

# ***Interactive comment on “Calcite and vaterite biosynthesis by nitrate dissimilating bacteria in carbonatogenesis process under aerobic and anaerobic conditions” by Marwa Eltarahony et al.***

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Response to peer-reviewers Firstly, we appreciate the reviewers' feedbacks and their careful reading of our manuscript, interest in our study and thoughtful comments that greatly improve the quality of the paper. Secondly, we did our best to respond to the points raised. The Referees have brought up some constructive suggestions and we appreciate the opportunity to clarify our research objectives and results. As indicated below, we have checked all the general and specific comments pointed out and have made the necessary changes accordingly to their indications. – Reviewer #3:

Specific comments:

C1)-The manuscript could benefit from a language check. Some sentences are almost impossible to understand. The partly confusing text structure can significantly be improved.

A1: We followed your respective suggestion and we revised the manuscript thoroughly and additionally, English native speaker checked English quality of revised manuscript. We rewrote the manuscript and totally reorganize its structure, as well, in particular the results and discussion section.

C2)-There are many literature references distributed in the results and discussion chapter that have little relation to the text before and after (for instance, Lines 174-179, 203-207, 298-300, 313-316). I don't see how these sentences support the discussion or how they can lead to the conclusions.

A2: All references were revised regarding to their suitability and fitness to the results or their interpretations.

â€” For lines 174-179: We referred to different bacterial groups (phylum Firmicutes, family Bacillaceae; phylum Proteobacteria, family Enterobacteriaceae and phylum Actinobacteria, family Actinomycetaceae) that related to the exact classification of our isolates and had the same scope (CaCO<sub>3</sub> production with various applications). So, we thought the importance for mentioning other studies that are similar to our results and support our vision. However, according to your notice and Reviewer's #1 recommendation, we stated such information in convenient way in the revised manuscript (line 227-230, page 10), particularly that there were different publications stated this point and dealt with their data with the same sort of discussion.

â€” For lines 203-207: We would manifest that we obtained CaCO<sub>3</sub> crystal with different colors and morphology by different bacterial species, so such variations were observed due inter-species differences. The exact observation was recorded by Montano-Salazar et al., (2017), who found that, *Rhodococcus qingshengii* M101 produce spherical brown crystals; *Arthrobacter crystallopoietes* produced irregular yellowish crystals

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and *Psychrobacillus psychrodurans* showed irregular white/beige crystals of  $\text{CaCO}_3$ . Such finding supports our results which were listed at page 12 (lines 258-262).

â€” For lines 298-300: Caicedo-Pineda et al., (2018) mentioned that the highly pure  $\text{CaCO}_3$  give EDX spectrum with Ca peak higher than C peak. That comes in agreement with our result. As observed in Figure 5, the peaks intensities of Ca are more than peak intensities of C in all examined samples, which finally implied pure  $\text{CaCO}_3$ .

â€” For lines 313-316: From our point of view, this reference stated that the size of the precipitated calcite by *Alternaria* sp. was ranged from less than 1- and not exceeded 10  $\mu\text{m}$ , which was agreed with the size of calcite crystals formed by strain *Raoultella planctica* (VIP), and under the same nitrate utilization conditions. Despite that, we followed your notice and the opinion of Reviewer #1 and deleted it in the revised manuscript.

C3)- Further, I couldn't read the figure because of the poor quality.

A3: We apologize for such unintended mistake. Respect your opinion. All figures were adjusted and subjected to improve the image quality using photo editing software. All figures become clear enough to read and perceive.

C4)-Figure 3: I would suggest to add two diagrams showing the parameters of the anoxic and oxic control media

A4: Respect your opinion. However, adding such diagrams with all parameters which will remain constant along 10 days incubation would not reveal any additional observation. Therefore, presence of such diagrams with fixed parameters will not add information for comparing or interpretation of data. Rather, it would minimize the figures sizes of the other experiments which contained important data about the process.

C5)-present all diagrams on the same size, and use the same colors for the same parameters.

A5: All diagrams at figure 3 were in the same size. All the same parameters had the same and unique colors/mark. For example:  $\text{NO}_2^-$  is blue line with rhombus mark,

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NO<sub>3</sub>- is red line with square mark, pH green line with triangular mark, etc. The lateral caption illustrated such details.

C6)-Line 95: If those bacteria support CaCO<sub>3</sub> precipitation, why was it possible to isolate them from a non-calcareous habitat?

A6: We focused our study on nitrate reduction mechanism and its main role in CaCO<sub>3</sub> precipitation under oxic/anoxic conditions. So, during screening and isolation process, we concentrate on finding organisms with such mechanism to ensure their validity in the CaCO<sub>3</sub> precipitation. On the other hand, the inverted pathway, screening from calcareous area ensures isolation of CaCO<sub>3</sub> precipitating organisms, but didn't ensure presence of nitrate reduction mechanism; they might exhibit CaCO<sub>3</sub> precipitation through urea degradation, deamination of proteins, carbonyle anhydrase or sulfate reduction (listed at introduction section).

C7)-Chapter 2.3: this chapter describes the experimental incubation conditions; I think this should be reflected in the title.

A7: Thanks for your suggestion. We followed it and added this note in the title (Page 7 & Page 11, Aerobic/ anaerobic CaCO<sub>3</sub> precipitation).

C8)-Line 134: maybe a short description about the anoxic incubation set-up?

A8: Thanks for your suggestion. We believed that it is well known and reported tremendously in other literatures. Despite, we mentioned what you suggested (Line 165-168; Page 8).

C9)-Chapter 2.5 this chapter should be merged with chapter 2.3.

A9: according to your recommendation and also Reviewer's #1 note, we merged both sections and clarified the studied items (Line 157, page 7).

C10)-Line 174-179: what does this information have to do with identification and isolation of the studied bacteria? These sentences seem lost there.

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A10: A17: We referred to different bacterial groups (phylum Firmicutes, family Bacillaceae; phylum Proteobacteria, family Enterobacteriaceae and phylum Actinobacteria, family Actinomycetaceae) that related to the exact classification of our isolates and had the same scope (CaCO<sub>3</sub> production with various applications). So, we thought the importance for mentioning other studies that are similar to our results. However, according to your notice, we stated such information in brief and in a convenient way in the revised manuscript (line 227-230, page 10), particularly there were different publications stated this point and dealt with their data with the same sort of discussion.

C11- Line 191: this title is too general. The whole paper is about CaCO<sub>3</sub> deposition. This chapter rather describes the relationship between NR, oxic conditions and biogenically induced carbonate precipitation. A11: We are grateful for your note. In the revised manuscript, this section was divided into two parts with two different titles (3.3- Aerobic / anaerobic CaCO<sub>3</sub> precipitation) which confirmed deposition of CaCO<sub>3</sub> crystals and described appearance differences between precipitated crystals under aerobic/anaerobic conditions. The other title is (3.4- Study of the parameters associated with CaCO<sub>3</sub> precipitation), which described the parametric changes during CaCO<sub>3</sub> deposition process under aerobic/anaerobic nitrate reduction conditions. C12-Line 211-213: this is an important general observation and explanation that is followed by description of the results. This text structure should be changed. A12: We are grateful for your recommendation. The changes were performed (Line 329-334, page 15, revised manuscript). C13-Line 226-228: I don't understand this sentence. Also, a new paragraph starts here. A13: We followed your recommendation and start new paragraph at such point. As observed in Figure 3, under aerobic conditions, periplasmic nitrate reductase was detected along 10 days of incubation even after complete depletion of NO<sub>3</sub><sup>-</sup> (nitrate reductase substrate). While, anaerobically, membrane-bound nitrate reductase was detected only in the presence of NO<sub>3</sub><sup>-</sup> (for 180 and 70 h for *L. sphaericus* and *R. planticola*, respectively). That means, both strains have two different types of nitrate reductases which were expressed under different aeration conditions and both have different physiological roles as indicated in the manuscript. The structure of sen-

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tence was improved to be more understandable and clearer (Line 284-296, page 13).

C14-The description and the discussion of the results is mixed up throughout this chapter. I would suggest to change that. A14: We are grateful for your recommendation. Reorganization and rearrangement of this section were performed. C15-Line 240-241: How is this information about a completely different microbe related to the former sentence? How does this information help you to get to your conclusions? I think this sentence, as it stands there, is not necessary.

A15: Firstly, we would like to declare that there was no previous report about our examined organisms in CaCO<sub>3</sub> deposition using nitrate reduction strategy, till our knowledge, so there was no more data available on the same organisms could be compared with our results. Secondly, we compare the overall process with its output (CaCO<sub>3</sub> formation) and such comparison includes time frame of formation, type of organism, polymorph, size of crystals and amount of precipitated CaCO<sub>3</sub>. We referred to such data at their proper position in manuscript. Where, at SEM we referred to the shape and size; at XRD we referred to the polymorphs. Whereas, at parametric changes study, we referred to time frame and amount of deposited crystals. Thus, our results are characteristic and strains produced considerable amount of CaCO<sub>3</sub> upon comparison with cited strains.

C16-Line 243-245: How much sense does it make to compare the CaCO<sub>3</sub> precipitation amounts (in different units!) from three completely different experiments? What can we learn from that? It is not productive to only list results from different experiments if no conclusion can be drawn from that.

A16: According to your valuable note, we made the required changes to confirm uniformity of unites according to standards (expressed the data in gm/ 100 mL). Herein, we thought that it is logic to compare the whole process (CaCO<sub>3</sub> precipitation) and its output with other works. It is true that are completely different strategies (ureolysis & nitrate reduction), but the same target was obtained. So, we displayed the performance

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of our strains under such conditions, compared the metabolic activity with expressed enzyme, and eventually compared the product either amount or polymorph, which all such data fall in the circle of CaCO<sub>3</sub> precipitation. Our main conclusion is the possibility of production of different polymorph of CaCO<sub>3</sub> with considerable amount and also within suitable frame of time, which will provide good impact at technological, environmental and medical levels. Such conclusion comes from comparison with other studies output.

C17-Chapter 3.4: I would recommend to further subdivide this chapter because this helps to build a proper text structure. A17: We are grateful for your recommendation. We followed it and subdivided into 3 sections (XRD (page 16), EDX (Page 19) and SEM (Page 20). C18- Line 298-300: How does this notion of another study stand in relationship to the sentences before and after? How does it impact your discussion? I don't think this information is helpful here. A18: We pointed out to other studies which either agreed or contrast our finding. This sentence supports our finding, in which the same shape of crystals with presence of obvious extracellular polymeric substances (EPS) was deposited. The presence of EPS is a characteristic feature. As mentioned above, the whole process with its characteristic output was compared regardless the producing organism as long as in the exact target of study. So, we thought that sentence present in a coherent way with the previous one. C19-Line 313-316: Another notion of a further study. How much sense does this short notion about a study with a fungus species make? How does this information help your argumentation? A19: From our point of view, this reference stated that the size of the precipitated calcite by *Alternaria* sp. was ranged from less than 1- and not exceeded 10  $\mu\text{m}$ , which was agreed with the size of calcite crystals formed by strain *Raoultella planticola* (VIP), and under the same nitrate utilization conditions. Overall, we quoted this study as fall in the same scope (CaCO<sub>3</sub> deposition) regardless the producing organism. Finally, we take your point of view in our consideration and removed it. C20-Line 320: what exactly was the finding of Li et al. (2012)? A20: Such reference highlighted to the presence of bacterial imprint on the surface of CaCO<sub>3</sub> crystals in the formed of holes, which is agreed with our results.

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Li and coworkers attributed such holes to the bacterial cells which act as nucleation site. The sentence was rewritten to be more clear (Line 479, page 21). C21- Line 321-323, 326-328: see comments above (Line 298-300, 313-316) A21: All sentences were revised and our reply indicated above. Line 321-323 (the answer of Reviewer's #1, C33, page 11). Line 326-328 (the answer of Reviewer's #1, C34, page 12). C22-Line 331: why the reference? Isn't this the conclusion of your study? A22: It is the conclusion of the study. The reference was deleted. The whole structure of this section (Results & discussion) was reformatted. C23-Line 375-376: see comments above (Line 298-300, 313-316) A23: In this sentence, we displayed reference that mentioned different ranges of pH at which calcite and vaterite were formed. Subsequently, this sentence boosts our finding. C24-Line 377- 379: see comments above (Line 298-300, 313-316) A24: In this sentence, we displayed the opposite finding to our study. We intended to display the diversity of conditions to obtain different polymorphs. Nonetheless, we deleted such sentence. C25-Line 490: maybe sum up likely reasons for the formation of the different CaCO<sub>3</sub> types. A25: Agreed with you. C26-Technical corrections Line 50: "microbial", not "Microbial". A26: Thanks for your note. The correction was performed (line 63, page 3). C27-Line 54: confusing reference A27: Thanks for your note. The sentence with reference was revised and the correction was performed (line 68, page 4). C28-Line 67: do you mean microorganisms with different metabolisms? A28: We meant different microbial group could synthesize different CaCO<sub>3</sub> polymorph (calcite, vaterite, aragonite, etc.). The sentence became clear after amendment and revision (Line 84, page 4) C29-Line 70: What exactly seems to be more abundant? A29: The heterotrophic one is more abundant. To prevent confusion and misunderstanding, we rewrote the sentence (line 87, page 4). C30-Line 98: CaCO<sub>3</sub> crystal A30: Thanks for your note. The correction was performed (line 123, page 6). C31-Line 106: where all three bacteria found in all three sampling sites? A31: Thanks for your note. We referred to such point in section 3.1 (line 214-216, page 10). C32-Line 118: 1  $\mu$ mol A32: The correction was performed (line 145, page 7). C33-Line 121: using 16S. A33: The correction was performed (line 149, page 7). C34-Line 122: what is PCR?

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A34: Polymerase chain reaction. It is well-known and the most widely used molecular technique. It is applied in identification and differentiation purposes. C35-Line 123: “in” instead of “elsewhere” A35: The correction was performed (line 152, page 7). C36-Line 124: what does BLAST mean? A36: In bioinformatics, BLAST (basic local alignment search tool) is an algorithm and program for comparing primary biological sequence information, such as the amino-acid sequences of proteins or the nucleotides of DNA and/or RNA sequences. By such tool, query (protein or nucleotide sequence) was compared with library or database of sequences, and identifies library sequences that resemble the query sequence above a certain threshold. C37-Line 131: What is a M9 media? A37: The media for precipitation. According to your opinion and Reviewer’s #1 opinion, the composition of media was listed (Line 162-164; Page 7-8). C38-Line 136: 10,000 g A38: The correction was performed (line 190, page 9). C39-Line 143: delete one parenthesis A39: The correction was performed (line 203, page 9). C40-Line 144: operates A40: The correction was performed (line 204, page 9). C41-Line 163: What is APHA? A41: American Public Health Association (APHA, 1999). It provides Standard Methods for the Examination of Water and Wastewater. C42-Line 175: What is MICCP? A42: Microbial induced calcium carbonate precipitation (paragraph 1, page 3) C43-Line 189: please rewrite. A43: The correction was performed (line 240-241, page 11). C44-Line 192: please add the reference after “preceding literature” A44: The reference was added (line 245-246, page 11). C45-Line 195: red semicolon A45: The correction was performed (line 247, page 11). C46-Line 197: different appearances: the strains or the crystals? Please be more specific. A46: We meant crystals. The correction was performed (line 249, page 11). C47-Line 198: please add parentheses around (Fig. 2) A43: The add parentheses around (Fig. 2) was added (line 250, page 11). C48-Line 201: delete “were”. A48: The correction was performed (line 255, page 12). C49-Appared in all anaerobic cultures? A49: Yes, appeared in all anaerobic cultures. We declared this point in the revised manuscript (Line 256, page 12). C50-Also, the comparison between irregular crystals in the anaerobic cultures and fine white powder in the supposedly aerobic culture appears strange. The former describes

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the morphology of crystals, the latter can roughly tell something about the crystal size. A50: Both expressions described general appearance (color, texture and size). We reported equal description for both types. Where, large and coarse beige or buff color and irregular crystals (anaerobically) were compared to fine white powder (aerobically). The description involved size (large coarse and fine), color (beige/buff and white) and texture (powder and crystal). Generally, any salt would be described morphologically either powder or crystal in chemistry point of view. The exact morphology and exact size were determined through SEM, which confirmed such optical observation. C51-Line 203: “. . .brown aggregated pellets.” Do you speak about the aerobic culture? A51: Yes, such point (aerobic culture of EM4) was manifested previously in section 3.2 (Line 239-241, page 11). C52-Line 209: were instead of “. . .was monitored.” A52: The correction was performed (line 265, page 12). C53-Line 249: “It is worth to mention. . .” Why is it worth to mention? A53: We thought that it is important to mention such information, where, EC used mainly in determination of the ureolytic efficiency in MICP and didn't use before in determination of nitrate reduction performance. So, we stated that it is general method could be used to give an insinuation about metabolic activity and overall mineralization process and it is worked and gave the required information. C54-Line 253: “by almost all of the parameters”? A54: Thanks for your note, we amended the sentence. Where, most of examined parameters and not all exhibited such observation. Because, there are some parameters were consumed completely before the end of experiment (10 days) such as NO<sub>3</sub><sup>-</sup> (in aerobic and anaerobic conditions), NR & NO<sub>2</sub><sup>-</sup> (anaerobic conditions). So, the rest of parameters exhibited such slight decline or stability state (line 354, page 16). C55-Line 255-258: this is difficult to understand A55: The sentence was revised to be easier for understanding (line 355-360, page 16). C56-Line 277: are A56: The correction was performed (line 423, page 19). C57-Line 281: “. . .EDX peaks that. . .” A57: The correction was performed (line 430, page 19). C58-Line 286-288: reference is missing A58: The reference was added (line 437, page 19). C59-Line 292: was instead of were A59: The correction was performed (line 448, page 20). C60-Line 352-354: please rewrite. A60: The structure of

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this point was completely reformatted. Some sentences were deleted. C61-Line 358-359: reference is missing. A61: This sentence is the result of our study, so no reference was added. Additionally, the structure of this point was completely reformatted. C62-Line 359-360: either delete or elaborate further. A62: This sentence is quote that supports our results. The quoted reference didn't mention the exact size of crystals at both cases regarding strain of *L. sphaericus*, to elaborate further. For clarification, the shaking conditions provides more aeration and homogeneity than static one (it is known information), so we quoted it as a reference for comparison between different levels of aeration (Line 483-487, page 21). C63-Line 363: ". . .is controlled by several factors." A63: The sentence was amended according to your recommendation (lines 380, page 17). C64-Line 367: this sentence is incomplete. A64: The sentence was revised (line 385-387, page 17). C65-Line 387: cations are always positively charged. A65: The whole manuscript was revised and rewrote and some sentences were adjusted or deleted. C66-Line 451-453: this sentence is incomplete. A66: The sentence was revised (line 497-499, page 22). C67- Line 453: better: vaterite formation. A67: The sentence was amended according to your recommendation (line 496, page 22). C68-Line 465: better for what? A68: For synthesis process and subsequent application. The natural synthesis methods and naturally produced products are always safe, biocompatible and ecofriendly. Any natural product is better than any synthetic one. Where, natural products were free from any traces of reactant substances. Such trace could cause undesired impact.

C69-Line 466-467: confusing sentence. A69: The sentence meant that the produced CO<sub>2</sub> and subsequently CO<sub>3</sub><sup>-</sup> are produced from the metabolic activity of bacteria and from their respiration, so it is truly biogenic process. Biomimetic process includes employment and addition of biologically origin molecules such as proteins and polymers in a synthetic reaction.

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