Enzyme kinetics approach to assess biocatalyst inhibition and deactivation caused by [bmim][Cl] ionic liquid during cellulose hydrolysis

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Abstract

The aim of this work was to study the inhibition and deactivation of commercial enzyme cocktail (Cellic® Htec2) in the presence of [bmim][Cl] ionic liquid employing model cellulosic substrate, carboxymethyl cellulose (CMC). It turned out from the experiements – relying on enzyme kinetics appproach – that [bmim][Cl] could act as a competitive inhibitor. Furthermore, depending on the process conditions i.e. contact of enzyme solution with high concentration [bmim][Cl], severe biocatalyst inactivation should be also taken into account as a potential risk during the enzymatic cellulose hydrolysis even in as short process times as few minutes.

Keywords: lignocellulose, pretreatment, ionic liquid, enzymatic hydrolysis, inhibition, deactivation

1. Introduction

Lignocellulose, as an inexpensive and abundant, renewable material is a trending feedstock for new generation energy production technologies. Among its constituents, cellulose is the most important one to be considered for biotechnological applications (Liu et al., 2012). Cellulose is a well-known polymer molecule, built-up by individual glucose monomers, which represent the primary source of fermentable sugars. Unfortunately, the direct conversion of lignocelluloses to valuable products i.e. biofuels is hindered by the limited access of biocatalysts to the cellulose regions (Kumar et al., 2015; Xu et al., 2016a). Therefore, to open up the complex, recalcitrant structure and enhance the solubilization of such organic matter, a pretreatment is recommended (Vancov et al., 2012). To accomplish this step, the use of ionic liquids (IL) has been widely proposed because of their recognized potential for the efficient structural transformation of the crystalline cellulose, which increases the efficiency of its consecutive enzymatic hydrolysis and thus, the glucose yield (Brandt et al., 2013; Maki-Arvela et al., 2010; Raj et al., 2016). Basically, two main routes to realize enzyme-catalyzed hydrolyzis after dissolving cellulose in IL have been suggested. In the first one, the cellulose is separated from the IL solution and this, so-called regenerated cellulose (having reduced degree of crystallinity and increased porosity) is subjected for subsequent enzymatic hydrolysis (Tan et al., 2011; Zhao et al., 2009). In the other one, referred as *one-pot or single-step* approach, the enzymatic hydrolysis of dissolved cellulose is conducted by adding cellulase enzymes directly to the IL phase in a water-based buffer solution (Gunny et al., 2014; He et al., 2016; Shi et al., 2011;

Xu et al., 2014, 2016b), after which the glucose released can be converted to biofuels or other alternative products such as glucose esters (Findrik et al., 2016).

To make sure that the best IL is chosen for the pretreatment of a particular lignocellulose, a screening is advised (Zavrel et al., 2009) since its appropriateness is dependent on factors such as the physicochemical properties, i.e. the anion and cation constituents of the particular IL (Raj et al., 2016). It was found that among the various ILs, those consisting of an imidazolium-ring, such as [bmim][Cl] are promising candidates to dissolve considerable amount of cellulose (Engel et al., 2012; Tan et al., 2011; Zhao et al., 2009). However, besides that high capacity, ILs should meet additional criteria e.g. biocompatibility with cellulase enzymes performing the cellulose saccharification (Li et al., 2010). In this regard, issues with ILs (including [bmim][Cl]) were observed attributed to their reportedly negative influence on hydrolytic enzyme activity and stability (Engel et al., 2012; Li et al., 2013; Lozano et al., 2011; Ouellet et al., 2011; Park et al., 2012; Salvador et al., 2010; Turner et al., 2003; Xiao et al., 2012; Zhao et al., 2009).

Although the enzyme inhibition and deactivation by ILs from these examples are known to occur, to our knowledge, no thorough study has been dedicated so far to evaluate these phenomena i.e. in terms of the mechanisms involved for the inhibition. Therefore, in this work, comprehensive enzyme kinetics (well-known approach of the biocatalysis area) was applied to get new insights to the enzymatic cellulose hydrolysis process using a model cellulose substrate (carboxymethyl cellulose, CMC) and a commercial enzyme solution (Cellic® Htec2) in the absence and presence of [bmim][Cl], as one of the most widely employed ILs for lignocellulose pretreatment.

The novelty of this investigation is the findings presented for the first time using the enzyme kinetic approach, which can thus have the potential to contribute to the international knowledge and further expansion of the research area regarding the application of ionic liquids in the (ligno)cellulose-based biorefinery concept.

2. Materials and methods

2.1. Enzyme kinetics

2.1.1. Cellulose hydrolysis in the absence of [bmim][Cl] ionic liquid – Michaelis-Menten kinetics

The enzymatic cellulose hydrolysis was considered as a multi-step process: (i) the free (cellulase) enzyme (E) binds to the substrate (S) as reversible reaction (E+S), subsequently (ii) an enzyme-substrate complex (ES) is formed and afterwards, as a results of irreversible (ES) breakdown, (E) and product (P) are released (Zhang et al., 2010), in accordance with the classical Michaelis-Menten kinetics (Johnson and Goody, 2011). This model was thus applied to assess the enzymatic hydrolysis reaction (Yeh et al., 2010) in the absence of [bmim][Cl] ionic liquid (**Eq. 1**).

$$V = \frac{Vmax\ [S]}{Ks + [S]} \quad (1)$$

where V and V_{max} are the actual (initial) and maximal (initial) product formation rates (g product/g enzyme-min), respectively, and *[S]* is the actual (initial) substrate concentration (g/L). K_s is denoted as the half-saturation constant (g/L), equaling to an *[S]* where $V=V_{max}/2$. Experimental conditions i.e. in terms of range of *[S]* tested can be found in Section 2.2.

2.1.2. Cellulose hydrolysis in the presence of [bmim][Cl] ionic liquid – inhibition kinetics

Reversible enzyme inhibition may occur via four different reaction mechanisms such as competitive (**Eq. 2**), uncompetitive (**Eq. 3**), linear-mixed (**Eq. 4**) and non-competitive (special case of Eq.4, where $K_i=K'_i$) (Marangoni, 2003).

$$V = \frac{V_{max}\,[S]}{Ks\left(1+\frac{[I]}{K_i}\right)+[S]} \quad (2)$$

$$V = \frac{V_{max}\,[S]}{Ks+\left(1+\frac{[I]}{K'_i}\right)[S]} \quad (3)$$

$$V = \frac{V_{max}\,[S]}{Ks\left(1+\frac{[I]}{K_i}\right)+\left(1+\frac{[I]}{K'_i}\right)[S]} \quad (4)$$

where K_i and K'_i are the enzyme-inhibitor and (enzyme-substrate)-inhibitor dissociation constants (g/L), respectively, while *[I]* is the actual (initial) inhibitor concentration (g/L).

To determine which of the above is actually involved when an inhibition phenomenon is observed, a comprehensive kinetic study was carried out, requiring sets of experiments at various initial substrate and inhibitor (in this investigation, bmim][Cl] ionic liquid) concentrations (Marangoni, 2003). The related measurement details are specified in Section 2.2.

2.1.3. Enzyme deactivation by undiluted [bmim][Cl] ionic liquid

To study whether [bmim][Cl] ionic liquid – besides its potential inhibitory effect on the cellulose hydrolysis process – causes the deactivation of the enzyme used thoroughly in this work (see below in Section 2.2.), a first-order kinetics was taken (**Eq. 5**). It is a widely used model in the literature to investigate enzyme deactivation (Lencki et al., 1992; Sadana, 1988) and in particular, deactivation of cellulase in the course of cellulose hydrolysis (Zhang et al., 2010).

$$A = \frac{a_t}{a_0} = e^{-k_{de\,1}t} \qquad (5)$$

where the relative activity of the enzyme is *A*. a_t refers to the activity measured in the presence of the ionic liquid (with a particular concentration, herewith 50 mg/L, see Section 2.2.) after treating the enzyme with undiluted ionic liquid for various times (*t*).

147 a_0 *is* the activity measured in the presence of the ionic liquid (with a particular
148 concentration, herewith 50 mg/L) without previous incubation of enzyme with
149 undiluted ionic liquid (t=0, to be taken into account as baseline activity). k_{de1} is
150 denoted as the first-order rate constant of cellulase deactivation (min^{-1}) and e is the
151 exponential term (2.718). By taking the natural logarithm of both sides in **Eq. 5** and
152 plotting ln A against t, k_{de1} is derived from the slope of the straight line (Lencki et al.,
153 1992).
154
155 **2.2. Cellulose hydrolysis assays**
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157 All cellulose hydrolysis measurements were performed in closed-top 250 mL
158 Erlenmeyer flasks (to prevent evaporation) with 100 mL total working volume,
159 ensuring vigorous stirring (400 rpm by magnetic bar) and constant 60 ^{o}C temperature.
160 Citrate buffer (100 mM, pH of 4.5) served as bulk phase thoroughly. The model
161 substrate used was low-viscosity carboxymethyl cellulose (CMC) (Sigma-Aldrich,
162 USA) with phisyco-chemical properties reported in the specification sheet available
163 for download at the manufacturer's official website, while Cellic® Htec2
164 (Novozymes®, Denmark) was utilized as enzyme source, which has an already
165 reported potential for the hydrolysis of cellulosic materials (Benjamin et al., 2014; Joe
166 et al., 2015; Song et al., 2014). Cellic® Htec2 is characterized with optimal working
167 pH and temperature of 4.5-5.5 and 60-75 ^{o}C, respectively (according to the application
168 sheet issued by the producer) and was provided in 600(±48) mg/L concentrations for

each tests. Data were always evaluated by considering the exact, actual mass of enzyme supplemented to the particular reaction vessel.

During the experiments without ionic liquid (Michaelis-Menten kinetics, Section 2.2.1.), substrate (CMC) concentrations investigated were 0.5, 2, 5 and 25 g/L.

In case of enzyme inhibition tests (Section 2.2.2.), definite amounts of [bmim][Cl] (99 % purity, IoLiTec, Germany) were added (without further purification) to the citrate buffer to get the desired ionic liquid concentration (50, 100, 150, 200 and 250 mg/L) along with substrate concentrations of 1, 2.5 and 5 g/L.

In the course of enzyme deactivation study (Section 2.2.3), 60(±4.8) mg enzyme was first mixed with 5 mg (undiluted) [bmim][Cl] ionic liquid and held together for treatment (contact) times such as 1, 2, 3 and 10 minutes at 60 ^{o}C. Subsequently, the whole mixture was loaded to the above described citrate buffer-based reaction medium (resulting in an initial [bmim][Cl] and enzyme concentrations of 50 mg/L and 600(±48) mg/L, respectively) containing 2.5 g substrate/L.

Quantitative analysis of glucose (as final end-product of multi-step cellulose hydrolysis) released from cellulose hydrolysis was monitored based on the refractive index (RI) change of the reaction mixture over time – relative to the RI of initial reaction mixture (background) – pumped continuously (10 mL min^{-1}) through Merck-Hitachi (RI-71) differential refractometer (at 37 ^{o}C), attached to the reaction vessel in a closed-loop design. Preliminary calibration – to establish the relationship of RI change and glucose concentrations – was accomplished by measuring the RI of reaction mixture with well-defined glucose (reagent grade, Sigma-Aldrich, USA) contents.

Experiments were always commenced by the injection of the enzyme solution to the reaction mixture. Kinetic parameters (i.e. K_s, V_{max}, K_i, kd_{e1}) were estimated by linear regression using the least squares method in Matlab software.

3. Results and Discussion

3.1. Cellulose hydrolysis process kinetics without [bmim][Cl] ionic liquid

The progress curves obtained during the measurements can be seen in **Fig. 1**. It seems to infer that product (glucose) formation showed a directly proportional trend with time, as reflected by the appreciably high R^2 values (**Table 1**). Though to some extent, various terms of experimental error e.g. data recording, observations, etc. (Bakonyi et al., 2015) may make the determination of apparent, initial reaction rates vague, replicates undertaken (**Fig. 1**) indicated that the results were fairly reproducible and consequently, basically reliable for further analysis. The confidential evaluation of V is supposed to be performed using a good mass of data acquired within a limited time, otherwise it can be misleading due to fact that initial cellulose hydrolysis rates decline remarkably as time passes (Carrillo et al., 2005). Hence, information recorded in the first 10-15 minutes of the enzymatic hydrolysis tests was only taken into account in this study. Similar period of time was considered by other researchers working in this field, as well (Yeh et al., 2010).

It can be concluded from the time profiles of cellulose hydrolysis experiments (**Fig. 1**) that enhanced V values (slope of the trendlines fitted, **Table 1**) could be achieved along with increasing substrate concentrations. Applying the well-known

Lineweaver-Burk double reciprocal plot (V^{-1} vs. $[S]^{-1}$), the kinetic parameters of the Michaelis-Menten model could be computed and as a result, K_s and V_{max} were found as 2.57 g/L and 0.068 g glucose/g enzyme-min, respectively. The value of K_s is in the same order of magnitude reported by Yeh et al. (2010) for a range of microcrystalline cotton cellulose substrates and a cellulase enzyme produced by *T. reesei* ATCC 26921.

Generally speaking, the use of Michaelis-Menten kinetics for discussing the experimental data requires a "mass of enzyme to mass of substrate ratio" <0.15, which ensures that the mechanistic model is applicable (Bezerra and Dias, 2004, 2005) and the substrate is not limiting. In our current investigation, apparent "mass of enzyme mass to mass of substrate ratios" such as 0.024, 0.12, 0.3 and 1.2 were obtained, being solely dependent on the initial substrate concentration because of the constant enzyme loadings (600 mg/L). Although seemingly not all of them meet the criteria referred, a decent correlation of the experimental outcomes with the fitted Michaelis-Menten kinetics was established. This contradiction can be explained by considering the properties of the enzyme source. Cellic® Htec2 is a commercial enzyme cocktail preparation and not a highly purified cellulase. Such products can be seen as a mixture of accessory enzymes such as cellulases and hemicellulases (Samayam and Schall, 2010), however, their exact composition and relative proportion of ingredients is unknown (Barr et al., 2012). Thus, once a given mass of this enzyme solution is used (e.g. 600 mg for every liter of reaction mixture in this study), only its limited portion was actually the cellulase enzyme participating in the hydrolysis reaction, while the rest consisted mainly of water, other enzymes i.e. endoxylanases (Benjamin et al., 2014; Joe et al., 2015; Song et al., 2014), etc.

Consequently, the real "cellulase enzyme mass to substrate mass ratio" was (probably orders of magnitudes) below the above defined threshold value (<0.15) even in the lower initial substrate concentration range (0.5-2 g/L), making the Michaelis-Menten theory valid within the respective experimental boundaries and leading to an adequate prediction of the hydrolysis process.

3.2. On the inhibition of enzymatic cellulose hydrolysis by [bmim][Cl] ionic liquid

It turned out in previous works examining cellulose hydrolysis in the mixture of aqueous buffer and IL that the cellulose hydrolysis can be negatively affected by ionic liquids (Kamiya et al. (2008; Turner et al., 2003). For instance, Kamiya et al. (2008) summarized that biocatalyzed cellulose saccharification (applying commercialized cellulase from *Trichoderma reesei*) was fully stopped in a citrate-buffer based reaction mixture composing of >40 vol.% [emim][dep], meaning that the proportion of IL is a determining factor of the process. Moreover, another research carried out by Turner et al. (2003) also confirmed the existence of IL-induced enzyme inhibition working with [bmim][Cl], which was associated with the high, dissociated Cl^- ion concentration in the media, causing the interference of cellulose with ionic liquid.

Overall, it is clear by the literature examples that enzyme-mediated cellulose hydrolysis can be sensitive to the presence of ILs, including [bmim][Cl] employed thoroughly herewith. However, it is still of question which of the possible inhibition mechanisms (i.e. competitive, uncompetitive, mixed, non-competitive) stands behind

once decreased reaction rates (as a side-effect of IL) are encountered. Therefore, a throughout kinetic study was devoted to get new insights on this subject.

The analysis of the progress curves and consecutive use of Lineweaver-Burk double reciprocal technique (Lineweaver and Burk, 1934) (V^{-1} vs. $[S]^{-1}$, along with various inhibitor concentrations *[I]*, 50-250 mg [Bmim][Cl]/L) yielded **Fig. 2**, which is a clear identification of the competitive-type inhibition (Marangoni, 2003). Thus, in the light of this diagnosis, the deviation of initial reaction velocities from those projected by the Michaelis-Menten model (no inhibition occuring) took place by the mechanism established in **Eq. 2**. When the data in **Fig. 2** are subjected for an estimation of the catalytic parameters, it can be observed that V_{max} remains unaffected in accordance with the feature of competitive inhibition, meanwhile K_S is influenced by the inhibitor concentration (*[I]*) and the enzyme-inhibitor dissociation constant (K_i) (**Eq. 2**). Since the experimental results suggest competitive inhibition caused by the [bmim][Cl] ionic liquid, it means that the inhibitor competes for the substrate-binding site of the enzyme and when an enzyme-inhibitor complex is formed rather than an enzyme-substrate one, the enzyme cannot express its catalytic activity. Overall, it seems to be the case that the cellulase enzymes present in Cellic® Htec2 suffer from an inhibition in the presence of [bmim][Cl], even at low *[I]*. General strategies to suppress this type of inhibition include the use of higher enzyme as well as substrate loadings to increase the probability of enzyme and substrate interactions rather than that of the enzyme and the inhibitor. In summary of the kinetic analysis on enzyme inhibition tests (**Fig. 2**, **Table 2**), numerical estimate for K_i was obtained as 0.163 g/L. In general, the increase of $[I]/K_i$ will more notably reduce the affinity of the enzyme to

284 the substrate, causing the concomitant raise of K_S. For instance, once the [bmim][Cl]
285 concentration in the reaction mixture reaches to K_i, it will double the original
286 (uninhibited) K_S value, in accordance with the mechanistic model for competitive
287 inhibition in **Eq. 2**.
288
289 ### 3.3. Cellulase enzyme deactivation by [bmim][Cl] ionic liquid
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291
292 The important work done by Turner et al. (2003) implied that not only an
293 inhibition, but even deactivation of cellulase may occur when they are contacted with
294 ionic liquids in the solution. As it was deduced, enzyme denaturation can be a possible
295 threat and in such a case, overcoming strategies may be unable to refold, reactivate the
296 cellulase. Deactivation (or in other words, irreversible inhibition) is one of the major
297 phenomena known to slow the cellulose hydrolysis rate down (Bansal et al., 2009),
298 leading essentially to a process of limited efficiency.
299 To judge whether the inactivation of cellulase enzymes in Cellic® Htec2 is to be
300 taken into account, tests (in accordance with Section 2.2.) were undertaken and the
301 enzyme solution was mixed and incubated with [bmim][Cl] for various durations,
302 followed by the determination of actual, initial reaction rates to reveal residual enzyme
303 activity. It can be drawn from **Fig. 3** that the treatment time of enzyme by IL
304 considerably affected the achievable V values, providing a good indication that contact
305 of the cellulase with undiluted [bmim][Cl] – even when it happened for short times
306 only e.g. 1 min – was accompanied by the relative loss of enzyme activity. Taking the
307 advantage of the formula expressed in **Eq. 5**, kinetic analysis was conducted to
308 compute the rate constant of deactivation. Since in the bottom part of **Fig. 3** a well-

309 fitting linear relationship can be found, the first-order kinetics to predict biocatalyst
310 deactivation seems to be valid (Lencki et al., 1992) and k_{de1} as 0.132 min^{-1} could be
311 determined. A correlation of similar shape between relative cellulase activity and
312 various residence times in 10 % [bmim][Cl] was communicated by Salvador et al.
313 (2010), as well. However, in that investigation (Salvador et al., 2010), residual enzyme
314 activities were much closer to the original, since even after 40 minutes of contact with
315 IL, reduction was only 13-14 %, suggesting that the inhibition was mostly of
316 reversible nature and ascribed primarily to the change of thermodynamic water activity
317 under the different conditions. However, it was also shown that increased IL
318 concentrations are severely disadvantageous from an enzyme activity point of view
319 (Salvador et al., 2010; Xiao et al., 2012). Significant loss of Celluclast® cellulase
320 enzyme activity was observed by Engel et al. (2010) too in reaction mixtures
321 comprising of commercialized ionic liquids (10 %), including [bmim][Cl].

322 Keeping this in mind together with our results, it would appear that when
323 cellulase enzymes (even for short times) are exposed to concentrated ILs, deactivation
324 effect may take over. This would explain why in our experiments the reaction rates
325 have notably dropped, causing that the enzyme lost approximately 25 % of its activity
326 relatively to its control (a_0) even after only 1 min of pre-incubation with undiluted
327 [bmim][Cl]. This phenomenon can be interesting when cellulase hydrolysis is
328 performed *in situ*, referred as *one-pot* process design. In this arrangement, the
329 cellulosic raw material is first pretreated with concentrated ionic liquid to help the
330 dissolution of the polysaccharide fractions and in that way, provide better accessibility
331 for the enzymes participating in their hydrolysis. Thereafter, the hydrolytic enzymes –

carried by an aqueous buffer solution – are loaded to the same vessel containing the cellulose being dissolved in the IL (Shi et al., 2013). Many ionic liquids, depending on their structural features, are hydrophilic (including [bmim][Cl]) and therefore soluble in water to certain degrees (Huddleston et al., 2001). In these cases, ILs can be more or less homogenously distributed in the whole reaction medium, which makes it possible to decrease their concentrations to a sufficiently low threshold level. However, appropriate dilution factor should be selected since even its low concentrations could exhibit a hindering impact on the hydrolysis for the particular example of [bmim][Cl] used in this work, as demonstrated by our results in Section 3.2. Besides dilution method (Li et al., 2013), the engineering and application of cellulases with satisfactory robustness to work under harsh conditions can be proposed (Nordwald et al., 2014; Raddadi et al., 2013) i.e. via the development of halophilic, IL-tolerant cellulases (Gunny et al., 2014; Xu et al., 2014; 2016b), their immobilization (Xu et al., 2016c) which may better withstand the negative impact of ILs. In other cases, when the IL is basically immiscible with the water-based solution, a biphasic (separated) system is formed (Kuroda et al., 2016), and saccharification can take place on the phase boundary, where the probability of an enzyme-concentrated IL encounter is higher, representing a threat on the time-stability of the process. For cellulose saccharification and subsequent utilization technologies relying on regenerated cellulose, ionic liquid residues should be removed as much as possible to avoid inhibition by a washing process (Li et al., 2013; Ouellet et al., 2011)

Conclusions

In this work, the effect of [bmim][Cl] ionic liquid on cellulose hydrolysis catalyzed by Cellic® Htec2 enzyme solution was studied. It was found that the enzymatic reaction could be fairly described by the Michaelis-Menten kinetics when [bmim][Cl] ionic liquid was absent. However, the presence of this IL in low concentrations significantly hindered the process via competitive inhibition, supported by the kinetic evaluation. Furthermore, it was demonstrated that contacting the enzyme with highly-concentrated IL even for short times could induce irreversible inhibition (deactivation), which should be considered as an important aspect of technology design.

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522

523

Figure Legends

Fig. 1 – Typical progress curves of cellulose hydrolysis (actual experimental conditions: absence of [bmim][Cl] ionic liquid, varied initial substrate concentration)

blue diamond and red square: [S]=0.5 g/L; green triangle and purple cross: [S]=2 g/L; blue asterisk and orange dot: [S]=5 g/L; pink dash: [S]=25 g/L

Fig. 2 – Kinetic evaluation on enzyme inhibition test results obtained during cellulose hydrolysis with different initial [bmim][Cl] ionic liquid (inhibitor) and substrate concentrations

blue diamond: no [bmim][Cl] added; red square: 50 mg/L [bmim][Cl]; green triangle: 100 mg/L [bmim][Cl]; purple cross: 150 mg/L [bmim][Cl]; blue asterisk: 200 mg/L [bmim][Cl]; orange dot: 250 mg/L [bmim][Cl]

Fig. 3 –Kinetic evaluation on enzyme deactivation experiments

blue dots: initial reaction rate; red square: natural logarithm of relative enzyme activity

Table 1 – Example for progress curve analysis to deliver the initial reaction rates (*V*) using the dataset of Fig. 1.

	Fitted trendline properties				
	1. repetition		2. repetition		
[S] (g/L)	slope (g glucose/g enzyme-s)	R^2	slope (g glucose/g enzyme-s)	R^2	*V* (g glucose/g enzyme-min)
0.5	0.000180	0.98	0.000181	0.91	0.011
2	0.000430	0.19	0.000425	0.95	0.026
5	0.000837	0.99	0.000843	0.99	0.051
25	0.001013	0.99			0.060

546 Table 2 – Characteristics of fitted trendlines (Fig. 2) as the function of [bmim][Cl]
547 concentration to estimate the maximal initial reaction rate (*Vmax*) and enzyme-
548 inhibitor dissociation constant (K_i) for competitive inhibition model. The
549 corresponding $[S]^{-1}$ range studied is presented in Fig. 2.

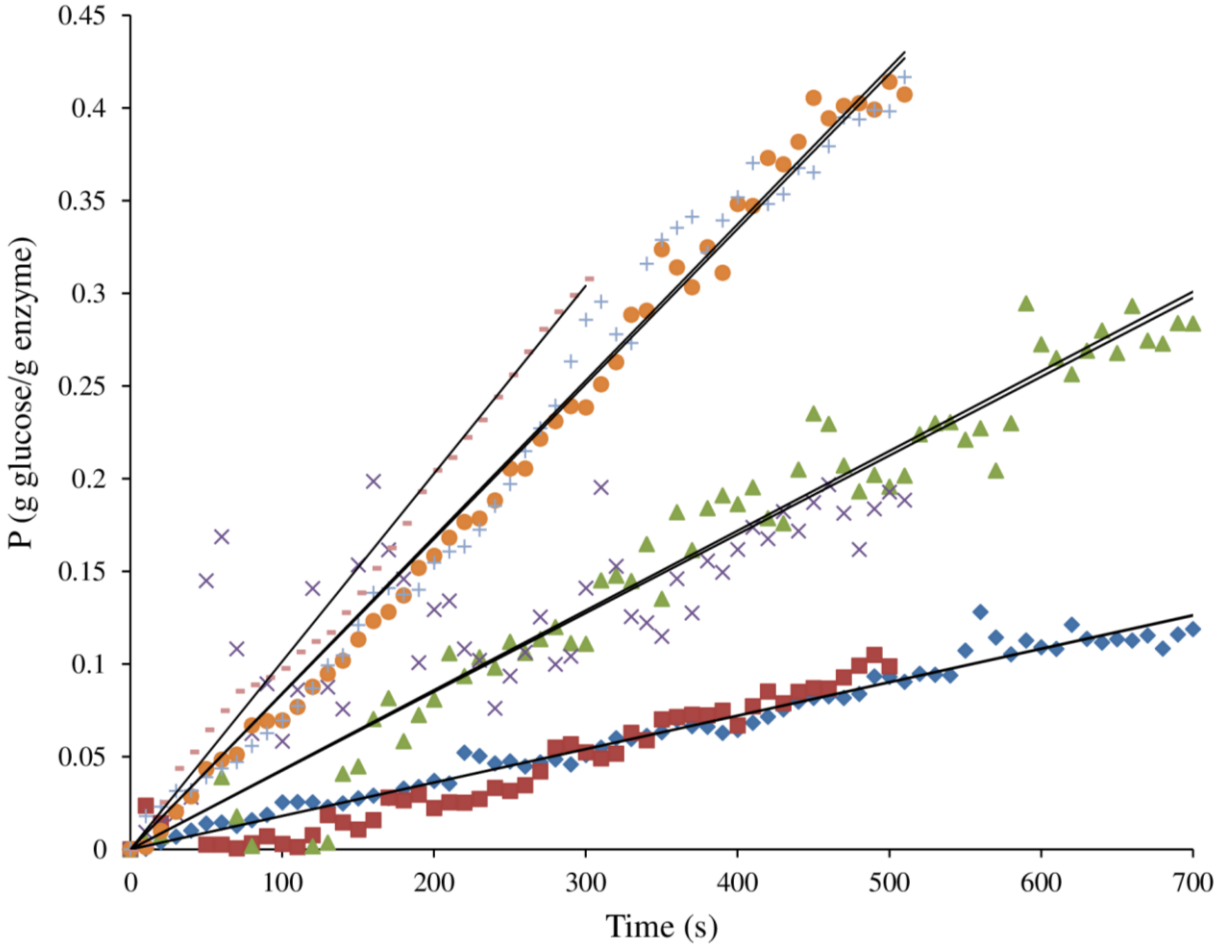
550

	[bmim][Cl] concentration (mg/L)				
	50	100	150	200	250
slope	45.71	54.71	63.99	81.04	96.27
intercept	14.82	14.82	14.82	14.82	14.82
R^2	0.98	0.97	0.94	0.99	0.99

551

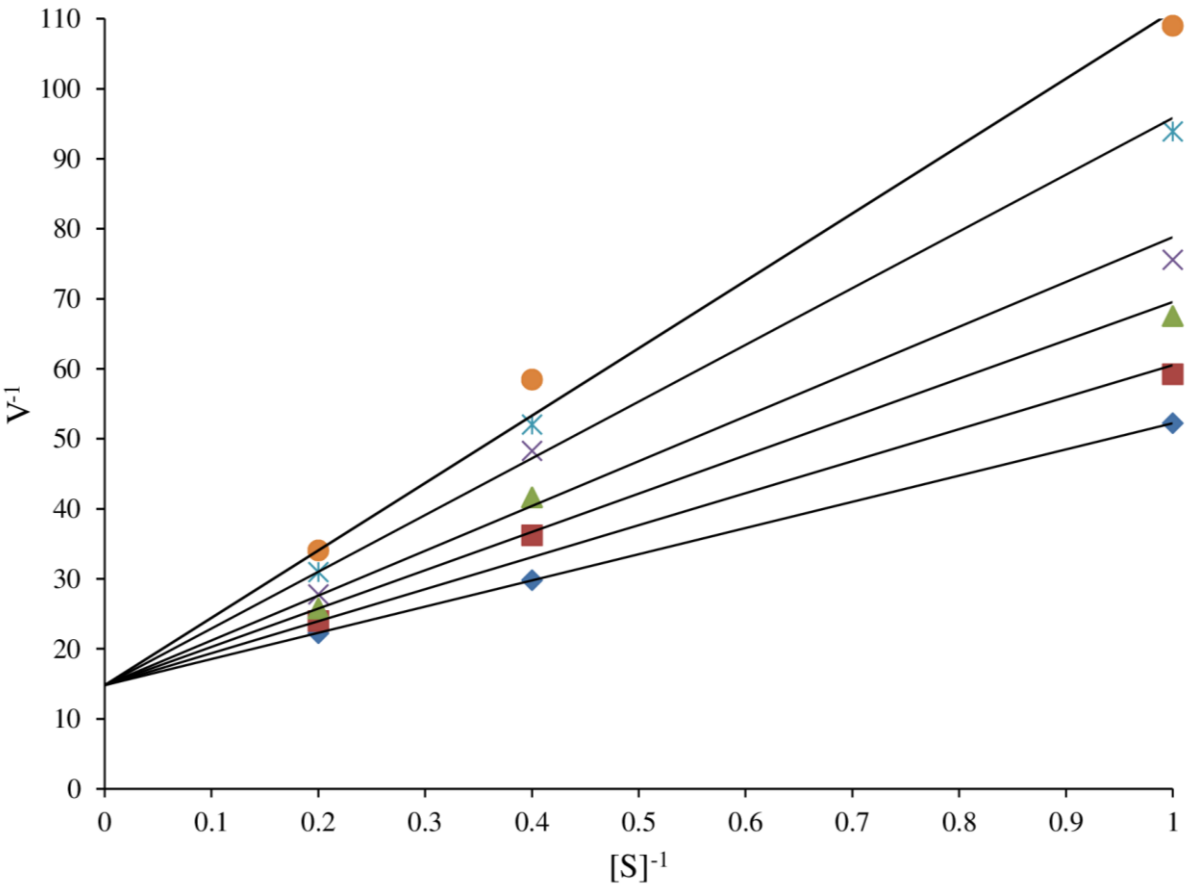
552

553 **Fig. 1**



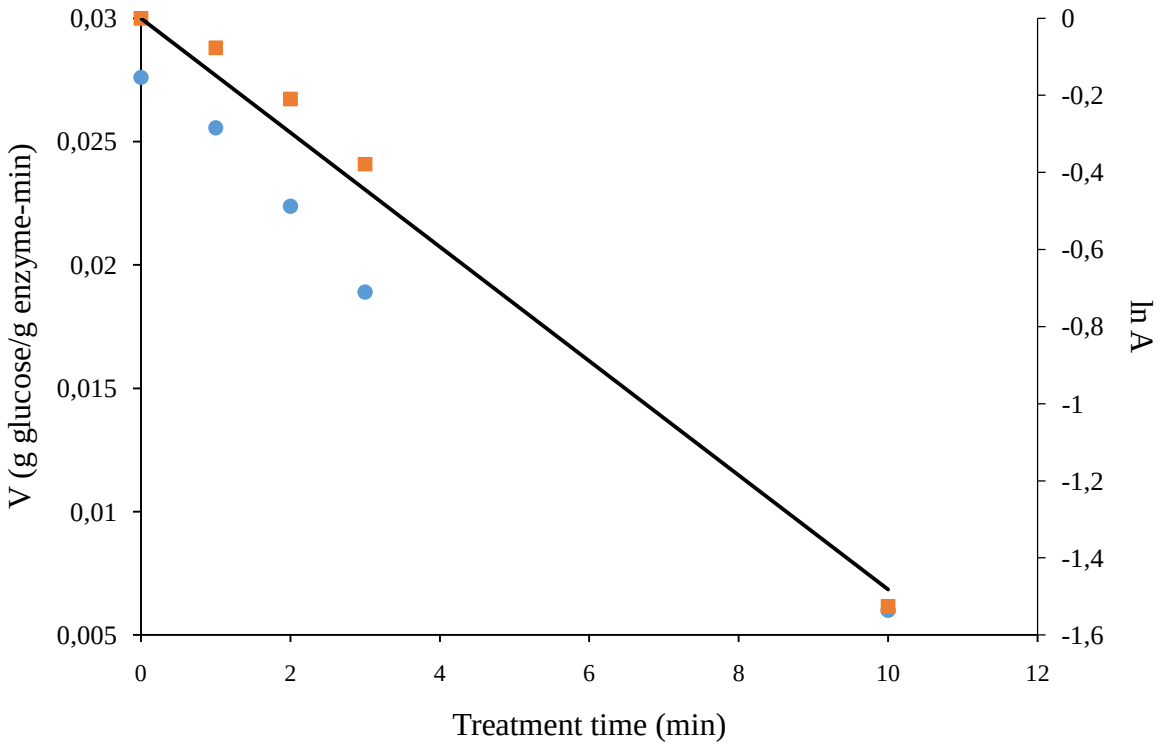
554
555

556 **Fig. 2**



557

558 **Fig. 3**


0,03
0,025
0,02
0,015
0,01
0,005
V (g glucose/g enzyme-min)
0
-0,2
-0,4
-0,6
-0,8
-1
-1,2
-1,4
-1,6
ln A
0
2
4
6
8
10
12
Treatment time (min)

559