ABSTRACT


6-deoxyerythronolide B (6dEB) is the macrocyclic core of erythromycin, a well-known broad-spectrum antibiotic. 6dEB analogues with various non-native and non-natural chemical functionalities can be engineered by chemobiosynthesis or heterologous biosynthesis using recombinant Escherichia coli. These analogues will be used as scaffolds for the discovery of new antibiotics with improved pharmacological properties.

This project intended to develop a biosynthesis process for intensified 6dEB and/or 6dEB-analogues production. Several high cell-density media and a fed-batch process were evaluated for enhanced 6dEB production in recombinant E. coli containing two plasmids with similar origins of replication. The effects of various medium components and induction strategies were studied in shaking-flask experiments. The stability of the incompatible dual-plasmids co-existing in this host was improved by strain purification and optimization of seed train protocol. The project outcomes included a preliminarily high-cell-density fed-batch fermentation procedure in a 2-L bioreactor using HCD medium, optimized induction strategy and critical process parameters, and several optimized media with improved 6dEB production compared to LB, such as CD6 (76.87 mg/L), PB1 (25.15 mg/L), PM1 (51.98 mg/L), EM1 (34.4 mg/L), and HCD (343.11 mg). Furthermore, a non-growth cell immobilization strategy, latex-based cell patch coating, was studied for overcoming the challenge of the poor stability of the dual-plasmids and improving the titer of 6dEB. 6dEB production with latex-coatings cell-patches was confirmed with improved level in LB medium, compared to shaking-flask methods.
Development of High Cell-Density Media, a Precursor Fed-Batch Fermentation Process, and Latex-immobilized Cell-patches for Enhanced Production of 6-deoxyerythronolide B (6dEB) using *Escherichia coli*

by
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A thesis submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Master of Science

Biomanufacturing

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APPROVED BY:

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Committee Chair

_______________________________
Dr. Driss Elhanafi
DEDICATION

I would like to dedicate this thesis to my family, who have always supported my endeavors in education, life and ambition no matter the circumstance, especially in remembrance of my grandpa who passed away this year before my birthday, when I could not even go back to have a last sight. Mom, Dad, and my Sister, thank you all for your unconditional love, encouragement and companionship on this journey with me. I love you all and you are forever in my heart.
BIOGRAPHY

Zhixiang Feng was a Teaching Assistant during his graduate studies in the Biomanufacturing Master’s of Science, the Master in Biological and Agricultural Engineering and a Master’s Minor in Biotechnology at North Carolina State University. He received his Bachelor’s of Engineering in Bioengineering from Sichuan University (Chengdu, China) in May 2008 and Master’s of Science in Microbial and Biochemical Pharmacy from Sichuan Industrial Institute of Antibiotics (Chengdu, China) in May 2011. Subsequently, his working experience in Institute of Plant Physiology and Ecology, SIBS, Chinese Academy of Sciences (Shanghai, China) as a synthetic biology researcher inspired him to further his education and study abroad in North Carolina State University. He was in a PhD program on biobutanol project in Biological and Agricultural Engineering for the first two years in NCSU. Later he discovered his true academic interest in biopharma instead of biofuel and hence changed the program. During his graduate studies, Zhixiang also completed courses more than required for a Master’s Minor in Statistics and gained experiences in Design of Experiments and statistical analysis & tools. He also worked as a summer R&D intern in Quality Control for a diagnostic startup company, BioMedomics Inc. (RTP, NC), gaining experiences in writing SOP, optimizing QC methods, data consolidation and analysis. He was the Public Relations Director of the NCSU student chapter of International Society for Pharmaceutical Engineering (ISPE) and the Secretary for Chinese Student and Scholars Friendship Association at NCSU. He was also an active volunteer in NCSU Engineering Career Fair and OIS-ISSERV program. Zhixiang is dedicated to applying his skill set in development and improvement of novel biologics and technologies for helping the lives of patients and the wellbeing of daily life.
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Many individuals have contributed greatly to the success of this project. I would first like to acknowledge Dr. Michael C. Flickinger, project advisor and thesis academic chair, for his direction, compassion, inspiration and all-around support in this project. I would further like to appreciate thesis committee members Dr. Gavin Williams and Dr. Driss Elhanafi for their guidance, forbearance and assistance with my experimentations and thesis composition. I would like to acknowledge the generous bestowing of the recombinant strain by Pfeifer’s group (University at Buffalo), the 6dEB synthetic standards by Krische’s group (University of Texas at Austin), and the composition of unpublished Production Medium B by Sherman’s group (University of Michigan). In addition, I appreciate the analysis support by Triad Mass Spectrometry in University of North Carolina at Greensboro (UNCG) and Mass Spectrometry Facility in Department of Chemistry (NCSU). Especially, I would like to appreciate my project co-worker, Dr. Christian Kasey, Mounir Zerrad, and Sarah Schultheis from Department of Chemistry who had contributed a lot in early data collection, development of extraction and analysis protocols. There were many additional supporters from BTEC staff and faculty, including Michele Ray, Michael Ray, Haiwei Zhang, Eric Safarz, Dr. Gisele Gurgel, Dr. Sarwat Khattak, and Dr. Nathaniel Hentz. Last but not least, I would like to appreciate a dear friend Michael J. Kroeplin (M. Ed.), who helped me proof-reading and correcting errors.
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CHAPTER 1. Introduction

1.1 Introduction of the Natural Products

Natural products or semi-synthetic derivatives with novel structures are the hot sources for discovering novel bioactivities for human therapeutics. Among many natural product-derived drugs approved in the United States, 38% were new small-molecule entities (Harvey, 2008). From 1981 to 2014, 49% of small molecule drugs were natural products or derivatives (Newman & Cragg, 2016). Among all types of natural small molecule, the polyketides (PKS) is a large class of complex compounds with broad and potent biological and medicinal properties (Williams, 2013). Examples of PKS include rapamycin (immunosuppressant), lovastatin (anti-cholesterol) (Boghigian, Zhang, & Pfeifer, 2011) and erythromycin (antibacterial), and the list goes on. These complex biomolecules are synthesized from simple building blocks by modular polyketide synthases (PKSs) through successive rounds of decarboxylative Claisen condensation reactions. The complexity of PKS multi-enzyme complex structure impedes the practical development of de novo synthesis (Gao, Woo, & Krische, 2013; B. A. Pfeifer & Khosla, 2001). Nowadays, the understanding and manipulation of PKS machinery offer a possibility for heterologous biosynthesis, which facilitates the development of a high-yield, cost-effective production process (B. A. Pfeifer & Khosla, 2001; Williams, 2013; Zhang, Wang, & Pfeifer, 2008).

Basically, reconstitution of a PKS metabolic pathway in a heterologous host requires (1) functional expression of the enzymatic machinery, (2) adequate chaperones and a posttranslational modification system for correct PKSs folding and assembly, (3) the availability of sufficient substrates or chaperons for PKS assembly, and (4) the tolerant ability of the host producer cell against the toxicity of the product (B. A. Pfeifer & Khosla,
To achieve a considerable titer of product expression, it is important to harness the intrinsic metabolic activity of proteins in surrogate host, including codon optimization, auto-inducer utilization, promoter enhancement, and gene copy number control (Zhang, Boghigian, & Pfeifer, 2010). Moreover, protein engineering has focused on substituting of individual enzymatic domains/modules for alternate enzymatic functions by introducing different building blocks into PKS analogs (Williams, 2013). These semisynthetic derivatives or analogs obtained by precursor-derived biosynthesis became a new reservoir for looking for novel, potent, and bioactive molecules to combat the growing threat of antibiotic resistance (Murli et al., 2005; Zhang, Wang, Wu, Skalina, & Pfeifer, 2010) For example, the ketolides (e.g., ABT773, telithromycin) are derivatives of macrolide antibiotic erythromycin and active against macrolide-resistant bacteria. Some other examples of erythromycin semisynthetic derivatives include azithromycin and clarithromycin (Whitman & Tunkel, 1992).

Erythromycin A is a historic example of type I PKS macrolide with potent bioactivity against bacterial infection. It is originally discovered from the soil-borne actinomycetes *Saccharopolyspora erythraea* (Zhang, Wang, et al., 2010). The industrial strains for producing erythromycin were domesticated to be remarkably high-yield, e.g. 8 g per liter (Minas, Brunker, Kallio, & Bailey, 1998), through many years of traditional domestication and process development. Given the slow-growing, intractable molecular biological tools, and intricate nature of *S. erythraea*, it is more practical to use a well-characterized heterologous host for expression or directed mutagenesis to produce analogs (B. Pfeifer, Hu, Licari, & Khosla, 2002). In this case, the macrolide core, bio-inactive 6-deoxyerythronolide B (6dEB), has become the center of most studies.
1.2 Previous Studies on Heterologous Expression of 6-deoxyerythronolide B

1.2.1 PKS biosynthesis of 6dEB

6dEB is the polyketide core, the key precursor of erythromycin A. The PKSs responsible for 6dEB synthesis is called 6-deoxyerythronolide B synthase (DEBS), one of the most extensively characterized modular polyketide synthases (Kinoshita, Pfeifer, Khosla, & Cane, 2003). DEBS enzyme is a large (2 MDa) $\alpha_2\beta_2\gamma_2$ multi-domain enzymatic complex with 28 distinct active sites, seven of which requires 4'-phosphopantetheine posttranslational modification (B. A. Pfeifer, Admiraal, Gramajo, Cane, & Khosla, 2001). It consists of three individual proteins (DEBS1, DEBS2, and DEBS3), catalyzes the conversion of 6dEB from one propionyl-CoA starter unit and six (2S)-methylmalonyl-CoA extender units as shown in Figure 1.1 (B. A. Pfeifer et al., 2001). The genes of these proteins are about 10 kb each in size and have been engineered into a heterologous host, such as *Escherichia coli*, by many individual groups (H. Y. Lee & Khosla, 2007; Murli, Kennedy, Dayem, Carney, & Kealey, 2003; Peiru, Menzella, Rodriguez, Carney, & Gramajo, 2005; B. A. Pfeifer et al., 2001).

Figure 1.1. Modular Synthesis of 6-Deoxyerythronolide B (B. A. Pfeifer et al., 2001)
As shown in Figure 1.1, each DEBS protein contains two modules. A module represents a physical location for a Claisen-like condensation reaction (B. A. Pfeifer & Khosla, 2001). A starter unit propionyl-CoA is loaded onto the ketosynthase (KS) of module 1 by the loading didomain, acyl transferase (AT) and acyl carrier protein (ACP). An extender unit methylmalonyl-CoA is loaded onto the ACP of module 1 by AT domain. The condensation reaction takes place between the two units, and ketoreductase (KR) reduces the resulting ketone group while the chain passes to the KS domain of the next module in a progressive fashion. At the end of polyketide chain extension and diversification, the C-terminal thioesterase (TE) domain is responsible for the chain release and cyclization (B. A. Pfeifer & Khosla, 2001). For erythromycin production, deoxysugar residues will be attached to the macrolactone product, 6dEB, through post-PKS tailoring enzyme system, such as glycosyltransferase, and P450-type oxygenases.

1.2.2 Selection of heterologous hosts

Heterologous expression became increasingly popular due to three main advantages, (1) overproduction of the natural product with amenable production platform, (2) to facilitate combinatorial biosynthesis due to handy genetic tools and physiological insights of model organisms, and (3) lateral transfer of genes and heterologous expression might cause phenotypic alternation of host for easier selection (B. A. Pfeifer & Khosla, 2001).

For 6dEB heterologous biosynthesis, three different hosts have been studied, *Streptomyces coelicolor* (Kao, Katz, & Khosla, 1994), *Streptomyces lividans* (Xue, Ashley, Hutchinson, & Santi, 1999), and *Escherichia coli* (B. A. Pfeifer et al., 2001). Both *S. coelicolor* and *S. lividans* belonged to the *Streptomyces* genus. While the former as a model organism, the extensive knowledge of *Streptomyces* and the intrinsic PKS synthesis
apparatus made it an ideal host for PKS heterologous production (B. A. Pfeifer & Khosla, 2001). However, *S. coelicolor* was limited by only malonyl-CoA-derived building blocks could be utilized for synthesis. Moreover, using high-copy-number vectors in this expression system led to instability for unknown reasons (B. A. Pfeifer & Khosla, 2001). On the other hand, *E. coli* used for heterologous expression, had many advantages such as many molecular toolbox methods, well-known genetic information, fast growth, and potential for development of a high-cell-density production process. Nevertheless, *E. coli* as a prokaryotic platform, at least four challenges must be overcome, (1) avoid the PKS proteins to accumulate as inclusion bodies due to overproduction by adopting judicious control of temperature, medium composition, and other conditions, (2) provide indispensable posttranslational modification such as pantetheinylation of soluble PKSs, (3) avoid codon bias in translation due to high G+C content in actinomycete’s PKS genes by optimizing codon usage, and (4) ensure adequate amount of PKS substrates by reconstitution of heterologous pathways for substrate generation (B. A. Pfeifer & Khosla, 2001). Now 6dEB production by *E. coli* had been accomplished and proven with greater potential for further process development (Lau, Tran, Licari, & Galazzo, 2004; B. A. Pfeifer et al., 2001).

1.2.3 Organism background

Pfeifer and his colleagues developed the *E. coli* strain BAP1 from BL21(DE3) (Studier & Moffatt, 1986) for the heterologous production of PKS (B. A. Pfeifer et al., 2001). This strain and its derivatives, such as TB3 (Zhang, Boghigian, et al., 2010), and K207-3 (Kennedy, Murli, & Kealey, 2003), had been used as the expression host for 6dEB. The K207-3/pKOS207-129/pBP130 was a proprietary strain, which was used for a high-cell-density fed-batch process and reported with a maximum yield of over 1g/L 6dEB (Lau et al.,
2004). For this project, the *E. coli* strain TB3 was pre-constructed and kindly provided by the Pfeifer’s group (State University of New York at Buffalo) through collaboration with the William’s group (Zhang, Boghigian, et al., 2010).

The development of BAP1 included the introduction of heterologous DEBS genes through vectors, and chromosome engineering of four metabolic pathways from three different organisms (B. A. Pfeifer et al., 2001). Firstly, the surfactin phosphopantetheinyl transferase (*sfp*) gene from *Bacillus subtilis* (Quadri et al., 1998) was inserted into the *prpRBCD* location of the BL21(DE3) chromosome for posttranslational modification of the DEBS enzymes. The insertion of *sfp* disrupted the *prp* operon to eliminate its propionate catabolism function. Secondly, a T7 promoter was inserted before the native *E. coli* propionyl-CoA ligase (*prpE*) gene for overexpression and enhancing conversion of exogenous propionate into propionyl-CoA. Finally, the propionyl-CoA carboxylase (PCC) genes from *S. coelicolor* were incorporated in vectors and expressed in the host strain to enable the conversion of propionyl-CoA into (2S)-methylmalonyl-CoA (B. A. Pfeifer et al., 2001). Since 6dEB had no known cell toxicity against *E. coli*, self-resistance genes were unnecessary.

TB3 was a derivative strain of BAP1 (Zhang, Boghigian, et al., 2010) constructed by P1 transduction from *E. coli* ECK2916 (Baba et al., 2006). The deletion of *ygfH* gene by inserting the kanamycin cassette was reported with two folds of 6dEB increased production levels (Boghigian et al., 2011; Zhang, Boghigian, et al., 2010). The generic backgrounds of all the strains mentioned above are summarized in Table 1.1.
### Table 1.1. Generic Background of the Host Strains for 6dEB Production

<table>
<thead>
<tr>
<th>E. coli strains</th>
<th>Description</th>
<th>Notes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL21(DE3)</td>
<td>F-ompT hsdS8 (rg-mB-) gal dcm (DE3)</td>
<td></td>
<td>(Studier &amp; Moffatt, 1986)</td>
</tr>
<tr>
<td>BAP1</td>
<td>BL21(DE3); ΔprpRBCD::T7prom-sfp-T7prom-prpE</td>
<td>Produces holo proteins</td>
<td>(B. A. Pfeifer et al., 2001)</td>
</tr>
<tr>
<td>TB3</td>
<td>BAP1; ΔygfH</td>
<td>Produces holo proteins</td>
<td>(Zhang, Boghigian, et al., 2010)</td>
</tr>
<tr>
<td>K173-145</td>
<td>BAP1; panD::panDS25A</td>
<td></td>
<td>(Kennedy et al., 2003)</td>
</tr>
<tr>
<td>K207-3</td>
<td>K173-145; ygfG::T7prom-accA1-T7prom-pccB-T7term</td>
<td>Produces holo proteins</td>
<td>(Kennedy et al., 2003)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Genes</th>
<th>Resistance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBP130</td>
<td>(from pET21c) T7prom-DEBS2-DEBS3-T7term</td>
<td>Carb</td>
<td>(B. A. Pfeifer et al., 2001)</td>
</tr>
<tr>
<td>pBP144</td>
<td>(from pET28) T7prom-PCC-rbs-ACC-T7prom-DEBS1-T7term</td>
<td>Kan</td>
<td>(B. A. Pfeifer et al., 2001)</td>
</tr>
</tbody>
</table>

The strain used in the study, TB3/pBP130/pBP144, was cloned with two plasmids pBP130 and pBP144. They were constructed with the genes required for DEBS complex and substrate of 6dEB biosynthesis (Peiru et al., 2005). The two plasmids are shown in Figure 1.2.

![Figure 1.2. General Construction Map of pBP130 and pBP144](image)

In brief, pBP130 (~26kb) derived from pET21c, which contained the DEBS2 (eryA2) and DEBS3 (eryA3) genes under a single T7 promoter. Plasmid pBP144 (~19kb) derived
from pET28, which contained DEBS1 (eryA1) gene under a T7 promoter and genes coding for the two subunits of the Streptomyces coelicolor propionyl-CoA carboxylase enzyme ACC (accA1) and PCC (pccB) (Rodriguez & Gramajo, 1999) under the control of second T7 promoter. The DEBS genes were all cloned from the native erythromycin producer, Saccharopolyspora erythraea (Cortes, Haydock, Roberts, Bevitt, & Leadlay, 1990; Donadio, Staver, McAlpine, Swanson, & Katz, 1991). The two plasmids were retained under selection pressure of 50 mg/L kanamycin and 100 mg/L carbenicillin, respectively.

1.2.4 Summary of previous studies on 6dEB production using E. coli

Many strategies have been tried to improve the production titer of 6dEB, such as improving the stability of the large plasmids harboring DEBS genes (Murli et al., 2003), utilizing alternative substrate pathways (Dayem et al., 2002), and high-cell-density bioprocess production (Lau et al., 2004). As Table 1.2 summarizes, BAP1, TB3, and K207-3 were studied for improving 6dEB titer with various approaches, including medium development (B. Pfeifer et al., 2002; Zhang, Boghigian, et al., 2010), optimization of IPTG induction level and trace elements through P-B screening (Pistorino & Pfeifer, 2009), scale-up and process development (Lau et al., 2004; B. Pfeifer et al., 2002) and high-cell-density fermentation (Lau et al., 2004).

As shown in Table 1.2, the highest reported titer (1.1g/L 6dEB) was from the proprietary strain K207-3 in a 1.5-L fed-batch, high-cell-density fermentation for 13 days (Lau et al., 2004). For strain TB3, the highest reported titer (527 mg/L 6dEB) was operated in a 1-L batch mode with optimized production medium for 5 days fermentation (Zhang, Boghigian, et al., 2010). Generally, high-cell-density, batch or fed-batch mode, and medium optimization were the possible approaches for enhancing the 6dEB production. Additionally,
low incubation temperature (22°C) after IPTG induction was found to be critical for DEBS1 and DEBS2 proteins to be produced in active form (B. A. Pfeifer et al., 2001).

**Table 1.2. Summary of Reported 6dEB Production Titters in Heterologous *E. coli***

<table>
<thead>
<tr>
<th>Strains</th>
<th>Scale</th>
<th>Mode</th>
<th>6dEB titers</th>
<th>Notes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP1</td>
<td>1 L</td>
<td>Shake-flask</td>
<td>23.2 mg/L</td>
<td>LB, ~80 h, 1mM IPTG</td>
<td>(B. A. Pfeifer et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>25 mL</td>
<td>Shake-flask</td>
<td>1 ~ 10 mg/L</td>
<td>F1 medium, 10 μM ~ 10 mM IPTG, 10mg~10g/L propionate</td>
<td></td>
</tr>
<tr>
<td>BAP1</td>
<td>2 L</td>
<td>Fed-batch</td>
<td>~100 mg/L</td>
<td>F1 medium, 140 h, 100 μM IPTG, 2 g/L propionate (fed every 48-h)</td>
<td>(B. Pfeifer et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>2 L</td>
<td>Fed-batch</td>
<td>~180 mg/L</td>
<td>Same as above, but host strain contains thioesterase (pGZ119EH)</td>
<td></td>
</tr>
<tr>
<td>BAP1</td>
<td>2.5 mL</td>
<td>Shake-tube</td>
<td>12.6 mg/L</td>
<td>LB, 3 days, 100 μM IPTG, 20 mM propionate</td>
<td>(Wang, Boghigian, &amp; Pfeifer, 2007)</td>
</tr>
<tr>
<td></td>
<td>200 μL</td>
<td>96-well microplate</td>
<td>160 mg/L</td>
<td>Enhanced medium (40g/L tryptone+14g/L glycerol), 5 days, P-B screening</td>
<td>(Pistorino &amp; Pfeifer, 2009)</td>
</tr>
<tr>
<td>BAP1</td>
<td>15 mL</td>
<td>Shake-flask</td>
<td>65 mg/L</td>
<td>Production medium (10g/L tryptone+15g/L glycerol), 5 days</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 L</td>
<td>Batch</td>
<td>206 mg/L</td>
<td>Production medium (10g/L tryptone+45g/L glycerol), 5 days, OD~20</td>
<td>(Zhang, Boghigian, et al., 2010)</td>
</tr>
<tr>
<td>TB3</td>
<td>15 mL</td>
<td>Shake-flask</td>
<td>129 mg/L</td>
<td>Same as BAP1 in shake-flask</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 L</td>
<td>Batch</td>
<td>527 mg/L</td>
<td>Same as BAP1 in batch, OD~20</td>
<td></td>
</tr>
<tr>
<td>K207-3</td>
<td>unknown</td>
<td></td>
<td>22.5 mg/L</td>
<td>5 mM propionate (PCC genes and mutase pathway were integrated into chromosome)</td>
<td>(Kennedy et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>30 mL</td>
<td>Shake-flask</td>
<td>10–13 mg/L</td>
<td>Production medium (10g/L Marcor+15g/L glycerol), 5 days</td>
<td></td>
</tr>
<tr>
<td>K207-3</td>
<td>2 L</td>
<td>Fed-batch</td>
<td>700 mg/L</td>
<td>Defined medium (ammonium sulfate + glucose), 11 days, OD~50-60</td>
<td>(Lau et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>1.5 L</td>
<td>Fed-batch</td>
<td>1.1 g/L</td>
<td>Defined medium (ammonium sulfate + glucose), 13 days, OD~60-120</td>
<td></td>
</tr>
</tbody>
</table>

1.2.5 Production of 6dEB analogs using *E. coli*

The significance of producing 6dEB analogs through chemobiosynthesis or precursor-directed biosynthesis was to find novel structures or derivatives of erythromycin for new bioactive compounds. Also, it created a novel paradigm by using any favorable precursor to enlarge the chemicals reservoir that integrates novelty, patentability and scalability (Zhang, Wang, et al., 2010). Precursor-directed biosynthesis of 6dEB used a version of mutant 6-deoxyerythronolide B synthase (DEBS) of which was deleted or had
replaced the loading or other early-stage modules, which allowed the modified building blocks to be incorporated (Frykman, Leaf, Carreras, & Licari, 2001; Murli et al., 2005; B. A. Pfeifer et al., 2001; Terasaka et al., 2003). For example, a chemobiosynthesis engineered DEBS route was established in both *E. coli* and *S. coelicolor* and fed with a variety of acyl-thioesters and several novel 15-R-6dEB analogues were produced (Murli et al., 2005).

Figure 1.3. Example of 6dEB Analogue Strategy

The Williams Group developed an AT-mutagenesis strategy (Figure 1.3) and engineered a set of biosynthetic components for the chemobiosynthesis of a panel of novel 6dEB analogues that are regioselectively modified with various non-native and non-natural chemical functionalities. These analogues had potentials for the discovery of new macrolide antibiotics with optimized pharmacological properties.

1.3 High Cell-density Fed-batch Fermentation Using *Escherichia coli*

*E. coli* is a facultative anaerobe, which means it can grow both aerobically and anaerobically. Because *E. coli* grows better as an aerobic organism, aerobic fermentation is preferred for reaching a higher cell density as well as higher product protein production. To
achieve a high cell-density fermentation, Fed-batch was one of most popular modes for bioreactor operation, along with batch and continuous operations (Faulkner et al., 2006; Yamanè & Shimizu, 2006). Batch operation was suitable for small productions and kinetic studies but usually unstable for extended process, because pH and dissolved oxygen, were usually left uncontrolled (Yee & Blanch, 1992). Fed-batch operations were defined by one or more nutrients being added in a predetermined or controlled fashion aiming for increasing the biomass concentration and the productivity, while the products were harvest at the end of the run (Cinar, Parulekar, Ündey, & Birol, 2003; Yee & Blanch, 1992). A high cell-density fermentation could improve productivity and stability, reduce reactor volumes, achieve higher volumetric productivities, simplify downstream processing, reduce wastewater and overall costs of production (Kangwa et al., 2015; S. Y. Lee, 1996).

Fed-batch usually had a tighter control of various Critical Process Parameters (CPPs) in favor of cell growth and volumetric yield of target metabolites, such as pH, dissolved oxygen, substrate & inhibitor levels, anti-foaming, and specific growth rate (Kangwa et al., 2015; Krause, Neubauer, & Neubauer, 2016). The highly concentrated feed usually was a growth-limiting nutrient, such as glucose or glycerol. The control of substrate feeding rate would affected the specific growth rate, substrate & metabolite inhibition effect, respiration rate and the product accumulation rate (Hewitt & Nienow, 2007; Krause et al., 2016). Other than glucose, oxygen supply was always a limitation factor in an aerobic processes because of the limited solubility of oxygen (Krause et al., 2016).
Figure 1.4 shows the concept of a typical fed-batch operations with or without feedback control (Moulton, 2014). The feeding mode could be bulk step-feed, constant feeding rate, or various feeding rate with feedback control.

For a feedback-controlled fed-batch, usually the dissolved oxygen, pH, and substrate concentrations were monitored and controlled in favor of cell growth. Normally, by changing the volumetric feeding rate, the concentration of growth-limiting substrate (e.g. glucose), was kept constant at a low level to avoid substrate inhibition effect and the formation of harmful by-products, e.g. acetic acids (Cinar et al., 2003; Moulton, 2014). The inhibiting concentrations of acetate for different strains of *E. coli* varied between 5 and 10 g/L (Kleman & Strohl, 1994). Moreover, this allowed the cells to grow at a desired slow specific growth rate to ensure proper expression, plasmids stability, less foaming or cell lysis, and a stable system for longer fermentation to accumulate more target product (Faulkner et al., 2006).
Normally there were four phases to the growth curve of bacterial culture like *E. coli*, which are lag phase, exponential growth phase, stationary phase, and death phase. Lag phase was usually eliminated during industrial production by using seed culture of mid-exponential phase as inoculum for bioreactor fermentation (Cinar et al., 2003; Moulton, 2014). A generation time was described as the time it took for the culture to double the total number of cells in the culture (Moulton, 2014). Under standard conditions, the generation time (or doubling time) of an *E. coli* culture was usually between 20 to 30 minutes. Generally, most recombinant *E. coli* had longer generation time due to growth stress by producing recombinant proteins as well as antibiotic selective pressure for maintaining heterologous vectors. In the growth curve, it meant the cell number (N), which was indicated by optical density (OD$_{600}$), was a function of time (T) (Moulton, 2014). With a few mathematical manipulations, the growth equation could be written in the well-known form of

\[ N = N_0 e^{\mu t} \]

where \( \mu \) was the growth rate constant and \( N_0 \) was the initial cell number. To calculate the growth rate (\( \mu \)), a relation between growth rate and the turbidity (optical density) of the cell culture over time could be written as

\[ \mu = \frac{\ln \text{OD}_{600}(t_2) - \ln \text{OD}_{600}(t_1)}{t_2 - t_1} \]

In practice, the measurement of optical density was linear only within lower range of less than 0.8 at A$_{600}$ and hence samples of growing culture must be diluted to this range prior measurement. The growth curve is plotted using the natural log of OD$_{600}$ against fermentation time t (h) and linear regression within the exponential phase used to determine the specific growth rate. After the stationary phase, the culture would eventually reach death.
phase because of severe nutrient starvation or inhibited growth due to the enrichment of toxic inhibitors or cell debris (Moulton, 2014).

As mentioned, in an ideal feedback-controlled fed-batch operation, the concentration of growth-limiting substrate (e.g. glucose) in culture was kept constant. Ideally, there should be only one growth-limiting factor, such as carbon source, while other factors, such as dissolved oxygen and pH, were controlled at sufficient level or ideal range for cell growth (Faulkner et al., 2006). Hence, the feeding rate of substrate F (L/h) was related to the concentration of substrate $c_{\text{glu,s}}$ (g/L) in production system. Meanwhile, the concentration of glucose was a function of the substrate uptake rate $qS$ (g/g•h$^{-1}$), initial reactor liquid volume $V_0$ (L), initial substrate concentration in system $c_{0,s}$ (g/L), feed volume $V_F$ (L), substrate concentration in feed $c_{\text{glu,F}}$ (g/L), the evaporation loss (usually negligible) and time $t$ (h). The feed rate $F$ (L/h) could be described in following equation

Equation 1.3.  

$$F \sim c_{\text{glu},s} = f (qS, V_0, c_{0,s}, V_F, c_{\text{glu,F}}, t)$$

The substrate uptake rate was dynamically changing, and the cell growth was also inhibited by acetate level once it reached threshold level. Lots of mathematical models and expressions for various fed-batch system, such as constant feeding and exponential feeding, were developed and described in the literature (Cinar et al., 2003; Faulkner et al., 2006; Xu, Jahic, & Enfors, 1999; Yamanè & Shimizu, 2006; Yee & Blanch, 1992).

The feedback control of a bioreactor was usually based on signals obtained from the on-line sensors for pH, DO, glucose and acetate levels. Moreover, the initiation of feeding began when the initial substrate was depleted or consumed to a very low level. Usually it was at the early to mid-exponential phase of cell growth. By keeping the glucose concentration at constant low level, the cells could grow at a desired specific growth rate which was below
the critical growth rate for acetate formation (Faulkner et al., 2006). The threshold values were found to be 0.35 h\(^{-1}\) for defined media (Jensen & Carlsen, 1990), and 0.2 h\(^{-1}\) for semi-defined media (Riesenberg, 1991; Riesenberg et al., 1991).

1.4 **Nanoporous Latex Coatings of *Escherichia coli***

In the later stage of this study, a new strategy was introduced to overcome the high plasmids loss, a problem during long fed-batch fermentation. This new strategy used a cell immobilization and process intensification biocoating approach pioneered by the Flickinger group using a nanoporous latex coatings as patches in miniature 6-well plate fermentations (Flickinger, Schottel, Bond, Aksan, & Scriven, 2007). The latex coatings technique was a cell immobilization technique that helps to preserve living microbial cells as stabilized and highly concentrated biocatalysts as thin films at ambient temperature. The latex coating technique was superior than the traditional bioconversion routes (e.g. submerged fermentation) in many ways, such as higher specific reactivity (viability and activity), stability at ambient conditions, and convenience for preservation and transportation (without refrigeration) (Flickinger et al., 2017; Flickinger et al., 2007). The earliest latex biocatalytic coatings containing active microbial were studied by Lawton, Bunning and Flanagen in 1980s (Bunning, Lawton, Klei, & Sundstrom, 1991; Flanagan, Klei, Sundstrom, & Lawton, 1990; Lawton, Klei, Sunstrom, Voronka, & Scott, 1986). By that time, little knowledge of the mechanism of polymer film formation and preservation of microbial viability were available. Swope and Flickinger (Swope & Flickinger, 1996) were the first ones to demonstrate gene expression of latex *E. coli* coatings with validated porosity and preserved viability. Over the past two decades of studies, latex microbial coatings had been found to be
more promising with many advantages over some other porous materials for cell immobilization, such as biocers (biological ceramics) and hydrogels (Flickinger et al., 2007).

Recent studies on nanoporous latex coatings developed synthetic microbial biofilms with consistent thickness, high porosity, mechanical integrity, wet adhesive properties, high cell entrapping capacity (50% by volume), high cell viability, longer reactive half-life and decreased sensitivity to system stress (Flickinger, Lyngberg, Freeman, Anderson, & Laudon, 2009; Flickinger et al., 2007; Gosse, Engel, Hui, Harwood, & Flickinger, 2010; Gosse & Flickinger, 2011). The latex coatings mimicked natural biofilms, which are hundreds of microns to several millimeters thick films made of self-produced extracellular polymeric substances with a complex matrix structure and thousands of live microbes embedded within (Flickinger et al., 2007). Moreover, unlike natural biofilms that often slough when the majority of cells start to die, latex coatings or other nontoxic adhesive polymers could be used to retain the biological activity of immobilize whole-cell biocatalysts and probably extend their active life by permanently entrapping and stabilizing a high density of any viable cells in thin nanoporous coatings (Flickinger et al., 2017; Flickinger et al., 2007). The ideal scenario was to keep the cells biologically alive, but without division or propagation.

During the coating and drying process, some challenges must be resolved in order to generate active biocatalyst films (Flickinger et al., 2007). (1) Generating nanoporosity. Simple latex paint emulsions do not generate porous films during the polymer particle coalescence and diffusion (drying) process. Thus, to quench the rate and degree of polymer particle coalescence during film formation, addition of porogens or glycerol and carbohydrates were found to be effective (Lyngberg, Ng, Thiagarajan, Scriven, & Flickinger, 2001). (2) Selection of polymer material and circumventing formation toxicity. Commercial
latex emulsions usually contained antimicrobial and antifungal biocides that should be avoided if possible. The coating & drying process might impose some toxicities, such as osmotically stress or oxygen starvation, towards the cells (Charaniya, 2004). (3) Generating thin coatings with uniform thickness. This was also determined by the coating process. A thinner and porous coating improved the mass transfer and surface area for more embedded cells. While uniform thickness was important to reduce edge effects (Flickinger, Fidaleo, et al., 2009) (4) Other factors should be considered, such as pH control, capacity of cell concentration, reducing outgrowth by applying a thin nanoporous sealant topcoat, and determination of cell viability.

Despite all the challenges mentioned above, adhesive nanoporous latex polymer coatings have now been proven to be a successful technique for process intensification and various types of microorganisms had been used as substrate-limited non-growth immobilized biocatalysts by latex coatings, such as numerous E. coli strains (Lyngberg et al., 2001; Swope & Flickinger, 1996; Thiagarajan, Huang, Scriven, Schottel, & Flickinger, 1999), Gluconobacter oxydans (Fidaleo et al., 2006), Rhodopseudomonas alustris (Gosse et al., 2007), Thermotoga maritima (Lyngberg et al., 2005) and Rhosopseudomonas palustris (Gosse et al., 2010).

1.5 Project Objectives, Overviews and Challenges

1.5.1 Project objectives

This major goal of this study was to develop an optimized bioprocess for the over production of 6dEB using a recombinant E. coli strain TB3/pBP130/pBP144. To overcome challenges of plasmids incompatibility and determination of CPPs, three objectives were developed during the progressing of project.
1. Development of an optimized medium formula for improving the 6dEB titer as well as cell growth with stable bioactivity.
   a) A growth kinetics study in shake-flasks for understanding growth characteristics and nutrients preferences of the recombinant *E. coli* host strains that were used.
   b) A plasmid stability study for optimizing medium with a higher preservation of plasmids during a longer fermentation (batch & fed-batch).
   c) A medium optimization and comparison based on the maximum cell density and 6dEB titer during shake-flask fermentation.

   a) Batch operation study for growth kinetics of the recombinant strain.
   b) Development of a fed-batch fermentation process and optimization of bioreactor control parameters to achieve a stable system and operation.
   c) Medium optimization for achieving high cell-density fed-batch fermentation and high 6dEB production titers

3. Preliminary study on process intensification using nanoporous latex coatings to prepare immobilized biocatalyst to achieve higher 6dEB productivity.
   a) Optimization of cell paste preparation protocols using modified seed train preparation with improved plasmids-retaining ratio.
   b) Development of a preparation method for latex-coating patches for miniature fermentation and a proof of concept study on the 6dEB production titer.
1.5.2 Project overviews and challenges

As an overview of this project and the challenges therein, an illustrative mind map was shown below (Figure 1.5). Figure 1.5 shows the main progress of this project from development of medium, fed-batch process, and nanoporous latex coating cell patches, as well as some challenges and corresponding strategies.

To improve 6dEB titer with our recombinant E. coli strain, we started from medium optimization to achieve better cell growth as well as higher 6dEB production in shake-flask study. The major media that were tested and modified are high cell-density medium (HCD) (Lau et al., 2004), Production medium B from Sherman's group with ~50mg/L 6dEB titer previous unpublished result (Sherman, 2014), and F1, PM1, EM1, etc. reported by Pfeifer's group (B. Pfeifer et al., 2002; Pistorino & Pfeifer, 2009; Zhang, Skalina, Jiang, & Pfeifer, 2012). After that, we started to develop a high cell-density fed-batch process with DO-feedback control strategy. Then we found that after long fermentation time (> 20 h), the dual-plasmids become unstable. To minimizing the plasmid loss and change in plasmid ratio, we developed plasmids stability test/assay (PST) using a CFU selective plating method and a rapid method of preparation of latex-coating cell patches and minimized fermentation. The latex coating cell patches improved the observed stability of plasmids for a longer time.

We tried to purify the original seed culture for dual-plasmids containing cells using a CFU selective plating method. And the “purified” strain was applied back to the medium development study and fed-batch development in a later stage. The 6dEB analysis assays were developed by my colleague Christian Kasey from the William’s group. The challenges of the 6dEB assay development led to different assays were applied at different stages. In addition, the bioreactor equipment system had some shortcomings, such as rudimentary
feedback feeding control system and no on-line sensors for detecting glucose, glycerol, & acetate levels. All these problems added challenges on process development.

Figure 1.5. Illustration of Project Mind Map and Challenges

Due to assay challenges, such as a limited amount of standards, and long turnaround time of LC-MS, other 6dEB assay methods were developed and applied at different stages. Thus, some datasets were noncomparable among each other because of inconsistent assay methods. To save 6dEB assays costs, there were no replicates results to perform an accurate statistical analysis. We only completed the proof of concept and make conclusions on our
objectives, but the process could be further improved with more consistent and comparable data in the future.

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In Table 1.3, “Y” indicates at least some batches of this experiment had been tested using the assay method listed in the first row. “S” indicates synthetic 6dEB standards, which we had only 0.9 g given by Krische’s group from UT Austin and quickly used up. While “B” represented a biological 6dEB standard which I extracted and purified from the 1-L PB1 ferment broth by flash chromatography and TLC and tested with LC-MS and NMR results. Based on NMR results, the biological standard looked very pure.

**Table 1.3. Summary of 6dEB Assays and Types of 6dEB Standards**

<table>
<thead>
<tr>
<th>6dEB sample sources</th>
<th>LC-MS (UNCG)</th>
<th>LC-MS (MSF, NCSU)</th>
<th>HPLC-ELSD (William’s)</th>
<th>Flash chromatography + TLC + NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shake-flask &amp; medium development</td>
<td>Y, S*</td>
<td>Y, S</td>
<td>Y, B*</td>
<td></td>
</tr>
<tr>
<td>2-L fed-batch bioreactor</td>
<td>Y, S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmid stability</td>
<td>Y, S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Latex-cell-patches</td>
<td>Y, B</td>
<td>Y, B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-L PB1 ferment broth extraction</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>

*“Y” = at least some samples (row) were tested by this method (column); “S” = used synthetic 6dEB standard for calibration; “B” = used home-made “biological 6dEB standard for calibration
Three LC-MS assay methods were utilized in this project. The oldest data was collected by the Triad Mass Spectrometry facility in UNCG, using simple supernatants of fermentation broth as samples. Other data was collected by the Mass Spectrometry Facility (MSF) in Department of Chemistry (NCSU), using extracted and concentrated 6dEB sample in methanol. The MSF developed two LC-MS methods, 15-min & 10-min mass spectrometry (see Appendix II). Some samples of later stage of this project were only tested by the HPLC-ELSD (Evaporative Light Scattering Detector) assay developed by Christian Kasey (William’s group). But the limitation of detection (LOD) of HPLC-ELSD was found to be much higher than LC-MS assay (to 3 orders of magnitude). More details were discussed in Chapter 5.
CHAPTER 2.  Medium Development for High-Cell-Density and Enhanced 6dEB Production

2.1 Development of Shake-flask Method

2.1.1 Seed train and shaking flask procedures

The original strain TB3/pBP130/pBP144 was kindly provided by Dr. Pfeifer from University at Buffalo. Firstly, the strain was cultured in Luria-Bertani (LB) broth medium (Bertani, 1951) and expanded into a working cell bank. The protocol of creating a working cell bank was explained in Protocol A (Appendix I). Each batch of shaking-flask fermentation retrieved a fresh cryovial of strain from the working cell bank and perform the seed rejuvenation and shaking-flask fermentation protocol as illustration in Figure 2.1 below, using LB medium as an example.

![Figure 2.1. Illustration of Shaking-flask Fermentation Procedure Using LB medium](image)

(1) The LB/Kan+/Carb+ was the LB medium supplemented with antibiotics of kanamycin (50 mg/L) and carbenicillin (100 mg/L) (B. Pfeifer et al., 2002; B. A. Pfeifer et al., 2001; Zhang, Boghigian, et al., 2010).

(2) Seed rejuvenation. A LB (Kan+/Carb+) agar plate was streaked with glycerol strain stock using a sterile inoculating loop. After 12-15 h of incubation at 37°C, a single colony was picked to inoculate a seed culture (3 mL of LB/Kan+/Carb+) and incubated at 37°C, 300 rpm for 2 to 4 hours to reach mid-exponential phase.
(3) Inoculate the seed culture into a fresh 50-mL LB//Kan\textsuperscript{+}/Carb\textsuperscript{+} liquid medium with an initial OD\textsubscript{600} of 0.1. The media were supplemented with 0.1\% of 50\% Antifoam B. For other medium studies, use the corresponding medium in this step with supplements of antibiotics mentioned above.

(4) Incubate 50-mL of culture at 37°C, 250 rpm and change temperature to 22°C at mid-exponential phase (for LB, OD\textsubscript{600} was about 0.6~1.0) for induction with 100 μM IPTG (isopropyl-beta-D-thiogalactoside), and 35 mM of sodium propionate as precursor (Lau et al., 2004; B. Pfeifer et al., 2002; Pistorino & Pfeifer, 2009; Zhang, Boghigian, et al., 2010).

(5) Keep fermentation for 5 days. Add 15 mM of sodium propionate per day after Day 3. Take 1 or 2 mL of culture samples at various timepoint for OD\textsubscript{600} and pH measurement, glucose analysis (Appendix I), PST, and 6dEB assay (Appendix II).

2.1.2 Growth kinetics determination

The cell growth kinetics usually had four stages (phases): lag, exponential (log), stationary, and death phases. There were at least four purposes to determine the growth kinetics. Frist determine the OD\textsubscript{600} of the beginning of the log-phase. The fermentation started from the initial OD\textsubscript{600} of 0.1~0.2 (for LB) which could eliminate the lag phase. Second, the seed culture was collected at mid-exponential phase to be used as an inoculum for large volume fermentations (shake-flask or bioreactor), because the cells were healthy and vigorous. Third, optimization of medium composition was aiming for a better cell growth kinetics, such as shorter doubling time and higher maximum OD\textsubscript{600}. And finally, to predict the length of fermentation at healthy conditions and determine the sampling timepoint for each growth stages were necessary for process development.
Using LB medium (with antibiotics, no temperature changes) as an example, the cell growth kinetics of TB3/pBP130/pBP144 *E. coli* was shown as Figure 2.2 below. Triplicated samples were collected from three parallel shaking flasks at each timepoint. As shown in the figure, the period from 3 to 6.5 hour was the exponential phase, where the cells grew at the maximum specific rate $\mu_{\text{max}}$ at 0.8742 h$^{-1}$ or doubling time $t_d$ of 0.79 h. The final OD$_{600}$ was about 2.7 for this study. Apparently, LB medium was not ideal because of a long doubling time and low cell density. Growth kinetics study was repeated for all other media.

### 2.1.3 Determination of dry cell density vs. optical density

The calibration curve for correlation between optical density and dry cell weight (DCW) was established using aluminum muffin cups according to an established protocol (BEC363-Lab4, 2014 Spring). The calibration curve was shown as below (Figure 2.3).
Figure 2.3. Correlation of Dry Cells Density vs. OD$_{600}$ for TB3/pBP130/pBP144

To determine the correlation of DCW vs. OD$_{600}$, a lot of *E. coli* cells must be prepared. About 2.5 liters LB fermented culture (overnight) were centrifuged and the cell paste were collected and washed using 1% NaCl and made into dilutions. More details were explained in Protocol B (Appendix I).

2.2 **Medium Development and Optimization**

The composition of media mentioned in this project are listed in Appendix III. The optimization of medium included modifying the levels of carbon & nitrogen source and types, various buffer & salt strengths, trace metals (TM) & vitamins levels, IPTG level, etc. Many media were experimented and modified in compositions. The previous reported media included the chemically-defined media, such as CD1, high-cell-density medium (HCD) (Lau et al., 2004) and F1 (B. Pfeifer et al., 2002), and some semi-defined media, including the Production Medium B (Sherman, 2014), OPMedia (BTEC propriety), Production Medium and Enhanced Medium (Pistorino & Pfeifer, 2009) were also evaluated.

\[
y = 0.3506x + 0.1798 \\
R^2 = 0.9999
\]
2.2.1 Main medium components optimization

The optimization started from a chemical defined medium (Lau et al., 2004), which was renamed as CD1 in this study. Based on the growth kinetics methods, we investigated the effects of various concentrations of glucose, ammonium sulfate, and pH buffer strengths.

2.2.1.1 Optimization of glucose & ammonium sulfate levels

From CD1 to CD4 medium, the glucose level was increased by four folds, while ammonium sulfate (nitrogen source) was increased by 10 folds. As shown in Figure 2.4, CD4 medium produced a final OD$_{600}$ that was 3.5-folds from LB, and 6.5 folds from CD1. The lag phase was eliminated by using an inoculum of mid-log phase to reach an initial OD of 0.1. The doubling time of CD4 was 1.2 h, which was between 0.79 h (LB) and 1.79 h (CD1).

![Growth Kinetics of Recombinant E. coli in LB, CD1 and CD4 (Shake-flask)](image)

**Figure 2.4. Growth Kinetics of Recombinant E. coli in LB, CD1 and CD4 (Shake-flask)**

Table 2.1 shows the optimization from CD1 to CD4 by adjusting the levels and ratio of carbon and nitrogen. Usually, a medium with a lower carbon-to-nitrogen ratio (C/N) would have a shorter doubling time because more nitrogen might achieve a fast growth rate.
However, this could lead to considerable amounts of ammonium by-products that was toxic and caused the medium to become basic (affect the pH). High growth rate might increase the probability of losing one or both plasmids during cell replication. On the other hand, cell growth in a medium with a higher C/N ratio could obtain a higher specific growth rate and final OD$_{600}$ (cell density) while leading to a lower pH depending on the type of carbon source. Cell growth in a medium using glycerol instead of glucose would have a longer doubling time. The levels and types of carbon and nitrogen sources were adjusted to achieve a desired specific growth rate and maximum cell density.

Table 2.1. Comparison Among Chemical Defined Media

<table>
<thead>
<tr>
<th>Media Name</th>
<th>LB</th>
<th>CD1</th>
<th>CD2</th>
<th>CD3</th>
<th>CD4</th>
</tr>
</thead>
<tbody>
<tr>
<td>max specific rate $\mu_{\text{max}}$ (h$^{-1}$)</td>
<td>0.8742</td>
<td>0.3879</td>
<td>0.4458</td>
<td>0.5518</td>
<td>0.5798</td>
</tr>
<tr>
<td>Doubling time $t_d$ (h)</td>
<td>0.79</td>
<td>1.79</td>
<td>1.55</td>
<td>1.26</td>
<td>1.20</td>
</tr>
<tr>
<td>PPS buffer (mM)</td>
<td>None</td>
<td>35</td>
<td>35</td>
<td>50</td>
<td>70</td>
</tr>
<tr>
<td>Glucose (g/L)</td>
<td>TOC = 0.6 -0.9</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$ (g/L)</td>
<td>TON = 1.7 - 1.9</td>
<td>0.4</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>C/N ratio</td>
<td>0.3 - 0.5</td>
<td>5.94</td>
<td>4.752</td>
<td>4.752</td>
<td>2.376</td>
</tr>
<tr>
<td>Final pH</td>
<td>$&gt; 8.5$</td>
<td>6.80 - 6.85</td>
<td>6.3 - 6.7</td>
<td>4.3 - 4.8</td>
<td>4.1 - 4.3</td>
</tr>
<tr>
<td>Final OD$_{600}$</td>
<td>2.6 - 2.8</td>
<td>1.4 - 1.6</td>
<td>5.4 - 5.7</td>
<td>5.10 - 7.03</td>
<td>9.78 - 10.73</td>
</tr>
<tr>
<td>Dry cell density (g/L)</td>
<td>0.92 - 0.99</td>
<td>0.50 - 0.57</td>
<td>1.9 - 2.0</td>
<td>1.80 - 2.49</td>
<td>3.46 - 3.80</td>
</tr>
</tbody>
</table>

TOC = total organic carbon; TON = total organic nitrogen; the data is calculated by addition of TOC or TON each component of LB medium (according to manufacturer’s product information)

2.2.1.2 pH buffer types and strengths

As we know, the pH was affected by C/N ratio and the types of medium components. Thus, the strengths of the phosphate buffer were adjusted to control pH neutral. As we know, the pH was affected by C/N ratio and the types of medium components. Thus, the strengths of the phosphate buffer were adjusted to control pH neutral. shows the pH changes of some media in shaking flask studies with no induction and incubation at a constant 37°C. In CD4
medium, the cells grew at a high specific rate of 0.57 h\(^{-1}\) and pH decreased quickly due to formation and accumulation of by-product acetate at the end of log-phase (after 9 h).

![Figure 2.5. pH Comparison of Recombinant E. coli in LB, CD1 and CD4 (Shake-flask)](image)

Because the glucose level was much higher in CD4 (20 g/L) than CD1 (5 g/L), more acetate was accumulated and hence lead to a lower pH. For LB, the pH rose above 8 because it was rich in the N-source with a low C/N ratio. To maintain a neutral pH environment, the strength of phosphate buffer was increasing to 150 mM (original 35 mM). Meanwhile, 100 mM HEPES buffer could also maintain the desired pH range (data not shown). Phosphate buffer (PPS) was the main choice for saving cost in this study by eliminating costly HEPES buffer.

2.2.1.3 Comparison of inorganic and organic nitrogen sources

Semi-defined medium usually referred to a medium that contained organic nitrogen source because growth and expression were not merely affected by the amount of nitrogen,
but also the composition of amino acids. Based on 6dEB titer analysis, further optimization
compared inorganic and organic nitrogen sources. LB was used as a control for comparing
6dEB titers of other media. Based on a semi-defined media, Production Medium B (Sherman,
2014), PA1 and PB1 media were defined and compared with LB and CD4. In which, PA1
was chemically-defined and derived from PB1 and CD4, with the main components
compared in Table 2.2 below.

Table 2.2. Comparison of various C- and N-sources

<table>
<thead>
<tr>
<th>Media Name</th>
<th>LB</th>
<th>CD4</th>
<th>PA1</th>
<th>PB1</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu_{\text{max}}$ (h$^{-1}$), BI*</td>
<td>1.181</td>
<td>0.493</td>
<td>0.371</td>
<td>0.688</td>
</tr>
<tr>
<td>$t_d$ (h), BI</td>
<td>0.59</td>
<td>1.41</td>
<td>1.89</td>
<td>1.01</td>
</tr>
<tr>
<td>$\mu_{\text{max}}$ (h$^{-1}$), AI*</td>
<td>0.222</td>
<td>0.077</td>
<td>0.047</td>
<td>0.088</td>
</tr>
<tr>
<td>$t_d$ (h), AI</td>
<td>3.12</td>
<td>9.06</td>
<td>14.84</td>
<td>7.89</td>
</tr>
<tr>
<td>pH buffer (mM)</td>
<td>n/a</td>
<td>PPS, 70</td>
<td>PPS, 150</td>
<td>PPS, 150</td>
</tr>
<tr>
<td>C-source (g/L)</td>
<td>n/a</td>
<td>glucose, 20</td>
<td>glycerol, 14</td>
<td>glycerol, 14</td>
</tr>
<tr>
<td>N-source (g/L)</td>
<td>n/a</td>
<td>(NH4)2SO4, 4</td>
<td>(NH4)2SO4, 4</td>
<td>Tryptone, 40; YE, 1</td>
</tr>
<tr>
<td>TM folds</td>
<td>0</td>
<td>1</td>
<td>1.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Vitamin folds</td>
<td>0</td>
<td>1</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Final OD$_{600}$</td>
<td>2.770</td>
<td>7.880</td>
<td>8.160</td>
<td>9.480</td>
</tr>
<tr>
<td>6dEB titer (mg/L) at 43 h</td>
<td>6.37 ± 0.95</td>
<td>0.04 ± 0.01</td>
<td>2.07 ± 0.78</td>
<td>25.15 ± 11.36</td>
</tr>
</tbody>
</table>

All results based on 300 µM IPTG induction & 35 mM propionate precursor; *BI = Before induction, AI = After induction; ** TM = trace metal solutions

As shown in Table 2.2, the compositions, growth and production of 4 media were
compared. The 6dEB titers were assayed by UNCG LC-MS facility, using triplicated
supernatant samples (not concentrated) after induction with 300 µM IPTG. CD4 and PA1 had
much lower 6dEB titers compared to either LB or PB1. Tryptone as an organic nitrogen
source seemed to have a positive effect on 6dEB production, which was also reported by
Pistorino and Pfeifer (2009).
2.2.1.4 Further optimization on semi-defined media for higher 6dEB titer

Some new semi-defined media were developed, including CD6, CD7, PB1-Int, HCD with modified feed, PM1, and EM1. The compositions of all media were listed in Appendix III. Figure 2.6 shows the growth kinetics and 6dEB production titers of various media in 50-mL shaking flask fermentation.

![Figure 2.6. Comparisons of Growth Kinetics on Various Media](image)

Figure 2.6. Comparisons of Growth Kinetics on Various Media

Figure 2.6 shows various media that were tested. In which, CD4, CD6, and CD7 were modified CD-series media originated from CD1 (Lau et al., 2004). PM1 and EM1 media were modified from Production Medium and Enhanced Medium reported by Pistorino and Pfeifer (2009), respectively. The major medium components & levels, key parameters of growth kinetics, and 6dEB titers were summarized in Table 2.3 below. In which, YE was the yeast extract and TM was trace metals solution. The 6dEB samples were assayed by LC-MS facility from Chemistry Department (NCSU), using nonreplicated 2.5x concentrated samples dissolved in methanol, which were extracted from the supernatants of cultures.
### Table 2.3. Summary of Major Optimized Media by Shake-flask Studies

<table>
<thead>
<tr>
<th>Media Name</th>
<th>LB</th>
<th>CD4</th>
<th>CD6</th>
<th>CD7</th>
<th>PB1-Int</th>
<th>PM1</th>
<th>EM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\mu_{\text{max}}$ (h$^{-1}$), BI$^*$</td>
<td>0.857</td>
<td>0.729</td>
<td>0.742</td>
<td>0.788</td>
<td>0.856</td>
<td>0.801</td>
<td>0.825</td>
</tr>
<tr>
<td>$t_d$ (h), BI</td>
<td>0.81</td>
<td>0.95</td>
<td>0.93</td>
<td>0.88</td>
<td>0.81</td>
<td>0.87</td>
<td>0.84</td>
</tr>
<tr>
<td>$\mu_{\text{max}}$ (h$^{-1}$), AI$^*$</td>
<td>0.226</td>
<td>0.049</td>
<td>0.024</td>
<td>0.022</td>
<td>0.013</td>
<td>0.067</td>
<td>0.065</td>
</tr>
<tr>
<td>$t_d$ (h), AI</td>
<td>3.07</td>
<td>14.15</td>
<td>28.88</td>
<td>31.51</td>
<td>53.32</td>
<td>10.35</td>
<td>10.66</td>
</tr>
<tr>
<td>pH buffer (mM)</td>
<td>n/a</td>
<td>PPS, 70</td>
<td>PPS, 150</td>
<td>PPS, 150</td>
<td>PPS, 150</td>
<td>HEPES, 100</td>
<td>HEPES, 100</td>
</tr>
<tr>
<td>C-source (g/L)</td>
<td>n/a</td>
<td>glucose, 20</td>
<td>glucose, 20</td>
<td>glucose, 20</td>
<td>glycerol, 14</td>
<td>glycerol, 15</td>
<td>glycerol, 14</td>
</tr>
<tr>
<td>N-source (g/L)</td>
<td>n/a</td>
<td>(NH$_4$)$_2$SO$_4$, 4.</td>
<td>casamino acids, 4; YE, 1.</td>
<td>tryptone, 4; YE, 1.</td>
<td>tryptone, 4; YE, 1.</td>
<td>tryptone, 10; YE, 5.</td>
<td>tryptone, 40; YE, 1.</td>
</tr>
<tr>
<td>TM folds</td>
<td>0</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>4.5</td>
<td>5.625</td>
<td>5.625</td>
</tr>
<tr>
<td>Vitamin folds</td>
<td>0</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.875</td>
<td>1.875</td>
</tr>
<tr>
<td>Final OD$_{600}$</td>
<td>2.760</td>
<td>12.480</td>
<td>10.180</td>
<td>7.100</td>
<td>3.160</td>
<td>8.040</td>
<td>7.680</td>
</tr>
<tr>
<td>6dEB titer (mg/L)</td>
<td>29.80</td>
<td>15.08</td>
<td>76.87</td>
<td>9.77</td>
<td>8.32</td>
<td>51.98</td>
<td>34.40</td>
</tr>
</tbody>
</table>

All results based on 1 mM IPTG induction & 35 mM propionate precursor; $^*$BI = Before induction, AI = After induction.

As shown in Figure 2.4, Figure 2.6, Table 2.1 and Table 2.3, the growth kinetics of CD-series media improved significantly from the original CD1 medium. The final optimized CD-series media were CD4, CD6 and CD7 with potentials to be developed for fed-batch process in the future. The advantages of these new optimized media including:

1. Relative short time (3 ~ 5 h) to reach a desired OD$_{600}$ for induction while growing at a high growth rate of 0.73 ~ 0.85 h$^{-1}$. The formation and accumulation of acetate was negligible for the fermentation duration time was short.

2. Desired low growth rate after temperature was changed from 37°C to 22°C after induction, which was around 0.2 h$^{-1}$, a reported critical threshold growth rate for inhibitory acetate to form (Faulkner et al., 2006; Riesenberg et al., 1991).
(3) Simple carbon & nitrogen sources, which were easy to be developed into initial and feed media for fed-batch process

(4) Final OD$_{600}$ was high (7 ~ 13). Relative high cell-density achieved at shaking-flask scale, indicating a possibility to achieve a high cell density in bioreactor.

(5) 6dEB titers in optimized defined media were comparable or higher than LB (29.80 mg/L), e.g. CD4 (15.08 mg/L), and CD6 (76.87 mg/L).

PM1, EM1 were semi-defined media derived from Production Medium and Enhanced Medium in a previous study (Pistorino & Pfeifer, 2009) with reduced salts or nutrients compared to the originals. PM1 and EM1 had very high 6dEB titers, which are 51.98 mg/L and 34.40 mg/L. Although they were lower than the reported titers of original media as shown in Table 1.2, our modified PM1 and EM1 still had several advantages:

(1) We used 50-mL liquid medium in 250 mL baffled shaking-flasks, which was larger in volume than both Production Medium (15 mL) and Enhanced Medium (200 μL). Smaller volume fermentations might be affected by system errors due to evaporation.

(2) Reduced nutrients were preferred to be used as initial medium for fed-batch process. There was a potential to reach much higher titers in batch or fed-batch. For example, the titer of Production Medium was improved by 3 folds from 15-mL shaking flask to 1-L batch bioreactor operation (Table 1.2)

(3) Relative high cell density (OD$_{600}$ ~ 8) was achieved in shaking-flask scale.
Figure 2.7. Glucose Consumption Analysis for CD4, CD6, and CD7
The other components modified were the trace metals and vitamins levels. Both were used in elevated levels for optimized media. We did not investigate thoroughly the levels of each components. However, it was reported that tryptone and the trace metals solution had a significant effect on 6dEB production (Pistorino & Pfeifer, 2009). In addition, some vitamins are also necessary for a healthy cell growth and metabolism, such as biotin (B. A. Pfeifer et al., 2001; Wenzel & Muller, 2005; Williams, 2013).

2.2.2 Glucose consumption

Glucose concentration of samples were measured off-line using the supernatant samples by a YSI 2900 Biochemistry Analyzer (Appendix I). The glucose consumption rate of media CD4, CD6, CD7 in the shaking-flask study were compared in Figure 2.7 above along with growth kinetics and 6dEB titer. All three media had an initial glucose concentration of 20 g/L and the cultures were induced by 1 mM IPTG with 35 mM propionate at 3.5~4 h, when cells were at mid-exponential phase. CD4 used ammonium sulfate, while CD6 and CD7 used organic N-sources. CD4 had a higher glucose consumption rate at 0.39 g•L⁻¹•h⁻¹ than CD6 (0.26 g•L⁻¹•h⁻¹) and CD7 (0.13 g•L⁻¹•h⁻¹).

The glucose consumption behavior of a batch-mode fermentation can be used to determine the feeding rate for a fed-batch process. For example, the feeding usually started when the glucose in the initial medium was depleted. In addition, the glucose consumption rate determined the feeding rate so that the concentration of glucose in the bioreactor could be kept at a low level. For example, there were reported feeding controls to maintain glucose within 5 and 15 g/L during the production phase (Lau et al., 2004), or below 1 g/L (B. Pfeifer et al., 2002). The purpose was to avoid substrate inhibition effect or the accumulation of inhibitory acetate.
2.2.3 IPTG levels

Three levels of IPTG were investigated, including 100 μM, 1 mM and 10 mM. The propionate precursor levels were same. As shown in Figure 2.8 below, overall speaking for a high cell density medium, the 6dEB titer of using 1 mM IPTG induction was usually higher than the other levels because the efficacy of inducer levels are related to the available IPTG per cell. While for LB, 100 μM IPTG was sufficient for all cells, and hence 1 mM IPTG did not improve the 6dEB production. For CD7, 6dEB titer at 10 mM IPTG induction was significantly decreased, which was probably due to the toxicity of a high IPTG concentration.

![IPTG Level Comparison for Higher 6dEB Titer](image)

**Figure 2.8. IPTG Level Comparison for Higher 6dEB Titer**

High IPTG level might also trigger excessive metabolic stress for cells at mid-exponential phase and lead to loss of plasmids, which was discussed in Chapter 5. As shown in Figure 2.9 below, the cell growth in LB at high IPTG induction level (10 mM) indicates a 45% shorter exponential phase and 30% lower maximum cell density than 1 mM induction. Excessive metabolic stress, which was related to available IPTG per cell, might slow down the cell growth and cause loss of plasmids. Culture with lower growth rate had less loss of...
plasmids and hence suggested a higher expression level. Thus, it was preferred to induce the culture at mid-exponential phase to reduce the IPTG inhibition and to ensure high level heterologous protein expression in healthy cells. In addition, the induction temperature was critical; determined to be 22°C for a correct folding of DEBS protein (B. A. Pfeifer et al., 2001).

![Figure 2.9. Effects of IPTG Levels on Cell Growth in LB Medium](image)

2.2.4 Precursor selection and feeding strategy

Usually, sodium propionate was used as the feeding substrate for conversion of precursors to 6dEB biosynthesis (Lau et al., 2004; B. Pfeifer et al., 2002; B. A. Pfeifer et al., 2001; Pistorino & Pfeifer, 2009; Zhang, Boghigian, et al., 2010). However, propanol which is used in the industrial production of erythromycins might also be used as a precursor substrate in this case because propanol provided a 3-carbon structure as a soluble metabolic intermediate that does not affect the culture pH.
Figure 2.10 compared the 6dEB production of using propionate and propanol. In this experiment, both precursor substrates were fed at 35 mM initially at induction, and 15 mM supplemented every 24 hours after IPTG induction. The appropriate amount of sterile 50% Antifoam B was added if there was a foaming problem during fermentation. Both conditions were induced by 1 mM IPTG level for all 4 media.

![Bar chart of Max. 6dEB titer (mg/L) for LB, CD7, PM1, and EM1 with propionate and propanol](image)

**Figure 2.10. Comparison of Precursor on 6dEB titer between propionate and propanol**

In general, propionate had a higher titer in 6dEB production. However, there was no statistically significant conclusion on the effects of propanol and propionate towards 6dEB titer. More data was needed for a solid statistical comparison. However, propanol might be more convenient in large-scale application because it was liquid under ambient environment. While propionate must be prepared into a concentrated master solution before use.

2.3 **Discussion and Conclusion**

Because 6dEB doesn’t have chromophore and hence is UV inactive, other assays should be developed to analyze 6dEB concentration, such as HPLC with evaporative light scattering detector (ELSD) and HPLC-Mass Spectrometry. In this study, both LC-MS and
HPLC-ELSD assays were employed and further discussed in Chapter 5. Most LCMS assays were developed and performed by MSF in NCSU and Christian Kasey from William’s group. In this project, it was unintended that multiple LC-MS assay methods were employed in medium optimization study. Therefore, some data from different testing facilities or assayed by different methods were not comparable and conclusions between different experiments cannot be rigorously compared in a strict manner.

Figure 2.11. Summary of 6dEB Production in Shaking-flask Studies

As shown in Table 2.2, 6dEB assay results from UNCG indicated that PB1 had the highest titer among the four media that was tested in the first batch. The titer in PB1 was 25.17 ± 11.36 mg/L, which was about 4~5 folds higher than the LB medium titer.

Table 2.3 and Figure 2.11 shows the results mainly obtained by MSF in the Department of Chemistry (NCSU), where the major optimized media were compared (with 1
mM IPTG induction). The “*” indicates 6dEB was assayed in UNCG MS facility (0.1 mM IPTG induction). The composition of each medium can be found in Appendix III.

During the development of CD-series media, we found a higher level of glucose caused a higher growth rate and final cell OD, but possibly led to acetate accumulation and a pH challenge. While high levels of ammonium sulfate usually led to possible early cell lysis and hence foaming problem (Cinar et al., 2003; B. Pfeifer et al., 2002; Wang et al., 2007). During the shake-flask studies, most chemically-defined media showed a foaming and cell-lysis situations, while the semi-defined medium PB1, PM1 and EM1 were less likely to have a severe foaming problem indicating less cell lysis.

The 6dEB titer of chemically-defined CD4 was about 2-folds less than LB medium. Organic N-sources such as tryptone were reported to have a positive effect on a high titer of 6dEB production (Pistorino & Pfeifer, 2009). Thus, we developed the semi-defined medium CD6 and CD7, of which casamino acids and tryptone were used, respectively. Interestingly, CD6 (76.87 mg/L) increased titer about 8-fold compared to CD7 (9.77 mg/L). By simply changing the type of N-source from tryptone into casamino acids, it was possible that casamino acids had a more positive effect in facilitating the expression of DEBS enzyme levels and hence a higher 6dEB titer, which was worth further investigation. CD6 was also the medium with the highest 6dEB titer among all optimized media in shaking-flask studies. Compared to LB shaking-flask studies, the 6dEB titer of CD6 was improved by 158%.

Based on PB1 medium, PB1-Int used 10 times less tryptone. As a result, PB1-Int medium displayed a lower 6dEB titer (8.32 mg/L) than PB1 (25.15 mg/L) or the original Production Medium B, ~50 mg/L (Sherman, 2014). Meanwhile, the modified PM1 and EM1 showed the most 6dEB production potential among all media because of relatively high
6dEB titers and less foaming issues. The high 6dEB titers were possibly accredited to either high tryptone level (EM1, 40 g/L) or high yeast extract level (PM1, 5 g/L). Due to the limitation of time (long turnaround of assay) and tested samples (saving cost), there was not enough data to perform a statistical analysis and conclusion. In the future, more data should be collected based on the suggestions mentioned in Table 2.4 below.

For the development of a fed-batch process, we started from development of HCD (based on CD4), and PB1 medium for high cell-density and enhanced 6dEB production. Table 2.4 shows the key points of medium compositions or fermentation process development studies in achieving a high 6dEB production at shaking-flask scale.

**Table 2.4. Key Points of Optimized Shaking-flask Protocol and Medium**

<table>
<thead>
<tr>
<th>Seed train</th>
<th>Plate cannot be over incubated (&lt;15 h) to pick single colony; 3-mL seed must be stopped within mid- to late-log phase (OD=0.6-1.2 for LB seed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculation</td>
<td>Initial OD&lt;sub&gt;600&lt;/sub&gt; = 0.1 ~ 0.2 to eliminate lag-phase</td>
</tr>
<tr>
<td>Induction</td>
<td>1 mM IPTG, 35 mM sodium propionate, change to temperature at 22°C</td>
</tr>
<tr>
<td>Substrate</td>
<td>Propionate and propanol are both feasible; the former may have a higher 6dEB titer; Supplement extra 15 mM substrate per day to ensure adequate supply</td>
</tr>
<tr>
<td>pH buffer</td>
<td>Phosphate buffer 150 mM; pH adjusted at 7.0</td>
</tr>
<tr>
<td>Antifoam B</td>
<td>Initially add 0.1% of 50% antifoam B; supplement more for high-cell-density medium during fermentation</td>
</tr>
<tr>
<td>N-source</td>
<td>Tryptone, casamino acids, and yeast extract are crucial for a high 6dEB titer; High levels of ammonium sulfate might lead to cell lysis and low yield</td>
</tr>
<tr>
<td>C-source</td>
<td>Glucose: faster growth; high cell-density, but more cell lysis; Glycerol: slower growth; have less foaming problem</td>
</tr>
<tr>
<td>Candidate for fed-batch</td>
<td>CD4, CD6, PB1-Int, PM1, EM1</td>
</tr>
</tbody>
</table>
CHAPTER 3. Development of Feedback Controlled Fed-batch Process in a 2-L BIOSTAT® B-Plus Benchtop Bioreactor

3.1 Growth Kinetics Study Under Batch Mode Operation

3.1.1 Pre-determined parameter:

Based on the results of shaking-flask studies and bioreactor operation principles, several CPPs had been determined and assigned with optimized initial values. These preset parameters were transferred for fermentation operation in a 2-L bench-top bioreactor, including

1. Use an optimized seed train procedure to prepare mid-log cells as inoculum for next step. In addition, the seed train procedure was modified after we investigated the plasmids stability (Chapter 5).

2. Inoculate a 50-mL shaking-flask LB medium with an initial OD\textsubscript{600} of 0.1 for seed expansion at 37°C, 6~12 h.

3. Collect the expansion culture as inoculum for bioreactor fermentation at an initial OD\textsubscript{600} of 0.1~0.5 and start fermentation at 37°C, 450~500 rpm.

4. The culture contained 150 mM PPS as pH buffer. The pH was maintained neutral by the feedback-control system with external feed of ammonium hydroxide (3M) and phosphoric acid (1M), for a feedback fed-batch operation.

5. Maintain the dissolved oxygen (DO) at 30% on bioreactor by feedback controlling on the agitation speed or stirring rate (STIRR, rpm), and initial inflow air was kept at 1.0 L/min.
(6) The initial Antifoam B (50%) level was 0.2% ~ 2% depends on the type of medium. Additional antifoam might be necessary for high cell-density stage and was added manually.

(7) Change the temperature to 22°C at 15 mins before IPTG induction, allowing the system to be cooled down and stabilized.

(8) Add 1 mM IPTG for induction and feed 35 mM sodium propionate as initial substrate, and supplement 15 mM sodium propionate per day after induction.

3.1.2 Growth kinetics study using PB1 and PB2 and batch control optimization

The seed train procedure was similar to that in the shaking-flask study. Only a seed expansion step in a 50-mL LB shaking-flask was placed before the main culture was inoculated into the 2-L BIOSTAT® B-Plus Benchtop Bioreactor to a starting OD$_{600}$ of 0.1 ~ 0.5. The system volume for a batch mode operation was 1 L autoclaved PB1 or PB2 medium containing 100 mg/L carbenicillin and 50 mg/L kanamycin. Before autoclaving of each batch, the DO and pH probes must be calibrated accordingly. After the medium was autoclaved with the bioreactor and cooled down, the probes were sitting in the medium as implemented for polarization and the bioreactor was running at 400 rpm for 4 ~ 6 hours until the system (agitation, temperature, pH & DO sensors) was stable. The BIOSTAT controller station utilized a proportional-integral-derivative (PID) controller to provide loop feedback controls on various CPPs. There was no pH control for a batch operation, but the initial pH was adjusted to between 7.3 and 7.6. The DO was controlled at 30% (low boundary) by cascade feedback on changing the stirring rate (STIRR, rpm). Temperature was controlled with a circulation cooling system integrated with BIOSTAT controller. The data of process was acquired by BioPAT® MCFS/win monitoring & controlling software. However, our
bioreactor system did not contain an antifoam sensor and feedback control, weighing system for measuring mass balance, an on-line probe (sensor) for the measurement of glucose concentration, and on-line or off-line assays for glycerol concentration. The basic operational parameters of a batch mode operation were shown in Table 3.1 below.

Table 3.1. Operational Parameters - Batch Mode

<table>
<thead>
<tr>
<th>Operational System</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture volume</td>
<td>1 L</td>
</tr>
<tr>
<td>Calibration</td>
<td>pH, DO probes</td>
</tr>
<tr>
<td>Probes stabilize</td>
<td>4 ~ 6 h</td>
</tr>
<tr>
<td>Initial OD600</td>
<td>0.1 ~ 0.5</td>
</tr>
<tr>
<td>Induction (if)</td>
<td>change temperature to 22°C</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Operational PID Parameters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>STIRR</td>
<td>&gt; 400 rpm</td>
</tr>
<tr>
<td>DO (pO2)</td>
<td>&gt;30%; cascade feedback on STIRR</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>pH</td>
<td>7.5</td>
</tr>
<tr>
<td>Inflow Air</td>
<td>1 L/min</td>
</tr>
</tbody>
</table>

For a batch fermentation at exponential phase, the cell growth could be simply considered by the following Equation 1.1 and Equation 1.2. Without consideration of substrate limitation, the mass balance of batch mode could be expressed as Equation 3.1.

While in consideration of substrate consumption (S), it became Equation 3.2.

Equation 3.1. \[ \frac{dX}{dt} = \mu(t)X, \text{with } X = X_0 \text{ at } t = t_0 \]

Equation 3.2. \[ \frac{dX}{dt} = \mu(S, t)X, \text{with } X = X_0 \text{ at } t = t_0 \]

To find out the growth rate values at any timepoint, several sigmoidal mathematic models were tested using PB1’s batch growth data, in which this Logistic model (Birch, 1999; Kahm, Hasenbrink, Lichtenberg-Frate, Ludwig, & Kschischo, 2010; Zwietering,
Jongenburger, Rombouts, & Vantriet, 1990) gave the best fit for our experimental data with a R-square of 0.9967 as shown in Figure 3.1

Equation 3.3.

\[
Ln \left( \frac{X}{X_0} \right) = \frac{A}{1+\exp \left( \frac{\mu_{max} A (k-t)+1}{k} \right)}
\]

The Logistic growth model is shown by Equation 3.3, where \( X \) is the biomass concentration at a given time, \( X_0 \) is the initial cell concentration, \( t \) as fermentation time, and three characteristic parameters: the length of lag phase \( k \), the maximum specific growth rate \( \mu_{max} \) (equal to ‘u’ in the Figure), and the maximum cell growth \( A \).

Figure 3.1. Time Course of Growth Rate and Kinetics of TB3 in PB1 Batch Operation

By differentiating the growth kinetics function, we obtained the time course plot of growth rate \( \mu \), as shown in Figure 3.1. By calculating the second derivative of the Equation 3.3 respect to \( t \) to find the inflection point, the maximum specific growth rate was found to be
0.9816 h\(^{-1}\) at 4.169 h. From the time course plot of growth rate, it rose up quickly between 2 ~ 4 hours, reached the maximum at 4.16 h, and began to decay from 4 to 7 hours. This plot described the growth behavior of strain TB3/pBP130/pBP144 in a PB1 batch mode at 37°C and no induction conditions, which meant minimal metabolic pressure for heterologous proteins expression. The initial OD\(_{600}\) of this batch was 0.15, while the final OD\(_{600}\) was 5.2. The result showed a lag phase for about 2.37 hours which indicated that the inoculum OD\(_{600}\) could be higher, or the inoculum was over incubated and wasn’t collected at mid-log phase.

Similarly, for a PB2 batch fermentation as shown in Figure A (Appendix V), the growth rate was analyzed by fitting the Logistic model and found with a maximum growth rate of 0.7342 h\(^{-1}\) at 5.73 hours. The growth reached a short range of “plateau” for maximum growth rate (> 0.732 h\(^{-1}\)) between 5.66 and 5.96 h, which was about 18 min. As mentioned before, it was preferable to have a specific growth rate lower than the critical value. But we also want the cells to keep growing at a constant high rate (healthy state) until it reached sufficient cell density before induction in fed-batch process. Thus, further optimization will be focused on manipulating the maximum growth rate and extending the “plateau” range.

Figure B-I (Appendix V) displays the changes of system parameters over time for PB1 batch fermentation recorded by BIOSTAT MCFS/win system. The history plot of PB1 indicated a stable control on the batch fermentation. Both pH and temperature were stable. DO was well controlled above 30%. The decrease of DO and increase of STIRR reflected the increasing demands to oxygen by the culture. This stage corresponded to the fast-growing state in log-phase. The death phase might begin after 12 h, where DO significantly rose up. But without a viable cell count assay, it was hard to determine.
Figure B-II (Appendix V) was the history plot of PB2 batch. Because PB2 had both glucose (3 g/L) and glycerol (20 g/L) as C-source and abundant N-source components, it significantly increased the final OD<sub>600</sub> up to 27.6. However, high OD<sub>600</sub> created lots of challenges for the control of a batch fermentation. The culture started to become foamy after 6 hours of fermentation, where STIRR reached its upper limit (1750 rpm), as shown in Figure A and B-II (Appendix V). Between 6 and 8 h, the DO could not be controlled at 30% because the STIRR was at maximum limit (Figure B-II). The culture suffered oxygen starvation and gradually self lysed (foamy) due to accumulation of inhibitory by-products. Thus, extra 0.1% Antifoam B (50%) was added four times manually and the inflow air was increased to 1.5 L/min at 8 h to bring up the DO to 30%. To ensure adequate supply of oxygen and well controlling of DO, the DO controller should be adjusted in PID cascade feedback on both STIRR and Air Flow (sparging rate).

3.1.3 Biomass yield coefficient and choice of nitrogen sources

The biomass yield coefficient $Y_{X/S}$ was the efficiency of conversion of substrate to biomass and is calculated as below. In which, the dry weight of biomass (namely DCW) was considered as the DCW of which the limiting substrate was depleted, or the beginning point of stationary phase.

$$\text{Biomass Yield Coefficient} = \frac{\text{Dry weight of biomass produced}}{\text{Weight of substrate used}}$$

Based on previous shaking-flask studies of CD series media, glucose level was investigated between 5 to 20 g/L. A preliminary correlation of biomass on glucose level was plotted as Figure 3.2. The slope was the biomass yield coefficient $Y_{X/S} = 0.13$ g glucose/g DCW.
When the glucose level was fixed at 20 g/L, three types of N-source were compared at the same level of 4 g/L, including ammonium sulfate, casamino acids, and tryptone. The contribution of different N-source on biomass production was shown in Figure 3.3 below.

**Figure 3.2. Biomass Yield Yₓₛ on Glucose Level**

**Figure 3.3. Comparison of Nitrogen Sources (4 g/L) on Biomass Yield**
Although ammonium sulfate had a slightly higher contribution than tryptone, in practice a high level of ammonium sulfate might be detrimental. CD5 medium contained 6 g/L of ammonium sulfate and lysed and stopped growing at a very early stage, which indicated that a high concentration of ammonium sulfate could be toxic to the strain (data not shown). However, both casamino acids and tryptone can be used up to at least 40 g/L.

3.1.4 Glucose consumption rate

OPM2 medium was the OMPMedium (BTEC proprietary) added with 20 g/L of glucose and reached a maximum OD over 20. OPM2 was a semi-defined medium. Compared to CD4 medium, OPM2 was easier to keep the cells growing healthily for a longer batch fermentation time. Thus, it was selected to perform the glucose consumption study.

Figure 3.4. Growth Analysis of E. coli TB3/pBP130/-BP144 with OPM2 Medium
As shown in Figure 3.4, the cells were induced at 4.25 h with 100 μM IPTG and 35 mM sodium propionate. The temperature was changed to 22°C after induction and hence the growth slowed down. Before induction, the maximum growth rate was at 0.6708 h⁻¹ for the 1st log-phase at 37°C. While after induction, the maximum growth rate was 0.1538 h⁻¹ for the 2nd log-phase at 22°C between 4.5 and ~23 hours. A double Boultzmann model was used to fit the growth kinetics (R² = 0.9957) for estimation of specific growth rate.

Equation 3.4. \[ \ln \left( \frac{X}{X_0} \right) = X_0 + A \left[ \frac{p}{1 + \exp \left( \frac{t - t_1}{k_1} \right)} + \frac{1-p}{1 + \exp \left( \frac{t - t_2}{k_2} \right)} \right] \]

Figure 3.5. Time Course of Glucose Consumption Rate of TB3 in OPM2 Medium

Meanwhile, the offline samples of culture were centrifuged, and the supernatants were analyzed for glucose concentration using a YSI 2900 Analyzer (Appendix I). As shown in Figure 3.4, glucose was depleted after 30 hours of fermentation. A Gompertz model was used to fit the experimental data (R² = 0.9826) and generated more simulated values.
Equation 3.5. \[ \ln \left( \frac{X}{X_0} \right) = A \times \exp \left[ -\exp \left( \frac{\mu_m}{A} \right. \left. e(k - t) + 1 \right) \right] \]

By differentiating Equation 3.5, the glucose consumption rate was plotted in Figure 3.5. Within 0~30 h, the average glucose consumption rate was found to be -0.4135 g·L⁻¹·h⁻¹.

3.2 Bioreactor settings for a fed-batch process

Similar modeling processes could be performed for other media fermentation tests under a fed-batch or batch operation. The important parameters, such as the biomass yield, glucose consumption rate, and specific growth rate could be obtained using suitable models. For example, a model was developed by Xu et al. (1999) to simulate the feeding strategy to maintain a constant desired growth rate (< 0.2 or 0.3 h⁻¹) based on biomass yield on limiting substrate is shown below as Equation 3.6.

Equation 3.6. \[ F(t) = \frac{\mu_D}{Y_{X/S}} \left( XV / S_{feed} \right) \exp[\mu_D(t - t_0)] \]

Where \( \mu_D \) was the desired specific growth rate (h⁻¹), \( Y_{X/S} \) was the biomass yield on glucose (g/g), \( X \) was the cell concentration (g/L), \( V \) was the culture volume (L), \( S_{feed} \) was the glucose concentration in the feed (g/L), and \( t_0 \) was the time at which the feed is started.

The fed-batch modeling process was discussed in this thesis for the possibility to develop a model-controlled process. However, because our equipment did not have the model-control capability and no accurate feeding equipment was available at the time, this study employed a non-model based PID control instead of a model-controlled process. The development of this bioreactor process was still an ongoing process, but the following strategies were optimized and modified from a previous study (Lau et al., 2004). Followed by the preparation of seed inoculum using triplicated 50-mL LB (Kan⁺/Carb⁺) shake-flask, the Fed-batch operation process was illustrated in Figure 3.6. The bioreactor setting up was shown in Figure C (Appendix V).
This study developed a preliminary fed-batch process for PB1 and HCD media. Taken HCD fed-batch as an example, the major process steps were as follows:

1. Preparation of bioreactor included medium preparation, pH & DO probes calibration, autoclave, cooling down, connecting the bioreactor to the controller, cooling system and feeding bottles, adjustment of initial pH and temperature, DO probe stabilization, PID parameterization, etc.

2. Pick a potent dual-plasmid containing seed from LB agar plate colony. Seed expansion and rejuvenation in triplicate 50-mL LB shake-flask cultures at 37°C. Collect the culture at mid-exponential phase (OD$_{600}$ < 1.0), usually 4~6 h incubation.
(3) Concentrate the cells and re-suspend in 50-mL of the same medium (PB1, HCD, etc.) used in 2-L bioreactor. Aseptically add adequate inoculum volume to make the initial OD<sub>600</sub> of bioreactor to be at 0.1~0.5.

(4) Take samples from the sampling port every hour or as needed. Use the same medium as diluent for high-cell-density samples during OD<sub>600</sub> measurement. Centrifuge the rest samples (at least 1.5-mL) and collect supernatants for pH and glucose assay. Samples were stored in -20°C freezer before assay.

(5) Induce the cells with 1 mM IPTG, and 35 mM sodium propionate at mid-log phase, 4~6 h, or when OD<sub>600</sub> = 10~20 for a high-cell-density fed-batch process. The induction time was at an adequate amount of biomass compared to maximum OD<sub>600</sub>, while not allowing 37°C incubation for a long time. The temperature was changed to 22°C after induction to reduce the growth rate to increase plasmid stability and ensure proper expression and folding of heterologous proteins. Additional 15 mM propionate was fed every 24 h in bolus feed after induction. 1 mM IPTG as well as 5X antibiotics were added in the feed substrate.

(6) Feeding starts when the initial glucose (glycerol for PB1) was depleted. This time point was indicated by the STIRR increased and then began to drop and usually followed by an oscillation or self-stabilizing-process (Figure 3.9).

(7) The controlling of feeding rate required an accurate feeding system (controller, peristaltic pumps and tubing). Either a model or an empirical step-feeding profile must provide accurate control of both the substrate concentration and the specific growth rate at the desired levels. This study developed a preliminary empirical step-feeding-rate profile for HCD (high-cell-density) fed-batch process.
In this study, tuning the feeding rate of each pump was a big challenge. The diameter of tubing, material rigidness and pump’s maximum speed were examined during the tuning of feeding speed. Each pump performed differently and must be calibrated individually. The maximum pump’s feeding speed was an experimentally determined value that was specific to each pump and tubing, and was used for calculating the pump’s aperture percentage of each step to achieve a desired feeding rate of substrate in g/L. Because the BIOSTAT B-Plus Bioreactor control system could not adjust feeding-rate accurately and conveniently for each pump, the feeding could accumulate some system errors over time.

Besides the development of an empirical step-feeding-rate profile, many operational details were optimized through troubleshooting during the process. Such as balancing the air sparging rate for oxygen supply and shear force & foaming issue, implement of extra pure oxygen feeding line for HCD fed-batch, dual-cascade DO control on both STIRR and air flow (L/min), foaming control and outlet air-filter clogging troubleshooting, optimization of circulation rate of coolant, prevention of failure of DO cascade feedback control, and even dealing with the power outage and shutdown of the cooling circulation system during fermentation. More details in operation control are discussed below.

3.3 HCD bioreactor run and 6dEB production

3.3.1 Operational PID parameterization

The fed-batch operation was developed based on batch mode operations, as mentioned. PID parameterization and operation optimization are required for developing a stably-controlled fed-batch process. First, some CPPs were monitored and controlled by the bioreactor’s build-in PID controllers with the PID parameterization shown in Table 3.2.
Table 3.2. PID Parameterization for CPPs

<table>
<thead>
<tr>
<th>CPPs</th>
<th>PV (initial value)</th>
<th>MIN%</th>
<th>MAX%</th>
<th>DEADB%</th>
<th>XP%</th>
<th>TI (s)</th>
<th>TD (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>37.0</td>
<td>0.0</td>
<td>62.0</td>
<td>0.0</td>
<td>20.0</td>
<td>200.0</td>
<td>0.0</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
<td>-100.0</td>
<td>100.0</td>
<td>0.2</td>
<td>30.0</td>
<td>30.0</td>
<td>0.0</td>
</tr>
<tr>
<td>pO2 (DO)</td>
<td>30.0%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cascade STIRR</td>
<td>450.0</td>
<td>30.0</td>
<td>95.0</td>
<td>0.5</td>
<td>150.0</td>
<td>100.0</td>
<td>5.0</td>
</tr>
<tr>
<td>SUBA &amp; SUBB</td>
<td>0.0</td>
<td>100.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Where PV was the initial setting value for CPPs. MIN% and MAX% were the adjustable range of operational component for each parameter. For example, -100% of MIN% for pH meant the 100% feeding rate (or pump aperture) for adding phosphoric acid. 95% of MAX% for STIRR meant 95% of equipment limit (2000 rpm), which was 1900 rpm. The DEADB% was the tolerant error of CPPs in feedback control. XP% was the proportional gain. TI was the integration time constant and TD was the derivative time constant. The relationship of PID parameters was shown below

Equation 3.7.

\[ u(t) = K_p e(t) + K_i \int_0^t e(\tau) d\tau + K_d \frac{de(t)}{dt}, \quad \text{where } K_i = \frac{K_p}{TI}, K_d = K_p \cdot TD \]

Where \( K_p, K_i, \) and \( K_d \) were the proportional gain, integral gain, and derivative gain, respectively. The \( e(t) \) was the error (signal), while \( u(t) \) was the response (output).

Figure 3.7 shows the transfer functions of the PID-controlled parameters. The signal-response time was the minimal required time for the maximum adjustment requirement within the operational range. For example, the maximum adjustment requirement for temperature was 0°C to 62°C. The minimal required time to reach 62°C from 0°C was about 5500 s (1.5 h). While in practice, the temperature of the water jacket and cooling circulation...
system affect the temperature control. For a temperature change from 37°C to 22°C, we found the actual stabilizing time was about 8~15 mins.

Figure 3.7. PID Parameterization and Signal-response Analysis

3.3.2 Development of substrate feeding profile

The SUBA & SUBB was the optional cascade controls for the dissolved oxygen. However, it was more important to control the concentration of substrate (glucose) lower than critical levels to minimize inhibition effects. Thus, the feeding rate was controlled by implementing an optimized step-feeding-rate profile on the BOSTAT B-Plus shown in Table 3.3. During the exponential phase from 0 to 50-h (Figure 3.10), the glucose feeding rate was escalating and after that, it was maintained at a constant level. The pump aperture of
each step was calculated for matching the desired feeding rate, based on an empirically estimated parameter of maximum feeding rate (6.2 mL/min) at 100% pump aperture.

Table 3.3. Feeding Profile for a 1000-mL High-Cell-Density (HCD) Feed

<table>
<thead>
<tr>
<th>Medium Feed time</th>
<th>Start feeding at 5.5 h, where OD=8.46, glucose depleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pump aperture (%)</td>
<td>hh:mm</td>
</tr>
<tr>
<td>100.0%</td>
<td>n/a</td>
</tr>
<tr>
<td>STEP 1</td>
<td>2.4%</td>
</tr>
<tr>
<td>STEP 2</td>
<td>2.4</td>
</tr>
<tr>
<td>STEP 3</td>
<td>4.8</td>
</tr>
<tr>
<td>STEP 4</td>
<td>4.8</td>
</tr>
<tr>
<td>STEP 5</td>
<td>5.5</td>
</tr>
<tr>
<td>STEP 6</td>
<td>5.5</td>
</tr>
<tr>
<td>STEP 7</td>
<td>0.0</td>
</tr>
<tr>
<td>STEP 8</td>
<td>0.0</td>
</tr>
<tr>
<td>STEP 9</td>
<td>3.0</td>
</tr>
<tr>
<td>STEP 10</td>
<td>3.0</td>
</tr>
<tr>
<td>Sum total (L)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.8. Feeding Profile for a 1-L HCD Feed

The feed medium was provided in Appendix III, which was mainly concentrated glucose (654 g/L). The build-in feeding profile setting only allowed for 10 steps. And there was no available feeding vessel at the 1-L scale. Thus, the feeding was conducted by two steps of 500-mL feedings. The transition of two steps was operated manually.
Figure 3.9. History Plot of HCD Fed-batch Process (0~50h)
The feeding profile was modified from Lau et al. (2004). In short, start feeding after glucose depletion: 0 ~ 21 h, 0.15 mL/min; 21 ~ 30 h, 0.30 mL/min; 30 ~ 40 h, 0.34 mL/min; after 40 h, maintain feeding at 0.2 mL/min until the rest feed was used up. The feeding profile was designed based on the growth kinetics and glucose depletion rate (Figure 3.10). However, the feed was paused for fixing the oxygen supply line because the DO was out of control during the late-log phase (Figure 3.9). The 1st feed was designed to stop at 45.5 h. The 2nd Feed was manually started from 48 h but paused until 50.5 h, which caused a 5-hours gap (Table 3.3). In fact, the 1st feed ran out between 36 h and 40 h according to the history plot, where DO was gradually reduced to zero. This was because the feeding pump was not accurately controlled for a desired feeding rate.

The temperature was maintained at 22°C after induction with 1 mM IPTG and the addition of 35 mM sodium propionate. Sodium propionate was subsequently added as a single bolus at a rate of 15 mM per 24 h. The initial agitation rate was adjusted to 600 rpm. And elevated aeration rate of 1.5 ~ 3 L/min were manually adjusted during the process to maintain 30% DO if agitation rate was approximate to operational range (Figure 3.10). Both pH and temperature were stably controlled. The foaming problem was controlled by manual addition of 50% (v/v) Antifoam B during the process as needed. However, DO control was a challenge in HCD fed-batch process, which was discussed in next section. Fed-batch operation was also developed for the PB1 medium. More details could be found in Figure D-I and D-II (Appendix V). The feeding profile of PB1 was shown in Figure D. (Appendix V).

3.4 Analysis of 6dEB production in fed-batch process

The growth kinetics, glucose consumption, feeding status, and 6dEB production of HCD fed-batch were shown in Figure 3.10 below. A 1-L initial HCD fed-batch medium and
1-L HCD feed were used for the process. The maximum specific growth rate was found to be 0.564 h\(^{-1}\) at 3.3 h. After the initial glucose was depleted at 5 h, the feeding process began with the step-feeding-profile control. At 9.5 h, OD\(_{600}\) reached 24.5. The PV (process values or known as “set point”) of temperature was changed to 22°C one half hour before induction for the system to stabilize. Then the culture was induced with 0.2 mM IPTG and 35 mM sodium propionate. An additional 15 mM of propionates were added at 33, 57, and 80 h.

![Figure 3.10. HCD Fed-batch Process and 6dEB Productivity](image)

Figure 3.10. HCD Fed-batch Process and 6dEB Productivity
The DO was initially controlled by a simple cascade feedback control on STIRR. Feeding fresh medium could help the DO to oscillate around 30% at the beginning. However, after 26 hours, the cell density was very high and hence the agitation rate (STIRR) quickly escalated and reached the operational limit (1750 rpm) to increase air sparging and dissolved oxygen. The air flow rate was increased to 3 L/min at 35 h, and STIRR was then reduced and the DO oscillated. As Figure 3.9 shows, after 36 hours, the culture exhausted the dissolved oxygen after the first 500-mL feed ran out at around 38 h. The 2nd Feed started at 48 h, which helped to bring back the DO to 30% for a short time. However, not enough dissolved oxygen was available to supply the system. At 52 h, an extra pure oxygen line was implemented, and the bioreactor controlled at a 12% ~ 15% GFR (gas flow ratio, or percentage of pure oxygen in total air inflow), the DO was finally brought back to 30% and the STIRR was reduced and stabilized. After 94 h, the DO quickly rose up to 100%, indicating the death phase of culture and end of the process. During the process, extra antifoam was also added at 25, 31, 35, 46, 50, 57, 80, and 82 h manually.

The bench scale bioreactor lacked many control capabilities. There was no automated foaming control (or probe) or on-line sensors for substrates. The substrate feeding control was not accurate and the 1st feed ran out much earlier than the theoretical time. The simultaneous cascade control on STIRR and GFR was not developed. Thus, manual control and adjustments were improvised during the development process such as adjusting air flow rate and implementing the pure oxygen line.

The Fed-batch process was also modified and implemented for using PB1, PB3, CD4, and OPM2 media. The 6dEB titer of the CD4 fed-batch was very low (data not shown). Other media are compared in Table 3.4 and Figure 3.11 below. In which, HCD medium
showed the highest per batch productivity, 343.1 mg (2-L), while PB1 medium showed the highest per unit biomass productivity, 25.1 g 6dEB per gram of biomass (DCW). The PB3 and OPM2 were less good than HCD or PB1 medium for 6dEB production in fed-batch process.

**Table 3.4. Maximum 6dEB Production of Various Fed-batch Media**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Time (h)</th>
<th>OD$_{600}$</th>
<th>Approx. real-time vol. (mL)</th>
<th>6dEB per batch (mg)</th>
<th>6dEB/DCW (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB1</td>
<td>46</td>
<td>7.84</td>
<td>1497</td>
<td>110.1</td>
<td>25.1</td>
</tr>
<tr>
<td>PB3</td>
<td>59</td>
<td>18.4</td>
<td>1409</td>
<td>37.6</td>
<td>4.0</td>
</tr>
<tr>
<td>OPM2</td>
<td>122</td>
<td>34.4</td>
<td>1782</td>
<td>27.1</td>
<td>1.2</td>
</tr>
<tr>
<td>HCD</td>
<td>82</td>
<td>200.4</td>
<td>1853</td>
<td>343.1</td>
<td>2.6</td>
</tr>
</tbody>
</table>

DCW (g) = (0.3506*($OD_{600}$) + 0.1798)

**Figure 3.11. Comparison of 6dEB Production of 4 Media in Fed-batch**

Tryptone and casamino acids were found to be crucial for 6dEB production according to shaking-flask studies. Both were organic N-sources. Use of a slow-growth-carbon source, such as glycerol, might help to reduce cell lysis and foaming problems, which was also
suggested by fed-batch experiments. For example, cells were growing very well in PB1 fed-batch process, and minimal foaming control was needed. On the other hand, by feeding glucose and keeping substrate at a low level, high-cell-density fed-batch fermentation could result in a high 6dEB production per batch (the HCD fed-batch approach).

3.5 Conclusions

Although this fed-batch process was not automated, and much trouble shootings occurred during the development. The 6dEB production achieved a very promising result. And some preliminary results were determined for identifying CPPs.

The maximum 6dEB production was 342.7 mg at 82 h. In titer representation this would be 177.6 mg/L. The maximum OD_{600} was observed at 74.5 hours, which was 214.8. Finally, Table 3.5 listed the key points of operating a fed-batch for high-cell-density fermentation and would be used as references for future optimization.

Table 3.5. Key Points for Developing a High-cell-density Fed-batch Process

<table>
<thead>
<tr>
<th>DO</th>
<th>Pure oxygen feed is necessary; maintaining sufficient DO% is critical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate feed</td>
<td>1. Require accurate diaphragm pump on controlling the feeding rate</td>
</tr>
<tr>
<td></td>
<td>2. Interruption of substrate feeding could cause system unstable</td>
</tr>
<tr>
<td></td>
<td>3. Adjust feeding profile based on growth kinetics and glucose depletion for each medium</td>
</tr>
<tr>
<td>Sensors</td>
<td>1. On-line glucose analysis might be necessary</td>
</tr>
<tr>
<td></td>
<td>2. Weight measurement is necessary for calculating mass balance and k_{La}</td>
</tr>
</tbody>
</table>

Table 3.6 compared the shaking-flask and fed-batch 6dEB results in this study. All the 6dEB results were assayed by using LC-MS in the Chemistry Department of NCSU. In which, CD6 was the best medium in shaking-flasks, which could be utilized for fed-batch production in the future. HCD in fed-batch reached the highest maximum cell density among all media and batch experiments. Compared to previous study on strain TB3/pBP130/pBP144 and a propriety strain K207-3 (Table 1.2), our best titer (177.58 mg/L) in HCD fed-batch was
66% less than TB3 in Production Medium (527 mg/L, OD~20, batch mode). Besides the reason of medium types, many other conditions, such as strain purity (plasmids stability), different 6dEB assay methods, calibration, and instruments, and process optimization, variations among different batches could also affect the results. For strain TB3/pBP130/pBP144 used in this study, compared with max 6dEB production in LB medium (29.80 mg/L, shaking-flask), the 6dEB titer of HCD fed-batch was improved by 460%, and of PB1 fed-batch by 147%.

Table 3.6. Summary of 6dEB Results in This Study

<table>
<thead>
<tr>
<th>Medium</th>
<th>Culture scale</th>
<th>max. 6dEB</th>
<th>max. OD</th>
<th>Assay</th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>50 mL SF</td>
<td>29.80 mg/L</td>
<td>2.62</td>
<td>LC-MS; HPLC-ELSD</td>
<td>70</td>
</tr>
<tr>
<td>CD4</td>
<td>50 mL SF</td>
<td>15.08 mg/L</td>
<td>12.48</td>
<td></td>
<td>46</td>
</tr>
<tr>
<td>EM1</td>
<td>50 mL SF</td>
<td>34.40 mg/L</td>
<td>7.68</td>
<td></td>
<td>78</td>
</tr>
<tr>
<td>PM1</td>
<td>50 mL SF</td>
<td>51.98 mg/L</td>
<td>7.46</td>
<td></td>
<td>78</td>
</tr>
<tr>
<td>CD7</td>
<td>50 mL SF</td>
<td>9.77 mg/L</td>
<td>6.8</td>
<td></td>
<td>78</td>
</tr>
<tr>
<td>CD6</td>
<td>50 mL SF</td>
<td>76.87 mg/L</td>
<td>10.18</td>
<td></td>
<td>70</td>
</tr>
<tr>
<td>PB1</td>
<td>2-L fed-batch</td>
<td>110.05 mg</td>
<td>7.84</td>
<td></td>
<td>46</td>
</tr>
<tr>
<td>PB3</td>
<td>2-L fed-batch</td>
<td>37.59 mg</td>
<td>18.4</td>
<td></td>
<td>59</td>
</tr>
<tr>
<td>OPM2</td>
<td>2-L fed-batch</td>
<td>27.11 mg</td>
<td>34.4</td>
<td>LC-MS</td>
<td>122</td>
</tr>
<tr>
<td>HCD</td>
<td>2-L fed-batch</td>
<td>343.11 mg</td>
<td>200.4</td>
<td></td>
<td>82</td>
</tr>
</tbody>
</table>

There were lots of limitations on our bench scale fermentation equipment. Thus, it would be possible in the future to further optimize the fed-batch process by providing more on-line sensors, an accurate feeding-rate control system, additional medium optimization, and more samples assayed in triplicate in the experimental design.
CHAPTER 4. Process Intensification by Nanoporous Latex-coating Cell-patches with Scaled-down 6-Well Plate Fermentation

4.1 Development of Latex-coating Method

4.1.1 Preparation of concentrated cell paste

To prepare immobilized cell-patches, lots of cells must be collected. The cell paste was prepared by collecting cells from duplicated 50-mL LB shaking-flask fermentation broths, which were at their mid-late log phase. The fermentation was incubated at 37°C for about 4 hours since inoculation from a cryovial strain. When OD$_{600}$ reached to about 0.6, the cultures were induced by 1 mM IPTG and temperature was changed to 22°C for 2 hours of pre-induction prior to cell harvest, at which OD$_{600}$ reached about 1.5 (early stationary phase). About 1.14 g of wet cell paste was collected by concentration. The preparation protocol of cell paste was important and optimized in many aspects:

1. Use 100 mM HEPES buffer in LB medium. Prepare 6 flasks of 50-mL LB
2. Medium contained 5X folds of antibiotics (kanamycin and carbenicillin)
3. Inoculation used 150 µL of “purified” seed strain (see Chapter 5) into each flask and incubated at 300 rpm, 37°C for 4~6 hours until OD$_{600}$ was 0.5 ~ 0.8
4. Change to 22°C incubation when the cultures were induced with only 1 mM IPTG, no addition of sodium propionate was evaluated in the cell preparation phase.
5. After another 2.5 hours induction, the cultures were harvested into sterile 50-mL tubes. Centrifuged the cultures at 24°C, 4000 min$^{-1}$, for 5 min.
6. Discard the supernatant and add 10 mL of LB/HEPES medium to wash the cells by using pipette to mix and break cell pellet from the inner wall gently. Combine the solutions into a new pre-weighted 50-mL sterile tube.
7. Centrifuge again as in Step 5. Discard the supernatant and added 10 mL of PBS buffer (1X) with 5X folds of antibiotics (Kan/Carb). Break cell pellet gently.

8. Centrifuge again and discard the supernatant. The final cell paste was weighted, about 1.53 g of cells was collected in this batch.

4.1.2 Formulation of latex coating emulsion,

In this project, the polymer latex binder 6776 was used for making an emulsion of E. coli cell paste. The formulation of cell paste was prepared according to a previous study (Gosse & Flickinger, 2011), which was 1.2 g of wet cell pellet, 1 mL latex emulsion, 350 μL of sucrose (0.58 g/mL), and 150 μL of neat glycerol. This step was performed in a biosafety hood but did not require sterile conditions because the organism (strain) was very concentrated. The cell paste was mixed with sucrose and glycerol at first by using a spatula to break the cell pellets thoroughly. Then the latex binder was added into the mixture.

The pH of the latex binder must be pre-adjusted to a neutral. The original pH of latex 6776 was ~9.0. Four methods were experimented and compared for pH adjustment performance as shown in Table 4.1 below

<table>
<thead>
<tr>
<th>Method</th>
<th>pH solution</th>
<th>pH</th>
<th>Amount required for 1 mL latex</th>
<th>Final pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>HCl, 0.1N</td>
<td>2.15</td>
<td>~ 700 μL</td>
<td>7.45</td>
</tr>
<tr>
<td>(2)</td>
<td>Boric acid, 0.5 M</td>
<td>2.85</td>
<td>~ 800 μL</td>
<td>7.6</td>
</tr>
<tr>
<td>(3)</td>
<td>Acetic acid, 0.01 N</td>
<td>3.00</td>
<td>2.2 mL</td>
<td>7.66~8.15</td>
</tr>
<tr>
<td>(4)</td>
<td>Acetic acid, 0.1 N</td>
<td>2.90</td>
<td>~ 400 μL</td>
<td>7.55</td>
</tr>
<tr>
<td>(5)</td>
<td>Boric acid, dry</td>
<td>n/a</td>
<td>24.73 mg</td>
<td>7.33</td>
</tr>
</tbody>
</table>

Method (1) generated many flakes of polymers (aggregates), while method (3) did not but the latex solution was very diluted. Method (2) and (4) did not cause any instant cloudy or flakes of polymers. Thus, these two methods were employed to adjust pH of latex for
preparation of cell-paste emulsion. Method (5) used dry material of boric acid. It was found that boric acid was the most amiable material to this latex 6776 in pH adjustment. Thus, adding dry materials did not cause flakes but also significantly reduced the dilution problem. 4.1.3 Coating and Drying procedure

The procedure was modified from a previous study (Gosse & Flickinger, 2011). In brief, cut a 4.5 x 6 inch transparent vinyl mask sheet and a 4 x 5.5 inch polyester substrate sheet. Then use a Metric Punch with hammer to cut 6 holes of 32 mm diameter in the vinyl mask as shown in Figure 4.1. Wipe clean the polyester substrate sheet with 70% ethanol and tape onto a work station with a roller to make the substrate sheet flat. Peel off the paper backing of the vinyl mask and carefully orient the mask and slowly place the mask sheet onto the polyester sheet with a roller to press the mask down and remove any trapped air bubbles. Use Kimwipes® paper and 70% ethanol to clean the surface of the mask and then tape the entire sheet (template) onto a Gardco ACCU-LAB Drawdown Machine in a Controlled Environmental Chamber Model 2398. The chamber had controlled temperature at 22°C and relative humidity at 60%. Ensure the surface of drawdown machine was level. Firmly roll the template onto the surface and wipe the mask & substrate sheets clean with Kimwipes® paper. Add 2 mL of formulation emulsion across the top of the mask as a long bead of liquid and use the #8 (wire size) applicator Meyer rod to apply the formulation by slowly pull the rod forward across the surface of template, allowing the liquid to fill in the circle wells which was formed by the substrate and mask sheets. More details about the application techniques have already been discussed in previous studies (Flickinger, Fidaleo, et al., 2009; Flickinger et al., 2007; Gosse & Flickinger, 2011). The rod must be removed for immediate clean off with warm water to prevent any coalescence of polymer onto the wire of the Mayer rod.
Figure 4.1. Coating and Drying Procedure for Cell-patches

The coating was dried in the chamber for 1 ~ 2 hours until the liquid was dried into a film. The drying time depended on the concentration of polymers and cells in the formulation. Usually, diluted polymer or more concentrated cell paste took longer time to dry. Finally, the mask was removed, and the cell-patch circles were punched out with the same 32 mm Metric Punch. The circular patches were placed into a 6-well plate (one per well) for the following studies. To each well was added with 5 mL of liquid medium and 5X folds of antibiotics. Samples were taken for PST, OD$_{600}$, pH and 6dEB analysis.
4.2 Fermentation and 6dEB production

4.2.1 Comparison of different media

As mentioned in Table 4.1, the pH adjusted latex by method (2) and (4) were selected to make formulations with cell paste, which were named as Formulation 2 and Formulation 3, respectively. The effects of different pH adjustment methods, boric acid and acetic acid, on the production of 6dEB by immobilized cell-patches were compared. Meanwhile, five types of media were selected for the 1st batch of study, including PB1, PM1, CD6, LB/HEPES, and a simple PBS media. Each well of the plate was added with a piece of circular cell-patch, 5 mL of medium and 5X folds of double antibiotics (kanamycin & carbenicillin) for enhanced selective pressure due to concentrated biomass in cell patches. The plates were placed on an orbital shaker for incubation at 150 rpm, 22°C.

Among the five media, only LB samples had a constantly detectable level of 6dEB by ELSD method. Cell-patches in other media did not obtain detectable 6dEB by ELSD, except one sample from PB1 medium at 12 h had a titer of 7.623 mg/L. The reason might be the repair ability of LB on damaged & shocked E. coli cells. However, without any live cell counting technique, the viable cells percentage was unclear.

4.2.2 Compare different formulations and induction strategies

The cell paste was pre-induced with 1 mM IPTG for 2 hours prior to the formulation preparation. During this preliminary study, different induction conditions were compared including extra 0.5 mM IPTG in 6-well plate or enhanced initial level (5x fold, 175 mM) of substrate, sodium propionate. Additional 15 mM of substrate was added to each well every 24 h. In addition, another type of latex binder SF012 (pH 7.3) was tested as well. But there was no 6dEB detected using ELSD in any induction conditions or medium types.
The 6dEB production results of various conditions on LB medium at 72 h were shown in Figure 4.2 below. The results were obtained by LC-MS 10-min protocol (Appendix II). According to an ANOVA in block design analysis ($\alpha = 0.05$), the F-stat (among blocks) was 0.0031, which was smaller than F-crit. = 18.5128. Thus, we concluded that no significant difference was observed between the two types of formulations. Because boric acid could be added in the form of dry material, the future experiments were tested by using Method (5) to make formulations for convenience and higher concentration of binder.

![Graph showing 6dEB production results](image.png)

**Figure 4.2. Comparison of 6dEB Production by Cell-patches in LB**

Moreover, extra 0.5 mM IPTG addition did not improve but decreased the 6dEB production. However, samples with higher initial level of substrate propionate (175 mM) were found to have 4~5 folds improved 6dEB titers than the normal condition at 35 mM. In Figure 4.2, LB-SF control was a 50-mL LB shaking-flask inoculated with the formulation cells (immobilized) and added with 35 mM propionate. It seemed that the cell-patch method had a lower 6dEB titer compared to the shaking-flask control by 3~4 folds at 35 mM propionate induction level.
4.2.3 6dEB production life span of cell-patches in LB

Due to evaporation problems and sample collection for OD$_{600}$ and 6dEB analysis, the initial 5-mL of medium per well would reduce to less than 3-mL after about 80 hours of fermentation. To find out the active life of cell-patches for 6dEB production, an extended cell-patch fermentation was performed in LB medium by continuously moving the cell-patch into fresh 5-mL of medium every 80 hours. The medium was the same LB with 100 mM HEPES buffer, and 15 mM propionate was added every 24 hours. As shown in Figure 4.3 below, sextuplicate cell-patch samples were assayed for 6dEB production over 240 hours by HPLC-ELSD method. The dual-plasmids retaining ratio (PS%) and OD$_{600}$ were also shown.

![Graph showing 6dEB Production Life Span of Cell-patch in LB](image)

**Figure 4.3. 6dEB Production Life Span of Cell-patch in LB**

Medium was replenished at 85, 158, and 230 h. Some sample points were assayed with PST (plasmids stability test) and shown as blue square dots. The figure indicates that
during the process, the PS% ratio varied between 43% and 80%. Samples from 85 h to 230 h were assayed by ELSD for 6dEB production detection. About 4~21 mg/L 6dEB titer was detected for cell-patches, in which the maximum titer (21.229 mg/L) was detected at 158 h. In consideration of the twice replacements of fresh medium, the total production of the method within 230 h and with 15 mL LB medium was 236.97 μg. Theoretically, using 50 mL medium by cell-patch production in 230 h, a total of 789.9 μg 6dEB was predicted. Compared to 50-mL LB in shaking-flask for 86 hours, which was the normal fermentation time of a LB-SF production, the total production was 558.62 μg 6dEB. The cell-patch method improved the production by 41% but a longer production time was required.

4.2.4 Confirmation of 6dEB production of cell-patches in LB

A confirmation experiment of cell-patch on LB was conducted. At the same time, the LB no immobilized control was a 50-mL LB shaking-flask inoculated with the cell paste of the same strain without formulation with latex binder. Trial 3 (LB control) was a 50-mL LB shaking-flask inoculated with the formulation (immobilized cells). Trial 1 and Trial 2 were the immobilized cell-patches.

![Figure 4.4. 6dEB Production of Immobilized Cell-patches and LB Controls](image)
The results were shown in Figure 4.4, which indicates that the immobilized cells might have a higher 6dEB production capabilities. The cell-patch samples gave a maximum titer of 31.05±5.22 mg/L and 36.97±3.92 mg/L for Trials 1 and 2 at 89 h, respectively. For Trial 3, the immobilized cells were cultured in liquid LB shaking-flask, the titer was 30.8±0.42 mg/L at 99 h. Compared to a titer of 8.31 mg/L by LB-control (before formulation), the immobilized cells had much higher 6dEB production titers. It was possible that the immobilized cells restricted the growth and plasmids loss of cells.

4.3 Discussion

As shown in Figure 4.3, during the cell-patch fermentation, there were still lots of cells that grew into the liquid medium. The growth was mostly occurred after 40 h. Because this was a preliminary study on immobilization of preparation of cell-patches. The nanoporous film was not covered with a polymer layer sealant to prevent the cell to outgrown. It was possible that the immobilized cells on the surface of patches could propagate and grow into the liquid by contacting the medium without nanoporous structure restrictions. However, because of the design of cell-patch method, only the immobilized cells were pre-induced and no additional IPTG was added in liquid medium, which means only the immobilized cells contain expressed DEBS enzymes. Although cells were outgrowing into liquid medium, we can assume the free submerged cells were not induced and hence unlikely to produce 6dEB.

This method proved that the cell-patches of immobilized strain could produce 6dEB and could be reusable for at least 240 hours. Unlike submerged fermentation, of which the cells usually entered death phase after 80 hours in shaking-flask, the immobilized cell-patches significantly improved the active life span of biocatalysts. As shown in Figure 4.4, the immobilized cell-patches method improved 6dEB titer by 309% compared to a no-
immobilized LB shaking-flask control. Although the productivity was still low, and the method did not work for other production medium, the cell-patch method has potential to be optimized and developed into a practical production process. The health status of cell pastes, the preparation protocol for reducing osmotic shock or stress, and the procedure and formula of making the coating formulation are all possible to be optimized for improving 6dEB production using immobilized cells.
5.1 Plasmid Stability Testing (PST) study

Kanamycin is an aminoglycoside antibiotic, while carbenicillin is a β-lactam antibiotic belong to the family of penicillins. Both antibiotics were added in medium as selection pressures to preserve the plasmids and prevent contamination by undesired fast-growing organisms. During the development of bioreactor process, we found that the dual-plasmids-retaining cells ratio was decreasing severely after a long-time fermentation. About 48% of cells retained dual plasmids after 3-days of fermentation in shaking-flask (LB), while only 25% after 4-days of fermentation in a fed-batch process (PB1).

Under none antibiotic selection pressure, the cells with no plasmids would quickly outgrow those with plasmids, because less replication burden was required (Schmid & Kayser, 1976). However, even when antibiotics were added, sometimes the plasmids would still be lost over time. A few reasons might be responsible for this situation. Firstly, the two plasmids were incompatible (Novick, 1987; Velappan, Sblattero, Chasteen, Pavlik, & Bradbury, 2007). The strain used in this study was constructed with two plasmids which share the same type of pBR322 replication origin. Hence, the two plasmids would compete in sharing the same replication systems of the host. Thus, the next generation of cells were likely to lose one or both plasmids, especially when cells grew at a high growth rate. Secondly, antibiotic resistance mechanism decreased the efficacy of antibiotics selection pressure, such as transporters, and β-lactamases degradation on carbenicillin (Korpimaki, Kurittu, & Karp, 2003; Schmid & Kayser, 1976; Walsh, Amyes, & Duffy, 2013). According to previous studies, the degradation time of 25 mg/L carbenicillin in β-lactamase secreted E.
coli culture was 3 hours. For 100 mg/L carbenicillin, we could estimate the complete degradation time as 11-12 hours. The host strain came from BL21(DE3). Thus, the cells should not secrete β-lactamase. Thirdly, chemical degradation of antibiotics occurs under cultural conditions, such as ionic strength, pH, and temperature. Basically, this depends on the chemical stability of antibiotics. Some empirical rules show that the most antibiotics in culture last for 2 to 4 days. Fourth, dilution of selective pressure can occur due to high cell density. Finally, some studies have found that induction might also facilitate plasmid loss (Collins et al., 2013).

![Diagram of Serial Dilution and PST Procedure]

**Figure 5.1. Illustration of Serial Dilution and PST Procedure**

To confirm the dual-plasmids retaining ratio and increase the plasmids stability, a Plasmids Stability Test (PST) was developed based on CFU (colony-forming units) counting methods. Although CFU methods had some limitations (Sutton, 2012), CFU counting was still the most commonly used technique in measuring plasmids stability (Sutton, 2006; Walia,
Deb, & Mukherjee, 2007). Firstly, 1-mL culture sample was collected from fermentation. Perform serial dilution as Figure 5.1 illustrated using 1% NaCl as the diluent. Secondly, a 0.1-mL dilution was applied to both non-antibiotic control LB plate (B) and dual antibiotic LB plate (K+C). Choose appropriate dilution range for plate spreading. For example, for a low cell density sample (OD$_{600}$ < 0.6), the dilutions of $10^{-3}$ to $10^{-5}$ was spread; for a high cell density sample (OD$_{600}$ > 1.0), the dilution of $10^{-5}$ to $10^{-7}$, or even $10^{-8}$ was spread. Thirdly, the plates were incubated in 37°C for 12 to 15 hours. Another blank control LB plate (no inoculation, open lid in biosafety hood during procedures) was also incubated for ensuring sterility of the medium and procedure. Finally, count the colonies forming numbers of each plate. The viable cell number per mL can be obtained with the following equation

$$N_{\text{cells/mL}} = \frac{\text{number of colonies (CFUs)}}{0.1 \times \text{dilution}}$$

The purpose of spreading multiple dilutions was to obtain a dilution of which CFUs could fall within 30 and 300 to reduce random errors. Plates with fewer than 30 colonies were designated as too few to count (TFTC). While plates with more than 300 colonies were designated as too numerous to count (TNTC). Both situations were considered as not accurate counting (Sutton, 2006, 2012). Then the plasmids ratio was calculated by dividing CFU of the K+C plate over CFU of the B plate. Meanwhile, the spreading and incubation time was critical. The ideal colonies must be tiny, distinguishable, and evenly distributed.

Big colonies forming after over incubation, and uneven spreading would give an underestimation in counting CFUs (see Figure E in Appendix V). The samples for plasmids stability assay (PST) was collected during fermentation as time course data.

To improve the plasmids stability, the seed cryovial was firstly “purified” by using a double-layer gradient antibiotic plate selection method, which was shown as Figure 5.2.
Figure 5.2. Double-layer Gradient Antibiotics Purification Method

A double-layer LB agar plate was prepared of which the lower layer contained 100X folds (10 g/L carbenicillin & 5 g/L kanamycin) of normal antibiotics usage and the upper layer had no antibiotics. The two layers of LB-agar were solidified as a slant in petri dishes to create the gradient of antibiotics from 0 to 100X levels. Figure 5.2 shows the side view and top view of a gradient plate and the procedure for purifying seed culture. The plasmids ratio of the original seed cryovial was assayed by the PST method. The seed was inoculated onto the gradient plate by streaking. After incubation, an expected colony forming situation was illustrated in the figure. A single colony at the end of higher antibiotic concentration would be picked to inoculate a new gradient plate for confirmation of the purity and resistance of the cells to double antibiotics. Once confirmed, the culture from the end of higher antibiotic concentration of the second gradient plate would be collected as the “purified culture”. Based on this method, the purified culture had an improved dual-plasmid retention ratio of 93%, much higher compared to the original seed strain (~64%). The new seed was amplified into a new working cell bank.
An experiment was designed to determine the plasmid loss rate of this strain w/ & w/o antibiotics addition over time. Three time-course PST studies were conducted. First, PST over 12.5 generations in 5-mL LB (Kan+/Carb+) fermentation (37°C). Second, PST over 12.5 generations in 5-mL LB (no antibiotics) fermentation (37°C), with reseeding mid-late log-phase culture into fresh 5-mL LB medium. Third, PST over 17.2 generations in 5-mL LB (Kan+/Carb+) fermentation (30°C), with reseeding mid-late log-phase culture into fresh 5-mL LB (Kan+/Carb+) medium.

**Figure 5.3. Dual-plasmids Depletion in LB w/ & w/o Antibiotics & Reseeding**
The number of generations was determined by the doubling time at the respective conditions. The time course results of dual-plasmids (PS ratio) depletion were shown in Figure 5.3. With antibiotics pressure, the PS ratio of the strain was 47% ~ 61% after 12.5 generations. The depletion might reflect the degradation and/or diluted selective pressure of the antibiotics. While in plot C, the reseeding procedure ensured the cells were keep growing in log-phase. Interestingly, after each reseeding and growing to an OD of 0.6~1.0, the PS ratio rose and reached as high as 63% ~ 89%. The reason might be that the cells were at mid-log phase, and medium had adequate active antibiotics. Plot B shown the cells kept growing in LB with no antibiotics at high growth rate (log-phase). The reseeding procedure did not affect much on PS ratio. In fact, the PS ratio was decrease from 70%~114% to 27%~47% from 2 to 6 generations.

The PS ratio depletion study suggested that for a fed-batch process which cells were kept growing in an extended log-phase with fresh antibiotics in feeding, the PS ratio of “purified” strain could be retained at 63% ~ 89%. After log-phase & feeding stopped, the dual-plasmids ratio might quickly decrease as well as production.

5.2 Glucose analysis

The glucose assay protocol was mentioned in Appendix I by using a YSI 2900 Biochemistry Analyzer. This was an off-line analysis using supernatant samples of the fermentation culture. For a further study, an on-line glucose monitor would be useful and necessary, such as YSI 2960 Analyzer. The glucose membrane contained immobilized glucose oxidase. The oxidation of glucose would generate signal to the detector probe and provide a voltage signal which is proportional to the concentration of glucose.

\[
\text{Glucose} + O_2 \xrightarrow{\text{Glu oxidase}} H_2O_2 + D-\text{Glucono-δ-Lactone}
\]
The sample size, diluent, calibration, linearity, linear range, precision, and typical working life are all included in the manual of YSI 2900 series.

5.3 HPLC-MS for 6dEB analysis

5.3.1 Changes in LC-MS assay methods

Because 6dEB was UV inactive, both LC-MS and HPLC-ELSD assays were developed and employed in this study. The assay methods were mostly developed by Kasey C. from William’s group. As mentioned in Chapter 1, Figure 1.5 and Due to assay challenges, such as a limited amount of standards, and long turnaround time of LC-MS, other 6dEB assay methods were developed and applied at different stages. Thus, some datasets were noncomparable among each other because of inconsistent assay methods. To save 6dEB assays costs, there were no replicates results to perform an accurate statistical analysis. We only completed the proof of concept and make conclusions on our objectives, but the process could be further improved with more consistent and comparable data in the future.

, multiple 6dEB assay methods were developed and applied at different stages. Thus, some datasets were noncomparable among each other because of inconsistent assay methods. There were three developed LC-MS assay methods applied in this project. One was developed and performed by the Triad Mass Spectrometry facility in UNCG, using supernatant samples for 6dEB analysis, and detecting [M+Na]^+ of which m/z within 409.2556 ~ 409.2576. The other two were developed and performed by the Mass Spectrometry Facility (MSF) in Department of Chemistry at NCSU, using extracted and semi-purified 6dEB samples and detecting [M+H]^+ of which m/z within 387.27218 ~ 387.27606. The last two LC-MS methods were different in gradient time, which were 15-min
& 10-min respectively (see Appendix II). The 10-min method was better than the older one (15-min) because the peak of 6dEB was more concentrated and narrower.

The protocol for 6dEB extraction and purification were explained in Appendix II. Several solvents (ethyl acetate, chloroform, dichloromethane, and ether), and a resin (Amberlite XAD-16) at various pH (3, 4, 7, 10) were compared in extraction performance. Results were shown in Appendix V. Considering the convenience, economy and efficiency, ethyl acetate was selected for extraction at neutral pH7. In short, the extraction protocol was a 3X times of equal volume extraction using ethyl acetate to extract 0.5 mL supernatant sample. The extracts were concentrated by a Rotovap or overnight evaporation in chemical hood, and re-dissolved in 100 ~ 200 μL methanol for LC-MS assay. Semi-purification steps by flash chromatography were available for obtaining a purer 6dEB material.

Figure 5.4. Performance Comparison of 10-min and 15-min LCMS Methods
The m/z filter range for 6dEB spectrogram was between 387.27218 and 387.27606 for 15-min protocol, while between 409.25401 and 409.25811 for 10-min protocol. The retention time of 6dEB was around 6.7~6.8 min for the 15-min protocol, and 4.94 for the 10-min protocol. The comparison in LC gradient and retention time of the two LC-MS methods were shown in Figure 5.4.

5.3.2 Deviation analysis of calibration curves for 6dEB estimation

A calibration curve of standard 6dEB should be determined along with the fermentation samples during each 6dEB assay. The standard samples were a series of 6dEB dilutions with known concentrations. The calibration curve was plotted as 6dEB standard concentration vs. peak area (or area under curve, AUC). The formula was determined by using either linear regression or polynomial regression depends on the results to give the best fit. The reason for different mathematical regression was mainly because of the concentration range of 6dEB standards. For the lower concentration range, usually 0 ~ 5 μM 6dEB, a linear regression could describe the calibration curve. However, for application of wider or higher concentration range (> 10 μM), a polynomial regression was preferred to fit the calibration. Nevertheless, both methods required the response value (peak area) of 6dEB sample (culture) to fall within the application range of relative calibration curves. If a sample had a high concentration of 6dEB and AUC was out of the range, it was necessary to dilute the sample and re-run the LC-MS assay again along with standards. Moreover, the three different LC-MS assays (different sample protocols, facilities, or assay methods) were very different during calibration determination, which added variation and/or deviation to estimate the 6dEB titers of relative samples. In addition, most 6dEB assays did not test triplicate standards.
(in order to save analysis costs), and some batches did not even include 6dEB standards.

Both added more difficulties in data analysis and further discussion.

![Calibration Curve Example of 6dEB LCMS assay in UNCG](image)

**Figure 5.5. Calibration Curve Example of 6dEB LCMS assay in UNCG**

Figure 5.5 shows the calibration curve obtained by a LCMS assay in UNCG, which indicates a linear range of $0 \sim 1$ mM 6dEB (or AUC $< 2E7$) with a $R^2$ of 0.9989. This curve was used to estimate 6dEB titers of some early stage shaking-flask studies, such as LB, CD4, PA1, and PB1 media. However, this calibration curve was not determined along with the batch of samples at the same time. Thus, the 6dEB titers of the four media were estimates and might be inaccurate.

Figure 5.6 shows the calibration curves for 15-min LCMS assay (old protocol) determined in the Department of Chemistry, NCSU. The results indicated that the calibration was linear within a range of $0 \sim 50$ μM 6dEB (or AUC $< 1E9$) with a $R^2$ of 0.998. For application of a wider range of calibration, a polynomial regression was fitted for $0 \sim 1$ mM 6dEB (or AUC $< 5.5E9$). These curves were used to estimate 6dEB titers of some shaking-flask studies and early stage of bioreactor fed-batch samples, such as PB1, PB3, and OPM2. In addition, these curves were developed using the synthetic 6dEB standards.
Figure 5.6. Calibration Curves for 15-min 6dEB assay in NCSU

Figure 5.7 shows the calibration curves used for 10-min LCMS assay (new protocol) determined in the Department of Chemistry, NCSU. The selection of standard dilutions, concentration ranges, and standard types (synthetic or biological) were all related to the determination of a proper calibration curve, either linear or polynomial regression.

In Figure 5.7-A, a calibration curve within 0 ~ 1 mM (or AUC < 6E9) was determined for HCD fed-batch samples. The dilutions of 6dEB synthetic standards were 0, 0.0025, 0.01, 0.025, 0.1, and 1 mM, which were condensed in lower range. Unevenly distributed data might not properly describe the actual calibration. While in Figure 5.7-B, two calibration curves within 0 ~ 15 μM (or AUC < 5.5E8) were developed for cell-patch samples. The selected dilutions of 6dEB biological standards were more evenly distributed.
Figure 5.7. Calibration Curves for 10-min 6dEB assay in NCSU

In Figure 5.7-C, a calibration curve within 0 ~ 0.25 mM (or AUC < 6E8) was determined for some SF samples, such as LB, PB1, CD4, and CD6. Figure 5.7-D shows the calibration of the same 6dEB biological standards but determined within a range of 0 ~ 5 μM (or AUC < 2.5E8) using linear regression (R² = 0.9763). Depends on the application AUC range of data, a linear or polynomial regression was determined and applied accordingly.
These curves were used to estimate 6dEB titers of some medium studies in shaking-flasks, HCD and PB1 fed-batches, and the cell-patch LB samples, respectively.

In short, the calibration curves had significant effect on the estimation of 6dEB titers. A series of properly prepared standard dilutions at evenly distributed concentrations, and triplicated samples tested along with the samples at the same time would generate a relatively accurate calibration curve.

5.4 Preparation of biological 6dEB standard

As mentioned in Due to assay challenges, such as a limited amount of standards, and long turnaround time of LC-MS, other 6dEB assay methods were developed and applied at different stages. Thus, some datasets were noncomparable among each other because of inconsistent assay methods. To save 6dEB assays costs, there were no replicates results to perform an accurate statistical analysis. We only completed the proof of concept and make conclusions on our objectives, but the process could be further improved with more consistent and comparable data in the future.

As shown in Table 5.1 and Figure 5.8, we had 0.9 g of synthetic 6dEB standards provided by Krische’s group and this standard was quickly used up after several fed-batch and some other studies. After a good batch of PB1 broth (1-L) was obtained, 26.7 mg of 6dEB was extracted and purified from this batch to be a “biological 6dEB standard”. This biological 6dEB standard was examined with LC-MS and NMR to confirm the peak purity and molecule entity. Unfortunately, there was not enough synthetic standard to assay against the home-made 6dEB standard to confirm purity. However, based on the results of NMR (Figure 5.9), the biological standard looked very pure. Then we assumed the biological 6dEB as a pure standard and comparable to the synthetic counterpart for the following experiments.
The extraction and purification protocols were similar to small-volume samples as explained in protocol B (Appendix II). They are modified for treating a large culture-volume as described below.

1. **Centrifugation.** Remove the cells and collect supernatant using 4,000 rpm, 10 ~ 20 min in a large volume centrifuge. Repeat the process until the liquid became clear enough. About 1-L supernatant was divided into 2 parts of 500-mL for extraction.

2. Add ~500 mL supernatant into a 2-L separatory funnel and adjust the pH to neutral if needed. Add equal volume of ethyl acetate. Mix solution well from end to end. Let the solution sit still at room temperature in a fume hood for 20 to 30 min until the liquid phases separated from each other.

3. Discard the lower phase (aqueous) and retain the upper phase (organic) solution. Repeat step 2 to 3 for the other 500 mL supernatant. Combine the total organic extractions in glass beaker.

4. Transfer the extractions into a round-bottom flask and use a Sigma-Aldrich IKA® RV 10 rotary evaporator to concentrate the sample until all ethyl acetate evaporates. Take caution to not over dry the samples.

5. Add 1~2 mL of methanol to re-dissolve the 6dEB inside of the round-bottom flask.

6. Use 2 steps of flash chromatography to purify the 6dEB sample including the 1\textsuperscript{st} one (15-35\% EtOAc/hexanes) and the 2\textsuperscript{nd} one (2-5\% MeOH/CH\textsubscript{2}Cl\textsubscript{2}).

7. Use a small TLC plate and potassium permanganate stain method to find out which fractions contained 6dEB (see protocol in Appendix II).

8. Combine the fractions with 6dEB and then perform a final polishing step of preparatory TLC (35\% EtOAc/hexanes) for getting the pure material.
9. Use $^{13}$C and $^1$H NMR to verify if the 6dEB sample was clean enough.

As shown in the Table 5.1 and Figure 5.8 below, the biological 6dEB standard was comparable to previous results of synthetic standard in mass spectrometry.

**Table 5.1. [M+Na]$^+$ Comparison of Biological and Synthetic 6dEB Standards**

<table>
<thead>
<tr>
<th>6dEB standard</th>
<th>[6dEB + Na] mass error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical</td>
<td>409.25664 0</td>
</tr>
<tr>
<td>Biological 6dEB (Kasey’s)</td>
<td>409.25528 -3.3</td>
</tr>
<tr>
<td>Synthetic 6dEB (Krische’s)</td>
<td>409.25525 -3.4</td>
</tr>
</tbody>
</table>

**Figure 5.8. Mass Spectrum Comparison of Synthetic and Biological 6dEB Standards**

As shown in Figure 5.9 and Table 5.2 below, the $^{13}$C and $^1$H NMR of biological 6dEB was clean and comparable to that of synthetic 6dEB standard. In summary, the home-made biological 6dEB standard was a very pure material and could be used for calibration.
Figure 5.9. $^{13}$C NMR of Biological 6dEB Standard

Table 5.2. Comparison of $^{13}$C NMR for Synthetic and Biological 6dEB

<table>
<thead>
<tr>
<th>Carbon #</th>
<th>Krische (CDCl$_3$, 500 MHz)</th>
<th>Kasey (CDCl$_3$, 500 MHz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>213.4</td>
<td>213.5</td>
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<tr>
<td>2</td>
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</tr>
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<td>6.9</td>
</tr>
<tr>
<td>21</td>
<td>6.2</td>
<td>6.3</td>
</tr>
</tbody>
</table>

Contaminant peak at 29.7
5.5 HPLC-ELSD for 6dEB analysis

The HPLC-ELSD methods were developed and optimized by Christian Kasey (William’s group) and explained in detail in protocol C (Appendix II). An ELSD detector was a mass-dependent detector, and the response signal depended on the physical properties (size, shape), but not spectral or chemical properties of the analyte (Vervoort, Daemen, & Torok, 2008). In this study, Agilent 1220 Infinity LC systems and 1260 Infinity ELSD were used. Through a liquid chromatographic system, the eluent of sample was nebulized into aerosol using a flow of nitrogen and transported through a temperature-controlled evaporator tube where solvent was removed at high temperature. The remaining solute particles were subsequently introduced into a detector chamber where a laser light source was directed onto the particles. Then the scattering of the incident light was detected by a photomultiplier and generated a signal proportional to the number of photons scattered. Meanwhile, the degree of light scattering was related to the concentration of analyte of interest in the sample (Agilent Technologies, 2012; Vervoort et al., 2008).

A UV response (absorbance) followed Beer’s law \( A = \varepsilon b c \), while the ELSD response followed an exponential relationship, which was described below (Agilent Technologies, 2012; Allgeier, Nussbaum, & Risley, 2003).

Equation 5.2. \[ A = am^x \]

where \( A \) was the peak area response (the intensity of scattered light), \( m \) was the mass of the scattering particles, and \( a \) and \( x \) were coefficients reflecting properties of the sample and the parameters of the chromatographic and detector systems. A log-log plot of peak area response versus quantity would be linear, which could be described as

Equation 5.3. \[ \log A = x \log m + \log a \]
5.5.1 Sensitivity of HPLC-ELSD for 6dEB analysis

According to protocol C (Appendix II), the retention time of 6dEB was usually 6.475 ~ 6.495 min. column. ELSD assay results of a production sample and 6dEB standards (biological) were shown in Figure 5.10 below.

Sample Info : 2P3-5, 73h

![ELSD Chromatogram for 6dEB Assay](image1)

Sample Info : 100 μM 6dEB

![ELSD Chromatogram for 6dEB Assay](image2)

Sample Info : 1000 μM 6dEB

![ELSD Chromatogram for 6dEB Assay](image3)

**Figure 5.10. Example of ELSD Chromatogram for 6dEB Assay**

Through method validation with 25 ~ 1500 μM 6dEB standards, the limit of detection (LOC) of this HPLC-ELSD assay was preliminarily determined to be ~100 μM of 6dEB (biological std.). Below that concentration, the peak at 6.475 ~ 6.495 min was not always
observed on the chromatogram. Compared to LC-MS, which could detect as low as 0.1 μM, the ELSD method was obviously much less sensitive. However, LC-MS was too expensive and took a much longer turnaround time and required highly trained personal to operate. This ELSD method was much cheaper, faster and more convenient to use in a routine R&D environment, as long as the target samples had sufficient quantities of analytes to be detected.

5.5.2 Preliminary method validation of 6dEB-ELSD calibration

Figure 5.11. Linearity and Accuracy of 6dEB-ELSD Calibration

Based on a preliminary study on the ELSD method, we found that the calibration was more suitable for sample with relatively high 6dEB titer. The linearity of calibration followed Equation 5.3. And shown in Figure 5.11, a calibration curve was generated by triplicated 6dEB standards tested at the beginning, middle and the end of an ELSD assay along with 6dEB samples. This curve suggested that ELSD method could usually generate a linear calibration within a range of 100~1500 μM 6dEB. The precision of this calibration CV% was 2~35%. According to this calibration curve, the accuracy of 6dEB standards at 125, 250, and
500 μM were shown in right plot of Figure 5.11. The estimates (using calibration) were lower than experimental (true) values by 2.4% to 14.8% in mg/L.

![Figure 5.12. Preliminarily Comparison of LCMS and ELSD Calibration for 6dEB](image)

To determine the accuracy of this ELSD method, a set of data (6dEB sample) was determined by two calibrations, LCMS and ELSD, and was preliminarily compared in Figure 5.12. High titer samples were diluted for assay. Each sample was different in treatment or harvest time. Thus, an ANOVA for block design was shown in Table 5.3 below.

<table>
<thead>
<tr>
<th>Table 5.3. ANOVA Test on Cell-patch 6dEB Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample no.</td>
</tr>
<tr>
<td>LCMS (mg/L)</td>
</tr>
<tr>
<td>ELSD (mg/L)</td>
</tr>
<tr>
<td>ANOVA</td>
</tr>
<tr>
<td>Source of Variation</td>
</tr>
<tr>
<td>Rows</td>
</tr>
<tr>
<td>Columns</td>
</tr>
<tr>
<td>Error</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>
Although LCMS had a much lower LOD than ELSD, for the samples with relative high concentrations, ELSD had comparable estimations on 6dEB titers compared to LCMS. Because the F-stat (0.016 among blocks) was smaller than F-crit. (6.608) by ANOVA test, there was no significantly difference between LCMS and ELSD ($\alpha = 0.05$).

Figure 5.13 shows the repeatability of 6dEB-ELSD calibrations obtained on two different days (5-days apart), which indicates the calibration was repeatable on different days.

![Figure 5.13. Repeatability of 6dEB-ELSD Calibration](image)

In summary, this ELSD method was usually linear within the range of 100~1500 μM 6dEB with good repeatability and accuracy. Within its applicable range of 6dEB assay, ELSD method can give comparable results to LCMS.

In this project, various analysis methods on 6dEB assay were applied. The main reasons for changing methods along the project were (1) saving cost, (2) method optimization, and (3) using ELSD method for faster and cheaper analysis.
CHAPTER 6. Conclusions

In this project, 6dEB production through recombinant *E. coli* strain TB3/pBP130/pBP144 had been enhanced through optimization of medium components, induction strategies, fed-batch fermentation and latex patch cell immobilization (cell-patches), etc. Through shaking-flask studies, several chemically-defined and semi-defined media with promising 6dEB productivity have been developed, including CD4 (15.08 mg/L), CD6 (76.87 mg/L), PB1 (25.15 mg/L), PM1 (51.98 mg/L), and EM1 (34.4 mg/L) media. During the study of medium components, we found that high levels of glucose causes a higher growth rate and final cell OD but led to a pH control challenge. High levels of ammonium sulfate might cause early cell lysis and foaming problem. Organic N-sources, such as tryptone and casamino acids, showed positive effects on enhancing 6dEB titers, as well as less foaming problem compared to ammonium sulfate. Through IPTG and propionate studies in shaking-flask and cell-patches, the induction levels were optimized to 1 mM IPTG, 175 mM sodium propionate, and extra 15 mM propionate per 24 h after induction. Propanol could be used as the substrate as well. The 150 mM phosphate buffer was optimized for pH control. Compared to the maximum 6dEB titer in LB (29.8 mg/L), CD6 shaking-flask improved by 158%.

During the development of a 2-L bioreactor fed-batch process, various CPPs were preliminarily determined and promising 6dEB titers were obtained from PB1 (110.05 mg) and HCD (343.11 mg) fed-batch processes. The seed train of rejuvenation and expansion were optimized and a “purified” strain with 93% dual-plasmids-retention ratio was obtained after PST study. A fed-batch strategy was found to be able to produce high cell density and high 6dEB titer. A preliminary step-feeding profile was developed for HCD fed-batch process.
The bioreactor set up was modified with extra pure oxygen inflow line, pH control feeds, foaming control, and PID parameterization in cascade feedback control on DO. In this study, compared with maximum 6dEB production in LB shaking-flask (29.80 mg/L), the 6dEB titer of HCD fed-batch was improved by 460%, and of PB1 fed-batch by 147%.

During the study of cell immobilization using cell-patches, only LB medium was consistently found to allow immobilized cells to produce 6dEB. The protocol of cell-patch preparation was preliminarily developed, and further optimization will be required, such as adding a nanoporous sealant cover layer. With pre-induced cells and an elevated level of antibiotics selection pressures with the addition of propionate substrate, the cell-patch method was found to be able to produce 6dEB for a longer life span in LB (at least 240 h). The immobilized E. coli methods improved 6dEB titer by 309% compared to a no-immobilized LB shaking-flask control, which was very potential to be optimized and developed into a practical production process.
CHAPTER 7. Future Work

7.1 Construction of compatible plasmids for recombinant protein expression

This project used the strain TB3/pBP130/pBP144, which was generously given by Pfeifer’s group. Because the two plasmids shared the same origin of replication, the dual-plasmids system was not very stable. If possible, the next researcher should start by developing a new expression system by using different vectors for the three DEBS genes. For example, the high production titer (> 1 g/L) of the propriety strain K207-3/pKOS207-129/pBP130 developed a compatible dual-plasmids system. Compatible plasmids system could sufficiently improve the 6dEB production titer.

7.2 Further optimization in medium and fed-batch process

Through shaking-flask studies, several medium components were found to have positive effects on enhancing 6dEB titer. In the future, more rigorous experimental design could be utilized in this study to optimize the levels of medium components, such as casamino acids, yeast extract, tryptone, trace metals and vitamins. Similarly, IPTG, induction OD_{600}, and propionate or propanol levels could also be considered for experiment design study. Triplicated samples of each control should be collected for 6dEB analysis at the same time.

Take CD6 medium in shaking-flask as an example for DOE study. CD6 had a maximum OD_{600} of 10.18 at 70 h. If the fermentation time was set constant as three days (72 hours), the response variable would be 6dEB titer (mg/L) and max. OD_{600}. Two DOE studies could be conducted.
For the first one, the factors and levels would be IPTG (100 mM ~ 2 mM), induction OD\textsubscript{600} (2 ~ 8, or 20% to 80% of max. OD\textsubscript{600} in general), propionate (35 ~ 350 mM), and propanol (0 ~ 350 mM).

A definitive screening design (DSD) (Errore, Jones, Li, & Nachtsheim, 2017; Jones & Nachtsheim, 2013) could be used to construct the experiments as shown in Table 7.1 below. DSD is suitable to use in early stages of experimentation when there are a large number of potentially important factors that may affect a response of interest and when the goal is to identify what is generally a much smaller number of highly influential factors.

**Table 7.1. Definitive Screening Design for Induction in CD6 Shaking-flask**

<table>
<thead>
<tr>
<th>Run</th>
<th>IPTG (mM)</th>
<th>Induction OD\textsubscript{600}</th>
<th>Propionate (mM)</th>
<th>Propanol (mM)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
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<td>2</td>
<td>35</td>
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</table>

The second DOE could be a Box-Behnken Surface Response Design (Box & Behnken, 1960) to investigate the levels and effects of casamino acids (0~40 g/L), yeast extract (0 ~ 15 g/L), and tryptone (0 ~ 40 g/L) to 6dEB titers. The design table could be constructed using JMP Pro 14 as shown in Table 7.2 below.
Further optimization of the bioreactor fed-batch process would focus on the operational parameters such as cascade control of DO on STIRR and GFR and the feeding rate control system. A more accurate feeding system including better fed-batch pumps and the addition of an on-line glucose/glycerol monitoring system would be needed to maintain the concentration of substrates. Beside those, an automatic foaming control and a weight monitor system are also necessary for a better control of fed-batch operations. In the future, the process can be scaled up into a 30-L bioreactor for a pilot study and 6dEB production.

### 7.3 Optimization in latex-coating cell-patches method

In this study, we found the latex-coating methods had some damage effects on cell viability. Only LB medium was found to produce detectable level of 6dEB production with cell-patches. Further confirmation would be required to optimize this protocol to preserve the cell viability with other medium, such as drying time, concentration of latex binder, pH, cell to binder ratio, temperature and RH. In addition, the cell-patches are preferable to be covered.

---

**Table 7.2. Box-Behnken Surface Response Design of N-sources in CD6 Shaking-flask**

<table>
<thead>
<tr>
<th>Run</th>
<th>Pattern</th>
<th>Casamino acids (g/L)</th>
<th>Yeast Extract (g/L)</th>
<th>Tryptone (g/L)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>−−0</td>
<td>0</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
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<td>4</td>
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<td>0</td>
<td>7.5</td>
<td>40</td>
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<tr>
<td>4</td>
<td>−+0</td>
<td>0</td>
<td>15</td>
<td>22</td>
</tr>
<tr>
<td>5</td>
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<td>20</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
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<td>20</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
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<tr>
<td>15</td>
<td>0</td>
<td>40</td>
<td>15</td>
<td>22</td>
</tr>
</tbody>
</table>
by a sealant layer to prevent the outgrowth. The characterization of cell patches would also be needed, including the diameter, thickness, storage time & conditions, and structure porosity.

Because the cell-patches method was performed in small scale (5-mL, 6-well plate), this method would be suitable for a DOE study on various factors. We can also compare the immobilized cells and free submerged cells fermentation in 6-well plate. Other factors of interest are IPTG levels, medium types (PM1, EM1, CD6, etc.), and cell viability and density in the cell-patches.

7.4 Optimization of 6dEB analysis and extraction

One of the largest challenges in this study was the inconsistent LC-MS methods and lack of 6dEB standards and calibration curves along with sample analysis. In the future, a single LC-MS method should be used as a standard analysis protocol. 6dEB calibration samples must be triplicate and evenly distributed in concentration for developing a calibration curve that covers the range of interest. Culture samples that out of range must be diluted and re-tested against a new set of calibration standards. At the same time, an ELSD method could be used as a screening protocol for analyzing sample of high 6dEB titers.

Meanwhile, the 6dEB extraction and purification protocols could be further optimized since the current method had only about 50% efficiency (Figure F-II). For extraction of large volume of culture broth, many details should be considered and optimized, such as the cell removal by centrifugation, emulsion problem in solvent extraction, extraction time and temperature, and the purification chromatography protocols.
REFERENCES


BEC363-Lab4. (2014 Spring). Laboratory Session 4: Correlation between Optical Density and Dry Weight Foundations of Recombinant Microorganisms for Biomanufacturing: Golden LEAF Biomanufacturing Training and Education Center (BTEC), NCSU.


Moulton, G. G. (2014). *Fed-batch fermentation a practical guide to scalable recombinant protein production in Escherichia coli* Woodhead publishing series in biomedicine number 42 (pp. 1 online resource.).


Appendix I. General Protocols

Protocol A  Working cell bank preparation

1. Clean the work station of a BSL-2 Biological Safety Cabinet (pre-sterilized by 30 minutes of UV-light exposure). Wear proper Personal Protection Equipment (PPE).

2. Obtain a cryovial of the original strain, TB3/pBP130/pBP144 from -70°C freezer. Thaw the vial with hands.

3. Use a sterile inoculation hood to inoculate the strain onto a prepared LB(Kan\(^+\)/Carb\(^+\))-agar plate (added with antibiotics, 50 mg/L kanamycin and 100 mg/L carbenicillin) by streaking.

4. Incubate the plate in a 37°C incubator overnight or for 12~15 hours.

5. Picking a single colony near the ending part of streaks and inoculate into 35 mL of LB (Kan\(^+\)/Carb\(^+\)) liquid medium in a 250-mL baffled Erlenmeyer flask. Shaking incubate at 37°C, 300 rpm for 4 to 6 hours.

6. Monitor and measure the optical density (OD\(_{600}\)) of the culture sample at a wavelength of 600 nm. Stop fermentation when OD\(_{600}\) reaches 1.0~1.5. This culture is referred as seed culture afterwards.

7. Prepare sterile 20% glycerol and 80 sterile 2-mL cryotubes by 121°C sterilization (autoclaving).

8. In a sterile biosafety hood, add 750 μL of sterile 20% glycerol into each cryotube. Then add 250 μL of seed culture into each tube.

9. Store the cryovials in a cryo-storage box and label accordingly. Keep the cryovials in -70°C freezer.
Protocol B  Determine DCW vs. OD600

1. About 2.5 liters LB cell culture were prepared overnight using shaking-flask fermentation protocol as mentioned in Chapter 2.

2. Collect the cell paste by centrifugation at 14,000 rpm and the cell paste were washed using 1% NaCl one time.

3. Dissolve the cell paste in 60 mL of 1% NaCl, which was the most condensed sample D1. A serial dilution of 15 mL cell solution with 45 mL fresh 1% NaCl was performed to make another four dilutions from D2 to D5, while D6 is blank control (1% NaCl). Measure the OD_{600} of dilution D5 and calculate the theoretical OD_{600} of the other concentrated samples D1~D4.

4. Each dilution was aliquot onto three aluminum pans with 15 mL each. All pans were dried out in an oven at 85°C for over 24 hours. The aluminum pans were pre-weighed.

5. Take out the pans with dried samples. Store in a plastic storage box with desiccates and let them cool down in room temperature.

6. Weigh the pans and calculate the net weight of each sample. Subtract the average weight of blank sample (1% NaCl) from each other samples to get the net dry cell weight.

7. Plot the results of dry cell weight against theoretical OD_{600}. 
Protocol C  Glucose assay using YSI 2900 Biochemistry Analyzer

Materials:

- System buffer YSI 2357
- Calibration standard YSI 2776 (2.5 g/L)
- Membrane YSI 2365
- Linearity standard YSI 1531, YSI 2777
- Salt solution YSI 2392

Methods:

1. Dissolve content of one buffer pouch in 475 ± 25 mL DI water. Rinse buffer bottle, filled with buffer, and install onto the working station of YSI 2900 Analyzer.

2. Install calibration solution YSI 2776 at position Cal 1A. Rinse the sipper with DI water before priming the fluid. Calibrate the reading (following manual).

3. Install linearity solution YSI 1531 at position Cal 1A. Perform linearity calibration by following the manual. For application requiring linearity performance to 25.0 g/L, use YSI 2777 for linearity calibration, and set sample size to be 10 μL.

4. Unscrew the probe and remove the old membrane. Rinse the probe with YSI 2392 salt solution. Open the membrane holder and rinse the membrane with a few drops of salt solution. Assemble the O-ring membrane YSI 2365 gently onto the enzyme probe 1A and finger tight the probe back.

5. Perform [FLUSH] operation to wash the probes with buffer several times. Let the machine sit in room temperature for 4~6 hours or overnight. Observe the baseline of probe current value until the value is below 6 nA and maintaining stable.

6. Troubleshooting. If a probe doesn’t have a current change, it is possible that the probe is malfunction or membrane is overdue or damaged (storage refrigerated, Do Not Freeze). Use a fresh new membrane and change to other probes.
Appendix II. Analysis Assays and Relevant Protocols

Protocol A  Potassium permanganate stain for TLC (35% EtOAc/hexanes)

Recipe (a typical lifetime for this stain is approximately 3 months):

KMnO₄, 1.5 g  K₂CO₃, 10 g
10% NaOH, 1.25 mL  DI water, 200 mL

Uses for detecting olefins and other readily oxidized groups

This staining method is excellent for functional groups which are sensitive to oxidation. Alkenes and alkynes will appear readily on a TLC plate following immersion into the stain and will appear as a bright yellow spot on a bright purple background. Alcohols, amines, sulfides, mercaptans and other oxidizable functional groups may also be visualized, however it will be necessary to gently heat the TLC plate following immersion into the stain. These spots will appear as either yellow or light brown on a light purple or pink background. Finally, circle these spots following visualization as eventually the TLC will take on a light brown color upon standing for prolonged periods of time.

Example:

Shortly after staining  Overnight sitting after staining
Sample preparation & semi-purification:

1. Obtain 500 μL supernatant of each sample to a new 1.5-mL EP tube. Centrifuge again at 14,000 rpm for 10 min to remove any precipitates.

2. Add & mix 1:1 of ethyl acetate (EA) with samples by vortex for 10 ~ 30 sec.

3. Let the mixture sit in room temperature for at least 5 min until two liquid phases separate. Centrifuge at 14,000 rpm for 15 ~ 30 sec again.

4. Pipette the upper (organic) phase carefully to a new 1.5-mL EP tube and label it.

5. Repeat step 2 to 4 for three times of extraction, and combine the extracted solution.

6. Concentrate the extractions by either open and lid of tubes and let them sit in a chemical fume hood overnight (or 1.5 days) or using a rotavapor.

7. Add 100 μL or 200 μL of methanol to re-dissolve the samples. Store the samples at 4°C refrigerator before submitting for 6dEB assays. Usually the samples were submitted for 6dEB assay after this step.

8. If a more purified 6dEB is required, three steps of purification will be performed, including a 1st flash column chromatography (15-35% EtOAc/hexanes), a 2nd flash column chromatography (2-5% MeOH/CH₂Cl₂), and a final prep. TLC (thin layer chromatography) polishing step to get the pure material.

LC-MS by Mass Spectrometry Facility in Department of Chemistry, NCSU

A. 15-min LC-MS method (old one)

Analysis was performed by LC-MS at the NCSU MSF. A Thermo Scientific Dionex UltiMate 3000 LC system was coupled to a Thermo Fisher Scientific Exactive Plus MS (benchtop full-scan Orbitrap™ mass spectrometer) for the experiments detailed here. The
system was operated in positive ion mode using the Heated Electrospray Ionization (HESI) technique. Full scan MS analysis at a resolution of 70,000 was performed using the Orbitrap mass analyzer at \( m/z \) range of 100-1000. The instrument was externally calibrated prior to sample analysis.

<table>
<thead>
<tr>
<th>HESI Source Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spray voltage</td>
</tr>
<tr>
<td>Capillary temperature</td>
</tr>
<tr>
<td>Heater temp</td>
</tr>
<tr>
<td>S Lens RF level</td>
</tr>
<tr>
<td>Sheath gas flow rate</td>
</tr>
<tr>
<td>Scan Range</td>
</tr>
</tbody>
</table>

### Materials:
- Water (OmniSolve, LC-MS grade)
- Acetonitrile (Burdick & Jackson, HPLC grade)
- Formic Acid (Fluka: 56302).

A gradient elution was performed at a flow rate of 0.5 mL/min with solvent A (H\(_2\)O with 0.1% HCOOH) and solvent B (ACN with 0.1% HCOOH) as the mobile phase system. A reverse phase C\(_{18}\) column (50 mm x 2.1 mm, 175 Å pore size, 1.9 μm particle size) was used for separation of sample components. The column temperature was maintained at 50°C. Injections of sample were 10 μL on column. One technical LC/MS run was performed on each sample. The LC gradient profile is described below. Retention time of 6dEB was detected at 6.7~6.8 min and 6dEB in the [M+H]\(^+\) form of C\(_{21}\)H\(_{39}\)O\(_6\).

<table>
<thead>
<tr>
<th>Retention Time (min)</th>
<th>% A</th>
<th>% B</th>
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<tbody>
<tr>
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<td>72</td>
<td>28.0</td>
</tr>
<tr>
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<td>72</td>
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</tr>
<tr>
<td>15.0</td>
<td>72</td>
<td>28.0</td>
</tr>
</tbody>
</table>
The mass spectrogram of a sample from PB1 medium was shown below.

B. 10-min LC-MS method (new one)

Analysis was carried out on a Thermo Fisher Scientific Exactive Plus MS, a benchtop full-scan Orbitrap™ mass spectrometer, using Heated Electrospray Ionization (HESI) technique. The sample was analyzed via LCMS injection into the mass spectrometer at a flow rate of 500 µL/min. The mobile phase B was acetonitrile with 0.1% formic acid and mobile phase A was water with 0.1% formic acid. The mass spectrometer was operated in positive ion mode. The LC column was a Thermo Hypersil Gold (50 mm x 2.1 mm, 1.9 µm particle size).

<table>
<thead>
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<th>LC Gradient</th>
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<tbody>
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<tr>
<td>5.0</td>
</tr>
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<td>7.0</td>
</tr>
<tr>
<td>8.0</td>
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<td>10.0</td>
</tr>
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</table>
### HESI Source Parameters

<table>
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<th>Parameter</th>
<th>Value</th>
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<tr>
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<td>Capillary temperature</td>
<td>350°C</td>
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<tr>
<td>Heater temp</td>
<td>300°C</td>
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<tr>
<td>S Lens RF level</td>
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<td>Sheath gas flow rate</td>
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<tr>
<td>Resolution</td>
<td>70,000</td>
</tr>
<tr>
<td>Scan Range</td>
<td>250-1200 m/z</td>
</tr>
</tbody>
</table>

The 6dEB samples were detected at a retention time of 4.94~5.05 min in [M+Na]$^+$ form of $C_{21}H_{38}O_6Na$. The mass spectrogram of 1 μM 6dEB standard sample was shown below.

6dEB assays (LC-MS) by Triad Mass Spectrometry in UNCG Chemistry & Biochemistry inject supernatant samples directly, and 6dEB was detected in [M+H]$^+$ form. The linear range of their calibration curve was up to 1 mM with $R^2 = 0.989$. Other details are not clear.
Protocol C  

6dEB HPLC-ELSD assay (method by William’s group)

An Agilent 1220 Infinity HPLC equipped to an Agilent 1260 Infinity ELSD equipped with an Agilent Poroshell C\textsubscript{18} column was used for HPLC analysis. ELSD evaporator temperature and nebulizer temperature were kept at 80 °C with a nitrogen gas rate of 1.6 SLM. Sample injections were 10 μL. Analysis was conducted using 1 minutes of 100% buffer A at 2 mL/min, and a gradient of 0%-100% buffer B for 10 min followed by 2 min of 100% buffer B. Buffer A is 0.1% formic acid in water and buffer B is 0.1% formic acid in acetonitrile. The retention time of 6dEB was usually 6.475 ~ 6.495 min.
Appendix III. Medium Compositions

The following master solutions were all filter sterilized using 0.22-micron PES membrane filters, except (1) vitamin B2 prepared in 20% DMSO was sterilized with 0.22-micron nylon membrane, and (2) propionate and 50% Antifoam B was autoclaved.

a. 1000x TM (trace metals) *

FeCl₃ · 6H₂O, 33.75 g/L  ZnCl₂ · 4H₂O, 2.5 g/L
CaCl₂ · 6H₂O, 2.5 g/L  concentrated HCl, 25 mL/L
Na₂MoO₄ · 2H₂O, 2.5 g/L  CuSO₄ · 5H₂O, 2.375 g/L
H₃BO₃, 0.625 g/L

*For solubility and stability concern, Fe³⁺, Zn²⁺, and Ca²⁺ were made into stock 1 (TM1) with concentrated HCl; MoO₄²⁻ was stock 2 (TM2); BO₃³⁻ and Cu²⁺ were stock 3 (TM3).

b. 1000x Vitamins, stock solution in aliquot 15 mL tube individually**

Riboflavin (B2) 0.525 g/L  Niacin (B3) 7.5 g/L
Pantothenic acid (B5) 6.75 g/L  Biotin (B7) 75 mg/L
Folic acid (B9) 50 mg/L  Pyridoxine (B6) 1.75 g/L

**Each vitamin was prepared and stocked separately. pH was adjusted to 7.6.

c. Antibiotics stock solution (1000x) (filter sterilized)

Kanamycin 50 mg/mL (in ddH₂O)  aliquot 1 mL per stock, -20°C storage
Carbenicillin 100 mg/mL (in ddH₂O)  aliquot 1 mL per stock, -20°C storage

d. Propionate stock solution (5M)

Sodium propionate 96.07 g  ddH₂O 200 mL

e. 50% Antifoam B

Antifoam B 25 mL  ddH₂O 50 mL
The following media were all added with 0.1% of 50% Antifoam B; pH adjusted to 7.0~7.6. Antibiotics (kanamycin and carbenicillin) and vitamins solution were added after medium autoclave. Glucose was filter sterilized using 0.22-micron PES membrane filters.

**CD1 (35 mM PPS buffer)**

- Glucose, 5 g
- MgSO\(_4\) · 7H\(_2\)O, 0.31 g
- (NH\(_4\))\(_2\)SO\(_4\), 0.4 g
- KH\(_2\)PO\(_4\), 1.5 g
- K\(_2\)HPO\(_4\), 4.34 g
- DI water, 1 L
- 1000x TM, 1 mL/L
- 1000x Vitamins, 1 mL/L

**CD2 (35 mM PPS buffer)**

- Based on CD1 composition, only modifications are:
- Glucose, 10 g
- (NH\(_4\))\(_2\)SO\(_4\), 1.0 g

**CD3 (50 mM PPS buffer)**

- Glucose, 20 g
- MgSO\(_4\) · 7H\(_2\)O, 0.31 g
- (NH\(_4\))\(_2\)SO\(_4\), 2.0 g
- KH\(_2\)PO\(_4\), 2.079 g
- K\(_2\)HPO\(_4\), 6.048 g
- DI water, 1 L
- 1000x TM, 1 mL/L
- 1000x Vitamins, 1 mL/L

**CD4 (70 mM PPS buffer)**

- Glucose, 20.0 g
- MgSO\(_4\) · 7H\(_2\)O, 0.31 g
- (NH\(_4\))\(_2\)SO\(_4\), 4.0 g
- KH\(_2\)PO\(_4\), 2.911 g
- K\(_2\)HPO\(_4\), 8.467 g
- DI water, 1 L
- 1000x TM, 1 mL/L
- 1000x Vitamins, 1 mL/L

**CD5 (100 mM PPS buffer)**

- Based on CD4, the only modifications are:
- (NH\(_4\))\(_2\)SO\(_4\), 6.0 g
- KH\(_2\)PO\(_4\), 4.158 g
- K\(_2\)HPO\(_4\), 12.069 g
**CD6 (150 mM PPS buffer)**

- Glucose, 20.0 g
- Casamino acids, 4.0 g
- 1000x TM, 4.5 mL/L
- **MgSO₄ • 7H₂O, 0.31 g**
- **KH₂PO₄, 6.3 g**
- **K₂HPO₄, 18.1 g**
- **Yeast Extract, 1.0 g**
- **DI water, 1 L**

**CD7 (150 mM PPS buffer)**

Based on CD6, the only modification is instead of casamino acids, use tryptone 4.0 g/L

**CD8 (150 mM PPS buffer)**

Based on CD6, the only modification is instead of casamino acids, use casein 4.0 g/L

**Production Medium B (150 mM PPS buffer)**

- Glycerol, 14.0 g
- Tryptone, 40.0 g
- 1000x TM, 1 mL/L
- **NaCl, 10.0 g**
- **KH₂PO₄, 6.3 g**
- **K₂HPO₄, 18.1 g**
- **Yeast Extract, 1.0 g**
- **DI water, 1 L**

**PB1-Int (150 mM PPS buffer)**

Modified from Production Medium B, for shaking-flask, only changes are:

- 1000x TM, 4.5 mL/L
- **1000x Vitamins, 1.5 mL/L**
- Tryptone, 4.0 g

**PA1 (150 mM PPS buffer)**

- Glycerol, 14.0 g
- KH₂PO₄, 6.3 g
- 1000x TM, 1.5 mL/L
- **(NH₄)₂SO₄, 4.0 g**
- **K₂HPO₄, 18.1 g**
- **MgSO₄ • 7H₂O, 0.47 g**
- **DI water, 1 L**

**OPMedium (OPM), BTEC propriety**

- NH₄Cl, 0.1%
- Na₂HPO₄, 0.6%
- Glucose, 3 ~ 20 g/L
- Bacto Yeast Extract, 2.5%
- **Tryptic Soy Broth, 1.5%**
- **KH₂PO₄, 0.3%**
PB2 (150 mM PPS buffer), based on PB1 and OPM

Glycerol, 20.0 g  Tryptic Soy Broth, 15.0 g  (NH₄)₂SO₄, 2.0 g
Glucose 3.0  Bacto Yeast Extract, 25.0 g  NaCl, 10.0 g
KH₂PO₄, 6.3 g  K₂HPO₄, 18.1 g  MgSO₄ • 7H₂O, 0.31 g
DI water, 1 L

PM1 (100 mM HEPES buffer), modified from “Production Medium”

Glycerol, 15.0 g  NaCl, 10.0 g  Yeast Extract, 5.0 g
Tryptone, 10.0 g  HEPES, 23.831 g  DI water, 1 L
1000x TM, 5.625 mL/L  1000x Vitamins, 1.875 mL/L

EM1 (100 mM HEPES buffer), “modified from “Enhanced Medium”

Glycerol, 14.0 g  NaCl, 10.0 g  Yeast Extract, 1.0 g
Tryptone, 40.0 g  HEPES, 23.831 g  DI water, 1 L
1000x TM, 5.625 mL/L  1000x Vitamins, 1.875 mL/L

For 2-L Bioreactor Fed-batch study, medium was developed into initial and feed media.

PB1-Int (150 mM PPS buffer)

Glycerol, 14.0 g  NaCl, 10.0 g  Yeast Extract, 1.0 g
Tryptone, 4.0 g  KH₂PO₄, 6.3 g  K₂HPO₄, 18.1 g
1000x TM, 4.5 mL/L  1000x Vitamins, 1.5 mL/L  DI water, 1 L

PB1 Feed (150 mM PPS buffer)

Glycerol, 600 mL/L  Tryptone, 40.0 g  1000x TM, 10 mL/L
1000x Vitamins, 10 mL/L  5X antibiotics level  1X IPTG level
DI water, volume as needed to final 1 L.
HCD (150 mM PPS buffer), modified from CD1 and report

- Glucose, 7.5 g
- MgSO₄ · 7H₂O, 0.47 g
- (NH₄)₂SO₄, 0.6 g
- KH₂PO₄, 6.3 g
- K₂HPO₄, 18.1 g
- DI water, 1 L
- 1000x TM, 4.5 mL/L
- 1000x Vitamins, 1.5 mL/L

HCD feed

- Glucose, 654.0 g
- MgSO₄ · 7H₂O, 12.0 g
- (NH₄)₂SO₄, 6.0 g
- KH₂PO₄, 1.5 g
- DI water, volume as needed to final 1 L
- 1000x TM, 15 mL/L
- 1000x Vitamins, 15 mL/L
- 5X antibiotics level
- 1X IPTG level

F1 (36 mM PPS buffer)

- Glucose, 5.0 g
- MgSO₄ · 7H₂O, 150.5 g
- (NH₄)₂SO₄, 0.4 g
- KH₂PO₄, 1.5 g
- K₂HPO₄, 4.34 g
- DI water, 1 L
- 1000x TM, 1.25 mL/L
- 1000x Vitamins, 1.25 mL/L

F1 feed

- Glucose, 430.0 g
- MgSO₄ · 7H₂O, 3.9 g
- (NH₄)₂SO₄, 110.0 g
- DI water, volume as needed to final 1 L
- 1000x TM, 10 mL/L
- 1000x Vitamins, 10 mL/L
- 1X IPTG level
- D5X antibiotics level
Appendix IV. Major Equipment

Thermos Scientific Revco Revco Ultima II Ultra-Low Upright Freezer (-70°C)
Fisher Scientific Isotemp™ General-Purpose Series Freezer (4°C and -20°C)
Fisher Scientific Isotemp™ Microbiological Incubator
Mettler Toledo AL104 Analytical Balance
Eppendorf® 5418 Microcentrifuge
Thermo Sorvall Legend Mach 1.R Refrigerated Centrifuge
Thermo Scientific GENESYS™ 20 Visible Spectrophotometer
Thermo Scientific Orion™ Versa Star Pro™ pH Benchtop Meter
Orion Research 611 Digital pH/Milivolt Meter
YSI 2900 Biochemistry Analyzer
SteriGARD® SG603A-HE ClassII type A2 Biosafety Cabinet
Sartorius CERTOMAT® BS-1 Programmable Shaking Incubator
INFORS HT Multitron Dual-Stack Incubator-Shaker
Bellco 7744-01010 Orbital Shaker
Sartorius BIOSTAT® B Plus 2-L Benchtop Bioreactor with Controller Station and Cooling System
Cole-Parmer Controlled Environmental Chambers 2398

Category: LABORATORY ACCESSORIES | Subcategory: MISC
Gardco ACCU-LAB Drawdown Machine
Sigma-Aldrich IKA® RV 10 Rotary Evaporator
Agilent 1220 Infinity LC Systems and 1260 Infinity ELSD
Appendix V. Additional Figures

Figure A. Time Course of the Specific Growth Rate and Growth Kinetics of TB3 with PB2 Medium Under Batch Mode Operation
Figure B-I. PB1 Batch Operational History Plot

Final OD\textsubscript{600} at 13 h was 5.28. Without viable cell count, it is unclear whether the death phase started from 12 h, where the DO increased significantly.
Figure B-II. PB2 Batch Operational History Plot

Final OD$_{600}$ at 9.5 h was 27.6. Between 6 ~ 8 h, DO cascade feedback exceeds the upper limit of STIRR. DO was failed to maintain above 30%. Added more Antifoam B and increased the air flow to 1.5 L/min to restore proper DO/STIRR control.
Figure C. Bioreactor Settings and Feed Connections of HCD Fed-batch Process
Figure D-I. Feeding Profile for PB1 500-mL Feed

<table>
<thead>
<tr>
<th>Medium</th>
<th>PB1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Feed time</strong></td>
<td><strong>Start feeding at 2 h, where OD &gt;1.1, glucose depleted</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tube par.</th>
<th>Pump aperture (%)</th>
<th>hh:mm</th>
<th>Flow rate (mL/min)</th>
<th>Step volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STEP 1</td>
<td>100.0%</td>
<td>n/a</td>
<td>6.2</td>
<td>n/a</td>
</tr>
<tr>
<td>STEP 2</td>
<td>2.4</td>
<td>0:01</td>
<td>0.15</td>
<td>0.1</td>
</tr>
<tr>
<td>STEP 3</td>
<td>3.2</td>
<td>15:00</td>
<td>0.20</td>
<td>133.8</td>
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<tr>
<td>STEP 4</td>
<td>3.2</td>
<td>15:01</td>
<td>0.20</td>
<td>106.9</td>
</tr>
<tr>
<td>STEP 5</td>
<td>5.5</td>
<td>24:01</td>
<td>0.34</td>
<td>0.3</td>
</tr>
<tr>
<td>STEP 6</td>
<td>5.5</td>
<td>37:00</td>
<td>0.34</td>
<td>265.6</td>
</tr>
<tr>
<td>STEP 7</td>
<td>0.0</td>
<td>37:01</td>
<td>0.00</td>
<td>0.0</td>
</tr>
<tr>
<td><strong>Sum total (L)</strong></td>
<td><strong>39-h feeding</strong></td>
<td><strong>507.0</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure D-II. History Plot of PB1 Fed-batch Process

The fed-batch operation started with PB1-Int as the initial medium. The initial agitation rate was adjusted to 500 rpm. Aeration rate of 1.5 L/min air supply were maintained. As shown in the history plot of PB1 fed-batch process, the dissolved oxygen was controlled at 30% by feedback agitation rate of between 500 and 950 rpm. Both pH and temperature were stably controlled. The foaming problem was controlled by manual addition of 50% (v/v) Antifoam B during the process as needed. Additional 500-mL feed was added after 50 h.
Figure D-III. Growth Kinetics of PB1 Fed-batch Process
Figure E. Effect of Spreading and Incubation Time on CFU Counting for PST Assay
Figure F-I. Comparison in 6dEB extraction with various solvents at pH 4 and 7

0.389 mM equal 6dEB standards were added in LB medium for extraction by ethyl acetate and resin XAD-16. The efficiency is % efficiency = \( \frac{6 \text{dEB assayed conc.} (\text{mM})}{0.389 \text{ mM}} \times 100 \)