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NARINGINASE IMMOBILIZED ON MODIFIED BANANA PEEL WITH POTENTIAL APPLICATION IN THE CITRUS INDUSTRY

Naringinasa inmovilizada en cáscara de banano modificada con potencial aplicación en la industria de cítricos

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Abstract

Banana peel after chemical ant thermal modification was used as an alternative support to immobilize the commercial enzyme naringinase (*Penicillum Decumbens*); an immobilization yields greater than 70% was observed at pH 7. The structural characteristics of the support were determined by scanning electron microscopy with elemental analysis, showing the presence of pores and elements such as carbon, oxygen, sulfur, and zinc, while the attachment of the enzyme was concluded by infrared spectroscopy. For the free and immobilized enzyme, the K_M and V_{max} values were 0.0006 molar and 2000 U, and 0.0003 molar and 1666 U, respectively. The temperatures of the greatest activity for the free was 70°C and for the immobilized enzyme was 50°C, respectively, and the best pH was 4.5 in both cases. It was found that, after the third use, the catalyst maintained 50% of the enzymatic activity. These results seem to suggest the potential of the synthesized material for its application in the debittering of citrus juices.

Key words: *enzyme, immobilization, naringinase.*

Resumen

La cáscara de banano después de modificación química y térmica se utilizó como soporte alternativo para inmovilizar la enzima comercial naringinasa (*Penicillum Decumbens*); obteniéndose un rendimiento de inmovilización superior al 70% a pH 7. La morfología del soporte se caracterizó por microscopía electronica de barrido con análisis elemental, mostrando la presencia de poros y elementos como carbono, oxígeno, azufre y zinc; la inmovilización de la enzima fue confirmada por espectroscopía infrarroja. Para la enzima libre e inmovilizada, los valores de K_M y V_{max} fueron 0,0006 molar y 2000 U, y 0,0003 molar y 1666 U, respectivamente. Las temperaturas de mayor actividad para la enzima libre e inmovilizada fueron 70°C y 50 °C, respectivamente, y el pH óptimo fue 4,5 en ambos casos. De las pruebas de reutilización se encontró que luego del tercer uso, el catalizador mantuvo el 50% de la actividad enzimática. Los resultados de esta investigación sugieren el potencial del material sintetizado para su aplicación en la industria alimentaria, específicamente en el desamargado de jugos de cítricos.

Palabras clave: *enzima, inmovilización, naringinasa.*

1. INTRODUCTION

Enzymes are selective biocatalysts with industrial applications fields as food [1], textiles [2] biosensors [3], and wastewater treatment [4-7]. However, the use of free enzymes generates drawbacks related to the difficult of reusing them, the effects of the environmental modifications and the recovery of the enzyme, which limits its use on a large scale. These problems can be solved using a support where it is possible to immobilize the enzyme [8] and although the enzymatic immobilization techniques in an insoluble support are expensive, they represent an important alternative, especially in continuous operation processes with possibilities of application at industrial level [9] since immobilized enzymes are more stable and have the advantage to eaiser recovery and reuse [10].

Enzyme immobilization on a support can be done by several ways: adsorption, covalent bonding, encapsulation/ entrapment, and crosslinking [11]. Weak binding forces are involved in the adsorption immobilization while in the other cases, immobilization implies covalent bond formation between the enzyme and the support [12]. Materials used as support of enzymes must have high surface area, high chemical and thermal resistance, no reactivity to microbial environment, no soluble in the solvent, low cost, [13]. Different types of materials have been used for supporting enzymes [14]; however, the use of alternative materials as supports in the immobilization of enzymes, considering the principles of green chemistry, constitutes one of the main challenges from the academic and industrial point of view. In this way, economic and environmental advantages can be obtained using agroindustrial wastes for attaching enzymes. Banana is one of the most popular fruits in the world owing to its nutritional value and cost effectiveness [15]. The peel waste is an available and low-cost material and can be used for commercial application. This waste could be used as a potential candidate in the immobilization of enzymes.

The complex enzyme naringinase is used in several industries as biotechnology and pharmaceuticals [16]; in the citrus juice industry it is useful because the reduction of the intensity of its bitter taste [17]. The naringinase was immobilized on zeolite ITQ-2 functionalized with glutaraldehyde and was tested in the hydrolysis of naringin giving a high conversion (> 90 %) and excellent selectivity. In the continuous debittering grapefruit juices during 300 h, excellent results were obtained [18].

In this study, we focused on the immobilization of narainginase on a material from banana peel. The solid was prepared by one step method, impregnated with $ZnSO_4.7H_2O$ and heat treated in a nitrogen atmosphere at 500 °C. The material was analyzed by scanning electron microscopy and infrared spectroscopy. Initial enzyme activity and enzymatic kinetic were evaluated for immobilized and not immobilized enzyme. The effect of the pH, time, and temperature parameters on the immobilization of enzyme was evaluated. Immobilized enzyme was reused aiming to determinate immobilization success.

2. MATERIALS AND METHODS

2.1. Materials

Banana peel was obtained from the local supplier, Medellín, Antioquia. Naringinase enzyme from Sigma, naringin (grapefruit extract) from L´eternel World, LLC; $ZnSO_4.7H_2O$ was analytical reagent grade from Merck, ethyl alcohol denatured 40 B was purchased from J. T. Baker. Nitrogen gas UPA (99.999 %) from Messer. All chemicals were used as they were received.

2.2. Methods

2.2.1. Pretreatment of banana peel

Once in the laboratory, the banana peels were washed with water and dried with absorbent paper and treated at 80 °C during 24 h. 1 g of pulverized peel dust was suspended in 20 mL of water and subjected to microwave (Mars 5, CEM) heating from room temperature to 50 °C (3 min). The residue was separated from the aqueous extract and treated at 80 °C during 24 h. The obtained material was mixed in an autoclave at 121° C and 0.13 MPa with 70 % ethanol solution using a 1:10 w/v ratio for

20 min, and then rapidly decompressed [19]. The solid material was treated with NaOH (2 M) in a banana peel/ NaOH 1:10 w/v and stirred for 24 h at room temperature; then, the solid was filtered and washed until pH 7.

Finally, the solid was dried for 24 h. Dried banana peel was mixed with an aqueous solution of $ZnSO_4.7H_2O$ (1.4 M) using an activating agent/peel weight ratio of 2:1 [20], and the system was kept at room temperature for 24 h at 500 rpm. Then, the resulting paste was dried in an oven at 100 °C for 18 h. The dried and pulverized peels were thermal treated in a nitrogen flow (100 mL/min) at 500 °C for 1 h. Finally, the solid was washed with ethanol and hot water and subsequently dried at 80 ºC for 4 h. This material was labelled as BS.

2.2.2. Support characterization

ATR-FTIR analysis between 400 and 4000 cm^{-1} (Frontier Spectrum 65, Perkin Elmer) was used for identified the functional groups on surface of support material. Material morphology was analyzed using Scanning electron microscopy (JEOL JSM 6490 LV); elemental compositions was identified by EDX (INCA PentaFETx3 Oxford Instruments). The samples were analyzed in a high vacuum scanning electron microscope operating at 20 kV, fixed on a graphite tape, and coated with gold.

2.2.3. Enzymatic assay

The reducing sugars concentration was evaluated by dinitrosalicylic acid (DNS) test at λ = 540 nm (Thermo Fisher model GENESYS 50 spectrophotometer). The naringin hydrolysis (0.5% w/v) was carried out in standard solutions in citrate buffer (50.0 mM), at pH 4.5. The enzyme activity was determined with Eq (1):

$$Activity(U/mL) = \frac{A * 1000 * V_T}{\varepsilon * l * t * EV} \quad (1)$$

where:

A = sample absorbance

l= light-path length (cm)

V_T = Reaction total volume (µL)

ε = molar extinction coefficient (µmol/mL*cm)

t = reaction time (min)

EV = Enzyme volume (µL)

With U the amount of enzyme necessary to convert 1 mol of DNS per minute.

2.2.5. Immobilization of naringinase on the banana peel suport (BS-NG)

The enzyme (1 mg/mL) was solubilized in a HPO_4^{2-}/H_2PO^{4-} buffer (25 mmol/L), pH 7. Then 2 mL of the enzymatic solution and 30 mg of SB were stirred for 1 h at 22°C in a roll bed. During the immobilization process, aliquots of the supernatant were removed. Bradford method [21] was used for protein quantification on the supernatant before starting the contact between enzyme and support and at the end to calculate the immobilization percentage of the enzyme on the support. Yield immobilization was calculated using Eq (2).

$$YI = \frac{1 - FP}{IP} * 100 \quad (2)$$

where:

YI = Yield immobilization (%)

FP = final protein content (mg/mL)

IP= initial protein content (mg/mL)

2.2.5. pH Influence on the enzymatic immobilization

To determinate the pH influence on the yield immobilization, YI was calculated by solubilizing the enzyme at 22 °C in buffer solutions between pH 4 and 8 that were prepared using 1 M citric acid ($C_6H_8O_7$) solution and a 2 M sodium phosphate (Na_2HPO_4) solution according to McIlvaine [22]. Buffer solutions between pH 9 and 10 were prepared using a bicarbonate buffer ($NaHCO_3/Na_2CO_3$).

2.2.6. Enzyme kinetics for free and immobilized enzyme

Enzyme kinetics was performed with different concentrations 0.1 – 2 mg/mL of naringin substrate at 22°C, enzyme load of 1 mg/mL and at 15 min. The enzymatic activity was calculated at each concentration and subsequently the V_o reported in mg/min/mg. K_M and V_{max} were calculated by the Lineweaver-Burk method [23].

2.2.7. Effect of pH and temperature on non-immobilized and immobilized naringinase

The enzyme behavior at different pHs was prolonged by measuring the enzyme activity at a constant temperature of 22 °C in a pH range between 2-10 and intervals of one. Buffer solutions between pH 2 and 8 were prepared using a 1 M citric acid ($C_6H_8O_7$) solution and a 2 M sodium phosphate (Na_2HPO_4) [19].

Buffer solutions between pH 9 and 10 were prepared using a bicarbonate buffer ($NaHCO_3/Na_2CO_3$). After knowing the optimum pH, the effect of temperature in the 22 – 90 °C range was analyzed by measuring the enzymatic activity at optimum pH. The measure of naringin activity was realized using the spectrophotometric technique previously described in 2.2.4. section.

2.2.8. Reusability of immobilized naringinase

The reuse was verified by the reaction of 0.5 g of SB derivative with 2 mL of naringinase solution 1 mg/mL in 50 mM sodium citrate buffer at pH 4.5, at 22 °C, and each cycle lasted until the amount of reducing sugars formed was constant with time. The derivative was then rinsed with solution of sodium acetate 100 mM (pH 5.0) at 25 °C, finally the biocatalyst was separated from the product by centrifugation and then submitted to a subsequent cycle.

3. RESULTS AND DISCUSSION

3.1. Support characterization

Figure 1 shows Fourier Transform Infrared (FTIR) spectra of free naringinase (Figure 1a), banana support (Figure 1b) and naringinase immobilised on banana support (Figure 1c). The signal observed in the FTIR spectrum of non-immobilized naringinase (Figure 1a) at 1650 cm^{-1} corresponding to amide I while the signal at 1546 cm^{-1} can be assigned to the amide II [24]. Additionally, the carbohydrate moiety of the enzyme shows the asymmetric and symmetric stretching vibrations of methylene at at 2933 cm^{-1} [25] and the band at 1029 cm^{-1} corresponds to C-O [26].

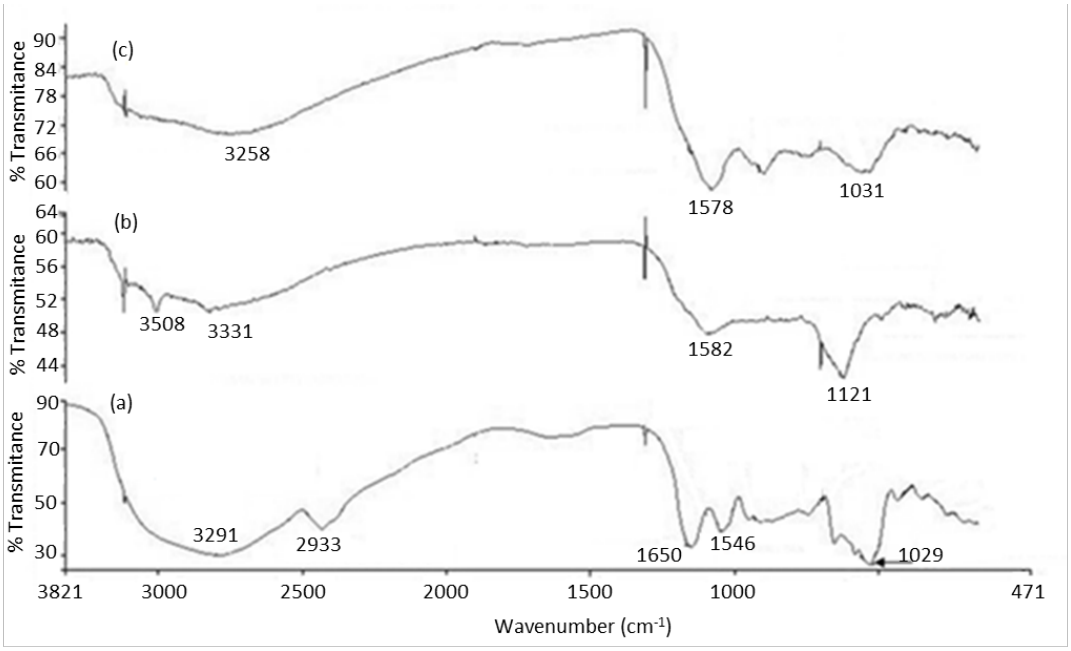
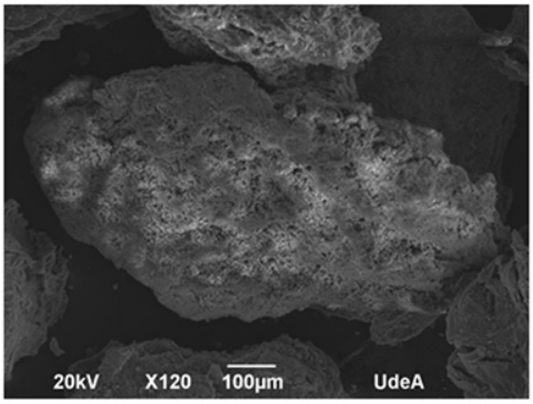
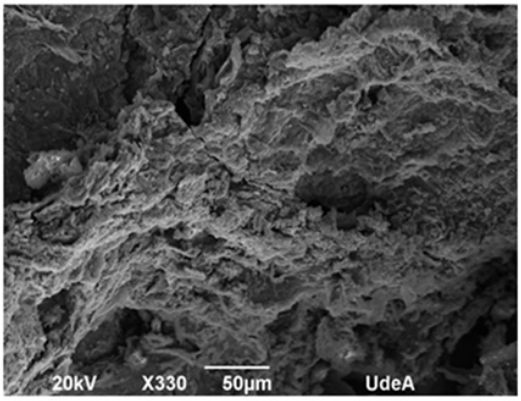


Figure 1. Infrared spectra of: (a) naringinase (NG), (b) BS, (c) BS-NG. **Source:** authors, 2023.





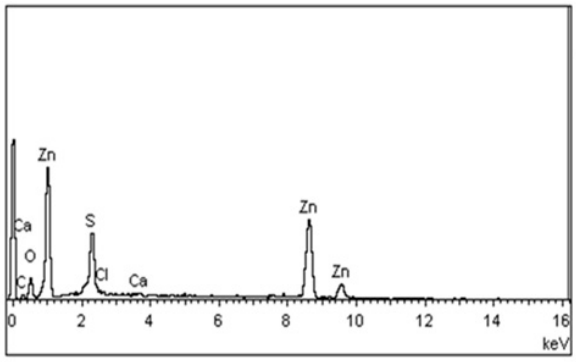


Table 1. Mean elemental composition

Element	Weight (%)
C	4.44
O	12.03
S	7.04
Al	0.89
Zn	75.36
Ca	0.38
Cl	0.59

Figure 2. SEM photograhs, EDX spectra and mean elemental composition of BS. **Source:** authors, 2023.

Furthermore, the FTIR spectrum of BS (Figure 1b) shows signal at 3508-3331 cm^{-1}, 1582 cm^{-1} and 1121 cm^{-1} can be assigned to O–H stretching, C= C stretching vibration in aromatic rings and asymmetric stretching of aromatic ethers, esters, and phenols [27]. Appearance and disappearance of some peaks in the spectrum of the naringinase immobilized on banana support (Figure 1c) can confirm the immobilization [28]. These results are comparable with those reported in the literature [29]. The morphology of the banana support before naringinase immobilization analyzed by SEM are shown in Figure 2.

The images indicated the presence of structure with opening in the material surface resembled the formation of pores. These pores can result of the activation process with $ZnSO_4.7H_2O$ and release of volatile compounds during the thermal treatment, it makes possible naringinase immobilization. The analysis of constituent elements was performed using EDX and this analysis showed that the banana support is formed by carbon, oxygen, sulfur, and zinc and calcium, aluminum, and chloride as minor elements.

3.2. Immobilization of naringinase on SB Support

The immobilization yield (YI) was determined at different pHs (see Figure 3a), and it was observed the maximum YI (above 70%) at pH 7. The residual protein during the immobilization process at pH 7 after 30 minutes decreased around 30% and 70% in 60 minutes (see Figure 3b). These values suggest that the enzyme is immobilized on the support through physical interactions such as electrostatic forces of van der Waals that corresponds to immobilization by adsorption.

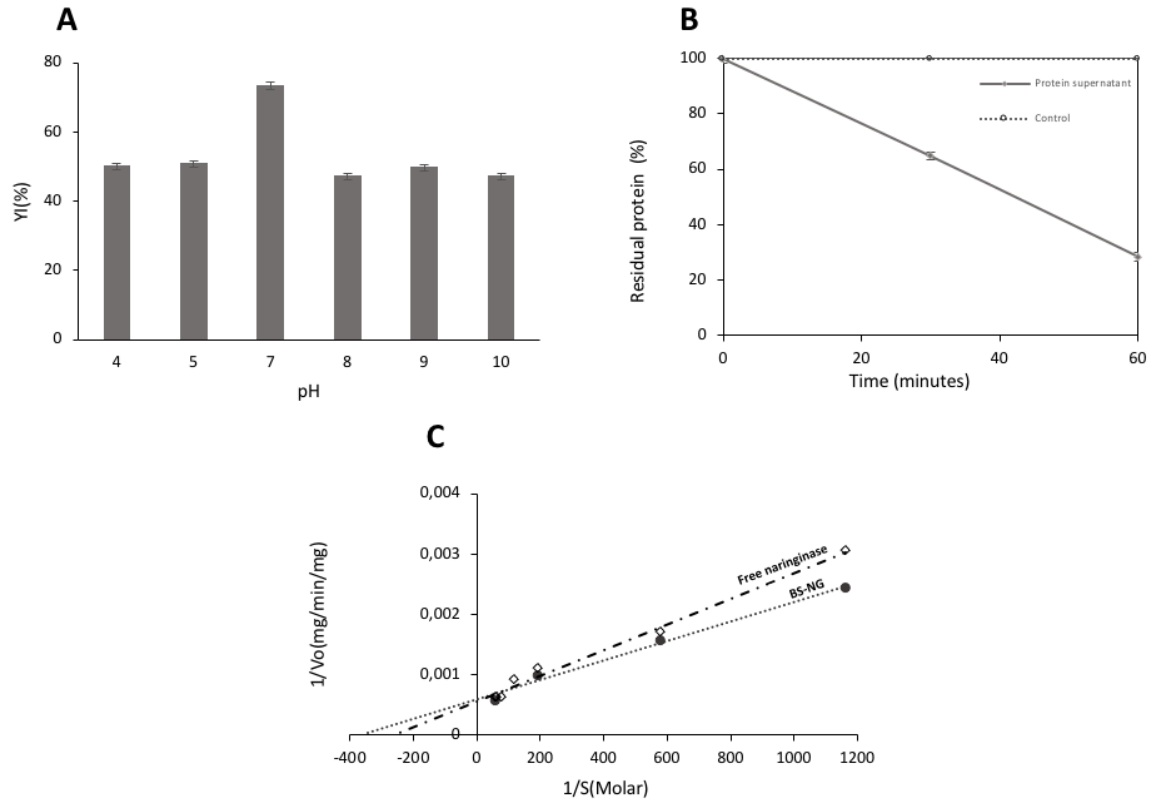


Figure 3. (a) pH influence on yield immobilization of naringinase, (b) quantification of residual protein of the supernatant during the immobilization of naringinase on BS at pH 7 and (c) Lineweaver-Burk graph for free naringinase and BS- NG.
Source: authors, 2023.

3.3. Michaelis-Menten Kinetics

The determination of K_M and V_{max} was done from various concentrations until a saturating concentration of the substrate was identified. The K_M and V_{max} values obtained by the Lineweaver-Burk method [23] for non-immobilized enzyme was 0.0006 molar and 2000 U, and 0.0003 molar and 1666 U, for the immobilized enzyme. The straight line obtained for the concentration of substrate with the free and the immobilized enzyme present a regression coefficient R^2 = 0.9819 and R^2 = 0.9876, respectively (see Figure 3c).

The lower value of K_M suggest that the immobilized enzyme has a higher affinity to the substrate that the non-immobilized enzyme enzyme, due to its lower value in K_M [30].

3.4. Variations of temperature and pH on the behavior of non-immobilized and immobilized naringinase

Figure 4 shows the variations of temperature and pH on the behavior of non-immobilized and BS-naringinase. It can be observed that 4.5 was the best value of pH for non-immobilized and SB- naringinase, which agrees with reported pH effect [31-34]. The behavior of both enzymes was very similar increasing from pH 2 to 4.5 as a maximum of relative activity and decreased from pH 6.5 to 10.

Variations of the temperature were different for free naringinase and BS-naringinase, the optimum temperature for free naringinase was 70 °C which agrees with reported values in the literature (60 – 70 °C) [31,32]. For BS- naringinase, 50 °C was the optimum temperature, nevertheless at 55 °C relative activity fluctuated slightly

retaining 95% of activity which suggest that the enzyme immobilization improved thermostability.

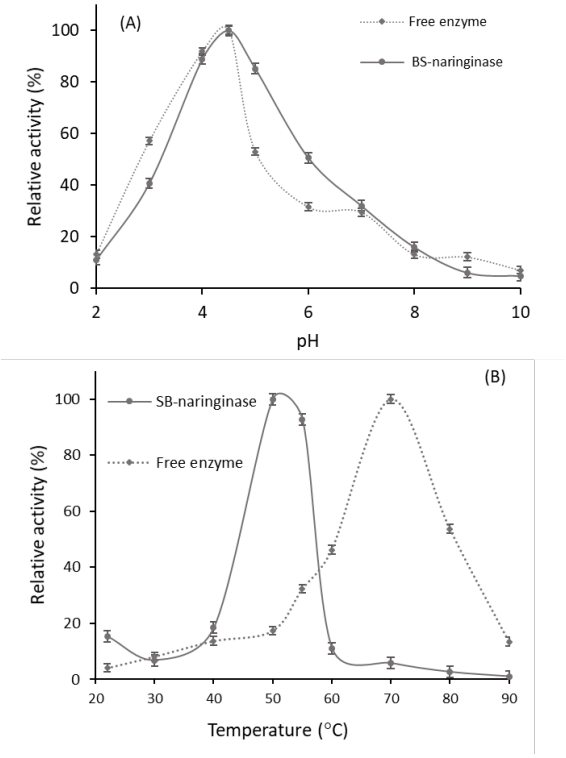


Figure 4. Variations of pH (A) and temperature (B) on the behavior of non-immobilized and immobilized naringinase. **Source:** authors, 2023.

3.5. Reusability of naringinase immobilized (BS-naringinase)

It was found that the SB-support was able to retain at least 100% in the first recycle. The naringinase activity decreased 50% in the second recycle, and in the fourth recycle the relative activity decreased drastically (see Figure 5). Enzyme leaching from the SB supports or denaturation of the naringinase during the intermediate processes between each reuse could be the causes of the activity lost. Successive studies must be carried out to improve the reusability of the BS-support. There are other characteristics that makes attractive the reported synthesis because the low cost of the material, the simple immobilization technique, the considerably required lower enzyme amount, and the relatively high yield of prunin and naringenin that can be obtained in a short period of time.

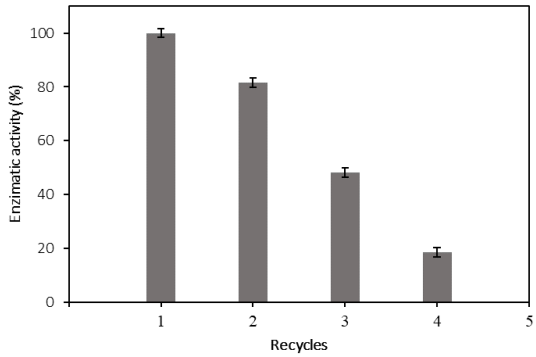


Figure 5. Reusability of BS-naringinase. Conditions: 0.5 g of BS; 2 mL of naringinase (1 mg/mL) in 50 mM sodium citrate buffer; pH 4.5; 22 °C. **Source:** authors, 2023.

4. CONCLUSIONS

This study shows the procedure for obtaining a support from banana peel for the immobilization of naringinase enzyme. The solid was prepared by chemical activation by $ZnSO_4.7H_2O$, then a thermal treatment in nitrogen atmosphere at 500 °C. The FT-IR analysis allowed us to confirm that the enzymatic immobilization process was carried out efficiently, while SEM-EDX showed the presence of pores and carbon, oxygen, sulfur, zinc as main elements. The methodologies applied to immobilize the commercial naringinase produced enzymatic derivatives with excellent kinetic properties. The value of yield of naringinase immobilized and its activity suggested that banana peel materials are promising matrices for immobilization of naringinase. The obtained material showed catalytic potential in the hydrolysis of naringin.

5. ACKNOWLEDGMENTS

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