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REVIEW

of the doctoral dissertation by Kamran Ayaz, MSc, Eng. entitled
„Application of Pure and Mixed Cultures of Bacteria Strains in Synthetic Dyes Removal Processes - Mechanisms and Optimisation of Process Conditions"
Supervisor: dr hab. Ewa Zabłocka-Godlewska, prof. at SUT

The doctoral dissertation submitted for review was prepared at the Faculty of Energy and Environmental Engineering at the Silesian University of Technology, within the Department of Air Protection, under the academic supervision of Dr hab. Ewa Zabłocka-Godlewska, Professor at the Silesian University of Technology. This review has been prepared in accordance with the applicable legal provisions, in particular taking into account the requirements defined in the Act of 20 July 2018 the Law on Higher Education and Science (consolidated text, Journal of Laws 2024, item 1571, as amended), as well as the standards adopted for the assessment of doctoral dissertations in the discipline of environmental engineering, mining and power engineering. The review was based on the copy of the doctoral dissertation provided.

Subject matter of the dissertation and topicality of the research problem

Synthetic azo dyes represent one of the most serious contemporary threats to the quality of surface water and industrial effluent. The textile industry discharges an estimated 10 to 15 per cent of the total quantity of dyes used into the environment in the form of untreated or inadequately treated effluent. Conventional municipal wastewater treatment plants do not remove these compounds because their chemical stability and anionic nature mean that conventional biological, physical and chemical methods are only of limited effectiveness.

The PhD student has accurately identified one of the key research problems: the mere loss of colour in wastewater, as assessed by the UV-Vis method, is an insufficient criterion for treatment effectiveness. Mechanisms of biosorption, physical adsorption and partial cleavage of the chromophore can lead to apparent decolourisation without any actual chemical transformation. The author has accurately articulated this problem, pointing out that effective treatment must result in a chemically confirmed transformation. This observation forms the core around which the entire research project is structured. The choice of Evans Blue as the dye is fully justified, as it is one of the most difficult anionic diazo dyes to remove. On the other hand, the use of microbial fuel cells (MFCs) for the simultaneous treatment of wastewater and recovery of electrical energy, is in line with the mainstream of green biotechnology. The subject matter of the dissertation aligns with the current priorities of the Horizon Europe programme in the areas of clean water technologies and industrial biotechnology, which underscores its practical value.

Formal and substantive assessment of the dissertation

The dissertation comprises 198 A4 pages. The main body of the dissertation, comprising the introduction, materials and methods, results and discussion, conclusions, directions for future research and supplementary materials, is set out on pages 1 to 168, followed by the bibliography. The dissertation was written in English. The attached abstract in Polish meets the formal requirements defined in the Act. The work has a clear, logically structured layout comprising seven main chapters. The author has designed a multi-stage experiment in which each part has a clearly defined function. The increasing complexity of the experimental set-ups, ranging from pure cultures on solid media, through optimised model cultures, to integrated bioelectrochemical systems, demonstrates a well-considered research logic. The introductory chapter serves as a comprehensive literature review and concludes with a precise formulation of the objectives and hypotheses. The 'Materials and Methods' chapter is very well structured and can be regarded as exemplary from the point of view of research reproducibility. The presentation and discussion of the results covers four experimental modules. Each successive stage is logically designed. The dissertation is not a compilation of four separate research procedures, but a coherent programme. The dissertation is rounded off by the chapters on conclusions, directions for future research and supplementary materials.

The objectives of the dissertation have been formulated precisely and with a high level of methodological awareness. The aim of the work was to develop and integrate methods for the removal of synthetic dyes, with particular emphasis on Evans Blue. The specific objectives and hypotheses were stated explicitly, rather than being reconstructed from the results, which is a hallmark of a well-planned, mature research programme. The literature review is comprehensive, well-structured and focused on issues closely related to the research topic. The author has demonstrated an in-depth understanding of the biochemical mechanisms of dye decolourisation, making a precise distinction between enzymatic biotransformation and electrochemical processes, biosorption and bioprecipitation. Chapters 1.5–1.6 are particularly valuable, discussing the mechanisms of decolourisation and dye degradation, as well as the factors influencing the efficiency of degradation. The author has accurately identified a research gap: the lack of studies combining the selection of microorganisms, mechanistic confirmation of chemical transformation, statistical optimisation and bioelectrochemical control of the process. The author has drawn on up-to-date literature (predominantly studies from 2022–2025). The bibliography is comprehensive and correctly formatted. A minor shortcoming is the rather cursory discussion of the aspects of scaling MFC systems and the economics of the process; however, given the scope and quality of the work, this reservation is merely hypothetical.

The methodological framework presented in Chapter 2 is impressive both in terms of its scope and the soundness of the techniques employed. Particularly noteworthy is the fact that the methods were not selected at random, but form a coherent body of evidence: UV-Vis spectroscopy to track chromophore depletion, HPLC to assess the parent compound degradation, GC-MS to identify transformation products, CV and EIS to characterise the biointerface, FTIR and XPS to confirm the stages of anode modification, SEM and CLSM to assess the morphology and viability of the biofilm, and 16S rRNA sequencing to interpret the function of the microorganisms. Such an integrated approach is rarely encountered in doctoral theses and deserves a very high rating. Particularly valuable is the use of RSM in the form of a full factorial design (four factors: time, dye concentration, carbon source concentration, temperature) to optimise the conditions for the biodegradation of Evans Blue by S. *oneidensis* MR-1. The author did not limit himself to optimising a single parameter, but simultaneously analysed main effects and interactions. I positively evaluate the execution of an independent validation experiment under optimal conditions. Measurements were conducted in triplicate and reported as mean ± SD. Confirmation of the actual chemical transformation of Evans Blue by S. *oneidensis* MR-1 using HPLC (disappearance of the parent dye signal) and GC-MS (identification of transformation products, including aromatic intermediates and low-molecular-weight 3-methylbutanoic and octanoic acids) provides strong evidence of biodegradation, rather than merely biosorption or physical adsorption. This is one of the most significant contributions to methodological rigour.

The main point of criticism is the incomplete identification of all polar, sulphonated intermediate products in the SCMFC–GC-MS system, which fails to capture the highly polar fraction. There is also a lack of a complete TOC/COD balance to confirm complete mineralisation. The author himself identifies these limitations and highlights them as areas for future research.

The evaluation of the results obtained and their discussion were organised into four modules. Module I (Chapter 3.1) presents the results of an assessment of the use of immobilised actinomycetes for the removal of azo dyes. The results of the actinomycetes screening are valuable not because they yielded the best results, but because they demonstrated why a simple model is insufficient. The demonstration of the role of physical sorption on straw supports, the partial desorption of the dye, and the disproportionately high efficiency of the 'straw + biomass' system compared to each component individually, highlights the interpretative pitfall of relying solely on UV-Vis spectroscopy.

Module II (Chapter 3.2). This is a very important chapter of the dissertation. The achievement of over 99% decolourisation of Evans Blue by S. *oneidensis* MR-1 under optimised conditions, combined with confirmation of the disappearance of the parent dye signal in HPLC and identification of the degradation pathway via GC-MS, provides the first such comprehensive evidence in the literature of the actual biodegradation of Evans Blue by a pure bacterial culture. Time and temperature were identified as the factors with the strongest influence. Reducing the process time from 14 days (Module I) to approximately 3 days represents a very significant improvement in efficiency. The RSM operating window (time: 24–72 h, dye concentration: 25–100 mg/dm³, carbon source: 250–1000 mg/dm³, temperature: 25–37°C) has direct practical significance.

Module III (Chapter 3.3). The transition to a DCMFC system coupled with an aerobic bioreactor is well justified. The results obtained are very good: decolourisation efficiency ranging from 90 ± 2% to 98 ± 1.9% for 100 mg/dm³ of Evans Blue and from 79 ± 2% to 87 ± 1% for 200 mg/dm³ over a period of 20–24 hours. A maximum power density of 119.2 mW/m² confirms that the system not only removes the contaminant but actually functions as an MFC. The identification of key microorganism species *(Geobacter* responsible for electron transfer; *Actinomarinicola* and *Thermochromatium* for the aerobic stage) based on 16S rRNA sequencing provides valuable information regarding the mechanisms of the process. The author correctly points out the cost of the improvement achieved: a long acclimatisation phase (approx. 30 days) and greater complexity of the process.

Module IV (Chapter 3.4). This is the most innovative aspect of the work. Here, the author does not alter either the microorganism or the basic concept of the process, but deliberately designs the microorganism–electrode phase boundary. It has been demonstrated that modifying carbon cloth (CC) with polyaniline (PANI), glucose and gelatine (MCC electrode) leads to a 50-fold increase in CSC (174.9 ± 10.2 vs. 3.3 ± 0.3 mC/cm²), biofilm coverage of 90.2 ± 0.2 per cent and a reduction in the Evans Blue decolourisation time to 13 hours at 150 mg/dm³, whilst maintaining efficacy at concentrations previously considered inhibitory (up to 450 mg/dm³). A key finding is that glucose located near the anode and stabilised by gelatine promotes the formation of an electrode-coupled biofilm. The results of FTIR, XPS and SEM analyses provide strong support for the successive stages of surface functionalisation, while EIS and CV correlate these changes with improvements in bioelectrochemical parameters.

The discussion is conducted thoroughly. The author has compared his own results with the literature, exercising interpretative caution by describing the proposed Evans Blue degradation pathway as a 'functional class pathway' rather than a fully identified pathway. It is worth noting that no high risk of aromatic amine formation was demonstrated, which is a significant factor in assessing the environmental safety of the process.

The conclusions have been formulated concisely and precisely. The author has presented a coherent picture of the process, highlighting the progressive improvement in three key parameters: decolourisation time (from

14 days to 13 hours), initial dye concentration (from 100 to 450 mg/dm³) and the degree to which chemical transformation has been confirmed. The conclusions are not merely a list of numerical values, but contain a substantive interpretation that takes into account the mechanisms of the processes.

The chapter on future research directions is breviloquent and exact. The author identifies four main areas: optimisation of the reactor and the MFC biointerface, a complete carbon balance and assessment of the toxicity of the final products, tests using actual textile wastewater, and scaling up the system. These are realistic priorities, arising directly from the limitations identified in the study itself. In my view, the author takes a realistic view of the developed technology – its application potential is high, but its readiness for implementation remains at the laboratory and development stage.

The dissertation is written to a standard appropriate for JCR-listed journals, as confirmed by the publication of three articles (IF ≥ 3.4). Technical and biological terminology is used consistently and correctly. The graphical presentation of the work is meticulous; mechanistic diagrams, such as the proposed degradation pathway of Evans Blue in the SCMFC-CC, integrate data from various analytical techniques into clear diagrams.

In some instances, the descriptive narrative is somewhat too elaborate at the expense of concise quantitative conclusions. The dissertation benefits where the author consistently distinguishes between the concepts of decolourisation, removal, degradation and mineralisation; however, this linguistic rigour is not maintained throughout.

In my view, the dissertation makes a significant and original contribution in four areas.

Firstly, this work is the first in the literature to provide comprehensive documentation, supported by HPLC and GC-MS analysis, of the biodegradation (rather than merely decolourisation) of the azo dye Evans Blue by a pure culture of S. *oneidensis* MR-1. This represents an interpretative and methodological breakthrough compared with previous studies, which generally relied solely on UV-Vis spectroscopy.

Secondly, the implementation of a DCMFC system with an oxygen-based post-treatment phase using a mixed culture of actinomycetes isolated from garden compost is an innovative approach. It has been demonstrated for the first time that a mixed culture of actinomycetes can fulfil this role in a DCMFC system. `

Thirdly, the demonstration that modification of the bioanode with a PANI-glucose-gelatine (MCC) leads to a 50-fold increase in CSC, improved biofilm adhesion and viability, and enables the degradation of Evans Blue at concentrations previously considered inhibitory (up to 450 mg/dm³), is a significant engineering achievement with scalability potential.

Fourthly, the methodological and interpretative systematisation of various pathways of colour removal (biosorption versus biodegradation) in systems with immobilised biomass, supported by evidence of dye desorption, constitutes a valuable methodological contribution with broader applications in environmental engineering.

The novelty of this work lies primarily in its integrative nature: the combination of microorganism selection, chemical validation, statistical optimisation and bioelectrochemical biointerface engineering within a single coherent programme is rarely found in doctoral theses in this field.

Critical comments and recommendations

I regard the following comments as constructive feedback rather than shortcomings that undermine the value of the dissertation:

1. The absence of a complete mass balance and full identification of polar, sulphonated intermediate products in the section on SCMFC. GC-MS does not detect highly polar fractions. The analytical work should be extended to include LC-MS/MS, TOC/COD balances for all major stages, and at least one standardised toxicity test before and after treatment.

2. There is a lack of validation of the long-term stability of the MCC layer during continuous operation and under hydraulic loads closer to those encountered in actual operation. It would be advisable to conduct continuous tests and assess fouling, ageing and resistance to changes in salinity and pH.
3. The studies were carried out on model solutions, not on actual textile effluent. It would be valuable to conduct tests using actual effluent containing salts, surfactants and dye mixtures.
4. The separation of the contributions of individual functional groups in mixed-culture systems (DCMFC) was insufficient. The 16S rRNA data should be linked to functional analysis or an electron budget.
5. There was no formal techno-economic analysis (CAPEX/OPEX, unit treatment costs). It would be advisable to estimate material costs, HRT and scaling requirements before proceeding to the pilot phase.

Having read the content of the dissertation, the following questions arise, which the PhD candidate should address during the defence of their dissertation:

1. Is there not a concern that the Evans Blue transformation products identified by GC-MS in the SCMFC stage (3-methylbutanoic and octanoic acids) are products of secondary metabolism of culture medium components (e.g. acetate), rather than direct metabolites of Evans Blue degradation? How does the author verify their origin?
2. The 16S rRNA analysis in the DCMFC stage revealed a significant proportion *of Thermochromatium* (4.82%) in the anode consortium. How does the author interpret the presence of this photosynthetic sulphate-reducing bacterium under anaerobic conditions without an external light source?
3. To what extent can the results from Module I (use *of Actinomycetes*) be parameterised and utilised for reactor design? Which key engineering parameters (substrate loading, biomass retention) would the author consider most important when scaling up the results?
4. What are the main barriers to transferring SCMFC-MCC technology to real-world textile wastewater conditions (complex matrix, variable pH and salinity), and how might, or might not, modification of the bioanode address these?

Conclusion

The PhD dissertation by Kamran Ayaz, MSc (Eng), entitled 'Application of Pure and Mixed Cultures of Bacterial Strains in Synthetic Dye Removal Processes: Mechanisms and Optimisation of Process Conditions' is a study of a very high scientific standard, characterised by well-thought-out research logic, a broad and sound methodological approach, an original experimental contribution and interpretative rigour. It combines the quantitative accuracy of data presentation with a mature mechanistic interpretation.

The dissertation meets all the criteria specified in Article 187(1) of the Act of 20 July 2018 – Law on Higher Education and Science: it presents an original solution to a scientific problem, demonstrates the PhD candidate's general theoretical knowledge in the scientific discipline, and demonstrates the ability to conduct independent scientific research.

In my opinion, the dissertation also possesses distinguishing features: very good coherence of the research programme and clear potential for development towards pilot studies. The comments and questions raised do not detract from the positive assessment of the dissertation, but rather point to areas worthy of further scientific work.

In view of the above, I conclude that the doctoral dissertation by Kamran Ayaz, MSc (Eng), meets the requirements for doctoral theses in accordance with the applicable legal provisions, and I recommend that the doctoral candidate be admitted to a public defence. At the same time, I recommend that the dissertation be awarded a distinction.

[handwritten signature] Ewa Wiśniowska