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Title: Increasing bacterial tolerance and metabolism of the biofuel, n-butanol using community-level evolution and functional genomics

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Increasing bacterial tolerance and metabolism of the biofuel, n-butanol using community-level evolution and functional genomics

Thesis Proposal for the Master's Degree of Kayley You Mak
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Abstract

n-butanol is a strong biofuel candidate since it is more energy dense and less volatile than ethanol. The bottleneck for industrial production of biobutanol is the toxicity; most microbes cannot survive about 1.5% v/v. We propose to develop bioremediation capability along with the biofuel technology to mitigate future spills and their consequences. Two main approaches were used: community-level screening and functional genomics, both followed by directed evolution to increase biodegradation. For the first approach, a bacterial library from historic bioremediation projects was re-isolated and re-characterized, then mixed into artificial communities of ~20 strains ("master mixes"). These master mixes were screened for tolerance to butanol, and continuously exposed to increase tolerance. Promising communities were then tested for growth with butanol as the sole carbon source. Secondly, we looked for bacteria with alcohol dehydrogenase enzymes and looked to increase butanol metabolism. We found that the tolerance for n-butanol may be improved with repeated exposure, but it was difficult to switch from tolerance to metabolism. Bacterial community dynamics may be influenced by n-butanol concentration, and there was putative butanol metabolism found with both research approaches.

Introduction

Modern life relies on burning fuel and with the climate crisis, there is growing demand for biofuels. The first widely produced alcohol, as opposed to biodiesel, were bioethanols from plants. Over time, biobutanol has become a better option for automotive fuel in internal combustion engines, while being able to utilize existing fuel pipelines[1]. Some reasons include biobutanol is more energy dense than bioethanol, less volatile, and can be mixed with gasoline at higher percentages.[1–3] Fuel spills when processing and transporting the quantities needed are inevitable. We could better prepare for future biofuel spills by simultaneously researching bioremediation.

Bioremediation is the use of microorganisms to extract or degrade pollutants. Traditionally, humans have removed contaminated waste to a less desirable area. However, this is not long term sustainable, thus bioremediation to transform or degrade toxic waste into less harmful compounds is desirable and can be more cost efficient. For things that rarely occur in nature, microorganisms have not had the evolutionary time to develop metabolic pathways. Thus, directed evolution techniques could be used on microorganisms to metabolize these novel, toxic carbon sources.

n-butanol, also known as 1-butanol, is a straight chain four-carbon alcohol compared to ethanol's two-carbon structure. n-butanol can be produced from biomass (biobutanol) or fossil fuels (petro-butanol).[4] Butanol is naturally fermented by *Clostridium acetobutylicum* and *Clostridium beijerinckii* with the acetone-butanol-ethanol fermentation that creates these compounds in a 3:6:1 ratio.[5,6] It is currently used as an industrial solvent, but being developed as

a biofuel. 1-butanol is toxic with concentrations of 1.5-2% being inhibitory for most organisms.[6–8] The butanol is polar and can therefore disrupt cell membranes by increasing their fluidity, leading to a loss of membrane function and cell growth.[9,10] Documented cellular responses to butanol include controlling membrane composition.[10–12]

This study aims to further explore the metabolic pathway and increase the rate of metabolism of the toxic n-butanol compound in bacteria. Several approaches were used; long-term growth assays, spatial evolution, directed community-level evolution, and functional genomics leading to single-strain experiments. Preliminary experiments were conducted to explore range-finding for time scales of bacterial community evolution to toxic compounds.

Basic Methods

A bacterial library was re-isolated and re-characterized from about 550 strains of bacterial glycerol stocks form historical bioremediation projects. The original glycerol stocks were grown in 28°C in Lennox Luria Broth (LB, BD Biosciences) liquid medium overnight or until visible biomass, then selected for single cell isolates on LB with 1.5% agar plates. A single colony was then picked and grown again in Lennox LB, then frozen into a fresh 10% glycerol stock and aliquoted for 16S sequencing. For genus-level identification, 16S sequencing was completed as either Sanger sequencing at GeneWiz (Azenta Life Sciences, Plainfield, NJ) or on a MiSeq 2000 at Los Alamos National Laboratory.

The re-isolated bacterial strains were randomly combined into 28 artificial communities ("master mixes") of 12-50 strains (mean=20), as well as putative genus-specific master mixes. The bacterial strains were predominantly *Enterobacter* (Table 1). Additionally, *Escherichia coli* K12 and *Pseudomonas putida* KT2440 were ordered from the American Type Culture Collection (ATCC) and used in this study.

Table 1. The number of single cell isolate bacterial cultures in each genus in the bacterial library.

Class	Genus	Number of isolates
Actinomycetia	*Brachybacterium*	6
	Micrococcus	1
	Arthrobacter	2
	Kocuria	1
Alphaproteobacteria	*Caulobacter*	2
	Methylobacterium	8
Bacilli	*Bacillis*	43
	Priestia	3
	Staphylococcus	4
Betaproteobacteria	*Comamonas*	2
	Cupriavidus	32
	Burkholderia	1
	Ralstonia	1
	Delftia	1
	Acidovorax	1
Flavobacteriia	*Flavobacterium*	1
	Chryseobacterium	8
Gammaproteobacteria	*Acinetobacter*	11

	Enterobacter	151
	Leclercia	1
	Pseudomonas	26
	Stenotrophomonas	2
-	Uncharacterized from 16S	254

Preliminary experiments with negative results

Long-term growth assays

Long-term growth assays with large communities were exposed to 3% or 5% v/v butanol. Triplicate flasks were inoculated with 1mL of high cell-count combinations of 16 master mixes that were grown overnight in minimal media with 25mM glucose. The flasks were monitored for long-term growth, with OD_{600} measurements taken more frequently as cultures began to grow (Figure 1a). Once growth was observed in 3% cultures, they were transferred to two fresh flasks with 3% or 4% butanol. The community composition in the highest growing flask in 3% butanol was characterized (Figure 1b). However, when transferred to new flasks again, this community did not continue to grow.

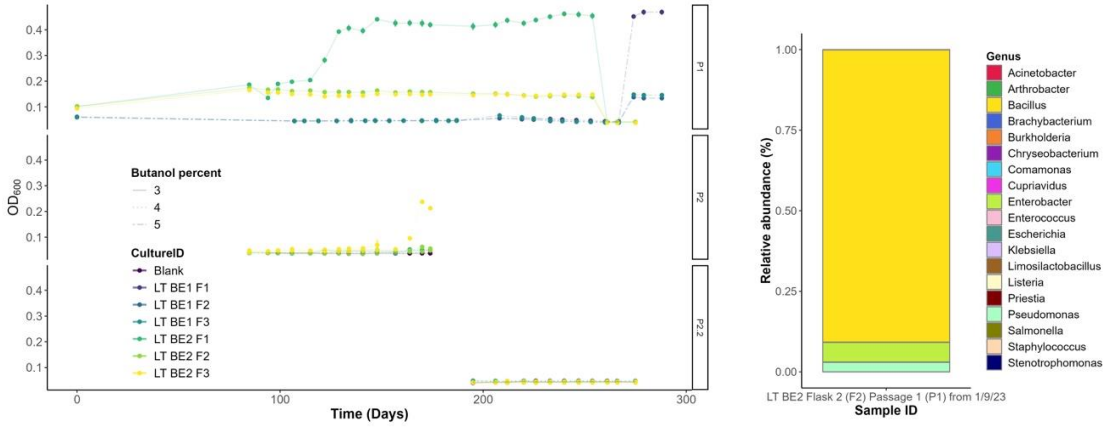


Figure 1. A) Growth curves measured with OD_{600} of each biological replicate flask over time B) Community composition of the best-performing community in minimal media with 3% butanol from 16S data.

Spatial evolution

Microbial Evolution and Growth Arena (MEGA) plates as introduced by Baym et al. 2016 offer a spatial agarose growth area to study microbial evolution.[13] Based off this work where antibiotic resistance evolved over spatial gradients by having a "swim agar" on the top, we designed 100% scale MEGA plates that are 10.5in W x 22.5in L (Figure 2). The MEGA plates have five underlying "bricks." Above the bricks we used a "mortar" layer to provide an even surface over which we poured a "face" of swimming agar (4g/L). The bricks and mortar were solid agar at 15g/L. Our 75% scale MEGA plates internal measurements are 17in W x 8in L. We used the bricks to create a linear gradient over which to evolve our bacteria.

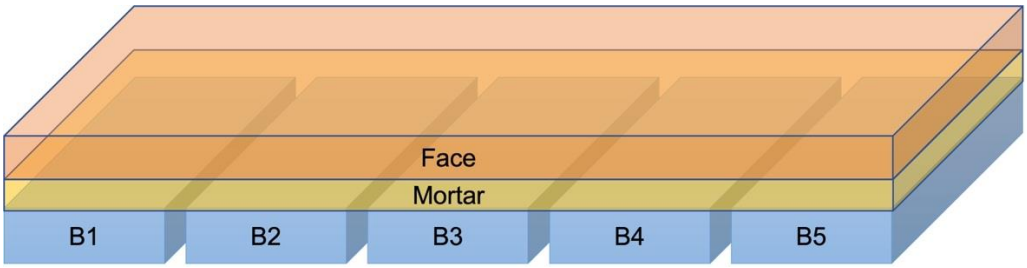


Figure 2. Schematic of the Microbial Evolution and Growth Arena (MEGA) plates.

To evolve butanol metabolism, we poured MEGA plates with a gradient from carbon-rich Lennox LB to Standard Minimal Medium with no glucose added. From left to right the bricks were: (1) 100% LB, (2) 66% LB + 33% Minimal Medium with 25mM glucose, (3) 33% LB + 66% Minimal Medium with 25mM glucose, (4) 100% Minimal Medium with 25mM glucose, and (5) 100% Minimal Medium with 0mM glucose. The mortar and face were also 100% Minimal Medium with 0mM glucose. The butanol did not spread evenly across a warm agarose solution, nonetheless, 3 MEGA plates were inoculated with bacterial master mixes and left to grow. Growth spread most easily over the edges as the (Figure 3). The culture was then taken into liquid medium with butanol and was not successfully grown (Figure 1Figure 4).

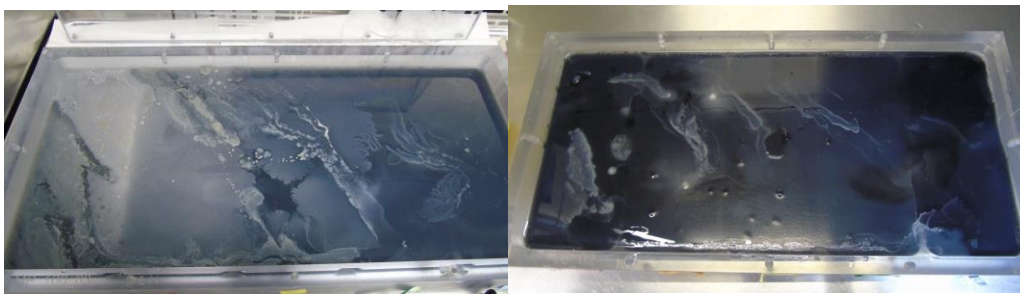
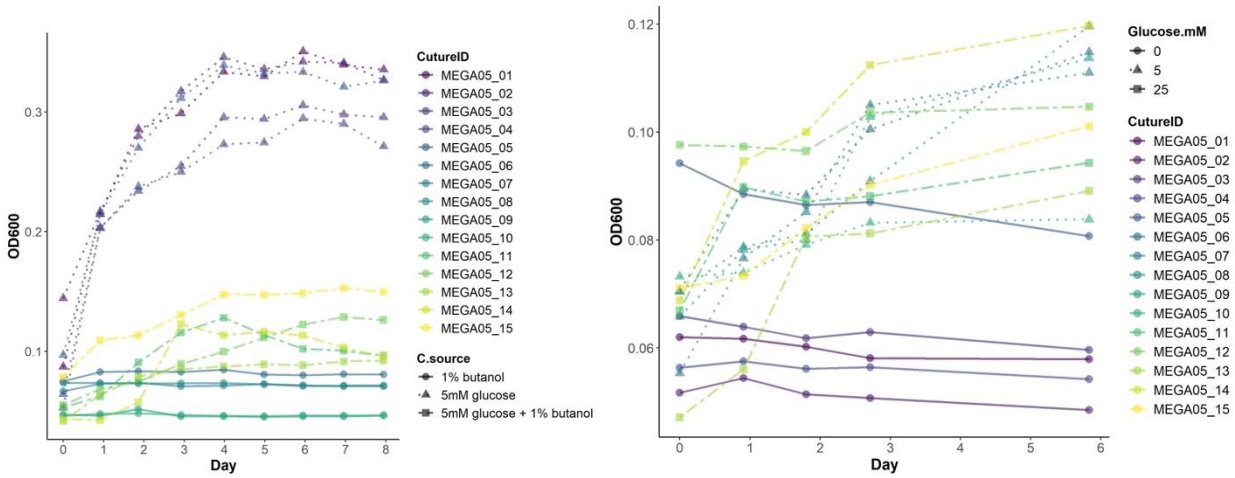


Figure 3. Overhead photograph of the 100% and 75% scale MEGA plates with a gradient from carbon-rich Lennox LB to standard Minimal Medium with no glucose added. Bacterial growth in white spreading across the heterogenous surface.



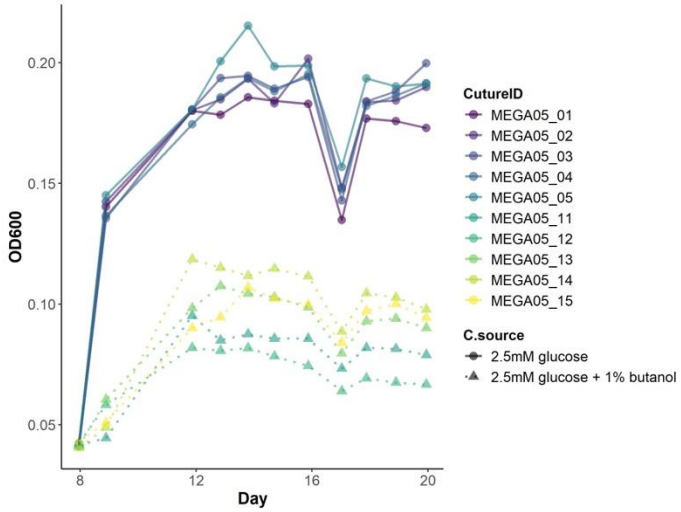


Figure 4. Growth curves of OD_{600} over time in days of bacterial cultures picked from MEGA plates with 1% butanol. (A) Cultures grew in minimal media with 5mM glucose, but not with 1% butanol nor 5mM glucose + 1mM butanol. (B) Cultures did not grow in minimal media with 1% butanol plus 0, 5, nor 25mM glucose. (C) Cultures grew in minimal media with 2.5mM glucose, but not with 2.5mM glucose + 1% butanol.

Community-level evolution for tolerance

Artificial communities (master mixes) of bacteria were created from the re-isolated library. These communities were then tested for tolerance to various concentrations of butanol, and exposed to that concentration for a community-level evolution for tolerance to butanol as these experiments were conducted in carbon-rich medium. The pre-evolved and evolved communities were compared. The evolved community was then attempted to be weaned off of the carbon-rich media, but was unable to successfully transition from tolerance of butanol to metabolism of butanol.

A tolerance assay was conducted on 7 bacterial master mixes, plus three individual strains: *Enterobacter* PB2, *Arthrobacter* A1, and *Escherichia coli* K12. A range of butanol concentrations (0%, 1%, 2%, 5%, or 10% v/v) was added to Lennox LB, a carbon-rich medium. Triplicate cultures were grown at 28°C on an orbital shaker at 120 RPM. Nothing grew in 10% butanol, but one master mix grew in 2% and several grew in 1% butanol (Figure 5a). From this experiment, the top three performing cultures that successfully grew in 1% butanol, the culture that grew in 2% butanol, and their corresponding 0% controls from the tolerance assay were treated as biological replicates for a community-scale evolution experiment. *E. coli* K12 served as a negative control. The three biological replicates of each culture were grown for 10 passages in LB ± 1% or 2% butanol. The control 0% cultures were diluted when they reached an $OD_{600} \geq 1$. The 1% and 2% butanol cultures were knocked back every 3-4 days. OD at 600nm was measured daily, and glycerol stocks were made at passages 1, 5, and 10 for 16S amplicon sequencing. A Zymo Research Quick-DNA Fungal/Bacterial Miniprep kit (catalog no. D6005) was used to extract DNA. QC was done with Qubit 3.0 and e-gel. Sequencing was done on Illumina MiSeq. Cultures continued to grow in their butanol conditions (Figure 5b) and community composition was shaped by butanol percentage (Figure 6).

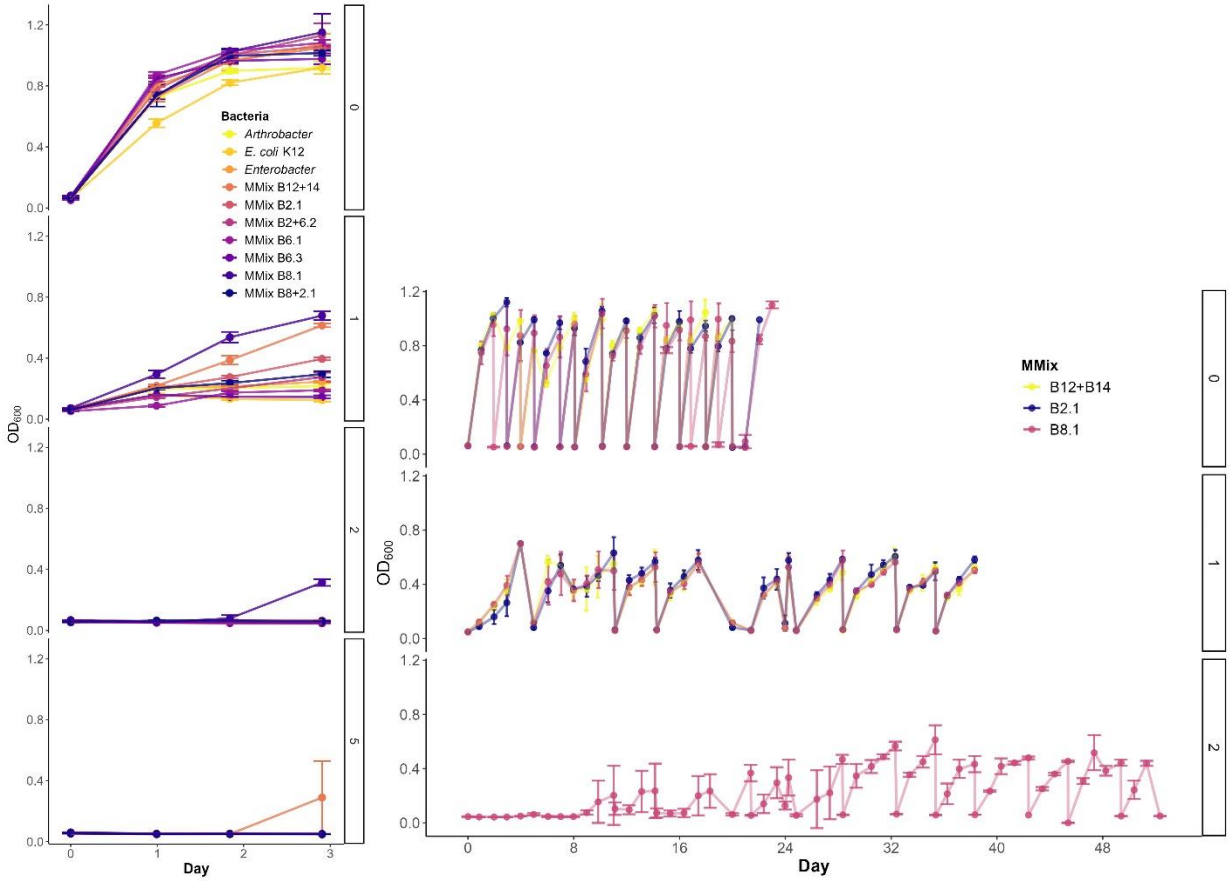


Figure 5. Growth curves of OD_{600} over time of (A) the tolerance assay of each bacterial single strain or master mix at 0%, 1%, 2%, and 5% butanol and (B) the top-performing master mixes grown in 0%, 1%, or 2% butanol for 10 passages.

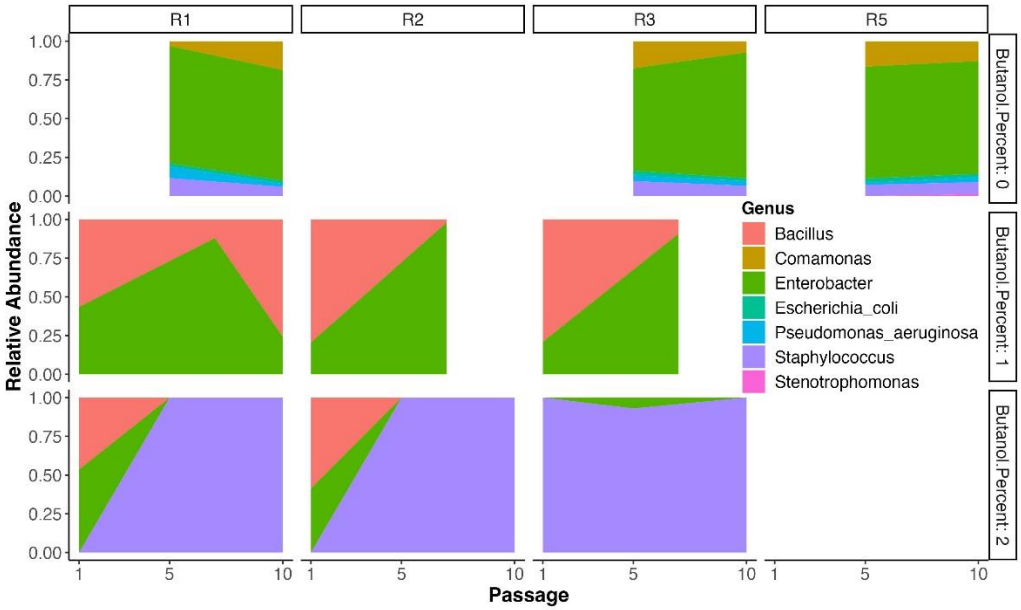


Figure 6. The community composition of the master mixes grown at 0%, 1%, and 2% butanol at the end of passages 1, 5, and 10.

To validate the continuous exposure, the three 2% master mix cultures were grown in triplicate flasks from the passage 1 and passage 10 glycerol stocks to compare differences in growth rate after the 10 passage community-evolution. The growth did appear faster after 10 passages (Figure 7). Furthermore, five sequential plate passages of single-cell isolates were completed on 9 plates showing 2 morphologies and 16S sequenced. These were identified as *Staphylococcus* and *Bacillus* but did not continue to grow in the presence of butanol as a single-strain isolate (

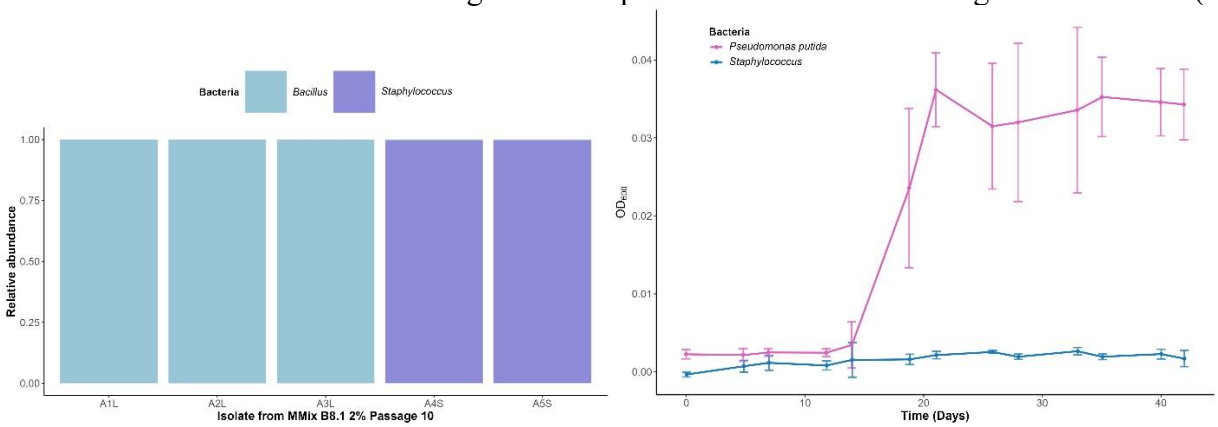


Figure 8).

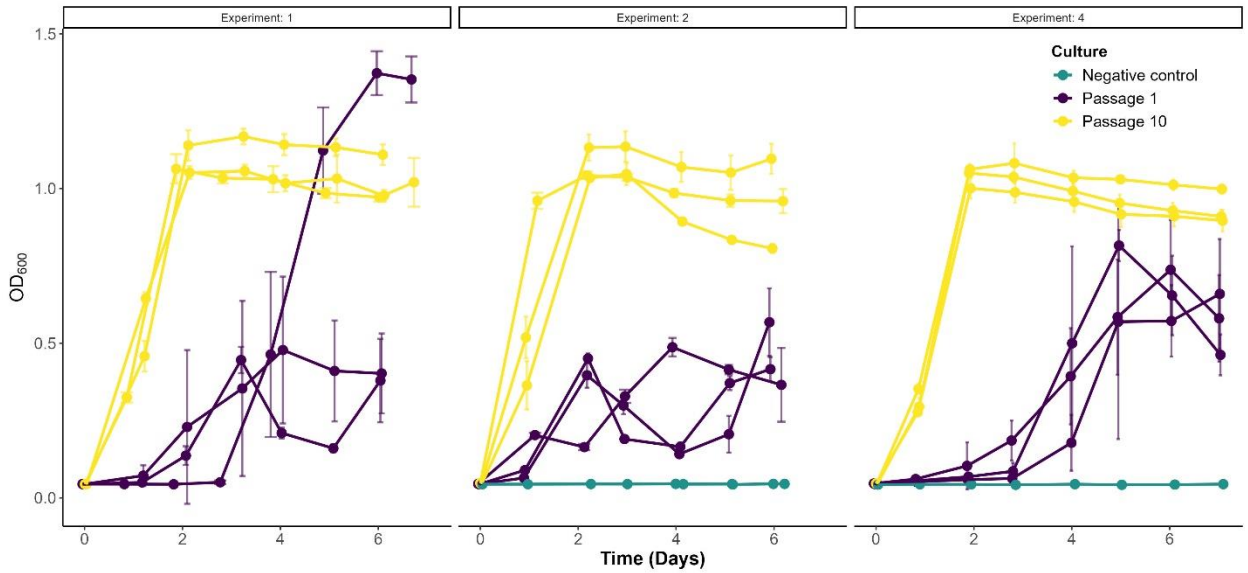


Figure 7. The comparative growth of master mix B8.1 in Lennox LB with 2% butanol after passage 1 (purple) and passage 10 (yellow) of the community evolution for tolerance experiment.

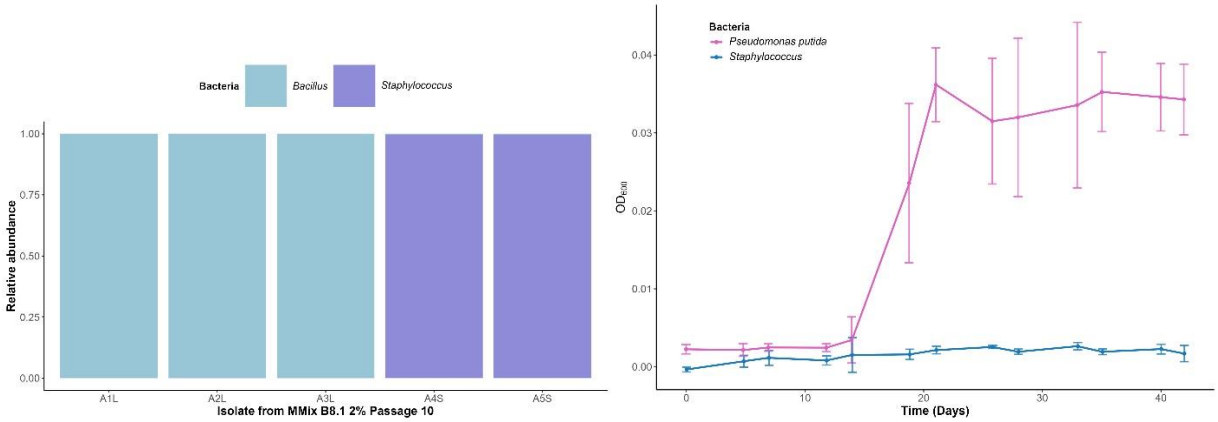
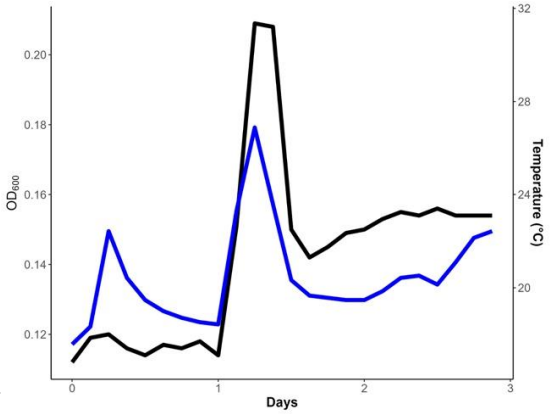


Figure 8. (A) The 16S data showing the genus-level identification of the two morphologies of bacteria isolated from passage 10 of master mix B8.1 in 2% butanol. The smaller colonies were *Staphylococus* (purple), and the larger colonies were *Bacillus* (blue). (B) As *Staphylococcus* was more predominant in the community, the single-strain was grown in Minimal Media with 1% butanol, but did not grow (blue).

The evolved master mix B8.1 from 2% butanol cultures were then attempted to be weaned off of LB plus butanol to a carbon-less Minimal Media plus butanol. A complete shift killed the cultures, so triplicate flasks were grown in nutrient-rich LB plus 2% butanol and transferred every 4 days to a lower percentage of LB combined with Minimal Media with 2% butanol. This was attempted twice as the cultures could not be grown in butanol without LB (Figure *9*). The three components of LB were tested by being added to Minimal Media with 2% butanol, to see which was the limiting factor. Using a LogPhase600 instrument containing four 96-well plates with 150uL of culture at 30°C shaking at 800 RPM, the OD_{600} was measured every 20 minutes. The OD_{600} measurements were correlated with the external temperature of the



lab, and did not lead to conclusive results (

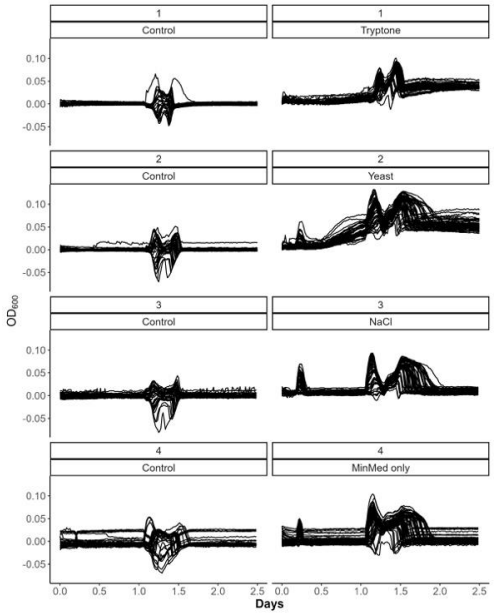
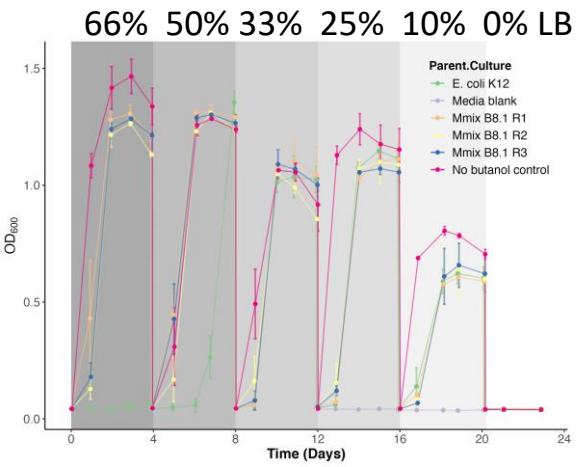


Figure 10). The community-level evolution approach appeared to increase tolerance to butanol, but could not shift from butanol tolerance to metabolism.



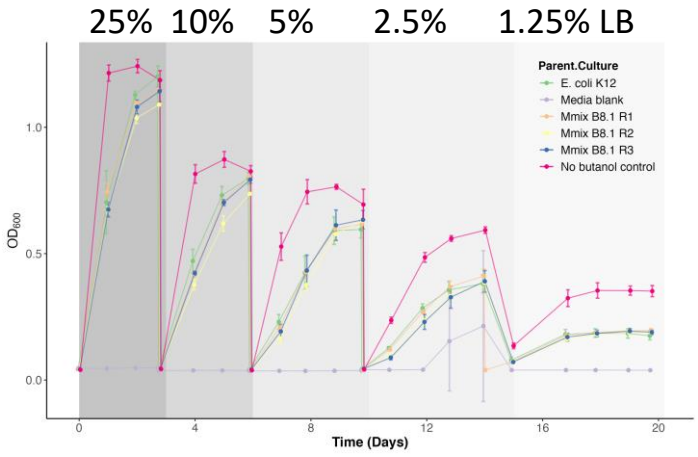


Figure 9. The growth of evolved master mix B8.1 in 2% butanol grown in mixtures of Lennox LB and Minimal Media with 2% butanol. Percentages indicate percent Lennox LB in each growth cycle.

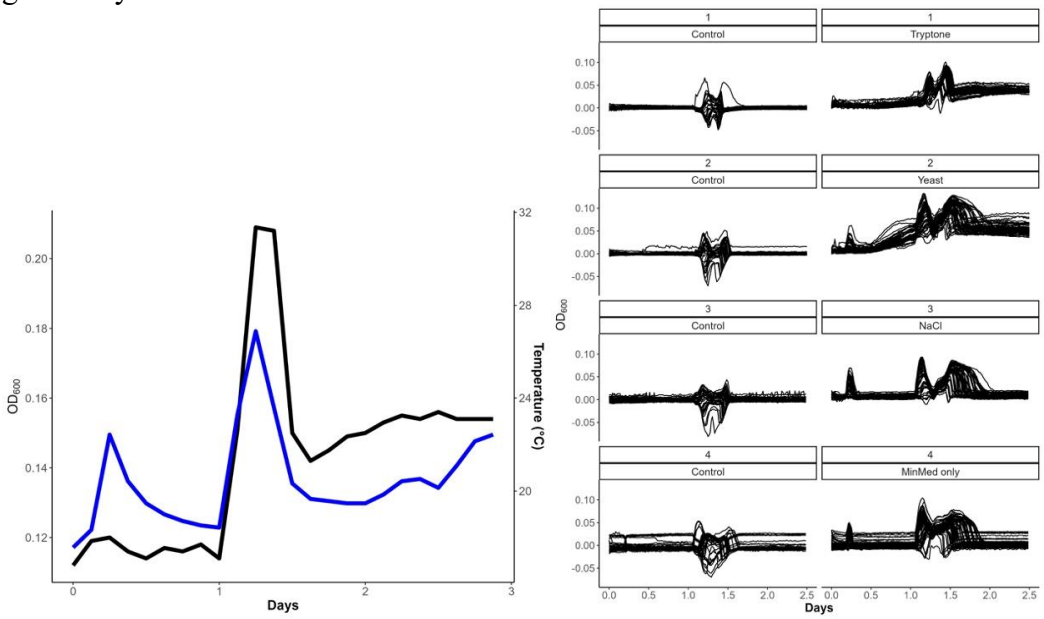


Figure 10. (A) The temperature in the lab (blue) compared to the measured OD from the LogPhase600 (black). (B) The blank subtracted growth curves from the evolved master mix B8.1 in 2% butanol in Minimal Media with 2% butanol plus or minus a component of LB.

Methods and Results

Community-level evolution for metabolism

The community-level evolution approach did appear to increase butanol tolerance of the community over 10 passages, the experimental approach was re-done with less carbon in the growth medium to promote butanol tolerance from the initial growth. Based off of all experiments, master mixes including *Pseudomonas*, *Bacillus*, and *Staphylococcus* were selected to create the initial community for the metabolism evolution experiment. The cultures were grown at 100mL in 250mL screw top glass Erlenmeyer flasks to reduce potential evaporation of butanol. All cultures were grown at 25°C, shaking at 120 RPM, and diluted by treatment when the flasks reached above OD_{600}≈0.25. The cultures were grown for 10 passages in Minimal Media with 5mM glucose plus 0%, 1%, or 2% butanol. The 1% butanol cultures were then continued into Minimal Media with no glucose plus 1% butanol for 10 passages, then 1.5% butanol (Figure 11). The growing cultures were also pushed to higher butanol percents (1%, 1.25%, and 1.5%) in Chi.Bio chemostats. The cultures are growing well, and in the sequencing queue for 16S community analysis over time. There is preliminary GC-FID data comparing the amount of butanol in bacterial flasks to their corresponding media blanks at the end of the growth cycle, as well as NMR data for the same comparison.

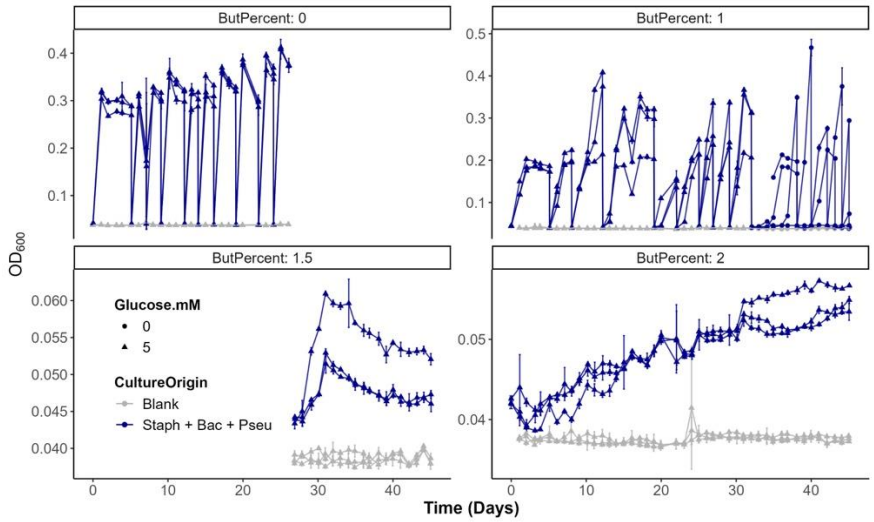


Figure 11. Growth curves of 3 master mixes including top-performing genera *Staphylococcus*, *Bacillus*, and *Pseudomonas* grown in Minimal Media with 5mM glucose for 10 passages with 0, 1, 1.5, or 2% butanol. 1% butanol cultures were then continued to be grown in Minimal Media with 0mM glucose with 1% butanol for 10 passages.

Functional genomics approach

To use a functional genomics approach, the pfam (protein families) database was searched for bacteria with multiple alcohol dehydrogenase enzymes. Alcohol dehydrogenase enzymes are the suggested first step to breaking down compounds like butanol to butyl aldehyde.[14] Searching for a readily available biosafety level 1 bacteria in a genera known to metabolize a variety of compounds with multiple alcohol dehydrogenases led us to the *Pseudomonas putida* strain KT2440. The KT2440 strain has been documented to degrade a wide variety of compounds including nicotinic acid[15], napthenic acids[16], and benzoate[17] and was purchased from ATCC (American Type Culture Collection, Manassas, VI).

KT2440 has been studied in terms of tolerance and degradation of butanol[14,18,19], but we were aiming to increase the metabolism and growth rate. In our experiments, KT2440 showed an aptitude for growing in minimal media with 1% butanol as the sole carbon source, although this growth was not consistent (Figure 12). Several experiments using KT2440 were completed in either 20mL chemostat reactors, 7mL in 13mL culture tubes, 100mL in screw-top pyrex 250mL Erlenmeyer flasks, or 200uL in 96-well plates. All experiments were done in Minimal Media with 1% butanol, 25mM organic compound A, or 1% butanol plus 25mM organic compound A as the carbon source. Orbital shaking was 120 RPM for flasks, 210 RPM for tubes, a stir rate of 0.4 for chemostats, and 355 cpm for 96-well plates. All experiments were conducted at 25°C.

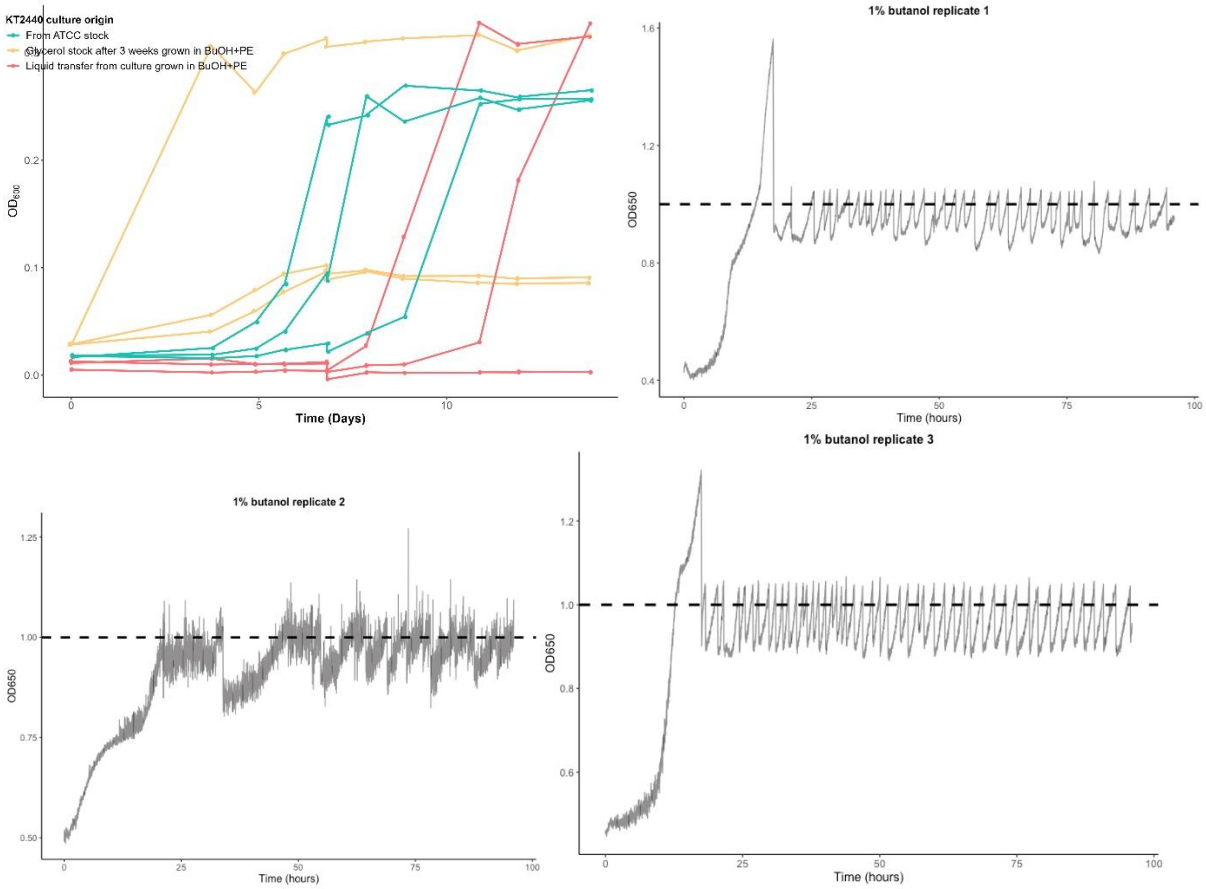


Figure 12. (A) An example of growth curves of KT2440 in 1% butanol + 25mM organic compound A directly from the ATCC stock, a pre-exposed glycerol stock, or a liquid transfer from the pre-exposure. Each biological replicate shown as a separate line. (B-D) Growth curves from 3 chemostat reactors with KT2440 in 1% butanol over time.

Based on inconsistent growth found at the beginning of an emerging co-metabolism that stabilized over time[20], we hoped to see a similar pattern and conducted a long-term growth study of KT2440 in butanol and co-metabolism with an organic compound A of interest. Co-metabolism has been shown to increase growth by using a target compound as a non-growth substrate[21,22]. Cultures were 7mL in polystyrene culture tubes in a 25°C incubator on an orbital shaker at 120 RPM in Minimal Media with either 1% butanol, 25mM of organic compound A, or 1% butanol plus 25mM organic compound A. Growth was inconsistent at first, so 500uL culture were transferred to fresh media every other week. Once cultures seemed to be reaching stationary phase, cultures were transferred every week. The culture in just organic compound A did not grow consistently, so was seeded from the co-metabolism culture as necessary. KT2440 may grow better in butanol with continuous exposure (Figure 13). The concentration of both butanol and organic compound A were close to expected at the end of every passage, as quantified with NMR (Figure 14).

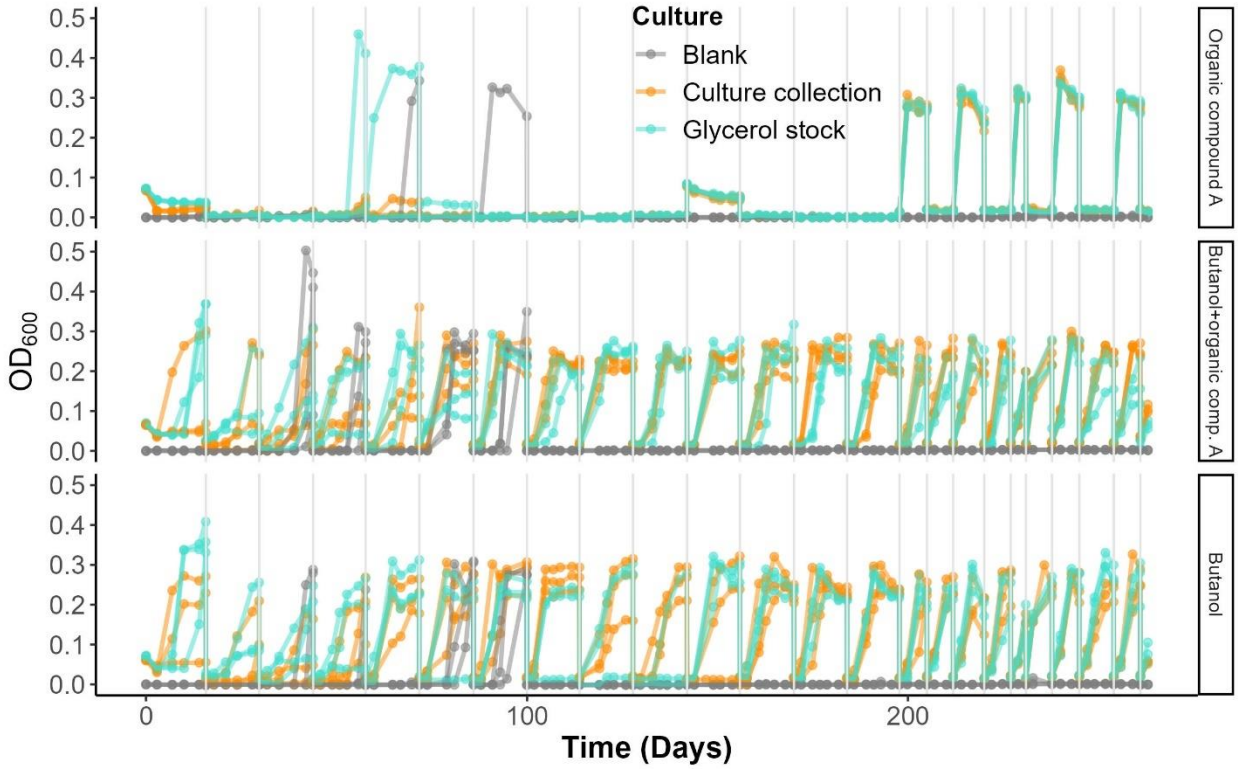


Figure 13. Long-term growth of *Pseudomonas putida* KT2440 in minimal media with 1% butanol, 25mM organic compound A, or 1% butanol + 25mM organic compound A over time. KT2440 directly from the ATCC stock ("Culture collection" in orange) or after a 2-week pre-exposure to butanol and organic compound A ("Glycerol stock" in green) were grown with media blanks (grey). Cultures were transferred every 2 weeks (grey vertical lines), until it seemed most cultures were reaching stationary phase growth and cultures were transferred every week.

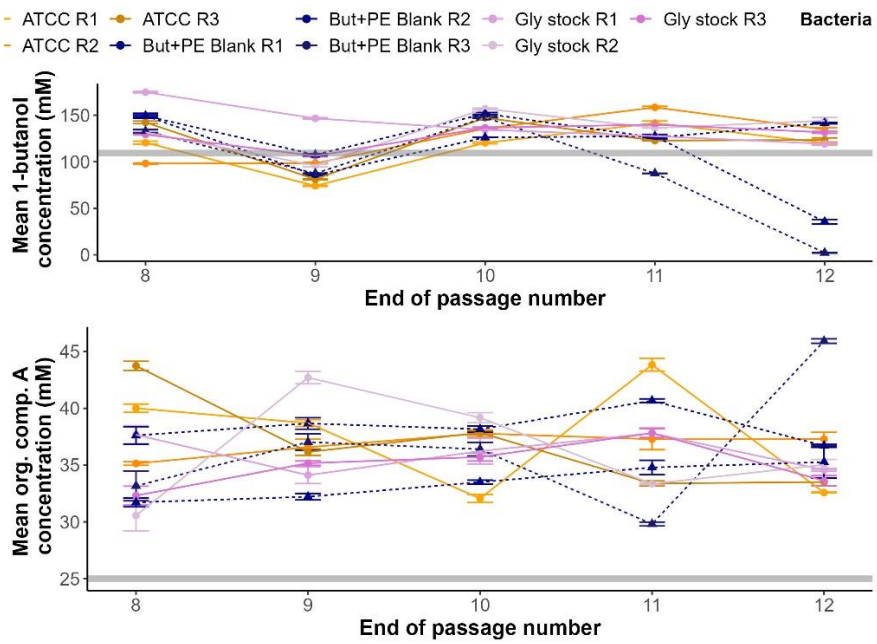


Figure 14. NMR quantification of butanol and organic compound A at the end of a passage in the bacterial cultures compared to the media blanks.

Future work

Experimental work

To finish the butanol evolution experiment, biomass pellets for 16S sequencing have already been submitted to look at the community composition over time. Additionally, samples for GC-FID have been saved as cultures in 4°C at time of transfer to compare the amount of butanol in bacterial cultures compared to media blanks at the end of growth cycles. This analysis was started but has been put on hold due to gas system repairs. One more experiment to compare the growth of the evolved versus the ancestor from the 1% evolution is planned to be completed as well. The analogous experiment for the long-term KT2440 cultures is also planned. The experiments should be completed by the end of February.

Data analysis/visualization and writing

Plotting has been done in R (version 4.2.2, R Core Team), using packages including: ggplot2, dplyr, ggpubr, ggpmisc, viridis, zoo, ggforce. Plots will be modified for readability and clarity. Genomic analyses were done in Qiime 2 using EDGE and CLC Genomics Workbench. Statistical data analysis will be completed in R. To test data normality, Shapiro-Wilk tests for conducted. Then growth rates will be compared with one-way ANOVA or Student's t-test if the data are normal, or the non-parametric Kruskal-Wallis test or Mann-Whitney U-test if not. From there, the full thesis writing can be completed. More background literature will be added to the introduction and discussion sections to tell a more comprehensive story about the importance of microbial bioremediation and successful approaches used.

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