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Review of the PhD thesis of Sunday Ocholi Samson MSc, entitled “Cyanobacterial biomass in biocatalysis and bioaccumulation processes”.

Introduction

The research leading to the PhD Thesis of Mr Sunday Ocholi Samson has been conducted at the Chemistry Department of the Wrocław Technical University under the joint supervision of Professor Ewa Żymaczyk-Duda and Dr Monika Serafin-Lewańczuk (assistant supervisor). The aim of the thesis was to assess the biotransformation potential of several selected cyanobacteria as well as the bioaccumulation of europium and gadolinium ions. Although the thesis was conducted in the discipline of science of the chemical sciences, it combines techniques from the field of microbiology and chemistry.

Information about PhD Thesis

The thesis under review has the form of a monograph, which theoretically spans over 172 pages of text. However, due to the selected format of references by the PhD student, its real volume is only 116 pages, of which 51 pages are dedicated to the introduction and literature review, 24 pages to the description of methodology, 39 pages to results and discussion, and finally 3 pages to conclusions. The thesis cites 250 citations. Interestingly, the results presented in the thesis were already published in two papers, while the third is under preparation, and they are accompanied by 3 Polish patent applications. Therefore, it is surprising that the actual results encompass only 35% of the thesis (or 26% if one counts the volume dedicated to references). One would expect that the content of 3 articles and 3 patent applications, when presented in detail, provides more scientific material and grounds for discussion with the literature.

Assessment of the scientific merit of the PhD thesis

The thesis is open with an extensive list of scientific achievements of the PhD student (13 papers, 6 conference presentations at Polish conferences, 3 patent applications, the list of awards as well as the list of tables (23 in total), figures (29 in total) and a list of abbreviations used by the author.

The **introduction** starts with a general description of the merits of using cyanobacteria as whole-cell photobiotransformers and bioaccumulators and the aims of the thesis. According to that section, the dissertation aimed to assess the photobiocatalytic capabilities of several selected cyanobacteria strains in the transformation of ester substrates, including those containing C-P bonds. The screening of the various strains was to be followed by chromatographic and spectroscopic evaluation of the obtained products, estimation of their antimicrobial activity, optimisation of the cultivation media with respect to reagent stability, as well as identification of the strains best performing under conditions modelling industrial application (high loading of the reactor with substrate). The second goal was to evaluate to what extent the cyanobacteria can be used for bioaccumulation of rare earth elements (europium or gadolinium ions), especially after their use for biotransformation.

This section is followed by an introduction of various types of biocatalytic or biotransformation strategies and a discussion of their merits compared to chemical homogeneous or heterogeneous catalysis. Furthermore, biocatalytic processes that have already found their way into industry and are relevant to the topic of the thesis are introduced, especially various types of kinetic resolutions and racemisation processes (which lead to dynamic kinetic resolution systems), asymmetric isomerisations conducted by flavin-based enzymes catalysing hydride transfer, reduction of ketone or aldehyde groups by alcohol dehydrogenases/ketoreductases as well as canonical and promiscuous reactions catalysed by hydrolases. This section is accompanied by comprehensive figures illustrating the described processes, which are very helpful in following the text. With small exceptions (see below), this is a very good part of the thesis. I especially like the description of the non-standard application of lipases developed by the Polish researchers, Prof. Ryszard Ostaszewski and Dr hab. Dominik Koszelewski (multicomponent Ugi, Passerini and Knoevenagel reactions).

The next section of the introduction is dedicated to whole-cell factories, with special focus on cyanobacterial systems. Several advantages and disadvantages of such systems are outlined comprehensively and concisely. Examples of both biotransformations as well as production of industrially attractive secondary metabolites are provided. I like how the author counterweighted the advantages of enzyme catalysis or whole-cell biotransformation processes with their inherent limitations (like narrow substrate scope or toxicity of substrates to living cells).

Finally, the capacity of cyanobacteria for rare earth elements recovery is introduced and compared to the already implemented chemical methods such as liquid extraction or adsorption. Despite citation of several works dedicated to the development of REE extraction or biosorption, the reader cannot gain quantitative insight from this section on the respective efficiencies of those approaches. The efficiencies of sorption are always provided in a relative manner (% of recovery), which is of course dependent on the concentration of the respective element in the solution, while overall robustness of microbial biosorption or bioaccumulation is described only in terms of 'promising efficiency and reduced environmental impact'. **Therefore, I would like to ask the PhD student to provide a comparative summary during**

defence on both approaches – i.e. quantitative data (if available) of adsorption of REE on chemical sorbents vs. cyanobacteria cell mass.

Introduction is followed by a chapter dedicated to methods and materials. It starts with an extensive list of all reagents used in the study and provides structures of chemical substrates used in biotransformation/bioaccumulation tests. This is followed by the list of strains used with respective strain codes/collection sites and information on instrumental techniques.

The next chapter is dedicated to Materials and Methods. It starts with an extensive list of all reagents used in the study and finally provides structures of chemical substrates used in biotransformation tests.

This is followed by the list of strains used, with respective strain codes/collection sites, followed by a detailed description of the media compositions that were used for the cultivation of bacteria. This section **is well written**, and one can see how to compose the media in a practical way. Also, I find **the section concerning the optimisation of the media composition** in order to stabilise the substrates used in the study **very interesting**. This type of fundamental preparation for biotransformation **is a hallmark of good microbial biotechnology**.

Surprisingly, the information on the cultivation of cyanobacteria is very scarce. Only **two paragraphs** are dedicated to that topic, with no example of the growth curve (OD vs time) or any data on biomass production. Also, in the results section, there are no additional data depicting the standard growth curves of the cyanobacteria used in the study (although one can find a specific growth curve in Fig. 5c-d when substrate stability was examined). This is surprising, as different media were tested in the course of the research, and I would expect that OD was monitored also during the biotransformation process. Therefore, it is quite difficult for somebody not well versed in the cultivation of cyanobacteria to estimate the robustness of the used methodology. How much biomass can be produced in 14- 21 days of cultivation, and how active would this biomass be for the transformation of the particular substrate? I would appreciate if the author provided some data in that regard that would connect OD with dry mass or any other estimate of cell mass quantity (more precise than '100 mL biocatalyst') that was used to calculate the specific efficiency of the biotransformation/bioaccumulation process. How did the author compare different experiments if the methodology usually provides only volume and age of the culture? I presume that OD was routinely measured as a cell quantity proxy, but this information is not clear. Furthermore, section 3.5.2. Dry mass determination tells us that the dry mass was evaluated for some cultures, which means that the author has established a connection between OD and dry mass of the cyanobacterial cells.

This section is followed by the most important methods of the thesis, which focus on biotransformation of 1-phenylethyl acetate and organophosphonates. Special interest is dedicated to dimethyl (1E)-3-hydroxyprop-1-enylphosphonate, for which purification from the biotransformation process as well as chemical synthesis is described together with analyses of its antimicrobial capabilities. I have almost no problems with this section. However, I was a bit puzzled why, in the agar well diffusion method, the control for the antibiotic was done by the disk-diffusion method ("The positive control involved placing an antibiotic disc (cefoperazone, 30 µg) directly onto the bacterial lawn"), not by the solution of the antibiotic introduced into

70 μ L volume into the 6 mm well. I was under the impression that in the well-diffusion method, the control has to be conducted similarly to the agent under evaluation due to diffusion of the added volume in the gel and the associated decrease of its concentration. So one should not mix the well-diffusion method with the disk-diffusion method. **I would like a comment on the reason for such an approach.**

Furthermore, after reading the materials and methods section, I have a few questions, as I was not able to find all the important information. I have also several request of clarification/correction of the thesis to be delivered during the PhD defence:

Estimation of the free phosphate:

- The methodology is in fact divided between the methods and results section (4.2.1), where the actual calibration line and the equation are provided. I have some doubts if the linear model is sensible up to an absorbance of 2.5 (one can already see a departure from linearity between the last two points). Furthermore, the use of a linear equation allows for the estimation of the error from the method. Based on the error of estimates from the linear regression, an error of concentration determination should be estimated, and those errors (of concentrations) should find their way into Table 13. This would allow determination of the statistical significance of the difference between various samples. **I request the addition of such statistical estimation.** I also would like to point out that **it is a methodological error** to use a linear model already departing from linearity within the calibration range to estimate concentration for samples with absorbance beyond such calibration range (e.g. Abs. above 6). Concentration estimated from such a high absorbance must be associated with significant error due to the different nature of the Abs(c) function beyond an absorbance of 1.5. In the results section, one can read that the samples were turbid, which increased the apparent absorbance. The table should actually provide the value which was measured and estimate the concentration for such a sample, not the value already multiplied (as I presume was done).

HRMS:

- Very little technical information was provided about that analysis. We are told that ESI(+)-TOF mode was used to confirm the mass of dimethyl (1E)-3-hydroxyprop 1-enylphosphonate. The small number of details does not allow reproduction of the experiment. Perhaps the analysis was not conducted directly by the PhD student, as the TOF analysis is usually done by a qualified analyst. If so, this should be explained, but still, the parameters of analysis should be described and the respective acknowledgement of the technical support provided.

Other analytical methods:

- The author writes that "Substrate conversion and product formation were monitored using chromatographic and spectroscopic analytical techniques, including HPLC, infrared spectroscopy (IR), high-resolution mass spectrometry (HRMS), and nuclear magnetic resonance (NMR) spectroscopy." However, I could not locate any methodological description of analytical HPLC (only preparative one) as well as infrared spectroscopy. As HPLC and FT-IR were used to produce data for the results section, the **methodology must be provided** (column, instrument, separation program, method of product quantification for HPLC, instrument type, mode of sample preparation for FT-IR). **I request amendment of this omission during the PhD defence.**

The chapter is closed by 3 sections dedicated to bioaccumulation, i.e., ecotoxicological screening, dry mass estimation and ICP-MS determination of accumulated heavy metal ions. In this section, OD750 and OD682 were used to quantify the cell number, and inoculum strength was provided in number of cells/mL of inoculum. As, according to the text, this quantity was again established based on optical method estimation, not direct cell counting, I wonder if the same method could not be used for quantification of all experiments in the thesis. I would also like to know why two wavelengths were used for measurement of OD and why only in this experiment (as previously a more standard OD600 was used)?

Chapter four is dedicated to the most important part of each PhD thesis, i.e., results and their scientific discussion. It starts with the problem of epoxymethyl dimethyl phosphonate stability in the cultivation media (especially SM and BG-11) – a first important challenge that was to be addressed by the PhD student. The experiment showed that modification of EMDP occurs in the media – the conclusion is apparently based on P NMR (Fig. 12a vs 12b), although no explanation is provided of what the expected signal is for non-modified EMDP. As a result, to the reader, this experiment is quite hermetic. Furthermore, Fig. 12d is very puzzling. It shows the decrease in OD of the culture in the modified BG-11 medium, but the growth curve starts at 0.8 instead of a value close to zero, as in the case of Fig. 12c. Although the author claims that both experiments were not, in fact, paired but still indicate that “modified BG-11 medium supported limited growth (Figure 12d), with both strains experiencing a progressive decline in OD₇₂₀”. I am not sure how the author understands growth of the colony, but the decline in the population of cells is not growth. I agree that the reason for such a situation may be metabolic stress due to a change of the medium, but to my non-expert eyes, the cells are dying faster than dividing. As a result, I am completely puzzled by this section, and I do not know which medium should be selected as a compromise between substrate stability and the well-being of the culture (either for growing or just biotransformation purposes). **I would like to ask for reformulation of this section presentation during the defence in such a way that the problem is clearly explained** and the experimental results answer, to some extent, those questions.

This section is followed by a study of divinylphosphonate biotransformation, which turned out to be hydrolysed only in a non-specific hydrolytic manner in the presence of bacteria. The process was investigated by P NMR (which indicated no change in the substrate) and determination of free phosphate by malachite green assay. As I wrote already, I have significant doubts about the quality of the conducted experiments, due to off-calibration-range values recorded, even for the control without any bacteria and substrate (SM control). If even the negative control provides the maximum value of ‘hydrolysis’, then the experiment is not correctly conducted, or the selected method is not appropriate for the given problem. The most puzzling result is a negative change in free phosphate observed over time (sequestration of phosphate by the bacteria?) even for control samples which did not have the substrate. Also, the lack of any error estimation, as well as standard deviation values, does not allow for estimation of the significance of this experiment. BTW, according to the Method section, the P NMR was also used quantitatively. Did changes in P NMR also agree with the changes indicated by UV-vis? The lack of a reference method and the very dubious result collected by the PhD student render the impression that the whole result is mostly noise of the method or that it was conducted in a not very sterile way (and super-turbid samples are a

result of microbial contamination by other fast-growing bacteria). **This section definitely needs an explanation during the PhD defence.**

The next section, on the other hand, provides a report on successful biotransformation of epoxymethyl dimethyl phosphonate (EMDP) to dimethyl (1E)-3-hydroxyprop-1-enylphosphonate (DMHPP). The chapter opens with the proposed scheme of the cascade, which is further supported by literature (i.e. assuming involvement of epoxide hydrolases in the epoxide opening process). The product was identified by means of NMR, IR and HRMS methods, supported by the chemical synthesis and isolation of the product standard. The bioconversion was conducted for 5 (not 6, as it is claimed in the text under Figure 19) cyanobacterial species, indicating *Nostoc cf-muscorum* as the least efficient and *Limnospira* sp. turned out to be the most efficient strain, which converted 99% of the substrate in a time below 1 day. However, in the biotransformation process, conversion is not the main indicator of strain robustness but the yield of product formation (as the substrate can also be utilised by the cells, for example, as a carbon source). In that regard, *Synechococcus* sp. turned out to be the most efficient, as at 61% conversion it produced 10% of the product (i.e. 0.1 mM). As a result, the strain was used for further optimisation, allowing production of 0.26 mM of the product from 4 mM substrate in 4 days. The relatively low efficiency of the system was attributed to potential sequestration of the phosphonates by the phospholipid bilayers of the cells. The study of DMHPP and EMDP is closed by antimicrobial studies that generally showed that DMHPP does not exhibit antimicrobial activity against tested species. Still, the negative results are also important and valuable, especially as the biomedical papers are biased toward success stories.

In section 4.2.5. the author investigates the bioconversion of racemic 1-phenylethyl acetate. The idea is to obtain an optically pure product, 1-phenylethanol, or the substrate by means of kinetic resolution. The reality, as is usually the case with biotransformation, is more complex. The ester indeed is being hydrolysed, and enantiomeric excess is observed for both the ester substrate and alcohol product, but also the product is further oxidised to acetophenone. This part of the research seems to be quite well designed, as several factors are taken into consideration besides the type of strain, such as light regime, age of culture used for biotransformation (7 or 14 days) and time of the reaction, or design of the photobioreactor. The author observed that older cultures and less intense illumination usually resulted in more efficient biotransformation, while the overoxidation to acetophenone decreased the enantiomeric excess of the alcohol product when high conversion of the substrate was reached. The most interesting results were obtained for *Leptolyngbya foveolarum*, for which high ee of the product was maintained during the whole biotransformation process. Nevertheless, the information collected in Table 17 is, in my opinion, insufficient to understand the whole process in full. For example, we are not informed of the reason for the increased enantioselectivity of the product. In the case of 96% substrate conversion occurring within 24 h, one would expect a racemic mixture as a product. However, we see 34% ee followed by 74% ee in the final product. Clearly, the hydrolysis of the ester is not the step at which kinetic resolution occurs (total conversion of the substrate) but rather subsequent oxidation to acetophenone by enantioselective alcohol dehydrogenases. The same conclusions are drawn by the author, but for some reason the author states that the

'hydrolytic activity remained dominant over competing oxidative pathways' and the effect is attributed to *Leptolyngbya foveolarum*. While I agree that hydrolytic activity was very high, resulting in quick hydrolysis of both enantiomers of the ester, the rise in enantiomeric excess of the alcohol product must be the result of the oxidative effect of a stereospecific dehydrogenase. Based on Table 17, one cannot determine which of the systems studied is indeed the most efficient, as the concentrations of the product(s) are not provided. Meanwhile, cyanobacteria catalysts may express both R- and S-specific dehydrogenases, so it is important to know how much and how pure the obtained product is. **Therefore, I would like to ask for an extension of the information presented in Table 17 by concentration of the product(s), as well as an indication of which strain of cyanobacteria was used in each experiment** (as, for some reason, only the genus part of the name was used in the table while the text refers to particular strains). **Based on that, I would like to know which strain the author of the Thesis deems to be the most efficient for the production of the enantiopure 1-phenylethanol.**

The final section of the results is dedicated to the study of bioaccumulation of REE. For this purpose, the PhD student employed bacterial cells previously used for biotransformation of phenylethanol acetate as well as epoxymethyl dimethyl phosphonate. These cells were compared with reference ones, which were not utilised in the biotransformation process. Interestingly, the microscopic investigation of cyanobacteria revealed no morphological changes in cells that were used in biotransformation. In the next stage, different cyanobacteria were grown in the presence of Gd and Eu ions in order to assess the influence of those elements on their growth. Fig. 25 provides results presenting inhibition of growth in (%). However, no definition of this value in the method is provided. As the measure is based on OD750 and OD682 or OD680 (depending on where one reads – in the method or results section), I presume it is the relative difference of those optical densities with respect to the control. In the thesis, one can read that "inhibition was calculated from differences in OD increase between treated and control cultures over time." In the Fig. 25 caption, one can find information that 72 h was the only time point analysed. **I would like to ask for a more precise definition and explanation of that value so it is unequivocally clear which time point of the experiment is used to produce the value presented in Fig. 25 and why only the last point was analysed.** Also, I would like to know what the initial ODs were of the cultures of the pre- and post-biotransformation cyanobacteria, and whether the ODs of the different strains were more or less in the same range as the time points were analysed, and what effect the presence of the REE had on the growth rate. I think that may actually be more informative than just one relative value abstracted from the real dynamics of the biomass change of the microbial colony. If such data are available, it would be interesting to see them during the PhD defence, especially to see if the hormetic-like responses are only visible at one point or rate of growth.

I was also surprised by the following statement:

Interestingly, in some cases, the lowest concentrations induced stronger inhibitory responses than higher concentrations, a phenomenon (hormesis) previously reported in toxicity studies where stress responses are triggered at sub-lethal levels, resulting in a non-linear response.

According to the definition of ‘hormesis’, it is ‘a two-phased dose-response relationship where low-dose exposures have a beneficial effect, and high-dose exposures are either inhibitory to growth or toxic’ (Hochberg et al. Am. J. Resp. Crit. Care Med. 2021, 204(6), 632-641). Therefore, I would like to discuss if the following effect is indeed hormesis or an **inverse hormesis** (Pearce et al, PNAS 2014, 111 (167) 5998-6003), where a small dose results in a detrimental effect while higher doses do not.

Regardless of the nomenclature issues, the PhD Thesis in the following parts focuses on the efficiency of REE accumulation by the investigated strains. To determine that Mr Sunday had to establish a reliable technique to measure the dry mass of cells and quantify the amount of rare elements. Fig. 27 delivers information on the biomass of the four investigated strains, which were exposed to REE either before or after the biotransformation process. It looks like the effect is very much strain-dependent, and no general conclusion can be drawn from these observations. However, the results of ICP-MS measurements are a bit more coherent – regardless of strain or type of accumulated ion, the post-biotransformation cyanobacteria exhibited higher specific bioaccumulation (mg/g of biomass). The obtained efficiencies equal or exceed those reported in the literature, which is indeed impressive. The author provides two hypotheses that could explain the observed phenomenon (increased LPS production or modification of bacterial surface/change in bacterial metabolism induced by biotransformations) but does not venture into testing any of the proposed hypotheses. On the other hand, I appreciate the comparative efficiency analysis provided in Table 22, which definitely puts those data into context. **As photobiotransformation has an effect both on the biomass of cyanobacteria and their capacity for REE accumulation, I would like to ask the PhD candidate to point out the most robust strain that could guarantee the most efficient accumulation (in mg per volume of the treated water) of REE.**

Surprisingly, at page 109, the information on REE accumulation is broken by an interlude – a stray paragraph on biotransformation product DMHPP. I think this part is accidentally located in the wrong section (vicinity of 4.2.3 seems to be more appropriate).

The thesis is closed by a conclusion section which wraps up the whole project. The conclusion is a nice summary of the experimental observations conducted in the project, although they rarely go beyond the explorative nature and usually does not venture into a deeper explanation of the observed phenomena.

Finally, I have found in the thesis several smaller mistakes which should be corrected but do not necessarily require comments during the defence:

- In Table 1, where different classes of biocatalysts were summarised, the author attributed the lack of genetic modifiability to isolated or cell-free enzymes. At the moment, the majority of biocatalytic processes are conducted by recombinant enzymes; therefore, the statement is not true. As long as a convenient recombinant expression system is available, genetic modifications are available for all 3 subtypes.
- In Fig. 4d something is wrong with the figure – the phenyl ring is lost in the final product, and the arrangement of the double bonds in the ring of the intermediate is not very probable.

- Page 30 – the paragraph on the promiscuous lipase activity was accidentally copied into the section dedicated to alcohol dehydrogenases. It is again available below.
- Page 98 - *Azoarcus sp. strain EbN1* is already (for the last 15 years) called *Aromatoleum aromaticum*

Summary

After reading the thesis, I am able to state that Mr Sunday Ocholi Samson MSc demonstrates the general theoretical knowledge of the person applying for the doctoral degree in chemical sciences. The dissertation demonstrates the ability of the PhD candidate to conduct scientific work in an independent manner, which yielded original scientific results. As a result, I state that the thesis meets the requirements described in art. 187 ust. 1-2 ustawy z dnia 20 lipca 2018 r. Prawo o szkolnictwie wyższym i nauce (tj. Dz.U. z 2024 poz.1571). Although, as I stated in my review of the thesis, many parts require further clarification by the PhD candidate, I express my positive assessment of the thesis. I am sure that my questions can be properly addressed during the PhD defence. Therefore, I recommend its further proceedings by the PhD commission of the Scientific Discipline Council for Chemical Sciences of the Wrocław University of Science and Technology.

Signed by /
Podpisano przez:



Maciej Szaleniec

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Prof. dr hab. Maciej Szaleniec