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A thermophilic enzymatic cocktail for galactomannans degradation

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ABSTRACT

The full utilization of hemicellulose sugars (pentose and exose) present in lignocellulosic material, is required for an efficient bio-based fuels and chemicals production. Two recombinant thermophilic enzymes, an *endo*-1,4-β-mannanase from *Dictyoglomus turgidum* (*Dtur*CelB) and an α-galactosidase from *Thermus thermophilus* (*Tt*GalA), were assayed at 80 °C, to assess their heterosynergystic association on galactomannans degradation, particularly abundant in hemicellulose. The enzymes were tested under various combinations simultaneously and sequentially, in order to estimate the optimal conditions for the release of reducing sugars. The results showed that the most efficient degree of synergy was obtained in simultaneous assay with a protein ratio of 25% of *Dtur*CelB and 75% of *Tt*GalA, using Locust bean gum as substrate. On the other hand, the mechanism of action was demonstrated through the sequential assays, i.e. when *Tt*GalA acting as first to enhance the subsequent hydrolysis performed by *Dtur*CelB. The synergistic association between the thermophilic enzymes herein described has an high potential application to pre-hydrolyse the lignocellulosic biomasses right after the pretreatment, prior to the conventional saccharification step.

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 leguminosae. 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 disarrayed [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

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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 galactosidases 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 different "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 (*Dt*urCelB) from *Dictyoglomus turgidum* and α-1,6-galactosidase (*Tt*GalA) from *Thermus thermophilus* on galactomannan substrates from Locust bean gum, Carob and Guar. *D. turgidum*, the hyperthermophilic gram-negative anaerobic bacterium, was isolated from a hot spring in the Uzon Caldera, in Russia and grows up to 80 °C [18], while *T. thermophilus* HB27, the thermophilic 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. 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 *Dtur*CelB, was synthetically produced and cloned into the *Nde*I/*Xho*I 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 genomic DNA and cloned into the *Nde*I/*Hind*III digested pMKE2 vector for the expression in *T. thermophilus* HB27:nar strain.The recombinant protein, bear a His-tag at their N-terminus, was purified by two steps: an anionic exchange chromatography on a Hi-trap Q HP column and an affinity chromatography on a His-Trap column [12].

2.3. Substrate specificity determination of DturCelB and TtGalA towards galactomannans

*Dtur*CelB and *Tt*GalA 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 *Dtur*CelB and *Tt*GalA the enzymes were tested simultaneously and sequentially using 1% of galactomannan substrates (Locust bean gum, Carob and Guar) dissolved in 50 mM citrate-phosphate buffer pH 6.0. For the simultaneous assay, various ratios of *Dtur*CelB and *Tt*GalA were tested (50% *Dtur*CelB–50% *Tt*GalA; 25% *Dtur*CelB–75% *Tt*GalA; 75% *Dtur*CelB–25% *Tt*GalA) 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 *Dtur*CelB or *Tt*GalA 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 concentration 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, synergism is calculated as ratio between the observed activity of the enzyme mixture and the theoretical sum of individual specific activity of the same enzymes. The degree of synergy (DS), between *Dtur*CelB and *Tt*GalA, was determined by the following equation:

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

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 indicate 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 *Dtur*CelB and *Tt*GalA were previously characterized for their biochemical catalytic features [18]. In this study, the hydrolytic *endo*-mannanase activity of *Dtur*CelB 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) branching out from the linear mannan backbones and causing steric hindrance to the enzymes (Fig. 1).

A similar behaviour was also demonstrated for *Clostridium thermocellum* Man5A [14]. Therefore, one way to improve the *Dtur*CelB hydrolysis of galactomannans is to combine its catalytic activity with an α-galactosidase acting on the branched glycosidic 1,6-α-bounds between galactose and mannose. As potential partner, it was chosen *Tt*GalA, an α-galactosidase from *T. thermophilus* performing its highest catalytic activity at 90 °C and pH 6.0 on synthetic *p*NP-α-D-galactopyranoside substrate (pNPG, Sigma) [12]. Assays conditions for the two enzymes were set at 80 °C and pH 6.0 because *Tt*GalA retained 98% of its catalytic activity at 80 °C. In this work *Tt*GalA was assayed towards Locust bean gum (4.4 U mg^{-1}), Carob (1.4 U mg^{-1}) and Guar (0.33 U mg^{-1}) galactomannans and displayed a specific activity lower

Table 1
Specific activity of *Dtur*CelB and *Tt*GalA on different galactomannan substrates.

Substrate	*Dtur*CelB Specific activity (U mg^{-1})	*Tt*GalA Specific activity (U mg^{-1})
Locust bean gum (G/M:1/4)	44.0	4.4
Carob (G/M:1/3.5)	40.3	1.4
Guar (G/M:1/2)	2.8	0.33

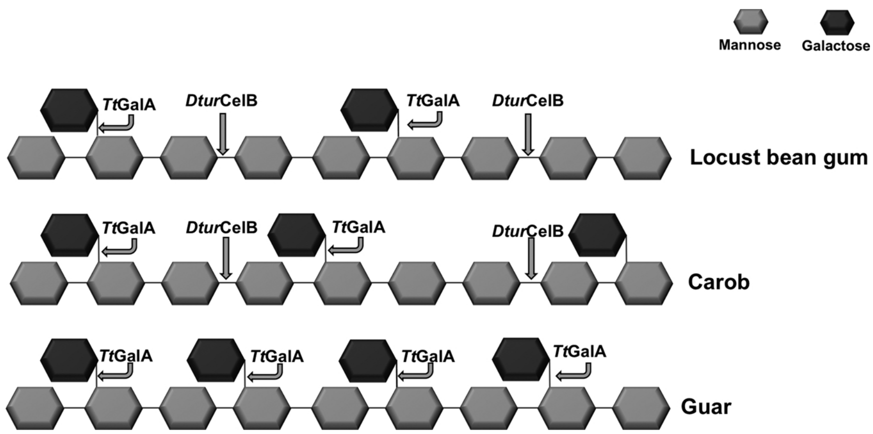

Fig. 1. Graphical representation of the galactomannans used in this study: Locust bean gum, Carob and Guar.

than that detected on pNPG substrate (338 U mg^{-1}). The different specific activities are in agreement with the preference of *Tt*GalA towards galactose-oligosaccharides over -polysaccharides, as for other GH36 members [10]. Nevertheless *Tt*GalA catalytic activity on polymeric substrates is not negligible (Table 1), indeed it is higher if compared to that of a GH36 AglC (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 *Tt*GalA and *Dtur*CelB 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 *Tt*GalA and *Dtur*CelB 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% *Tt*GalA–50% *Dtur*CelB; 75% *Dtur*CelB–25% *Tt*GalA and 25% *Dtur*CelB–75% *Tt*GalA), while in sequential assays it was used the same ratio (50% *Dtur*CelB–50% *Tt*GalA).

Locust bean gum is the most important galactomannan used as stabilizing agent in food and non-food industries [15], it is purified from endosperm of carob tree seeds [16] and is the lowest galactose containing polymer (G/M: 1/4) among the substrates tested (Fig. 1). All the conditions led to an increase of the release of reducing sugars compared to that achieved by the two enzymes alone (Fig. 2). Using this substrate, the enzymes exhibited synergism under all combinations with a DS of 1.8, 1.3 and 1.1 using a ratio of 25% *Dtur*CelB–75% *Tt*GalA, 75% *Dtur*CelB–25% *Tt*GalA and 50% *Dtur*CelB–50% *Tt*GalA, respectively (Fig. 2A). To get further insight into the observed synergistic action, we performed sequential assays. When *Dtur*CelB was added as first, the DS = 1.1 (Fig. 2B) turned out to be identical to that obtained with simultaneous assays (Fig. 2A). Conversely, the DS raised up to 1.4 when *Tt*GalA was added as first (Fig. 2B). These results demonstrate that *Tt*GalA significantly supported *Dtur*CelB activity by removing galactose branches on the polymer that would have sterically hindered *Dtur*CelB.

Locust bean gum and Carob are both isolated from *Ceratonia siliquia*. 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 *Tt*GalA 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 *Dtur*CelB and *Tt*GalA 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 *Tt*GalA not completely productive, thus in turn inhibiting the *Dtur*CelB 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 *Dtur*CelB to *Tt*GalA 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

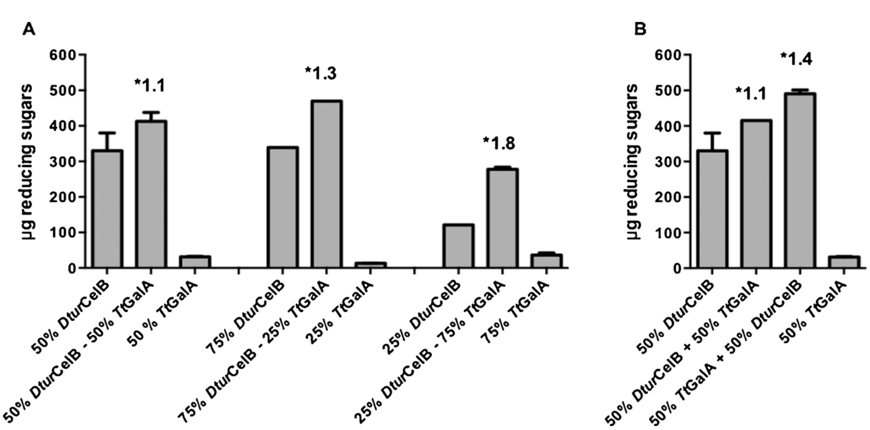

Fig. 2. Simultaneous (A) and sequential (B) assays of *Tt*GalA and *Dtur*CelB using 1% Locust bean gum. Various combinations of recombinant enzymes were tested and protein ratio was expressed as relative percentage. The degree of synergy is highlighted with an *asterisk*. Values were presented as mean values ± S.D. (n = 3).

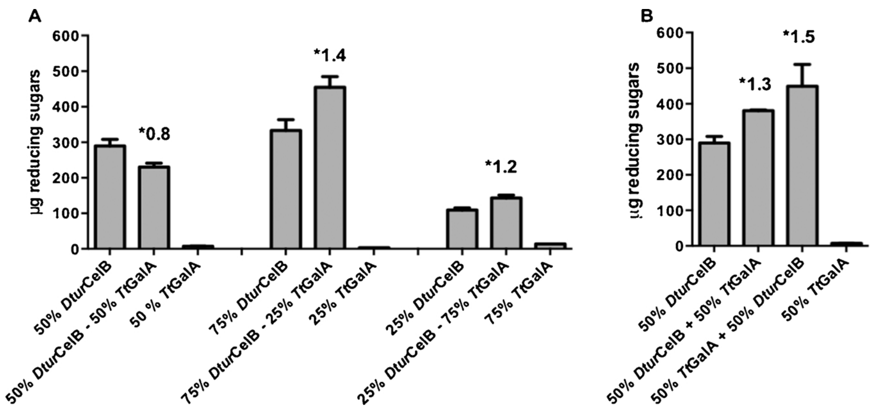

Fig. 3. Simultaneous (A) and sequential (B) assays of *Tt*GalA and *Dtur*CelB using 1% Carob. Various combinations of recombinant enzymes were tested and protein ratio was expressed as relative percentage. The degree of synergy is highlighted with an *asterisk*. Values were presented as mean values ± S.D. (n = 3).

(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 *Tt*GalA on Carob, since the affinity of *Dtur*CelB on Carob and Locust bean gum is almost the same (Table 1). Our data highlighted the role of *Tt*GalA, that plays a major function in enhancing the *Dtur*CelB 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 *Dtur*CelB and *Tt*GalA 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 *Tt*GalA by removing galactose substituents, increases the release of reducing sugar by *Dtur*CelB (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 *Dtur*CelB and *Tt*GalA 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 *Tt*GalA activity, which removes galactose branches from the galactomannan polymers, then improving the accessibility of the linear mannan backbone to *Dtur*CelB. Our finding also revealed that the 25%–75% ratio of *Dtur*CelB and *Tt*GalA 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 *Tt*GalA and *Dtur*CelB 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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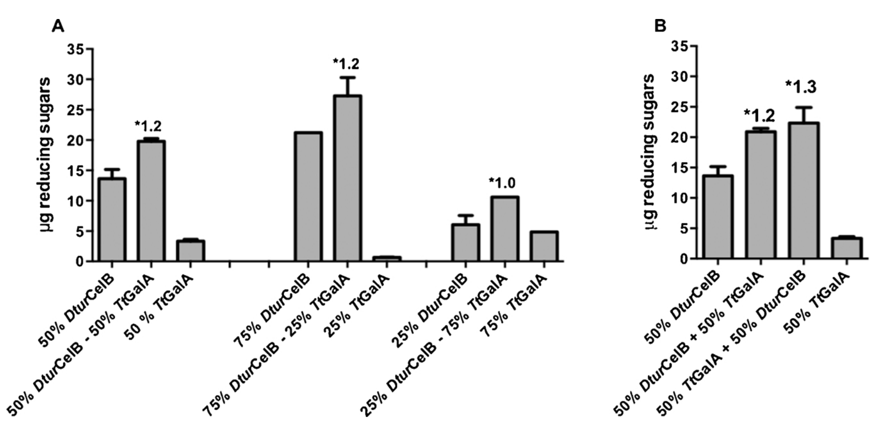

Fig. 4. Simultaneous (A) and sequential (B) assays of *Tt*GalA and *Dtur*CelB using 1% Guar. Various combinations of recombinant enzymes were tested and protein ratio was expressed as relative percentage. The degree of synergy is highlighted with an *asterisk*. Values were presented as mean values ± S.D. (n = 3).

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