β-Galactosidase activity in mixed micelles of imidazolium ionic liquids and sodium dodecylsulfate: A sequential injection kinetic study


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An automated methodology for the kinetic study of β-galactosidase activity in sodium dodecylsulfate (SDS)/ionic liquid (IL) mixed micelles was developed. The main objective of the work was the evaluation of mixed micelles as reaction media for the industrial synthesis of glyco-oligosaccharides. Enzyme activity was evaluated by means of a model reaction with the fluorescent substrate 4-methylumbelliferyl-α-D-galactopyranoside (MUG). The assay was implemented in a sequential injection analysis (SIA) system and enzyme activity was studied in SDS/bmim [BF₄] and SDS/hmim [Cl] mixed micelles with variable concentrations of both components. In order to perform a critical evaluation of the obtained results, CMC and average micellar size of SDS/hmim [Cl] mixed micelles were evaluated by fluorescence and dynamic light scattering, respectively. In the micelle characterization assays it was observed that the CMC of the mixed micelles increased with hmim [Cl] concentration up to 1 mol L⁻¹. In the presence of higher IL concentrations there were no evidences of micelle formation. Regarding micellar size, it was maximum for an IL concentration of 0.09 mol L⁻¹. The kinetic assays evidenced that SDS/bmim [BF₄] and SDS/hmim [Cl] mixed micellar systems can lead to an increase of enzyme activity. This increase is dependent on the variation of the average micellar size that occurs with the increase of IL concentration up to 0.09 mol L⁻¹. It was also noticed that the most promising systems are those incorporating SDS and IL in concentrations under 50 mmol L⁻¹ and 0.5 mol L⁻¹, respectively. These results evidenced that the studied ILs can modify the physico-chemical properties of the surfactant solution in a favourable way regarding β-galactosidase activity being an important achievement for the future implementation of industrial processes catalyzed by this enzyme, mainly the synthesis of glyco-oligosaccharides. Indeed, surfactant/IL mixed micelles proved to be an interesting alternative to conventional organic solvents in this field enabling the implementation of the processes in a relatively hydrophobic media with enhanced enzyme activity.

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1. Introduction

Ionic liquids (ILs) have been studied in the last two decades as promising solvents in Green Chemistry [1,2]. These organic salts exhibit low vapour pressure and their polarity can be modulated by the nature of the side chains. Hence, they are very attractive as alternatives to conventional organic salts in a variety of processes such as organic synthesis, extractions, chromatographic separations, electrochemistry and biocatalysis, among others [3,4]. To further increase their applicability in “green” industrial processes, in which the replacement of harmful and volatile organic solvents is nowadays mandatory, in the last years we assisted to the association of ILs to other environmentally benign systems such as supercritical fluids, polymers and surfactant-based systems [5–7].

There has been an increasing and particular interest on the issue of micelle formation mainly on alkylimidazolium ILs and considerable progress has been made in this filed [8,9]. These compounds have pronounced hydrophilic and hydrophobic molecular fragments and thus are very similar to classical surfactants [10,11]. However, besides the possibility of self-assembly in solutions [12–14] one must also consider their ability to act as modulators of the aggregative behaviour of classical surfactants either in mixed [5–7,15,16] or reverse micelles [17,18], which opens novel and broad perspectives regarding their industrial application. This is partially related with ILs solvation ability that facilitates the interaction with classical surfactants; the solvatochic interaction between the IL and the hydrocarbon portion of the surfactant results in the formation of mixed micelles of IL and surfactant with enhanced solvation characteristics. The amphiphilic nature of ILs has also been outlined as a major point during micelle formation since it can determine important interfacial and aggregation phenomena [19]. Thus, the main characteristics of micellar aggregates such as critical micelle

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concentration (CMC), aggregation number or micelle size can be modified by association with ILs with tunable hydrophobicity [7]. This idea of micelles with tailored physico-chemical properties in ILs solutions is very promising and has already been considered economically convenient. Several authors have been studying the properties of mixed micelles of imidazolium ILs and surfactants, mainly SDS [6,16]. In particular it seems that imidazolium ILs affect the aggregative behaviour of SDS, namely its CMC. This effect is stronger than the one observed in the presence of inorganic salts and is dependent on the length of the alkyl chain and generally, mixed micelles are considered more versatile than single surfactant systems. Considering the peculiar characteristics of SDS/IL mixed micelles and the previous interesting results obtained with other surfactant/IL/organic reverse micelles [17,18,20] it seems that the first ones offer promising possibilities regarding their application in enzyme based transformations. Indeed, biocatalysis in organ- ized assemblies, mainly micellar biocatalysis, has been a field of intensive research in the last decades and its potential for biotechno- logical applications is well demonstrated [21]. Two main driven forces explain these efforts: the search for models that can mimic the enzyme natural environment (bio membranes) and the need to solubilize water-insoluble substrates. Also in the field of bio- catalysis, ILs have received growing attention as a reaction media for enzymatic transformations and generally, enzyme activity in ILs is comparable, or higher than, that measured in conventional organic solvents [4,22,23]. Moreover, there are also evidences of enzyme’s enhanced thermal and operational stability as well as good selectivity in these solvents [22]. All this can results in a dra- matic decrease of the energy consumption due to the enhancement of the processes’ yield. Additionally, and as mentioned above, there are recent reports that evidence the adequacy of reverse micelles, incorporating not only surfactants and organic solvents but also ILs, for biocatalytic assays [17,18,20].

In this work, we explored for the first time the potentialities of using mixed micelles composed only by imidazolium ILs and SDS in β-galactosidase catalyzed processes by means of a model reaction based on the fluorescent substrate 4-methylumbelliferyl-β-galactopyranoside (MUG) [24]. This enzyme is largely used in the industrial synthesis of glyco-oligosaccharides by means of transglycosylation reactions [25]. These reactions are well implemented in the food industry since they offer advantages over chemical methods namely high specificity, efficiency and green nature. However, in aqueous media there is a high tendency for the reaction equilib- rium to be shifted to favour hydrolysis over synthesis, leading to a reduction of the reaction yield [26,27]. To overcome this problem the activity of β-galactosidase has been evaluated in a variety of systems namely organic solvents, micelles, reverse micelles and ILs [26,28–31]. The utilization of organic media offers several advan- tages over aqueous environments but its routine utilization shall be avoided in accordance with Green Chemistry principles. Fur- thermore, there were reports of reduced enzyme stability in those environments [26,28]. The addition of surfactants has resulted in an increase of the initial rate of the transglycosylation in organic and aqueous media [32]. Reverse micelles proved to be an interesting option as they enable to work with both water-soluble and water-insoluble compounds in pseudo-homogenous phase [33–35]. In the presence of ILs the enzyme exhibited an activity which is similar or higher than that observed in organic solvents such as ethanol and acetonitrile [30]. Moreover, there were evidences of enzymes’ enhanced stability and selectivity due to interactions between the IL and the charged groups of the enzyme [31].

In this context, it seems appropriate to study the behaviour of β-galactosidase in mixed micelles of SDS and imidazolium ILs to investigate the influence of these systems on the enzyme in order to evaluate them as potential reaction media for transglyco- sylation reactions. Thus, a methodology for the kinetic evaluation of β-galactosidase activity was implemented and optimized in a sequential injection analysis (SIA) system. This flow strategy has been largely applied to the automation of enzyme-based assays and was already proposed as a generic tool for the study of enzyme reactions in ILs [36–38]. The versatility of the selection valve and the peculiar mode of operation of the system enable the exquisite control of the reagent conditions as well as the implementation of kinetic assays, with the reaction product being measured by stopping the reaction in the flow cell. The activity of the enzyme was studied in mixed micelles containing variable concentration of SDS and bmim [BF4] and hmim [Cl] and the kinetic parameters were calculated and discussed. As there was no sufficient data regarding the characteristics of mixed micelles of SDS and bmim [Cl] the CMC and the micelle size of this system were properly determined.

This work can be of the most importance for the future uti- lization of β-galactosidase in industrial synthesis and can be a starting point for the study of the influence of surfactant/IL mixed micelles on other enzymes involved in important biotechnological processes.

2. Experimental

2.1. Reagents

All solutions were prepared using chemicals of analytical reagent grade and high purity water (Milli-Q) with a specific con- ductance <0.1 µS cm⁻¹.

The carrier solution of the flow system was a phosphate–citrate buffer 50 mmol L⁻¹ pH 5, with NaCl 50 mmol L⁻¹.

Daily, a solution of β-galactosidase from Aspergillus oryzae (EC 3.2.1.23, Sigma) 0.5 U ml⁻¹ was prepared in phosphate–citrate buffer pH 5, from a 50 U ml⁻¹ stock solution. This solution was stored in the refrigerator and remained stable for about 2 days.

A stock solution of 4-methylumbelliferyl α-D- galactopyranoside (MUG) 25 mmol L⁻¹ was prepared daily in DMSO. MUG working solutions (0.30–3.0 mmol L⁻¹) were pre- pared immediately before the kinetic assays by dilution of the stock solution in mixed micelles with variable concentra- tions of sodium dodecylsulfate (SDS; 0.09–120 mmol L⁻¹) and 1-butyl-3-methylimidazolium tetrafluoroborate (bmim [BF4]; 0.5–1.5 mol L⁻¹; Sigma) or 1-hexyl-3-methylimidazolium chloride (hmim [Cl]; 0.5–1.5 mol L⁻¹; Fluka).

2.2. Apparatus

Spectrofluorimetric measurements were made in a Jasco FP 2020 spectrofluorimeter. The SIA system (Fig. 1) consisted of a Gilson Minipuls 3 peristaltic pump, equipped with PVC pumping tube (1.2 mm i.d.) and a 8-port multiposition Vici Valco selection valve. Manifold components were connected by means of 0.8 mm i.d. PTFE tubing which was also used for the holding and reaction coil (4 and 0.15 m, respectively).

Analytical system control, including the operation of the per- istaltic pump and selection valve, was achieved by means of an Advantech USB-4711 interface card and a Pentium-I based micro- computer. Software was developed in Microsoft Visual-Basic and permitted the control of flow rate, flow direction, valve position, sample and reagent volume as well as data acquisition and processing. During optimisation, the analytical signals were also recorded on a Kipp & Zonen BD 111 strip chart recorder. In the studies on the influence of temperature on enzyme activity, the reaction coil was immersed on a water bath thermostatted with a Medingen ES temperature controller.
2.3. Sequential injection procedure

The analytical cycle established for the implementation of the kinetic evaluation of β-galactosidase activity, in the SIA system started with the sequential aspiration of 85 μL of MUG (in micellar media) and 25 μL of enzyme solution to the holding coil. Then, the flow was reversed and the reaction zone propelled by the carrier solution through the reaction coil, directly to the detector at a flow rate of 2.2 ml min⁻¹, during 11 s. Then the flow was stopped for 120 sec and the variation of the analytical signal was acquired in the computer. After the stop period, the peristaltic pump was activated to clean the flow cell.

2.4. Initial rate measurement

After the aspirated zones were directed to the flow cell a stopped-flow period of 90 s was implemented with fluorescence readings being continuously taken (4 readings per second). On completion of the stop period, the pump was reactivated and the contents of the flow cell washed out, thus being ready for the next analytical cycle. Within this stopped-flow period, a fixed data collection interval was chosen (8–40 s) during which the slope of the response curve (yielding the reaction rate (dIF/dt)) was evaluated. Reaction rates (determined in ΔIF s⁻¹ units) were then calculated according to zero-order kinetics by linear least-squares regression of the data obtained over the respective period. This relationship was considered linear after visual inspection of the graphs (number of points >50) and when the correlation coefficient was greater than 0.995. The apparent Michaelis–Menten constant (𝐾𝑀) and maximum reaction rate (𝑉max) were obtained by nonlinear regression to a plot of the initial rate values (𝑉0 − ΔIF s⁻¹) against [5] by using the GraphPad Prism 5 program for Windows. Theoretically, 𝐾M is the concentration of substrate at 1/2 𝑉max and represents the affinity of the substrate for the enzyme; the catalytic constant was calculated using the expression: 𝑘cat = 𝑉max/[enzyme]; the catalytic efficiency represents the ability of the enzyme to convert the substrate in a final product and is calculated as 𝑘cat/𝐾M.

2.5. Characterization of SDS/hmim [Cl] mixed micelles

2.5.1. Determination of CMCs of mixed micelles of SDS/hmim [Cl] (light scattering)

The CMC of SDS/hmim [Cl] mixed micelles was determined by light scattering according to the procedure described by Reis et al. [39]. Fluorometric measurements for the determination of CMC were performed in a Perkin Elmer LS50B spectrofluorimeter. In all the determinations the slit was fixed at 5 nm and the excitation and emission wavelengths were set at 400 nm. Measurements were performed on mixed micelles of SDS with variable concentration of hmim [Cl] (0.09, 0.5, 1 and 1.5 mol L⁻¹). For each IL concentration the concentration of SDS was varied between 0.025 and 400 mmol L⁻¹ (Fig. 2).

2.6. Measurements of particle size

Particle size and distribution was determined by dynamic light scattering (DLS), using a BI-MAS size Analyzer (Brookhaven Instruments, USA). Mixed micelles with SDS 50 mmol L⁻¹ and hmim [Cl] between 0.01 and 1 mol L⁻¹ were analyzed and the available software was used to correlate the intensity of scattered light (at a backscattering angle of 173°) with the hydrodynamic radius of the spherical particle. For each sample, the mean diameter ± standard deviation of at least three determinations was calculated applying multimodal analysis.

3. Results

In this work, a kinetic evaluation of β-galactosidase activity was performed in a SIA system. Enzyme activity was studied in mixed micelles of SDS and two imidazolium ionic liquids with different alkyl chain length: bmim [BF₄] and hmim [Cl]. The kinetic parameters were calculated for all the studied reaction media. These studies aimed the evaluation of these organized assemblies as possible solvents for the industrial synthesis of glyco-oligosaccharides. In the following sections the optimization of the SIA methodology and the parameters affecting enzyme activity as well as the kinetic assays in mixed micelles of SDS and ILs will be discussed.

3.1. Optimization of the SIA kinetic assay

The activity of β-galactosidase was evaluated by means of the degradation of MUG with release of the fluorescent product, 4-methylumbelliferone [24]. The parameters affecting enzyme activity and the performance of the system were evaluated in micellar media (SDS 8.5 mmol L⁻¹) by the univariate approach. The results were analyzed in terms of initial reaction rate expressed as ΔIF s⁻¹.

3.2. Reaction protocol

Reaction coil length, propulsion flow rate and sequence of aspiration were previously fixed considering the particularities of the assay. Thus, a 0.15 m reaction coil was inserted between the selection valve and the detector to enable overlapping of the aspirated
zones on their way to the flow cell. The flow rate was fixed at 2.2 ml min⁻¹ in order to get a fast and reproducible transport of the reaction zone to the detector (rsd <5% in SDS micelles, n = 5). Regarding the analytical cycle, it was established that the enzyme aliquot should be aspirated immediately before the flow reversal to reduce its dispersion. The other parameters were studied afterwards; the range of the studied parameters and the selected final conditions are summarized in Table 1. The volumes of MUG and enzyme showed to influence the initial rate of reaction up to 150 and 50 μL, respectively. The final protocol involved the sequential aspiration of 85 μL of MUG and 25 μL of enzyme as a compromise between sensitivity and zone overlapping. Indeed, both volumes were as low as possible considering eventual mixing problems. The issue of mixing efficiency was considered due to the viscosity of the mixed micelles to be tested.

In a SIA kinetic assay the transport of the reaction zone to the detector is of the most importance for the successful implementation of the study since it defines the portion that will be stacked in the flow cell for reaction monitoring. In this case the selection of the reaction zone to be monitored was performed in terms of time during which the reaction zone was propelled to the detector, at 2.2 ml min⁻¹, before stopping the flow for data acquisition. The initial reaction rate increased with the delivery time until 4.5 s; after this there was a decrease of the reaction rate indicating that the reaction zone was partially sent to waste due to an excessive propulsion time (Fig. 3). Thus, the reaction protocol involved the propulsion of the aspirated zones to the flow cell during 4.5 s at 2.2 ml min⁻¹.

In the abovementioned experimental conditions the concentration of β-galactosidase was studied between 0.1 and 5 U ml⁻¹ (Fig. 4). It was observed that the reaction rate increased with enzyme concentration up to 1 U ml⁻¹ and above this concentration a steady-state profile was attained. To guarantee zero-order conditions (reaction rate proportional to enzyme concentration) a value of 0.5 U ml⁻¹ was selected.

The influence of pH on the initial reaction rate was studied between 4 and 6 by changing the pH of the citrate–phosphate buffer
used as carrier in the flow system and for the preparation of enzyme solutions. It was noticed that the reaction rate increased slightly until 5 units; buffer solutions with higher pH led to a significant decrease of the reaction rate so that the kinetic studies proceed with citrate–phosphate buffer pH 5 (Fig. 5).

The evaluation of enzyme behaviour at variable temperature revealed that no significant variation on the initial reaction rate was observed by increasing the temperature until 40 °C. Thus, the kinetic assays were implemented at room temperature.

### 3.3. Characteristics of SDS/IL mixed micelles

It was previously defined that the discussion of the obtained results should be based on the correlation of the kinetic parameters with the characteristics of the tested mixed micelles. Thus, the discussion of our results was partially supported by literature data concerning the physicochemical properties (CMC, aggregation number and average micellar size) of mixed micelles of SDS and bmim [BF₄] [6]. In the case of SDS/hmim [Cl] mixed micelles, the available data [16,19] was not enough to support the discussion of the obtained results since micelle characterization was performed for low IL concentrations (in the mmol L⁻¹ range) and then was not applicable to our studies (performed in the mol L⁻¹ level). Thus, as a complement we performed a simple characterization of the micelles in terms of CMC and micellar size. The results of these studies are compiled in Table 2 where we also inserted literature data concerning SDS/bmim [BF₄] mixed micelles. It is worth mentioning that our results for micelle diameter of aqueous SDS is similar to those published in literature [6,40,41].

### Table 2

<table>
<thead>
<tr>
<th>IL concentration (mol L⁻¹)</th>
<th>CMC (mmol L⁻¹)</th>
<th>Particle diameter (nm)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.0⁺</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>hmim [Cl]–0.01</td>
<td>nd</td>
<td>2.9 ± 0.6</td>
</tr>
<tr>
<td>hmim [Cl]–0.09</td>
<td>0.44</td>
<td>18.5 ± 3.3</td>
</tr>
<tr>
<td>hmim [Cl]–0.5</td>
<td>0.75</td>
<td>16.0 ± 0.6</td>
</tr>
<tr>
<td>hmim [Cl]–0.75</td>
<td>Nd</td>
<td>6.8 ± 0.5</td>
</tr>
<tr>
<td>hmim [Cl]–1</td>
<td>10.6</td>
<td>5.0 ± 0.3</td>
</tr>
<tr>
<td>bmim [BF₄]–0.02⁺</td>
<td>1.5</td>
<td>2.9</td>
</tr>
<tr>
<td>bmim [BF₄]–0.09⁺</td>
<td>1.0</td>
<td>10.3</td>
</tr>
<tr>
<td>bmim [BF₄]–0.44⁺</td>
<td>8.5</td>
<td>9.1</td>
</tr>
<tr>
<td>bmim [BF₄]–0.88⁺</td>
<td>50</td>
<td>5.7</td>
</tr>
<tr>
<td>bmim [BF₄]–1.33⁺</td>
<td>110</td>
<td>4.5</td>
</tr>
</tbody>
</table>

nd: not determined.  
⁺ Literature data [6].  
² Assays in hmim [Cl] with SDS 50 mmol L⁻¹ (above CMC for the all IL concentrations).

Some unexpected results on both the assays of CMC determination of mixed micelles of SDS/hmim [Cl] as well as on particle measurements on micelles of the same composition were obtained. On the CMC assay, with hmim [Cl] 1.5 mol L⁻¹ one could not reach a fluorescence response profile that enabled the graphical determination of CMC of those solutions, indicating that in some of the studied systems there were no micelles presented. On the other hand, during size measurements, performed with SDS 60 mmol L⁻¹ to guarantee concentration above the CMC for all IL concentrations, there was no detection of particles and a size value was not obtained in the assays with hmim [Cl] 1.5 mol L⁻¹. Moreover, in the automated kinetic assays in the presence of mixed micelles with SDS 120 mmol L⁻¹ there was no variation of the initial rate of reaction with MUG concentration, for all the IL concentrations tested. All this suggested that there were no evidences of micelle formation in solutions of SDS above 50 mmol L⁻¹ in the presence of hmim [Cl].

As can be seen in Table 2 there is a strong influence of both ILs on the properties of the micelles. Regarding hmim [Cl] there is a significant decrease of the CMC by addition of IL up to 0.5 mol L⁻¹. However, higher concentrations result in a strong increase of CMC. There were also significant changes on the micellar size in the presence of hmim [Cl] and a maximum diameter of 18.5 nm was attained with 0.09 mol L⁻¹. It is our belief that the addition of high concentrations of hmim [Cl] (0.5–1 mol L⁻¹) to aqueous SDS led to an increase of CMC similarly to polar cosolvents. Indeed, the increase of CMC is probably associated with the reduction of IL dissociation, as the water concentration decreases, resulting on a cosolvent effect. This effect was also related by Beyaz et al. regarding the influence of bmim [BF₄] on SDS micelles [16]. On the other hand, the effect of small amounts of IL (up to 0.5 mol L⁻¹) is equal to that observed for common electrolytes (reduction of CMC and increase of micellar size). Probably, the electrostatic attraction between the imidazolium moiety and the anionic group of SDS reduces the repulsion between the head groups of the surfactant which allows more molecules to form micelles, reducing the CMC.

The exposed data for mixed micelles with bmim [BF₄] evidences also a strong increase of the CMC with the increase of IL concentration. It can also be noticed an increase of the micelles size with the IL concentration up to 0.09 mmol L⁻¹.

### 3.4. Kinetic assays in SDS/bmim [BF₄] and SDS/hmim [Cl] mixed micelles

It is known that ILs can modify the physicochemical properties of aqueous surfactants in a favourable way [5,19,42,43]. Thus, ILs can enable the formation of micelles with desirably modified properties which can broaden the application field of these organized media, namely to biocatalytic processes. Thus, in the optimized assay conditions the influence of mixed micelles composed of SDS and bmim [BF₄] or hmim [Cl] on the kinetic behaviour of β-galactosidase was tested in order to evaluate these assemblies as possible reaction media for the synthesis of glyco-oligosacharides. The variation of the initial reaction rate with substrate concentration was evaluated for MUG concentrations between 0.30 and 3.0 mmol L⁻¹. The kinetic assays were performed in the presence of micelles with variable concentration of both SDS (8.5–120 mmol L⁻¹) and IL (0.09–1.5 mol L⁻¹). The assays were performed by fixing the concentration of SDS and varying IL concentration. The kinetic parameters were calculated for all the assayed conditions (Tables 3 and 4 and Fig. 6). In the absence of IL a decrease of Km and an increase in the catalytic efficiency were observed for SDS concentrations up to 25 mmol L⁻¹ (Fig. 6). Micelles with higher concentration of surfactant result in a reduction of enzyme activity. Regarding enzyme activity in SDS/IL micelles it was noticed that for all the SDS concentrations and for both bmim [BF₄] and hmim [Cl] there was, in most cases, a decrease of Km accompanied by an increase of the catalytic
efficiency, mainly for the lower IL concentrations tested (0.09 and 0.5 mol L\(^{-1}\)). Generically, the enhancement of enzyme activity is particularly evident in the assays with SDS 8.5 and 25 mmol L\(^{-1}\) (IL: 0.09–0.5 mol L\(^{-1}\); Fig. 6). In the studies with bmim [BF\(_4\)], an increment of enzyme activity between 15 and 55% was attained (Table 3). Regarding the assays with SDS/hmim [Cl] mixed micelles similar results were obtained for lower SDS concentrations. However, it is evident that in the presence of hmim [CI] the catalytic efficiency of β-galactosidase is lower regarding the assays with bmim [BF\(_4\)] under similar experimental conditions, namely in the assays with the same SDS concentration (Table 4). It is also evident that mixed micelles with SDS above 25 mmol L\(^{-1}\) do not constitute an interesting option as possible reaction media for β-galactosidase due to the lower catalytic efficiencies observed for all the assay conditions, even in the absence of IL (Fig. 6).

4. Discussion

As it was stated above, the discussion of the obtained results was performed by correlating the changes in the kinetic parameters not only with micelle composition (in terms of IL concentration) but also with the changes induced by the ILs in the particular characteristics of the micelles, namely CMC and average micelle size.

Regardless of the kinetic parameters in the absence of ILs, the results are not surprising as anionic surfactants are well known enzyme denaturants at high concentrations. In particular, the effect of SDS on several enzymes, including β-galactosidase, has also been described as concentration dependent, so that it is able to enhance or reduce enzyme activity [44–46]. Thus, the observed reduction of enzyme activity for SDS concentrations above 25 mmol L\(^{-1}\), in the absence and in the presence of ILs, is probably related with enzyme denaturation and alteration of its secondary structure caused by the interaction of the enzyme not only with the micelles but also with surfactant monomers [45,46]. Taking in consideration the results of the kinetic assays in mixed micelles incorporating ILs (Tables 3 and 4, Fig. 6) as well as the contents of Table 3, the majority of the obtained results are probably related with the increase of the average micellar size induced by bmim [BF\(_4\)] and hmim [CI] in concentrations up to 0.5 mol L\(^{-1}\). However one must also consider the interaction of the enzyme with IL molecules since as it is widely accepted this can result in enhanced activity and stability due to ILs high hydrogen bond capacity that can be an important factor on preventing enzyme unfolding. Moreover, the reduction of water in the reaction media can decrease side reactions and reduce the hydrolysis of the reaction products and thus increase the reaction yield.

The abovementioned changes of the average micellar size, caused by the presence of both ILs in the mixed micelles and evidenced experimentally for mixed micelles of hmim [CI] and described in literature [6,19], can result in deep changes not only

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**Table 3** Kinetic parameters of β-galactosidase in SDS/hmim [BF\(_4\)] mixed micelles.

<table>
<thead>
<tr>
<th>Composition of micelles</th>
<th>(K_a) (mmol L(^{-1}))</th>
<th>(k_{cat}) (s(^{-1}))</th>
<th>Catalytic efficiency (s(^{-1}) mmol L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS 8.5 mmol L(^{-1})</td>
<td>1.08 ± 0.09</td>
<td>0.039 ± 0.001</td>
<td>0.036 ± 0.002</td>
</tr>
<tr>
<td>SDS 8.5 mmol L(^{-1}) + bmim [BF(_4)] 0.09 mmol L(^{-1})</td>
<td>0.82 ± 0.01</td>
<td>0.035 ± 0.001</td>
<td>0.042 ± 0.001</td>
</tr>
<tr>
<td>SDS 8.5 mmol L(^{-1}) + bmim [BF(_4)] 0.5 mmol L(^{-1})</td>
<td>0.48 ± 0.04</td>
<td>0.027 ± 0.001</td>
<td>0.056 ± 0.002</td>
</tr>
<tr>
<td>SDS 8.5 mmol L(^{-1}) + bmim [BF(_4)] 1 mol L(^{-1})</td>
<td>0.71 ± 0.09</td>
<td>0.034 ± 0.008</td>
<td>0.048 ± 0.006</td>
</tr>
<tr>
<td>SDS 8.5 mmol L(^{-1}) + bmim [BF(_4)] 1.5 mol L(^{-1})</td>
<td>0.75 ± 0.01</td>
<td>0.025 ± 0.004</td>
<td>0.033 ± 0.004</td>
</tr>
<tr>
<td>SDS 25 mmol L(^{-1})</td>
<td>1.04 ± 0.07</td>
<td>0.032 ± 0.001</td>
<td>0.031 ± 0.001</td>
</tr>
<tr>
<td>SDS 25 mmol L(^{-1}) + bmim [BF(_4)] 0.09 mmol L(^{-1})</td>
<td>1.09 ± 0.09</td>
<td>0.040 ± 0.008</td>
<td>0.042 ± 0.003</td>
</tr>
<tr>
<td>SDS 25 mmol L(^{-1}) + bmim [BF(_4)] 0.5 mmol L(^{-1})</td>
<td>0.97 ± 0.01</td>
<td>0.040 ± 0.001</td>
<td>0.041 ± 0.001</td>
</tr>
<tr>
<td>SDS 25 mmol L(^{-1}) + bmim [BF(_4)] 1 mol L(^{-1})</td>
<td>1.03 ± 0.01</td>
<td>0.029 ± 0.001</td>
<td>0.028 ± 0.002</td>
</tr>
<tr>
<td>SDS 25 mmol L(^{-1}) + bmim [BF(_4)] 1.5 mol L(^{-1})</td>
<td>1.01 ± 0.01</td>
<td>0.025 ± 0.011</td>
<td>0.025 ± 0.012</td>
</tr>
<tr>
<td>SDS 50 mmol L(^{-1})</td>
<td>1.24 ± 0.12</td>
<td>0.030 ± 0.001</td>
<td>0.025 ± 0.003</td>
</tr>
<tr>
<td>SDS 50 mmol L(^{-1}) + bmim [BF(_4)] 0.09 mmol L(^{-1})</td>
<td>1.95 ± 0.09</td>
<td>0.045 ± 0.002</td>
<td>0.023 ± 0.001</td>
</tr>
<tr>
<td>SDS 50 mmol L(^{-1}) + bmim [BF(_4)] 0.5 mmol L(^{-1})</td>
<td>0.86 ± 0.04</td>
<td>0.031 ± 0.001</td>
<td>0.035 ± 0.003</td>
</tr>
<tr>
<td>SDS 50 mmol L(^{-1}) + bmim [BF(_4)] 1 mol L(^{-1})</td>
<td>0.92 ± 0.05</td>
<td>0.029 ± 0.001</td>
<td>0.031 ± 0.001</td>
</tr>
<tr>
<td>SDS 50 mmol L(^{-1}) + bmim [BF(_4)] 1.5 mol L(^{-1})</td>
<td>0.66 ± 0.04</td>
<td>0.017 ± 0.001</td>
<td>0.026 ± 0.001</td>
</tr>
<tr>
<td>SDS 120 mmol L(^{-1})</td>
<td>2.38 ± 0.12</td>
<td>0.035 ± 0.001</td>
<td>0.015 ± 0.001</td>
</tr>
<tr>
<td>SDS 120 mmol L(^{-1}) + bmim [BF(_4)] 0.09 mmol L(^{-1})</td>
<td>1.03 ± 0.26</td>
<td>0.034 ± 0.005</td>
<td>0.033 ± 0.003</td>
</tr>
<tr>
<td>SDS 120 mmol L(^{-1}) + bmim [BF(_4)] 0.5 mmol L(^{-1})</td>
<td>0.88 ± 0.31</td>
<td>0.032 ± 0.003</td>
<td>0.036 ± 0.004</td>
</tr>
<tr>
<td>SDS 120 mmol L(^{-1}) + bmim [BF(_4)] 1 mol L(^{-1})</td>
<td>1.20 ± 0.07</td>
<td>0.030 ± 0.003</td>
<td>0.025 ± 0.001</td>
</tr>
<tr>
<td>SDS 120 mmol L(^{-1}) + bmim [BF(_4)] 1.5 mol L(^{-1})</td>
<td>1.09 ± 0.25</td>
<td>0.024 ± 0.001</td>
<td>0.022 ± 0.002</td>
</tr>
</tbody>
</table>

\(K_a\) – concentration of substrate at 1/2 \(V_{max}\), represents the affinity of the substrate for the enzyme; \(k_{cat}\) (catalytic constant) = \(V_{max}/\text{[enzyme]}\); catalytic efficiency = \(k_{cat}/K_a\), represents the ability of the enzyme to convert the substrate in a final product.

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on the enzyme itself but also on the microenvironment around it. Firstly, the increase of the micelle size induced by both ILs may imply changes in the substrate, such as molecular orientation or molecular density, which can result in an improvement of the reaction yield. Secondly, the increase of the interfacial area can affect the partitioning of the reaction substrate and product towards the micelle and this can also influence favourably the dynamics of the overall process. On a distinct perspective, one cannot forget the already described possibility of modulate \( \beta \)-galactosidase activity through interaction with membranes \([45-47]\). Thus, we postulate that the modification of the micellar interface, and in particular the increase of the micelle size induced by ILs, might result in steric modifications that can alter the enzyme flexibility facilitating the catalysis. These theories should not be applied exclusively but must rather be considered as integrated as a part of a possible explanation for the observed changes on \( \beta \)-galactosidase behaviour in SDS/IL micelles.

A global analysis of the results indicates a slight reduction of the enzyme catalytic efficiency in mixed micelles of SDS/hmim [Cl] comparing with SDS/bmim [BF\(_4\)]. Considering all the aspects exposed above regarding the main characteristics of both micellar systems one could not explain this observation. Indeed, the characteristics of the micelles formed by ILs incorporation are very similar, mainly regarding particle size which was considered above the main factor affecting enzyme activity and its kinetic parameters. Thus, it became evident that the different enzyme behaviour observed in the two mixed micelles should be related with intrinsic characteristics of the ILs. Even though there are many aspects of a specific IL that can define its influence on enzyme behaviour, in this case it is more likely that the slight reduction in enzyme activity is related with distinct viscosities and alkyl chain lengths. Comparing with bmim [BF\(_4\)], hmim [Cl] exhibits higher viscosity (due to its longer alkyl side chain) that may lead to mass transfer limitations and consequently to a lower reaction rate. This aspect has already been explored by some authors and it was concluded that the most prominent consequence of the increase of the alkyl chain of the cation regarding enzyme activity is the increase of viscosity and not the reduction of polarity \([4\] . On a distinct perspective the increase of the side chain length of hmim [Cl] can be a potant structural element affecting enzyme inhibition. A few articles regarding enzyme inhibition by ILs demonstrated that the most inhibiting compounds exhibit longer side chains \([48-50]\). This is possibly related with steric restrictions caused by the alkyl chain that hinder the access of the substrate to the active site of the enzyme, lowering enzyme activity as was observed in this work.

5. Conclusions

The developed SIA methodology for the kinetic evaluation of \( \beta \)-galactosidase activity proved to be a valuable tool for the study of enzyme behaviour in SDS/IL media. The automated assay enabled the evaluation of enzyme activity in a wide range of surfactant and IL concentrations by means of a simple protocol with reduced consumption of reagents and good repeatability (sd < 6.6%, n = 10), considering the viscosity of SDS/IL mixed micelles.

The obtained results revealed that SDS/IL mixed micelles can be an interesting reaction media for \( \beta \)-galactosidase catalyzed processes, namely for the synthesis of glyco-oligosaccharides. Indeed, enzyme activity can be enhanced up to 55% in the studied mixed micellar media with good selectivity, evidenced by a decrease of \( K_M \). The most promising systems were those incorporating SDS and IL in concentrations under 50 mmol L\(^{-1}\) and 0.5 mol L\(^{-1}\), respectively. It was stated and confirmed that the selected ILs can modify the main characteristics of the micelles in a concentration dependent mode. The enhancement of enzyme activity was then associated not only to a stabilizing effect of ILs due to their hydrogen bond capacity but
also to the observed increase in the micelles size induced by both ILS.

The utilization of SDS/IL mixed micelles in industrial processes catalyzed by β-galactosidase will result in the reduction of the water amount in the reaction media avoiding the shift of the equilibrium towards hydrolysis of the reaction product, a main issue to overcome in this kind of processes. This will led to an enhancement of the process’s yield. Moreover, the utilization of surfactant/IL mixed micelles can be a significant improvement for enzyme catalyzed synthesis mainly regarding the current concerns of Green Chemistry since they enable the substitution of toxic organic solvents by surfactant/IL mixed micellar systems with reduced toxicity guaranteeing enhanced enzyme selectivity and activity. Notwithstanding, much work needs to be done in this field to take advantage of the full potential of surfactant/IL mixed micelles. Thus, studies like this should be extended to other enzymes and surfactant/IL systems in order to create tailored reaction media for specific purposes aiming high yield greener processes.

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References