April 9th, 2016

Dear Dr. Olgica Nedić,

Thank you for your e-mail and review of our manuscript. We have now addressed and clarified each issue raised by the reviewer to the best of our ability.

The reviewer comments are in **BOLD** font and the author’s responses are in NORMAL font. Changes have been highlighted in yellow color in revised manuscript.

We believe that the manuscript is considerably improved due to reviewers’ suggestions, and we do hope that you will find everything in order.

Kind regards,

Lidija Djokic

**REVIEWER B**

**The authors report a whole cell system that incorporates 4-OT mutants (Arg-1, Lys-1, and Lys-1, Lys-2) to carry out a Michael reaction with aldehydes and nitroolefins to produce nitroaldehydes. The report has 3 major flaws (listed below), which preclude publication.**

**REVIEWER B QUERY 1:**

**The authors never actually show or provide any evidence that the 4-OT mutants (in the whole cells) proceed through the mechanism shown in Scheme 1A (where the N-terminal amino group forms an enamine). They indicate that it is reasonable to assume it does, but it is not. In *E. coli*, there is a correlation between the removal of the initiating methionine and the identity of the second amino acid. Hence, if Gly, Ala, or Pro is in the second position, Met-1 (the initiating methionine) is removed. If Arg or Lys is in the second position, Met-1 is not removed. (See Hirel, P.H., Schmitter, M.J., Dessen, P., Fayat, G., and Blanquet, S. (1989) Extent of N-terminal methionine excision from Escherichia coli proteins is governed by the side-chain length of the penultimate amino acid, Proc. Natl. Acad. Sci. USA 86, 8247-8251.). This means that the authors are not examining Lys or Arg in the N-terminal position, but they are examining methionine.**

**AUTHOR REPONSE TO QUERY 1:**

Firstly, I would like to acknowledge and thank the reviewer for time and effort that it was put into revision of this study. It really brings back the faith in peer review process. Secondly I would like to address the issue of methionine excision which is certainly an interesting one and opens up a point for discussion. After carefully going through suggested reference and numerous other references including:

1: Giglione C, Boularot A, Meinnel T.Protein N-terminal methionine excision. Cell Mol Life Sci. **2004** Jun;61(12):1455-74.PubMed PMID: 15197470

2: Frottin F, Martinez A, Peynot P, Mitra S, Holz RC, Giglione C, Meinnel T. The proteomics of N-terminal methionine cleavage. Mol Cell Proteomics. 2006 Dec;5(12):2336-49. Epub **2006** Sep 8. PubMed PMID: 16963780.

3: Xiao Q, Zhang F, Nacev BA, Liu JO, Pei D.Protein N-terminal processing: substrate specificity of Escherichia coli and human methionine aminopeptidases. Biochemistry. **2010** Jul 6;49(26):5588-99. doi: 10.1021/bi1005464. PubMed PMID: 20521764

4: Lowther WT, Matthews BW. Structure and function of the methionine aminopeptidases. Biochim Biophys Acta. **2000** Mar 7;1477(1-2):157-67.PubMed PMID: 10708856

5: Piatkov KI, Vu TT, Hwang CS, Varshavsky A.Formyl-methionine as a degradation signal at the N-termini of bacterial proteins. Microb Cell. **2015**;2(10):376-393. PubMed PMID: 26866044

6: Liao YD, Jeng JC, Wang CF, Wang SC, Chang ST. Removal of N-terminal methionine from recombinant proteins by engineered E. coli methionine aminopeptidase. Protein Sci. **2004** Jul;13(7):1802-10.PubMed PMID: 15215523

we could only conclude that the question of the methionine aminopeptidase (MAP) activity and specificity is still very open and that without experimental data, it would be quite difficult to state the extent of methionine presence in our biocatalyst. It has been agreed that the extent of methionine excision is likely to reflect the catalytic efficiency of MAP, as specified by the penultimate N-terminal residue of its substrate. The results obtained by Frottin show that the extent of excision decreases when the size of the introduced second amino acid increases. In addition, several physical parameters may characterize the size of an amino acid: accessible surface area, side-chain volume, gyration radius, etc. However, even after 25 years of extensive research, we are still only talking about the % of cleavage probability. Studies have shown cleavage probablility to be highest for Gly (97%) followed by Ala, Thr (90%), Pro, Ser, and Val (84%). Cleavage was less probable for the substrates Cys (71%), Ile (18%), Asp, Leu, and Asn (16%). On the other side, authors have been reporting “variable cleavage”, especially for *E. coli* MAPs.

Clarifying the extent of methionine presence in each of the biocatalysts within the current study would outweigh the purpose of the study, having in mind that this is ‘follow-up’ study that relies largely on our previous work whereby we have included numerous controls that suggest that the biocatalyst responsible for Michael-type additions is actually recombinantly expressed 4-OT (please see the answer to Reviever B, Query 3). Therefore, a discussion point has been added in the Results and discussion part of the revised manuscript to cover the possibility of the methionine presence (pg 8, section Protein modeling, paragraph 2, highlighted in yellow) and three references have been added.

**REVIEWER B QUERY 2:**

**The in-silico analysis does not take the initiating methionine into account.**

**AUTHOR REPONSE TO QUERY 2:**

This is correct and we do state that in-silico analysis by definition allows to pick and choose paramethers, so the intention was to examine the effect of the Pro1 substitutions with Arg1 and Lys1. Therefore, this section of the manuscript has not been changed.

**REVIEWER B QUERY 3:**

**For the reasons above, it is not clear to me what (in the whole cell system) is generating product, but it’s not the N-terminal amino group. For lysine, it might be the side chain, but this should be demonstrated. For arginine, the side chain is not sufficiently nucleophilic to carry out a reaction.**

**Just a general comment: the side chain of lysine can be charged or neutral in the active site of an enzyme. It can function as a general base (neutral) or general acid (charged). It can also engage in hydrogen bonding and electrostatic interactions. Arginine is rarely neutral in an active site. It is almost always charged and can participate in hydrogen bonding and electrostatic interactions.**

**AUTHOR REPONSE TO QUERY 3:**

The current study is actually follow up of our previous work with the similar biocatalyst, and the number of controls have been used throughout the experiments, which clearly demonstrate that the host strain *Escherichia coli* BL21(DE3) with and without the vector pREST-B is not capable of catalyzing the Michael-type additions of nitroolefins and aldehydes (Narancic T, Radivojevic J, Jovanovic P, Francuski D, Bigovic M, Maslak V, Savic V, Vasiljevic B, O'Connor KE, Nikodinovic-Runic J. Highly efficient Michael-type addition of acetaldehyde to β-nitrostyrenes by whole resting cells of *Escherichia coli* expressing 4-oxalocrotonate tautomerase. Bioresour Technol. 2013, 142:462-8. and J. Radivojevic, G. Minovska, L. Senerovic, K. O'Connor, P. Jovanovic, V. Savic, Z. Tokic-Vujosevic, J. Nikodinovic-Runic, V. Maslak, *RSC Adv*. **4** (2014) 60502).

Furthermore, while carrying out these ‘control’ experiments, we have actually concluded that this host can carry out the reduction of conjugated nitroalkenes: Jovanovic P, Jeremic S, Djokic L, Savic V, Radivojevic J, Maslak V, Ivkovic B, Vasiljevic B, Nikodinovic-Runic J. Chemoselective biocatalytic reduction of conjugated nitroalkenes: new application for an *Escherichia coli* BL21(DE3) expression strain. Enzyme Microb Technol. 2014 Jun 10;60:16-23.

**REVIEWER B QUERY 4:**

**Table 2 reports percent yield, but should report the amount of product (in milligrams).**

**AUTHOR REPONSE TO QUERY 4:**

The suggested change has been introduced in the revised Table 2.

**REVIEWER B QUERY 5:**

**The English is poor. An annotated copy will be attached.** **There are too many English mistakes to list.**

**AUTHOR REPONSE TO QUERY 5:**

We apologize for the numerous grammar and spelling mistakes. We have revised the manuscript to the best of our ability and the appropriate changes/suggestions have been introduced throughout the manuscript. Changes are too numerous to list, so they have been highlighted in yellow.

**REVIEWER F**

**In this article authors studied efect of substitutions of terminal proline of 4-OT and activity towrds different substrates and compared it with activity of organocatalyst such as lithium salt of lysine. Three different mutants were constructed and tested. Mutants showed increased activity towards some of the substrates comaperd to the wild type of the enzyme and organocatalyst. Improoved activities were explanied by modelling studies. Since development of biocatalysts for organic synthesys and green chemistry is of high importance and generated mutants are step forward for Michael addition of branched aldehydes to β-nitrostyrenes I recommend publications of this aricle after minor revisions.**

**REVIEWER F QUERY 1:**

**1. Authors should include in introduction section some comments on previous work if any in literature on muatational studies of 4-OT**

**AUTHOR REPONSE TO QUERY 1:**

Previously we performed mutational studies on 4-OT where N-terminal prolyne enriched mutants were generated, one with additional N-terminal proline (4-OT\_P), as well as a variant with two substituted amino acids to proline, namely Ala3Pro and Gln4Pro (4-OT\_2P) (Narancic T, Radivojevic J, Jovanovic P, Francuski D, Bigovic M, Maslak V, Savic V, Vasiljevic B, O'Connor KE, Nikodinovic-Runic J. Highly efficient Michael-type addition of acetaldehyde to β-nitrostyrenes by whole resting cells of *Escherichia coli* expressing 4-oxalocrotonate tautomerase. Bioresour Technol. 2013, 142:462-8.). We included coments on this work in the introduction section (pp. 4, Introduction section, paragraph 3, highlighted in yellow).

**REVIEWER B QUERY 2:**

**2. If there are previous mutational studies of 4-OT they should compare their results with previous ones in results and discussion section.**

**AUTHOR REPONSE TO QUERY 2:**

Results from this work are compared with previous mutational studies on 4-OT(please see response to REVIEWER F QUERY 1) and a discussion point has been added in the Results and discussion part of the revised manuscript (pp. 10, Result and discussion, section Biotransformation of **1**, **2** and **3** with 4-OT wild type biocatalyst and 4-OT lysine mutants, paragraph 3, highlighted in yellow*).*