

IMMOBILIZATION OF CELLULASES ON CHITOSAN: APPLICATION FOR SUGARCANE BAGASSE HYDROLYSIS

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In this work, a commercial cellulolytic cocktail was immobilized on glutaraldehyde activated chitosan gel. The chitosan concentration in the gel preparation, pH, immobilization time and enzymatic loading were evaluated. Immobilized cellulases showed better hydrolysis performance when an enzyme loading of 134 mg protein/g carrier was used for immobilization at pH 9.0 for 30 minutes. Hydrolysates with a glucose content of 13.43 and 10.35 g/L were obtained when Avicel and pretreated sugarcane bagasse were used as substrate, respectively. Immobilized cellulase lost 60% of its hydrolysis performance after 8 cycles using Avicel, and 75% after 6 cycles for sugarcane bagasse. The hydrolysis performance associated with the reuse of the immobilized cellulases indicates that an improvement in the immobilization of cellulases, coupled with an improvement in the pretreatment of lignocellulosic biomass, will allow the development of a continuous hydrolysis system with the enzyme retained in the reactor.

Keywords: organosolv pretreatment, enzyme reuse, enzyme immobilization, lignocellulosic biomass, chitosan

INTRODUCTION

The enzymatic hydrolysis of cellulose from pretreated biomass is an established process for the release of glucose, which can be converted into different chemical compounds, for example, biofuels, such as bioethanol, bio-butanol, biogas, biohydrogen and bio-methane,¹⁻⁷ and carboxylic acids,⁸⁻¹¹ as well as other valuable products, such as 5-hydroxymethylfurfural – HMF – and 5-methylfurfuryl alcohol – MFA.¹¹ The main enzymes used in this process are called cellulases (classified into endoglucanases, exoglucanases and beta-glucosidases), and their cost has a significant impact on the final cost of the product. In order to reduce the cost of the use of these cellulases, several enzyme immobilization strategies have been studied, which have led to increases in enzyme stability and its reuse.¹²⁻¹⁵

Enzyme immobilization consists in transforming a homogeneous biocatalyst into a heterogeneous one by binding the enzyme to a solid support. The most commonly used immobilization techniques for cellulase immobili-

zation include: adsorption, covalent attachment, entrapment and cross-linking.¹⁶⁻¹⁸ A wide variety of polymers, water insoluble polysaccharides, such as chitosan and alginate, and magnetic nanoparticles have been used as a support matrix for enzyme immobilization.¹⁹⁻²² This latter approach using nanomaterials has become the focus of recent research in this area due to the characteristics of these materials. However, the application of such nanomaterials is limited because of the effectiveness of their recovery process.²³

Because the cellulases act on solid substrates, the immobilization techniques to be used should avoid cellulase confinement techniques, for example, porous particulate immobilization, encapsulation, and crosslinking, since the enzymes immobilized within a matrix would not have access to the insoluble substrate.²⁴ Moreover, during the process of hydrolysis of the biomass, the enzymes can leach, compromising the recycling process. Therefore, the covalent

immobilization of cellulases on the surface of very small and non-porous particles seems to have more advantages.^{24,25} Other techniques, such as the use of ionic liquids (ILs) for saccharification mediated by cellulases, leading to the breakdown of cellulose crystallinity and a decrease in lignin content, thus improving cellulose solubility and cellulase accessibility due to increased hydrolysis surface area, and reducing the adsorption of non-productive enzymes, have been used for the degradation of lignocellulosic materials using this type of enzymes.^{26,27} However, most cellulases are partially or completely inactivated in the presence of ILs. In addition, the difficult separation of the sugars produced, the relatively high cost of the ionic liquids themselves, lack of knowledge in terms of process considerations for a biorefinery based on these solvents, are the main disadvantages present in the use of ILs.²⁸⁻³⁰

Another promising immobilization strategy involves the use of chitosan gel activated by glutaraldehyde, which has adequate characteristics for the immobilization of these enzymes, even in large-scale production processes and biotransformations on an industrial scale. One of the advantages of using glutaraldehyde is its availability in commercial quantities and its low cost.^{25,31} For this reason, the use of chitosan gel activated with glutaraldehyde has suitable characteristics to be used as a support for cellulase immobilization for the enzymatic hydrolysis of lignocellulosic materials.³²

In addition to the use of a strategy that allows conditions of repeated use of the enzyme, another important factor is the type of the enzyme cocktail that will be used during the full hydrolysis of the cellulose, because of the recalcitrance of the biomass. This makes the development of new enzyme cocktails a current challenge, considering their determinant role in an efficient degradation of a lignocellulosic material.³³

It is known that several factors influence the activity of a cellulase cocktail, among them, the type of substrate, mass relationship between the cellulases present in the cocktail, the reaction temperature, hydrolysis time, substrate concentrations and the final product. Therefore, the optimization of these variables will make it possible to produce more efficient cocktails, capable of hydrolysing lignocellulosic biomass in a profitable way at the industrial level.^{33,34}

The commercial enzyme cocktail used in this study has cellulolytic components in sufficient titers. This balance must be maintained after

immobilization to ensure maximum performance of the immobilized enzymes. Thus, this study utilized glutaraldehyde activated chitosan gel as the ideal support for cellulase immobilization. The main objective was to establish the optimal immobilization conditions to apply this derivative in pretreated sugarcane bagasse hydrolysis.

EXPERIMENTAL

Materials and methods

Cellic CTec 3 enzymatic cocktail, provided by Novozymes A/S, was used as a model enzymatic complex for the immobilization of cellulases. Medium molecular weight chitosan from Sigma Aldrich was used as support for immobilization, and the activating agent employed was 25% (v/v) glutaraldehyde (Vetec).

Whatman n° 1 filter paper, cellobiose, Avicel and carboxymethylcellulose (CMC) were used as substrates for measuring the hydrolytic activity of the enzymatic cocktail. To evaluate the performance of immobilized cellulases, the sugarcane bagasse previously treated by organosolv, provided by Embrapa Agroenergia, and microfibrillated Avicel, obtained by processing a 10% (m/v) solution of Avicel in a Turrax for 30 minutes at 15,000 rpm, were used as substrates.

Preparation of substrates for enzymatic hydrolysis

The sugarcane bagasse was treated using an ethanol-water (50% v/v) solvent system with a liquid-solid ratio of 6 gg⁻¹. The organosolv process was carried out in a batch reactor at 180 °C for 4 h. The solid fraction rich in cellulose obtained after the pretreatment was separated from the liquid fraction through filtration. Then, the solid material was exhaustively washed with ethanol solution (70% v/v), dried at 30 °C and stored at room temperature before being used as substrate in the enzymatic hydrolysis assays. The microfibrillated cellulose paste was prepared from Avicel and water. A mixture of Avicel and water in a solid:liquid ratio of 1:10 was kept under shaking in a Turrax at 1200 rpm for 1 h.³⁵

Lignocellulosic biomass composition

The composition of the sugarcane bagasse pretreated by the organosolv process was determined according to a protocol described by Sluiter³⁶ and Gouveia.³⁷

Analysis of the structural sugars was performed by high performance liquid chromatography, using an Aminex HPX-87H column (45 °C) and a mobile phase of 5 mM H₂SO₄ at a flow rate of 0.6 mL/min with an RID detector (40 °C).

Measurement of cellulase activity

The free enzymes activity was determined using the reaction conditions recommended by Ghose,³⁸ and Xiao.³⁹ The total activity of the cellulolytic cocktail

was measured using Whatman n°1 filter paper, while the activities of exoglucanase and endoglucanase were measured substituting the filter paper with 4% (m/v) Avicel or 2% (w/v) CMC, respectively, following the same procedure recommended by Xiao.³⁹ The total reducing sugars were quantified by means of the 3,5-dinitrosalicylic acid method.⁴⁰ The activity of beta-glycosidase (cellobiase, specifically) was carried out as described by Ghose.³⁸ The glucose released in the reaction was quantified using the Bioclin enzymatic glucose dosing kit (Brasil).

Immobilization by covalent attachment on chitosan

Chitosan gel activated with glutaraldehyde was prepared as previously described.⁴¹ The 1% (m/v) powdered chitosan was solubilized in 2% (v/v) acetic acid, and it was gently stirred for 40 minutes at 50 °C. Then, 20 mL of 10 mM KOH solution was added to form a chitosan gel, and this mixture was kept under stirring for another 30 minutes at 50 °C. To the chitosan gel suspension, 1% (v/v) glutaraldehyde was added, and this mixture was kept under stirring for another 20 minutes at 50 °C. After that, the activated chitosan gel was vacuum filtered and thoroughly washed with deionized water.

To the activated support, a solution of Cellic CTec 3, prepared in sodium citrate/citric acid buffer 0.1 M, pH 7, was added in the ratio of 1:10. The mixture was kept under stirring for 2 hours.

Immobilization performance

To determine the enzyme immobilization yield, the protein content in the supernatant of the immobilization solution was analyzed before and after contact with the activated support, according to Equation (1). Protein quantification was performed using bicinchoninic acid – BCA, according to the Thermo Scientific – Pierce® BCA Protein Assay kit manual, using bovine serum albumin as standard.

$$\text{Immobilization yield} = \frac{\text{Protein}_{\text{initial}} - \text{Protein}_{\text{final}}}{\text{Protein}_{\text{initial}}} \times 100 \quad (1)$$

Analysis of operational parameters for immobilization

Cellulase immobilization was performed following the methodology described above, varying the amount of chitosan used to prepare the support (0.5 to 2.0%), pH (7 to 10), time immobilization (10 to 120 minutes) and enzyme loading (40.2 to 1340 mg protein/g support). The experimental conditions are shown in Table 1.

Enzymatic hydrolysis and reuse

In order to study the performance of immobilized cellulase under different operating conditions, the enzymatic hydrolysis was performed in a 5 mL microtube, in medium containing 0.1M citric acid/sodium citrate buffer, pH 5.0, with a concentration of 2.5% (m/v) of pretreated sugarcane bagasse. The assays were run in triplicate using a shaker at 200 rpm, 50 °C for 48 hours. The catalyst concentration in the reaction medium was 1% m/v. To verify the possibility of reuse of the immobilized cellulase, at the end of each hydrolysis assay, the reaction medium was centrifuged at 10,000 rpm for 5 minutes to recover the solid (unhydrolyzed biomass + immobilized cellulase) and this was applied as a catalyst in a new hydrolysis process, under the same conditions as in the first hydrolysis cycle.

In order to study the reuse of the immobilized cellulase under optimized conditions, the enzymatic hydrolysis followed the same methodology described above, with the difference of being carried out in jacketed reactors with a final volume of 50 mL and a mass of catalyst used of 0.75% m/v. The hydrolysis was performed with both pretreated sugarcane bagasse and microfibrillated Avicel.

Table 1
Experimental conditions of chitosan and enzyme concentration for different time and pH

| Chitosan concentration (% m/v) | Time (min) | pH | Enzyme loading (mg protein/g support) |
|-------------------------------------|-----------------|--------------|--|
| 0.5; 1.0; 2.0 | 60 | 9 | 134 |
| Optimized chitosan concentration | 10; 30; 60; 120 | 7; 8; 9; 10 | 134 |
| Optimized chitosan concentration | Optimized time | Optimized pH | 40.2; 67; 134; 268; 536; 1340 |

The glucose released in the hydrolysis assays was determined using a HPLC instrument, equipped with an Aminex HPX-87H column and a refractive index detector (RID). The mobile phase was 5 mM H₂SO₄ at a flow rate of 0.6 mL/min. The column oven was maintained at 45 °C.

RESULTS AND DISCUSSION

Sugarcane bagasse pretreated by the organosolv process (SBPO) was used as model biomass in this study to evaluate the performance of immobilized cellulases in cellulose hydrolysis. The SBPO used was composed of cellulose

(59.6%), hemicelluloses (16.3%) and lignin (18.6%).

The most desirable characteristics for immobilized enzymes are that these enzymes exhibit high activity and stability, thus enabling their reuse. Data previously reported in the scientific literature indicate that immobilized cellulases can release up to 38.8 g/L of reducing sugars when pretreated lignocellulosic biomass is used as a substrate,²¹ and that these can be reused up to 5 cycles maintaining 50% of their initial hydrolysis performance in the case of steam-exploded straw.⁴²

Liang *et al.*⁴³ and Yu *et al.*⁴⁴ also performed 5 recycles maintaining the performance at 83.1% and 52% relative to the initial hydrolysis of corn straw and cotton yarn, respectively, while Jiang *et al.*⁴⁵ reused for up to 10 cycles maintaining 70% of initial corn stalk hydrolysis performance.

The enzymatic cocktail used in this study was Cellic Ctec 3, which contains protein at a concentration of 268 g/L, and has the following activities: FPase (223.4 FPU/mL), Carboxymethylcellulase (1155 IU/mL), Avicelase (335.4 IU/mL) and Cellobiase (6482 IU/mL). In order to achieve high yields of cellulose hydrolysis, it is necessary, for both soluble and

immobilized enzymes, that all types of enzyme (endoglucanases, betaglycosidases and exoglucanases, mainly) are present during the hydrolysis, since the absence of one of these enzymes prevents the complete conversion of cellulose to glucose.⁴⁶

Therefore, the method chosen for the immobilization of cellulases shall not be selective; it should allow the immobilization of all types of enzymes present in the cocktail.

It is noteworthy that before initiating the immobilization tests, the enzymatic extract model (Cellic Ctec 3) was evaluated for stability against pH variation. No significant loss of biomass hydrolysis performance was observed after incubation of the enzyme extract for 48 h at room temperature, and pH values ranging from 5 to 10, as can be seen in Figure 1.

Aiming at the immobilization of cellulases, which consist of a mixture of enzymes of different size and chemical structure, glutaraldehyde was used as the activating agent of the support. This activating agent can react with different functional groups of proteins and bind them covalently to the amino groups on the surface of the immobilization support.⁴⁷

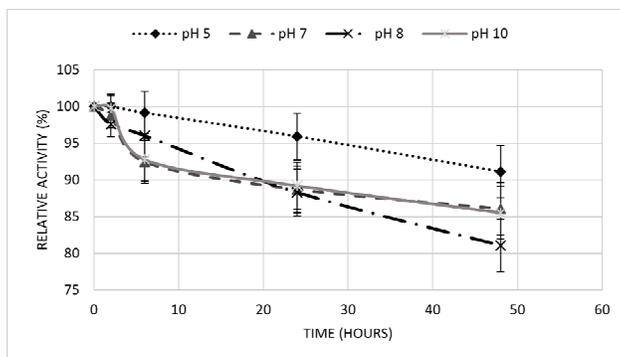


Figure 1: Enzymatic stability at 28 °C for different incubation pH values

The content of glutaraldehyde plays an important role in the overall recovery of activity. According to Qi *et al.*⁴⁸ better immobilized activities are obtained when 1% (v/v) of glutaraldehyde is used, while lower values cause a lower availability of glutaraldehyde for covalent attachment and higher values than these significantly decrease activity. The negative effect of high glutaraldehyde concentrations can be attributed to the fact that glutaraldehyde causes protein denaturation due to conformational changes. In addition, excess crosslinkages

between the enzyme molecules result in increased stiffness of the enzyme, negatively affecting its catalytic activity. Due to these facts, the glutaraldehyde concentration was maintained at 1% (v/v) in all experiments.

The chitosan mass used for the preparation of the support, the enzymatic loading, time and pH of immobilization are important parameters in the optimization of the immobilization methodology for finding better cost benefits and possible industrial application.

Adjustment of chitosan concentration

A significant variable for the effective immobilization of cellulases is the size of the carrier particle. It seems unlikely that cellulases immobilized on insoluble particles are capable of acting on insoluble substrates, however, it is known that large cellulolytic complexes (cellulosomes) bound to the cell wall, such as *Clostridium thermocellum*, are able to catalyze the hydrolysis of crystalline cellulose.⁴⁹ Knowing that the *C. thermocellum* cell is approximately 0.3-0.4 μm wide by 2-4 μm long,⁵⁰ it is expected that any such particle is also effective for hydrolysis of cellulose. In this work, solutions with different concentrations of chitosan were prepared, aiming to obtain particles with different sizes for the immobilization of cellulases.^{51,52}

The biomass hydrolysis performance, using the chitosan gel immobilized cellulases with different particle sizes, was investigated and the results are shown in Figure 2. The immobilization yield and hydrolysis performance are dramatically reduced when the chitosan concentration during the preparation of the gel is increased from 0.5 to 2.0% m/v. The increase in particle size hinders

the access of the enzyme and its action during the hydrolysis of cellulose. The existence of particles with dimensions similar to that of a *C. thermocellum* cell is one indication that cellulases are acting on the hydrolysis of cellulose in the immobilized and non-soluble form.

pH adjustment and immobilization time

The quantity of active enzymes bound to the surface of the support after immobilization depends on the operating conditions of this process.^{53,54} Mild conditions of temperature and agitation were used in this study in order to avoid loss of enzymatic activity, while pH and immobilization time were evaluated, aiming to increase the immobilization efficiency and the performance of immobilized enzymes in the hydrolysis of pretreated biomass. The immobilization yield at pH 7 and 8 was slightly higher, when compared to the immobilization performed at pH values 9 and 10 (Fig. 3A). On the other hand, enzymes immobilized at pH 9 presented higher operational stability, when compared to enzymes immobilized at pH values 7, 8 and 10.

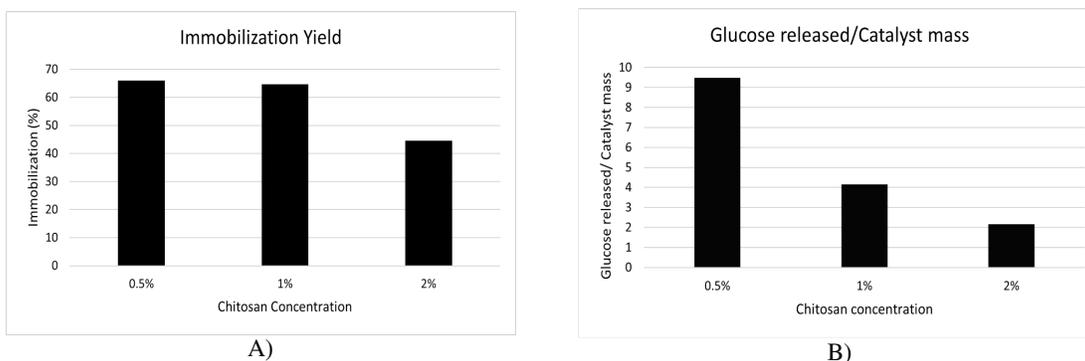


Figure 2: A) Immobilization yield and B) Glucose released by mass of catalyst used in the hydrolysis for different percentages of chitosan in the formation of the support

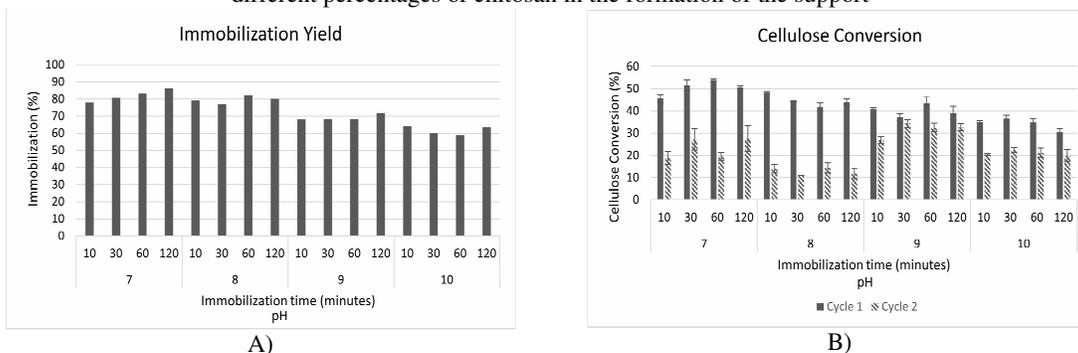


Figure 3: A) Immobilization yield and B) Percentage of cellulase conversion at different pH and immobilization time

This is evidenced by the lower loss of hydrolysis performance presented by enzymes immobilized at pH 9, when comparing the second and the first hydrolysis cycles (orange bar, Fig. 3B).

Probably, during immobilization at pH 7 and 8, most of the enzymes were only adsorbed, or reversibly bound to the support⁵⁵ and, as a result, these enzymes were removed from the reaction medium after the first cycle of hydrolysis, while at pH 9 most of the enzymes were covalently bound to the support. It was expected that the immobilization procedure performed at pH 10 would result in higher immobilization yield and higher hydrolysis performance, compared to those at the other pH values, since the cellulases used are stable at pH 10, however this was not observed. It is possible that an irreversible immobilization of enzymes at pH 10 occurred very quickly on the surface of the chitosan gel, preventing better distribution of the enzymes throughout the surface of the support. It is desired that the immobilization process be slow enough, so that the enzymes are distributed and accommodated on the surface of the support in order to maintain the catalytic activity, avoiding problems of steric hindrance.⁵⁶

Regarding the immobilization time at pH 9, it was observed that 30 minutes is sufficient time to reach the maximum enzyme binding yield and stabilization, since significant gains were not observed after this interval. In spite of a slightly higher immobilization yield in 10 minutes, it is likely that some of these enzymes were only adsorbed or single-bonded (only one bond between the same enzyme and the support), since

there was a strong reduction in hydrolysis performance in the second cycle in which this catalyst was used. Apparently, after 30 minutes, possible multipoint bonds (two or more bonds between the same enzyme and the support) had already occurred, and the catalyst maintained practically the same performance in the second hydrolysis cycle.

Enzyme load adjustment

After adjustment of the variables, such as chitosan concentration, pH and immobilization time, the enzymatic loading was also established, so that the catalyst reached its maximum hydrolysis performance. In the case of immobilization of cellulases, a mixture with at least 17 different enzymes, with molecular weights varying from 10 to 800 kDa, each one of these enzymes having different types and amounts of functional groups on its surface, it is essential to establish the maximum protein load permissible value, so that the greatest possible diversity of enzymes is immobilized, since the absence of one of the enzyme types may prevent cellulose hydrolysis. In this way, the excess of protein offered for the immobilization could induce a selective immobilization, generally favoring the immobilization of proteins with low molecular mass. In this study, the protein load investigated for immobilization ranged from 40.2 to 1340 mg protein/g support. Despite reaching the maximum immobilization yield for 40.2 mg protein/g support (80%), the maximum hydrolysis performance was only reached when the protein load was increased to 134 mg protein/g support (Fig. 4).

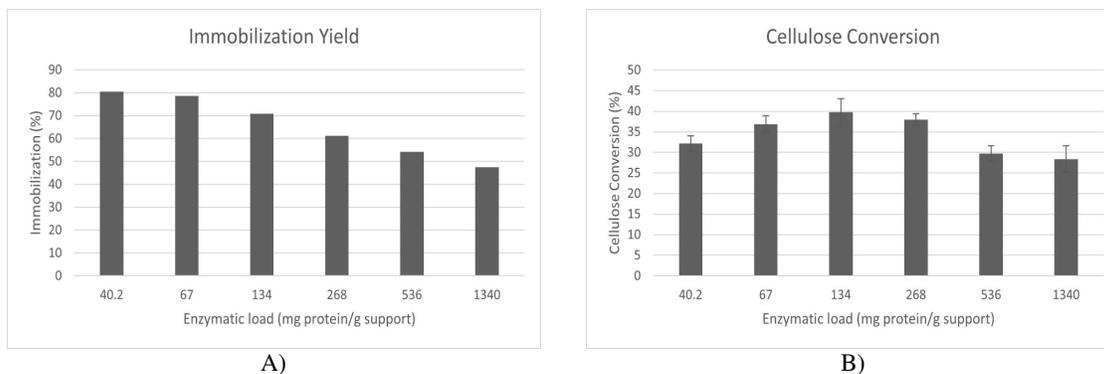


Figure 4: A) Immobilization yield and B) Percentage conversion of cellulase at different enzymatic charges

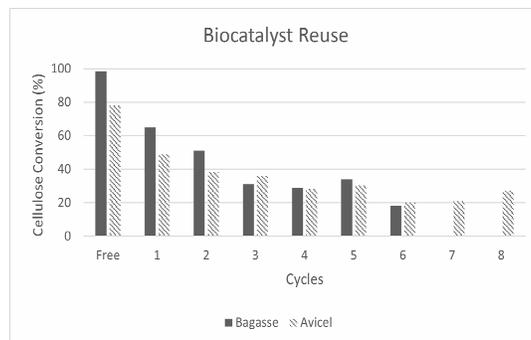


Figure 5: Percentage conversion of cellulose during hydrolysis with enzyme in its free form and recycled immobilized enzyme

When very low protein loads are offered, the hydrolysis performance is impaired, probably because some of the enzymes undergo inactivation, rendering the cellulolytic cocktail unbalanced. On the other hand, the excess may lead to preferential immobilization, also harming cellulose hydrolysis. For the conditions established in this study, the optimum load for cellulase immobilization is 134 mg protein/g support.

Reuse

After adjustment of the main immobilization parameters, the catalyst obtained was evaluated for its reuse potential. In addition to the pretreated sugarcane bagasse, the immobilized enzymes were tested on microfibrillated Avicel. The latter was used to avoid the accumulation of solid during the hydrolysis cycles, a phenomenon that is observed when pretreated sugarcane bagasse is used, because of the presence of approximately 20% of lignin in its composition, a component that is not hydrolyzed and remains insoluble after hydrolysis of cellulose and hemicelluloses. The remaining solid from the hydrolysis cannot be separated from the catalyst (also solid) because of their similar physical characteristics, and it is difficult to differentiate the particles. The accumulation of this solid throughout the hydrolysis cycles hinders the homogenization of the reaction medium and consequently interferes with the performance of the catalyst, masking its operational stability and leading to low cellulose conversions.

When pretreated sugarcane bagasse was used, only 6 cycles of hydrolysis were possible, each cycle of hydrolysis lasting 48 h, totaling 288 h of enzyme use. After this time interval, approximately 75% of the initial hydrolysis performance had been lost (blue bars in Fig. 5).

When microfibrillated Avicel was used, 8 cycles of hydrolysis were performed, totaling 384 h of enzyme use. In this case, there was a 60% loss of the initial hydrolysis performance up to the sixth cycle, and in the following cycles the hydrolysis performance was maintained (Fig. 5), indicating that there is a subpopulation of highly stable enzymes that would probably remain stable for several cycles.

The results obtained in this study are unprecedented, considering the number of long cycles of hydrolysis in which immobilized cellulases could be reused when a solid substrate was used. Similar results were obtained by Jiang *et al.*,⁴⁵ when cellulase immobilized on magnetite carboxymethylchitosan alginate/calcium alginate – cellulase bioconjugate (MCCCB) was reused 10 times in 24 h cycles, in the hydrolysis of corn stalk, while maintaining the performance of 70%, totaling 240 hours of enzyme use, which is lower than that obtained in this study.

The results obtained in this work generate some insights in the search for viability in the use of immobilized cellulases. Firstly, a subpopulation of highly stable enzymes that could be reused several times has been found. Considering the improvement in the immobilization process to increase the amount of the highly stabilized enzymes, it would be possible to apply them in the hydrolysis process, together with a small replacement of soluble enzymes, thus reducing the cost of applying these enzymes. Another challenge would be the co-immobilization of other enzymes, in addition to the cellulases, so that there would be complete liquefaction of the pretreated biomass after the enzymatic hydrolysis. This would make possible the reuse of the catalyst, since there would be no solid accumulation in the medium. Secondly, it has been proved by the possibility of reuse and by

the size of chitosan particles that cellulases can act in the immobilized form. To increase the hydrolysis efficiency of immobilized cellulases on chitosan particles, it is still necessary to prepare particles with a more uniform size distribution and preferably less than 10 µm, since the catalyst must have the particle size large enough to be recovered by centrifugation or filtration and small enough in order to avoid mass transfer limitations.

CONCLUSION

The immobilized cellulases can catalyze the hydrolysis of cellulose, as long as they are attached to sufficiently small particles, with dimensions similar to that of a bacterial cell, for example. The immobilization of cellulases in glutaraldehyde activated chitosan gel allows obtaining a catalyst containing a subpopulation of highly stable cellulolytic enzymes with potential for use in several cycles.

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REFERENCES

- ¹ R. R. Singhania, in “Biofuels”, edited by A. Pandey *et al.*, Academic Press, 2011, p. 177-201, <https://doi.org/10.1016/B978-0-12-385099-7.00008-5>
- ² M. Prussi, M. Padella, M. Conton, E. D. Postma and L. Lonza, *J. Clean. Prod.*, **222**, 565 (2019), <https://doi.org/10.1016/j.jclepro.2019.02.271>
- ³ N. M. D. Huzir, M. D. M. A. Aziz, S. B. Ismail, B. Abdullah, N. A. N. Mahmood *et al.*, *Renew. Sustain. Energ. Rev.*, **94**, 476 (2018), <https://doi.org/10.1016/j.rser.2018.06.036>
- ⁴ R. Lukajtis, I. Holowacz, K. Kucharska, M. Glinka, P. Rybarczyk *et al.*, *Renew. Sustain. Energ. Rev.*, **91**, 665 (2018), <https://doi.org/10.1016/j.rser.2018.04.043>
- ⁵ S. Rezanian, M. F. M. Din, S. M. Taib, J. Sohaili, S. Chelliapan *et al.*, *Int. J. Hydrog. Energ.*, **42**, 20955 (2017), <https://doi.org/10.1016/j.ijhydene.2017.07.007>
- ⁶ S. Rezanian, B. Oryani, J. Cho, A. Talaiekhozani, F. Sabbagh *et al.*, *Energy*, **199**, 1174572 (2020), <https://doi.org/10.1016/j.energy.2020.117457>
- ⁷ C. L. Gomes, E. Gonçalves, C. A. G. Suarez, D. S. Rodrigues and I. C. Montano, *Cellulose Chem. Technol.*, **55**, 101 (2021), <https://doi.org/10.35812/CelluloseChemTechnol.2021.55.10>

- ⁸ A. Onda, T. Ochi, K. Kajiyoshi and K. Yanagisawa, *Appl. Catal. A: Gen.*, **343**, 49 (2008), <https://doi.org/10.1016/j.apcata.2008.03.017>
- ⁹ J. Albert, R. Wolfel, A. Bosmann and P. Wasserscheid, *Energ. Environ. Sci.*, **5**, 7956 (2012), <https://doi.org/10.1039/C2EE21428H>
- ¹⁰ A. T. Quitain, M. Faisal, K. Kang and H. Daimon, *J. Hazard. Mater.*, **99**, 6207 (2002), [https://doi.org/10.1016/S0304-3894\(02\)00024-9](https://doi.org/10.1016/S0304-3894(02)00024-9)
- ¹¹ A. Yopez, A. Pineda, A. Garcia, A. A. Romero and R. Luque, *Phys. Chem. Chem. Phys.*, **15**, 12165 (2013), <https://doi.org/10.1039/C3CP50707F>
- ¹² X. Han, Y. Guo, X. Liu, Q. Xia and Y. Wang, *Catal. Today*, **319**, 2 (2019), <https://doi.org/10.1016/j.cattod.2018.05.013>
- ¹³ N. Srivastava, M. Srivastava, P. K. Mishra, V. K. Gupta, G. Molina *et al.*, *Renew. Sustain. Energ. Rev.*, **82**, 2379 (2018), <https://doi.org/10.1016/j.rser.2017.08.074>
- ¹⁴ R. Ahmad and S. K. Khare, *Bioresour. Technol.*, **252**, 72 (2018), <https://doi.org/10.1016/j.biortech.2017.12.082>
- ¹⁵ I. N. Ahmed, X. L. Yang, A. A. Dubale, R. F. Li, Y. M. Ma *et al.*, *Bioresour. Technol.*, **270**, 377 (2018), <https://doi.org/10.1016/j.biortech.2018.09.077>
- ¹⁶ A. Sassolas, L. J. Blum and B. D. Leca-Bouvier, *Biotechnol. Adv.*, **30**, 489 (2012), <https://doi.org/10.1016/j.biotechadv.2011.09.003>
- ¹⁷ S. Datta, L. R. Christena and Y. R. Rajaram, *3 Biotech*, **3**, 1 (2013), <https://doi.org/10.1007/s13205-012-0071-7>
- ¹⁸ C. Mateo, J. M. Palomo, G. Fernandez-Lorente, J. M. Guisan and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, **40**, 1451 (2007), <https://doi.org/10.1016/j.enzmictec.2007.01.018>
- ¹⁹ V. L. Sirisha, A. Jain and A. Jain, *Adv. Food Nutr. Res.*, **79**, 179 (2016), <https://doi.org/10.1016/bs.afnr.2016.07.004>
- ²⁰ R. Ahirwar, J. G. Sharma, P. Nahar and S. Kumar, *Biocatal. Agric. Biotechnol.*, **11**, 248 (2017), <https://doi.org/10.1016/j.bcab.2017.07.014>
- ²¹ K. Saha, P. Verma, J. Sikder, S. Chakraborty and S. Curcio, *Renew. Energ.*, **133**, 66 (2019), <https://doi.org/10.1016/j.renene.2018.10.014>
- ²² L. Zang, J. Qiu, X. Wu, W. Zhang, E. Sakai *et al.*, *Ind. Eng. Chem. Res.*, **53**, 3448 (2014), <https://doi.org/10.1021/ie404072s>
- ²³ K. Khoshnevisan, E. Poorakbar, H. Baharifar and M. Barkhi, *Magnetochemistry*, **5**, 36 (2019), <https://doi.org/10.3390/magnetochemistry5020036>
- ²⁴ Y. Wang, D. Chen, G. Wang, C. Zhao, Y. Ma *et al.*, *Chem. Eng. J.*, **336**, 152 (2018), <https://doi.org/10.1016/j.cej.2017.11.030>
- ²⁵ G. H. Podrepsek, M. Primozi, Z. Knez and M. Habulin, *Chem. Eng. Trans.*, **27**, 235 (2012)
- ²⁶ Y. X. An, M. H. Zong, S. Q. Hu and N. Li, *Chem. Eng. Sci.*, **161**, 48 (2017), <https://doi.org/10.1016/j.ces.2016.12.014>

- ²⁷ G. Papa, T. Feldman, K. L. Sale, F. Adani, S. Singh *et al.*, *Bioresour. Technol.*, **241**, 627 (2017), <https://doi.org/10.1016/j.biortech.2017.05.167>
- ²⁸ D. Feng, L. Li, F. Yang, W. Tan, G. Zhao *et al.*, *Appl. Microbiol. Biotechnol.*, **91**, 399 (2011), <https://doi.org/10.1007/s00253-011-3263-x>
- ²⁹ D. Hu, L. Xiao, L. Li, C. Zhong, X. Ju *et al.*, *ACS Sustain. Chem. Eng.*, **4**, 4922 (2016), <https://doi.org/10.1021/acssuschemeng.6b01229>
- ³⁰ D. Klein-Marcuschamer, B. A. Simmons and H. W. Blanch, *Biofuel. Bioprod. Biorefin.*, **5**, 562 (2011), <https://doi.org/10.1002/bbb.303>
- ³¹ S. Romo-Sanchez, C. Camacho, H. L. Ramirez and M. Arevalo-Villena, *Adv. Biosci. Biotechnol.*, **5**, 517 (2014), <https://doi.org/10.4236/abb.2014.56062>
- ³² M. L. Verma, S. Kumar, A. Das, J. S. Randhawa and M. Chamundeeswari, in "Sustainable Agriculture Reviews", edited by G. Crini and E. Lichtfouse, vol. 35, Springer, 2019, pp. 147-173, https://doi.org/10.1007/978-3-030-16538-3_4
- ³³ F. Contreras, S. Pramanik, A. M. Rozhkova, I. N. Zorov, O. Korotkova *et al.*, *Int. J. Mol. Sci.*, **21**, 1 (2020), <https://doi.org/10.3390/ijms21051589>
- ³⁴ S. A. Teter, K. B. Sutton and B. Emme, in "Advances in Biorefineries", edited by K. W. Waldron, Woodhead Publishing, Cambridge, 2014, pp. 199-233
- ³⁵ L. Mesa, E. González, C. Cara, M. González, E. Castro *et al.*, *Chem. Eng. J.*, **168**, 1157 (2011), <https://doi.org/10.1016/j.cej.2011.02.003>
- ³⁶ A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter *et al.*, *Laboratory Analytical Procedure (LAP)*, Colorado, July 8, 2011, pp. 1-15
- ³⁷ E. R. Gouveia, R. T. Nascimento and A. M. Souto-Maior, *Quim. Nova*, **32**, 1500 (2009), <https://doi.org/10.1590/S0100-40422009000600026>
- ³⁸ T. K. Ghose, *Pure Appl. Chem.*, **59**, 257 (1987), <http://dx.doi.org/10.1351/pac198759020257>
- ³⁹ Z. Xiao, R. Storms and A. Tsang, *Biotechnol. Bioeng.*, **88**, 832 (2004), <https://doi.org/10.1002/bit.20286>
- ⁴⁰ G. L. Miller, *Anal. Chem.*, **31**, 426 (1959), <https://doi.org/10.1021/ac60147a030>
- ⁴¹ S. Budriene, N. Gorochovceva, T. Romaskevicius, L. V. Yugova, A. Miezeliene *et al.*, *Cent. Eur. J. Chem.*, **3**, 95 (2005), <https://doi.org/10.2478/BF02476241>
- ⁴² Y. U. Zhang, J. Xu, D. Li and Z. Yuan, *Biocatal. Biotransfor.*, **28**, 313 (2010), <https://doi.org/10.3109/10242422.2010.516391>
- ⁴³ W. Liang and X. Cao, *Bioresour. Technol.*, **116**, 140 (2012), <https://doi.org/10.1016/j.biortech.2012.03.082>
- ⁴⁴ Y. Yu, J. Yuan, Q. Wang, X. Fan, P. Wang *et al.*, *Eng. Life Sci.*, **13**, 194 (2013), <https://doi.org/10.1002/elsc.201200086>
- ⁴⁵ J. Jiang, J. Zhao, C. He, B. Cui, J. Xiong *et al.*, *Carbohydr. Polym.*, **166**, 358 (2017), <https://doi.org/10.1016/j.carbpol.2017.03.003>
- ⁴⁶ M. Kostylev and D. Wilson, *Biofuels*, **3**, 61 (2012), <https://doi.org/10.4155/bfs.11.150>
- ⁴⁷ A. A. Mendes, P. C. D. Oliveira, H. F. D. Castro and R. D. L. C. Giordano, *Quim. Nova*, **34**, 831 (2011), <http://dx.doi.org/10.1590/S0100-40422011000500019>
- ⁴⁸ B. Qi, J. Luo and Y. Wan, *Bioresour. Technol.*, **268**, 577 (2018), <https://doi.org/10.1016/j.biortech.2018.07.115>
- ⁴⁹ E. A. Ximenes and C. R. Felix, *Univ. Ciências da Saúde*, **1**, 335 (2003), <https://doi.org/10.5102/ucs.v1i2.516>
- ⁵⁰ K. Sato, M. Tomita, S. Yonemura, S. Goto, K. Sekine *et al.*, *Biosci. Biotechnol. Biochem.*, **57**, 2116 (1993), <https://doi.org/10.1271/bbb.57.2116>
- ⁵¹ E. C. Rodrigues, B. T. C. Bezerra, B. V. Farias, W. S. Adriano, R. S. Vieira *et al.*, *Adsorpt. Sci. Technol.*, **31**, 17 (2013), <https://doi.org/10.1260/0263-6174.31.1.17>
- ⁵² D. G. Borges, J. A. Baraldo, C. S. Farinas, R. L. C. Giordano and P. W. Tardioli, *Bioresour. Technol.*, **167**, 206 (2014), <https://doi.org/10.1016/j.biortech.2014.06.021>
- ⁵³ L. Poon, L. D. Wilson and J. V. Headley, *Carbohydr. Polym.*, **109**, 92 (2014), <https://doi.org/10.1016/j.carbpol.2014.02.086>
- ⁵⁴ O. Barbosa, C. Ortiz, Á. Berenguer-Murcia, R. Torres, R. C. Rodrigues *et al.*, *RSC Adv.*, **4**, 1583 (2014), <https://doi.org/10.1002/chin.201421290>
- ⁵⁵ I. Migneault, C. Dartiguenave, M. J. Bertrand and K. C. Waldron, *Biotechniques*, **37**, 790 (2004), <https://doi.org/10.2144/04375RV01>
- ⁵⁶ Y. F. Fang, X. J. Huang, P. C. Chen and Z. K. Xu, *BMB Rep.*, **44**, 87 (2011), <https://doi.org/10.5483/BMBRep.2011.44.2.87>