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Original Article

Biochemical Characterization of *Candida rugosa* Lipase Immobilized on Green Synthesized Gold Nanoparticles

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Abstract

Introduction: Lipases are one of the most essential enzymes in biological systems and industries. The use of free lipase is not cost-effective because of its low half-life. Enzyme immobilization on different supports leads to enhanced stability, reusability, ease of product separation, and low cost. Lipases have been immobilized on various organic and inorganic supports, but recently, nanoparticles have been used because of their high surface-to-volume unit. The present study aims to green synthesize gold nanoparticles, covalently immobilize lipase, and compare the biochemical characteristics of free and immobilized enzymes.

Materials and Methods: After synthesis of the nanoparticles and immobilization of lipase, nanoparticles and enzyme immobilization were characterized and confirmed by SEM microscopy, Raman spectroscopy, and DLS. The biochemical characteristics, such as optimal pH and temperature, thermal stability, and storage stability of free and immobilized enzymes, were then determined.

Results: The SEM results showed that the diameter of the synthesized nanoparticles was less than 50 nm. The Raman diagram of immobilized lipase showed two characterized peaks at 1468.44 cm^{-1} and 1639.61 cm^{-1} wavelength, confirming the immobilization process. Toward the free enzyme, the optimum pH of the immobilized enzyme shifted 0.5 units to the acidic range, whereas the optimum temperature did not change. Immobilized lipase showed higher thermal stability at 55 and 60 °C. Storage stability of the immobilized enzyme increased compared with the free enzyme. The immobilized enzyme could be used 10 times under optimum conditions.

Conclusions: It appears that the immobilized lipase has improved characteristics for application in different industries.

Keywords: Nanoparticle, Gold, *Candida rugosa* Lipase, Covalent Immobilization

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Introduction

With the advancements in nanotechnology and its expansion to different sciences, including biology and biochemistry, extensive research with remarkable results has recently been conducted on stabilizing different enzymes on chemical nanoparticles to promote their quality and efficiency. Because of their short life, low efficiency, and instability of their structure, enzymes are stabilized on nanoparticles and used for industrial, medical, and pharmaceutical purposes.[1-4] In this study, an enzyme was immobilized on green nanoparticles and the biochemical parameters were examined. So far, enzyme immobilization has mainly been performed on chemical nanoparticles. The question was, "Can nanoparticles synthesized by using plants be a good substrate for the stabilization of enzymes?" Enzymes are protein-based catalysts that accelerate biological reactions by reducing the activation energy and remain unchanged after the reaction.[5] The enzyme used in this study was *Candida rugosa* lipase. Lipases are multipurpose hydrolase enzymes of plant, animal, or microbial origin.[6] They have various applications, but their stability in different environments is very low. Also, they are expensive, and their use in free form is not cost-effective. Because their half-life is short and there are limitations to their repeated usage, the stabilized form of the enzyme is used.[7] Enzyme stabilization means stabilizing the enzyme on the surface. This is performed with various carriers and by using different methods. Usually, a method that incurs the most minor damage to the enzyme, is more accessible, safer, and less costly is preferred.[8] In this study, the enzyme was stabilized on a nanoparticle using covalent bonds. The covalent bond of the enzyme to the carrier has the advantage of firmly fixing the enzyme, so that the possibility of purging in an aqueous environment is minimized, and there will be no protein contamination in the product. Nanoparticles are one of the most important

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substances for this purpose, with a size of 10-100 nm.[9] Green synthesized gold nanoparticles were used in this study because gold nanoparticles, which are 1000 times smaller than cells, are used for different medical purposes, including cancer diagnosis, radiation therapy, gene transfer, sensors and biosensors, and the control and suppression of bacterial growth.[10-13] In addition, using green synthesize nanoparticles is very attractive and rather unknown.[13] Following chemical methods, some toxic reagents usually remain on the nanoparticles. This is why the use of plants as stable and accessible resources in preparing biocompatible nanoparticles has attracted the attention of many researchers in recent years. The merits of this method include its non-toxicity, biocompatibility, low cost, and high purity nanoparticle production.[14] Compared with free enzymes, immobilized enzymes have recently found more applications due to their remarkable merits such as higher stability, continuous use, retrieval, lower reaction volume, reduced environmental contamination, and cost-effectiveness.[15-17]

This study aimed to immobilize *C. rugosa* lipase on green synthesized gold nanoparticles, determine the protein concentration and activity of the stabilized enzyme, examine the biochemical parameters such as temperature, pH, storage stability, reusability, and thermal stability, and compare all these parameters with those of the free enzyme.

Materials and Methods

Materials

The materials included lipase originating from *C. rugosa*, potassium chloride (KCl), Mercaptooctanoic acid (MOA), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), p-nitrophenyl palmitate (pNPP), dipotassium phosphate, monopotassium phosphate, sodium carbonate, Triton X-100, gum arabic, Tris, Ethanol, Isopropanol, and MES (2-(N-morpholino) ethanesulfonic acid) buffer. All materials were prepared by Sigma.

Gold Nanoparticle Synthesis Using Pennyroyal Extract

Preparation of the Pennyroyal Extract

First, 2 g of the fine powder of dried pennyroyal leaves was added to 100 ml of distilled water and boiled for 5 min with stirring at a moderate speed. The extract was then centrifuged at 8000 g for 10 min; the supernatant was separated and kept in the refrigerator.[18]

Green Synthesis of Gold Nanoparticles

A combination of 1 mM $HAuCl_4.3H_2O$ and different ratios of the plant extract (1:1-1:5) was used to obtain the best concentration in terms of synthesis speed and nanoparticle type. The 1 mM $HAuCl_4.3H_2O$ solution was placed on a stirrer at a high speed. When the solution reached boiling point, the extract was added. In a short period, the solution changes color to deep red, indicating the synthesis of gold nanoparticles. The nanoparticles were separated from the centrifugation device at 8000 g for 10 min, washed with distilled water in triplicate, and placed in a dry oven for 24 h at 60 °C.[19] All the analyses related to the green synthesis of nanoparticles, including the UV test to confirm gold nanoparticles' synthesis by using gold absorption wavelength, FITR (HORIBA scientific, Kyoto, Japan) to determine the functional groups on the nanoparticles' surface, X-ray diffraction (XRD) for determining the atomic and molecular structure of nanoparticles, and scanning electron microscopy (SEM) (leica Inc., Foster city, GA) to determine size and shape of nanoparticles were performed.

Enzyme Immobilization on Green Gold Nanoparticles

Substrate Preparation

First, 1 ml of green synthesized gold nanoparticles was mixed with 100 µl of distilled water and 80 µl of mercapto-enoic acid. This solution should never be exposed to light. Therefore, the solution was poured into a vial, covered with foil, and kept in the refrigerator for 24 h.

The next day, the solution was centrifuged for 10 min at 10000 rpm. Then, the supernatant was discarded and 0.4 ml of the MES buffer was added to the sediment. It was then placed in a beaker on a shaker to suspend the sediment. Next, to this solution, 0.1 ml NHS was added. After 10 min, 0.08 ml EDAC was added, immediately followed by adding 0.2 ml lipase. After 1 hour, the solution, which including the immobilized enzyme, was centrifuged for 10 min at 10000 rpm. Subsequently, the sediment was dissolved in 0.3 ml of PBS and used in the next steps.[20]

Measurement of the Immobilization Yield

To calculate the amount of immobilized protein (lipase), the total amount of protein used in the immobilization process can be subtracted from the amount of protein in the supernatant after centrifugation and washing. Then, the immobilization yield was calculated according to (Eq. (1)).

$$\text{Immobilization Yield (\%)} = \frac{C_1 - C_2}{C_1} \times 100 \qquad \text{Eq. (1)}$$

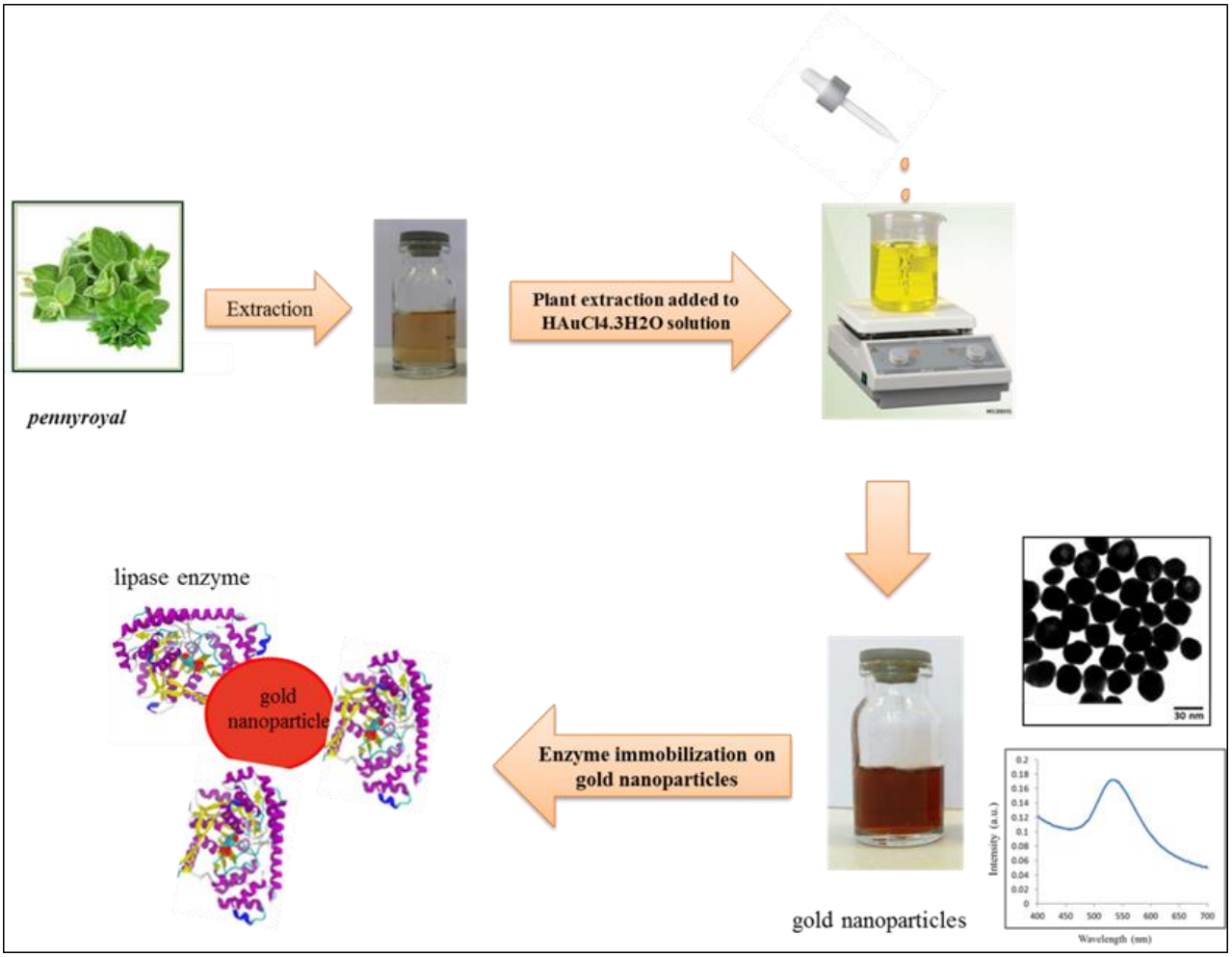
C_1: initial protein concentration (mg/ml)
C_2: final protein concentration (mg/ml)

Where C_1 and C_2 are the concentrations of the free enzyme used in the immobilization and in the supernatant and washing solutions after immobilization, respectively.

Measurement of Lipase Activity Using the pNPP Substrate

In this method, pNPP was used as the lipase substrate to measure enzyme activity. This is a valid, rapid, and frequently used method, and its end product is fatty acid and p-nitrophenol.

For substrate preparation, two solutions of A and B were



Scheme 1. The Schematic Green Synthesis Gold Nanoparticles and Immobilization of Lipase Enzyme onto Herbal Gold Nanoparticles.

used. To prepare solution A, 1 mg of p-nitrophenyl palmitate was weighed and dissolved in 250 µl of isopropanol. To prepare solution B, 50 mM Tris base buffer, Triton X-100 (5%), and gum arabic (1%) were required. Initially, 60 mg of Tris was weighed, and 8 ml of distilled water was added. Then, 0.05 ml Triton X-100 was added. Next, 10 mg of gum arabic was weighed and added to the solution. Finally, the volume was increased to 10 ml using distilled water. In the next step, 1 unit of solution A was dissolved in 9 units of solution B, and the substrate was prepared.

Note that p-nitrophenol is yellow and its photoabsorption is read at a wavelength of 400-410 nm. Activity was measured by comparing the absorption value read at 410 nm on the spectrophotometer with the standard curve of p-nitrophenol. The standard curve was plotted based on different p-nitrophenol concentrations (0.1, 0.05, 0.01, 0.005 mM).

To measure lipase activity in the desired samples, 900 µl of the pNPP substrate and 100 µl of PBS (0.1 M) at pH 7.4 were added to each Eppendorf capped vial. Then, 10 µl of the free or stabilized lipase enzyme was added to the vials, and after complete mixing using a tube shaker, the vials were re-incubated for 5 min at the optimal temperature. The enzymatic reaction was then terminated with sodium carbonate (0.1 g/ml), and the photoabsorption of the samples was read at 410 nm by the spectrophotometer.[21]

Temperature Profile of the Free and Immobilized Enzymes

To determine the effect of temperature on lipase activity, samples of free and immobilized enzymes were incubated in the vicinity of the pNPP substrate at 30, 35, 40, 45, 50, 55, and 60 °C in a water bath for 10 min. Then, the absorption of the samples was read at a wavelength of 410 nm in the spectrophotometer, and the lipase activity in these samples was determined.

Effect of pH on Free and Immobilized Enzymes

Changes in the activity of free and immobilized enzymes were examined at different pH levels, from 6 to 8 (6, 6.5, 7, 7.5, and 8). In this method, the substrate is prepared at different pH levels. The substrate is poured onto the enzyme and incubated for 10 min at optimal temperature. At the end of the reaction, their absorption is read using a spectrophotometer at 410 nm.

Examining Thermal Stability in Free Mode and Immobilized on Gold Nanoparticles

In this study, enzyme activity was assessed for 30 min at 55 and 60 °C. Enzyme product absorption was measured with the spectrophotometer at different temperatures, and its activity was assessed at different temperatures (in 5-min intervals) based on its activity percentage.

Examining the Storage Stability of Free Enzymes and Enzymes Immobilized on Gold Nanoparticles

In the first step, the free and stabilized enzymes were prepared in Tris buffer with optimum pH and kept in the refrigerator. In 24 h intervals, the activity of free and stabilized enzymes was assessed.

To measure storage stability, the substrate was freshly prepared and used for both types of enzymes every day.

Evaluation of the Re-use Ability of the Immobilized Enzyme

To this end, 20 ml of the stabilized enzyme and 80 ml of the substrate were added. After 10 min, the enzymatic reaction was completed under optimum conditions. Enzyme activity was measured and taken as 100% activity. To re-use this enzyme, the vial containing the enzyme and the product was centrifuged for 10 min. The supernatant was removed. The enzyme sediment was washed twice with the buffer and re-assessed upon adding the substrate. This was continued as long as the enzyme activity did not exhibit a marked reduction.

Results and Discussion

Infrared Spectroscopy of Green Gold Nanoparticles

FTIR was performed to identify the secondary metabolites as the covering agents of the synthesized nanoparticles and to determine the functional groups on the surface of the nanoparticles. Based on Figure 1, the peak at 3464 belongs to NH, amides, or OH bonds. The peak at 2078 is associated with the CH bonds of alkanes. The peak at 2357 indicates the type-1 amine bond or O═C═O bonds.

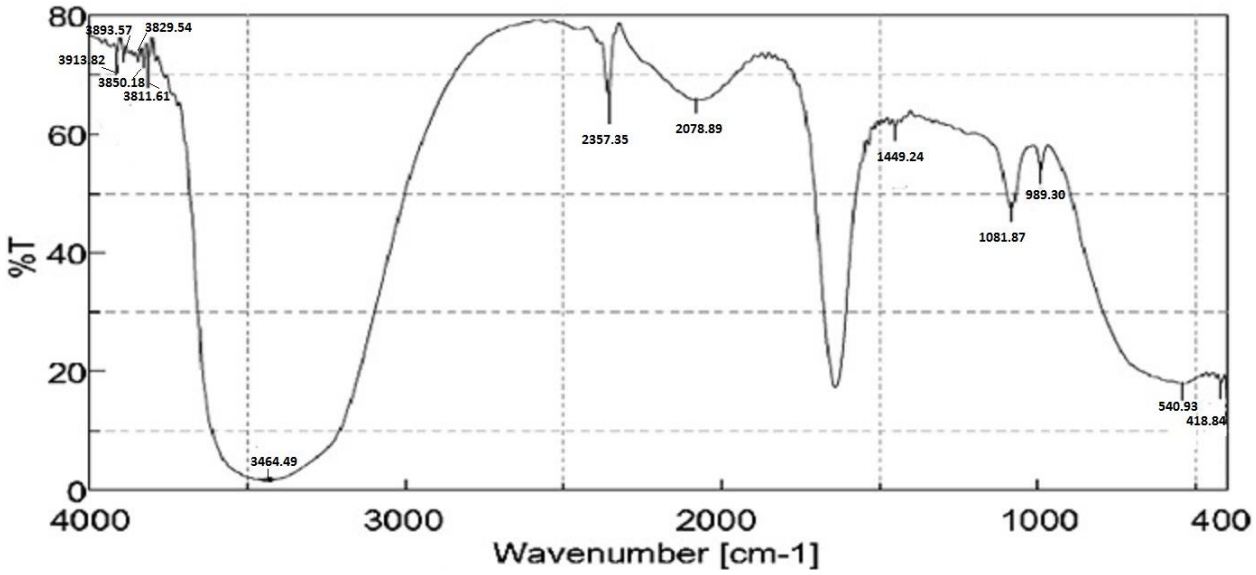


Figure 1. FTIR Spectra of the Green Gold Nanoparticles.

SEM Image of Green Gold Nanoparticles

Based on Figure 2, which depicts the shape and size of the nanoparticles, the shape of the green gold nanoparticles is spherical, and their size is <50 nm. These spherical nanoparticles are suitable for enzyme immobilization because of their high surface-to-volume ratio and the presence of biological agents on their surface.

Raman Spectroscopy of Green Gold Nanoparticles

Based on Figure 3, the Raman spectrum of the green gold nanoparticles was obtained. Evidently, the gold nanoparticles at the 100-200 cm^{-1} region have strong peaks, which may indicate the unique feature of the gold nanoparticles synthesized from pennyroyal leaves.

Raman Spectroscopy of Lipase Immobilized on Green Gold Nanoparticles

Based on Figure 4, Raman spectra of the enzyme, nanoparticles, and enzyme immobilized on nanoparticles were prepared. In the Raman spectrum of the enzyme, in the 1048 cm^{-1} region, a strong peak is observed, which belongs to the N–H group in proteins. A similar peak in the Raman spectrum of the enzyme immobilized on nanoparticles was less strong and moved to higher values. This is because of the enzyme's bond to the carboxyl group of the nanoparticle and the formation of a peptide bond. Moreover, in the Raman

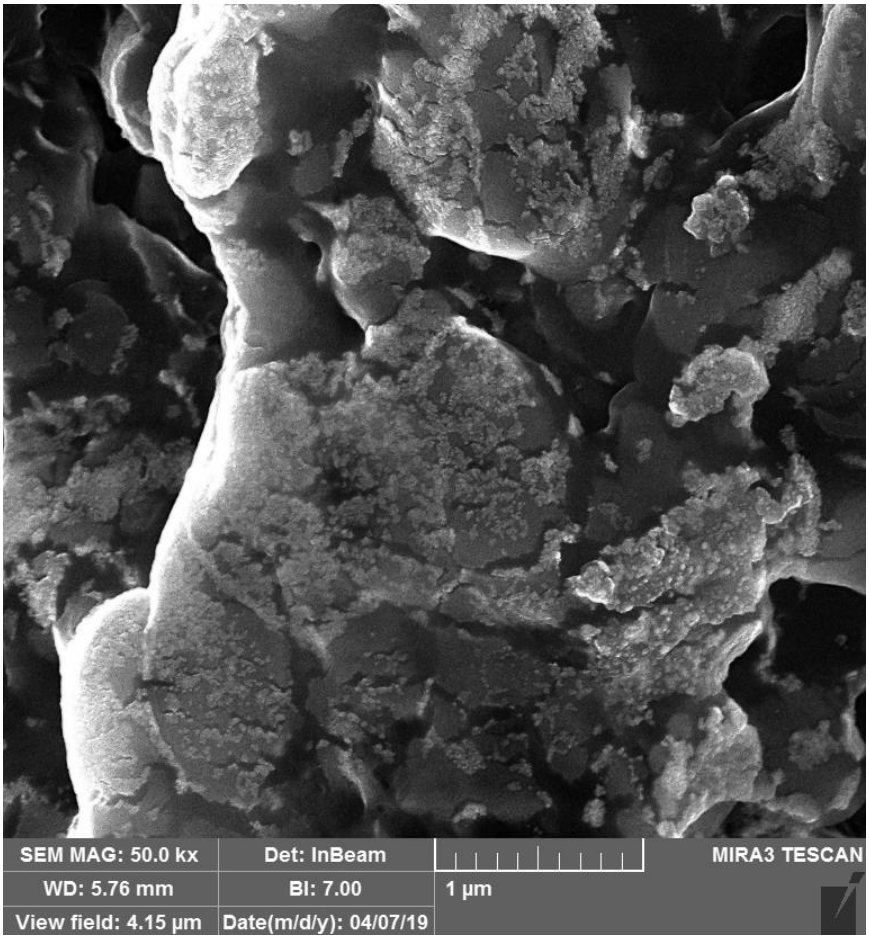


Figure 2. SEM Image of the Green Gold Nanoparticles.

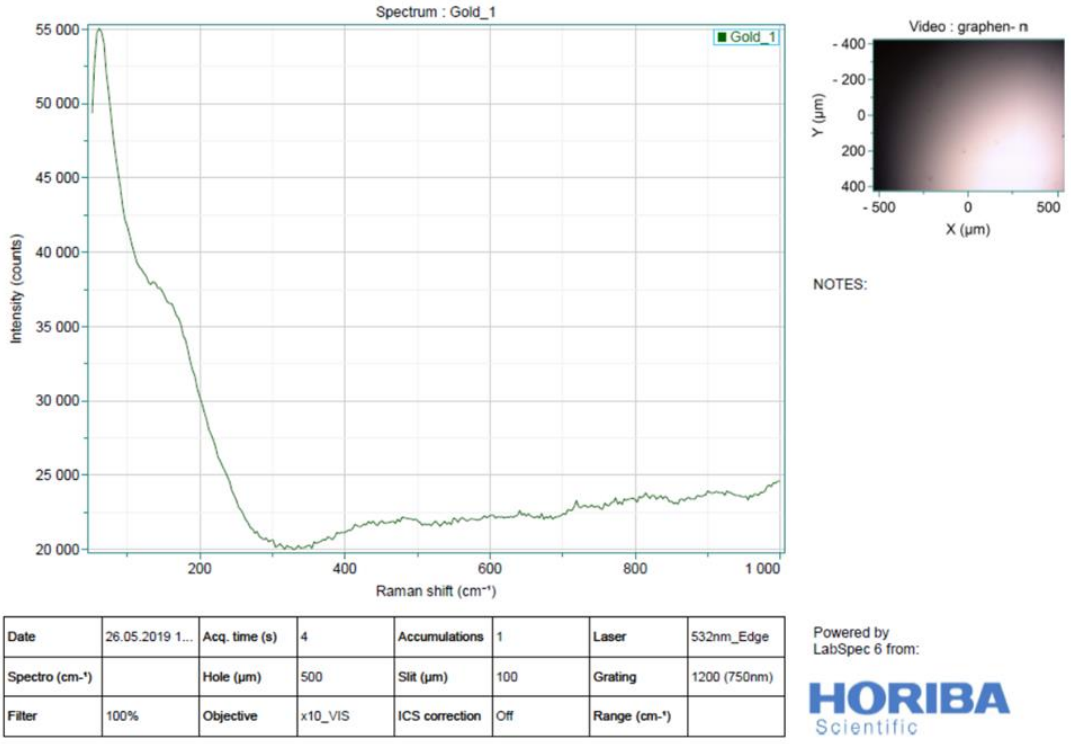


Figure 3. Raman Spectroscopy of the Green Gold Nanoparticles.

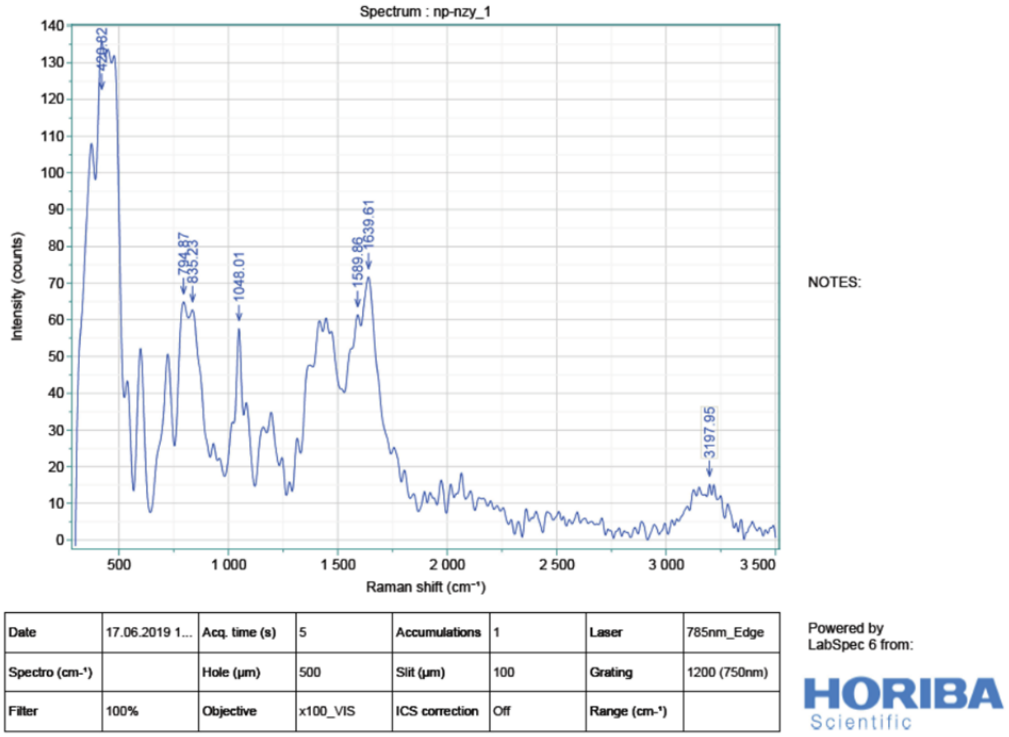


Figure 4. Raman Spectroscopy of Lipase Immobilized on Green Gold Nanoparticles.

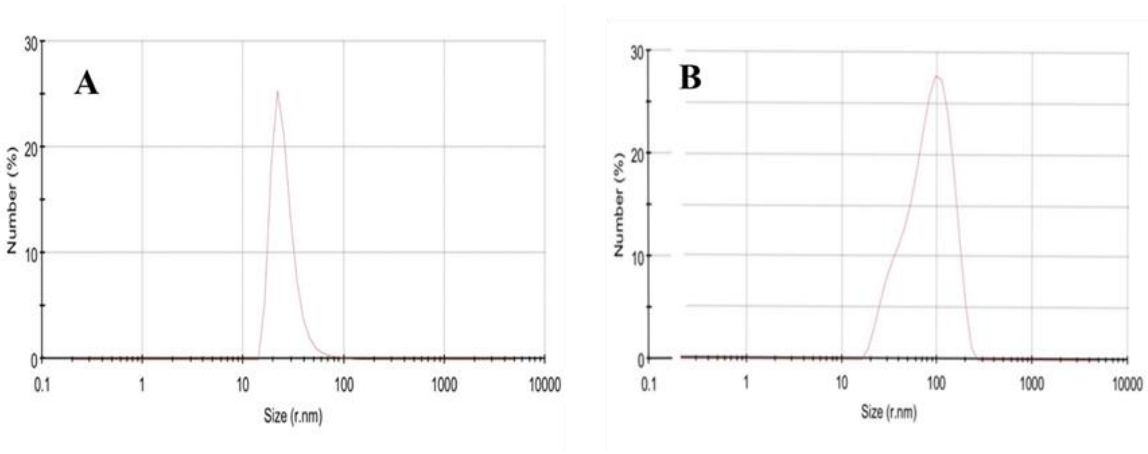


Figure 5. DLS Diagram of the A) gold nanoparticles synthesized from pennyroyal leaf extract; B) enzyme immobilized on the gold nanoparticles.

spectrum of the nanoparticles, a relatively strong bond in the 1450 cm^{-1} region is seen, which belongs to the C–N group. The gold nanoparticle also has strong peaks in the 1000-2000 cm^{-1} region which are highly weakened in the enzyme immobilized on the nanoparticle spectrum and during bonding to the enzyme.

DLS Diagram of Gold Nanoparticles Synthesized from Pennyroyal Leaf Extract

Based on Figure 5, nanoparticle size indicates the suitable and acceptable size distribution of the nanoparticles. The size of the nanoparticles was 10-100 nm before stabilization (Figure 5A) and reached 20-500 nm after stabilization (Figure 5B).

Immobilization Yield

First, the absorption (at a wavelength of 280 nm) of the supernatant obtained from enzyme immobilization was obtained. The protein concentration in the supernatant solution was then determined using the prepared standard diagram. The protein concentration in the supernatant was subtracted from the total protein concentration to obtain the concentration of protein immobilized on the nanoparticle. To investigate enzyme immobilization, the following formula was used:

$$\text{Immobilization yield} = \frac{\text{immobilized protein}}{\text{total used protein}} \times 100$$

The immobilization yield was 48% according to the

above calculations.

Effect of Temperature on Enzyme Activity

Based on the Figure 6 diagram, the free enzyme at 37 °C and the immobilized enzyme at 40 °C had the best activity. The thermal stability of the immobilized enzyme was higher than that of the free enzyme. The process of reducing enzyme activity in the immobilized enzyme is slower than that in the free enzyme. Thus, the immobilized enzyme at 50°C lost 20% of its initial activity after 8 h, but in the free enzyme under these conditions, almost 38% of the enzyme's activity decreased. The results obtained in this research showed that the enzyme immobilization on the studied nanoparticles did not change much at the optimal temperature. It can be said that at higher temperatures, free lipase is easily denatured, whereas immobilized lipase is protected from denaturation. Therefore, because of the stability of the enzyme structure, its catalytic activity is also maintained.

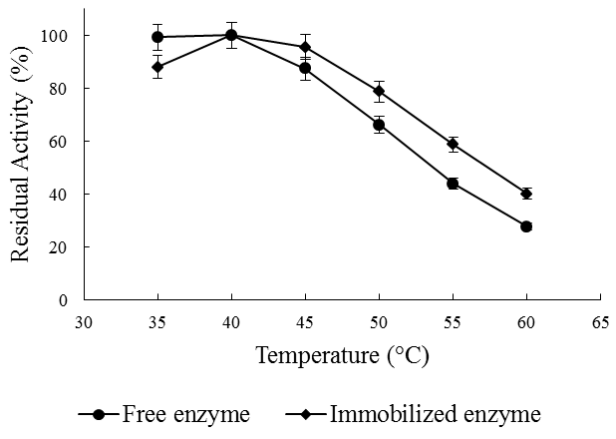


Figure 6. Temperature Diagram for Free and Immobilized Lipase Enzyme on Herbal Gold Nanoparticles.

Effect of pH on Enzyme Activity

In general, immobilization modifies the optimal pH of the enzyme. Based on the Figure 7 diagram, the optimum pH of the free enzyme compared with the immobilized enzyme was 7-6.5, which means that the immobilized enzyme with the highest level of activity has a lower pH compared with the free enzyme. At a certain pH, the enzyme has an original native conformation and suitable enzymatic activity. At a certain pH, the enzyme has an original native conformation. The change in pH may be due to strong interactions between the lipase enzyme and the substrate surface. The charge of gold nanoparticles synthesized from pennyroyal extract is negative, and because of the stabilization of the enzyme on the nanoparticles, the pH has shifted toward the acidic range. This result indicates that the immobilized enzyme is unstable at higher pH, which is consistent with the results of various studies.[22-24]

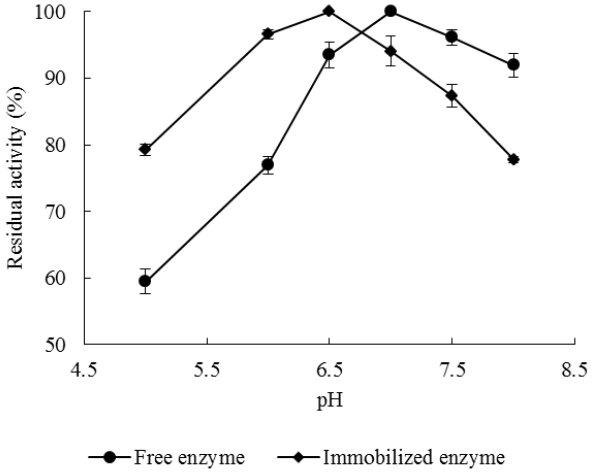


Figure 7. pH Diagram for Free and Immobilized Lipase Enzyme on Herbal Gold Nanoparticles.

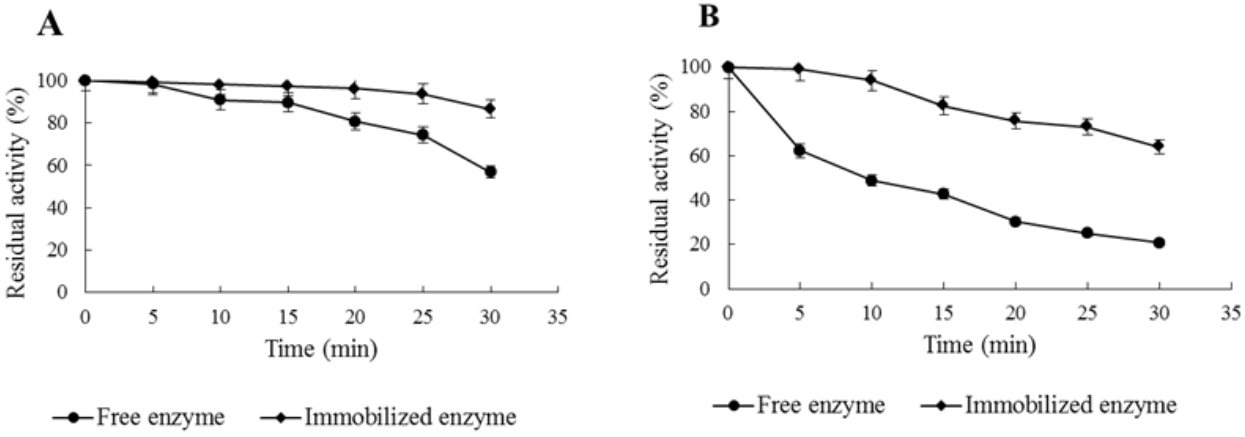


Figure 8. Diagram of Thermal Stability of Free and Immobilized Lipase Enzyme on Herbal Gold Nanoparticles at A) 55 °C and B) 60 °C.

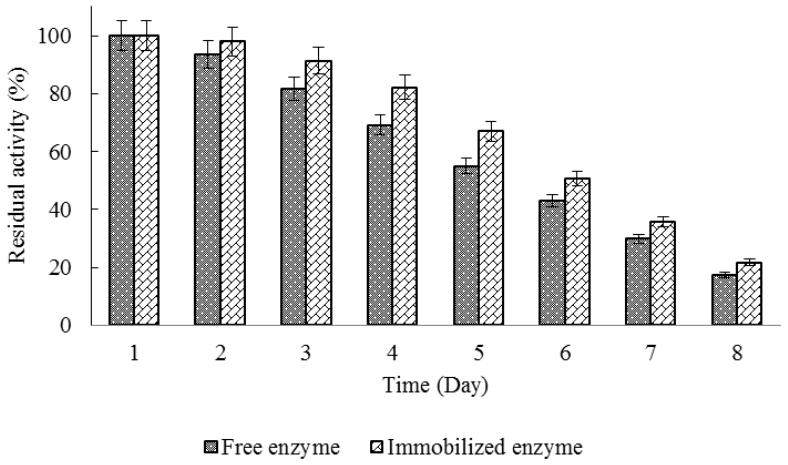


Figure 9. Diagram of Storage Stability for Free and Immobilized Lipase Enzyme on Herbal Gold Nanoparticles.

Thermal Stability

Based on the Figure 8 diagram, the free enzyme had an activity reduction of 44% at 55 °C and 79% at 60 °C during 30 min. However, the immobilized enzyme had a 14% and 36% activity reduction, respectively, at the mentioned times and temperatures (Figure 8A, B). It can be concluded that the immobilized enzyme was more stable than the free enzyme. The increase in the thermal stability of the immobilized lipase could be attributed to the structure of the support, which might provide protection to the enzyme molecules and prevent the conformation transition of the enzyme at high temperatures.[25]

Storage Stability

Based on the results (Figure 9), it is evident that the immobilized enzyme had more storage stability than the free enzyme. The free enzyme decreased by 83% and the immobilized enzyme by 80% over 8 days. Various studies have been conducted on the storage stability of immobilized lipase enzyme on different types of nanoparticles. For example, the immobilized lipase enzyme on magnetic iron nanoparticles coated with chitosan could be used for 12 days without losing its activity, but the free lipase enzyme had lost about 78% of its activity in 10 days.[26] Also, the storage stability of the lipase-immobilized enzyme on zinc oxide nanoparticles showed that the immobilized enzyme had lost 94% of its activity after 6 hours, while the free enzyme had lost 75% of its initial activity after 4 h.[27]

Reuse of the Immobilized Enzyme

According to Figure 10, the immobilized enzyme on green synthesized gold nanoparticles lost its activity after 10 times of use, so the immobilized enzyme can be used up to 10 times. The results showed that the immobilized enzyme retained 87% of its activity after 5 cycles. These results show that the use of nanoparticles for enzyme immobilization is very effective in improving enzyme stability and performance. For this purpose, the use of gold nanoparticles is a good choice.

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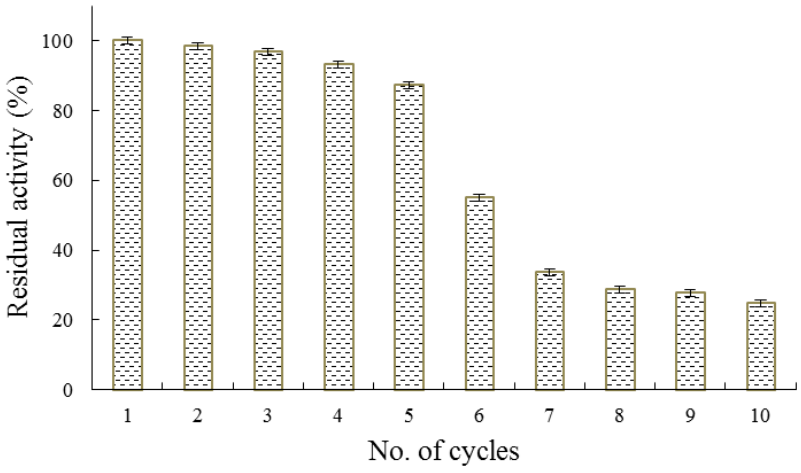

Figure 10. Diagram of Reusability of Immobilized Enzyme on Herbal Gold Nanoparticles.

Conclusion

In this work, green synthesis of gold nanoparticles was carried out using pennyroyal leaves extract and lipase enzyme from *Candida rugosa* was immobilized covalently on the nanoparticles. The results of electron microscope imaging, Raman spectroscopy and DLS technique showed the successful immobilization of the enzyme. The comparison of free and immobilized enzyme activity showed a change in the optimal temperature from 37 °C to 40 °C. also changing the optimal pH of the immobilized enzyme to the acidic range can make it a suitable candidate for use in industries that require acidic conditions such as paper, leather making and food industry. Increased storage stability and reuse of immobilized enzymes are other advantages of immobilization. It appears that the immobilized lipase has improved characteristics for application in different industries.

Authors' Contributions

MJ: Enzyme immobilization experiments; MG: Supervision, Project administration, Biochemical characterization, Analyzed and interpreted the data, Writing- review & editing; KE: Project administration, chemical characterization; writing—review & editing; analyzed and interpreted the data; ED: Synthesis of support nano-carrier, review & editing. All authors have read and agreed to the published version of the manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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