Studies of adsorptive capacity of bacterial β-glucosidases on lignocresol aiming the enzymatic recycling in bioprocesses

Mariana T. Barduco Ferreira[a], Thiago B.B. Zuccari[a], Wanius Garcia[b], Mario de Oliveira Neto[a],*

[a] *São Paulo State University - Unesp, Department of Physics and Biophysics, Institute of Biosciences, Botucatu, São Paulo, Brazil*
[b] *Center for Natural and Human Sciences (CCNH), Federal University of ABC (UFABC), Santo André, Brazil*

ABSTRACT

Enzymes are essential in many biological processes, including second-generation ethanol production. However, enzymes are one of the main expenses for the industrial process in these days. Several studies have been done to maximize cost savings, however, many processes are still economically infeasible. In this study, we report the synthesis of a suspension of lignocresol for recycling or reuse of enzymes in bioprocesses. In this way, it was performed the adsorption assays between lignocresol and β-glucosidases from *Thermotoga petrophila*, belonging to the families GH1 and GH3, for the development of a lignocresol-enzyme complex. Our results show that lignocresol maintains greater adsorptive capacity for β-glucosidases than lignin. This capacity can be explained both by its great hydrophobicity and also by electrostatic characteristics. Therefore, all these results demonstrate good adsorption of the enzymes to the lignocresol, demonstrating great potential for enzymatic recycling.

© 2019 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Enzymes are used as natural catalysts of life-critical chemical reactions. Which is highly explored in industrial scale for fermentation, food manufacturing, animal nutrition, cosmetics, medicines, textile industry, paper industry, bioethanol production, among others [1,2]. Costs involving the application of a range of natural catalysts in various bioprocesses is one of the main expenses for industry. As an example, the production of bioethanol where the cost of enzymes has a big impact on the price of the final product [3]. Enzymes called cellulases, which are, endo-1,4-β-D-glucanase (EC3.2.1.4), exo-1,4-β-D-glucanase (EC3.2.1.176), exo-1,4-β-D-glucanase (EC3.2.1.91) and β-glucosidase (EC3.2.1.21) degrade the lignocellulosic material by working synergistically acting on the cellulose polymer and are generally produced by micro-organisms [4]. β-glucosidases are essential in the cellulolytic system, breaking down cellobiose in glucose monomers and can also work synergistically optimizing the action of other cellulolytic enzymes, increasing the yield of the process, since it avoids the inhibition of other enzymes by accumulated cellobiose [5,6]. Although cellulases are present in many industrial applications, the high cost of its acquisition makes enzymatic processes costly.

In this context, enzyme immobilization leads to more economical methods due to the possibility of reuse of the enzymes. The lignocellulosic components have promising characteristics for their use as raw material in the production of chemical products or fuels because it is renewable, available in abundance in the environment and relatively low cost. [7].

Lignin is a limiting factor in the enzymatic saccharification of cellulose, reducing the accessibility of the hydrolytic enzymes to the cellulosic fibers [8,9]. This process is called unproductive adsorption and occurs through hydrophobicity, electrostatic bonding or hydrogen bonding [10,11]. However, we can use unproductive adsorption as a positive point, due to the high affinity with biopolymers and to its hydrophobic characteristic that can bind to these enzymes [12]. From lignin, a new class of polymers can be obtained, lignocresol [7].

Lignocresol (LC) is a cellulosic compound derived from lignin, which appears as an industrial alternative since much of lignin is discarded in industrial processes. LC was developed by Funaoka [13], which is synthesized from lignin using the phase separation system, that primarily consists of the use of a concentrated acid and a phenol derivative, p-cresol [12–15]. LC preserves the hydrophobic characteristics of lignin, since, lignin is protected from the action of concentrated sulfuric acid by cresol solvation [13]. LC has a high immobilization capacity for enzymes, due to the hydrophobic regions of the molecule; this characteristic is of great economic importance since it can be used for the recycling of

* Corresponding author.
E-mail address: mario.oliveira@unesp.br (M. de Oliveira Neto).

enzymes used in bioprocesses [12]. The present study reports the synthesis of a suspension of LC to promote enzymatic adsorption between LC and β-glucosidases from *Thermotoga petrophila*, and the development of a LC-enzyme complex, enabling enzyme recycling in bioprocesses.

2. Experimental

2.1. Cloning, expression and purification of recombinant β-glucosidases

Cloning, expression and purification of the *Thermotoga petrophila* β-glucosidases from families GH1 and GH3, respectively (*Tp*Bgl1) (GenBank: ABQ46970.1) and *Tp*Bgl3 (GenBank: ABQ46916.1) were carried out as described previously [16,17]. The purity of the final products was checked 15% SDS-PAGE. 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 were calculated using the ProtParam tool (http://web.expasy.org/protparam). The theoretical coefficients employed were ε_{280nm} = 121,240 $M^{-1} cm^{-1}$ for *Tp*Bgl1 and ε_{280nm} = 102,930 $M^{-1} cm^{-1}$ for *Tp*Bgl3. The final purified products were then frozen and stored at $-80\,°C$ and were melted on ice before use.

2.2. LC preparation

LC was synthesized from a standard Indulin AT lignin (kindly provided by MeadWestvaco Corporation), extracted from the residual liquor resulting from the Kraft pulping process of *Pinus* spp. The methodology performed to synthesis, called phase separation, was adapted from Funaoka [15]. For LC synthesis, a total of 20 mL of 72% sulfuric acid was added to 1 g of lignin with 10 mL of p-cresol. The mixture was stirred vigorously for one hour at room temperature; following centrifugation for 20 min at room temperature (4000 rpm). The supernatant was dropwise into 200 mL of cold ethyl ether under vigorous stirring. The aqueous precipitate formed was recovered by centrifugation at 5 °C (3500 rpm) for 20 min and the supernatant discarded. The LC was extracted with 80 mL of acetone and volume was reduced to 10 mL at a rotary evaporator. Afterwards, all volume was dropped in 200 mL of cold ethyl ether under vigorous stirring and centrifuged for 20 min at room temperature (3500 rpm). The supernatant was discarded, and the LC pellet was oven dried at the stove (50 °C for 12 h).

2.3. Adsorption of the β-glucosidases onto LC

The adsorptions assays were carried out by determining β-glucosidases concentrations after incubation with *Pinus* spp. LC or unmodified lignin (LG). The reaction was done in a range of concentration of 0.06–1.6 and 0.2–0.5 mg/mL, respectively to *Tp*Bgl1 and *Tp*Bgl3, in 50 mM sodium citrate buffer (pH 6.0 for *Tp*Bgl1 and pH 4.0 for *Tp*Bgl3). According to Cota *et. al.* [16], *Tp*Bgl1 has an optimum activity within the pH range of 6–7, whereas *Tp*BGL3 has optimum activity around pH 4.0. The adsorption experiments were conducted according to Nonaka *et. al.* [18]. LC and LG suspensions were performed with 50 mM sodium citrate buffer (pH 6.0 for interaction with *Tp*Bgl1 and pH 4.0 for interaction with *Tp*Bgl3) in a microtube by ultrasonication (Sharp UT-205S, 200 W) to achieve a final interaction concentration of 10 mg/mL. Enzymatic dilutions were then mixed to the LC, and LG suspensions and samples were could for 20 min in stir and then centrifuged for 15 min at 5 °C (10,000 rpm). According to Nonaka et al. [18] in the previous study, cellulase adsorption reaches equilibrium in 10 min.

The amount of enzyme adsorbed on LC or LG was calculated by subtracting the amount of enzyme remaining in the supernatant from the total amount of β-glucosidases added and then converted to adsorptive capacity in percentage. The residual enzyme concentration in the supernatant after centrifugation was measured by the Bradford method [19].

2.4. Adsorption of β-glucosidases in LC in a glucose-rich medium

The adsorption assays simulating a medium in the presence of hydrolysates were done with a complex LC-enzymes. The final concentration of the enzymes *Tp*Bgl1 and *Tp*Bgl3 in the complex was 0.2 mg/mL. The experiments were done as described in the section 2.3 with the addition of a glucose + CMC solution in different concentrations (4 mg/mL glucose + 0,1% CMC, 10x diluted). The residual enzyme concentration in the supernatant after centrifugation was measured by Bradford method [19].

2.5. β-Glucosidases activity after complex formation

After enzyme adsorption on LC, the supernatant was carefully removed using a pipette. The enzymatic assay for β-glucosidase activity adsorbed on LC was carried, the pellet was mixed with 50 μl of buffer solution adjusted at different pH values (pH 6 for *Tp*Bgl1 and pH 4 for *Tp*Bgl3) were used and 250 μL of 2 mM 4-nitrophenyl- β-D-glucopyranoside (pNPG) was used as substrate. Following incubation at 50 °C during 15 min, the reaction was stopped by addition of 1000 μL of 1 M Na_2CO_3 and the releasing of p-nitrophenol was monitored colorimetrically at 410 nm using a microplate reader. One enzyme unit is defined as the amount of enzyme releasing 1 mmol of p-nitrophenol per min under the specific condition.

2.6. Electrophoretic light scattering (ELS)

Electrophoretic light scattering (ELS) measurements were used to determine the average zeta potential (ζ) of the LC and LG, which were collected using a Zetasiser Nano-ZS at 20 °C [20,21]. In this study, the zeta potential was measured for LC and LG preparations (at 20 °C) in 20 mM acetate–borate–phosphate buffer adjusted to the different pH values. This instrument measures the electrophoretic mobility (μe) and converts the value to a ζ potential (mV) through Henry's equation: $\mu e = [2\varepsilon\zeta F(\kappa a)]/3\eta$, where ε is the dielectric constant of water and η is the viscosity. Furthermore, F (κa) is the Henry's function, which was calculated through the Smoluchowski approximation F(κa) = 1.5. The isoelectric point is given by the pH value at which the zeta potential is approximately zero. Knowledge of the electrophoretic mobility enables one to calculate the average number of charges per molecule (N_c) from the Lorenz– Stokes relationship $Nc = \frac{6\pi\eta e}{e} R_s \mu_e$, where μe is expressed in $\mu m\ cm\ s^{-1}V^{-1}$ and the value in the denominator corresponds to the elementary charge (e = 1.602 × 10^{-19} C) [20,21].

3. Results and discussion

3.1. Adsorption of LC and LG with Tp*Bgl1 and Tp*Bgl3

The results obtained between adsorption of LC and LG with *Tp*Bgl1 and *Tp*Bgl3 can be confirmed due to the absence of the enzyme in the supernatant at some concentration points. Fig. 1 shows that LC has a higher adsorption capacity than LG. The best adsorption point (LC suspension at 10 mg/mL) was observed for *Tp*Bgl1 and *Tp*Bgl3 at initial enzyme concentrations below 0.3 and 0.4 mg/mL, respectively (Fig. 1). The existence of a better point in the adsorptive capacity can be explained by the large surface area of LC molecule.

LC when adsorb to the β-glucosidases, is entirely occupied by the enzymes, thus preventing adsorption from new enzymatic charges if added. This discussion is effective since the LC suspension concentration was maintained at approximately 10 mg/mL throughout the interaction time. In summary, 1 g of LC from *Pinus* spp. could adsorb at maximum 40 mg of *Tp*Bgl1 and 30 mg of *Tp*Bgl3 in solution.

Lower adsorption of LG was expected for both enzymes since the synthesized LC is subjected to the phase separation system [22]. According to Funaoka [13,14], this methodology allows physical-chemical modifications of the LG, leading to an increase of phenolic hydroxyl in its composition, which results in very hydrophobic LC polymers. Thus, it is assumed that such modifications help in the adsorption moment with the enzymes, possibly by the contribution to the surface hydrophobic characteristic to the polymer [23].

In a study of LC from softwood and from hardwood in interaction with an enzymatic cocktail, showed that the adsorption capacity of LC from Eucalyptus (softwood) was lower than that of Hinoki (hardwood), probably because LC of Eucalyptus is more hydrophilic due to the lower molecular weight and linear structure with more phenolic hydroxyl groups [24].

3.2. Influence of pH on the β-glucosidases adsorption to LC and LG

ELS (Electrophoretic Light Scattering) measurements were conducted to determine the average zeta-potential (ζ) of the LG and LC samples. The ELS (Fig. 2) shows that LC exhibits more negative potential-zeta values than LG. Da Silva et al. [25] found the same results in unmodified sugarcane and eucalyptus lignins; both were negative in the pHs observed in our work. In the same study, the authors report the surface charge of β-glucosidases (*Tp*Bgl1 e *Tp*Bgl3) is affected by the pH. At pH 7 and 6, both β-glucosidases acquire very similar negative charges, while at pH 4 the β-glucosidases acquire a positive charge. Therefore, considering that electrostatic characteristics influence the interactions between the polymers and the enzymes, and not only by surface hydrophobicity as discussed previously. Fig. 1 shows that at a certain point LG has higher adsorption than LC on *Tp*Bgl3, which can be explained by the pH that the assays were performed. At pH 4, LG and LC show the same zeta potential and charge, as observed in Fig. 2. However, *Tp*Bgl1 has higher negative zeta potential on LC than LG at pH 6 (Fig. 2), indicating that the adsorption of *Tp*Bgl1 on LG and LC is highly different. As previously reported by Da Silva et al. [25], the zeta potential of *Tp*Bgl1 and *Tp*Bgl3 at pH 4 was positive and at pH 6
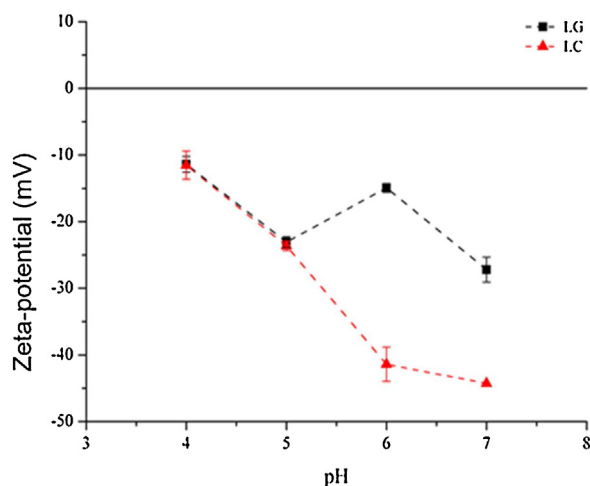
Fig. 2. Zeta- Potential of lignocresol (LC) and lignin (LG) as a function of pH.

was negative, confirming the difference of adsorption. These results imply that the strength of enzyme interaction toward LG or LC is highly dependent of pH value and substrate surface charges.

It can be noted that the adsorption of *Tp*Bgl3 to LC (40 mg/g) is higher compared to *Tp*Bgl1. This could be related by comparing the size of both enzymes, in which *Tp*Bgl3 has larger surface area than *Tp*Bgl1. Additionally, *Tp*Bgl3 has the fibronectin-like domain (FnIII) [25], which its role on substrate adsorption is not completely elucidated [26,27]. This domain can be related to recognition of small substrates and in the binding of large polymers [26,27]. Da Silva et al. [25] showed that this domain at pH 4, possibly acquires positive charges and adsorption may be explained mostly by electrostatic interactions between the lignin preparations used at experiments with *Tp*Bgl3. This is an indicative that this additional area in *Tp*Bgl3 the may contribute to the hydrophobic surface interaction between enzyme and LC better than *Tp*Bgl1. Studies with FnIII from *Aspergillus niger*'s β-glucosidase *An*Bgl1 show that this domain is essential for anchoring the enzyme to the lignin matrix. Although there is no specificity in the complexation between FnIII and lignin. In addition, in FnIII domain exist important lignin dimers binding sites forming a hydrophobic cage by aromatic residues and arginines [22]. In this way, the data shown in this paper are consistent with previous model of how β-glucosidases adsorb in the LC where electrostatic and hydrophobic interactions contribute to the adsorption mechanism.

3.3. Influence of a glucose-rich medium on the β-glucosidases-LC adsorption

The adsorption of β-glucosidases to LC with different concentrations of glucose was analyzed to determine if hydrolysis products would affect the LC adsorption mechanism. The choice for glucose concentrations was based on our enzyme hydrolysate studies (data not shown). Fig. 3 shows that the adsorption of the enzymes is not affected by the presence of glucose in the medium.

Maurer et al. [28] show that glucose does not compete with the enzymes for the active binding sites present on the surface of the cellulosic substrates, consequently, not interfering in the adsorption of the enzymes. In our studies, we showed that the same occurs for LC.

3.4. Enzymatic activity from β-glucosidases onto LC

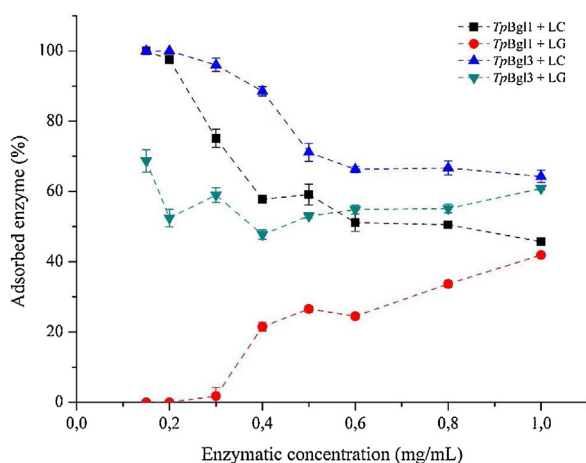After the adsorption of β-glucosidases onto LC, was determined the enzymatic activity of LC-enzymes complex (Fig. 4).

Fig. 1. Evaluating the adsorptive capacity of lignocresol and lignin to *Tp*Bgl1 and *Tp*Bgl3. LC-Lignocresol, LG- Lignin.
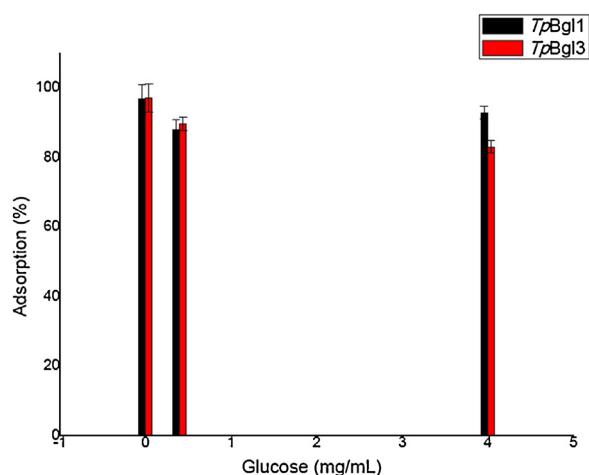
Fig. 3. Evaluating the adsorptive capacity of lignocresol and to *Tp*Bgl1 and *Tp*Bgl3 in presence of glucose + CMC.
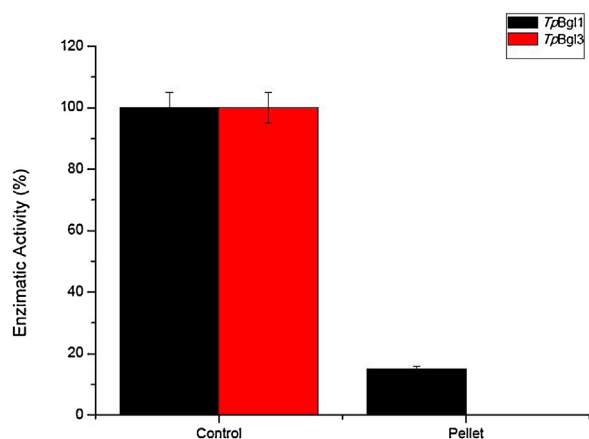

Fig. 4. Effect of adsorption effect on the enzymatic activity of β-glucosidases.

LC-*Tp*Bgl1 complex maintains relatively activity: about 20% of the enzymatic activity of the free enzyme. This data suggests that *Tp*Bgl1 does not entirely lose function and enzymatic activity when immobilized. On the other hand, *Tp*Bgl3 completely loses functionality. This total loss of activity may be explained by the stable binding of *Tp*Bgl3 to LC by the hydrophobic and electrostatic interactions as shown in our results.

Possibly in *Tp*Bgl1 occurs detachment of these enzymes from de complex with LC when mixed in the buffer due to their weaker interaction. Nonaka *et.al.,* [29] in previous studies reports that a small release of cellulase from LC when the complex is mixed in the fresh acetate buffer. But the author affirms that enzymatic activity is estimated using LC-cellulase complex rather than the cellulase detached from the solution [29]. Previous studies with filter paper hydrolysis of cellulases adsorbed onto LC, show that the enzymatic activity was approximately 30% and 45% (softwood and hardwood LC respectively) compared to free native cellulase. However, when enzymes are immobilized their physical and chemical properties may change, effects on stability, kinetic properties and specificity beyond enzyme productivity should be considered [30]. There are differences in the behavior of the immobilized enzyme as to its native form, and are the following factors: conformational modification of the enzyme molecule due to the alteration in the tertiary structure of the active site; stereochemical effects - a portion of the enzyme molecule is immobilized at a position such that the active site is relatively inaccessible; mass transfer effects -

arise from the diffusion resistance of the substrate to the catalytic site of the enzyme, and from the reaction product; microenvironmental effects - resulting from the immobilization method used or from the presence and nature (hydrophobic or hydrophilic) of the carrier in the vicinity of the enzyme [30]. These factors may influence the properties of the immobilized enzyme that acquire new kinetic properties, leading to a different body than expected. The enzymatic activity of the complex is low, but even so, the LC is efficient in enzymatic immobilization. However, further studies of enzyme activity will be evaluated in our future studies.

4. Conclusion

In conclusion, the data showed that β-glucosidases could adsorbent onto LC through different mechanisms such as by hydrophobic and electrostatic interactions. The glucose in the reaction medium do not interfere the adsorption of the enzymes to LC. *Tp*Bgl1 bound to LC maintains a residual enzymatic activity. In this way, LC demonstrates a great potential for enzymatic recovery and recycling. However, it still necessary efforts to increase enzyme activity after the adsorption onto LC. These studies can be useful in the field of plant structural polysaccharides conversion into bioenergy.

Declaration of interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

Acknowledgment

References

[1] J. Adrio, L. Demain, Microbial cells and enzymes - a century of progress, Methods in Biotechnology, Microbial Enzymes and Biotransformation, 17, Humana Press, Totowa, 2005, pp. 27 1.
[2] S. Li, X. Yang, S. Yang, M. Zhu, X. Wang, Technology prospecting on enzymes: application, marketing and engineering, Comput. Struct. Biotechnol. J. 2 (2012) e201209017.
[3] S.T. Merino, J. Cherry, Progress and challenges in enzyme development for biomass utilization, Adv. Biochem. Eng. Biotechnol. 108 (2007) 95–120.
[4] C. Florencio, A.C. Badino, C.S. Farinas, Current challenges on the production and use of cellulolytic enzymes in the hydrolysis of lignocellulosic biomass, Quim. Nova 40 (2017) 1082–1093.
[5] M.A. Lima, M. Oliveira-Neto, M.A.S. Kadowaki, F.R. Rosseto, E.T. Prates, F.M. Squina, A.F.P. Leme, Skaf, S. Munir, I. Polikarpov, Aspergillus niger beta-Glucosidase Has a Cellulase-like Tadpole Molecular Shape Insights into glycoside hydrolase family 3 (gh3) beta-glucosidase structure and function, J. Biol. Chem. 288 (2013) 32991–33005.
[6] O.G. Korotkova, M.V. Semenova, V.V. Morozova, I.N. Zorov, L.M. Sokolova, T.M. Bubnova, O.N. Okunev, A.P. Sinitsyn, Isolation and properties of fungal -glucosidases, Biochemistry 74 (2009) 569–577.
[7] K. Mikame, M. Funaoka, Polymer structure of lignophenol I - Structure and function of fractionated lignophenol, Polym. J. 6 (2006) 585–591.
[8] H. Palonen, F. Tjerneld, G. Zacchi, M. Tenkanen, Adsorption of trichoderma reesei cbh i and eg ii and their catalytic domains on steam pretreated softwood and isolated lignin, J. Biotechnol. 107 (2004) 65–72.
[9] A. Akimkulova, A. Zhou, X. Zhao, D. Liu, Improving the enzymatic hydrolysis of dilute acid pretreated wheat straw by metal ion blocking of non-productive cellulase adsorption on lignin, Bioresour. Technol. 208 (2016) 110–116.
[10] S. Nakagame, R.P. Chandra, J.F. Kadla, J.N. Saddler, The isolation, characterization and effect of lignin isolated from steam pretreated Douglas-fir on the enzymatic hydrolysis od cellulose, Bioresour. Technol. 102 (2011) 4507–4517.
[11] H. Lou, J.Y. Zhu, T.Q. Lan, H. Lai, X. Qiu, pH-Induced Lignin Surface Modification to Reduce Nonspecific Cellulase Binding and Enhance Enzymatic Saccharification of Lignocelluloses, ChemSusChem. 6 (2013) 919–927.
[12] Y. Nagamatsu, M. Funaoka, Design of recyclable matrixes from lignin-based polymers, Green Chem. 5 (2003) 595–601.

[13] M. Funaoka, I. Abe, Rapid separation of wood into carbohydrate and lignin with concentrated acidphenol system, Tappi J. 72 (1989) 145–149.

[14] M. Funaoka, A new type of phenolic lignin-based network polymer with the structure-variable function composed of 1,1-diarylpropaneunits, Polym. Int. 47 (1998) 277–290.

[15] M. Funaoka, Sequential transformation and utilization of natural network polymer "LIGNIN", React. Funct. Polym. 73 (2013) 396–404.

[16] J. Cota, T.L.R. Corrêa, A.R.L. Damásio, J.A. Diogo, Z.B. Hoffman, W. Garcia, L.C. Oliveira, R.A. Prade, F.M. Squina, Comparative analysis of the hyperthermophilic GH1 and GH3 family members with industrial, N. Biotechnol. 32 (2015) 13–20.

[17] F.M. Squina, R.A. Prade, H. Wang, M.T. Murakami, Expression, purification, crystallization and preliminary crystallographic analysis of an endo-1,5-α-l-arabinanase from hyperthermophilic Thermotoga petrophila, Acta Crystall. Sect. F 65 (2009) 902–905.

[18] H. Nonaka, H. Tanaka, M. Funaoka, Adsorption of Trichoderma reesei cellulase on softwood lignin-based lignophenol, J. Jpn. Inst. Energy 90 (977) (2011) 981.

[19] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 7 (1976) 248–254.

[20] T. Pozzo, J.L. Pasten, E.N. Karlsson, D.T. Logan, Structural and functional analyses of β-glucosidase 3B from Thermotoga neapolitana: a thermostable three-domain representative of glycoside hydrolase 3, J. Mol. Biol. 397 (2010) 2724–2739.

[21] R.P. McAndrew, J.I. Park, R.A. Heins, W. Reindl, G.D. Friedland, P. Dıhaeseleer, Northen T, K.L. Sale, B.A. Simmons, P.D. Adams, From soil to structure, a novel dimeric β-glucosidase belonging to glycoside hydrolase family 3 isolated from compost using metagenomic analysis, J. Biol. Chem. 288 (2013) 14985–14992.

[22] M.A. Lima, M. Oliveira-Neto, M.A.S. Kadowaki, F.R. Rosseto, E.T. Prates, F.M. Squina, A.F.P. Leme, Skaf, S. Munir, I. Polikarpov, Aspergillus niger beta-Glucosidase Has a Cellulase-like Tadpole Molecular Shape Insights into glycoside hydrolase family 3 (gh3) beta-glucosidase structure and function, J. Biol. Chem. 288 (2013) 32991–33005.

[23] D.W. Sammond, J.M. Yarbrough, E. Mansfield, Y.J. Bomble, S.E. Hobdey, S.R. Decker, L.E. Taylor, M.G. Resch, J.J. Bozell, M.E. Himmel, T.B. VInzant, M.F. Crowley, Predicting enzyme adsorption to lignin films by calculating enzyme surface hydrophobicity, J. Biol. Chem. 289 (2014) 20960–20969.

[24] A. Kobayashi, H. Nonaka, M. Funaoka, Comparison of Softwood and Hardwood Lignocresol-immobilized Cellulases, J. Jpn. Inst. Energy 91 (2014) 992–997.

[25] V.M. Da Silva, A.S. De Souza, D. Negrão, I. Polikarpov, F.M. Squina, M. Oliveira-Neto, J.R.C. Muniz, W. Garcia, Non-productive adsorption of bacterial β-glucosidases on lignins is electrostatically modulated and depends on the presence of fibronectin type III-like domain, Enzyme Microb. Technol. 8788 (2016) 1–8.

[26] T. Pozzo, J.L. Pasten, E.N. Karlsson, D.T. Logan, Structural and functional analyses of β-glucosidase 3B from Thermotoga neapolitana: a thermostable three-domain representative of glycoside hydrolase 3, J. Mol. Biol. 397 (2010) 2724–2739.

[27] R.P. McAndrew, J.I. Park, R.A. Heins, W. Reindl, G.D. Friedland, P. Dıhaeseleer, T. Northen, K.L. Sale, B.A. Simmons, P.D. Adams, From soil to structure, a novel dimeric β-glucosidase belonging to glycoside hydrolase family 3 isolated from compost using metagenomic analysis, J. Biol. Chem. 288 (2013) 14985–14992.

[28] S.A. Maurer, C.N. Bedbrook, C.J. Radke, Cellulase Adsorption and Reactivity on a Cellulose Surface from Flow Ellipsometry, Ind. Eng. Chem. Res. 51 (2012) 11389–11400.

[29] A. Nonaka, H. Tanaka, M. Funaoka, Adsorption of Trichoderma raasei cellulase on softwood lignin basead lignophenol, J. Jpn. Inst. Energy 90 (977) (2011) 981.

[30] L.C. Cardoso, Q.B. Cass, Immobilization of the enzymes on chromatographic supports: a tool to research of inhibitor compounds, Química Nova 32 (175) (2009) 187.