Częstochowa, 14 April 2026

Dr Ewa Wiśniowska, Eng., Prof. PCz.
Department of Sanitary Networks and Installations
Faculty of Infrastructure and Environment
Częstochowa University of Technology

REVIEW OF THE DOCTORAL DISSERTATION

"Determination of the mechanism and optimisation of the conditions of the process of removing colored aromatic compounds by selected Basidiomycota"
Author: Ruchi Manishkumar Upadhyay, M.Sc.
Supervisor: Prof. Wioletta Przystaś, PhD, Eng.
Unit: Silesian University of Technology, Faculty of Energy and Environmental Engineering

This review of the doctoral dissertation by Ruchi Manishkumar Upadhyay, M.Sc., was prepared at the request of the Discipline Council for Environmental Engineering, Mining and Power Energineering, Mining and Energy, based on the submitted manuscript of the dissertation entitled: *"Determination of the mechanism and optimisation of the conditions of the process of removing coloured aromatic compounds by selected Basidiomycota"*, conducted at the Faculty of Energy and Environmental Engineering of the Silesian University of Technology, under the supervision of Prof. Wioletta Przystaś, PhD, Eng.

This review has been prepared in accordance with applicable legal provisions, in particular taking into account the requirements stipulated in the Act of 20 July 2018 the Law on Higher Education and Science (i.e. 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 submitted copy of the doctoral dissertation, including its descriptive section and the results of the PhD candidate's own research.

The doctoral dissertation submitted for review addresses a topic of high scientific relevance and significant practical importance, namely the removal of synthetic dyes from the aquatic environment using biological methods. This issue falls within one of the key areas of contemporary environmental engineering, focused on the development of effective, low-emission and sustainable wastewater treatment technologies. In an era of tightening environmental regulations and growing pressure to reduce industry's negative impact on the natural environment, issues related to the elimination of persistent organic pollutants, which include synthetic dyes, are taking on particular significance.

The author accurately and convincingly demonstrates that the problem of the presence of dyes in the environment is global in nature and stems both from the very large scale of their production and the insufficient effectiveness of conventional industrial wastewater treatment methods. Due to their complex chemical structure, high stability and resistance to biodegradation, synthetic dyes constitute a group of pollutants that is particularly difficult to remove. Their presence in the aquatic environment leads not only to a deterioration in water quality through changes in optical properties, but also to a range of negative ecological effects, including the inhibition of photosynthesis, disruption of food chains, and potential toxic and mutagenic effects on living organisms. In this context, the search for alternative methods of dye removal based on biological processes should be considered a fully justified area of research. Biological methods, including in particular the use of microorganisms capable of biodegrading xenobiotic compounds, represent a promising alternative to traditional physicochemical methods, which are often associated with high operating costs and the generation of secondary pollutants. The use of white rot fungi, characterised by a broad spectrum of enzymatic activity and the ability to degrade complex aromatic compounds, is in line with current trends in the development of environmental biotechnology, which focus on the use of natural metabolic mechanisms in environmental remediation processes.

It is particularly worth emphasising that the author does not limit herself solely to assessing the effectiveness of the dye removal process, but attempts to understand the mechanisms underlying it, which significantly enhances the scientific value of the dissertation. Unlike many strictly applied works, which focus mainly on achieving high pollutant removal efficiency, the author makes an effort to understand the fundamental processes occurring at the biochemical and cellular levels.

The scope of the work encompasses both the optimisation of process conditions (including an analysis of the impact of key operating parameters), an attempt to identify the mechanisms of biodegradation and biosorption, an assessment of the enzymatic activity of biological systems, ecotoxicological studies to evaluate the environmental impact of the process, as well as omics analyses (transcriptomic and proteomic) aimed at capturing the response of organisms at the molecular level. Such a multi-level research approach, encompassing both the macroscopic scale (process efficiency) and the microscopic scale (molecular mechanisms), should be regarded as consistent with current trends in research development within environmental engineering and biotechnology, where increasing emphasis is placed on the integration of process and biological data. This approach also demonstrates the author's high level of research awareness and her commitment to a comprehensive understanding of the problem under analysis. It should also be emphasised that the inclusion of ecotoxicological studies constitutes a significant complement to classical analyses of process efficiency, allowing for an assessment of the actual impact of the achieved results on the environment, rather than merely on technological parameters. However, a broad and multi-faceted approach to the problem carries with it the natural risk of diluting the main focus of the analysis and resulting in an uneven level of treatment of individual issues. It is evident from the work that not all research areas have been developed with the same methodological and interpretative depth, which stems both from their diverse nature and from experimental limitations. This applies in particular to the analysis of the mechanisms of the processes taking place, based on omics analyses, in which – despite the great cognitive potential – the scope and method of data processing do not always allow for the formulation of unambiguous and fully substantiated conclusions.

The research methodology has been developed in detail and encompasses a wide range of experimental techniques, which should be viewed positively both in terms of the comprehensiveness of the research approach and the potential for a multifaceted interpretation of the results obtained. The author utilises two species of fungi belonging to the group of so-called white rot fungi, namely *Trametes versicolor* and *Pleurotus ostreatus*, which are widely recognised as organisms with high degradation potential for aromatic compounds. The choice of these biological models should be considered justified, as it allows for a comparison of the degradation capabilities of organisms differing in their enzymatic profile and xenobiotic metabolism strategy. The study analyses the ability of the tested strains to remove dyes representing different chemical classes, which significantly broadens the scope of the research. This approach enables an assessment of the versatility of the method used and the identification of potential relationships between the chemical structure of the dye and the mechanism of its removal. Particular attention should be paid to the consideration of both biodegradation and biosorption as co-occurring mechanisms of pollutant removal. This approach is consistent with the current state of knowledge, according to which the removal of dyes by microorganisms is a complex, multi-stage process, involving both physicochemical processes (adsorption on the surface of biomass) and enzymatic reactions leading to the transformation of compounds. Including both these mechanisms in the analysis allows for a more realistic representation of the process and avoids oversimplification.

Another key element of the methodology is the wide range of process parameters analysed. The author investigates the influence of key operational factors, such as pH, temperature, the type and availability of carbon and nitrogen sources, culture conditions (static and dynamic), as well as the method of biomass immobilisation. This approach enables the identification of conditions conducive to maximising process efficiency and provides a basis for potential technological optimisation. In particular, taking into account different forms of biomass (free, immobilised and inactivated) allows for the assessment of the influence of the system's physical and biological properties on the course of the dye removal process. The use of a multi-stage approach, encompassing both laboratory-scale studies and attempts to verify them under conditions close to real-world scenarios (bioreactor systems), also deserves positive recognition. This approach demonstrates an attempt to link basic research with its potential practical application, which is particularly important in the field of environmental engineering. The research results presented in the dissertation are comprehensive, multifaceted and, for the most part, properly documented, which attests to a significant amount of experimental work and consistency in the implementation of the adopted research programme. The author demonstrated a high removal efficiency of the tested dyes, reaching approximately 96% under conditions deemed optimal, which should be regarded as a very good result and indicative of the significant potential of the biological systems employed in the treatment of wastewater containing coloured compounds. In particular, the results obtained confirm that white rot fungi can be an effective tool in the removal of compounds with a complex aromatic structure, which are difficult to eliminate using conventional methods.

At the same time, it should be noted that despite the wide range of methods employed, not all elements of the methodology have been developed in equal detail, and in some cases there is a lack of full consistency between the scope of the experiment and the conclusions drawn. This applies in particular to the methods used to distinguish between process mechanisms and to link experimental results with molecular analyses, which will be discussed in more detail later in this review. It should also be emphasised that the interpretation of the results obtained requires greater caution and a clearer distinction between the observed process effects and the actual chemical transformations occurring in the system. The primary indicator of process efficiency adopted in this study is the degree of decolourisation determined by the UV-Vis spectrophotometric method, based on measuring changes in the solution's absorbance. Although this method is widely used and useful as a preliminary assessment tool, it is indirect in nature and does not allow for unequivocal confirmation of the degradation of chemical compounds, let alone their complete mineralisation. Notably, it should be borne in mind that a change in the colour intensity of the solution may result from various processes, such as dye sorption on the surface of biomass, changes in its structure leading to the loss of chromophore properties, or the formation of intermediate products with lower absorption intensity in the analysed wavelength range. In each of these cases, we are dealing with a different process mechanism, which does not necessarily imply the actual breakdown of the compound into forms that are less harmful to the environment. In the study, the decrease in colour intensity is at times equated with biodegradation, which, from a methodological point of view, constitutes a simplification and may lead to an overinterpretation of the results obtained. The failure to supplement the analyses with more direct methods for identifying reaction products, such as chromatographic techniques (e.g. HPLC, LC-MS) or the determination of summary parameters (TOC, COD), limits the ability to unambiguously determine the extent of the actual chemical transformation of the compounds under investigation. Consequently, it must be acknowledged that the presented results reliably document the high efficacy of the process in terms of the decolourisation of dye solutions; however, conclusions regarding their complete biodegradation or mineralisation should be formulated with greater caution and explicitly referenced to the limitations of the research methodology employed.

Certain reservations may also be expressed regarding the section of the work concerning degradation mechanisms, which – despite its conceptual value – has not been fully supported by sufficiently reliable experimental data. The author claims to have identified the mechanisms of dye removal based on the results of enzymatic and omics analyses, which in principle constitutes a modern approach in line with current research trends. However, the actual evidential value of these data, in the current form of the study, is limited and does not always justify drawing unequivocal conclusions. To be specific, attention should be drawn to the proteomic section, which – although potentially very valuable in terms of knowledge – was conducted in a manner that significantly limits the possibility of a reliable interpretation of the results. As indicated in the description of the methodology, the proteomic analyses were performed without taking biological variability into account ("only a single sample… without biological replicates", p. 47). From the perspective of proteomic research standards, particularly in quantitative analyses, this prevents the conduct of a reliable statistical analysis and the assessment of the biological variability of the systems under study. Consequently, it is not possible to reliably determine whether the observed differences in protein expression levels are genuine or result from experimental or technical fluctuations. Furthermore, the author notes the absence of statistical filtering of results ("no p-value-based filtering was performed", p. 47), while simultaneously applying an arbitrary differentiation criterion based on changes in expression levels (log2FC). Such an approach increases the risk of interpreting random changes as biologically significant and does not meet the standards applicable in proteomic analyses. Consequently, the proteomic results obtained cannot serve as a basis for quantitative differential analysis or for drawing generalised conclusions. They should be treated solely as exploratory observations, indicating potential directions for further research, but requiring confirmation in experiments using rigorous statistical analysis methods. Despite these limitations, the study presents conclusions regarding 'metabolic reprogramming' and specific cellular responses to the presence of the dyes under investigation, which, given the current state of the data, should be regarded as an overinterpretation of the results. Formulating such conclusions requires not only qualitatively sound omics data, but also their statistical validation and – equally importantly – correlation with independent experimental results (e.g. quantitative analysis of metabolites or enzymatic activity). In summary, it must be stated that this mechanistic part of the work constitutes an interesting and ambitious attempt to interpret processes occurring at the molecular level; however, in its current form, it is preliminary and exploratory in nature. It requires a clear limitation of the scope of the conclusions drawn and an unambiguous indication of methodological limitations, so as to maintain proper scientific rigour in the interpretation of the results obtained.

In the case of transcriptomic analyses, the situation is more favourable, though significant limitations arise here as well. The author applies a significance criterion based on a 'raw p-value < 0.05' (p. 44), without explicitly indicating the use of multiple correction testing. In analyses involving thousands of genes, the absence of such a correction leads to an increased risk of false-positive results, which significantly limits the reliability of the conclusions. The consequence of this is the limited reliability of functional analyses, including the identification of enriched metabolic pathways or the interpretation of the organism's cellular response to the presence of dyes. Conclusions regarding the involvement of specific groups of genes or biological processes should therefore be formulated with greater caution and explicitly refer to the analytical limitations applied.

Another significant methodological issue is the method of distinguishing between biosorption and biodegradation mechanisms, which is a key element in the interpretation of the results obtained. The author uses a desorption procedure in a methanol solution ("70% (v/v) methanol solution", p. 33), treating it as a tool for assessing the contribution of sorption processes. It should be emphasised, however, that this method is only approximate and does not allow for an unambiguous determination of the mechanism of contaminant removal. The use of methanol as a desorption agent may, in fact, affect not only the compounds adsorbed on the surface of the biomass, but also the structure of the biomass itself, including its cell wall components, as well as the intermediate products formed during biodegradation. Consequently, the substances released into the solution need not correspond exclusively to the originally adsorbed dye, but may also include its transformed forms. Furthermore, the lack of a complete mass balance and the failure to identify reaction products makes it impossible to determine explicitly which part of the dye has actually undergone degradation and which has merely been transferred to another phase. As a result, the distinction between biosorption and biodegradation mechanisms presented in this study should be regarded as qualitative and approximate. Conclusions in this regard require clear limitations and supplementation with additional analytical methods, such as the identification of reaction products, which would allow for a more unambiguous determination of the nature of the transformations taking place.

Critical remarks also concern the statistical section of the dissertation, which – despite the purportedly wide range of analytical tools employed – has not always been conducted with due methodological rigour and interpretative clarity. The author refers to the use of a range of statistical methods; however, in many cases there is no clear attribution of specific tests to the analysed data, nor a detailed justification for their selection in the context of the nature of the variables and the structure of the experiment. The employment of one-tailed t-tests (p. 57) raises particular concerns. The use of such tests is methodologically justified only when the research hypothesis has a clearly defined direction (e.g. a predicted increase or decrease in the analysed parameter), and the choice of test was made prior to data analysis. However, the study lacks a clear formulation of directional hypotheses that would justify the use of one-tailed tests. Consequently, there is a risk that the choice of statistical method may have been dictated by the results obtained, which, from the point of view of the validity of statistical inference, constitutes an undesirable approach. Furthermore, no information is provided regarding the verification of the basic assumptions of the tests used, such as the normality of the data distribution or the homogeneity of variances. The absence of these elements limits the ability to assess the validity of the methods used and affects the reliability of the results obtained. In the context of the numerous analyses conducted for various combinations of process parameters, one would also expect a reference to the issue of multiple testing and the potential accumulation of statistical error.

In the ecotoxicological section of the study, there is a discrepancy between the cited testing standards and the actual conduct of the experiments. The author refers to various standards, including OECD 211 and ISO 6341, yet the description of the procedure indicates that a short-term test was conducted with readings taken after 24 and 48 hours ("After 24h and 48h…", p. 49), which is characteristic of the OECD 202 acute toxicity test. This inconsistency indicates a lack of methodological precision and may introduce ambiguity regarding the scope and interpretation of the results obtained. It should be emphasised that the selection of an appropriate testing standard is crucial for the interpretation of ecotoxicological test results, as different procedures address distinct aspects of a substance's impact on living organisms (acute vs. chronic toxicity). Following this, incorrect or imprecise application of methods may lead to unjustified conclusions regarding the actual impact of the tested systems on the environment. As a result, it must be acknowledged that the ecotoxicological section of the study, although providing a valuable complement to the process studies, requires more cautious interpretation and a clear indication of the limitations of the methodology employed.

Also worthy of note is the limited applicability of the results obtained, stemming primarily from the conditions under which the experiments were conducted. The studies were carried out in highly controlled systems, under aseptic conditions (p. 27), which – although justified from the point of view of repeatability and control of the experimental process – significantly limits the possibility of directly relating the results to the actual operating conditions of wastewater treatment plants. Under laboratory conditions, the presence of natural associated microflora is eliminated, whereas in real-world technological systems this microflora plays a significant role in shaping the course of biodegradation processes. In actual industrial and municipal wastewater, there is a complex and dynamic community of microorganisms that can both compete with introduced organisms for substrates and influence their metabolic activity through symbiotic or antagonistic interactions. The presence of this microflora can lead to significant changes in process efficiency, both in terms of a reduction (e.g. through competition for carbon sources) and potentially its modification (e.g. through the interaction of various metabolic pathways). Additionally, it should be noted that real-world wastewater is characterised by high variability in its physicochemical composition, which may affect both the stability of the biological system and the course of sorption and enzymatic processes. In particular, the variability of parameters such as pH, conductivity, pollutant load or oxygen availability may lead to different effects than those observed under laboratory conditions. In view of the above, it should be recognised that the results presented in this study are primarily exploratory in nature and form the basis for further research; however, their direct translation to an industrial scale requires additional verification under conditions approximating real-world scenarios. In particular, it would be advisable to conduct studies using actual wastewater and in non-sterile systems, which would allow for an assessment of the stability and effectiveness of the process under conditions corresponding to engineering practice.

Despite the reservations noted, it must be clearly emphasised that the dissertation constitutes a valuable scientific study, in line with current trends in the development of environmental biotechnology. Among its most significant achievements is a comprehensive research approach, integrating process analysis with an attempt to interpret mechanisms at the molecular level, which represents a significant step towards a better understanding of pollutant biodegradation processes.

Specific comments

p. 33 – The use of UV-Vis spectrophotometry as the main indicator of process efficiency (determination of the degree of decolourisation) does not allow for unequivocal confirmation of the degradation of the tested compounds, let alone their mineralisation. This method merely reflects changes in the optical properties of the solution, which may result from chemical transformation, sorption of the dye onto the biomass surface, or the formation of intermediate products with altered absorption properties. Therefore, it would be appropriate to supplement the studies with the determination of summary parameters (e.g. TOC, COD) or the identification of reaction products, which would allow for a more unambiguous assessment of the nature of the transformations taking place.

p. 33 – The use of the methanol desorption procedure as a tool to distinguish between the contribution of biosorption and biodegradation is not a conclusive method. Methanol may affect both the adsorbed compounds and the structure of the biomass as well as the transformation products, which hinders an unambiguous interpretation of the results obtained. Consequently, the distinction between mechanisms presented should be regarded as approximate and requiring confirmation by other methods.

p. 42 – The limited scope of the transcriptomic analyses, in particular the lack of a full set of experimental variants and the lack of reference to all the biological systems studied, restricts the possibility of generalising the results. Conclusions regarding the cellular response of organisms should be explicitly restricted to the experimental conditions analysed.

p. 44 – The lack of clear information regarding the use of multiple testing corrections (e.g. FDR) in RNA-seq analyses constitutes a significant methodological limitation. In analyses involving a large number of simultaneous statistical tests, the use of uncorrected p-values leads to an increased risk of false positives, which may affect the interpretation of differential gene expression and functional analyses.

p. 47 – The absence of p-value-based filtering of proteomic results ("no p-value-based filtering was performed") and the use of criteria based solely on changes in expression levels increases the risk of interpreting random fluctuations as biologically significant changes.

p. 57 – The lack of justification for the use of one-tailed t-tests and the lack of clearly formulated directional hypotheses undermines the reliability of the statistical analyses. Furthermore, no information was provided regarding the verification of test assumptions (normal distribution, homogeneity of variance), which makes it difficult to assess the validity of the methods used.

p. 27 – Conducting research under aseptic conditions, whilst justified from the point of view of experimental control, limits the possibility of directly relating the results to real-world conditions. Failure to account for the influence of natural microflora and the variability in wastewater composition may lead to an overestimation of the process's efficiency in relation to practical conditions.

pp. 48–49 – Methodological inconsistency with regard to ecotoxicological standards (reference to OECD 211 while simultaneously applying a procedure characteristic of a 24/48-hour short-term test) indicates a lack of precision in the selection and description of research methods. This is of significant importance for the interpretation of the results obtained.

p. VIII (Abstract) – The statement regarding the 'environmental safety' of the samples following the treatment process is too categorical. A reduction in toxicity in short-term tests is not synonymous with full environmental safety, particularly in the absence of an analysis of transformation products and long-term effects.

p. 168 – The interpretation of the decrease in process efficiency in the bioreactor system ("decrease likely indicates…") is speculative and is not supported by additional measurements (e.g. changes in physicochemical parameters, enzymatic activity or the composition of reaction products). Conclusions in this regard should be clearly marked as hypotheses requiring further verification.

In view of the comments presented in this review, I kindly ask the Author to address the following issues during the public defence of the dissertation:

1. Distinction between decolourisation and degradation of dyes

Please provide a clear explanation of how the Author distinguishes the process of decolourisation from actual degradation, and in particular the mineralisation of dyes. What evidence – apart from UV-Vis absorbance measurements – allows one to conclude that the chemical compounds have actually decomposed? Did the author consider the use of additional analytical methods (e.g. TOC/COD determinations, identification of reaction products) that could confirm the nature of the changes taking place?

2. Criteria for transcriptomic data analysis

Please clarify whether multiple testing correction (e.g. FDR/Benjamini–Hochberg) was applied in the RNA-seq analyses. If not, how does the author assess the impact of using uncorrected p-values ("raw p-value < 0.05") on the reliability of the identification of differentially expressed genes? How would the interpretation of the results change if such correction were applied?

3. Interpretation of proteomic results

Given the lack of biological replicates in the proteomic analyses, please indicate which of the proposed conclusions regarding the mechanism of the process the author considers most reliable, and which should be treated solely as research hypotheses. How does the author justify the possibility of drawing conclusions regarding degradation mechanisms given the limited statistical analysis of the data?

4. Limitations of the desorption method in assessing the mechanisms of the process

Please discuss the limitations of the desorption method used in a 70% methanol solution as a tool for distinguishing between biosorption and biodegradation. How does the author rule out the influence of the solvent on the structure of the biomass and the presence of intermediate products? Is it possible to use more unambiguous methods to assess the contribution of individual mechanisms?

5. Statistical approach used

Please justify the use of one-tailed tests in the analysis of experimental data. What were the directional hypotheses justifying their use? Were the basic assumptions of the statistical tests (normality of distribution, homogeneity of variance) verified?

6. Interpretation of ecotoxicological results

Please clarify the basis on which the author draws the conclusion regarding the "environmental safety" of the samples following the treatment process. How does the author relate the results of short-term tests to potential long-term effects, including the possibility of bioaccumulation and the impact of transformation products on living organisms?

7. Methodological consistency regarding ecotoxicological standards

Please explain the discrepancies between the cited testing standards (e.g. OECD 211) and the experimental procedure actually used (24/48-hour test). How does the author assess the impact of this inconsistency on the interpretation of the results?

8. Applicability of the results under real-world conditions

Please assess the applicability of the results obtained to real-world conditions in a wastewater treatment plant. How does the author assess the impact of the presence of natural microflora, variations in wastewater composition and the presence of other contaminants on the process's effectiveness?

9. Operation of the system at bioreactor scale

Please elaborate on the interpretation of the results obtained in the bioreactor studies, in particular the reasons for the observed decline in process efficiency in subsequent cycles. What additional measurements should be carried out to unequivocally confirm the proposed explanations?

10. Directions for further research

In the context of the methodological limitations presented, please indicate which research activities the Author considers key to the further development of the presented concept, particularly with regard to confirming the mechanisms of biodegradation and increasing the applicability of the proposed solution.

Final conclusion

I conclude that the doctoral dissertation by Ruchi Manishkumar Upadhyay, MSc, entitled: *"Determination of the mechanism and optimisation of the conditions of the process of removing colored aromatic compounds by selected Basidiomycota"* **meets the requirements defined in Article 187 of the Act of 20 July 2018 on Higher Education and Science (Journal of Laws, as amended) for candidates applying for the award of a doctoral degree.** The dissertation presents an original solution to a scientific problem, and the PhD candidate has demonstrated appropriate theoretical knowledge in the discipline of environmental engineering and the ability to conduct independent scientific research. Despite the critical comments noted in the review, the dissertation makes a significant contribution to the development of knowledge in the field of biotechnological methods for removing pollutants from the aquatic environment. I therefore request the Discipline Council for Environmental Engineering, Mining and Power Engineering to admit Ms Ruchi Manishkumar Upadhyay to the subsequent stages of the procedure for the award of a doctoral degree in the field of engineering and technical sciences, within the discipline of environmental engineering, mining and power engineering.

[handwritten signature]

Ewa Wiśniowska, PhD, Eng.,
Prof at the University of Częstochowa