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Design and Optimization of Laccase Immobilization in Cellulose Acetate Microfiltration Membrane for Micropollutant Remediation

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Abstract: The industrial and environmental applications of laccase, especially in wastewater treatment, have gained focus in recent years. Therefore, developing the proper laccase immobilization techniques, which could improve the stability of the enzymes and simplify the required downstream processes, is needed. A novel two-step immobilization process was developed, resulting in cross-linked enzyme aggregates (CLEA) in the pores of the membrane. Laccase adsorption on a biodegradable cellulose acetate microfiltration membrane along with cross-linking was investigated to maximize the enzyme load and immobilization efficiency. The optimization was done regarding the: pH, temperature, enzyme concentration, adsorption time, cross-linker concentration, and temperature. It was concluded that the highest immobilization efficiency (76%) could be achieved in acidic buffers at 29 °C with high surface activity (1174 U·m^{-2}) at the cost of partial denaturation and membrane fouling. The membrane was successfully utilized for the enzymatic treatment of diclofenac, and 58% removal efficiency was achieved. The results indicated that cellulose acetate is a suitable carrier for adsorption-based immobilization of laccase for the potential for environmental utilisation.

Keywords: enzymatic membrane bioreactor; micropollutant; laccase; immobilisation; diclofenac

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1. Introduction

Organic micropollutants in water bodies are widely monitored in the European Union [1], and their removal from aquatic matrices has become of growing interest [2]. One of the possible technological solutions to reduce the emission of these pollutants is to use oxidoreductase enzymes such as laccase [3] and horseradish peroxidase [4]. Micropollutants could be transformed into less toxic organic molecules with these enzymes [5]. In order to ease industrial costs and accessibility, the production of these on a large scale has been developed [6].

Along with efficient production [7], the immobilisation techniques [8] are the keys to a more cost-efficient use since these can enhance stability and reusability [9]. In the case of bioremediation, it is beneficial to use laccase in an immobilised form, such as adsorbed, entrapped or covalently bonded to a carrier. It is important to consider the application for choosing the immobilisation method [10]. Immobilisation of laccase on different adsorbent materials can ease the catalyst's separation process while having unique advantages. The synergic effect of adsorption and enzymatic transformation could enhance the efficiency of the overall removal process [11,12]. Similarly, entrapment of the laccase is beneficial due to its simplicity and the carrier matrix could also adsorb the pollutants and the reaction products as well [13]. However, entrapment may severely decrease the apparent activity of the catalysts by limiting substrate diffusion transfer to the enzyme [14]. In that aspect, the immobilisation of nanoparticles is advantageous, due to the homogeneous dispersion of the particles in the liquid media increasing the substrate accessibility of laccase [15]. Covalently

bonding laccase to the carrier can improve the stability of the enzyme along with excellent retention. However, chemical modification of the laccase structure may result in a decrease in activity. For example, Piao et al. [16] achieved complete encapsulation of laccase in hydrogel microparticles, while the activity recovery was 18.9%.

Carrier-free immobilisation techniques were developed to propagate industrial uses, including cross-linked enzyme aggregates (CLEA) [17]. By cross-linking methods, intermolecular bonds formed between enzyme molecules result in high catalyst density, long shelf life and improved operational stability [18], while maintaining sufficient activity recoveries [19]. Furthermore, from a practical standpoint, a higher concentration of catalysts is possible in the reactor since no bulk material must be fed along with the enzyme [20]. On the other hand, the main weakness of using CLEA is the difficulty of preparation since controlling the sizes of the aggregates is required, which may result in varying performances and separation issues [21]. In order to overcome this, enzymes could be immobilised inside so-called ordered mesoporous materials in which enzyme accessibility and stability are greatly improved by interconnected pores [22].

Membranes are related to these materials, and many could be used as enzyme carriers. The porous membranes could be made from materials which can withstand operational conditions, although chemical modification of the membrane materials is often required to immobilise laccase. It can be done via the functional groups of the membrane surfaces [23]. Using membrane processes is a generally effective strategy for biocatalytic process intensification [22] due to increased degradation efficiency by the synergetic effect of membrane rejection, adsorption, and enzymatic oxidation in membrane reactors [24]. Laccase-catalysed reactions may result in dimers and oligomers with different water solubility [25]. Even precipitates may form from this [5]. These could be separated from the solution using membrane separation processes like membrane distillation [26] or microfiltration [27].

Enzyme aggregates could be trapped in membranes for continuous conversion of the substrate [28] or retained based on the molecular size in the reactor with ultrafiltration [29]. Composite membranes could be made by deploying an adsorbent layer on the membrane to immobilise laccase both physically or chemically. For example, laccase was adsorbed on a gelatin layer deployed in the pores of microfiltration ceramic membrane [30]. However, the specific activity was reported as $7.10^4 \pm 5.10^3$ U·m^{-2}, and the authors highlighted the opportunity to improve the activity further and lower surface area. Laccase could be immobilised in the pores of commonly used membrane materials like polyethersulfone [21]. Chitosan-coated membranes could be made from polysulfone capillary membranes, successfully utilised for phenol removal [31]. Although specific activity was not reported, 83.7% of 143 U was immobilised on a 4.84 cm^2 active membrane area. Despite the excellent operational performance of polyethersulfone membranes, they are possible sources of microplastic pollution, which must be considered in membrane material selection [32]. High surface activity (4.47 U·cm^{-2}) was achieved by covalently bonding laccase to multi-walled carbon nanocomposite polyvinylidene fluoride membranes. Such coating may result in a relatively high specific activity, however high deactivation was also reported [33]. Dip-coating with TiO_2 and functionalisation with silanization of microfiltration membranes could increase the enzyme load on the membrane, although densification of the surface has to be considered to maximise specific activity [34].

Despite widespread utilisation of oxidoreductases in different applications [35], there is still a lack of use in wastewater treatment due to high costs and low efficiency. The complexity of enzyme immobilisation methods and the specific activity play an essential role in the viability of the treatment process [36]. Therefore, developments are aiming to find a simple, low-cost method that can ensure the retention of the enzymes are essential [37]. Several factors have to be considered to provide high operating and storage stability without decreasing the enzyme activity by structural changes, diffusion limitations [38].

The aim of this study was to develop and optimise a novel CLEA-based immobilisation method for utilising laccases in an integrated membrane bioreactor. In order to decrease wastes and the risk of microplastic release at the end of the membrane life-cycle,

biodegradable [39] cellulose acetate microfiltration membranes were chosen as carriers, and the enzyme activity losses were minimised. The utilisation of cellulose as the carrier is possible [28] due to its relatively high protein adsorption capacity, therefore it could be used without functionalisation, which could minimize the loss of activity in the immobilisation process. Despite these benefits, there is a lack of research using it as a membrane for laccase immobilisation. Therefore, in order to investigate the application of the enzymatic membrane as a catalyst, the reaction rate measure by ABTS was used as a response parameter for optimisation and reaction kinetics were determined. Along with the characterisation of the membrane, the micropollutant removal was also measured by the degradation of a commonly detected pharmaceutical, diclofenac (DCF).

2. Results

The immobilization includes adsorption on cellulose acetate microfiltration membrane and cross-linking with glutaraldehyde. In order to maximize the enzyme load and efficiency, the two steps had to be investigated separately. Screening experiments were performed regarding the effect of pH on the adsorption process and the stability of free-form laccase during the experiments. Based on the screening experiments, the process of laccase adsorption has been optimized with RSM, and the effect of the cross-linker was studied only afterwards.

The design of the experiments near the optimum was done using a central composite design for the adsorption. Based on this, the quadratic model was fitted with linear regression to determine the optimal adsorption conditions. After optimization, the characterization of the catalytic membrane was carried out by measuring the membrane activity and investigating the enzyme kinetics of the conversion of a model substrate (ABTS). The morphology of the CLEAs on the surface by scanning electron microscopy (SEM) was also inspected. After a detailed characterization of the catalytic membrane, an application to micropollutant removal was also investigated with DCF.

2.1. Effect of the pH

The effect of pH on the adsorption efficiency was investigated with fixed laccase concentration and temperature. Increasing pH had a negative effect on adsorption efficiency, as shown in Figure 1. The lowest pH (pH4) resulted in the highest enzyme load when 78% (SD = 4%) of the initial activity was adsorbed on the membrane (416 U·L^{-1}, SD = 39.7). In contrast, the adsorption efficiency was substantially lower at higher pH (7%, SD = 12%).

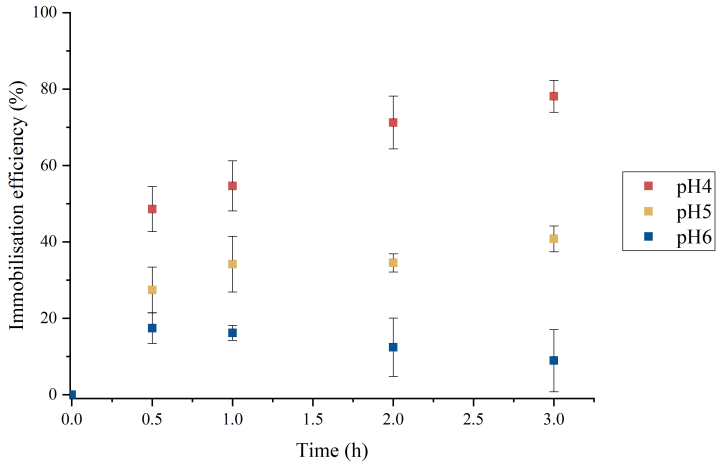

Figure 1. Effect of pH on the adsorption process. (Conditions used for the experiments are: laccase concentration: 1 mg·mL^{-1}, temperature: 20 °C, adsorption time: 3 h. pH = 4, 5, 6).

2.2. Thermal Denaturation of the Laccase

In order to consider the stability of laccase during the immobilization process, blank experiments were performed without membranes. Kinetic experiments with the highest laccase concentration according to experimental design (3 mg·mL^{-1} laccase in pH = 4) were carried out to investigate the effect on the activity at different temperatures (15–40 °C). Despite laccases being reported to be stable between 4 and 40 °C for a short time [40], a relatively high loss of enzyme activity above 30 °C occurred after 6 h (Figure 2). The decay constants were calculated for 35 °C and 40 °C as 0.0358 h^{-1} and 0.0463 h^{-1}, respectively. It means the half-life of the enzyme in case of 35 °C is 19.4 h and for 40 °C is 14.9 h. Therefore, during a nine-hour immobilization procedure, approximately 28% and 34% of activity would be lost due to high temperature. On the contrary, no decrease in activity was detected in blank experiments when temperatures were below 30 ° C. In order to achieve high adsorption efficiency, 35 °C was considered as the upper-temperature limit for the optimization, and the losses were included in the calculations.

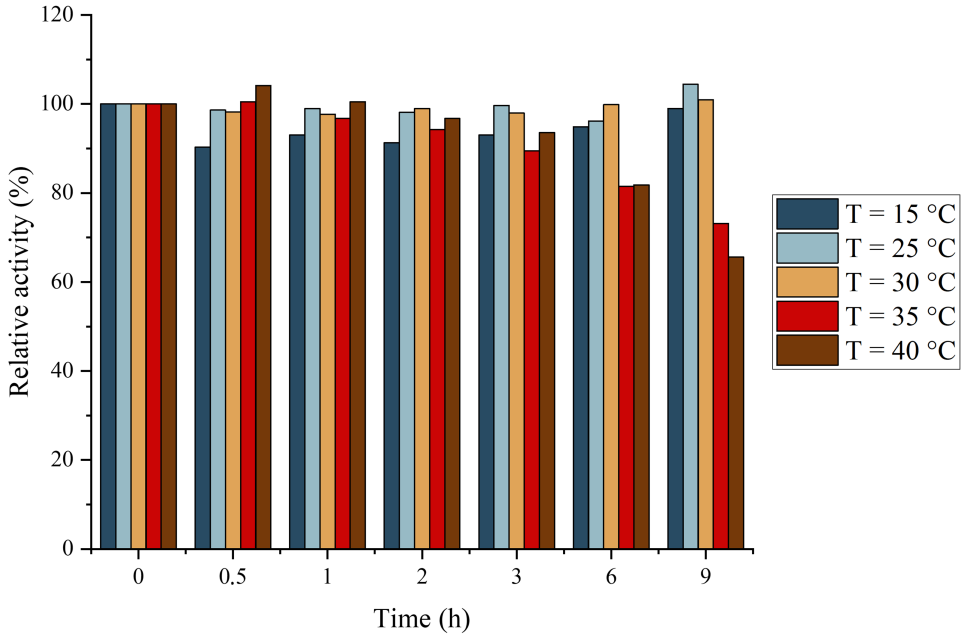

Figure 2. Change of relative activity at different temperatures (Conditions: laccase concentration = 3 mg·mL^{-1}, pH = 4, constant mixing in thermostatic cabinet without membrane, initial activity was considered as 100%).

2.3. Optimization of Laccase Adsorption on the Membrane

To achieve maximum enzyme load on the membranes, experimental conditions were chosen for extended adsorption times and concentrations considering the effect of thermal denaturation of laccase (Table 1). Five repetitions were performed in the central point of the design for the determination of experimental error. These runs resulted in a standard deviation of 2% for immobilization efficiency and 12 U·L $^{-1}$ for immobilized activity, which was considered an acceptable error, as it is similar to the deviation of the measurements of the activity in triplicate.

Curve fittings were performed on the measured data after correction with the blank experiments according to Equation (3). Fitting resulted in a regression coefficient of 0.966. All of the chosen main factors had a significant effect ($\alpha < 0.05$) on the adsorption process, while the factors considering quadratic effects were insignificant, except for the adsorption time (Table A2). However, they were not omitted from the model to achieve a more accurate determination of the optimum. Including these effects, the adjusted R-squared resulted in 0.923 and the ANOVA test (Table A3) resulted in a significant model (p value = 0.0001). In the central point, the difference between the measured and the calculated activity was

13 U·L^{-1} which is close to the experimental error. Therefore, the model was declared to be adequate for optimization purposes. After fitting the constants of Equation (3), a visual representation of the model results as surfaces were provided in Figure 3.

Table 1. Optimization of laccase adsorption on cellulose acetate membrane.

No.1	Laccase Concentration (mg·mL^{-1})	Adsorption Time (h)	Temperature (°C)	Immobilisation Efficiency	Immobilized Activity (U·L^{-1})
1	1	3	15	0.38	108
2	3	3	15	0.20	172
3	1	9	15	0.57	162
4	3	9	15	0.42	358
5	1	3	35	0.65	185
6	3	3	35	0.44	374
7	1	9	35	0.71	201
8	3	9	35	0.54	463
9	1	6	25	0.81	229
10	3	6	25	0.52	446
11	2	3	25	0.57	321
12	2	9	25	0.77	437
13	2	6	15	0.56	319
14	2	6	35	0.62	351
15	2	6	25	0.66	375
16	2	6	25	0.64	364
17	2	6	25	0.61	346
18	2	6	25	0.62	350
19	2	6	25	0.62	352

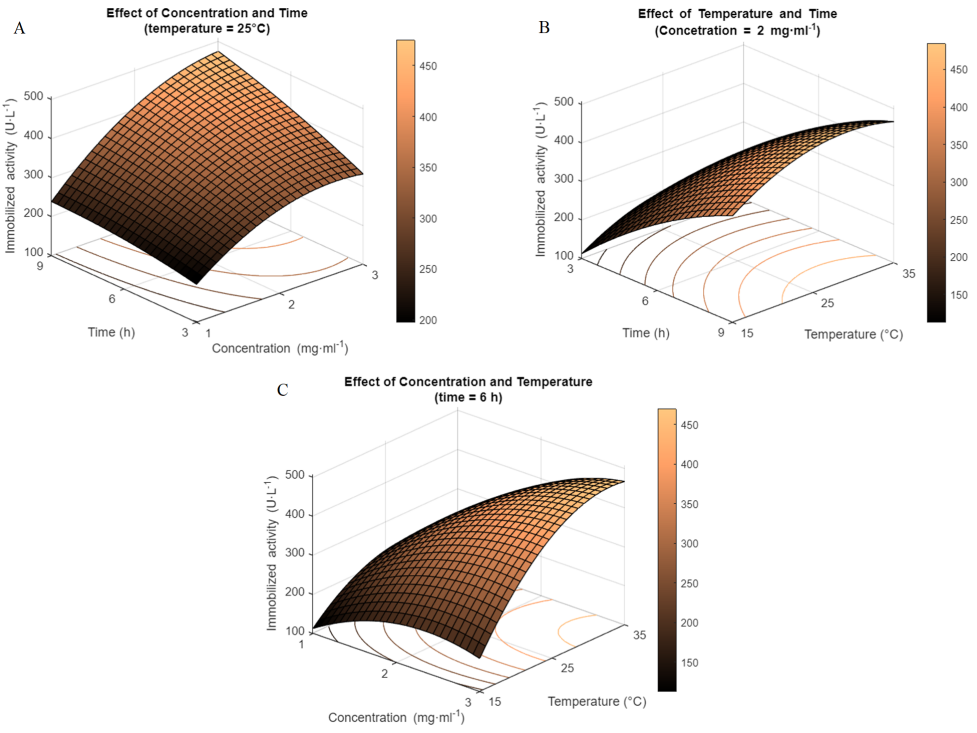

Figure 3. Effect of parameters on enzyme adsorption. Visual representations were based on the polynome fitted to our results with one of the three parameters fixed as indicated above each surface plot. (**A**) Effect of concentration and time with temperature fixed at 25 °C, (**B**) Effect of temperature and time with concentration fixed at 2 mg·mL^{-1} (**C**) Effect of concentration and temperature with time fixed at 6 h.

The optimization study resulted in the maximum time and temperature allowed by the thermal stability of the enzyme. Despite the slight thermal denaturation of the laccase, increasing temperature promoted the adsorption, as it was visible on the fitted surfaces (Figure 3). The temperature might improve the diffusion of the particles and propagate the access of the protein molecules to the adsorption sites inside the pores of the membrane material. Additionally, the higher temperature decreased the viscosity of the solution, which might aid in better mixing. However, due to the complexity of the protein adsorption on solid phases, white box models are barely applicable. Therefore, experimental optimization was done. It was concluded that an increase of temperature to 29 °C results in an overall higher adsorption rate with acceptable thermal denaturation.

The optimal conditions were determined by finding the maximum of Equation (3) numerically. This calculation resulted in 485 $U \cdot L^{-1}$ immobilized activity in case of a laccase concentration of 3 $mg \cdot mL^{-1}$, adsorption time of 9 h, and temperature of 29 °C. Then, in order to test the estimated optimum conditions, five more experiments were performed with these settings. These resulted in 538 $U \cdot L^{-1}$ (SD = 55 $U \cdot L^{-1}$) immobilized activity and adsorption efficiency of 76% (SD = 7%). Since these values are higher than any of the previously measured results, the optimization of the adsorption step was declared successful.

Measurement for the immobilized protein and activity was also based on Bradford protein concentration measurement. 1.58 $mg \cdot cm^{-2}$ protein was immobilized on the membrane during the adsorption. For calculating specific activity, measurements with known dry weight and membrane surface resulted in 44.4 $U \cdot mg^{-1}$ or 42.8 $U \cdot cm^{-2}$, respectively.

2.4. Effect of Cross-Linker Concentration

The effect of glutaraldehyde concentration on activity was assessed (Figure 4) as a next step since the cross-linking may play a major role in the catalytic activity and stability of the CLEAs [41].

Experiments were performed with 50–500 mM GA concentrations in triplicates at two different temperatures. The positive effect of lower temperature during the cross-linking was noticeable. In some cases, the membrane activity almost doubled with the lowering of the temperature. On the other hand, the influence of cross-linker concentration was dependent on the temperature. The difference was insignificant at 20 °C, but an optimum could be determined at the lower temperature. Comparing the results of the runs, it was concluded that the best performance could be achieved by performing the cross-linking with 100 mM of GA at 4 °C for 2 h.

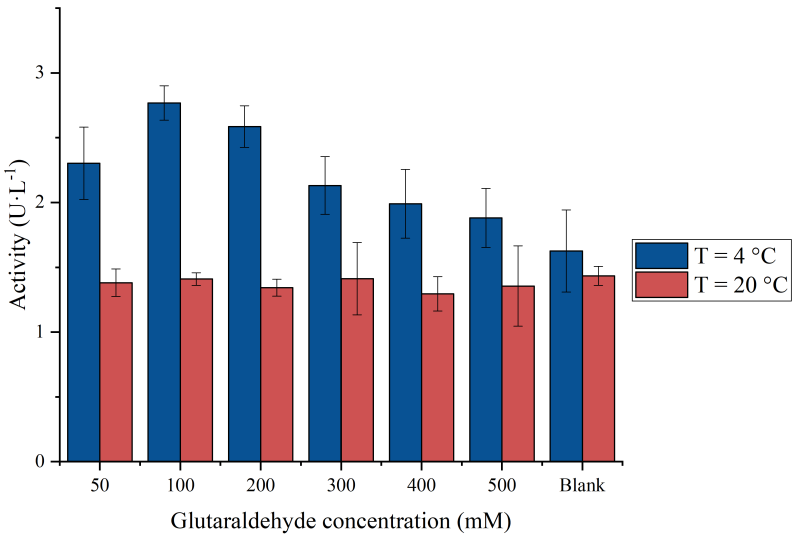

Figure 4. Effect of GA concentration and temperature on the enzymatic activity of the membrane. Measured with the following settings: adsorption: 3 $mg \cdot mL^{-1}$ laccase in pH4 for 9 h at 29 °C, cross-linking: 50–500 mM at 4 °C (blue) and 20 °C (red), reaction time: 2 h.

2.5. Storage Stability

Investigation of storage stability was performed with the membrane produced by the optimized method for seven days (Figure 5). During these experiments, the membranes were tested with the same method described above for activity measurement. The membranes showed a slight decrease (19%), in conversion during the investigations.

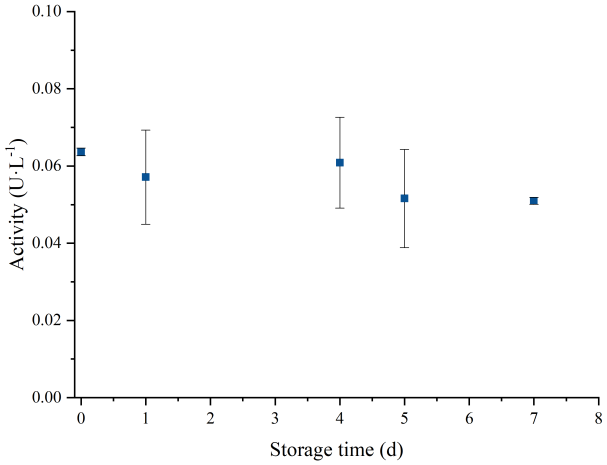

Figure 5. Effect of storage on the conversion of ABTS with enzymatic membrane. Enzymatic membranes were prepared by using the optimal conditions for adsorption (conditions: laccase concentration: 3 mg·mL^{-1}, adsorption time: 9 h, temperature of adsorption: 29 °C, time of cross-linking: 2 h, temperature of cross-linking: 4 °C, GA: 100 mM, temperature of storage: 4 °C).

2.6. Membrane Activity and Enzyme Kinetics

Experiments performed at various substrate concentrations (0.006–0.075 mg·mL^{-1}) of ABTS revealed that the Michaelis–Menten equation could formally describe the enzymatic reaction rate (Figure 6). The reaction rate of the conversion was calculated by Equation (4). The measured flow rate of the ABTS solution was 0.95 cm^3·s^{-1} (SD = 0.07 cm^3·s^{-1}, n = 5). Therefore, residential time is approximately 0.13 s. Based on contact time estimations, the specific activity of the membrane-bound laccase was 1174 U·m^{-2}. Fitting the Michaelis–Menten kinetics using this information resulted in kinetic constants: v_{max} = 577.1 µM·s^{-1}, K_M = 48.7 µM.

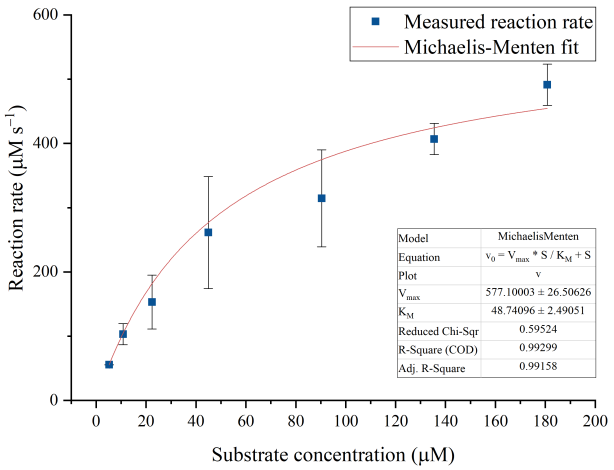

Figure 6. Effect of substrate concentration on the reaction rate. The orange line indicates the calculated rates of Michaelis–Menten kinetics fitted to the measured reaction rates (blue) at the first filtration cycle. Error bars represent the standard deviation of triplicate measurements.

2.7. Surface Analysis by Electron Microscopy

In order to investigate morphological changes and pores, SEM recordings were collected (Figure 7). Graphical analysis of the pictures revealed that the cellulose-acetate membrane used as a carrier has an average pore size of 0.19 µm (SD = 0.1 µm, n = 176) on its surface, which slightly shifted during the immobilization to 0.24 µm (SD = 0.1 µm, n = 477). Furthermore, SEM observations revealed small globular particles attached to the side of the pores with an average size of 0.39 µm (SD = 0.09 µm, n = 82). These objects are most likely to be the cross-linked laccase aggregates formed during the immobilization, but due to low concentration on the surface, it was not measurable with EDS.

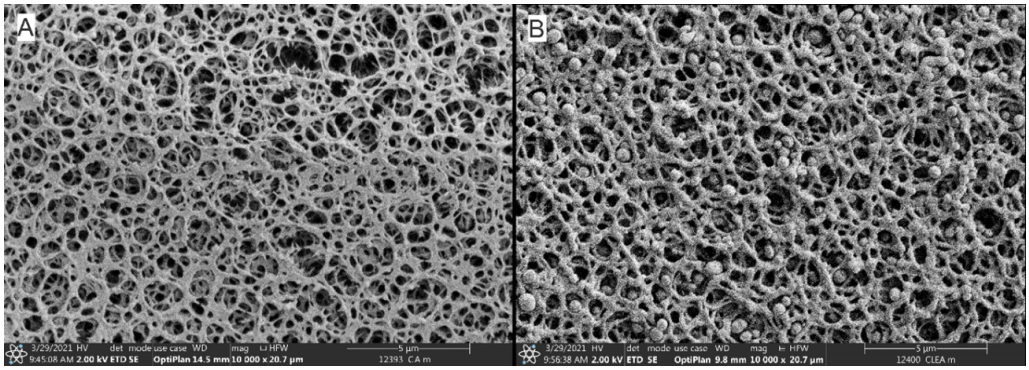

Figure 7. Scanning Electron Microscopy images of the cellulose acetate membrane before (**A**) and after (**B**) immobilization in 10,000 times magnification.

2.8. Effect of Transmembrane Pressure on Flux and Activity

Transmembrane pressure was set to 1 bar to determine the flux of the enzymatic and the raw membrane with distilled water. The flux of the enzymatic membrane was 5.65×10^{-4} $m^3m^{-2}{\cdot}s^{-1}$ (SD = 5.83×10^{-5} $m^3m^{-2}s^{-1}$, n = 9), while in the case of the raw membrane was 8.90×10^{-1} $m^3m^{-2}s^{-1}$ (SD = 0.12×10^{-5} $m^3m^{-2}s^{-1}$, n = 3). That means the flux of the membrane was decreased by 36% due to the immobilization. As shown in Figure 8, a decrease in the flow rates compared to the raw cellulose-acetate membrane was observed in different transmembrane pressure.

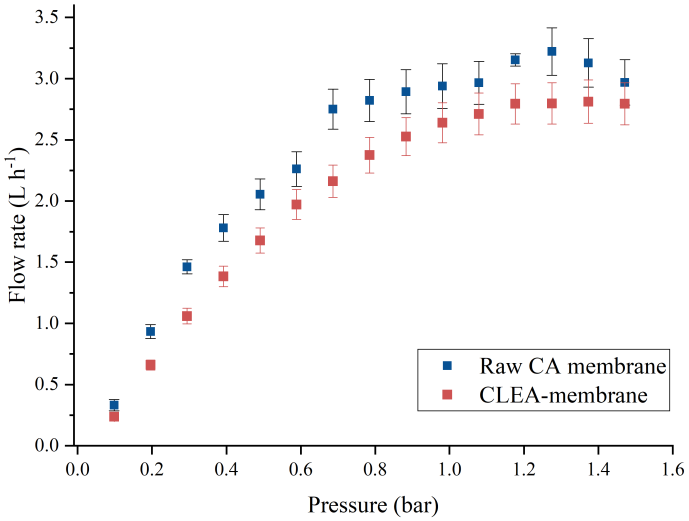

Figure 8. Effect of transmembrane pressure on membrane flux. Raw cellulose acetate membrane was used without any pre-treatment except washing with distilled water. Error bars represent the standard deviation of multiple flow measurements with specific settings.

2.9. Degradation of Diclofenac

The optimized enzymatic membrane was used to evaluate the efficiency of DCF transformation in pH = 5 buffer solution at 10 mg·L^{-1} initial concentration. The flow rate was kept constant at 0.018 L·h^{-1}. As a result, 57.9% (SD = 0.6%) removal efficiency was reached by using dead-end filtration through the membrane without recirculation. Furthermore, the change of the UV spectrum between 200 and 340 nm, and the shift of the peak wavelength from 278 nm to 250 nm also indicates the successful conversion of DCF (Figure 9).

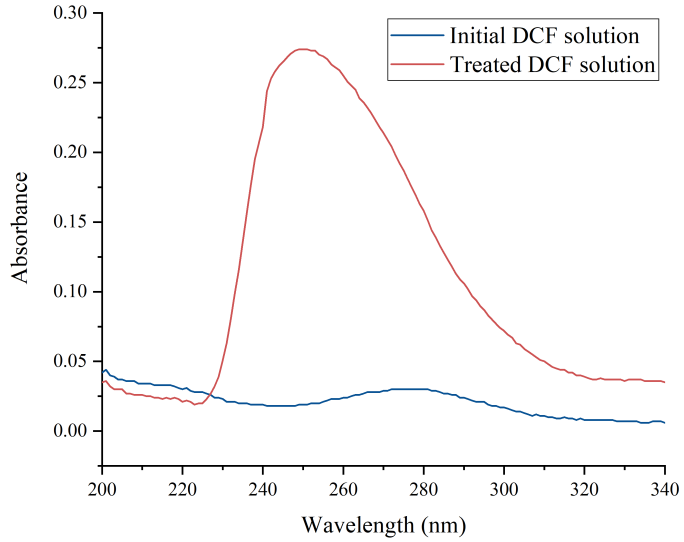

Figure 9. Change of the maximal peak wavelength in the UV spectrum as a result of treatment.

3. Materials and Methods

3.1. Materials

Laccase enzyme from *Trametes versicolor* (catechol activity > 0.5 U·mg^{-1}) and ABTS substrate (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)), DCF, HPLC eluent grade methanol, trifluoro-acetic acid and Bradford protein tests were purchased from Sigma-Aldrich, St. Louis, MO, USA. Glutaraldehyde (48–52 w%) cross-linking reagent, citric acid and disodium hydrogen phosphate were purchased from VWR International Ltd., Radnor, PA, USA, Solid phase extraction cartridges (Oasis HLB 200 mg, 6 cm^3) were purchased from Waters. Hydrophilic cellulose-acetate membrane discs with a nominal pore size of 0.1 µm were purchased from Hawach Scientific, Shanghai, China.

3.2. Activity Measurement

Laccase activity units (U) were defined as the amount of enzyme capable of oxidizing 1 µM ABTS substrate in one minute. The dissolved enzyme activity was measured using ABTS as substrate in a pH5 buffer solution [42]. Briefly, ABTS substrate (0.05 mg·mL^{-1}) was mixed with the laccase containing the sample and the change of absorbance at 420 nm ($\epsilon = 3.6 \times 10^4$ M^{-1}cm^{-1} [43]) was measured for the calculation of the initial reaction rate for 2 min. Each activity measurement was performed in triplicate and the average activity was represented as U·L^{-1}. For specific activity, the weight used was the amount of protein determined by the Bradford assay [44].

In the case of determining the specific activity of the membrane-bound laccase, ABTS-containing buffer solution was mixed with small discs of the enzymatic membranes. The change in absorbance of the liquid phase was measured for 5 min and the activity was calculated as described above. For specific activity, the weight and the surface area of the membrane were used.

3.3. Immobilisation of Laccase

Flat sheets of cellulose-acetate membranes were immersed in laccase solution in closed vessels and mixed at 100 RPM on an orbital shaker. The temperatures of the experiments were set between 15 and 40 °C by a thermostatic cabinet (±0.1 °C). The protein adsorption was investigated by the solution depletion technique [45]. Briefly, samples were taken from the solution periodically to determine the change in enzyme activity.

The immobilized enzyme activity (A_{im}) was calculated by measuring the change in enzyme activity (A) between the beginning of the experiments (A_0) and after the specified time (A_t). The change determined in the blank experiments (A_{blank}) due to inactivation was considered in Equation (1).

$$A_{im}(U/L) = A_0 - A_t - (A_{blank,0} - A_{blank,t}) \quad (1)$$

In order to indicate the loss of activity used for the immobilization, the efficiency (η_{im}) was calculated with Equation (2).

$$\eta_{im} = \frac{A_{im}}{A_0} \quad (2)$$

As a next step, the membrane was placed into a glutaraldehyde solution for cross-linking. Different concentrations of glutaraldehyde (50–500 mM) were tested by dilution with distilled water. All the cross-linking reactions were performed at constant temperature (4–20 ° C) for 2 h and mixed as described above.

After cross-linking, the enzymatic membranes were put into the stainless-steel, dead-end filtration module. Before use, 10 mL of distilled water was filtered through the membranes at least nine times to remove unbound laccase. The activity of the membranes was measured as described before. All membranes were freshly used or stored in distilled water for a maximum of 24 h after preparation.

3.4. Optimisation

In order to determine the effects of pH, time and temperature on the adsorption and cross-linker concentration on the immobilization process, screening experiments were performed prior to optimization. The effect of pH on the adsorption process was tested first by dissolving laccase (1 mg·mL^{-1}) in McIlvaine buffer (pH 4–6). The range of pH was chosen by considering the recommendation for the membrane material and the possible range of this buffer. During the experiment, samples were taken for activity measurements after 0.5, 1, 2, and 3 h. Parameters achieving the highest immobilized activity were used for subsequent experiments.

Next, thermal denaturation during the adsorption step was determined. Experiments were performed with 3 mg·mL^{-1} laccase dissolved in pH = 4 buffer at different temperatures (15–40 °C), and samples were collected periodically for 9 h, and the decay of activity was calculated.

Optimization of the immobilization procedure was designed according to the central composite design scheme (Table A1) with the response surface method (RSM). The aim of the optimization was to maximize the effect of the main factors on the immobilized activity (Equation (1)). Factor ranges were chosen based on screening experiments and literature research data as laccase concentration: 1–3 mg·mL^{-1} adsorption time: 3–9 h and temperature: 15–35 °C. Specific factor levels were presented in the standard order table (Table 1). Each run was performed in triplicates, and experimental uncertainty was determined by five repetitions in the central point.

The constants (b) of a quadratic Equation (3) were determined to fit the measured activity using linear regression in Matlab. The main factors labelled as A, B, and C are concentration, reaction time and temperature, respectively. Since central composite design has three levels of the main factors, it was possible to investigate both the linear and quadratic effects on the immobilization process. Optimal levels of the main factors to maximize the response variable (Y) were determined by the Optimisation Toolbox of Matlab.

$$Y = b_0 + b_1A + b_2B + b_3C + b_{12}AB + b_{13}AC + b_{23}BC + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 \quad (3)$$

3.5. Surface Analysis

After the immobilization, the surface analysis was made by scanning electron microscopy (SEM) with ThermoFisher Apreo S scanning electron microscope. Images of the membranes were taken before and after immobilization. The samples were dried and surface coated with gold prior to analysis. The sizes of the pores on the surface were determined by measuring the pores on SEM images.

3.6. Effect of Substrate Concentration

For the purpose of studying the kinetic properties of the enzyme, the reaction rates of the conversion of ABTS were determined by changing the feed concentration between 0.006–0.075 mg·mL^{-1}. Instead of mixing laccase with ABTS, the substrate solution was filtered through the membranes in dead-end operation by applying 1 bar excess pressure. The conversion was determined by comparing the ABTS radical concentration (c) of the feed and the permeate. The reaction rate (r) was calculated according to Equation (4). Based on that, the specific activity of the membranes-bound laccase could be defined as U per m^2 of the membrane surface and U per mg membrane weight.

$$r = \frac{(c_{permeate} - c_{feed}) \cdot Q}{V_m \cdot \phi} \quad (4)$$

Estimating the pore volume was based on the measured membrane porosity (ϕ) and membrane volume (V_m). Membrane porosity was determined in duplicates using the dry-wet weight method [46]. As a simplification, the residential time of the substrate solution in the membrane pores was used as reaction time. It was calculated using the flow rates (Q) and estimated pore volumes. The flow rate was calculated by measuring the weight of permeate at one-second intervals using an analytical balance. The transmembrane pressure was kept constant during the measurement by controlling a regulator of a synthetic air gas cylinder connected to a membrane module. Based on these results, Michaelis–Menten kinetic equation was fit using Origin.

3.7. Storage Stability

The storage stability was tested by storing the enzymatic membranes in 20 mL of deionized water at 4 °C for two weeks. The membrane activity measurements were repeated after 1, 4, 5 and 7 days, and the decay of activity (k) was calculated using Equation (5). It describes the process as a first-order decay.

$$\frac{A_t}{A_0} = e^{-kt} \quad (5)$$

3.8. Transformation of Diclofenac Using Enzymatic Membrane

The degradation of DCF was tested with the enzymatic membrane made by the optimized method. The DCF-containing feed was prepared in a pH5 buffer to set a 1 mg·L^{-1} initial concentration. The lab-scale unit contained a flat-sheet membrane with a surface area of 12.57 cm^2. Instead of maintaining constant pressure, 60 mL of synthetic wastewater was fed to the membrane at a constant flow rate using a peristaltic pump. Samples were collected from the permeate and analyzed by UV-Vis spectroscopy and HPLC-UV. The samples were then enriched with solid phase extraction (Oasis HLB 6 cm^3/200 mg) using protocols recommended by JRC Technical report [47] and measured with HPLC-UV at 278 nm using gradient elution. All experiments were done in triplicates, and averages were shown.

4. Discussion

Results regarding the effect of pH were similar to previously published results [48]. As they concluded that a more stable layer of laccase and higher load could be achieved by lowering the pH during laccase adsorption on cellulose and lignin surfaces. It was shown that a pH far from the isoelectric point (pI) of laccase from Trametes Versicolor (pI = 5.8–6.1 [49]) promotes the adsorption process. These results provide support for Norde's theory [50] that adsorptions of proteins on hydrophilic surfaces could be driven by the electrostatic attractions resulting from a structural rearrangement in the protein molecule. Since cellulose acetate membranes have a slightly negative surface charge which varies only a little with pH [51], protonation of the laccase amino-acid groups might determine the immobilization efficiency. Interestingly, the optimal conditions were determined close to the laccase activity optimum around pH = 4–4.3 [41].

Intermolecular bonds formed by cross-linking can chemically stabilize the protein and increase the rigidity of protein structure [52], but might result in decreased activity. Therefore, the cross-linker concentration and temperature have a definitive effect on the level of cross-linking. Mainly because these directly affect the chemical reaction rate.

Investigation of the blank experiments of cross-linking, it was concluded that the lower temperatures could stabilize the laccase on the membrane during the experiment, but the differences were neglectable. On the other hand, the effect of the cross-linker concentration was highly dependent on the temperature. In the case of 4 °C and 100 mM GA concentration, the activity was significantly higher than at 20 °C. Interestingly, the activity of the membrane was higher than in the blank after cross-linking. This phenomenon could result from the higher stability of the laccase in the form of cross-linked aggregates inside the pores [53]. Therefore, less laccase was removed during the washing step after cross-linking.

After optimizing the method, the specific activity of the membrane-bound laccase was determined. In order to specify the activity, it could be expressed as units bound to the membrane surface in $U \cdot m^{-2}$ [54]. Membrane activity was measured by the conversion of ABTS substrate, similar to the case of the free enzyme. However, the ABTS solution was filtered through the membrane instead of mixing the catalysts with the substrate. In our study, a simplified method for calculating the membrane activity was presented. However, it must be mentioned that the kinetics of enzymatic substrate conversion could be described with more advanced models [55] for modelling purposes. The membrane activity could be best used to compare different enzymatic membranes. As a result of our optimization study, 1174 $U \cdot m^{-2}$ activity was achieved. This value is lower than it was achieved by chemical immobilisation methods as in the case of gelatin-based immobilisation on ceramic membranes [30], which highlights the advantages of chemical immobilisation methods. Compared to kinetic studies of the dissolved form of laccase the K_M value of 48.7 µM is higher than was published by Lorenzo et al. [56] (38 µM). It is indicating that the affinity of the immobilised laccase is lower than in free form, which could be the result of lower accessibility of the immobilised enzyme [57]. In a similar study, when laccase was immobilised on a microfiltration cellulose membrane, the K_M value was higher. This could indicate higher affinity to ABTS in our study, although they utilised laccase from different laccase sources (*M. thermophila*) on lower pH and elevated temperature [57], which increased the activity and made the direct comparison difficult. Generally, this is a common limitation of any enzymatic decontamination process, that when lower substrate concentrations are present in a solution, longer retention times would be required due to lower reaction rates [58]. However, it could be overcome by applying higher enzyme concentration or performing the treatment where the highest concentrations of the pollutant appear. This concept was successfully tested in municipal wastewater by Spina et al. [59]. They reported significant differences in removal efficiency when using enzymatic treatment in different stages of the wastewater treatment plants.

The surface analysis made by SEM indicated that the CLEA formed on the surface and inside the pores, similar to the previously published work with lipase [60]. In addition,

uniformly sized spherical aggregates were detected with a comparable diameter of the membrane pores. That could be the main reason for decreased membrane flux, which generally occurs during biocatalytic membrane preparation [61].

Compared to the substrate used for optimization, the rate and the mechanism of DCF removal is different, since the transformation of ABTS has the highest reaction rate among other substrates [62]. The degradation of DCF was performed in buffering solutions, so the changes in pH were eliminated. Because the membrane system was open to the air through the feed vessel and permeate side, the dissolved oxygen remained sufficiently high and did not inhibit catalysis without additional aeration.

However, 57.9% (SD = 0.6%) removal of DCF was achieved; this removal corresponds to specified conditions, which is a dead-end filtration without recirculating the effluents. Given in a time-unit, approximately 0.1 mg DCF could be removed hourly using 19.9 mg of enzyme immobilised in membrane pores. The same laccase used in the form of suspended CLEA was able to transform 15.4 μg $g_{laccase}^{-1}$ within 24 h, achieving 60% removal of DCF [63]. However, these results are not directly comparable due to different initial concentrations of DCF and reactor configurations. Nair et al. [64] used a similar two-step immobilisation procedure on functionalised silica spheres involving adsorption and cross-linking. These were retained in an integrated membrane reactor, and over 70% of DCF removal was achieved by continuous recirculation over 80 h. Encapsulated laccase in electrospun material was tested to transform naproxen and diclofenac with similar experimental conditions [65] resulting in 80% removal efficiency and less toxic effluents within 24 h. Masjoudi et al. [33] achieved 95% under 4 h using 5 ppm DCF initial concentration at 25 °C, pH = 5 utilisation of multi-walled carbon nanotubes. In our study, high removal was achieved despite the enzyme-substrate contact time being short compared to these studies. It indicates the potential improvement with repeated filtration of the treated solution to increase the removal efficiency.

In the case of phenolic pharmaceuticals, molecules with low solubility and precipitates might be produced by enzymatic conversion [27]. Reaction products of conversion were not detected in the effluent by HPLC-UV measurement on 278 nm wavelength. However, the slight shift in maximum absorbance of the reaction mixture in the UV range indicates the formation of some reaction product, which was published for diclofenac removal with immobilised laccase [66]. A slight yellow discolouration of the membrane was observed, which was common in the case of transformation of diclofenac with free laccase [67], but the precipitate formation was minimal during DCF removal. The accumulation of reaction products on the membrane could significantly modify the reaction kinetics over time and change the efficiency. The formation of these reaction products is highly dependent on the process conditions and the initial DCF concentrations [27]. Generally, the ecotoxicity of the diclofenac solution is reduced by the treatment with laccase [65]. In addition, the partial removal of reaction products with membrane separation could further reduce the ecological risks. Although our results regarding the micropollutant removal application are promising, further research on the optimal operating conditions to maximise the removal of the reaction product and investigation of the effect of membrane fouling and inhibition would be beneficial for a scaled-up version of the integrated process.

5. Conclusions

Optimal conditions of the enzyme adsorption on the membrane were determined by a central composite design scheme and linear regression. The optimal condition of the process is to perform the adsorption step with 3 mg·mL^{-1} laccase solution dissolved in pH4 buffer, with an adsorption time of 9 h and a temperature of 29 °C. After adsorption, the membrane must be cooled down to 4 °C for 2 h of cross-linking with 100 mM GA could result in the highest possible activity (1174 U·m^{-2}). The enzymatic membrane was characterized using a model substrate (ABTS) and showed apparent Michaelis–Menten kinetics (v_{max} = 577.1 μM·s^{-1}, K_M = 48.7 μM). Scanning Electron Microscopy analysis showed that enzyme aggregates were detected on the surface and within the membrane's pores of cellu-

lose acetate membrane. Despite a slight decrease in the flux, the biocatalytic membrane made using the optimized immobilization process showed high activity towards ABTS and was able to catalyze the conversion of DCF. Our work demonstrated that biodegradable cellulose acetate microfiltration membranes can be used as a carrier for laccase immobilization for micropollutant remediation.

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Abbreviations

The following abbreviations are used in this manuscript:

A	laccase enzyme activity measured with ABTS
A_{actual}	Activity measured during the experiment (t).
A_{intial}	Activity measured at the beginning of the experiment (t = 0).
ABTS	2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
$c_{permeate}$	concentration in the permeate (µM)
c_{feed}	concentration in the feed (µM)
EMR	enzymatic membrane reactor
k	first-order decay constant
K_M	Michaelis–Menten kinetic constant (µM)
OFAT	one factor at time method
Q	mass flow rate ($g{\cdot}s^{-1}$)
RSM	response surface methodology
S	membrane surface (cm^2)
SD	standard deviation
t	time (s)
V_m	membrane volume (cm^3)
v_{max}	maximal reaction rate ($\mu M{\cdot}s^{-1}$)
ϵ	Extinction coefficient ($M^{-1}cm^{-1}$)
Φ	membrane porosity

Appendix A

Table A1. Central Composite design scheme. Factors coded as laccase concentration (A), temperature (B), adsorption time (C).

A	B	C
−1	−1	−1
1	−1	−1
−1	1	−1
1	1	−1
−1	−1	1

Table A1. *Cont.*

A	B	C
1	−1	1
−1	1	1
1	1	1
−1	0	0
1	0	0
0	−1	0
0	1	0
0	0	−1
0	0	1
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0

Table A2. Regression table for linear regression model.

Estimated Coefficients	Estimate	SE	t Stat	*p* Value
(Intercept)	371.23	10.524	35.276	4.5644×10^{-10}
A	92.8	9.0903	10.209	7.2736×10^{-6}
B	46.1	9.0903	5.0714	0.00096339
C	45.5	9.0903	5.0053	0.0010458
A:B	25.625	10.163	2.5213	0.035734
A:C	23.875	10.163	2.3492	0.046743
B:C	−16.875	10.163	−1.6604	0.13541
A^2	−51.01	17.39	−2.9333	0.018903
B^2	−9.5103	17.39	−0.54688	0.59938
C^2	−53.51	17.39	−3.077	0.015185
A:B:C	−7.375	10.163	−0.72565	0.48873

Linear regression model: Activity $1 + A + B + C + A \times B + A \times C + B \times C + A^2 + B^2 + C^2 + A \times B \times C$. Number of observations: 19, Error degrees of freedom: 8. Root Mean Squared Error: 28.7. R-squared: 0.966, Adjusted R-Squared: 0.923. F-statistic vs. constant model: 22.7, p-value = 8.47×10^{-5}.

Table A3. ANOVA table of the linear regressions.

	Sum SQ	DF	Mean SQ	F	*p* Value
1. Total	1.9391×10^5	18	1.0773×10^4	NaN	NaN
2. Model	1.8730×10^5	10	1.8730×10^4	22.6663	0.0001
3. Linear	1.2807×10^5	3	4.2691×10^4	51.6633	0.0000
4. Nonlinear	5.9226×10^4	7	8.4608×10^3	10.2390	0.0019
5. Residual	6.6107×10^3	8	826.3318	NaN	NaN
6. Lack of fit	6.0435×10^3	4	1.5109×10^3	10.6549	0.0208
7. Pure error	567.2000	4	141.8000	NaN	NaN

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