

Dear Editor and Reviews,

We would like to thank the *International Journal of Molecular Sciences* for giving us the opportunity to revise our manuscript. We are truly grateful for the critical comments and thoughtful suggestions of the reviewers on our manuscript, Efficient *de novo* Biosynthesis of Heme by Membrane Engineering in *Escherichia coli* (No. ijms-2021322). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our research. Based on the comments and suggestions, we have made careful modifications to the original manuscript. These changes will not influence the content and framework of the paper. All changes made to the text are marked by “Trace Changes” in the manuscript. We hope the new manuscript will meet your standards. You will find our point-by-point responses to the reviewers’ comments/questions written in the “Response To Reviews”.

With best regards,

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The following is a point-to-point response to the reviewer’s comments.

(Manuscript Number: ijms-2021322)

Reviewer 1:

The present work describes successful overexpression of heme by manipulating genes arrangements involved in the heme biosynthesis. The experiments are well designed and the results are solid and reliable. The manuscript is well written and readable. I think that this interesting work would certainly advance our understanding of the overexpression of heme in *E. coli*. I recommend this paper for publication in the Journal. However, I raise some concerns that need to be addressed before publication. If those concerns are adequately address in the revised manuscript, this interesting report would be significantly strengthened.

Response: Thanks very much for the positive comments. We appreciate your time and effort in reviewing our manuscript, and we have made revision in the revised manuscript according to your comments.

1. I think references are biased rather to food sciences. I would suggest the authors to add brief comments covering research fields of heme and heme-bound proteins in the first paragraph of Introduction by citing the following papers for general readers to understand the important role of heme. This is essential for publication of the present interesting paper. *Chem. Rev.* **2015**, 115, 6491. Heme-based gas (O₂, NO, CO) sensors. *Crit. Rev. Oncol. Hemato.* **2018**, 126, 121. Heme toxicity. *Chem. Soc. Rev.* **2019**, 48, 5624. Heme-responsive sensors. *Free Rad. Biol. Med.* **2019**, 133,

88. Heme movement. *JACS Au* **2021**, 9, 1296. Various heme proteins. *BBA Mol. Cell Res.* **2021**, 1868, 118881. Heme trafficking. *Coord. Chem. Rev.* **2022**, 452, 214286. Bacterial heme biosynthesis. *Coord. Chem. Rev.* **2022**, 472, 214793. Heme acquisition. Please let me remind that the present journal's name is "...Molecular Sciences". Short comments about heme from "the Molecular/Chemical Science's points of view" citing those papers would be beneficial/useful to significantly improve this novel paper.

Response: Thank you. The suggestion is valuable and very helpful for revising and improving our paper, we have included these papers in our references.

2. Overexpression in *E. coli* of many heme-proteins generates already-heme-bound proteins, but does not need to overexpress the heme iron complex itself separately. How do the authors rationalize the present study? Also please explain the superiority of the present method compared to extraction of heme from bloods and meats.

Response: Thanks very much for the comment. Artificial meat is one of the hottest topic in the field of food science recently, however its acceptance was affected by the color. So, we hope to improve its color by applying heme in it. Biosynthesized heme can satisfy the need of color improvement as well as iron supplement. Therefore, we overexpressed the heme iron complex in recombinant *E. coli*, laying the foundation for massive preparation of heme.

Heme can be extracted from animal blood by organic solvent or enzymatic hydrolysis,

that is against to the animal welfare. We consider animal-free and biotechnological production of free-heme molecules is essential for high-yield, economical, and eco-friendly production.

The revised sentences are shown as follow (Lines 38-45):

“Artificial meat is one of the hottest topic in the field of food science recently, however its acceptance was affected by the color. So, we hope to improve its color by applying heme in it. Heme can be extracted from animal blood by organic solvent or enzymatic hydrolysis, that is against to the animal welfare. Biosynthesized heme can satisfy the need of color improvement as well as iron supplement. Therefore, animal-free and biotechnological production of free-heme molecules is essential for high-yield, economical, and eco-friendly production.”

References:

Palmer, C., Engineer Dinner—Save the Environment? *Engineering* 2020, 6, 213-215.

<https://doi.org/10.1016/j.eng.2020.01.002>

Ko, Y. J.; Kim, M.; You, S. K.; Shin, S. K.; Chang, J.; Choi, H. J.; Jeong, W. Y.; Lee, M. E.; Hwang, D. H.; Han, S. O., Animal-free heme production for artificial meat in *Corynebacterium glutamicum* via systems metabolic and membrane engineering. *Metab. Eng.* 2021, 66, 217-228. <https://doi.org/10.1016/j.ymben.2021.04.013>

3. Heme is not soluble in water. How did the authors address this problem? Is heme incubated/overexpressed into the cell membrane? Title says “... by Membrane Engineering...”, but I don’t see how membrane engineering works in the

manuscript. Please emphasize this somewhere or in Conclusion.

Response:

(1) Given the hydrophobic nature of heme, we used acid acetone assay to extract and determine the heme content of the sample. In this method, acetone: HCl (95:5) buffer and NaOH was used to extract heme from the corrupted cell pellets. The operation was provided in 3.6. *Analytical methods*.

(2) We knocked out the genes *CysG*, *CyoE* and *hemX* to down-regulate ALA outward transport, and enhanced the dipeptide transport system substrate-binding protein and heme exporter protein A of heme outward transport regulated by the genes *DppA* and *CcmA*. Among which, dipeptide transport system substrate-binding protein and heme exporter protein A are membrane proteins. In order to describe more accurately, we added the description “dipeptide transport system substrate-binding protein and heme exporter protein A are cell membrane proteins” in the manuscript as follows (Lines 337-342):

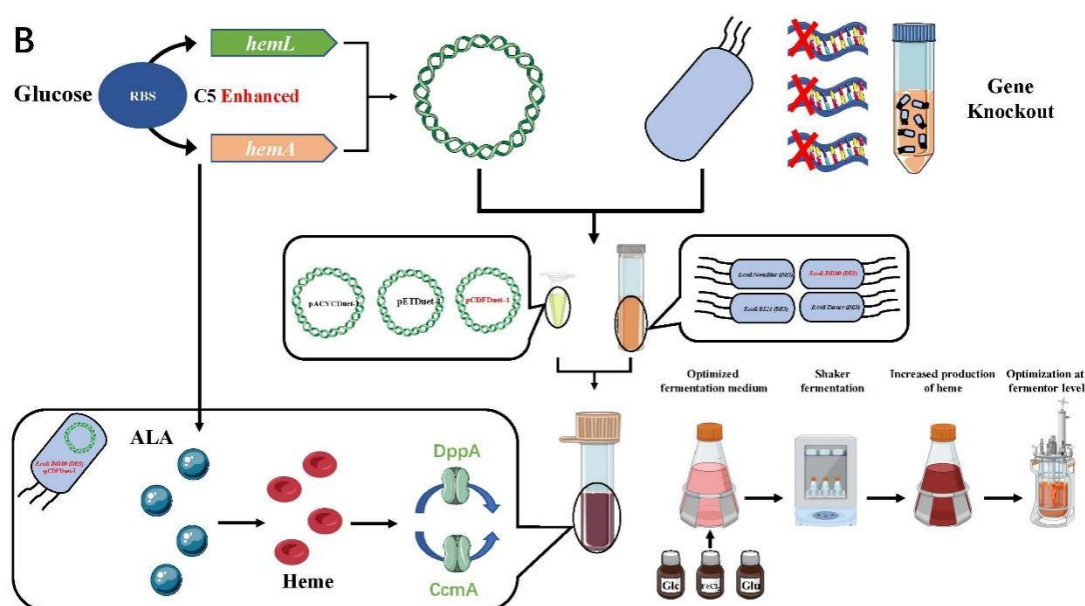
“Finally, membrane engineering was attempted by enhanced putative heme exporters. Enhanced the dipeptide transport system substrate-binding protein and heme exporter protein A of heme outward transport regulated by the genes *DppA* and *CcmA*. It was demonstrated that overexpression of the genes *CcmA* and *DppA* encoding heme export proteins also enhanced heme production.”

References: Espinas, N.A., Kobayashi, K., Takahashi, S., Mochizuki, N., Masuda, T., 2012. Evaluation of unbound free heme in plant cells by differential acetone extraction.

Plant Cell Physiol. 53, 1344–1354. <https://doi.org/10.1093/pcp/pcs067>

4. I would suggest the authors to make a new figure describing genome organization of genes involved in heme biosynthesis. Please describe cartoon depicting general features composing heme-related genes such as *hemA*, *hemB*, *hemC*, *hemD*, *hemE*, *hemF*, *hemG*, *hemH*, *hemL*, *hemX*, *CyoE*, *CysG*, *CcmA*, *DppA* (discussed in the text) in order to rationally and clearly explain relationships among those genes for general readers to grasp the points. Also please visually explain your experiments and results in this figure in order to clearly demonstrate the present important findings. Figs. 1 and 2B are not enough.

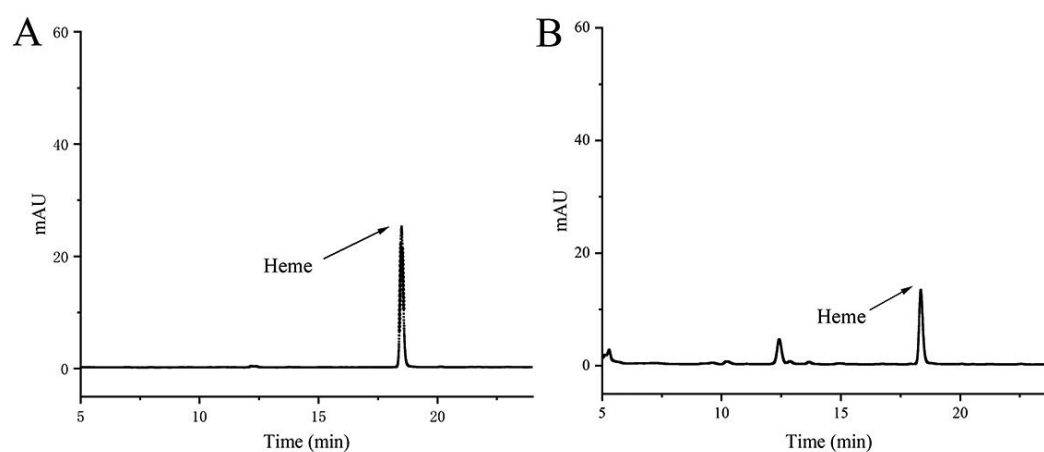
Response: Thank you for the comment, we have added Figure 1B in the manuscript.



5. Papers of heme and heme-proteins always provide absorption spectra. I don't see any optical absorption spectra (340-700 nm) of cells overexpressing Fe³⁺ heme complex under various conditions and those of purified (semi-?) Fe³⁺ heme

complex in the present paper. Please incorporate spectra into the Supplementary Information.

Response: Thanks very much for your comments about our manuscript work. Heme was detected using high performance liquid chromatography (HPLC) with VWD detector at 405 nm. The HPLC chromatogram of the heme standard and the heme extracted from the sample were shown as follows. The separation effect was satisfactory. We've put the HPLC chromatograms into the supplementary information (Fig. S2).



HPLC chromatograms of heme. (A) the chromatograms obtained from standard heme (20.0 mg/L); (B) the chromatograms obtained from sample.

Reference:

Ko, Y. J.; Kim, M.; You, S. K.; Shin, S. K.; Chang, J.; Choi, H. J.; Jeong, W. Y.; Lee, M. E.; Hwang, D. H.; Han, S. O., Animal-free heme production for artificial meat in *Corynebacterium glutamicum* via systems metabolic and membrane engineering. *Metab. Eng.* **2021**, *66*, 217-228. <https://doi.org/10.1016/j.ymben.2021.04.013>

6. I would suggest the authors to consider to use Supplementary Information in order

to simplify the main text and to emphasize important points in the main text about the present interesting study. For example, Table 1 might be moved to Supplementary Information. Also, some of experimental results shown in Table 2 and Fig. 2 might be moved to Supplementary Information for the same reasons.

Response: Thank you for the comment. We have moved Table 1 to supplementary information Table S1. Also, some of the data in Table 2 and the single gene expressed recombinant strain in Figure 2 have been moved to supplementary information Table S3 and Figure S1.