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Review of the PhD thesis of Mr. Jason DEXTER

The dissertation presented by Mr Jason Dexter for the degree of Doctor of Philosophy is entitled "***Heterologous Microcystinase Expression as an Industrially Relevant Model System for Development of Cyanobacterial Biotechnology***". The scientific field of the thesis is biotechnology of microalgae. The work has been supervised by Dr. hab. Dariusz Dziga, prof. JU, from the Laboratory of Metabolomics, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University.

1. Topic of the thesis

As important primary producers, cyanobacteria constitute a key component of aquatic ecosystems. They are also well recognized as sources of bioactive compounds, including acute toxins, allelochamicals and candidates for drug development. Microcystins (MCs) are the most widely occurring and at the same time the most widely studied group of cyanotoxins. In humans they can cause symptoms such as abdominal pain, skin rash and cough. In extreme cases, death incidents were recorded. In some countries (e.g. China, USA), removal of toxic cyanobacteria and MCs from drinking water sources is a problem of high priority. For this reason, development of innovative methods and modified organisms capable of removing the harmful agents and reducing the risk of human and animal intoxication is a socially important topic of studies.

Mr Jason Dexter undertook a challenging task to heterologously express protein (enzyme) capable of degrading microcystins in photosynthesizing host, so that commercialization of the enzyme could be more realistic. The PhD candidate tried to meet the objectives of the thesis in a systematic and well planned way.

2. Organization and content of the PhD thesis

The structure of the thesis follows the usual pattern of scientific work. Initial sections include, among others, a list of abbreviations which I find very useful as in the work the abbreviations are numerous and used frequently. In abstracts (in English and Polish), the main goals and scope of the work are presented. It is emphasised that the main bottleneck in biotechnological application of

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cyanobacteria or their products is the slow growth rate of these organisms. This fact usually makes the biotechnological processes based on cyanobacteria cost ineffective.

2.1 Introduction

The Introduction provides a comprehensive overview of the chain of processes from host selection, genetic manipulation to development of commercial product. In this part of the thesis, Mr Jason Dexter justifies the choice of the host organism. *Synechocystis* sp. PCC6803 (hereafter 6803) is a good model system for heterologous gene expression, and the author had documented experience in the work with this strain. Though, the doubling time of 6803 is rather low, compared to other picocyanobacteria. In the thesis, this strain was used for heterologous expression of microcystinase MlrA. Unfortunately, in the Introduction there is a lack of comments about the natural sources, significance and properties of MlrA. These elements are discussed later in the thesis. Of course, it is a PhD candidate decision how to organize the text. However, more comments in the Introduction on MlrA, not only on methods, would even better justify the purpose of the work.

According to the Mr Jason Dexter opinion, microcystinase MlrA could be produced for commercial purposes and applied for microcystins removal. To make the process feasible for implementation, certain factors should be optimized, including efficiency of the process of enzyme production and stability of enzyme activity.

In further parts of the Introductions, Mr J. Dexter presents a detailed discussion on various aspects of the thesis. Among others, he justifies the choice of the two promoters controlling transcription, $PcpcB_{560}$ and $Ptrc_{10}$, and compares their advantages and limitations. An important element of this evaluation is the statement that despite the great significance of the promoters in research, their use at industrial scale is not economically feasible. Other topics covered in the Introduction include the methods of heterologous expression used in the thesis, effect of N-terminal peptide modification on target expression. Moreover, problems and challenges related to heterologous gene expression in cyanobacteria were presented and supported by selected examples. Finally, the significance of heterologous MlrA expression and enrichment of the enzyme in cyanobacterial biomass for microcystin remediation (e.g. reduction of the toxin in irrigation waters) was presented. It was pointed out that cyanobacteria growth can be supported by waste waters, which can make the process of MlrA production more cost-effective.

Introduction ends with presentation of specific questions or rather problems related to heterologous MlrA expression in 6803. In his thesis, the PhD candidate tried to address the problems and, in my opinion, this efforts were successful.

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From the text in the Introduction, I understand that the overall goal of the work was the integration of *mlr*A into PCC6803 genetic material and determination of stability of the protein during storage. The Introduction, in fact, could be a part of a comprehensive review article on heterologous MlrA expression in cyanobacteria. The author demonstrated his extended and up-to-date knowledge on biotechnological significance of the process.

In my opinion, the value of this chapter is reduced due to numerous typos and style inconsistencies. Sentences such as: "Thus, given (1) the non-requirement for eukaryotic heterologous expression, and (2) *Synechocystis* sp. PCC6803 is the first oxygenic photosynthetic organism to have the full genome sequenced, is naturally transformable, and is the most well-characterized cyanobacterial system to date, 6803 was selected as the initial strain"(p. 28) are unclear for me. In Table 1, the growth rates of different strains are compared, but no vales (e.g. doubling time or mg/V/time) are given. At the end of each section, the content of the next section is reviewed. I do not think it is necessary.

I cannot agree with the sentence "A primary difference between cyanobacteria (prokaryotic) and microalgae (eukaryotic) chassis application for expression of heterologous target is the extensive capacity for post-translation peptide modification present in eukaryotic systems" (p. 27). Post-translation modifications of peptides also take place in prokaryotic organisms. In addition, the publication by Mathieu-Rivet et al. 2020 refers to the glycosylation in microalgae (including cyanobacteria) and therefore, it is cited in a wrong place.

The Materials and Methods section contains thirty pages. Of these, significant part is dedicated to the integration of *mlr*A into CyanoGate. In M&M section, complete data on kind and source of materials, reagents and some parameters of the applied methods are lacking.

2.2 Results and Discussion

The results obtaining by PhD candidate are significant and well documented. Part of the work (section 3.1) has already been published in Environmental Pollution (IF-9.988) and Mr J. Dexter is the first author of the article. The work was done in cooperation with partners from the College of Life Science and Technology, Beijing University of Chemical Technology and New Energy Research Centre, China University of Petroleum (Beijing). I would like to emphasised the fact that the method described in this article was patented by Mr J. Dexter.

In the thesis, the attempts were made to obtain the MlrA expressing *Synechocystis* sp. PCC6803 isolates. For this purpose, two approaches were used: integration of the designed cassette with genome by recombination and introduction of *mlr*A in plasmids obtained by CyanoGate methods

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(by exoconjugation). The fact that cell lysate of 6803mlrAsec+ (transformed *Synechocystis* sp. PCC6083) was active against MC-LR confirmed the successful recombination and heterologous gene expression in *Synechocystis* sp. PCC 6803. The activity was located in cytoplasm (not in periplasm) and was higher than in *Sphingomonas* (the natural MlrA producer) and lower than in *E.coli* (another host).

The growth rate of the transformant (compared to the 6803 wild type) and the stability of the MlrA activity were studied. The growth rate was found to be slightly reduced, but the difference was in fact minor, especially when the 48-h average was taken into account. Mr Dexter suggested that, at least partially, it could be caused by the differences in the initial OD values of the two cultures (WT and 6803mlrA+).

Further comparison of the two different hosts (*E. coli* BL21_pET21a-mlrA and 6803mlrAsec+) showed that in 6803mlrAsec+ the MlrA activity is more persistent, though the activity of *E.coli* was higher. Also the whole-cell activity of two MlrA6803 isolates expressing MlrA were compared. They were found to be similar and also the growth rates of the strains were similar.

Previously, the attempts to transfer the *mlr*A gene to *E.coli* BL21 and effectively produce MlrA have been made. In fact, the transformed strain degraded microcystin faster than native bacterium, but activity of the immobilized *E.coli* quickly dropped. In the thesis, successful heterologous expression of *mlr*A gene in *Synechocists* strain 6803 resulted in more stable activity/production of the enzyme. It is assumed to be attributed to the addition of the PilA N-terminus tag. More stable MC-degrading activity of transformed 6803 was also documented in the work under semi-natural conditions.

According to the PhD candidate, additional positive aspect of the studies performed with the application of *Synechocists* strain 6803 is the fact that picocyanobacteria (primary producers), when used in water management to remove microcystins, would be less harmful elements of ecosystem than *E.coli*. One of the additional advantages is that picocyanobacteria, due to their surface to volume ratio, are known to remove different contaminants from water bodies (e.g. heavy metals).

One of the important findings of the work is that MlrA-containing extracts ($MlrA_{CE}$) from 6803mlrAsec+ and from heterologous MlrA expressing *E. coli* were more active than the whole cells of the strains (samples normalized to culture density). When results were normalized to total soluble protein (TSP), lysate from *E. coli* C41_pET21a_*mlr*A was found more active than lysate from 6803mlrAsec+.

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As an important comment to the thesis, Mr J. Dexter mentioned that in the MCs removal from natural waters other components of the microbial *mrlA* degradation pathway may also play some role. Therefore, heterologous expression of MlrB and MlrC , simultaneously with MlrA, can increase the efficiency of the process. As it was proved by some authors, these enzyme can also degrade other chemicals.

In the thesis, the expression cassette responsible for the synthesis of MlrA was integrated into the 6803 chromosome with two different promoters: PcpcB560 (native 6803 promoter) and Ptrc (non-native promoter). Both are strong promoters and no statistically important differences in expression level of the promoters on whole cell activity was found.

Part 4.2 of the Discussion section ends with short, but interesting comments on how the MC-degrading enzyme could be possibly applied to the aquatic system: as MlrA-producing cells or MlrA-enriched cell extracts ($MlrA_{CE}$) – with the latter option being more promissing.

The PhD candidate made attempts to clone the Ptrc-PilA-MlrA expression cassette into the native 6803 plasmid pCA2.4. Although the cloning was successful, no colonies of the transformed strain were produced. The possible reasons for this failure and solutions are presented in part 4.3 of Discussion.

In subsequent section, the effect of modifications in the MlrA N-terminal peptides on 6803 MlrA activity was explored. These studies were performed in co-operation with Prof. Alistair J. McCormick from Institute of Molecular Plant Sciences, School of Biological Sciences, University of Edinburgh, UK and with application of the CyanoGate, a cloning suite for engineering cyanobacteria. In this important part of the thesis Mr J. Dexter well proved his ability to use the most advanced tools of synthetic biology.

Final section of the dissertation is dedicated to stability of MlrA activity. As it was emphasised in different parts of the thesis, besides activity, the development of stable form of MC-degrading enzyme is a challenge. As could be expected, the lyophilization improves the stability of $MlrA_{CE}$ activity. What is important, the recovered $MlrA_{CE}$ showed similar activity as before lyophilization. As many factors can have an impact $MlrA_{CE}$ stability (crucial for commercial application), the experiments to improve this factor will probably be continued.

The thesis ends with Conclusions which are presented on 4.5 pages and summarized in 8 objectives. They reflects the complexity of the work done within this thesis and the ambitious and challenging goals set by Mr J. Dexter. The developed and refined method of MlrA expression in *Synechocistis* sp. PCC6803 presented in the thesis constitute a significant step forward to

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commercial application of this micocystins-degrading enzyme. As it was mentioned in the thesis, in the situation when the access to safe (uncontaminated) drinking water resources is limited, development of new and cost-effective methods of water treatment and toxins removal is of high priority. What increases the value of the work is the fact that different stages of the work were done at different universities: in China, UK and finally in Poland, in the laboratory headed by Dr. hab. Dariusz Dziga, prof. UJ. So, Mr Dexter had a chance to learn from the renown experts in different aspects of the work.

My only objections concern the style of Mr Dexter writing which, in my opinion, in some places hinders understanding of the text and full recognition of the high value of the work. Some sentences are so long that their meaning becomes unclear. E.g. "It was in consideration of this unexpected, novel effect of PilA N-terminus tag on enabling 6803 MlrA activity expression combined with contemporary reports noting the utility of other N-terminal peptide modifications that motivated further exploration N-terminal modifications on mlrA activity."

In conclusion, I highly value the contribution of Mr Jason Dexter to the development of the microcystinase MlrA as an enzyme potentially used in water treatment processes, as MlrA enriched cyanobacteria extract. In the thesis, he proved his ability to master different methods used in cyanobacteria engineering and effectively cooperated with research groups from different laboratories. The results of his work are of high scientific value. Therefore, I **propose the Members of the Biology Research Discipline Council to accept the PhD thesis of Mr Jason Dexter. Personally, I consider the work to be outstanding and worth recognition.**

W podsumowaniu, wnioskuję do Rady Dyscypliny Nauk biologicznych Uniwersytetu Jagiellońskiego o dopuszczenie mgr Jasona Dextera do dalszych etapów postępowania o nadanie stopnia doktora w dziedzinie nauki ścisłe i przyrodnicze, w dyscyplinie nauki biologiczne. Jednocześnie, ze względu na wysoki poziom naukowy pracy, proponuję ją wyróżnić.

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