Revalorization of cellulosic wastes from *Posidonia oceanica* and *Arundo donax* as catalytic materials based on affinity immobilization of an engineered β-galactosidase

María José Fabra[1], Isabel Seba-Piera[1], David Talens-Perales[1], Amparo López-Rubio[1], Julio Polaina[1], Julia Marín-Navarro[1,2] *

[1]*Instituto de Agroquímica y Tecnología de Alimentos, CSIC, Valencia, Spain*

[2]*Departamento de Bioquímica y Biología Molecular, Universidad de Valencia, Spain.*

*Corresponding author.

E-mail address: juvicma@uv.es

Abstract

Catalytic materials obtained by enzyme immobilization have multiple potential applications in the food industry. The choice of the immobilization method and support may be critical to define the properties of the immobilized enzyme compared to the soluble form. Although the use of immobilized enzymes shows multiple advantages, their catalytic efficiency is compromised in many instances. Molecular engineering techniques have been used to generate hybrid proteins where the enzyme of interest is fused to a module with affinity to a specific biopolymer. Binding of the hybrid TmLac-CBM2 protein, in which the β-galactosidase from *Thermotoga maritima* is fused to a carbohydrate-binding module from *Pyrococcus furiosus*, to cellulosic material from aquatic biomass wastes (such as *Posidonia oceanica* and *Arundo donax*) has been assayed. Both species generate environmental wastes that could be revalorized if converted into bioactive materials. Cellulose cryogels, but not films, from *P. oceanica* were able to bind the TmLac-CBM2 hybrid, with a higher immobilization yield (90 %) than that from *A. donax* cellulose cryogels (60 %). However, fractions containing also hemicellulose were less effective as immobilization supports in both cases, with yields of 47 % and 30 %, respectively. Cellulose cryogels loaded with β-galactosidase were able to hydrolyse lactose with the same efficiency as the free form of the enzyme. In contrast, enzyme-loaded cellulose films were inactive. This study represents a proof of concept for the valorisation of cellulosic wastes as bioactive materials. Furthermore, it provides information about the interaction specificity between the binding module and the cellulosic support, useful for other enzymes.

Keywords

Lactase, cellulose, hemicellulose, enzyme bioadsorption, carbohydrate-binding module, bioaffinity-based immobilization

1. Introduction

The development of catalytic materials through enzyme-immobilization is of great interest for the food industry (Yushkova et al., 2019). Nowadays, immobilized enzymes are preferred over their corresponding free forms due to several technical and economic advantages, including improved stability and recovery of products with greater purity. Moreover, enzyme-activated supports can be implemented in continuous processes, applied as biosensors or used in the production of bioactive packaging (Khan, Wen, Huq, & Ni, 2018; Nguyen, Lee, Lee, Fermin, & Kim, 2019; Sharma & Leblanc, 2017; Sheldon & van Pelt, 2013). Several methods are employed for immobilization, mainly based on the adsorption of the enzyme to the support through chemical interactions (either covalent or non-covalent) (Bilal & Iqbal, 2019; Datta, Christena, & Rajaram, 2013; Jesionowski, Zdarta, & Krajewska, 2014; Sirisha, Jain, & Jain, 2016; Yushkova et al., 2019).

In the food industry, β-galactosidases (EC 3.2.1.23) are biotechnology relevant enzymes with multiple applications related to the production of lactose-free milk and milk derivatives. These enzymes catalyse the hydrolysis of terminal non-reducing β-D-galactose residues from di-, oligo- and conjugated saccharides, including lactose (Husain, 2010). Therefore, β-galactosidases with lactase activity are currently employed to generate lactose-free milk products (Adam, Rubio-Texeira, & Polaina, 2004), with an increasing demand due to the high prevalence of lactose intolerance among the world population (Harrington & Mayberry, 2008; Itan, Jones, Ingram, Swallow, & Thomas, 2010). Based on their hydrolytic properties, β-galactosidases are also used in large scale industrial processes for the treatment of cheese-whey derived from dairy industries to reduce pollution load and to produce sweeteners (Andler & Goddard, 2018; Husain, 2010; Oliveira, Guimarães, & Domingues, 2011). Furthermore, these enzymes also

68 catalyse transglycosylation reactions, which can be used to produce added-value
69 products from lactose, such as health beneficial prebiotics (e. g. lactulose or
70 galactooligosaccharides) (Andler & Goddard, 2018; Gänzle, 2012). However, there are
71 some important factors limiting the utilization of β-galactosidases in food applications,
72 for instance, the difficulties associated with recovering enzymes from the final product.
73 Different immobilization techniques and supports have been applied to β-galactosidases
74 (Marín-Navarro, Talens-Perales, Oude-Vrielink, Cañada, & Polaina, 2014; Ricardi et
75 al., 2018; Sen, Nath, Bhattacharjee, Chowdhury, & Bhattacharya, 2014; Urrutia, Bernal,
76 Wilson, & Illanes, 2018; Wolf, Gasparin, & Paulino, 2018; Yushkova et al., 2019).
77 Enzyme immobilization through covalent cross-linking providing multi-point
78 attachment usually results in improved stability (Sheldon & van Pelt, 2013). However,
79 the catalytic efficiency is compromised in many cases. Physical adsorption through
80 electrostatic interactions (e.g. using activated carbon supports) is a simple method and,
81 in general, it affects enzyme activity to a lesser degree than technologies mediated
82 through covalent crosslinking (Jesionowski et al., 2014). Even in this case, enzyme
83 confinement within the immobilization matrix may affect catalytic activity since the
84 conditions of the internal milieu may differ from the bulk solution. Both the chemical
85 characteristics of the solid support and the restrictions imposed to diffusion of substrates
86 and products contribute to the change in the microenvironment of the immobilized
87 enzyme, compared to the free form (Bolivar & Nidetzky, 2019). A particular case of
88 physical adsorption, referred as bioadsorption or affinity immobilization, is based on the
89 affinity of a protein or protein module to a specific substrate (Estevinho et al., 2018;
90 Mislovičová, Masárová, Vikartovská, Gemeiner, & Michalková, 2004). In this context,
91 molecular engineering techniques can be used to create hybrid proteins by fusion of the
92 enzymes to a module with affinity for a specific biopolymer. Such is the case of

carbohydrate-binding modules (CBM), which are found in different glycoside hydrolases and are able to bind specific polysaccharides. They are classified into different families by sequence homology, which is related to their substrate specificity (Boraston, Bolam, Gilbert, & Davies, 2004). In a recent work, Estevinho *et al.* (2018) showed the suitability of hydrated and freeze-dried bacterial cellulose (BC) to immobilize a hybrid enzyme composed by a β-galactosidase from *Thermotoga maritima* (TmLac) and a CBM2 from *Pyrococcus furiosus*, with high specificity compared to the non-engineered enzyme. The CBM2 provided a stable attachment of the hybrid enzyme to the BC support at high temperatures. However, initial enzymatic activity of the immobilized enzyme was lower (around 52 %) than that of the free form, probably because of diffusion constraints within the highly compact matrix of BC. Thus, cellulosic materials with different structures may be explored to circumvent this limitation. Moreover, the production cost of BC is usually quite high whereas multiple cellulose sources from agricultural, food or aquatic wastes are available and represent eco-friendly and low-cost alternative materials (Khiari & Belgacem, 2017; Rao & Rathod, 2018).

The marine plant *Posidonia oceanica*, which generates a large amount of coastal waste, and, the invasive plant *Arundo donax* are both recognized as important environmental problems (Khiari & Belgacem, 2017; Lambert, Dudley, & Saltonstall, 2010). These species show a high content of cellulose and hemicelluloses (Khiari & Belgacem, 2017), which may be used as immobilization supports. The strategy used in this work takes advantage of the potential of these natural resources in line with the current policies focused in bioeconomy and circular economy (Khiari & Belgacem, 2017; Pilavtepe, Celiktas, Sargin, & Yesil-Celiktas, 2013). This study is focused on investigating the potential of lignocellulosic materials obtained from biomass wastes to

generate bioactive supports through the immobilization of the hybrid enzyme TmLac-CBM2. Initially, the binding yield of the hybrid enzyme was tested on cellulosic fractions with different degrees of purity, and prepared in different physical forms (film vs. cryogel). On a second stage, the activity of the immobilized enzyme was analysed and compared to that of the free form. This study can be considered a proof of concept relative to the generation of catalytic materials, while taking into consideration different immobilized enzymes through specific interactions between the CBM2 module and the cellulosic support.

2. Materials and methods

2.1 Preparation of hybrid TmLac-CBM2 β-galactosidases

The hybrid enzyme used in this work (TmLac-CBM2) was constructed by fusing a β-galactosidase from *Thermotoga maritima* (TmLac) (Marín-Navarro et al., 2014) with a carbohydrate binding module (CBM2) from *Pyrococcus furiosus* chitinase (Nakamura et al., 2008). The genetic construct encoding the hybrid protein, as well as the production of both TmLac-CBM2 and the wild type TmLac by *Escherichia coli*, have been described in a previous work (Estevinho et al., 2018). Because both TmLac and TmLac-CBM2 are thermoresistant proteins, purification was carried out by heat shock treatment of crude extracts at 85 ^{0}C during 10 min. Endogenous *E. coli* proteins precipitated whereas most of the recombinant protein remained in the soluble fraction. This single step purification procedure was selected because it is much more cost-effective and less time-consuming than chromatographic methods. The soluble fraction was dialyzed against phosphate buffer 50 mM pH 6.5 and concentrated by ultrafiltration using a membrane with a 20 KDa cutoff (Thermo Fisher Scientific). Enzyme concentration was determined as the ratio of β-galactosidase activity (expressed in μmol

galactose · min^{-1} · ml^{-1} sample) divided by the intrinsic activity of TmLac-CBM2 (28 μmol galactose·min^{-1}·mg $enzyme^{-1}$), as previously described (Estevinho et al., 2018).

2.2 Preparation of cellulosic fractions

Posidonia oceanica leaves were collected directly from beaches located in Calpe (Valencian Community, Spain) and the stems of *Arundo donax* (common cane) were obtained from a freshwater environment in Buñol (Comunidad Valenciana, Spain). All the materials were thoroughly washed with water to remove impurities like sand/soil and salts.

In order to sequentially eliminate the different components of the cell wall and obtain the different lignocellulosic fractions, a purification procedure previously applied to other plant resources was carried out (Benito-González, López-Rubio, & Martínez-Sanz, 2018; Lu & Hsieh, 2012b, 2012a). Initially, a Soxhlet extraction step was carried out to remove wax, pigments and oils. 40 g of ground wet *Posidonia* leaves or *Arundo donax* stem biomass (corresponding to approximately 4 g of dry material) were extracted with 800 mL of toluene/ethanol (2:1 v/v) during 24h. The resulting material was dried at room temperature overnight to obtain the first fraction (referred to as F1). Subsequently, the lignin was eliminated by submitting the previously ground de-waxed material (F1) to a 5 h incubation at 70 ° C with 700 mL of a 1.4% $NaClO_2$ solution, having the pH adjusted to 3 with acetic acid. After that, the reaction was stopped by quenching with ice, and the excess liquid was decanted. The yellowish solid was collected and repeatedly washed with distilled water by vacuum filtration until the filtrate became approximately neutral, thereby obtaining the second fraction (referred to as F2). The de-lignified material was then treated with 400 mL of 5% KOH solution for

24 h at room temperature, followed by 2 h at 90 ° C, in order to remove the hemicelluloses, yielding pure cellulose (referred to as F3).

2.3 Formation of hydrated cellulose, films and cryogels

Initially, 0.25 g of material were resuspended in 50 mL of 50 mM phosphate buffer at pH 6.5 and dispersed by mild sonication, followed by ultra-turrax homogenization until obtaining homogeneous, viscous suspensions, which were used to prepare hydrated cellulose, films and cryogels.

Hydrated cellulose was collected after centrifugation (1500 g for 15 minutes) of the viscous solutions and removal of the excess of solvent by carefully pipetting the supernatant.

Films were obtained by filtering the viscous solution using a vacuum pump (Comecta, S.A.) and PTFE filters with 0.2 μm pore size to remove the phosphate buffer. The solid material remaining in the filter was then dried at room temperature overnight. The formed films were peeled off the filters and stored at 0% relative humidity. For comparative purposes, cellulose films were also prepared after incubating 2.5 g of hydrated cellulose (F3) with 4.2 mL of enzyme (as described in section 2.4, immobilization step). After removal of the unbound enzyme, 50 mM phosphate buffer at pH 6.5 was added (up to 50 mL), and the film was formed by filtration as previously described. These films prepared with enzyme-bound cellulose were referred as Enzy-films.

The cryogels were prepared by freeze-drying the previously obtained viscous solutions from each of the lignocellulosic phases.

2.3 Enzyme immobilization

192 The immobilization of the hybrid β-galactosidase (TmLac-CBM2) on the different
193 cellulosic matrices was carried out by incubating 30 mg of the material with 0.5 mL of
194 enzyme (0.5 mg/mL) in phosphate buffer 50 mM pH 6.5 under constant stirring (180
195 rpm) for 4 h at 37 ºC. Afterwards, unbound enzyme was directly recovered from the
196 supernatant. The lignocellulosic support was then washed once with 1 mL of assay
197 buffer (50 mM phosphate pH 6.5, 10 mM NaCl, 1 mM $MgCl_2$) to remove the unbound
198 enzyme and the lactase activity of the immobilized enzyme was determined in the
199 lignocellulosic support. Control samples of enzyme without lignocellulosic supports
200 were incubated in parallel under the same conditions. All the assays were performed in
201 triplicate.
202 Immobilization yield (IY) was calculated according to the following formula:
203 $IY = [(C - UB)/C] \cdot 100$
204 where C and UB correspond to the β-galactosidase activity of the control and unbound
205 samples, respectively.
206
207 **2.4 Enzyme assays**
208 The quantification of β-galactosidase activity was determined at 75 ºC using as
209 substrate a 5 mM solution of *p*-nitrophenyl β-D-Galactopyranoside (PNPGal) in the
210 assay buffer (50 mM phosphate pH 6.5, 10 mM NaCl, 1 mM $MgCl_2$). Enzymatic
211 activity was quantified spetrosphotometrically at 400 nm (NanoDrop ND-1000)
212 corresponding to the maximum absorption of the *p*-nitrophenyl.
213 The lactase activity of the free and immobilized enzyme was tested by incubating the
214 lignocellulosic supports with 5 % (w/v) of lactose solution in assay buffer at 75 ºC in a
215 final volume of 20 mL. Control samples containing similar enzyme amounts of soluble
216 enzyme were analysed in parallel. The amount of glucose released was quantified using

a glucose assay kit (P7119-10CAP, Sigma- Aldrich, Madrid, Spain) by measuring with a UV/Vis spectrophotometer (BeckMan DU530, GMI, Minnesota, USA) at 450 nm. One unit (IU) is defined as the amount of enzyme releasing 1 μmol of glucose per minute.

2.5 Scanning Electron Microscopy (SEM) analysis.

The microstructural analysis of the cryogels was carried out by means of a Scanning Electron Microscope (Hitachi S-4800). Films were cryo-fracturated after immersion in liquid nitrogen and randomly broken to investigate the cross-section of the samples. Cryo-fracturated films and cryogel samples were mounted on aluminium stubs and fixed on the support using a double-size adhesive tape. Finally, samples were gold–palladium coated and observed using an accelerating voltage of 10 kV and a working distance of 10 mm.

2.6 Statistical analysis

The statistical analysis of data was carried out by means of IBM SPSS Statistics software (v.23) (IBM Corp., USA) through the analysis of variance (ANOVA). Tukey's Honestly Significant Difference (HSD) was used at the 95% confidence level for multiple comparison tests

3. Results and Discussion

3.1. Effect of the physical state of the cellulosic support on the binding yield of the enzyme mediated by the CBM2 module

The binding yield of the hybrid TmLac-CBM2 to pure cellulose (F3) from *P. oceanica* in three different physical states: hydrated cellulose, cryogel or film was compared. As shown in Figure 1, the enzyme was successfully immobilized on pure cellulose both in the format of hydrated cellulose and cryogel, with a somewhat higher yield in the latter case. In contrast, the hybrid enzymes were unable to bind to the cellulosic film. This is likely due to the highly compact structure of the film, compared to the cryogel (see Figure 2). The high density of hydrogen bonds interconnecting cellulose fibres within the film probably hampers the interaction with the CBM2 module. Therefore, the cryogel format was selected for further studies.

The binding yield of the cellulose cryogel from *P. oceanica* (7.5 mg enzyme/g of support) obtained after incubation of 30 mg of support with 0.25 mg of enzyme was within the range of the values reported for the freeze-dried bacterial cellulose (3.6 - 9.2 mg enzyme/g of support), assayed under similar conditions (incubation of 30 mg of support with 0.15 - 0.35 mg of enzyme) (Estevinho et al., 2018). This result suggests that the available number of binding sites in both supports is comparable. In the previous study, immobilization of the TmLac-CBM2 to bacterial cellulose was stable at high temperature, with negligible enzyme leaching after incubation at 75 °C for 3 hours (Estevinho et al., 2018). This indicates that the CBM2 module shows a strong affinity for cellulose, allowing reusability of immobilized chimeric enzymes as long as the catalytic module remains active. A similar result would be expected for *P. oceanica* cellulose.

3.2. Analysis of the binding yields of the TmLac-CBM2 hybrid to different cellulosic fractions from *P. oceanica* and *A. donax*.

The suitability of less purified cellulosic fractions from *P. oceanica* and *A. donax* for the development of enzymatically active material was also evaluated. Three different cellulosic fractions from *P. oceanica* and *A. donax* were compared as immobilization supports for the TmLac-CBM2 hybrid. Fraction F1 was obtained after the elimination of lipids, pigments and oils from the original sample. Subsequently, F2 was isolated after removal of lignin and finally, F3 was obtained as pure cellulose after separation from hemicelluloses. Fraction F1 from both sources was discarded because it could not be recovered as a cryogel form after freeze-drying, giving rise to a powder which was not easily handled.

Figure 3 shows the affinity of TmLac-CBM2 for F2 and F3 supports obtained from both sources. Immobilization yield mediated by the CBM2 module was more efficient with pure cellulose than with the fraction containing hemicellulose. This is a relevant result since CBMs belonging to family 2 are present in a wide range of cellulolytic enzymes including cellulases, chitinases, xylanases and mananases (Lombard, Golaconda Ramulu, Drula, Coutinho, & Henrissat, 2014). Thus, carbohydrates as cellulose, chitin, xylan and manan are potential substrates for these modules. Specifically, the CBM2 from *P. furiosus* used in the current work was able to bind both cellulose and chitin (Estevinho et al., 2018; Talens-Perales, Marín-Navarro, Garrido, Almansa, & Polaina, 2017) but its affinity for hemicelluloses has not been previously studied. The fact that the binding yield was higher for F3 (pure cellulose) than for F2 (containing both cellulose and hemicellulose) suggests that the CBM2 used here (from *Pyrococcus furiosus*) is more specific for cellulose than it is for hemicellulose. This behaviour may rely on the different chemical structure of cellulose and hemicellulose. While cellulose is composed solely by glucose units interconnected in a linear polysaccharide, which is able to form highly structured fibres, hemicelluloses display a higher diversity of

chemical compositions and structures (including linear or branched) (Chen, 2014). Indeed, pure cellulose fraction (F3) showed a more fibrous structure than F2, probably because of the higher heterogeneity of the latter (Figure 4). Binding to the CBM2 module is based on the interaction of the linear polysaccharide with three structurally aligned tryptophan residues (Nakamura et al., 2008). Thus, the branching sites in hemicelluloses may interfere with the interaction at this planar binding platform (Figure S1 in Supplementary Material).

Another conclusion from the results presented in Figure 3 is that the cellulosic fraction extracted from *P. oceanica* is a better substrate for the immobilization of the hybrid enzyme than that from *A. donax*. Substrate specificity of the CBMs not only relies on the chemical composition but also in the high-order structure of the polysaccharide. Thus, some CBMs are specific for amorphous cellulose while others bind preferentially to crystalline cellulose (Boraston et al., 2004). The polysaccharides extracted from *P. oceanica* and *A. donax* may show distinct molecular structural arrangements, not detected by SEM analysis, that explain the differences in TmLac-CBM2 binding yields.

3.3. Comparative analysis of the lactase activity of free and immobilized enzyme with different cellulosic supports.

After characterizing the suitability of each cellulosic fraction as a support for the enzyme, the activity of enzyme loaded cryogels from both *P. oceanica* and *A. donax* was compared to that of the enzyme in the free form. Furthermore, the activity of the enzyme immobilized in a cellulose film, here named Enzy-film, was also assayed. Since TmLac-CBM2 was not attached to a cellulose film when it was added exogenously (Figure 1), Enzy-film was obtained by enzyme immobilization on the hydrated cellulose

from *P. oceanica* and subsequent generation of the film. Such preparation would be more easily handled than the cryogel form.

Initial lactase activities (after incubation for 30 minutes) were roughly the same for the enzyme immobilized in the cellulose cryogel from *P. oceanica* or *A. donax* and the free form (around 40 IU/mg enzyme). Taking in account the different immobilization yields of both materials, this represents an activity of 210 and 300 IU/g of support for *A. donax* and *P. oceanica* cellulose, respectively. The immobilization efficiency (defined as the fraction of observed activity after immobilization from the total immobilized activity) (Sheldon & van Pelt, 2013) is 100 % in both cases. However, the Enzy-film was inactive under the assayed conditions (Table S1 in Supplementary material). This may be explained because the dense network of interactions among cellulose fibres in the film may difficult the accessibility of lactose to the enzyme within this matrix. The result obtained with the cellulose cryogel from *P. oceanica* and *A. donax* was better than that reported for bacterial cellulose, which showed immobilization efficiencies of 27 % (freeze-dried) and 52 % (hydrated) for the same enzyme (Estevinho et al., 2018). The high crystallinity of bacterial cellulose (Benito-González et al., 2018; Martínez-Sanz, Lopez-Rubio, & Lagaron, 2011), may provide a compact structure where substrate diffusion is a limiting factor for optimal activity.

The hydrolysis of lactose with free and immobilized enzyme was followed for a longer period of incubation, up to 8 hours, using 30 mg of the enzymatically active cryogels and the corresponding amount of enzyme in the free form (Figure 5). Again, no differences were found between free and bound enzymes neither for *P. oceanica* nor for *A. donax* cellulose. Lactose hydrolysis was more efficient with the catalytic cryogel from *P. oceanica*, as a result of the higher immobilization capacity of this support (Figure 3).

4. Conclusions

This work shows that the efficiency of bioadsorption of hybrid enzymes mediated by the CBM2 module relies on both the chemical nature and the supra-molecular structure of the polysaccharides used as the support. On the other hand, to assure an efficient action of the bound enzyme, substrate diffusion into the immobilization matrix should not be hampered by a dense network of interactions connecting the polysaccharide fibres. The cellulose extracted from *P. oceanica* and *A. donax* fulfils the requisites for efficient enzyme immobilization and operation. Thus, this approach represents an economic and environmentally-friendly revalorization of a waste material as enzyme support.

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Figure legends.

Figure 1. Immobilization yield of TmLac-CBM2 to cellulose (F3) from *P. oceanica* in different physical states. Error bars represent standard deviation of triplicates. Homogeneous groups are indicated with lower case letters (significance level α=0,05); nd: not detected.

Figure 2. SEM images of a film (A) and cryogel (B) obtained from cellulose (F3) from *P. oceanica*

Figure 3. Immobilization yield of TmLac-CBM2 to cellulose fractions (F2 and F3) from *P. oceanica* and *A. donax* in the form of cryogel. Error bars represent standard deviation of triplicates. Homogeneous groups are indicated with lower case letters (significance level α=0,05).

Figure 4. SEM images of cryogels obtained from fractions 2 (F2P and F2C) and 3 (F3P and F3C) isolated from *P. oceanica* (F2P and F3P) or *A. donax* (F2C and F3C).

Figure 5. Kinetics of lactose hydrolysis with bioactivated cryogels from *P. oceanica* (PO) or *A. donax* (AD) or with the equivalent amounts of enzyme in the free form (CP and CO, respectively). Error bars represent standard deviation of triplicates.

Figure 1

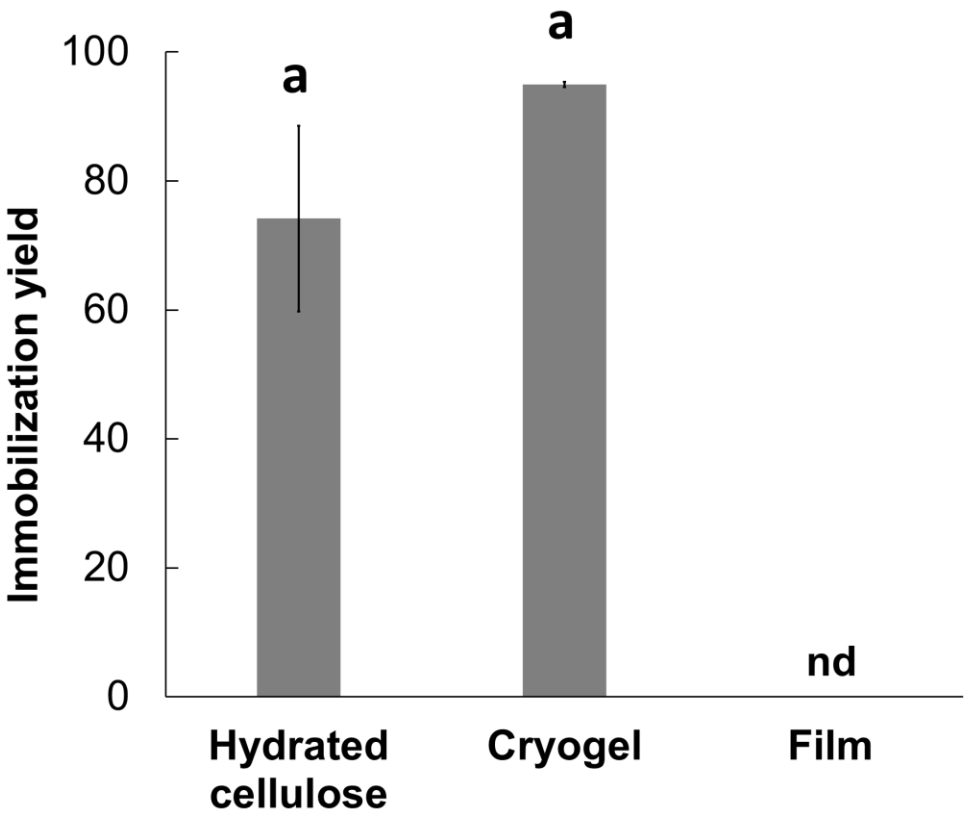

Immobilization yield
100
80
60
40
20
0
a
a
nd
Hydrated cellulose
Cryogel
Film

Figure 2

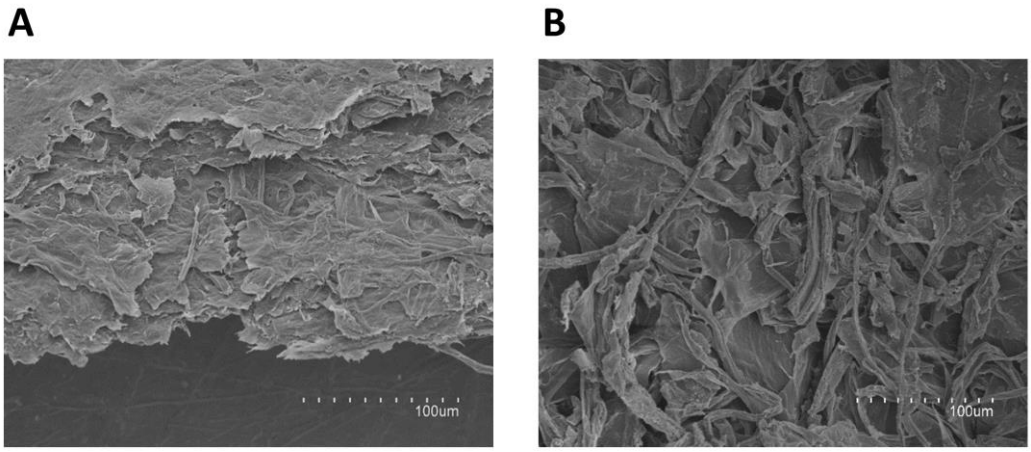


Figure3

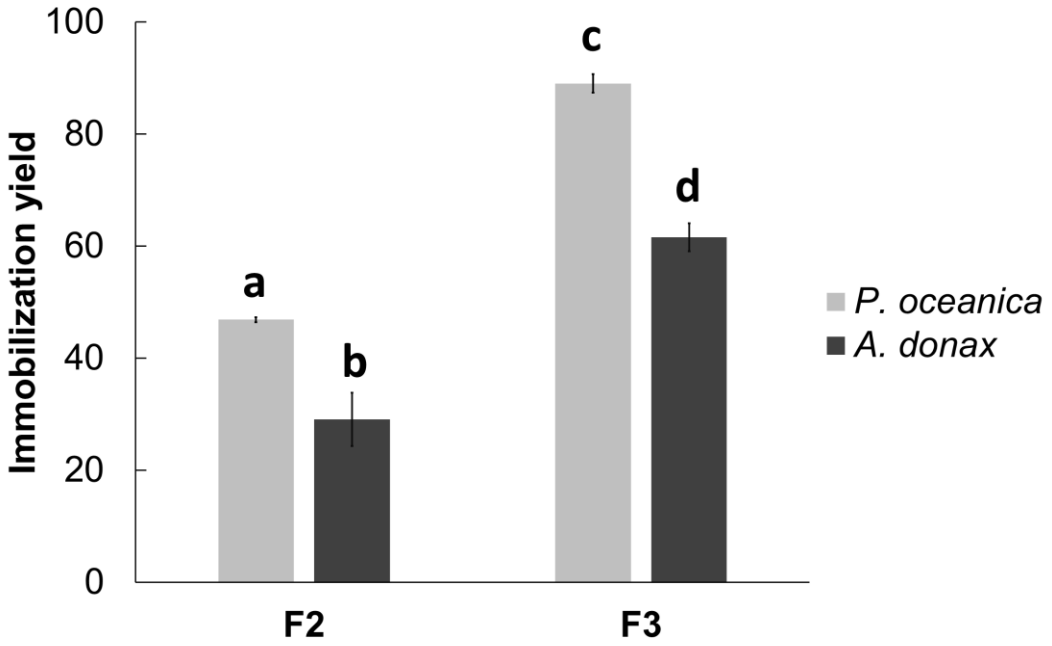
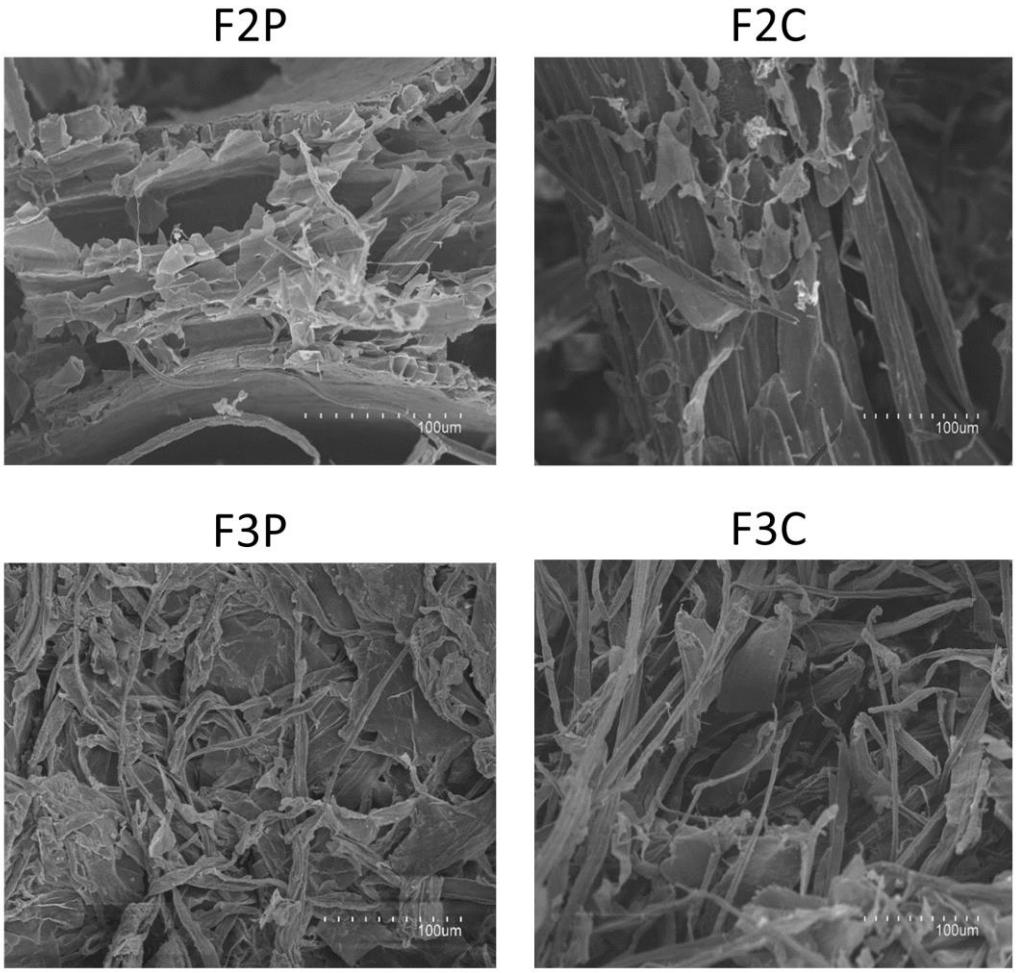
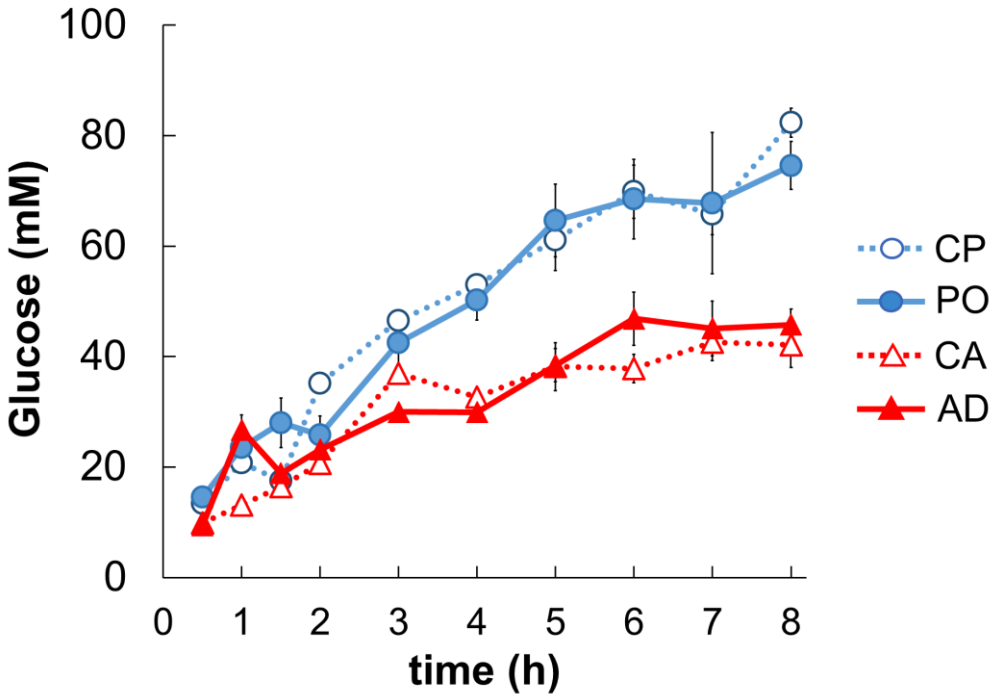

100
80
60
40
20
0
Immobilization yield
a
b
c
d
F2
F3
P. oceanica
A. donax

Figure 4



579 **Figure 5**



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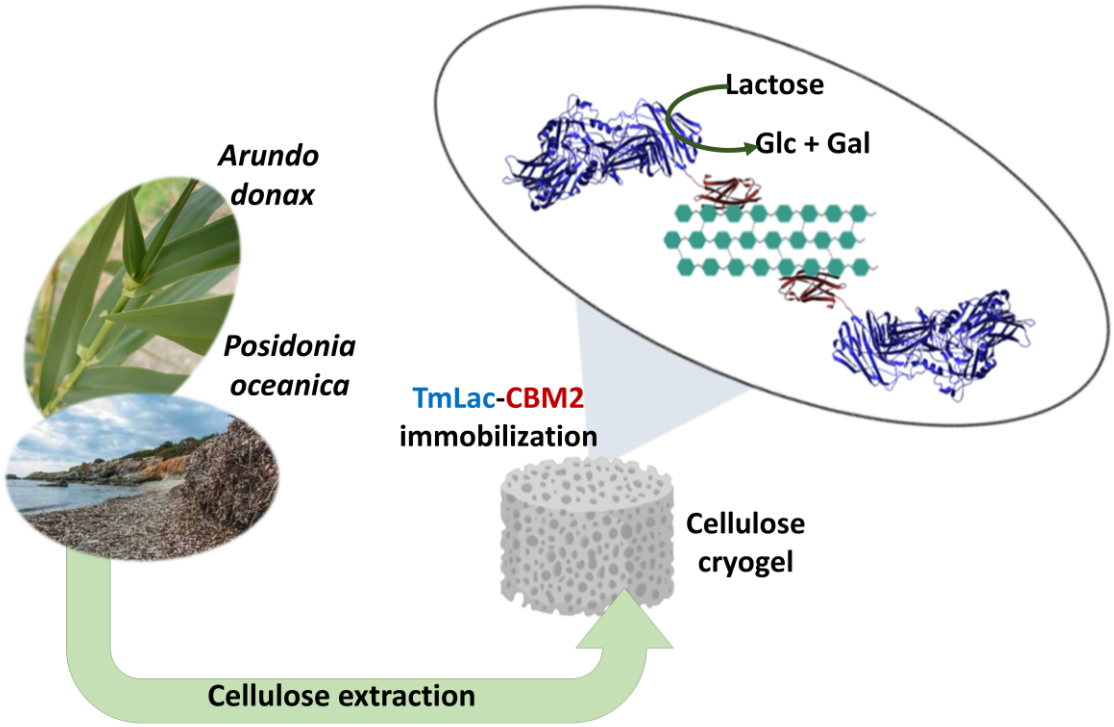
581 **Graphical Abstract**



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