Warsaw, 11.09.2022

Dr hab. Iwona Jasser, prof. UW
Department of Ecology and Environmental Conservation
Institute of Environmental Biology,
Faculty of Biology, Biological and Chemical
Research Centre (CNBCh UW)
University of Warsaw

Revue of the doctoral dissertation of M.Sc. Jason Dexter titled:

"Heterologous Microcystinase Expression as an Industrially Relevant Model System for Development of Cyanobacterial Biotechnology"

completed under supervision of dr hab. Dariusz Dziga at Faculty of Biochemistry, Biophysics and Biotechnology, Laboratory of Metabolomics at Jagiellonian University

Our present economy is based on one-way exploitation of the environment through the use of natural resources, and production based on them, without paying attention to the consequent damage to the environment, or to the management of various resulting types of waste. This destructive economy contributes to severe ecological imbalance, loss of biodiversity, environmental pollution, and climate change, coupled in negative feedback. In opposition to this, outdated, economic model is circular economy. This new model is based on three principles: elimination of wastes, circulation of products and materials (at their highest value) and regeneration of nature. The circular economy approach implies a change of priorities by implementing nature-based solutions aiming at protecting, sustainably managing, or restoring natural ecosystems. This is possible to apply thanks to modern technologies such as microbial biotechnology.

The doctoral dissertation by M.Sc. Jason Dexter titled *"Heterologous Microcystinase Expression as an Industrially Relevant Model System for Development of Cyanobacterial Biotechnology"* presented for evaluation fits in perfectly with the principles of circular economy with usage of advanced microbial biotechnology.

Before, reviewing the dissertation further, I would like to point out that while the dissertation presents original, so far mostly unpublished results, M. Sc. Jason Dexter has published two scientific papers in highly rated journals as first author:

(1) Dexter J, Dziga D, Lv J, Zhu J, Strzalka W, Maksylewicz A, Maroszek M, Marek S, Fu P. Heterologous expression of mlrA in a photoautotrophic host - Engineering cyanobacteria to degrade microcystins. Environ Pollut. 2018; 237:926-35. (Journal Impact Factor = 8.071).

(2) Dexter J, McCormick AJ, Fu P, Dziga D. Microcystinase - a review of the natural occurrence, heterologous expression, and biotechnological application of MlrA. Water Res. 2021; 189:116646. (Journal Impact Factor = 11.236).

Additionally, Mr. Dexter is also an author of patent: Biocatalyst comprising photoautotrophic organisms producing recombinant enzyme for degradation of harmful algal bloom toxins. US10787489B2 granted patent (2020). WO2018017828A1, and presently he and his co-authors prepared a manuscript for submission with the title: "New tools for effective production and long-term stabilization of microcystinase (MlrA) - a biotechnological perspective towards hepatotoxic microcystins remediation" by Dexter J, Łobodzińska A, Tlałka A, Fu P, Dziga D.

M. Sc. Jason Dexter is also the author of a project "Algae-Microbial Fuel Cell employing cyanobacteria for the simultaneous production of electricity and valuable enzyme from wastewater - a pilot study", source of funding: The Priority Research Area BioS under the program Excellence Initiative – Research University at the Jagiellonian University in Krakow.

Synechocystis sp. PCC 6803, *Synechococcus elongatus* sp. PCC 7942 and *Synechococcus* sp. PCC 7002 are among cyanobacteria that have been already used in synthetic biology studies for phototoautotrophic production of different chemicals such as free fatty acids, isoprene, 1-butanol, alkanes, hydrogen and many others. An important aspect of cyanobacterial biotechnology is that these photoautotrophic, oxygen evolving prokaryotic microorganisms are able to grow on wastewaters utilizing nutrients from them as well as CO_2 from atmosphere. In this way cyanobacteria are perfect and valuable examples of nature-based solutions and circular economy.

Cyanotoxins and among them microcystins are the most commonly occurring toxins connected with the harmful algal blooms in freshwater environment, although recently recorded massive bloom of *Prymnesium parvum* in the Odra River proved that they are not the only ones. However, blooms of cyanobacteria are reoccurring in eutrophic waters and

often pose threat to humans and ecosystem. To mitigate cyanobacterial blooms various methods are used with hydrogen peroxide demonstrating effectiveness. Nonetheless when dosages of H_2O_2 used to mitigate the cyanobacterial blooms are low, safe for the other aquatic organisms, extracellular microcystins are not degradable. Thus, there is a need of accelerating the decomposition of these toxins, which was proposed by Dziga et al (2019).

In his dissertation, Jason Dexter presents a new direction of research in cyanobacterial biotechnology, aimed at the production and extraction from cyanobacterial chassis of the MlrA peptide capable of decomposing microcystins from a toxic cyclic form to a less toxic linear form. The dissertation comprises several experiments involving genetic engineering, cyanobacterial transformations, production of the desired peptide, verification of activity and stabilization of the protein product applied to answer hypotheses.

He hypothesizes that heterologous microcystinase expression can be achieved in a commercially pertinent cyanobacterial model system. To achieve this, the Author applied successive steps from (1) chassis selection, (2) cultivation, downstream processing such as (3) harvest, (4) extraction, (5) fractionation and (5) stabilization. Steps 1 – chassis selection - and 6 – the stabilization of the extracted product - are the only ones described in detail in the dissertation, however. The Author set himself eight objectives to verify the research hypothesis cited above.

1) Can MlrA be expressed within cyanobacterial chassis during photoautotrophic growth (it was before expressed in E. coli)
2) Can the transformation of cyanobacterial chassis be obtained by both strategies – the double homologous recombination and self-replicating vector
3) Can usage of non-native promoter Ptrc increase expression MlrA comparing with cyanobacterial promoter PcpcB560?
4) Can transformation by pCA2.4 native small plasmid increase MlrA expression via gene copy effect comparing with transformation using the chromosomal slr0271 locus?
5) What is the expression on based cyanobacterial chassis comparing with *E. coli* based?
6) Can N-terminal peptide modification of MlrA positively modify MlrA activity, or support peptides of increased industrial applicability

7) can MlrA and N-terminal genetic sequences be integrated into established synthetic biology DNA assembly platforms?
8) Can MlrA activity be stabilized towards commercial/industrial relevance?

For his research, the Author chose *Synechocystis* sp. PCC 6803 and Synechococcus elongatus UTEX 2973 compared in some experiments to E. coli chassis. He positively verified that a cyanobacterial chassis 6803 can express heterologous MlrA activity. The transformation can be achieved using two strategies; the double homologous recombination and self-replicating vector. The second strategy was accomplished after using CyanoGate vectors. Because the production of desired peptides is connected with promoters used for transformation the Author verified if non-native promoter Ptrc may reinforce the MlrA expression when compared with PcpcB560 promoter. He found out that the effects of promoters as similar and Ptrc does not enhances the expression of MlrA. The objective 4, a natural transformation with the pCA2.4trcMlrA vector, was not reached.

A significant result of the presented work is the demonstration that MrlA activity in cyanobacterial chassis is comparable (though lower) to *E. coli* chassis exhibiting longer activity in experimental conditions.

Another important achievement of the work presented in the dissertation is the proposition, and positive verification, of the genetic sequences of MlrA and N-terminal modifications to such a degree that they can already be integrated into established DNA assembly platforms in synthetic biology such as CyanoGate.

The last objective of the dissertation was to obtain stabilized protein product (MlrA) activity after extracting and stabilization via lyophilization. This is a very important aspect, from both the commercial and ecological point of view. As the Author himself admits, under present regulations it is not acceptable to use live biomass of transformed cyanobacteria in a natural environment in order to degrade microcystins. The Author has managed to acquire an end product that is commercially significant and is in the form of a freeze-dried product that can be easily distributed. He has also verified the rate of activity recovery of the lyophilized MlrA enzyme proving that storage in 4 ° C is sufficient. The nature of Mr. Jason Dexter's work is not only innovative but practical. By avoiding storage at -20°C the enzyme is easier to handle and possible to reduce operating costs.

Although it seems that the production process still requires scaling up, the author is well aware of this.

Another issue connected with the achievements of the discussed doctoral work is the usage of the lyophilized enzyme in natural conditions. Although, as demonstrated by Dziga et al. (2019), MlrA enzyme extracted from *E. coli* chassis reduced extracellular microcystins concentrations is mesocosms experiments in Lake Ludoš in Serbia, further studies in various mesocosm and macrocosm experimental conditions in the natural environment should be performed before such a stabilized enzyme can be widely used commercially.

The doctoral research presented in the dissertation is innovative and implementing. The dissertation is very well written and sufficiently illustrated. It contains an extensive introduction, covering all aspects of cyanobacterial biotechnology necessary to achieve the objectives of the work. The results chapter is succinctly and clearly written. The discussion supports the results, points to novel findings, and addresses and explains the undermined objectives.

I have a few questions concerning the presented research:

1) What is the reason for the lower growth rate of transformed *Synechococcus* chassis (6803mlrAsec+) compared to wild strain of WT 6803? Can it be overcome?

2) Has the Author managed to recover exconjugant colonies from transformation 2973 chassis (after Covid) or plans to do it still?

3) Is it feasible to use *Synechococcus* sp. PCC 11901, a marine strain, as chassis for the commercially important Mlra production or any other valuable enzyme product using wastewaters? Presently many countries are faced with the production of saline wastewater from industries including chemical or mining, which are the cause of environmental disasters, such as in the case of the Odra River. The use of such wastewaters would, to some extent at least, allow for its partial utilization within the circular economy paradigms, even if it would not solve the problem entirely.

The objectives of the dissertation were mostly achieved and the research hypothesis was positively verified. The Author also discussed the limitations of the methods used, which proves the knowledge, experience, and scientific maturity of the doctoral student. He presented a commercially relevant product, and provided genetic sequences which can be integrated into established synthetic biology DNA. The scientific output of the research has already been partially published, patented and presented at various conferences. I am applying for the admission of M.Sc. Jason Dexter to the next stages of the doctoral dissertation.

Because of the extreme importance of research on genetic modifications of cyanobacteria and the use of the obtained end products in bioremediation in environmentally friendly biotechnological applications, the advanced methods used in the research, as well as the broad collaboration initiated by the Author, I am applying for the award of the peer-reviewed work with an appropriate distinction.