Non-productive adsorption of bacterial β -glucosidases on lignins is electrostatically modulated and depends on the presence of fibronectin type III-like domain

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ABSTRACT

Non-productive adsorption of cellulases onto lignins is an important mechanism that negatively affects the enzymatic hydrolysis of lignocellulose biomass. Here, we examined the non-productive adsorption of two bacterial β -glucosidases (GH1 and GH3) on lignins. The results showed that β -glucosidases can adsorb to lignins through different mechanisms. GH1 β -glucosidase adsorption onto lignins was found to be strongly pH-dependent, suggesting that the adsorption is electrostatically modulated. For GH3 β -glucosidase, the results suggested that the fibronectin type III-like domain interacts with lignins through electrostatic and hydrophobic interactions that can partially, or completely, overcome repulsive electrostatic forces between the catalytic domain and lignins. Finally, the increase of temperature did not result in the increase of β -glucosidases adsorption, probably because there is no significant increase in hydrophobic regions in the β -glucosidases structures. The data provided here can be useful for biotechnological applications, especially in the field of plant structural polysaccharides conversion into bioenergy and bioproducts.

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1. Introduction

Lignocellulose biomass is a complex material composed of cellulose, hemicellulose, and lignin with an approximate ratio of 2:1:1, which provides a useful starting feedstock for industrial processes [1,2]. Cellulose is a recalcitrant biopolymer composed of linear chains of D-glucose linked by β -1,4-glycosidic bonds, whereas hemicelluloses are linear or branched heteropolysaccharides [1]. The third main component of the biomass, lignin, is an aromatic complex polymer composed of phenylpropane units, that is, guaiacyl propanol (G), syringyl propanol (S), and *p*-hydroxyphenyl propanol (H) [3,4]. The lignin composition varies from plant to plant, and the proportion of G and S units differ significantly in softwood and hardwood [3]. Softwood lignin is principally composed of G units, while both G and S units are present in hard-

wood. Furthermore, H units in minor amounts, associated with G and S units, are also present in grasses and compression wood lignin [3].

The bioconversion of lignocellulose biomass into bioethanol normally involves three main steps: (i) a pretreatment to decrease substrate recalcitrance and partially fractionate the biomass, (ii) enzymatic hydrolysis of the cellulose and hemicellulose to hexoses (as glucose) and pentoses, and (iii) fermentation of simple sugars into ethanol. Several pretreatment technologies are currently employed to remove selectively hemicellulose and lignin from the lignocellulose biomass [2,5–7]. For example, untreated sugarcane bagasse contains about 35% of cellulose and similar amounts of hemicellulose (25%) and lignin (22%). After acid pretreatment using 1% H₂SO₄, most of the hemicellulose fraction was removed (25–7.8%), however, the lignin relative percentage in the sample increased slightly (22–30%). Then, after basic pretreatment using NaOH, under concentrations between 0.5% and 2%, the lignin relative percentage decreased progressively from 30% to 10% [2].

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Fig. 1. Three-dimensional models of the β -glucosidases from *Thermotoga petrophila* and zeta-potential analysis. (A) β -Glucosidase GH1 from *T. petrophila* (*TpBGL1*). (B) β -Glucosidase GH3 from *T. petrophila* (*TpBGL3*). The C-terminal FnIII-like domain is highlighted in black. The three-dimensional models of the *TpBGL1* and *TpBGL3* were built by SWISS Model Server [35] using as template the available structures of the β -glucosidases from *Thermotoga maritima* (*TmBGL1*, PDB 1OIN, 99% sequential identity) [14] and *Thermotoga neapolitana* (*TnBGL3*, PDB 2 $\times$ 41, 88% sequential identity) [16], respectively. (C) Zeta-potential of the β -glucosidases (*TpBGL1* and *TpBGL3*) and lignin preparations (SL and EL) as a function of pH.

The complete enzymatic hydrolysis of cellulose to glucose requires at least three types of enzymes working synergistically: endoglucanases (EC 3.2.1.4) that randomly cleave the β -1,4-glycosidic linkages of cellulose, cellobiohydrolases (EC 3.2.1.91) which cleave off cellobiose units from the reducing or nonreducing end of cellulose chain, and β -glucosidases (EC 3.2.1.21) responsible for hydrolysis of the released cellobiose to glucose [8–10]. Endoglucanases and cellobiohydrolases are frequently inhibited by cellobiose, therefore, β -glucosidases are essential to avoid the decrease the hydrolysis rates of cellulose over time due to cellobiose accumulation [10].

The presence of residual lignin after pretreatments may affect negatively the enzymatic hydrolysis of cellulose to glucose. Cellulases bind to lignin, deactivating the enzymes and reducing the overall enzymatic activities [3,4,11]. In this study, we examined the non-productive adsorption of two β -glucosidases from *Thermotoga petrophila* [12,13], belonging to the families GH1 and GH3, on the lignin preparations from both sugarcane (grasses) and eucalyptus (hardwood). These two β -glucosidases (*TpBGL1* and *TpBGL3*) are enzymes with potential for applications in the field of plant structural polysaccharides conversion into bioenergy and bioproducts [12]. *TpBGL1* has a common (β/α)₈-barrel structure, while *TpBGL3* is arranged in three distinct domains (Fig. 1) [13–15]. The N-terminal domain is an (α/β)₈ triose phosphate isomerase (TIM) barrel, followed by a five-stranded α/β sandwich domain, and the

C-terminal domain has a fibronectin type III fold (FnIII-like domain) [16–18].

2. Materials and methods

2.1. Materials

Imidazole, kanamycin, LB medium, isopropyl- β -D-thiogalactopyranoside (IPTG), nickel-nitrilotriacetic acid resin (Ni-NTA) and 4-nitrophenyl- β -D-glucopyranoside (pNPG) were purchased from Sigma-Aldrich. Protein standards used as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) markers were purchased from Sigma. All chemicals and reagents used in this study were of the highest purity analytical grade.

2.2. Expression and purification of recombinant β -glucosidases

The expression and purification of recombinant β -glucosidases were carried out as described previously with minor modifications [13]. Briefly, 1 L of LB medium containing kanamycin (50 μ g/mL), inoculated with *Escherichia coli* BL21(DE3) containing the expression plasmid construct, was incubated at 37 $^{\circ}$ C in shake flasks until attaining an optical density of 0.5 at 600 nm. IPTG was added to a final concentration of 0.5 mM, and incubation continued at 37 $^{\circ}$ C for approximately 4 h. After incubation, the cells were immediately harvested by centrifugation at 5000 $\times$ g for 20 min at 4 $^{\circ}$ C, resuspended in 20 mM acetate–borate–phosphate buffer adjusted at pH 7.4, and ruptured by sonication. The suspension was then centrifuged at 5000 $\times$ g for 20 min at 4 $^{\circ}$ C to separate the cell debris. The supernatant was then heat treated at 70 $^{\circ}$ C during 30 min, to precipitate heat-labile proteins, and centrifuged again at 5000 $\times$ g for 20 min and 4 $^{\circ}$ C. Finally, the supernatant containing the recombinant β -glucosidase (*TpBGL1* or *TpBGL3*) was applied to a nickel-affinity column equilibrated with 20 mM acetate–borate–phosphate buffer adjusted at pH 7.4. The recombinant β -glucosidase was eluted from the column with the same buffer containing 150 mM imidazole. The purity of the final products was verified by 15% SDS-PAGE. The resulting β -glucosidases were exhaustively dialyzed in 20 mM acetate–borate–phosphate buffer adjusted at pH 7.4 for elimination of imidazole. The concentrations of the recombinant β -glucosidases were determined by UV absorbance at 280 nm using a theoretical extinction coefficient based on the amino acid composition. The extinction coefficients (ϵ) were calculated for the monomers using the ProtParam tool [19]. The theoretical coefficients employed were $\epsilon_{280\text{nm}} = 121,240 \text{ M}^{-1} \text{ cm}^{-1}$ for *TpBGL1* and $\epsilon_{280\text{nm}} = 102,930 \text{ M}^{-1} \text{ cm}^{-1}$ for *TpBGL3*. The final β -glucosidases were stored at 4 $^{\circ}$ C and used until five days after the purification.

2.3. Preparation of lignins from sugarcane (SL) and eucalyptus (EL)

The lignin preparations from sugarcane and *Eucalyptus* spp. were isolated as described previously [20]. Briefly, lignins were precipitated from Kraft black liquors at pH 5 as described below. For sugarcane, the Kraft black liquors were oven-dried at 30 $^{\circ}$ C till become powder. For *Eucalyptus* spp., the Kraft black liquors (pH 14) were heated at 55–65 $^{\circ}$ C. Subsequently, the pH values of the liquors were slowly acidified with 3 M H₂SO₄. After reaching the desired pH, the liquors were incubated undisturbed during 12 h in the dark at room temperature. After the incubation period, the supernatants were discarded and the solid lignins were collected, dried at 55–60 $^{\circ}$ C, and stored at room temperature and isolated from interaction with light.

2.4. Electrophoretic light scattering (ELS)

ELS measurements were used to determine the average zeta-potential (ζ) of the β -glucosidases and lignin preparations, which were collected using a Zetasiser nano-ZS at 20 $^{\circ}$ C [21]. The zeta-potential were measured using fixed concentrations of enzymes (*TpBGL1* and *TpBGL3*) and lignin preparations (SL and EL) of 1 mg/mL in 20 mM acetate–borate–phosphate buffer adjusted to the different pH values. This instrument measures the electrophoretic mobility (μ_e) and converts the value to a zeta-potential (mV) through Henry's equation: $\mu_e = [2\epsilon\zeta F(\kappa a)]/3\eta$, where ϵ is the dielectric constant of water and η is the viscosity [21]. Furthermore, $F(\kappa a)$ is the Henry's function, which was calculated through the Smoluchowski approximation with $F(\kappa a) = 1.5$. The isoelectric point is given by the pH value at which the zeta-potential is approximately zero.

2.5. β -Glucosidases adsorption

Adsorption analyses were performed by pulling down β -glucosidases, after incubation with different lignins concentrations, and measuring its residual enzymatic activity in the supernatants [18]. The adsorptions of the β -glucosidases, *TpBGL1* or *TpBGL3*, to lignins were conducted with 6 μ M of enzyme in 20 mM acetate–borate–phosphate buffer adjusted to different pH values, and different lignin preparations (SL or EL) at concentrations varying from 0 to 25 mg/mL. The samples (vol. 1 mL) were incubated at 20 $^{\circ}$ C or 70 $^{\circ}$ C for 2 h under continuous agitation and subsequently centrifuged at 14,000 $\times$ g for 20 min. The β -glucosidase activity in the supernatant was measured on pNPG as described below. β -glucosidases (6 μ M) also were incubated with lignins (15 mg/mL) in the presence of surfactant (1% Triton X-100). Controls lacking β -glucosidases or lignin preparations

were used as reference. For each analysis, two independent experiments were done with enzymes purified in different days. Each independent experiment was done in triplicate, and the averaged values are reported. In addition, the free β -glucosidases content in the supernatant (after incubation with different lignin concentrations) were determined employing the method described by Bradford [22]. The Bradford assays were carried out in triplicate.

2.6. β -Glucosidase activity

The standard enzymatic assay for β -glucosidase activity was performed according to previously described method [12,23]. Briefly, 100 ng of enzyme, 40 μ L of buffer solution adjusted at different pH values (pH 6 for *TpBGL1* and pH 4 for *TpBGL3*) were used and 50 μ L of 1 mM 4-nitrophenyl- β -D-glucopyranoside (pNPG) was used as substrate. Following incubation at 70 °C during 4 min, the reaction was stopped by addition of 100 μ L of 1 M Na₂CO₃ and the releasing of *p*-nitrophenol was monitored colorimetrically at 405 nm using a microplate reader. One unit of β -glucosidase activity was defined as the amount of enzyme that released *p*-nitrophenol at rate of 1 μ mol min⁻¹ under standard conditions. All β -glucosidase assays were performed in triplicate, and the averaged values are reported.

3. Results

3.1. Influence of pH on the net charge of the β -glucosidases and lignins

Zeta-potential, which is a measurement of the charge that a molecule acquires in particular environment, is an important parameter for understanding electrostatic colloidal dispersion [13,21]. In order to better understand the influence of pH on the net charge, zeta-potential of the β -glucosidases and lignin preparations were determined as a function of pH (Fig. 1C). As can be seen in Fig. 1C, the net charge on β -glucosidases (*TpBGL1* and *TpBGL3*) is affected by the pH as recently demonstrated [13]. The enzymes can become more negatively or positively charged due to the loss or gain of protons, respectively. At pH 7 and 6, both β -glucosidases acquire very similar negative charges, while at pH 4 the β -glucosidases acquire positive charge. Likewise, the net charge of sugarcane lignin (SL) and eucalyptus lignin (EL) preparations are also pH-dependent, however, both acquire negative charges in the entire studied pH range (Fig. 1C). Previous studies showed that lignins isolated from others sources also acquired negative charges at pH values below 7 [24,25]. For SL preparation, the negative charges were found to increase with increasing of pH value. At pH 4, the negative charge was significantly higher in EL preparation when compared to SL preparation.

3.2. Influence of pH on the β -glucosidases adsorption to lignins

The adsorption of β -glucosidases to different lignin preparations were analyzed as a function of pH at 20 °C. Fig. 2 shows the adsorption of *TpBGL1* (6 μ M) as a function of the sugarcane lignin (SL) concentration at different pH values. For pH values of 7 and 6, the results showed very low adsorption of *TpBGL1* on the SL at concentrations up to about 25 mg/mL (Fig. 2A and B). At pH 4, only a low adsorption of *TpBGL1* was observed. The supernatant residual β -glucosidase activity remained around 70% even at the highest SL concentration used in our experiment, indicating low capacity of *TpBGL1* binding to the SL at pH 4 (Fig. 2C). The adsorption of *TpBGL1* as a function of the eucalyptus lignin (EL) concentration at different pH values is also shown in Fig. 2. At pH 7 and 6, the adsorption of *TpBGL1* is very low, even at high concentrations of EL (Fig. 2D and E). However, at pH 4 very strong adsorption of *TpBGL1* on the EL can be observed at concentrations above 1 mg/mL (Fig. 2F). In addition, the free *TpBGL1* content in the supernatant was also monitored as a function of the SL and EL concentrations (Supplemental Fig. 1) employing the method described by Bradford [22]. At pH 7 and 6 were not observed significant changes in *TpBGL1* content even at high concentrations of SL and EL. However, at pH 4 significant changes in the protein concentrations were observed. At pH 4 the

free *TpBGL1* content in the supernatant was reduced dramatically at EL concentrations above 1 mg/mL. These results are in agreement with the β -glucosidase activities described above.

The adsorption of *TpBGL3* (6 μ M) as a function of the SL concentration at different pH values is given in Fig. 3. At all pH values, the *TpBGL3* enzymatic activity in the supernatant decreased significantly with the increase of SL concentrations applied to the solutions, indicating strong adsorption of *TpBGL3* on the SL. At the highest SL concentration used in our experiment, the supernatant relative residual activities observed at all pH values were between 10% and 30% (Fig. 3A, B and C). Fig. 3 also shows the adsorption of *TpBGL3* as a function of the EL concentration at different pH values. At all pH values, the results showed strong adsorption of *TpBGL3* on the EL. At pH 6 and 7, the supernatant relative residual activities of 30% were observed at the highest EL concentration used. At pH 4 very strong adsorption of *TpBGL3* on the EL at concentrations above 1 mg/mL can be observed (Fig. 3F). At pH 4 and for higher EL concentrations the relative residual activity measured for the supernatant was of about 10%. In addition, the free *TpBGL3* content in the supernatant was also monitored as a function of the SL and EL concentrations (Supplemental Fig. 2). At all pH values, the free *TpBGL3* content in the supernatant showed a progressively reduction with increasing lignins concentration. These results are in agreement with the β -glucosidase activities described above. Our data are in agreement with recent studies where another GH3 β -glucosidase (*Aspergillus niger* β -glucosidase, a highly important cellulase) preferentially adsorbs to lignin [18,26,27].

3.3. Influence of surfactant and temperature on the β -glucosidases adsorption to lignins

Previous studies reported that nonionic surfactants could reduce enzyme adsorption because the hydrophobic portion of the surfactant binds to lignin [26,28,29]. Therefore, we examined the non-productive adsorption of β -glucosidases on the lignin preparations in the absence and presence of surfactant. Fig. 4 shows the adsorption of *TpBGL1* and *TpBGL3* to SL and EL preparations (15 mg/mL) in the absence and presence of surfactant Triton X-100 (TX) at 20 °C. For *TpBGL1*, the most significant change was observed at pH 4 (Fig. 4B), where the addition of 1% TX reduced significantly the adsorption of *TpBGL1* on the EL preparation (from 10% to about 50%). Thus, suggesting that at pH 4 the hydrophobic interactions play an important role in non-productive adsorption of *TpBGL1* on the EL. The decrease in *TpBGL1* adsorption at pH 4, therefore, was most likely due to the competitive adsorption of TX onto hydrophobic EL surface. For *TpBGL3*, at all pH values, the addition of 1% TX also reduced the adsorption of *TpBGL3* on the SL and EL preparations (Fig. 4C and D), which suggests that hydrophobic interactions also contribute to the non-productive adsorption of *TpBGL3* on the lignins.

Previous studies showed that an increase of temperature resulted in an increase in the adsorption of cellulases onto lignins, probably due to increase of hydrophobic interactions [30–32]. Therefore, the effect of temperature on bacterial β -glucosidases adsorption to SL and EL preparations (5 mg/mL) were evaluated by performing the adsorption experiments at 20 °C and 70 °C (Fig. 5). For *TpBGL1* and *TpBGL3*, similar adsorption profiles were observed at both temperatures.

4. Discussion

4.1. *TpBGL1* adsorption is electrostatically modulated

Lignin adsorption is a major inhibitory mechanism of cellulases, xylanases, and β -glucosidases [3,11]. The enzyme adsorption

Fig. 2. Effect of pH on *TpBGL1* adsorption on the SL and EL. Adsorption of *TpBGL1* (6 μ M) as a function of the SL or EL concentration (0–25 mg/mL) at three different pH values.

Fig. 3. Effect of pH on *TpBGL3* adsorption on the SL and EL. Adsorption of *TpBGL3* (6 μ M) as a function of the SL or EL concentration (0–25 mg/mL) at three different pH values.

mechanism can be induced by electrostatic and hydrophobic interactions [4,11,26,33]. Furthermore, lignin sources affect enzyme adsorption using structural features and lignin composition [3]. *TpBGL1* binding to SL and EL preparations were found to be strongly pH-dependent, suggesting that electrostatic forces are involved in enzyme-lignins interactions (Fig. 2). In general, *TpBGL1* binding decreased with increasing pH value. Both β -glucosidases

were negatively charged at pH 7 and 6, while at pH 4 the enzymes acquired positive charge (Fig. 1C). However, both lignin preparations were negatively charged in the entire pH range studied. Hence, very low *TpBGL1* adsorption at pH 7 and 6 may be explained by repulsive electrostatic interactions between lignin preparations and *TpBGL1* (Fig. 2). Furthermore, strong *TpBGL1* adsorption at pH 4 may be explained by attractive electrostatic interactions between

Fig. 4. Effect of surfactant (1% Triton X-100) on *TpBGL1* and *TpBGL3* (6 μ M) adsorption on the lignin preparations (15 mg/mL).

lignin preparations (SL and EL) and *TpBGL1* (Fig. 2C and F). At pH 4, EL preparation is significantly more negative charged when compared to SL preparation (Fig. 1C). This difference may, at least partially, explain why more *TpBGL1* was bound onto EL preparation.

4.2. The C-terminal FnIII-like domain increases *TpBGL3* adsorption

The function of FnIII-like domain is not completely clear. Previous studies indicated that the position of this domain in the structure excludes its direct participation in the recognition of small substrates, however, it may be involved in the anchoring of the enzyme on large polymeric substrates, as well as, in thermo stabilization of the entire protein [16,17]. Besides, functional analysis of hybrid enzymes generated by FnIII-like domain shuffling confirms its role in cell wall adhesion [34]. Recently, Lima et al. suggested that FnIII-like domain from *A. niger* β -glucosidase is important for anchoring the domain to the lignin matrix [18]. The presence of the FnIII-like domain (Fig. 1B) indeed increased non-productive adsorption of *TpBGL3*. At the entire pH range considered in this study, significantly more *TpBGL3* than *TpBGL1* was bound onto lignin preparations, both SL and EL (Figs. 2 and 3). The theoretical pI values determined for *TpBGL3* Δ FnIII (catalytic domain, gray in Fig. 1B) and FnIII-like domain (black in Fig. 1B) are 5.1 and 8.7, respectively [19]. Thus, FnIII-like domain possibly acquires positive net charge in the entire pH range considered. On the contrary, *TpBGL3* Δ FnIII possibly acquires negative charges at

pH 7 and 6, while at pH 4 acquires positive charge. Hence, it is likely that at pH 7 and 6, FnIII-like domain interacts with lignin preparations through electrostatic and hydrophobic interactions that can partially, or completely, overcome repulsive electrostatic forces between the catalytic domain (*TpBGL3* Δ FnIII) and lignin preparations. At pH 4, both *TpBGL3* domains possibly acquire positive charges and adsorption may be explained mostly by attractive electrostatic interactions between the lignin preparations (SL and EL) and *TpBGL3*. The data provided in this study are consistent with a model where electrostatic and hydrophobic interactions contribute to the adsorption mechanism of β -glucosidases, which is summarized in Fig. 6.

4.3. Increase of temperature did not result in an increase in the adsorption of β -glucosidases onto lignins

An increase in temperature may affect the structure of mesophilic enzymes leading to exposure of more hydrophobic regions to the solvent and, therefore, enhancing the hydrophobic interactions [30–32]. However, it was recently shown that there were no significant changes in the secondary, tertiary and quaternary structures of the hyperthermophilic bacterial β -glucosidases *TpBGL1* and *TpBGL3* for temperatures below 80 °C [13]. Thus, probably even at 70 °C there is no significant increase in hydrophobic regions on β -glucosidases structures exposed to the solvent. This could explain why an increase in temperature from 20 °C to

Fig. 5. Effect of temperature (20 °C and 70 °C) on *TpBGL1* and *TpBGL3* (6 μ M) adsorption on the lignin preparations (5 mg/mL).

Fig. 6. Schematic model of the interaction between β -glucosidases and lignins. The results described in this study are consistent with a model where electrostatic interactions strongly contribute to the adsorption mechanism. F_E : electrostatic force. F_H : hydrophobic force.

70 °C did not result in a significant change in the fraction of β -glucosidases adsorbed to the SL and EL preparations (Fig. 5).

4.4. *TpBGL1* can be recycled via the liquid phase at pH of optimum activity

A recent study reported that *TpBGL1* reached its optimum activity within the pH range of 6–7, while *TpBGL3* reached its optimum activity around pH 4 [12]. At all pH values, attractive electrostatic interactions strengthen the non-productive adsorption of *TpBGL3*. Furthermore, the higher adsorption was observed at pH 4, condition where *TpBGL3* reaches its optimum activity. However, at pH 7 and 6, electrostatic repulsion weakens the non-productive adsorption of *TpBGL1* to lignins, conditions where *TpBGL1* reaches its optimum activity. Therefore, the results indicate that *TpBGL1* could be recycled via the liquid phase at pH where its activity is maximum.

5. Conclusions

In conclusion, the data showed that β -glucosidases can adsorb to lignins through different mechanisms. *TpBGL1* adsorption onto lignins was found to be strongly pH-dependent, suggesting that the adsorption is electrostatically modulated. For *TpBGL3*, the results suggest that FnIII-like domain interacts with lignins through electrostatic and hydrophobic interactions that can partially, or completely, overcome repulsive electrostatic forces between the catalytic domain and lignins. Finally, the increase of temperature did not result in the increase of β -glucosidases adsorption, probably because there is no significant increase in hydrophobic regions in the β -glucosidases structures. These studies can be useful in the field of plant structural polysaccharides conversion into bioenergy.

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Conflict of interest

The authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

Author contributions

VMS, ASS: carried out the production and purification of *TpBGL1* and *TpBGL3*. DN, MON: carried out the isolation of lignin preparations. VMS, ASS, WG: carried out the experiments. WG: coordinated the overall study, and contributed to the analysis of the results and finalizing the paper. VMS, ASS, DN, MON, JRCM, FMC, IP, WG: all authors suggested modifications to the draft and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.enzmictec.2016.02.007>.

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