Degradation of Fluorene and Phenanthrene Using Recombinant E.coli

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1. PURPOSE

Conduct a series of biotransformation experiments to measure the efficacy of the engineered strains containing phenanthrene and fluorene degradation synthetic pathway.

2. CLONE DESCRIPTION

Phenanthrene catabolic pathway:
P insert 1: Synthetic phnF, phnE, phnC, phnD + P insert 2: Synthetic phnAc, phnAd, phnB

Fluorene catabolic pathway:
F insert 1: Synthetic flnB, dbfA1, dbfA2 + F insert 2: Synthetic flnE, flnD1, ORF16, flnC

Part Name	Clone No.	Clone Description	Strain	Resistance gene	Vector Backbone
K2491013	CCA-48	CCA-30 [Fluorene insert 1_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23100_BBa_B0034 EcoRI/SpeI] as EcoRI/SpeI CCA-36 [Fluorene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23100_BBa_B0034 EcoRI/SpeI] as XbaI/PstI Vector pSB3T5 as EcoRI/PstI	E.coli BL-21	Tetracycline	pSB3T5
K2491025	CCA-51	CCA-30 [Fluorene insert 1_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23101_BBa_B0034 EcoRI/SpeI] as EcoRI/SpeI CCA-38 [Fluorene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23101_BBa_B0034 EcoRI/SpeI] as XbaI/PstI Vector pSB3T5 as EcoRI/PstI	E.coli BL-21	Tetracycline	pSB3T5
K2491026	CCA-54	CCA-30 [Fluorene insert 1_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23110_BBa_B0034 EcoRI/SpeI] as EcoRI/SpeI CCA-40 [Fluorene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23110_BBa_B0034 EcoRI/SpeI] as XbaI/PstI Vector pSB3T5 as EcoRI/PstI	E.coli BL-21	Tetracycline	pSB3T5
K2491027	CCA-57	CCA-23 [Promoter BBa_J23100 /RBS_BBa_B0034 EcoRI/SpeI + Phenanthrene insert 1_Ter_BBa_B0015 XbaI/PstI] as SpeI/PstI CCA-42 [Phenanthrene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23100_BBa_B0034 EcoRI/SpeI] as XbaI/PstI	E.coli BL-21	Chloramphenicol	pSB1C3
K2491028	CCA-60	CCA-26 [Promoter BBa_J23101 /RBS_BBa_B0034 EcoRI/SpeI + Phenanthrene insert 1_Ter_BBa_B0015 XbaI/PstI] as SpeI/PstI CCA-44 [Phenanthrene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23101_BBa_B0034 EcoRI/SpeI] as XbaI/PstI	E.coli BL-21	Chloramphenicol	pSB1C3
K2491029	CCA-64	CCA-29 [Promoter BBa_J23110 /RBS_BBa_B0034 EcoRI/SpeI + Phenanthrene insert 1_Ter_BBa_B0015 XbaI/PstI] as SpeI/PstI CCA-46 [Phenanthrene insert 2_Ter_BBa_B0015 XbaI/PstI + Promoter BBa_J23110_BBa_B0034 EcoRI/SpeI] as XbaI/PstI	E.coli BL-21	Chloramphenicol	pSB1C3

3. CALIBRATION

As directed by IGEM, we use LUDOX-S40 as a single point reference to obtain a ratiometric conversion factor to transform the absorbance data into a standard OD 600 measurement. The path-length correction was turned off. To measure the standard LUDOX Abs 600 we have used the same plate and volumes (100 uL) used in the cell based assays.

Materials:
1ml LUDOX (provided in kit)
Water

96 well plate with flat with transparent bottom

Method:

√ Add 100 µl LUDOX into wells A1, B1, C1, D1 (or 1 mL LUDOX into cuvette)
√ Add 100 µl of H 2 O into wells A2, B2, C2, D2 (or 1 mL H 2 O into cuvette)
√ Measure absorbance 600 nm of all samples in all standard measurement modes in instrument
√ Record the data in the table below or in your notebook
√ Import data into Excel (OD600 reference point tab)

Result:

The calibration was done in the context of the interlab experiment. The correction was calculated to be 5.05.

4. BIOTRANSFORMATION MEASUREMENT

4.1. Materials

Cultures of clones in transformed in *Escherichia coli* strain BL21(DE3)
LB (Luria Bertani) media
Minimal medium
Fluorene
Phenanthrene
Chloramphenicol
Tetracyclin
Ampicillin
IPTG
Baffled flasks
50 ml Falcon tube with vented cap
Incubator at 37°C
1.5 ml Eppendorf tubes for sample storage
Ice bucket with ice
Pipettes
96 well plate with flat transparent bottom Devices
Shaker

4.2. Methods

Instrument.

√ Set instrument to read OD600 (as OD calibration setting)
√ Measure OD600 of the overnight cultures
√ Record data in notebook
√ Import data into Excel **Dilution Calculation**
√ Dilute the cultures to a target OD 600 of 0.02 (see the volume of preloading culture and media in Excel (**Dilution Calculation**) Sheet_1) in **4 mL** LB medium + Chloramphenicol or tetracycline or both in 14 mL falcon tube
√ Incubate the cultures at 37°C and 220 rpm.
√ Take 450 µL samples of the cultures at various time points. At each time point, take a sample from each clone, two colonies per clone
√ Place samples on ice.
√ At the end of sampling point, measure samples
√ Use the same instrument settings for all time points

√ Pipette 100 µl of each sample into each well.

Growth. Recombinant constructs in *Escherichia coli (E. coli)* were routinely grown and maintained on Luria-Bertani (LB, 1.0% tryptone, 0.5% yeast extract, 1.0% sodium chloride) on agar plates (1.5% agar) or broth liquid amended with the corresponding antibiotics.
Minimal medium M9 was used to grow recombinant E.coli BL21(DE3) supplemented with the corresponding antibiotic and supplemented with glucose, fluorene, and/or phenanthrene. M9 is a minimal defined growth medium [42mM sodium phosphate, dibasic, 24mM potassium phosphate, monobasic, 9mM sodium chloride, 19mM ammonium chloride, 1mM Thiamine, 0.2mM magnesium sulfate, 0.1mM calcium chloride with 1.5% agar for solid medium and without agar for liquid medium]. M9 medium presents the advantage of having low auto-fluorescence (when excited at 488nm) and low absorbance. Glucose (0.4%) or PAH (1, 0.5, 0.1 mg/mL) were added as a carbon source to supply carbohydrates. LB broth was used as a complete medium for growing *E.coli* strains before transferring it to minimal medium. Solid medium contained 1.5% agar.
For liquid medium, stock solutions of 10 or 100 mg/mL of fluorene and phenanthrene solubilized in DMSO or methanol were added to the medium. Fluorene and phenanthrene were provided as crystals in the lid Petri dishes.
When needed, carbenicillin, tetracycline, or chloramphenicol were added at 100, 12.5, and 34 µg/mL respectively. Cells were grown at 37°C or at 20-25°C for experiments conducted at room temperature.

4.3. BIOTRANSFORMATION RESULTS IN PRESENCE OF FLUORENE AND PHENANTHRENE DISSOLVED IN METHANOL OR DMSO

4.3.1. EXPERIMENTAL DESIGN

In order to assess whether the newly engineered E.coli strains containing either the fluorene catabolic pathway or the phenanthrene catabolic pathway were able to degrade their respective PAH, they were grown in minimal medium supplemented with fluorene or phenanthrene as a sole source of carbon. For controls, the strains were grown in presence of glucose. In addition, E.coli strains containing the corresponding vector without insert was also grown in parallel.
Fluorene and phenanthrene were prepared as stock solution of 10 mg/mL and were initially dissolved in methanol. Because the stock solution showed a slight precipitate, stock solutions of 10 mg/mL were also prepared in the organic solvent dimethyl sulfoxide (DMSO). Growth comparisons using these 2 solvents were performed in parallel.

4.3.2. CULTURE SETUP

Cultures were started from glycerol stock in 4 mL of medium and incubated at 37°C. The OD readout of the overnight cultures was determined using a spectrophotometer according to the protocol shown above. All cultures were then diluted to 0.02 using the volume below and OD measurements were determined at the indicated time points.

Table 1. OD measurement and dilution scheme of overnight cultures to initiate biotransformation studies.

target Abs600		**0.02**	
target volume (mL)		**4**	
sample	Abs600 Reading	Volume of Preloading Culture (mL)	Volume of Preloading Media (mL)
pSB1C3	0.473	0.185	3.815
P1P2_100=clone 57	0.526	0.165	3.835
P1P2_101=clone 61	0.524	0.166	3.834
P1P2_110=clone 64	0.486	0.180	3.820
pSB3T5	0.520	0.167	3.833
F1F2_100 =clone 48	0.267	0.355	3.645
F1F2_101 =clone 51	0.419	0.211	3.789
F1F2_110 =clone 54	0.272	0.347	3.653
media	0.041		

4.3.3. RESULTS AND DISCUSSION

a. RESULTS AND DISCUSSION – Culture growth of recombinant E.coli BL21(DE3) in minimal medium in presence of phenanthrene and fluorene only

The data show all absorbance measurements obtained during the biotransformation of phenanthrene and fluorene by our recombinant E.coli in minimal medium supplemented with PAHs. In order to evaluate whether the recombinant cells had the ability to transform PAHs, growth experiments were set up with various clones expressing the fluorene or phenanthrene catabolic pathway. The clones described above with the catabolic pathway under the control of 3 different constitutive were set in cultures using minimal medium supplemented with fluorene (0.1 mg/mL) and phenanthrene (0.1 mg/mL) as sole source of carbon (figures below). Antibiotics were added as appropriately.

Clones containing the catabolic pathway exhibited higher cell density at 48 hours compared to their respective controls (vector alone) with the strongest promoter given a greater advantage (clone 48 for fluorene and clone 57 for phenanthrene). PAHs dissolved in DMSO appeared slightly more available than PAHs dissolved in methanol. This may be explained by the fact that PAHs stock solutions prepared in methanol exhibited some precipitates not observed with DMSO-based stocks.

Table 2. Raw Data of OD measurement at 600 nm of cultures at various time points of 3 Fluorene (F) catabolic clones and control-vector clone when grown on minimal medium supplemented with Fluorene 0.1 mg/mL dissolved in methanol (M) or DMSO (D).

Plate Layout Fluorene

T=0 hour			1	2	3	4	5	6
pSB3T5	Clone 51	MM	0.065	0.064	0.061	0.060	0.041	0.041
pSB3T5	Clone 51	MM+Fd	0.067	0.067	0.068	0.076	0.042	0.048
pSB3T5	Clone 51	MM+Fm	0.068	0.065	0.068	0.067	0.070	0.040
Clone 48	Clone 54		0.060	0.060	0.061	0.060	0.048	0.047
Clone 48	Clone 54		0.067	0.068	0.060	0.065	0.048	0.048
Clone 48	Clone 54		0.065	0.067	0.061	0.069	0.047	0.048
empty	empty	empty	0.048	0.048	0.048	0.047	0.049	0.048
empty	empty	empty	0.048	0.048	0.048	0.048	0.048	0.048

24 hour			1	2	3	4	5	6
pSB3T5	Clone 51	MM	0.083	0.072	0.087	0.086	0.041	0.048
pSB3T5	Clone 51	MM+Fd	0.082	0.086	0.087	0.124	0.048	0.041
pSB3T5	Clone 51	MM+Fm	0.091	0.090	0.083	0.085	0.040	0.039
Clone 48	Clone 54		0.090	0.087	0.083	0.088	0.048	0.048
Clone 48	Clone 54		0.163	0.111	0.196	0.125	0.048	0.047
Clone 48	Clone 54		0.087	0.087	0.090	0.089	0.048	0.048
empty	empty	empty	0.047	0.048	0.048	0.048	0.048	0.049
empty	empty	empty	0.048	0.048	0.047	0.048	0.048	0.049

48hour			1	2	3	4	5	6
pSB3T5	Clone 51	MM	0.109	0.104	0.087	0.087	0.041	0.042
pSB3T5	Clone 51	MM+Fd	0.095	0.097	0.176	0.194	0.041	0.041
pSB3T5	Clone 51	MM+Fm	0.090	0.098	0.152	0.147	0.039	0.039
Clone 48	Clone 54		0.085	0.085	0.076	0.074	0.049	0.047
Clone 48	Clone 54		0.345	0.365	0.295	0.282	0.048	0.047
Clone 48	Clone 54		0.155	0.158	0.154	0.154	0.049	0.049
empty	empty	empty	0.050	0.048	0.048	0.048	0.048	0.048
empty	empty	empty	0.049	0.048	0.048	0.048	0.048	0.048

Table 3. Averages of converted measurement of cultures at 600 nm at various time points of 3 fluorene (F) catabolic clones and control-vector clone when grown on minimal medium (MM) supplemented with PAH 0.1 mg/mL dissolved in methanol (M) or DMSO (D).

Media	Clone	OD Average	OD Conversion		Clone	OD Average	OD Conversion		Clone	OD Average	OD Conversion
MM	Clone Cont	0.064	0.116		Clone 51	0.0607	0.098		No clone	0.041	0.208
MM+Fd	Clone Cont	0.067	0.109		Clone 51	0.0719	0.135		No clone	0.045	0.228
MM+Fm	Clone Cont	0.066	0.057		Clone 51	0.0678	0.064		No clone	0.055	0.278
MM	Clone 48	0.060	0.094		Clone 54	0.0608	0.094				
MM+Fd	Clone 48	0.067	0.113		Clone 54	0.0626	0.113				
MM+Fm	Clone 48	0.066	0.055		Clone 54	0.0649	0.055				
MM	Clone Cont	0.077	0.164		Clone 51	0.0861	0.209		No clone	0.045	0.226
MM+Fd	Clone Cont	0.084	0.200		Clone 51	0.1053	0.308		No clone	0.044	0.224
MM+Fm	Clone Cont	0.090	0.257		Clone 51	0.0835	0.223		No clone	0.039	0.199
MM	Clone 48	0.088	0.220		Clone 54	0.0859	0.434				
MM+Fd	Clone 48	0.137	0.469		Clone 54	0.1606	0.811				
MM+Fm	Clone 48	0.087	0.238		Clone 54	0.0893	0.451				
MM	Clone Cont	0.106	0.327		Clone 51	0.0868	0.229		No clone	0.042	0.210
MM+Fd	Clone Cont	0.096	0.277		Clone 51	0.1851	0.727		No clone	0.041	0.207
MM+Fm	Clone Cont	0.094	0.276		Clone 51	0.1493	0.556		No clone	0.039	0.198
MM	Clone 48	0.085	0.220		Clone 54	0.0751	0.169				
MM+Fd	Clone 48	0.355	1.585		Clone 54	0.2880	1.247				
MM+Fm	Clone 48	0.157	0.594		Clone 54	0.1540	0.580				

Table 4. Raw Data of OD measurement at 600 nm at various time points of 3 Phenanthrene (P) catabolic clones and control-vector clone when grown on minimal medium supplemented with phenanthrene 0.1 mg/mL dissolved in methanol (M) or DMSO (D).

Absorbance at 600 nm Raw data

Plate Layout Phenanthrene

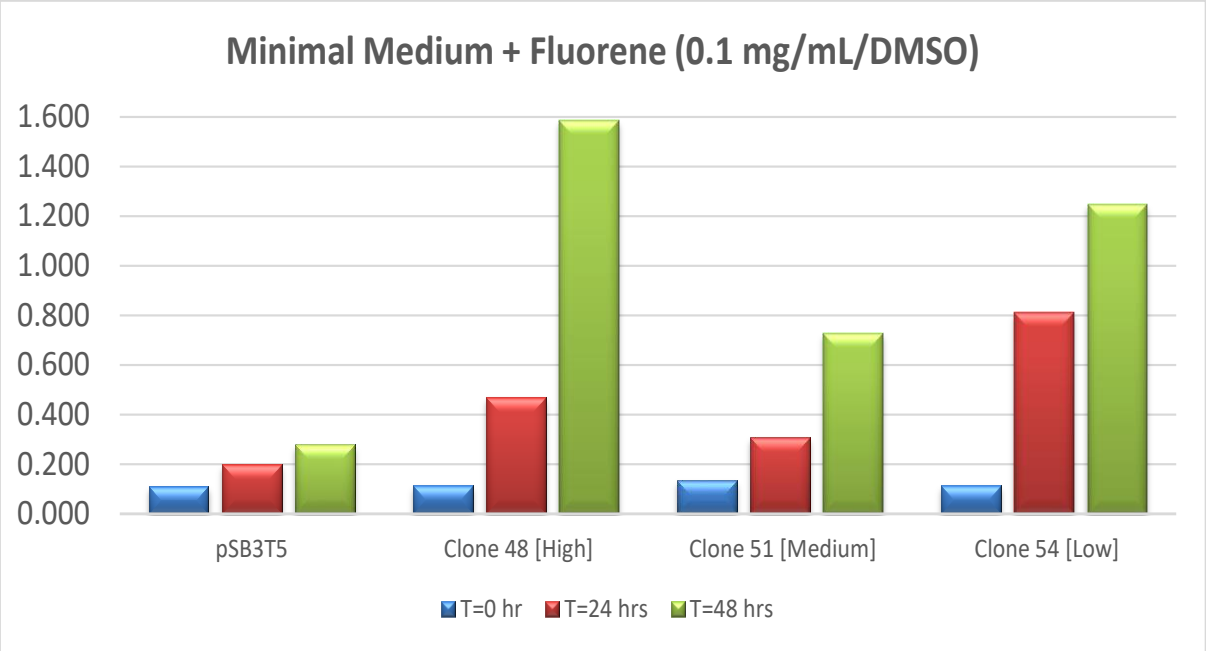
T=0 hour			7	8	9	10	11	12
pSB1C3	Clone 60	MM	0.065	0.064	0.062	0.062	0.041	0.041
pSB1C3	Clone 60	MM+Fd	0.061	0.067	0.064	0.067	0.040	0.043
pSB1C3	Clone 60	MM+Fm	0.069	0.067	0.065	0.075	0.050	0.052
Clone 57	Clone 64		0.070	0.070	0.059	0.059	0.048	0.048
Clone 57	Clone 64		0.060	0.069	0.063	0.061	0.048	0.048
Clone 57	Clone 64		0.063	0.065	0.063	0.063	0.048	0.048
empty	empty	empty	0.048	0.048	0.048	0.048	0.049	0.048
empty	empty	empty	0.048	0.048	0.047	0.048	0.048	0.053

T=24 hour			7	8	9	10	11	12
pSB1C3	Clone 60	MM	0.083	0.083	0.105	0.104	0.041	0.042
pSB1C3	Clone 60	MM+Fd	0.087	0.086	0.094	0.092	0.040	0.039
pSB1C3	Clone 60	MM+Fm	0.089	0.089	0.083	0.109	0.039	0.039
Clone 57	Clone 64		0.127	0.128	0.111	0.111	0.048	0.049
Clone 57	Clone 64		0.121	0.143	0.106	0.108	0.048	0.048
Clone 57	Clone 64		0.102	0.111	0.117	0.116	0.048	0.048
empty	empty	empty	0.049	0.048	0.048	0.049	0.048	0.048
empty	empty	empty	0.049	0.048	0.047	0.048	0.048	0.049

T=48 hour			7	8	9	10	11	12
pSB1C3	Clone 60	MM	0.084	0.082	0.113	0.110	0.041	0.041
pSB1C4	Clone 60	MM+Fd	0.093	0.098	0.233	0.236	0.039	0.039
pSB1C5	Clone 60	MM+Fm	0.098	0.096	0.265	0.256	0.039	0.039
Clone 57	Clone 64		0.138	0.139	0.126	0.120	0.048	0.048
Clone 57	Clone 64		0.271	0.301	0.236	0.233	0.048	0.048
Clone 57	Clone 64		0.238	0.244	0.212	0.237	0.048	0.048
empty	empty	empty	0.048	0.048	0.038	0.048	0.048	0.049
empty	empty	empty	0.048	0.048	0.047	0.048	0.048	0.048

Table 5. Averages of converted measurement at 600 nm at various time points of 3 Phenanthrene (P) catabolic clones and control-vector clone when grown on minimal medium supplemented with phenanthrene 0.1 mg/mL dissolved in methanol (M) or DMSO (D).

Time Point	Media	Clone	OD Average	OD Conversion		Clone	OD Average	OD Conversion		Clone	OD Average	OD Conversion
T=0 hr	MM	Clone Contr	0.064	0.119		Clone 60	0.062	0.108		No clone	0.041	0.206
	MM+Pd	Clone Contr	0.064	0.111		Clone 60	0.065	0.120		No clone	0.042	0.211
	MM+Pm	Clone Contr	0.068	0.085		Clone 60	0.070	0.097		No clone	0.051	0.256
	MM	Clone 57	0.070	0.145		Clone 64	0.059	0.145				
	MM+Pd	Clone 57	0.065	0.117		Clone 64	0.062	0.117				
	MM+Pm	Clone 57	0.064	0.067		Clone 64	0.063	0.067				
T=24 hr	MM	Clone Contr	0.083	0.211		Clone 60	0.104	0.319		No clone	0.041	0.209
	MM+Pd	Clone Contr	0.087	0.239		Clone 60	0.093	0.271		No clone	0.039	0.199
	MM+Pm	Clone Contr	0.089	0.250		Clone 60	0.096	0.287		No clone	0.039	0.199
	MM	Clone 57	0.127	0.435		Clone 64	0.111	0.559				
	MM+Pd	Clone 57	0.132	0.466		Clone 64	0.107	0.540				
	MM+Pm	Clone 57	0.106	0.339		Clone 64	0.117	0.588				
T=48 hr	MM	Clone Cont	0.083	0.213		Clone 60	0.111	0.356		No clone	0.041	0.207
	MM+Pd	Clone Cont	0.095	0.286		Clone 60	0.234	0.988		No clone	0.039	0.196
	MM+Pm	Clone Cont	0.097	0.292		Clone 60	0.260	1.116		No clone	0.039	0.198
	MM	Clone 57	0.138	0.492		Clone 64	0.123	0.415				
	MM+Pd	Clone 57	0.286	1.247		Clone 64	0.234	0.987				
	MM+Pm	Clone 57	0.241	1.017		Clone 64	0.224	0.934				



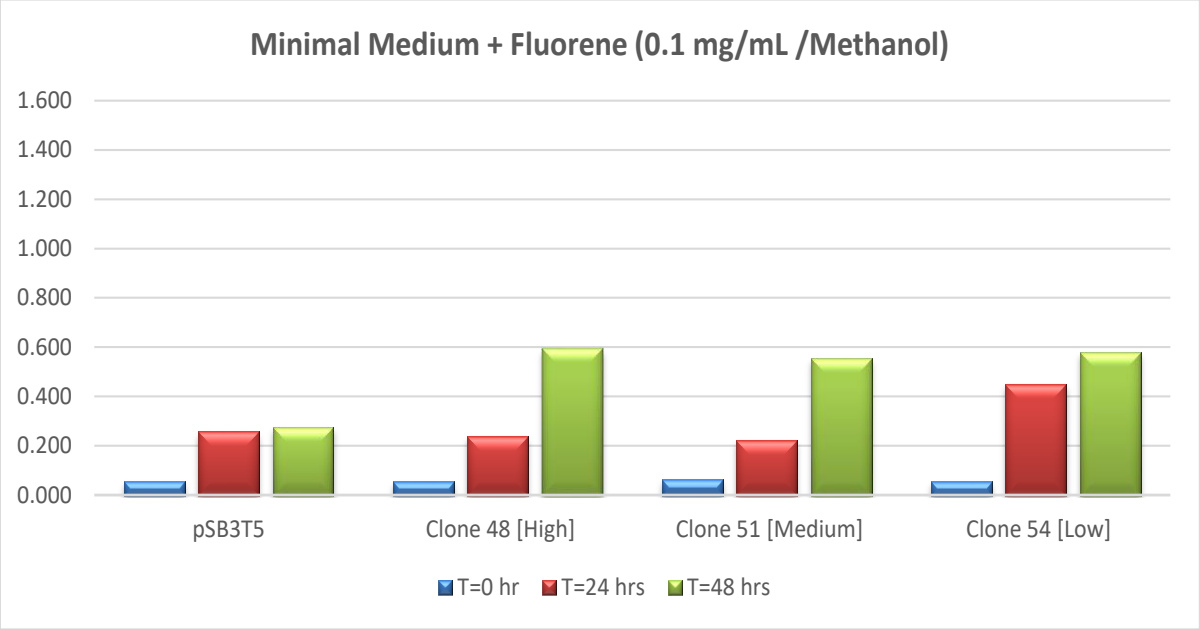
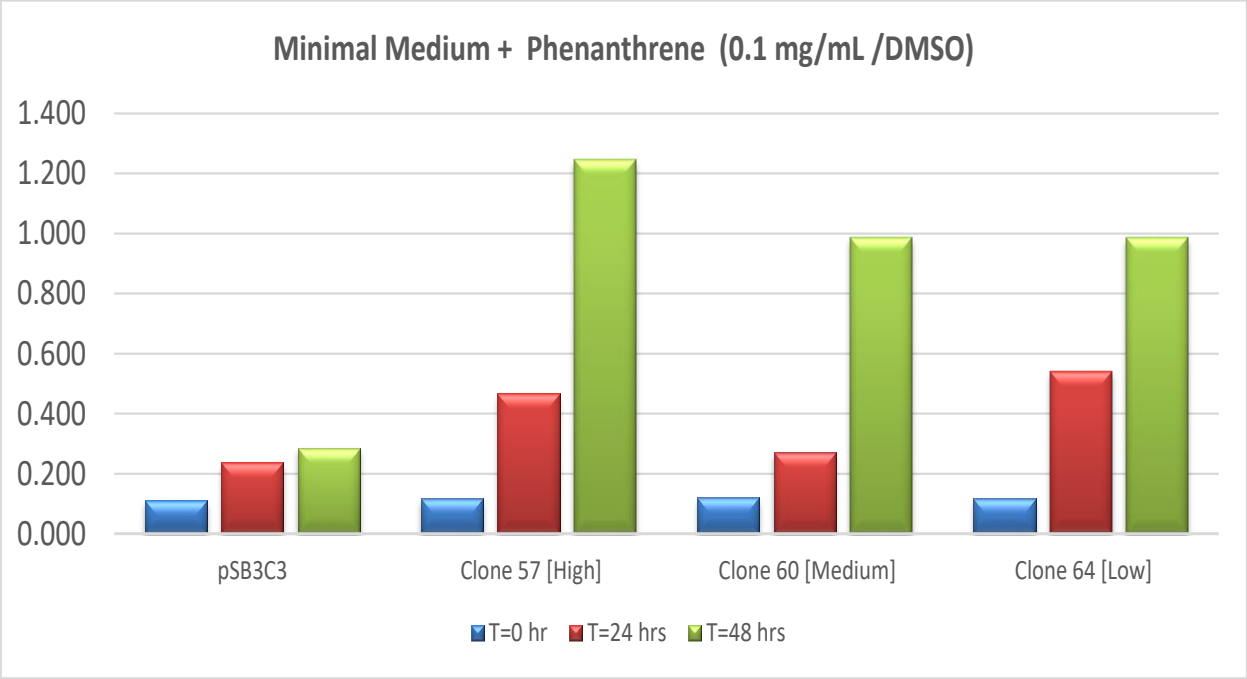


Figure 1. Growth of recombinant E.coli BL21DE3 cultures harboring the control plasmid pSB3T5 or the fluorene pathway under the control of 3 different constitutive promoters: BBa_J23100 (clone 48), BBa_J23101 (clone 51), and BBa_J23110 (clone 54) cloned into pSB3T5. Data points represent value averages of duplicate of OD at 600 nm taken over time for 2 independent colonies per clone. Recombinant clones were grown in minimal medium supplement with tetracycline (15 µg/mL) and fluorene (0.1 mg/mL). Fluorene was dissolved in 100% DMSO (Top panel) or 100% methanol (Bottom panel).



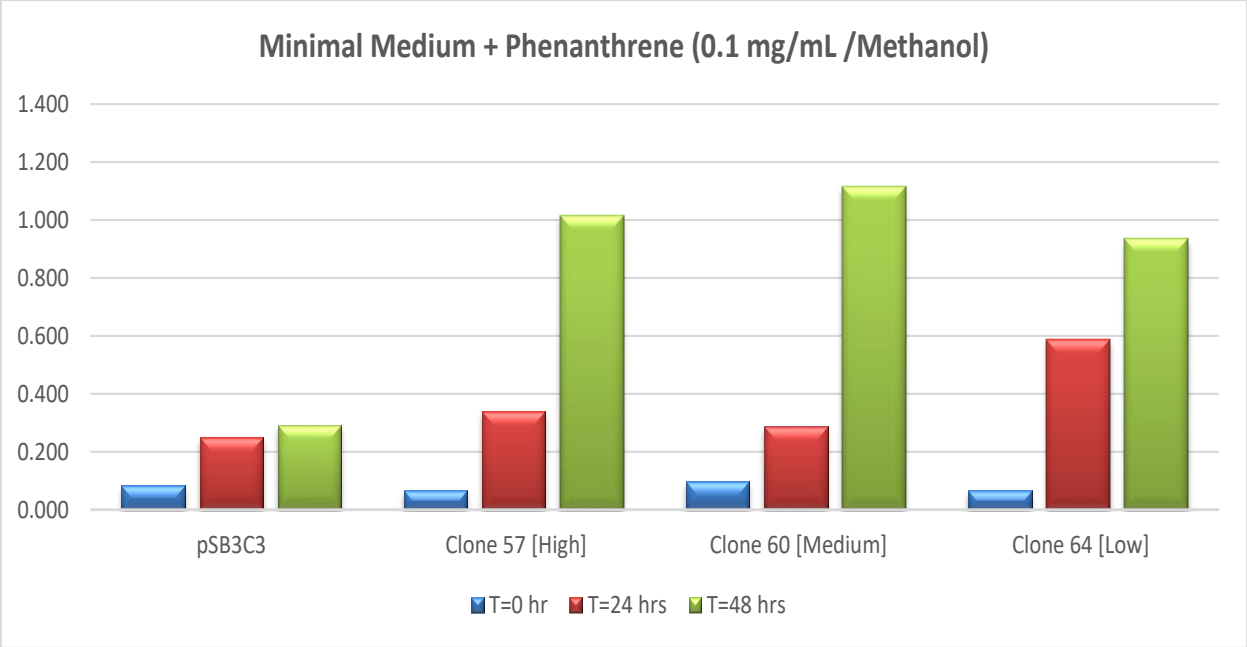


Figure 2. Growth of recombinant E.coli BL21DE3 cultures harboring the control plasmid pSB1C3 or the phenanthrene pathway under the control of 3 different constitutive promoters: BBa_J23100 (clone 57), BBa_J23101 (clone 60), and BBa_J23110 (clone 64) cloned into pSB1C3. Data points represent value averages of duplicate of OD at 600 nm taken over time for 2 independent colonies per clone. Recombinant clones were grown in minimal medium supplement with chloramphenicol (34 µg/mL) and phenanthrene (0.1 mg/mL). Phenanthrene was dissolved in 100% DMSO (Top panel) or 100% methanol (Bottom panel).

b. RESULTS AND DISCUSSION – Culture growth of recombinant E.coli BL21(DE3) in minimal medium in presence of phenanthrene, fluorene, and glucose

Growth comparison of recombinant E.coli in minimal medium supplemented with PAHs and glucose. In order to evaluate the role of toxicity and/or the metabolic burden caused by the PAH catabolic genes and the PAHs, namely fluorene and phenanthrene, cells were grown in minimal medium together with fluorene or phenanthrene and glucose as carbohydrate sources (figures below).

It appears that all 3 clones carried by a low copy plasmid number with the fluorene catabolic pathway under the control of 3 promoters of various strengths behaved similarly to the control strain harboring the corresponding vector pSB3T5 with no insert. The fluorene genes independently of their expression level do not appear to impact cell growth when carried by a low copy vector. In addition, fluorene (0.1 mg/mL) in presence of glucose is not toxic to the cells.

It appears that all 3 clones carried by a high copy plasmid number with the phenanthrene catabolic pathway under the control of 3 promoters of various strengths behaved differently to the control strain harboring the corresponding vector pSB1C3 with no insert. The phenanthrene genes independently of their expression level appear to impact cell growth at least during the initial phase. Phenanthrene (0.1 mg/mL) in presence of glucose does not appear to be toxic to the cells as the control cell grew.

Table 6. Averages of converted measurement at 600 nm of cultures aliquoted at various time points of 3 Phenanthrene (P) catabolic clones and control-vector clone when grown on minimal medium supplemented with phenanthrene 0.1 mg/mL dissolved in methanol (M) or DMSO (D).

Growth curve in M9 minimal media	M9 Minimal Medium + Glucose		0 hr		2 hr		4 hr		6 hr		24 hr	
Colony 1	3	4	1	2	5	6	7	8	9	10	11	12
pSB3T5	0.041	0.042	0.072	0.079	0.085	0.088	0.119	0.126	0.167	0.162	0.233	0.236
Clone 48	0.048	0.048	0.067	0.072	0.085	0.089	0.124	0.126	0.175	0.175	0.393	0.238
Clone 51	0.048	0.048	0.064	0.070	0.072	0.074	0.104	0.102	0.154	0.142	0.452	0.271
Clone 54	0.048	0.048	0.066	0.070	0.092	0.083	0.124	0.117	0.178	0.162	0.341	0.227
pSB1C3	0.048	0.048	0.066	0.070	0.094	0.088	0.137	0.133	0.187	0.172	0.267	0.264
Clone 57	0.048	0.048	0.067	0.070	0.062	0.065	0.062	0.065	0.072	0.073	0.291	0.290
Clone 60	0.048	0.049	0.067	0.069	0.063	0.062	0.063	0.063	0.071	0.071	0.298	0.369
Clone 64	0.048	0.048	0.066	0.068	0.063	0.064	0.064	0.064	0.075	0.076	0.330	0.268

			0 hr		2 hr		4 hr		6 hr		24 hr	
Colony 2	3	4	1	2	5	6	7	8	9	10	11	12
pSB3T5	0.041	0.042	0.071	0.078	0.085	0.086	0.120	0.126	0.168	0.163	0.227	0.229
Clone 48	0.048	0.048	0.067	0.071	0.086	0.088	0.126	0.128	0.177	0.176	0.375	0.230
Clone 51	0.048	0.048	0.064	0.070	0.072	0.073	0.106	0.102	0.156	0.143	0.434	0.259
Clone 54	0.048	0.048	0.066	0.072	0.092	0.083	0.126	0.117	0.180	0.165	0.328	0.218
pSB1C3	0.048	0.048	0.066	0.069	0.094	0.086	0.138	0.134	0.188	0.174	0.257	0.254
Clone 57	0.048	0.048	0.067	0.069	0.061	0.064	0.062	0.064	0.071	0.073	0.271	0.275
Clone 60	0.049	0.048	0.067	0.069	0.062	0.061	0.063	0.063	0.072	0.072	0.279	0.347
Clone 64	0.048	0.048	0.065	0.067	0.062	0.064	0.064	0.064	0.076	0.077	0.318	0.264

Table 7. Top panel: Averages of converted OD measurement at 600 nm of cultures aliquoted at various time points for 2 values of 3 fluorene catabolic clones (Clones 48, 31, and 54) and control-vector (pSB3T5) when grown on minimal medium supplemented with fluorene (0.1 mg/mL). Lower panel: Averages of converted OD measurement at 600 nm at various time points for 2 values of 3 phenanthrene catabolic clones (Clones 57, 60, and 64) and control-vector (pSB1C3) when grown on minimal medium supplemented with phenanthrene (0.1 mg/mL).

	Corrected Values [Colony 1]				
Clone	0 hr	2 hr	4 hr	6 hr	24 hr
pSB3T5	0.147	0.203	0.384	0.595	0.950
Clone 48	0.115	0.206	0.398	0.647	1.358
Clone 51	0.104	0.132	0.286	0.512	1.591
Clone 54	0.110	0.207	0.373	0.624	1.198
pSB1C3	0.099	0.215	0.440	0.664	1.097
Clone 57	0.102	0.077	0.076	0.123	1.224
Clone 60	0.101	0.072	0.075	0.117	1.440
Clone 64	0.095	0.076	0.080	0.139	1.266

	Corrected Values [Colony 2]				
Clone	0 hr	2 hr	4 hr	6 hr	24 hr
pSB3T5	0.142	0.197	0.386	0.602	0.917
Clone 48	0.114	0.206	0.406	0.656	1.291
Clone 51	0.104	0.131	0.290	0.520	1.515
Clone 54	0.114	0.206	0.378	0.636	1.144
pSB1C3	0.098	0.212	0.443	0.670	1.046
Clone 57	0.100	0.073	0.075	0.121	1.137
Clone 60	0.100	0.069	0.076	0.120	1.338
Clone 64	0.093	0.074	0.081	0.143	1.228

Table 8. Averages of converted OD measurement at 600 nm at various time points for 2 colonies of each clones.

	Corrected Values - Average 2 colonies per construct				
Clone	0 hr	2 hr	4 hr	6 hr	24 hr
pSB3T5	0.145	0.200	0.385	0.599	0.934
Clone 48	0.115	0.206	0.402	0.652	1.325
Clone 51	0.104	0.131	0.288	0.516	1.553
Clone 54	0.112	0.207	0.375	0.630	1.171
pSB1C3	0.098	0.213	0.442	0.667	1.072
Clone 57	0.101	0.075	0.075	0.122	1.181
Clone 60	0.101	0.070	0.076	0.119	1.389
Clone 64	0.094	0.075	0.080	0.141	1.247

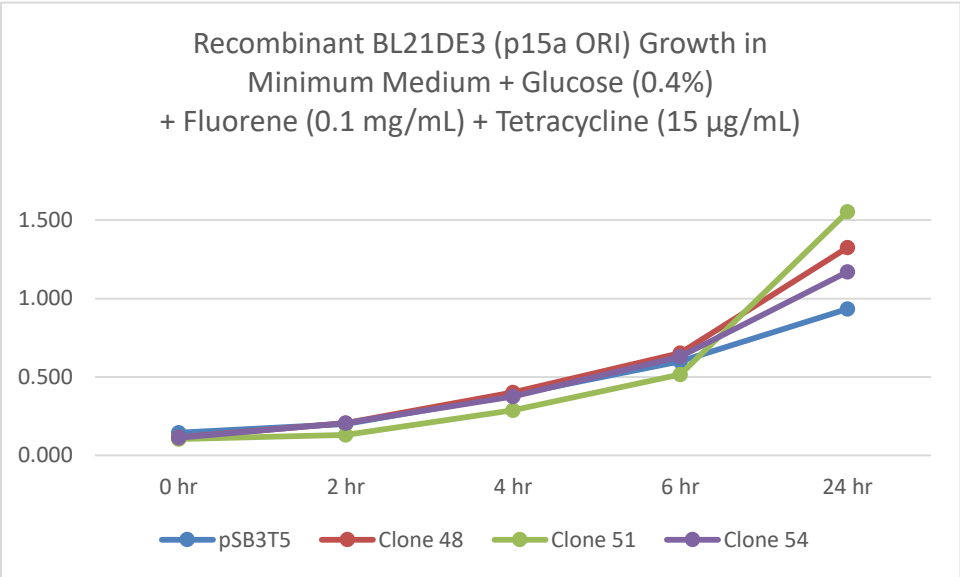


Figure 3. Fluorene biotransformation experiment using recombinant E.coli BL21DE3 harboring the control plasmid pSB3T5 or the fluorene pathway under the control of 3 different constitutive promoters: BBa_J23100 (clone 48), BBa_J23101 (clone 51), and BBa_J23110 (clone 54) cloned into pSB3T5. Data points represent value averages of duplicate of OD at 600 nm taken over time for 2 independent colonies per clone. Recombinant clones were grown in minimal medium supplement with tetracycline (15 µg/mL), fluorene (0.1 mg/mL), and glucose (0.4%).

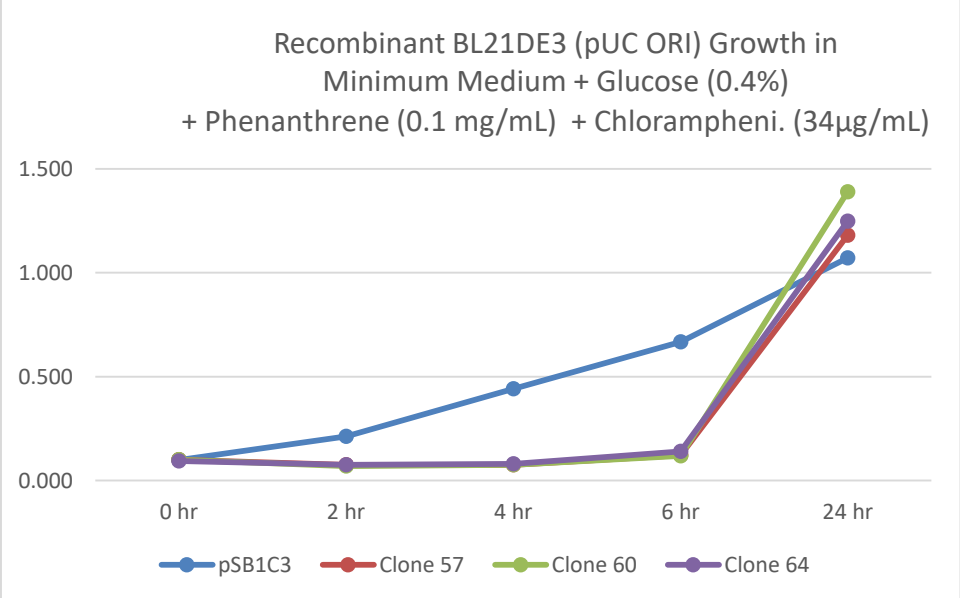


Figure 4. Phenanthrene biotransformation experiment using recombinant E.coli BL21DE3 harboring the control plasmid pSB1C3 or the phenanthrene pathway under the control of 3 different constitutive promoters: BBa_J23100 (clone 57), BBa_J23101 (clone 60), and BBa_J23110 (clone 64) cloned into pSB1C3. Data points represent value averages of duplicate of OD at 600 nm taken over time for 2 independent colonies per clone. Recombinant clones were grown in minimal medium supplement with chloramphenicol (34 µg/mL), phenanthrene (0.1 mg/mL), and glucose (0.4%).

4.4. BIOTRANSFORMATION RESULTS IN PRESENCE OF FLUORENE AND PHENANTHRENE DISSOLVED IN DMSO

4.4.1. EXPERIMENTAL DESIGN

In order to assess whether the newly engineered E.coli strains containing either the fluorene catabolic pathway or the phenanthrene catabolic pathway were able to degrade their respective PAH, they were grown in minimal medium supplemented with fluorene or phenanthrene as a sole source of carbon. For controls, the strains were grown in presence of glucose. In addition, E.coli strains containing the corresponding vector without insert was also grown in parallel.

4.4.2. CULTURE SETUP

Cultures were started from glycerol stock in 4 mL of medium and placed at 37°C. The OD readout of the overnight cultures was determined using a spectrophotometer according to the protocol shown above. All cultures were then diluted to 0.02using the volume below and OD measurements were determined at the indicated time points.

Table 9. OD measurement and dilution scheme of overnight cultures to initiate biotransformation studies.

target Abs600		**0.02**	
target volume (mL)		**4**	
sample	Abs 600 Reading	Volume (mL) of Preloading Culture	Volume (mL) of Preloading Media
pSB3T5_colony 1	0.256	0.374	3.626
pSB3T5_colony 2	0.274	0.345	3.655
Fluorene_Colony 1	0.224	0.440	3.560
Fluorene_Colony 2	0.229	0.428	3.572
media	0.042		

target Abs600		**0.02**	
target volume (mL)		**4**	
sample	Abs600 Reading	Volume (mL) of Preloading Culture	Volume (mL) of Preloading Media
pSB1C3_colony 1	0.446	0.198	3.802
pSB1C3_colony 2	0.458	0.192	3.808
Phe_Colony 1	0.269	0.352	3.648
Phe_Colony 2	0.267	0.356	3.644
media	0.042		

4.4.3. RESULTS AND DISCUSSION – Growth in presence of phenanthrene (0.1, 0.5, and 1.0 mg/mL) and fluorene (0.1, 0.5, and 1.0 mg/mL)

Biotransformation of phenanthrene and fluorene by recombinant E.coli in minimal medium supplemented with PAHs and surfactant. Strains naturally involved in bio-remediation typically secrete biosurfactants aiming at optimizing the access of microorganisms to PAHs. To mimic a biosurfactant, biotransformation experiments were conducted with surfactant such as Tween-20. In order to evaluate whether the recombinant cells had the ability to transform PAHs, growth experiments were set up with various clones expressing the fluorene or the phenanthrene catabolic pathway. The clones described above with the catabolic pathway under the control of the strongest constitutive were set in cultures using minimal medium supplemented with fluorene (0.1, 0.5 and 1.0 mg/mL) and phenanthrene (0.1, 0.5 and 1.0mg/mL) as sole source of carbon (figures below) in presence of Tween (0.1%). Antibiotics were added as appropriately. The PAHs were solubilized in DMSO.
As illustrated in the table below, the ratio indicates that bacteria containing the biodegradation pathway could utilize fluorene or phenanthrene.

Table 10. Average absorbance values measured in quadruplet at 600 nm of cultures of 2 independent colonies of control (vector) and catabolic plasmid (clone 57) 24 hours after inoculation of minimal media supplemented with phenanthrene at 0.1, 0.5, and 1 mg/mL with or without glucose (0.4%). All media contained the surfactant Tween-20 (0.1%). MM= Minimal Medium, CHL= Chloramphenicol, Glu= Glucose; OD=Optical Density; SD= standard deviation.

Medium		OD Average	OD SD	Ratio Clone/Vector
MM_Chl	Vector	0.078	0.007	
MM_Chl	Clone-57	0.087	0.014	1.12
MM_Chl_Phenanthrene 1 mg/mL	Vector	0.016	0.002	
MM_Chl_Phenanthrene 1 mg/mL	Clone-57	0.065	0.005	4.00
MM_Chl_Phenanthrene 0.5 mg/mL	Vector	0.039	0.004	
MM_Chl_Phenanthrene 0.5 mg/mL	Clone-57	0.158	0.003	4.05
MM_Chl_Phenanthrene 0.1 mg/mL	Vector	0.137	0.020	
MM_Chl_Phenanthrene 0.1 mg/mL	Clone-57	0.245	0.001	1.79
MM_Glu_Chl	Vector	1.353	0.020	
MM_Glu_Chl	Clone-57	1.135	0.037	0.84
MM_Glu_Chl_Phenanthrene 1mg/mL	Vector	0.452	0.064	
MM_Glu_Chl_Phenanthrene 1mg/mL	Clone-57	0.715	0.065	1.58

Table 11. Average absorbance values measured in quadruplet at 600 nm of cultures of 2 independent colonies of control (vector) and catabolic plasmid (clone 48) 24 hours after inoculation of minimal media supplemented with fluorene at 0.1, 0.5, and 1 mg/mL with or without glucose (0.4%). All media contained the surfactant Tween-20 (0.1%). MM= Minimal Medium, Tet= Tetracycline, Glu= Glucose; OD=Optical Density; SD= standard deviation.

Medium		OD Average	OD SD	Ratio Clone/Vector
MM_Tet	Vector	0.085	0.022	
MM_Tet	Clone 48	0.084	0.001	0.99
MM_Tet_Fluorene 1 mg/mL	Vector	0.074	0.013	
MM_Tet_Fluorene 1 mg/mL	Clone 48	0.253	0.017	3.44
MM_Tet_Fluorene 0.5 mg/mL	Vector	0.082	0.002	
MM_Tet_Fluorene 0.5 mg/mL	Clone 48	0.273	0.006	3.33
MM_Tet_Fluorene 0.1 mg/mL	Vector	0.072	0.003	
MM_Tet_Fluorene 0.1 mg/mL	Clone 48	0.446	0.067	6.22
MM_Glu_Tet	Vector	1.232	0.005	
MM_Glu_Tet	Clone 48	0.999	0.241	0.81
MM_Glu_Tet_Fluorene 1mg/mL	Vector	1.006	0.019	
MM_Glu_Tet_Fluorene 1mg/mL	Clone 48	0.862	0.041	0.86

Table 12. Raw Data of OD values at 600 nm for 2 colonies of clone 48 (Fluorene), clone 57 (Phenanthrene) and their respective vector controls.

PAH:		Fluorene											
Timepoint:		24 hours											
Absorbance:		600 nm											
Volume:		100 uL											
Spetrophotometer:		Molecular Devices Spectra Max											
Conditions:	Raw A to D	MM_Tet	MM_Tet	MM_Tet	MM_Tet_F 1 mg/mL	MM_Tet_F 1 mg/mL	MM_Tet_F 1 mg/mL	MM_Tet_F 0.5 mg/mL	MM_Tet_F 0.5 mg/mL	MM_Tet_F 0.5 mg/mL	MM_Te t_F 0.1 mg/mL	MM_T et_F 0.1 mg/mL	MM_T et_F 0.1 mg/mL
	Raw E to H	MM_Glu_Tet	MM_Glu_Tet	MM_Glu_Tet	MM_Glu_Tet_F 1mg/mL	MM_Glu_Tet_F 1mg/mL	MM_Glu_Tet_F 1mg/mL	Empty wells	Empty wells	Empty wells	Empty wells	Empty wells	Empty wells
Colony:	2												
96-well plate readout													
	Temperature(°C)	1	2	3	4	5	6	7	8	9	10	11	12
	25.7	0.0411	0.0526	0.0529	0.0469	0.0643	0.0903	0.0453	0.0634	0.0905	0.0412	0.0536	0.139
		0.0396	0.0526	0.0598	0.0423	0.0595	0.0907	0.0414	0.0604	0.0915	0.0393	0.0526	0.1309
		0.0395	0.0526	0.0561	0.0432	0.0552	0.0934	0.0432	0.0542	0.0902	0.0396	0.0532	0.1268
		0.04	0.0529	0.0531	0.0438	0.0573	0.0944	0.0421	0.0545	0.0949	0.0405	0.0546	0.1221
		0.0397	0.2731	0.2001	0.0575	0.2534	0.2215	0.0484	0.0479	0.0483	0.0483	0.048	0.0482
		0.0401	0.2842	0.2067	0.0568	0.2531	0.2277	0.0483	0.0468	0.0481	0.0486	0.0485	0.0481
		0.0396	0.2906	0.2014	0.0581	0.252	0.2172	0.0496	0.048	0.048	0.0485	0.0484	0.0484
		0.041	0.291	0.2086	0.0547	0.2547	0.2206	0.0482	0.0477	0.0471	0.0478	0.0481	0.0478

	MM_Tet	MM_Tet	MM_Tet	MM_Tet_F 1 mg/mL	MM_Tet_F 1 mg/mL	MM_Tet_F 1 mg/mL	MM_Tet_F 0.5 mg/mL	MM_Tet_F 0.5 mg/mL	MM_Tet_F 0.5 mg/mL	MM_Tet_F 0.1 mg/mL	MM_Tet_F 0.1 mg/mL	MM_Tet_F 0.1 mg/mL
	Control	Vector	CCA-48	Control	Vector	CCA-48	Control	Vector	CCA-48	Control	Vector	CCA-48
OD600 Average:	0.040	0.053	0.055	0.044	0.059	0.092	0.043	0.058	0.092	0.040	0.054	0.130
OD600 SD:	0.001	0.000	0.003	0.002	0.004	0.002	0.002	0.005	0.002	0.001	0.001	0.007
OD with Blank:		0.013	0.015		0.015	0.048		0.015	0.049		0.013	0.090
Corrected OD Values:		0.069	0.085		0.083	0.265		0.083	0.268		0.073	0.493

	MM_Glu_Tet	MM_Glu_Tet	MM_Glu_Tet	MM_Glu_Tet_F 1mg/mL	MM_Glu_Tet_F 1mg/mL	MM_Glu_Tet_F 1mg/mL	Empty wells					
	Control	Vector	CCA-48	Control	Vector	CCA-48						
OD600 Average:	0.040	0.285	0.204	0.057	0.253	0.222	0.049	0.048	0.048	0.048	0.048	0.048
OD600 SD:	0.001	0.008	0.004	0.001	0.001	0.004	0.001	0.001	0.001	0.000	0.000	0.000
OD with Blank:		0.245	0.164		0.197	0.165		-0.001	-0.001		0.000	0.000
Corrected OD Values:		1.235	0.829		0.992	0.833		-0.005	-0.004		0.000	-0.001

PAH: Fluorene
Timepoint: 24 hours
Absorbance: 600 nm
Volume: 100 uL
Spetrophotometer: Molecular Devices Spectra Max

Conditions:	Raw A to D	MM_Tet	MM_Tet	MM_Tet	MM_Tet _F 1 mg/mL	MM_Tet _F 1 mg/mL	MM_Tet _F 1 mg/mL	MM_Tet _F 0.5 mg/mL	MM_Tet _F 0.5 mg/mL	MM_Tet _F 0.5 mg/mL	MM_Tet _F 0.1 mg/mL	MM_Tet _F 0.1 mg/mL	MM_Tet _F 0.1 mg/mL
	Raw E to H	MM_Glu _Tet	MM_Glu _Tet	MM_Glu _Tet	MM_Glu _Tet_F 1mg/mL	MM_Glu _Tet_F 1mg/mL	MM_Glu _Tet_F 1mg/mL	Empty wells	Empty wells	Empty wells	Empty wells	Empty wells	Empty wells
Colony:	1												

96-well plate readout

Temperature(°C)	1	2	3	4	5	6	7	8	9	10	11	12
25.3	0.041	0.059	0.057	0.047	0.059	0.088	0.045	0.061	0.099	0.041	0.054	0.110
	0.039	0.058	0.057	0.046	0.059	0.089	0.042	0.062	0.091	0.039	0.052	0.113
	0.039	0.058	0.053	0.045	0.056	0.089	0.043	0.054	0.090	0.039	0.052	0.119
	0.040	0.059	0.053	0.046	0.057	0.093	0.043	0.055	0.095	0.041	0.054	0.108
	0.040	0.275	0.277	0.049	0.242	0.222	0.048	0.048	0.048	0.048	0.048	0.048
	0.040	0.284	0.265	0.048	0.251	0.226	0.048	0.047	0.048	0.048	0.048	0.048
	0.039	0.289	0.274	0.052	0.252	0.222	0.048	0.048	0.048	0.048	0.048	0.048
	0.041	0.285	0.271	0.049	0.262	0.235	0.048	0.048	0.047	0.048	0.048	0.048

	MM_Tet	MM_Tet	MM_Tet	MM_Tet _F 1 mg/mL	MM_Tet _F 1 mg/mL	MM_Tet _F 1 mg/mL	MM_Tet _F 0.5 mg/mL	MM_Tet _F 0.5 mg/mL	MM_Tet _F 0.5 mg/mL	MM_Tet _F 0.1 mg/mL	MM_Tet _F 0.1 mg/mL	MM_Tet _F 0.1 mg/mL
	Control	Vector	CCA-48	Control	Vector	CCA-48	Control	Vector	CCA-48	Control	Vector	CCA-48
OD600 Average:	0.040	0.058	0.055	0.046	0.058	0.090	0.043	0.058	0.094	0.040	0.053	0.113
OD600 SD:	0.001	0.001	0.003	0.001	0.002	0.002	0.001	0.004	0.004	0.001	0.001	0.004

OD with Blank:	0.018	0.015		0.012	0.044		0.015	0.050		0.013	0.072
Corrected OD Values:	0.101	0.083		0.064	0.241		0.081	0.277		0.070	0.398

	MM_Glu_Tet Control	MM_Glu_Tet Vector	MM_Glu_Tet CCA-48	MM_Glu_Tet_F 1mg/mL Control	MM_Glu_Tet_F 1mg/mL Vector	MM_Glu_Tet_F 1mg/mL CCA-48	Empty wells					
OD600 Average:	0.040	0.283	0.271	0.050	0.252	0.226	0.048	0.047	0.048	0.048	0.048	0.048
OD600 SD:	0.001	0.005	0.005	0.002	0.008	0.006	0.000	0.001	0.001	0.000	0.000	0.000
OD with Blank:		0.243	0.232		0.202	0.176		-0.001	0.000		0.000	0.000
Corrected OD Values:		1.228	1.170		1.020	0.890		-0.003	-0.001		0.000	0.000

PAH: Phenanthrene
Timepoint: 24 hours
Absorbance: 600 nm
Volume: 100 uL
Spetrophotometer: Molecular Devices Spectra Max

Conditions:	Raw A to D	MM_Chl	MM_Chl	MM_Chl	MM_Chl_P 1 mg/mL	MM_Chl_P 1 mg/mL	MM_Chl_P 1 mg/mL	MM_Chl_P 0.5 mg/mL	MM_Chl_P 0.5 mg/mL	MM_Chl_P 0.5 mg/mL	MM_Chl_P 0.1 mg/mL	MM_Chl_P 0.1 mg/mL	MM_Chl_P 0.1 mg/mL
	Raw E to H	MM_Glu_Chl	MM_Glu_Chl	MM_Glu_Chl	MM_Glu_Chl_P 1mg/mL	MM_Glu_Chl_P 1mg/mL	MM_Glu_Chl_P 1mg/mL	Empty wells	Empty wells	Empty wells	Empty wells	Empty wells	Empty wells

Colony: 1
96-well plate readout

Temperature(°C)	1	2	3	4	5	6	7	8	9	10	11	12
26.2	0.041	0.054	0.054	0.042	0.046	0.054	0.042	0.048	0.068	0.045	0.052	0.096
	0.039	0.053	0.053	0.042	0.044	0.053	0.040	0.047	0.068	0.047	0.050	0.094
	0.039	0.052	0.053	0.045	0.045	0.053	0.039	0.045	0.068	0.049	0.050	0.092

	0.040	0.054	0.056	0.042	0.047	0.057	0.039	0.046	0.069	0.042	0.051	0.095
	0.040	0.306	0.255	0.055	0.159	0.219	0.048	0.048	0.048	0.048	0.048	0.048
	0.039	0.304	0.262	0.100	0.161	0.208	0.048	0.047	0.048	0.048	0.048	0.048
	0.039	0.302	0.255	0.044	0.160	0.208	0.048	0.048	0.048	0.048	0.048	0.048
	0.041	0.308	0.266	0.047	0.161	0.215	0.048	0.051	0.047	0.048	0.048	0.048

	MM_Chl Control	MM_Chl Vector	MM_Chl CCA-57	MM_Chl_P 1 mg/mL Control	MM_Chl_P 1 mg/mL Vector	MM_Chl_P 1 mg/mL CCA-57	MM_Chl_P 0.5 mg/mL Control	MM_Chl_P 0.5 mg/mL Vector	MM_Chl_P 0.5 mg/mL CCA-57	MM_Chl_P 0.1 mg/mL Control	MM_Chl_P 0.1 mg/mL Vector	MM_Chl_P 0.1 mg/mL CCA-57
OD600 Average:	0.040	0.053	0.054	0.043	0.046	0.054	0.040	0.047	0.068	0.046	0.051	0.094
OD600 SD:	0.001	0.001	0.001	0.002	0.001	0.002	0.001	0.001	0.001	0.003	0.001	0.002
OD with Blank:		0.013	0.014		0.003	0.011		0.007	0.028		0.005	0.049
Corrected OD Values:		0.067	0.071		0.013	0.056		0.235	0.143		0.025	0.246

	MM_Glu_Chl Control	MM_Glu_Chl Vector	MM_Glu_Chl CCA-57	MM_Glu_Chl_P 1mg/mL Control	MM_Glu_Chl_P 1mg/mL Vector	MM_Glu_Chl_P 1mg/mL CCA-57	Empty wells					
OD600 Average:	0.040	0.305	0.259	0.062	0.160	0.212	0.048	0.048	0.048	0.048	0.048	0.048
OD600 SD:	0.001	0.002	0.005	0.026	0.001	0.005	0.000	0.002	0.000	0.000	0.000	0.000
OD with Blank:		0.265	0.219		0.099	0.151		0.001	0.000		0.000	0.000
Corrected OD Values:		1.339	1.108		0.498	0.762		0.003	0.000		0.000	-0.001

PAH:		Phenanthre											
Timepoint:		24 hours											
Absorbance:		600 nm											
Volume:		100 uL											
Spetrophotometer:		Molecular Devices Spectra Max											
Conditions:	Raw A to D	MM_Chl	MM_Chl	MM_Chl	MM_Chl_P 1 mg/mL	MM_Chl_P 1 mg/mL	MM_Chl_P 1 mg/mL	MM_Chl_P 0.5 mg/mL	MM_Chl_P 0.5 mg/mL	MM_Chl_P 0.5 mg/mL	MM_Chl_P 0.1 mg/mL	MM_Chl_P 0.1 mg/mL	MM_Chl_P 0.1 mg/mL
	Raw E to H	MM_Glu_Chl	MM_Glu_Chl	MM_Glu_Chl	MM_Glu_Chl_P 1mg/mL	MM_Glu_Chl_P 1mg/mL	MM_Glu_Chl_P 1mg/mL	Empty wells	Empty wells	Empty wells	Empty wells	Empty wells	Empty wells
Colony:	2												
96-well plate readout													
	Temperature(°C)	1	2	3	4	5	6	7	8	9	10	11	12
	25.7	0.041	0.055	0.059	0.041	0.046	0.054	0.041	0.051	0.069	0.049	0.052	0.095
		0.039	0.054	0.058	0.046	0.048	0.054	0.040	0.048	0.069	0.062	0.050	0.093
		0.039	0.055	0.056	0.047	0.046	0.053	0.039	0.045	0.069	0.050	0.050	0.108
		0.040	0.055	0.057	0.042	0.049	0.066	0.040	0.046	0.070	0.042	0.050	0.100
		0.040	0.314	0.264	0.058	0.161	0.220	0.048	0.048	0.048	0.048	0.048	0.048
		0.040	0.308	0.275	0.159	0.166	0.220	0.048	0.047	0.049	0.048	0.048	0.048
		0.039	0.308	0.269	0.048	0.166	0.210	0.048	0.048	0.048	0.048	0.048	0.048
		0.041	0.313	0.272	0.071	0.164	0.214	0.048	0.051	0.047	0.048	0.048	0.048

		MM_Chl	MM_Chl	MM_Chl	MM_Chl_P 1 mg/mL	MM_Chl_P 1 mg/mL	MM_Chl_P 1 mg/mL	MM_Chl_P 0.5 mg/mL	MM_Chl_P 0.5 mg/mL	MM_Chl_P 0.5 mg/mL	MM_Chl_P 0.1 mg/mL	MM_Chl_P 0.1 mg/mL	MM_Chl_P 0.1

	Control	Vector	CCA-57	Control	Vector	CCA-57	Control	Vector	CCA-57	Control	Vector	mg/mL CCA-57
OD600 Average:	0.040	0.055	0.058	0.044	0.047	0.056	0.040	0.048	0.069	0.051	0.050	0.099
OD600 SD:	0.001	0.001	0.001	0.003	0.002	0.006	0.001	0.003	0.000	0.008	0.001	0.007
OD with Blank:		0.015	0.018		0.003	0.012		0.008	0.029		0.000	0.048
Corrected OD Values:		0.076	0.090		0.016	0.063		0.038	0.147		-0.001	0.244

	MM_Glu_Chl Control	MM_Glu_Chl Vector	MM_Glu_Chl CCA-57	MM_Glu_Chl_P 1mg/mL Control	MM_Glu_Chl_P 1mg/mL Vector	MM_Glu_Chl_P 1mg/mL CCA-57	Empty wells					
OD600 Average:	0.040	0.311	0.270	0.084	0.164	0.216	0.048	0.049	0.048	0.048	0.048	0.048
OD600 SD:	0.001	0.003	0.005	0.051	0.002	0.005	0.000	0.002	0.001	0.000	0.000	0.000
OD with Blank:		0.271	0.230		0.081	0.133		0.001	0.000		0.000	0.000
Corrected OD Values:		1.367	1.161		0.407	0.669		0.003	0.001		0.000	-0.001

4.5. TIME COURSE BIOTRANSFORMATION RESULTS IN PRESENCE OF FLUORENE AND PHENANTHRENE

4.5.1. EXPERIMENTAL DESIGN

This study was aimed at determining the growth rate of the recombinant strains containing the phenanthrene catabolic pathway or the fluorene catabolic pathway alone or together when using phenanthrene or fluorene as sole source of carbon. Recombinant cells were grown in minimal medium supplemented with fluorene or phenanthrene as a sole source of carbon. For controls, the strains were grown in presence of glucose. In addition, E.coli strains containing the corresponding vector without insert was also grown in parallel. PAHs were dissolved in DMSO.

4.5.2. CULTURE SETUP

Cultures were started from glycerol stock in 4 mL of medium and placed at 37°C. The OD readout of the overnight cultures was determined using a spectrophotometer according to the protocol shown above. All cultures were then diluted to 0.02 using the volume below and OD measurements were determined at the indicated time points.

Table 13. OD measurement and dilution scheme of overnight cultures to initiate biotransformation studies.

target Abs600		**0.02**	
target volume (mL)		**4**	
sample	Abs600 Reading	Volume (mL) of Preloading Culture	Volume (mL) of Preloading Media
pSB3T5_colony 1	0.2559	0.374	3.626
pSB3T5_colony 2	0.2743	0.345	3.655
Fluorene_Colony 1	0.2241	0.440	3.560
Fluorene_Colony 2	0.2294	0.428	3.572
media	0.0423		

target Abs600		**0.02**	
target volume (mL)		**4**	
sample	Abs600 Reading	Volume (mL) of Preloading Culture	Volume (mL) of Preloading Media
pSB1C3_colony 1	0.4458	0.198	3.802
pSB1C3_colony 2	0.4581	0.192	3.808
Phe_Colony 1	0.2695	0.351	3.649
Phe_Colony 2	0.2668	0.356	3.644
media	0.0418		

target Abs600	**0.02**
target volume (mL)	**4**

sample	Abs600 Reading	Volume (mL) of Preloading Culture	Volume (mL) of Preloading Media
pSB1C3/pSB3T5_colony 1	0.3247	0.283	3.717
pSB1C3/pSB3T5_colony 2	0.3277	0.280	3.720
Flu/Phe_Colony 1	0.2589	0.369	3.631
Flu/Phe_Colony 2	0.2633	0.362	3.638
media	0.0422		

4.5.3. RESULTS AND DISCUSSION: Time course study of culture growth of recombinant E.coli BL21(DE3) in minimal medium in presence of phenanthrene (0.1 mg/mL) and fluorene (0.1, mg/mL) with a Biosurfactant (0.1%) at room temperature (25◦C) and at 37◦C.

In order to evaluate whether the recombinant cells had the ability to transform PAHs at room temperature (25°C), growth experiments were set up with clones CCA-48 (=clone 48) and CCA-57 (= clone 57) expressing the fluorene or the phenanthrene catabolic pathway respectively at two temperatures. The clones described above with the catabolic pathway under the control of the strongest constitutive were set in cultures using minimal medium supplemented with fluorene (0.1 mg/mL) and phenanthrene (0.1 mg/mL) as sole source of carbon in presence of Tween (0.1%). Antibiotics were added as appropriately.

As illustrated in the table below reporting the average of 8 data points (Optical Density) of cultures, the E.coli bacteria containing the biodegradation pathway could utilize fluorene (clone CCA-48) or phenanthrene (clone CCA-57) whereas the control bacterial containing the vector with no insert could not. Minimal media was used as medium for culture. Strains could all grow in presence of glucose when used as a carbon source demonstrating that the cells were viable.

The E.coli strain containing both plasmids CCA-48 and CCA-57 did not grow very well. It may be due to the fact that 2 antibiotics were added in the medium to maintain the 2 plasmids (chloramphenicol for CCA-57 and tetracycline for CCA-48) thus slowing down the growth rate. Optical density may have increase at a later time point.

Table 14. Average absorbance values measured in quadruplet at 600 nm of cultures of 2 independent colonies of control (vector) and catabolic plasmid (clone CCA-48 for fluorene) or (clone CCA-57 for phenanthrene) at 2, 4, 24, and 48 hours after inoculation of minimal media supplemented with fluorene at 0.1 mg/mL or phenanthrene at 0.1 mg/mL or both with or without glucose (0.4%) at 25. All media contained the surfactant Tween-20 (0.1%). Cultures were grown at 25°C or 37°C. MM= Minimal Medium, Glu= Glucose; Ave.= Average; OD=Optical Density; SD= standard deviation.

Minimal Media without Glucose.

Incubation Temperature: 25°C				
Minimal Media	Vector		CCA-57	
Phe_0.1 mg/mL	OD_Ave.	OD_SD	OD_Ave.	OD_SD
2 hours	0.042	0.007	0.099	0.003
4 hours	0.069	0.008	0.155	0.010
24 hours	0.095	0.016	0.184	0.006
48 hours	0.071	0.014	0.182	0.014

Incubation Temperature: 37°C				
Minimal Media	Vector		CCA-57	
Phe_0.1 mg/mL	OD_Ave.	OD_SD	OD_Ave.	OD_SD
2 hours	0.103	0.003	0.163	0.007
4 hours	0.136	0.005	0.207	0.002
24 hours	0.132	0.008	0.259	0.024
48 hours	0.086	0.039	0.674	0.057

Incubation Temperature: 25°C				
Minimal Media	Vector		CCA-48	
Flu_0.1 mg/mL	OD_Ave.	OD_SD	OD_Ave.	OD_SD
2 hours	0.061	0.003	0.134	0.008
4 hours	0.145	0.015	0.197	0.004
24 hours	0.139	0.004	0.237	0.002
48 hours	0.166	0.000	0.424	0.007

Incubation Temperature: 37°C				
Minimal Media	Vector		CCA-48	
Flu_0.1 mg/mL	OD_Ave.	OD_SD	OD_Ave.	OD_SD
2 hours	0.085	0.007	0.181	0.003
4 hours	0.087	0.014	0.276	0.034
24 hours	0.113	0.016	0.471	0.075
48 hours	0.141	0.056	0.700	0.231

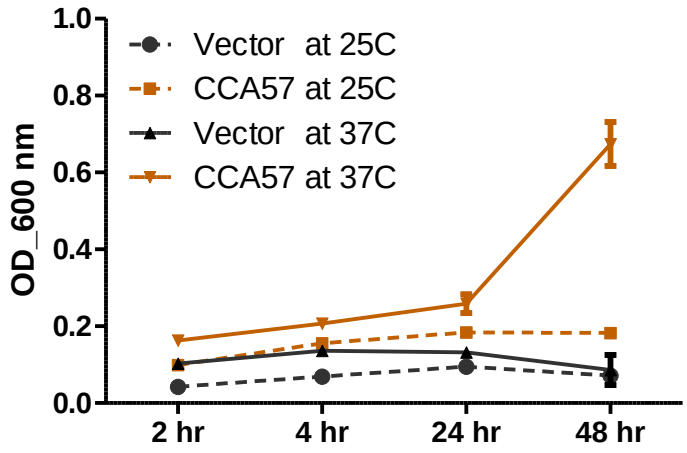
Incubation Temperature: 25°C				
Minimal Media	Vector		CCA-57 +CCA-48	
Flu_Phe_0.1 mg/mL	OD_Ave.	OD_SD	OD_Ave.	OD_SD
2 hours	0.114	0.016	0.202	0.009
4 hours	0.194	0.000	0.239	0.022
24 hours	0.179	0.179	0.349	0.019
48 hours	0.169	0.019	0.389	0.007

Incubation Temperature: 37°C				
Minimal Media	Vector		CCA-57 +CCA-48	
Flu_Phe_0.1 mg/mL	OD_Ave.	OD_SD	OD_Ave.	OD_SD
2 hours	0.220	0.012	0.241	0.014
4 hours	0.084	0.009	0.170	0.010
24 hours	0.147	0.056	0.269	0.014
48 hours	0.201	0.010	0.277	0.025

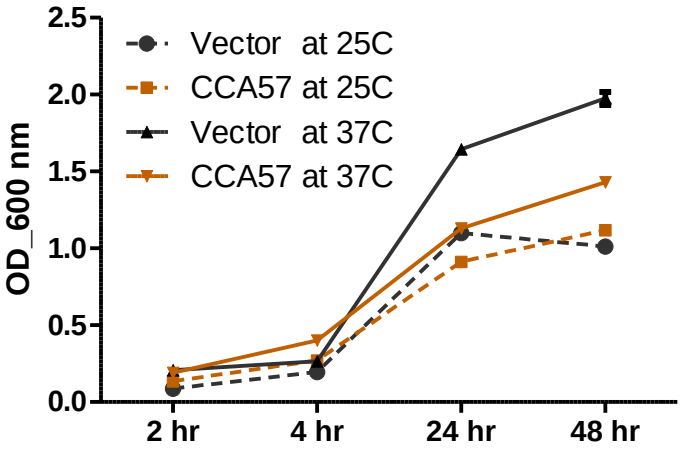
Minimal Media with Glucose.

Incubation Temperature: 25°C					**Incubation Temperature: 37°C**				
Minimal M + Glucose	Vector		CCA-57		**Minimal M + Glucose**	Vector		CCA-57	
Phe_0.1 mg/mL	OD_Ave.	OD_SD	OD_Ave.	OD_SD	**Phe_0.1 mg/mL**	OD_Ave.	OD_SD	OD_Ave.	OD_SD
2 hours	0.087	0.001	0.134	0.012	2 hours	0.208	0.002	0.190	0.011
4 hours	0.195	0.003	0.270	0.004	4 hours	0.266	0.001	0.400	0.026
24 hours	1.098	0.020	0.911	0.030	24 hours	1.645	0.030	1.130	0.017
48 hours	1.011	0.013	1.118	0.014	48 hours	1.975	0.043	1.430	0.012
	Incubation Temperature: 25°C					**Incubation Temperature: 37°C**			
Minimal M + Glucose	Vector		CCA-48		**Minimal M + Glucose**	Vector		CCA-48	
Flu_0.1 mg/mL	OD_Ave.	OD_SD	OD_Ave.	OD_SD	**Flu_0.1 mg/mL**	OD_Ave.	OD_SD	OD_Ave.	OD_SD
2 hours	0.161	0.010	0.278	0.005	2 hours	0.067	0.008	0.121	0.003
4 hours	0.217	0.033	0.445	0.024	4 hours	0.169	0.027	0.188	0.006
24 hours	1.151	0.013	0.907	0.004	24 hours	1.675	0.005	1.103	0.014
48 hours	1.331	0.002	2.159	0.075	48 hours	1.558	0.014	1.229	0.089
	Incubation Temperature: 25°C					**Incubation Temperature: 37°C**			
Minimal M + Glucose	Vector		CCA-57 +CCA-48		**Minimal M + Glucose**	Vector		CCA-57 +CCA-48	
Flu_Phe_0.1 mg/mL	OD_Ave.	OD_SD	OD_Ave.	OD_SD	**Flu_Phe_0.1 mg/mL**	OD_Ave.	OD_SD	OD_Ave.	OD_SD
2 hours	0.410	0.032	0.459	0.000	2 hours	0.343	0.007	0.391	0.007
4 hours	0.345	0.016	0.395	0.035	4 hours	0.527	0.029	0.668	0.012
24 hours	1.175	0.011	1.034	0.010	24 hours	1.658	0.005	1.061	0.015
48 hours	1.583	0.017	1.037	0.026	48 hours	1.747	0.061	1.836	0.063

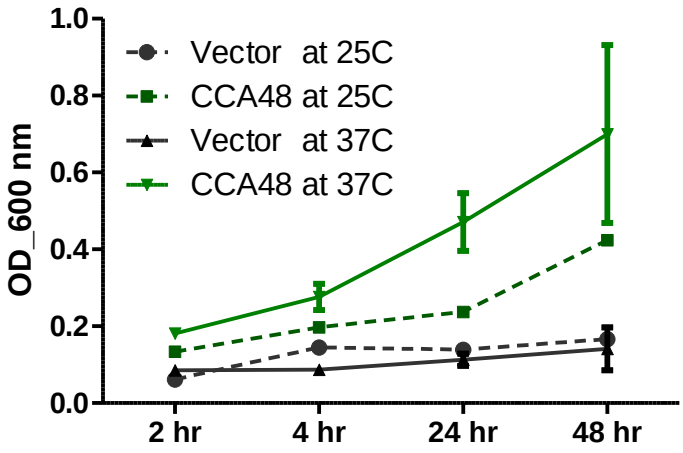
Biotransformation of Recombinant E.coli BL21 on Minimal Medium + Phenanthrene 0.1 mg/mL



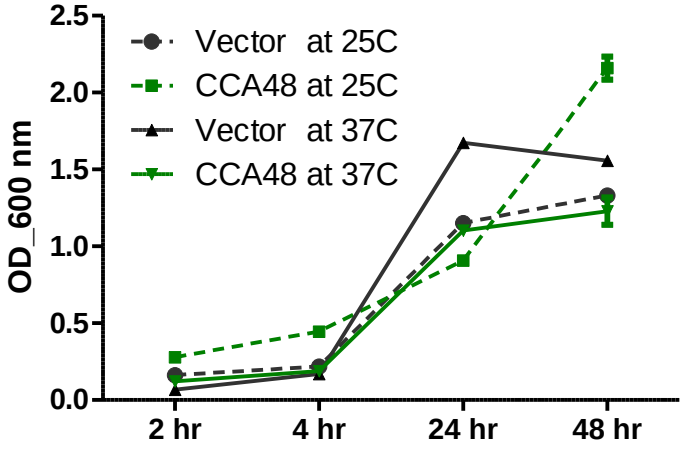
Biotransformation of Recombinant E.coli BL21 on Minimal Medium + Glucose (0.4%) + Phenanthrene 0.1 mg/mL



Biotransformation of Recombinant E.coli BL21 on Minimal Medium + Fluorene 0.1 mg/mL



Biotransformation of Recombinant E.coli BL21 on Minimal Medium + Glucose (0.4%) + Fluorene 0.1 mg/mL



4.6. BIOTRANSFORMATION RESULTS IN PRESENCE OF CRUDE OILS

4.6.1. EXPERIMENTAL DESIGN

This study was aimed at determining the growth rate of recombinant E.coli BL21(DE3) strains containing the phenanthrene catabolic pathway and the fluorene catabolic pathway together when using minimal medium and crude oil as a source of carbon (0.01%). Controls consisted of E.coli strain containing the corresponding vectors with no insert and culture growth on minimum medium with or without glucose (0.4%).

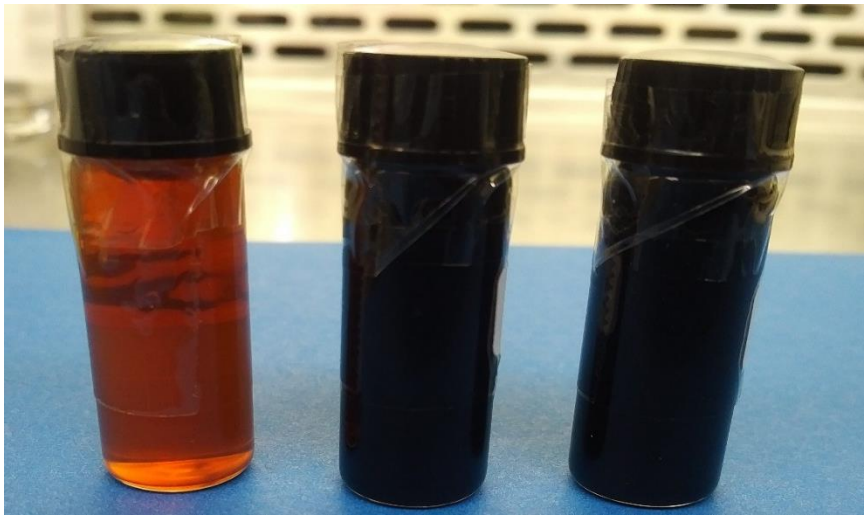


Figure 5. Crude oil samples from Pennsylvannia, Ecuador, and Saudi Arabia (left to right).

Table 15.Crude-oils description.

Properties	**Pennsylvania**	**Ecuador (EC), Saudi Arabia (SA)**
Type of crude oil	paraffin based crude oil	asphalt based crude oil
Color	green-brown , sweet	brown-black, sour
Density	light, 0.810 g/mL	heavy, over 0.910 g/mL
Sulfur	0.14 %	EU=1.31%, SA=2.48%
Composition	paraffin, hydrocarbons	Aromatics, asphalt
Products after Refining	gasoline, kerosene	gasoil, asphalt

4.6.2. CULTURE SETUP

E.coli BL21(DE3) recombinant cells containing both plasmids CCA-57 and CCA-48 were first grown at 37°C from glycerol stock in 4 mL of LB medium with both chloramphenicol and. CCA-57 harbored the phenanthrene pathway on vector pSB1C3 and clone CCA-48 harbored the fluorene pathway on vector pSB3T5. Recombinant cell cultures of the corresponding vectors with no catabolic inserts were initiated in a similar fashion. Overnight cultures were spun and resuspended in 4 mL of minimal medium. OD of all cultures was measured at 600 nm using a spectrophotometer. All cultures were then diluted to 0.02 using the volume determined in the table below.

Growth experiments were initiated in minimal medium with no carbohydrate source, with glucose (0.4%), with phenanthrene and fluorene (0.1 mg/mL each) from a stock solution of 100 mg/mL prepared in DMSO, or with crude oil (0.01%). No antibiotics were added to the minimum medium. Three sources of crude oils were tested: Pennsylvania, Ecuador, and Saudi Arabia.

Table 16.OD measurement and dilution scheme of overnight cultures to initiate biotransformation studies.

target Abs600	**0.02**	
target volume (mL)	**4**	
Sample	Abs600 Reading	Volume of Preloading Culture
pSB3T5_pSB1C3_colony 1	0.1538	0.704	3.296
pSB3T5_pSB1C3_colony 2	0.1500	0.729	3.271
CCA48_CCA57_Colony 1	0.1221	0.977	3.023
CCA48_CCA57_Colony 2	0.1189	1.017	2.983
Pseudomonas_Colony 1	0.1199	1.003	2.997
Pseudomonas_Colony 2	0.1194	1.010	2.990
media	0.0402		

Temperature(°C)					
22.8		pSB3T5_pSB1C3	CCA48_CCA57	Media	No media
	Colony_1	1	2	3	4
		0.1520	0.1264	0.0414	0.0474
		0.1544	0.1143	0.0395	0.0481
		0.1534	0.1133	0.0394	0.0475
		0.1554	0.1342	0.0404	0.0478
	Colony_2	0.1526	0.1209	0.0414	0.0476
		0.1506	0.1185	0.0395	0.0477
		0.1505	0.1139	0.0396	0.0478
		0.1461	0.1221	0.0401	0.0483

		pSB3T5_pSB1C3	CCA48_CCA57	Media	No media
Colony_1	Average	0.154	0.122	0.040	0.048
	SD	0.001	0.010	0.001	0.000
Colony_2	Average	0.150	0.119		
	SD	0.003	0.004		

4.6.3. RESULTS AND DISCUSSION: Time course study of culture growth of recombinant E.coli BL21(DE3) in minimal medium in presence of crude oils

In order to evaluate whether the recombinant cells had the ability to transform PAHs in crude oil samples, growth experiments were set up with a recombinant clone expressing both the fluorene and the phenanthrene catabolic pathways. The clone with the catabolic pathway under the control of the strongest constitutive promoter [CCA-48 for fluorene and CCA-57 for phenanthrene] was set in cultures using M9 minimal medium supplemented with fluorene (0.1 mg/mL) and phenanthrene (0.1 mg/mL) as sole source of carbon in presence of Tween (0.1%), or with crude oils from three sources.

As illustrated in the table above reporting the average of 8 data points (Optical Density) of cultures, the recombinant E.coli containing the biodegradation pathway could utilize crude oils whereas the control bacteria containing the vector only could not. Minimal media was used as medium for culture. Strains could all grow in presence of glucose when used as a carbon source.

Table 17. Average absorbance values of E.coli BL21 recombinant bacterial cultures measured in quadruplet at 600 nm of cultures of 2 independent colonies of control (vector pSB3T5 and pSB1C3) and catabolic plasmids (clone CCA-48 for fluorene and clone CCA-57 for phenanthrene) at 0, 24, 48, and 72 hours after inoculation of M9 minimal media supplemented with crude oil samples (0.01%) from Pennsylvania, Ecuador, and Saudi Arabia, or fluorene and phenanthrene at 0.1 mg/mL or with or without glucose (0.4%) at 37°C. All media contained the surfactant Tween-20 (0.1%). MM= Minimal Medium, Glu= Glucose; Ave.= Average; OD=Optical Density; SD= standard deviation.

pSB3T5_pSB1C3	Minimal Media (MM)		Minimal Media + Glucose		MM + Fluorene +Phenanthrene		MM + Oil Pennsylvania		MM + Ecuador Oil		MM + Saudi Arabia Oil	
	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
0 hours	0.109	0.000	0.118	0.012	0.106	0.001	0.107	0.001	0.123	0.008	0.111	0.008
24 hours	0.028	0.002	0.751	0.024	0.022	0.001	0.020	0.001	0.014	0.002	0.023	0.001
48 hours	0.079	0.000	1.843	0.054	0.074	0.006	0.060	0.002	0.036	0.001	0.063	0.003
72 hours	0.074	0.003	1.796	0.049	0.080	0.008	0.053	0.002	0.033	0.002	0.056	0.003

CCA48_CCA57	Minimal Media (MM)		Minimal Media + Glucose		MM + Fluorene +Phenanthrene		MM + Oil Pennsylvania		MM + Ecuador Oil		MM + Saudi Arabia Oil	
	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
0 hours	0.118	0.015	0.099	0.003	0.108	0.001	0.086	0.009	0.107	0.005	0.094	0.010
24 hours	0.025	0.000	0.517	0.025	0.558	0.004	0.471	0.001	0.570	0.005	0.531	0.018
48 hours	0.067	0.002	1.320	0.086	1.511	0.045	1.332	0.041	1.576	0.056	1.480	0.086
72 hours	0.063	0.001	1.299	0.057	1.527	0.022	1.185	0.005	1.450	0.040	1.370	0.078

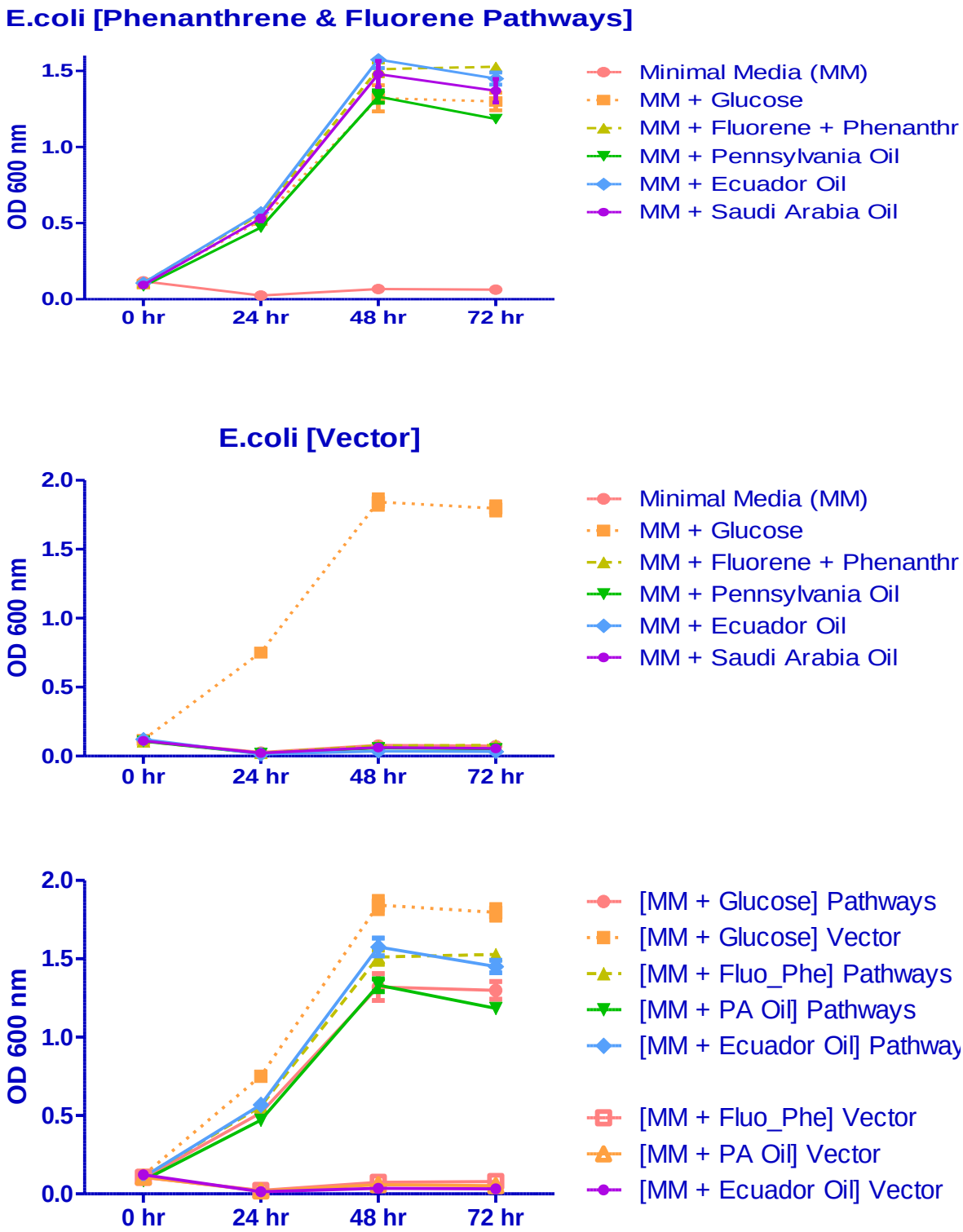


Figure 6. Time course biotransformation experiments using crude oil samples from Pennsylvania (PA), Saudi Arabia, and Ecuador, measuring absorbance at 600nm of E.coli BL21 recombinant cultures containing the fluorene and phenanthrene catabolic pathways or control vectors. MM=M9 minimal medium.

Table 18. Raw Data of biotransformation experiment using crude oils: Absorbance measured at 600nm using a spectrophotometer.

Time point: 0 hours Absorbance 600 nm Volume: 100 uL

Spectrophotometer: Molecular Devices Spectra Max

	Minimal Media (MM)			MM + Glucose			MM + Fluorene+Phenanthrene			
	Control Bact	Bacteria (48+57)	Media	Control Bact	Bacteria (48+57)	Media	Control Bact	Bacteria (48+57)	Media	Average blank
Raw Data (No Blank)	0.0626	0.0622	0.0415	0.0637	0.0626	0.041	0.0616	0.0654	0.0413	0.040
	0.0607	0.0602	0.0395	0.0647	0.0577	0.0395	0.0603	0.0592	0.0391	
	0.0607	0.0605	0.0391	0.0634	0.0562	0.0388	0.0617	0.0584	0.0395	0.040
	0.0617	0.0614	0.0404	0.0676	0.0595	0.0402	0.0612	0.0621	0.0404	
	0.0603	0.0613	0.0395	0.0616	0.0611	0.0398	0.0605	0.0631	0.04	0.040
	0.0606	0.0696	0.0398	0.0625	0.0572	0.0395	0.0607	0.0605	0.0399	
	0.0603	0.0696	0.039	0.0602	0.0603	0.0391	0.0619	0.0591	0.0391	
	0.0642	0.0611	0.0404	0.0612	0.0609	0.0402	0.0606	0.0634	0.0407	
OD -Blank	0.023	0.022	0.002	0.024	0.023	0.001	0.022	0.025	0.001	
	0.021	0.020	0.000	0.025	0.018	0.000	0.020	0.019	-0.001	
	0.021	0.021	-0.001	0.024	0.016	-0.001	0.022	0.018	-0.001	
	0.022	0.022	0.001	0.028	0.020	0.000	0.021	0.022	0.000	
	0.020	0.021	0.000	0.022	0.021	0.000	0.021	0.023	0.000	
	0.021	0.030	0.000	0.023	0.017	0.000	0.021	0.021	0.000	
	0.020	0.030	-0.001	0.020	0.021	-0.001	0.022	0.019	-0.001	
	0.024	0.021	0.001	0.021	0.021	0.000	0.021	0.023	0.001	
Corrected OD	0.115	0.113	0.008	0.121	0.115	0.006	0.109	0.128	0.007	
	0.105	0.103	-0.002	0.126	0.091	-0.001	0.103	0.097	-0.005	
	0.105	0.104	-0.004	0.119	0.083	-0.005	0.110	0.093	-0.003	
	0.110	0.109	0.003	0.141	0.100	0.002	0.107	0.112	0.002	
	0.103	0.108	-0.002	0.110	0.108	0.000	0.104	0.117	0.000	
	0.105	0.150	-0.001	0.115	0.088	-0.001	0.105	0.104	-0.001	
	0.103	0.150	-0.005	0.103	0.104	-0.003	0.111	0.096	-0.005	
	0.123	0.107	0.003	0.108	0.107	0.002	0.104	0.118	0.004	

	Minimal Media (MM)		MM + Glucose		MM + Fluorene+Phenanthrene	
	Average	SD	Average	SD	Average	SD
pSB3T5_pSB1C3_colony 1	0.109	0.005	0.127	0.010	0.107	0.003
pSB3T5_pSB1C3_colony 2	0.108	0.010	0.109	0.005	0.106	0.003
CCA48_CCA57_Colony 1	0.107	0.005	0.097	0.014	0.107	0.016
CCA48_CCA57_Colony 2	0.129	0.024	0.102	0.009	0.109	0.010
media	0.001	0.005	0.001	0.005	0.000	0.005
media	-0.001	0.003	-0.001	0.002	0.000	0.003

Time point	Minimal Media (MM)		MM + Glucose		MM + Fluorene+Phenanthrene	
0 hours	Average	SD	Average	SD	Average	SD
pSB3T5_pSB1C3	0.109	0.000	0.118	0.012	0.106	0.001
CCA48_CCA57	0.118	0.015	0.099	0.003	0.108	0.001
Media	0.000	0.002	0.000	0.001	0.000	0.001

0 hours

MM + Crude-oil Pennsylvania			MM + Crude-oil Ecuador			MM + Crude-oil Saudi Arabia			
Control Bact	Bacteria (48+57)	Media	Control Bact	Bacteria (48+57)	Media	Control Bact	Bacteria (48+57)	Media	Average blank
0.0623	0.0589	0.0414	0.0617	0.0601	0.0409	0.0618	0.0605	0.0414	0.040
0.0601	0.0555	0.0394	0.0602	0.0586	0.0395	0.0601	0.0563	0.0392	
0.0602	0.0575	0.0392	0.0686	0.0598	0.0387	0.0604	0.0579	0.0391	0.040
0.0613	0.0614	0.0401	0.0611	0.063	0.0404	0.0611	0.0656	0.0407	
0.0603	0.057	0.0397	0.0694	0.0603	0.0394	0.0612	0.0607	0.0402	0.040
0.062	0.0553	0.0401	0.0603	0.0626	0.0391	0.0604	0.0537	0.0399	
0.0613	0.0541	0.0392	0.0698	0.0605	0.0391	0.0695	0.0561	0.0402	
0.0618	0.0564	0.0405	0.0612	0.0633	0.0402	0.0609	0.0587	0.0405	
0.022	0.019	0.002	0.022	0.020	0.001	0.022	0.021	0.001	
0.020	0.016	-0.001	0.020	0.019	0.000	0.020	0.016	-0.001	
0.020	0.018	-0.001	0.029	0.020	-0.001	0.020	0.018	-0.001	
0.021	0.022	0.000	0.021	0.023	0.001	0.021	0.026	0.001	
0.020	0.017	0.000	0.030	0.021	0.000	0.021	0.021	0.000	
0.022	0.015	0.000	0.021	0.023	-0.001	0.020	0.014	0.000	
0.021	0.014	-0.001	0.030	0.021	-0.001	0.030	0.016	0.000	
0.022	0.017	0.001	0.021	0.024	0.000	0.021	0.019	0.001	
0.113	0.096	0.008	0.111	0.103	0.006	0.110	0.104	0.007	
0.102	0.079	-0.003	0.103	0.095	-0.001	0.102	0.082	-0.004	
0.103	0.089	-0.004	0.146	0.101	-0.005	0.103	0.090	-0.005	
0.108	0.109	0.001	0.108	0.117	0.003	0.107	0.129	0.004	
0.103	0.086	-0.001	0.150	0.104	-0.002	0.107	0.105	0.001	
0.112	0.078	0.001	0.104	0.115	-0.003	0.103	0.069	-0.001	
0.108	0.072	-0.004	0.152	0.105	-0.003	0.149	0.081	0.001	
0.111	0.083	0.003	0.108	0.119	0.002	0.106	0.094	0.003	

MM + Crude-oil Pennsylvania		MM + Crude-oil Ecuador		MM + Crude-oil Saudi Arabia	
Average	SD	Average	SD	Average	SD
0.106	0.005	0.117	0.019	0.105	0.004
0.108	0.004	0.128	0.026	0.116	0.022
0.093	0.013	0.104	0.009	0.101	0.021
0.080	0.006	0.111	0.008	0.087	0.015
0.001	0.005	0.001	0.005	0.001	0.006
0.000	0.003	-0.002	0.003	0.001	0.001

MM + Crude-oil Pennsylvania		MM + Crude-oil Ecuador		MM + Crude-oil Saudi Arabia	
Average	SD	Average	SD	Average	SD
0.107	0.001	0.123	0.008	0.111	0.008
0.086	0.009	0.107	0.005	0.094	0.010
0.000	0.001	-0.001	0.002	0.001	0.000

Timepoint: 24 hours
Absorbance: 600 nm
Volume: 100 uL
Spectrophotometer: Molecular Devices Spectra Max

	Minimal Media (MM)			MM + Glucose			MM + Fluorene+Phenanthrene			
	Control Bact	Bacteria (48+57)	Media	Control Bact	Bacteria (48+57)	Media	Control Bact	Bacteria (48+57)	Media	Average blan
Raw Data (No Blank)	0.0469	0.0458	0.0415	0.1972	0.1536	0.0414	0.0454	0.1504	0.0414	0.04
	0.045	0.0439	0.0398	0.1899	0.1334	0.0394	0.0435	0.1416	0.0391	
	0.0451	0.0444	0.0391	0.1882	0.1332	0.0389	0.0438	0.1464	0.0393	0.04
	0.0462	0.0457	0.0404	0.1921	0.1628	0.0402	0.0451	0.1609	0.0408	
	0.0447	0.0453	0.0395	0.1886	0.1436	0.0395	0.0439	0.1543	0.04	0.04
	0.0459	0.0454	0.0402	0.1853	0.1372	0.0392	0.0449	0.1497	0.0399	
	0.0443	0.0436	0.0392	0.185	0.1354	0.0387	0.0446	0.1486	0.0386	
	0.0463	0.0457	0.0408	0.1815	0.1383	0.0404	0.0436	0.1516	0.0403	
OD -Blank	0.007	0.006	0.001	0.157	0.114	0.002	0.005	0.110	0.001	
	0.005	0.004	0.000	0.150	0.094	0.000	0.004	0.102	-0.001	
	0.005	0.004	-0.001	0.148	0.093	-0.001	0.004	0.106	-0.001	
	0.006	0.006	0.000	0.152	0.123	0.000	0.005	0.121	0.001	
	0.005	0.005	-0.001	0.149	0.104	0.000	0.004	0.114	0.000	
	0.006	0.005	0.000	0.146	0.097	-0.001	0.005	0.110	0.000	
	0.004	0.004	-0.001	0.145	0.096	-0.001	0.005	0.109	-0.001	
	0.006	0.006	0.001	0.142	0.099	0.001	0.004	0.112	0.000	
Corrected OD	0.035	0.029	0.007	0.795	0.575	0.009	0.028	0.558	0.007	
	0.025	0.019	-0.001	0.758	0.473	-0.002	0.018	0.513	-0.004	
	0.025	0.022	-0.005	0.750	0.472	-0.004	0.020	0.538	-0.003	
	0.031	0.028	0.002	0.770	0.622	0.002	0.026	0.611	0.004	
	0.023	0.026	-0.003	0.752	0.525	-0.001	0.020	0.578	0.000	
	0.029	0.027	0.001	0.735	0.492	-0.003	0.025	0.554	0.000	
	0.021	0.018	-0.004	0.734	0.483	-0.005	0.024	0.549	-0.007	
	0.031	0.028	0.004	0.716	0.498	0.003	0.019	0.564	0.002	

	Minimal Media (MM)		MM + Glucose		MM + Fluorene+Phenanthrene	
	Average	SD	Average	SD	Average	SD
pSB3T5_pSB1C3_colony 1	0.029	0.005	0.768	0.020	0.023	0.005
pSB3T5_pSB1C3_colony 2	0.026	0.005	0.734	0.015	0.022	0.003
CCA48_CCA57_Colony 1	0.025	0.005	0.535	0.075	0.555	0.041
CCA48_CCA57_Colony 2	0.025	0.005	0.500	0.018	0.561	0.013
media	0.001	0.005	0.001	0.006	0.001	0.006
media	-0.001	0.004	-0.001	0.004	-0.001	0.004

Time point	Minimal Media (MM)		MM + Glucose		MM + Fluorene+Phenanthrene	
24 hours	Average	SD	Average	SD	Average	SD
pSB3T5_pSB1C3	0.028	0.002	0.751	0.024	0.022	0.001
CCA48_CCA57	0.025	0.000	0.517	0.025	0.558	0.004
Media	0.000	0.001	0.000	0.002	0.000	0.002

24 hours
600 nm
100 uL
Molecular Devices Spectra Max

	MM + Crude-oil Pennsylvania			MM + Crude-oil Ecuador			MM + Crude-oil Saudi Arabia			
	Control Bact	Bacteria (48+57)	Media	Control Bact	Bacteria (48+57)	Media	Control Bact	Bacteria (48+57)	Media	Average blank
Raw Data (No Blank)	0.0454	0.1455	0.0413	0.0443	0.1615	0.0412	0.046	0.161	0.0411	0.040
	0.0439	0.1264	0.0395	0.0421	0.1498	0.0396	0.044	0.1349	0.0392	
	0.0436	0.1313	0.039	0.0413	0.1497	0.0392	0.0436	0.1443	0.0393	0.040
	0.0441	0.1307	0.0401	0.0427	0.1521	0.0406	0.0454	0.1507	0.0406	
	0.0439	0.1377	0.0394	0.0418	0.1528	0.0398	0.0446	0.1491	0.0399	0.040
	0.0442	0.1338	0.0398	0.0422	0.1509	0.0392	0.0452	0.1438	0.0403	
	0.0426	0.1289	0.0392	0.0416	0.1538	0.0388	0.0434	0.1367	0.0387	
	0.045	0.1323	0.0406	0.0431	0.1502	0.0398	0.0443	0.1406	0.0403	
OD -Blank	0.005	0.105	0.001	0.005	0.122	0.001	0.006	0.121	0.001	
	0.004	0.086	-0.001	0.002	0.110	0.000	0.004	0.095	-0.001	
	0.004	0.091	-0.001	0.002	0.110	-0.001	0.004	0.104	-0.001	
	0.004	0.091	0.000	0.003	0.112	0.001	0.005	0.111	0.001	
	0.004	0.098	-0.001	0.002	0.113	0.000	0.005	0.109	0.000	
	0.004	0.094	0.000	0.002	0.111	-0.001	0.005	0.104	0.000	
	0.003	0.089	-0.001	0.002	0.114	-0.001	0.003	0.097	-0.001	
	0.005	0.092	0.001	0.003	0.110	0.000	0.004	0.101	0.000	
Corrected OD	0.027	0.532	0.006	0.023	0.615	0.008	0.031	0.611	0.006	
	0.019	0.436	-0.003	0.012	0.556	-0.001	0.021	0.480	-0.004	
	0.018	0.461	-0.005	0.008	0.555	-0.003	0.019	0.527	-0.003	
	0.020	0.458	0.000	0.015	0.568	0.004	0.028	0.559	0.003	
	0.019	0.493	-0.003	0.011	0.571	0.000	0.024	0.551	0.000	
	0.021	0.473	-0.001	0.013	0.561	-0.003	0.027	0.525	0.002	
	0.013	0.449	-0.004	0.010	0.576	-0.005	0.018	0.489	-0.006	
	0.025	0.466	0.003	0.017	0.558	0.000	0.022	0.508	0.002	

	MM + Crude-oil Pennsylvania		MM + Crude-oil Ecuador		MM + Crude-oil Saudi Arabia	
	Average	SD	Average	SD	Average	SD
pSB3T5_pSB1C3_colony 1	0.021	0.004	0.015	0.006	0.024	0.006
pSB3T5_pSB1C3_colony 2	0.020	0.005	0.012	0.003	0.022	0.004
CCA48_CCA57_Colony 1	0.472	0.042	0.573	0.028	0.544	0.055
CCA48_CCA57_Colony 2	0.470	0.018	0.567	0.008	0.518	0.026
media	0.000	0.005	0.002	0.005	0.001	0.005
media	-0.002	0.003	-0.002	0.002	-0.001	0.004

	MM + Crude-oil Pennsylvania		MM + Crude-oil Ecuador		MM + Crude-oil Saudi Arabia	
	Average	SD	Average	SD	Average	SD
pSB3T5_pSB1C3	0.020	0.001	0.014	0.002	0.023	0.001
CCA48_CCA57	0.471	0.001	0.570	0.005	0.531	0.018
Media	-0.001	0.001	0.000	0.003	0.000	0.001

Timepoint: 48 hr
Absorbance: 600 nm
Volume: 100 uL
Spectrophotometer: Molecular Devices Spectra Max

	Minimal Media (MM)			MM + Glucose			MM + Fluorene+Phenanthrene			
	Control Bact	Bacteria (48+57)	Media	Control Bact	Bacteria (48+57)	Media	Control Bact	Bacteria (48+57)	Media	Average blank
Raw Data (No Blank)	0.0568	0.0536	0.0413	0.4078	0.3249	0.0412	0.0556	0.3463	0.0411	0.040
	0.0553	0.0523	0.0393	0.4191	0.3023	0.0396	0.0587	0.3454	0.0395	
	0.0542	0.0525	0.0388	0.4113	0.2797	0.0391	0.0545	0.3568	0.0392	0.040
	0.0556	0.0534	0.0404	0.4114	0.3461	0.0407	0.0533	0.3333	0.0409	
	0.0544	0.0539	0.0394	0.4135	0.3047	0.0396	0.054	0.3295	0.0397	0.040
	0.0549	0.0534	0.0399	0.3974	0.283	0.0391	0.0533	0.329	0.0401	
	0.0528	0.051	0.0395	0.3924	0.2828	0.0392	0.0534	0.3333	0.0387	
	0.0603	0.0552	0.0412	0.3855	0.2859	0.0403	0.0545	0.3395	0.0407	
OD -Blank	0.017	0.014	0.001	0.368	0.285	0.001	0.016	0.306	0.001	
	0.015	0.012	-0.001	0.379	0.262	0.000	0.019	0.305	0.000	
	0.014	0.013	-0.001	0.371	0.240	-0.001	0.015	0.317	-0.001	
	0.016	0.013	0.000	0.372	0.306	0.001	0.013	0.293	0.001	
	0.014	0.014	-0.001	0.374	0.265	0.000	0.014	0.290	0.000	
	0.015	0.013	0.000	0.358	0.243	-0.001	0.013	0.289	0.000	
	0.013	0.011	0.000	0.353	0.243	-0.001	0.013	0.293	-0.001	
	0.020	0.015	0.001	0.346	0.246	0.000	0.015	0.300	0.001	
Corrected OD	0.085	0.069	0.007	1.858	1.440	0.007	0.079	1.547	0.006	
	0.077	0.062	-0.003	1.915	1.325	-0.001	0.094	1.542	-0.002	
	0.072	0.063	-0.006	1.876	1.211	-0.004	0.073	1.600	-0.004	
	0.079	0.068	0.002	1.876	1.547	0.004	0.067	1.481	0.005	
	0.073	0.070	-0.003	1.887	1.337	-0.001	0.071	1.462	-0.001	
	0.075	0.068	0.000	1.806	1.228	-0.004	0.067	1.460	0.001	
	0.065	0.056	-0.002	1.780	1.227	-0.003	0.068	1.481	-0.007	
	0.103	0.077	0.006	1.746	1.243	0.002	0.073	1.513	0.004	

	Minimal Media (MM)		MM + Glucose		MM + Fluorene+Phenanthrene	
	Average	SD	Average	SD	Average	SD
pSB3T5_pSB1C3_colony 1	0.078	0.005	1.881	0.024	0.078	0.012
pSB3T5_pSB1C3_colony 2	0.079	0.016	1.805	0.060	0.070	0.003
CCA48_CCA57_Colony 1	0.066	0.003	1.381	0.145	1.543	0.049
CCA48_CCA57_Colony 2	0.068	0.009	1.259	0.053	1.479	0.024
media	0.000	0.006	0.002	0.005	0.001	0.005
media	0.000	0.004	-0.002	0.003	-0.001	0.004

Time point	Minimal Media (MM)		MM + Glucose		MM + Fluorene+Phenanthrene	
48 hours	Average	SD	Average	SD	Average	SD
pSB3T5_pSB1C3	0.079	0.000	1.843	0.054	0.074	0.006
CCA48_CCA57	0.067	0.002	1.320	0.086	1.511	0.045
Media	0.000	0.000	0.000	0.002	0.000	0.001

48 hours
600 nm
100 uL
Molecular Devices Spectra Max

	MM + Crude-oil Pennsylvania			MM + Crude-oil Ecuador			MM + Crude-oil Saudi Arabia			
	Control Bact	Bacteria (48+57)	Media	Control Bact	Bacteria (48+57)	Media	Control Bact	Bacteria (48+57)	Media	Average blank
Raw Data (No Blank)	0.0531	0.3139	0.0417	0.0487	0.3693	0.0409	0.0541	0.3657	0.0415	0.040
	0.0506	0.2953	0.0394	0.0465	0.3641	0.0437	0.0523	0.3104	0.0392	
	0.05	0.2907	0.0387	0.0461	0.3495	0.0385	0.0516	0.3406	0.0393	0.040
	0.0525	0.2922	0.0404	0.0471	0.356	0.0406	0.0535	0.3637	0.041	
	0.0502	0.3224	0.0393	0.0458	0.353	0.0395	0.0521	0.321	0.0396	0.040
	0.0518	0.3131	0.04	0.0466	0.348	0.0394	0.0526	0.3282	0.04	
	0.0538	0.3005	0.0391	0.0463	0.3325	0.0393	0.0501	0.3061	0.0392	
	0.0529	0.3019	0.045	0.0481	0.3424	0.0407	0.0528	0.3286	0.0405	
OD -Blank	0.013	0.274	0.002	0.009	0.329	0.001	0.014	0.326	0.002	
	0.011	0.255	-0.001	0.007	0.324	0.004	0.012	0.270	-0.001	
	0.010	0.251	-0.001	0.006	0.310	-0.001	0.012	0.301	-0.001	
	0.013	0.252	0.000	0.007	0.316	0.001	0.014	0.324	0.001	
	0.010	0.282	-0.001	0.006	0.313	0.000	0.012	0.281	0.000	
	0.012	0.273	0.000	0.007	0.308	0.000	0.013	0.288	0.000	
	0.014	0.261	-0.001	0.006	0.293	-0.001	0.010	0.266	-0.001	
	0.013	0.262	0.005	0.008	0.303	0.001	0.013	0.289	0.001	
Corrected OD	0.066	1.383	0.009	0.045	1.664	0.005	0.071	1.645	0.008	
	0.054	1.289	-0.003	0.034	1.637	0.019	0.062	1.366	-0.004	
	0.051	1.266	-0.006	0.032	1.564	-0.007	0.059	1.518	-0.003	
	0.063	1.274	0.002	0.037	1.597	0.004	0.068	1.635	0.005	
	0.052	1.426	-0.003	0.030	1.581	-0.002	0.061	1.419	-0.002	
	0.060	1.379	0.000	0.034	1.556	-0.002	0.064	1.455	0.000	
	0.070	1.316	-0.004	0.033	1.478	-0.003	0.051	1.344	-0.004	
	0.065	1.323	0.025	0.042	1.528	0.004	0.065	1.457	0.003	

	MM + Crude-oil Pennsylvania		MM + Crude-oil Ecuador		MM + Crude-oil Saudi Arabia	
	Average	SD	Average	SD	Average	SD
pSB3T5_pSB1C3_colony 1	0.058	0.007	0.037	0.006	0.065	0.006
pSB3T5_pSB1C3_colony 2	0.062	0.008	0.035	0.005	0.060	0.006
CCA48_CCA57_Colony 1	1.303	0.054	1.615	0.044	1.541	0.130
CCA48_CCA57_Colony 2	1.361	0.052	1.536	0.044	1.419	0.053
media	0.000	0.007	0.005	0.011	0.001	0.006
media	0.004	0.014	-0.001	0.003	-0.001	0.003

	MM + Crude-oil Pennsylvania		MM + Crude-oil Ecuador		MM + Crude-oil Saudi Arabia	
	Average	SD	Average	SD	Average	SD
pSB3T5_pSB1C3	0.060	0.002	0.036	0.001	0.063	0.003
CCA48_CCA57	1.332	0.041	1.576	0.056	1.480	0.086
Media	0.002	0.003	0.002	0.004	0.000	0.002

Timepoint: 72 hours
Absorbance: 600 nm
Volume: 100 uL
Spectrophotometer: Molecular Devices Spectra Max

	Minimal Media (MM)			MM + Glucose			MM + Fluorene+Phenanthrene			
	Control Bact	Bacteria (48+57)	Media	Control Bact	Bacteria (48+57)	Media	Control Bact	Bacteria (48+57)	Media	Average blank
Raw Data (No Blank)	0.0557	0.0528	0.0408	0.4097	0.3222	0.0407	0.0567	0.3541	0.0407	0.040
	0.0551	0.0515	0.0394	0.4071	0.2842	0.0396	0.0593	0.3453	0.0393	
	0.0541	0.0522	0.039	0.4012	0.2794	0.0393	0.0544	0.3503	0.0391	0.040
	0.0539	0.0515	0.0395	0.3906	0.3337	0.0401	0.0568	0.3311	0.0399	
	0.0538	0.0532	0.0396	0.3975	0.3049	0.0397	0.0563	0.3391	0.0402	0.040
	0.0536	0.0523	0.0392	0.3859	0.285	0.0386	0.0528	0.3337	0.0396	
	0.0529	0.0502	0.0394	0.3841	0.2821	0.0392	0.0551	0.34	0.0383	
	0.0546	0.0529	0.0402	0.3865	0.2831	0.0397	0.0535	0.3429	0.0407	
OD -Blank	0.016	0.013	0.001	0.370	0.283	0.001	0.017	0.314	0.001	
	0.015	0.012	0.000	0.367	0.245	0.000	0.020	0.306	0.000	
	0.014	0.013	-0.001	0.362	0.240	0.000	0.015	0.311	-0.001	
	0.014	0.012	0.000	0.351	0.294	0.000	0.017	0.291	0.000	
	0.014	0.014	0.000	0.358	0.265	0.000	0.017	0.299	0.000	
	0.014	0.013	0.000	0.346	0.245	-0.001	0.013	0.294	0.000	
	0.013	0.011	0.000	0.344	0.242	0.000	0.015	0.300	-0.001	
	0.015	0.013	0.001	0.347	0.243	0.000	0.014	0.303	0.001	
Corrected OD	0.081	0.066	0.006	1.869	1.427	0.005	0.086	1.588	0.005	
	0.078	0.060	-0.001	1.856	1.235	0.000	0.099	1.543	-0.002	
	0.073	0.063	-0.003	1.826	1.211	-0.002	0.074	1.568	-0.003	
	0.072	0.060	-0.001	1.772	1.485	0.002	0.086	1.471	0.001	
	0.072	0.068	0.000	1.807	1.340	0.000	0.084	1.512	0.002	
	0.071	0.064	-0.002	1.749	1.239	-0.005	0.066	1.485	-0.001	
	0.067	0.053	-0.001	1.740	1.225	-0.002	0.078	1.516	-0.007	
	0.076	0.067	0.003	1.752	1.230	0.000	0.070	1.531	0.005	

	Minimal Media (MM)		MM + Glucose		MM + Fluorene+Phenanthrene	
	Average	SD	Average	SD	Average	SD
pSB3T5_pSB1C3_colony 1	0.076	0.004	1.831	0.043	0.086	0.010
pSB3T5_pSB1C3_colony 2	0.071	0.004	1.762	0.031	0.074	0.008
CCA48_CCA57_Colony 1	0.062	0.003	1.340	0.137	1.543	0.051
CCA48_CCA57_Colony 2	0.063	0.007	1.258	0.055	1.511	0.019
media	0.000	0.004	0.002	0.003	0.000	0.004
media	0.000	0.002	-0.002	0.003	0.000	0.005

Time point	Minimal Media (MM)		MM + Glucose		MM + Fluorene+Phenanthrene	
72 hours	Average	SD	Average	SD	Average	SD
pSB3T5_pSB1C3	0.074	0.003	1.796	0.049	0.080	0.008
CCA48_CCA57	0.063	0.001	1.299	0.057	1.527	0.022
Media	0.000	0.000	0.000	0.002	0.000	0.000

72 hours
600 nm
100 uL
Molecular Devices Spectra Max

	MM + Crude-oil Pennsylvania			MM + Crude-oil Ecuador			MM + Crude-oil Saudi Arabia			
	Control Bact	Bacteria (48+57)	Media	Control Bact	Bacteria (48+57)	Media	Control Bact	Bacteria (48+57)	Media	Averag blan
Raw Data (No Blank)	0.052	0.2902	0.0417	0.048	0.3422	0.0411	0.0525	0.3497	0.0414	0.04
	0.0491	0.2688	0.0391	0.0459	0.3353	0.0429	0.0505	0.2896	0.0391	
	0.0492	0.264	0.0389	0.0455	0.3179	0.0388	0.0503	0.3123	0.0395	0.04
	0.0517	0.2714	0.0404	0.0465	0.3337	0.0404	0.052	0.3365	0.0406	
	0.049	0.28	0.0394	0.0456	0.3198	0.0396	0.051	0.3001	0.0398	0.04
	0.0497	0.2793	0.0401	0.0459	0.3269	0.0393	0.0508	0.3065	0.0398	
	0.0495	0.2719	0.0391	0.0454	0.3147	0.0392	0.0486	0.2901	0.0391	
	0.0511	0.2691	0.0451	0.047	0.3233	0.0407	0.0514	0.304	0.0403	
OD -Blank	0.012	0.251	0.002	0.008	0.303	0.001	0.013	0.310	0.002	
	0.009	0.229	-0.001	0.006	0.296	0.003	0.011	0.250	-0.001	
	0.010	0.224	-0.001	0.006	0.278	-0.001	0.011	0.273	0.000	
	0.012	0.232	0.001	0.007	0.294	0.001	0.012	0.297	0.001	
	0.009	0.240	0.000	0.006	0.280	0.000	0.011	0.260	0.000	
	0.010	0.240	0.000	0.006	0.287	0.000	0.011	0.267	0.000	
	0.010	0.232	-0.001	0.006	0.275	0.000	0.009	0.250	-0.001	
	0.011	0.229	0.005	0.007	0.284	0.001	0.012	0.264	0.001	
Corrected OD	0.062	1.265	0.010	0.042	1.528	0.008	0.065	1.565	0.008	
	0.048	1.157	-0.003	0.032	1.493	0.017	0.054	1.262	-0.003	
	0.048	1.133	-0.004	0.030	1.405	-0.004	0.053	1.377	-0.001	
	0.061	1.170	0.004	0.035	1.485	0.004	0.062	1.499	0.004	
	0.047	1.214	-0.001	0.030	1.415	0.000	0.057	1.315	0.000	
	0.051	1.210	0.002	0.032	1.451	-0.002	0.056	1.347	0.000	
	0.050	1.173	-0.003	0.029	1.389	-0.002	0.045	1.264	-0.003	
	0.058	1.159	0.028	0.037	1.433	0.005	0.059	1.335	0.003	

	MM + Crude-oil Pennsylvania		MM + Crude-oil Ecuador		MM + Crude-oil Saudi Arabia	
	Average	SD	Average	SD	Average	SD
pSB3T5_pSB1C3_colony 1	0.055	0.008	0.035	0.006	0.059	0.006
pSB3T5_pSB1C3_colony 2	0.051	0.005	0.032	0.004	0.054	0.006
CCA48_CCA57_Colony 1	1.182	0.058	1.478	0.052	1.426	0.134
CCA48_CCA57_Colony 2	1.189	0.027	1.422	0.026	1.315	0.036
media	0.002	0.007	0.006	0.009	0.002	0.005
media	0.007	0.014	0.000	0.003	0.000	0.002

	MM + Crude-oil Pennsylvania		MM + Crude-oil Ecuador		MM + Crude-oil Saudi Arabia	
	Average	SD	Average	SD	Average	SD
pSB3T5_pSB1C3	0.053	0.002	0.033	0.002	0.056	0.003
CCA48_CCA57	1.185	0.005	1.450	0.040	1.370	0.078
Media	0.004	0.003	0.003	0.004	0.001	0.001