Lublin, 6.09.2024 r.

dr hab. Anna Pytlak
Institute of Agrophysics,
Polish Academy of Sciences
Doświadczalna 4
20-290 Lublin
a.pytlak@ipan.lublin.pl

Review of the Doctoral Thesis
of Aleksandra Gęsicka, M.Sc. Eng

Title: Conversion of methane into selected polyhydroxyalkanoates with the use of methanotrophic microorganisms

Supervisors:
Prof. Piotr Oleśkowicz-Popiel
Mateusz Łężyk, PhD

This review has been prepared on the basis of the resolution of the Council of the Discipline of Environmental Engineering, Mining and Energy, Poznań University of Technology, dated 02.07.2024, and the letter of the Chairman of the Council, Mr Prof. Zbigniew Nadolny, PhD, dated 08.07.2024 (WISIE.63.37.2024).

The thesis prepared by Aleksandra Gęsicka, M.Sc., addresses very important and topical issues in the field of environmental engineering. The dissertation is focused on biosynthesis of polyhydroxyalkanoates (PHAs) - bio-based polymers with potential to substitute traditional plastics produced from fossil raw materials. Importantly, PHAs are degradable under composting conditions. Their use would contribute to reducing the accumulation of plastic in the environment as well as restrain the spread of micro- and nanoplastics. Moreover, the studied process relies on methane as the primary carbon substrate for microorganisms. Bearing in mind that methane is a potent greenhouse gas, reducing its emission to the atmosphere through biological sequestration comprises a further positive aspect of the process and strengthens importance of the results presented in the dissertation.

In this regard, the subject of the dissertation is perfectly in line with the scope of the discipline, which is dedicated to the rational management of natural resources, prevention, but also remediation of the effects of human impact on the environment. In addition, the focus of the research on the increase in the proportion of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) (characterized by better physical properties than poly-3-hydroxybutyrate (PHB)) in the overall pool of PHAs indicates that the subject matter is in close relation to the practical needs of the industry.

Based on the content of the dissertation, I conclude that the Author possesses an adequate theoretical knowledge related with the discipline. Confirmation of the above is definitely Chapter 2, presenting an outline of information on methanotrophic bacteria and in-depth information on their biotechnological potential. The high level of knowledge in the field of biotechnology of methanotrophic bacteria is also indicated by the discussion of experimental results presented in chapters 3-5 of the dissertation, in which the Author refers to current knowledge on methane-based processes.

The dissertation also confirms that the Author has the ability to plan and effectively conduct the research process. Apart from the theoretical introduction (Chapters 1 and 2), the dissertation consists of three manuscripts describing the experimental work (Chapters 3-5), with a joint conclusion and a summary in Chapter 6. This structure of the dissertation deviates from the traditional dissertation structure, but is formally understandable given that two of the presented manuscripts have already been published. The PhD student has skilfully assembled the manuscripts into one coherent whole, presenting a continuum of thought directed towards solving the research problem (which is the development of an efficient PHA production technology) and illustrating the progress of research work during her PhD studies. Each of the manuscripts is accompanied by an author contribution statement. According to the declaration on page 13 of the dissertation, the PhD student was the primary author of all of them, responsible for conceptualisation, performing the research, collection, formal analysis and visualisation of the data. Furthermore, the research procedures used were meticulously documented by the PhD student in the methods section of each of the subsections describing the experiments.

Noteworthy is the multitude and diversity of research techniques used. The Author has demonstrated the ability to work with advanced microbial culture systems, to perform

quantitative and qualitative determinations of biopolymers and the ability to apply cutting-edge methods in the field of microbial molecular ecology.

The objectives of the study, formulated by the Author, were aimed at gaining new knowledge on the possibility of biopolymer production by a community of microorganisms growing on methane, with the addition of other carbon substrates as a factor increasing the proportion of PHBV. The originality of the approach was based, inter alia, on the fact that the object of study was newly isolated communities from environmental samples. The essence of environmental enrichment cultures is that each time they offer the chance to reach for a community of new, unknown microorganisms, potentially useful in biotechnological processes. Furthermore, in the dissertation, for the first time, a PHA-producing mixed methanotrophic culture was used to study the PHBV accumulation in a continuous stirred-tank reactor (CSTR) under a sequential feast-famine regime. Concluding, the dissertation confirms an original approach of the Author to the important problem of efficient and stable production of biopolymers from methane.

Comments and questions for discussion

The work submitted for assessment is of a high quality, but there are a some issues that need to be discussed or clarified.

1. On page 28 of the dissertation, the Author indicates that 'Representatives of methanotrophs can be found in three phyla: Proteobacteria (the largest faction), Verrucomicrobia and newly separated candidate phylum NC10'. In fact, methanotrophy as a life strategy also occurs in representatives of Archaea, the family Methanoperedenaceae (although based on fundamentally different enzymatic processes). In a further description, the Author points out that there are two known pathways of C assimilation by methanotrophic bacteria (i.e. RuMP and serine cycles). Indeed, aerobic methane oxidizers affiliated to Proteobacteria (so-called "canonical" methanotrophs) utilize either RuMP or serine cycle. However, in the known methanotrophic representatives of phylum NC10 (anaerobic), the Calvin-Benson-Bassham (CBB) cycle plays a dominant role in C assimilation. It is understandable that the expression used in the dissertation is a shorthand. However, in order to be in line with the current state of knowledge, in the submitted dissertation, the research object should be consistently defined as 'aerobic methanotrophic bacteria' or, alternatively,

all methanotrophs should be characterised, including those belonging to the Archaea and taking into account the metabolic diversity within the Bacteria domain.

2. Description of the taxonomic classification of methanotrophic bacteria belonging to the Alphaproteobacteria omits genus *Methyloferula*. In fact, its occurrence is limited to acidic wetlands and soils and it has not yet been the subject of biotechnology research, but it should be mentioned.

3. What was the motivation for selecting environments to isolate methanotrophic communities? The ability to accumulate PHA is an element of microorganism adaptation to unbalanced environmental conditions. How could the conditions within the source ecosystems have influenced the ability of the isolated communities to accumulate PHAs?

4. The microbial identification techniques used by the Author are based on long-reads of the 16S rRNA gene sequences, which is undoubtedly an advantage of the work as it indicates a commitment to using the most up-to-date and accurate research methods. Application of long-reads, compared to commonly used methods based on amplification of short fragments of the 16S rRNA gene, increases taxonomic resolution. However, a drawback of the approach presented in the dissertation is that the available sequence databases, to which the results are mapped, still contain relatively few records. Particularly when it comes to full operon sequences. In this dissertation, especially in Chapters 4 and 5, there is a lack of information on the contribution of microorganisms whose taxonomic assignment has failed at the species level, or have been assigned to so-called uncultured taxa. Meanwhile, as the results of Chapter 3 indicate, the AS_10 community that was the starting point of the other experiments contained a significant proportion of micro-organisms that were not assigned to any known taxa at the genus level. One of the advantages of environmental enrichment cultures is the ability to observe relationships between microorganisms including those that are not known in pure culture form. This is a way to glimpse into the microbial 'dark matter' and an opportunity to isolate new, potentially biotechnologically useful microorganisms. This raises the question - within the sequences obtained in the experiments described in Chapters 4 and 5, were there microorganisms not assigned to species or assigned to uncultured microorganisms? If so, what was their share in the whole bacterial community ?

5. It also seems to me that there is an error in the description of the methodology for the identification of microorganisms in Chapters 4 and 5. There is an information about the amplification of the V3 -V4 region of 16S rRNA gene given, whereas the primers and the

subsequent description of the methodology describe the sequencing of long fragments of rrn operon.

6. My next question is whether the effect of the timing of co-substrate addition and changes in substrate load on accumulation of 3HV fraction in PHA could be tested in the same bioreactor operating at continuous mode? As shown by the Author (Figure 4.5), the composition of the community in the bioreactor changes over the course of the experiments and was significantly different at the start of the experiment and when the amount of carbon substrate supplied (both valerate and methane) was altered. Considering the above, is it possible to assess the influence of the above factors on the rate of PHA accumulation by the microbial community? Was it considered to investigate the effect of the amount of CH_4 and valerate added in separate bioreactors with the same starting culture.

7. Finally, I would like to ask the Author to comment on the results reported by Adrian Ho and co-workers (Ho, A., de Roy, K., Thas, O. et al. The more, the merrier: heterotroph richness stimulates methanotrophic activity. ISME J 8, 1945-1948 (2014)) indicating that not only is the methanotroph diversity directly correlated to methanotrophic activity, but also the richness of heterotroph interacting partners is relevant to enhance methane oxidation. In the experiments presented in the dissertation, did the biodiversity within methanotrophic or heterotrophic bacteria influence the rate of carbon substrate assimilation and PHA production?

Editorial comments

The dissertation would benefit in terms of readability if the text used indentation in paragraphs and increased the font in diagrams, especially those presenting the composition of the microbial community. Also, the captions under the illustrations could have been more descriptive, following the principle that the illustration and its description should be self-explanatory.

Final conclusions

The thesis represents an original approach to a scientifically significant and methodologically challenging research problem. The Author has demonstrated that she has sufficient general theoretical knowledge and the competence to conduct scientific work.

I, hereby, declare that the reviewed dissertation by Aleksandra Gęsicka, M.Sc. Eng meets the criteria pursuant to art. 187 of Act of 20 July 2018 The Law on Higher Education and Science (Journal of Laws of 2018, item 1668, as amended) and request that the Research Discipline Council accepts Aleksandra Gęsicka, M.Sc. Eng for further stages of doctoral proceedings in the discipline of Environmental Engineering, Mining and Energy.