Results

CLONE DESCRIPTION (Details for what clone number means what in testing)

For more information on rawdata or experiments - please visit the lab notebook section and look for the testing notebook.

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

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

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.

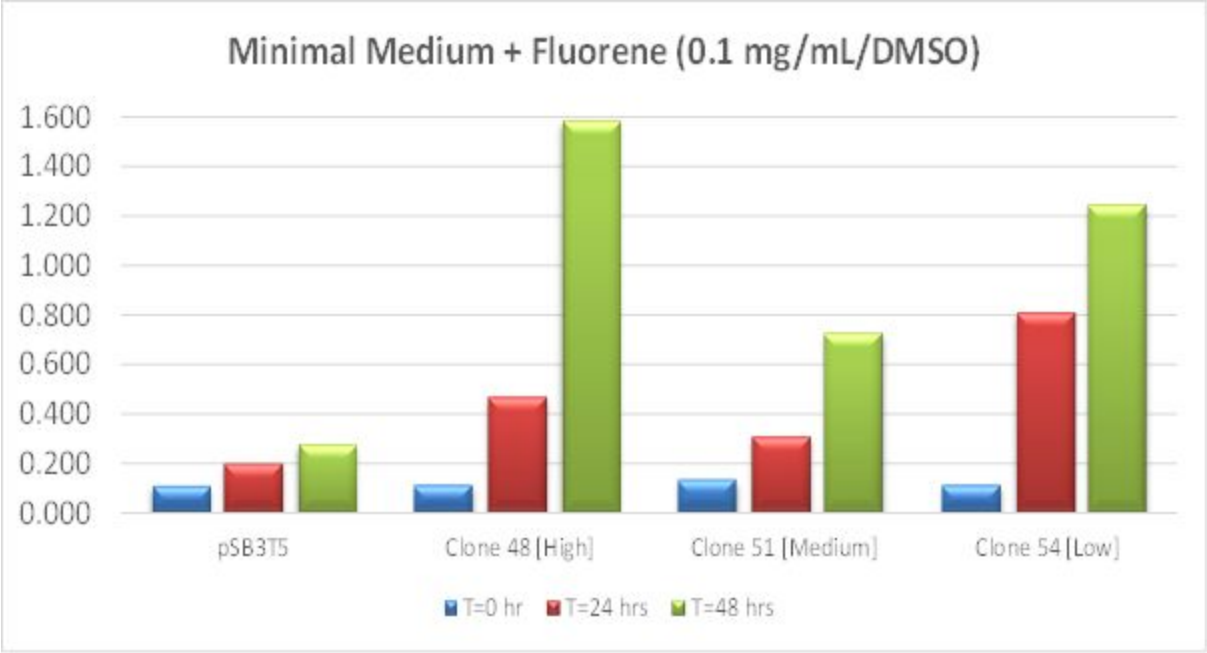
CULTURE SETUP

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.

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.



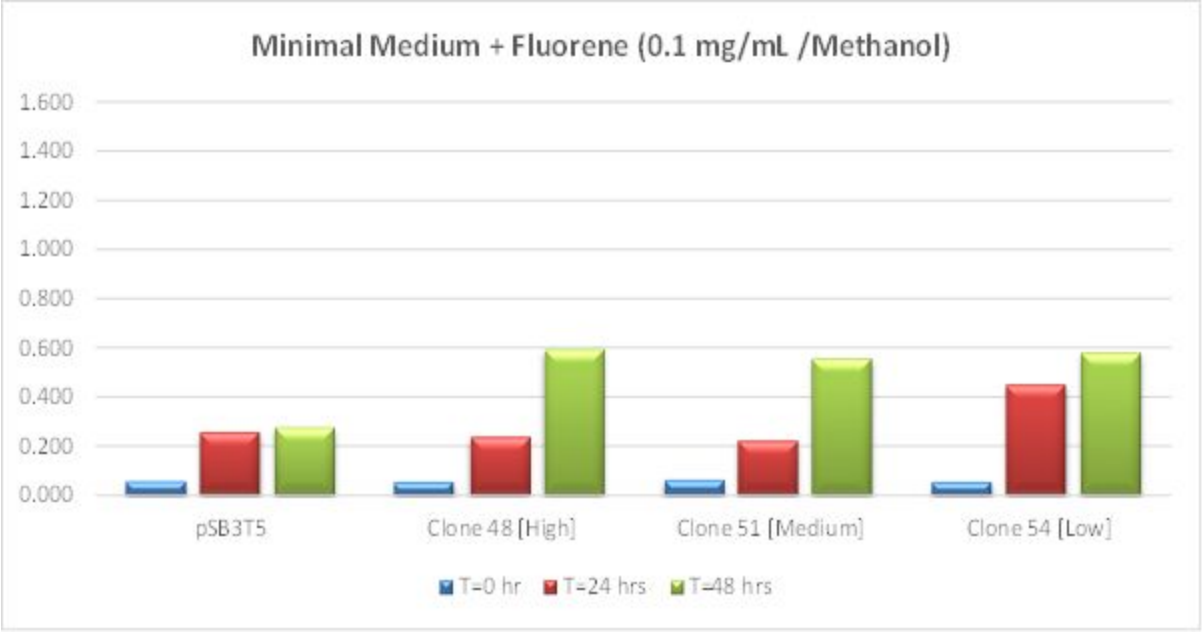
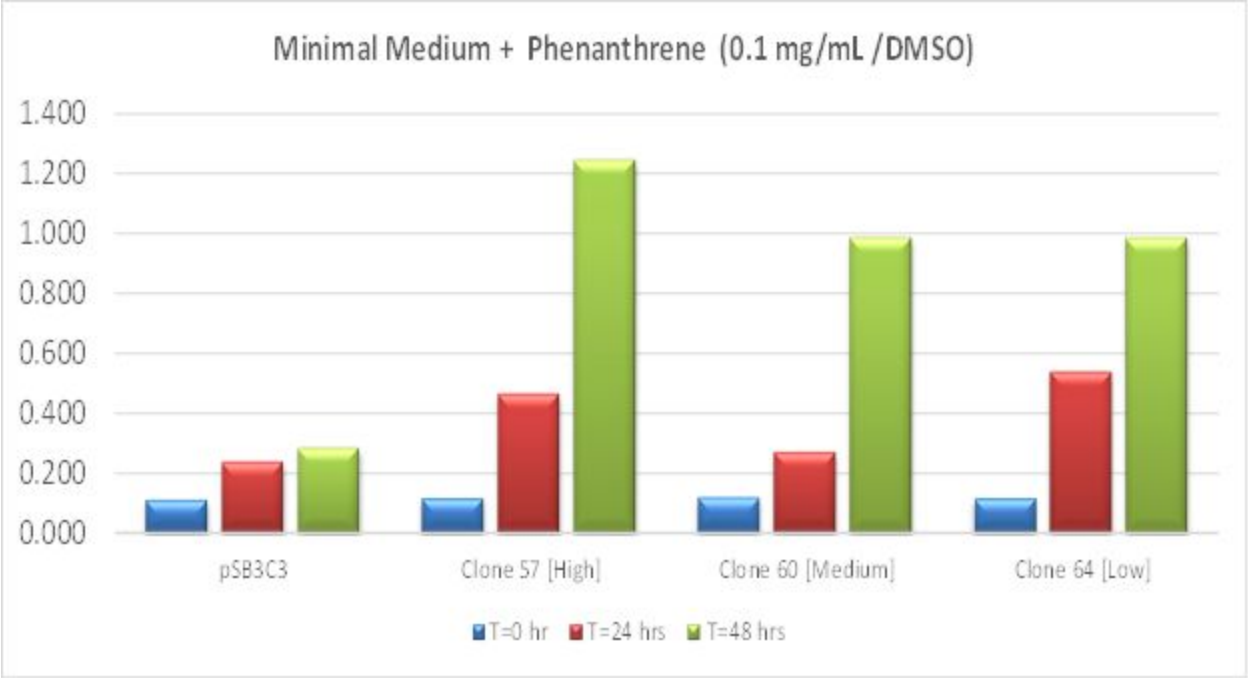


Figure 1. Growth of recombinant E.coli BL21DE3 cultures harboring the control plasmid pSB3T5 or the fluorene pathway under the control of 3 different constitutive promoters: BBa_J23100 (clone 48), BBa_J23101 (clone 51), and BBa_J23110 (clone 54) cloned into pSB3T5. Data points represent value averages 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).



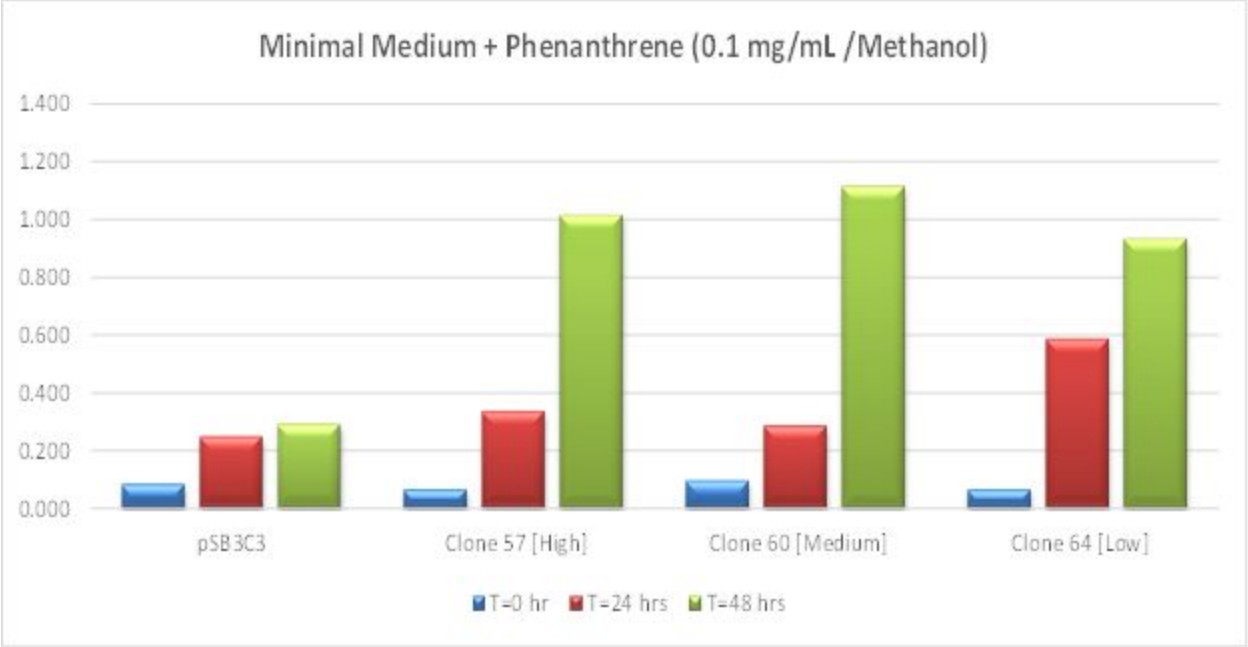


Figure 2. Growth of recombinant E.coli BL21DE3 cultures harboring the control plasmid pSB1C3 or the phenanthrene pathway under the control of 3 different constitutive promoters: BBa_J23100 (clone 57), BBa_J23101 (clone 60), and BBa_J23110 (clone 64) cloned into pSB1C3. Data points represent value averages 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).

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.

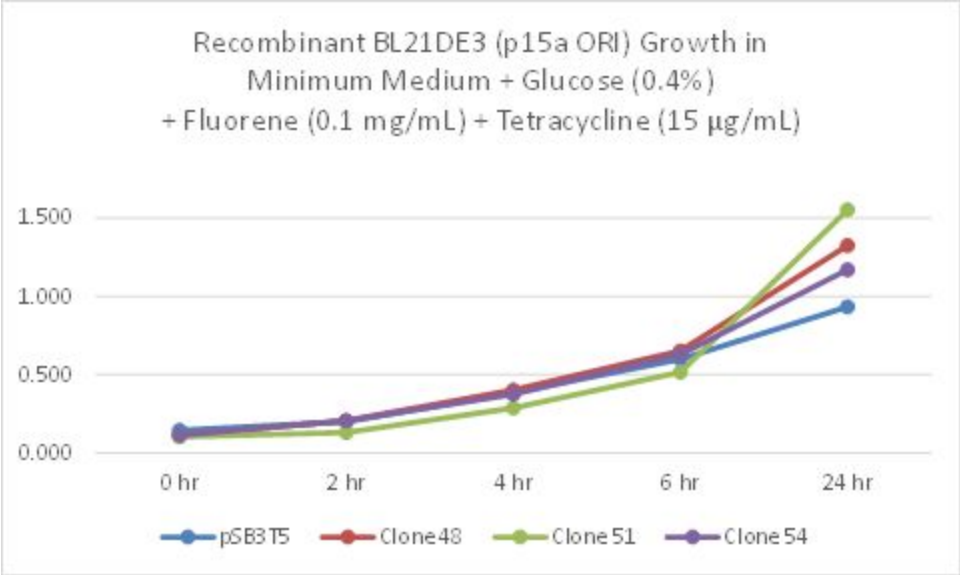


Figure 3. Fluorene biotransformation experiment using recombinant E.coli BL21DE3 harboring the control plasmid pSB3T5 or the fluorene pathway under the control of 3 different constitutive promoters: BBa_J23100 (clone 48), BBa_J23101 (clone 51), and BBa_J23110 (clone 54) cloned into pSB3T5. Data points 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 supplemented with tetracycline (15 µg/mL), fluorene (0.1 mg/mL), and glucose (0.4%).

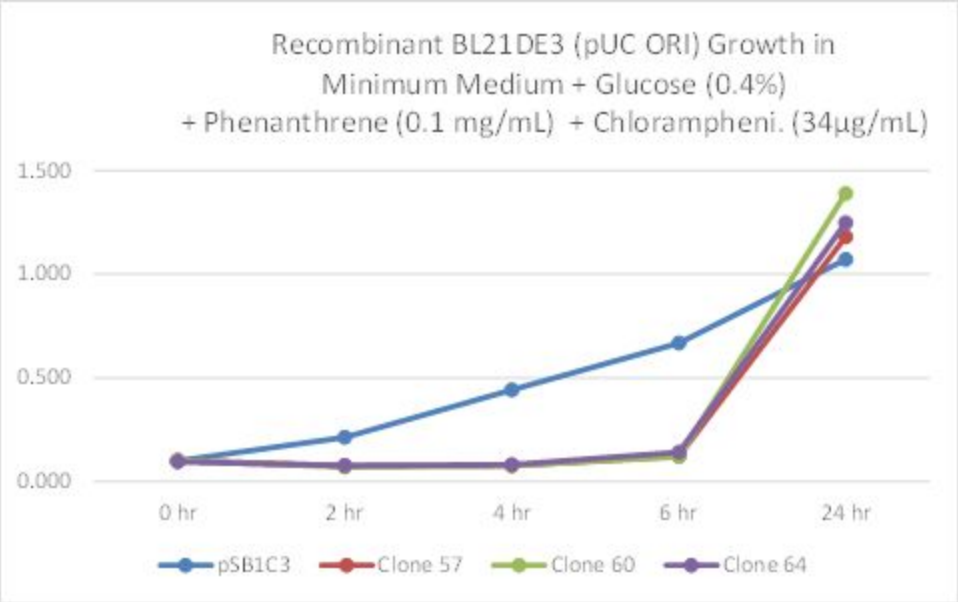


Figure 4. Phenanthrene biotransformation experiment using recombinant E.coli BL21DE3 harboring the control plasmid pSB1C3 or the phenanthrene pathway under the control of 3 different constitutive promoters: BBa_J23100 (clone 57), BBa_J23101 (clone 60), and BBa_J23110 (clone 64) cloned into pSB1C3. Data 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%).

TIME COURSE BIOTRANSFORMATION RESULTS IN PRESENCE OF FLUORENE AND PHENANTHRENE

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.

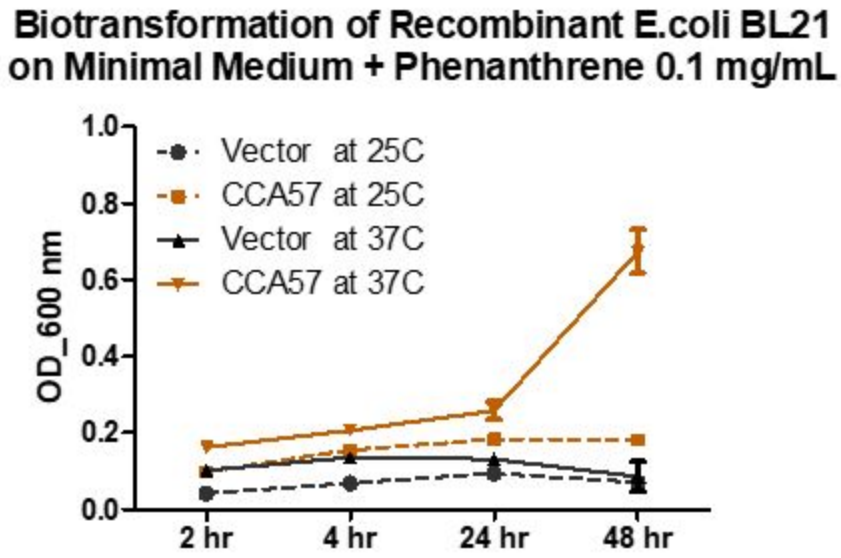
CULTURE SETUP

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

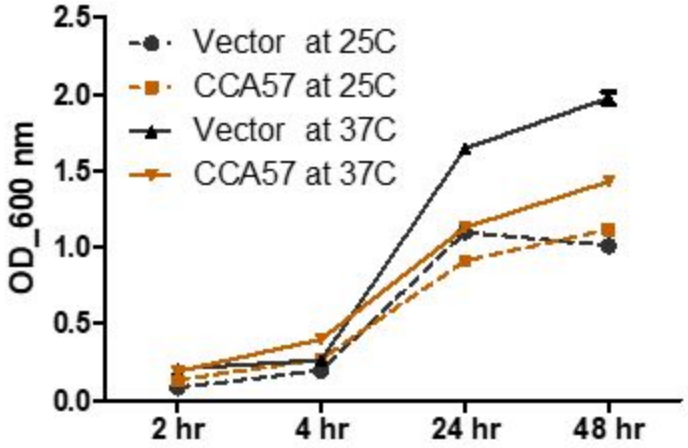
protocol shown above. All cultures were then diluted to 0.02 using the volume below and OD measurements were determined at the indicated time points.

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.

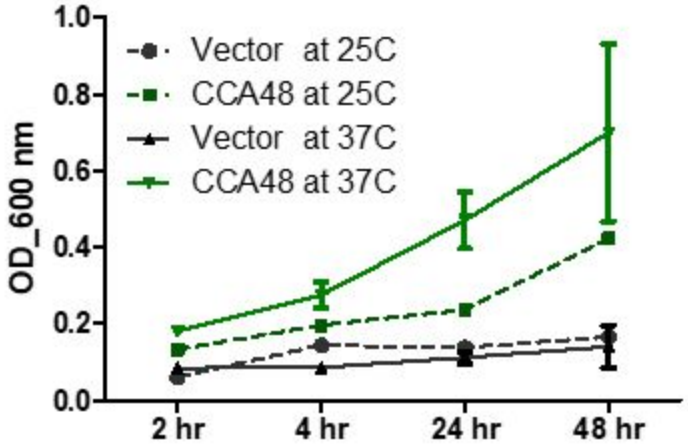
In order to evaluate whether the recombinant cells had the ability to transform PAHs at room temperature (25°C), growth experiments were set up with clones CCA-48 (=clone 48) and CCA-57 (= clone 57) expressing the fluorene or the phenanthrene catabolic pathway respectively at two temperatures. The clones 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.

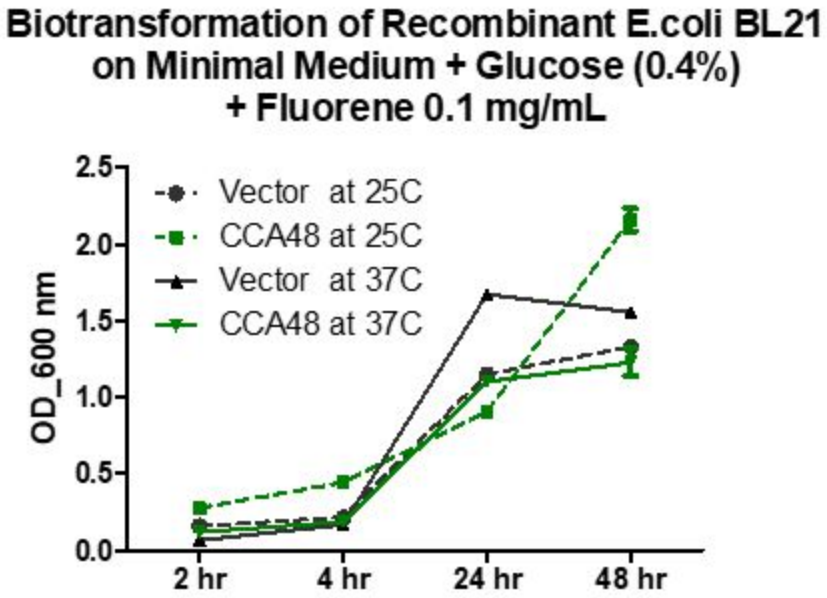


Biotransformation of Recombinant E.coli BL21 on Minimal Medium + Glucose (0.4%) + Phenanthrene 0.1 mg/mL



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





BIOTRANSFORMATION RESULTS IN PRESENCE OF CRUDE OILS

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%).

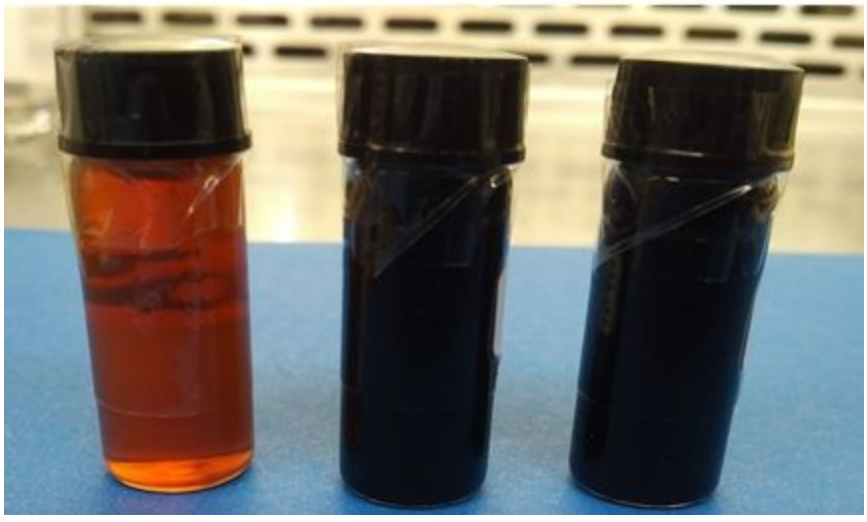


Figure 5. Crude oil samples from Pennsylvannia, Ecuador, and Saudi Arabia (left to right).

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

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.

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.

This acts as a proof of concept because the fact that our bacteria are degrading PAHs are an indication of our methods' effectiveness.

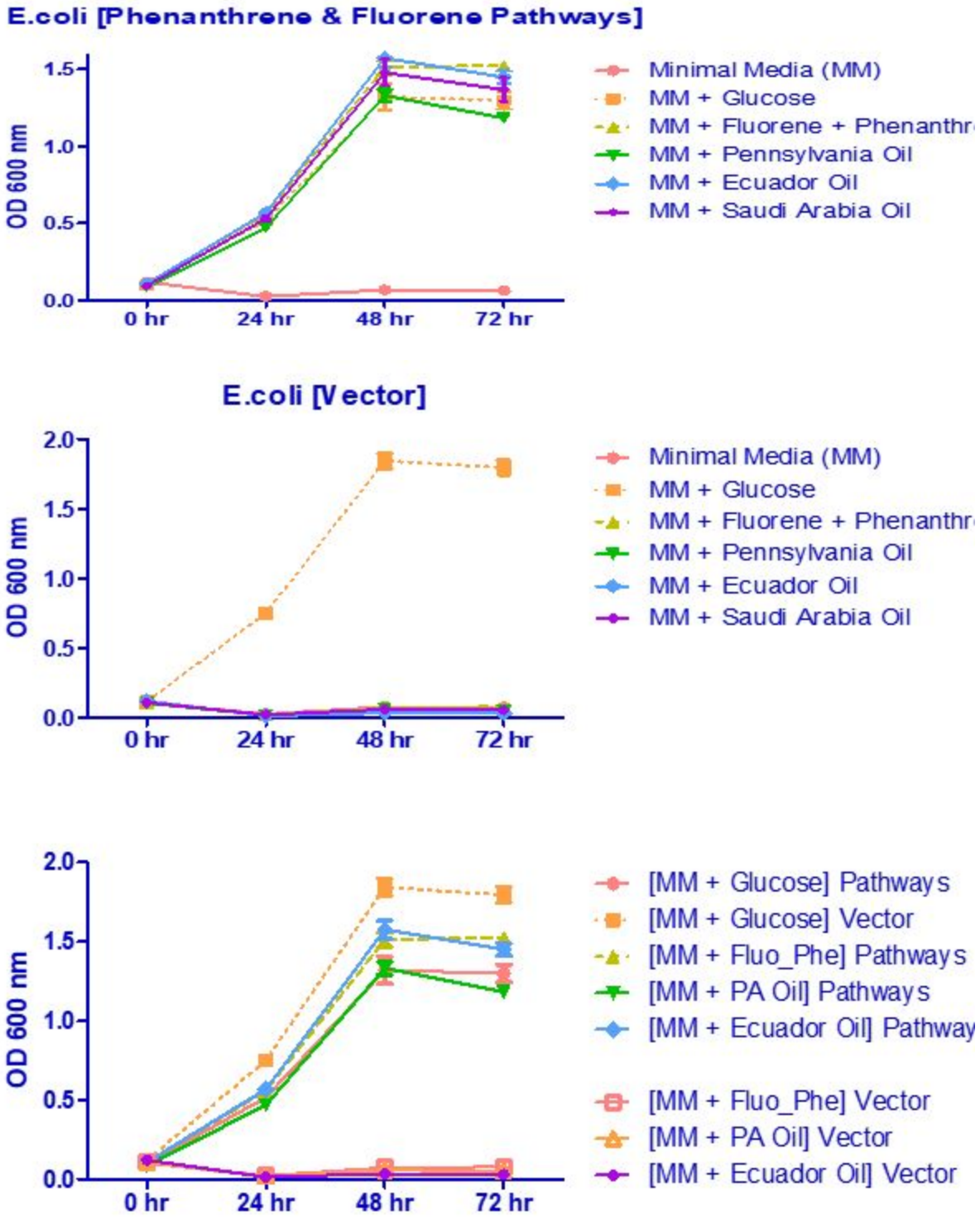


Figure 6. Time course biotransformation experiments using crude oil samples from Pennsylvania (PA), Saudi Arabia, and Ecuador, measuring absorbance at 600nm of E.coli BL21

recombinant cultures containing the fluorene and phenanthrene catabolic pathways or control vectors. MM=M9 minimal medium.

1.1. General Conclusions

Our biotransformation (degradation) tests using the two PAHs fluorene or phenanthrene as sole carbon source clearly demonstrate that our recombinant strains containing the corresponding catabolic pathways are able to degrade fluorene or phenanthrene efficiently. The growth curves and bar graphs indicate that the recombinant bacteria containing the catabolic pathways are growing at significant faster rates than recombinant bacteria containing the corresponding vector alone. Since bacteria need a source of carbon to grow, these data indicate that the recombinant E.coli strains containing the fluorene and the phenanthrene catabolic pathway must be degrading PAH and use it as carbon source.

When compared with bacteria growing on minimal media containing glucose as sole source of carbon, the recombinant strains containing the catabolic pathways exhibited a growth of similar rate and peak and time frame than control recombinant strains. This indicates that the recombinant bacteria use PAH as a carbon source just as effectively as glucose, a non-toxic carbon source.

When comparing the expression level of the catabolic pathways under the control of 3 different constitutive promoter of various strengths (parts BBa_J23100, BBa_J23101, and BBa_J23110), there is a minimal difference in growth potential. However, promoter BBa_J23100 is the most effective as it balances out the energy utilized for protein expression and the degradation rate of the PAH. This allows for an optimal growth for the bacteria.

What is more astonishing is that a recombinant E.coli strain containing the two pathways – fluorene and phenanthrene - is able to use a source of crude oil rich in polycyclic aromatic hydrocarbons just as effectively as glucose or pure fluorene and phenanthrene.

All these data indicate that our project is successful and meets our main objective of degrading PAHs in crude oils.