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Review of the doctoral dissertation by Kamran Ayaz, MSc, entitled "Application of pure and mixed cultures of bacterial strains in synthetic dye removal processes – mechanisms and optimisation of process conditions" Supervisor: Dr Ewa Zablocka-Godlewska, Professor at the Silesian University of Technology

The basis for this review is a letter from Prof. Krzysztof Labus, PhD, Chairman of the Discipline Council for Environmental Engineering, Mining and Power Engineering at the Silesian University of Technology in Gliwice, dated 1st April 2026 (RIE-BD.512.17.2026) concerning the preparation of an assessment of the aforementioned doctoral thesis.

The doctoral dissertation under review by Mr Kamran Ayaz, MSc, was written in English, with the exception of the abstract, which is in both English and Polish.

At the outset of this review, it is worth emphasising that a large proportion of the results included in the doctoral dissertation have appeared in four publications, three of which are research articles published in renowned journals with high impact factors (IF) ranging from 3.4 to 7.2. I am referring in particular to the publications in *the Journal of Water Process Engineering* and *the Journal of Environmental Chemical Engineering*. These publications appeared between 2022 and 2026. In all the aforementioned publications, Mr Kamran Ayaz is the first author, and in two of the three scientific articles he is also the corresponding author, which indicates his key role in their creation.

Synthetic dyes released into the natural environment pose a huge threat to both the environment itself and to humans. These substances are generally persistent, toxic and carcinogenic, a significant proportion of them, as much as 60% of the initial dye mass, may not be utilised in technological processes and ends up in wastewater. The textile industry is prominent among other sectors due to its very high consumption of water and synthetic dyes. It is estimated that the majority of these, between 65 and 75%, are azo dyes. Chemical, physical and biological methods are used to remove azo dyes from wastewater. The latter are cheaper and more environmentally friendly than the other two groups, which is why they have attracted considerable interest for many decades among both scientists and practitioners. Although the processes of biological degradation of azo dyes under aerobic and anaerobic conditions are fairly well understood, new solutions are still being sought to develop innovative technologies or to advance existing ones. The aim of these efforts is to improve the efficiency of treating wastewater that contains synthetic dyes by utilising previously unknown capabilities of microorganisms, or by optimising process parameters, which is made possible by advances in physicochemical, biochemical and microbiological analysis techniques. I therefore believe that Mr Kamran Ayaz, in his work on the microbial degradation of synthetic dyes, and in particular the diazo dye Evans' Blue (EB, Direct Blue 53), chose an appropriate topic for his doctoral thesis. This choice is fully justified by scientific considerations, as well as the potential for future practical application of the research results obtained in the dissertation.

The primary objective of the reviewed doctoral dissertation was to develop and validate biological and bioelectrochemical strategies to accelerate the removal of synthetic dyes, particularly Evans' Blue, with the use of pure and mixed bacterial cultures, while determining the biochemical transformations that occur during the microbial degradation of these dyes. To achieve this objective, the doctoral dissertation was divided into four main research stages. The aim of the first stage was to evaluate the decolourisation efficiency of three dyes, namely diazo Evans' Blue (EB), triphenylmethane brilliant green (BG) and crystal violet (CV), in processes involving isolated strains of actinomycetes. The second stage aimed to determine the optimal process conditions, including time, temperature, electron donor, and the biodegradation of Evans' Blue by the electrochemically active *Shewanella oneidensis* MR-1 bacteria. Response Surface Methodology (RSM) was used for optimisation. The primary objective of the third stage was to evaluate the effectiveness of Evans' Blue

biodegradation in a dual-chamber microbial fuel cell (DCMFC) combined with the second stage of pollutant degradation, the so-called post-treatment, taking place under aerobic conditions with the participation of isolated actinomycete strains. Mixed cultures of microorganisms were used in the DCMFC, and their composition was estimated with the use of molecular biology techniques. The aim of the fourth stage of the research was to evaluate the biodegradation of Evans' Blue in a single-chamber microbial fuel cell (SCMFC) with a modified anode surface. The modifications were carried out in such a way as to achieve the best possible effect of microbial degradation of the dye with the use of a pure culture of the *Shewanella oneidensis* MR-1 bacteria. The author also mentioned the fifth stage, in which he intended to synthesise his findings and conclusions from the four research stages mentioned above and describe the optimal method, or even a strategy, for removing Evans' Blue from textile wastewater. The research hypothesis was formulated on the basis of the research objectives. It is comprehensive and divided into five parts. Each of these corresponds to the five sub-objectives of the doctoral dissertation mentioned earlier. I consider the scope of the work undertaken by the PhD candidate to be extensive and appropriate for a doctoral thesis. The research conducted is comprehensive and demonstrates scientific novelty, particularly the studies conducted using a two-chamber microbial fuel cell combined with an additional stage of pollutant degradation under aerobic conditions. All the research conducted by the PhD candidate serves to address the scientifically recognised but still challenging problem of decolourisation of textile wastewater with the use of biological methods. The research results can be utilised in practice to optimise the microbial degradation of azo dyes. I would like to emphasise that the PhD candidate successfully verified the hypothesis put forward by conducting extensive research, analysing the results and drawing appropriate conclusions.

The doctoral dissertation is divided into 7 chapters, preceded by abstracts, a list of the author's own publications on the subject of the doctoral thesis, and a list of abbreviations and variables used in the thesis. The abstracts are detailed, and I have rarely come across doctoral dissertation abstracts exceeding three typed pages. The list of abbreviations and variables is comprehensive and clearly presented. In Chapter 6, the PhD candidate included appendices, and the final chapter, Chapter 7, contains a list of cited publications. I believe that the appendices should be placed at the very end of the dissertation and should not constitute a separate chapter. Apart from this one organisational comment, I consider the proposed structure of the doctoral dissertation to be logical and appropriate for this type of academic work.

In my opinion, the doctoral candidate correctly compiled and illustrated the research results obtained in both graphs and the tabular form. The doctoral dissertation under review contains 36 figures and 10 tables. In addition, the author included 11 figures and 4 tables in the appendix. In my opinion, all the tables are clear and easy to understand. However, I believe that some of the figures could be better prepared in terms of layout. Some figures, such as the multi-panel Figure 3.5 or Figure 3.11, are too small, which makes them difficult to analyse for the reader. Figure 3.15 should be reformatted, as it is illegible, although I understand that the results of the analysis of the microbial composition are not easy to visualise. In general, the captions for the figures and tables were edited correctly, but in my opinion the author used a font that is too small, which does not make reading easier for the recipient. The vast majority of the figures (with the exception of, for example, Figs. 3.2 and 3.17) included in the doctoral dissertation are taken directly from scientific articles published by the PhD candidate, but I would like to emphasise that the author indicates the source of each illustration in the caption. References in the text to figures and tables are correct and were placed in the appropriate locations.

Apart from the editorial comments mentioned above, I consider the doctoral dissertation to be well prepared in terms of editing, particularly with regard to the layout of the text. The numbering of chapters, subchapters, figures and tables is, in my view, correct. The same applies to the numbering of equations. However, the regression equation on page 91 should be numbered, or the number of the general form of this equation from page 71 should be cited. Abbreviations and symbols are explained in the list at the beginning of the thesis, as well as upon their first appearance in the text, which aids in reading the thesis. The broad, interdisciplinary nature of the research conducted as part of the doctoral dissertation required the PhD candidate to use and master numerous symbols and abbreviations, of which, according to my estimates, there were over a hundred. I believe the PhD candidate coped very well with this task. The PhD candidate's work is not entirely free of minor editorial errors, such as the absence of superscripts or subscripts in some places, or unnecessary spaces, but these do not affect the value of the dissertation or my assessment of it. In formal terms, I assess the

dissertation as well prepared.

The language used by the author in the dissertation is, in my opinion, correct. It is scientific language, appropriate for a doctoral dissertation. I consider that the PhD candidate does not use the term 'dye load' most accurately in a few places (e.g. on p. 80), as this term should relate the dye concentration, for example, the concentration of microbial biomass and time; however, the term actually refers to the dye concentration, which is why I believe it should be named as such. As I have already mentioned, this was not a common practice throughout the thesis, so this is merely a minor comment on the terminology used. Overall, I found the doctoral dissertation to be written in clear, substantive and scholarly language. The abstract in Polish contains stylistic errors and unfortunate phrasing, but this is understandable, as the author does not know and is not required to know the Polish language.

The literature review was titled 'Introduction' by the PhD candidate and forms the first chapter of the thesis. The author divided it into ten sub-chapters, in which he characterises, among other things, several groups of synthetic dyes, including the azo and triphenylmethane dyes that are the subject of experimental research; he discusses methods of biological degradation of synthetic dyes, factors influencing these processes, and presents microbial fuel cells as a potential solution for the decolourisation of wastewater. Throughout Chapter 1, the author endeavours to justify the need for further research into the removal of synthetic dyes, particularly azo dyes, from wastewater. This demonstrates the PhD candidate's maturity and scientific awareness; amidst a large number of scientific reports on the issues he chose as the subject of his PhD, he endeavours to find — and ultimately does find — a suitable research niche. Undoubtedly, both the selection of topics and the choice of literature sources were appropriate and helped the PhD candidate to formulate the research problem (sub-chapter 1.9), and subsequently the aim and scope of the work, as well as the research hypothesis (sub-chapter 1.10).

Mr Kamran Ayaz, MSc, drew on a total of approximately 313 English-language literature sources throughout the thesis, the majority of which were academic articles published in reputable journals. Such a large number of cited publications indicates the PhD candidate's inquisitiveness and also attests to a thorough analysis of the literature. I would also like to emphasise that the vast majority of the cited works – over three-quarters (approximately 77%) – were published between 2016 and 2026. This means that the PhD candidate drew upon the latest, up-to-date scientific achievements related to the topic of his thesis. I consider the selection of literature sources to be comprehensive, accurate and up-to-date. The list of cited literature sources presented in Chapter 7 should have been prepared more carefully. In at least a few of the cited sources, the authors' surnames are missing. The bibliography also contains editorial errors, e.g. the year of publication is sometimes written in bold and sometimes not. The method of citing publications in the text is inconsistent. Usually, these consist of the authors' surnames and the year of publication, but in some cases the authors' surnames are preceded by the first letter of their first names. I believe that the authors' surnames and the year of publication alone would suffice.

In the second chapter of the doctoral dissertation under review, the PhD candidate discussed the research materials and methods used in the experimental work. The topics were divided into 16 subchapters and, in my opinion, presented appropriately in a conventional manner. Here, the PhD candidate described, among other things, the synthetic dyes selected for the research, the pure and mixed cultures of microorganisms he used, the culture media and other substances employed in the laboratory studies, as well as the equipment used, including microbiological fuel cells, methods of physicochemical analysis (e.g. liquid and gas chromatography combined with mass spectrometry), microbiological analyses (e.g. PCR, 16S rRNA sequencing on the Illumina MiSeq platform, determination of bacterial viability with the use of a laser confocal microscope) and electrochemical methods. An overview of these analytical methods highlights the interdisciplinary nature of the experimental work and the need for the PhD candidate to master knowledge from various scientific fields, including microbiology, molecular biology, chemistry and electrochemistry, as well as the ability to operate the various instruments required for the analysis and evaluation of the course of microbial dye degradation processes. To process the data, the candidate utilised software available through the Python Software Foundation, as well as MS Excel, OriginPro (OriginLab), ImageJ visual analysis suite, and software for the analysis and simulation of impedance spectra. The skilful combination of various experimental techniques, including bioelectrochemistry, and analytical methods indicates the high scientific standard of the work and, in my opinion, deserves great recognition.

The research results and their discussion were combined into a single chapter (Chapter 3) and constitute the most extensive part of the doctoral dissertation under review. I have no objections to the combination of results and discussion. I believe that this approach is frequently adopted in scientific works in environmental engineering and was a sound decision on the part of the PhD candidate. Chapter 3 was divided into four subchapters, the content of which corresponds to the first four objectives of the thesis. I have already listed all the objectives of the dissertation earlier in this review, so I shall not repeat them here. However, I felt that a fifth sub-chapter was missing from Chapter 3, in which the author, as announced in the objectives of the thesis, would have integrated and compared all his findings from the experimental work conducted. Of course, a summary of these findings is included in the subsequent chapter of the thesis, namely in the conclusions, and therefore the objective has been fully achieved. In my view, a well-prepared tabular overview of the results obtained in the dissertation regarding the removal of Evans' Blue, which compares the degree of removal of this dye using various methods, different microorganisms, different experimental set-ups and process conditions, would be very valuable and would also assist the PhD candidate in formulating conclusions that would directly follow from such a comparison. This is merely a suggestion for the PhD candidate, which he may wish to take into account in the future.

Looking at the presentation of the research results as a whole, I consider that they were very well presented in the figures and tables. In each of the sub-chapters, the author made an effort to justify the sequence of the work performed, pointing out the advantages and disadvantages, or rather the limitations, of the dye removal methods used. The content was organised in a logical, substantive manner and to a high academic standard. The discussion of the results was thorough. The PhD candidate compared his results with numerous studies published by other research teams, highlighting similarities and differences, and in the case of the latter, sought to explain their causes.

In the conclusions (Chapter 4), the PhD candidate began by referring to the aim of the thesis, noting that it had been achieved, which I, as a reviewer, confirm without any doubt. However, he did not refer directly to the research hypotheses put forward. Admittedly, these were correlated with the aims of the thesis; perhaps this is why the PhD candidate did not feel the need to do so. In the subsequent paragraphs of the conclusions, the author highlights the most significant achievements from each of the four stages of the experimental work. In my view, he does so clearly, emphasising the originality of the research conducted and his own contribution to the development of environmental engineering.

In my opinion the PhD candidate demonstrated that bioelectrochemical methods can be successfully applied to the removal of azo dyes, but do not always lead to the complete mineralisation of these dyes. Therefore, a second stage of degradation of the intermediate products, mainly aromatic amines, is required, which, as the PhD candidate showed, can be carried out with the involvement of *Actinomycetes*. The PhD candidate demonstrated that the choice of the microbial fuel cell, its design (single- or dual-chamber cell), the material from which the electrodes are constructed, the selection of microorganisms immobilised on the electrodes (pure or mixed cultures of microorganisms, source of origin of the microorganisms), and the process conditions, including the initial dye concentration, are extremely important. I consider the formulation of guidelines for optimising the removal and biodegradation of synthetic dyes using microbial fuel cells to be the PhD candidate's most significant achievement that expands knowledge in the field of environmental engineering. Mr Kamran Ayaz, MSc, demonstrated that it is possible to remove and at least partially mineralise the degradation products of Evans' Blue in microbial fuel cells at a high concentration of this dye, amounting to 100 mg/l or higher, within a timeframe not exceeding one day. These achievements can be utilised in the design of microbial fuel cells for the treatment of textile wastewater.

I have several substantive comments on the reviewed dissertation, which I have divided into four groups. I would ask the PhD candidate to address these during the defence of his doctoral thesis. They are as follows.

1. The first of these concerns the results of the biodegradation of Evans' Blue by *Shewanella oneidensis* MR-1 bacteria and their statistical analysis in particular. Could you please explain how the analysis of variance (ANOVA) was applied to assess the fit of the model predicting the degree of dye removal (equation xi), and whether this was a one-way or two-way ANOVA? How should we interpret the fact that the parameter D representing temperature is significant, but D^2 is not significant (Table 3-1)? When comparing the removal values predicted by the model (equation xi) with the actual values (Table S6-1, pages 158–159), significant

differences are evident, suggesting that the model may not be the best fit. Furthermore, some of the estimated removal efficiency values are negative, and some exceed 100%.

2. The second set of questions concerns the studies conducted in DCMFC which involved post-treatment under aerobic conditions. In Chapter 3.3.2 the PhD candidate writes that the results obtained, and in particular the analysis of absorption spectra, indicate the complete biodegradation of aromatic amines and other degradation products formed under anaerobic conditions. Figure 3.11a) shows absorption spectra in the wavelength range from approximately 300 to 1000 nm, whereas for an aromatic ring the absorption maximum is below 300 nm. Therefore, are the spectra shown in Fig. 3.11a) sufficient evidence of the complete biodegradation of aromatic amines, or does the PhD candidate have other spectra at his disposal which he did not include in his doctoral thesis?

3. Further questions relate to the research conducted in SCMFC. Why were concentrations of Evans' Blue of 150, 300 and 400 mg/l chosen here, rather than testing a concentration of 100 mg/l? Had this been selected as one of the concentrations, it would have been easier to compare the biodegradation efficiency of Evans' Blue with the results obtained in previous stages of the experimental work, and it would have also been easier to compare SCMFC with DCMFC. In my opinion, the PhD student correctly described the changes in the degree of Evans' Blue removal over time in the SCMFC using pseudo-first-order kinetics (Fig. 3.25 c)), but this figure or a separate table should show the approximation equations and the values of the correlation coefficient R^2 for each concentration tested. COD removal values not exceeding 90% (Fig. 3.25 d)) indicate that some of the Evans' Blue degradation products have not undergone biodegradation. This is also indicated by the results of the GC-MS chromatographic analysis. Therefore, should an additional removal stage for these contaminants not be applied?

4. Finally, I would like to ask the PhD student to summarise and, referring to the main objective of the thesis, to suggest what the strategy or approach should be for the removal of synthetic dyes, particularly azo dyes, from wastewater. Which single- or two-chamber microbial fuel cell, made of what materials and using which microorganisms, would, in the PhD candidate's opinion, work best for the decolourisation of azo dyes and the biodegradation of their degradation products, mainly aromatic amines, on a full-scale basis.

Despite the comments and questions raised above, I consider the doctoral dissertation under review to be, as a whole, a work of very high quality. It successfully meets the requirements for doctoral theses and makes an original contribution to the development of environmental engineering, while the comments presented are largely of a speculative nature.

In conclusion, I find that Mr Kamran Ayaz, MSc, submitted a doctoral dissertation that meets the requirements specified in the relevant provisions of the Act of 20 July 2018 - Law on Higher Education and Science (Journal of Laws of 2024, item 1571, as amended). I therefore request the Discipline Council for Environmental Engineering, Mining and Power Engineering at the Silesian University of Technology in Gliwice to admit Mr Kamran Ayaz, MSc, to the subsequent stages of the doctoral procedure, as provided for by the regulations.

Taking into account the very high quality of the research conducted, its interdisciplinary nature and the publication of a large part of the research results in renowned scientific journals with a high impact factor ranging from 3.4 to 7.2, e.g. the research article published in *the Journal of Environmental Chemical Engineering,* which has an IF of 7.2, I hereby submit a request for this doctoral dissertation to be awarded a distinction.

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