Synthetic Microbial Consortium for Isobutanol Production
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Abstract

Lignocellulosic biomass may be a potential solution to the world’s energy dilemma after corn ethanol is accused of rising grain prices\textsuperscript{1}. Coming from agriculture residues, such biomass can be fermented to generate biofuels such as ethanol. However, the fermentation process is so complicated that it is difficult to be completed by any one single microbe. Thus, microbial consortia, which consist of multiple microbial populations, could be utilized to carry out such processes. Being a frontier microbial technique, microbial consortia have been applied to wastewater treatment\textsuperscript{2}, programmed pattern formation\textsuperscript{3} and human genome project\textsuperscript{4}. The goal of this project is to construct part of a synthetic tunable microbial consortium, which will allow us to engineer multiple species to work together to generate isobutanol, a promising next generation biofuel, from lignocellulosic biomass.

Introduction

We would like to control the synthetic microbial consortium via the exchange of essential metabolites, such as the amino acids tryptophan and tyrosine, so that we can tune the growth rate and composition of the consortium. Manipulating the coculture growth rate and composition would then allow us to tune the production of desired byproducts. A similar system\textsuperscript{5} has already been constructed by Alissa Kerner, Lin’s Lab, UM PhD student. Auxotroph strain W3 can secrete the amino acid tryptophan, which is required by strain Y3, while Y3 secretes tyrosine, which is
required back by W3. Arabinose serves as an inducer to tune the secretion of tryptophan from strain W3, and sodium propionate is the inducer for tuning Y3’s tyrosine production. (Clear mechanism is shown in graph 1) Since the other strain is the only source of vital molecule require for growth, the growth rate of the auxotroph can be tuned by the amount of inducer adding.

The goal of this research is to construct isobutanol production auxotroph strains based on Alissa’s experiment; and also build up part of the lignocellulosic biofuel production microbial consortia using such strains. For the 10-week research period, we worked on construction and growth studies of normal C6(use glucose as sugar source) and C5(xylose) specialist auxotroph strains; analyzed the productivity of isobutanol and the consumption of sugars.
Results and Discussion

Our way to construct the microbial consortia is by setting up a genetic circuits in two E.coli strains: strain C6/W(#1), an auxotroph overproduces tyrosine[Tyr] and mainly utilizes six-carbon sugar source; and strain C5/Y(#4), an auxotroph overproduces tryptophan[Trp] while utilizing five-carbon sugar source. The genetic construction process is very similar to the one Alissa Kerner, Lin's Lab, UM PhD student conducted, even under the conditions when isobutanol production plasmids (pSA55 and pSA69, they are provided from the Liao Lab, UCLA) are inserted and some oligo-related knockout on the C5 strain (ΔptsG, Δglk, ΔmanX) which was done by another lab member, Jeremy Minty16. For the C6/W(#1) strain, a two-step construction is needed to lead to Tyr overproduction: 1) over express yddG gene which manipulate YddG protein, an aromatic amino acid exporter, to export more Tyr, 2) knockout tyrR, a gene shows inhibition on the express of yddG6. For the C5/Y(#4) strain, construction is relatively more complicated since utilization of five-carbon sugar source is not a wild type phenotype, modifications is needed for this part. However, Trp overproduction is well studied and gene construct is commercially synthesized6. In previous studies, we tried to use P1 transduction17, a process which utilize the special property of P1 phage to recombine genes into transduced strain, and vector pCP20 to knockout tyrA, which was not successful; so we designed another pathway, which starts with P1 transduction to insert a ΔRed-cat-sacB cassette. This cassette contains the ΔRed genes, which promote Red-mediated homologous recombination14.
After transduction, only the selection plate (cm 25) with both E.coli strain and P1 virus had colonies, which was as expected and suggested that transduction was successful. To further prove the result, colonies were streaked out twice (to prevent phage attached on strain cells), stored for PCR verification. We ran PCR with both bioanalyzer and EtBr, however the results did not prove that the transduction was 100% successful. Only J4 (one of the C5/Y(#4) transductant strains) shows consistent results: weak band around ~3000bp with the lambda-cat-sacB-del primers, and high bp bands similar to the positive control strain TUC1 (graph 2).

*graph 2. PCR verification with EtBr gel*

strains from left to right: (+)TUC01, (-)JCL260 C5, 1kb ladder, (-)K12, K1, K2, K3, K4, J1, J2, J3, J4. (All K and J are transductants after P1 transduction) Notice that the upper two bands of (+)TUC01 and J4 are very similar, which means J4 had the correct transduction.

Besides the genetic circuits construction, we also worked on estimating the growth
rates, sugar consumption and isobutanol production of the C5/C6 strains without tuning. Two flask experiments were run at the end of winter semester and early summer. OD reads and plate counts yielded the growth rates data, and the saved samples were analyzed lately using HPLC to yield sugar and isobutanol composition data. Below are the summarized results, growth rates results are as expected (graph 3), wild type K12 has the highest growth rate at around 0.87e-6 hr⁻¹ followed by C6 strain grows in glucose media, then C6 strain in xylose media, and the lowest was C5 strain grows in xylose media with around .31~.34e-6 hr⁻¹. Because we modified C5 strains more than C6 strains and since each modification may significantly affect growth rates, the results are reasonable.

![Growth rate comparisons, all have pSA55/69](image)

graph 3
Sugar consumption and isobutanol production results, on the other hand, revealed great necessity of improvement, especially for the C5 strain. For the C6 strain, utilization of glucose was high (~35g/L in 100 hrs, *graph 5*) compared with wild type K12 (~9g/L in 100 hrs, *graph 6*) and produced decent amount of isobutanol (~8g/L in 100hrs, *graph 5*). For the C5 strain however, utilization of xylose and isobutanol production were poor and from *graph 7* and *graph 8* we can see that even C6 strain (~30g in 60hrs) utilizes more xylose than C5 (~23g in 60hrs). In future studies, we could either modify the oligo pathway of our current C5 strain, or try to use a different C5 strain; the Liao Lab from UCLA mentioned a new C5 strain called NV3 (r1, r2)\textsuperscript{13} in a new published paper, which might be helpful to our studies.
Average JC6 in M9IPG+YE

- **glucose**
- **isobutanol**

**Graph 5**

K12

- **K12 1**
- **K12 2**

**Graph 6**
Graph 7

Graph 8. JC5 HPLC analysis from 4-12-2012
graph 9. JC5 HPLC analysis from 5-30-2012

graph 10
The goal of our study is to construct a tunable microbial consortia. The next step of our work will be inserting the inducer manipulation gene to the isobutanol-produced strains. Then we will be able to control the composition of these two strains by changing the amount of inducers added. If this is successful, we could combine the outcome with other research projects in the Lin Lab, which focus on constructing cellulolytic specialist specie, to generate a whole circuit of producing lignocellulosic biofuel, which is also known as consolidated bioprocessing (CBP)\textsuperscript{7}. Lignocellulosic biomass, which is added to the circuits, will be hydrolyzed by the enzyme secreted from the cellulolytic specialist; then the product pentose, glucose, and mono- and oligosaccharides will be fermented by our auxotroph strains to produce final isobutanol product. (Clear process is shown as \textit{graph 11})
Methods and Materials

Strains  E.coli MG1655 (K12) was used as (-) control in ΔRed-cat-sacB PCR verification and growth rate estimation flask experiments; E.coli TUC01 was used as (+) control in ΔRed-cat-sacB PCR verification and donor strain in P1 transduction. E.coli JCL260 C5 (ptsG::FRT glk::FRT manX::FRT) was used as (-)control in PCR verification and infected strain in P1 transduction.

Media  Luria Broth (LB) media with 25 mg/ml Cm antibiotics was used in transduction selection process to test ΔRed-cat-sacB cassettes; M9IPG and M9IPX mixed with Yeast Extract were used in C5/C6 flask experiments.

P1 transduction  E.coli JCL260 C5 (ptsG::FRT glk::FRT manX::FRT) and MG1655((-)control) were grown overnight under 37C, after centrifuged condensed cell pellets were mixed with MC buffer and TUC01 P1vir lysate. After 30 mins incubation under 30C, 100 µL of 1M Na Citrate (stored in 4C) and 1mL LB were added and cells were incubated under 30C for another 3hr. 100 µL of 1M Na Citrate were added and samples were poured on dried LB Cm 25 plates for selection. After 1 day of 30C incubation, ~10 colonies were found on plate with both cells and P1vir, while none of the control plates had colony showed up.
HPLC Analysis

500 µl of each sample collected from flask experiments were spin down with centrifuge, 12,000 rpm for 1 min, after filtered with 0.2 µm filter (Restek), 100 µl of each sample were aliquot to the labeled HPLC small volume vial. The injection volume of the HPLC was set to be 5 µl, flow rate to 0.5 ml/min, pressure to < 70 bar, column temp to 60°C and VWD – default 210 nm; RID.

PCR verification

Three PCRs were used to test correct transduction of \( \Lambda \text{Red-cat-sacB} \). Both Bioanalyzer and EtBr gel methods were used. Used primers included \( \text{lambda-cat-sacB-del, lambda_cat_sacB_fwd} \) and \( \text{lambda_cat_sacB_rev} \), enzyme was PHU HS.

**Primer design and Analysis:**

\( \lambda \text{-cat-sacB:} \) ~11,600 bp (+)control and transductants

\( \text{cat-sacB:} \) ~3000 bp (+)control and transductants

\( \text{WT:} \) ~2,312 bp (-)control
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