HORIZON 2.0 (CCA_San_Diego iGEM Team)

PAH Degradation and Biohydrogen Production From Sequential Dark Fermentation and Photofermentation

Set Goal - After PAH degradation, convert the resulting organic intermediaries to hydrogen via metabolic engineering of Escherichia coli and Rhodobacter sphaeroides

We performed extensive research in order to select target PAHs, find ideal gene targets, determine optimal degradation conditions, and research protocols and methods utilized by other scientists. The information gained from our research proved invaluable throughout the course of Horizon 2.0. Here we summarize the key insights and data presented by pertinent literature

Main Purpose - Develop novel constructs to promote biohydrogen synthesis from fermentation pathways, as well as novel PAH degradation pathways.

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Acenaphthene, Fluorene

Bioremediation of polycyclic aromatic hydrocarbon (PAH) compounds: (acenaphthene and fluorene) in water using indigenous bacterial species isolated from the Diep and Plankenburg rivers, Western Cape, South Africa[1]

Experimental process:

- simulated batch scale experiments
- optimum temperature for efficient degradation of both compounds determined in a shaking incubator after 14 days
- testing at 25 °C, 30 °C, 35 °C, 37 °C, 38 °C, 40 °C and 45 °C
- followed by experiments in a Stirred Tank Bioreactor
- 16S target region was amplified using the universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTACGACTT-3′).

Results:

- *Raoultella ornithinolytica*, *Serratia marcescens, Bacillus megaterium* and *Aeromonas hydrophila* efficiently degraded both compounds at 37 °C, 37 °C, 30 °C and 35 °C respectively
- The degradation of fluorene was more efficient and rapid compared to that of acenaphthene
- degradation at Stirred Tank Bioreactor scale was more efficient for all treatments
- *Raoultella ornithinolytica*, *Serratia marcescens*, *Bacillus megaterium* and *Aeromonas hydrophila* degraded a mean total of 98.60%, 95.70%, 90.20%, and 99.90% acenaphthene, respectively and 99.90%, 97.90%, 98.40% and 99.50% fluorene

Comparison (to our lab):

- Microorganisms were extracted from rivers in Africa and tested for possibility in use of PAH degradation
    - We're using genetically modified Escheria coli (E. coli) and rhodobacter sphaeroides instead of microorganisms extracted from the ocean.

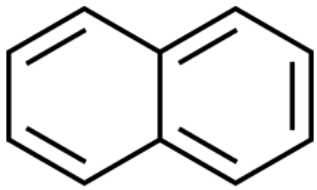
[1] "Bioremediation of polycyclic aromatic ... - NCBI - NIH." 24 Nov. 2016, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5470342/. Accessed 18 Oct. 2019.

Naphthalene

Naphthalene is another PAH we used. Naphthalene is an aromatic hydrocarbon and two fuzed benzenes. Prior research showed that the bacterium Pseudomonas Aeruginosa was able to degrade naphthalene in a bioreactor environment. In the research, P. Aeruginosa were injected into a bioreactor and grown. Gradually, synthetic wastewater containing naphthalene was inputted into the reactor. The researchers changed variables including temperature, PH, salinity, nitrogen concentration to study the differences over different environmental conditions. Final degradation was measured with gas chromatography.
The bacteria was able to survive naphthalene concentrations of up to 20 mg/L. As the byproduct build up increased, the bacteria's efficiency decreased. Higher media PH led to higher degradation efficiency.

Naphthalene | $C_{10}H_8$[2]:

- Naphthalene is an aromatic hydrocarbon with two fused benzenes[3]



Biodegradation of naphthalene using Pseudomonas aeruginosa by up flow anoxic–aerobic continuous flow combined bioreactor[4]:

Experimental Process:

- Summary: Novel Pseudomonas aeruginosa counted and injected on vertical anoxic–aerobic continuous flow combined bioreactor used
- Pseudomonas aeruginosa were grown on R2A medium with 30 mg/L naphthalene
- Synthetic wastewater containing naphthalene was gradually entered into the reactor. Naphthalene was added to deionized water and stirred for 12–14 h to prepare a saturated solution temperature, pH, nitrogen
- concentration, salinity, inoculum concentration, different naphthalene concentration and SCOD were variables that were changed

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[2] "Naphthalene | C10H8 - PubChem - NIH." https://pubchem.ncbi.nlm.nih.gov/compound/Naphthalene. Accessed 18 Oct. 2019.
[3] "Naphthalene | C10H8 - PubChem." https://pubchem.ncbi.nlm.nih.gov/compound/Naphthalene. Accessed 19 Oct. 2019.
[4] "Biodegradation of naphthalene using Pseudomonas ... - NCBI." 26 Mar. 2015, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4391106/. Accessed 18 Oct. 2019.

- After incubation, degradation measured with gas chromatography
- GC Method and COD were used for analysis

Results:

- Bacteria able to survive naphthalene concentration of up to 20 mg/L
- With increased organic buildup (usage of bacteria), bioreactor efficiency decreases
- Bacterial anoxic/aerobic processes were more effective at degradation than the control reactor
- Higher pH led to better degradation

Naphthalene degradation by Pseudomonas sp. HOB1: in vitro studies and assessment of naphthalene degradation efficiency in simulated microcosms[5]:

Experimental Process:

- Medium with naphthalene was inoculated with 1% sediment suspension and incubated with automatic shaking at 150 rpm at 30 C with repetitive transfers
- Bacteria isolated and analyzed individually
- Genomic data extracted and underwent PCR and analyzed with BLAST
- Bacteria inoculated with naphthalene and underwent tolerance studies, Response Surface Methodology, Analysis of Surfactants, and other analysis techniques

Results:

- Pseudomonas sp. HOB1 can tolerate naphthalene up to 60,000 ppm
- Naphthalene degrades best in alkaline pH and at temps of 35 to 37 degrees C
- Can function well when other microorganisms are present

[5] "PubMed - NCBI." 24 Dec. 2008, https://www.ncbi.nlm.nih.gov/pubmed/19167154. Accessed 18 Oct. 2019.

Bacterial Metabolism of Naphthalene: Construction and Use of Recombinant Bacteria To Study Ring Cleavage of 1,2-Dihydroxynaphthalene and Subsequent Reactions[6]:

Experimental Process:

- recombinant bacteria carrying genes cloned from plasmid NAH7 were investigated
- Pseudomonas aeruginosa with pathway enzymes were incubated with naphthalene
- Degradation products separated by chromatography and identified with spectroscopy adn gas chromatography

Results:

- HCCA was detected as the first reaction product
- Slow isomerization of product occurred spontaneously, resulting in an equilibrium mixture with the same composition
- Metabolism of tHBPA to salicylaldehyde is catalyzed by a single enzyme encoded by a 1-kb MluI-StuI restriction fragment.
- E. Coli carrying the KpnI-BgI fragment catalyzed the rapid equilibration of HCCA and tHBPA.
- The tHBPA formed from HCCA is metabolized by a hydratase-aldolase to salicylaldehyde and pyruvate

Naphthalene Degradation and Incorporation of Naphthalene-Derived Carbon into Biomass by the Thermophile Bacillus thermoleovorans[7]:

Experimental Process:

- Bacteria incubated in mineral salt medium along with naphthalene at 60 degrees C
- Isolate found and inoculated with naphthalene in mineral salt medium
- Batch experiments performed with CO2 trapper valve.

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[6] "Bacterial metabolism of naphthalene: construction and use of ... - NCBI." https://www.ncbi.nlm.nih.gov/pubmed/1447127. Accessed 18 Oct. 2019.
[7] "Naphthalene Degradation and Incorporation of ... - NCBI - NIH." https://www.ncbi.nlm.nih.gov/pmc/articles/PMC91857/. Accessed 18 Oct. 2019.

- 3 ml naphthalene put into batch reactor w/ 1 ml cell suspension, which was then shook until liquid turned yellow

Results:

- Optimal growth at 60 degrees C and increase in bacteria correlates with increased degradation
- Between 25 to 50 hours cell growth is highest, and thus, degradation is highest. Afterwards, growth/degradation hits a plateau
- Naphthalene had high 13C incorporation, meaning the bacteria used naphthalene-derived carbon for its amino acid synthesis
- molecular peaks showed a shift of 0 to 5 atomic mass units, indicating an incorporation of up to 5 13C atoms per molecule

Chemotaxis of Pseudomonas spp. to the Polyaromatic Hydrocarbon Naphthalene[8]:

Experimental Process:

- Two naphthalene-degrading bacteria, Pseudomonas putida G7 and Pseudomonas sp. strain NCIB 9816-4, were chemotactically attracted to naphthalene in drop assays and modified capillary assays.

Results:

- Growth on naphthalene or salicylate induced the chemotactic response. P. putida G7 was also chemotactic to biphenyl
- other polyaromatic hydrocarbons that were tested did not appear to be chemoattractants for either Pseudomonas strain
- strains that were cured of the naphthalene degradation plasmid were not attracted to naphthalene.

[8] "Chemotaxis of Pseudomonas spp. to the ... - NCBI - NIH." https://www.ncbi.nlm.nih.gov/pmc/articles/PMC168726/. Accessed 18 Oct. 2019.

Desaturation and Oxygenation of 1,2-Dihydronaphthalene by Toluene and Naphthalene Dioxygenase[9]:

Experimental Process:

- Bacterial strains expressing toluene and naphthalene dioxygenase were used to examine the sequence of reactions involved in the oxidation of 1,2-dihydronaphthalene.
- Cell extracts were prepared by passage of cell suspensions through a chilled French pressure cell
- Ethyl acetate extracts containing metabolites produced by strain F39/D were purified by radial dispersion chromatography on silica gel plates

Results:

- recombinant E. coli strains expressing NDO and TDO activity oxidize compound I to the same products in almost the same ratios as those formed by the mutant strains
- TDO and NDO have different specificities when dihydronaphthalene is used as a substrate
- The reaction sequence A3B3D3H involving the formation of compound V through the arene hydrate intermediate compound II can therefore be eliminated

[9] "Desaturation and oxygenation of 1,2-dihydronaphthalene by ...." https://jb.asm.org/content/177/20/5799. Accessed 18 Oct. 2019.

PAHs Concentration in Crude Oil Contaminated water samples[10]:

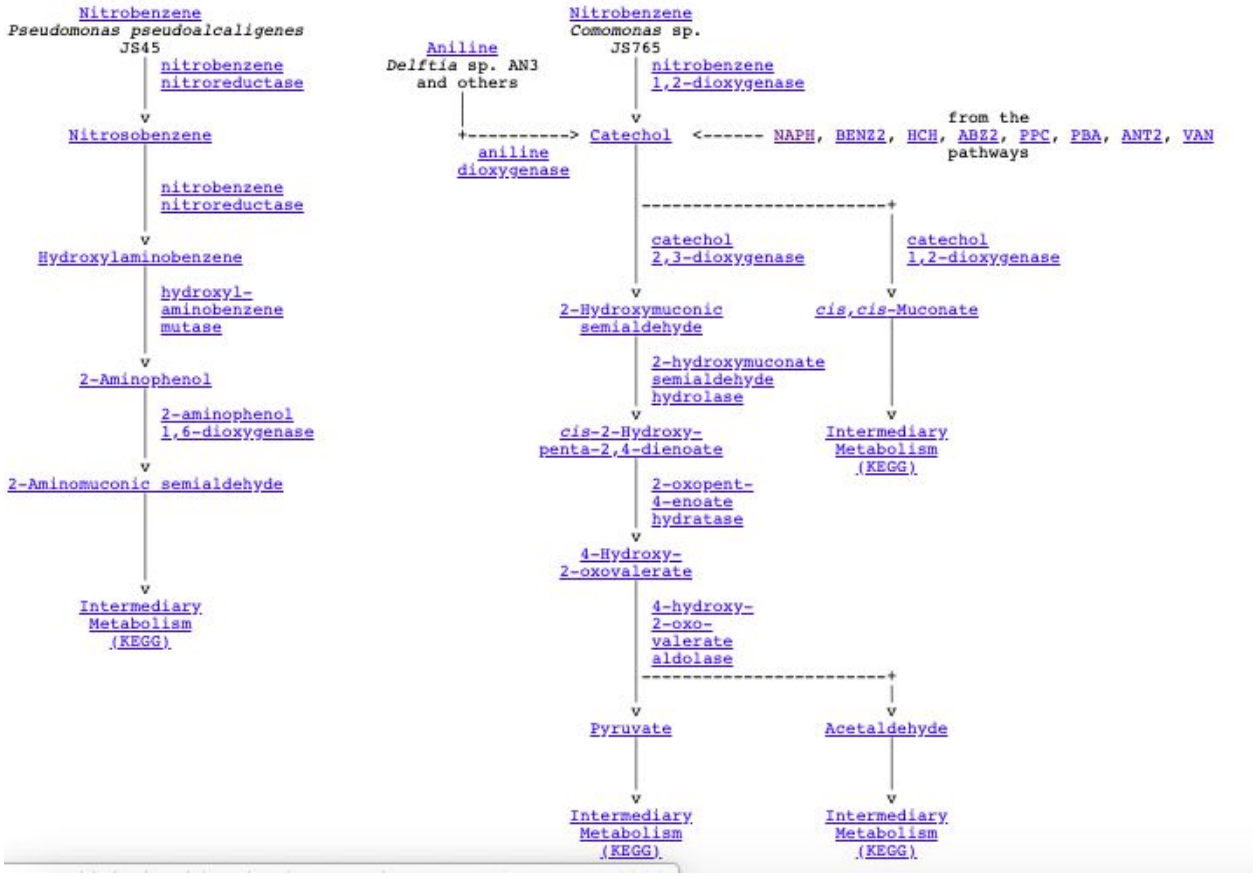
S/No.	PAH in water sample	Abbr.	Conc. (ppm)	Abundance (%)	No. Of Aromatic rings	∑PAHs in ppm and (%)
1	Naphthalene	NAP	58.67	15.397	2	
2	Naphthalene, 2-methyl-		48.05	12.610	2	
3	Biphenylene		0.07	0.018	2	106.79(28.0)
4	Acenaphthene	ACE	57.54	15.100	3	
5	Triphenylene		0.83	0.218	3	
6	Phenanthrene	PHE	16.08	4.220	3	74.45(19.54)
7	Fluorene	FLR	45.45	11.927	4	
8	Fluoranthene	FLT	113.16	29.696	4	
9	Pyrene	PYR	14.08	3.695	4	
10	Benz[a]anthracene	BaA	1.86	0.488	4	174.55(45.81)
11	Benzo[b]fluoranthene	BbFA	16.08	4.220	5	
12	Benzo[a]pyrene	BaP	1.73	0.454	5	
13	Dibenz[a,h]anthracene	DBahA	4.72	1.239	5	22.53(5.91)
14	Indeno[1,2,3-cd]pyrene	IP	0.65	0.171	6	
15	Benzo[ghi]perylene	BghiP	2.09	0.548	6	2.74(0.72)
	∑PAH15		381.06	100		

- Shows that naphthalene is the most prevalent in PAHs

Naphthalene degradation pathway[11]:

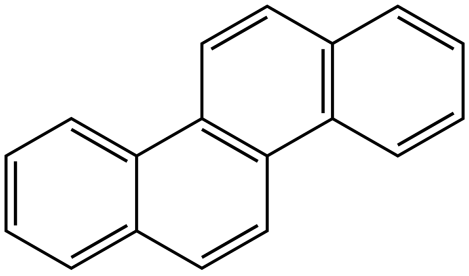
[10] "Polycyclic Aromatic Hydrocarbons (PAHs) - Sryahwa ...." https://www.sryahwapublications.com/annals-of-ecology-and-environmental-science/pdf/v3-i1/2.pdf. Accessed 18 Oct. 2019.

[11] "Nitrobenzene Degradation Pathway - eawag-bbd." http://eawag-bbd.ethz.ch/nb/nb_map.html. Accessed 18 Oct. 2019.



Chrysene

One key PAH (Polycyclic Aromatic Hydrocarbon) our team focused on is chrysene, with chemical notation as C18H12.

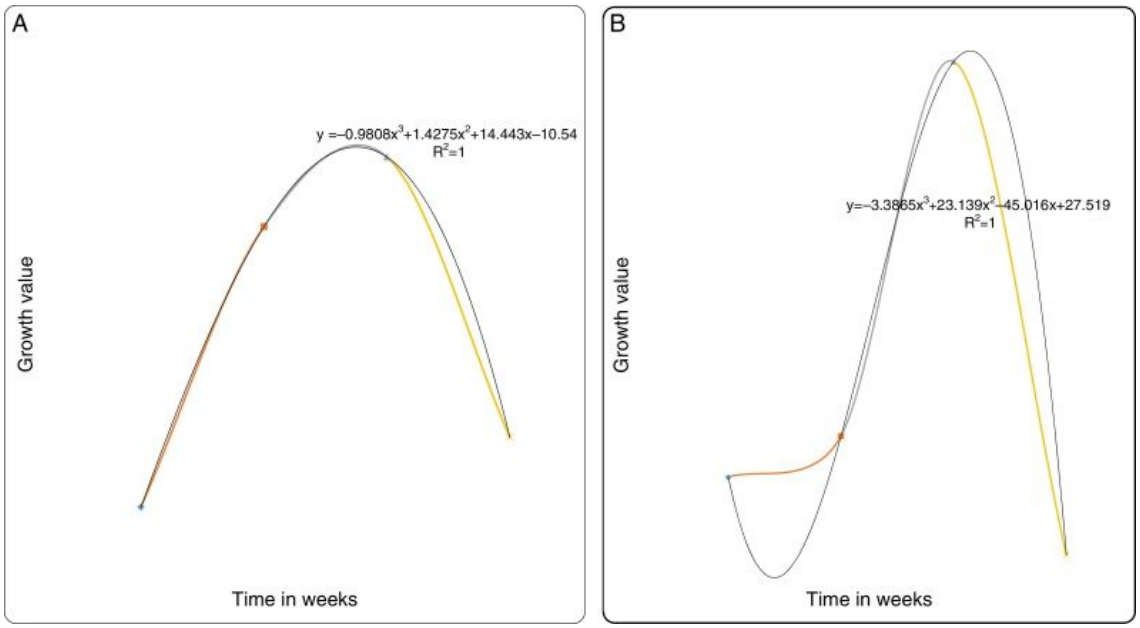


Chrysene is formed when gasoline or garbage burns; it is also known to be a key constituent of tobacco smoke. It combines with dust particles in the air, is carried into water and soil, and eventually finds its way onto crops as well. Chrysene is found in the coal tar pitch that industry uses to join electrical parts. It is also found in creosote, a chemical used to preserve wood. It can cause catastrophic damage to the ecosystem and is widely thought to be carcinogenic to humans. Chrysene is further found significantly in toxic oil compounds.[12]

[12] "Chrysene - Wikipedia." https://en.wikipedia.org/wiki/Chrysene. Accessed 19 Oct. 2019.

Pseudomonas fluorescens (PF), a common Gram-negative, rod-shaped bacterium, has been shown to degrade chrysene as a source of energy for itself. In an experiment, 2 strains of PF consumed chrysene as an energy source, with a chrysene concentration of 79 ppm. After a 21 day incubation period, the strains consumed an average of 14-16% of chrysene in their media. However, after a certain point, the bacteria began to die off. The reason is unknown but it could be due to harmful byproducts of chrysene degradation that built up in the media.

Predictive growth models[13]:

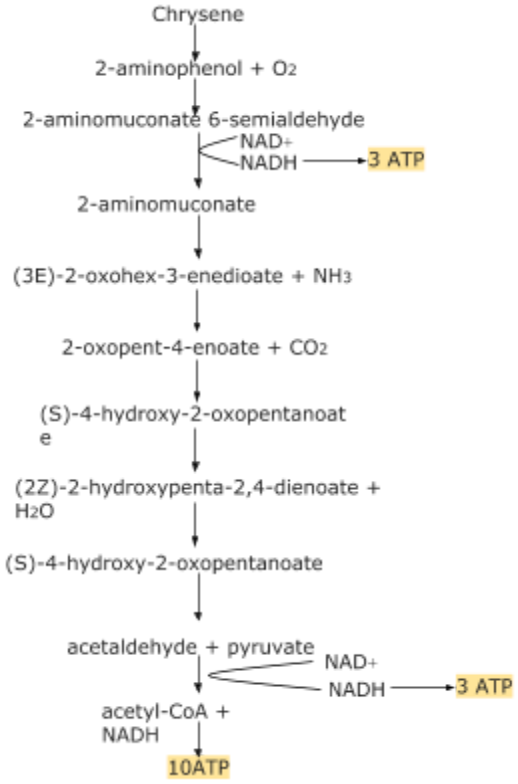


Genes implicated in PF degradation are:

1. amnA: ferrous iron binding, oxidoreductase activity
2. amnB: ferrous iron binding
3. amnC; oxidoreductase activity
4. amnD; Aromatic hydrocarbons catabolism, hydrolase activity
5. amnE; Aromatic hydrocarbons catabolism, lyase activity
6. amnG; Manganese, Metal-binding
7. amnH; oxidoreductase activity, NAD

[13] "Degradation of polynuclear aromatic ... - NCBI - NIH." 24 Apr. 2016, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4927684/. Accessed 18 Oct. 2019.

The reactants and products of these genes are also succinctly outlined in our pathway for Chrysene below[14]. The chrysene Degradation pathway was further modeled by us using the Simbiology package in MATLAB.



Chrysene degradation by two strains of Pseudomonas (PB-1 and PB-2)[15]

Experimental Process

- Degradation studies conducted in the balch tubes
- Tubes were inoculated with the different bacterial cultures, crimp sealed and incubated horizontally on the shaker table at ambient temperature for 21 days
- 5 mL of hexane was added, vortexing for 1–2 min and subsequently, mixed continuously on a tube rotator for 12 h to stop the degradation study.
- Beckman GS-6 series centrifuge at 2190 rpm for 20 min was used to separate the hexane fraction and the aqueous phase.

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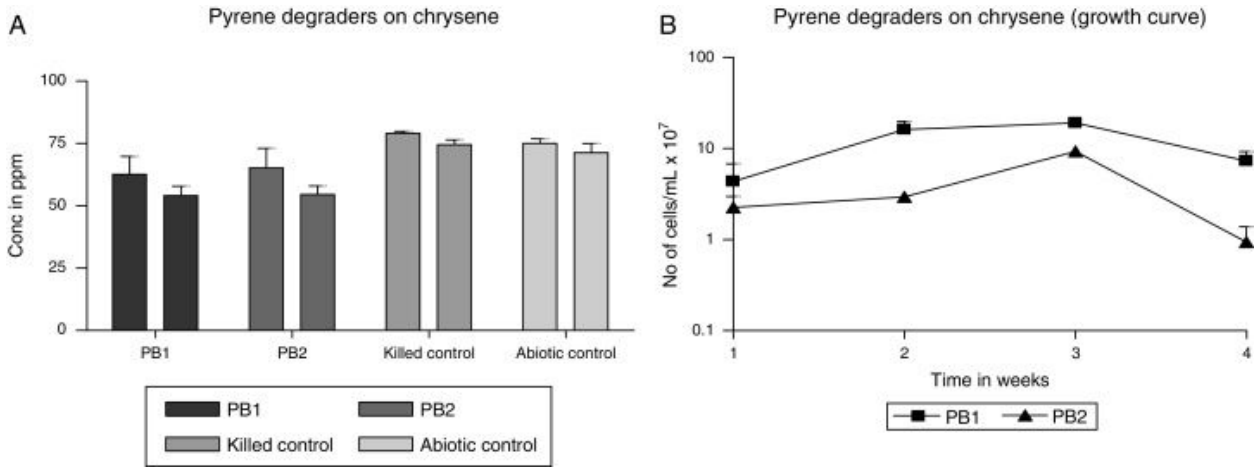
[14] "Degradation of polynuclear aromatic ... - NCBI - NIH." 24 Apr. 2016, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4927684/. Accessed 18 Oct. 2019.
[15] "Oculopharyngeal myopathy with distal and ... - NCBI - NIH." https://www.ncbi.nlm.nih.gov/pmc/articles/PMC492768/. Accessed 18 Oct. 2019.

- Hexane extracts were stored in target vials with a headspace of 1 mL and crimp sealed using an 11 mm Teflon rubber stopper and preserved at 4 °C prior to analysis.

Results

- Strains PB-1 and PB-2 consumed chrysene as a source of its energy and carbon sources
- strain PB-2 was able to degrade more of the chrysene than strain PB-1
- At the end of the 21 days incubation period, the strains degraded about 14–16% of chrysene
- Final concentration (mean and standard deviation) assessed was 54 ppm
- Strain PB-1 consumed the chrysene at 14% at volume biodegradation rate of 0.017 ± 0.011 mg L−1 h−1
- Strain PB-2 utilized 16% of chrysene at the volume biodegradation rate of 0.021 ± 0.009 mg L−1 h−1.



(A) Degradation of chrysene by MS-benzoate grown cells of PB-1 and PB-2, incubated for 21 days. Data represent the mean and standard deviation of triplicate determination of initial and final concentration respectively. The error bars (Standard Deviation) were due to differential response of cells in triplicate tubes. (B) Chrysene dependent growth and cell numbers distribution of strains PB-1 and PB-2, in chrysene incubated for 21 days. Data represent the mean of replicates tubes for initial time (0) cell density represented as (1) and final time (21 days) represented as (4) respectively. The x-axis value range was chosen as such to allow for even spread of the growth curve. The error bars (Standard Deviation) were due to differential response of cells in triplicate tubes.

Degradation of Chrysene by Enriched Bacterial Consortium[16]

Experimental Process

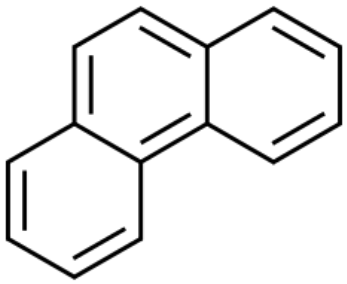
- Development, enrichment and characterization of bacterial consortium ASDC, consisting of Rhodococcus sp., ASDC1; Bacillus sp. ASDC2; and Burkholderia sp. ASDC3
- Chrysene was utilized as a sole source of carbon and energy by the consortium, having maximum degradation rate of 1.5 mg/L/day and maximum growth rate of 0.125/h, under optimized conditions of pH 7.0, 37°C under aeration of 150 rpm on gyrating shaking.
- Chrysene degradation unaffected in presence of other PAHs like pyrene, fluoranthene, naphthalene, phenanthrene, benzene, toluene and xylene, individually as well as in mixture

Results

- Peptone, ammonium nitrate, sodium succinate enhanced chrysene degradation rate during the first 24 h of experimentation
- HPLC studies suggested that chrysene was degraded through phthalic acid pathway by the consortium ASDC
- enriched consortium ASDC exhibited maximum degradation (96%) in polluted, non-sterile soil sediment

Phenanthrene

Phenanthrene, a 3 ring angular PAH known to be a skin photosensitizer and promoter of DNA translocation, is one of the 3 most abundant polycyclic aromatic hydrocarbons (PAH) found in crude oils (see table below)[17].



[16] "Degradation of Chrysene by Enriched Bacterial ... - NCBI." 26 Jun. 2018, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6036299/. Accessed 18 Oct. 2019.
[17] "Phenanthrene | C14H10 | ChemSpider." http://www.chemspider.com/Chemical-Structure.970.html. Accessed 19 Oct. 2019.

Table 1. Major constituent of 48 crude oils and 2 Northern sea crude oils.

Crude oil	48 different crude oils			North Sea	Goliat
PAH	Minimum mg/kg oil	Maximum mg/kg oil	Mean mg/kg oil	mg/kg oil	mg/kg oil
Naphthalene	1.2	3700	427	1169	1030
Fluorene	1.4	380	70.34	265	75
Phenanthrene	0	400	146	238	175
Anthracene	0	17	4.3	1.5	*

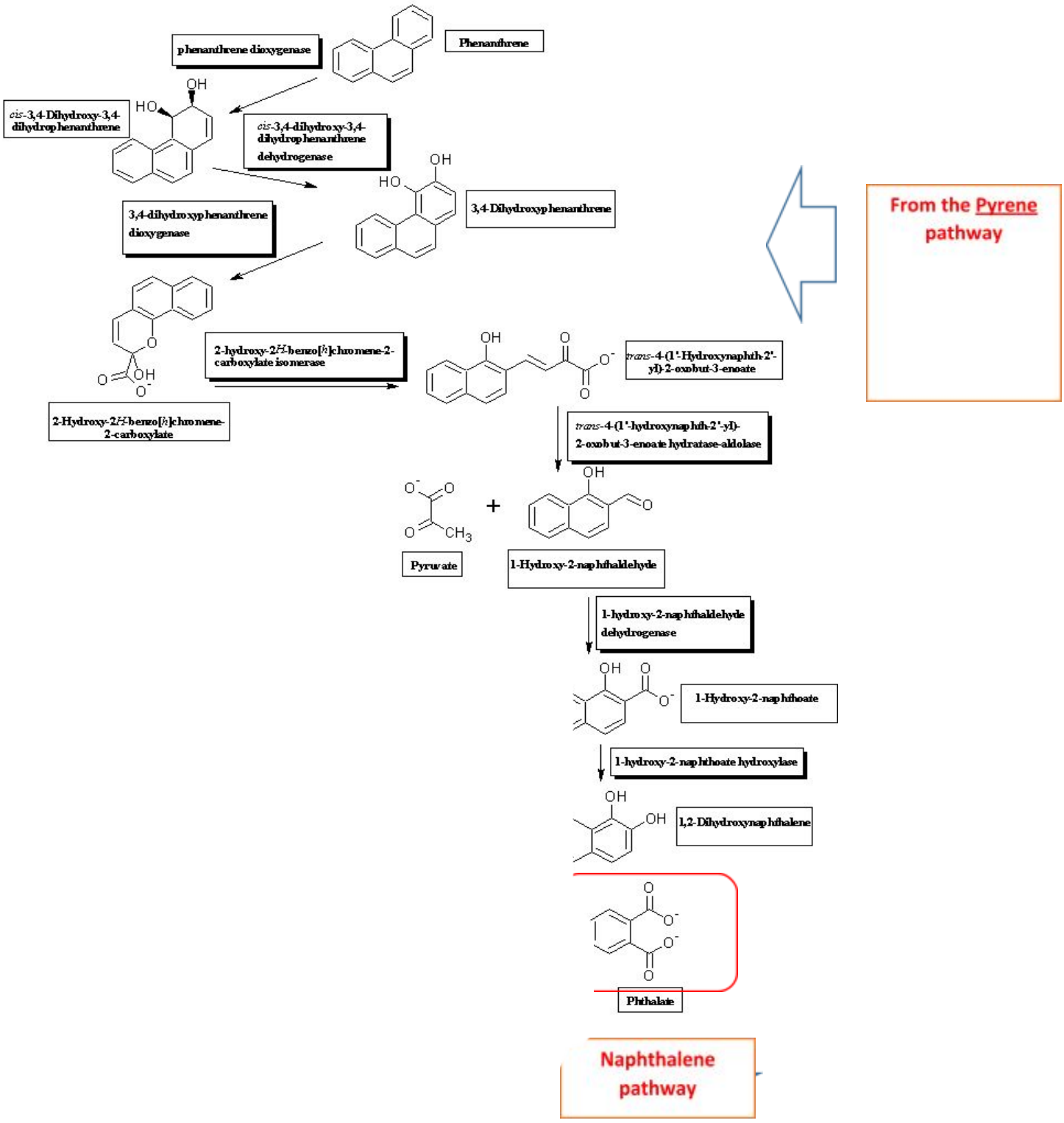
Source: Polycyclic Aromatic Hydrocarbons a Constituent of Petroleum: Presence and Influence in the Aquatic Environment, Pampanin et al., 2013, Hydrocarbon

Phenanthrene Catabolic Pathway

The pathways for phenanthrene metabolism by bacteria includes the action of a dioxygenase with subsequent oxidation to form 4-dihydroxyphenanthrene which then undergoes meta-cleavage and is converted to 1-hydroxy-2-naphthoic acid. This is the common upper pathway. 1-hydroxy-2-naphthoic acid can be degraded in different ways.
Phenanthrene degradation can be accomplished by two distinct routes, via either phthalate or salicylate. Genes for the phenanthrene metabolism pathway via salicylic acid and catechol have been isolated from several strains. The interesting aspect of the phenanthrene degradation pathway is that it can be at the crossroads of degradation of many other PAHs such as pyrene and naphthalene.

Figure 1 (Below). The phenanthrene upper catabolic pathway showing pathways convergence with other PAHs. [18,19,20,21]

[18] "Phenanthrene Graphical Pathway Map - eawag-bbd." http://eawag-bbd.ethz.ch/pha/pha_image_map_1.html. Accessed 18 Oct. 2019.
[19] "Naphthalene degradation - Reference ... - KEGG PATHWAY." https://www.kegg.jp/pathway/map00626+M00534. Accessed 18 Oct. 2019.
[20] "KEGG PATHWAY: Polycyclic aromatic hydrocarbon ...." https://www.genome.jp/kegg-bin/show_pathway?map=map00624&show_description=show. Accessed 18 Oct. 2019.
[21] "List of All Pathways - EAWAG-BBD." http://eawag-bbd.ethz.ch/servlets/pageservlet?ptype=allpathways. Accessed 18 Oct. 2019.

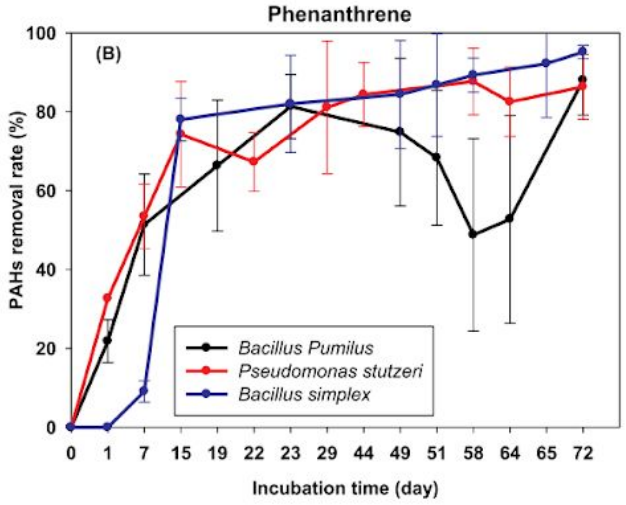

phenanthrene dioxygenase
Phenanthrene
cis-3,4-Dihydroxy-3,4-dihydrophenanthrene
cis-3,4-dihydroxy-3,4-dihydrophenanthrene dehydrogenase
3,4-Dihydroxyphenanthrene
3,4-dihydroxyphenanthrene dioxygenase
2-Hydroxy-2H-benzo[h]chromene-2-carboxylate
2-hydroxy-2H-benzo[h]chromene-2-carboxylate isomerase
trans-4-(1'-Hydroxynaphth-2'-yl)-2-oxobut-3-enoate
trans-4-(1'-hydroxynaphth-2'-yl)-2-oxobut-3-enoate hydratase-aldolase
Pyruvate
1-Hydroxy-2-naphthaldehyde
1-hydroxy-2-naphthaldehyde dehydrogenase
1-Hydroxy-2-naphthoate
1-hydroxy-2-naphthoate hydroxylase
1,2-Dihydroxynaphthalene
Phthalate
From the Pyrene pathway
Naphthalene pathway

Bacillus strains isolated from oil spill sites[22]

Experimental Procedure

- Twenty-seven bacterial strains were isolated from contaminated site by oil spills.
- PAHs-degrading bacteria were screened to select high tolerant species for ensuring an efficient bioremediation
- Each of the isolated bacterial strains were grown under different PAHs concentrations (250, 500, 1000 and 1500 mg/L)
- Bacterial strains with the most bioremediation activity were identified by VITEK MS™ MALDI-TOF mass spectrometry
- total DNA was amplified with the 16S forward 5-AGAGTTTGATCMTGGCTCAG-3′ and 16S reverse 5′-GGMTACCTTGTTACGAYTTC-3′ primers (Drago et al., 2011), and the following PCR program: 94 °C/3min, 29 cycles 94 °C/40s, 55 °C/50s and 72 °C/2 min; and finally, 72 °C/10min
- DNA Amplicons were separated on 1% agarose gel. Electrophoresis was performed at 100 V for 2 h using 1XTris-borate-EDTA. The gels were stained with GEL-RED (Biotium, Canada) and visualized by using the GelDoc (BioRad, France).

Results

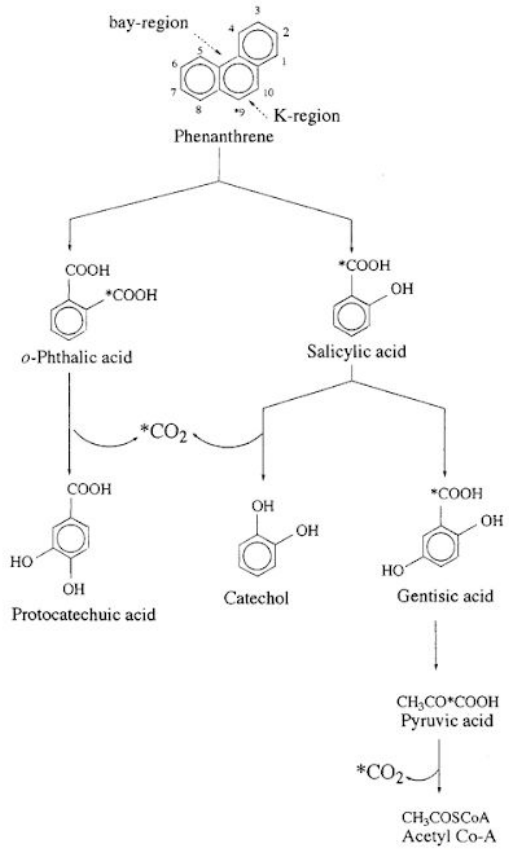


- Among the 27 strains, 8 resulted to be resistant to high concentration level of PAHs (1500 mg/L) and thereof can use PAHs as sole source of carbon and energy
- Rapid degradation of fluorene and phenanthrene were observed in early stage during the first week
- Phenanthrene, a low molecular weight PAHs, was degraded efficiently (about 86-95%) within 72 days of test organism incubation
- The biodegradation kinetics of both fluorene and phenanthrene were fit a first order rate with R^2 values ranging from 0.88 to 0.92

[22] "Degradation of fluorene and phenanthrene in PAHs ... - NCBI." 17 Nov. 2018, https://www.ncbi.nlm.nih.gov/pubmed/30453222. Accessed 18 Oct. 2019.

Degradation of phenanthrene by different bacteria: evidence for novel transformation sequences involving the formation of 1-naphthol[23]

Experimental Procedure



- Four polycyclic aromatic hydrocarbon (PAH)-degrading bacteria, namely Arthrobacter sulphureus RKJ4, Acidovorax delafieldii P4-1, Brevibacterium sp. HL4 and Pseudomonas sp. DLC-P11, capable of utilizing phenanthrene as the sole source of carbon and energy, were tested for its degradation using radiolabeled phenanthrene.
- Microorganisms grown on mineral salts medium (MSM) with 100 mg/l phenanthrene
- For growth on plates, phenanthrene was sprayed as a 5% (w/v) solution in diethyl ether over the surface of MSM agar previously inoculated with the test microorganisms
- Degrading ability confirmed by the presence of clearing zones around the inoculated regions on the plates
- Following growth of microorganisms on phenanthrene, aliquots were taken at different time intervals, supernatants were obtained and a half-volume of Folin-Ciocalteu reagent was added.
    - Samples kept in the dark for 1 h at room temperature
    - Absorbance measured at 750 nm
- Effects of different PAHs upon [9-14C]phenanthrene degradation were investigated by adding fluorene, fluoranthene and pyrene in addition to phenanthrene at a ratio of 1:1:1:1 (total concentration 100 mg/l)

[23] "Degradation of phenanthrene by different bacteria: evidence ...." http://crdd.osdd.net/eprints/archives/open/documents/disk0/00/00/03/32/01/jain99.pdf. Accessed 18 Oct. 2019.

Results

- Within 18 h of incubation, 30.1, 35.6, 26.5 and 2.1% of the recovered radiolabeled carbon was degraded to 14CO2 by RKJ4, P4-1, HL4 and DLC-P11, respectively
- All four microorganisms are capable of degrading radiolabelled phenanthrene into $^{14}CO_2$
- Four different microorganisms, viz. RKJ4, P4-1, HL4 and DLC-P11, are reported (for the first time) as being to use phenanthrene (and other PAHs) as the sole source of carbon and energy
- RKJ4, P4-1 and DLC-P11 degrade phenanthrene via o-phthalic acid whereas HL4 uses the salicylic acid pathway
- HL4 degrades phenanthrene via 1-hydroxy-2-naphthoic acid, 1-naphthol and salicylic acid
- DLC-P11 degrades phenanthrene via the formation of 1-hydroxy-2-naphthoic acid, 1-naphthol and o-phthalic acid

BioHydrogen Synthesis

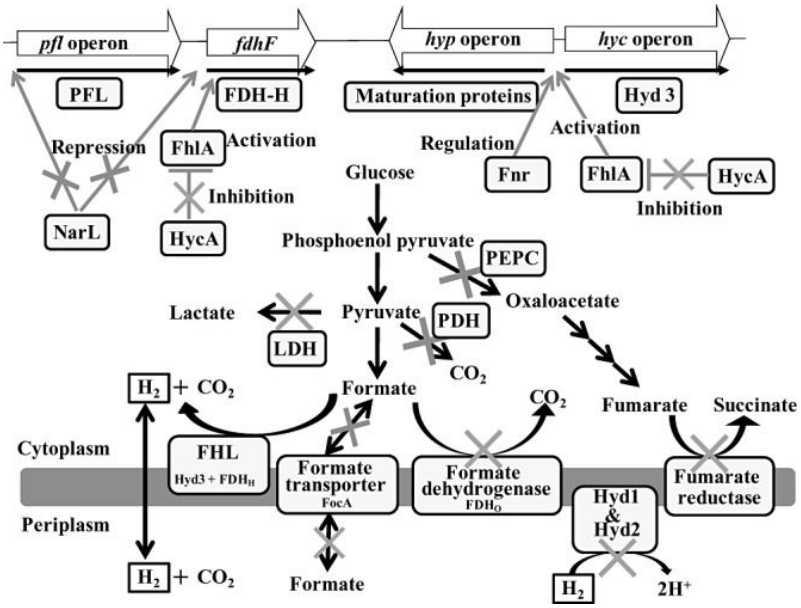
Hydrogen production by recombinant Escherichia coli strains[24]

Strategies to increase hydrogen production in E. coli can be done through metabolic engineering, heterologous gene expression, adaptive evolution, and protein engineering. Escherichia coli, is a coliform bacteria commonly found in food, the environment, and the intestines of animals. Due to its ability to survive in variable growth conditions and its ease in reproducing and growing rapidly, E. coli has become a model organism used to help scientists perform effective biological research.

Metabolic engineering from formate metabolism can be done using E. coli native hydrogenases, since hydrogen is formed through the reaction $HCOO^- + H2O \leftrightarrow H2 + HCO_3^-$. One strategy to improve hydrogen production from formate in E. coli is by inactivating HycA repressor of FHL and by overproducing the FhlA activator of FHL. Hydrogen activity can also be increased by deleting *hycA* and overexpressing *fhlA*. Possible combinations (previously engineered) *hyaB*, *hybC*, *hycA*, *fdoG*, *ldhA*, *frdC* and *aceE*.
Biohydrogen was a portion of our project that allows for the degradation of crude oil to be repurposed into clean energy via bacteria fermentation. In certain species of bacteria, including

[24] "Hydrogen production by recombinant Escherichia coli ... - NCBI." 20 Feb. 2012, https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3815781/. Accessed 18 Oct. 2019.

Escherichia coli, the anaerobic process of mixed acid fermentation converts formate to hydrogen and carbon dioxide via the formate hydrogen lyase (FHL) complex. The FHL complex is regulated by fhlA, which activates transcription of the fdhF, hyp, hyc, and hydN-hypF operons[25]



hyfR is a homolog of fhlA that transcribes the hyf operon (hydrogenase-4) and has also been found to enhance expression of fdhF [10, 4]. Hydrogenase-4, along with hydrogenase-3 (hyc operon), is the hydrogen-producing unit of the FHL complex[26].

The fnr and arc systems work in concert to regulate anaerobic respiration[27]. In the absence of oxygen, both ArcA and FNR repress aerobic processes (ETC "dampers") and promote the transcription of anaerobic enzymes[28] [29], including fhlA. FNR is activated by its oxygen-sensitive [4Fe-4S] clusters and appears to be specific to anaerobic respiration and fermentation pathways. ArcA is phosphorylated by its counterpart ArcB and exerts a broader range of control over

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[25] "Bacterial formate hydrogenlyase complex | PNAS." https://www.pnas.org/content/111/38/E3948. Accessed 18 Oct. 2019.
[26] "Regulation of the hydrogenase-4 operon of Escherichia coli ...." https://www.ncbi.nlm.nih.gov/pubmed/12426353. Accessed 18 Oct. 2019.
[27] "Anaerobic activation of arcA transcription in ... - NCBI." https://www.ncbi.nlm.nih.gov/pubmed/8022271. Accessed 18 Oct. 2019.
[28] "FNR Is a Global Regulator of Virulence and Anaerobic Metabolism in ...." https://jb.asm.org/content/189/6/2262. Accessed 18 Oct. 2019.
[29] "ArcA overexpression induces fermentation and ... - Nature." 19 Sep. 2017, https://www.nature.com/articles/s41598-017-12144-6. Accessed 18 Oct. 2019.

various intracellular redox conditions[30]. Both systems are sensitive to different levels of oxygen in the environment, meaning the level of expression of target operons varies depending on the degree of air saturation[31].

In anaerobic conditions, both FNR and ArcA induce the transcription of pyruvate formate-lyase, the enzyme responsible for converting pyruvate into formate[32] [33]. Thus, overexpression of the fnr and arc systems may increase formate concentrations during fermentation and consequently increase biohydrogen production via the FHL complex.

There is a lack of experimental evidence for the combined effects of FNR and ArcA on fhlA or hyfR expression. However, it is possible that the binding of the FNR enzyme nearby the hyf sequence may minimize the ability of the larger hyfR protein to bind to its upstream target sequence[34]. This possibility may be tested in our experiments.

Our goal with the beyond constructs was to target more hydrocarbons in crude oil for the purpose of expanding the Horizon project. To decide which hydrocarbons we wanted to target we analyze which hydrocarbons were possible to degrade, but also are harmful to the environment. We decided to focus on three of the most prevalent hydrocarbons in crude oil: the long chained hydrocarbons including alkanes, alkenes, and alkynes.

Alkanes are the most simple type of hydrocarbon, following the formula C_nH_{2n+2}. By reading literature that studied bacterial degradation, we determined the genes and enzymes necessary for the degradation of these compounds. One article that looked in n-alkane biodegradation mechanisms highlighted the bacteria Pseudomonas Putida[35]; specifically, we looked at the Alk gene system of Pseudomonas putida. This operon encodes seven proteins, of which three are involved in alkane degradation[36]. To determine which exact genes were necessary we looked at

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[30] "The Arc Two-component Signal Transduction System ...." 30 Mar. 2001, http://www.jbc.org/content/276/13/9917.full. Accessed 18 Oct. 2019.
[31] "Effect of microaerophilic cell growth conditions on ... - NCBI - NIH." https://www.ncbi.nlm.nih.gov/pubmed/8576043. Accessed 18 Oct. 2019.
[32] "Anaerobic induction of pyruvate formate-lyase gene ... - NCBI." https://www.ncbi.nlm.nih.gov/pubmed/1592804. Accessed 18 Oct. 2019.
[33] "Specific transcriptional requirements for positive ... - NCBI." https://www.ncbi.nlm.nih.gov/pubmed/7934836. Accessed 18 Oct. 2019.
[34] "Expression and Regulation of a Silent Operon, hyf, Coding for ...." https://jb.asm.org/content/186/2/580. Accessed 18 Oct. 2019.
[35] "Structural insights into diversity and n-alkane ... - Frontiers." https://www.frontiersin.org/articles/10.3389/fmicb.2013.00058/full. Accessed 18 Oct. 2019.
[36] "The Pseudomonas oleovorans alkBAC operon encodes two ...." https://europepmc.org/abstract/med/2647719. Accessed 18 Oct. 2019.

the degradation pathway for octane, a type of alkane. The necessary enzymes were alkane monooxygenase (coded for by the gene AlkB), rubredoxin-2 (coded for by the gene AlkG), and rubredoxin-Nad(+) reductase (coded for by the gene AlkT). These enzymes all work in conjunction to degrade octane into 1-octanol. The enzymes already present in *E. coli* strain K12 can then degrade the new product of 1-octanol all the way down to Octanoyl-CoA, which the bacteria can use as an energy source. The functions and names of these three enzymes present in *E. coli* can be seen in the degradation diagram below.

Alkenes are a type of hydrocarbon that follows the formula C_nH_{2n}. During our initial research, we discovered an article that examined the bacteria Xanthobacter Strain Py2 and the enzyme Alkene Monooxygenase[37]. The Xamo operon present in this bacteria plays an integral role in the degradation of alkenes. From the Eawag Biocatalysis/Biodegradation Database, we obtained a degradation pathway for propylene. The first enzyme in the pathway was alkene monooxygenase, which degrades propylene (also known as 1-propene) into 1,2-epoxypropane. This enzyme is coded for by XamoA-F.

The enzymes XecA, XecD, and XecC, all from Xanthobacter, work separately to degrade 1,2-epoxypropane all the way down to acetoacetate. Acetoacetate can be broken down by *E. coli* into 2 acetyl-CoA molecules, which can be directly inserted into the Krebs Cycle for ATP production. Therefore, by using a combination of enzymes present in Xanthobacter, in conjunction with enzymes already present in *E. coli*, an alkene can be broken down into a compound that eventually the bacteria can use to produce energy.

Alkynes are a hydrocarbon which follows the formula C_nH_{2n-2}.

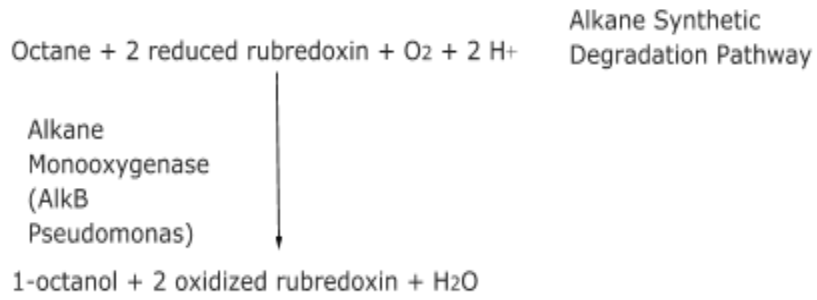

Figure 2: Degradation of Alkane into intermediate compound using recombinant gene.

[37] "The Alkene Monooxygenase from Xanthobacter Strain ... - NCBI." https://www.ncbi.nlm.nih.gov/pmc/articles/PMC91225/. Accessed 18 Oct. 2019.

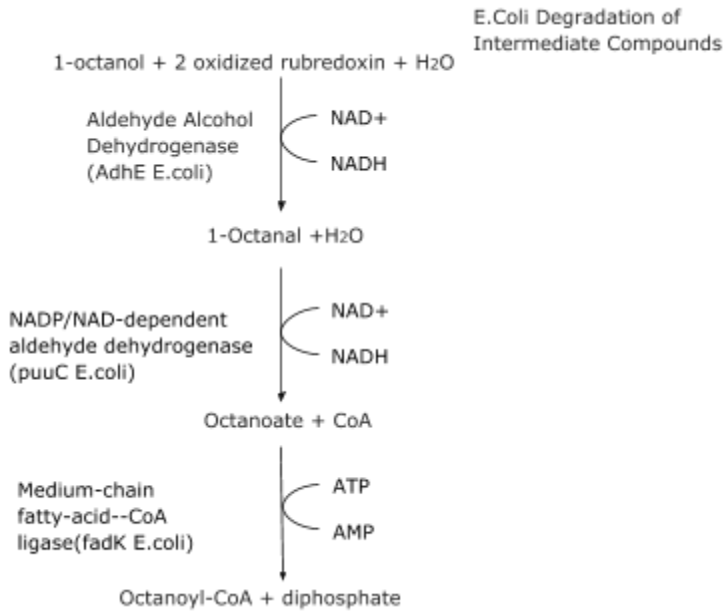

Figure 3: Conversion of intermediate compound into usable energy form within the E Coli genome.

Summary

Biosynthesis of hydrogen can be accessed by dark and light fermentation processes which are optimized by strategic upregulation and inhibition of regulatory E. Coli mechanisms and engineering of an idealized protein lyase.