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**Engineering *Ralstonia eutropha* for Production of Isobutanol (IBT) Motor Fuel from
Carbon Dioxide, Hydrogen, and Oxygen
DE-FOA-0000206**

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I. Accomplishments and Milestone Update

The goals and milestones for the entire project of the ARPA-E Electrofuels funding, from the MIT standpoint, center around the *Ralstonia eutropha* strain development and optimization for isobutanol (IBT) production, increase in our knowledge of key pathway enzymes and nodes in biosynthesis of IBT, study and improve product tolerance, develop and utilize metabolic modeling to identify possible cofactor limitations, reduce O₂-induced metabolic inhibition and improve carbon fixation efficiency. Figure 1 illustrates our desired goal for the strain construction portion of the project. The parental strain utilized here produces polyhydroxybutyrate (PHB) as an intracellular carbon storage material during nutrient stress in the presence of excess carbon.

As previous reports stated, we have redirected the excess carbon from PHB storage to the production of IBT and 3-methyl-1-butanol (3MB), both branched-chain alcohols can directly substitute for fossil-based fuels and be employed within the current infrastructure. To achieve *de novo* production of IBT and 3MB, we evaluated various mutant strains of *R. eutropha* with isobutyraldehyde dehydrogenase activity, in combination with the overexpression of plasmid-borne, native branched-chain amino acid biosynthesis pathway genes and the overexpression of heterologous ketoisovalerate decarboxylase gene for the biosynthesis of IBT and 3MB. Study of cellular growth, metabolism, and product production revealed that the branched-chain alcohol production was initiated during either nitrogen or phosphorus limitation in the engineered *R. eutropha*. One engineered strain (Re2401/pJL26) not only produced over 180 mg/L branched-chain alcohols in flask culture, but also was significantly more tolerant of IBT toxicity than wild-type *R. eutropha*. After the elimination of genes encoding three potential carbon sinks (valine-specific transaminase, branched-chain keto acid dehydrogenase complex, and the pyruvate dehydrogenase complex), the production titer improved to 270 mg/L IBT and 40 mg/L 3MB. Semicontinuous flask cultivation was utilized to minimize the toxicity caused by IBT while supplying cells with sufficient nutrients. Under this semicontinuous flask culture, the engineered *R. eutropha* (Re2425/pJL26) grew and produced a normalized total of more than 14 g/Liter fermenter of branched-chain alcohols over the duration of 50 days. These results demonstrated that *R. eutropha* carbon flux can be redirected from PHB to IBT and 3MB, and that engineered *R. eutropha* can be cultivated over prolonged periods of time for product biosynthesis and isolation.

During year three, in order to increase IBT and 3MB productivity in engineered strains, batch, fed-batch, and two-phase fed-batch cultures were carried out. A maximum 380 mg/L of total alcohols production was observed when using 2g/L initial NH₄Cl concentration in batch cultures. A pH-stat control strategy was utilized to investigate the optimum carbon source feeding levels during fed-batch cultures to obtain higher cell density. In cultures of *R. eutropha* strains that did not produce PHB or alcohol, a maximum cell dry weight of 36 g/L was observed using a fed-batch strategy, when 10 g/L carbon source was fed into the culture medium. Finally, 790 mg/L total alcohols were obtained from the engineered strain Re2410/pJL26 in a two-phase fed-batch culture system with pH-stat control. IBT made up over 95% of the total branched-chain alcohols produced in these fermentation studies.

During the final quarter, we continued to validate that the rate-controlling step of *de novo* IBT production is the relatively low activity and the feedback inhibition of the IlvBH (acetohydroxyacid synthase) enzyme complex. While much is already known about branched-chain amino acid production in *Corynebacterium glutamicum* [1-3], a similar pathway exists in *R. eutropha* and we have been characterizing it. In year one and two, we overexpressed the necessary branched-chain amino acid (BCAA) production genes via plasmid-borne gene duplications in *R. eutropha* to help direct the flow of carbon through this portion of the IBT production pathway (see Figure 1 for details). We demonstrated enzyme activity of all BCAA pathway gene products that are necessary for IBT production, and have refined the relative gene expressions for optimal product formation. We also examined the performance of a chromosomally encoded IBT production operon. To further improve IBT production and eliminate the production bottleneck, we identified a carbon flux regulatory enzyme and analyzed various ribosome-binding site sequences to balance the production enzyme

activities.

The metabolic model of *R. eutropha* that has been previously constructed has been extended with the autotrophic and nitrates metabolism of the strain and the branched-chain alcohol production pathway. Simulations based on different scenarios have been carried out in order to identify limiting steps of branched-chain alcohols production in terms of yields and derive solutions to overcome these bottlenecks. We were able to determine the optimized pathway for IBT production, considering only one NADPH-dependent enzyme. It is important to note that with fructose, substrate catabolism is associated with NADPH generation. This is why the IBT production pathway should keep one enzyme that is NADPH-dependent, thereby avoiding a redox imbalance. Transhydrogenase or other enzymes that increase the cellular pool of NADPH are necessary. Alternatively a NADPH-dependent enzyme could be exchanged with a NADH-dependent one.

In year three, we continued to examine mechanisms of IBT tolerance in wild-type and engineered *R. eutropha* strains. Two IBT-tolerant strains, Re2432 and Re2433 (derived from *R. eutropha* strains H16 and Re2051, respectively), were previously isolated using a sequential transfer method and tested for tolerance to various IBT concentrations up to 1.5% (v/v). We were able to identify mutations in several efflux proteins that could contribute to IBT tolerance. We have examined global gene expression throughout the IBT production cycle in heterotrophic cultures, and determined that global gene expression upon entry into IBT biosynthesis is different than that during PHA biosynthesis, even though IBT production is closely related to nutrient limitation. We have also examined the expression of carboxysomes in *R. eutropha* strains and concluded that carboxysomes were expressed in *R. eutropha* and could be utilized to increase carbon fixation efficiency and decrease any O₂-related byproduct formation by RuBisCO, a key enzyme in CO₂ fixation. We have done autotrophic fermentation with formate as the 1-C carbon and energy source and were able to achieve high growth of *R. eutropha* under the studied conditions.

As previously reported, our manuscript detailing our previous strain construction efforts was published in the journal Applied Microbiology and Biotechnology. These findings prompted coverage from mainstream media outlets, such as Scientific American and others (<http://www.scientificamerican.com/article.cfm?id=scientists-engineer-bacteria-to-make-fuel-from-co2>; <http://www.dailybrink.com/?p=4968>; <http://ethanolproducer.com/articles/9071/researchers-tweak-microbe-to-turn-carbon-dioxide-into-isobutanol>). The manuscript on fed-batch fermentation was published in the journal Biomass and Bioenergy. One additional manuscript on carbonic anhydrase in *R. eutropha* has been submitted for review.

Figure 1 - Overall schematic of project goals for construction of an IBT-producing strain of *R. eutropha*. The reactions in red represent the redirection of carbon flux from 2-ketoisovalerate to IBT.

Year 1-3 Milestones and Metrics	Actual Performance
Task 1: Identify and remove IBT enzyme production pathway bottlenecks.	<ul style="list-style-type: none"> we have eliminated four major carbon sinks present in the <i>R. eutropha</i> production strain we evaluated IBT production pathway enzymes production enzyme activity balance is being achieved by overall gene overexpression and engineered ribosome-binding-site optimization synthetic sRNA has been designed and created to eliminate several carbon sinks
Task 2: Identify limiting cofactor and minimize the energy requirement for such cofactor production.	<ul style="list-style-type: none"> metabolic modeling was developed and utilized to evaluate the production strain and its cofactor usage NADPH was determined to be the limiting cofactor during IBT production cellular concentration of NAD^+, NADH, NADP^+, and NADPH were determined transhydrogenase, malic enzyme, and glyceraldehyde-3-phosphate

2. Insertion and expression of a heterologous gene, *kivD* from *Lactococcus lactis*, into *R. eutropha*, and reporting of KivD activity in the resulting strain.
3. Construction of *ilvBHCD* and *kivD* genes in an operon on a plasmid to enhance gene dosage and, consequently, IBT biosynthesis.
4. Demonstrated the value of a plasmid stability system (known as a metabolic addiction system) in which the *proC* gene (for proline biosynthesis) is removed from the *R. eutropha* chromosome and placed *in trans* on an expression vector.
5. Examined branched-chain amino acid pathway enzymes in detail. Methods in which we can alter expression or activity of these genes to affect IBT production have been determined. Allosteric regulation of certain pathway genes has been determined, also.
6. Demonstrated that the constitutive expression of an alcohol dehydrogenase (*adh*) gene, locus tag H16_A0757, is suitable for producing IBT.
7. Construction of a strain, Re2410 (Table 1), which with the *kivD* gene and a second *ilvBHCD* operon added *in trans* (see Table 2 for a list of plasmids), has been shown to produce and secrete IBT. We worked with Re2410 with designs on improving its IBT productivity. Most importantly, we demonstrated *de novo* IBT biosynthesis.
8. Demonstrated initial IBT production.

Year 2

1. Evaluation of IBT efflux systems, which constitutes a native *R. eutropha* transporter. This transporter is unknown, but whole genome microarray analysis provides clues about its potential identity.
2. Demonstrated biofilm formation in *R. eutropha* Δ *wspF* (robust) and in wild-type (to a lesser extent) strains.
3. Increased IBT production from the first year by identification and elimination of three carbon sinks (besides PHB production pathway enzymes already eliminated year 1): valine-specific transaminase, keto acid dehydrogenase, and pyruvate dehydrogenase complex enzymes Identification of carbon sinks in IBT production: We have identified various carbon sinks during IBT production. Carbon sink elimination improved IBT production by ~80%.
4. Evaluated the relevant branched-chain amino acid (BCAA) biosynthesis enzymes in the IBT production strain and demonstrated activity over the course of a 96h culture [4].
5. Demonstrated prolonged >50 days growth and IBT production >14 g/L with the engineered *R. eutropha* strain

Year 3

1. Growth of production strain in bioreactors: We cultivated the engineered IBT-producing *R. eutropha* in batch, fed-batch, and two-phase fed-batch fermentation cultures and achieved production of ~800 mg/L IBT and 3MB. Small-scale growth experiments of *R. eutropha* using CO₂, H₂ and O₂ were performed in coordination with the ARPA-E funded group at the neighboring Wyss Institute for Biologically Inspired Engineering. We demonstrated the autotrophic production of IBT at these small scales.

2. Enhancement of IBT production: We identified and evaluated a global gene expression regulator producer (RelA) in the IBT-production strain for its potential to alleviate nitrogen-associated gene expression suppression, mRNA destabilization, and protein degradation. In addition, we calculated the binding-energy of, and incorporated several ribosome-binding sites in front of the IBT-production pathways genes to improve production. Lastly, we evaluated *R. eutropha*'s codon usage and bias, constructed strains overexpressing rare codons to improve heterologous gene expression.
3. Whole genome microarray analysis on IBT production strain: We sampled biomass from several key time points in heterotrophic IBT-producing cultures to harvest whole cell RNA. We performed the microarray analysis to examine potential alcohol stress gene expression and alcohol production/utilization gene expression.
4. Small synthetic sRNA could be used to regulate gene expression in *R. eutropha* to further maximize carbon flux towards isobutanol production. We demonstrated an initial use of a designed sRNA regulation system in *E. coli*.
5. Improve alcohol tolerance of the production strain: Whole genome microarray analysis has provided us an efflux gene/protein candidate (DctA; locus tag H16_A3590). The full role of this gene in IBT efflux has been identified as a candidate for further study. Experimental evolution was performed on several strains of *R. eutropha*, including engineered IBT-producing strains. We achieved an IBT-tolerant *R. eutropha* strain and were able to identify several efflux proteins that are responsible for such tolerance.
6. Evaluation of *udhA* and *gapN* for IBT Production Strain: The amounts of reducing cofactors were determined in *R. eutropha* wild type and the engineered strain without PHB production enzymes. Transhydrogenase (UdhA) from *E. coli*, Malic enzyme of *R. eutropha*, and GapN (glyceraldehyde-3-phosphate dehydrogenase) were overexpressed and evaluated in *R. eutropha* individually for their ability to increase supply of NADPH pool.

Table 1: List of *R. eutropha* strains constructed and/or used by the Sinskey and Worden groups throughout the entire ARPA-E Electrofuels project.

Strain name	Genotype	Reference
H16	Wild type, Gentamicin resistant, PHB ⁺	ATCC 17699
Re2061	H16 Δ <i>phaCAB</i> (PHB ⁻)	This work
CF17	H16 constitutively expressing <i>adh</i> (H16_A0757)	[5, 6]
CF303	H16 constitutively expressing <i>adh</i> (H16_A0757)	[5, 6]
DJ21	H16 constitutively expressing <i>adh</i> (H16_A0757)	[5, 6]
Re2401	DJ21 Δ <i>phaCAB</i>	This work
Re2402	Re2401 Δ <i>ilvE</i>	This work
Re2403	CF17 Δ <i>phaCAB</i>	This work
Re2407	CF303 Δ <i>phaCAB</i>	This work
Re2408	H16 Δ <i>bkdAB</i>	This work
Re2409	Re2061 Δ <i>bkdAB</i>	This work
Re2410	Re2402 Δ <i>bkdAB</i>	This work
Re2047	H16 Δ <i>fliC</i>	This work
Re2419	H16 Δ <i>cheB</i> (<i>wspF</i>)	This work
Re2425	Re2410 Δ <i>H16_A1753</i>	This work
Re2426	Re2410 Δ <i>cheB2</i> (<i>wspF</i>)	This work
Re2427	H16 Δ <i>can</i>	This work
Re2428	H16 Δ <i>caa</i>	This work
Re2429	H16 Δ <i>can</i> Δ <i>caa</i>	This work
Re2430	H16 Δ <i>cag</i>	This work
Re2431	H16 Δ <i>can</i> Δ <i>cag</i>	This work
Re2437	H16 Δ <i>can2</i>	This work
Re2427_CAN	Re2427/pCAN	This work
Re2428_CAA	Re2428/pCAA	This work
Re2430_CAG	Re2430/pCAG	This work
Re2437_CAN2	Re2437/pCAN2	This work
Re2432	IBT tolerant H16 strain (see below)	This work
Re2433	IBT tolerant Re2061 strain (see below)	This work
Re2434	H16 Δ <i>can</i> Δ <i>caa</i> Δ <i>cag</i>	This work
Re2440	H16 Δ <i>can</i> Δ <i>can2</i> Δ <i>cag</i>	This work
Re2436	H16 Δ <i>can</i> Δ <i>can2</i> Δ <i>caa</i> Δ <i>cag</i>	This work
Re2438	H16 Δ <i>H16_A3357</i> (<i>acrA6</i>)	This work
Re2439	Re2425 Δ <i>H16_A3357</i> (<i>acrA6</i>)	This work
Re2442	H16 Δ <i>H16_A3729</i> (<i>acrA</i>)	This work
Re2443	Re2438 Δ <i>H16_A3729</i> (<i>acrA</i>)	This work
Re2444	Re2439 Δ <i>H16_A3729</i> (<i>acrA</i>)	This work
Re2445	Re2425 Δ <i>H16_A3729</i> (<i>acrA</i>)	This work
Re2435	Re2425:: <i>ilvBHCDkivD</i> (inserted into Δ <i>phaCAB</i> locus)	This work
Re2436_CAN	H16 Δ <i>can</i> Δ <i>can2</i> Δ <i>caa</i> Δ <i>cag</i> /pCAN	This work
Re2436_CAN2	H16 Δ <i>can</i> Δ <i>can2</i> Δ <i>caa</i> Δ <i>cag</i> /pCAN2	This work
Re2436_CAA	H16 Δ <i>can</i> Δ <i>can2</i> Δ <i>caa</i> Δ <i>cag</i> /pCAA	This work
Re2436_CAG	H16 Δ <i>can</i> Δ <i>can2</i> Δ <i>caa</i> Δ <i>cag</i> /pCAG	This work
H16_CAN	H16 /pCAN	This work
H16_CAN2	H16/ pCAN2	This work
H16_CAA	H16/ pCAA	This work
H16_CAG	H16 /pCAG	This work
Turner(DE)	<i>E. coli</i>	Commercial

Table 2: List of plasmids constructed and/or used by the Sinskey and Worden groups throughout the entire ARPA-E Electrofuels project.

Plasmid name	Relevant characteristics	Source/reference
pBBR1MCS-2	Gene expression plasmid for <i>R. eutropha</i> . Uses T4 and T7 promoters flanking MCS.	[7]
pBBR1MCS-C	pBBR1MCS-2 containing <i>proC</i> gene	This work
pJL17	pBBR1MCS-2 expressing <i>udhA</i> from <i>E. coli</i>	This work
pJL18	pBBR1MCS-2 expressing <i>gapN</i> from <i>R. opacus</i>	This work
pJL19	pBBR1MCS-2 expressing <i>maeA</i> from <i>R. eutropha</i>	This work
pKivd1	pBBR1MCS-2 expressing <i>kivD</i> from <i>L. lactis</i>	This work
pJL26	pBBR1MCS-2 expressing <i>ilvBHCD</i> from <i>R. eutropha</i> and <i>kivD</i> from <i>L. lactis</i> in a synthetic operon	This work
pJL27	pBBR1MCS-2 expressing <i>alsS</i> from <i>Bacillus subtilis</i> , <i>ilvCD</i> from <i>R. eutropha</i> and <i>kivD</i> from <i>L. lactis</i> in a synthetic operon	This work
pJL28	pBBR1MCS-2 expressing <i>ilvDBNC</i> from <i>Corynebacterium glutamicum</i> and <i>kivD</i> from <i>L. lactis</i> in a synthetic operon	This work
pJL29	pBBR1MCS-2 expressing <i>ilvBNCD</i> from <i>Corynebacterium glutamicum</i> and <i>kivD</i> from <i>L. lactis</i> in a synthetic operon	This work
pJL30	pBBR1MCS-2 expressing <i>ilvBNCD</i> from <i>Corynebacterium glutamicum</i> and <i>kivD</i> from <i>L. lactis</i> in a synthetic operon; inserted in reverse orientation compared to pJL29	This work
pCan	pBBR1MCS-2 containing <i>can</i> gene (H16 A0169)	This work
pCan2	pBBR1MCS-2 containing <i>can2</i> gene (H16 B2270)	This work
pCaa	pBBR1MCS-2 containing <i>caa</i> gene (H16 B2403)	This work
pCag	pBBR1MCS-2 containing <i>cag</i> gene (H16 A1192)	This work
pCbbLS	pBBR1MCS-2 containing <i>cbbLS</i> genes (H16_B1395, H16_B1394)	This work
pCbbLSX	pBBR1MCS-2 containing <i>cbbLSX</i> genes (H16_B1395, H16_B1394, H16_B1393)	This work
pETCan	pET14b containing <i>can</i> gene (H16 A0169)	This work
pETCan2	pET14b containing <i>can2</i> gene (H16 B2270)	This work
pETCaa	pET14b containing <i>caa</i> gene (H16 B2403)	This work
pETCag	pET14b containing <i>cag</i> gene (H16 A1192)	This work
pStrepCan	pET51b containing <i>can</i> gene (H16 A0169)	This work
pStrepCaa	pET51b containing <i>caa</i> gene (H16 B2403)	This work
pBBR1MCS_prpR_PprpD2	pBBR1MCS-2 containing the <i>Corynebacterium glutamicum prpR</i> gene and the <i>prpD2</i> promoter.	This work
pPilvBHCDkivD	pBBR1MCS_prpR_PprpD2 containing the synthetic IBT production operon genes <i>ilvBHCDkivD</i>	This work
pBBad	pBBR1MCS-2 containing the <i>araC-P_{BAD}</i> arabinose-inducible expression system	[8]
pCarboxy	pBBad containing the carboxysome operon of <i>Halothiobacillus neapolitanus</i> in the MCS	This work
pSG1	pJL26 containing <i>can</i> gene (H16 A0169)	This work
pSG2	pJL26 containing <i>can2</i> gene (H16 B2270)	This work
pSG3	pJL26 containing <i>caa</i> gene (H16 B2403)	This work
pSG4	pJL26 containing <i>cag</i> gene (H16 A1192)	This work
pSG5	pJL26 containing <i>cbbLS</i> genes (H16_B1395, H16_B1394)	This work
pSG6	pJL26 containing <i>cbbLSX</i> genes (H16_B1395, H16_B1394, H16_B1393)	This work
pRARE	Plasmid containing <i>E. coli</i> rare anticodon genes	Novagen

Year 3 Tasks 1-6 results

Task 1: Growth of production strain in bioreactors

1. Heterotrophic cultures

a. Fed-batch cultures for branched-chain alcohols production by Re 2410/pJL26

From the results of the batch cultures in the fermenters described in our previous report, it is necessary to supply more carbon source to maintain the production of branched-chain alcohols. An additional carbon source in fed-batch cultures would presumably be helpful in enhancing the production titer of alcohols. Two culture feeding strategies were performed by adding either 10 or 20 mL of the described feeding solution into two different fermenters to avoid carbon source exhaustion. The Feeding solution was fed into fermenters at 31 h and 55 h during the fed-batch cultivation, respectively. The time-courses of CDW, residual carbon source, and total alcohol production (ROH) for the 72 h fed-batch culture are shown in Figure 2. The CDW reached 10.8 g/L in experiment #2 by adding 20 mL carbon source solution, but with lower overall alcohol production (Figure 2B). Although the highest CDW was more than 10 g/L, alcohols production in experiment #2 was only 120 mg/L. In contrast, the total alcohols production was nearly 450 mg/L in experiment #1 by feeding 10 mL carbon source solution (Figure 2A). The results presented here have demonstrated that fed-batch culture was useful for efficient production of total alcohols in the cultivation of Re 2410/pJL26. However, the maximum CDW in experiment #1 was only 3.1 g/L. These results lend evidence to the conclusion that higher alcohol concentrations will inhibit the cell growth, as suggested from previous flask culture results [9-11]. It needs to be pointed out that higher CDW was achieved with 20 mL feeding strategy, whereas 10 mL feeding strategy resulted in higher alcohol production. The above result indicates simple fed-batch feeding strategy is not the ideal method to enhance both total CDW and alcohol production. Therefore, further research on optimization of the fed-batch fermentation processes would need be carried out for any further increase in cell density without the alcohols inhibitory effect.

Figure 2. Time profile of cell dry weight (CDW), residual carbon source, and total alcohols (ROH) production by Re 2410/pJL26 in fed-batch cultures. The initial concentration of carbon source (fructose) and nitrogen source (NH_4Cl) was 20 g/L and 5 g/L, respectively. Feeding solution was composed of 500 g/L fructose. Feeding volume of 10 mL (in Experiment #1, A) or 20 mL (in Experiment #2, B) was manually fed into two different fermenters when fructose concentration was lower than 10 g/L.

b. Fed-batch culture for high cell density of Re2410/pBBRMCS-2 with pH-stat control

Although higher branched-chain alcohol production was achieved by using fed-batch culture strategies, cell growth is potentially inhibited in fermentations by the presence of increased concentrations of total alcohols. In order to avoid this, a two-phase culture system could be used as a viable alternative solution. In the first phase, cell density would be enhanced to high level without alcohols production, which could then be

accomplished by triggering in the second phase through induction mechanisms. The induction systems for controlling expression of alcohols production genes could be achieved by altering culture medium compositions. Therefore, branched-chain alcohol production would be improved due to the high cell density obtained in the first phase, and growth inhibition by alcohol present in the culture media should be less of a concern in the second phase. In order to mimic the culture condition of the first phase in a two-phase fed-batch culture, a strain Re2410/pBBR1MCS-2 (incapable of producing branched-chain alcohols) was constructed and employed to achieve higher cell density in the absence of alcohol production.

A low nutrient concentration in culture medium compared to the carbon supply will limit cell production. Thus, the fed-batch strategy in that feeding fructose and NH_4Cl during fermentation should be adopted to avoid the inhibitory nutrient concentration levels in culture medium. An unbalanced consumption rate between the carbon source and nitrogen source often causes the accumulation of ammonium ions, which will result in culture pH increase. By using PID control system, certain amount of nutrient solution could be transferred into fermenters automatically without nutrient limitation. To accomplish a high cell density culture of Re2410/pBBR1MCS-2, a pH-stat modal fed-batch culture under different carbon source concentrations with intermittent feeding was carried out. Feeding volume of 10, 20, and 40 mL (corresponding to 10, 20, and 40 g/L fructose, respectively and corresponding to 5, 10, and 15 g/L NH_4Cl) was automatically added to different fermenters respectively, when the pH value became higher than pH 6.85. The maximum CDW of 36 g/L and biomass yield of 0.45 g/g were achieved when 10 g/L fructose was fed in the fed-batch cultures and a biomass productivity of 0.51 g/L/h was obtained. It is obvious that a higher carbon source concentration in the culture medium exhibits an inhibitory effect on cell growth, which was found in the fed-batch cultures feeding 20 or 40 mL nutrient solution into the fermenters. These results indicate that the optimal feeding volume should be controlled at 10 mL during the pH-stat modal fed-batch culture. The time profile of fed-batch culture feeding 10 g/L carbon source can be seen in Figure 2, no carbon source limitation effect was observed during this fed-batch culture. Therefore a pH-stat fed-batch culture with nutrient volume control of 10 mL could be utilized for branched-chain alcohols in a two-phase fed-batch culture system.

Figure 3 Time profile of cell growth of Re2410/pBBR1MCS-2 in the fed-batch cultures with pH-stat control. The initial concentration of carbon source (Fructose) and nitrogen source (NH_4Cl) was 20 g/L and 5 g/L, respectively. Feeding solution was composed of 500 g/L fructose and 150 g/L NH_4Cl . Feeding volume of 10 mL was automatically fed into fermenter when pH was up to 6.85 during the fermentation.

c. Two-phase fed-batch culture for branched-chain alcohol production by Re 2410/pJL26 with pH-stat control

Since branched-chain alcohol production is controlled by the limitation of nitrogen source [20], a two-phase fed-batch culture was performed for high alcohols productivity by adding two feeds with different carbon/nitrogen (C/N) ratios. Nitrogen-excess culture condition was maintained by feeding lower C/N ratio nutrient solution (500 g/L fructose with 150 g/L NH_4Cl) during the first phase culture to facilitate biomass accumulation. When the culture OD_{600} reached 30 at 72h, the first phase feed was replaced by a nutrient solution with a higher C/N ratio (500 g/L fructose with 12.5 g/L NH_4Cl) for the branched-chain alcohol production phase. As shown in Figure 4A, maximum CDW of 21 g/L and OD_{600} of 36 were observed in this two-phase fed-batch cultivation. A maximum total branched-chain alcohol (ROH) of 790 mg/L was then obtained toward the end of the second phase of the two-phase fed-batch culture (Figure 4B). By using pH-stat modal control strategy, 20 times of CDW and 4 times of alcohol production were achieved comparing with the results from flask cultures [11]. These results indicated that both higher CDW and alcohol production could be accomplished in this two-phase fed-batch culture. The compositions of total alcohols obtained in this two-phase fed-batch cultivation were analyzed by gas chromatography (GC) which can be seen in Figure 5. The total branched-chain alcohols were composed of isobutanol and 3-methyl-1-butanol, with over 95% of the total alcohols produced determined to be isobutanol. It is clear in Figure 4 that the amount of total alcohols produced decreased significantly at 168 h when the carbon source was exhausted. These results may be due to the consumption of isobutanol in high cell density conditions, which is in agreement with data collected from our flask cultures [11]. Since cells could utilize the isobutanol as a carbon source, a feeding delay was also observed (Figure 4B). However, isobutanol consumption stopped when carbon source was supplied into the fermenter.

d. Conclusion

Three cultivation modes (batch, fed-batch and two-phase fed-batch culture) were investigated for improving the production of branched-chain alcohols by recombinant *R. eutropha*. For the first time, pH-stat modal two-phase fed-batch cultivation was described for the production of branched-chain alcohols. By controlling the C/N ratio of the feeding solution, a titer of branched-chain alcohols of 790 mg/L and cell dry weight of 21 g/L was achieved with pH-stat modal feeding. The production of branched-chain alcohols was improved by more than 60% comparing with the results of batch cultures. Future efforts could focus on the control of the alcohol inhibition and consumption effects during the cultivation. Further development of other advanced culture systems could be also considered for branched-chain alcohol production, such as membrane cell recycling continuous culture system. In this system, cells would be recovered by a hollow fiber membrane and the culture medium containing alcohol products would pass through the membrane and be collected in containers for alcohol recovery. It should be noted that more than 95% of the total alcohols produced by Re2410/pJL26 were isobutanol, which could provide higher energy density than other advanced drop-in biofuels because of its high octane number.

Figure 4. Time profile of cell growth (A) and total alcohols (B) production by Re2410/pJL26 in a two-phase fed-batch culture. The initial concentration of carbon source (Fructose) and nitrogen source (NH_4Cl) was 20 g/L and 5 g/L, respectively. The first feeding solution composed of 500 g/L fructose with 150 g/L NH_4Cl was used in the first phase culture (0-72h). The second phase feeding solution composed of 500 g/L fructose with 12.5 g/L NH_4Cl was applied at 72h of this two-phase fed-batch culture. Feeding volume of 10 mL was automatically fed into fermenter when pH was up to 6.85 during the fermentation.

Figure 5. The concentration and compositions of branched-chain alcohols produced by Re2410/pJL26 in a two-phase fed-batch culture.

2. Autotrophic cultures

Due to space constraints and safety regulations at MIT (see Section III, Changes in Approach), we formulated alternative plans for growth of *R. eutropha* autotrophic cultures:

- a. anaerobic jar (in collaboration with Wyss Institute of Biologically Inspired Engineering (Harvard))

In collaboration with the neighboring laboratory of Professor Pamela Silver at Harvard Medical School and Dr. Jeffrey Way at the Wyss Institute of Biologically Inspired Engineering (Harvard), we were able to perform autotrophic flask/tube cultures of *R. eutropha* strains in a controlled environment, using jars typically designed for anaerobic cultures. Figure 6 The gas mixture was controlled by pressure measurement using a manometer, and the calculated mixture gives us a gas ratio of $H_2:CO_2:O_2$ (8:1:1). The minimal media cultures without added carbon source, containing different concentrations of ammonium chloride, were used for testing autotrophic growth. The agitation was provided by a stir bar inside the culture flask. The jar containing cultures was incubated at 30°C.

Figure 6. Jars (<http://www.almore.com>) used for the autotrophic growth tests in the Silver laboratory at Harvard Medical School.

Cultures were performed over a 48 h period. In order to extract a sample for the 24 h time point, the jars were opened and fresh gas mixture was injected. Growth of our current IBT production strain (Re2425/pJL26) was tested using two different concentrations of nitrogen (0.5 g/L and 0.25 g/L). Cultures were grown over a 96 h period, and sampled at 48 h. We were able to grow cells that produced biomass and alcohols using these growth methods. The amount of branched chain alcohols produced was low (~5 mg/L), roughly the limit of detection of our GC method at that time.

In short, the autotrophic growth method allowed us to produce biomass and IBT using recombinant *R. eutropha*.

b. Microbioreactor (Pharyx, Inc. Boston) for the Sinskey laboratory at MIT

Another alternative plan to do autotrophic cultures on CO₂, H₂, O₂ was to use microbioreactor technology [12-15] for growth of *R. eutropha* autotrophic cultures at MIT. When working with mixtures of gases including H₂ and O₂, explosion is always a risk, should an ignition source be introduced. Thus, it would be advantageous to work with the gas mixtures in very small volumes. The microbioreactor technology was developed at MIT and currently being produced by Pharyx, Inc. of Boston, MA. We applied for internal (MIT) funding for this purpose as of the beginning of 2013, however the proposal was not approved.

c. Autotrophic fermentations using formate as a safe replacement of CO₂, H₂, O₂ gas mixture.

Awaiting adaption of the fermenter for use with a mixture of H₂, CO₂ and O₂, we tested the autotrophic growth of *R. eutropha* using formate as the carbon and energy source, instead of CO₂ and H₂. The energy and reducing power derived from the oxidation of this substrate are used by the organisms to assimilate CO₂ through the Calvin cycle. Also, the assimilated CO₂ need not necessarily arise from the oxidation of the C₁-compounds. This chemoorganoautotrophic metabolism can thus be considered an unusual type of mixotrophy. It differs from the common mixed substrate mixotrophy in that the organic substrate primarily serves as the energy source [16]. Previously, tests using formate as a carbon source were performed in flask cultures using minimal media and 0.2% (v/v) formic acid.

There are many studies aiming to improve lithoautotrophic growth of *Ralstonia eutropha* and high cell densities up to 25 g dry weight per liter have been reached by carefully investigating the macro- and micronutrient requirements of *R. eutropha* [17]. Growth up to 91.3 g dry weight (CDW) per liter have been reached by developing special agitation systems and adjusting the gas composition [18].

Few studies exist that focus on improving organoautotrophic growth of *R. eutropha* on formic acid. All publications focus either on formate metabolism [19] or on the proteomic examination of *Ralstonia* in response to formic acid [20]. Improving growth conditions with formic acid as the sole energy source was generally neglected and only between 0.8 and 2 g cell dry weight per liter have been reached so far [19, 21, 22].

It was a goal to develop a working fermentation strategy for organoautotrophic growth of *R. eutropha* on formic acid to reach higher cell densities of approximately 25 g cell dry weight per liter. With such a system we could simulate autotrophic growth without the safety issues of “traditional” autotrophic growth on hydrogen and oxygen. We were able to reach an OD₆₀₀ of 17, corresponding to approximately 7 g cell dry weight (CDW) per liter (see Report Y3Q1) with the pH-controlled fed-batch fermentation we developed for organoautotrophic growth on formic acid (pH-stat). To improve upon this, further feeding optimization was needed.

i. Optimization of the feeding solution

We were able to establish a culture strategy resulting in an almost constant level of NH₄Cl by employing a feeding solution with 50 % (w/v) formic acid and 13.5 g/L NH₄Cl. To maintain a constant concentration of formic acid, an additional manual feeding of pH corrected-formic acid was necessary. However the aim was to achieve a completely automated fermentation without the need of a manual control. To support an automated supply of sufficient amounts of formic acid, the feeding solution was optimized.

In order to feed more formic acid with the pH-controlled fermentation, NH₄OH or NH₃(aq) was added to the feed to slightly increase its pH. Therefore more formic acid would be fed to correct the pH increase of the fermentation medium. Furthermore ammonium hydroxide also serves as a nitrogen source. It is advantageous over NH₄Cl, because no Cl⁻ ions are accumulated. The amount of NH₃(aq) was calculated on the basis of the previous nitrogen feed with 13.5 g/L NH₄Cl, which is an amount of nitrogen of 252.4 mM. The new feeding solution thus contained 50% (wt/vol) formic acid and 252.4 mM or 4.3 g/L ammonium hydroxide as NH₃(aq).

The application of the new feeding solution containing 252.4 mM NH₃(aq) instead of NH₄Cl was proven successful. The pH increase from the formic acid feed balanced the basic NH₃(aq). Through careful feeding-design, the basic NH₃ was just enough to neutralize the additional formic acid that was necessary to replenish the consumed substrate. The formic acid concentration as well as the nitrogen concentration in the fermenter maintained could then be maintained at an almost constant level.

Even though it was possible to establish a fermentation in which a constant level of formic acid and nitrogen could be maintained, the maximum OD₆₀₀ reached was still only 17. The growth always ceased at some point, even though enough formic acid and nitrogen were present, which was verified by HPLC and ammonia enzyme assay, respectively. Additionally the growth curves were never exponential, but rather linear, which indicated that the culture was either limited or inhibited by one or more components.

To exclude a nutrient limitation, a new minimal medium derived from Repaske et al. (1976) was tested. No improvement in cell growth could be observed through the periodical addition of nutrients according to Repaske et al. (data not shown). Therefore a limitation of the tested nutrients could also be excluded as the reason for the non-exponential growth of *Ralstonia eutropha* on formic acid.

ii. Effect of different initial concentrations of formic acid

Another reason for the linear growth behavior of *R. eutropha* on formic acid could be an inhibition, due to formic acid itself. Formic acid is toxic to cells since it acts as an uncoupler of the proton electrochemical gradient across the bacterial cell membrane, especially at higher concentrations and with decreasing pH [23]. It has already been shown by Lee et al. (2006) that formic acid shows a concentration-dependent manner of growth inhibition. They have shown that the growth yield of *R. eutropha* on formic acid will be lower at a concentration of 5 g/L compared to 2 g/L formic acid. For this reason the aim was to keep the formic acid initial concentration in the medium below 2 g/L. We decided to test concentrations that are even lower than 2 g/L to see if an inhibitory effect could be relieved.

Three different initial concentrations of (pH corrected) formic acid, 0.5 g/L, 1 g/L and 2 g/L were tested. We

could show that the formic acid concentration in the medium will stay roughly constant when starting with an initial concentration of 2 g/L. Thus we hoped to achieve the same behavior when starting with an even lower initial concentration (0.5 g/L or 1 g/L).

The following conditions were used for the fermentation: pHset: 6.7 ± 0.1 (dead band), airflow: 2 vvm, pO₂ setpoint: 25%, stirrer: 200-1000 rpm (pO₂ cascade). The initial amount of NH₄CL was 1 g/L. The feed contained 50% (wt/vol) formic acid and 4.3 g/L NH₃(aq). Fermenters were inoculated to an initial OD₆₀₀ of 0.3.

Figure 7 - Comparison of different initial concentrations of formic acid (pH 6.5). A) Growth curves (OD₆₀₀) and concentration of formic acid [g/L] in the fermenter for three different initial concentrations of formic acid (pH 6.5): 0.5 g/L, 1 g/L and 2 g/L. B) Ammonium concentration in the fermenter. C) Overall biomass yield for *Ralstonia eutropha* on formic acid [Cmol/Cmol]. All fermentations have been done as duplicates (n=2) and the average values are shown. (pH_{set}: 6.7 ± 0.1 (dead band), airflow: 2 vvm, pO₂ setpoint: 25 %, stirrer: 200-1000 rpm (pO₂ cascade). The initial amount of NH₄CL was 1 g/L. The feed contained 50 % (w/v) formic acid and 4.3 g/L NH₃(aq). Fermenters were inoculated to an initial OD₆₀₀ of 0.3.)

Looking at the growth curves of *R. eutropha* and the formic acid concentration (Figure 7A), one can see huge differences for the growth behavior dependent on the initial amount of formic acid (pH 6.5) in the medium. The maximal growth rates (μ) of the cultures were calculated for the exponential growth phase of the cultures (Table 3). Exponential growth lasted from 12.5 h to 28 h for the fermenters with an initial formic acid concentration of 0.5 g/L and 1 g/L. For the fermenter containing an initial 2 g/L the exponential growth ended at 24 h, since the culture had already reached a 2.5 fold higher OD₆₀₀ at that time point.

The fermenter with the highest initial concentration of formate (2 g/L) showed the highest growth rate of $0.181 \text{ h}^{-1} \pm 0.001$. A reason for this could be the higher availability of the carbon source for the microorganism. Since the free acid molecules can quickly penetrate the cell membrane [23], a concentration-dependent uptake rate of formic acid into the cell can be assumed. Thus a higher initial concentration of formic acid leads to a higher availability of the substrate for the cells.

Table 3 - Growth rates in the exponential growth phase of the culture

Initial conc. of formic acid [g/L]	Exponential growth phase	$\mu \text{ [h}^{-1}\text{]}$
0.5	12.5 h - 28 h	0.152 ± 0.003
1	12.5 h - 28 h	0.131 ± 0.002
2	12.5 h - 24 h	0.181 ± 0.001

However the fermenter with the lowest initial concentration of formic acid (0.5 g/L) had a higher growth rate ($0.152 \text{ h}^{-1} \pm 0.003$) than the fermenter with an intermediate initial formate concentration of 1 g/L ($0.131 \text{ h}^{-1} \pm 0.002$). This result seems to stand in contrast to the theory of a concentration-dependent availability of formic acid for the cell. However when looking at the biomass concentration (OD_{600}) between 12.5 and 28 h (Figure 7 A) both cultures are behaving very similarly.

Comparing the development of the formic acid concentration (Figure 7A) and the nitrogen concentration (Figure 7B) in the medium, one can also observe a very different behavior dependent on the initial concentration of formate (pH 6.5). With an initial formate concentration of 2 g/L, we were able to maintain a relatively constant level of both formic acid and nitrogen in the medium. However when using initial concentration of 1 g/L or 0.5 g/L, the concentrations of formic acid and nitrogen will decrease over the time of the fermentation.

These results show that the pH-controlled feeding of formic acid is dependent on the initial concentration of formate that was added to the medium. The initial addition of formate (pH 6.5) to the medium is necessary to induce the pH controlled feeding system. Upon consumption of the formate, the pH will increase and thus new formic acid will be fed to replenish the carbon supply. The more pH corrected formic acid was added in the beginning, the bigger will be the pH increase and thus more formate will be added. As we can see in Figure 8, much more formic acid was fed within the same time period, when a higher initial formic acid concentration was employed. Within 50 h a total of 74 g formic acid (100 %) was added when starting with an initial concentration of 2 g/L. This is almost twice as much, compared to the fermenter containing an initial amount of 1 g/L pH-corrected formic acid (38.5 g formic acid in 50 h) and even 5.5 fold compared to the fermenter with an initial concentration of 0.5 g/L (13.4 g in 50 h).

Figure 8 - Total amounts of fed formic acid. Three different initial concentrations of pH-corrected formic acid (pH 6.5) were compared: 0.5 g/L, 1 g/L and 2 g/L

The amount of fed formic acid is dependent on the biomass concentration in the medium. The more cells in the reactor, the faster the formic acid is metabolized. Thus the pH will increase at a higher rate and the flow rate of the feed will increase. In order to compare how efficient the cells could metabolize the formic acid to produce biomass, the overall biomass yield (Y_x) was calculated for every time point (Eq. 1 - Eq. 4).

	$Y_x = \frac{X_{total}}{S_{total}}$	Eq. 1
	$X_{total} = CDW(t) * V_{medium}(t) + \sum X_{sample} - X_{initial}$	Eq. 2
	$V(t)_{medium} = V_{initial} + V_{feed, total}(t) - \sum V_{sample}$	Eq. 3
	$S_{total} = S_{feed, total}(t) - S_{medium}(t) - \sum S_{sample}$	Eq. 4
Y_x = biomass yield [g/g] or [Cmol/Cmol], X_{total} = total biomass produced [g], S_{total} = total substrate (formic acid) consumed [g], $CDW(t)$ = cell dry weight at the given time point [g/L], $V_{medium}(t)$ = Volume of the medium at the given time point [L], $\sum X_{sample}$ = Total amount of biomass taken as samples [g], $X_{initial}$ = Initial biomass at $t=0$ [g], $V_{initial}$ = initial volume of the medium [L], $V_{feed, total}(t)$ = Total volume of added feed at the given time point [L], $\sum V_{sample}$ = Total volume taken as samples [L], $S_{feed, total}(t)$ = Total amount of fed formic acid at the given time point [g], $S_{medium}(t)$ = Amount of substrate in the medium at the given time point [g], $\sum S_{sample}$ = Total amount of substrate taken as samples [g]		

The unit used for Y_x was Cmol of biomass (produced) per Cmol of formic acid (consumed). Literally it says how much carbon derived from the substrate (HCOOH, formate) will be converted to the biomass of *R. eutropha*.

Comparing the overall biomass yields (Figure 7C) we can again see a clear concentration-dependent behavior. The lower the initial concentration of formic acid, the higher is the biomass yield. The maximal biomass yield for the medium that contained an initial concentration of 0.5 g/L was 0.16 Cmol/Cmol. For 1 g/L initial formic acid it was 0.15 Cmol/Cmol and for 2 g/L the highest biomass yield was only 0.12.

Looking at the fermentation with an initial concentration of formic acid of 0.5 g/L, one can clearly see that the carbon feed becomes limiting after approximately 30 h (Figure 7A). Formic acid is still fed in the fermenter

(Figure 8) but there is no residual concentration in the bioreactor from 30 h - 42 h. In this time period the highest biomass yield of 0.16 Cmol/Cmol is achieved for this fermentation. This yield is high compared to the literature, where yields between 0.094 and 0.138 were achieved [19, 24].

In general, organoautotrophic growth on formic acid requires a lot of energy and has a very low maximal theoretical yield. As seen on Figure 9, already 9 moles of formic acid (HCOOH) are necessary to produce one mole of 3-phosphoglycerate (C₃H₇O₇P), the product of the Calvin cycle. For this initial step the maximal theoretical yield is only 0.33 Cmol/Cmol.

1)	Formate + NAD ⁺ → CO ₂ + NADH
2)	3 CO ₂ + 8 ATP + 5 NADH → 3-PG + 8 ADP + 5 NAD(P) ⁺
3)	½ O ₂ + NADH + H ⁺ → 2 ATP + H ₂ O
2+3)	9 NADH + 3 CO ₂ + 2 O ₂ + 4 H ⁺ → 3-PG + 8 ADP + 5 NAD ⁺ + 4 H ₂ O
1+2+3)	9 Formate + 4 NAD ⁺ + 2 O ₂ + 4 H ⁺ → 3-PG + 6 CO ₂ + 8 ADP + 4 H ₂ O

Figure 9 - Stoichiometric calculation for the amount of formic acid that is necessary to obtain one molecule of 3-phosphoglycerate (3PG). 1) Reaction catalyzed by formate dehydrogenase 2) Overall equation of the Calvin cycle 3) Equation of the electron transport chain

Further energy and carbon will be lost from 3-phosphoglycerate when producing important metabolites that are necessary for biomass production. This will further decrease the maximal theoretical biomass yield. Therefore a complete metabolic model has been constructed (see. Task 6 for more details), and considering the whole reaction system as described in Annex 1 and 3, for a Y_{ATP,X} of 14 g_X·mole_{ATP}⁻¹, during aerobic growth, the theoretical biomass yield is between 0.18 and 0.20 Cmole·Cmole⁻¹ depending on NADPH generation pathway. This means that experimentally, we were able to reach 80 to 89% of the theoretical yield which was very good performances.

The results of this experiment indicate a concentration-dependent manner of growth inhibition by formic acid. A similar reduction of the growth yield was shown upon an increase of the formic acid concentrations from 2 g/L to 5 g/L by Lee et al. [24].

In terms of a constant supply of formic acid and nitrogen, the fermentation with an initial formic acid concentration of 2 g/L seems to be best choice. However the maximum biomass yield was higher when using lower concentrations of formic acid. Unfortunately it was not possible to maintain a constant level of formic acid when starting with 1 g/L or 0.5 g/L. It could be possible to carefully raise the pH feed of the feeding solution to maintain a constant level of formic acid and nitrogen at a concentration of 1 g/L or even 0.5 g/L. The results of this experiment show that even slight changes of the initial concentration of formic acid have a significant impact on the whole pH-controlled feeding strategy. It is thus very hard to maintain the same baseline conditions for multiple fermentations.

Task 2: Enhancement of IBT production

1. Global regulation via the stringent response

Previously we determined that the production of IBT and 3MB in engineered *R. eutropha* is related to nutrient limitation, similar to the native carbon storage (PHB production). One possibility is that the initiation of PHB accumulation occurs as a result of the stringent response. This process is a mechanism that provides protection against nutrient stress in a wide range of organisms. The stringent response is governed by the

nucleotide guanosine tetraphosphate (ppGpp), which destabilizes the RNA polymerase σ^{70} holoenzyme and thus strongly reduces the transcription levels of genes under the control of σ^{70} or its homologs in other organisms. This inhibition of σ^{70} controlled genes results in a strong induction of genes under the control of alternative σ factors, such as σ^{54} . Additionally, ppGpp has been shown to directly inhibit translation. This process could potentially reduce alternative carbon sinks, thus trigger PHB accumulation. We utilized microarray data and available genome sequence to elucidate the enzyme that controls ppGpp production under stringent conditions. Our results indicated that when RelA (enzyme that produces ppGpp) was eliminated from wild type *R. eutropha*, the cells no longer produce PHB even under stringent conditions with all PHB production enzymes intact. Branched-chain amino acid pathways are tightly regulated via RNA suppression, protein degradation, etc. Such regulations could be initiated by the ppGpp, which is synthesized by RelA during nutrient limitation, specifically nitrogen. We eliminated RelA encoding gene from *R. eutropha* in order to bypass this regulation and examined the resulting strains' ability to produce IBT and 3MB. Engineered strain without *relA* was able to produce IBT at ~300 mg/L and 3 MG at ~60 mg/L, slightly higher than strain without *relA* deletion; however the improvement is not significant and further research is needed to identify and bypass nutrient induced regulation in *R. eutropha* for IBT production.

2. Synthetic ribosome-binding site design and implementation

Low production of IBT and 3MB is largely due to the limitation of branched-chain amino acid pathway regulations and translation of various pathway enzymes. One rationale to address this is to increase the translation efficiency by optimizing the ribosome-binding site (RBS) sequences. The IBT production operon (pJL26) utilized contain the same sequence of RBS for each production genes. We utilized the RBS calculator program (<https://www.denovodna.com/software/forward>, [25]) to determine the RBS sequence for maximum translation efficiency. The determining factor is largely dependent on the translation initiation rate (TIR), which directly correlated with the efficiency of protein translation. As shown in Table 3, the TIR can potentially improve 14 to 123 times for IBT pathway enzymes with low activities (IlvBH, IlvC, and KivD). Therefore a new operon was constructed with synthetic RBSs that could yield the maximum TIR and remains to be evaluated in engineered *R. eutropha* for IBT and 3MB production.

Table 3: Translation initiation rate of RBS sequences calculated for pJL26, pJL41a and the maximum achievable rate with the possible folds of improvement. The IBT pathway enzymatic activity in pJL26 were listed to indicate low activities in IlvBH, IlvC, and KivD.

Gene	TIR (pJL26)	TIF (pJL41a)	TIR (max)	Improvement
ilvBH (pBBR1MCS-2)	--	1,377	169,943	123
ilvC	--	5,557	75,594	14
ilvD	898,412	898,412	3,625,577	4
kivD	36,660	36,660	550,584	15
adhA	--	38,487	212,319	6
Enzyme		Activity in pJL26 (mU/mg)		
IlvBH		9.3		
IlvC		84		
IlvD		832		
KivD		67		
AdhA		239		

3. Engineer codon-optimized *R. eutropha* strain

Condon usage bias is a phenomenon when synonymous codons are used with different frequencies and it is a defining characteristic of each genome. Codon biases vary dramatically between organisms. It is generally accepted that the speed at which ribosomes decode a codons depends on the cellular concentration of the tRNA that recognize it. Also, it is important to note that the most abundant codons pair with the most

abundant tRNAs and vice versa. As a result, gene codon bias strongly correlates with gene expression levels in organism. Relating to our research, when the mRNA of target genes is overexpressed in *R. eutropha*, differences in codon usage can impede translation due to the demand for one or more tRNAs that may be rare or lacking in the population. Consequently, the insufficient tRNAs pools can lead to translational stalling, premature translation termination, translation frame shifting and amino acid misincorporation, which ultimately result in the depression of the target protein synthesis. This is especially true when a gene encodes clusters of numerous rare *R. eutropha* codons.

It is highly desirable to minimize the number of codons that are underrepresented in *R. eutropha* genes. Some of the methods proposed to alleviate codon usage expression issues include the modification of culture conditions such as lowering the temperature or changing media composition. However, based on several studies which reported that the levels of most of the tRNA isoacceptors corresponding to rare codons remain unchanged at different growth rates, we hypothesized that the codon usage-based expression problems can be improved by supplying the limiting amino acid in the culture medium. There is a high probability that by elevating the cognate tRNA levels, the expression yields of proteins whose genes contain rare codons can be remarkably improved. One of the suggested ways to elevate the tRNA levels is by increasing the copy number of the respective tRNA gene. This can be accomplished by inserting the wild type tRNA gene on a multiple copy plasmid. The tRNA gene is either inserted into the expression vector itself or inserted in the genome of the host organism. Here, we focused on the effect of increasing the gene dosage of *kivD* (ketoisovalerate decarboxylase in IBT production pathway) on expression yield, plasmid stability and cell viability.

Among other possibilities to enhance the expression yield of protein are by assembling various combinations of rare tRNA gene to optimize the expression of genes isolated from organisms with genomes that have corresponding codon usage bias. These actually have been tested, for example, in the experiment to overcome the codon bias in *E. coli* [26]. Therefore using the idea of combinations used in the *E. coli* experiment to yield the same result in overcoming the codon bias of *R. eutropha* for enhanced protein expression holds promise.

Task 3: Whole genome microarray analysis on IBT production strain

The third task in year three was to evaluate gene expression in our engineered *R. eutropha* IBT production strain. We have previously performed microarray analysis of *R. eutropha* strains grown on fructose vs. triacylglycerols as a carbon source [27] and throughout the PHB cycle [28]. Strain of Re2425 (DJ21 Δ *phaCAB* Δ *ilvE* Δ *bkdAB* Δ *aceE*) with plasmid of pJL26 (pBBR1MCS-2 with *ilvBHCDkivD*) was chosen for this microarray analysis. (see Figure 10) Procedures and results are detailed in the previous Y3Q2 report. This study confirmed gene expression in our engineered IBT strain has been altered in favor of IBT production (confirmation of the deletion of *phaCAB* in Re2425/pJL26 and that *ilvBHCD* genes expression are constitutive in accordance with the chosen promoter (P_{lac}) in *Ralstonia*). We were also able to identify a gene homologous to a transporter protein with a regulation pattern that suggests involvement in IBT transport from the cell: a putative C4 dicarboxylate transporter gene (*dctA* locus tag H16_A3590) and a major facilitator superfamily (MFS) transporter gene (H16_B2297)

Figure 10. Heat map of overall gene expression changes in *R. eutropha* strain Re2425/pJL26 sampled during growth (16h), nitrogen starvation/early IBT production (26h), peak IBT production (50h), and late IBT production (90h). The blue color indicates low expression levels, the yellow color indicates moderate expression levels and the red color indicates high expression levels. Full discussion of these findings are in the Y3Q2 report.

Some follow-on questions also rose from these data: Upon entry into nitrogen starvation, wild-type *R. eutropha* exhibits massive changes in expression in many groups of genes, including DNA replication, translation, cell division, and motility genes [28]. For several groups of genes, the expression changes of Re2425/pJL26 entering into nitrogen starvation appeared very different from those of wild type. In H16 (WT), expression of several putative nitrogen responsive genes significantly increases upon entry into nitrogen starvation, whereas Re2425/pJL26 shows no change in those genes expression. Also in H16, expression of a putative nitrogen scavenging operon (locus tags H16_A0175 – H16_A0187) is dramatically upregulated during PHB production. During nitrogen starvation for Re2425/pJL26, this increase in gene expression is not seen. These observations suggest a potential change in how carbon flux is regulated in Re2425/pJL26 in the absence of the polyhydroxybutyrate (PHB) biosynthesis stress response. What does this change in response mean for the IBT production strain(s)? One possibility is that IBT production in Re2425/pJL26, and similar strains, is not controlled in the way that PHB biosynthesis is controlled in wild type *R. eutropha*. To determine if this is the case, we have made repeated attempts to construct regulatory mutants of Re2425. It has been demonstrated that the RelA protein, which produces the alarmone ppGpp, plays a prominent role in PHB production in *R. eutropha* H16 [28]. However, multiple attempts at deleting the *relA* gene in strain Re2425 and derivatives to examine the effect of the gene, and moreover the enzyme and its product ppGpp, on IBT production remain inconclusive.

We also examined the expression of a putative operon that is significantly upregulated from growth to late IBT production. This operon contains a putative regulator (H16_A1563), 3-hydroxybutyrate dehydrogenase-

like protein (H16_A1562), hypothetical protein (H16_A1561), and a putative aldolase (H16_A1560). While the exact role of this operon in IBT biosynthesis is still very much a mystery, the observation that the entire gene cluster is upregulated with the same expression pattern is compelling evidence to suggest a role in IBT homeostasis. Potentially, given the presence in the operon of putative aldolase and dehydrogenase genes, this gene cluster could play a role in IBT catabolism.

We continued to examine the role of certain genes in IBT tolerance in *R. eutropha* (see Task 5). It has been documented that lesions in certain genes increase alcohol tolerance in *E. coli*, and lesions in certain *R. eutropha* homologs also bestow IBT tolerance (see previous report). Table 4 lists genes we have studied (and their expression) for their roles in IBT tolerance in *R. eutropha*. Genes H16_A0381, H16_A3378, and H16_A3413 exhibit constitutive expression throughout growth and IBT production. Gene H16_A3378, a *marA* homolog, exhibits only background levels of expression, suggesting the gene may not be expressed at all under the conditions surveyed here. Gene H16_A3357 exhibits a significant increase in expression during transition to nitrogen limitation, but expression levels decrease starting at peak IBT production. Gene H16_A3729, an *acrA* homolog, exhibits a significant increase in expression at peak IBT production, perhaps suggesting a role for this gene product in IBT tolerance.

Table 4: Expression values of genes potentially affecting IBT tolerance in *R. eutropha*.

<i>R. eutropha</i> locus tag	<i>E. coli</i> homolog	Description	Log ₂ gene expression values			
			16h	26h	50h	90h
H16_A3413	<i>marC</i>	Putative Transporter	8.9	7.9	8.4	8.5
H16_A3378	<i>marA</i>	AraC family transcriptional regulator	4.6	4.5	4.4	5.3
H16_A3357	<i>acrA6</i>	Cation/Multidrug efflux system, membrane-fusion component	5.5	6.7	5.5	5.4
H16_A3729	<i>acrA</i>	Acriflavin resistance protein A	7.7	7.5	8.4	8.5
H16_A0381	<i>yhbJ</i>	Hypothetical protein	10.0	10.4	10.7	10.9

A loop of Re2425/pJL26 cells from a single colony grown on TSB agar plate was used for the seed cultures in glass tubes with 5 mL TSB with 10 µg/mL gentamicin on a roller drum at 30°C for 18 h. The seed cultures were harvested and centrifuged at 3500 rpm and then resuspended in sterile 0.85% saline for inoculation into flask cultures. Minimal medium containing 2% fructose and 0.2% NH₄Cl was utilized for the flask cultures in 250-mL flasks. Culture volumes were collected at 16, 26, 42, 50, 68 and 90 h. Total RNA of Re2425/pJL26 was extracted from these samples based on the RNA extraction protocol in our laboratory [27, 28].

To explore the changes in gene expression of Re2425/pJL26 during growth and alcohol production in flask cultures, several sampling times were chosen. The sample times: pre-alcohol production (16 h); early, middle and late stage alcohol production (26, 42, and 50 h, respectively), and late stage (post-) alcohol production (90 h). As shown in Figure 11, alcohol biosynthesis and secretion was observed at 26 h and the highest alcohol concentration (245 mg/L) was observed at 50 h. The maximum OD₆₀₀ of the culture was found at 42 h (8.1±0.31).

Figure 11 Cell growth and total alcohol production by Re2425/pJL26 in flask cultures with minimal culture medium (n=3). The samples prepared for microarray analysis were taken at 16, 26, 50 and 90h (arrows) during the cultures.

The preparation and extraction of RNA samples were completed according to the standard protocol of our lab. Final RNA samples were sent to the MIT BioMicro Center (<http://openwetware.org/wiki/BioMicroCenter>) for further analyses. Total RNA integrity data from *R. eutropha* Re2425/pJL26 samples are listed in Table 5. Concentrations of RNA from each sampling time were in the range from 500~1400 ng/μL. The 260/280 ratio suggests that the samples from this work are in the range of “pure” RNA and the number of RNA integrity (RIN) indicates the RNA samples are acceptable for microarray analysis. Therefore, RNA hybridizations for whole genome microarray analysis were employed using these RNA samples.

Table 5: Results of Prokaryote total RNA Bioanalyzer assay (n=3)

Sample time ^a	260/280 ^b	RNA integrity number (RIN) ^c	Concentration (ng/μL)
16h	2.09±0.03	9.50±0.00	1424.53±729.14
26h	2.09±0.03	8.77±0.45	835.67±536.16
42h	1.78±0.57	8.83±0.12	457.33±315.31
50h	2.09±0.02	8.80±0.10	647.93±315.58
68h	2.13±0.02	8.83±0.06	516.60±255.23
90h	2.07±0.02	8.47±0.21	684.03±251.38

^a The samples prepared for microarray analysis were taken at 16, 26, 50 and 90h during the cultures. The 42h and 68h samples were not used for analysis.

^b A ratio of ~2.0 is generally accepted as “pure” for RNA

^c RIN: A value of 8.5 to 10 is generally accepted as good quality for RNA use in microarray hybridizations.

Triplicate RNA samples were used to hybridize *R. eutropha* gene expression microarray chips (Affymetrix). RNA hybridization and signal quantitation were performed by MIT BioMicro Center (<http://openwetware.org/wiki/BioMicroCenter>) using standard protocols. Statistical analysis was performed as described previously [27, 28] using ArrayStar software (DNASar, Madison, WI, USA). Overall, a maximum of 2409 genes exhibited significant ($p < 0.01$) changes in gene expression when compared to gene expression during cell growth (16h). Figure 10 shows a heat map of the gene expression changes in Re2425/pJL26 that

occur throughout growth and IBT production. As has been demonstrated during PHB production [28], many changes in gene expression occur upon transition from growth into nitrogen starvation. This can be seen by the change in banding pattern of the 26h sample in Figure 10, compared to the 16h sample.

Task 4: Identification of carbon sinks in IBT production

We have identified various carbon sinks during IBT production. The four major sinks identified: PHB production pathway enzymes, valine-specific transaminase, keto acid dehydrogenase, and pyruvate dehydrogenase complex enzymes were all eliminated from the engineered IBT-production strain. Carbon sink elimination improved IBT production by ~80% as explained in reports (year 2)

While working on this task of **Identification of carbon sinks in IBT production**, we have further developed an engineering plan to use sRNA to optimize the flow of carbon around them. This plan, although outside the deliverables for this current project, we believe holds great promise for future work. We therefore present our thinking and design here in some detail.

Fine tuning of expression of genes responsible for carbon sinks in *R. eutropha* through synthetic sRNA
We worked on developing a system using small synthetic sRNA to regulate gene expression in *R. eutropha* to maximize carbon flux towards isobutanol production. The group of Na et al. [29] have just recently shown the successful use of small regulatory RNA for metabolic engineering in *Escherichia coli*. Using synthetic sRNA they were able to produce an engineered *E.coli* strain capable of producing 2 g/L of tyrosine. Furthermore, using a library of 130 synthetic sRNAs they were able to identify chromosomal gene targets to increase cadaverine production up to 55%.

1. General design and function of the synthetic sRNA

The general design and function of the synthetic sRNA is very simple. It consists of a target-sequence and a scaffold-sequence:

- 1) The target sequence of the sRNA is the reverse-complement of the first 24 bp of the target gene. The sRNA will thereby bind to the mRNA of the target gene and repress its translation.
- 2) The second part is a scaffold sequence that is necessary for the binding of the mRNA chaperon Hfq. Hfq facilitates the interaction between the sRNA and the mRNA.

E.coli	MA-KGQSLQDPFLNALRRERVPVSIYLVNGIKLQGQIESFDQFVILLKNTVSQMVKHAI	59
R.eutropha	MSNKGQLLQGPFLNALRKEHVPVSIYLVNGIKLQGNIESFDQYVVLRLNTVTQMVKHAI	60
	*: *** *.*****:*.*****:*****:*.**.*:*****	
E.coli	STVVPSPRPVSHHSNNAGGGTSSNYHHGSSAQNTSAQQDSEETE	102
R.eutropha	STVVPARAVNFRVDDAAEA-----	79
	*****:*.**.:*:.	

Figure 12 - Sequence alignment for Hfq protein from *R. eutropha* and *E. coli*

So far no endogenic sRNA, including the scaffold sequence of these sRNAs, has been annotated for *R. eutropha* yet. However due to the high AA-sequence similarity between the *Ralstonia eutropha* Hfq and the *E. coli* Hfq, we think that the endogenous *R. eutropha* Hfq might be able to recognize *E. coli* sRNA scaffold sequences. Thus we use the same scaffold-sequence that Na, et al. (2013) have used for their synthetic sRNA constructs, which was the most efficient in *E.coli*. The scaffold sequence comes from a natural *E.coli* sRNA called MicC.

If the endogenous *Ralstonia eutropha* Hfq does not recognize the MicC scaffold from *E.coli*, we will coexpress the *E. coli* Hfq on the same plasmid.

Figure 13 - General design principle of the synthetic sRNA

2. Targets for synthetic sRNA mediated repression in the branched-chain amino acid pathway

We have targeted the genes of three different enzymes (**ilvA**, **tdcB** and **panB**) that represent carbon sinks within the branched-chain amino acid pathway used for isobutanol production. By repressing the translation of these genes the carbon flux towards isobutanol and 3-methylbutanol will be increased, thus hopefully increasing the isobutanol and 3-MB production titer and yield.

Figure 14 - Three target enzymes encoded by the genes *ilvA*, *tdcB*, and *panB* have been identified. They represent carbon sinks within the branched-chain amino acid pathway.

The first two targets genes: *tdcB* and *ilvA* encode both for the enzyme threonine dehydratase. It catalyzes the dehydration reaction of threonine to 2-ketobutyrate, the intermediate precursor of L-isoleucine biosynthesis. It has been shown that the deletion of *ilvA* leads to L-isoleucine auxotrophy. However the inducible expression

of the synthetic sRNA against *ilvA* allows for a time controlled repression of this gene in an IBT production phase that is separate from the preceding growth phase. Thus our system is advantageous over traditional knock-out methods.

We test the synthetic sRNA mediated repression of *tdcB* and *ilvA* using an enzymatic assay that determines threonine dehydratase activity, developed by Guillouet et al. [1].

The third target gene *panB* encodes for another carbon sink associated with the branched-chain amino acid pathway. It encodes for the enzyme ketopantoate hydroxymethyltransferase (KPHMT) which converts 2-ketoisovalerate to 2-dehydropantoate, an intermediate precursor of pantothenate production.

Upon repression of the translation of the selected target gene mRNAs we hopefully enhance the carbon flux towards isobutanol and 3-methylbutanol production and thus enhance the yield of biofuel production. If the synthetic sRNA mediated metabolic engineering of *R. eutropha* proves to be successful we can design a plasmid that expresses multiple synthetic sRNAs to target multiple genes at the same time. Then we can potentially design a library of synthetic sRNA against a variety of targets in the metabolic pathway associated with isobutanol and 3-MB production. These targets include enzymes that represent potential carbons sinks, as well as possible regulatory proteins (repressors) in order to enhance the production of enzymes of the isobutanol production pathway.

3. Design of the plasmids carrying the synthetic sRNA

We have constructed a plasmid for the synthetic sRNA that features two different promoters: P_{lac} and P_{BAD} . The P_{lac} promoter is constitutively expressed in *R. eutropha*. Downstream of P_{lac} lays a multiple cloning site (MCS) for the insertion of constitutively expressed genes. We have inserted the isobutanol (IBT)-operon from the plasmid pJL26 with the genes *ilvBHCD* and *kivD* for isobutanol production via the branched-chain amino acid pathway.

We also constructed a plasmid carrying either GFP or RFP in the same MCS. The fluorescent proteins will be employed for initial tests of the sRNA mediated repression of gene expression in *R. eutropha* (see below).

Furthermore the Hfq chaperon of *E. coli* is optionally inserted in front of the IBT operon, GFP or RFP. If the endogenous *R. eutropha* Hfq is not able to mediate the binding of the synthetic sRNA to the mRNA of the target gene, the *E. coli* Hfq might be able mediate this interaction.

Further downstream of the plasmid we have introduced a second, inducible promoter: P_{BAD} for the transcription of the synthetic sRNA. The construct consist of the MicC scaffold sequence from *E. coli* and the transcription terminators (T1/T2). In front of the MicC scaffold two unique restriction sites: SbfI and NheI were inserted for a conveniently simple insertion of a target binding sequence. Thus this construct can be used to construct a library of plasmid with synthetic sRNA against a large number of targets.

The inducible expression of the synthetic sRNA with L-arabinose allows for a time controlled repression of genes that are essential for growth and which could hence not be targeted by conventional knock-out methods. Thus we can down-regulate the expression of the target genes during an IBT production phase, which is separate from the preceding growth phase.

Figure 15 – Original Design (#1) of the plasmid carrying a constitutive promoter (P_{lac}) for GFP/RFP or the IBT operon and an inducible P_{BAD} promoter for the transcription of the synthetic sRNA

4. Initial test of synthetic sRNA mediated repression in *Ralstonia eutropha*

In order to prove the functionality of the synthetic sRNA mediated repression of gene expression we have chosen to target the fluorescent proteins GFP and RFP. They are expressed on a plasmid together with the synthetic sRNA targeting the mRNA of the fluorescent proteins. Upon induction of the P_{BAD} promoter with L-arabinose, synthetic sRNA against GFP or RFP will be transcribed, which should in theory bind to the mRNA of the respective fluorescent protein and hopefully repress its translation. A significant reduction of the fluorescence should be observed.

Before testing the sRNA mediated repression in *Ralstonia eutropha* we have decided to try the system in *Escherichia coli* first, since the functionality of the sRNA mediated repression for this organism has already been shown by Na et al.

The constructed plasmids were transformed into *E.coli* NEB10 cells. The cells were grown in tube cultures (LB medium + 50 $\mu\text{g/mL}$ Kanamycin) overnight till they reached stationary growth. In the “+ara” cultures 0.5 % (w/v) arabinose was added from the beginning of the cultivation (0 h). No arabinose was present in the “-ara” cultures. After approx. 16 h the fluorescence of the cells was measured. Measured intensities were adjusted by subtracting the autofluorescence intensity from cells without a fluorescent protein. Each culture was measured as a duplicate ($n=2$).

Figure 16 - Initial experiment to validate sRNA mediated repression of GFP or RFP expression. The fluorescence intensity of equal amounts of culture broth was measured after 16h. GFP/RFP: psRNA plasmid with GFP or RFP inserted in the MCS behind P_{lac} ; _asGFP or _asRFP: synthetic sRNA with a target binding sequence against GFP or RFP, respectively.

Figure 17 - GFP/RFP: psRNA plasmid with GFP or RFP inserted in the MCS behind P_{lac} ; _asGFP or _asRFP: synthetic sRNA with a target binding sequence against GFP or RFP, respectively. The capital “H” in front of the annotations stands for the Hfq protein from *E.coli*, which is inserted at the beginning of the MCS, upstream of GFP or RFP

For the culture with the plasmid carrying GFP a reduction of the fluorescence can be observed for the arabinose-induced cultures (ara+). However the fluorescence reduction appears both for the plasmid carrying the synthetic sRNA against GFP (GFP_asGFP) as well as for the negative control, carrying the synthetic sRNA against RFP (GFP_asRFP). For the strains with RFP on the plasmid no significant the reduction of the fluorescence in presence of arabinose was observed.

For the strains carrying the *E.coli* Hfq protein on the plasmid in front of RFP or GFP (HGFP_asGFP, HGFP_asRFP, HRFP_asGFP, HRFP_asRFP) no significant results can be observed upon induction with L-arabinose.

5. Redesign of the synthetic sRNA sequence part and test in flask culture with E.coli NEB 10

Since the original design of the plasmid did not work as anticipated, the complete synthetic sRNA part of the psRNA plasmid was revised in order to resemble the functional design used by Na et al. The revised plasmid without GFP or RFP and without a target-binding sequence is called psRNA2.

Compared to the previously published design by Na et al, First, the NheI restriction site between the target binding sequence and the MicC scaffold of the synthetic sRNA has been removed. It is possible that 6 bp gap within the synthetic sRNA hinders the functionality of the synthetic sRNA.

Additional 20 bp upstream of the target binding sequence (including the SbfI restriction site) have been removed in order to move the start of transcription directly to the first base of the target-binding sequence of

the synthetic sRNA. It is possible that the 20bp at the 5' end of the synthetic sRNA were hindering its binding to the target mRNA.

Figure 18 - Redesign of the psRNA plasmid. The NheI and SbfI restriction sites, as well as additional 14 bp in front of the SbfI site have been removed in order to improve the construct design.

After these modifications, the structure of the synthetic sRNA was very similar to the construct from Na et al. In contrast to our construct, Na et al. are using the **P_R promoter**, which is repressible by the **lambda cI repressor** (cI857). Additionally they are using a T1/TE transcription terminator instead of the T1/T2 terminator. However these differences should have no effect on the functionality of the construct.

Figure 19. Comparison of the design of the synthetic sRNA sequence part with the construct from Na et al. (2013)

The new psRNA2 plasmids with the revised synthetic sRNA region were tested in a flask culture experiment. The plasmids were again transformed into *E.coli* NEB10 cells. 250 ml baffled flask were prepared with 25 mL LB+Kan50 and 1mM IPTG. The flasks were inoculated from overnight cultures (16h) with 1% (v/v). They were incubated at 37°C, shaking at 200 rpm. After 2 h, different concentrations of arabinose (0, 0.05, 0.1 0,5 or 1%) were added to the flask to induce the transcription of the synthetic sRNA against GFP or RFP. At 2h, 4h, 6h, 8h and 10h both the optical density (OD₆₀₀) as well as the fluorescence of the cells were measured.

Figure 20. Optical density (OD₆₀₀) after 8 h. The addition of arabinose reduces the final OD₆₀₀ in the stationary growth phase. psRNA2: plasmid without GFP or RFP and without target-binding sequence, GFP2/RFP2: psRNA2 plasmid with GFP or RFP inserted in the MCS behind Plac; _asGFP or _asRFP: synthetic sRNA with a target binding sequence against GFP or RFP, respectively.

Figure 21 - Flask culture experiments for the test of the 2nd synthetic sRNA constructs. After 2 h L-arabinose (0 to 1%) was added to the flasks to induce the transcription of the synthetic sRNA against GFP or RFP. The fluorescence of the cells was normalized by the optical density (FU/OD₆₀₀). GFP2/RFP2: psRNA2 plasmid with GFP or RFP inserted in the MCS behind P_{lac}; _asGFP or _asRFP: synthetic sRNA with a target binding sequence against GFP or RFP, respectively.

The experiment shows that the OD₆₀₀ was influenced by the addition of arabinose (Figure 20). The higher the arabinose concentration (added after 2h), the lower the growth rate and the final OD₆₀₀ in the stationary phase after 8h. Therefore all fluorescence measurements were normalized by dividing by the OD₆₀₀ values.

As one can see in Figure 20, the FU/OD₆₀₀ value is reduced upon addition of arabinose for the plasmids carrying the GFP protein (pGFP2_asGFP and pGFP2_asRFP). However the reduction of the GFP fluorescence can be observed for both the positive control (pGFP2_asGFP) as well as the negative control (pGFP2_asRFP). A slightly stronger fluorescence decrease can be observed at an arabinose concentration of 0.1 % for the strain carrying the plasmid pGFP2_asGFP compared to pGFP2_asRFP, which could indicate a target-specific repression of the gene expression.

Unfortunately no significant target-specific sRNA mediated repression could be observed for the plasmids expressing the RFP protein. The negative control (pRFP2_asGFP) shows the same (weak) repression of the fluorescence as the positive control (pRFP2_asRFP).

6. Second revision of the plasmid construct

We identified two approaches to troubleshoot the non-functional synthetic sRNA plasmid constructs.

The first approach is to exchange the inducible promoter P_{BAD} with the constitutive P_{tac} promoter. Although the design of our construct is almost identical to the construct from Na et al. (2013), no successful repression of the fluorescent protein expression was observed. There is reasonable cause to suspect that the P_{BAD} promoter is not inducing the transcription of the synthetic sRNA upon induction with L-arabinose. In order to exclude this possibility we will exchange the P_{BAD} promoter with the constitutive P_{tac}. We should be able to see a reduced fluorescence for the strains with the sRNA targeting the complementary fluorescent protein mRNA (pGFP3_asGFP and pRFP3_asRFP) compared to the negative controls with non-target specific sRNAs (pGFP3_asRFP and pRFP3_asGFP).

The second approach is the introduction of a second terminator region behind the end of the *araC* regulator sequence (the *araC* gene lies in reverse direction). The arabinose operon contains two promoter: P_{BAD} and P_c. The L-arabinose inducible P_{bad} promoter is necessary for the transcription of the synthetic sRNA. A second promoter, P_c that lies upstream of P_{bad} and which is facing in the reverse direction is responsible for the transcription of the *araC* regulator protein. However downstream of *araC* (relative to the transcription initiated by P_c) is the MCS of the plasmid in which the GFP or RFP genes are inserted (in forward direction). Thus it is possible that the complete *antisense* mRNA of the GFP and RFP genes are transcribed at the 3' end of the *araC* mRNA. It is possible that the reverse complementary mRNA of the GFP or RFP genes (initiated by P_c) binds to the regularly transcribed mRNA (initiated by P_{LAC}) and thus reduced its translation.

Figure 22 - The P_c promoter initiates the transcription of mRNA that is reverse complementary to the mRNA transcribed behind P_{Lac}. In order to prevent the formation of a antiGFP/antiRFP-mRNA, we will insert a terminator sequence behind the *araC* gene.

It has been shown that upon L-arabinose addition, the activity of P_c increases up to 10-fold for about 10 minutes [30]. Although this is a much weaker increase compared to the 300-fold increased transcription behind P_{bad} , it could still be sufficient to explain the reduced GFP/ RFP fluorescence due to the antisense-mRNA formation initiated by P_c upon addition of L-arabinose.

To prevent the formation of the reverse complementary mRNA of GFP or RFP a terminator region behind the *araC* gene would need to be introduced.

Task 5: Improve Alcohol Tolerance of Production Strain

1. Experimental Evolution

In order to obtain *R. eutropha* strains able to survive in the presence of increased IBT concentrations, H16 and Re2061 were subjected to an experimental evolution experiment. An experiment of this type began with the inoculation of those strains to a media containing an initial concentration of IBT that would not significantly disrupt cell growth or survival (0.5% (v.v⁻¹) alcohol). *R. eutropha* cells were subcultured every day into a fresh media containing an equal or higher IBT concentration. The extracellular IBT concentration was increased at regular intervals, every 15 days, helping to facilitate the selection of strains that evolved and became able to survive in the presence of IBT concentrations higher than 0.5% (v/v). After 75 days, *R. eutropha* strains were obtained capable of growing on culture media containing up to 2.5% IBT (v.v⁻¹).

2. Evaluation of isobutanol tolerance in evolved strains

The improvement in tolerance on strains Re2432 and Re2433 from H16 and Re2061 respectively was measured by two different tolerance assays: The first evaluates the difference in growth curves under increasing IBT concentrations. Therefore, the assay will show if the strains will still be able to grow once IBT biosynthesis starts. In the second assay, the amount of cells (in CFU/mL) capable of survival after incubation in the presence of different concentrations of IBT is determined, and the ability of strains to survive when in contact with IBT is evaluated.

In all cases, biomass production decreases significantly as extracellular IBT concentration increases. However, strain Re2433 exhibits more robust growth in 0.5% and 1.0% IBT (v v⁻¹) when compared to its parental strain Re2061 (Figure 23). No improvement is seen for the strain Re2432 compared to its parent strain H16. When analyzing the tolerance of the evolved strains by determination of viable cell counts (in CFU/mL) after IBT exposure, the improvement of cell survival in the evolved strains is clearly noticed compared to H16, especially at higher concentrations of IBT (1.75% and 2.0% (v v⁻¹)) (Figure 24).

Figure 23. Growth of Re2432 and Re2433 and respective parental strains H16 and Re2061 A) 0%, B) 0.5%, C) 1%, D) 1.5% IBT (v v⁻¹).

Figure 24. Tolerance of *R. eutropha* (H16) and evolved strains (Re2432 and Re2433) in the presence of IBT. A) Strains were exposed for 12 h to different concentrations of IBT (1%, 1.25%, 1.5%, 1.75% and 2% (v v⁻¹)) in TSB media. Tolerance was determined in terms of CFU/mL of culture. B) Example of TSB plates containing serial dilutions of H16, Re2432 and Re2433 used for counting. Average values from three experiments were plotted with error bars representing the standard deviation.

3. Identification of genes mutation in evolved strains

Previous works using genome sequencing, followed by gene expression studies, in different alcohol tolerant *E. coli* strains identified several genetic lesions in the genome. Mutations such as *acrA(B)*, *gatY*, *tnaA*, *yhbJ*, *marC(RAB)*, *hfq*, *mdh*, *gatYZABCD*, and *rph* were described as being primarily responsible for increased IBT tolerance. These findings are consistent with the complex, multigenic nature of solvent tolerance, and, in general, the genotypic adaptations observed suggest mechanisms of adaptation to alcohol stress. An analysis of the evolved strains obtained in this study was also performed in order to screen for mutations that are related to IBT tolerance. Based on those previous studies of IBT tolerance in *E. coli*, we selected the 5 genes that had mutations identified in both prior studies and that had homologues with similar function present in *R. eutropha*, in order to screen for genetic lesions (Table 6).

Table 6 Potential genes related to IBT tolerance and their respective location and description.

<i>E.coli</i> gene	<i>R. eutropha</i> locus tag	Description
<i>marC</i>	H16_A3413	Putative transporter
<i>marA</i>	H16_A3378	AraC family transcriptional regulator
<i>acrA6</i>	H16_A3357	Cation/multidrug efflux system, membrane-fusion component
<i>acrA</i>	H16_A3729	Acriflavin resistance protein A
<i>yhbJ</i>	H16_A0381	Hypothetical protein

The five genes were amplified by PCR, using genomic DNA from Re2432, Re2433, and their parental strains (H16 and Re2061) as templates. Results were analyzed by agarose gel electrophoresis and gene sequencing, which identified genes that acquired mutations during experimental evolution. Two such genes incurring lesions during the experimental evolution process, *acrA* (H16_A3729) and *acrA6* (H16_A3357), are putatively part of a multidrug efflux transport system, which is described as being responsible for the protection of cells against various stresses, including antibiotics and organic solvents. An inactivation of the components of these efflux systems has previously been shown to increase IBT tolerance in *E. coli*.

4. Study of deletion mutants of identified genes

In-frame deletions of genes H16_A3357 and H16_A3729 (*R. eutropha* homologous for *acrA6* and *acrA*, respectively) were created in wild-type (H16) and IBT production strains (Re2425) of *R. eutropha*, and the resulting strains were evaluated in terms of ability to grow in the presence of IBT, ability to survive in elevated IBT concentrations, IBT production and IBT consumption.

a. Isobutanol tolerance in engineered strains

The AcrAB-TolC multidrug efflux system is an important mechanism of tolerance, protecting cells against various stresses, including antibiotics and organic solvents, by exporting these substances outside of the cell. It is composed of a transporter (AcrB), a periplasmic accessory protein (AcrA) and an outer membrane protein (TolC). Various studies showed that the deletion of *acrA* increased cell susceptibility to different solvents. However other studies illustrate a different response for IBT stress: the presence of extracellular IBT causes quinone depletion, which significantly induces *acrAB-tolC* transcription, leading to an increase in quinone flux leading to a decrease in activity for enzymes that utilize it for their electron-carrier capability. Therefore, deletion of AcrAB-TolC units could increase IBT tolerance by reducing quinone depletion as was seen previously in *E. coli* (Atsumi, et al., 2010b).

IBT tolerance was tested in engineered strains using the same methods described for evolved strains, but

using different IBT concentrations. Our study has shown that this mechanism might be similar in *R. eutropha* response to IBT stress. The deletion of H16_A3357 and H16_A3729 genes, separately or combined, caused an increase in the number of cells able to survive after 12 h under exposure to high concentrations of IBT (> 0.5%) , in both wild-type (H16) and Re2425 strain backgrounds. Growth assay results indicate no significant difference in growth between parental and engineered strains (Figure 25). However, the exposure assay shows that all engineered strains exhibited greater survivability in the presence of IBT (Figure 26). Similar results were observed in the evolved strain Re2432, which showed the highest number of viable cells surviving in the presence of 1.75% and 2.0% IBT ($v v^{-1}$), but exhibited no improvement in growth using IBT as carbon source when compared to its parental strain (H16), even though Re2432 came from a selected population that was able to grow in the presence of IBT.

Figure 25. Growth of engineered strains Re2438 (H16 Δ H16_A3357 (*acrA6*)), Re2442 (H16 Δ H16_A3729 (*acrA*)), Re2443 (Re2438 Δ H16_A3729 (*acrA*)), with their parental strain H16 growing in different IBT concentrations on minimal media with 2% (w v⁻¹) fructose: A) 0% IBT, B) 0.5% IBT C) 1% IBT (v v⁻¹). Growth of Re2425 (DJ21 Δ phaCAB Δ ilvE Δ bkdAB Δ H16_A1753) and its derivative engineered strains Re2439 (Re2425 Δ H16_A3357 (*acrA6*)), Re2444 (Re2425 Δ H16_A3357 (*acrA6*) Δ H16_A3729 (*acrA*)) and Re2445 (Re2425 Δ H16_A3729 (*acrA*)) in the presence of different IBT concentrations on minimal media with 2% (w v⁻¹) fructose: D) 0% IBT, E) 0.5% IBT F) 1% IBT (v v⁻¹). Graphs show no significant difference between engineered and parental strains. Average values from three experiments were plotted with error bars representing the standard deviation.

Figure 26. Viable cell counts of H16, Re2438, Re2442, Re2443, Re2425, Re2439, Re2444, Re2445 showing survival after exposure to A) 0.5%, B) 1.0%, C) 1.5%, D) 2.0% IBT (v/v^{-1}) for a duration of 12 h. Average values from three experiments were plotted with error bars representing the standard deviation.

b. Isobutanol production by engineered strains

The *kivD* gene from *L. lactis* and *ilvBHCD* genes from the valine biosynthesis pathway in *R. eutropha* H16 were overexpressed on plasmid pJL26 (Table 2), that was later inserted in all *R. eutropha* engineered strains derived from Re2425 (Re2439, Re2444, Re2445) as well as in strains Re2425 and Re2405, (Table 1). Those strains containing pJL26 were cultivated in minimal media with 2% fructose and 0.05% NH_4Cl . The IBT produced by these strains was measured by gas chromatography, showing that strain Re2445 is able to produce a great amount of IBT, even higher than the amount produced by our control strains (Figure 27A).

Figure 27. A) Isobutanol and B) 3MB productions in mg/L by parental and engineered production strains Re2405, Re2425, Re2439, Re2444 and Re2445 containing plasmid pJL26 during 72 h. Strain Re2445 show elevated IBT production when compared with either parental as other engineered strains.

c. Isobutanol consumption by engineered strains

One aspect to be considered when choosing a suitable strain for IBT production is its ability to consume product, which is incompatible with large scale production. In order to evaluate this IBT consumption, strains (Re2425, Re2439, Re2444 and Re2445) were cultivated in minimal media containing only IBT as carbon source. Results showed that all tested strains were able to grow using IBT as a carbon source, illustrating that deletion of *acrA* and/or *acrA6* did not affect this characteristic.

These experiments suggest that AcrA in *R. eutropha* has the same mechanism as in *E. coli*, and same stress response to IBT in *R. eutropha*, and that once it is deleted, lead to similar results in increased IBT tolerance. Deletion of *acrA* resulted in an increase of up to 18.75E+5 CFU/mL in 2% (v/v) IBT. This study also confirmed that the improved tolerance leads to an increase in IBT production from 76.2 mg/L to 231.9 mg/L. Further studies must be performed to understand the physiological role of AcrA in *R. eutropha* in order to optimize tolerance in production strains. It is worth mentioning that a further study involving another regulatory system, the *marCRAB* operon, is also important to the understanding of IBT tolerance in *R. eutropha*. In similar studies with *E. coli*, the best results in growth improvement in the presence of IBT were achieved when an *acrA* deletion was combined with deletion of *marCRAB*, which decreases *AcrAB-TolC* expression. However, in *R. eutropha*, *marCRAB* is not located in a single operon, and there are >13 potential homologues, thus providing a significant challenge in determining its role in *R. eutropha*.

Task 6: Evaluation of *udhA* and *gapN* for IBT Production Strain

Work on this task started with a study of the intracellular redox levels and their effect on metabolic fluxes. The principal intracellular anabolic reductant is NADPH. This coenzyme is necessary for two enzymes of the IBT production pathway: the KARI (see Figure 1) and the ADH (as we have determined in our alcohol dehydrogenase studies, see previous reports).

A combined approach of stoichiometric modelling and strain engineering has been developed to assess intracellular redox levels and their effect on metabolic yields and fluxes:

1. Summary of metabolic modeling results

The metabolic model of *R. eutropha* has been previously constructed considering heterotrophic metabolism (4 carbon sources: Glucose, Butyric acid, Propionic acid, Acetic Acid) and one electron acceptor (O₂) to produce biomass and PHA (PHB and PHB-co-HV) [31]. For this project of Isobutanol production, the autotrophic and nitrates metabolism of the strain and the alcohols production pathway (see Figure 1) have been implemented (See Annex 1 to 4 for the complete list of reactions used). Simulations based on different scenario have been carried out in order to identify limiting steps of alcohols production in term of yields and to propose solutions to overcome the bottlenecks by choosing the best combination of genes to introduce in *R. eutropha*.

More than 10 scenarios were considered. Simulation results showed that considering the actual coenzyme for the enzyme of the IBT production pathway, NADPH was limiting. Two kinds of solutions can be considered:

- the demand for NADPH should be reduced by changing the IBT pathway enzyme from NADPH to NADH-dependent; but for an efficient strain on fructose one enzyme should be kept NADPH-dependent since fructose catabolism is associated with NADPH generation. For autotrophic Isobutanol production, the whole pathway can be NADH dependent to increase yields.
- or the NADPH generation should be increased via the expression of a transhydrogenase like *UdhA* from *E. coli* or with a non-phosphorylating glyceraldehyde 3-phosphate dehydrogenase (*GapN*) from *Rhodococcus opacus*. But with *GapN*, the generation of an additional NADPH is done at the expense of one ATP and NADH, leading to more important risk of ATP limitation of the strain.

2. Summary of evaluation of in vivo intracellular redox levels and effects of *udhA*, *gapN* and *maeA* genes expression in *R. eutropha*

As demonstrated with the stoichiometric modeling, an additional source of NADPH in the cell might alleviate the pathway constraints. We have inserted the *gapN*, *udhA*, and *maeA* genes separately into pBBR1MCS-2 for expression in *R. eutropha* and enzymatically quantified the intracellular redox cofactor pool (cofactor cycling assay see previous report).

While the presence of an empty expression plasmid had no effect on the shifting of the reducing equivalent between NADH and NADPH, the expression of *udhA*, *gapN*, and *maeA* genes in *R. eutropha* increased the pool of NADPH and at the same time lowered concentration of NADH. These observations represent the first demonstration of the enzymatic activity of *GapN*, and suggest that we can supply additional anabolic reducing cofactor NADPH, using any of the three enzymes, if this cofactor became limiting in the IBT production strain.

Nevertheless, according to the metabolic modeling, the focus should be on *udhA* utilization to increase NADPH pool for IBT production.

For the intracellular redox levels and their effect on metabolic fluxes, the principal intracellular anabolic reductant is NADPH. As we have determined in our alcohol dehydrogenase studies, this cofactor plays an important role in the isobutanol production pathway. The effect of having an additional source of NADPH in the cell might alleviate the pathway constraints. The main source of NADPH in the cell is the pentose phosphate pathway. Since glucose-6-phosphate is a flexible node, change of flux through glucose-6-phosphate dehydrogenase can be difficult to control on an enzymatic level. Additionally, *R. eutropha* has no active 6-phosphobluconate dehydrogenase, thus harbors an incomplete pentose phosphate pathway. Some of the alternative NADPH generation strategies are listed below. A soluble pyridine nucleotide transhydrogenase (UdhA) from *E. coli* [32] has been expressed in *R. eutropha*. High levels of UdhA can transfer reducing equivalents between NADH and NADPH, thus increase NADPH availability. Non-phosphorylating glyceraldehyde 3-phosphate dehydrogenase (GapN) from *Rhodococcus opacus* bypasses 1,3-bisphospho-D-glycerate in glycolysis and generates an additional NADPH at the expense of one ATP and NADH. GapN has also been studied in *R. eutropha* for its effect on additional reductant availability. Malic enzyme catalyzes the conversion of malate to pyruvate and is part of a metabolic shunt that also includes pyruvate carboxylase and malate dehydrogenase. NADPH is produced in this shunt with an expense of one ATP and NADH as well. Malic enzyme can be overexpressed in *R. eutropha* to increase cellular reductant level. We have inserted *gapN*, *udhA*, and *maeA* into pBBR1MCS-2 for expression in *R. eutropha* and enzymatically quantified the intracellular redox cofactor pool.

A cofactor cycling assay was employed to measure the amount of intracellular NAD^+ , NADH, NADP^+ , and NADPH. As shown in Figure 28, substrate ethanol or glucose-6-phosphate was used with its corresponding oxidizing enzyme alcohol dehydrogenase or glucose-6-phosphate dehydrogenase. The intracellular NAD^+ , NADH, NADP^+ , and NADPH were isolated separately and added to this assay. The reducing equivalent is cycled through phenazine ethosulfate to form a reduced demethylthiazole diphenyltetrazolium and was detected at 570nm. The reaction rate was fitted to standard curve of known concentrations of these cofactors.

Figure 28. Schematic of the cofactor cycling assay. A: assay for detection of NAD^+ and NADH. B: detection of NADP^+ and NADPH.

Figure 29 summarizes the amount of redox cofactor detected via the cofactor cycling assay. Strain H16/pBBR1MCS-2 and Re2061/pBBR1MCS-2 are controls in which empty pBBR1MCS-2 plasmid is inserted in wild type *R. eutropha* H16 and H16Δ*phaCAB*. While the empty expression plasmid had no affect on shifting the reducing equivalent between NADH and NADPH, expression of *udhA*, *gapN*, and *maeA* in *R. eutropha* increased the pool of NADPH and at the same time lowered concentration of NADH. This demonstrated that we could supply in the IBT production strain additional anabolic reducing cofactor NADPH as needed.

Figure 29. Intracellular concentration of NAD⁺, NADH, NADP⁺, and NADPH in pM per CFU. Error bar indicates standard deviation with n=3.

The Worden laboratory at Michigan State University has constructed and is testing a unique Bioreactor for Incompatible Gases (BIG) and an integrated IBT recovery system (see below). In the fourth quarter of the third year, MSU team members dedicated their efforts to mathematical simulation of the system performance to identify the most crucial operational conditions, identifying the rate-limiting factors in autotrophic production of IBT and quantitating the rate of IBT catabolism.

Milestones and Metrics	Actual Performance
<p>Q12 Milestones</p> <p>Performance of BIG system measured during continuous run with alternating heterotrophic growth on fructose and autotrophic product formation on H₂, CO₂, and O₂.</p> <p>Mathematical model used to analyze and optimize system performance during continuous operation.</p> <p>Measure performance of column packed with resin having high affinity for IBT during adsorption.</p> <p>Characterize the mass transfer of IBT across hollow fiber membrane</p> <p>Use mathematical model to predict system performance for scaled-up BIG system</p>	<p>We have demonstrated both heterotrophic and autotrophic production of IBT in BIG system during continuous operation with heterotrophic and autotrophic stages.</p> <p>An unsteady-state structured model has been developed to account for changes in cells' physiological state and describe cell growth under both autotrophic and heterotrophic growth conditions as well as product formation (see Y2Q4 progress report). We are now incorporating the structured kinetic model with the mass transfer model (see Y2Q1 and Y2Q2 progress reports)</p> <p>The performance of the adsorption column for IBT recovery from the gas phase has been measured, and IBT desorption using methanol has been demonstrated.</p> <p>The apparent diffusion coefficient of IBT in the spongy layer of XM-50 hollow fiber membranes has been measured at various lumen liquid flow rates. The experiment is simulated in COMSOL to validate the results</p> <p>A COMSOL model has been constructed to simulate BIG system performance in both batch and continuous mode.</p>

Year 1, 2 and 3 Tasks Completed.

In Year 1 of the project, we designed, assembled the BIG system, and obtained the hardware required for the Opto22-based control network. By acquiring a novel microbubble generator design, the need to use potentially toxic surfactants was eliminated. We also selected the hollow fiber material suitable for the application and constructed small scale hollow fiber reactors to measure the growth rate of immobilized cells. In Year 2 of the project, we completed the cross-platform control network, measured the cell growth rate and substrate mass transfer rate in the hollow fiber membrane. Several IBT recovery strategies were explored and resin adsorption was determined to be optimal for lab scale operations. Mathematical models were constructed to help understand the cellular kinetics, mass transfer in the hollow fiber and adsorption process in the resin column.

Year 1

- Detailed process flow chart for BIG system developed
- Components (Opto22 control hardware, Sartorius fermentation system, Bruker gas chromatograph, gas sensors) for the BIG system ordered and assembled
- A variety of commercially available hollow fiber membranes compared with regard to their pore sizes, affinity to cells and interference to cell viability assays
- Small scale hollow fiber reactor constructed in-house and tested with heterotrophic growth of immobilized *R. eutropha*
- Microbubble generator designed, constructed and performance validated

Year 2

- Procedure for hollow fiber reactor inoculation and cleaning established
- Membrane diffusivity of O₂ and CO₂ measured
- Opto22 based control network constructed and communications across Sartorius fermentation system and Bruker gas chromatograph established via OPC protocol
- Gas stripping and resin adsorption both explored as potential IBT recovery approach
- IBT adsorption capacity of resin column measured, mathematical model built to describe the adsorption process
- MATLAB model constructed to describe the mass transfer of heterotrophic and autotrophic substrates
- A structured kinetic model constructed to describe the competition between cell mass generation and IBT production
- The reactor scaled up from single fiber to a membrane area of 180 cm² and further to 1 ft²

Year 3 Tasks

Task 1: Characterizing IBT mass transfer across the membrane

Experiments were conducted to measure the IBT diffusion coefficient in the BIG spongy layer. The experimental system is shown in Figure 30.

Figure 30. System configuration for measuring IBT apparent diffusion coefficient across the XM-50 membrane

The experimental system consisted of a hollow fiber module (1018-1.0-45-XM50 hollow fiber reactor) and a 200 mL reservoir that acted as a CSTR and contained an IBT aqueous solution. The IBT solution had an initial concentration, C_0 , of 5g/L (0.068M), and was continuously recycled through the fiber lumen at a flow rate of 80 mL/min. Since the water is not recycled, the driving force for IBT diffusion is only a function of IBT concentration in the lumen side. Under these conditions, an unsteady-state mass balance on IBT in the lumen predicts that a plot of the natural log of IBT concentration (C_{IBT}) vs. time (t), should be linear with a slope that would allow the apparent diffusion coefficient (D_{app}) to be calculated using the membrane area (A), total liquid volume (V) and membrane thickness (Δr), as shown in Equation 1.

$$\ln C_{IBT} = -\frac{AD_M}{V\Delta r}t + \text{constant} \quad \text{Equation (1)}$$

Experimental data were collected with an initial IBT concentration of 5 g/L and liquid flow rate of 80 mL/min on both the lumen and shell sides. IBT sample from the inlet and outlet of the lumen, and the outlet of the shell, were taken at several times: 0min, 15min, 30min, 45min, 60min and 90min. This experiment was run in duplicate. The samples were analyzed using a Bruker 450-GC gas chromatograph. The experimental data were compared to a mathematical model that was solved using COMSOL Multiphysics, and the results are being used to validate the mathematical model.

The results (Figure 31) showed a high degree of linearity ($R^2=0.994$), and gave a D_{app} value of 3.09×10^{-6}

cm²/s. The experiment was repeated for various lumen flow rates ranging from 22 mL/min to 184 mL/min (Figure 30). Flow rates higher than 132 mL/min did not seem to have a significant impact on the D.

The observed dependence of D_{app} on liquid flow rate is typically explained using liquid film theory. For a solute to diffuse from the well mixed bulk fluid into the membrane, it has to travel through a stagnant liquid film, whose thickness is affected by the local Reynolds number. The total mass transfer resistance from lumen to shell consists of the resistance from the stagnant liquid film and the membrane itself, as described in Equation 2, where l is the thickness of the membrane, $D_{membrane}$ is diffusion coefficient of IBT in the membrane, and k is the mass transfer coefficient of IBT in the stagnant liquid film. Since the flow path around the fibers in the shell side is complex, we assumed that the liquid is well mixed, with negligible liquid film resistance.

$$\frac{l}{D_{app}} = \frac{l}{D_{membrane}} + \frac{1}{k} \quad (2)$$

During laminar flow through the lumen, k is expected to vary with the flow velocity (v_0) as shown in Equation 3 [33], where Sh is Sherwood number, d is the diameter of the tube, D is the solute's diffusivity in water and L is the fiber length.

$$Sh = \frac{kd}{D} = 1.62 \left(\frac{d^2 v_0}{LD} \right)^{1/3} \quad (3)$$

Equations 2 and 3 can be combined and rearranged to obtain Equation 4, which expresses D_{app} as a function of v_0 .

$$D_{app} = \frac{l}{\frac{l}{D_{membrane}} + \frac{1}{1.62} \left(\frac{dL}{D^2 v_0} \right)^{1/3}} \quad (4)$$

Figure 31. C_{IBT} in the reservoir versus time data plotted as shown in Equation 1, so that the slope can be used to calculate D_{app} .

Figure 32. Measured D_{app} versus flow rates and simulated data (dashed line).

The best fit of Equation 4 to the experimental data, shown as the dashed line in Figure 32, predicts a weaker dependence on v_0 than is reflected in the data. This deviation can be attributed to the pulsatile flow of the peristaltic pump used to circulate the liquid. Krasuk et. al[34] have demonstrated that mass transfer coefficients in laminar pipe flow increase with pulsation frequency. With peristaltic pumps, a higher flow rate is achieved by increased rotating speed of the pump head. Thus, the higher pressure pulsation frequency needed to achieve higher v_0 values would be expected to increase the D_{app} values more than would be predicted by Equation 4.

Effect of shell side flow rate on measured IBT membrane diffusivity

Previously, the IBT diffusivity measurements were carried out with shell side flow rate at 80mL/min, and it was assumed that the shell flow rate does not significantly affect the measured IBT membrane diffusivity because the shell side geometry is highly irregular, and enough turbulence is generated to achieve a uniform concentration profile. In order to verify these assumptions, a few follow-up measurements were made with shell flow rate at 184mL/min (Figure 33), and the resulting IBT membrane diffusivities were compared with the earlier results in Table 7. The calculated p-value was higher than 0.05, indicating that the results from the two shell side flow rates are not significantly different.

Figure 33. Plot of the natural log of IBT concentration at the inlet as a function of time for the collected experimental data. The slope has been calculated by fitting a trendline to the data. Standard deviation error bars were also included for each data point.

Table 7. Comparison of measured apparent diffusivities at two different shell flow rates

Shell flow rate (mL/min)	Lumen flow rate (mL/min)	Calculated D_{app} (cm ² /s) (Rigorous)	p-value
80	80	$2.24\text{E-}6 \pm 1.52\text{E-}7$	0.17
184	80	$2.61\text{E-}6 \pm 1.88\text{E-}7$	

Validate the membrane diffusivity calculations in COMSOL simulation

The IBT membrane diffusivity experiments were simulated using COMSOL Multiphysics. The simulation was used to estimate the membrane diffusivity of IBT, and the results were compared with those from diffusivity calculations based on two different sets of assumptions.

Figure 34. Geometry of a single fiber in the hollow fiber reactor. The model is axisymmetric and has the following dimensions: lumen radius=0.57mm, membrane thickness=0.33mm, shell thickness=0.30mm, fiber length=42.5cm. The reactor contained 63 fibers.

For the previous diffusivity measurements, the diffusivities were calculated using Equation 5. This equation was derived using the following simplifying assumptions: 1) the thickness of the membrane is significantly smaller than its inner radius and therefore the effect of the membrane curvature is negligible; 2) all the liquid in the system is well mixed and has the same concentration at any given time, which means there is no concentration gradient in the axial direction (along the length of the fiber).

$$\text{Slope} = -\frac{AD_M}{V\Delta r} \quad (5)$$

The variables in Equation 5 (denoted the “simplistic” model) are defined below:

- A = Lumen-side membrane surface area [m^2]
- D_M = Membrane diffusion coefficient [m^2/min]
- V = System volume [m^3]
- Δr = Membrane thickness [m]

While the above-mentioned assumptions greatly simplify the derivation, they are not strictly valid. First, the thickness the XM-50 hollow fibers is 0.033cm and is not significantly smaller than the ID at 0.11cm; second, even though a significant portion of the liquid in the system is well-mixed in the reservoir, the rest of the liquid is in the lumen space and doesn’t have the same concentration; third, at lower lumen flow rates, there is a significant drop in concentration between the inlet and outlet. Taking all of these factors into account but still assuming the IBT solution is well-mixed in the axial direction, Equation 6 (denoted the “more rigorous” model) was derived analytically:

$$\text{Slope} = \frac{F_L}{V} \left[\exp \left(-\frac{2\pi L D_M}{\left(\frac{F_L}{N}\right) \ln \left(\frac{OD}{ID}\right)} \right) - 1 \right] \quad (6)$$

where:

- F_L = Flow rate of liquid in the lumen [m^3/min]
- V = Reservoir volume [m^3]
- L = Fiber length [m]
- D_M = Membrane diffusion coefficient [m^2/min]
- N = Number of fibers
- OD = Fiber outer diameter [m]
- ID = Fiber inner diameter [m]

Table 8. Comparison of the assumptions made in three different types of calculation

Type of Calculation	Assumptions
Simplistic (Equation 5)	1) No membrane curvature 2) No concentration gradient in the radial and axial direction 3) All the liquid in the system is well mixed (no dead volume in the reactor lumen)
More rigorous (Equation 6)	1) Membrane curvature accounted 2) No concentration gradient in the radial direction in the lumen 3) Concentration gradient in the axial direction 4) Dead volume in the reactor lumen
COMSOL	1) Membrane curvature accounted 2) Concentration gradient in lumen radial direction 3) Concentration gradient in the axial direction 4) Dead volume in the reactor lumen

An even more accurate third model was developed based on the assumptions that the flow was fully developed laminar flow in the lumen, while the shell side was assumed to have a uniform velocity throughout. The model (denoted the COMSOL model) was solved numerically using COMSOL Multiphysics software. The simulation assumed a lumen flow rate at 80mL/min and shell flow rate at 184mL/min. The natural log of the average inlet concentration was plotted against time to allow comparison with the experimental data. A comparison of the assumptions made in the two types of calculation and COMSOL is summarized in Table 8. Since the COMSOL model used the fewest simplifying assumptions, its predictions are considered the most accurate of the three.

Because COMSOL does not have a data-fitting functionality, the optimal diffusivity value was determined by interpolation, as described below. The COMSOL model was solved for three different membrane diffusivities ($D_M = 3.54 \times 10^{-8} \text{ m}^2/\text{min}$, $1.77 \times 10^{-8} \text{ m}^2/\text{min}$, and $8.85 \times 10^{-9} \text{ m}^2/\text{min}$). The natural log of IBT concentrations were then plotted against time (Figure 35). Slopes of linear trend lines to model the data were calculated, and plotted against the diffusivity (See Figure 36) to give an almost linear relationship. A linear trend line was then fit to the plotted points, and the equation for the line was used to estimate the apparent diffusivity of the experiment.

Figure 35. Plot of the natural log of the average IBT concentration at the inlet as a function of time at three different membrane diffusivities, using COMSOL Multiphysics. A trendline has been fitted to the data to calculate the slopes at each membrane diffusivity.

Figure 36. Plot of slope against membrane diffusivity based on the results from COMSOL. The slope for each membrane diffusivity was from that found in Figure 35.

The resulting equation for the slope as a function of membrane diffusivity for the COMSOL model was

$$\text{Slope} = -(1.113 \times 10^6) D_M - 0.0119 \quad (7)$$

Given the slope of the experimental data (seen in Figure 33), the membrane diffusivity for each computation model was calculated using each of the models Equation 5-7. The calculated values for each method were then compared in Table 9.

Table 9 Comparison of computation methods and the calculated membrane diffusivity.

Computation Method	Equation	D_M [m ² /min]	% Error
Simplistic	(5)	1.93×10^{-8}	105%
More rigorous	(6)	1.25×10^{-8}	33%
COMSOL	(7)	0.94×10^{-8}	

Assuming the membrane diffusivity value from COMSOL model is an accurate value, the simplistic and more rigorous models gave values that were 33% and 105% too high, respectively. Although the COMSOL method is most accurate, it requires highly specialized software, whose computations are highly time-consuming. The rigorous calculation given by equation (6) provided a reasonably close approximation, and was much quicker to use, so it was used to correlate the experimental data.

Updated O₂, CO₂ and IBT membrane diffusivities

Using the rigorous calculation (Equation 6), the earlier diffusivity data for CO₂, O₂ and IBT were updated and applied the dimensional analysis as described in Equation 2-4. The results are summarized below in Figure 37-Figure 39.

Figure 37. Updated O₂ apparent diffusivity versus lumen flow rate. The dashed line is the simulation data with the best fit to the results of rigorous calculation. The obtained membrane diffusivity is 8.56E-6cm²/s.

Figure 38. Updated CO₂ apparent diffusivity versus lumen flow rate. The dashed line is the simulation data with the best fit to the results of rigorous calculation. The obtained membrane diffusivity is 4.93E-6cm²/s.

Figure 39. Updated IBT apparent diffusivity versus lumen flow rate. The dashed line is the simulation data with the best fit to the results of rigorous calculation. The obtained membrane diffusivity is 4.84E-6cm²/s.

Year 3 Major Task 1 completed.

Task 2 Construction of a dynamic model in MATLAB and COMSOL Multiphysics to simulate system performance

In an earlier progress report (Y2Q4), we presented the simulation results from a structured kinetic model constructed in MATLAB. The simulation described the impact of substrate availability on cell growth and product formation in a planktonic batch culture, but it didn't account for IBT re-uptake by the cells. We have seen extensive experimental evidence of IBT catabolism and believe it significantly influences the bioreactor's productivity of IBT. Thus, the MATLAB simulation was modified to include IBT catabolism and examine its effect on reactor performance. We assumed the cells need oxygen as electron acceptor to catabolize IBT, so the rate of IBT re-uptake was assumed to exhibit saturation kinetics for both IBT and oxygen concentrations, as shown below:

$$r_d = -k_d M \frac{C_o}{K_o + C_o} \frac{C_{ibt}}{K_{ibt} + C_{ibt}}$$

where k_d is the rate constant for IBT catabolism, M is the cell concentration, and K_o and K_{ibt} are the saturation constants for oxygen and IBT, respectively. The model was solved to show the effect of catabolism on IBT concentrations during batch reactor operation for three k_d values and six initial cell concentrations (Figure 40). The simulated results show that a hallmark trend of IBT degradation is an initial increase in IBT concentration as cell concentration increases, followed by a relatively rapid drop in IBT concentration as M passes a threshold level. For higher initial cell densities, the rise in IBT concentration to the maximum value and the onset of the decline in IBT concentration both occur sooner, and the final IBT concentration is lower. This same trend is observed for the highest and intermediate catabolism rate constants (a and b), but the IBT concentrations reach a higher maximum and stay at the elevated levels longer, and the steady state IBT concentrations are also higher for the intermediate rate constant (b). However, in the absence of catabolism (c), the IBT accumulates to a maximum value, which is determined by IBT toxicity to the cells, regardless of the initial cell density.

Figure 40. Isobutanol concentrations at various initial cell density and isobutanol re-uptake rates.

The MATLAB model simulations including catabolism exhibited trends similar to our experimental observations, namely the rapid increase in IBT concentration followed by a relatively rapid decrease. However, this model is too simple to describe the interplay between the mass transfer through the immobilized cells and the cells' structured kinetics. In the BIG system, the substrates are transferred to the cells only by diffusion from two different sides of the membrane. To understand performance of the BIG system, it is important to account for uneven distribution of substrate gases in the radial and axial directions.

We previously incorporated the structured kinetic model (see Y2Q4 progress report for details) with mass transfer and hydrodynamics and made the model a time-dependent 2-dimensional system in cylindrical coordinates with 5 chemical species (CO_2 , H_2 , O_2 , NH_4Cl and IBT). As this system of coupled partial differential equations is difficult to solve using MATLAB software, COMSOL® Multiphysics software is equipped with modules specifically designed to simulate coupled physical and chemical processes. Last quarter we adapted our dynamic model of the BIG system in COMSOL, and this quarter we further refined the model and used it to help interpret experimental results, identify rate-limiting steps, and optimize system performance. The improved model would be suitable to help design and scale up practical BIG reactors for IBT production.

The model is axisymmetric and includes 3 domains (Figure 41): lumen, biofilm and shell, whose boundaries vary in the r dimension according to the hollow fiber module manufacturer's specifications. The lumen and biofilm domains contain an aqueous phase, while the shell contains a gas phase. Equilibrium is assumed to exist between the liquid-phase concentrations at the outer edge of the biofilm and the gas-phase concentration in the shell, as predicted by Henry's Law. The boundary condition at the right hand side is assumed to be of Dirichlet type (the gas concentrations are set to equal the bulk composition). As with the MATLAB model, the IBT re-uptake is assumed to be dependent on the local IBT concentration, oxygen concentration and cell density.

Simulations were carried out for two modes of operation: "once-through" and "recirculation." In the once-through mode, the liquid medium is passed through the lumen once without recirculation. In the recirculation mode, the medium exiting the outlet is directly recirculated back to the lumen inlet. The once-through mode is better suited for showing instantaneous behavior of the system, whereas the recirculation mode is better to show long-term performance of the BIG system as the various system properties evolve over time due to cell growth, product accumulation, etc.

Figure 41 The 3 domains in hollow fiber simulation

We have substituted reasonable values of model parameters into the model and are assessing the model's ability to predict how concentration profiles of the reactants and products evolve after inoculation.

Effect of initial cell density

The COMSOL model's predicted time-dependent profiles have a similar trend to the planktonic culture MATLAB simulations described above. The IBT concentration in recirculation mode is predicted to increase

initially and then to drop suddenly. The IBT accumulation and onset of the IBT decline both occur earlier for higher initial cell densities (Figure 42 and Figure 43). These same trends occur in both once-through and recirculation modes. Even though the initial cell density is varied over three orders of magnitude, the peak cell density occurs in a relatively narrow time frame of five to 23 h. Detailed examination of the computed results indicates that IBT catabolism is responsible for the shape of the curve. The higher the initial cell loading, the more rapidly hydrogen mass transfer becomes rate-limiting and significant net IBT catabolism begins. While the rate of IBT production is limited by hydrogen mass transfer, IBT catabolism is not. The higher the cell concentration, the more rapidly IBT will be catabolized. Also, as hydrogen becomes depleted, IBT production slows, leaving more oxygen available for IBT catabolism.

Figure 42. IBT concentration versus time at different initial cell densities in once-through mode. The rate constant for isobutanol catabolism is assumed to be 10 h^{-1} .

Figure 43. IBT concentration versus time at different initial cell densities in a recirculation mode. The rate constant for isobutanol catabolism is assumed to be 10 h^{-1} .

Effect of initial ammonium concentration

Figure 44 summarizes the simulation results in recirculation mode at various ammonium concentrations. Two distinctly different shapes of the trend lines are predicted. At the highest initial ammonium concentrations of 1 g/L and 5 g/L , the ammonium never gets fully depleted in the 50 hour time frame, allowing cell growth to exceed the point where hydrogen depletion occurs and rapid IBT catabolism takes place. As a result, the IBT concentration peaks and decays rapidly, as was reported above. However, for the ammonium concentrations of 0.01 and 0.1 g/L , the nitrogen source becomes depleted, and cell growth stops 5.6 h and 12.8 h after inoculation, respectively. After these times, the cell densities decrease slowly due to endogenous metabolism, causing the IBT levels to gradually drop. However, because the cell density never achieves levels at which hydrogen becomes depleted throughout much of the biofilm, the rapid drop in IBT concentration attributed to catabolism does not occur. These results suggest that an intermediate cell density would maximize IBT production when using cell strains that catabolize IBT, and that the IBT productivity could be very sensitive to the effect of ammonium concentration. This result makes in-situ control of ammonium level crucial to optimizing the system IBT productivity. Therefore, we plan to implement an ammonium/nitrogen sensor and control loop for on-line control of ammonium levels in future experiments.

Figure 44. IBT concentration at various initial ammonium concentrations

Effect of H_2 diffusivity

Since H_2 has the lowest solubility and the highest stoichiometric coefficient among the three gaseous substrates, it is intuitively most likely to be the rate-limiting reactant. To help guide experimental efforts to increase reactor productivity, we conducted simulations to study the effect of the membrane's H_2 diffusivity on BIG performance in once-through mode (Figure 45).

Surprisingly, changes up to 8-fold in H_2 membrane diffusivity did not have a significant impact on the IBT productivity during the first 12 hours. Examination of the hydrogen and oxygen profiles at the reactor outlet at the 12 hour time point (Figure 46 and Figure 47) revealed that as the hydrogen diffusivity increases, hydrogen concentration becomes elevated throughout the membrane, but the oxygen concentration profiles remain the

same. The H_2 concentration affects the reaction rate through a Monod term $\frac{C_{H_2}}{C_{H_2} + K_A}$, and Monod constant for H_2 K_A is assumed to be $1 \times 10^{-3} \text{ mol/m}^3$, two orders of magnitude smaller than the H_2 concentration in the membrane (ranges between $0.1\text{-}0.9 \text{ mol/m}^3$), so the rate of reaction is not affected by the H_2 concentration in this range.

However, after 12 hours, as the cell mass increases and the demand for H_2 continues to rise, in the scenario with lowest H_2 diffusivity (dashed blue line in Figure 45 H_2 concentration decreases on the lumen side of the membrane, and IBT production slows down, leaving more oxygen available for IBT catabolism (outlet H_2 and O_2 profile at 20h is shown in Figure 48 and Figure 49). As IBT catabolism increases, the rate of increase in IBT concentration slows down (between 12 h and 14 h in Figure 45). Eventually H_2 becomes completely depleted in part of the membrane, causing cell growth and IBT production to stop in that region. This onset is

manifested as an inflection point at 14h in Figure 45. Beyond this point, IBT concentration start to decrease, but since the IBT concentration and cell mass are both decreasing, the rate of IBT degradation also drops. Figure 50 shows how the O₂ and H₂ profiles across the membrane affect the relative rates of IBT production and catabolism and, in turn, the sign of IBT net reaction rate. This graph captures the value of the model in elucidating how the complex interplay of reaction and diffusion of multiple species to achieve IBT production.

With a higher H₂ membrane diffusivity ($2.0 \times 10^{-9} \text{ m}^2/\text{s}$, red solid line in Figure 45), the H₂ mass transfer can sustain the cell growth and IBT production longer, but at around 16 hour, the same decrease of IBT concentration occurs.

With the two highest H₂ membrane diffusivities, the drop in IBT concentration doesn't occur within the 20 hour duration of the simulation, but with sufficient ammonium source and continued cell mass growth, the same transition in IBT concentration would eventually take place. Also, in these two scenarios, since H₂ concentration is high throughout the membrane, O₂ becomes depleted on the shell side of the membrane and limits IBT catabolism. The oxygen profiles are identical because the reaction rate becomes insensitive to the change in H₂ concentration.

In summary, having H₂ in excess is a good strategy for minimizing IBT catabolism, but enhancing the H₂ mass transfer beyond a certain level will only result diffusion of H₂ entirely through the membrane and into the fluid flowing through the lumen.

Figure 45. Average IBT outlet concentration with various H₂ membrane diffusivities

C_{H_2} vs. radius, outlet at 12h

Figure 46. H_2 profile at the reactor outlet at 12h. The grey box represents the membrane.

C_{O_2} vs. position, outlet at 12h

Figure 47. O_2 profile at the reactor outlet at 12h. The grey box represents the membrane.

Figure 48. H_2 concentration profile at the reactor outlet at 5h. Radius 0.58mm to 0.7mm is the membrane with immobilized cells. The grey box represents the membrane.

Figure 49. O_2 concentration profile at the reactor outlet at 5h. Radius 0.58mm to 0.7mm is the membrane region with immobilized cells. The grey box represents the membrane.

Figure 50. O_2 and H_2 profiles as well as IBT production, catabolism and net reaction rates within the membrane

Effect of flow rates through the lumen

In most of our BIG experiments, oxygen has been provided through the liquid flowing through the lumen. Therefore, the lumen's liquid flow rate affects the oxygen availability to cells immobilized in the membrane. Simulations were conducted to explore the effect of lumen flow rate on BIG performance. The simulation result for once-through operation (Figure 51) shows that increased oxygen availability accelerates the IBT catabolism and causes the net IBT production to drop. Although a two-fold increase in flow rate could explain a two-fold decrease in IBT concentration due to the shortened liquid residence time, Figure 51 shows a roughly 100-fold decrease in IBT concentration following a change in flow rate from 160 mL/min to 320 mL/min, and a million-fold decrease in IBT concentration following a change in flow rate from 160 mL/min to 640 mL/min. On the other side of the membrane, where O_2 availability is limited but H_2 is abundant, catabolism is much slower than IBT generation, so the local IBT concentration increases modestly with lumen flow rate (Figure 52). IBT catabolism not only competes with IBT production for oxygen, it also makes increasing oxygen availability an unsuitable strategy for increasing IBT yield.

The previously unexpected trend that increasing the oxygen concentration can decrease the net IBT production is evident in Figure 51. The extent to which this effect occurs is determined by the relative magnitudes of the rate constant for IBT catabolism compared to that of IBT production. If IBT catabolism occurred at a lower rate, increased oxygen availability would have a significant positive impact on IBT productivity. Experiments are underway to measure the specific isobutanol catabolism rate and thereby improve the accuracy of simulation results.

Figure 51. IBT concentration at various flow rates through the lumen in continuous mode. The inset figure indicates the point where IBT concentration is plotted; in this case it is the center of the reactor outlet.

Figure 52. IBT concentration versus time at the membrane outer surface near outlet.

Effect of biofilm thickness

Since all the gas substrates are delivered to the immobilized cells via diffusion through the membrane, membrane thickness would be expected to be an important process parameter. Immobilized-cell biocatalysts exhibiting standard Monod kinetics exhibit an optimum biofilm thickness. Biofilms thicker than the optimum are diffusion-limited, and biofilms thinner than the optimum are kinetically limited. The addition of IBT catabolism further complicates the calculations and data interpretation. As the biofilm gets thicker, more severe hydrogen limitation results in more IBT catabolism, thereby reducing net IBT production. These trends are evident in Figure 53, which shows simulation results for three biofilm thicknesses. Surprisingly, the thinnest biofilm simulated gave highest IBT concentration, although a much longer time was required for the cells to grow to a high-enough cell density within the thin membrane to achieve maximum IBT concentration.

The interdependence of reaction kinetics and diffusion has been analyzed in traditional reaction engineering using dimensionless groups (effectiveness factor and Thiele modulus). However, such analysis is not directly useful for our case, because of the complexity of the kinetics and the fact that the incompatible gaseous reactants H_2 and O_2 diffuse to the cells from opposite directions. Nevertheless, we are investigating whether a similar dimensional analysis may be useful to correlate results and identify an optimal biofilm thickness for our BIG system.

Figure 53. IBT concentration at various biofilm thicknesses

Incorporation of mixed-substrate metabolism into the predictive mathematical model

In the IBT-feeding experiments (Y3Q4 progress report) it was observed that *R. eutropha* can also grow on IBT. The structured kinetics in the previous model accounts for cell growth and isobutanol production from CO_2 , H_2 and O_2 as well as IBT catabolism in the presence of O_2 . Therefore, mixed-substrate (mixotrophic) metabolism, in which the cell simultaneously uses H_2/CO_2 and IBT as carbon and energy sources, was incorporated in the updated model.

Several models have been proposed for mixotrophic growth, and, as reviewed by Kovárová-Kovar and Egli [1], most of them were modified from Monod-type saturation kinetics. One of the models [2] defines the mixotrophic specific growth rate to be the function of Monod parameters from single-substrate growth and the percentage of each substrate in the mixture. This model turned yielded the best fit for *E. coli* growth on multiple substrates. Therefore this form was adopted in the *R. eutropha* model to account for the mixotrophic growth. Modifying our structured growth model to include mixotrophic metabolism gives the resulting model equations:

$$\mu = \mu_{max} \left(\frac{\alpha C_{H_2}}{\alpha C_{H_2} + (1 - \alpha) C_{IBT}} \right) \left(\frac{C_{CO_2}}{C_{CO_2} + K_c} \cdot \frac{C_{H_2}}{C_{H_2} + K_h} \cdot \frac{C_{O_2}}{C_{O_2} + K_o} \cdot \frac{C_{NH_3}}{C_{NH_3} + K_n} \right) \left(1 - \frac{C_{IBT}}{C_{IBT,max}} \right) \quad (1)$$

$$k_n = k_{n,max} \left(\frac{\alpha C_{H_2}}{\alpha C_{H_2} + (1 - \alpha) C_{IBT}} \right) \left(\frac{C_{CO_2}}{C_{CO_2} + K_c} \cdot \frac{C_{H_2}}{C_{H_2} + K_h} \cdot \frac{C_{O_2}}{C_{O_2} + K_o} \cdot \frac{C_{NH_3}}{C_{NH_3} + K_n} \right) \left(1 - \frac{C_{IBT}}{C_{IBT,max}} \right) \quad (2)$$

$$\mu_{IBT} = \mu_{max,IBT} \left(\frac{(1 - \alpha) C_{IBT}}{\alpha C_{H_2} + (1 - \alpha) C_{IBT}} \right) \left(\frac{C_{IBT}}{C_{IBT} + K_{IBT}} \cdot \frac{C_{O_2}}{C_{O_2} + K_o} \cdot \frac{C_{NH_3}}{C_{NH_3} + K_n} \right) \left(1 - \frac{C_{IBT}}{C_{IBT,max}} \right) \quad (3)$$

$$k_{n,IBT} = k_{n,max,IBT} \left(\frac{(1 - \alpha) C_{IBT}}{\alpha C_{H_2} + (1 - \alpha) C_{IBT}} \right) \left(\frac{C_{IBT}}{C_{IBT} + K_{IBT}} \cdot \frac{C_{O_2}}{C_{O_2} + K_o} \cdot \frac{C_{NH_3}}{C_{NH_3} + K_n} \right) \left(1 - \frac{C_{IBT}}{C_{IBT,max}} \right) \quad (4)$$

$$r_M = \mu M + \mu_{IBT} M - k_s s N \quad (5)$$

$$r_N = k_n s N + k_{n,ibT} s N - k_d N \quad (6)$$

$$r_s = [1 + s] [\mu - k_n s + \mu_{IBT} - k_{n,ibT} s + k_d] - k_s s \quad (7)$$

$$r_{IBT} = k_{IBT} s N \left(\frac{C_{CO_2}}{C_{CO_2} + K_c} \cdot \frac{C_{H_2}}{C_{H_2} + K_h} \cdot \frac{C_{O_2}}{C_{O_2} + K_o} \right) \left(1 - \frac{C_{IBT}}{C_{IBT,max}} \right) - Y_{IBT/cell} \mu_{IBT} - k_{d,IBT} s N \frac{C_{IBT}}{C_{IBT} + K_{IBT}} \frac{C_{O_2}}{C_{O_2} + K_o} \left(1 - \frac{C_{IBT}}{C_{IBT,max}} \right) \quad (8)$$

where M is cell mass density, N is cell number density, and s is excessive cell mass. C_{H_2} , C_{CO_2} , C_{O_2} , C_{NH_3} are concentrations of H_2 , CO_2 , O_2 and NH_3 ; K_h , K_c , K_o , K_n are the corresponding Monod constants; $C_{IBT,max}$ is the highest IBT concentration the strain can tolerate; μ_{max} and $\mu_{max,IBT}$ are the maximum specific autotrophic growth rate and maximum specific growth rate on IBT respectively; $k_{n,max}$ and $k_{n,max,IBT}$ represent the maximum specific cell division rates using CO_2 and IBT as the only carbon source. α indicates the strain's relative preference to use H_2 as energy source, with 0 meaning the strain does not utilize H_2 at all and 1 meaning the strain doesn't utilize IBT at all. The immobilized cells grow both autotrophically and on IBT in the presence of NH_3 . Since H_2 is typically the limiting substrate in autotrophic growth, the contributions of growth on autotrophic substrates and IBT in mixotrophic metabolism are determined by the local ratio of H_2 and IBT concentrations. Growth on H_2 and IBT are both inhibited by IBT. IBT is also catabolized by the cells in the presence of O_2 .

With mixotrophic metabolism incorporated into the model, the predictions of bioreactor performance show exhibit similar trends to those reported in Y3Q4 progress report. Simulations are underway to predict the system's performance when IBT catabolism pathway is suppressed through future metabolic engineering efforts. The resulting lower tendency for the cells to utilize IBT, can be simulated by using a higher α value.

The simulation results also indicate that the immobilized biofilm never reaches a true steady state. As the cells in the biofilm grow, the rate of consumption of both O_2 and H_2 increase, causing their gradients to become steeper. As a result, the region where both O_2 and H_2 are available becomes narrower, and only cells in this "overlap" region continue growing. Consequently, the biofilm becomes thinner and more concentrated. (Figure 54). As O_2 concentration gradually drops along the length of the reactor, the "overlap" region shifts towards the lumen side. (Figure 54-Figure 56) The H_2 -deprived region expands with time and productivity drops as IBT catabolism increases. However, this trend only occurs when there is abundant nitrogen source available, indicating that process control schemes based on intelligent *in situ* NH_3 regulation could be used to

maintain the biofilm at a desired density and thickness.

Figure 54 Model-predicted evolution of cell mass profile at the bioreactor inlet

Figure 55 Model-predicted evolution of cell mass profile at the middle of bioreactor

Line Graph: immobilized cell density [weight/volume] (g/L)

Figure 56 Model-predicted evolution of cell mass profile at the bioreactor outlet

Year3, Major Task 2 completed. Using the mathematical model, we are able to explain our experimental observations and predict bioreactor performance under a wide range of experimental conditions.

Task 3: BIG system operated continuously with integrated IBT recovery system

In the first three quarters of Year 3, we have consistently obtained conclusive evidence of autotrophic IBT production in the BIG system, and we have tested various combinations of experimental conditions. In all our experiments, we observed an initial higher IBT concentration that soon decreased, and we have observed little impact of H₂ pressure on IBT productivity. We also observed that increasing the O₂ availability by increasing the lumen flow rates has a relatively small effect on IBT yield, but IBT productivity is more sensitive to NH₄⁺ concentration. As previously stated, COMSOL simulations that incorporated IBT catabolism were able to reproduce most of our observed trends, providing strong support that cells in H₂-poor regions of the BIG catabolize IBT produced in H₂-rich regions. Hence, this quarter, we focused on experimentally characterizing the effect of IBT catabolism by the *R. eutropha* on reactor performance.

Growth and IBT production of Re2425/pJL26 and Re2426/pJL26 sparging with pure O₂

To study cell growth and IBT production in presence of pure O₂, two types of batch experiments have been conducted in pH controlled Sartorius reactor that was retrofitted to provide pure O₂, either by recirculating liquid from the Sartorius vessel or through the lumen of a hollow fiber module having oxygen on the shell side, or by sparging the gas directly into the vessel. Two strains provided by Dr. Sinskey's lab, Re2425/pJL26 and Re2426/pJL26 (a putative biofilm overproducer), were used for experiments. In these experiments, fructose was initially used as a carbon source, and pure O₂ was sparged directly into liquid at fixed low flow rate of 0.05 L/min. pH was controlled by NaOH, and dissolved O₂ was monitored by a dissolved oxygen (DO) probe. The Sartorius reactor was kept as a closed system to decrease loss of IBT vapor. For safety purposes, a pressure gauge was connected to the Sartorius vessel to monitor possible pressure increases in the reactor due to the supplied pure O₂ or metabolically produced carbon dioxide. Under these conditions, the DO was maintained at around 100% up to a cell OD around 4. After that, DO started to decrease but never went to less than 40%, indicating that O₂ was not limiting in these experiments.

Figure 57. Heterotrophic growth and IBT production by Re2425/pJL26 in presence of pure O₂

Figure 58. Heterotrophic growth and IBT production by Re2426/pJL26 in presence of pure oxygen

Both the strains grew well under these conditions, with a specific growth rate in the exponential growth phase of 0.21 h^{-1} and 0.22 h^{-1} for Re2425/pJL26 and Re2426/pJL26, respectively (Figure 57 and Figure 58). IBT production was induced by nitrogen limitation for both the strains. After 30 h, both strains gave an IBT concentration of about 70 mg/L, along with about 8 mg/L of 3-methyl,1-butanol (isoamyl alcohol). These

experiments confirmed that pure O₂ can be used for cell growth and IBT production instead of air in an effort to minimize DO limitation.

Autotrophic IBT production in suspension culture integrated with resin column for IBT adsorption

In previous quarters, we reported that the BIG system produced low levels of IBT, even when the porous membrane was inoculated with high cell *R. eutropha* cell densities. Additional experimental and modeling work has suggested the hypothesis that the low IBT levels were due to IBT catabolism by cells located in H₂-depleted regions of the BIG. To test this hypothesis, we grew the *R. eutropha* strains in suspension culture under conditions designed to mimic the H₂-depleted, autotrophic conditions. To achieve such conditions, a 500 mL working volume Sartorius bioreactor vessel was used as the liquid reservoir. A *R. eutropha* culture in fructose-free media was recirculated through the lumen (for Re2425) or shell (for 2426) of the BIG's hollow fiber cartridge at a flow rate of 100-400 mL/min. The liquid effluent from the hollow fiber reactor (HFR) was recycled to the Sartorius vessel using two different setups. In Setup 1, the recycled liquid returning from the HFR was delivered into the headspace above the reactor vessel liquid, so that any H₂-containing gas bubbles generated within the HFR would disengage from the returning liquid stream and enter the Sartorius headspace without being entrained into the agitated liquid in the Sartorius vessel. In Setup 2, the recycled liquid was injected directly into the liquid within the Sartorius vessel, so that any H₂-containing gas bubbles generated within the HFR would be entrained into the agitated liquid in the Sartorius vessel. This difference would make it unlikely in Setup 1 that any H₂ gas contained in the recycle stream would be dissolved into the liquid and consumed by the cell suspension. However, in Setup 2, H₂ gas recycled to the Sartorius vessel would be distributed into the liquid of the vessel, increasing the likelihood that this H₂ would be consumed by the *R. eutropha* cells suspended in the liquid. The DO probe of the Sartorius vessel was calibrated using the gas that was delivered (either air or pure O₂).

In Setup 1, when using Re2425, air (at 0.5 L/min) and CO₂ (at 0.1-0.3 L/min) were sparged into the reservoir liquid, which was recirculated through the lumen of the HFR; H₂ gas was delivered into the shell side. When strain Re2426 was used with Setup 1, liquid was recirculated through the shell side, and H₂ was delivered into the lumen side. In Setup 2, H₂ was delivered to the lumen side for both strains. H₂ pressure was maintained at 15 psig using a pressure controller. The effluent gas stream from the liquid reservoir was passed through an Optipore SD-2 resin packed bed column and then passed through the gas analyzer. The Opto22 control system was programmed to stop H₂ flow to the shell side if the H₂ concentration in the exhaust gas from the liquid reservoir exceeded a threshold value (4%). The ammonia concentration was measured periodically and added as needed to prevent its concentration from dropping to zero. Samples were collected from the culture vessel for measurement of cells, IBT and ammonia. Also, methanol was periodically used to recover IBT from the resin column and measure the amount of IBT adsorbed. Two resin columns were alternately used for either capturing IBT or IBT removal and regeneration.

Figure 59. Autotrophic growth and IBT production by Re2425/pJL26 (Setup 1)

Early in the experiment with the Re2425/pJL26 strain, the specific growth rate during autotrophic growth 0.053 h^{-1} , and the IBT production rate was $48 \text{ mg}/(\text{L} \cdot \text{day})$ when ammonia was depleted at 70 h (Figure 59 and Figure 60). At this time, the cell OD was 6.8. To increase the productivity, cell growth was encouraged by adding NH_4Cl . Cells resumed growing at slower growth rate, but the net production of IBT become very low even after ammonia became depleted. At 129 h, to confirm that minerals were not rate limiting, CaCl_2 , MgSO_4 and trace elements were added, but there was no increase in IBT production. At 170 h, we replaced the culture medium with fresh medium to test the hypothesis that some unknown inhibitory component had accumulated in the liquid during fermentation that was inhibiting IBT production. This step was achieved by collecting the liquid contents of the reactor, spinning down the culture, resuspending the cells into an equivalent amount of fresh medium and then returning the cell suspension to the reactor vessel. However, medium replacement had virtually no effect on IBT formation. To test the hypothesis that the lack of apparent IBT production was caused by high cell density, we diluted the cells to an OD of 2.2 at 199 h. After dilution, the cells began growing autotrophically again at a rate of 0.057 h^{-1} and producing IBT. The IBT productivity peaked at about $60 \text{ mg}/(\text{L} \cdot \text{day})$ at an OD of about 8, even though ammonia concentration was still low. The cells were diluted again at 263 h to an OD of 0.76, leading to resumption of cell growth at a specific rate of 0.065 h^{-1} and IBT production. A peak IBT production rate of about $68 \text{ mg}/(\text{L} \cdot \text{day})$ was measured when the ammonia become limiting at an OD of about 6 (Figure 59 and Figure 60). These results provide strong support for the hypothesis that high cell density causes the drop in IBT productivity, and are consistent with other experimental results suggesting that IBT catabolism greatly reduces the net IBT production rate under conditions in which hydrogen becomes depleted. In this case, as the cell density increases beyond a threshold value, IBT generation drops precipitously. Similar trends were observed in the MATLAB and COMSOL simulations above.

Figure 60. Growth of Re2425/pJL26 under autotrophic conditions (Setup 1)

A similar experiment was also carried out with Re2426/pJL26 strain. The cells initially grew autotrophically at a specific growth rate of 0.08 h^{-1} . At 113 h, when ammonia become limiting at OD of 7, the cells produced IBT at a rate of $210 \text{ mg}/(\text{L day})$ and 3 methyl 1 butanol (3M1B) at a rate of $60 \text{ mg}/(\text{L day})$ (Figure 61 and Figure 62). However, as cell growth continued to an OD of 9.5, the net IBT dropped significantly. While this trend of a peak in IBT productivity at an OD of about 7 followed by a rapid decline was observed with both strains, the Re2426/pJL26 strain appeared to give a higher IBT productivity than Re2425/pJL26.

Figure 61. Autotrophic growth of Re2426/pJL26 (Setup 1)

Figure 62. Autotrophic IBT production by Re2426/pJL26 (Setup 1)

Similar experiments were then conducted using Re2425/pJL26 and Re2426/pJL26 in Setup 2. In Setup 2, a 500 mL working volume Sartorius bioreactor vessel was used as the liquid reservoir. A growing *R. eutropha* culture in fructose-free medium was recirculated through the shell side of the BIG's hollow fiber cartridge at a flow rate of 100-200 mL/min. The outlet liquid of hollow fiber reactor (HFR) was directly injected into the liquid in the reactor vessel, rather than dispensing it into the gas headspace above the liquid, as was done in Setup 1. The DO was controlled at 80%-100% using a mixture of air and pure O₂. Air (0.4 L/min), CO₂ (0.2-0.3 L/min) and pure O₂ were sparged into the reservoir liquid, and H₂ gas was metered into the lumen side of the HFR at a pressure of 15 psig. As with Setup 1, a packed-bed column of polymeric resin was used to capture IBT from the gas effluent of the reactor. The Opto22 control system was programmed to control H₂ pressure and concentration of effluent gas. Assays for pyruvic acid, ammonia, cell growth and alcohol production were conducted.

Figure 63. Autotrophic growth of Re2425/pJL26 (Setup 2)

Figure 64. Autotrophic IBT production by Re2425/pJL26 (Setup 2)

Using Setup 2 (Figure 63 and Figure 64), the initial specific growth rate of Re2425/pJL26 under autotrophic conditions was 0.11 h^{-1} , which is higher than the value measured using Setup 1. The peak volumetric productivity of IBT was of 325 mg/(L day) , and that for 3M1B was 50 mg/(L day) . Both of these values are higher than those achieved in Setup 1. However, again, when the OD reached about 10, the net IBT production plummeted, even during ammonia-limiting conditions. At around 92 h, when the BIG was is not producing IBT, the CO_2 sparging rate, O_2 sparging rate into vessel culture liquid and liquid flow rate through shell side of HFR were increased, but no increase in IBT production was observed. Pyruvic acid was produced at a level of about 8 g/L at low cell density, but this product was also taken up at higher cell density.

Figure 65. Autotrophic growth of Re2426/pJL26 (Setup 2)

When strain Re2426/pJL26 was used in Setup 2 (Figure 65 and Figure 66), the initial specific growth rate of under autotrophic conditions was 0.12 h^{-1} . The peak volumetric productivities were $1030 \text{ mg}/(\text{L day})$ for IBT and $175 \text{ mg}/(\text{L day})$ for 3M1B. All of these values are higher than those measured using Setup 1. However, again, when the OD reached a value of around 9, IBT levels dropped to near zero. Pyruvic acid was produced around 4.5 g/l at lower cell density but it was taken up at higher cell densities. After adding small amount of NH_4Cl at 55.3h cell productivity was increased.

Figure 66. Autotrophic IBT production by Re2426/pJL26 (Setup 2)

Results from this set of autotrophic IBT-production experiments showed that both strains generated higher product levels when used in Setup 2 than Setup 1. The major difference between the setups is the point at which the liquid recycle from the HFR was released into the vessel. Injection of the recycled liquid directly into the reservoir liquid allowed small bubbles of H_2 from the HFR to be dispersed throughout the liquid contents of the vessel, thereby achieving some degree of H_2 transfer to the culture in the vessel as well as the HFR.

Experiment to quantify IBT catabolism by *R. eutropha* (Re 2425pJL26.)

We previously reported an experiment to demonstrate IBT catabolism by *R. eutropha*. This quarter we attempted to quantify key parameters describing IBT catabolism that could be used in the model (e.g., the cell yield coefficient on IBT and specific IBT catabolism rate). The approach used a hollow fiber module to oxygenate the liquid phase without stripping IBT produced in the fermentation. It also leveraged the Sartorius bioreactor's ability to measure dissolved O_2 concentration on-line and used that information to add IBT. The schematic diagram of the experimental setup is shown in Figure 67. A Re 2425pJL26 culture was inoculated in the Sartorius bioreactor vessel and recirculated at 180 mL/min through the lumen of an HF module while O_2 gas was maintained at a pressure of 10 psig in the shell side of the HFR. Periodically, we added IBT or fructose based on evidence that the carbon substrate had been depleted (i.e., a sudden spike in the DO). The ammonia concentration was measured and added as needed to prevent its concentration from dropping to zero. Samples were collected from the culture vessel for off-line measurement of cell, NH_4Cl , fructose and IBT concentrations.

Figure 67. Schematic diagram of the fed-batch bioreactor for carbon feed on demand without using a gas feed stream in the bioreactor vessel.

Figure 68. Fructose, NH₄Cl and IBT profiles during fructose or IBT consumption

When the fructose concentration reached zero at 34.5 h, as indicated by the spike in the DO value to 100%, fructose was added. The resumption of fructose catabolism increased the oxygen consumption and resulted in a drop in the DO values. At 63 h, when the fructose concentration dropped to zero again and the DO spiked, IBT was added as the carbon source. From then, on, IBT was added on demand in response to spikes in the DO to values near 100%, indicating that the IBT had been depleted. At 233 h, when the OD approached 30, the cell concentration was diluted to an OD of 2.

Figure 69. OD and DO profiles during fructose or IBT consumption

Based on the data collected during *R. eutropha* catabolism of IBT during hours 233-350, the calculated maximum cell yield and average cell yield on IBT values were 0.6 g cell/g IBT and 0.47 g cell/g IBT, respectively. The maximum specific IBT uptake rate was 0.062 g IBT/(g cell h). Under O₂ limiting conditions, the uptake rate was 0.04 g of IBT/(g cell h), and in presence of trace amounts of fructose and sufficient O₂ it was 0.02 g IBT/(g cell h) (Figure 68 and Figure 69).

Year 3, Major Task 3 done. Volumetric productivities of IBT at 325 mg/(L day) and of 3M1B at 50 mg/(L day) were achieved, which were comparable to that achieved under heterotrophic conditions.

Task 13: Economic analysis

In the task 13 we estimated the capital and operating costs of the process to convert hydrogen, oxygen, and carbon dioxide to isobutanol (C₄H₁₀O) based on 10⁶ gal/year isobutanol.

Capital cost

Reactor cost

Basis: 1 million gallon IBT /year production

$$\begin{aligned}
 \text{IBT production} &= 1 \text{ million gallon/year} \\
 &= 3.78 \times 10^6 \text{ L/year} \\
 &= 3.78 \times 10^6 \times 802 / (365 \times 24 \times 3600) \text{ g/s} \quad (\text{density of IBT} = 802 \text{ g/L}) \\
 &= 96.2 \text{ g IBT/s} \quad (\text{molecular weight} = 74) \\
 &= 1.3 \text{ mole IBT/s}
 \end{aligned}$$

Radius of lumen of reactor=0.50 mm = 0.0005 m

Velocity of liquid in lumen=1 cm/s (assumed)

From modeling and available reactor configuration available commercially, length of the hollow fiber reactor is assumed to be 1.5 m.

Inner surface area of each fiber= $2\pi rL = 2 \times 3.14 \times 0.0005 \times 1.5 = 4.71 \times 10^{-3} \text{ m}^2$

Average IBT production flux based on modeling = $3.0 \times 10^{-3} \text{ g}/(\text{m}^2\text{s})$ (from Jan., 2012 progress report)

Assumed IBT percentage in liquid exiting the fiber=1%

Required total fiber internal surface area = $96.2 / (3 \times 10^{-3}) \text{ m}^2$
 $= 32000 \text{ m}^2$

Total number of fibers required = $32000 / (4.71 \times 10^{-3})$
 $= 6.81 \times 10^6$

Approximate fiber cost per unit surface area for lab-scale commercial HFR system = \$50/m² (vendor estimate)

Membrane lifetime = 10 years

Annualized membrane capital cost = \$5/(m² year)

Annual hollow fiber cost = $32000 \text{ m}^2 \times \$5/(\text{m}^2 \text{ year})$
 $= \$ 160000/\text{year}$

Sources:

Desalination 280 (2011) 120–126

<http://www.spectrumlabs.com/cell/MaxCarts.html>

http://www.celltrends.com/services_cellculture.htm

Operating cost

Pump cost for micro-bubble generator:

Mole of IBT production = $96.2 \text{ g/s} / (74 \text{ g/mole})$ (molecular weight of IBT=74)
 $= 1.3 \text{ mole/s}$

Molar O₂ requirement = $1.3 \times 12 \text{ mole O}_2/\text{mole IBT} = 16 \text{ moles/s}$

(based on assumed stoichiometry of $4 \text{ CO}_2 + 36 \text{ H}_2 + 12 \text{ O}_2 \rightarrow \text{C}_4\text{H}_{10}\text{O} + 31 \text{ H}_2\text{O}$) from original proposal

Assuming 90% of oxygen is utilized, the O₂ requirement is $= 16 / 0.9 = 17 \text{ moles/s}$

Volume of gas flow (assuming ideal gas law, 30°C, 1 atm) = $17 \times 0.082 \times 303 / 1 = 430 \text{ L/s}$

Assuming the gas hold up in reactor is 0.3 (volume of gas/total volume)

Liquid flow = $430 \times (1 - 0.3) / 0.3 = 1000 \text{ L/s} = 1 \text{ m}^3/\text{s}$

Assume liquid velocity in microbubble generator = 20 ft/s = 6.1 m/s (personal experience)

Assume inner pipe diameter of microbubble generator = 1 cm = 0.01 m (nominal value used industrially)

Number of microbubble generators required = $1 \text{ m}^3/\text{s} / [(\pi/4) \times (0.01 \text{ m})^2 \times 6.1 \text{ m/s}] = 2130$

Reynolds number (NRe) = $(0.01 \times 6.1 \times 1000 / 0.001) = 60960$

Darcy friction factor $f = 0.3164 \text{ NRe}^{-0.25} = 0.02$

Assume each pipe length = 1 m (conservative upper bound)

Frictional pressure drop in each pipe line = $v^2 f L \rho / 2D$
 $= 6.1^2 \times 0.02 \times 1 \times 1000 / (2 \times 0.01)$
 $= 37000 \text{ Pa}$
 $= 5.4 \text{ psi}$

Pumping power requirement for each microbubble generator = pressure drop x volumetric flow rate
 $= 5.4 \text{ psi} \times [(\pi/4) \times (0.01 \text{ m})^2 \times 6.1 \text{ m}^3/\text{s}]$
 $= 5.4 \times 703.68 \text{ (kg}_f/\text{m}^2) \times 0.000479 \text{ m}^3/\text{s}$

$$\begin{aligned}
 &= 1.818 \text{ kg}_f \text{ m/s} \\
 &= 1.818 \times 9.807 \text{ watt} \\
 &= 17.83 \text{ watt}
 \end{aligned}$$

Total pump power requirement = $2130 \times 17.83 \text{ watt} = 37975.32 \text{ watt} = 37.98 \text{ kW}$
 Assume electrical charge = \$0.1/kwh
 Electrical power supply cost for running the Pump per year = $\$ 37.98 \times 365 \times 24 \times 0.1$
 = \$ 33200 (annual cost)

Pressure drop in fiber

The velocity in the fiber = 0.01 m/s (1 cm/s assumed value used in previous modeling results)
 Diameter of fiber = 1 mm = $1 \times 10^{-3} \text{ m}$
 $\text{NRe in fiber} = 1 \times 10^{-3} \times 0.01 \times 1000 / 0.001 = 10$, indicating laminar flow
 Darcy friction factor in fiber, $f = 64 / \text{NRe} = 6.4$
 So the pressure drop in fiber = $v^2 f L \rho / 2D = 480 \text{ Pa} = 0.07 \text{ psi}$

This pressure drop is very low compared to that in microbubble generator. Therefore, the microbubble generator pump is assumed to be sufficient to drive liquid flow through the hollow fiber reactor.

Gas cost

H_2 requirement per year = $1.3 \text{ mole IBT/s} \times 36 \text{ mole H}_2/\text{mole IBT}$ (from stoichiometry)
 Total H_2 cost per year = $\$1/\text{kg} \times 1.3 \text{ mole/s} \times 36 \times 2 \text{ g/mole} \times 10^{-3} \text{ g/kg} \times 3600 \times 24 \times 365 = \$ 2947000$
 (Assume H_2 cost = \$1/kg)

CO_2 requirement per year = $1.3 \text{ mole IBT/s} \times 4 \text{ mole CO}_2/\text{mole IBT}$ (from stoichiometry)
 Total CO_2 cost per year = $\$03/\text{kg} \times 1.3 \text{ mole/s} \times 4 \times 44 \text{ g/mole} \times 10^{-3} \text{ g/kg} \times 3600 \times 24 \times 365 = \$ 216000$
 (Assume CO_2 cost = \$0.03/kg)

O_2 requirement per year = $1.3 \text{ mole IBT/s} \times 12 \text{ mole O}_2/\text{mole IBT}$ (from stoichiometry)
 Total O_2 cost per year = $\$0.2/\text{kg} \times 1.3 \text{ mole/s} \times 12 \times 32 \text{ g/mole} \times 10^{-3} \text{ g/kg} \times 3600 \times 24 \times 365 = \3149000
 $\$ 0.2 \times 545888160 \times 32 \times 10^{-3}$
 (Assume O_2 cost = \$0.2/kg)

Purification cost (adsorption into resin and then desorption by methanol and then distillation to separate IBT from methanol)

Distillation cost of separation of methanol to isobutanol

IBT production as bottom product of the distillation column

IBT production = 1 million gallon/year
 = $3.78 \times 10^6 \text{ L/year}$
 = $3.78 \times 10^6 \times 802 / (365 \times 24) \text{ g/hr}$ (density of IBT = 802 g/L)
 = 4.68 kmole IBT/hr (molecular weight = 74)

Feed composition of distillation column : 10 mole% of IBT and 90% of methanol

Total feed flow rate (IBT+Methanol) = 46.8kmole/hr

According to ASPEN simulation 99% IBT will be recovered with 11 stages.

Aspen: condenser heat duty: 494 kW, Reboiler heat duty 498 kW

Total heat load=992 kw

Yearly power requirement for distillation cost = $992 \times 365 \times 24$ kwh/year
=8689920 kwh/year

Electrical power supply cost for distillation per year = \$ 8689920 x 0.1 [Assume electrical charge=\$0.1/kWh]
=\$ 869000 (annual cost)

Results from ASPEN PLUS v8.2

Figure 70. Schematic diagram for distillation process used in ASPEN

Table 10 Heat duty for distillation based on ASPEN using NRTL model.

The screenshot displays the Aspen Plus software interface. The left-hand navigation tree shows the project hierarchy, with 'Results Summary' selected. The central pane shows the 'Dist' (Distillation) results summary for unit B1. The table below contains the data from this summary.

Dist	
Name	B1
Property method	NRTL
Henry's component list ID	
Electrolyte chemistry ID	
Use true species approach for electrolytes	YES
Free-water phase properties method	STEAM-TA
Water solubility method	3
Number of stages	11
Feed stage	6
Reflux ratio	0.19
Distillate to feed mole ratio	0.8999
Condenser pressure [atm]	1
Reboiler pressure [atm]	1
Condenser duty [kW]	493.537
Reboiler duty [kW]	497.341
Feed stage temperature [C]	67.7755
Top stage temperature [C]	64.7677
Bottom stage temperature [C]	104.35
Feed quality	9.1062E-08
Total feed stream CO2e flow [kg/hr]	0
Total product stream CO2e flow [kg/hr]	0
Net stream CO2e production [kg/hr]	0

Table 11 Results summary for distillation using ASPEN

Preheater cost for distillation

Assume initial temperature of methanol and IBT mixture coming from resin column is 30°C , and that the feed to distillation column is saturated liquid. The boiling point of 10% IBT and 90% methanol mixture is 67°C (From Aspen)

Yearly power requirement for pre heating to 67 °C from 30 °C = $mC_p(T_2-T_1)$

[as C_p of methanol = 79.5 J/(mol. °K), IBT=222 J/(mol. °K),

$C_{p\text{ mix}} = 79.5 \times 0.1 + 222 \times 0.9 = 93.75 \text{ J/(mole K)}$] from

(http://en.wikipedia.org/wiki/Methanol_%28data_page%29

http://www.solvents.basf.com/portal/load/fid228773/Isobutanol_e_03_08.pdf)

Power for preheating = $46.8 \times 24 \times 365 \times 93.75 \times (67-30) \text{ kJ}$

= 1422076500 kJ

= $1422076500 \times 2.7778 \times 10^{-4} \text{ kWh}$

= 395052 kWh/year

Electrical power supply cost for preheater per year = \$ 395052 $\times$ 0.1 [Assume electrical charge=\$0.1/kWh]

= \$ 39500 (annual cost)

Total electric cost for separation = \$ 869000 + \$ 39500 kWh/year

= \$ 908500 kWh/year

Cost of separation and purification of IBT = \$ 908497/10⁶ gallon = \$ 0.91/gallon

Summary of key annual costs:

Annualized capital cost (not including distillation and resin):

Reactor cost: \$ 160000/year

Capital cost per gallon = \$0.16/gallon

Annual operating cost (microbubble + separation and purification costs):

Pump power for microbubble generator + separation

= \$134000 + \$ 908000 = \$1042000

Operating costs/gallon = \$1.04/gallon

Raw material cost (H₂, CO₂ and O₂)

H₂ gas: \$ 2947000

CO₂ gas: \$ 216000

O₂ gas cost: \$ 3149000

Total gas costs: \$3163000 (not including O₂, assuming air will be used)

Raw materials cost/gallon = \$3.16/gallon

Task 14: Extended Strain/gene/experiment Library and ARPA-E restricted *R. eutropha* Wiki.

A strain/gene/experiment library was drafted with the intent to hold various strain and gene development work being done on this project. A screen shot in Figure 71, showing the library adapted from an earlier prototype, suggests how it could be developed to allow users to navigate through the developed strain hierarchy, examining the strain's genetic components and deletions, and to examine experiments in which the

strain was tested, as well as links to research references and experimental procedures used. The prototype was not adopted for use by the researchers, was never populated beyond initial sample data, nor underwent any structured use case or system testing. In practice, our library of strains, experiments, and protocols were maintained in shared files, principally using Microsoft Office Word and Excel, on a shared file server.

However, we did populate a Wiki to facilitate communication of protocols and exchange of ideas between the researchers and other ARPA-E awardees (Figure 72). Access to the wiki is restricted to invited members and any ARPA-E awardee can request an invitation. Initially all ARPA-E awardees working on *R. eutropha* were invited to join the wiki, currently all ARPA-E awardees are invited to participate.

Figure 71 Integrated strain/gene/experiment library.

Inserting heterologous genes into the *R. eutropha* genome at the *phaC* locus - Ralstonia eutropha - Windows Internet Explorer

https://wiki.mit.edu/confluence/display/Reutropha/Inserting+heterologous+genes+into+the+R.+eutropha+genome+at+the+ph...

File Edit View Favorites Tools Help

Google Search

Inserting heterologous genes into the R. ...

Dashboard > Ralstonia eutropha > Discussion Home > SOPs Browse John Quimby Search

> Inserting heterologous genes into the *R. eutropha* genome at the *phaC* locus

Inserting heterologous genes into the *R. eutropha* genome at the *phaC* locus Edit Add Tools

Added by [Christopher Brigham](#), last edited by [Christopher Brigham](#) on Dec 15, 2010 12:00

This is a general method for "knock-in" of PHA synthases from other organisms into the *Ralstonia eutropha* chromosome at the *phaC* (deletion) locus. This procedure serves as a general guideline. Individual experiences may vary.

1. Amplify the PHA synthase gene of choice by PCR. The gene can consist of the synthase ORF, or the ORF and a ribosome binding site. Engineer the PCR primers to contain restriction sites that will be used to facilitate cloning.
2. Digest the vector pJV7 (ask Sinskey lab for vector, see MTA on this website) with the restriction enzyme *Swa*I. (Note: pJV7 is similar to the plasmid pGY46, which was used to knock out the *R. eutropha* *phaC* gene. The difference is that pJV7 has a *Swa*I restriction site inserted between the 5' and the 3' flanking regions of homology surrounding the *phaC* gene. Additionally the *Bam*HI fragment in pJV7 is in the opposite orientation to the rest of the vector compared to pGY46)
3. If necessary, digest the PCR product (i.e. the synthase gene) with a restriction enzyme that leaves blunt ends (Note: the restriction site must be engineered into the PCR product. Typically, the restriction site is added to the primers used to amplify the gene). [Alternatively, clone the PCR product via TOPO cloning prior to excising the gene]
4. Ligate the synthase gene and the vector using T4 DNA ligase.
5. Transform competent *E. coli* using an aliquot of the ligation mixture. Select for transformants on LB + 50 µg/mL Kanamycin.
6. Select at least 6 Kanamycin resistant clones, and grow them in LB + 50 µg/mL Kanamycin broth.
7. Extract the plasmid in the Kanamycin-resistant clones by mini-prep.
8. Examine the map of pJV7, and examine the DNA sequence of the PHA synthase gene insert to determine what restriction enzyme to

Figure 72 *R. eutropha* (now ARPA-E Electrofuels) SOP Wiki.

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Annex 1: Reactions considered during autotrophic growth

Specific reaction for formic acid as substrate:

Specific reaction for CO₂ /H₂ as substrate (According to Bongers, litho-autotrophic metabolism has a P/O of 2 which is equivalent to the NADH):

Annex 2: Reactions considered during heterotrophic growth on Fructose

Annex 3: Reactions of the respiratory chain

O₂ as electron acceptor (according to Ishaque and Aleem (1970), NADH has a P/O of 2 and succinate has a P/O of 1):

Nitrate as electron acceptor:

ATP generation and dissipation:

Annex 4: Reactions of Isobutanol production

Annex 5: Additional reaction for NADPH supply

UdhA:

GapN:

II. Issues, Risks, and Mitigation

Issue #1: (MIT) Sparse lab space and safety regulations are challenges that must be overcome before autotrophic growth of *R. eutropha* can begin at MIT.

- ✓ We have looked at microbioreactors as possible autotrophic growth systems for *R. eutropha* to test for IBT production at MIT. Furthermore, we have entered into collaboration with the Way/Silver groups from Harvard's neighboring Wyss Institute of Biologically Inspired Engineering. We examined small autotrophic batch cultures of *R. eutropha* using their autotrophic growth setup. And we developed autotrophic fermentation with formic acid as carbon and energy source
- ✓ This issue has delayed examination of autotrophically grown *R. eutropha* gene expression via microarray analysis.

Issue #2: (MSU) All the commercial hollow fibers we have tested either have very tight skin layers or narrow pore structures, which makes it very difficult to embed the bacterial cells in the pores.

- ✓ We are now considering the development of biofilms either on the inside or on the outside surface of hollow fibers, possibly via surface coating the fiber to enhance attachment.

Issue #3: (MSU) The DCU OPC Server for Sartorius fermenter has not yet been installed properly on the computer so as to achieve data interchange.

- ✓ Due to the lack of technical support we are getting for the standalone Sartorius OPC server, we decided to upgrade to the full version control software Sartorius MFCS with OPC server.

Issue #4: (MSU) The shipment of Opto22 hardware components was delayed by 3 months due to the company's error.

- ✓ We are working on the installation to make up for the delay in progress.

Issue #5: (MSU) Due to the quality inconsistency in different batches of silicalite adsorbent, we are unable to pelletize silicalite using the established protocol.

- ✓ We decided to use commercially available polymeric resin as the adsorbent.

Issue #6: (MSU) The inherent safety concerns associated with the use of hydrogen gas presents challenges to scale-up.

- ✓ A joint research team consisting of scientists and engineers from Michigan State University and the adjacent Michigan Biotechnology Institute has been developing plans to scale up these fermentations. An extensive roadmapping activity identified risks and developed strategies to mitigate the risks. As a result of this analysis, the scale up initiative was approved by all parties involved in is advancing with high priority.

III. Changes in Approach

MIT: In our original task list, the deliverable of determining the necessity of NADPH for the Calvin Cycle was assigned. Since microbial Calvin Cycle pathways use NADH, as opposed to NADPH [35], that task as written no longer applies. However, NADPH is still predicted to play an important role in the IBT production pathway, as evidenced by our alcohol dehydrogenase studies. Therefore, we determined the value of a large intracellular NADPH pool not just for the Calvin Cycle, but for the whole IBT production pathway. We have also studied the cellular NADH/NADPH pools this quarter (starting January 2013), as efforts to produce IBT autotrophically are still challenging.

MIT: We have evaluated the use of microbioreactors for autotrophic growth experiments using *R. eutropha* strains. The purchase of a larger size bioreactor for autotrophic growth experiments is not feasible due to space constraints and safety concerns. But the technology is not yet ready for this purpose. Furthermore, we have entered into collaboration with the Way/Silver groups from Harvard's Wyss Institute of Biologically Inspired Engineering. We examined small autotrophic batch cultures of *R. eutropha* using their autotrophic growth setup.

MSU: Our new microbubble generator can generate microbubbles in the absence of surfactants. Methods are being explored to use hydrodynamics to prevent bubble coalescence. If successful, this approach will eliminate the need for surfactants, thereby reducing operating costs for the scaled-up process.

MSU: We have discovered that the polymeric resin is effective at removing IBT from both the gas and liquid phases, and that methanol is effective for desorbing IBT from the resin. As a result, we have expanded work on that separation strategy and reduced work on the alternative IBT separation strategy of gas absorption.

MSU: We have obtained strong evidence that IBT catabolism significantly limits product formation under autotrophic conditions, both when the cells are immobilized in the hollow fiber membrane and when they are grown in suspension culture. As a result, we have begun exploring bioreactor-engineering solutions to this problem, and in July, 2013 we submitted an invention disclosure on a promising approach.

IV. Key Personnel

Here is a list of personnel that have joined our group since the beginning of the project:

- Claudia Gai, a postdoctoral associate, joined the MIT group on November, 2010, and left the group on November of 2012.
- Daan Speth, a Master's Degree student, joined the MIT group on December, 2010 and left the group on August of 2011.
- Sean Elliott, a visiting scholar from Boston University, joined the MIT group as of September, 2011 and left the group on January of 2012.
- John Qiang Fei, a postdoctoral associate has joined the MIT group on November of 2011, and left the group in January 2013.
- Amanda Bernardi, a visiting undergraduate student, joined the MIT group on April 2012 and left the group in December 2012.
- Sophia Li, an MIT undergraduate student, has joined the MIT group as of June 2012, and left the group in May 2013.
- Stephan Grunwald, a visiting Master's degree student, has joined the MIT group as of September 2012
- Estelle Grousseau, a postdoctoral scholar, has joined the MIT group as of September 2012.
- Soumen Maiti, a postdoctoral associate has joined the MSU group as of September, 2011.

V. Project Output

A. Journal Articles:

Brigham, C.J., C.S. Gai, J. Lu, D.R. Speth, R.M. Worden, and A.J. Sinskey (2012) Engineering *Ralstonia eutropha* for Production of Isobutanol (IBT) Motor Fuel from CO₂, H₂, and O₂. Advanced Biofuels and Bioproducts (J.W. Lee, Ed.). DOI: 10.1007/978-1-4614-3348-4_39.

Copy Sent to Program Director? ____yes (sent to B. Haendler)_____

Lu, J., C.J. Brigham, C.S. Gai, and A.J. Sinskey (2012) Studies on the production of branched-chain alcohols in engineered *Ralstonia eutropha*. Appl Microbiol Biotechnol. 96(1): 283-297.

Copy Sent to Program Director? ____yes (sent to B. Haendler)_____

Fei, Q., Brigham C.J., Lu, J., Fu, R., Sinskey, A.J. Production of branched-chain alcohols by recombinant *Ralstonia eutropha* in fed-batch cultivation. 2013 Biomass and Bioenergy 56: 334-341

Copy Sent to Program Director? ____yes (sent to B. Haendler & C. Haynes)_____

Maiti, S. K., Liu, Y. C., Sinskey, A.J., Worden, R.M. Isobutanol Production in Bioreactor for Incompatible Gases (BIG): 1. Experimental Studies (in preparation)

Liu, Y. C., Maiti, S. K., Worden, R.M. Isobutanol Production in a Bioreactor for Incompatible Gases (BIG): 2. Simulation Studies (in preparation)

Maiti, S. K., Liu, Y. C., Sinskey, A.J., Lira, C.T., Worden, R.M. In-situ Isobutanol Recovery from an Autotrophic Fermentation in a Bioreactor for Incompatible Gases (in preparation)

B. Papers:

Bernardi, A.C., C.S. Gai, C.J. Brigham, J. Lu, and A.J. Sinskey. Isobutanol tolerance in *Ralstonia eutropha*. Boston Bacteria Meeting, 2012, Cambridge, MA.

Brigham, C.J., Lu, J., Gai, C.S., and Sinskey, A.J. *Ralstonia eutropha* and the de novo synthesis of isobutanol. SBE Electrofuels, 2011, Providence, RI.

Gai C.S., Brigham, C.J., Lu, J. and Sinskey, A.J. Carbonic Anhydrases: key enzymes in CO₂ metabolism in *Ralstonia eutropha*. SBE Electrofuels, 2011, Providence, RI.

Gai C.S., Lu J., Speth D.R., Brigham C.J., and Sinskey A.J. Characterization of Carbonic Anhydrases in *Ralstonia eutropha*. Boston Bacteria Meeting, 2011, Cambridge, MA.

Lu, J., Brigham, C.J., Gai, G.S., and Sinskey, A.J. Isobutanol production in recombinant *Ralstonia eutropha*. Boston Bacteria Meeting, 2011, Cambridge, MA

Lu, J., Brigham, C.J., Gai, G.S., and Sinskey, A.J. Characterization and modification of the 2-ketoisovalerate biosynthesis enzymes in *Ralstonia eutropha* H16. SBE Electrofuels, 2011, Providence, RI.

Worden, R.M., Liu, Y, and Maiti, S, “Mass Transfer Issues in Gas-Intensive Fermentations,” SBE Electrofuels, 2011, Providence, RI.

Copy Sent to Program Director? ___Y _____

C. Status Reports:

Quarterly update to ARPA-E. October 15, 2010 (First status report);
Quarterly update to ARPA-E. January 15, 2011 (Second status report);
Quarterly update to ARPA-E. April 15, 2011 (Third status report);
Quarterly update to ARPA-E. July 15, 2011 (Fourth status report);
Quarterly update to ARPA-E. October 15, 2011 (Fifth status report);
Quarterly update to ARPA-E. January 15, 2012 (Sixth status report);
Quarterly update to ARPA-E. April 15, 2012 (Seventh status report);
Quarterly update to ARPA-E. July 15, 2012 (Seventh status report);
Quarterly update to ARPA-E. October 15, 2012 (Eighth status report)
Quarterly update to ARPA-E. January 15, 2013 (Ninth status report)
Quarterly update to ARPA-E. April 15, 2013 (Tenth status report)
Quarterly update to ARPA-E. July 15, 2013 (Eleventh status report)
Quarterly update to ARPA-E. Oct 15, 2013 (Twelfth status report)

D. Media Reports:

Title: Teaching a Microbe to Make Fuel

Written by: David Chandler, MIT News Office

URL: <http://web.mit.edu/newsoffice/2012/genetically-modified-organism-can-turn-carbon-dioxide-into-fuel-0821.html>

Press release: <http://web.mit.edu/press/2012/genetically-modified-organism-can-turn-carbon-dioxide-into-fuel.html> (Contact = Sarah McDonnell)

E. Invention Disclosures:

Title: Reactor for incompatible gaseous reactants

Date Submitted: May 10, 2013

Agency Submitted to: MSU Technologies

PI Name: R. Mark Worden

Title: Using GapN (TadD) enzyme from *Rhodococcus opacus* PD630 to modulate intracellular reduced cofactor pool size.

Date Submitted: Disclosure document in preparation

Agency Submitted to: MIT TLO, Massachusetts Institute of Technology

PI Name: Anthony J. Sinskey

Title: Construction and application of a hyper-biofilm-forming strain of *Ralstonia eutropha*.

Date Submitted: Disclosure document in preparation

Agency Submitted to: MIT TLO, Massachusetts Institute of Technology

PI Name: Anthony J. Sinskey

F. Patent Applications:

Title: *Catalytic Bioreactors and Methods of Using Same*

International Application No. PCT/US2012/053958

Date: Filed September 6, 2012

PI Name: Robert Mark Worden

Title: Bioreactor for Incompatible Gases

Date Submitted: October 11, 2010

Agency Submitted to: MSU Technologies, Michigan State University

PI Name: Robert Mark Worden

Invention disclosure has been converted into a provisional patent application and submitted to the US Patent Office.

G. Licensed Technologies:

None

H. Networks/Collaborations Fostered

Name of Network/Collaboration: ARPA-E *R. eutropha* Discussion Group (Wiki)

Entities Involved: MIT (principal), MSU, OPX-Bio, NREL, Lawrence Berkeley Labs, Ohio State University, Battelle, Inc., Ginkgo BioWorks, Harvard University, ARPA-E

Date of Agreement: N/A

Brief Description of Network/Collaboration: We have set up a Wiki-based collaboration with other ARPA-E awardees. This collaboration platform will help exchange protocols and ideas, and potentially lead to future partnerships in this burgeoning field of electrofuels.

Name of Network/Collaboration: Carbonic Anhydrase (Akermin)

Entities Involved: MIT (principal), Akermin, Inc.

Date of Agreement: TBD

Brief Description of Network/Collaboration: We are beginning discussions for evaluation of *R. eutropha* carbonic anhydrase activities and properties. The partner involved in this discussion is Akermin, Inc.

Agreement and Material Transfer Agreements (MTA) to be established.

Name of Network/Collaboration: MIT/Harvard ARPA-E collaboration

Entities Involved: MIT, Wyss Institute of Biologically Inspired Engineering (Harvard).

Date of Agreement: TBD

Brief Description of Network/Collaboration: We are beginning discussions on a collaboration between the Sinskey group (MIT) and the Silver/Way group (Harvard) involving projects related to the electrofuels project. Discussions have commenced in the form of joint group meetings.

Name of Network/Collaboration: Facility to Scale Up Gas-Intensive Fermentations

Entities Involved: MSU, Michigan Biotechnology Institute

Date of Agreement: TBD

Brief Description of Network/Collaboration: Special facilities and expertise needed to engineer and scale up fermentations that consume large amounts of gas per unit of product are being assembled at MSU and the adjacent Michigan biotechnology Institute. Emphasis is being placed on fermentations based on flammable and or explosive gaseous fermentation substrate. The intent is to provide a central site for engineering and scale up of such fermentations. A mass-transfer column has been designed to measure mass transfer coefficients for various types of microbubble generators, and a 300-L fermentor is being equipped for microbubble mass transfer experiments under realistic bioreactor conditions.

Name of Network/Collaboration: MIT/LBL ARPA-E collaboration

Entities Involved: MIT, Lawrence Berkeley Laboratories, Logos Technologies, Boston University, others.

Date of Agreement: TBD

Brief Description of Network/Collaboration: We are beginning discussions on a collaboration between the Sinskey group (MIT) and Steven Singer's group (LBL) involving projects related to second round (scale up) funding for the electrofuels project. Discussions have commenced in the form of skype meetings. Reagents have been exchanged between the Sinskey group and the Singer group, and the Singer group have constructed an electrofuel-producing strain based on these reagents, which has been sent to Logos for fermentation.

I. Websites Featuring Project Work or Results

Name of Website: None

Specific Webpage: None

Brief Description: N/A.

J. Other Products (e.g., Databases, Physical Collections, Audio/Video, Software, Models, Educational Aids or Curricula, Equipment or Instruments)

Brief Description: A Wiki-based collaboration has been set up, and is up and running currently.

Date of Release: December 7, 2010

Provided to: See part H, and others TBD

K. Awards, Prizes, and Recognition

Jingnan Lu has received a 2013 MIT Graduate Women of Excellence award from MIT in part for research done in this project.

VI. Follow-On Funding

Additional funding committed or received from other sources (e.g., private investors, government agencies, nonprofits) after effective date of ARPA-E Award.

Source	Funds Committed or Received
None	\$0.00

VII. Recipient and Principal Investigator Disclosures

None of the referenced conditions exist for the Prime Recipient, Subrecipient, or Principal Investigators (including Co-PIs).

VIII. Conflicts of Interest Within Project Team

There are no conflicts of interest within the Project Team.

IX. Performance of Work Within the United States

100% of the Total Project Cost is being and has been performed within the United States, specifically in Massachusetts and Michigan.

VII. Project Schedule Status

Task Title	Schedule				
	Start Date	Finish Date	Actual Start	Actual Finish	Estimated % Complete
Remove IBT Production Pathway Bottlenecks	7/15/10	1/15/12	7/15/10		95%
Deletion of PHA Production Genes	7/15/10	10/15/10	7/15/10	9/15/10	100%
Optimization of <i>kivd</i> Expression and Enzyme Activity	7/15/10	1/15/11	7/15/10	1/15/13	100%
Insertion of <i>kivd</i> into production strain	7/15/10	10/15/10	9/15/10	10/15/10	100%
Optimization of Relevant Branched-Chain Amino Acid Production Pathway Genes/Enzymes	7/15/10	4/15/11	9/15/10		90%
Optimization and Heterologous Expression of Alcohol Dehydrogenase Gene	7/15/10	1/15/11	8/15/10	1/15/12	100%
Construction of a Synthetic “IBT Production Operon”	10/15/10	1/15/12	7/15/10	7/15/12	100%
Eliminate ATP Requirement to Convert NADH to NADPH via Transhydrogenase	1/15/11	7/15/11	10/15/12		80%
Determine Need for NADPH for <i>R. eutropha</i> Calvin Cycle	1/15/11	4/15/11	10/15/12	1/15/11	100%
Assess IBT production yield depending on NADPH generation pathway	1/15/2013	4/15/2013	1/30/2013	4/15/2013	100%
Assess Utility of UdhA for NADH Conversion	1/15/11	10/15/11	10/15/12		70%
Assess Utility of GapN for NADH Conversion	1/15/11	10/15/11	10/15/12		70%
Form <i>R. eutropha</i> Biofilm	10/15/11	1/15/12	4/15/11	1/15/12	100%
Reduce Oxygen-Induced Inhibition of <i>R. eutropha</i> Metabolism	7/15/11	7/15/12			50%
Expression of carboxysome	7/15/11	7/15/12	10/15/2012		50%
Increase <i>R. eutropha</i> Tolerance to IBT	7/15/10	7/15/12	1/15/11		90%
Gene Expression Analysis of IBT-treated <i>R. eutropha</i> Cultures	7/15/10	1/15/11	10/15/10	10/15/12	100%
Assess IBT/Alcohol Efflux Systems	10/15/10	10/15/11	7/15/11	10/15/11	100%
Increase Alcohol Tolerance of <i>R. eutropha</i> Strains	7/15/10	10/15/11	1/15/11		90%

Major tasks outlined in Proposal (Control #: 0206-1526) shaded in grey.

Task Title	Schedule				
	Start Date	Finish Date	Actual Start	Actual Finish	Estimated % Complete
Construct an Efficient, Scalable Autotrophic Fermentation Bioreactor	7/15/2010	1/15/2012	7/15/2010		
Develop detailed process flow diagram for BIG system	7/15/2010	10/15/2010	7/15/2010	10/1/2010	100%
Order bioreactor system components	7/15/2010	10/15/2010	7/15/2010	01/16/2012	100%
Validate operation of walk-in fume hood in Worden lab	7/15/2010	10/15/2010	7/15/2010	10/31/2010	100%
Assemble BIG components in walk-in hood	10/15/2010	1/14/2011	12/15/2010	2/10/2012	100%
Design and order components of Opto-22 based monitoring and control system	7/15/2010	1/15/2010	7/15/2010	01/10/2012	100%
Design and order components of microbubble generator	7/15/2010	10/15/2010	7/15/2010	11/30/2010	100%
Purchase and install gas chromatograph	7/15/2010	10/15/2010	7/15/2010	10/15/2011	100%
Measure rate of <i>Ralstonia eutropha</i> growth on fructose inside the spongy layer of various hollow fibers	10/15/2010	1/15/2011	9/15/10	10/15/2012	100%
Select type of hollow fiber to be used for BIG system studies	1/15/2011	4/15/2011	1/15/2011	05/15/2011	100%
Measure rate of <i>Ralstonia eutropha</i> growth in medium containing various non-toxic surfactants	1/17/2011	7/15/2011			No longer necessary due to improved technology
Select surfactant to be used for BIG system studies	4/15/2011	7/15/2011			No longer necessary
Measure rate of oxygen mass transfer between lumen-side liquid and spongy layer of hollow fibers	4/15/2011	10/15/2011	04/15/2011	12/15/2011	100%
Measure rate of gas mass transfer between shell side and spongy layer of hollow fibers	10/15/2011	1/15/2012	10/10/2011		90%
Characterize rate of fructose and isobutanol mass transfer between tube side and spongy layer of hollow fibers	10/15/2011	4/15/2012	01/16/2012		80% Filtration inoculation method eliminated need to measure fructose
Develop and validate mathematical model describing mass transfer	1/15/2012	4/15/2012	09/01/2011	06/01/2013	95% Not all modeling constants could be measured independently
Develop and validate	1/15/2012	7/15/2012	01/15/2012		95%

mathematical model describing <i>R. eutropha</i> growth and product formation kinetics within the spongy layer of hollow fibers					Not all modeling constants could be measured independently
Install Opto 22 system capable of measuring and controlling key variable for continuous operation of BIG system	4/15/2011	7/15/2011	04/15/2011	04/15/2012	100%
Tune Opto22 control system	7/15/2011	1/15/2012	04/10/2012	05/15/2012	100%
Measure performance of BIG system during continuous run with alternating heterotrophic growth on fructose and autotrophic product formation on hydrogen, carbon dioxide and oxygen	1/15/2012	7/15/2012	04/10/2012		95% Fructose feeding was not required during the autotrophic IBT production experiments
Develop an In-Situ IBT Recovery System for the Bioreactor					
Measure performance of column packed with resin having high affinity for isobutanol during adsorption from water stream and regeneration using heated air stream	1/15/2011	4/15/2011	01/15/2011	04/01/2013	100%
Measure performance of trickle-bed column using countercurrent flow of aqueous isobutanol stream and air stream	7/15/2011	1/15/2012	11/15/2011		30%, but no longer necessary due to improved technology
Use mathematical models suitable for process scale-up to analyze key performance properties adsorption and desorption kinetics, height of a theoretical plate, etc.) for isobutanol separation systems	1/15/2012	7/15/2012	1/15/2012	09/17/2013	100%
Integrate BIG system operated continuously with isobutanol recovery system	7/15/2012	1/15/2013	02/01/2013	07/30/2013	100%
Use mathematical models to analyze and optimize system performance during continuous operation	1/15/2013	4/15/2013	01/15/2013	09/30/2013	100%
Use mathematical models to predict system performance for scaled-up BIG system	1/15/2013	7/15/2013	01/15/2013	09/30/2013	100%

VIII. Budget Status Lead Recipient

Final Lead Recipient Budget Status is to be found in the ePIC reporting system.

IX. Budget Status Subrecipient

Final Subrecipient Budget Status is to be found in the ePIC reporting system.

X. Certification of Compliance

Persons receiving funding under DE-FOA-0000206 (Electrofuels), DE-FOA-0000207 (BEEST), DE-FOA-0000208 (IMPACCT), DE-FOA-0000288 (ADEPT), DE-FOA-0000289 (BEETIT), and DE-FOA-0000290 (GRIDS) are required to complete and sign the following certification.

I have the authority to make the following certification on behalf of the Lead Recipient named above. On behalf of the Lead Recipient, I certify that this project – i.e., the entirety of the work performed under the Award, including all work performed by the Lead Recipient, subrecipients, and contractors -- is in compliance with the “Equipment Purchases” and “Performance of Work in the United States” requirements set forth in Award Attachment 1, Clauses 18 and 20. On behalf of the Lead Recipient, I further certify that the information provided in this Quarterly Progress Report is accurate and complete as of the date shown below. I understand that false statements or misrepresentations may result in civil and/or criminal penalties under 18 U.S.C. § 1001.

SIGNATURE:

DATE: 10/15/2013

TYPED NAME: John Quimby

TITLE: Lab Manager

ORGANIZATION: MIT