

Kinetics of Arab Light crude oil degradation by *Pseudomonas* and *Bacillus* strains

Original Submission
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Recommendation: Major Revision

Overall Manuscript Rating (1-100): 50

Reviewer Blind Comments to Author:

General: This manuscript entitled on "Kinetics of Arab Light crude oil degradation by *Pseudomonas* and *Bacillus* strains" provides a general viewpoint for hydrocarbons degradation rates by bacteria. Authors presented a great effort to prepare this manuscript, however, the reviewer has some queries about this paper. I would strongly suggest to check this manuscript by English experts. There is no set for 'control' experiment. Authors should have prepared at least one control set with only crude oil in MSM without containing any bacterial strain. The experimental data obtained from microbial degradation must be compared with control data which strengthen the reliability of the research. Also, all experiments must be done with at least 3 replicates for data repeatability. I can see the authors only did one replicate for each experiment. This is the main fault of this paper. Please explain. I suggest that this manuscript can be acceptable with major revision. Other comments are as follows;

Control has now been included in Figure 8, it was not included in the original manuscript because kinetics is not affected (lines 144 and 425). GC chromatograms were repeated twice (line 143) and OD was measured by triplicate (line 166). Thanks.

- The English language of this manuscript has several grammatical errors and it must be checked and corrected by native English experts.

English expressions have been revised and English language has been checked and corrected in the whole manuscript.

- The abstract should be improved by including more numerical data.

Numerical data have been included in lines 14 and 17.

- Line 8-9: Please revise the first sentence to be understandable for readers. Similar in Line 38-40. What is the consequence of what? Please explain.

Both sentences have been rewritten and revised (lines 9 and 40).

- Line 15: Did the authors use chloromethane for extraction? Or they used dichloromethane (DCM)? This is not explained in Methodologies section.

It was confused in the old version of the manuscript. Now the use of DCM has been better explained (section 2.3. Experimental degradation assays, line 168). Thanks.

- Line 20: What does the first order kinetic coefficient mean? Is this value unitless? The first order rate constant should have a unit of time^{-1} .

It was an error, now corrected (line 21). Many thanks.

- There is lack of extensive literature review in this paper. Authors need to improve introduction section by citing several published literatures. For e.g., I could not see any citing references in the first paragraph (line 29-37), which contains several information. Revise the entire introduction section.

- I would suggest the authors to do an extensive literature review (cited) and improve the introduction section.

Some references were not included in the first paragraph of the manuscript. In the new version, we included 3 references about distribution of hydrocarbons in light crude oils and most abundant n-alkanes.

Introduction section has been revised and improved, but we think 41 references in the article (17 in the introduction) is a correct number of citations for a research article, otherwise too many references will be included, I think.

Literature review about oil composition, AlkH enzymes and kinetics of *ex situ* biodegradation of petroleum hydrocarbons was done several times and we found the articles cited in the manuscript (most important ones). Could be with more citations but we tried to select the more relevant articles (for that topics).

Thanks for indication.

- Line 40-44: Please make this sentence simpler to be easily understandable for readers. Sentence has been divided in two and reorganized (lines 42-48). Thanks
- The flow of writing in ascending paragraphs in the introduction should be connected with each other. What are the limitations of previous studies and why this study is important? Also, the specific objectives should be more intensified according to the research gaps in this field. It should be clarified by the authors.

The structure of the introduction is divided in 3 parts. Firstly composition of Arab Light is described, related to number of carbons in n-alkanes (1st paragraph). In accordance with this composition, the intention is to describe the enzymatic system (AlkH), which is the key for petroleum biodegradation, and we think, interesting for readers (paragraphs 2 and 3). Connected to these paragraphs, we cite the difficulty on oil biodegradation and describe the initial oxidation. And finally we conduct the argumentation to the lack of kinetic data on *ex situ* biodegradation and the importance to use it in bioremediation.

Some points of view about petroleum biodegradation have not been included, it is true, but we tried to focus the problem to the lack of kinetic data and the importance it has to restore oil spills.

Specific studies on biodegradation kinetics of petroleum have been described later, at the end of the article, comparing with results obtained (lines 433-436, references 37-41).

The specific objectives have been intensified (lines 80-84).

Thanks for effort.

- Line 113, 119: AGILENT ----→ Agilent
This error has been corrected (lines 113 and 119). Thank you.
- Line 141: Authors stated that they measured oil concentration several time as week by GC. But it is not specified that how many times a week they measured. Please revise with exact sampling time. In the scientific researches/experiments, everything must be clear about the experimental plans and procedures that have done and sampling time is the most important in research.

The exact sampling time has been corrected (line 142). Thanks.

- I would like to know about volume of sample during sampling (in MSM) and its processing (extraction with some solvents) for crude oil characterization or measurements by GC-MS or GC-FID. Also, there should be internal standard added during extraction or processing of samples and standard solution. Explain.

In lines 167-169 (materials and methods) and lines 265-267 (results) the volumes of aqueous samples and organic extractor have been included. Internal standard was not used, alkanes were evaluated by GC-FID using C7-C30 Sigma-Aldrich standard with the ChemStation calibration procedure [16, 17].

- Section 3.1 (Crude oil characterization) and 3.2 (Sample analysis) with Figure 3 should be moved to section 2 (Materials and methods) or supporting information.

But excuse me, Figure 3 is the composition of Arab Light, is a result that has been included in section 3.1. Crude oil characterization.

Sections 2.2.1. Crude oil characterization (methods) and 2.2.2. Crude oil measurements have been improved in the new version of the manuscript (lines 112-128).

- Figure 3: Xilene -----> xylene. Xylene is the correct English word for this chemical. Also, mention which xylene is present in this oil. How did authors calculate the % weight or individual abundance for the composition of aliphatic hydrocarbons? Please explain how the percentage of C7-C12 compounds over aliphatic alkanes distribution is 46.4%.
Xylene has been corrected (typographic error) in Figure 3. We have not information by GC-MS about which isomer of xylene it is (alfa-, meta- or para-). 46.4 % is calculated by summing percentages of C7-C12 alkanes and comparing with the total sum of percentages C7-C29 (in the table of Figure 3, below). Now this point has been better explained in the figure legend (line 214). Thanks.
- In Fig. 3 (upper, for standard solution), they mentioned chromatogram for C7-C30, however one can see only up to C29. It would be better if there is C30 peak.
C30 was not detected in GC-MS chromatogram for oil (Figure 3), it was not included in the spectra (Standard and sample). An explanation has been included in the new version of the manuscript (line 212). Good point because in Arab Light, alkanes over C27 are very scarce (Figure 3, green spectrum). Thank you.
- I wonder why authors do not marked above all peaks (corresponding n-alkane with C number) in all Figures?
To mark all numbers of carbons makes figures to be confused (too many numbers: "the trees do not permit to see the wood"). We decided to mark only the most representative n-alkanes to identify the important ones in biodegradation and the ones which seem to be recalcitrant (C7-C9 and C21, other peaks are not n-alkanes, are isomers). Thanks for the indication.
- Figures 5 and 6 do not represent the actual kinetics of Arab Light degradation. They are only representative chromatogram. Kinetics should be evaluated in concentration or other data regarding rates or rate constants. Please revise.
You are right. Now this is corrected in the legends of the figures 5 and 6. Thank you.
- Line 311-316: Authors shown that low molecular alkanes are less degraded while medium chain alkanes were completely degraded within 5 weeks. In contrast, Bajagain et al. (2018, 2020) argued that mid-chain hydrocarbons are less readily biodegraded in soil than low molecular hydrocarbons. See the following literatures (and also other papers) and discuss them in the present paper;
 - 1) Feasibility of oxidation-biodegradation serial foam spraying for total petroleum hydrocarbon removal without soil disturbance. Science of the Total Environment 626, 2018, 1236-1242.
<https://doi.org/10.1016/j.scitotenv.2018.01.212>
 - 2) Biodegradation and post-oxidation of fuel-weathered field soil. Science of The Total Environment 734, 2020, 139452.
<https://doi.org/10.1016/j.scitotenv.2020.139452>
 These references have been included in the new version of the manuscript and an explanation of the topic you mentioned has been included too (lines 350-352, references 27 and 28). Reference 27 is also cited in line 392 (TPH). Interesting papers, thank you.
- Figure 7: Please describe about the characterization method for bacterial microphotographs in M&M section.
Section 2.4. Optical microscopy photographs (lines 171-174) has been included in the new version of the manuscript, it was missing before. Thank you for this point.
- Equation 1 to 6: There are no nomenclatures of different variables or constants (e.g. S, S₀, r, k, t). Please define all the terms with their units after writing the equations, not in the Nomenclature section. Also, where do Eqs 1 and 2 arise from? Please explain.
In accordance with your indications, which are coincident with other referee comments, in the new version of the manuscript section 2.5. Crude oil biodegradation kinetics has been included, explaining in more detail the equations and variables. Please read lines 176-196.

- Line 414: It seems that the authors calculated degradation rates using equation 7 and 8 as they obtained degradation rates of 843 mg/Ld and 705 mg/Ld using $S_{oArabLight} = 8.6$ g/L. However, the Equations have the term ' S ', not the ' S_0 '. I am confused why authors used different equations? Is the terms ' S ' and ' S_0 ' have same meaning or ' S ' is the concentration at time t ? Authors must respond it.

Lines 176-196 describe equations and variables S (substrate concentration, petroleum) and S_0 (initial substrate concentration). In the new version of the manuscript in results, lines 446-451 explain how this degradation rates are obtained. S_0 is TPH initial value, a particular case of S , the higher value of TPH and the higher degradation rate (equation 1).

Degradation rates are negative, because TPH are decreasing, but normally, degradation rates are expressed in absolute values because they indicate the amount of substrate degraded in the unit of volume and time.

Thanks for your efforts on correcting the manuscript, very interesting comments to improve the article.

Reviewer Confidential Comments to Editor:

Optional - please do not duplicate your comments to the author here
