

# Immobilization and Performance of *Cellulase* on Recyclable Magnetic Hydrotalcites

Tran Boi An, Duong Huynh Thanh Linh, Nguyen Phung Anh, Tran Thi Tuong An, Nguyen Tri\*

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## Abstract

mHT(Zn) and mHT(Mg) hydrotalcites were fabricated by coprecipitation of  $Zn^{2+}/Al^{3+}$  and  $Mg^{2+}/Al^{3+}$  salt mixtures in the presence of  $Fe_3O_4$  and used as supports for immobilizing cellulase to form cell@mHT(Zn) and cell@mHT(Mg). The structure and properties of mHT(Zn), mHT(Mg), cell@mHT(Zn), and cell@mHT(Mg) were characterized by Fourier-transform infrared spectroscopy, X-ray diffraction, filtering electron microscopy. The effect of pH, cellulase concentration, and the number of supports on the immobilization of cellulase onto supports were carefully investigated. The enzyme activity of free cellulase, immobilized cellulase, and immobilization efficiency was analyzed by determining reduced glucose using DNS as a color indicator. The highest immobilization efficiency obtained was 94.9 % when carried out on mHT(Zn) at pH 6.5 and 95.3 % on mHT(Mg) and the concentration of cellulase in 0.1mg/mL at the pH of 5.5, using 0.2 g of supports. Cell@mHT(Zn) and cell@mHT(Mg) show high enzyme activity when reacting with 1 % CMC solution at 50 °C with relative enzyme activity of 78.0% and 70.4 %, respectively.

**Keywords:** Immobilization, Cellulase, Recyclable, Magnetic, Hydrotalcite

## Introduction

Nowadays, in the food and pharmaceutical industries, enzymes have become essential catalysts, with great potential for many

**Tran Boi An, Duong Huynh Thanh Linh, Nguyen Phung Anh**

Institute of Chemical Technology-Vietnam Academy of Science and Technology, Ho Chi Minh City, Vietnam.

**Tran Thi Tuong An**

Faculty of Chemical Engineering, Ho Chi Minh City University of Technology (HCMUT, VNU-HCM), Ho Chi Minh City, Vietnam.

**Nguyen Tri\***

Institute of Chemical Technology-Vietnam Academy of Science and Technology, Ho Chi Minh City, Vietnam.

Faculty of Biotechnology, Ho Chi Minh City Open University, Ho Chi Minh City, Vietnam.

\*E-mail: ntri @ ict. vast. vn

applications. Enzymes are well-known as highly efficient biocatalysts for many reactions due to their high selectivity and reactivity, reducing the number of reactions and toxic solvents. So that the reaction is less expensive and eco-environmental. The most important and widely used technique is enzyme immobilization. The enzyme is immobilized on highly stable and insoluble support in the reaction medium. The most significant advantage of immobilization is significantly improving the stability of biomolecules in different reaction conditions and enhancing the reusability in catalytic cycles (Aehle, 2007).

Cellulose is the main component of plant cells present in scraps of raw materials, fruits, vegetables, and horticultural and forestry industries. For humans and animals, it aids digestion, but in large amounts, it interferes with digestion because humans and animals cannot break down cellulose. However, many strains of microorganisms can convert cellulose into degradable products due to the hydrolysis of cellulose (Minovska *et al.*, 2005).

The adsorption method has the advantage of maintaining high enzyme activity. Cellulase immobilization has been widely investigated on supports such as multilayer carbon-nanotubes (Mubarak *et al.*, 2014), Ag, Au nanoparticles (Mishra & Sardar, 2015), clay minerals (Sinegani *et al.*, 2005), modified chitosan (Dinçer & Telefoncu, 2007), copolymers (Taça *et al.*, 2015), graphene oxide (Zhang *et al.*, 2020), and activated carbon (Anuradha Jabasingh & Valli Nachiyar, 2012). The weak physical bond causes the enzyme to be completely desorbed after use and cannot be reused. Covalent bonds play a significant role in binding enzymes on the support. However, this binding reduces the enzyme activity by changing the conformation of the enzyme molecule (Cass *et al.*, 1998). Cellulase was immobilized by covalent bonds due to its high strength and stability so that it has very high reusability (Li *et al.*, 2013; Zang *et al.*, 2014; Qi *et al.*, 2015; Wang *et al.*, 2015). With this advantage, the enzyme immobilization by covalent bonds is greatly promising for industrial applications. Hydrotalcite meets the support requirements such as high mechanical strength, insoluble in the reaction medium, not inactivating the enzyme, and selective adsorption.

Hydrotalcite (HT) has the general formula as  $[M_{1-x}^{2+}M_x^{3+}(OH)_2]^{x+}[(A^{n-})_{x/y}.mH_2O]^{x-}$  and organic anions or high molecular weight polymers as  $M^{2+}$ : Mg, Zn, Ca...  $M^{3+}$ : Al, Cr, Fe..., and  $A^{n-}$ :  $SO_4^{2-}$ ,  $CO_3^{2-}$ ,  $Cl^-$ . Combining these two salts

by co-precipitation in the presence of  $\text{Fe}_3\text{O}_4$  produces magnetic and porous support for enzyme immobilization. Several enzymes were immobilized on HT and used for biosensor application such as *dextranase* (Ding *et al.*, 2018), *peroxidase* (Baccar *et al.*, 2011; Baccar & Hafaiedh, 2011; Wang *et al.*, 2015; Hidouri *et al.*, 2021), *superoxide-dismutase* (Szilágyi *et al.*, 2018), *laccase* (Camacho Córdova *et al.*, 2009), *lactate-dehydrogenase* (Djebbi *et al.*, 2016), *tyrosinase* (Soussou *et al.*, 2017). Starch hydrolytic enzymes such as *amylase* (Bruna *et al.*, 2015; Sahutoglu & Akgul, 2015), *lipase* (Dias *et al.*, 2019), *glucosidase*, and *cellulase* (Zhang *et al.*, 2020) are attracted by research groups. The research results show the immobilization efficiency obtained was more than 90%, and the activity of immobilized enzymes was more than 50%. Dextranase was immobilized on MgFe-HT by an adsorption mechanism (Ding *et al.*, 2018). The protein immobilization utilizing 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) as a buffer at pH 7 achieved the highest adsorption parallel at 1.38 mg/g (416.67 U/mg). Histidine and phenylalanine also affected the adsorption process. The MgAl-HT was also studied to immobilize some enzymes with high immobilization efficiency, such as laccase (92% immobilization efficiency) (Camacho Córdova *et al.*, 2009; Soussou *et al.*, 2017) or horseradish peroxidase (HRP) applied as biosensor (Baccar & Hafaiedh, 2011). Lactate-dehydrogenase was immobilized on HT by anion exchange and co-precipitation methods, then determined the immobilized enzyme activity (Djebbi *et al.*, 2016). The immobilization efficiency depends on the immobilizing methods. A comparative study showed that the co-precipitation method was controllable and effective for bulk enzyme immobilization. The immobilized enzyme activity was investigated to conclude that the structure/microstructure correlates with immobilized enzyme activity. Furthermore, tyrosinase was immobilized on CoAl-HT and applied as an electrochemical sensor to detect polyphenols in green tea extract (Soussou *et al.*, 2017). This biosensor has high responsiveness, large working scale (up to 1000 ng/mL), and less detection limit (0.33 pg/mL for oxidation, and 0.03 pg/mL for reduction).

There are many announcing about the enzyme immobilization on inorganic supports with both advantages and disadvantages. Cellulase was immobilized on HT, especially the MgAl-HT, but the immobilization on ZnAl-HT is still limited. Compared to MgAl-HT, ZnAl-HT was synthesized by co-precipitation at higher pH, and the electrostatic charge on the ZnAl-HT surface is also higher than MgAl-HT. So that, ZnAl-HT is more sustainable than MgAl-HT. In this study, the cellulase was immobilized on the magnetic ZnAl-HT and MgAl-HT (notated mHT (Zn) and mHT(Mg)) to compare as well to improve the efficiency of enzyme catalyst use due to its magnetic properties, thermal and pH stability. Then, the immobilized enzyme was investigated to be reused for reaction with CMC 1 % to investigate the reuse ability of the immobilized enzyme.

## Materials and Methods

### Materials

All the utilized reagents  $\text{Zn}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ ,  $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ ,  $\text{FeCl}_3$ ,  $\text{FeCl}_2$ ,  $\text{NaOH}$ ,  $\text{HCl}$ , carboxymethylene cellulose (CMC), and dinitrosalicylic acid (DNS) were expository grade reagents. Protein cellulase (extricated from *Trichoderma longibrachiatum*) was displayed by the Biomass research facility, Ho Chi Minh City College of Technology.

### Preparation of Magnetic Hydrotalcite (mHT (Zn) and mHT (Mg))

Mixture of  $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$  and  $\text{Zn}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  with  $\text{Zn}^{2+}/\text{Al}^{3+}$  ( $n_{\text{Zn}^{2+}}/n_{\text{Al}^{3+}} = 2.0$ ) or mixture of  $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$  and  $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  ( $n_{\text{Zn}^{2+}}/n_{\text{Al}^{3+}} = 3.0$ ) was dissolved in 100 mL distilled water and then co-precipitated into the 100 ml of  $\text{Fe}_3\text{O}_4$  dispersion (0.5 % wt/wt). The pH of the reaction solution was kept at  $9.5 \pm 0.1$ . Then the co-precipitated mixture was aged at 80 °C for 24 hrs. mHT (Zn) and mHT (Mg) magnetic hydrotalcites were neutralized and dried at 60 °C for 6 hrs to obtain mHT (Zn) and mHT(Mg) powder.

mHT(Zn) and mHT(Al) samples were characterized by Fourier-transform infrared spectra, X-ray diffraction, and checking electron microscopy with the operating parameter concurring with the previous study (Nguyen *et al.*, 2021).

### Immobilization of Enzyme Cellulase on Magnetic Hydrotalcites

*Cellulase* was dispersed in acetate buffer solution (pH 5.5) with different concentration (0.05, 0.075, 0.1 and 0.2 mg/mL). A proper amount of mHT (Zn) and mHT (Mg) was added to 20 mL of *cellulase* solution, continuing shaking for 6 hrs. The effect of pH, enzyme concentration, and amount of support was studied. The procured immobilized protein was isolated by centrifugation and washed with distilled water, conserved in the refrigerator at 5 °C for further use.

### Cellulase Activity

The special protein activity of free and immobilized *cellulase* and the immobilization efficiency was resolved by the DNS method (Ghose, 1987). A cellulase movement unit (U) was recognized as the sum of *cellulase* that catalyzes CMC to create 1 mg glucose per diminutive at room temperature. An exact 1 ml of *cellulase* solution was reacted with 3 ml of 1% CMC solution in acidic - sodium acetate buffer (pH 4.8) in a water shower at 40 °C for 1 hour. After activation, 0.5 ml of the above solution was used to determine the reduced glucose concentration with the DNS as an indicator. The sum of diminished glucose was identified at 540 nm by a UV spectrophotometer utilizing equation (1) (Ghose, 1987).

$$E = G \times \frac{1000}{M_G} \times \frac{1}{t} \times \frac{1}{v} \times F \quad (1)$$

Where E has alluded to protein activity, it alludes to the response time, G is glucose concentration (mg/L),  $M_G = 180$ , v is *cellulase* solution volume, F is disintegration ratio. The amount of glucose

is calculated by the equation  $A_{560} = 0.0783C_{\text{glu}} - 0.0064$ , acquired from a standard curve (not shown).

### Cellulase Immobilization Efficiency

The cellulase immobilization on carriers was calculated from the disparity between the starting enzyme action ( $E_0$ ) and the ultimate enzyme action ( $E_f$ ). It was expressed in terms of loading efficiency ( $H_{im}$ ) (%) shown in equation (2) (Minovska *et al.*, 2005):

$$H_{im} (\%) = \frac{E_0 V_0 - E_f V_f}{E_0 V_0} \times 100 \quad (2)$$

Where  $V_0$  and  $V_f$  are the initial and final volume of enzyme solution.

### Activity of Immobilized Cellulose on Magnetic Hydrotalcites

The determined activity of immobilized cellulase is as follows: A precise amount of 0.1 g cell@mHT was included to 10 ml of 1% CMC, then heated and at that point kept at distinctive temperatures for different times. After that, the solution was filtrated and boiled to quench the cellulose degradation reaction. The reduced glucose was determined for calculating the immobilized enzyme activity, which was compared with free cellulase activity and expressed in terms of relative enzyme activity as equation (3) (Costa-Silva *et al.*, 2015):

$$E_R = \frac{E_{im}}{E_{cell}} \times 100 \quad (3)$$

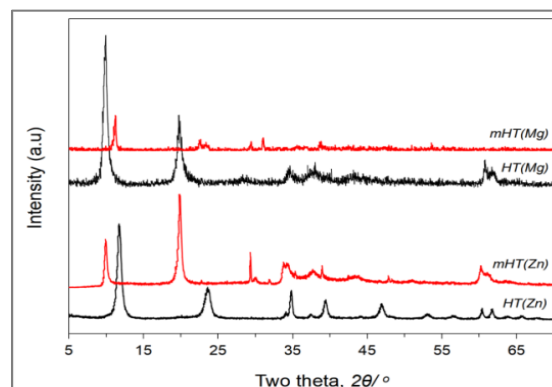
Where  $E_R$  is relative enzyme activity compared with the highest free cellulase activity (%);  $E_{im}$  is the immobilized cellulase activity (UI/g), and  $E_{cell}$  is the highest free cellulase activity (UI/g).

## Results and Discussion

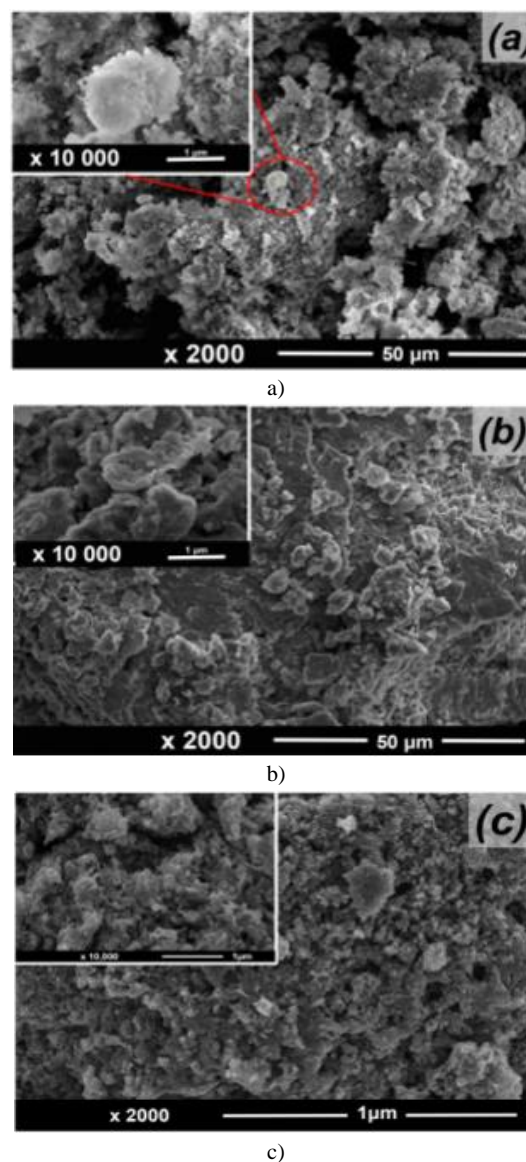
### Supports Characterization

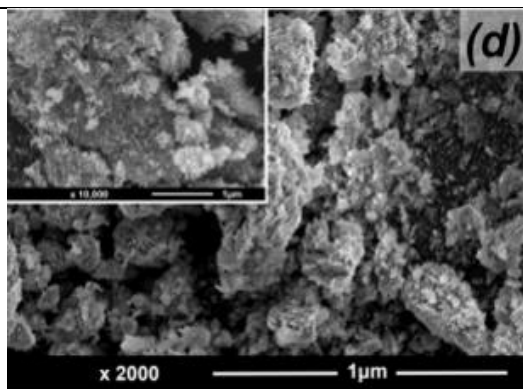
**Figure 1.** The characteristics peak of HT(Zn), HT(Mg), mHT(Zn), mHT(Mg), and samples are the pattern of XRD, mHT(Zn), mHT(Mg), indicate that all have layered structures with the  $\text{NO}_3^-$  anion in the interlayer. In particular, HT(Zn) was characterized by  $2\theta = 11.8^\circ, 23.2^\circ, 34.5^\circ, 39.1^\circ, 60.4^\circ,$  and  $61.2^\circ$ ; and HT(Mg) was characterized by  $2\theta = 10.6^\circ, 20.2^\circ, 35.1^\circ, 38.9^\circ, 60.8^\circ,$  and  $61.2^\circ$  according to the diffraction of (003), (006), (009), (015), (012), (110) and (113). XRD pattern of HT is consistent with that of authors (Wiyantoko *et al.*, 2015; Boukhalfa *et al.*, 2017). Both mHT (Zn) and mHT (Mg) samples have characteristics peaks of HT and few feature crest of  $\text{Fe}_3\text{O}_4$  at  $2\theta = 30.1^\circ$  and  $35.5^\circ$ . In addition, the diffraction of (009) has been divided into two sub-peaks for both mHT (Zn) and mHT (Mg). The front peak belongs to the hydrotalcite characteristic, and the back one shows the magnetite core deposited on the

surface of the hydrotalcite. XRD spectra of mHT are consistent with that of Triastuti Sulistyarningsih *et al.* (2015).



**Figure 1.** The XRD pattern of mHT(Mg), HT(Mg), mHT(Zn), and HT(Zn).

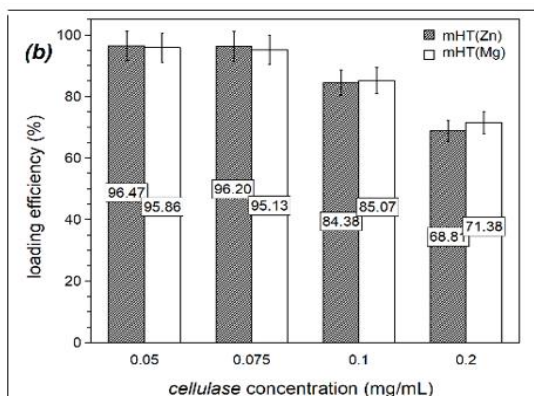
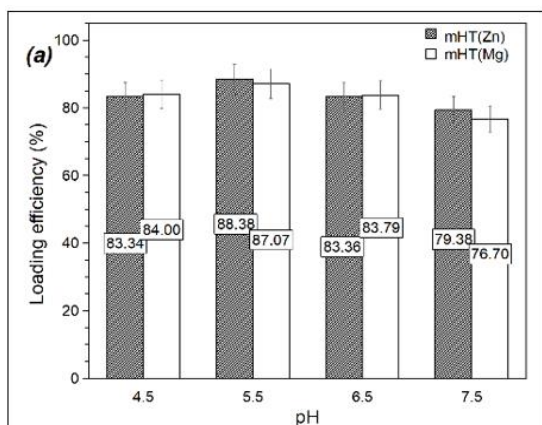




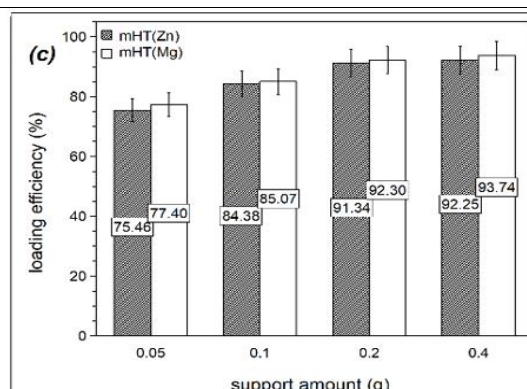
**Figure 2.** The SEM images of a) HT(Zn), b) mHT(Zn), c) HT(Mg), and d) HT(Mg)

SEM images observed the morphology of samples in **Figure 2**. The morphologies of both HT(Zn) and HT(Mg) samples are flakes shape, and the morphologies of mHT(Zn) and mHT(Mg) samples are porous consisting of multilayers, and Fe<sub>3</sub>O<sub>4</sub> particles have covered the HT surface. This result is entirely consistent with the results of structural analysis by significantly reduced X-ray diffraction.

*Immobilize Cellulase on mHT(Zn) and mHT(Mg) Supports*



b)



c)

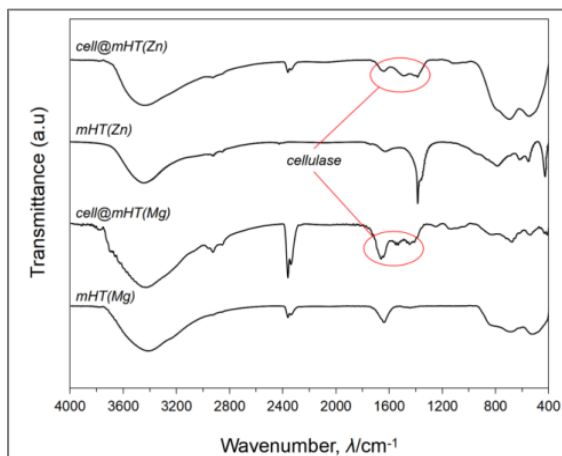
**Figure 3.** Effects of pH (a), cellulase concentration (b), and support amount (c) on cellulase immobilization on mHT(Zn) and mHT(Mg)

The pH value of enzyme medium is an imperative figure that influences enzyme activity and greatly affects the enzyme immobilization efficiency due to the surface characteristics of the supports. **Figure 3a** shows the effect of pH on cellulase immobilization efficiency. When immobilizing cellulase on both mHT(Zn) and mHT(Mg), the H<sub>im</sub> tent increase with the pH from 4.5 to 5.5. But, at higher pH 6.5 and 7.5, the H<sub>im</sub> decreased. In particular, when immobilizing at pH 4.5, 5.5, 6.5 and 7.5, the H<sub>im</sub> has obtained 83.34 %, 88.38 %, 83.36 %, and 79.38 % for immobilization on mHT(Zn), while the H<sub>im</sub> has obtained 84.00 %, 87.07 %, 83.79 %, and 76.70 % for mHT(Mg). So that, the immobilization of cellulase on mHT(Zn) and mHT(Mg) will be performed at pH 5.5 for the higher H<sub>im</sub>.

The effect of cellulase concentration on immobilization is shown in **Figure 3b**. H<sub>im</sub> decreased when increasing the cellulase concentration. When immobilizing in cellulase solution with 0.05, 0.075, 0.10, and 0.20 mg/mL, the H<sub>im</sub> of mHT(Zn) reached 96.5%, 96.2%, 84.4%, and 68.8%, respectively. And, that of mHT(Mg) did 95.9%, 95.1%, 85.1%, and 71.4%. This can be explained that both mHT(Zn) and mHT(Mg) have limited adsorption capacity; thus, the increase of cellulase concentration can not enhance the H<sub>im</sub> value. In general, when immobilizing with 0.075 mg/ml cellulase solution, the H<sub>im</sub> is higher than the remaining concentration cellulase solution. Therefore, the suitable enzyme solution for enzyme immobilization is 0.075 mg/mL for both samples.

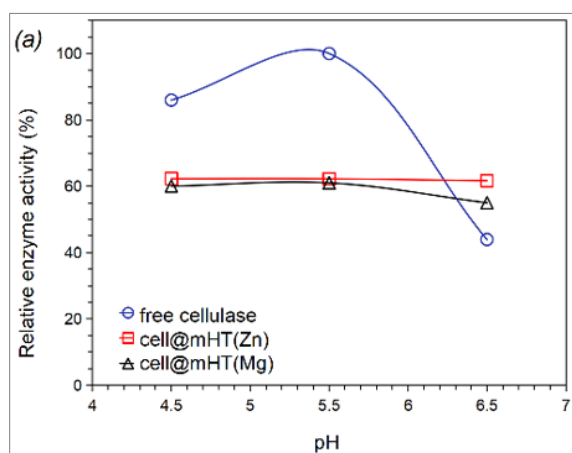
The amount of mHT(Zn) and mHT(Mg) affects the rate of enzyme immobilization, and the results are shown in **Figure 3c**. The H<sub>im</sub> was increased when increasing the amount mHT(Zn) and mHT(Mg) by 0.05, 0.1, 0.2, and 0.4 grs. In particular, H<sub>im</sub> obtained 75.5 %, 84.4 %, 91.3 %, and 92.3 % when using mHT(Zn) as support, and H<sub>im</sub> obtained 77.4 %, 85.1 %, 92.3 %, and 93.7 % when using mHT(Mg) as support. Thus, H<sub>im</sub> obtained the highest when using 0.4 grs of both mHT(Zn) and mHT(Mg) as supports, but it is not much higher than using 0.2 grs of supports, and the H<sub>im</sub> nearly reached saturation. Because the adsorption capacity of both mHT(Zn) and mHT(Mg) has limited. So that, the increase of support amount has not enhanced

immobilization efficiency when it reaches saturation. Therefore, the suitable mHT(Zn) and mHT(Mg) mass for immobilization were 0.2 grs.

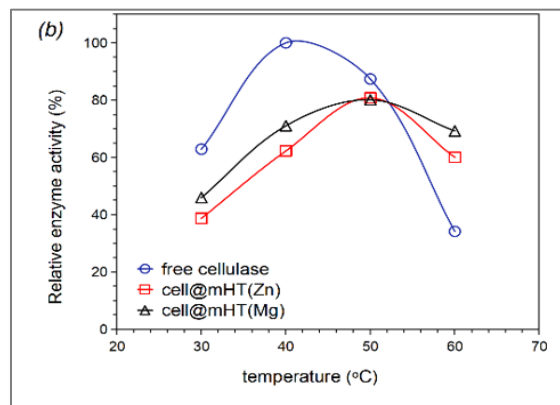


**Figure 4.** “FT-IR spectra of magnetic hydrotalcite with and without immobilizing cellulase.”

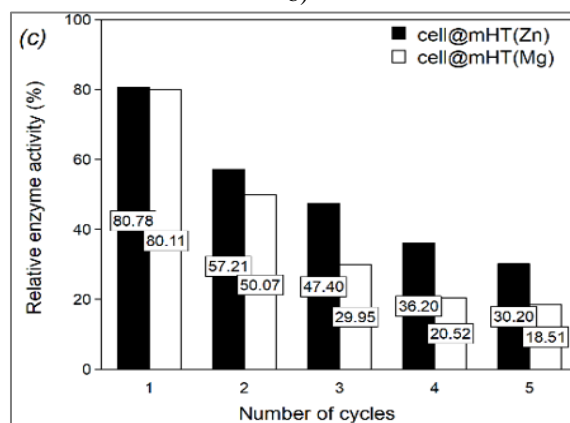
“FT-IR spectra of magnetic hydrotalcite with and without immobilizing cellulase.” (Figure 4) shows that the wideband at about  $3463\text{ cm}^{-1}$  is characteristic for vibration of  $-\text{OH}$  groups from lattice and the alternating layer of water. The weak adsorption at about  $1643\text{ cm}^{-1}$  is the vibration of  $-\text{OH}$  in the  $\text{H}_2\text{O}$  structure attached to the interlayer.  $1385$  is assigned for  $-\text{NO}_3$  vibration, and shaped assimilation peak, which is also present for mHT(Zn) and mHT(Mg) interlayers. The peaks at  $802$ ,  $617$ ,  $447$ , and  $409\text{ cm}^{-1}$  are due to  $\text{M}-\text{O}$  vibration ( $\text{Al}-\text{OH}$ ,  $\text{Zn}-\text{OH}$ ,  $\text{Mg}-\text{OH}$ ,  $\text{Fe}-\text{O}$ , and  $\text{Fe}-\text{O}-\text{Fe}$ ). And the peaks at about  $995$ ,  $1033$ , and  $1063\text{ cm}^{-1}$  are assigned for the cellulase characterization. This proved that cell@mHT(Zn) and cell@mHT(Mg) were successfully synthesized.



a)



b)



c)

**Figure 5.** Immobilized enzyme activity at different pH (a), temperature (b), and the number of reused cycles (c).

#### Activity Cellulase of on Magnetic Hydrotalcites

The activity of cell@mHT(Zn) and cell@mHT(Mg) were investigated and compared with free cellulase; the results are shown in Figure 5. When activated at  $40\text{ }^\circ\text{C}$  and pH 5.5, cell@mHT(Zn) obtained the highest activity with the  $E_R$  at 61.0%. On the other hand, cell@mHT(Mg) also received the highest activity with  $E_R$  is 62.3% (Figure 5a). But when performed at pH 6.5, the activity of free cellulase decreased (44.0%) and was lower than both cell@mHT(Zn) and cell@mHT(Mg) (61.7% and 55.0%). This could be clarified by the isoelectric point (pI) of the mHT(Zn) on the pH 11–12 and of the mHT(Mg) is about 8.5–10 (Dai *et al.*, 2007), while the pI of cellulase is about 4.5 (Rekha & Srivastava, 2019). Thus, the 5.5 pH level is optimum, mHT(Zn) and mHT(Mg) substrates are in a positive, acidic ionized charge, while cellulase is in the alkaline ion state, carrying a negative charge. As a result, there is a solid electrostatic impact between the emphatically charged mHT(Zn) and mHT(Mg) substrates and the contrarily charged cellulase, and help increase the strength of the covalent bond between the enzyme and the mHT substrate. This result shows that both samples are more sustainable than free cellulase.

Compared with free cellulase, both cell@mHT(Zn) and cell@mHT(Mg) have lower activity, but they intend to enhance thermal stability. The effect of immobilization temperature was

conducted in different temperatures in 30–60 °C at pH 5.5 (**Figure 5b**). Free *cellulase* is sensitive to temperature; the enzyme activity obtained the highest when activated at 40 °C. At the lower and higher temperature, rapidly decreased free cellulase enzyme activity with the  $E_R$  is 62.9%, 87.4%, and 34.2%, corresponding to activated at 30, 50, and 60 °C. cell@mHT(Zn) started at 50 °C obtained higher enzyme activity than cell@mHT(Mg). In detail, at 50 °C,  $E_R$  of cell@mHT(Zn) and cell@mHT(Mg) obtained 80.8% and 80.1%. Increasing immobilization temperature decreases enzyme activity, but the  $E_R$  of both cell@mHT(Zn) and cell@mHT(Mg) is higher than free *cellulase*. This is due to the mHT(Zn) and mHT(Mg) characteristics of the multilayer structure, which can be an ideal substrate for enzymes (Mousty & Prévot, 2013). Besides, the structure of mHT(Zn) and mHT(Mg) is double-layer with the intercalating-spacing that the enzyme can be intercalated to reduce the mobility and enzyme activity (Barbosa *et al.*, 2014). This improves the enzyme activity's stability due to the interaction of electrostatic charge on the enzyme and the emphatically charged layers of mHT(Zn) and mHT(Mg). However, there is a difference between cell@mHT(Zn) and cell@mHT(Mg) when immobilizing at high temperatures. cell@mHT(Mg) obtained higher  $E_R$  than cell@mHT(Zn), which are 69.2% and 60.1%, respectively. This can be due to the thermal stability of mHT(Mg) at high temperatures. In summary, cell@mHT(Zn) and cell@mHT(Mg) provide high  $E_R$ , which obtained 80.8% and 80.1% when activated at 50 °C at pH 5.5. Both cell@mHT(Zn) and cell@mHT(Mg) enhanced stability when activated at either higher temperature or higher pH.

Both cell@mHT(Zn) and cell@mHT(Mg) samples were also studied to reuse for five cycles of reaction with CMC 1 %. The decrease of activity of cell@mHT (Zn) and cell@mHT(Mg) after each cycle can be explained by the release of *cellulase* (**Figure 5c**). So that, the amount of *cellulase* immobilized on support also decreased after each cycle. In particular, after 5 cycles, the  $E_R$  of cell@mHT(Zn) decreased in sequence 80.8%, 57.2%, 47.4%, 36.2%, and 30.2%. Similarly, the  $E_R$  of cell@mHT(Mg) decreased in sequence 80.1%, 50.1%, 29.9%, 20.5%, and 18.5%. On the other hand, cell@mHT(Zn) always obtained higher  $E_R$  than cell@mHT(Mg), which proves that *cellulase* is more stable when immobilized on mHT(Zn) than on mHT(Mg) samples.

## Conclusion

The influence of Zn and Mg in mHT structure on the cellulase immobilization was carried out with various pH, cellulase concentrations, and mHT amounts. Both mHT (Zn) and mHT(Mg) can load most enzymes as immobilization at pH 5.5. The lower and the higher will decrease loading efficiency. The cellulose immobilization was studied on two supports, mHT (Zn) and mHT (Mg). Similarly, cellulase concentration and amount of mHT also affect immobilization efficiency, and both have limits for higher  $H_{im}$ . Specifically, the suitable cellulase concentration was 0.075 mg/mL, and the maximum amount of mHT was 0.2 g. While the free cellulase activity is decreased if used at temperatures above 40 °C and pH above 5.5, both cell@mHT (Zn) maintained higher activity at pH 6.5, and cell@mHT (Mg) maintained higher activity 60 °C. In conclusion, besides the

ability to recover by a magnetic field, cell@mHOT (Zn) promises to enhance the pH and thermal stability of biocatalyst used in practice.

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**Conflict of interest:** None

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**Ethics statement:** None

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