Biotechnology Reports 28 (2020) e00526

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Synergic activity of Cel8Pa β-1,4 endoglucanase and Bg1Pa β-glucosidase from *Paenibacillus xylanivorans* A59 in beta-glucan conversion

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ARTICLE INFO

Article history:
Received 19 May 2020
Received in revised form 2 September 2020
Accepted 3 September 2020

Keywords:
Endoglucanse
GH8
Beta-glucosidase
GH1
Paenibacillus
Beta-glucan
Saccharification

ABSTRACT

In the efficient bioconversion of polysaccharides from lignocellulosic biomass, endoglucanases and β-glucosidases are key enzymes for the deconstruction of β-glucans. In this work, we focused on a GH8 endoglucanase (Cel8Pa) and a GH1 β-glucosidase (Bg1Pa) from *Paenibacillus xylanivorans* A59. Cel8Pa was active on a broad range of substrates, such as β-glucan from barley (24.5 IU/mg), lichenan (17.9 IU/mg), phosphoric acid swollen cellulose (PASC) (9.7 IU/mg), carboxi-methylcellulose (CMC) (7.3 IU/mg), chitosan (1.4 IU/mg) and xylan (0.4 IU/mg). Bg1Pa was active on cellobiose (C2) and cello-oligosaccharides up to C6, releasing glucose as the main product. When both enzymes were used jointly, there was a synergic effect in the conversion rate of polysaccharides to glucose. Cel8Pa and Bg1Pa presented important properties for simultaneous saccharification and fermentation (SSF) processes in second generation bioethanol production, such as tolerance to high concentration of glucose and ethanol.

1. Introduction

Paenibacillus xylanivorans A59 is a bacterial strain with capacity of degrading polysaccharides (xylan, cellulose, chitin and starch) and high tolerance to salt (growth up to 7% NaCl), previously isolated from decaying soil of a pristine forest [1,2]. Based on unique and distinctive features, it has been recently classified as a novel species (*Paenibacillus xylanivorans*) related to *P. taichungensis* and *P. pabuli*, with A59 as the type strain [3]. Many *Paenibacillus* strains from diverse habitats that can produce enzymes and metabolites at an industrial scale have been described in recent years, which reveals the high biotechnological potential of the genus [4,5]. We have extensively studied the xylanolytic extracellular system of *P. xylanivorans* A59, which is induced when the bacteria is grown on xylan as sole carbon source [2,6]. Under these xylanase-inducing conditions, enzymes with potential activity on β-glucans from CAZy families GH6, GH8 and GH16 were also secreted [6].

Glucose polymers, or glucans, represent the most widespread polysaccharides in nature. They present different types of glycosidic linkages and anomeric configurations generating linear and branched α- or β- glucans with varied degrees of polymerization (DP). Cellulose, linear (1,4)-β-D-glucan, is the main constituent of plant cell walls. Also, in the endosperm cell wall of cereal grains such as oat and barley, and to a lesser extent in wheat and rye, linear (1,4) (1,3)- mixed linked β-D-glucans are abundant. Lichenan is a linear mixed-linkage β-D-glucan with a similar structure to cereal β-glucans commonly found in the lichen *Cetraria islandica* [7,8].

Cellulose conversion to glucose has been studied for decades in the context of the production of second-generation (2 G) biofuels, which are based on non-edible feedstocks, mainly residual biomass. Enzymes acting on cellulose and xylan degradation have been extensively studied and some commercial preparations are readily available [9,10]. However, the exploitation of polysaccharides other than cellulose, such as lichenan, xyloglucan, glucomannan and β-glucan, is also crucial for the feasibility of lignocellulosic biorefineries [11].

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https://doi.org/10.1016/j.btre.2020.e00526

Among these enzymes, β-glucanases are being widely studied for their use in several applications, such as the improvement of the digestibility of feed for monogastric animals (poultry and swine) and the brewing industry [12,13]. β-glucans are among the major non-starch polysaccharides (NSP) present in cereal-based feed but their viscosity produces an anti-nutritional effect [14]. This viscosity could be reduced through β-glucan enzymatic hydrolysis in order to improve feed's nutritional properties. Thus, the assessment of enzymes with improved activity against glucans would be of interest to develop tailor-made enzymatic cocktails that meet the requirements of each process.

Cellulose is degraded to glucose, according to the classical scheme, by the concerted action of endo-β-1,4-glucanases (EC 3.2.1.4), exo-β-1,4-glucanases (non-reducing and reducing end-acting cellobiohydrolases, EC 3.2.1.91 and EC 3.2.1.176, respectively) and β-glucosidases (EC 3.2.1.21). These enzymes act synergistically: endo-acting enzymes generate new reducing and non-reducing chain ends for the exo-acting enzymes, which release cellobiose that is further converted to glucose by β-glucosidases [15]. The above-mentioned enzymes involved in cellulose hydrolysis are also active on mixed-linked β-glucans along with lichenases (β-1,3−1,4-glucanase, EC 3.2.1.73), which specifically cleave β-1,4 glycoside bonds in 3-O substituted glucosyl residues, and β-1,3(4)-glucanases (EC 3.2.1.6), which act on β-1,3 or β-1,4 linkages when the glucose residues are substituted at C3 [16]. The enzymes that break down carbohydrates are grouped, based on their structural similarity, into several glycoside hydrolase (GH) families in the CAZy database [17]. β-glucosidases (EC 3.2.1.21), which are responsible for the processing of small oligosaccharides, have been classified in several GH families to date (GH1, 3, 5, 9, 30, 39 and 116), although the largest number of characterized β -glucosidases belongs to GH1 family [18]. The endo- and exo-acting glucanases as well as lichenases are mostly members of the GH5, 6, 7, 8, 9, 12, 16, 44, 45 and 48 families [19].

The aim of this work was to understand the polysaccharide utilization system of *P. xylanivorans* A59 and its applications, by characterizing two key enzymes with a potential role in β-glucans degradation. A GH8 endoglucanse (Cel8Pa) was selected because it is the only GH8 encoded in the genome and its secretion was induced by growth on xylan, which could indicate broad substrate specificity [6]. We also selected an intracellular GH1 (Bg1Pa), the only GH1 encoded in the genome with homology to β-glucosidases, to explore the boosting interaction between these two enzymes if combined in bioprocesses

2. Materials and methods

2.1. Sequence and phylogenetic analysis

The protein sequences of Cel8Pa (GenBank WP_053779698.1) and Bg1Pa (GenBank WP_053780132.1) were aligned using protein BLAST (BLASTP) against protein sequences from Protein Data Bank (PDB) and reference proteins (refseq_protein) available in the NCBI database (www.ncbi.nlm.nih.gov). Protein parameter calculations and signal peptide prediction were performed using ProtParam [20] and Signal P v5.0 servers [21], respectively.

Closely related sequences to Cel8Pa and Bg1Pa (with more than 90 % identity and coverage) were retrieved from the NCBI database and phylogenetic studies were conducted using MEGA v6.0 [22]. Sequences of GH8 and GH1 from other *Paenibacillus* sp. previously characterized (either biochemically or structurally) were obtained from the CAZy database [17] and included in the analysis. The evolutionary history was inferred by using the Maximum Likelihood method. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a Jones-Taylor-Thornton (JTT) model, and then selecting the topology with superior log likelihood value.

Homology modeling by Iterative Threading ASSEmbly Refinement (I-TASSER) [23] was used to generate a molecular model of Cel8Pa and Bg1PA mature proteins using multiple templates. Protein structures were retrieved from a PDB library (http://www.rcsb.org) by LOMETS server included in I-TASSER (https://zhanglab.ccmb.med.umich.edu/ITASSER). The principal PDB templates selected by LOMETS engine to construct the model of Bg1Pa were 5OGZ (*Ruminiclostridium Thermocellum*, β-Glucosidase A), 6QWI (*Paenibacillus polymyxa*, β-glucosidase), 1DWF (*Sinapis alba*, Myrosinase MA1), 1E4I (*Bacillus polymyxa*, β-glucosidase) and for Cel8Pa; 5XD0 (*Paenibacillus* sp. × 4, β-1,3−1,4-glucanase). The generated molecular models displayed a C-score (typically in the range −5, 2) of 1.85 and 1.42 and a TM- score of 0.98 and 0.91 for Cel8Pa and Bg1Pa, respectively. Model manipulation and imaging was performed using Chimera software [24].

2.2. Cloning, expression *in* Escherichia coli *and purification of recombinant proteins*

Open-reading frames (*orfs*) for Cel8Pa (without signal peptide) and full Bg1Pa were amplified from *P. xylanivorans* A59 total genomic DNA, using specific oligonucleotides PaGH8f aaaggatcccttctgattccggcaggattcgct; PaGH8r aaactcgagttagtatgtccaccagttaccgga and PaBG1f aaaggatccatgacgatttttcaatttcca; PaBG1r aaactcgagctaaattcgtgtttccaacca (restriction sites BamHI in forward primers and XhoI in reverse primers underlined). The amplification products (1167 and 1344 base for mature Cel8Pa and Bg1Pa, respectively) were first cloned into pGEMT Easy vector (Promega). Plasmids from selected colonies were isolated; the *orf*s were released with restriction enzymes *Bam*HI and *Xho*I and cloned into pET28a vector (Novagen) to generate N-terminal 6xHis tag fusion proteins. All cloning procedures were carried out using *E. coli* DH5-α competent cells. Sequence identity was confirmed in selected plasmids by DNA sequencing and *E. coli* Rosetta(DE3) competent cells (Novagen) were transformed with the corresponding pET28a-Cel8Pa or pET28a- Bg1Pa plasmids. The induction conditions that resulted in the highest yield of soluble protein were 0.1 mM IPTG, 16 h, 28 °C, in both cases. After cell lysis by sonication, recombinant proteins were purified by IMAC using a Ni-NTA agarose resin (Qiagen) with standard protocols (The QIAexpressionist, Qiagen), using 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole as elution buffer. Purified protein concentration was estimated using Bradford reagent (BIORAD) with BSA as standard. Purification resulted in a yield of 5–8 mg total purified protein from 100 mL induced cultures.

2.3. Enzyme activity

To determine substrate specificity, the activities of Cel8Pa and Bg1Pa were assayed on several commercial polysaccharide substrates (all at 0.5 % final concentration, unless otherwise indicated): β-glucan from barley (low viscosity) (Megazyme), lichenan (Megazyme), carboxymethyl cellulose (low viscosity) (SIGMA), laminarin (SIGMA), xyloglucan (XG) (Megazyme), xylan from beechwood (SIGMA), Avicel (Fluka), PASC (Phosphoric Acid Swollen Cellulose [25], chitosan (high molecular weight) (0.25 %) (Wako Chemicals), bacterial microcrystalline cellulose (BMCC) (0.625 %) (laboratory stock) and filter paper N° 1 (6 mm discs) (FP) (Whatman). Oligosaccharides and *para*-nitrophenol (*p*NP)- linked substrates were assayed at 2.5 mM: cellobiose, *p*NP-β-D-xylopyranoside (*p*NPX), *p*NP-β-D-glucopyranoside (*p*NPG) and *p*NP-β-D-cellobioside (*p*NPC) (all from SIGMA). Reactions were performed in citrate-phosphate buffer 50 mM, with 0.1 mg/mL enzyme, 15 min (unless otherwise indicated), in a final volume of 100 μL, with agitation at 500 rpm, at 40°, pH 4.5, for Cel8Pa and at 30 °C, pH 6.5, for Bg1Pa (based on preliminary assays on β-glucan and *p*NPG, respectively). Release of total reducing sugars from

polysaccharides was calculated by reduction of 3,5-dinitrosalicylic acid (DNS), detected by absorbance at 540 nm, with standard reference curve of glucose (for xylan, a xylose reference curve was used) [26]. Enzyme activity was defined, for each substrate, as μmol of product released per minute of reaction (international units, IU), per mg of enzyme (specific activity $IU_{(\mu mol/min)}$/mg). Negative results were confirmed in overnight reactions (16 h). For *p*NP- conjugated substrates, reactions were carried out in 100 μl total volume, with 0.1 mg/mL enzyme for 15 min and stopped with 0.5 mL of 2% Na_2CO_3. The μmol of *p*-nitrophenol (*p*N) released were determined by absorbance at 410 nm with a *p*N curve as standard reference [6]. For cellobiose hydrolysis, glucose was determined with the enzymatic glycemia kit from Wiener Lab (Argentina) using a glucose standard curve. In all assays, controls of substrate without enzyme and enzyme without substrate were included. The enzymatic hydrolysis of cello-oligosaccharides (COS) (1 mg/mL final concentration), with degrees of polymerization (DP) ranging from 2 to 6 (Megazymes), was evaluated by thin layer chromatography (TLC) after 1 h incubation at optimal enzyme conditions [27]. Glucose was also included as control for trans-glycosidase activity. The TLC was run in silica gel plates (SIGMA), using butanol/acetic acid/water (2:1:1) as solvents and revealed by spraying with a solution of water/ethanol/ sulfuric acid (20:70:3) with 1% (v/v) orcinol, and then heating slightly over flame [6].

2.4. Optimal reaction conditions, inhibition and kinetic parameters

Activity of both enzymes was evaluated in a pH range from 3 to 9 in citrate-phosphate buffer (for pH 3–6), phosphate buffer (pH 6–8) (SIGMA reference guide) and Glycine-NaOH buffer (pH 9) and a temperature range from 4 to 50 °C, by 3,5-dinitrosalicylic acid (DNS) assays for reducing sugars. Thermal stability and halotolerance were tested by pre-incubating enzymes at increasing temperatures or NaCl concentration (0–5 M), followed by enzymatic activity quantification by DNS assay at successive time points. Final product inhibition (glucose and/or cellobiose) or ethanol tolerance (0–50%) were determined by increasing their concentration in the enzymatic reactions (at optimal conditions for each enzyme). In inhibition assays, activity of Cel8Pa was determined with K-CellG3 endocellulase kit, that employs benzylidene blocked 2-chloro-4-nitrophenyl-β-D-cellotrioside (BCNPG3) as substrate and releases 2-chloro-4-nitrophenol (CNP) which is detected by absorbance at 400 nm, following indications of manufacturer (Megazyme). Activity of Bg1Pa was assayed on *p*NPG as previously indicated. To determine kinetic parameters V_{max} and K_M, initial velocity conditions were established for linear reactions on barley β-D-glucan (Cel8Pa) (linear between 5–20 min) and cellobiose and *p*NP-β-D-glucopyranoside (*p*NPG) for Bg1Pa (linear between 1–15 min). Activity assays were conducted at 15 min with increasing concentrations of barley β-D-glucan (1–25 mg/mL) for Cel8Pa. For Bg1Pa, 10 min reaction time was used, with increasing concentration of cellobiose (0.15–35 mM) or *p*NP-β-D-glucopyranoside (*p*NPG) (0.5–5 mM). Blank reactions with no substrate (indicated as 0) were also included. Calculations were done with GraphPad Prism for Windows (version 5.00) (www.graphpad.com).

2.5. Synergism assays

β-glucan from barley (0.5 %), lichenan (0.5 %), CMC (1%) and PASC (0.5 %) were used as model substrates for evaluating Cel8Pa and Bg1Pa synergism in glucan conversion to glucose. Reactions (100 μL final volume) were incubated in a thermomixer (Eppendorf) for 3 h (β-glucan and lichenan) or 24 h (CMC and PASC). Enzymes were used at 0.1 mg/mL (both enzymes for all substrates), except Cel8Pa that was used at 0.3 mg/mL in the case of CMC and PASC. The synergistic action of the two enzymes was evaluated by simultaneous incubation at pH 4.5, 40 °C. The final concentration of glucose was quantified using a glucose oxidase-peroxidase reaction (GOD-POD) based kit (Wiener Lab). Synergic ratio (SR_g) was defined as the amount of glucose released by the activity of the combined enzymes divided by the sum of glucose obtained by the individual enzymes: $SR_g = Glu_{(Cel8Pa+Bg1Pa)}/(Glu_{(Cel8Pa)}+Glu_{(Bg1Pa)})$. $SR > 1$ indicate positive enzyme interactions for boosting the release of the measured product (in this case, glucose), whereas ratios ≤ 1 represent no significant boosting effect [28]. Two independent assays were conducted, with three replicates for each condition. The results from only one of the assays are shown, given that the results obtained by each of them were equivalent.

3. Results

3.1. Cel8Pa and Bg1Pa sequence and phylogenetic analysis

Paenibacillus xylanivorans A59 secretes multiple glycoside hydrolases when grown on xylan, CMC or lignocellulosic biomass [6]. Among these, we identified a GH8 enzyme, which we named Cel8Pa, (NCBI WP_053779698.1; UniProtKB A0A0N0C5Q2) in the secretome from xylan cultures, which was the only GH8 encoded in the genome. GH8 are inverting enzymes, found mostly in bacteria, that can cleave β-1,4 linkages from β-1,4 or β-1,3–1,4 mixed linked glucans, lichenans, xylans (or xylo-oligosaccharides) and chitosans (www.cazypedia.org). Even though Cel8PA was identified only in the secreted fraction of xylan cultures, it had high sequence identity (over 95 % in almost 100 % coverage) and grouped with predicted endoglucanases from *Paenibacillus* sp. (Fig. S1a). Moreover, the closest related sequence corresponded to a characterized and crystalized broad substrate endo-glucanase from *Paenibacillus* sp. × 4, BGlc8H (PDB: 5XD0_A) (95.6 % identity in 100 % coverage) [29].

Other important enzymes involved in the final stages of cello-oligosaccharides (COS) conversion to glucose are GH1 β-glucosidases, which are abundant in several bacterial genomes (CAZY database). An analysis of the *P. xylanivorans* A59 genome revealed 10 coding-sequences for GH1 (Fig. S1b). We selected Bg1Pa (NCBI WP_053780132.1; UniProtKB A0A0N0UI50), because it was the only GH1 with predicted β-glucosidase activity, based on identity with previously characterized GH1 enzymes. Bg1Pa presented 75 % identity (with 99 % coverage) with a crystalized and characterized GH1 β-glucosidase (BglA) from *Paenibacillus polymixa* (PDBs: 1BGG_A, 1TR1_A, 1UYQ_A 1E4I_A, 6QWI_A). The lack of a signal peptide sequence in Bg1Pa suggests its intracellular localization. Moreover, it is encoded immediately downstream from a GH95 α-L-fucosidase (also with no signal peptide sequence) (WP_053780133.1). These findings suggest that these enzymes could form a cluster involved in the final intracellular degradation of oligosaccharides.

In structural molecular models, we identified the active site and catalytic residues of each enzyme (Fig. S2). Cel8Pa presented a regular $(\alpha/\alpha)_6$ barrel structure formed by six internal and 6 external α helix with the catalytic residues in an open cleft, characteristic of endo-acting enzymes. In contrast, the active site of Bg1Pa was located in a deep and narrow cavity (Fig. S2).

3.2. Substrate specificity of Cel8Pa and Bg1Pa and mode of action

To study the contribution of these enzymes to *P. xylanivorans* A59 β-glucans utilization system and to correctly assign them an Enzyme Commission (EC) number, we produced recombinant Cel8Pa and Bg1Pa in *E. coli* (Fig. S3) and then characterized their activity.

Table 1
Substrate specificity of Cel8Pa and Bg1Pa. Activity of Cel8Pa on polysaccharides (0.5 % unless otherwise indicated) was determined in 15 min reactions followed by total reducing sugars assay (DNS), with glucose or xylose as reference, according to the substrate (*16 h reaction, **30 min reaction). Cellobiose and *p*NP- substrates were used at 2.5 mM. Activity on cellobiose was determined by glucose released, using a GOD-POD glycemic enzymatic assay kit (Weiner). Activity on *p*NP-substrates was assayed colorimetrically at 410 nm, with a *p*NP reference curve. Enzymes were used at 0.1 mg/mL. Specific activity was defined as μmol of product released per minute of reaction (international units, IU), per mg of enzyme. References: Glc: glucose, Xyl: xylose, GlcNAc; N-acetyl glucosamine, GlcN: glucosamine, Gal: galactose, PASC: phosphoric acid swollen cellulose, BMCC: bacterial microcrystalline cellulose, CMC: Carboxy Methyl Cellulose, GlcAOMe: methylated glucuronic acid, pN; *para* nitro phenol, *p*NPG: *p*NP-β-D-glucopyranoside., *p*NPC: *p*NP-β-D-cellobioside, *p*NPX: *p*NP-β-D-xylopyranoside, ND: not detected.

Substrate	Main linkages	Specific activity (IU/mg)	
		Cel8Pa	Bg1Pa
Barley β-glucan	$[Glcβ-1,4-Glcβ-1,4-Glcβ-1,3-Glcβ-1,4]_n$	24.52 ± 0.30	0.31 ± 0.07 *
Lichenan	$[Glcβ-1,4-Glcβ-1,3-Glcβ-1,4]_n$	17.87 ± 0.05	0.16 ± 0.08 *
PASC	Glc β-1,4	9.73 ± 0.77	ND
CMC	Glc β-1,4 (40 % CM substitutions)	7.35 ± 0.02	ND
Beechwood xylan	Xyl β-1,4 (13 % GlcAOMe substitutions)	0.40 ± 0.05 **	ND
Chitosan (0.25 %)	GlcN β-1,4-GlcNAc β-1,4	1.38 ± 0.01	ND
BMCC (0.625 %)	Glc β-1,4	ND	ND
Avicel	Glc β-1,4	ND	ND
Filter Paper (6 mm discs)	Glc β-1,4	ND	ND
Laminarin	Glc β-1,3 (Glc β-1, 6 interstrand)	ND	ND
Xyloglucan	Glc β-1,4 (Xyl, Xyl-Gal α -1,6)	ND	ND
Cellobiose	$(Glcβ-1,4)_2$	ND	40.20 ± 0.10
*p*NPG	Glc β-1,4- *p*N	ND	46.81 ± 0.20
*p*NPC	Glc β-1,4- Glc β-1,4-*p*N	ND	18.41 ± 0.30
*p*NPX	Xyl β-1,4- *p*N	ND	1.50 ± 0.20

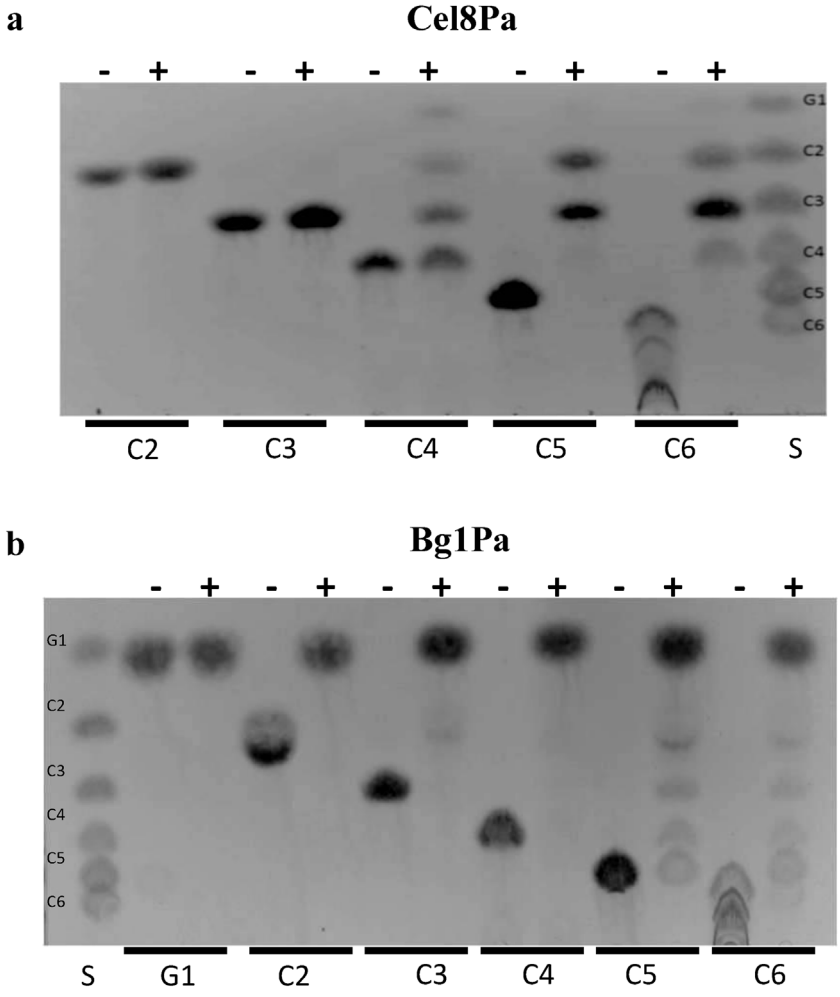

Fig. 1. Cel8Pa and Bg1Pa COS hydrolysis. Thin layer chromatography (TLC) of hydrolysis products of reactions on cello oligosaccharides by Cel8Pa (a) and Bg1Pa (b). Substrates were C2 to C6, used at 1 mg/mL. Assays were performed with 0.1 mg/mL enzyme (+) or buffer (-), for 1 h at pH 6.5, 30 °C (Cel8Pa) or pH 4.5, 40 °C (Bg1Pa). Standards (S): mix of G1: glucose, C2: cellobiose, C3: cellotriose, C4: cellotetraose, C5: cellopentaose and C6: cellohexaose. Activity on glucose (G1) was included for Bg1Pa to evaluate trans-glycosidase activity. The TLC was run on Silica Plates using butanol/acetic acid/water (2:1:1) as solvents and revealed by spraying (and gentle flame heating) with a solution of water/ethanol/ sulfuric acid (20:70:3), 1% (v/v) orcinol.

Cel8Pa was active on β-1,4 linkages from glucan substrates, such as β-glucan from barley (24.52 ± 0.30 IU/mg), lichenan (17.87 ± 0.05 IU/mg), Phosphoric Acid Swollen Cellulose (PASC) (9.73 ± 0.77 IU/mg) and CMC (7.35 ± 0.02 IU/mg), with a Michaelis-Menten kinetics (assayed on β-glucan from barley; V_{max}: 52.84 ± 3.15 IU/mg; K_M of 6.41 ± 0.93 mg/mL) (Fig. S4a). Cel8Pa also had activity on chitosan (assayed at 0.25 %) and beechwood xylan (1.38 ± 0.01 IU/mg and 0.40 ± 0.05 IU/mg, respectively). No activity was observed on laminarin (β-1,3 glucan linkages with β-1,6 interstrand), xyloglucan (tamarind), crystalline cellulose (Avicel, BMCC, filter paper), *p*NPC (exoglucanase activity), *p*NPG (β-glucosidase activity) or *p*NPX (β-xylosidase activity) (Table 1). When assayed on soluble cello-oligosaccharides (COS), Cel8Pa showed activity on cellohexaose (C6), cellopentaose (C5) and cellotetraose (C4), but not on shorter oligosaccharides, cellobiose (C2) and cellotriose (C3) (Fig. 1a). These assays unequivocally confirmed the predicted endo-acting nature of Cel8Pa, with preference for internal β-1,4 bonds in β-1,3−1,4 mixed glucans and COS equal or larger than cellotetraose (DP ≥ 4). Thus, Cel8Pa can be classified as an endo-β-1,4-glucanase (EC 3.2.1.4) with broad substrate specificity (with additional activities lichenase EC 3.2.1.73, chitosanase EC 3.2.1.132 and xylanase EC 3.2.1.8).

Bg1Pa had maximum activity on *p*NPG (46.8 ± 0.20 IU/mg) and cellobiose (40.2 ± 0.10) (Table 1), with Michaelis-Menten kinetics on both substrates (K_M 4.62 ± 0.24 mM and K_M 0.59 ± 0.02 mM; V_{max} 48.40 ± 0.88 IU/mg and 47.68 ± 0.32 IU/mg, for *p*NPG and cellobiose respectively) (Fig. S4 b, c). It was also active on *p*NPC (18.4 ± 0.30 IU/mg), slightly on *p*NPX (1.5 ± 0.20 IU/mg) and showed minimal release of glucose from β-glucan from barley and lichenan in long incubation reactions (16 h) (0.31 ± 0.07 and 0.16 ± 0.08 IU/mg, respectively) (Table 1). Hydrolysis of soluble linear COS from DP2 to DP6 was observed with the release of glucose as the main final product (Fig. 1b). These results confirmed the β -glucosidase activity (EC 3.2.1.21) of Bg1Pa, with an exo-acting mode.

3.3. Reaction conditions for bioprocesses

The application of these recombinant proteins in bioprocesses requires, first of all, an evaluation of their performance under the corresponding desired conditions. Different reaction conditions were evaluated for Cel8Pa and Bg1Pa using β-glucan and *p*NPG as model substrates, respectively. Cel8Pa showed maximum activity at 40 °C and pH 4.5 and Bg1Pa had optimal activity at 30 °C and pH 6.5 (Fig. 2 a, b). Noteworthy, both enzymes retained activity (between 30–40%) at temperatures between 5 and 20 °C (Fig. 2b). Although both enzymes tolerated more than 1 h at their optimal reaction temperatures, Bg1Pa lost almost all activity after 6 h (in solution without substrate) (Fig. 2c). An important trait to evaluate that relates to withstanding harsh conditions is tolerance to salt. Cel8Pa was active up to 3 M NaCl (50 % activity) and tolerated up to 16 h pre-incubation at this concentration. However, Bg1Pa was only active up to 0. 5 M NaCl (Fig. 2d).

In addition, we tested other important parameters for β-glucan conversion with focus in the process conditions for lignocellulosic bioethanol production, such as inhibition of enzymes by the product of the reaction (glucose and/or cellobiose) and tolerance to ethanol, which is the final product of the process. Cel8Pa was

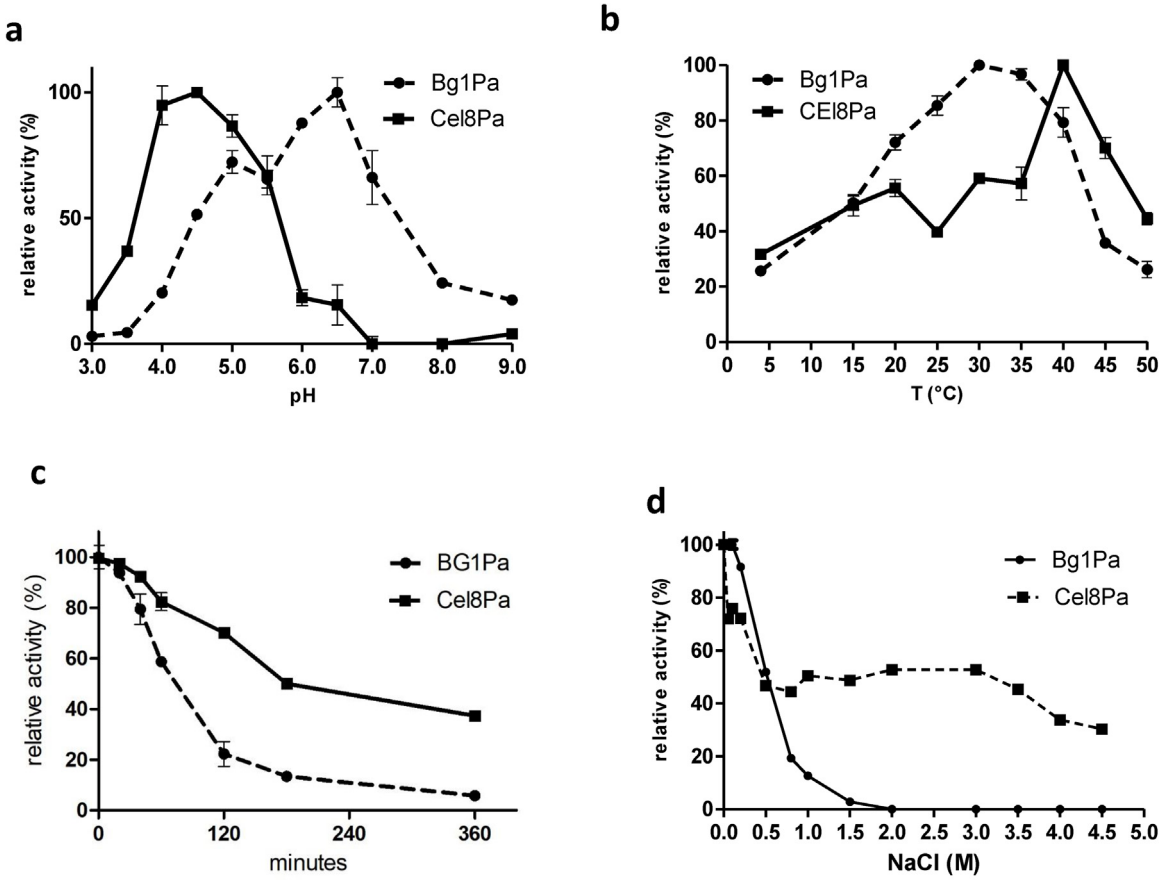

Fig. 2. Optimal parameters for Cel8Pa and Bg1Pa enzymatic activity. Optimal pH (a), temperature (b), thermal stability after pre-incubation at optimal reaction conditions (pH 6.5, 30 °C for Bg1Pa and pH 4.5, 40 °C for Cel8Pa) (c) and halotolerance to increasing concentrations of NaCl (d) were determined by DNS assay on β-glucan for Cel8Pa or colorimetric determination at 410 nm of *p*-NP release from *p*NPG for Bg1Pa. Enzymes were used at 0.1 mg/mL.

incubated with increasing concentration of cellobiose, glucose or ethanol and endoglucanase activity was determined by the CellG3 method (Megazyme). Similarly, Bg1Pa was incubated with increasing concentrations of glucose or ethanol, and activity was determined colorimetrically on *p*NPG substrate. Both enzymes retained more than 50 % activity at high glucose concentrations (0.9 M), while Cel8Pa also showed 50 % activity even at 0.25 M cellobiose (Fig. 3 a–b). Ethanol tolerance was remarkably high for both Cel8Pa and Bg1Pa, which retained more than 80 % activity at 5% final ethanol concentration in the reaction. Particularly, Bg1Pa retained around 50 % of activity at 20 % ethanol (Fig. 3 c). These results suggest that Cel8Pa and Bgl1Pa have a high potential for application in simultaneous saccharification and fermentation (SSF) 2 G ethanol processes.

3.4. Cel8Pa and Bg1Pa synergism for glucose release from polysaccharides

The synergistic action of Cel8Pa and Bg1Pa was tested on β-glucan, lichenan, PASC and CMC, by measuring the total amount of glucose released from polysaccharides using the enzymes separately or simultaneously. Considering conversion to COS as the limiting step, the reaction conditions were adjusted to maximize Cel8Pa activity. Simultaneous presence Cel8Pa and Bg1Pa resulted in an increase in the polysaccharide to glucose conversion rate respect of Cel8Pa alone with a synergy index (SR_g) of 5.50 for barley β-glucan, 3.69 for lichenan, 1.85 for PASC and 1.29 for CMC (Fig. 4a). By TLC analysis we confirmed the almost complete conversion of COS, released by Cel8Pa, to glucose when both enzymes were used, especially for barley β-glucan and PASC (Fig. 4b).

4. Discussion

Paenibacillus xylanivorans A59 was previously isolated by our group as an (hemi)cellulolytic strain from a bacterial soil consortium. We sequenced its full genome and analyzed its complex secretome under several conditions [1,2,6,3]. Although the number of available sequences for *Paenibacillus* enzymes has grown in the last few years, mostly through shotgun sequencing techniques, the activity characterization of these enzymes has not followed this trend. To study *P. xylanivorans* A59 glucan utilization system, we performed a thorough analysis to identify those genes coding for enzymes involved in glucan or cellulose deconstruction. In CAZY database, only five GH8 enzymes (NCBI ALP73600.1, BAL46897.1, BAB64835.1, AFP58892.1, AGV55649.1) and three GH1 (NCBI AAA22263.1, AAA22264.1, AEI42200.1) from several *Paenibacillus* strains are indicated as fully characterized to date. Therefore, we selected an endoglucanase -Cel8Pa- and a β-glucosidase -Bg1Pa- for recombinant expression and activity characterization.

Cel8Pa was the only GH8 encoded in the bacterial genome and was identified in the secretome of *P. xylanivorans* A59 grown on xylan as only carbon source, even though it was predicted to be an endoglucanase, by sequence analysis [6]. In a previous study describing the secretome profile of another *Paenibacillus* strain (sp. O199), no GH8 were secreted when the bacteria were grown in wheat straw and cellulose [30]. GH8 are bacterial enzymes which have been characterized mainly as endoglucanases and chitosanases [19] as well as xylanases [31]. GH8 endoglucanases have been widely studied as part of Bacterial Cellulose Synthase (Bcs) complexes involved in production and export of the glucan

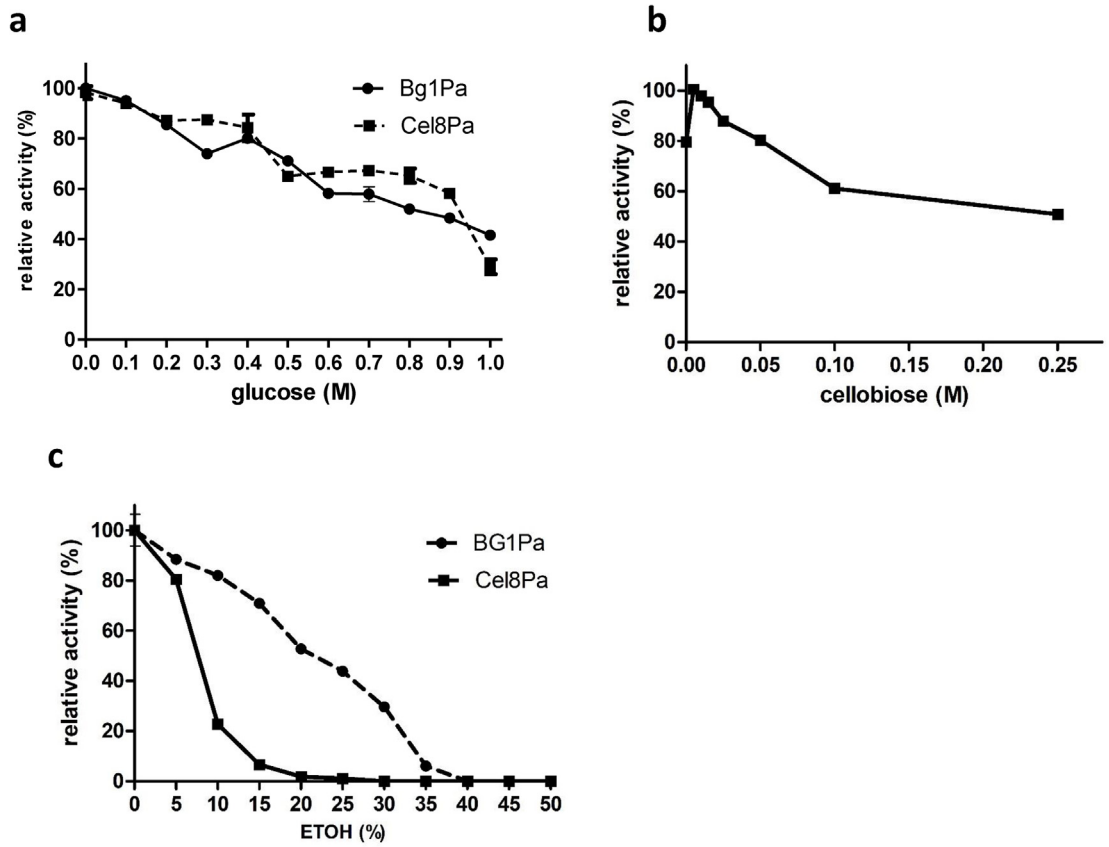

Fig. 3. Enzymatic inhibition by final product accumulation. Cel8Pa and Bg1Pa tolerance to (a) glucose, (b) cellobiose (only Cel8Pa) and (c) ethanol were tested by increasing the concentration of each compound in the enzymatic reactions at optimal conditions. Enzymes were used at 0.1 mg/mL. Cel8Pa activity was assayed with K-CellG3 method kit (Megazyme) and Bg1Pa was assayed by colorimetric determination at 410 nm of *p*-NP released from *p*NPG.

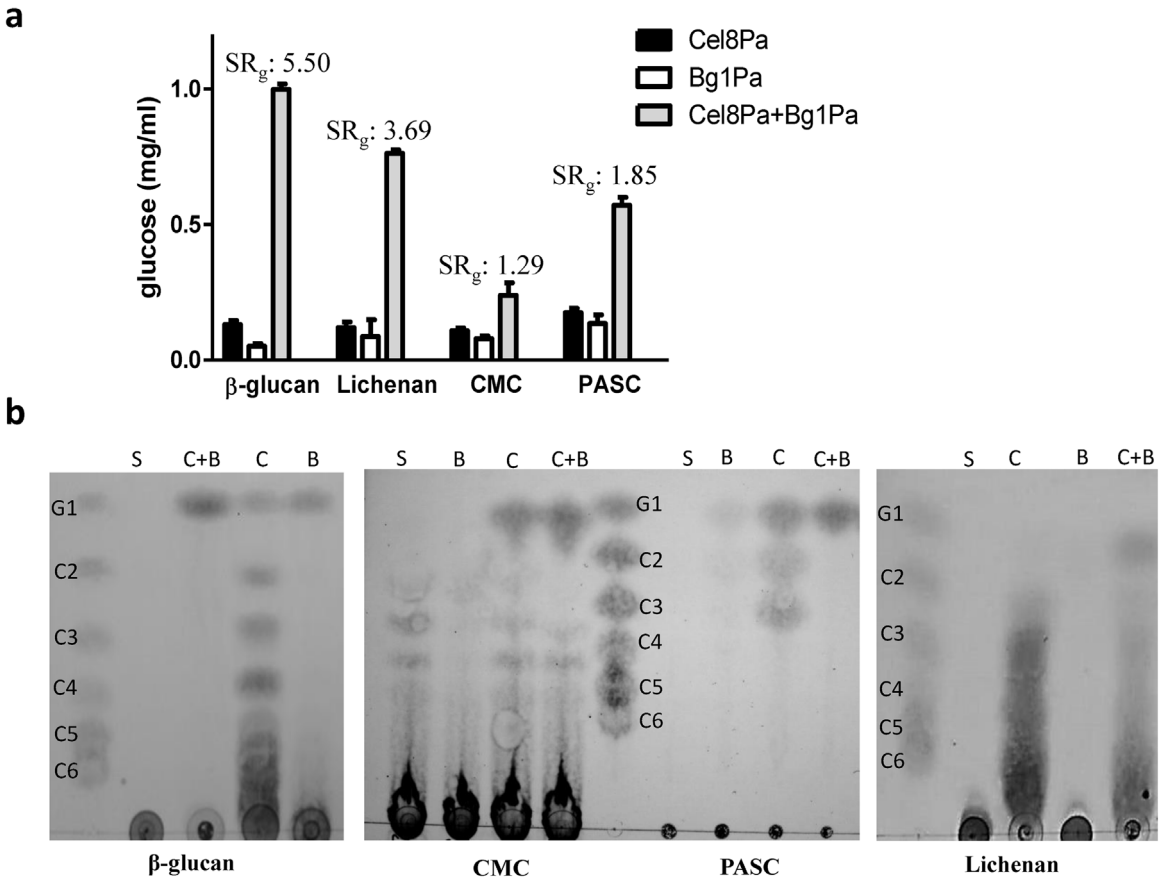

Fig. 4. Synergistic action of Cel8Pa and Bg1Pa on model substrates. (a) Glucose released form β-glucan, lichenan, CMC and PASC by Cel8Pa, Bg1Pa or Cel8Pa + Bg1Pa (used jointly). Glucose was quantified by GOD-POD enzymatic glycemia determination kit (Wiener Lab). Synergic ratio (SR_g) for each polysaccharide is indicated above. (b) TLC analysis of total hydrolysis products on β-glucan, CMC, PASC and lichenan by C: Cel8Pa, B: Bg1Pa or C + B: both enzymes jointly. TLC was run on Silica Plates using butanol/ acetic acid/water (2:1:1) as solvents and revealed by spraying (and gentle flame heating) with a solution of water/ethanol/ sulfuric acid (20:70:3), 1% (v/v) orcinol. S: control substrate without enzyme. Reference for standard mix: G1, glucose; C2, cellobiose; C3, cellotriose; C4, cellotetraose; C5, cellopentose and C6, cellohexose.

extracellular matrix in different bacteria [27,32] and also as part of polysaccharide degrading systems, with CelA from the anaerobic bacterium *Clostridium thermocellum* as one of the most studied ones [33]. In this regard, Cel8Pa had high identity with *Paenibacillus* sp. × 4 endoglucanase BGlc8H [29], with a difference of only thirteen aminoacids in the mature protein. Recombinant BGlc8H also presented activity at acidic pH (pH5) and moderate temperature (50 °C) as well as broad substrate specificity, although there were differences in substrate preference. For example, while BGlc8H had activity on β-1,3 glucan linkages (laminarin), Cel8Pa was not active on this substrate. Previously, other GH8 enzymes from the *Panibacillus* sp. genus had been characterized mostly as chitosanases [34] or chitosanases/glucanases [35,36]. Even though there are sequence differences, such as a conserved phenilalanine (Phe183) present in BGlc8H and Cel8Pa and absent from the other mentioned enzymes, we could not identify in this work structural differences that would explain the differences observed in substrate preferences.

Regarding Bg1Pa, it was the only GH1 annotated as β-glucosidase from the 10 GH1 enzymes encoded in the genome of *P. xylanivorans* A59. It had high identity with *Paenibacillus* sp. HC1 Bg1A (BAE48718.1), an intracellular β-glucosidase identified in a *Paenibacillus* strain when grown in rice bran [37,38]. BglA presented an extended cavity, which could accommodate β-1,4 oligosaccharides longer than cellobiose. A third sub-site was encountered, which could be related to the transglycosylating activity associated to this enzyme [39]. We did not observe transglycosidase activity in Bg1Pa, in accordance with absence of the third subsite. Bg1Pa was active on cellobiose and short oligosaccharides, releasing glucose. Its optimal reaction conditions were at neutral pH and 30 °C and showed low overall thermal stability, which makes it unsuitable for processes that require high temperatures.

However, moderate temperature and pH are desired conditions for simultaneous saccharification and fermentation (SSF) processes in 2 G ethanol production, in which hydrolysis and fermentation are carried out jointly, at the conditions that are optimal for the fermenting yeast (typically 30 °C, pH 5) [40]. At these conditions, Cel8Pa and Bg1Pa retain most of their activity. Moreover, the tolerance of both enzymes to glucose (up to almost 1 M) and the remarkable tolerance of Bg1Pa to ethanol (active even over 20 % ethanol) further support the potential application of these enzymes in SSF bioethanol production, where enzymatic inhibition due to product accumulation is a bottleneck.

The fact that Cel8Pa was active on a wide range of substrates indicates that it could also be evaluated in other processes in which degradation of mixed linked β-glucans are required, such as brewing and feed industries [12,13]. Besides showing Cel8Pa and Bg1Pa individual potential for their use in industrial bioprocesses, we demonstrated their synergistic effect in achieving a higher glucan conversion to glucose from several polysaccharides. Cel8Pa released soluble short oligosaccharides from polysaccharides, which were

completely converted to glucose when Bg1Pa was included in the reaction, resulting in an overall increase of polysaccharide to glucose conversion. This increase can be explained as a reduced inhibition of Cel8Pa by its reaction products, allowing further endo-glucanase activity, and the direct activity of Bg1Pa on the oligosaccharides, rendering higher amount of glucose.

5. Conclusion

Cel8Pa and Bgl1Pa have the potential to be used as an enzymatic cocktail for the saccharification of β-glucans in processes at low or moderate temperatures, such as SSF for 2 G ethanol.

CRediT authorship contribution statement

Silvina Ghio: Conceptualization, Investigation, Writing - original draft, Formal analysis. **María B. Bradanini:** Investigation, Formal analysis. **Mercedes M. Garrido:** Methodology, Writing - review & editing. **Ornella M. Ontañon:** Methodology. **Florencia E. Piccinni:** Methodology, Writing - review & editing. **Ruben Marrero Diaz de Villegas:** Methodology, Visualization. **Paola M. Talia:** Writing - review & editing. **Eleonora Campos:** Conceptualization, Supervision, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

EC, OO and PT are Researchers from the National Council of Research (CONICET). SG, RM and PT are researchers from INTA. FEP and MG have fellowships from CONICET. MG is a PhD student from FBMBC department, FCEN, Univ. of Buenos Aires (UBA). MB contribution was as an undergraduate student from the University of San Martín (UNSAM), Buenos Aires, Argentina. This work was supported by project grants MINCYT-ANPCYT PICT 2016-4695, PIP 11220150100121CO and INTA-I116, I149 and I152 (Argentina). Authors thank Barry McCleary, from Megazyme, for kindly supplying the K-CellG3 kit and Dr. Julia Sabio y García for English language editing and critical reading of the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2020.e00526.

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