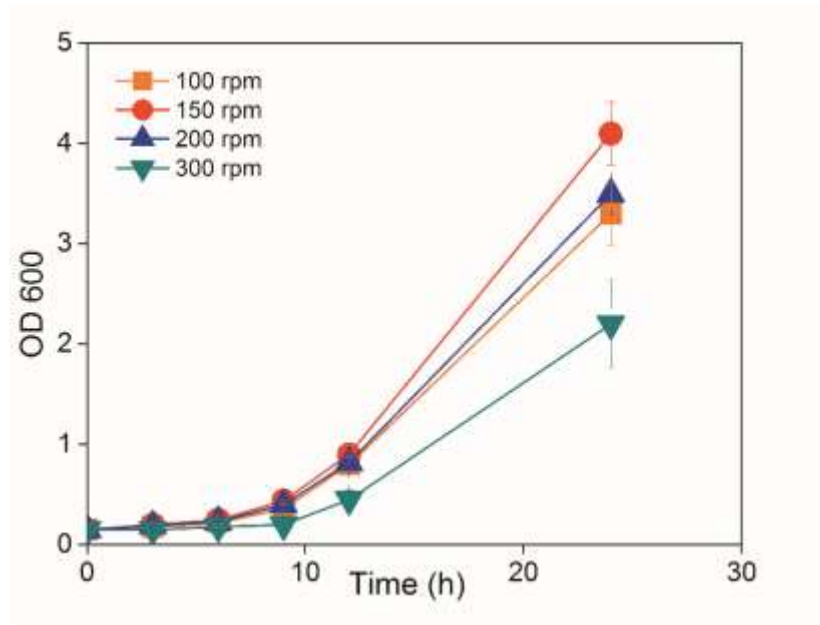


Figure 9. The effect of agitation speed on the cell growth of *Clostridium tyrobutyricum*.

4.6 Effect of pH

The effect of pH on the fermentation performance was investigated. The initial pH of the fermentation after culture inoculation was around 6.8. Throughout the fermentation process, the pH was continuously monitored and concentrated NaOH (2.0 M) was added automatically when necessary to keep the pH from falling below the pre-set value. As shown in Figs. 7A and 7B, when the pH of the fermentation was set at 4.0, only about half of the glucose was consumed,

with 10.9 g L⁻¹ BA was produced. When the pH was set at 4.5, around 80% (41.1 g L⁻¹) of the glucose was consumed, with 17.8 g L⁻¹ BA was produced. While when the pH was set at 5.0 or above, all 50 g L⁻¹ of glucose could be consumed within 48 hours. There was no significant influence of pH (when > 5.0) on BA production either; around the same amount of BA (21.9 - 22.6 g L⁻¹) was generated under these conditions.

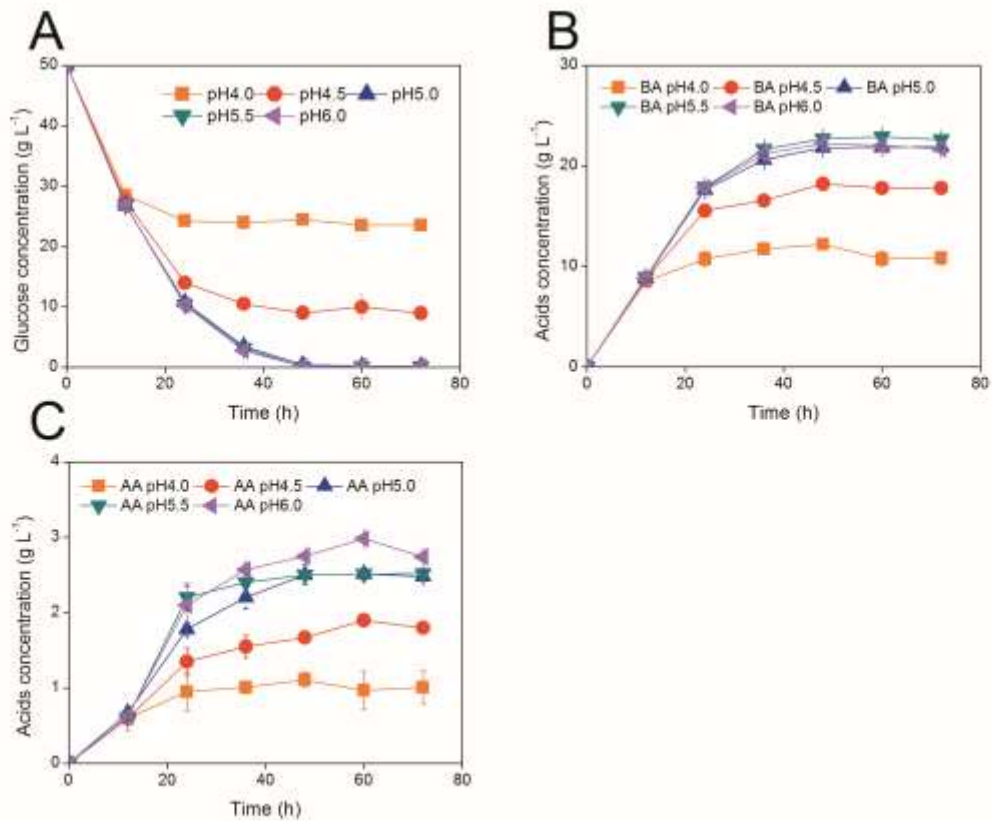


Figure 10. The effect of pH on the fermentation with *Clostridium tyrobutyricum*.

A. The glucose consumption profiles in the fermentation.

B. The acids production kinetics in the fermentation (BA: butyric acid).

C. The acids production kinetics in the fermentation (AA: acetic acid).

BA has a pKa of 4.82 at room temperature. The lower pH results in more undissociated form of BA present in the solution, and thus can facilitate the esterification for BB production (we tested the effect of pH on the esterification efficiency of LCS and found that there was no significant influence of pH ranging from 4.0-6.0; data not shown). However, lower pH (and thus more undissociated BA) may severely inhibit the bacterial cell growth and lead to poor fermentation performance (Maddox et al., 2000; Tang et al., 1989), as was actually shown in Fig. 10 when $\text{pH} < 4.5$. Therefore, to achieve the optimized fermentation (good cell growth and BA production) and efficient BB production through *in situ* esterification, we selected pH 5.0 and 5.5 for further evaluation in the extractive fermentation in the following steps.

It is worthwhile to notice that, under all the tested pH conditions, whether or not the substrate was completely consumed, the BA yield was about the same, which is around 0.46 g g^{-1} . This is comparable to the highest BA yield numbers as reported in the literature (Wu and Yang, 2003), demonstrating the hyper-butyrate producing capability of *C. tyrobutyricum*.

4.7 BB Production under Optimized Conditions

Based on the optimized conditions as discussed above, batch fermentations were performed to achieve efficient *in situ* BB production. Specifically, the fermentation was carried out in bioreactors with 1.0 L of fermentation medium and 0.5 L of hexadecane as extractant, with 5.0 g L^{-1} of LCS was employed to catalyze the esterification. Exogenous BOH was supplemented from the beginning of the fermentation. In order to achieve a steady BOH level throughout the fermentation, samples were taken frequently as necessary (more frequently at the early stage and

less frequently at the late stage of the fermentation), and the aqueous phase of the sample was analyzed immediately by HPLC. Based on the results, whenever the measured BOH concentration dropped more than 1 g L^{-1} below the intended concentration (either 5.0 or 10.0 g L^{-1}), 1 g L^{-1} additional BOH was supplemented into the reactor to maintain the desired BOH level in the fermentation (either 5.0 or 10.0 g L^{-1}). Fermentation was first carried out with 50 g L^{-1} glucose as sole carbon source, pH was set at 5.0 , and BOH level was maintained at 5.0 g L^{-1} . As shown in Fig. 11A, the fermentation generated 18.2 g L^{-1} BB along with 12.1 g L^{-1} BA, and 14.3 g L^{-1} of glucose was left unconsumed. This result seemed discrepant from the previous results for the pH influence experiment and the BOH toxicity test in which 50 g L^{-1} glucose could be completely consumed at pH 5.0 and 5.0 g L^{-1} BOH supplementation did not significantly inhibit the cell growth. The co-existence of BOH and BA along with other reagents and the generated BB in the extractive fermentation conditions brought about additive inhibition to the cells and the cell metabolism appeared to be less robust under these conditions. We then carried out the fermentation with the pH set at 5.5 . With the BOH level was maintained at 5.0 g L^{-1} (Fig. 11B), the fermentation generated 17.7 g L^{-1} BB along with 16.0 g L^{-1} BA left in the medium. The result was not satisfying either; especially a high level of BA was left unreacted. Then, we decided to increase the BOH level for the further fermentation attempting to achieve a better performance. When the BOH level was maintained at 10.0 g L^{-1} (pH was kept at 5.5 ; Fig. 11C), the BB production reached 34.7 g L^{-1} . Under both conditions at pH 5.5 , nearly all the 50 g L^{-1} of glucose was consumed (3.5 g L^{-1} and 7.6 g L^{-1} left, respectively), indicating the potential of this strain to consume more substrate in the extractive fermentation for elevated BB production. Therefore, we conducted additional experiments at pH 5.5 by increasing the initial glucose concentration to 80 g L^{-1} . When the BOH level was set at 5.0 g L^{-1} , most of the sugar was consumed (with $7.6 \text{ g$

L⁻¹ was left) and the BB production reached 16.2 g L⁻¹; meanwhile, a high level of BA (29.2 g L⁻¹) was observed at the end of the fermentation (Fig. 11D). While when the BOH level was kept at 10.0 g L⁻¹, although the sugar consumption was incomplete (leaving 23.0 g L⁻¹ unconsumed), BB titer was improved to the highest of 36.9 g L⁻¹ (Fig. 11E). In Table III, we compared our results with the results from the previous reports with the similar system for BB production through extractive fermentation. To our best knowledge, the BB production from this study is the highest among the ever reported from the batch fermentation. Xin et al. used 4 g L⁻¹ LCR in a similar extractive fermentation system and a BB production of 22.4 g L⁻¹ was achieved (Xin et al., 2016). LCR is in the solid powder form and LCS that we used in this study is in aqueous solution. Therefore, it is unfair to compare the enzyme loading solely based on the enzyme concentration used (in g L⁻¹). According to the product information from Novozymes, LCR has 200 times more enzymatic units than LCS at the same weight. In this sense, the esterification for BB production presented in this study was much more efficient than that was reported by Xin et al. (Xin et al., 2016).

Table III. Comparison of the butyl butyrate production in this study with the previous reports.

Strains	Extractants	Carbon sources		Enzymes		Butyl butyrate production (g L ⁻¹)	References
		Types	Concentration (g L ⁻¹)	Types*	Loading (g L ⁻¹)		
<i>Clostridium sp.</i> Strain BOH3	Bio-OSR	Xylose	70	LCR	4	22.4	(Xin et al., 2016)
<i>C. beijerinckii spo0A</i> mutant	Hexadecane	Glucose	50	LCS	8.33	3.32	(Seo et al., 2016)
<i>C. acetobutyricum</i>	Hexadecane	Glucose	40	LCA	2.21	4.9	(van den Berg et al., 2013)
<i>C. tyrobutyricum</i>	Hexadecane	Glucose	80	LCS	5	36.9	This study

*LCR: Lipase from *Candida rugosa*, as solid power;

LCS: Lipase from *Candida sp.*, recombinant, expressed in *Aspergillus niger*, in aqueous solution, also named as Novozymes Lipozyme® CALB;

LCA: Lipase immobilized on acrylic resin from *Candida antarctica*, recombinant, expressed in *Aspergillus niger*, also named as Novozymes 435.

It should be noticed that among all the fermentation conditions, the AA production was at negligible levels ($< 3.0 \text{ g L}^{-1}$; Fig. 10C), as well as lactic acid ($< 1.0 \text{ g L}^{-1}$, data not shown), and the other potential esters' production was not detected. This further demonstrated the high selectivity for BA production in *C. tyrobutyricum*.

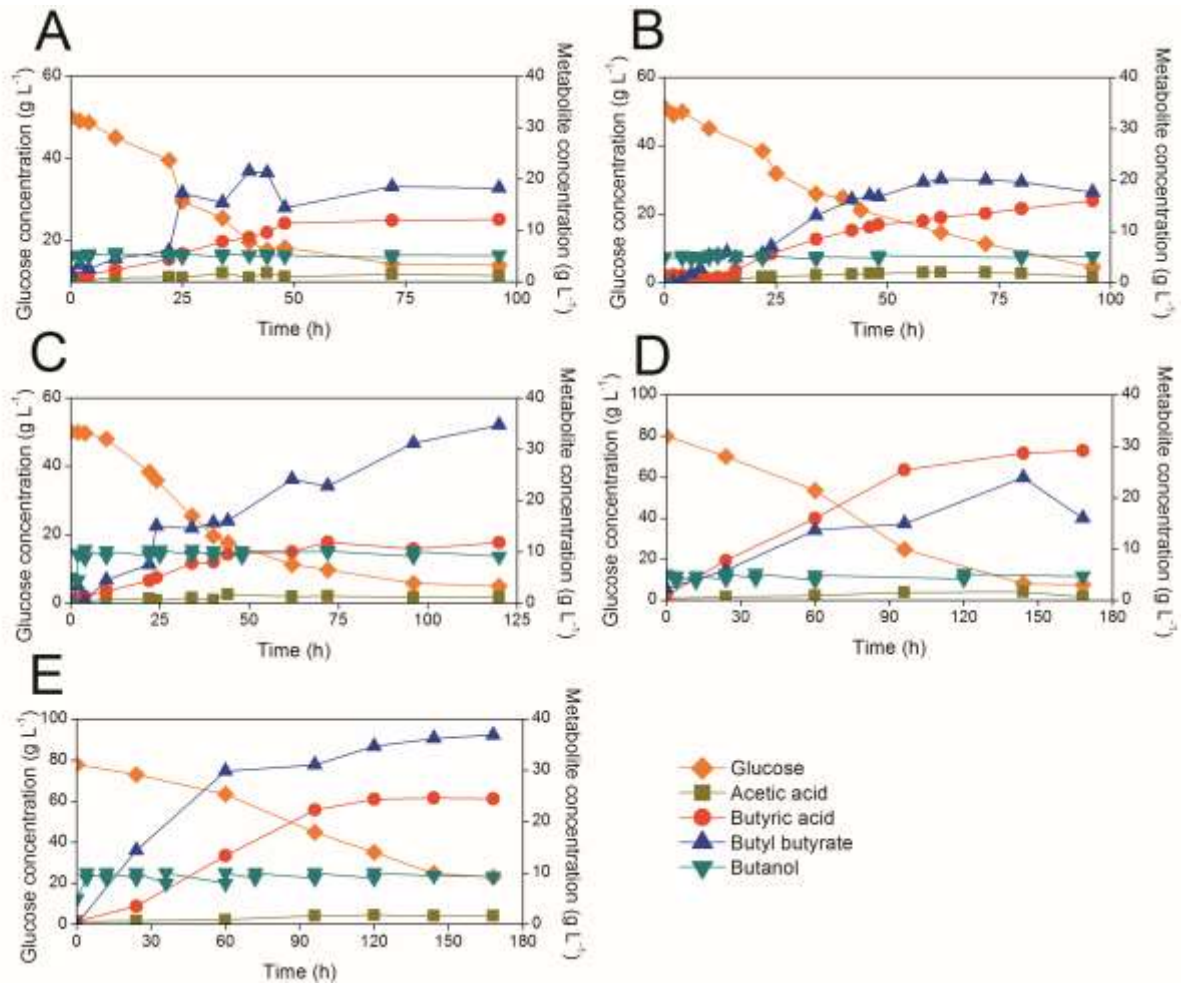


Figure 11. Butyl butyrate production in the extractive fermentation with *Clostridium tyrobutyricum* under various conditions.

A. The glucose consumption and metabolites production in the fermentation in which 50 g L^{-1} glucose was used as the substrate, supplemented butanol was maintained at 5.0 g L^{-1} , and pH was kept at 5.0.

B. The glucose consumption and metabolites production in the fermentation in which 50 g L⁻¹ glucose was used as the substrate, supplemented butanol was maintained at 5.0 g L⁻¹, and pH was kept at 5.5.

C. The glucose consumption and metabolites production in the fermentation in which 50 g L⁻¹ glucose was used as the substrate, supplemented butanol was maintained at 10.0 g L⁻¹, and pH was kept at 5.5.

D. The glucose consumption and metabolites production in the fermentation in which 80 g L⁻¹ glucose was used as the substrate, supplemented butanol was maintained at 5.0 g L⁻¹, and pH was kept at 5.5.

E. The glucose consumption and metabolites production in the fermentation in which 80 g L⁻¹ glucose was used as the substrate, supplemented butanol was maintained at 10.0 g L⁻¹, and pH was kept at 5.5.

4.8 Equilibrium Constant of the Esterification Reaction for BB Production

Based on the data that we obtained in previous shaking bottle reactions for lipase selection, an equilibrium constant, $K_{\text{biphasic}} = 348.1 \pm 45$ was calculated. This constant was used to predict final BB titers in our extractive fermentations. As shown in Table IV, the predicted BB titers were generally close to the ones observed in the extractive fermentations at various conditions, except for the last one (pH 5.5 with 10 g L⁻¹ BOH and 80 g L⁻¹ glucose). Two factors might contribute to the difference between the predicted and observed values in this case. Firstly, we stopped the reaction when we assumed the fermentation was ended (the stop of cell growth and sugar

consumption). However, the esterification was not necessarily ended at this point (although the reaction rate could be low). Therefore, if enough longer time were given and steady BOH supplementation were maintained, more BB could be continuously produced. Secondly, the predicted equilibrium constant K_{biphasic} was obtained based on the results from the shaking bottle experiments with lower reactant concentrations. Especially for the BA, for the equilibrium constant determination purpose, although we tried to mimic fermentation conditions in the shake bottle reactions by adding 1-20 g L⁻¹ of BA, the BA decreased quickly and significantly to be converted to BB. On the other hand, a high BA concentration in the actual extractive fermentation was generally maintained (more than 20 g L⁻¹ was left at the end of fermentation for the particular condition, pH 5.5 with 10 g L⁻¹ BOH and 80 g L⁻¹ glucose), which might have caused substrate inhibition to the lipase activity.

Table IV. Prediction of the butyl butyrate production using the equilibrium constant under various fermentation conditions in this study.

Fermentation conditions	BB titer (g L ⁻¹)	
	Observed	Predicted
pH 5.0 with 5 g L ⁻¹ BOH and 50 g L ⁻¹ glucose	18.3	16.9
pH 5.5 with 10 g L ⁻¹ BOH and 50 g L ⁻¹ glucose	17.1	22.2
pH 5.5 with 5 g L ⁻¹ BOH and 80 g L ⁻¹ glucose	34.7	40.6
pH 5.5 with 10 g L ⁻¹ BOH and 80 g L ⁻¹ glucose	36.9	68.2

Chapter 5 Conclusions and Future work

5.1 Conclusions

In this study, we supplemented lipase and butanol into *C. tyrobutyricum* fermentation, achieving efficient BB production through *in situ* esterification. We also tested three commercially available lipases among which the one from *Candida sp.* (recombinant, expressed in *Aspergillus niger*) was identified with highest catalytic activity for BB production. We then optimized fermentation and esterification conditions, including the extractant type, enzyme loading, agitation, pH, and butanol supplementation strategy (5.0 g L⁻¹ of enzyme loading, pH at 5.5, butanol kept at 10.0 g L⁻¹), obtaining 34.7 g L⁻¹ BB with complete consumption of 50 g L⁻¹ glucose as the starting substrate.

5.2 Future Work

To our best knowledge, the BB production achieved in this study is the highest among the ever reported from the batch fermentation process (> 60% higher than the previously reported highest from the similar extractive fermentation processes). Our results demonstrated an excellent biological platform for renewable BB production from low-value carbon sources. However there are several issues and pitfalls that needs to be addressed in future research in order to further improve the productivity.

5.2.1 Metabolic Engineering of *Clostridium tyrobutyricum* for *in vivo* Butyl Butyrate Production

The major limitation of this study is that one of the substrate has to be supplemented into the fermentation medium, as well as the lipase enzymes. This could raise the cost of this

process to be unrealistic for further scale up. Here we propose one method to simplify the whole complex supplementation process, which is metabolic engineering.

Metabolic engineering is the practice of conferring genetic and regulatory processes within cells to increase the cells' production of a certain product; in many cases, the product could be one that the host cannot naturally produce.

Our unpublished data proves that the *C. tyrobutyricum* can be engineered to produce astonishing level of butanol. And allogeneic lipase can be expressed *in vivo*. On the one hand we can boost the expression level of lipase in order to catalyze esterification reaction efficiently. Since the 5 g L⁻¹ supplementation level is very high considering the relative cell mass within the reactor, the robustness of the lipase gene remains to be investigated and improved. On the other hand, we can tailor the metabolic pathway to have the intermediate products reacting, instead of having produced acid and alcohol and then esterify them.

5.2.2 Simultaneous Fermentation and Esterification for Other Esters with Different Microorganisms

The preliminary data in our lab shows the various lipases that we tested in this study can not only catalyze esterification of butyric acid and butanol, but also catalyze efficient esterification between acetic acid and butanol, butyric acid and ethanol with the existence of the extractant layer. All the end products of these reactions, namely ethyl butyrate, butyl acetate, are valuable chemicals in industry. Meanwhile, there are a variety of hyper acids and alcohol producing microbes. With the guideline and methods provided in this study, more of the short chain esters can be produced with lipase catalyzed esterification and extractive fermentation.

5.2.3 Enzyme Immobilization

Another promising way of cutting the cost of the process is to use immobilized lipase, such as LCA in this study. Although it is not comparable in the catalytic activity with non-immobilized LCS, it can be collected and washed for several reuses, reducing the cost significantly.

Catalytic activity of immobilized enzyme can be limited by many factors, since the LCA was not originally produced and optimized for esterification applications rather than lysis, the immobilization matrix, bead size etc. can be investigated for better esterification efficiency.

5.2.4 Real-time Monitoring of Substrate Level for Automated Supplementation

From industrial engineering and process engineering stand point, the semi-batch/fed batch process used in this study can be turned into continuous fermentation and extraction. The major obstacle would be monitoring the substrate level in the reactor. Currently we used dense sampling and HPLC measurement, which is labor intensive and potentially inaccurate. On-line micro GC analysis can be applied to the bioreactor, collecting and analyzing gas exhaust in order to deduce alcohol and ester levels.

Once we can have a more precise reading of the substrate level in real-time, we can set the threshold level for a pump to automatically feed the needed substrate into the reactor, reducing labor intensity and further improve the productivity and titer.

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