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A LOW-COST LACCASE BIOSENSOR FOR ON-SITE MONITORING OF PHENOLIC POLLUTANTS IN AQUATIC ENVIRONMENTS

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Abstract
Phenolic compounds are toxic environmental pollutants commonly present in industrial wastewater and textile dye effluents. In this study, a laccase-based paper biosensor was developed for the rapid and cost-effective detection of phenolic compounds in water samples. The biosensor was fabricated using fungal laccase immobilized on Whatman filter paper with MBTH as a chromogenic substrate. In the presence of phenolic compounds, sa visible pink to maroon coloration was produced due to enzymatic oxidation reactions. Optimization studies showed that 2 mg/mL laccase and 24 mM MBTH provided the highest colour response. The biosensor exhibited sensitivity over a guaiacol concentration range of 1–512 µM with a detection limit of 1 µM. Sewage water and textile dye effluent samples collected from Faisalabad were successfully analyzed using the developed biosensor. Colour intensity was quantified through RGB image analysis using ImageJ software. The results demonstrated that the developed biosensor is rapid, portable, inexpensive, and suitable for environmental monitoring of phenolic pollutants in wastewater samples.

INTRODUCTION

The issue of water pollution becomes one of the critical environmental problems associated with the increase in economic activities. One of such issues is the problem of discharging industrial waste to water bodies, which include phenolic compounds. Such compounds are poisonous, carcinogenic, and mutagenic; they are capable of causing harm even in small amounts to both humans and animals and aquatic organisms, do not decompose, and also have a harmful impact on the environment (Zhou et al., 2020).

Phenolic compounds are produced endogenously in the decayed material of plants and animals, as well as exogenously from anthropogenic activities. Many goods and products are manufactured and sold that pose health and environmental dangers to their consumers without people's knowledge. Also, purposeful release of toxicants continues to occur to the environment through different human activities, but also sometimes occurs accidentally during spills (Galletti et al., 2019). At the same time, despite such problems existing, at the moment, there is no universally effective and sustainable approach to solving the problem of chronic aquatic contamination. Environmental pollution, especially water pollution, was stimulated by industrialization (Chen et al., 2022). Phenolic compounds (organic pollutants) are one of the leading contaminants of

water and soil (Singh et al., 2022). Surface waters are the most threatened by organic pollution due to the use of these substances as component of industrial waste, which are used in various industries such as the pharmaceutical industry, agriculture, dye-making, and the petrochemical industry (Vosoughi et al., 2017).

As noted above, phenolic compounds can be both endogenously produced and obtained exogenously. PC are widely used in various industrial areas, including the production of oil, pharmaceutical products, food products, pesticides, dyeing, and printing. Exogenous PCs are considered dangerous for the environment, being characterized by such properties as high toxicity and non-biodegradable, they can cause tumorigenesis, neurotoxicity, endocrine disruption, and reproductive toxicity (Zhu et al., 2022). In view of the above, there is a need for analytical studies for the detection of phenolic compounds. The main analytical methods used in the study of the phenolic compound include gas chromatography, high-performance liquid chromatography (HPLC), and electroanalysis (Umapathi et al., 2025). The advantage of this method is its high accuracy; however, it is extremely inconvenient in terms of practicality due to the need for complex equipment (Salcedo et al., 2019). In addition, these methods can determine only individual phenols in the water, but not phenolic mixtures. Thus, the rapid tests for determining the content of phenols is needed.

Water is the most natural product of vital importance. Nevertheless, modern industrialization, the expansion of the urban space, and the development of agriculture leads to the contamination of many resources. phenolic compounds appear in water through various production technologies, waste from agricultural production, or the destruction of natural products (Karim et al., 2021). Given the dangers of such pollutants for the environment and human health, the relevance of these compounds should be noted (Hernández et al., 2017). The control of phenolics is conducted by regulatory bodies including WHO and EPA for both health reasons and environmental considerations. The established concentration limit values for phenolic compounds include concentrations from 1 µg/L in drinking water and from 0.03-4000 µg/L in surface water (Ramos et al., 2021). However, current regulations do not make the distinction between specific phenolic compounds, thus emphasizing the need for stricter standards based on environmental and health hazard assessments. Therefore, phenolic compound detection in surface and groundwater is viewed as necessary to ensure environmental and health safety.

The laccase enzyme, classified under phenol oxidases subgroup among multicopper oxidases (PCOs), is characterized by its high redox power as well as ability to oxidize a wide array of organic pollutants (Pérez et al., 2022). Its catalytic activity has been widely recognized in applications in different biotechnological processes, namely, food production processes (Lambré et al., 2024), the textile industry (Peng et al., 2024), pharmaceuticals industries (Xu et al., 2024), and nanobiotechnology (Aghaee et al., 2024). Several researchers have reported laccase's ability to convert several organic pollutants to other forms through molecular oxygen oxidation reaction in the presence of both aromatic and non-aromatic hydrogen acceptors (Ishak et al., 2025). Although the enzyme has been proven useful in several biotechnological applications, there exist several disadvantages associated with the use of laccase which include low stability, high costs, and unavailability for repeated uses. Immobilizing the laccase enzyme using carriers can help overcome some of the aforementioned drawbacks through confining the enzyme to a certain matrix leading to increase its stability and reuse. Carrier selection is an important aspect of enzyme immobilization, and thus, carrier properties play a key role in the process (Dong et al., 2024). Laccases produced from fungi have been shown to be capable of degrading many classes of xenobiotic including phenolic compounds, dye materials, polycyclic aromatic hydrocarbons, pharmaceutical agents, cosmetics and personal care products, pesticide chemicals, plasticizer compounds, and related substances (Sodhi et al., 2024).

The primary goals of this research were to measure the phenolic chemicals found in sewage water and textile dyes and textile dyeing effluents, developed laccase-based paper biosensor. O-quinone molecules were quantified using MBTH as the chromogenic agent. In the presence of laccase, phenol is first oxidized to o-quinones before reacting with MBTH to produce a pink colour.

MATERIALS AND METHODS

2.1 Place of work:

This research done in the lab of biochemistry in Ripha international university of Faisalabad.

2.2 Material:

Laccase enzyme, MBTH (3-methyl 2-benzothiazolinone hydrazine) chromogenic substrate, PBS (phosphate buffer), Guaiacol, Whatmann paper, Wastewater sample, Vacuum desiccator, ImageJ, Smartphone camera, Beaker, Measuring cylinder, Micropipette, Petri dishes, Volumetric flask

2.3 Construction of laccase-based paper biosensor

Whatman filter paper was cut into the same size (1cm×1cm) for each use and it was autoclaved before use (Oktem et al., 2016).

2.3.1 Laccase enzyme:

The fungal laccase enzyme (*Termetes Vesicolor)* was purchased and used for the development of the laccase-based paper biosensor.

2.3.2 Laccase assay:

The laccase activity was measure by guaiacol oxidation assay, with minor adjustments to the process outlined by (Kalra et al., 2013). 1 mL of 2 mM guaiacol, 3 mL of 10 mM phosphate buffer at pH 7.0, and 1 mL of the enzyme solution synthesize the reaction mixture. 1 ml of distilled water was used in place of the enzyme to produce a blank. For fifteen minutes, the reaction mixture was incubated at 30 °C ± 2 °C. A UV spectrophotometer was used to quantify the absorbance at 450 nm in order to quantify the oxidation of guaiacol, a reddish-brown color produced by laccase activity (Hussain et al., 2025).

2.3.3 Phosphate buffered saline (PBS) preparation

To prepare Phosphate Buffered Saline (PBS, pH 7.0), sodium chloride (9.95g/L), potassium chloride (0.25g/L) and disodium hydrogen phosphate (1.14g/L) and potassium dihydrogen phosphate (0.25g/L) was dissolved in distilled water. The solution was thoroughly stirred to ensure complete dissolution. (Oktem et al., 2016).

2.4 Optimization studies

2.4.1 Optimization of Laccase concentration

Four different concentrations of laccase (2.0, 4.0, 8.0, 12 and 24mg/mL) were prepared in PBS (pH 7.0). The enzyme solutions were added to the filter paper (Whatman No.1, 1cm × 1cm) and left to dry for appropriate time in a vacuum desiccator at 37 °C ± 2 °C. This was repeated with the addition of 1 mL of MBTH (24 mM) and the enzyme loaded paper was dried under the same conditions once more. Thereafter, different concentrations of guaiacol solutions were applied and 20µl of each of the concentration (32µM, 64µM, 128µM, 256µM and 512µM) were applied. The papers were put in the desiccator and incubated at 37 ± 2 °C for drying. The amount of pink maroon coloring used led to the determination of optimum enzyme concentration. Therefore, optimal concentration of enzyme had been achieved by keeping the concentration of MBTH fixed as 24Mm. (oktem et al., 2012).

2.4.2 Optimization of MBTH Concentration

A series of MBTH solutions: 6, 12, 24, 48, 96 mM were made in PBS (7.0). The strips of filter paper utilized in each MBTH were 1cm x 1cm. The activity of the laccase, solution was applied on a pre-loaded paper and heated to 37 ± 2 °C for appropriate time period to get dried in the vacuum desiccator. Five different concentrations of guaiacol (32, 64, 128, 256 and 512 µM) were added as individual samples. The papers then were placed in a desiccator at 37 ± 2 °C to incubate for drying. A color of pink maroon was used as an index to identify the optimum level of MBTH, as well as to determine the effect of its level (Malleswari et al., 2016).

2.4.3 Evaluate the Sensitivity and Detection Limit

For further analysis, the ideal amounts of MBTH and laccase were used. The guaiacol solutions (20 µL solution with different concentrations: 0.1, 1, 2, 4, 8, 16, 32, 64, 128, 256 and 512 µM) which prepared were applied on the created biosensors strips. Then, the papers were incubated for appropriate time at 37 ± 2 °C in a vacuum desiccator and drying. Sensitivity was calculated by the range of colors observed when a change in concentration occurred, the lowest concentration observed to show coloration with measuring pink maroon coloration was used as the detection limit (Oktem et al., 2016).

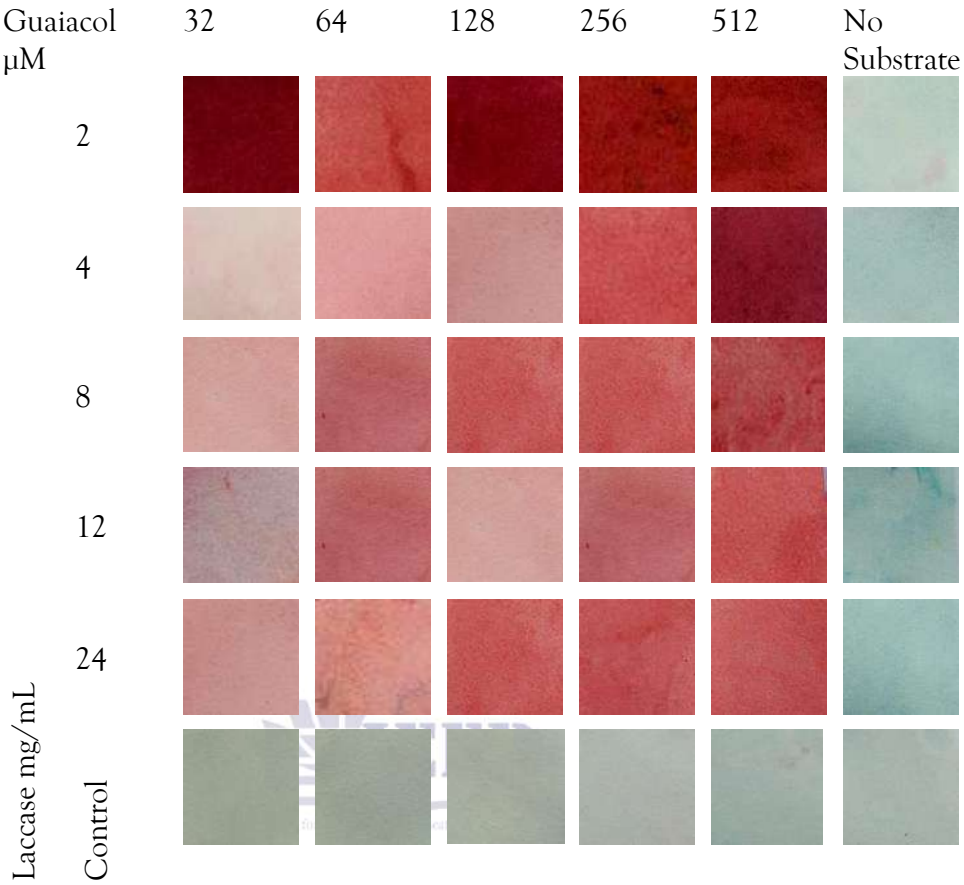
2.5 Application on phenols and waste water

The laccase-based paper biosensor was used to find phenolic chemicals in various wastewater samples and reference phenol solutions. To assess the biosensor's sensitivity and color response, standard guaiacol solutions with different concentrations were used.

Samples of sewage water, and textile industrial effluents were collected from various parts of Faisalabad for wastewater analysis. Before being analyzed, the obtained samples were filtered to get rid of suspended particles. Under ideal experimental conditions, the prepared biosensor strips were subsequently by the pink to maroon hue that appeared in positive samples.

2.5.1 Colour intensity analysis

Samples of papers were made with the optimum amounts of enzyme and MBTH for further testing. The various water samples were applied to the paper and dried, which was followed by scanning using a scanner or a smartphone camera. The quantitative data of the change of color obtained on the paper biosensor is then analyzed by the software called ImageJ (Malleswari et al., 2016).



RESULTS AND DISCUSSION

The findings of the production and assessment of the cost-effective laccase-based paper biosensor for the detection of phenolic contaminants in aquatic environments are presented in this chapter. The generated biosensor's sensitivity, selectivity, detection limit, and suitability for actual water samples were observed. To showed the performance of the devised approach, the results are compared with previously published research.

3.1 Construction of paper biosensor

Whatman No. 1 filter paper was used as the supporting matrix for enzyme fibrication in the development of the paper-based biosensor. Whatman No.1 filter paper was cut into the same size (1cm×1cm) for each use and it was autoclaved before use. The fungal laccase enzyme (*Termetes Vesicolor)* was used for the development of the laccase-based paper biosensor.

3.2 Optimization studies

3.2.1 Optimization of laccase concentration

The optimum concentration of laccase enzyme to be utilized in the laccase based paper biosensor has been tested by using different concentration of laccase enzyme from 2mg/ml-24mg/ml to get the highest colour response for the phenolic compounds. Biosensor strips were prepared with different amount of laccase (0-0.001 g/L) in all strips, and all other variables were kept constant. In the presence of guaiacol, the concentration of 2 mg/mL laccase exhibited the best pink maroon colour and also had the highest sensitivity. Hence 2mg/mL concentration of laccase was chosen as the best concentration for the preparation of the paper biosensor (Malleswari et al., 2016).

Figure:3.1 *Optimize concentration of laccase over guaiacol*

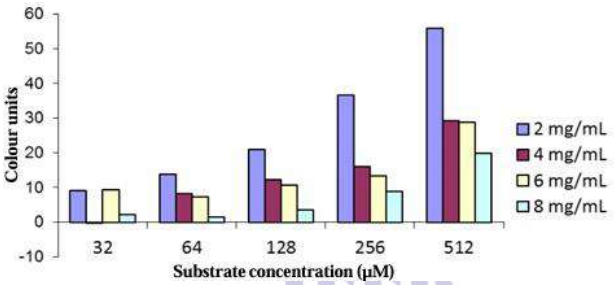


Figure:3.2 *The effect of enzyme concentration on paper biosensor over guaiacol 32-512*

The graph shows the influence of enzyme concentration on the biosensor reaction of a laccase paper biosensor using guaiacol as the substrate. As seen from the graph, at any enzyme concentration, color intensity is proportional to the concentration of the substrate, that is, the increase in substrate concentration leads to more biosensor reaction.

At all substrate concentrations, the biosensor reaction is maximized when the enzyme concentration is 2 mg/mL. Color intensity is also maximized at about 52 color units at the 512 µM guaiacol concentration. An increase in enzyme concentration above 2 mg/mL causes a decrease in color intensity at all substrate concentrations; with the 8 mg/mL enzyme concentration, the biosensor reaction is minimized. The reason for this may be the formation of clusters of the enzyme at high enzyme concentrations on the paper strip, limiting color formation on the biosensor. As such, it is evident that 2 mg/mL is the most suitable enzyme concentration for the paper biosensor. An increase in guaiacol concentration will lead to better sensitivity of the biosensor.

3.2.2 Optimization of MBTH concentration

Different MBTH concentrations ranging from 6–96 mM were evaluated for this optimization to get the highest colour response for the detection of phenolic compound using laccase based paper biosensor. To prepare the biosensor strips, different concentrations of MBTH were used and all experimental conditions were kept the same. 24 mM MBTH was the most intense pink maroon coloration and most sensitive when guaiacol was present of the tested concentrations. 24 mM was chosen as the best concentration of MBTH for the laccase-based paper biosensor to be fabricated.

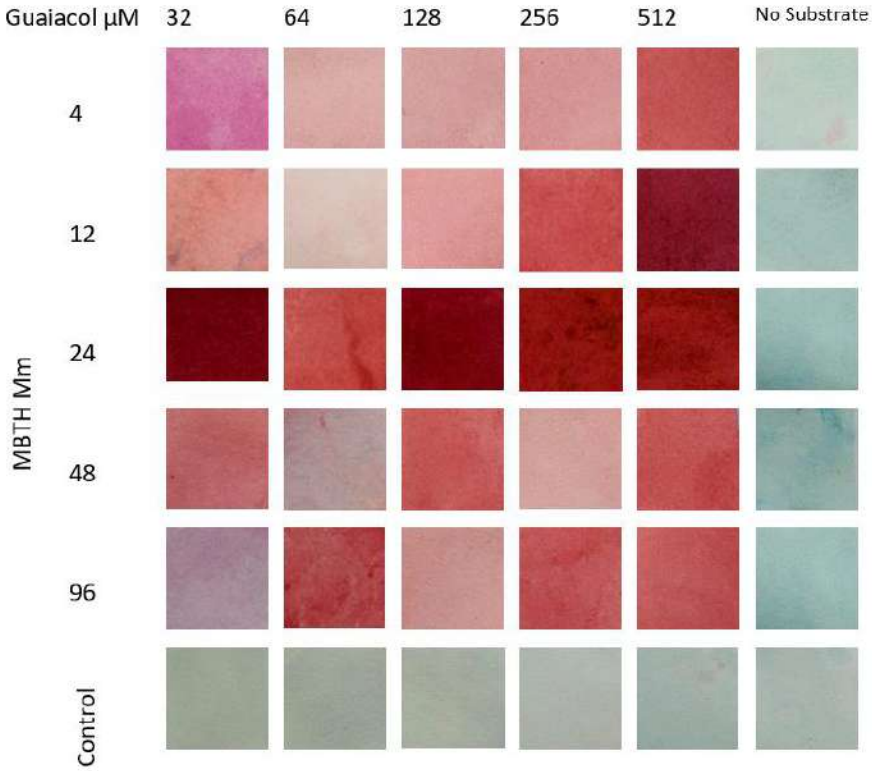


Figure:3.3 Effect of MBTH consentration over guaiacol

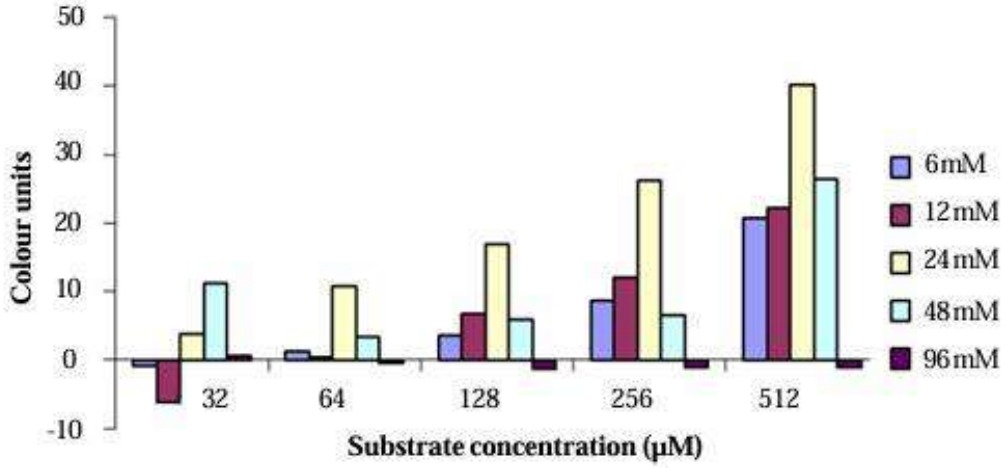


Figure:3.4 The effect of MBTH concentration on paper biosensor over guaicol 32-512

In the graph, the influence of different concentrations of MBTH on the sensitivity of the biosensor to different concentrations of substrates is discussed. In addition, the graph shows the intensity of color produced in the reaction of the biosensor. From the figure 4.2, it can be observed that the intensity of color produced increases with the concentration of the substrate. The highest intensity of color was recorded when the concentration of MBTH was 24 mM especially for substrate concentrations of 256 µM and 512 µM. When the concentration of MBTH was increased to 96 mM, it was observed that the intensity of color obtained was low to negative. Therefore, the optimum concentration of MBTH in terms of producing high intensity of color is 24 mM.

3.2.3 Optimization of substrate concentration

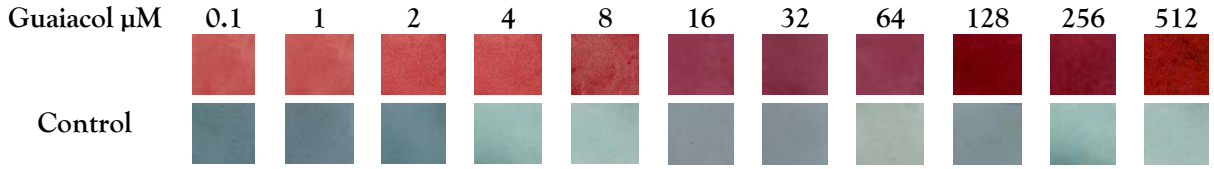


Figure 4.5: ***Optimization of substrate concentration***

Figure 4.5: *Optimization of substrate concentration*

To find the optimum concentration where the maximum color was obtained, different concentrations of guaiacol were tested to optimize the concentration of the substrate used in the laccase based paper biosensor. The optimized conditions of laccase and MBTH were used to test the different concentrations of substrates in the range of 1-512 µM. The color of the biosensor was seen to increase with the concentration of guaiacol, ranging from light pink to maroon. Biosensor was found to be sensitive throughout the range tested with the lowest detection limit of 1µM. The optimized range of substrate concentration showed that the biosensor is suitable for a rapid detection of phenolic compounds in water samples (Malleswari et al., 2016.

Sample	Shades developed on biosensor
Control (Guaiacol)	
Sewage Water 1	
Sample 2	
Sample 3	
Textile Dyeing effluents 1	
Effluents 2	
Synthetic Textile Dye effluents	

3.3 Application of Biosensor

3.3.1 Laccase-based paper biosensor application on sewage water

The laccase-based paper biosensor was effectively utilized to identify phenolic chemicals in sewage water samples that were collected from different areas of Faisalabad. The biosensor strips were submerged in the different samples of collected sewage water and their color development was monitored. The presence of phenolic pollutants in the sewage water samples was indicated by a notable pink, pinkish brown and brown coloration on the biosensor strips. These colored strips compared with standard guaiacol solution. Depending on the amount of phenolic chemicals in each produced color's intensity varied. imageJ or trigit software was used to further evaluate the color response for quantitative RGB assessment. The blank sample prepared in distilled water. From sewage water samples 1,2 and 3 have colour intensity 50,56 and 44 respectively. The results showed that the laccase-based

paper biosensor is an easy-to-use, quick, affordable, and efficient instrument for on-site monitoring of phenolic pollutants in sewage water from Faisalabad's different places (Jędrzejczak et al., 2022).

3.3.2 Laccase-based paper biosensor application on textile dye effluents

The developed laccase-based paper biosensor was used to determine phenolic contaminants in various textile dyes effluents sample 1 and 2 and textile dye samples that were collected from Faisalabad's textile industries. To assess the biosensor strips' reaction to phenolic pollutants frequently found in textile effluents, they were subjected to a variety of dye samples. The presence of phenolic and related oxidizable chemicals in the textile dyes was confirmed by the biosensor's observable light pink to maroon coloring upon contact with the dye samples. Different dye samples showed variations in color intensity because of variations in their phenolic content and chemical makeup. Textile dye effluents 1 and 2 samples have pink brown and pinkish maroon colour respectively. Synthetic textile dye showed dark maroon colour. ImageJ or trigit software was used to objectively examine the produced color response RGB colour intensity. Textile dye effluents 1 and 2 have colour intensity 102 and 107 respectively. Synthetic dye effluents have 135 colour intensity. The colour intensity increase by increasing the phenolic contents. The results demonstrated that the laccase-based paper biosensor offers a quick, affordable, portable, and environmentally acceptable way to track phenolic contaminants in textile dye and synthetic textile dye samples. Thus, biosensor used in environmental monitoring and textile effluent analysis in industrial regions (Jędrzejczak et al., 2022)

Table:3.1 Application of biosensor on different samples

Sample	Shades developed on biosensor
Control (Guaiacol)	
Sewage Water 1	
Sample 2	
Sample 3	
Textile Dyeing effluents 1	
Effluents 2	
Synthetic Textile Dye effluents	

3.3.3 Colour intensity analysis:

The enzymatic reaction occurs on the paper strips is observed by color intensity analysis in a laccase-based paper biosensor. The color that observed after applying the sample is directly proportional to the concentration of the analyte or the activity of the enzyme. A colored chemical is produced when the laccase enzyme oxidizes the chromogenic substrate, such as MBTH–guaiacol system. Increased the concentration of pollutants or an enzymatic reaction showed an increase in color intensity (Caleb et al., 2026

Samples	R	G	B	Mean RGB	Color Intensity (I_B-I_S)	S.D	Observation
Blank	200	210	190	200	0	10	Light yellow color
Sewage water sample 1	194	122	135	150	50	38.37	Pink brown
Sample 2	184	125	128	144	56	33.23	Brown
Sample 3	201	131	135	156	44	39.31	Pink
Textile dye effluents1	140	73	80	98	102	36.83	Pinkish maroon
Effluents2	130	65	76	93	107	34.79	Dark brown
Effluents3	95	44	54	65	135	27.02	Dark maroon

Where:

I_B = Mean RGB of blank

I_S = Mean RGB of sample

Standard Deviation(SD) formula:

$$S.D = \sqrt{\frac{\sum(x_i - \bar{x})^2}{n-1}}$$

Where:

$\bar{x}$ = Sample average

x = Individual value of sample

n = Number of samples

Colour intensity ∝ concentration of ezyme or pollutants

RGB intensity Value

RGB intensity formula = $\frac{R+G+B}{3}$

Blank colour intensity formula: $I_B - I_S$

Table:3.2 *RGB colour intensity of different samples*

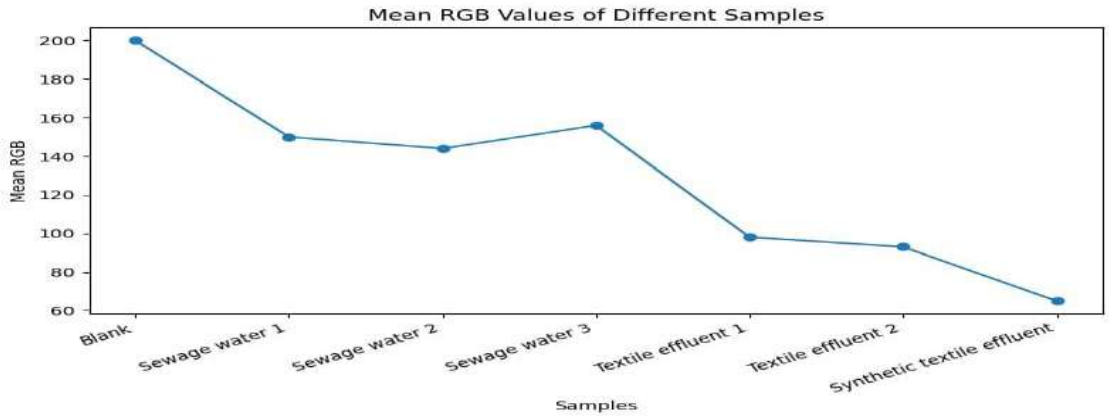


Figure 4.6: *Mean RGB values of different samples*

High colour intensity indicates the stonger enzymatic oxidation reaction and dark maroon colour give lower RGB value and higher colour intensity. Hence lower RGB value gives the higher intensity. The blank strips is act as control or reference for all calculation. The colour intensity of the laccase-based paper biosensor was determined by RGB image analysis through ImageJ (Caleb et al., 2026).

This table shows the RGB color intensity measurement of various wastewater and dye effluent samples using a paper-based biosensor that uses laccase enzymes. The color parameters R, G, and B, average RGB, color intensity, and standard deviation (S.D.) were used to measure biosensor activity. The blank sample had the highest mean RGB value (200) and color intensity of zero, indicating no phenolic compounds were present because there was no oxidation process. The natural color of the biosensor material is light yellowish-green. The color intensity ratings for sewage water samples ranged from 44 to 56. Compared to other sewage water samples, Sample 2 had the highest color intensity (56) with brown coloration, suggesting higher levels of phenolic or oxidizable chemicals. The standard deviation values (33.23 to 39.31) are reasonable, showing that the RGB measurements had some variation. For textile dye effluent, Samples 1 and 2 had higher color intensities (102 and 107, respectively), with dark brown and pinkish maroon coloration. This means higher phenolic concentration in textile dye effluents leads to more enzymatic oxidation. The synthetic textile dye effluent sample had the lowest RGB value (65), the highest color intensity (135), and dark maroon coloration. The great sensitivity of the paper biosensor to synthetic dyes in contaminated environments is shown by the decrease in mean RGB and increase in color intensity, which indicates effective color formation due to efficient dye oxidation catalyzed by immobilized laccase enzymes. Regarding RGB measurement, the computed S.D. values show a good level of consistency in the data. Because of their different compositions and color formations, sewage and textile samples had larger standard deviation values than the blank solution (10). The findings show that phenol and dye chemicals are successfully detected by the laccase immobilized on the paper biosensor through visual changes and RGB intensity measurement. Higher color intensity levels mean higher pollutant concentrations. Textile and synthetic dye effluent samples have more oxidizable elements than sewage water samples, so they show more vivid coloration. Therefore, the laccase-immobilized biosensor can be seen as an eco-friendly and practical tool for analyzing wastewater samples (Kavaliauskas et al., 2025).

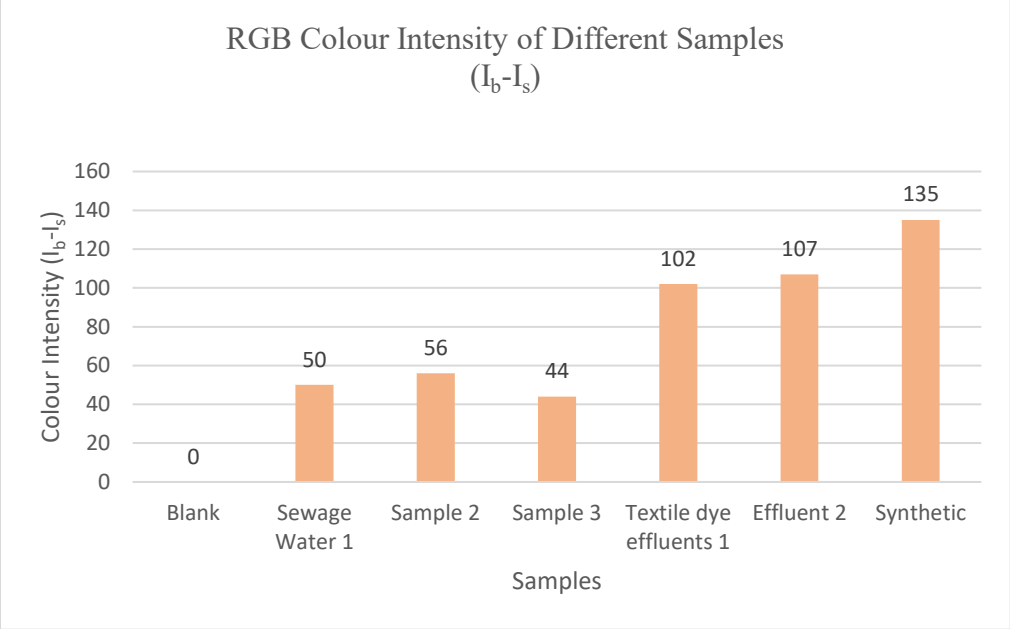


Figure 4.7: ***RGB colour intensity of different samples (I_b-I_s)***

As shown in graph figure 4.6, the amount of oxidizable pollutants in the samples went up, along with the color getting darker. Industrial dye waste and synthetic samples showed a stronger reaction than sewage water samples, because the laccase-based paper biosensor is good at detecting phenolic compounds and dyes. The biosensor was able to detect both low and high levels of pollutants by looking at the change in color of the RGB. The goal of this research was to design a laccase-based paper biosensor for detecting phenolic compounds from

different types of wastewater. Maroon and green colors were formed on phenolic substrates when laccase, immobilized with MBTH, was applied to Whatman paper. After drying in a vacuum desiccator, the paper with laccase and MBTH was ready for use. The best concentration of MBTH was found to be 24 mM, and the ideal laccase concentration on the paper that gave the strongest color and least background color was 2 mg/ml. The biosensor was sensitive to guaicol, and as its concentration increased, so did the color

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