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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