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Characterization of the cellulase-secretome produced by the Antarctic bacterium *Flavobacterium* sp. AUG42

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ABSTRACT

Flavobacterium sp. AUG42 is a cellulase-producing bacterium isolated from the Antarctic oligochaete *Grania* sp. (Annelida). In this work, we report that AUG42 produces a glycoside hydrolase cocktail with CMCase, PASCase and cellobiase activities (optimum pHs and temperatures ranging from 5.5 to 6.5 and 40 to 50 °C, respectively). The time-course analyses of the bacterial growth and cellulase production showed that the cocktail has maximal activity at the stationary phase when growing at 16 °C with filter paper as a cellulosic carbon source, among the tested substrates. The analyses of the CAZome and the identification of secreted proteins by shotgun Mass Spectrometry analysis showed that five glycoside hydrolyses are present in the bacterial secretome, which probably cooperate in the degradation of the cellulosic substrates. Two of these glycoside hydrolyses may harbor putative carbohydrate binding modules, both with a cleft-like active site. The cellulolytic cocktail was assayed in saccharification experiments using carboxymethylcellulose as a substrate and results showed the release of glucose (a fermentable sugar) and other reducing-sugars, after 24 h incubation. The ecological relevance of producing cellulases in the Antarctic environment, as well as their potential use in the bio-refinery industry, are discussed.

1. Introduction

The term cellulase refers to a group of enzymes which, acting together, hydrolyze cellulose mainly yielding cellobiose and glucose molecules. They are usually defined as β-1,4 glucanohydrolases that catalyze the hydrolysis of cellulose and some related polysaccharides. The cellulases are mainly classified as endoglucanases (E.C. 3.2.1.4), exoglucanases also called cellobiohydrolases (E.C. 3.2.1.176, E.C. 3.2.1.91, E.C. 3.2.1.74) and β-glucosidases or cellobiases (E.C. 3.2.1.21), according to the position of the hydrolyzed *o*-glycosidic linkage (Watanabe and Tokuda, 2010; Medie et al., 2012; Peralta et al., 2017).

Worldwide, the photosynthetic fixation of carbon dioxide (CO_2) is mainly responsible for the production of cellulose, hemicellulose and lignin and, the degradation of these compounds from the vegetal biomass, in turns, release CO_2 to the atmosphere. In this carbon cycling, cellulases play an important role, because the released oligo- and monomeric carbohydrate molecules from the vegetal biomass are used as carbon or/and energy source by many organisms, thus contributing to the degradation of the carbon pool from any environment (Horwath, 2015). In this regards, Antarctica is not an exception; the vegetation of the Antarctic environment is characterized by low coverage and low productivity, being mainly composed of two vascular plants (*Deschampsia antarctica* and *Colobanthus quitensis*), as well as other phototrophic organisms such as algae, and lichens (Borchhardt et al., 2017; Ogaki et al., 2019; Schultz and Rosado, 2019).

Cellulases are also widely used in many different industries including animal feed processing, food and brewery production, textile processing, detergent production and laundry, paper pulp manufacture and production of biofuels and chemicals from renewable resources, among others (Juturu and Wu, 2014). They constitute 10% of the worldwide industrial enzyme demands and in addition, they represent 48% of the ethanol sales price in the biofuel industry (Liu et al., 2016). The global market of enzymes has been expected to reach USD 10,519 million by 2024 (Global Enzymes Market Expected to Reach Global Enzymes Market Expected to Reach $10, 20190,519 Million by 2024, n.d) and the factors that drive this growth include the upsurge in demand mainly for cellulosic- and amylosic- based biofuels, followed by paper and pulp manufacturer, among others. Globally, it has been projected that the biofuel consumption will increase to USD 57.8 billion

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gallons by 2020 (Market Research Report Collections, 2019- WWW.StrategyR.com), a process that has boosted the demand for cellulolytic enzymes. Considering this market demand, the search for novel cellulases with improved properties is continuously growing.

Most of the enzymes used in industrial processes are actually produced by mesophilic microbes (microorganisms with optimum growth temperature ranging from 20 °C to 45 °C) that produce the so-called "mesophilic enzymes" (maximal enzyme activities between 50–60 °C). On the other side, the cold-loving microbes (psychrophilic and cold-tolerant microbes, with optimum growth temperatures of 10 °C or lower, and 20 °C, respectively) produce psychrophilic enzymes. These ones have maximal temperatures of activities around 30–40 °C and are easily heat inactivated (thermolability) (D'Amico et al., 2002; Feller and Gerday, 2003). These two properties (activity at low temperature and thermolability) place psychrophilic enzymes as good candidates for some biotechnological applications, including the design of new products and/or processes. Psychrophilic enzymes play an important role in the global market due to their potential applications in enzymatically unfavorable industrial conditions. More information and examples of commercial psychrophilic enzymes are available in Martínez-Rosales et al. (2012), Sharma (2019), Yarzábal (2016). An interesting example constitutes the potential use of psychrophilic cellulases in the biofuel industry (bioethanol production), in a simultaneous saccharification and fermentation (SSF) process (Paulova et al., 2015). Thus, our aim was to contribute to the biofuel industry, by using cellulases produced by a cold-tolerant bacterium.

In a previous work, we explored the cellulolytic potential of microorganisms that inhabit the Antarctic oligochaete *Grania* sp. (Annelida) (Herrera et al., 2017). Among 31 cellulase-producing yeast and bacteria isolated from this worm, the bacterium identified as *Flavobacterium* sp. AUG42, a cold-tolerant microbe, produced halos of hydrolysis when growing in plates with growth medium supplemented with carboxymethylcellulose (CMC) at low temperatures (10 °C and 20 °C). This result encouraged us to attempt the production of cellulolytic enzymes from strain AUG42 using different cellulosic substrates, the partial biochemical characterization of the cell-free supernatant, and the analysis of its saccharification potential for the future design of SSF processes. In addition, this work reports the CAZome from AUG42 and the identification of secreted carbohydrases by shotgun Mass Spectrometry analysis. The CAZome describes the collection of carbohydrate-active enzymes (CAZymes) encoded by the genome of an organism and provides an insight into the nature and extent of the metabolism of complex carbohydrates of the species (Carbohydrate active enzymes, 2019a; Carbohydrate active enzymes, 2019b). The different categories of CAZymes (glycoside hydrolases, GHs; glycosyltransferases, GTs; polysaccharide lyases, PLs; carbohydrate esterases, CEs; auxiliary activities, AAs with redox activity that act in conjunction with other CAZymes) and carbohydrate-binding modules (CBMs) are fully described in the CAZY (Carbohydrate-Active enZYme) database (Carbohydrate active enzymes, 2019a; Carbohydrate active enzymes, 2019b). In summary, we aimed to identify the cellulases produced by AUG42, to perform the partial biochemical characterization of the cellulolytic-supernatant and to analyze their saccharolytic potential.

2. Materials and methods

2.1. Bacterium and growth media

The cold-tolerant cellulase-producing *Flavobacterium* sp. AUG42 strain was used in this work (Herrera et al., 2017). The strain was maintained in Luria-Bertani broth (LB) and stored in 15% (v/v) glycerol at −80 °C.

2.2. Time-course of cellulase production: growth rate versus enzymatic activities

Pre-cultures of strain AUG42 (5 mL of LB) were transferred to 2 L flasks containing 500 mL of a modified GS2 medium (without resazurin and with an adapted mineral micronutrient solution) at pH 7.0 (Artzi et al., 2015). The GS2 medium contained 5 g per liter of yeast extract as carbon and energy source, and the following minerals: 53 μM $CaCl_2$, 10 nM $CuSO_4$, 10 nM $ZnSO_4$, 10 nM $Na_2B_4O_7$, 80 nM $MnCl_2$, 30 nM $CoCl_2$, 3 nM Na_2MoO_4. The growth medium was supplemented with different cellulosic substrates at 0.5% (w/v): Carboxymethylcellulose (CMC, SIGMA, C5678), Avicel (SIGMA, 11363), Grade 1 Whatman filter paper and microcrystalline cellulose (SIGMA, 435236). The flasks were incubated in aerobic conditions (200 rpm) at different temperatures (11 °C, 16 °C and 21 °C) and the time-course of cell growth was monitored by the determination of colony-forming units (CFU) in Petri dishes containing LB medium. The cellulolytic preparation or cell-free supernatant was obtained by centrifugation of cultures (10,000 rpm, 30 min, 4 °C), filtered through 0.45 μm Millipore filters and finally stored at 4 °C until use. When indicated, the preparation was lyophilized. The cell-free supernatant obtained from AUG42 cells grown at 16 °C with filter paper as a cellulosic carbon source, was used for the partial characterization of the cell-free supernatant as indicated below.

2.3. CMCase and PASCase activities

CMC and phosphoric acid-swollen cellulose (PASC) were used as cellulosic substrates for the determination of CMCase and PASCase activities, respectively. PASC was produced by treatment of Avicel by phosphoric acid (7.5 mg/ml) as described by Moraïs et al. (2010). The cellulolytic preparations were incubated with 1% (w/v) CMC or 60% (v/v) PASC in 50 mM acetate buffer at pH 5.5 during 1 or 3 h at 40 °C, respectively. The mixture was stirred under orbital agitation (1000 rpm), cooled in an ice bath and then centrifuged (14,000 rpm, 5 min, 4 °C). The released reducing sugars in the supernatant were analyzed by the 3,5-dinitrosalicylic acid (DNS) assay as described by Miller (1959) with minor modifications. Essentially, the supernatant was mixed with an equal volume of DNS solution, boiled for 10 min in a water bath, cooled in an ice bath, and the absorbance was measured at 550 nm. Glucose was used as a standard. One unit of enzyme activity (U) was defined as the amount of enzyme required for releasing 1 μmol of reducing sugars (expressed as glucose) per hour, under the assay conditions.

2.4. Cellobiase activity

Cellobiase activity was determined using D-(+) cellobiose (SIGMA, C7252) as the substrate, dissolved in the "buffer mix" described by Ellis and Morrison (1982) (0.05 M acetic acid, 0.05 M MES, and 0.1 M TRIS), at pH 5.5 and 40 °C. The cell-free supernatant (400 μL) was incubated with the substrate (100 μl of 40 mM cellobiose, prepared in buffer mix), for 2 h under stirring at 1000 rpm in an orbital shaker and then, boiled during 5 min in water bath. The concentration of the released glucose was determined using the GLUCOSE-TR Kit (from SPINREACT, Ref 1001191). One unit of enzyme activity (U) was defined as the amount of enzyme required for releasing 1 μmol of glucose per hour under the assay conditions.

2.5. Effects of pH and temperature on enzyme activities

The pH effect on CMCase, PASCase and cellobiase activities was determined in the range of 3.5–10, using the "buffer mix" described above. The effect of temperature on the enzymatic activities was determined by fixing the pH at 5.5 and varying the temperature in the range 10 °C–60 °C. The thermal stability of the enzymes was also evaluated, by measuring the residual activities in the cell-free supernatant

after 30 days of incubation at different temperatures, and after lyophilization.

2.6. Electrophoretic analyses

SDS-PAGE was carried out as described by Laemmli (1970), using 10% and 5% acrylamide for the resolving and the stacking gels, respectively. Proteins were visualized by staining with Coomassie Brilliant Blue R-250. Isoelectric focusing was done in the Pharmacia PhastSystem equipment (Pharmacia, Uppsala, Sweden) according to the manufacturer´s instructions, by using the broad isoelectric point (pI) calibration kit run on PhastGel IEF 3–9 (GE Healthcare), and proteins were silver stained. For zymographic experiments two SDS-PAGE and two isoelectric focusing gels were run in parallel; one gel was commonly stained for protein visualization, and the other was blotted to a 10% acrylamide gel containing 0.14% (w/v) CMC (CMC-gel) by soaking in 0.05 M sodium acetate buffer pH 5.5 during 1 h at 37 °C. Specific stain for activity was performed by soaking the CMC-gel in 0.1% (w/v) Congo-Red for 1 h and then washing the gel with 1.0 M NaCl. Active bands became clearly visible as a yellow band(s) against a red background.

2.7. CAZome analysis

The genome of strain AUG42 was sequenced, annotated and functionally categorized as described by Morel et al. (2016). Briefly, a highly pure genomic DNA sample was sequenced at Macrogen using a Hiseq2000 Illumina system by shotgun library sequencing. The quality of reads was analyzed using the FASTQC program, the raw material was assembled using the SPAdes 3.13.0 assembly software (SPAdes – Center for Algorithmic Biotechnology, 2019) and contigs were build using ABACAS (ABACAS, 2019). The draft genome was annotated using the RAST Version 2.0 (Rapid Annotation using Subsystem Technology) server (Aziz et al., 2008) (the manuscript regarding AUG42 draft genome is currently in preparation). The predicted proteins from AUG42 were analyzed using the web tool PFAM (PFAM, 2019). The PFAM (Finn et al., 2015) is a database that performs the search based on domains similarities, for the identification of proteins. For the particular identification of carbohydrate-active enzymes, all the predicted proteins were analyzed by dbCAN (database for the automated Carbohydrate-active enzyme annotation) (Yin et al., 2012), this web server is commonly used for the identification of CAZymes. The results obtained by dbCAN were also analyzed through BLASTP using the UniProtKB-SwissProt and PDB database (BLAST: Basic Local Alignment Search Tool, n.d.).

2.8. Shotgun Mass Spectrometry analysis of secreted proteins (secretome)

Cell-free supernatant fluids were loaded onto an SDS-PAGE 10% acrylamide gel allowing a short (1 cm) migration, Coomassie G-stained and those portions of gels containing proteins were excised for shotgun Mass Spectrometry (MS) analysis. Protein digestion and MS analysis were performed at the Proteomics Core Facility CEQUIBIEM, at the University of Buenos Aires/CONICET (National Research Council), as described by Ghio et al. (2018), using a Thermo Scientific Q Exactive hybrid quadrupole-Orbitrap mass spectrometer. The raw data were processed using the Proteome Discoverer software (version 1.4 from Thermo Scientific) and searched against the *Flavobacterium* sp. AUG42 protein sequence database obtained by genome annotation. The results obtained by shotgun of secreted proteins and by the dbCAN were compared to identify possible cellulases.

2.9. Protein modeling

The *in silico* protein structure from cellulases 265 and 976, in particular, the catalytic domains were performed using the homology-modeling Swiss-model server (SWISS-MODEL, 2019). The proteins with the highest scores were chosen as a template to model both AUG42 proteins. Those structures with the highest GMQE (Global Model Quality Estimate) were chosen and the position of amino acids was analyzed by Ramachandran plot, in the same server (Swiss-model). The active sites were identified using the Chimera 1.13.1 program (UCSF Chimera Home Page, 2019) by comparison with a “seed model” cellulase using the MatchMaker tool. Then, the active site and a putative carbohydrate substrate (a β-1,4 cellotetraose and β-1,4 cellobiose) were open and prepared using the Dock Prep tool in the Chimera program. Finally, the docking was performed using the AutoDock Vina tool.

2.10. Saccharification assays

The assay was performed by using 5% CMC as the substrate, in 20 mL vials containing 15 mL of ING medium (a medium commonly used in ethanol fermentation; chemical composition as follows: 7 mM KH_2PO_4, 26 mM K_2SO_4, 4,5 mM $(NH_4)_2SO_4$ and 10 mM $MgSO_4.7H_20$, pH 5.5). Vials were injected with a volume of AUG42 water-reconstituted lyophilized cell-free supernatant containing 44.3, 3.1 and 4.5 U/mL (final concentration at the vials) of CMCase, PASCase and cellobiase activities, respectively. The mixture was incubated for 24 h at 200 rpm and 30 °C. The efficiency of the saccharification process was monitored by determining the amount of released reducing sugars as well as the glucose concentration, as described above.

2.11. Nucleotide sequence accession numbers

The glycoside hydrolases sequences detected by the CAZome analysis were submitted to the GenBank. Accession numbers are as follows: MH510337 (gene 265), MH510338 (gene 266), MH510339 (gene 439), MH510340 (gene 649), MH510341 (gene 890), MH510342 (gene 891), MH510343 (gene 976), MH510344 (gene 1992), MH510345 (gene 2218) and MH510346 (gene 3439).

2.12. Statistics

At least three technical and three biological replicates were performed for each experiment. The statistical correlation of data was analyzed by the GraphPad Prism (6 version) software. Data are reported as means ± S.E.

3. Results

The production of extracellular cellulolytic enzymes from the Antarctic bacterium *Flavobacterium* sp. AUG42 was evaluated, when the bacterium was growing in GS2 liquid medium supplemented with different cellulosic carbon sources. The time-course of bacterial growth (log CFU/ml vs time) and the production of cellulases (evaluated by CMCase and PASCase activities) are shown in Figs. 1a and 2 . The results showed that AUG42 cells grew in the tested cellulosic carbon sources, while almost no growth was observed in the non-supplemented GS2 medium (just yeast extract as carbon and energy source, as described in Materials and Methods), suggesting that this bacterium may produce extracellular enzymes involved in the degradation of these cellulosic carbon sources. The curves exhibited a similar growth pattern, reaching always the stationary phase at 40 to 48 h of growth (Fig. 1a).

The analyses of the time-course of CMCase and PASCase production (Fig. 2a and b, respectively) revealed that both activities were maximal at the stationary phase when growing on filter paper, compared with other cellulosic carbon sources. Both activities were 4-fold and 6-fold higher respectively, compared to the activities observed when using cellulose or Avicel as a cellulosic carbon source.

Hence, the time-course of growth and production of cellulases at different temperatures were both attempted using filter paper as the

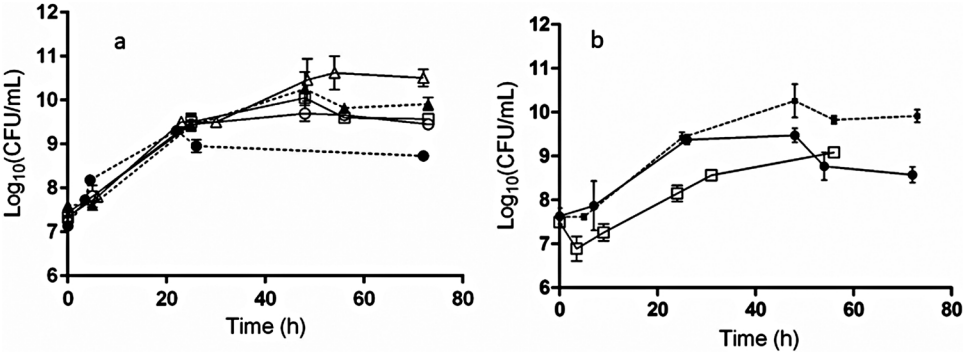

Fig. 1. Growth characteristics of *Flavobacterium* sp. AUG42: a) on different cellulosic substrates at 16 °C and b) at a different temperatures using filter paper as cellulosic substrate.
Mean and SDs were obtained from three separate measurements in each experiment, at least three experiments were performed for each substrate. Error bars were in some cases smaller than the size of the symbol. a) GS2 medium supplemented with: CMC (△), Microcrystalline Cellulose (○), Avicel (□) and Filter paper (▲). Control: GS2 medium (●). b) Growth at 11 °C (–□–), 16 °C (--▪--), and 21 °C (–●–).

carbon source. The results showed that AUG42 cells did not grow at 30 °C, but they grew at similar rates at 16 °C and 21 °C, and at a significantly slower rate at 11 °C (Fig. 1b; Table 1), reinforcing that strain AUG42 is a cold-tolerant rather than a psychrophilic bacterium. At the stationary growth phase, the values of CMCase and PASCase activities were significantly higher when growing at 16 °C compared at 11 °C and 21 °C (Table 1), suggesting that the production of cellulases was higher at 16 °C. Interestingly, there was cell growth when using GS2 medium without a mineral solution (with filter paper as carbon source); however, we did not detect any cellulase activity, suggesting that at least one or more of these salts are important for cellulase production or activity (data not shown). Although the time-course of cellobiase production was not specifically evaluated, this activity was detected (0.50 ± 0.05 U/mL) in the cell-free supernatant fluids from AUG42 collected at the stationary phase (growth at 16 °C). All these results together suggest that AUG42 produces a cocktail of glycosyl-hydrolases. Based on these findings, the cell-free supernatant produced by AUG42 cells growing in GS2 medium supplemented with 0.5% (w/v) filter paper at 16 °C, were used for further experiments (biochemical characterization and saccharification experiments).

A number of proteins with cellulase activity and their apparent molecular masses were determined by electrophoretic analysis coupled to zymographic gels (Figs. 3 and 4). Isoelectric focusing gel electrophoresis coupled to CMC-gels (zymogram) showed that the cell-free supernatant fluids contain at least four proteins with cellulase (CMCase) activity, with pIs ranging from 5.8 to 7.4 (Fig. 3). The results obtained after blotting of proteins from the SDS-PAGE gel to the CMC-gel also showed at least four protein bands with CMCase activity. Comparison of both gels (SDS-PAGE and CMC-zymographic gels) indicates that these cellulases may have apparent molecular masses ranging from 30 kDa to 100 kDa (Fig. 4).

Table 1
Growth characteristics and cellulase production at different temperatures.

Temperature of growth (°C)	Generation time (h)	Stationary phase at (h)	CMCase (U/mL)	PASCase (U/mL)
21	3.8 ± 0.3	25	0.87 ± 0.10	0.00 ± 0.01
16	3.3 ± 0.3	48	8.41 ± 0.40	1.20 ± 0.20
11	5.0 ± 0.3	48	3.07 ± 0.10	0.05 ± 0.10

The generation times (tg) were calculated from the specific growth rate (μ) corresponding to the exponential phase, according to the following equation: $tg = \ln 2 / \mu$. Values for CMCase and PASCase activities were determined at the stationary phase stage.

The effect of pH and temperature on the cellulolytic activity of the cell-free supernatant fraction showed optimal activities at pH 6.5 and 5.5 for CMCase and PASCase, respectively, and in both cases at 50 °C, a temperature commonly associated to mesophilic enzymes. However, both activities abruptly decreased at 60 °C (10% remaining activities, compared to values at 50 °C), suggesting that these enzymes are thermolabile, a feature mostly associated to psychrophilic enzymes. Cellobiase activity was highest at pH 5.5 and 40 °C, with a total inactivation at 50 °C, suggesting the psychrophilic character of this enzymatic activity. The data regarding the effect of pH and temperature on these enzymatic activities are shown as Supplementary Material.

At this point, we centered our attention into the analysis of the saccharolytic potential of the cell-free supernatant fluid, showing that after 24 h incubation at 30 °C and pH 5.5, the amount of total reducing sugars and glucose raised from 1,4 ± 0,3 to 17,0 ± 2,0 and from 0 to 21 ± 1 μmol/mL, respectively. This amount of glucose represents the release of 3.8 g/L of a fermentable sugar.

The analysis of the draft genome of strain AUG42 (manuscript in

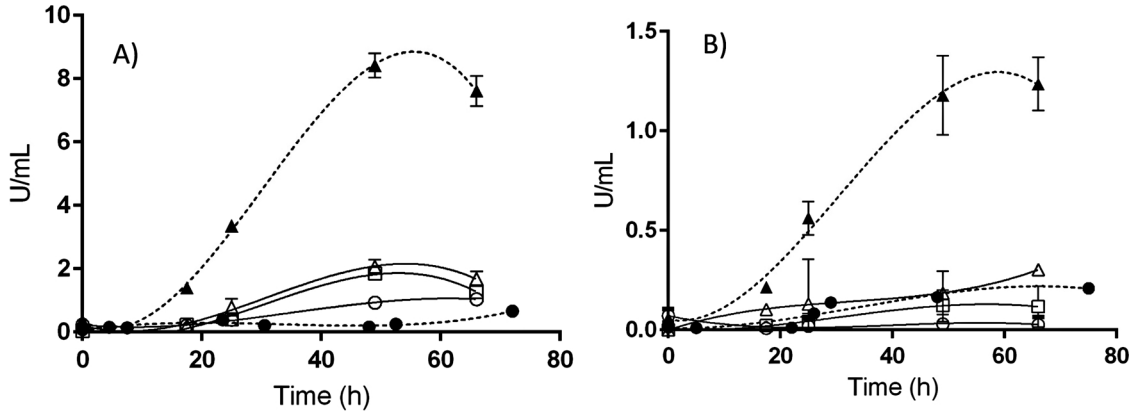

Fig. 2. Production of cellulose-degrading enzymes during the growth of *Flavobacterium* sp. AUG42.
A) CMCase and B) PASCase activities measured at 40 °C and pH 5.5. Mean and SDs were obtained from three separate measurements of a representative experiment, at least three experiments were performed for each condition. Error bars were in some cases smaller than the size of the symbol. GS2 medium supplemented with CMC (△), Microcrystalline Cellulose (○), Avicel (□), Filter paper (▲). Control: GS2 medium (●).

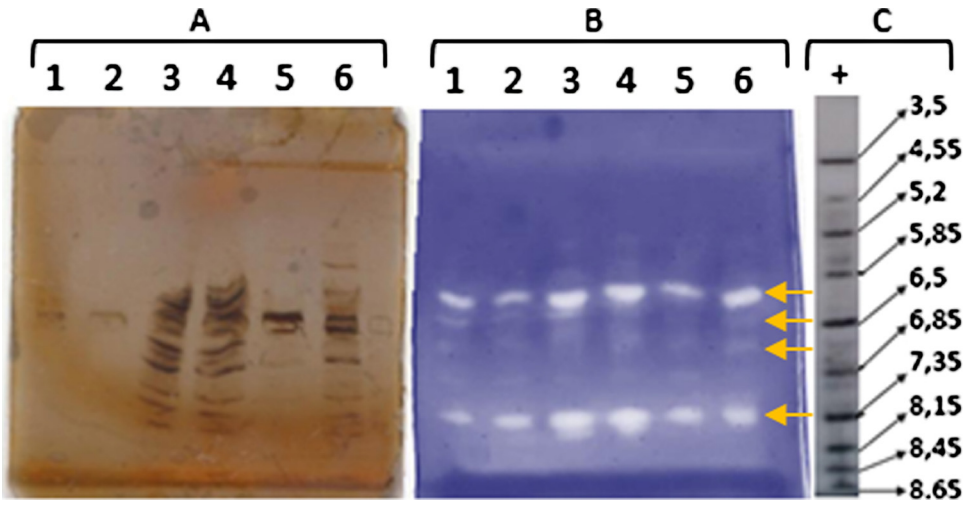

Fig. 3. Isoelectric focusing of the cell-free supernatant of *Flavobacterium* sp. AUG42 coupled to CMC-gels.
A) Isoelectric focusing gel, silver stained; B) zymographic CMC-gel stained with Congo-Red; and C) pI markers. Lanes 1, 2 and 5 were loaded with 1 µL of the cellulolytic cocktail (2-fold concentrated by lyophilization); lanes 3, 4 and 6 were loaded with 1 µL of the cellulolytic cocktail (12-fold concentrated by lyophilization). Arrows show the position of the active bands.

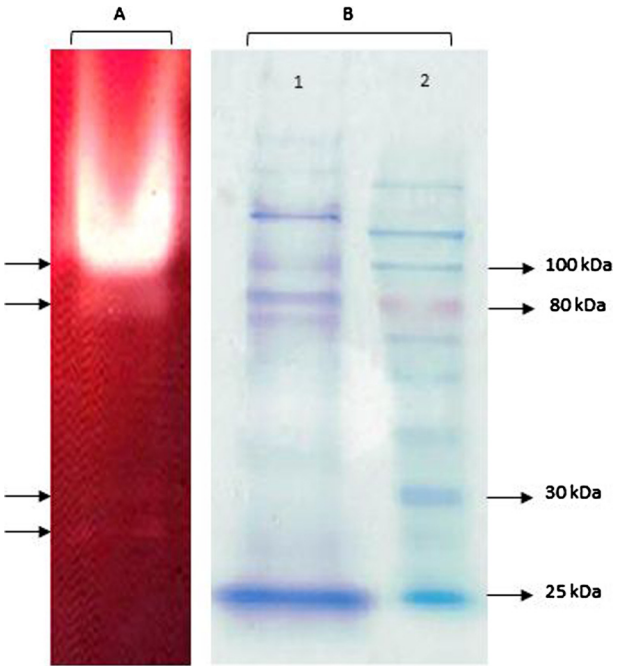

Fig. 4. SDS-PAGE of the cell-free supernatant of *Flavobacterium* sp. AUG42 coupled to CMC-gels.
A) Zymographic CMC-gel after blotting from the SDS-PAGE gel; arrows show the position of the active bands. B) SDS-PAGE gel. Lane 1 was loaded with 5 µL of the cellulolytic cocktail (2- fold concentrated by lyophilization) and lane 2 was loaded with 5 µL of the molecular marker Color Plus NEB (selected band sizes are indicated in the gel).

preparation) allowed the identification of at least ten open reading frames (ORFs) related to glycoside hydrolases probably involved in the hydrolysis of cellulosic materials (Table 2). The shotgun Mass Spectrometry analysis of secreted proteins showed that from these ten proteins, five GHs were present in the secretome of strain AUG42 under the assayed conditions. These glycoside hydrolases belong to the GH3, GH5, GH9 and GH30 families (Table 2). The search for carbohydrate binding modules (CBM) showed that glycoside hydrolases 265 (GH9) and 976 (GH5) harbor putative CBMs that may recognize cellulose, N-acetylglucosamine and xylans (Fig. 5). When the *in silico* modeling was performed using the entirely 265 and 976 proteins, they did not blast with any crystallized protein deposited in the database. However, it was possible to model the catalytic sites of both proteins, as well as the CBM of the 265 protein. The modeling and docking analyses of both catalytic sites seem to display cleft or groove-shaped active sites (Fig. 6), resembling those exhibited by endoglucanase enzymes (Gupta, 2016). The catalytic amino acids and the potential hydrogen bonds between the catalytic amino acids and the substrate are also shown (Table 3).

4. Discussion

Although fungi play an important role as biomass-degrading microorganisms, being responsible for the degradation of most biomass in nature (Payne et al., 2015), bacteria stand out like recalcitrant matter-degrading microorganisms. Among bacteria, members from the genus *Flavobacterium* are recognized as natural cellulosic fiber degraders (Lednická et al., 2000). In the present work, we report the production of cellulases and the characterization of the cellulolytic cocktail produced by the Antarctic cold-tolerant bacterium *Flavobacterium* sp. AUG42. The implications of the production of cellulases from an environmental and industrial point of view are also analyzed.

First, we attempted to answer the following questions: which is the preferred substrate and growth temperature of AUG42 to produce cellulolytic enzymes? and which kind of cellulases are produced by AUG42? The results showed that AUG42 produces glycoside hydrolases with CMCase, PASCase and cellobiase activities with a major production at the stationary growth phase when growing on filter paper as a cellulosic carbon source and 16 °C, among tested conditions. These activities were not detected when cells were grown in cellulosic-substrate-free medium, suggesting that the cellulosic material is needed for the enzymes production. Many reports showed that different cellulosic sources induce the production of cellulases at different extent; *i.e.* (Liang et al., 2014) informed that the bacterium *Paenibacillus terrae* requires the addition of CMC to the growth medium in order to produce CMCases, but the production showed a fivefold increase in the presence of wheat bran, as compared with the control experiment without cellulosic source. As AUG42 does, fungi from different genera prefer filter paper over CMC as an inducer of cellulase production during growth (Cai et al., 1999). Cellulases were mainly detected during the stationary phase, probably due to their participation in the secondary metabolism, as reported by (Sanchez et al., 1999).

Our next step was to answer whether this cellulolytic cocktail has biochemical properties which resemble those from psychrophilic enzymes. As reviewed by D'Amico et al. (2002), mesophilic and psychrophilic enzymes show maximal temperatures of activities close to 30 °C and 60 °C, respectively; hence, the cellulases produced by AUG42 might not be strictly classified as psychrophilic or cold-adapted enzymes; however, they are produced by a cold-tolerant microorganism, as described by Herrera et al. (2017). Although the optimum temperature for both CMCase and PASCase activities was close to 50 °C,

Table 2
CAZome or functional carbohydrate-active enzymes (CAZymes) from AUG42.

Protein identification	Blastp/Uniprot-Swissprot Access code (% coverage/%identity)	Blastp/PDB Access code (%coverage/%identity)	PFAM Domains	DBCAN	pI/MW
265*	Endoglucanase E-4 P26221.2 (52/54)	Chain A, endoexocellulase:cellobiose from *Thermomonospora* 1JS4 (51/54)	PF00759.18 GH9 PF00942.17 CBM3	GH9 CBM3	7.13 / 125643.81
266*	Cellulase E1 P54583.1 (42/45)	Chain A, *Acidothermus cellulolyticus* Endocellulase E1 1ECE (42/46)	PF00150.17 Cellulase	GH5	6.22 / 91976.75
439	Beta-hexosaminidase P40406.1 (99/28)	Beta-n-hexosaminidase (ybbd) From Bacillus subtilis 3BMX (99/28)	PF00933.20 GH3	GH3	5.71 / 59206.93
649	Beta-N-acetylglucosaminidase/beta-glucosidase Q7WUL3.1 (38/28)	Crystal structure of mutant (d318n) *Bacillus subtilis* Family 3 glycoside hydrolase 4GIJ (51/30)	PF00933.20 GH3 PF00144.23 Beta lactamase PF01915.21 GH3_C	GH3	8.25 / 112589.87
890*	Glycosyl hydrolase family protein 3B A7LXU3.1 (99/46)	*Bacteroides ovatus* Xyloglucan Pul Gh3b with bound glucose 5JP0 (96/47)	PF00933.20 GH3 PF01915.21 GH3_C PF14310.5 FN3 like	GH3	5.72 / 83592.27
891*	Acid beta-glucosidase Q9BDT0.1 (86/33)	Acid-Beta-Glucosidase 2V3D (86/33)	PF02055.15 GH30 PF17189.3 GH30_C	GH30	7.60 / 54726.14
976*	Cellulase Z P07103.2 (41/59)	Cellulase Cel5 from *Erwinia chrysanthemi* 1EGZ (36/65)	PF00150.17 Cellulase	GH5 CBM60 CBM5	5.75 / 85670.49
1992	Cellulase 2 P37701.1 (77/41)	Endoglucanase A from *Clostridium thermocellum* at atomic resolution 1IS9 (72/39)	PF01270.16 GH8	GH8	8.76 / 53495.18
2218	Cellobiase P33363.2 (94/42)	Crystal structure of GH3 beta-glucosidase from *Bacteroides thetaiotaomicron* 5XXL (94/46)	PF00933.20 GH3 PF01915.21GH3_C PF14310.5 FN3 like	GH3	5.85 / 84425.56
3439	Probable mannan endo-1,4-beta-mannosidase Q0C8J3.1 (21/29)	Native structure Of Endo-1,4-Beta-D-Mannanase from *Thermotoga petrophila* Rku-1 3PZ9 (24/25)	PF00150.17 Cellulase PF02836.16 GH2_C	GH5	8.69 / 61002.69

The identification of carbohydrases using different databases (PFAM, dbCAN and UniprotKB-SwissProt), and a few theoretical properties, such as isoelectric point (pI) and Molecular Weight (MW), are shown. Proteins with asterisks (*) were been identified in the secretome from AUG42, by shotgun as described in Materials and Methods.

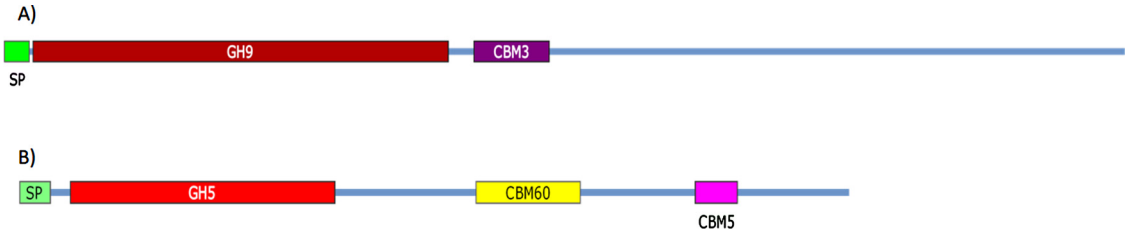

Fig. 5. Modular structure of cellulases 265 and 976.
A) Cellulase 265 (1178 amino acids) showing a GH9 catalytic domain located between amino acids 31 and 467 and a CBM3 domain located at the C-terminal (putative recognition of cellulose, between amino acids 496 and 574). B) Cellulase 976 (807 amino acids) showing a GH5 catalytic domain located between aminoacids 50 and 307 and two putative CBMs, a CBM60 (putative recognition of N-acetylglucosamine, located between amino acids 446 and 547) and a CBM5 (recognition of xylans, located between amino acids 660 and 700). SP represents the signal peptides found by search in the SignalP 4.1 server.

their thermolability resembles a psychrophilic enzyme. Notwithstanding, this will be elucidated after the out coming recombinant production, purification, and characterization of each enzyme.

Another question we arise was the identification of the cellulases present in the cell-free supernatant by comparing the data from the Mass Spectrometry study of the secreted proteins and the genome sequencing analysis. The results suggested that AUG42 produces a cocktail composed by five enzymes involved in the degradation of cellulosic matter, mainly endocellulases and a beta-glucosidase, in agreement with the CMCase, PASCase and cellobiase activities already found in the cell-free supernatant. The production of cellulolytic cocktails has also been reported for microorganisms such as *Aspergillus fumigatus* (Das et al., 2013) and *Bacillus licheniformis* (van Dyk et al., 2009), among others. Hence, the production of enzymatic cocktails may stand as a strategy to degrade complex polymeric substrates in their natural environment.

Interestingly, two cellulases with carbohydrate binding modules were identified in the secretome from AUG42, the so-called cellulases 265 and 976. When exploring their structural features, we found that these cellulases did not entirely blast with any previously crystallized protein deposited in the homology-modeling Swiss-model server. The herein reported results encourage us to face soon the resolution of their crystallographic structures. The *in silico* structure modeling of cellulases 265 and 976 revealed interesting protein features. The catalytic module-3D structure of 265 resembles the structure of proteins from the GH9 family, a barrel constituted by 12 α-helix in an $(\alpha/\alpha)_6$ arrangement, and a characteristic catalytic site able to accommodate an oligosaccharide substrate containing at least six glucose units. When modeled with its CBM, the 265 structure showed that the catalytic and cellulose-binding domains interact, as it has been described for the protein 1js4 (a cellulase from *Thermomonospora fusca* with both exo- and endocellulase activities) by Sakon et al. (1997). The authors also reported that 1Js4 is a processive enzyme, able to hydrolyze the cellulose chain releasing oligosaccharides containing four glucose as the CBM progresses in the cellulose strand. Thus, according to the shape of the catalytic site, we infer that 265 could be a processive endoglucanase. This cellulase also may possess a Ca^{2+} binding site, as found in the template 1js4.

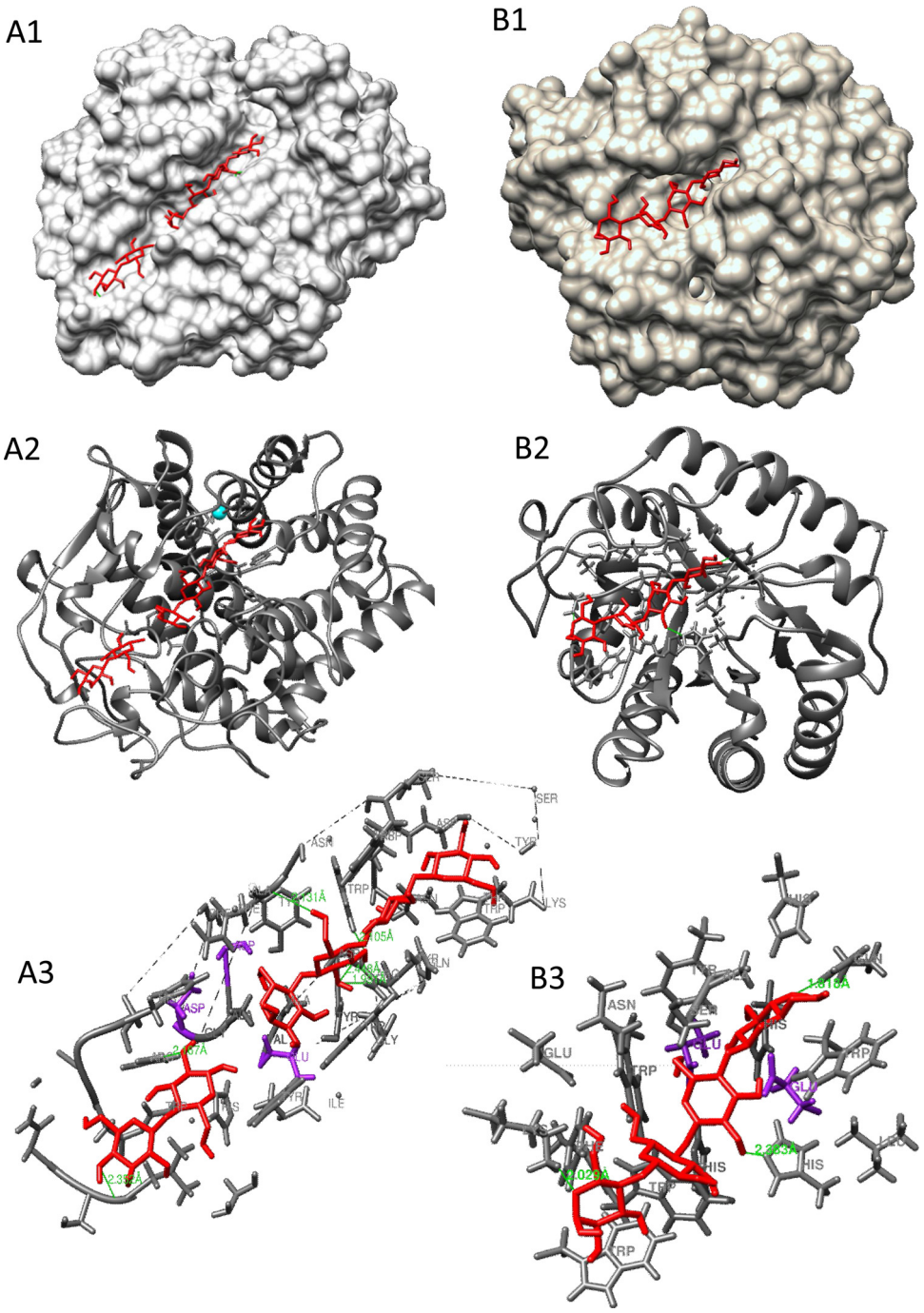

Fig. 6. Building a homology model for cellulases: 265 (A) and 976 (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
A1 and B1 are the surface representation; A2 and B2 are the Ribbon diagrams. A3 and B3 show the amino acids and hydrogen bonds that may interact with a substrate (shown in red).

The catalytic module-3D structure of 976 resembles the structure of a typical GH5 cellulase, a barrel constituted by eight external α chains and eight internal β or a $(\alpha/\beta)_8$ arrangement. The catalytic site seems to be located at the end of the α chains and the beginning of the β ones, thus the substrate can be attacked by following the Koshland double-displacement mechanism [*i.e.* two glutamic acids, one acting as nucleophile and the other as electrophile (Cazypedia, 2019)]. In addition, structural features of 976 protein are quite similar to those present in the template 4m1r, an enzyme reported as an endocellulase (Alvarez et al., 2013) (Table 3).

The next challenge dealt with the potential use of the cellulolytic cocktail during a saccharification process. The cocktail works at neutral or slightly acidic medium, a property that may be of interest in industrial applications (*e.g.* simultaneous saccharification and fermentation processes, clarification of fruit juice, *etc.*). Both, the optimum pH and temperature values of the enzymatic cocktail suggest that this could be used as an alternative tool for conventional and/or simultaneous saccharification and fermentation processes. Based on the theoretical yield of ethanol production from glucose (51.1% w/w) (Zhang and Bao, 2012) it can be inferred that a production of 3.8 g/L glucose may yield an amount of 2 g ethanol per liter, which represents a 0.2% w/w ethanol production. Clearly, the efficiency of saccharification is low and

Table 3
General information of modelling.

Cellulase	Modelling parameters					Docking			
	Template; Sequence identity; Coverage	GMQE	QMEAN	Amino acids in favored regions of the Ramachandran plot	Seed model	Substrate	Hydrogen bonding: amino acid, and distance to the substrate in Å	Amino acids involved in the coordination of Calcium	Catalytic amino acids
265	1js4; 63.74 %; 99 %	0,83	−1,88	94,94 %	1js4	Cellotetraose and cellobiose	F(228) 2,131 W(284) 2,105 R (341) 1,991 R (341) 1,409 L (413) 2,353 R (403) 2,167	S (237) D (241) E (242)	D (81) D (84) E (453)
976	4m1r; 71,66 %; 96 %	0,85	−0,04	94,72 %	2a3h	cellotetraose	H (140) 2,383 K (300) 2,028 Q (212) 1,818	No detected	E (261) E (173)

Catalytic amino acids, hydrogen bonding interactions involved in the substrate positioning in the catalytic site, amino acids involved in calcium coordination; the position of amino acids within the protein are indicated in parenthesis. GMQE mains Global Model Quality Estimation, and QMEAN mains Qualitative Model Energy Analysis.

needs to be upgraded; as an alternative, we are currently facing a strategy that includes the recombinant production of these enzymes for their use in SSF processes, particularly those proteins 976 and 265 with putative endo-cellulase activity (Table 2).

Recent works reported the saccharification of laminarin by an immobilized laminarinase from *Flavobacterium* sp. strain UMI-01 (Mitsuya et al., 2017) and the seaweed saccharification by a recombinant produced alginate lyases from *Flavobacterium* sp. FALy (Manns et al., 2016), among others; however, to the best of our knowledge this is the first report regarding the characterization of a cellulolytic cocktail produced by an Antarctic *Flavobacterium* isolate, with a potential application in the saccharification of cellulosic matter for the biofuel industry.

We finally discussed the potential role of AUG42 in the natural environment. Based on the complexity of plant cell walls, most biomass-degrading microorganisms display a battery of enzymes with a synergistic function to break down complex polysaccharides. As expected, AUG42 produces a set of enzymes with synergic activity to degrade the cellulosic material. By shotgun Mass Spectrometry analysis of the secreted proteins, we herein identified five glycoside hydrolyses which probably cooperate in the degradation of different cellulosic substrates. The *in silico* analysis of the identified secreted proteins suggests that these enzymes may recognize a set of polysaccharides such as cellulose, chitin (a long-chain polymer of N-acetylglucosamine, component of cell walls in fungi, the exoskeletons of insects, among others) and xylans (a group of hemicelluloses found in plant cell walls and some algae). Guo et al. (2017) reported that an enzymatic extract (a cocktail of cellulases, xylanase and laccase) obtained from a biomass-degrading bacterium after growth in a mineral salt medium containing wheat bran as a carbon source, efficiently hydrolyzes algal cell walls, facilitating the release of compounds involved in the carbon and nitrogen cycles. Although we did not focus our work on the cellulases produced by AUG42 cells in the natural environment (in association with the *Grania* worm), the results suggest that *Flavobacterium* sp. AUG42 produces a group of cellulases that might assist into the decomposition of algae. This ability may have a central role in the feeding of the worm *Grania* sp. and in the carbon cycling, thus contributing to the decomposition and nutrient recycling in the Antarctic ecosystem.

5. Conclusions

In summary, we showed that the Antarctic bacterial isolate *Flavobacterium* sp. AUG42 possess the genetic potential to produce ten carbohydrate-degrading enzymes and at least five of them were identified in the secretome by the shotgun proteins analysis. The degradation of algae by AUG42 cellulases need still to be evaluated, although the results suggest that this bacterium may provide available carbon sources from algae, thus improving the sustainability and nutrient balance of the Antarctic ecosystem through the decomposition of cellulosic materials. Finally, the enzymatic cocktail has biochemical properties that deserve attention from an industrial point of view, mainly as a resource for the biofuel industry.

Conflict of interest and ethical approval

The authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

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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.micres.2019.03.009.

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