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Revision of our manuscript submitted to Journal of Visualized Experiments:

'A toolkit to enable hydrocarbon conversion in aqueous environments'

by Eva K. Brinkman, Kira Schipper, Nadine Bongaerts, Mathias J. Voges, Alessandro Abate, S. Aljoscha Wahl

Dear Mrs. Meghan Berryman,

We would like to thank the reviewers for their input and critical evaluation of our manuscript. In the following pages, we have addressed the comments point-by-point, furthermore we have indicated the changes in the manuscript with colored typeset.

We hope that the revised version meets the quality standards for publication in JoVE

With kind regards from Delft,

Aljoscha Wahl.

Reviewer 1:
Summary
This submission describes an interesting research project, but does not really describe a novel technique or method in the sense outlined above. As such, it did not immediately strike me as an obvious choice for JoVE. It does include several standard techniques used to test the constructs made (gas chromatography for hydrocarbon analysis, enzyme activity assay for dehydrogenases, OD measurement, etc. I think perhaps a way forward, if the authors agree, would be to recast it slightly and make a video showing how the basic concept of BioBrick design and assembly works, using this project as an example. The HIM aspect may also be novel and interesting, but is further from my expertise.

Major Concerns
As noted above, this is a great research project, but does not really define a novel technique, as far as I can see, at least as far as the assays are concerned. Perhaps, in addition, if it included a video summary of the basic BioBrick assembly strategy used to make the organism; this would add something for everyone who is interested in synthetic biology and its promises for dealing with environmental problems.

RE:
The aspect of BioBrick assembly has now been included into the manuscript. In particular, we included the protocol for BioBrick assembly from existing parts (using two-way ligation) and a short note on the construction of new parts based on DNA synthesis and optimization (see modification on pages 7 and 8).

Minor Concerns
Nothing to add at this stage.

Additional Notes to the Author
I think this is a very nice piece of work, which could be published in a microbiology or biotechnology journal.

Reviewer 2:
Summary:
The article " A toolkit to enable hydrocarbon conversion in aqueous environments" by Brinkman et al describes results from an iGEM project from Delft Technical University. The results are interesting, but I have some doubts. The authors have selected a tricky field, alkane degradation, and combine this with additional work on the carbon catabolite repression of a specific promoter and experiments regarding growth in the presence of hexane. The set of methods described here lacks therefore a certain stringency – it is not clear to me what a section on CRP-effects contributes to this paper.

Concerns:
-The authors suggest to carry out resting cell assays over 24h and then to calculate rates from that. I find this doubtful given the fact that it is not clear whether the enzymes are active anywhere near that long. Therefore, distributing conversion over 24 h is only bringing rates down.

It is correct that choosing a long incubation time might lead to underestimation of alkane conversion rates. We want to point out that the main focus of the study was to show that there is activity, as well as to compare the conversion to wild-type activity. Both samples are treated for the same time and, because of the low activity in the wild-type, a long incubation was required to measure concentration changes.
We included a discussion on the incubation time and full characterization on p. 22 of the manuscript:

'The conversion activity has been measured by a comparative study (WT vs. mutant) after a given time. For a full characterization, additional studies have to be performed on the activity of the AH-system over time and the kinetics of the system.'

-In section two they heat a mix of alkane and water up to 100 degrees to help dissolving. Frankly, I cannot imagine a more efficient way to evaporate alkanes.

The heating was used especially for medium and long-chain alkanes, in this case Hexadecane (C16H34). This alkane has a boiling point of 287C, which is much higher than water. Furthermore, the flask was sealed to minimize evaporation.

Additionally, the assay is again a comparison of conversion between the wild-type and the mutant strain. In case the preparation did lead to a changed concentration in the starting mixture, it is the case for the wild-type and mutant.

On page 9 (now protocol three), we added a note on the heating step:
3.8) Heat the mixture at 100 °C for 5 minutes. Note: Perform this step only for medium-long chain alkanes with high boiling point. (e.g. C16: 287°C).

-In section 4, they report a protocol for activity measurements of enzymes that simply does not work (see their own results).

-The tolerance assay is very strange: They add hexane up to 10%, which in my experience gives a two phase system, which makes OD measurements – the prime readout – unreliable.

-In addition, they do the mixing in a microtiter plate, which is notoriously unreliable when it comes to mixing.

This assay has been performed as a comparative study between wild-type vs. solvent tolerant strain. Even if there have been effects of phase-separation, as mentioned by the reviewer, the effect would be the same for the wild-type and for the solvent tolerant strain. The experiments do show a clear difference between wild-type and mutant strain under the same conditions (same plate, temperature, solvent concentration). The error bars in the graph represent the standard deviation of the mean of three independent experiments (different wells), which shows that the experiment is reproducible.

We added a note in the tolerance protocol (p. 13):
7.4) Load a 96 well plate with 180 µL M9 medium containing the appropriate antibiotics and the proper final concentration of the toxic compound (e.g. 0, 4, 8, 10% of n-hexane) in triplicate. Because alkane-water mixtures could lead to two-phase systems, it is essential to have appropriate controls on the plate (e.g. different strains and the respective blank experiments).

-Finally, the model is rather mysterious: In the results, only GFP and biomass are recorded and modeled. The model has many more parameters, which are no longer considered – what happened?

It is correct that the model has several additional state variables. Because of the focus of the journal on experimental procedures, the model is not described in detail here. We have included a web-link to the detailed model description with more simulation details and outcomes.
Please see the additions on pages 16 and 17 of the manuscript.