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CELLULASE IMMOBILIZATION ON POLY(METHYL METHACRYLATE) NANOPARTICLES BY MINIEMULSION POLYMERIZATION

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Abstract - Cellulases are efficient enzymes for the conversion of cellulose into glucose. Their use in immobilized form enables them to be reused in successive cycles in many biotechnological processes. Unlike conventional methods of immobilization by covalent bonding, in miniemulsion polymerization the immobilization of enzyme and the synthesis of polymer nanoparticles (support) occur simultaneously. Based on these aspects, the immobilization of cellulose on poly(methyl methacrylate) (PMMA) nanoparticles by miniemulsion polymerization was studied. The surfactant type (non-ionic and ionic) and latex pH showed great influence on cellulase activity. High activity values were obtained only when non-ionic surfactant (Lutensol AT50) and buffering agent ($NaHCO_3$) were used simultaneously. MMA polymerization rate and final monomer conversion were not affected by the presence of cellulase. The maximum immobilization efficiency (60%) was obtained when 6 wt.% of cellulase was used and stable PMMA nanoparticles (133 nm) were obtained. The relative activity profile of immobilized cellulase, for pH as well as temperature, was similar to that reported for the free form. Immobilized enzyme keeps its activity throughout seven days when stored at 4 ºC and phosphate buffer pH 6.0. Based on the results obtained in this work, miniemulsion polymerization as a method for cellulase immobilization on PMMA nanoparticles showed to be a promising technique with high possibility of industrial application.

Keywords: Cellulase, Immobilization, Polymeric nanoparticles, Miniemulsion polymerization.

INTRODUCTION

Due to their biotechnological potential, cellulases contribute to the improvement of several processes, including food, textile, paper and cellulose industries, agriculture and, more recently, second-generation ethanol production. For this reason, these enzymes have been studied by several research groups both in the academic and industrial scope (Kuhad and Singh, 2011). Although the use of cellulase is widespread in several areas, its use in free form, as well as other enzymes, has some unfavorable aspects such as low stability in solution, high isolation and purification costs, and especially the difficulty of recovery from

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the reaction medium for subsequent reuse. As a result, the industrial application of cellulases is still a costly process (Daoud et al., 2010; Sheldon and Van Pelt, 2013). By biocatalyst immobilization, many of these drawbacks can be overcome, reflecting in economic benefits.

Nanoscale supports are very attractive for immobilization of enzymes since they can improve significantly biocatalyst efficiency. The reduced size provides a larger surface area for immobilization, enabling an increased amount of enzyme per particles and reducing the diffusion boundaries (Ansari and Husain, 2012; Jia et al., 2003). The most common ways to link an enzyme to nanoparticles are through electrostatic adsorption, covalent attachment to surface modified nanoparticles, direct conjugation to the nanoparticle surface and conjugation using specific affinity of protein (Ahmad and Sardar, 2015; Sheldon and Van Pelt, 2013; Elnashar, 2010). These approaches generally involve at least two steps, one for obtaining the support and another for enzyme immobilization, which usually requires a lot of time. Thus, miniemulsion polymerization is emerging as a promising technique to immobilize enzymes on polymer nanoparticles in a single-step without the use of organic solvents. In other words, the synthesis of polymeric support and immobilization of enzyme occur concurrently, eliminating the extra steps for functionalization and immobilization.

Miniemulsion polymerization is defined as a relatively stable dispersion of oil droplets in water in a size range of 50-500 nm. The oil nanodroplets are obtained by application of high shear in a system containing oil, water, surfactant and costabilizer (Antonietti and Landfester, 2002; Landfester et al., 1999). The high stability of the polymer nanoparticles obtained is a result of the combined effects of surfactant and cosurfactant, which suppress coalescence and Ostwald ripening, respectively (Crespy and Landfester, 2010). Thus, the proper choice of surfactant and a stabilizer as well as their amounts are very important to obtain stable nanoparticles for long periods.

Cipolatti et al. (2014) reported efficient immobilization of lipase CalB on PEGylated nanoparticles of poly(urea-urethane) (PUU) during step growth miniemulsion polymerization. Immobilized enzyme showed higher enzymatic activity and higher thermal stability than free CalB enzyme. Valério et al. (2015) immobilized lipase CalB on well-defined PMMA core-shell nanoparticles during free radical miniemulsion polymerization. The authors showed that, by applying this technique it was possible to obtain 75% of immobilization yield. Immobilized enzyme had a higher operational stability than free enzyme.

Despite the aforementioned promising works, this approach still requires further studies, because the effects of reaction conditions on enzyme activity are not yet entirely elucidated (Cipolatti et al., 2014; Valério et al., 2015). For instance, due to the quite specific actuation of each enzyme, different enzymes may present distinct behaviors, as well as effects on miniemulsion polymerization. The few works found in the literature employ miniemulsion polymerization as a technique for immobilization of enzymes and they are restricted to immobilization of lipase (Cipolatti et al, 2014; Valério et al., 2015; Chiaradia et al., 2016). Thus, aiming to contribute with the development of effective and innovative cellulase immobilization techniques, this paper presented a study of immobilization of a commercial cellulase on PMMA nanoparticles during miniemulsion polymerization.

MATERIALS AND METHODS

Materials

Free cellulase (Cellusoft CR) was kindly donated by Novozymes Brasil (Araucária, PR, Brazil). In the miniemulsion polymerization Crodamol GTCC, a triglyceride of capric and caprylic acids, used as costabilizer, was provided by Alfa Aesar and either Lutensol AT50 from BASF, or sodium dodecyl sulfate (SDS, Sigma-Aldrich) was used as a surfactant. Methyl methacrylate (MMA, 99.5%) monomer, initiator potassium persulfate (KPS, P.A) and buffer sodium bicarbonate ($NaHCO_3$, P.A) were provided by Sigma-Aldrich. Sodium hydroxide (NaOH, P.A) was acquired from Lafan Ltda. Potassium and sodium tartrate (P.A) were both provided by Synth. Glucose D(+) anhydrous dextrose (P.A), 3,5-dinitrosalicylic acid (DNS, P.A), monobasic anhydrous potassium phosphate (P.A), dibasic sodium dihydrogen phosphate (P.A), and sodium carboxymethylcellulose (CMC) were all provided by Sigma-Aldrich. Cellulose acetate membranes (Unifil, 47 mm, 0.45 μm) and qualitative filter paper was used to determine enzymatic activity. Amicon® Ultra Centrifugal Filters (0.5 mL, 100.000Da) were used in the sample centrifugation. All reagents were used as received without previous purification.

Cellulase immobilization by miniemulsion polymerizationon PMMA nanoparticles

The synthesis of PMMA nanoparticles followed the methodology previously described by Valério

et al. (2015), with some modifications. The aqueous phase (distilled water, surfactant (Lutensol AT50 or SDS) and enzyme) and organic phase (Crodamol and monomer, MMA) were prepared according to the formulation shown in Table 1. Initiator (KPS) and sodium bicarbonate ($NaHCO_3$) (when used) were dissolved in an aliquot of the water phase and added to the dispersion after sonication, beginning the polymerization process. Reactions were carried out under constant magnetic stirring at 70 ºC for 3 h, in a jacketed borosilicate glass reactor (50 mL).

With the immobilization process ended, the final colloidal suspension of PMMA-cellulase (latex) was used to determine the enzyme activity and thermal stability at 4 ºC and room temperature, in order to determine the nanoparticles stability and the immobilized enzyme storage stability. To determine the immobilization efficiency, the final colloidal suspension of PMMA-cellulase (latex) was centrifuged at 11.300 g for 30 min in an Eppendorf - MiniSpin centrifuge, making use of ultrafiltration devices (Amicon® filters - 0.5 mL, 100.000Da), and the permeate was used for the analysis. Fig. 1 presents schematically the described procedure.

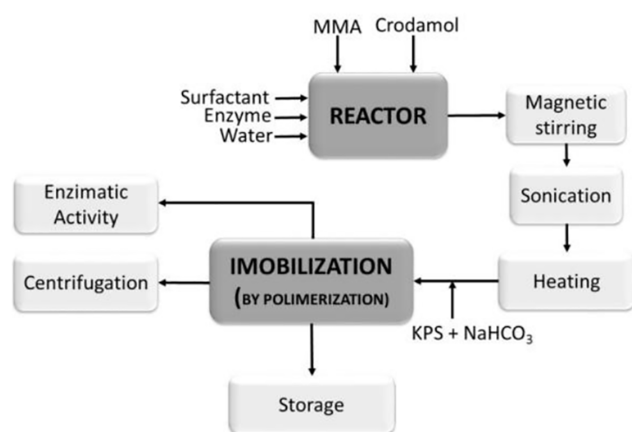

Figure 1. Schematic procedure for cellulase immobilization by miniemulsion polymerization

Enzyme activity assays

The cellulase activity was determined by the DNS method, following the procedure described: 900 µL of 4% (m/v) CMC solution, in phosphate buffer (0.05 M, pH 6.0), and 100 µL of PMMA-cellulase colloidal suspension were incubated at 55 ºC for 30 minutes. After the incubation time, 1.5 mL of DNS was added. The resulting solution was heated in a boiling water bath for 5 minutes and cooled in an ice bath, followed by water addition for sample dilution. These samples were then filtered in qualitative filter paper and cellulose acetate membranes for particulates removal. Finally, the glucose amount produced was determined from the absorbancea t540 nm. The relation between absorbance and concentration was achieved through a calibration curve, using glucose as standard.

In order to avoid interferences, a control solution was prepared for all experiments, adding 1.5 mL of DNS to the CMC and PMMA-cellulase solution at the beginning of enzymatic hydrolysis. One cellulose activity (U) unit was defined as glucose amount generated per minute (µmol/min). All measurements were performed in duplicate. The relative activity (AR) (Equation 1), where noted, was expressed as percentage of enzyme activity at a specific value (AE_i) relative to the maximum activity (AE_{max}). Maximum activity is the highest activity among all activities values obtained for the variable under study.

$$AR = \frac{AE_i}{AE_{\max}} \text{x}100 \qquad (1)$$

Free enzyme activity was determined in accordance with procedures and conditions used to determine the activity of the immobilized enzyme, excluding only the filtration steps.

Table 1. .Experiments performed to study the effect of pH, surfactant type and concentration on enzymatic activity and polymer particle size.

Exp.	SDS (g)	Lutensol AT50 (%)*	$NaHCO_3$(g)	Latex pH	Dp**(nm)	PDI	EA (U/mL)
1	-	2.7	0.01	6.5	131 ± 0.3	0.145	368.9 ± 2.8
2	-	2.7	-	3.0	128 ± 1.1	0.164	16.8 ± 3.1
3	0.060	-	0.01	5.1	***	***	37.8 ± 11.5
4	0.060	-	-	3.0	***	***	1.8 ± 0.26
5	-	1.4	0.01	6.5	193 ± 1.4	0.398	319 ±3.0
6	-	4.0	0.01	6.3	112 ± 0.7	0.185	386 ±6.0

wt.% related to the total weight of the reaction medium.
** Values expressed as mean ± standard deviation.
*** Unstable emulsion.
All reactions were performed at 70 °C during 3 h under constant magnetic stirring with: 3.09 g MMA, 3.03 g Crodamol, 0.30 g cellulase, 0.03 g KPS, 24 g water.

Immobilization efficiency

The amount of cellulase immobilized (Y_{imob}) on the PMMA nanoparticles was determined using Equation 2.

$$Y_{imob}(\%) = \frac{A_{free} - A_{supernatant}}{A_{free}} \quad (2)$$

in which A_{free} is the free enzyme activity before the immobilization process (U/mL); $A_{supernatant}$ is the supernatant activity after immobilization (U/mL).

Polymer characterization

The monomer conversion (MMA) was determined by gravimetric method. Latex samples were taken at different time intervals and added to previously pre-weighted aluminum capsules containing 0.2 g of hydroquinone aqueous solution (1 wt.%) to immediately stop reaction. The capsules were then dried at 60 ºC in a forced convection oven until constant weight. Conversion was calculated relating the polymer mass present in the reactor and the fed monomer mass.

The intensity average diameter (Dp) of the PMMA nanoparticles was determined by the dynamic light scattering technique (DLS - Malvern Instruments, Zetasizer Nano S); for the measurements, samples were diluted in distilled water. Scanning electronic microscopy with field emission (MEV-FEG - JEOL JSM-6701, LCME UFSC) was used in order to verify the morphology of PMMA nanoparticles. Sample preparation was performed by dropping the diluted latex (1:23 in distilled water) on a stub. After complete drying, the samples were sputter coated with a thin gold layer and analyzed.

Attenuated total reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR- Bruker TENSOR 27) in the range from 600 to 4000 cm^{-1} was used to confirm the chemical structure of the PMMA.

Effect of pH and temperature on enzymatic activity

In order to determine the influence of pH and temperature on free and immobilized cellulase, the activity assays were carried out in a range of pH from 4.0 to 8.0, and in a range of temperature from 35 to 75 ºC. Thermal stability was determined by measuring enzymatic activity of free and immobilized cellulase using the previously described procedure at different incubation times (30-300 min).

Storage stability

The storage stability of free and immobilized cellulose at 4 ºC was monitored by measuring the enzyme activity during 7 days. For this analysis free enzymes were kept in phosphate buffer (0.05M, pH 6.0), and the final latex was used as prepared (latex storage).

RESULTS AND DISCUSSION

Influence of the surfactant type and pH on the enzymatic activity

The influence of pH and surfactant type (anionic - SDS or non-ionic - Lutensol AT50) used in the miniemulsion polymerization, on the cellulase enzymatic activity, was evaluated using different polymerization formulations. The results are shown in Table 1. For experiments conducted with different surfactants and at the same pH (experiments 2 and 4), it was observed that the enzymatic activity was almost 10 times higher when non-ionic surfactant (Lutensol AT50) was used. The negative influence of ionic surfactants on cellulase hydrolysis was also reported by Eriksson et al. (2002) and Ueda et al. (1994).

All reactions were performed at 70 ºC during 3 h under constant magnetic stirring with: 3.09 g MMA, 3.03 g Crodamol, 0.30 g cellulase, 0.03 g KPS, 24 g water.

Comparing the experiments performed with the same surfactant and at different pH (experiments 1 and 2), it is noted that the enzymatic activity was much greater when $NaHCO_3$ was employed. A possible explanation for these results is the latex pH was under the enzyme stability range, causing its denaturing. From experiment 1, the latex pH was in the optimum range of enzyme activity (determined previously for free enzyme) upon adding only 0.01 g of buffering agent ($NaHCO_3$). According to these results, it can be concluded that the concomitant use of a non-ionic surfactant and a buffer is necessary to obtain high enzymatic activities.

Effect of surfactant concentration

The effect of Lutensol AT50 concentration on nanoparticle diameter was investigated. According to Table 1, the nanoparticle diameter (Dp) and dispersion (PDI) decreased upon increasing the surfactant concentration. The interfacial tension decreases with

increasing surfactant concentration, facilitating the dispersion of the monomer droplets during sonication, thus reducing nanoparticle diameter (Antonietti and Landfester, 2002; Bechthold et al., 2000; Romio et al., 2009; Valério et al., 2013). The polymeric nanoparticle surface area per surfactant molecule (A_{surf}) was reduced from approximately 1.77 to 1.02 nm^2/surfactant molecule when increasing the surfactant concentration from 1.4 to 4.0 wt.%. It means that the higher surfactant concentration increased the packing density of surfactant molecules at the particle surface. For non-ionic surfactant, steric stabilization is the main mechanism for the nanoparticle stabilization and it arises from a physical barrier. The surfactant molecules adsorbed on the surface of the nanoparticles extend into the continuous phase, providing a volume restriction or a physical barrier for particle interactions that prevents aggregation or coalescence and hence stabilizes emulsions. The higher the packing density of surfactant molecules at the particle surface, more effective is the physical barrier and higher is the nanoparticle stability, as was observed.

Influence of cellulase concentration

The effect of enzyme concentration on the relative activity during miniemulsion polymerization, immobilization efficiency, particle diameter and monomer conversion was also studied. According to the results shown in Fig. 2, it can be verified that the relative activity decreased during the polymerization for all concentrations of enzymes tested. A similar behavior was reported by Valérioet al. (2015) using the miniemulsion polymerization technique for CalB lipase immobilization.

Due to the immobilization process, part of the enzyme catalytic activity can be compromised. However, the relative activity obtained by the immobilization method used in the current work (64-78.7%) is equal to or higher than those reported for other immobilization methods by covalent bonds (Jordan et al., 2011; Silva et al., 2012; Abd El-Ghaffar and Hashem, 2010).

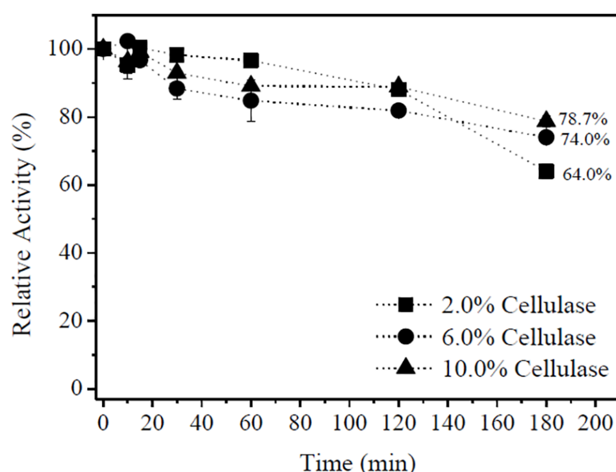

Figure 2. Influence of cellulase concentration on relative activity during miniemulsion polymerization using 2.7 wt.% of Lutensol AT50.

By measuring the supernatant enzyme activity, it could be observed that the highest immobilization efficiency was obtained when 6 wt.% of cellulase was used. At this concentration, approximately 60% of the initial enzyme activity was retained on PMMA nanoparticles. As shown in Table 2, at 10 wt.% of enzyme an expressive reduction in immobilization efficiency was observed. At low enzyme concentration the packing factor is lower than at higher enzyme concentration. However, there is a saturation point where the surface of the nanoparticle is fully covered by enzyme and the packing factor is maximum. Thus, increasing enzyme concentration above this point, a greater non-immobilized enzyme concentration is detected in the supernatant, leading to a decrease in immobilization efficiency.

According to Table 2, different concentrations of enzyme did not affect the particle size. Furthermore, there was not any evidence of the formation of coagulum during the polymerization reaction. These results suggest that, for all enzyme concentrations tested, the miniemulsions were stable. The enzyme concentration did not affect MMA conversion and for all reactions MMA conversion was above 90%. Similar behavior was observed by Valério et al. (2015).

Table 2. Influence of cellulase concentration on immobilization yield and nanoparticle diameter (Dp) and dispersion (PDI).

Cellulase Concentration (relative to MMA, wt.%)	Immobilization Yield (%)	Dp (nm)*	PDI
0		132±1.0	0.154
2	47±2	136±0.6	0.197
6	59±1	133±0.8	0.176
10	22±2	138±0.5	0.168

*values are expressed as mean ± standard deviation

Stability of PMMA-cellulase nanoparticles

The average size of PMMA nanoparticles was monitored during 60 days (Fig. 3). A colloidal dispersion of PMMA-cellulase nanoparticles was stored at room temperature and at 4ºC to determine the effect of temperature on nanoparticle stability.

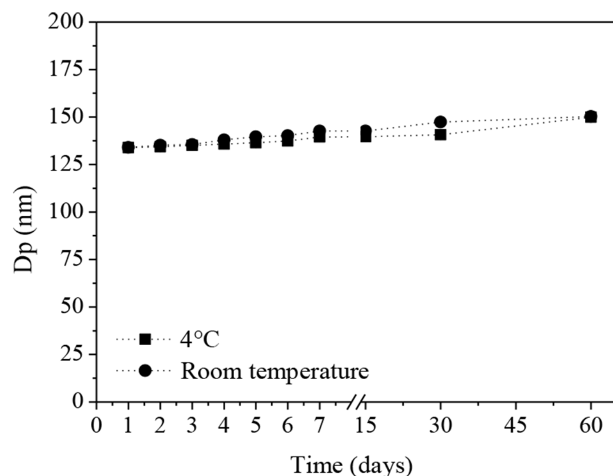

Figure 3. PMMA-cellulase nanoparticle (synthesized using 2.7 wt.% of Lutensol AT50 and 6 wt.% of cellulase) stability at 4 °C and at room temperature.

As observed in Fig. 3, the nanoparticle diameter slightly increased for both temperatures after 60 days of storage and the formation of coagulum was not observed indicating that the dispersion was stable and could be stored for long periods.

The MEV-FEG image (Fig. 4a) shows that PMMA nanoparticles had a spherical morphology and the particle size is in the same range as that obtained by DLS. As cellulase is predominantly lipophobic and it is dispersed in the water phase, the interaction between the lipophilic groups of the enzyme and PMMA occurs only at the surface of the nanoparticle, resulting in a core-shell structure where the immobilized enzyme remains at the nanoparticle surface. The TEM image (Fig. 4b) shows a polymer nanoparticle with a shadow around it that could be attributed to cellulase.

The chemical structure of PMMA wasverified by ATR-FTIR and the PMMA spectrum is shown in Fig. 5. The peak at 1147 $cm^{-1}$is assigned to the stretching vibration mode of C-O-C of the ester group. The peak at 1726 cm^{-1} is attributed to stretching of the carbonyl group of PMMA. The absorption bands in the range 3000-2800 cm^{-1} are attributed to stretching of C -H bonds of PMMA (Feuser et al., 2015; Matsushita et al., 2000). The absence of peaks at 1640 cm^{-1} is an evidence of complete polymerization of the monomer. This peak is attributed to C=C groups that are present in the monomer molecules that are directly involved in the polymerization (Otsuka and Chujo, 2010).

Effect of pH and temperature on enzyme activity

The effect of pH and temperature on the activity of free and immobilized enzyme is shown in Figs. 6 and 7. As can be observed, both free and immobilized enzymes are sensitive to pH and temperature variations. After immobilization, the relative activity profile for pH as well as temperature behavior was similar to that reported for free enzymes. Besides that, the maximum activity for free and immobilized enzymes was obtained at pH 6.0 and temperature of 55 ºC (optimal values). Similar observations were

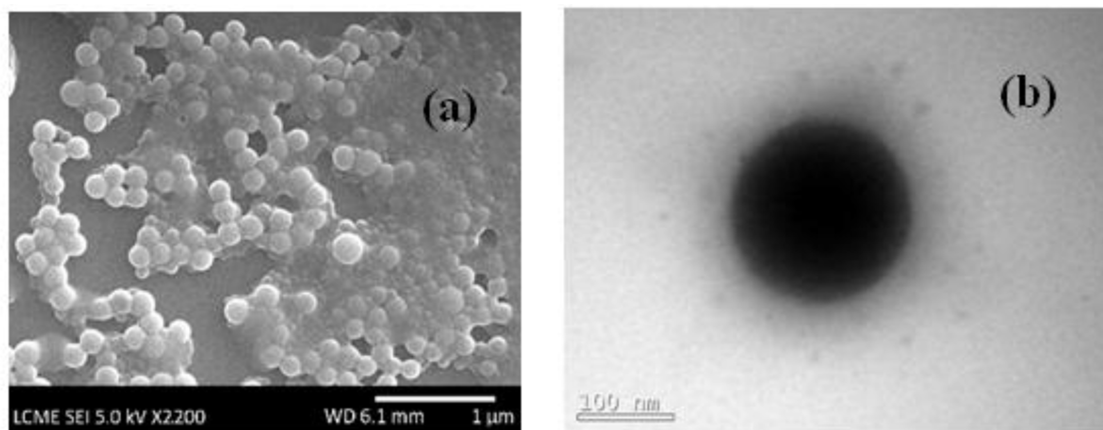

Figure 4. MEV-FEG (a) and TEM (b) images of PMMA-cellulase nanoparticles synthesized using 2.7 wt.% Lutensol AT50 and 6 wt.% cellulase.

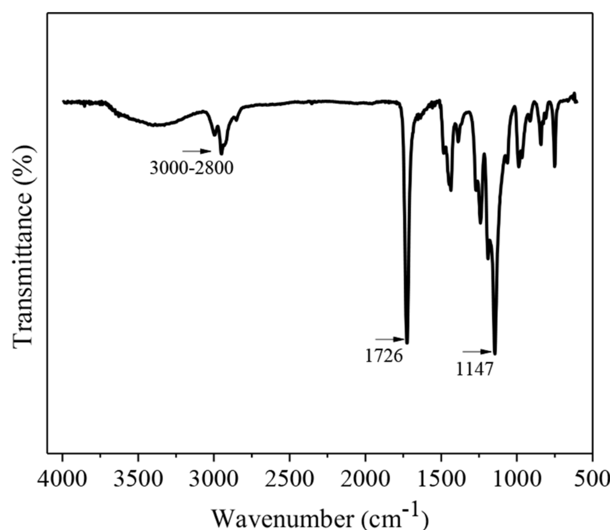

Figure 5. FTIR spectrum of PMMA nanoparticles synthesized using 2.7 wt.% of Lutensol AT50.

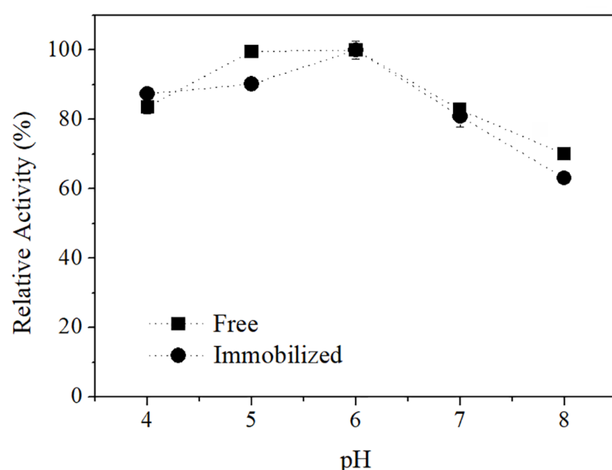

Figure 6. Effect of pH on enzymatic activity of free and immobilized cellulose at 55 ºC (PMMA-cellulase nanoparticles synthesized using 2.7 wt.% Lutensol AT50 and 6 wt.%).

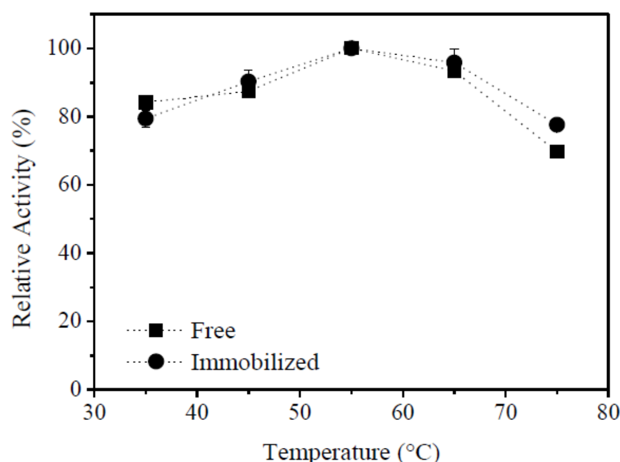

Figure 7. Effect of temperature on enzymatic activity of free and immobilized cellulase at pH 6.0 (PMMA-cellulase nanoparticles synthesized using 2.7 wt.% Lutensol AT50 and 6 wt.% cellulase)

reported by Liang and Cao (2012) for immobilized cellulase on polyacrylate copolymer.

Thermal stability

The thermal stabilities of free and immobilized cellulase were compared by measuring their activities over time at constant temperature (55 ºC). Fig. 8 shows that free and immobilized cellulase activity decreased gradually with time. Although the immobilized enzyme is inserted in a medium consisting of various components (water, ions, surfactant, etc.) that may affect its activity, its thermal stability was not affected.

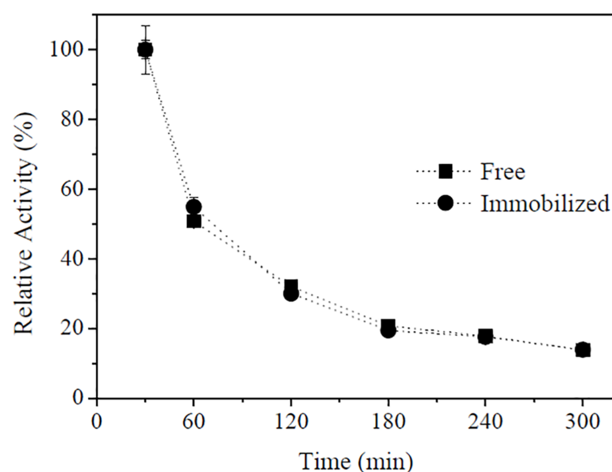

Figure 8. Thermal stability of free and immobilized cellulase at 55 °C and pH 6.0 (PMMA-cellulase nanoparticles synthesized using 2.7 wt.% Lutensol AT50 and 6 wt.% cellulase).

Storage stability

The storage stability of an enzyme is one of the main factors to evaluate its technological feasibility. Fig. 9 shows the stability of free and immobilized cellulase stored in phosphate buffer (0.05 M, pH 6.0) at 4 ºC. It was observed that the relative activity remained constant during 7 days for free and immobilized cellulase. According to Cavaco and Gübits (2003), temperature is a critical factor during enzyme storage, in both their solid and liquid forms. The results suggest that the latex did not interfere in the activity of immobilized cellulase under the tested conditions and these conditions (phosphate buffer pH 6.0 at 4 ºC) were suitable for enzyme conservation. Furthermore, at this temperature the microbial degradation can be minimized.

The immobilized cellulase was evaluated for four successive cycles and 22% of the initial activity was kept, showing the possibility of reusing the immobilized biocatalyst prepared here.

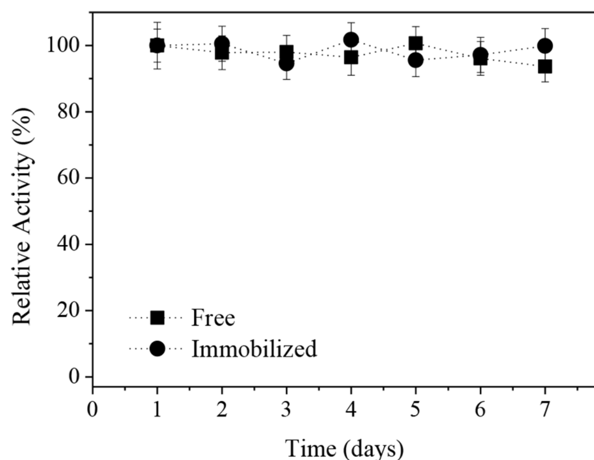

Figure 9. Storage stability of free and immobilized cellulase at 4 °C (PMMA-cellulase nanoparticles synthesized using 2.7 wt.% Lutensol AT50 and 6 wt.% cellulase).

CONCLUSIONS

In this work, miniemulsion polymerization was used as a technique for cellulase immobilization on poly(methyl methacrylate) (PMMA) polymeric nanoparticles. Polymerizations with non-ionic surfactant, Lutensol AT50, concomitantly with a buffering agent, $NaHCO_3$, led to high enzymatic activity values. From the study of the effect of surfactant concentration, 2.7 wt.%of Lutensol AT50 was sufficient to obtain stable nanoparticles with a high enzyme activity value. The increase in enzyme concentration in the polymerization reactions led to higher relative activity values at the end of the reactions. A cellulase concentration study indicated that the maximum immobilization yield was 60%, obtained when 6 wt.% of cellulase was added. Both free and immobilized enzymes presented the same behavior in relation to thermal stability, having their relative activity values reduced to 50% after 60 min of hydrolysis at 55 ºC and pH 6.0. In relation to storage stability, it was verified that immobilized enzyme keeps its activity throughout seven days when stored at 4 ºC in phosphate buffer, pH 6.0. Based on the results presented in this work, the immobilization of cellulase on PMMA polymeric nanoparticles by miniemulsion polymerization can be seen as a promising, feasible and innovative technique, which aims to cooperate with improvement, in both an economic and environmental sense, of several productive industrial processes.

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