

Immobilization of β -Glucosidase on Different Supports and Evaluation of Its Activity in Cellobiose Hydrolysis

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Abstract

β -glucosidase was used as catalyst for the hydrolysis of cellobiose, immobilized on different supports, including two silicas (MSNS and MCM-41) and three carbon supports (GAC, BDH and Norit). It was observed that mesoporous nanoparticles are better candidates as supports for the β -glucosidase, and that silica has a better performance, probably due to the presence of silanol groups. The MSNS showed better recovered activity compared to other supports, showing an optimum temperature at 70 °C and an optimum pH of 5. The enzyme immobilized in MSNS showed results 80% to 98% better than those immobilized on other supports. However, for the recycling test, from the second cycle onwards, the MSNS showed a performance drop of around 40%, reaching a relative activity of 41.9% for both the fourth and the final cycle. MCM-41, on the other hand, did not show as much discrepancy in the recycling test. However, its relative activity in the first cycle was 20% of the activity achieved by MSNS, at its optimum temperature of 60 °C. Such results indicate that the smaller particle size favours enzymatic activity. Notwithstanding, a larger available intra-pore surface area protects the enzyme from denaturation.

Introduction

Lignocellulosic biomass is considered the most promising renewable raw material for the production of biofuels. Ethanol, for example, due to its high octane number, heat of vaporization and compatibility with vehicle engines, stands out in the sector. (12) Lignocellulosic materials are considered advantageous, as they are sources of renewable energy, ecologically favourable, and are found in abundance, which reflects their low cost. They are basically composed of an aromatic structure called lignin and carbohydrate polymers, homopolysaccharides commonly called cellulose, and heteropolysaccharides, hemicellulose. (9)

Cellulose is the major component of herbal cell walls, making up approximately half of the woods, both coniferous and hardwood, and also the most common organic compound in nature. (7). It consists solely and exclusively of several molecules of glucose (monomer) and cellobiose (glucose dimer), which build an organized and partially crystalline structure by forming bonds between them. (2)

Enzymes play an important role in contemporary industry; however, there are difficulties in applying free enzymes in industry, such as the short catalytic duration, reuse and low thermal stability. To solve these problems, the recommendation is enzymatic immobilization. (6) Enzymatic immobilization can be carried out by simply binding the enzyme to the activated matrix or using a binding agent. Immobilization is a convenient method, since it increases the thermal and pH stability of the enzyme, brings a lower production cost and makes reuse easy to handle and separate. Indeed, enzymatic immobilization can provide many important advantages over the application of enzymes, such as reuse, continuous operation, controlled product formation, easy separation, use in unconventional media and simple processing. Immobilization can also increase the stability of enzymes. (8). Furthermore, enzymatic catalysis results in cleaner reactions and easy recovery of the product, when compared to traditional catalysis. As long as the stability of the enzyme can be improved, recycling costs may be reduced.

Enzymatic immobilization may be the answer to the application of biocatalysis on an industrial scale. (1).

For immobilization to be possible, a biocompatible matrix is needed. Such matrix must not interact with the biological activity and the natural structure of the protein. (3)

Enzymes can be immobilized through different methods, such as: adsorption, covalent bonding, encapsulation, among others. There is no universal method or support that fits all enzymes; hence, methods must be studied for each case due to the different properties of the substrates and the physical and chemical characteristics of the enzymes, as well as the product applications. Each method has advantages and limitations; therefore, the optimal conditions of immobilization for a given enzyme are determined empirically in the laboratory, by a process of trial and error. (10)

This work is inserted in the context of innovations in the production process of second generation ethanol, giving priority to technologies that allow milder operating conditions of temperature, pressure and pH, with regard to the biomass hydrolysis stage. Due to the high cost of the biocatalyst used, it is important to investigate solutions that allow its recyclability. Its objective is to study the immobilization of β -glucosidase on different supports and to evaluate its performance in the reaction of cellobiose hydrolysis to yield glucose units.

The method used to immobilize the enzyme was covalent bonding. In order to immobilize the enzyme by the covalent bonding method, it is necessary to prepare the support for the task in which the functional groups of the support are modified to produce reactive intermediates.

Two binding agents were used in this article. APTES (3-aminopropyltriethoxysilane), which is commonly used in functionalization to promote the Si-O-Si bond with the silanol group present in the support. (5) and glutaraldehyde, the preferred cross-linking agent due to its low cost, availability, ease of handling and ability to make covalent bonds with most enzymes. (13). The glutaraldehyde molecule reacts with the support and the enzyme, and these are immobilized by covalent bonding on the support through its amino groups (α -NH₂ of the terminal chain, ϵ -NH₂ of the lysine and / or NH₂ from chemical amination), which bind to the aldehyde groups on the support. (10)

Materials And Methods

3-aminopropyltriethoxysilane (APTES) (99%), anhydrous toluene (99.8%), glutaraldehyde solution (25%), almond β -glycosidase (3.4 U · mg⁻¹), mesoporous silica (MSNS and MCM-41), carbon supports (GAC, BDH and NORIT) and cellobiose were purchased from Sigma-Aldrich.

FUNCTIONALIZATION OF THE SUPPORTS

To prepare the supports to immobilize the enzyme, two steps were necessary. The first was functionalization with APTES (3-aminopropyltriethoxysilane), to form amino groups in each support. For

this purpose, 2g of support was used for a solution of 100 mL of toluene (dry) and 1% v / v APTES. The procedure was performed under reflux for 3 hours. The volumetric flask was immersed in an oil bath at a temperature of 105°C, with an agitation rate of approximately 400 RPM. Then, the material underwent washing with toluene (three times) in resistant tubes - TFE / PTFE Nalgene® and centrifuged at 20,000 xg for 30 minutes. (Thermo Scientific - Sorval LYNX 4000 Centrifuge) Finally, each support was dried in an inert atmosphere and stored under the same conditions.

In the second stage of preparation of the supports, the binding of the amino groups of APTES with glutaraldehyde was carried out. For the binding of the amino group with the homo bifunctional aldehyde, 40mg of the support was added to a solution of 5 ml of deionized water and 400 µL of 2% v / v glutaraldehyde. For this reaction, the solution remained for 24 hours in a New Brunswick™ Innova® 42 incubator at 200 RPM shaking at 30°C. Then, the samples were washed several times with distilled water and centrifuged for 30 minutes at 20,000 xg rotation.

IMMOBILIZATION OF B-GLUCOSIDASE

The enzyme was immobilized using 2 mg/mL for each 40 mg of each support, in a volume of 5 mL of 5 mM citrate buffer solution of pH 5. The immobilization occurred through an incubator (New Brunswick™ Innova® 42), shaking at 200 RPM, for 24 hours at a temperature of 30°C. After this period, the samples were washed and centrifuged for 40 minutes at 20,000 xg. This washing was repeated 3 times, being the supernatant discarded and replaced with 5 mL of the pH 5 buffer solution.

TESTS OF PH, TEMPERATURE AND RECYCLE

The pH test aims to verify the effect of pH on the performance of β-glucosidase activity. For the present work, the activity was analyzed in the pH range from 4 to 6. The samples underwent hydrolysis for 45 minutes at a fixed temperature of 50°C in an incubator (New Brunswick™ Innova® 42), shaking at 200 RPM.

In the optimal temperature test, the performance of β-glucosidase activity was analyzed using the same incubator conditions (New Brunswick™ Innova® 42), in a temperature range from 30 to 70°C, at a fixed pH of 5 by 45 minutes shaking at 200 RPM.

Likewise, for the recycle test, hydrolysis was carried out by subjecting the immobilized enzymes to a 45-minute hydrolysis in a New Brunswick™ Innova® 42 incubator, with agitation of 200 RPM, at its optimum pH and temperatures. Samples were washed and centrifuged for 30 minutes at 20,000 xg rotation. This test was repeated 4 times.

All hydrolysis reactions were carried out by suspending the immobilized enzymes in a buffer solution containing 25 g/L cellobiose. At the end, the samples were centrifuged (Thermo Scientific - Sorval LYNX 4000 Centrifuge) at 20,000 xg for 40 minutes and the supernatant was stored at 4°C. The activity was calculated based on the formation of glucose, analyzed in HPLC - Waters 2489, UV / Visible Detector, Hi-Plex H column, 300 x 77.7mm.

SUPPORT CHARACTERIZATION

Supports for enzyme immobilization were characterized using nitrogen adsorption and infrared methods. Textural properties were measured in TriStar II 3020, a fully automated, three station, surface area and porosity, at a bath of -195.850°C. The infrared transmission and reflection (FTIR) was analysed using a Tri-Range FT-IR Spectrometer after the samples were properly dried.

Results And Discussion

SUPPORT CHARACTERIZATION

One of the most important characteristics of the supports for enzyme immobilization are the textural properties thereof. In this work, two silicas (MSNS and MCM-41) and three carbons (GAC, BDH and Norit) were analyzed. Table 1 shows that the silicas have specific surfaces significantly smaller than the carbons. Carbons also have a larger pore volume than silicas. Among all, the MSNS has less BET surface area and less total pore volume. Evidently, the specific area is larger for the carbons due to their superior porosity, which means this is the intraporous surface area.

Table 1
Supports specific surface and pore volume

SUPpORTS	BET – specific surface	total pore volume
MSNS	61 m ² /g	0,09 cm ³ /g
MCM-41	84 m ² /g	0,18 cm ³ /g
GAC	646 m ² /g	0,39cm ³ /g
BDH	845 m ² /g	0,77 cm ³ /g
Norit	1031 m ² /g	0,62 cm ³ /g

It can be seen, in Table 2, that the average pore diameter of each of the supports is in the mesoporous range (between 2 nm and 50 nm). Taking into account that β -glucosidase has an average diameter of 6.7 nm, the only supports on which the enzyme could be deposited inside the pores would be BDH and MCM-41. However, according to Carvalho (2018), microporous materials would be better candidates as a support for β -glucosidase, as they have a high external area. This external support area would be available for immobilization. As β -glucosidase has larger dimensions, it could be trapped inside the mesoporous, which can lead to the deactivation of the enzyme. Causes of this deactivation could be steric obstruction or pore blockage caused by glutaraldehyde. (5)

Table 2
Supports average pore diameter

SUPpORTS	BJH – Average Pore Diameter (adsorption)
MSNS	61,54 Å
MCM-41	93,71 Å
GAC	52,61 Å
BDH	70,10 Å
Norit	48,15 Å

The use of infrared transmission and reflection (FTIR) is very efficient tool for the identification of bands in the vibrational spectra of silicas, pointing out well-defined bands, both for MSNS and MCM-41. Figure 1 shows the band referring to silanol groups in the 939 cm^{-1} range for MSNS. Similarly, MCM-41 also presented a 939 cm^{-1} silanol band (Si-OH), which can be seen in Fig. 2. The presence of silanol groups is positive because it allows the formation of bonds with APTES, thus preparing the support for the functionalization with glutaraldehyde.

IMMOBILIZATION RESULTS

The immobilized enzymes were used as catalysts for the hydrolysis reaction under different pH conditions to evaluate their performance, thus determining what would be the optimal pH for the conversion of cellobiose into glucose. The buffer solutions were prepared with distilled water, citric acid and sodium citrate, at pHs 4, 5 and 6. According to the literature, free β -glucosidase performed better at a temperature of 50°C . Therefore, this temperature value was maintained for the optimal pH test. It can be seen, in the results presented in Fig. 3, that the MSNS performed significantly better than the other tested supports. Anyhow, however bad was the performance of the other 4 supports tested, all showed an improvement in the conversion of cellobiose into glucose at pH 5.

For the optimal temperature test, it was assumed that the ideal medium for hydrolysis with immobilized enzymes would be pH 5. Once again, the MSNS mesoporous silica showed the best result, indicating that its optimum temperature is 70°C . The temperatures chosen for evaluation ranged from 30°C to 70°C . Temperatures below 30°C and above 70°C have not been tested due to the difficulty of experimental control.

The other supports showed results from 80–98% lower than the result obtained with the enzyme immobilized in MSNS. The MCM-41 shows, in Fig. 4, the second best result reaching about 20% of the activity achieved by the MSNS, at a temperature of 60°C . It is believed that this fact is due to the Si-OH groups identified in the FTIR of the silicas, which are not present in the carbons, thus allowing bonds with APTES and glutaraldehyde to happen. Such bonds make the enzyme immobilization more effective.

Among the results obtained for silicas, the concept of external surface as a function of particle size is quite relevant. The superiority of MSNS as a support can be attributed to the available external surface area, due to the smaller particle size. The particle size of MSNS is in the range of 75 nm (5), particles smaller than 100 nm are considered nano particles. MCM-41 has an average particle size of 400 nm (4). Although MCM-41 has a larger total area according to BET, a large part of this area is present inside the pores. Hence, substrate and product must undergo intrapore diffusion, which hinders the reaction.

As expected, the recycle test indicated a reduction in relative activity after the second cycle. The results of the five supports tested are depicted in Fig. 5. The support that presented the best result, MSNS, indicates a drop of almost 40% in its activity for the second cycle, reaching a relative activity of 41.90% for the fourth cycle. MCM-41, on the other hand, did not undergo major changes from one cycle to the next, maintaining a relative activity of 82.43% in the last cycle.

The high discrepancy between the first and the second cycle for MSNS may indicate denaturation of the immobilized enzyme, or even desorption of proteins present on the external surface of the support. As previously discussed, the pores present in the MSNS are not the ideal size for β -glucosidase to penetrate them, so if the support suffers loss of enzymes bound to the surface, it also loses much of its activity. MCM-41, on the other hand, which has pores large enough to accommodate β -glucosidase, suffered less loss of relative activity, because it offers protection from external conditions, such as pH and temperature, that could cause denaturation. Apparently, the enzyme is located inside the pores, being protected.

Among the carbons, GAC and BDH showed a false increase in relative activity in the fourth and second cycles, respectively. This is due to the imprecision of the HPLC equipment for very small concentrations, since both carbons showed very low glucose formation. The conversion rate of cellobiose to glucose in the first 40 minutes of the reaction, where the rate is faster, can be seen in Table 3. It is observed that both silicas have a faster rate compared to carbons. Naturally, the MSNS showed better results, about 70% faster than the MCM-41, support that came in second place.

Table 3
Glucose formation rate of the immobilized enzyme in different supports

Supports	reaction RATE (mol/s)
MSNS	$3,7016 \cdot 10^{-6}$
MCM-41	$1,1064 \cdot 10^{-6}$
GAC	$2,6512 \cdot 10^{-7}$
Norit	$1,4748 \cdot 10^{-7}$
BDH	$4,3843 \cdot 10^{-7}$

Conclusions

The present work aimed to study the immobilization of β -glucosidase and its performance on different supports, these being two silicas and three carbons. It was observed that materials with smaller pore diameter and smaller particle size would be better supports for β -glucosidase, since only the external area of the support is available for immobilization.

The silicas performed better in the immobilization of β -glucosidase. FTIR analysis revealed the presence of silanol (Si-OH) for both MSNS and MCM-41. This indicates that the binding with APTES and then with glutaraldehyde was facilitated by the presence of silanol groups, resulting in a better immobilization result.

Regarding the parameters that define the relative activity, it was noted that the support that obtained the best performance in the tests of both optimal pH and optimal temperature was MSNS. As for recyclability, the MCM-41 showed the best result, because it maintained approximately the same result for all cycles, with a small drop in activity. In general, at pH 5, the supports provided better results, with greater activity. The same did not happen with the temperature, since each support presented a better activity at different temperatures; therefore, the optimal temperature test was defined separately for each one. Interestingly, the optimal temperature for the MSNS proved to be quite high, equal to 70°C. This result may be interesting for future studies where β -glucosidase is to be used in extreme temperature conditions. The recyclability test was then carried out at the optimum temperature and pH of each support. The best result for this test was for the MCM-41. This silica has a larger pore diameter, enough to accommodate the enzymes inside the pores, differently from MSNS, which protects the enzyme from denaturation.

Results indicate that best supports for immobilization of β -glucosidase must present a high external surface area, therefore nanoparticles are recommended. Also, the existence of mesopores seems to be interesting, especially the ones with pores larger than 6.7 nm, which is the average size of β -glucosidase, since the enzyme would be protected from denaturation inside the pores. The combination of the two characteristics would present a better support for immobilization of β -glucosidase, for it would present a better activity towards higher temperatures and it would enforce the stability in the recycling process.

Declarations

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Authors contributions Conceptualization, YC, EFSA; Methodology, YC; Investigation, KCSS; Analysis, KCSS; Writing original draft, KCSS; Review and editing, YC, EFSA; Supervision, YC, EFSA. All authors have read and agreed to the published version of the manuscript.

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Availability of data and material All data generated or analysed during this study are included in this published article.

Code availability Not applicable.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent to publish Not applicable.

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Figures

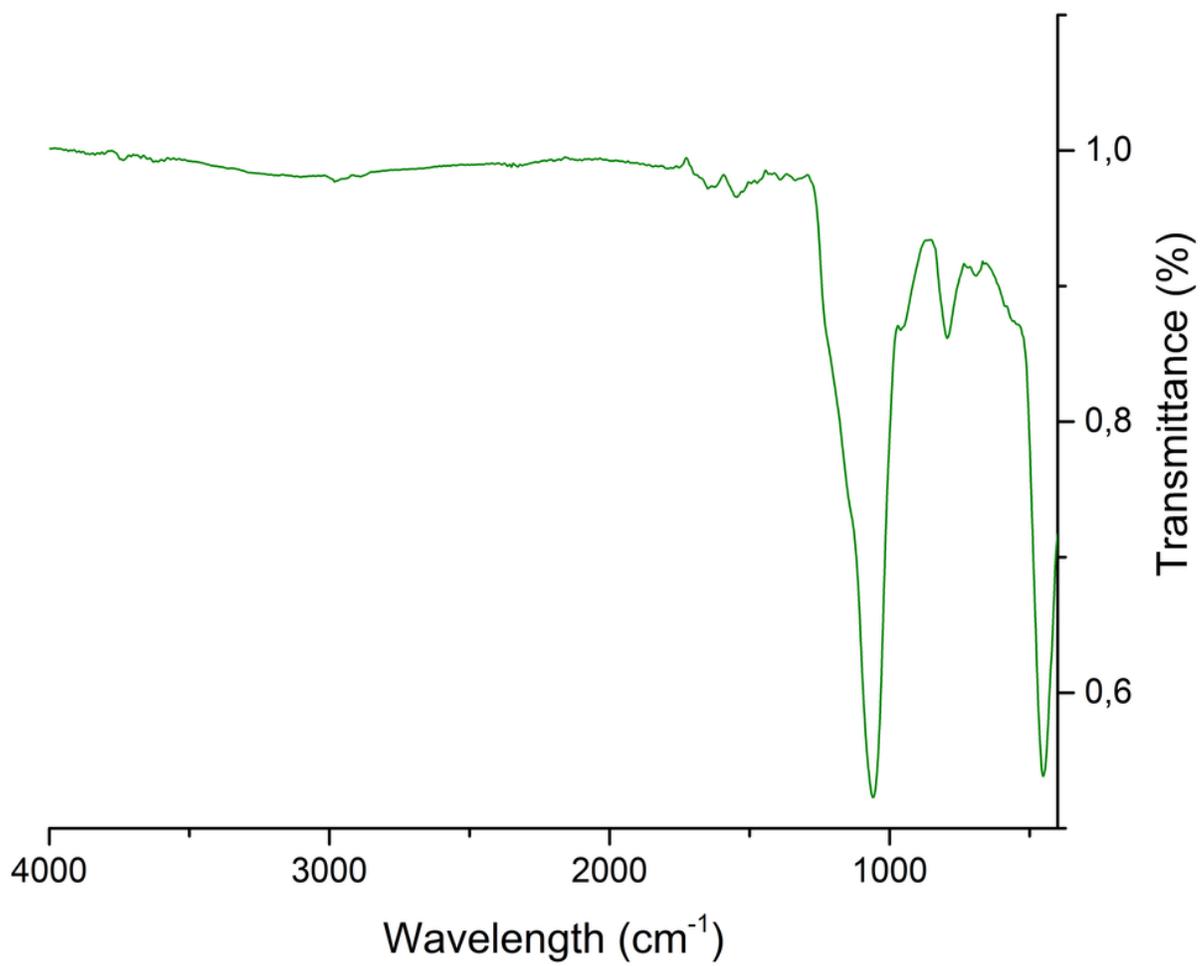


Figure 1

Fourier Transform Infrared (FTIR) spectrum of MSNS

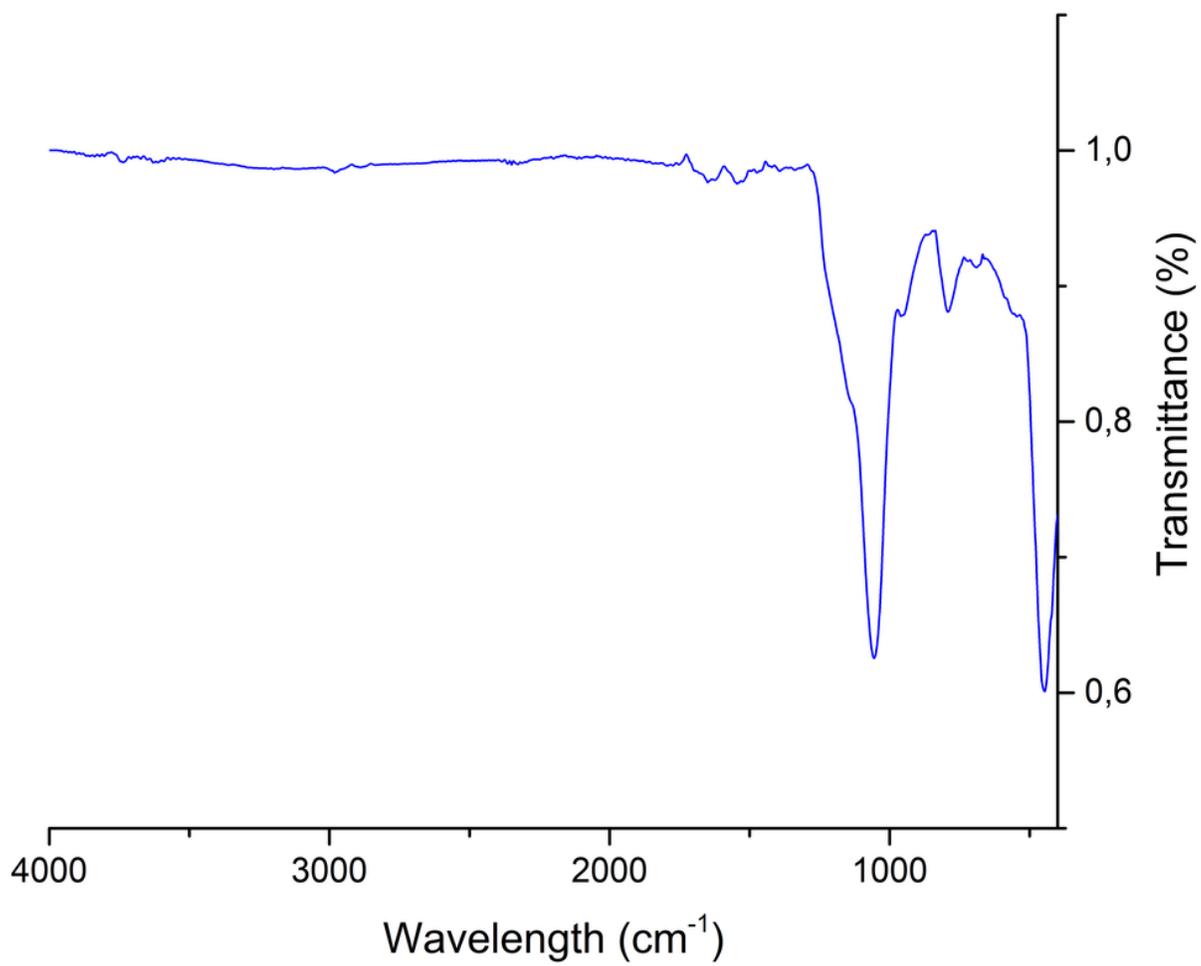


Figure 2

Fourier Transform Infrared (FTIR) spectrum of MCM-41

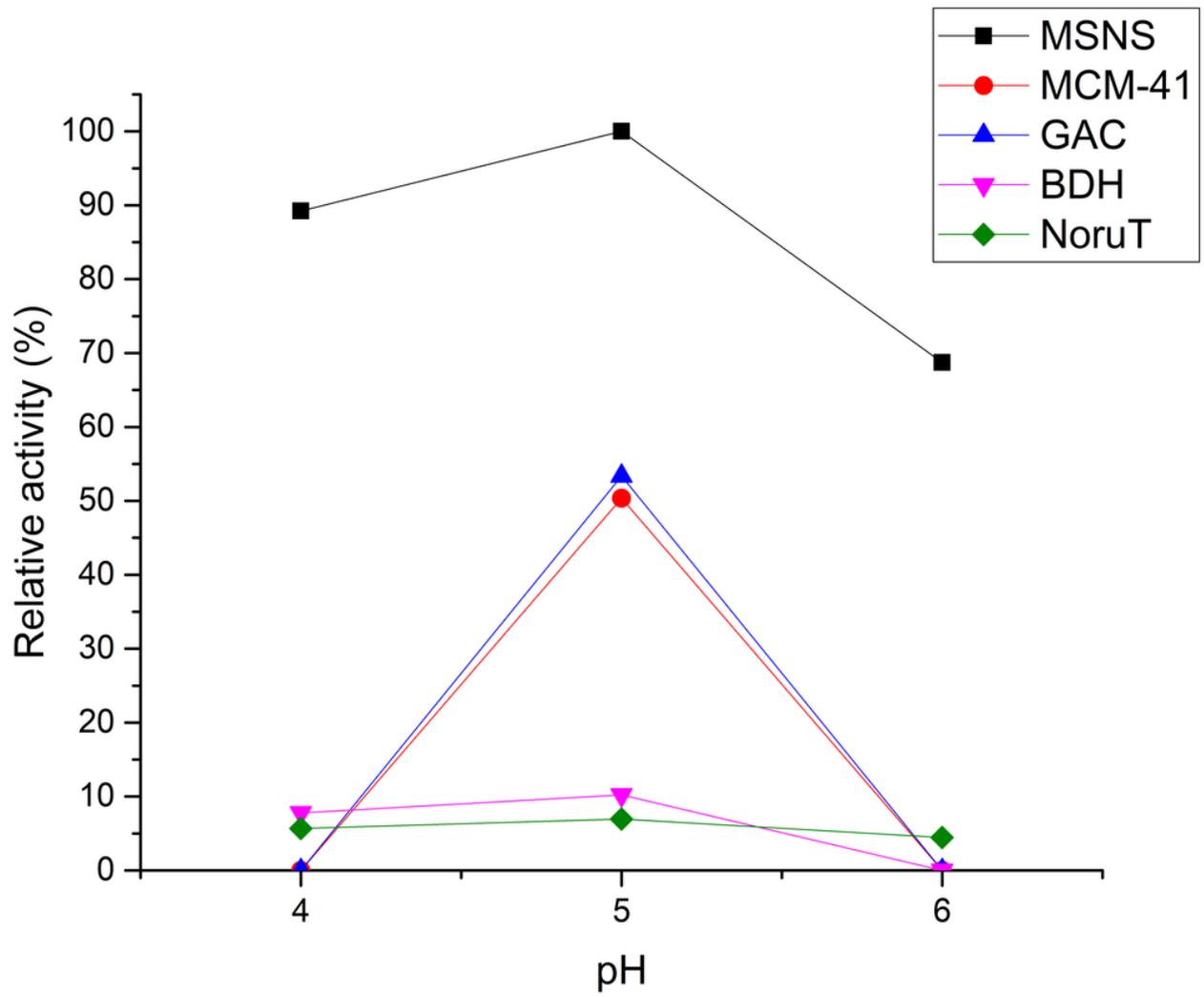


Figure 3

Relative activity of immobilized β -glucosidase on MSNS, MCM41, GAC, BDH and NORIT in different pH values

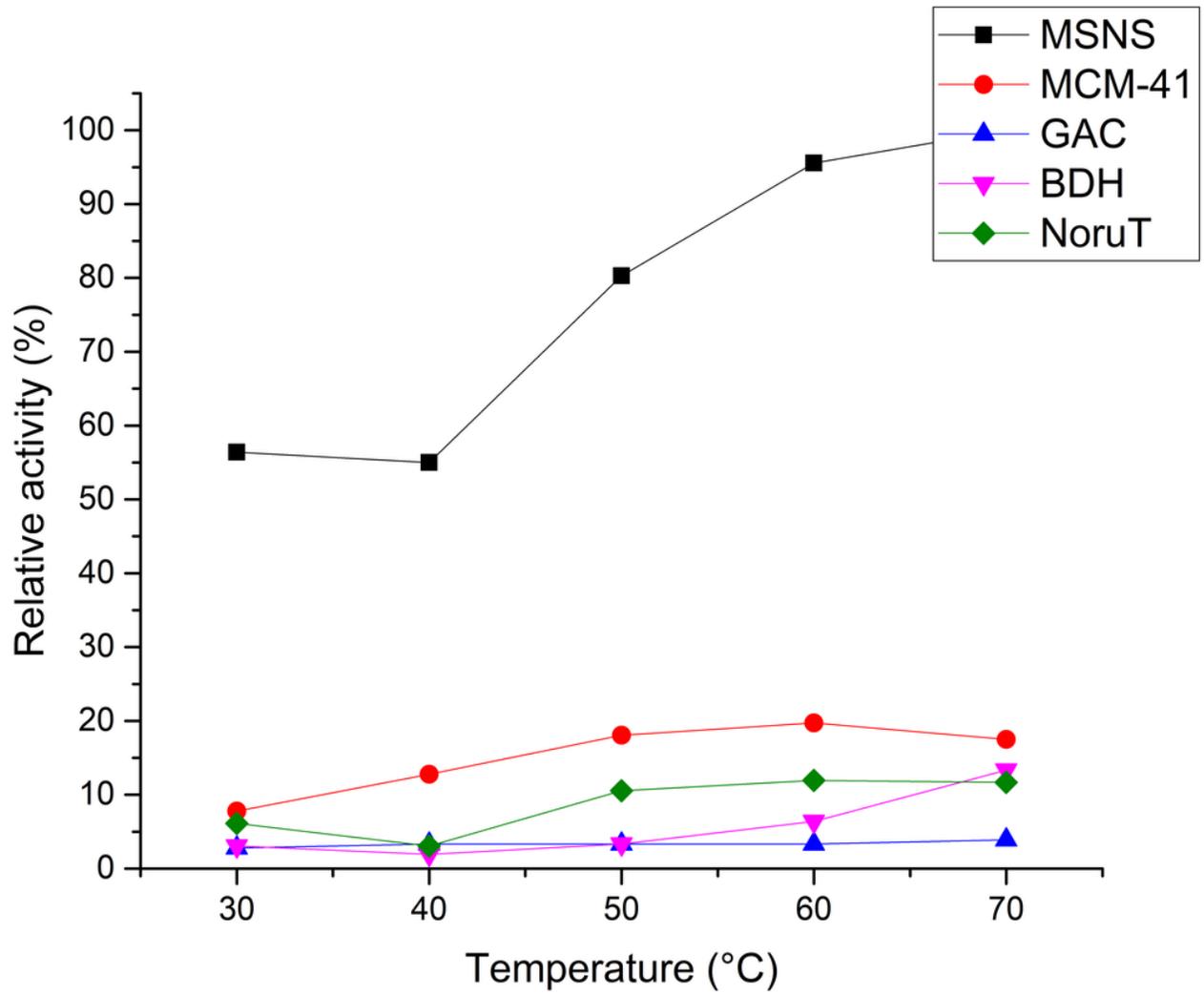


Figure 4

Relative activity of immobilized β -glucosidase on MSNS, MCM-41, GAC, BDH and NORIT in different temperatures

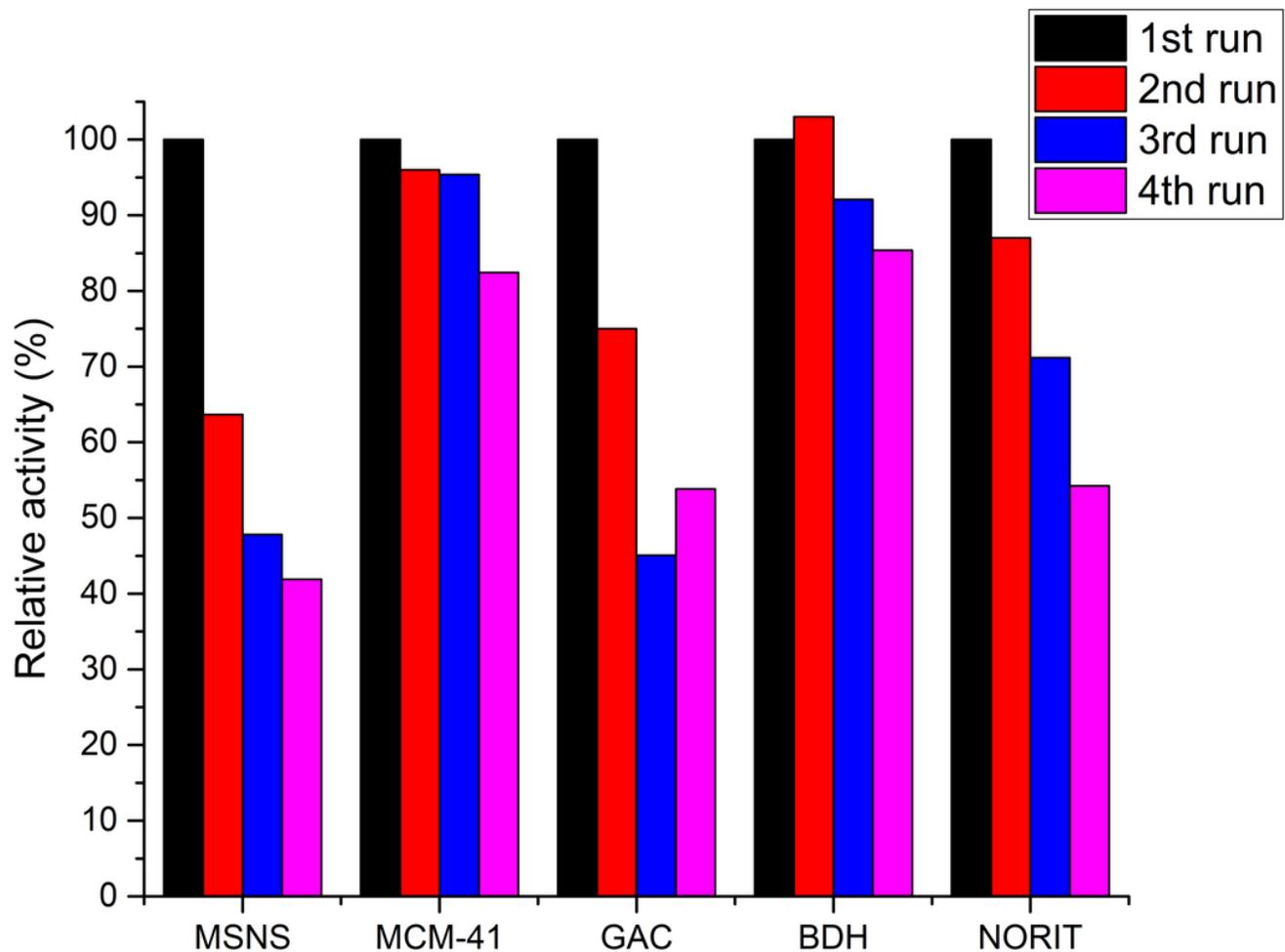


Figure 5

Recycling test of immobilized β -glucosidase in different supports, in their optimum pH and temperatures