

Immobilization of two endoglucanases from different sources

Rosalba Sarcina¹, Concetta Valeria L. Giosafatto¹, Vincenza Faraco¹, Licia Lama²,
Marilena Esposito¹, Loredana Mariniello¹

¹Department of Chemical Sciences, University of Naples “Federico II”, Complesso Universitario Monte Sant’Angelo, via
Cinthia 4, 80126 Naples, Italy

²ICB-CNR, via Campi Flegrei 34 80078, Pozzuoli, Naples, Italy

Abstract— Cellulases are a important family of hydrolytic enzymes which catalyze the bond of cellulose and other related cello-oligosaccharide derivatives. Industrial applications require enzymes highly stable and economically viable in terms of reusability. These costs can be reduced by immobilizing the cellulases, offering a potential solution through enzyme recycling and easy recovery. The covalent immobilization of enzymes is reported here: one is commercial cellulase from *Aspergillus niger* and other one is recombinant enzyme, named CelStrep it because was isolated from a new cellulolytic strain, *Streptomyces* sp. G12,. The optimal pH for binding is 4.6 for both cellulases and the optimal enzyme concentrations are 1 mg/mL and 5 mg/mL respectively. The support for immobilization is a polyacrylic matrix. Experiments carried out in this work show positive results of enzyme immobilization in terms of efficiency and stability and confirm the economic and biotechnical advantages of enzyme immobilization for a wide range of industrial applications.

Keywords — *Aspergillus. niger*, endoglucanases, immobilization, *Streptomyces*.

I. INTRODUCTION

Cellulases (E.C. 3.2.1.-) are enzymes that hydrolyze β -1,4 glycosidic bonds in cellulose. Namely, they can be distinguished in endoglucanases (E.C. 3.2.1.4), exoglucanases (E.C.3.2.1.91) and β -glucosidases (E.C.3.2.1.21). They are mainly used in various industries including pulp and paper, textile, laundry, food and feed industry, brewing, and agriculture [1]. They are important also for the production of biofuel, for which there is an important demand to have a more sustainable life style worldwide. However, biomass refineries are not spread out in the world because the cost of cellulase highly affects the production of cellulosic-derived ethanol. To overcome this problem many researches are dedicated to both find new cellulases and also produce immobilized enzymes to make more inexpensive biofuel production. Cellulases are enzymes synthesized by a variety of microorganisms: bacteria and fungi (yeasts and molds)

during their growth on cellulosic materials [2][3]. From the structure point of view, all of them contain a catalytic domain (Glycoside Hydrolase, GH) that can/may be linked through a simple sequence to a Carbohydrate Binding Domain (CBD) o Module (CBM). The catalytic domain is recognized as a Concanavalin-like domain that is common in both bacterial and fungal cellulases, while the CBD is present mostly in bacterial cellulases. Some bacteria possess “cellulosomes”, cellulase complexes with both type of enzymes (with and without the CBD). In this work we have used two different endoglucanases. The first one is the commercial cellulase from the fungus *Aspergillus niger* (*A. niger*), that possesses only the Concanavalin-like domain, while the second one is a recombinant protein isolated from a new cellulolytic bacterial strain, identified as belonging to *Streptomyces* sp. G12, isolated from mature compost obtained from agro-industrial wastes and named CelStrep by the authors [4]. Thus, the aim of this work was to immobilize both *A. niger* and Cel Strep cellulases and examine the activity of immobilized enzymes. In fact, enzyme immobilization provides the advantage of both re-use and increased enzyme stability and, consequently, reducing costs of specific industrial process (E. Cherian et al., 2015), i.d. biofuel production. The used covalent immobilization method recurs to Immobeads, namely COV-2 (150P), that have a polyacrylic matrix bringing an high amount of epoxide groups which bind the thiol, amine and hydroxyl groups of enzymes [5]. Furthermore we have studied the stability of the immobilized enzymes over the time.

II. MATERIALS AND METHODS

Materials

The supports used for immobilization process were Immobeads IB-COV-2-150P, (ChiralVision B.V., The Netherlands). Two different Cellulases (EC 3.2.1.4 - 1,4- β -endoglucanase) were used : the first from *Aspergillus niger* (*A. niger*, purchased by Sigma Aldrich – USA), and the second, a recombinant enzyme named CelStrep (Amore et al. 2012). *A. niger* cellulase activity was determined by using a kit purchased from Megazyme

(Megazyme International Ireland) using AZO-CM-cellulose. CelStrep activity was determined according to Ghose (1987)[6] using 3,5-dinitrosalicylic acid (DNSA) purchased from Merck. Sodium acetate trihydrate, zinc acetate and other reagents were purchased from Sigma Aldrich (USA).

Methods

Immobilization procedure

The cellulases were covalently linked to epoxy groups on the beads (IB-COV-2-150P). These were equilibrated with sodium acetate buffer (0.1 M pH 6.2). 400 µL of 1 mg/mL enzyme solution from *A. niger* and 400 µL of 5 mg/mL enzyme solution from Celstrep were added to 100 mg of beads, in 2 mL vials and suspended in sodium acetate buffer (0.1 M, pH 4.6).

The beads-enzyme mixture was shaken for 3 h at 4 °C on the rotary mixer to allow the reaction. After that, the vials were let stand overnight at 4°C in order to promote the natural decantation of the beads.

Then the supernatants were removed from the sedimented beads and used to determine enzyme activity. Finally beads were washed on the rotary mixer four times with 1.5 mL sodium acetate buffer (0.1 M pH 4.6): first wash was performed for 1 min; second and third washes for 15 min; fourth wash for 5 min. The amount of immobilized and active enzyme (U_s) was determined by subtracting enzyme activity resolved in supernatants and washes (U_f) from the total enzymatic activity before immobilization (U_0).

Endoglucanase Assay using Megazyme kit

The enzymatic activity of free or immobilized endoglucanase from *A. niger* was measured using AZO-CM-Cellulose as a substrate according to the supplier instructions (Megazyme).

Endoglucanase Assay using DNSA

The enzymatic activity of free or immobilized endoglucanase Celstrep was measured using the 3,5-dinitrosalicylic acid based method (DNSA) [6].

Determination of protein concentration

Protein determination was carried out by the Bio-Rad Protein Assay (Bio-Rad), using bovine serum albumin as standard [7].

Calculation of the parameters of immobilization

The calculation of the parameters of immobilization and immobilized protein were calculated as follows according to R.A. Sheldon et al. [8].

Immobilized Protein Yield (IPY) (also defined as *Protein Loading*) - percentage of immobilized protein based on difference of proteins subjected to immobilization (P_0)

and the proteins remaining in residual liquid after immobilization (P_f), divided by P_0 according to eq. 1.

$$IPY (\%) = [(P_0 - P_f) / P_0] \times 100 \text{ (eq. 1)}$$

Enzyme Immobilization Yield (IY) - percentage of immobilized enzyme based on the difference of enzyme subjected to immobilization (U_0) and that one remained in the liquid after the immobilization (U_f) divided by enzyme subjected to immobilization U_0 according to the eq. 2.

$$IY (\%) = [(U_0 - U_f) / U_0] \times 100 \text{ (eq. 2)}$$

Activity Recovery (AR) - percentage of immobilized enzyme in the support (U_s) divided by U_0 according to the eq 3. AR represents the percentage of active immobilized enzyme over the total enzyme subjected to immobilization.

$$AR (\%) = [U_s / U_0] \times 100 \text{ (eq.3)}$$

Lost Activity (LA) - percentage of the lost enzymes in the total immobilization process, due to the difference of U_0 and the sum of U_s and U_f , according to the eq. 4.

$$LA (\%) = [(U_0 - (U_{support} + U_f)) / U_0] \times 100 \text{ (eq. 4)}$$

Efficiency (E) also named Recovered Activity (RA) - percentage of immobilized enzyme in the support (U_s) divided by the difference between U_0 and U_f , according to the eq. 5.

$$E (\%) = [U_s / (U_0 - U_f)] \times 100 \text{ (eq. 5)}$$

The efficiency can be considered more precise immobilization yield than IY since the first is based on the enzyme activity measured in the support (immobilized enzymes). There is a loss of activity in the immobilized enzyme, or not all enzymes remain active in the support.

Stability of immobilized enzyme

The stability of immobilized enzyme was monitored over the time, from time 0 (immobilization day) until 45 days after immobilization, using Efficiency parameter as equal to 100% of cellulase activity at time 0. The immobilized enzymes were stored at 4°C.

Analysis of protein sequences

Information on the sequences and domain composition of *A. niger* and of *Streptomyces* cellulase has been taken from UniProt database (codes A2R322_ASPNC and I7L8N7_9ATCN, respectively) [9].

III. RESULTS AND DISCUSSION

In this study we have covalently immobilized two different cellulases, the first one from the mold *A. niger* commercialized from Sigma while, the second is a recombinant form previously cloned from the cellulolytic strain *Streptomyces* sp. G12 isolated from compost [4]. The immobilization procedure has been realized using the same protocol reported in Fig. 1. The support for immobilization are beads made of a polyacrylic matrix with epoxide groups that promote covalent binding to amino, thiol or hydroxyl groups of the enzyme. In Table 1 are reported immobilization parameters obtained with the two different enzymes. Immobilization Yield, calculated as reported in M&M, is approximately the same for both enzymes, reaching 67% for *A. niger* and 55% for CelStrep. This parameter is calculated measuring U_0 and U_f , without taking into account whether the enzyme molecules that have been bound to the support are all active. The last information is given from the Activity Recovery which is calculated as a ratio between the enzyme bound to the support (U_s) and the Enzyme subjected to immobilization (U_0). This parameter is equal to 53 % for *A. niger* and to 13 % for CelStrep. Conversely the Lost Activity is 14 % for the first cellulase and 42 % for the second one. Efficiency, which represents the percentage of the functional immobilized enzyme over the total (functional and not functional) immobilized enzyme is 78 % for *A. niger* and 23% for CelStrep, while the Immobilization Protein Yield (also known as Protein loading) is equal to 42 % for *A. niger* and 70% for CelStrep. These results could be related to both molecular weight and sequence of the two different enzymes. In fact, fungal cellulase is smaller (26 kDa) than the bacterial one (which is 37 kDa). In fact, *A. niger* cellulase contains only the Concavalin-like Domain, while the Carbohydrate Binding Domain is missing. The Concavalin-like Domain contains 113 residues out of 223 that possesses amino, thiol and hydroxyl groups, the ones classified as polar amino acids that are able to bind the epoxide groups of the Immo-beads 150P which represent 50.64% of the entire amino acid sequence (Table 2). On the contrary, the CelStrep contains both Concavalin-like Domain and carbohydrate Binding Domain that are made of 228 and 103 amino acids respectively, among which polar residues represent 45.13 and 39.78 % (Table 2). Thus, the latter enzyme exhibits an higher protein loading but a lower efficiency because most probably more residues are involved in the covalent binding to the support influencing enzyme functionality. In fact, it has been reported that high epoxide group content (typical of the Immo-beads 150P) provides quick binding but also multi-point attachment that is detrimental for some enzymes [5]. On the contrary for *A. niger*,

even if the IPL is lower, the covalent binding occurs involving residues without effecting the tertiary structure and thus the enzymatic activity. Similar results have been described in studies devoted to assess the immobilization efficiency in regard to the protein size [5]. In terms of stability, both immobilized endocellulases retained 100% activity after 45 days compared to the free enzymes (Fig. 2). In fact, *A. niger* lost 20% of its activity, while CelStrep exhibited only 60% of activity compared to the immobilized enzyme. The higher stability over the time of the free *A. niger* cellulase is not surprising since it is well known that fungal enzymes are usually more stable than bacterial ones [10].

IV. CONCLUSIONS

This study compares the activity of immobilized endocellulases of different origin. While *A. niger* endocellulase has been already immobilized by Huang et al 2014 [11], CelStrep was immobilized for the first time. The paper by Huang et al (2014)[11] refers to *A. niger* immobilized by recurring to magnetic nanoparticles that allow a re-use of the enzyme during hydrolysis of rice straw to produce ethanol. The process was judged efficient even if the immobilized enzyme only retained 80% of activity compared to the free one. CelStrep is a bacterial endocellulase isolated from a cellulolytic strain present in agro-industrial waste-derived compost by Amore et al 2012 [4] with the aim of selecting effective enzyme to treat cellulosic materials. Both endocellulases were immobilized on a polar polyacrylic matrix, previously used successfully in our laboratories to immobilize aspartic protease from *Carduus defloratus* [12], and retained the same activity compared to the free counterparts. Moreover, both enzymes exhibit a good stability over the time while, between the two different free enzymes, the *A. niger* cellulase retains 80% activity after 45 days, although CelStrep was 60% active in respect to the activity at time 0.

ACKNOWLEDGEMENTS

This study was supported by grant from the Ministero dell'Università e della Ricerca Scientifica Industrial Research Project B Development of green technologies for production of BIOchemicals and their use in preparation and industrial application of POLImeric materials from agricultural biomasses cultivated in a sustainable way in Campania region—BioPoliS PON03PE_00107_1/1, funded in the frame of Operative National Programme Research and Competitiveness 2007–2013. We are grateful to Maria Fenderico for technical assistance and to Prof. Anna Marabotti for helpful discussion on sequences of cellulases.

REFERENCES

- [1] Kuhad, R.C., Gupta, R., and Singh, A. (2011). Microbial Cellulases and Their Industrial Applications. *Enzyme Research*, 2010: 10 pages, Article ID 280696, doi:10.4061/2011/280696.
- [2] Kubicek, C.P., Messner, R., Gruber, F., Mandelss, M. and Kubicek-Pranz, E.M. (1993). Triggering of Cellulase Biosynthesis by Cellulose in *Trichoderma reesei*. *The Journal of Biological Chemistry*, v 268(26):19364-19368.
- [3] Sang-Mok, L. and Koo, Y-M. (2001). Pilot-scale production of cellulase using *Trichoderma reesei* Rut C-30 in fedbatch mode. *Journal of Microbiology and Biotechnology*, 11(2):229-233.
- [4] Amore, A., Pepe, O., Ventorino, V., Birolo, L., Giangrande, C., Faraco, V. (2012). Cloning and recombinant expression of a cellulase from the cellolytic strain *Streptomyces* sp. G12 isolated from compost. , 26;11:164.
- [5] Mateo, C., Grazu, V., and Guisan, J.M. Guisan, J.M. (2013). Immobilization of Enzymes on monofunctional and heterofunctional epoxy-activated supports in Guisan, J.M.(ed.) *Immobilization of Enzymes and Cells: Third edition. Methods in Molecular Biology*. Springer Science+Business Media, New York, 1051:43-57.
- [6] Ghose, T.K. (1987). Measurement of cellulase activities. *Pure & Applied Chemistry*, 59:257-268.
- [7] Bradford, M. (1976). A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Analytical Biochemistry*, 72:248-254.
- [8] Sheldon, R.A. and van Pelt, S. (2013). Enzyme Immobilisation in biocatalysis: why, what and how. *The Royal Society of Chemistry*, 42:6223-6235.
- [9] The UniProt Consortium. UniProt: the universal protein knowledgebase.(2017) *Nucleic Acids Research*, 45: D158-D169 (2017)].
- [10] Polaina, J. and A. P. MacCabe, A.P. editors (2007). *Industrial Enzymes Structure, Function and Applications* Springer, The Netherlands.
- [11] Huang, P.J., Chang, K.L., Hsieh, J.F. and Chen, S.T. (2014) Catalysis of rice straw hydrolysis by the combination of immobilized Cellulase from *Aspergillus niger* on β -cyclodextrin-Fe₃O₄ nanoparticles and ionic liquid". *BioMed Research International* 2015:9 pages, Article ID 409103, doi:10.1155/2015/409103
- [12] Esposito, M., Di Pierro, P., Dejonghe, W., Mariniello, L. and Porta, R. (2016). Enzymatic milk clotting activity in artichoke (*Cynara scolymus*) leaves and alpine thistle (*Carduus defloratus*) flowers. Immobilization of alpine thistle aspartic protease". *Food Chemistry*, 204:115-121.

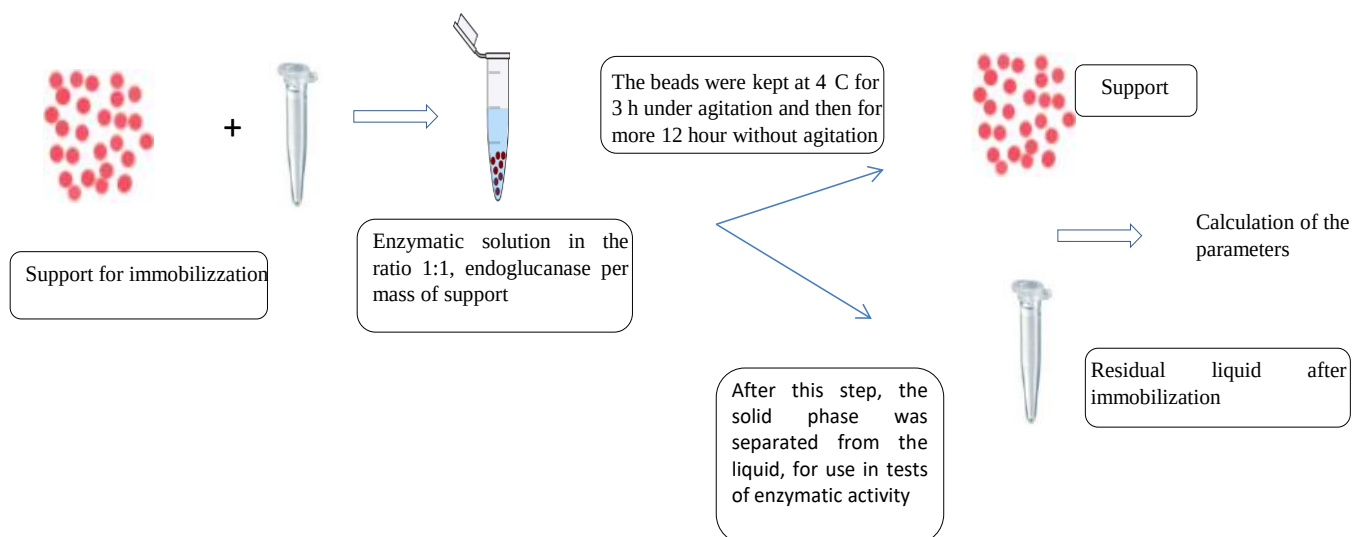


Fig.1: Flowchart of the enzyme immobilization process

Table.1: Parameters of immobilized cellulases

	IY (%)	AR (%)	LA (%)	E (%)	IPY (%)
<i>Aspergillus niger</i>	67.01 ± 3.78	53.57 ± 4.23	14.28 ± 1.89	78 ± 6.22	42.57 ± 5.07

CelStrep	55.44 ± 3.63	13.04 ± 5.11	42.41 ± 1.45	23.23 ± 7.69	70.41 ± 5.76
----------	--------------	--------------	--------------	--------------	--------------

Table.2: Percentage of polar amino acids (aa) in each domain of the studied endocellulases

	ConA-like Domain	CB Domain	Total number of aa
<i>Aspergillus niger</i>	223 aa	-	223 aa
% of polar aa	50.64	-	50.64
CelStrep	228 aa	103aa	331 aa
% of polar aa	45.13	39.78	43.5

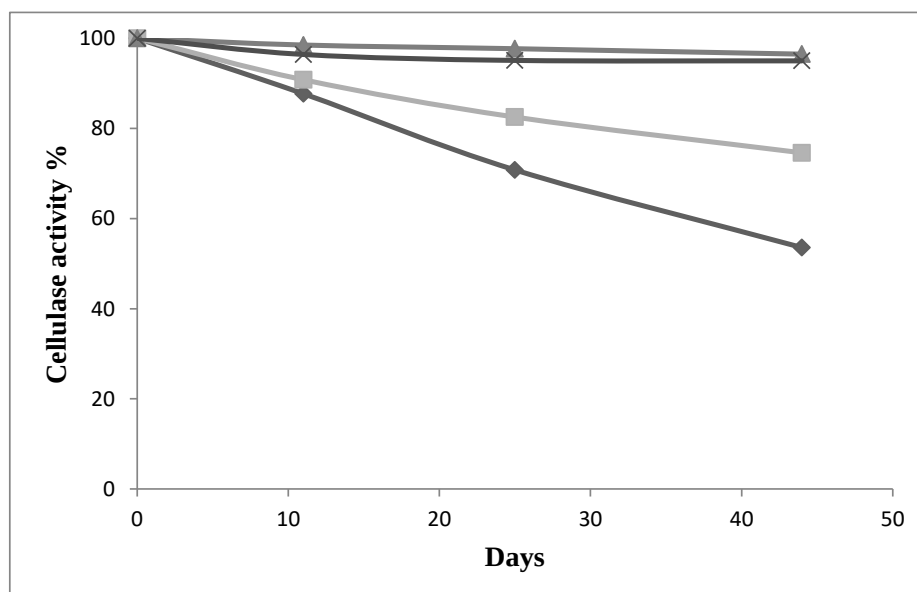


Fig.2: Stability of immobilized cellulases from *Aspergillus niger* (x) and Celstrep (▲) and of free enzymes, Celstrep (◆) and *A.niger* (■) during storage (at 4°C)