DEGRADATION OF REACTIVE DYES USING IMMOBILIZED PEROXIDASE PURIFIED FROM *NIGELLA SATIVA*

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ABSTRACT

The goal of the current work was to use *Nigella sativa* seeds' free and immobilised peroxidase to degrade textile dyes that pollute the environment and water. The enzyme was extracted for 15 minutes using a 1:20 (w:v) ratio, sodium acetate buffer at 0.2 M, and pH 5.0 after the optimal conditions for extracting the enzyme from Nigella seeds were determined. This yielded the highest specific activity of the enzyme, 1750 units/mg protein. The enzyme was purified in two stages: sucrose concentration and Sephadex G-150 gel filtration. With a 35% enzyme yield, the findings demonstrated a 2,8-fold increase in final purification folds. The entrapment method with Ca-alginate was used to immobilise peroxidase, and the immobilisation ratio was 49%. Under optimum circumstances, pH 5, temperature 37oC after 3 hours with textile dyes such as yellow, red, black, and blue dyes, the removal efficacy of dyes by crude enzyme (free, immobilized) and partially purified peroxidase were tested. With crude peroxidase, dye removal efficiency peaked at (76.9, 88.7, 91, and 88) %, respectively. The efficiency of the crude immobilised enzyme in removing the four dyes was roughly equivalent to the efficiency of the purified enzyme (70, 81, 72, and 56.4%, respectively).

Keywords: Plant, enzyme, Black seed, Textile dyes

مجلة العلوم الزراعية العراقية -2021 :52 (6):1365-1374 عزيز وآخرون

تفكيك الصبغات التفاعليه بوساطة انزيم البيروكسديز المقيد والمنقى من نبات حبة البركة

غازي منعم عزيز سحر ارحيم حسين سرى ضامن عباس ايه ليث ابراهيم دعاء كامل عباس
استاذ استاذ مساعد باحث باحث باحث
قسم التقنيات الاحيائية/ كليه العلوم/ جامعة بغداد

المستخلص

هدفت الدراسه الحاليه الى استغلال البيروكسيديز من بذور حبه البركه بصورته الحر والمقيد في تفكيك بعض صبغات معامل الانسجه الملوثه للبيئه والمياه. حددت الظروف المثلى لاستخلاص الانزيم من بذور حبه البركه وتم الحصول على اعلى فعاليه نوعيه للانزيم 1750 وحدة / ملغم بروتين عند استخلاص الانزيم من البذور المطحونه بنسبه 20:1 (وزن : حجم) بدارئ خلات الصوديوم بتركيز0.2 مولار ورقم هيدروجيني 5.0 لمدة 15 دقيقه. نقي الانزيم باستخدام خطوتين شملت التركيز باستخدام السكروز والترشيح الهلامي باستخدام مادة السيفادكس –ج 150، بينت النتائج زيادة في عدد مرات التنقيه النهائيه اذا بلغت 2.8 بحصيله انزيميه وصلت 35%.قيد انزيم البيروكسيديز بوساطه طريقه الحجز باستخدام الجنات الكالسيوم حيث بلغت نسبه التقييد 49%، كما قدرت كفاءة الانزيم الخام (الحر والمقيد) والمنقى في ازاله انواع مختلفه من صبغات معامل الانسجه مثل الصبغه الصفراء والحمراء والسوداء والزرقاء عند رقم هيدروجيني 5 ودرجه حرارة 37 مئويه بعد 3 ساعات من المعامله. بينت نتائج الدراسه ان الانزيم الخام الحر هو الاكفأ في ازاله الصبغات الاربعه اذ بلغت كفاءته (76.9 و88.7 و 88 و91) % على التوالي. وكانت هذه النتائج مقاربه الى كفاءة الانزيم المنقى في ازاله الصبغات الاربعه، بينما بلغت كفاءة الانزيم الخام المقيد في ازاله الصبغات حوالي (70 و81 و72 و 56.4) %على التوالي.

كلمات مفتاحية: نبات، انزيم، حبه البركه، صبغات الاقمشة.

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INTRODUCTION

Peroxidase enzyme (donor: H2O2 Oxidoreductase, EC. 1.11.1.7) is widely distributed in plant, animal, and microbial cells. In 1936, peroxidase was discovered in the fig tree. In 1941, the horseradish (HRP) enzyme was isolated and characterized (4).From 1942 to 1956, the enzyme was isolated from a variety of sources, including wheat, legumes, yeast, potato, and Japanese radish. Due to its varied applications in diagnostics and analysis, peroxidase has gained significant industrial and medical significance. ELISA (Enzyme-Linked Immuno Sorbent Assay), which identifies antibodies or antigens in immunological responses by targeting these enzymes on solid surfaces, is one of its most important applications. Due to the conditions that must be encountered (32), the ease of detection effectiveness configuring outputs of colour. This application may be the most significant due to the absence of a technique to assess the stages separating from substrates, the high persistence during storage, the cheap costs for production and purification, and its relevance in the analyses of the stigma blot tests and in pigmentation tissue. The amount of hydrogen peroxide created by many systems, such as the oxidation of glucose, amino acids, cholesterol, etc., is also measured using it in biochemical analysis (23). Additionally, by examining the antioxidant content of food extracts including ascorbic acid, phenols, flavonoids, and tannins, which experienced several modifications throughout the production and storage processes, the relevance of these enzymes in the processing of fruits and vegetables was discovered. The palm side-off products have many uses in addition to the dates' economic importance; furthermore, the question of exploiting the surplus dates' nucleus for the purpose of producing materials with a high economic value would be extremely advantageous and raise the value of the palm and its products (11). Immobilisation of enzymes may provide them with extra benefits. The immobilised enzymes can be used repeatedly or consistently in a variety of reactors for the efficient recovery of costly enzymes, and they can be readily extracted from reaction systems for reuse, thereby facilitating the work-up and ensuring the protein purity of the final product. In addition, as stated in clause (18), immobilised enzymes may exhibit greater selectivity and specificity. The goal of this research is to extract, purify, immobilize, and decolorize certain textile colors using *Nigella sativa* peroxidase.

MATERIAL AND METHODS

Plant: The source of the peroxidase enzyme was the black seed (*Nigella sativa*), throughout this investigation, was locally available in a market.

Optimal peroxidase extraction conditions

***Nigella sativa* seed treatment:** For peroxidase extraction, two procedures of black seed (*Nigella sativa*) were employed. These techniques included soaking the seeds for 24 hours and grinding them, with a sodium phosphate buffer being used to extract the enzyme from each kind of treatment. One gram of black seed was blended for 15 minutes at 30 degrees Celsius with a 1:5 (w:v) solution of 0.2 M sodium phosphate buffer. Before centrifuging at 8000 rpm for 10 minutes, the sediment was filtered through gauzes to remove any leftover cell debris. In each experiment, the crude extract was examined for enzyme activity, the amount of protein, and specific activity using the clear supernatant.

Peroxidase assay and protein concentration

The enzyme activity of peroxidase was calculated using guaicol as a substrate, as reported by Silva and Koblitz (28). After 3 minutes, the oxidation of guaicol was measured using a VIS-spectrophotometer by monitoring the increase in absorbance at 470 nm. The quantity of enzyme needed to oxidise one μmol of substrate per minute was known as one unit of enzyme activity. Protein concentration was determined using the Bradford technique (6).

Type of extraction buffer: In order to extract peroxidase, black seed was homogenised at 30^oC for 15 minutes with various buffers. These buffers consist of pH 3, 4, 5, and 6: 0.2 M sodium acetate buffer, pH 7: 0.2 M sodium phosphate buffer, and pH 8: 0.2 M Tris-base buffer. Each experiment measured the activity of enzymes, concentration of protein, and specific activity.

Ratio of extraction: A variety of amounts of 0.2 M sodium acetate buffer, pH 5, were added

to one gramme of black seed and homogenized for 15 minutes at 30°C to extract the peroxidase. The extraction ratios were as follows: 1:5, 1:10, 1:15, 1:20, 1:25, 1:30, and 1:40 (w: v). In every experiment, it was determined the enzyme's activity, the quantity of protein, and its specific activity.

Period of extraction

Black seeds (1 g) were homogenised in 0.2 M sodium acetate buffer pH 5 for 15, 30, and 60 minutes at 30 degrees Celsius. Each experiment included the calculation of enzyme activity, protein concentration, and specific activity.

Purification of peroxidase

Enzyme from black seed was purified through dialysis for concentration of enzymes, then gel filtration.

Concentration of enzymes by sucrose

Dialysis tubes were used to concentrate the crude enzyme solution using sucrose, and the resulting afterwards, the activity of enzyme, amount of protein, and specific activity were determined.

Gel filtration chromatography

Sephadex G-150 Preparation: In order to ensure that the gel beads swell, at 90 °C, 20 g of sephadex-G150 was immersed in 500 ml of purified water and stirred for three hours. They were then placed in a cool place at 4 °C overnight. This was done in accordance with the manufacturer's instructions (Pharmacia-Sweden). The gel was re-suspended after being rinsed twice with 0.2 M sodium phosphate buffer at pH 7.0. The gel was gently packed in a glass column (21×1.6 cm) with the same buffer amount, degassed using a vacuum pump, and equilibrated using the same sodium phosphate buffer.

Enzyme separation using Sephadex G-150 column: The elution step followed the enzyme's passage through a sephadex G-150 column was performed with a flow rate of 20 ml/h and a fraction amount of 3 ml of sodium phosphate buffer solution 0.2M, pH 7.0. At a wavelength of 280 nm, the enzyme activity of each protein fraction was measured, and the active fractions were collected. The total volume was then assessed, the activity and amount of proteins were determined, and the solution was concentrated, split, frozen, and kept for future studies.

Peroxidase immobilization

Calcium alginate-based entrapment: Five millilitres of sterile sodium alginate solution (3%), two millilitres of crude peroxidase (244 U/ml), and ten minutes of gentle stirring completed the reaction. To create 1 mm-diameter beads, the resultant mixture was extruded drop-by-drop using10 ml sterile syringe into a 0.2M cooled CaCl2 solution. The beads were then kept for an hour. The calcium alginate enzyme beads were kept at 4 oC (30) in 0.5% (w/v) CaCl2. To eliminate any non-immobilized enzyme, a cooled CaCl2 solution was used to rinse the beads. The activity of immobilized enzymes was determined according to Silva and Koblitz's (28) method.

Immobilized peroxidase activity

Immobilised peroxidase activity was determined replacing 0.1 ml of enzyme with 30 mg of immobilised enzyme in accordance with the Silva and Koblitz method (28). Using a VIS spectrophotometer, the rise in absorbance at 470 nm after three minutes allowed for the detection of guaicol oxidation. The quantity of enzyme needed to oxidize 1 μ mol of substrate per minute was used to define one unit of enzyme activity.

Peroxidase application

Decolorization of Dyes: In the present investigation, reactive black, red, blue, and yellow textile dyes have been bought from Al-Diwaniyah textile mill and used for decolorization studies. The dye degradation reaction mixture is made according to Alam et al. (3)'s instructions, with a few changes. It contains 1 gm of immobilised enzyme, 2 ml of an enzyme solution containing 244 U/ml (crude and purified peroxidase each) and 5 ml of (30 mg/l) dye solution for each dye. At 37°C, the reaction mixture was shaken and rotated at 120 rpm in a shaker incubator. In the control experiment, calcium alginate (1 gm) was used in place of the immobilised enzyme and distilled water (1 gm) was used in place of the enzyme solution. The degradation of a particular dye was studied throughout a range of incubation durations (0, 1, 2, 3, and 24) hours. According to Zhang et al, (33), the percentage of elimination effectiveness was computed using the absorbance at maximum for each dye.

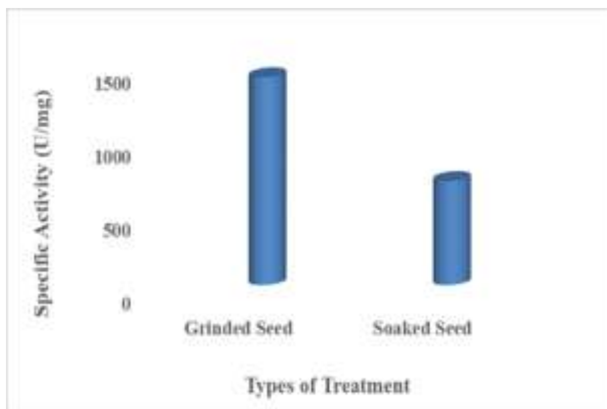

Figure 1. The Influence of Two *Nigella sativa* Seed Extraction Methods on the Extraction of Peroxidase 15 minutes at pH 7 and 30 °C using 0.2 M Phosphate Buffer

RESULTS AND DISCUSSION

Optimal peroxidase extraction conditions

For maximum enzyme extraction, many bioprocess variables impacting peroxidase extraction from black seed were optimised; some of these conditions include type of treatment, type of buffer, extraction ratio, and extraction time, among others. As a result, improving these conditions lowers the cost of extraction and raises peroxidase enzyme production.

Nigella sativa Seeds Treatments

Black seed was extracted using phosphate buffer at 30 oC for 15 minutes, with two treatments: seeds soaking for 24 hours and seeds grinding, to examine the effects of two peroxidase extraction techniques. As shown in figure (1), grinding resulted in the greatest peroxidase extraction, with a specific activity of 1,409 U/mg, while soaking resulted in a specific activity of 704.5 U/mg. Rudrappa et al. (24) determined that the specific activity of peroxidase extracted from red beet's hairy root culture reached 600 U/mg protein.

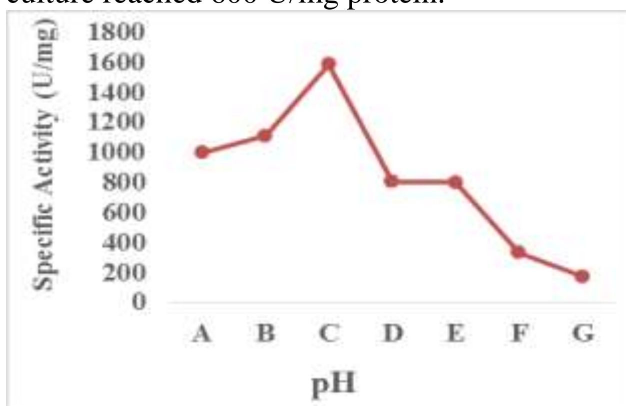

Figure 2. Influence of Buffer Types on the Extraction of Black Seed Peroxidase at 30 °C for 15 min

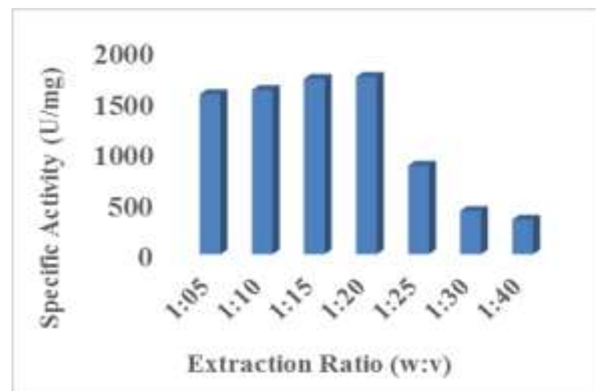

Figure 3. Impact Ratio of Extraction on Black Seed Peroxidase Extraction at 30°C for 15 minutes

Type of Extraction Buffer

Following extraction using various buffers, the specific activity of peroxidase was calculated, and the results are shown in (Fig. 2). These findings demonstrate that the optimum extraction buffer, sodium acetate buffer (0.2 M, pH 5.0), has a specific activity of 1584 U/mg protein. Other buffers with differing pH levels, on the other hand, demonstrated minimal particular action. Because various amino acid residues have distinct ionisation states, pH effects on enzymatic activity and stability may be explained by the fact that the acidity and alkalinity of the solution change enzyme protein structure (20). During the extraction operation, adding an appropriate buffer solution to a protein mixture may improve the stability of protein molecules when they are exposed to different stresses aimed to separate them for study. The pH balance of a buffer solution must match that of the live cell in order to protect the integrity of proteins while isolating them from other integrated cell components. It is prudent to choose a buffer solution that can maintain protein stability throughout the entire extraction process (13), so as to keep the procedure efficient and timely and to avoid the need to alter buffer solutions during the procedure (13). Many research have employed different buffers to extract peroxidase from various sources. Harco and colleagues (14) referred to potassium phosphate buffer (50 mM, pH 7.5) for extraction of peroxidase from *Nicotiana tabacum*.

A: Acetate Buffer with a pH of 3.0,
B: Acetate Buffer with a pH of 4.0,
C: Acetate Buffer with a pH of 5.0**,**
D: Acetate Buffer with a pH of 6.0,

E: Phosphate Buffer with a pH of 7.0,
: Tris Buffer with a pH of 8.0 and
G: Tris Buffer with a pH of 8.0

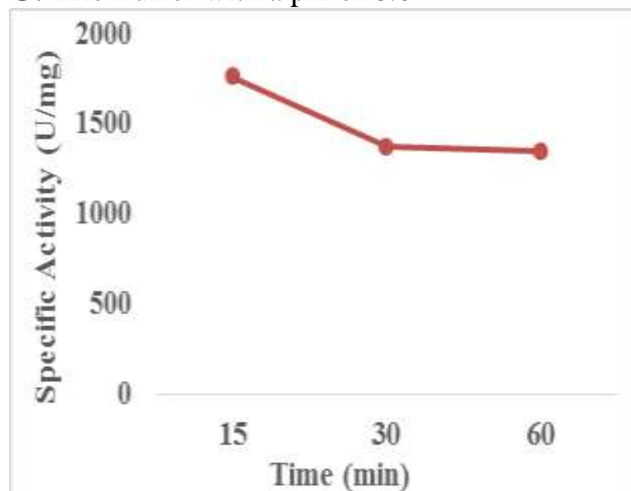

Figure 4. Impact of Extraction period on Black Seed Peroxidase Extraction at 30˚C for 15 min

In order to determine the optimal peroxidase extraction ratio using sodium acetate (0.2 M, pH 5.0), seven extraction ratios (1:5, 1:10, 1:15, 1:20, 1:30, and 1:40 (w: v) were chosen. The ratio of 1:20 produced the highest specific activity for the crude extract, which measured 1750 U/mg protein (Figure 3). Other ratios produced 1580, 1620, 1730, 875, 428, and 342 U/mg protein, respectively. The ideal peroxidase extraction ratio from eliminated mushroom beds, according to Nidadavolu et al. (22), was 1:1 and 1:2, whereas *Arthomyces ramosus* peroxidase was extracted with a 1:5 ratio.

Period of extraction

To find the optimal extraction time of peroxidase using sodium acetate (0.2 M, pH 5.0), three extraction periods were selected (15, 30, and 60) minutes. Crude extract had the highest specific activity after 15 minutes, reaching 1766 U/mg protein, compared to lower specific activity after 30 and 60 minutes, 1377.3 and 1350 U/mg protein, respectively (Fig. 4).

Purification of peroxidase

After homogenizing *Nigella sativa* with acetate buffer (0.1 M, pH 5.0), the peroxidase enzyme was extracted with cooling the mixture and centrifuging it at 8000 rpm for 10 minutes. Concentration by sucrose, gel filtering by (Sephadex -G150), and other techniques were used to separate the crude enzyme from the undesired proteins and other components as follows:

Concentration of peroxidase using sucrose

Table 1 demonstrates that 61.2% of enzyme was obtained with a purification fold of 2.5 when the crude extract initially concentrated with sucrose. Historically, sucrose was widely used for protein concentration, primarily as a cost-effective method of precipitating and concentrating a protein extract, sucrose acts on the enzyme as a nucleophilic effector (activator). By dialyzing against a concentrated sucrose solution and eliminating water molecules from the enzymatic solution, Bajaj and Singh (5) established a technique for concentrating enzymes. Ammonium sulphate, ethanol, and acetone were some other substances that could be used to concentrate the enzyme by precipitation. Dialyzing against an external solution of 96% ethyl alcohol or acetone allows for rapid volume reduction of the active ingredient (12).

Gel Filtration chromatography

In equilibrium with 0.2 M of pH 7 phosphate buffer, the size of a sephadex-G150 gel filtration column are (21 x 1.6) cm was used to filter the concentrated enzyme solution. The active fractions were recovered because the eluted fractions from the column showed four protein peaks and one peak of peroxidase activity (Fig. 5). According to (table 1), fractions (14-31) had a single peak of peroxidase activity, and the specific activity reached 3875 U/mg protein with a purification fold of 2.8 and a yield of 35%. By gel filtering on Bio-Gel P-60, Sessa and Anderson (25) purified soybean peroxidase. Khurshi (17) used $(NH4)_2SO_2$ precipitation, dialysis, and gel filtration using sephadex-G75 to purify horseradish peroxidase. Purification processes enhanced the activity of horseradish peroxidase from 6.3 to 9.9 U/ml. In the last phase, it obtained 45.77 purification folds.

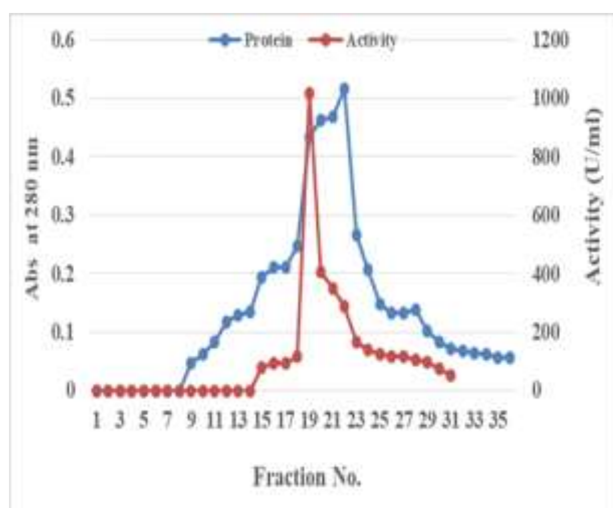

Figure 5. Gel filtration chromatography using a 21 x 1.6 cm Sephadex G150 column, calibrated and eluted with a phosphate buffer (0.2 M, pH 7) at a flow rate of 20 ml/hr, and a volume of 3 ml of each fraction for purification of peroxidase from *Nigella sativa*.

Figure 6. *Nigella sativa*-Extracted Peroxidase Trapped in Calcium Alginate Beads

Table 1. Steps for purification of peroxidase from black Seed

Sample	Volume (ml)	Enzyme Activity (U/ml)	Protein Conc. (mg/ml)	Specific Activity (U/mg)	Total Activity (U)	Purification Fold	Yield (%)
Crude Extract	30	1331	0.95	1401	39930	1	100
Sucrose concentration	10	2444.2	0.7	3491.7	24442	2.5	61.2
Gel Filtration Chromatography with (Sephadex G-150)	18	775	0.2	3875	13950	2.8	35

Peroxidase immobilization using the entrapment method: Calcium alginate was used to immobilize crude *Nigella sativa* peroxidase (Fig. 6). The immobilization ratio of peroxidase entrapped in calcium alginate attained 49%. Calcium alginate has several features for entrapping *Nigella sativa* peroxidase, which may be attributable to differences in constitution, composition, and porosity between calcium alginate and other matrices. One of the easiest, least costly, and safest ways to immobilize an enzyme is by entrapping it in alginate, which also offers moderate and physiological conditions for enzyme entrapment (21). The findings of the other studies revealed that immobilising peroxidase in calcium alginate was the most effective method of entrapment since it shielded the enzyme from its surroundings (including heat, osmotic pressure, and chemicals). It was also noted to be safe, simple, easy, quick, inexpensive, and to provide good mechanical strength (7, 10).Alginate concentration, enzyme to alginate ratio, CaCl2 concentration, and bead size all affect how peroxidase immobilizes in calcium alginate (2). Peroxidase from a *Saccharum spontaneum* leaf was immobilized by Shaffiqu et al. (26) on a hydrophobic matrix.

The use of peroxidase

Dye decolorization: The production of textiles is one of the earliest and most technologically advanced industries. Population growth has also contributed to an increase in textile production. Textile factories and their effluent water have increased proportionally, resulting in a global pollution crisis. Two-thirds of the dyestuff market is accounted for by the textile industry. 10-15% of the dyes used during the dying procedure are discharged into the effluent water. It is recognised as the leading cause of pollution in the environment (16). In addition to numerous industrial contaminants, textile dyes are

exceedingly toxic and possibly carcinogenic (27). thereby contributing to environmental degradation and various animal and human diseases (16).The ability of peroxidase (crude, purified, and immobilised) to degrade various (yellow, red, black, and blue) textile dyes was studied at dye concentrations of 30 mg/l, pH 5.0, and 37oC for 24 hours, as shown in Table (2) and Figures 7, 8, and 9. At the ideal wave length for each dye, the absorbance of each was measured. The fact that each dye's absorbance reduced as incubation time rose and stabilised after 3 hours and even after 24 hours is proof that peroxidase can break down a variety of dyes, in contrast to the absorbance of the control, which did not change during 24 hours. Due to the fact that each dye has a unique structure that affects the peroxidase enzyme's capacity for degradation, significant variations in the rate of degradation are seen in table (2). Removal efficiencies for the textile (black, yellow, and blue) dyes were 92%, 80.3%, and 77.5%, respectively. The purified peroxidase exhibited the best capacity to degrade textile red, with a maximum elimination extent of 94.6% after three (table 2). When compared to pure enzyme, crude peroxidase has almost the same efficiency in degrading dyes. Because the crude enzyme and the purified enzyme had equal efficacy in removing dyes, the crude enzyme was immobilised and used for eliminating the colours from the textiles. According to Kokol et al. (19), crude enzyme that has not been purified is cheaper and more stable than purified peroxidase. Using crude peroxidase, on the other hand, could reduce the expense of an industrial enzymatic decolorization procedure. These positive findings imply that peroxidase will soon be used in industry. Additionally, these dyes did not undergo the same degree of decolorization, which may be explained by variations in redox potentials and the compatibility of their steric structures with the enzyme's active site (29). The source of the enzyme and the chemical composition of the dye both affect the rate of the decolorization activity (1, 9). It has been noted that peroxidase's decolorization efficacy is restricted to a few azo dye structures, and that dyes' chemical structures have a significant impact on how quickly it may be removed from surfaces (8).The results also showed that, when compared to the other 3 dyes, the red dye saw a greater rate of enzyme immobilization-induced degradation, with a maximum removal efficiency of 81% after 3 hours. The intricacy of the dye structure has an impact on the peroxidase's capacity to degrade dyes, which causes variations in that ability. Hussein (15) observed that enzymatic oxidation was not effective against heterocyclic dyes. Shaffiqu et al. (26) used a hydrophobic matrix to immobilise pure peroxidase from a *Saccharum spontaneum* leaf. With a starting concentration of 50 mg/L, various heterocyclic textile dyes were completely degraded (100%) within one hour, whereas the other dyes were degraded by more than 70 percent within one hour.

Table 2. Dyes Degradation % by Peroxidase enzyme (Crude, Purified and Immobilized) from *Nigella sativa* at 37°C in pH 5.0 after 24 hr.

Dyes	Dyes Degradation		
	Crude Peroxidase	Purified Peroxidase	Immobilized Peroxidase
Textile Reactive Yellow	76.9	80.3	70
Textile Reactive Red	88.7	94.6	81
Textile Reactive Blue	88	77.5	56.4
Textile Reactive Black	91	92	72

Figure 7. Decolorization of Dyes by Crude Peroxidase 24 hours after extraction at pH 5.0 and 37°C with a 30 mg/l concentration from *Nigella sativa*.

Figure 8. Decolorization of Dyes via Purified Peroxidase 24 hours after extraction at pH 5.0 and 37°C with a 30 mg/l concentration from *Nigella sativa*.

Figure 9. Decolorization of Dyes by Immobilized Peroxidase 24 hours after extraction at pH 5.0 and 37°C with a 30 mg/l concentration from *Nigella sativa*

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