

Abstract

Oil spills continue to cause significant environmental damage because existing remediation approaches are often slow, inefficient, or environmentally disruptive. The engineering design goal investigated whether the fast-growing, commonly available inexpensive baker's yeast, *Saccharomyces cerevisiae*, can be genetically engineered to degrade hydrocarbons to advance sustainable bioremediation aligned with UN Sustainability Development Goal 9.

CRISPR gRNAs were designed in-silico using ChopChop and Benchling platforms to target two genes in *S.cerevisiae*. The alkane hydroxylase (*alkM*) gene from *Acinetobacter baumannii* was codon-optimized for yeast, commercially synthesized and cloned into a yeast expression plasmid with independent promoters and terminators for CRISPR/Cas9-mediated Knock-in. The ADE2 gene in *S.cerevisiae* was designed for knock-out via CRISPR/Cas9 homology-directed repair, producing red colonies that enabled visual and quantitative transformation efficiency assessment. Hydrocarbon degradation was evaluated gravimetrically over a 20-day incubation in sterile diesel-amended soil inoculated with the yeast having Cas9-only control plasmid and transformed with Cas9+gRNA plasmid. All in-vitro experiments were conducted under BSL1 conditions using commercially prepared competent yeast cells and plasmids.

Transformation efficiency exceeded 75% based on red versus white colony counts. Expressions of *AlkM* alone did not yield efficient hydrocarbon degradation. Co-expression of *AlkG* (rubredoxin) and *AlkT* (rubredoxin reductase) genes with *AlkM* into a single multigene plasmid (re-synthesized) enabled measurable diesel degradation (~49% with 95% confidence interval of +/- 3%).

Limited degradation efficiency reflects the absence of optimized downstream β -oxidation pathways for long-chain hydrocarbons in yeast. Future work could integrate peroxisomal β -oxidation pathway engineering. Results demonstrate the feasibility of engineering fast-growing yeast for scalable, innovative hydrocarbon bioremediation.

INTRODUCTION

- The **United Nations Sustainable Development Goals (UNSDG)** are a blueprint to achieve a better and more sustainable future for all. **UN SDG 9** is centered around Industry, Innovation and Infrastructure: Build resilient infrastructure, promote sustainable industrialization and foster innovation.
- Oil contamination is one of the most dangerous pollution factors known today** requiring sustainable and innovative cleanup techniques in alignment with UN SDG 9
- Oil spills have been a major issue across decades. The **Taylor Energy oil spill**, initiated by Hurricane Ivan on September 16, 2004, is the **longest-running offshore oil disaster in U.S. history**, with almost 0.03- 0.05 tonnes oil/per day estimated to leak and continue even today near the Louisiana coast
- A major **diesel oil spill** occurred as recent as **June 2025 in Baltimore's Inner Harbor** that released nearly 5000 gallons of oil into the water. Diesel engine oil, which is one of the major products of crude oil, constitutes a major source of pollution in our environment through wrecks of oil tankers carrying diesel oil, cleaning of diesel tanks by merchants and war ships carrying diesel oil.
- Scientists have found many ways to clean oil spills up but these **traditional techniques don't clean up spills efficiently**, which is a big problem because the leftover oil could lead to drastic side effects such as reduced plant growth in diesel oil contaminated soils
- Acinetobacter baumannii is a marine bacterium** which can utilize the hydrocarbons in oil as **they have special hydrocarbon breaking genes called alkane hydroxylase (AlkM) genes**
- Marine bacteria**, which naturally degrade oil, **grow slower** than other bacterial strains and yeast cells like the **inexpensive baker's yeast, Saccharomyces cerevisiae** (commonly available).
- CRISPR** allows researchers to **precisely cut and alter the DNA of a cell**. It consists of two components: **Cas9**, an enzyme that cuts DNA and is often referred to as molecular scissors, and a **synthetic RNA molecule called a gRNA**, which can be programmed to recognize specific sites in the genome

The Engineering goal for this research project is **"to use CRISPR-CAS9 to genetically transform Baker's Yeast Cells (Saccharomyces cerevisiae) with AlkM Gene from Acinetobacter baumannii to degrade hydrocarbons in order to clean up oil spills thereby working towards sustainable bioremediation"**.

There are two research objectives / design success criteria for the research project as below

Primary - *S. cerevisiae* that has been genetically transformed (having the *AlkM* gene knocked-in via CRISPR/Cas9) and inoculated into diesel-amended soil should demonstrate a hydrocarbon degradation efficiency of at least 50% compared to the control group.

Secondary - The ADE2 gene in *S. cerevisiae* designed for knock-out via CRISPR/Cas9 should produce red colonies and have a transformation efficiency of at least 75% when counted against original white colonies of the control group (non-transformed yeast).

METHODS

Final Design Procedures (Dry & Wet Lab)

Following are final and revised design of gRNA (Dry Lab)

The gRNA matches the gene that we want to change and tells the Cas9 protein where to cut. The only requirement is that the gene have two guanine(G) nucleotides or two cytosine(C) nucleotides next to each other. This is referred to as the **PAM or Protospacer Adjacent Motif** and is indicated by the sequence NGG or CCN where "N" means any nucleotide. URA3 is a gene on chromosome V in *S. cerevisiae* yeast cell. The plasmids to be transformed will contain a **URA3 selection marker**.

ADE2 Gene Knock-out for secondary research objective :

Use the Saccharomyces Genome Database (www.yeastgenome.org) to analyze the ADE2 gene sequence.Go to the website <http://chopchop.cbu.uib.no/> and choose following - For 'In', choose Saccharomyces cerevisiae, For 'Target', choose ADE2 , For 'Using', choose CRISPR/CAS9 , in 'For' choose knockout

Once you click find target sites, choose from among the ranking of gRNAs based on how unique they are (so other places in the genome aren't accidentally cut) and also other properties that make good gRNA. Since we are just trying to knock-out the gene it is best to choose the gRNA that occurs earliest in the gene.

AlkM Gene Knock-in for primary research objective : Analyze and download *alkM* DNA sequence from NCBI GenBank. In revised design, co-express *AlkG* (rubredoxin) and *AlkT* (rubredoxin reductase) genes with *AlkM*. Copy the genomic DNA sequence of all the bacterial genes into a new Benchling project page and codon-optimize for *S.cerevisiae* to enhance translational efficiency & reduce codon bias. Promoter and terminator sequences fragments were amplified from *S. cerevisiae* genome

Following are final design procedures for the Wet Lab

There are three steps in the Wet Lab Design Procedures :

Step 1 - Commercial Synthesis of plasmids (pre-express Cas9 in yeast, Empty control for Cas9 experiments and gRNA designs)

Step 2 - Preparation of Competent Yeast Cells for transformation

The following steps are accomplished at room temperature.

- 1.Prepare 10 mL YPD broth with raw ingredients from BSL1 lab
- 2.Grow yeast cells at 30°C in 10 mL YPD broth until mid-log phase (~5 x 10⁶ - 2 x 10⁷ cells/mL or OD600 of 0.8-1.0).
3. Pellet the cells at 500 x g for 4 minutes using the centrifuge in BSL1 lab and discard the supernatant.
4. Add 10 mL Zymoresearch Frozen-EZ Yeast Solution 1 to wash the pellet. Repellet the cells and discard the supernatant.
5. Add 1 mL Frozen-EZ Yeast Solution 2 to resuspend the pellet. The competent cells are stored frozen at -70°C for future use

Step 3 - CRISPR-Cas9 Gene Editing in "Competent" Yeast Cells

1. Add 10 μ l competent yeast cells to the CRISPR-Cas9 and gRNA plasmids from (0.2-1 μ g), mix by tapping or lightly vortexing; add 100 μ l Frozen-EZ Yeast Solution 3 and mix thoroughly.
2. Incubate at 30°C for 60 minutes. Mix vigorously 2-3 times during this incubation.
3. Take a CM minus URA yeast media plate (prepared by BSL1 lab researcher) out of the fridge and let it warm up to room temperature. Using the pipette, add 100 μ L of your CRISPR transformation mixture on top of an CM -URA Yeast media plate
4. Using an inoculation loop, gently spread the transformation mixture onto the plate & incubate the plate at 30°C for 3 days

Test Procedures for Secondary Research Objective

- 1.Control Setup - 3 plates of yeast cells having Cas9-only (control plasmid)
- 2.Experimental Setup - 3 plates of yeast cells transformed with Cas9+ ADE2 gRNA plasmid
- 3.Count the pink/red and white colonies on each of the experimental setup plate (3 trials) and use these data to calculate the transformation efficiency percentage: [(red colonies) ÷ (red + white colonies)] × 100

Test Procedures for Primary Research Objective

- 1.Control Setup - 12 samples of 15 g sterilized soil mixed with 1 mL (0.848 g) of Sterile Gulf diesel engine oil + 0.2 mL of sterile distilled water + 0.2 mL of the yeast cells having Cas9-only control plasmid
- 2.Experimental Setup - 12 samples of 15 g sterilized soil mixed with 1 mL (0.848 g) of Sterile Gulf diesel engine oil + 0.2 mL of the yeast cells transformed with Cas9 and the single multigene gRNA plasmid cassette containing Co-expression of *AlkG* (rubredoxin) and *AlkT* (rubredoxin reductase) genes alongside *AlkM* gene.
- 3.Baseline Setup - 12 samples of 15 g sterilized soil mixed with 1 mL (0.848 g) of Sterile Gulf diesel engine oil + 0.2 mL of sterile distilled water. This establishes baseline oil weight (no microbial activity)
- 4.The samples were set up in triplicate in Erlenmeyer flasks, which were incubated at 30°C for 20 days.
- 5.At five-day intervals, triplicate flasks of control, experimental, and baseline setup were removed from the incubator for capturing data over 3 trials of the experiment.
- 6.Mix each of the 15 g soil treatment samples with 40 mL of Acetone, place in a separating conical flask, shake vigorously for 3 min and allow to settle for 5 min
- 7.Filter through Whatman No. 1 filter paper with anhydrous sodium sulphate (to remove moisture)
- 8.Collect liquid phase in a pre-weighed 50 mL beaker
- 9.Evaporate Acetone mixture at 50°C in oven
- 10.The beaker with the residual diesel oil was allowed to cool to room temperature and weighed to determine the quantity of residual diesel oil by difference
- 11.Biodegradation (%)= (Weight of oil (baseline)-Weight of oil (degraded)) / Weight of oil (baseline)×100

RESULTS

Primary Research Objective - Bio-Degradation Efficiency Results : Data Table for Experiment Setup						
# Days of Incubation	Metrics Based on Mass of the Sample	Trial 1	Trial 2	Trial 3	Average of the 3 trials	Std. Dev of the 3 trials
5 days	Mass of Diesel Oil Extracted from Experiment	0.782 g	0.787 g	0.790g	0.786g	0.004g
	Mass of Diesel Oil Extracted from Baseline	0.848 g	0.848 g	0.848 g	0.848 g	0 g
	Mass of Oil Degraded (RE=Baseline - Exp)	0.066 g	0.061 g	0.058g	0.062g	0.004g
	Exp Cleanup Efficiency (RE/Baseline) *100	7.78%	7.19%	6.84%	7.27%	0.48%
10 days	Mass of Diesel Oil Extracted from Experiment	0.655 g	0.668 g	0.666 g	0.663 g	0.007 g
	Mass of Diesel Oil Extracted from Baseline	0.848 g	0.848 g	0.848 g	0.848 g	0 g
	Mass of Oil Degraded (RE=Baseline - Exp)	0.193 g	0.187 g	0.202 g	0.185 g	0.007 g
	Exp Cleanup Efficiency (RE/Baseline) *100	22.76%	22.05%	23.82%	22.82%	0.91%
15 days	Mass of Diesel Oil Extracted from Experiment	0.472 g	0.460 g	0.463 g	0.465 g	0.006 g
	Mass of Diesel Oil Extracted from Baseline	0.848 g	0.848 g	0.848 g	0.848 g	0 g
	Mass of Oil Degraded (RE=Baseline - Exp)	0.376 g	0.388 g	0.385 g	0.383 g	0.006 g
	Exp Cleanup Efficiency (RE/Baseline) *100	44.34%	45.75%	45.4%	45.17%	0.73%
20 days	Mass of Diesel Oil Extracted from Experiment	0.421g	0.443g	0.433g	0.416g	0.011 g
	Mass of Diesel Oil Extracted from Baseline	0.848 g	0.848 g	0.848 g	0.848 g	0 g
	Mass of Oil Degraded (RE=Baseline - Exp)	0.427g	0.405 g	0.415 g	0.432 g	0.011 g
	Exp Cleanup Efficiency (RE/Baseline) *100	50.35%	47.76%	48.94%	49.02%	1.30%

Primary Research Objective - Bio-Degradation Efficiency Results : Data Table for Control Setup

#Days of Incubation	Metrics Based on Mass of the Sample	Trial 1	Trial 2	Trial 3	Average of the 3 trials	Std Dev. of the 3 trials
5 days	Mass of Oil Degraded (RE=Baseline - Ctrl)	0.015 g	0.023 g	0.019 g	0.019 g	0.004 g
	Ctrl Cleanup Efficiency (RE/Baseline) *100	1.77 %	2.71 %	2.24 %	2.29%	0.47%
10 days	Mass of Oil Degraded (RE=Baseline - Ctrl)	0.038 g	0.045 g	0.049 g	0.044 g	0.006 g
	Ctrl Cleanup Efficiency (RE/Baseline) *100	4.48 %	5.31 %	5.78 %	5.17%	0.66%
15 days	Mass of Oil Degraded (RE=Baseline - Ctrl)	0.095 g	0.111 g	0.106 g	0.104 g	0.008 g
	Ctrl Cleanup Efficiency (RE/Baseline) *100	11.2%	13.1%	12.5%	12.32%	0.95%
20 days	Mass of Oil Degraded (RE=Baseline - Ctrl)	0.141 g	0.138 g	0.129 g	0.136 g	0.006 g
	Ctrl Cleanup Efficiency (RE/Baseline) *100	16.63%	16.27%	15.21%	15.98%	0.73%

Secondary Research Objective (Data Table for CRISPR / CAS9 Transformation Efficiency Results)

Setup	Metrics Based on Red & White Colonies	Plate 1 / Trial 1	Plate 2 / Trial 2	Plate 3 / Trial 3	Average of the 3 trials (Sample)	Std Dev. of the 3 trials (Sample)
Control Setup - Yeast Cells with Cas9 only Control	Number of Red Colonies (R)	0	0	0	0	0
	Number of White Colonies (W)	121	117	127	121.67	5.03
	Transformation Efficiency R/(R+W)*100	0%	0%	0%	0%	0%
Experimental Setup - Yeast Cells with Cas9 only Control	Number of Red Colonies (R)	97	99	94	96.67	2.52
	Number of White Colonies (W)	20	25	22	22.33	2.52
	Transformation Efficiency R/(R+W)*100	82.91%	79.84%	81.03%	81.26%	1.55%

