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A thermophilic enzymatic cocktail for galactomannans degradation

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1. Background

Lignocellulose is the most abundant available feedstock produced every-day on the Earth and it is constituted by cellulose (35-50%), hemicellulose (26-35%) and lignin (14-21%), as well as by other minor components [1]. Lignin provides the structural integrity of the plant, encapsulating the microfibrils of hemicellulose and cellulose, to withstand the herbivores and pathogens attacks. Hemicellulose is the second most abundant biopolymer present in lignocellulosic-feedstocks [2]. Unlike cellulose, a linear homopolymer of b(1,4)-linked D-glucose residues, hemicellulose is a branched heteropolymer composed by pentoses (i.e. xylose and arabinose), hexoses (i.e. glucose, galactose, mannose) and also by sugars in acidified form (glucuronic acid and galacturonic acid) [3]. Mannans are the major source of secondary cell wall found in hemicellulose fraction of conifers (softwood) and leguminoses. On the basis of their sugars components they are classified in: mannans, glucomannans, galactomannans and galactoglucomannans [4]. During the detrital food webs, the polysaccharides hydrolysis is carried out by saprophytes and detritivores, as the natural process for the deconstruction of biomasses [5]. Since lignocellulosic feedstock is clean and available in large amount, the biomass is currently used to produce value added-products such as bio-fuels and chemicals [1,6]. In the industrial processes, the deconstruction is performed using chemical and physical pretreatments upon which the lignin is dis-arrayed [7]. The resulting polysaccharides (i.e. cellulose and hemicellulose) are subsequently hydrolyzed by enzymatic mixture to produce fermentable sugars. This latter process, also named saccharification, involves an array of (hemi)cellulases, auxiliary enzymes and proteins to obtain an effective hydrolysis [8].

In nature plant biomass degradation is accomplished by the complex action of various glycosyl hydrolases (GH) enzymes. To achieve an efficient hydrolysis of galactoglucomannans, the presence of multiple GHs such as β-glucosidases (EC 3.2.1.21), endo-mannanases (EC 3.2.1.78), mannosidases (EC 3.2.1.25) and α-galactosidases (EC 3.2.1.22), is needed [9]. Therefore, the optimization of enzymatic mixtures to improve the conversion of biomasses into fermentable sugars is needed for biorefinery purposes. Nevertheless, a major issue in this context is to set up the right reaction conditions to achieve a synergistic interaction among enzymes that act on the same complex substrate. Moreover, enzymes belonging to diverse families can display synergistic and/or antisynergistic interaction due to their own substrate specificities. A synergistic association between two or more enzymes is present when the degree of synergy (DS) is greater than 1.0 and therefore produces a degradation yield greater than that obtained from enzymes acting separately. Synergy among mannanolytic enzymes is classified in two types: i) homosynergy between two main-chain
enzymes or two side-chain enzymes; ii) heterosynergy between side-
and main-chain enzymes [4].

Previous studies showed that galactomannans could be effectively
degraded by the combined action of a main-chain-cleaving mannanase
and a side-chain-cleaving galactosidase compared to when mannanases
or galactosidas were used alone [10]. Since the pretreatment step is
performed at high temperature (90°–120 °C), the development of
thermophilic enzymatic mixtures which could operate at high temperature
is needed to reduce the whole process cost [11]. However, knowledge
about thermophilic enzymatic cocktails is scarce. Therefore, it is
interesting to study the synergistic action of enzymes derived from dif-
ferent “hot” sources that can be employed in biomasses hydrolysis right
after the pretreatment.

The main objective of this work has been to study the synergistic
effect of the thermophilic endo-1,4-β-mannanase (DturCelB) from
Dictyoglomus turgidum and α-1,6-galactosidase (TtGalA) from Thermus
thermophilus on galactomannan substrates from Locust bean gum, Carob
and Guar. D. turgidum, the hyperthermophilic gram-negative anaerobic
bacterium, was isolated by a Japanese hot spring and shows optimal temperature of grow at 74 °C
[12].

2. Methods

2.1. Substrates

Locust bean gum was purchased from Sigma-Aldrich. Galactomannans (Carob, Low viscosity and Guar, Medium viscosity) were purchased from Megazyme.

2.2. Expression and purification of recombinant enzymes

Dtur_0671 gene, encoding DturCelB, was synthetically produced and cloned into the Ndel/XhoI digested pET30b (+) vector to express protein in E. coli BL21 DE3 strain. The transformant cells, grown until stationary phase, were induced by 0.5 mM IPTG for 18 h at 25 °C. The protein was purified by two steps: a heat-treatment at 70 °C for 15 min and an affinity chromatography on a His-Trap column [18]. TTP0072 gene, encoding TtGalA, was amplified by PCR from T. thermophilus HB27, the thermo-
philic and aerobic gram-negative bacterium, was isolated from water at a Japanese hot spring and shows optimal temperature of grow at 74 °C
[12].

2.3. Substrate specificity determination of DturCelB and TtGalA towards galactomannans

DturCelB and TtGalA activities were determined using Locust bean gum, Carob and Guar as polymeric substrates. The reaction mixtures (1 mL) containing one of the purified enzymes (1 μg) were assayed using 1% galactomannan substrates dissolved in 50 mM citrate-phosphate buffer pH 6.0. The reaction was carried out at 80 °C for 30 min and the concentration of reducing ends was determined following the Nelson-Somogyi (NS) method, using mannose as standard [13]. All enzyme assays were performed in triplicate. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of product per min, under the above assay conditions.

2.4. DturCelB and TtGalA synergistic action

To evaluate the degree of synergy between DturCelB and TtGalA the enzymes were tested simultaneously and sequentially using 1% of ga-
lactomannan substrates (Locust bean gum, Carob and Guar) dissolved
in 50 mM citrate-phosphate buffer pH 6.0. For the simultaneous assay, various ratios of DturCelB and TtGalA were tested (50% DturCelB–50% TtGalA; 25% DturCelB–75% TtGalA; 75% DturCelB–25% TtGalA) for a total amount of 2 μg. The assays were carried out as described above
through NS method.

For the sequential assay 1 μg of DturCelB or TtGalA was incubated at
80 °C for 30 min in the reaction mixture described above. Afterwards, the mixture was boiled for 10 min to inactivate the first enzyme. After
ice-cooling, the second enzyme (1 μg) was added to the mixture and the reaction was carried out under the same conditions (80 °C for 30 min).
Reactions containing only one of the heat-inactivated enzyme were
used as a negative control. All the samples were analyzed for the concent-
ration of reducing ends by NS method using mannose as standard. All enzyme assays were run in triplicate.

2.5. Synergy studies

To investigate the interaction between two or more enzymes, sy-
ergism is calculated as ratio between the observed activity of the en-
zyme mixture and the theoretical sum of individual specific activity of
the same enzymes. The degree of synergy (DS), between DturCelB and
TtGalA, was determined by the following equation:

$$DS = \frac{Y_{1+2} - (Y_1 + Y_2)}{(Y_1 + Y_2)}$$

where $Y_{1+2}$ indicates the yield (μg) of reducing sugars achieved by the
two enzymes working simultaneously or sequentially, $Y_1$ and $Y_2$ in-
icate the yields (μg) of reducing sugars achieved by each enzyme when
working separately.

3. Results and discussion

3.1. Determination of specific activity of DturCelB and TtGalA on different galactomannans

The recombinant enzymes DturCelB and TtGalA were previously
characterized for their biochemical catalytic features [18]. In this study,
the hydrolytic endo-mannanase activity of DturCelB was assayed at
80 °C and pH 6.0 towards Locust bean gum (44.0 U mg⁻¹), Carob
(40.3 U mg⁻¹) and Guar (2.8 U mg⁻¹) (Table 1).

The different catalytic efficiency can be explained by the increasing
number of galactose residues (Guar > Carob > Locust bean gum) branch-
ning out from the linear mannan backbones and causing steric
hindrance to the enzymes (Fig. 1).

A similar behaviour was also demonstrated for Clostridium thermo-
cellum Man5A [14]. Therefore, one way to improve the DturCelB hy-
drolysis of galactomannans is to combine its catalytic activity with an
α-galactosidase acting on the branched glycosidic 1,6-α-bounds be-
tween galactose and mannose. As potential partner, it was chosen
TtGalA, an α-galactosidase from T. thermophilus performing its highest
catalytic activity at 90 °C and pH 6.0 on synthetic pNP-α-α-galacto-
pyranoside substrate (pNP, Sigma) [12]. Assays conditions for the two
enzymes were set at 80 °C and pH 6.0 because

\[ \text{Table 1} \]

<table>
<thead>
<tr>
<th>Substrate</th>
<th>DturCelB Specific activity (U mg⁻¹)</th>
<th>TtGalA Specific activity (U mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Locust bean gum (G/M:1/4)</td>
<td>44.0</td>
<td>4.4</td>
</tr>
<tr>
<td>Carob (G/M:1/3.5)</td>
<td>40.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Guar (G/M:1/2)</td>
<td>2.8</td>
<td>0.33</td>
</tr>
</tbody>
</table>
than that detected on pNPG substrate (338 U mg\(^{-1}\)). The different specific activities are in agreement with the preference of TtGalA towards galactose-oligosaccharides over polysaccharides, as for other GH36 members [10]. Nevertheless TtGalA catalytic activity on polymeric substrates is not negligible (Table 1), indeed it is higher if compared to that of a GH36 AgaC (1.0 U mg\(^{-1}\)) from Aspergillus niger and very similar to that of a GH27 Aga27A from Cyamopsis tetragonolobus (3.7 U mg\(^{-1}\)) [10]. Therefore, the synergistic association between these two thermophilic enzymes might be functional to improve the hydrolysis of hemicellulose as already demonstrated in other systems [4,10].

3.2. Heterosynergistic studies of TtGalA and DturCelB towards three different galactomannans

The aim of this study was centred on the setting up of reaction conditions suitable to achieve heterosynergy between TtGalA and DturCelB to ameliorate the galactomannans hydrolysis. The synergistic interaction between the recombinant enzymes was assessed through the quantification of the reducing sugars released during the degradation of the three galactomannan substrates. These contained a different ratio of galactose- versus mannose- residues to assess how the activity and synergistic interactions of TtGalA and DturCelB were influenced by the extent of galactose substitution on the mannan backbone (Fig. 1). In simultaneous assays the enzymes were added to the reaction mixture at the same time, varying their relative ratio (50%–75%) no synergy was exhibited first (Fig. 2B). These results demonstrate that TtGalA significantly supported DturCelB activity by removing galactose branches on the polymer that would have sterically hindered DturCelB.

Locust bean gum and Carob are both isolated from Ceratonia siliqua. These galactomannan polymers display different chemical and rheological properties depending on their geographic origin [17]. The reported G/M ratio of Carob is slightly lower (1/3.5) than Locust bean gum (Fig. 1) and our data indicate that the specific activity of TtGalA on Carob is 30% of that on Locust bean gum (Table 1). Therefore, we resolved to perform a comparative synergy study of the two thermophilic enzymes also using this substrate. In fact, when DturCelB and TtGalA were assayed in combination of 50%–50% no synergy was exhibited (DS = 0.8) (Fig. 3A). This result might be explainable with a complex nature of the Carob substrate (purity degree, extent of galactose ramifications) that renders the binding of TtGalA not completely productive, thus in turn inhibiting the DturCelB hydrolysis when they are present in the enzymatic mixture in a similar amount. However, a similar degree of synergy (DS = 1.4 on Carob vs DS = 1.3 on Locust bean gum) was achieved when the enzymes were assayed simultaneously, with a protein ratio of DturCelB to TtGalA 75%–25% and the total amount of reducing sugars released was also comparable (467 µg vs 454 µg) (Figs. Figure 3A and Figure 2A). Yet, the highest DS obtained on Carob
(DS = 1.4) was indeed lower than that measured on Locust bean gum (Fig. 2A, DS = 1.8), indicating that the two enzymes performed their synergistic catalytic activity, less efficiently on this substrate (Figs. Figure 2A and Figure 1A). This result can be only explained by the low specific activity of TtGalA on Carob, since the affinity of DturCelB on Carob and Locust bean gum is almost the same (Table 1). Our data highlighted the role of TtGalA, that plays a major function in enhancing the DturCelB activity, improving the linear mannan chain accessibility. Accordingly, results from the sequential assay show clearly that also on Carob the synergistic association, between the two enzymes, benefits (DS = 1.5) by the previous action of the debranching enzyme.

The Guar backbone is composed of a linear chain of mannose residues (Fig. 1), where the galactose residues branch at every second mannose residue. The specific activity of DturCelB and TtGalA on Guar was lower than that obtained on Locust bean gum (Table 1), due to the higher extent of galactose substitutions (Fig. 1). Accordingly, the total yield of reducing sugars obtained on this substrate was much lower than that on Locust bean gum and Carob (Fig. 2–4). Nevertheless, in simultaneous assays our data clearly indicate that the synergistic interaction between the two enzymes occurred also using Guar as substrate (i.e. DS ≥ 1.0) under all the conditions tested (Fig. 4A). The sequential assays further confirmed that the prior action of TtGalA by removing galactose substituents, increases the release of reducing sugar by DturCelB (Fig. 4).

4. Conclusions

One of the major factor contributing to increase the yield of the efficient lignocellulose biomass conversion yield, resides in understanding how different enzymes may cooperate to degrade complex polymeric substrates. Both the new isolated thermophilic DturCelB and TtGalA enzymes performed a better catalytic activity working in synergy rather than alone, preferring the low galactose-polysaccharides than the highly galactose decorated polymers used in this study. In fact, a good degree of heterosynergy relationship with each other on galactomannan degradation was clearly demonstrated on all the substrate tested at high temperature (80 °C) and in a relatively short time (30 min) compared to other studies [10]. Based on the sequential assays, the synergy was a result of TtGalA activity, which removes galactose branches from the galactomannan polymers, then improving the accessibility of the linear mannan backbone to DturCelB. Our finding also revealed that the 25%–75% ratio of DturCelB and TtGalA is the best combination to attain a compromise between a good degree of synergy and the highest yield of reducing sugars released. The strength point of this enzymatic cocktail resides in the thermophilicity and thermostability of both the TtGalA and DturCelB enzymes [12], that allows to foresee their employment during the gradual cooling right after the pretreatment of lignocellulosic material. The addition of thermophilic enzymes earlier in this step would result in time savings and improved conversion efficiency of the whole process, compared to the use of mesophilic thermophilic enzyme cocktails.

Conflict of interests

None.

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