Optimisation of xylanases production by two *Cellulomonas* strains and their use for biomass deconstruction

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**Abstract**

One of the main distinguishing features of bacteria belonging to the *Cellulomonas* genus is their ability to secrete multiple polysaccharide degrading enzymes. However, their application in biomass deconstruction still constitutes a challenge. We addressed the optimisation of the xylanolytic activities in extracellular enzymatic extracts of *Cellulomonas* sp. B6 and *Cellulomonas fimi* B-402 for their subsequent application in lignocellulosic biomass hydrolysis by culture in several substrates. As demonstrated by secretomic profiling, wheat bran and waste paper resulted to be suitable inducers for the secretion of xylanases of *Cellulomonas* sp. B6 and *C. fimi* B-402, respectively. Both strains showed high xylanolytic activity in culture supernatant although *Cellulomonas* sp. B6 was the most efficient xylanolytic strain. Upscaling from flasks to fermentation in a bench scale bioreactor resulted in equivalent production of extracellular xylanolytic enzymatic extracts and freeze drying was a successful method for concentration and conservation of the extracellular enzymes, retaining 80% activity. Moreover, enzymatic cocktails composed of combined extra and intracellular extracts effectively hydrolysed the hemicellulose fraction of extruded barley straw into xylose and xylooligosaccharides.

**Key points**

- Secreted xylanase activity of *Cellulomonas* sp. B6 and *C. fimi* was maximised.
- Biomass-induced extracellular enzymes were identified by proteomic profiling.
- Combinations of extra and intracellular extracts were used for barley straw hydrolysis.

**Keywords** *Cellulomonas* · Lignocellulose · Waste valorisation · Lyophilisation · Enzymatic hydrolysis · Secretome analysis

**Introduction**

Lignocellulosic residues can serve as abundant, cheap, and renewable resources for the production of chemicals, platform molecules, fuels and energy to enhance the development of a sustainable bio-economy (De Bhowmick et al. 2018). Deconstruction of lignocellulosic resources into easily fermentable sugars is very important for the efficient valorisation of biomass. Microbial enzymes with cellulolytic and hemicellulolytic activities have a huge potential to be applied in bioprocesses that use lignocellulosic raw materials (Ballesteros 2010; Maitan-Alfenas et al. 2015). Decomposition of cellulose requires the concerted action of endo-β-1,4-glucanases (EC. 3.2.1.4), reducing end-acting cellobiohydrolases (EC 3.2.1.176), exo-β-1,4-glucanases (EC. 3.2.1.74), cellulase 1,4-β-cellobiosidases (non-reducing end) (EC. 3.2.1.91) and β-glucosidases (EC. 3.2.1.21)
belonging to the cies (Islam 2019). The (hemi)cellulolytic potential of strains aerobic or anaerobic culture conditions, depending on the spe-

eral advantages including high cell growth rate, robustness and low nutritional requirements, resulting in efficient and cheap processes for enzyme production (Maki et al. 2009; and low nutritional requirements, resulting in efficient and conservation of the enzymatic extract.

Materials and methods

Microorganisms

C. fimi B-402 was obtained from NRRL Agricultural Research Service Culture Collection (IL, USA). Cellulomonas sp. B6 was isolated from a bacterial consortium obtained from a preserved native subtropical forest soil sample (Piccinni et al. 2016). Strain Cellulomonas sp. B6 has been deposited in public collections DSMZ and NCIMB, as DSM 107934 and NCIMB 15124, respectively. The strains were maintained for long term in Luria-Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) (Bertani 1951) with 20% w/w glycerol at −80 °C. Before use, a loopfull was spread in LB agar plates (LB supplemented by 15 g/L agar) for single colonies isolation.

Model substrates and biomass feedstocks

Different carbon sources were tested for cell growth and enzyme secretion. Solka-floc (SF, kindly donated by Lund University, Lund, Sweden) and carboxymethyl cellulose of low viscosity (CMC, Sigma-Aldrich, STL, USA) were used as model substrates. The selection of CMC was due to its high purity and solubility in aqueous solutions. SF is a model substrate obtained from milled pinewood through several extraction steps and it has around 76% w/w cellulose and 12% w/w hemicellulose, in terms of dry matter content, and which is insoluble in water (Sipos et al. 2010). Wheat bran (WB), pre-treated sweet corn cob (PSCC) and pre-treated waste paper (PWP) were used as lignocellulosic carbon sources. Sweet corn cob pre-treated by alkali extrusion was provided by INRA (Toulouse, France). Waste paper consisted of corrugated cardboard pieces, which were cut into small pieces (smaller than 0.5 cm), mixed with distilled water to set 10% w/w dry matter content, homogenised with a hand blender for 10 min and autoclaved (121 °C for 30 min). Wheat bran was purchased at a dietary shop.

For enzymatic hydrolysis experiments, extruded barley straw (EBS) was used. EBS was obtained from the Babetreal5 project (https://www.babet-real5.eu/) and provided by CIEMAT (Madrid, Spain). The extrusion conditions were 100 °C, 4.5% NaOH/dry barley straw and neutralisation with H3PO4 (Duque et al. 2017).
Structural carbohydrates (glucan, xylan and arabinan) and the acid insoluble solids of WB and PWP were determined as described by the National Renewable Energy Laboratory (DEN, USA) (Sluiter et al. 2012). Relative composition of PSCC was provided by INRA. The structural carbohydrate analyses of WB and PWP were carried out in triplicates. Starch content of WB was also determined by using enzymatic treatment with α-amylase as detailed by Bedő et al. (2019). Composition of PSCC, WB and PWP is detailed in Table 1.

**Enzyme production in shake flask**

Bacteria (*C. fimi* and *Cellulomonas* sp. B6) were grown on minimal medium (MM) (1.67 g/L dipotassium hydrogen phosphate (K2HPO4), 0.87 g/L potassium dihydrogen phosphate (KH2PO4), 0.05 g/L sodium chloride (NaCl), 0.1 g/L magnesium sulphate (MgSO4×7H2O), 0.04 g/L calcium chloride (CaCl2), 0.004 g/L iron(III) chloride (FeCl3), 0.005 g/L sodium molybdate (NaMoO4×2H2O), 0.01 g/L biotin, 0.02 g/L nicotinic acid, 0.01 g/L pantothenic acid, 1 g/L ammonium chloride (NH4Cl)) supplemented with 1 g/L yeast extract and L nicotinic acid, 0.01 g/L pantothenic acid) with the substrates was sterilised in autoclave at 121 °C for 20 min, after which microelements (FeCl3, NaMoO4×2H2O, biotin, nicotinic acid and pantothenic acid) were added to the indicated concentration. Starter cultures were obtained by inoculating single colonies (from fresh agar plates) in 10 mL LB medium and incubating at 30 °C and 220 rpm for 24 h, in the case of *Cellulomonas* sp. B6, and for 72 h, in the case of *C. fimi*. Cultures were inoculated from the starter cultures to obtain an initial cell concentration that corresponds to an OD of 0.05. Cultures were first grown as starter cultures in LB medium and then inoculated into the MM supplemented with biomass to get an initial cell concentration that corresponds to an OD of 0.05. Enzyme productions, in duplicates, were accomplished at 30 °C for 72 h with daily sampling. The bioreactors were equipped to be able to control dissolved oxygen (DO) level by adjusting the agitation and air flow rate, and to monitor the pH in the culture medium. DO level was kept at 20% of saturation in order to ensure that the culture was maintained in aerobic conditions.

**Production of extracellular enzyme fraction and intracellular enzyme fraction**

When the culture of enzyme production reached late exponential phase (72 h), the remaining solid biomass and the obtained cell mass were separated from the supernatant by centrifugation (6000×g, 10 min). The supernatants are referred to as extracellular enzyme (EE) fractions. EE fractions were supplemented with 0.04% w/w sodium azide and kept at 4 °C until use. The cells and remaining biomass (centrifugation pellet) were resuspended in citrate buffer (100 mM, pH 6) in a ratio of 1:10 regarding the initial culture volume, ultrasonicated on ice (six pulses of 10 s, 28% amplitude) and centrifuged (10000×g, 30 min). After centrifugation, filtered supernatants were used as intracellular enzyme (IE) fractions.

**Lyophilisation of EE and IE fractions**

EE and IE fractions were frozen at −80 °C. Then, they were taken into the freeze-dryer (EDWARDS Super Modulyo Freeze Dryer, Thermo Electron Corp., Waltham, MA, USA), treated at −30 °C under 1 × 10−1 mbar vacuum for 48 h and stored at 4 °C. The lyophilised enzyme fractions were referred to as LEE and LIE in the cases of extracellular and intracellular enzyme fractions, respectively. For enzymatic assays, both fractions were resuspended with sterile water to the original volume. Ten times concentrated enzyme fraction of LEE (LEE (10×)) was also produced by dissolving LEE in appropriate amount of sterile water. Recovery of the xylanase activity was calculated as the ratio between xylanase activity after and before lyophilisation and expressed as a percentage. Lyophilisation experiments were performed in triplicates.
Enzymatic activity measurements

Xylanase and CMCase activities were assayed in microtubes using beechwood xylan (1% w/w) or CMC (2% w/w) as substrates, respectively (Ghio et al. 2012). For these assays, 0.1 mL of appropriately diluted extracts (EE, IC, LEE, LIE (10×) or LIE) were added to 0.1 mL of each substrate prepared in citrate buffer (pH 6). Hydrolysis reactions were carried out at 40 °C, 400 rpm for 10 min. These conditions were established in a previous work (Piccinini et al. 2019). Reducing sugars released from the reactions were measured by dinitrosalicylic acid (DNS) method (Miller 1959) using glucose or xylose standard curves.

β-Glucosidase, cellobiohydrolase, β-xylosidase and α-L-arabinofuranosidase activities were assayed, using 5 mM p-nitrophenyl-β-D-glucopyranoside (pNPG), p-nitrophenyl-β-D-cellobioside (pNPC), p-nitrophenyl-β-D-xylopyranoside (pNPX) and p-nitrophenyl-α-L-arabinofuranoside (pNPA) (Sigma-Aldrich, STL, USA) as substrates, according to previously established protocols (Ontañón et al. 2018). In brief, reactions were performed by combining 100 μL of LEE (10×) or LIE fraction with 100 μL of 2.5 mM substrate in citrate buffer (pH 6, 100 mM), and incubating at 40 °C for 20 min. Reactions were stopped with 500 μL of 2% w/w sodium carbonate and absorbance was determined at 410 nm. A p-nitrophenol (pNP) curve was used as a standard.

All enzymatic assays were conducted in triplicates and controls of enzyme without substrate and substrate without enzyme were included. In all cases, one international unit (U) was defined as the amount of enzyme required to release 1 μmol of product per minute under the assay conditions.

Analysis of secretome

Secretomic analysis was performed using flasks cultures of Cellulomonas sp. B6 in WB and C. fimi in PWP, in duplicate according to the protocol previously described by Piccinini et al. (2019). Briefly, total proteins contained in cell-free supernatants were quantified by Bradford assay (Promega, Biodynamics, CABA, Argentina), precipitated with 10% w/w trichloroacetic acid and then resuspended in ultrapure water (resistivity of 18.2 MΩ cm) to a final concentration of 1 mg/mL. Protein digestion and mass spectrometry analysis were performed at CEQUIBIEM (http://cequibiem.qb.fcen.uba.ar/). Proteins were reduced with dithiothreitol 10 mmol/L for 45 min at 56 °C, alkylated with iodoacetamide (55 mmol/L) for 45 min in the dark and digested with trypsin (PromegaV5111; Promega, Fitchburg, WI, USA) overnight at 37 °C. The digestes were analysed by nano LC-MS/MS in a Thermo Scientific Q-Exactive Mass Spectrometer coupled with a nano HPLC EASY-nLC 1000 (ThermoFisher Scientific, Waltham, MA, USA). Peptide hits were filtered for high confidence peptide matches with a maximum protein and peptide false discovery rate of 1% calculated by employing a reverse database strategy. A minimum of two unique peptides was considered as confident detection. For the estimation of relative abundance, we used the protein abundance index emPAI calculated by Sequest using protein identification data. The equation emPAI/Σ (emPAI) × 100 was used to calculate the protein content in mol.% (emPAI%) (Ishihama et al. 2005; Shinoda et al. 2009). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD022718.

Enzymatic hydrolysis of EBS and analysis of the hydrolysates

Enzymatic hydrolysis experiments on EBS were performed by using LEE fraction and a combination of LEE and IE fractions with different mixing ratios and dilutions. Enzymatic hydrolysis experiments were carried out in a reaction mixture containing 5% w/v dry EBS in citrate buffer (pH 6, 100 mM). The hydrolysis reactions were carried out at 45 °C, 220 rpm for 72 h and stopped by boiling for 5 min. The supernatant was clarified by centrifugation (6000×g, 15 min) and then used to measure the xylose released from the biomass employing d-xylose assay kit (Megazyme, Bray, Ireland). The hydrolysis products were analysed by thin layer chromatography (TLC) in silica gel plates using butanol/acetic acid/water (2:1:1) as solvents and revealed by water/ethanol/sulfuric acid (20:70:3) with 1% v/v orcinol solution over flame.
Results

Xylanase and CMCase activities of EE fractions obtained using different biomass feedstocks as carbon source

*Cellulomonas* sp. B6 and *Cellulomonas* sp. B6 were cultivated on MM supplemented with different lignocellulosic residues as carbon source in order to study the production and secretion of polysaccharide active enzymes (CAZYmes). The carbon sources evaluated in this study were Solka-floc (SF) and carboxymethyl cellulose (CMC) as model cellulosic substrates, and pre-treated waste paper (PWP), wheat bran (WB) and pre-treated sweet corn cob (PSCC) as lignocellulosic materials. The most suitable substrates for culture were selected based on the enzymatic activities measured in the EE fractions. In the case of *Cellulomonas* sp. B6, the highest xylanase activity (3.06 U/mL) was obtained using WB. Xylanase activities produced using PWP and PSCC were 60.6% and 50.5%, respectively, compared to the maximal activity (100%) reached on WB while SF and CMC (model cellulosic substrates) resulted in 21.4% and 12.1% relative xylanase activities, respectively (Fig. 1a).

The highest xylanase activity (1.32 U/mL) of the EE fractions produced by *C. fimil* was achieved by using PWP as the carbon source (Fig. 1b). Surprisingly, the second highest xylanase activity was obtained when SF was used, corresponding to 76.1% activity of the activity achieved on PWP (100%) (Fig. 1b). WB and PSCC resulted in relative xylanase activities of 41.5% and 47.6%, respectively, and the lowest relative xylanase activity (17.4%) was observed with CMC (Fig. 1b). These results suggested that the cellulose-rich, chemically not-modified substrates (SF and PWP) were good inducers for the extracellular production of xylanases by *C. fimil*.

CMCase (glucanase) activities of the EE fractions obtained with the different carbon sources by *C. fimil* and *Cellulomonas* sp. B6 were below 0.2 U/mL in all the cases and were therefore not further studied.

Enzyme production in bench-top bioreactor

Based on the results described in the previous section, WB and PWP were selected as the best carbon sources for extracellular xylanase production by *Cellulomonas* sp. B6 and *C. fimil*, respectively. Hence, scale-up experiments from shake flask to a bench-top bioreactor (300 mL culture volume) were performed only with these raw materials. The culture profile of *Cellulomonas* sp. B6 using 1% w/w WB is shown in Fig. 2. Apart from small deviations in the first 20 h, the pH remained nearly constant (pH 6.8) throughout the experiment; which suggests the absence of pH active by-products, at least in considerable amounts. After 24 h, the DO level started to increase, indicating a decrease of cell growth and/or metabolic activity of the cells. After 48 h, the DO level and CO2 concentration in the exhaust gas became nearly constant (Fig. 2).

The xylanase activity displayed a significant increase after 24 h and reached the highest activity at 48 h for *Cellulomonas* sp. B6 and at 72 h for *C. fimil* (Fig. 3). Under these conditions, the extracellular xylanase activity of *C. fimil* was lower than that of *Cellulomonas* sp. B6, which is consistent with the results obtained in flasks.

The extracellular xylanase activity for *Cellulomonas* sp. B6 grown in bench-top bioreactor (~ 3 U/mL) was similar to that obtained when grown in flasks (3.06 U/mL), after 72 h of fermentation using WB. For *C. fimil*, the outcome was similar: similar xylanase activities either grown in shake flask or bioreactor using PWP (~ 1.4 U/mL). These results prove the scalability of the process. After 2 days of fermentation, the xylanase activity in the EE fraction of *Cellulomonas* sp. B6 was three times higher than that of in the EE fraction of *C. fimil*.

Secretome analysis

With the aim to correlate the observed xylanase activity with the specific secreted proteins, extracellular extracts (EE) were analysed by mass spectrometry, in duplicate independent...
cultures for each strain. In the WB extracellular fraction of *Cellulomonas* sp. B6, 197 proteins were detected in both samples. In the case of *C. fimi* PWP extracellular fraction, 360 total proteins were identified in both samples. From all of these proteins, 28 and 25 corresponded to glycoside hydrolases (GH) in *Cellulomonas* sp. B6 and *C. fimi*, respectively (Table 2). *Cellulomonas* sp. B6 secreted a repertoire of xylanases (7 GH10 and 1 GH11), which could account for the high xylanase activity in the culture supernatant. In particular, 4 of these xylanases (3 GH10 and 1 GH11) were amongst the most abundant proteins in the extract (from 0.77 to 10% of total proteins secreted), based on the index of relative abundance (emPAI%). The most abundant putative CAZymes in the WB secretome were a GH62 α-L-arabinofuranosidase (a debranching enzyme), two GH10 xylanases, a GH6 exoglucanase and a GH9 endoglucanase. As in previous studies, a high abundance of extracellular components of putative ABC transporters were also identified (Piccinni et al. 2019). By culture in PWP, *C. fimi* secreted 4 GH10 (out of the 5 GH10 encoded in the genome) and 1 GH11 (the only one predicted). The most abundant protein was a xylanase (UNIPROT id F4H4N7) which represented around 15% of total proteins, followed by a GH9 endoglucanase (about 3%) and three other xylanases (0.5 to 1.5% of total proteins) (Table 2).

### Activities of lyophilised extracts of *Cellulomonas* sp. B6

As *Cellulomonas* sp. B6 presented the highest xylanase activity in the secreted fraction, and this activity correlated with a higher number of xylanases identified in the secretome, this strain was chosen for further studies. Lyophilisation of the enzymatic extracts (EE and IE fractions) produced by *Cellulomonas* sp. B6 growing on WB was analysed in order to provide an efficient method to preserve enzymatic activities and concentrate the fractions. Lyophilisation of EE fraction (LEE) of *Cellulomonas* sp. B6 resulted in 80% recovery of xylanase activity and could be concentrated 10 times (LEE (10×)). The IE fraction was not further concentrated after lyophilisation (LIE). Beyond the main xylanase activity, β-xylosidase, α-arabinofuranosidase, CMCCase, β-glucosidase and cellobiohydrolase activities of LEE (10×) and LIE fractions were also determined (Table 3). While xylanase activity was more than 10 times lower in LIE fraction compared to that

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**Fig. 2** Profile of *Cellulomonas* sp. B6 cultures on wheat bran (WB) in bench-top bioreactor (3L). Temperature and pH were maintained at 30 °C and 7, respectively.

**Fig. 3** Extracellular xylanase activities during the aerobic culture of *Cellulomonas* sp. B6 (grey bars) and *C. fimi* (black bars) on WB and PWP, respectively, in bench-top bioreactor. WB: wheat bran, PWP: pre-treated waste paper.
### Table 2
Glycoside hydrolases (GH) identified in the culture supernatants of *Cellulomonas* sp. B6 and *Cellulomonas fimi* grown on wheat bran (WB) and pre-treated waste paper (PWP), respectively, (72 h, duplicate cultures). Proteins were identified by mass spectrometry and the relative abundance index (emPAI%) was calculated in each sample (named as 1 and 2) by Sequest using protein identification data.

| Accession | UNIPROT | CAZY domains and protein probable function description | emPAI% |
|-----------|---------|--------------------------------------------------------|--------|
| A0A0V8TB53 | GH10 | β-xylanase | 9.67 B6 (WB)1 |
| A0A0V8TA57 | GH9-CBM4 | endo-glucanase | 8.67 B6 (WB)2 |
| A0A0V8S5R6 | GH6 | exo-glucanase | 7.47 |
| A0A0V8SBR8 | GH62-CBM13 | α-L-arabinofuranosidase | 5.58 |
| A0A0V8SU5 | GH10-CBM2 | β-xylanase | 4.81 |
| A0A0V8SMW4 | GH10-CBM13 | β-xylanase | 2.02 |
| A0A0V8TC27 | GH6-CBM2 | endo-glucanase | 1.35 |
| A0A0V8ST89 | GH10-CBM9 | β-xylanase | 1.10 |
| A0A0V8SFL7 | GH11-CBM2 | endo-1,4-β-xylanase | 0.77 |
| A0A0V8SF9 | GH48-CBM2 | exoglucanase | 0.62 |
| A0A0V8SJ9 | GH9 | endoglucanase | 0.58 |
| A0A0V8S95 | GH18-CBM2 | chitinase | 0.56 |
| A0A0V8TC27 | GH6-CBM2 | endo-glucanase | 1.35 |
| A0A0V8T944 | GH9-CBM2 | endoglucanase | 0.36 |
| A0A0V8S2E5 | GH5 | endoglucanase | 0.32 |
| A0A0V8SBG4 | GH16-CBM13 | 1,3-β-glucanase | 0.29 |
| A0A0V8SK7 | GH5 | β-mannosidase | 0.28 |
| A0A0V8TC25 | GH27-CBM13 | α-galactosidase | 0.18 |
| A0A0V8SMY7 | GH43-CBM13 | β-xilosidase | 0.14 |
| A0A0V8TAG7 | GH74 | xyloglucanase | 0.13 |
| A0A0V8ST72 | GH10 | β-xylanase | 0.12 |
| A0A0V8S3C7 | GH10 | β-xylanase | 0.10 |
| A0A0V8S6Y6 | GH51 | α-L-arabinofuranosidase | 0.09 |
| A0A0V8S1S2 | GH51 | α-L-arabinofuranosidase | 0.08 |
| A0A0V8S6I1 | GH3 | β-glucosidase | 0.09 |
| A0A0V8TK6 | GH3 | β-glucosidase | 0.06 |
| A0A0V8TD5 | AA10 | LPMO | 0.05 |
| A0A0V8TB3 | GH43-CBM13 | β-xilosidase | 0.05 |
| C. fimi (PWP)1 | | | 0.19 |
| F4H4N7 | GH10-CBM2 | β-xylanase | 14.50 |
| P14090 | GH9 | endoglucanase | 2.46 |
| F4H710 | GH11 | endo-1,4-β-xylanase | 1.12 |
| F4H454 | GH10-CBM2 | β-xylanase | 0.74 |
| F4GZV4 | GH10-CBM13 | β-xylanase | 0.49 |
| P50899 | GH48-CBM2 | exo-glucanase | 0.31 |
| F4H413 | GH6 | endoglucanase | 0.27 |
| F4H2N5 | GH13 | α-1,6-glucosidase | 0.26 |
| F4H3U7 | GH9 | endoglucanase | 0.25 |
| F4H0M7 | GH26-CBM23 | endo-1,4-β-mannosidase | 0.19 |
| F4GZL0 | GH5-CBM2 | endoglucanase | 0.09 |
| F4GZY2 | GH6-CBM2 | endoglucanase | 0.12 |
| F4GY46 | GH10 | β-xylanase | 0.12 |
| F4GY55 | GH3-CBM11 | β-glucosidase | 0.07 |
| P50401 | GH6-CBM2 | endoglucanase | 0.07 |
| F4HT8 | GH3 | β-glucosidase | 0.03 |
| F4GZV3 | GH62-CBM13 | α-L-arabinofuranosidase | 0.03 |
obtained in LEE (10×), additional activities relevant to hemi-
celluloses deconstruction, such as α-L-arabinofuranosidases
and β-xylosidases were similar in both fractions (Table 3).

**Enzymatic hydrolysis of EBS by *Cellulomonas* sp. B6
enzymatic extracts**

Enzymatic hydrolysis of EBS using the xylanolytic extracts of
*Cellulomonas* sp. B6 grown on WB was performed to test
their hydrolytic efficiency and applicability in lignocellulosic
bioprocesses. For this purpose, LEE alone or in combination
with IE were used. As no concentration was required in the
case of the intracellular extract, the fresh fraction of IE (before
lyophilisation) was used, which had shown the same enzymat-
ic activity as LIE. The different enzyme mixtures tested in
enzymatic hydrolysis of EBS contained LEE and IE fractions
with the following xylanase activity: 30 U/mL of LEE, 15 U/

Table 3  Activities of the lyophilised extracellular and intracellular
enzyme fractions of *Cellulomonas* sp. B6 grown on WB. LEE (10×): 10 times concentrated extracellular enzyme obtained from
lyophilised sample

| Activity (U/mL)                     | LEE (10×) | LIE        |
|------------------------------------|-----------|------------|
| Xylanase                           | 32 (5.1)  | 1.9 (0.8)  |
| CMCase                             | 3.6 (0.5) | 0.5 (0.2)  |
| α-arabinofuranosidase              | 1 (0.3)   | 1.1 (0.3)  |
| β-xylosidase                       | 0.4 (0.1) | 0.5 (0.0)  |
| β-glucosidase                      | < 0.1     | < 0.1      |
| Cellobiohydrolase                  | 0.1       | < 0.1      |

LIE lyophilised intracellular enzyme, CMCase carboxymethyl cellulase, WB wheat bran. Average values and standard deviations (in parentheses) were calculated from biological triplicates

Fig. 4  Released xylose during hydrolysis of extruded barley straw (EBS)
by using different mixtures of extracellular (LEE) and intracellular (IE)
fractions of *Cellulomonas* sp. B6 grown on wheat bran (WB). The en-
zyme mixtures were the following: 30 U$_{\text{xylanase}}$/mL of LEE (A), 15 U$_{\text{xylanase}}$/mL of LEE (B), 7.5 U$_{\text{xylanase}}$/mL of LEE (C), 15 U$_{\text{xylanase}}$/mL of LEE + 1.5 U$_{\text{xylanase}}$/mL of IE (D), 7.5 U$_{\text{xylanase}}$/mL of LEE + 0.75 U$_{\text{xylanase}}$/mL of IE (E), and 3 U$_{\text{xylanase}}$/mL of LEE + 0.3 U/mL xylanase of IE (F). G: Substrate control without enzymes. Xylose concentration was determined by enzymatic colorimetric assay from D-xylose Kit (Megazyme)
zymes in the model strain reported to induce the secretion of only some of these enzymes (GH6, GH9, GH48) encoded in extracellular xylanases (GH10, GH11) and cellulose-active enzymes (GH11), respectively. Proteomic analysis revealed that all the C. fimi sp. B6 genome were secreted by growth on WB. These findings, along with previously reported assay, confirm that complex substrates can induce the production of xylanases in C. fimi (Wakarchuk et al. 2016). The use of residues and by-products as substrates for microbial growth has the advantage of being an inexpensive strategy for inducing the production of enzymes (Lopes et al. 2018). Interestingly, we found that low-value substrates of wheat bran (WB) and pre-treated waste paper (PWP) were the best inducers for the extracellular production of xylanases by C. fimi. In addition, although the growth on complex biomass has served as a successful strategy to enhance xylanolytic activity in several microorganisms (Saratale et al. 2010; Takenaka et al. 2019), it has also been proven that cellulosic substrates can induce the production of xylanases in C. fimi (Wakarchuk et al. 2016). The use of residues and by-products as substrates for microbial growth has the advantage of being an inexpensive strategy for inducing the production of enzymes (Lopes et al. 2018).

In the current work, we studied the feasibility of using different carbon sources—purified polysaccharides and cheap agro-industrial residues—as selective inducers of (hemi)cellulolytic activity in C. fimi B-402 and Cellulomonas sp. B6, already known as lignocellulose-active bacteria. Altogether, both strains showed greater xylanolytic than cellulolytic activity. While Cellulomonas sp. B6 showed higher activity on lignocellulosic complex biomass, cellulose-rich (chemically not-modified) substrates (SF and PWP) were better inducers for the extracellular production of xylanases by C. fimi. In addition, although the growth on complex biomass has served as a successful strategy to enhance xylanolytic activity in several microorganisms (Saratale et al. 2010; Takenaka et al. 2019), it has also been proven that cellulosic substrates can induce the production of xylanases in C. fimi (Wakarchuk et al. 2016). The use of residues and by-products as substrates for microbial growth has the advantage of being an inexpensive strategy for inducing the production of enzymes (Lopes et al. 2018).

Interestingly, we found that low-value substrates of wheat bran (WB) and pre-treated waste paper (PWP) were the best inducers for xylanase activity in Cellulomonas sp. B6 and C. fimi, respectively. Proteomic analysis revealed that all the extracellular xylanases (GH10, GH11) and cellulose active enzymes (GH6, GH9, GH48) encoded in Cellulomonas sp. B6 genome were secreted by growth on WB. These findings, along with previously reported assay, confirm that complex substrates, such as WB, are highly suitable for the production of CAZymes, especially those active on hemicellulose, by Cellulomonas sp. B6 (Piccinii et al. 2019). Eleven enzymes belonging to families GH6, GH48, GH9, GH10 and GH11, commonly associated with endo-xylanase and endoglucanase activity, were also secreted by C. fimi growing on PWP. By contrast, other substrates like CMC, WB or xylan have been reported to induce the secretion of only some of these enzymes in the model strain C. fimi ATCC 484 (Wakarchuk et al. 2016; Spertino et al. 2018). Moreover, as described by Wakarchuk et al. (2016) the genome of C. fimi ATCC 484 encodes 5 GH10 and 1 GH11 predicted xylanases. In the current study, PWP induced the secretion of 4 GH10 and 1 GH11, which is the maximum amount of secreted xylanases reported for C. fimi to date.

All of the above-mentioned carbohydrate-active enzymes have a broad range of industrial applications. Therefore, their production, catalytic efficiency and activity-preservation are essential factors from a biotechnological perspective. Bench-scale bioreactors have resulted to be useful tools for the identification and validation of key parameters involved in the scaling-up of bioprocesses (Marques et al. 2010). In the current study, the xylanolytic activity of both strains growing in a stirred tank bioreactor employing their preferred substrates was comparable with the one obtained in shake flask cultures. Conversely, some researchers have reported a significant yield loss by transferring the enzyme fermentation to bench-scale bioreactor (Kumar et al. 2009; Garai and Kumar 2013). Oxygen limitation, reduced availability of insoluble substrate, shear induced by agitation, dilution, substrate and product concentration are amongst the main listed drawbacks (Yegin et al. 2017). Remarkably, Cellulomonas sp. B6 and C. fimi showed a good performance in bench-scale bioreactors, foreseeing a great chance of efficient enzyme production on an industrial scale. It is important to note that 1% w/w PWP resulted in a dense and slimy suspension, which was a difficult medium to work with, especially because it can easily cause inhomogeneity in the bioreactor. The xylanolytic activity in the culture broth of C. fimi continuously increased during the fermentation. Thus, longer fermentation times should be examined to determine the maximum xylanolytic activity that may be achieved by C. fimi grown on PWP in a bioreactor. However, increasing fermentation time also increases the production costs. Process optimisation, including different agitation rates, addition of antifoam or modifications in substrate concentration, could be accompanied by improved performance and reduced operating times. The profiles of CO2 level in exhaust gas, DO concentration and xylanase activity in the culture medium suggest that the xylanolytic production by Cellulomonas sp. B6 on WB might have a growth-associated characteristic. A similar trend was observed by Xu et al. (2005) when the production of xylanase extract by Pseudomonas sp. WLUN024 was studied.

Concentration of enzymes is an important step in their production, especially if it helps to overcome the need for preservation and transportation at low temperatures. Many commercial enzyme preparations consist of concentrated culture broth with additives that stabilise enzymatic activity (Poletto et al. 2015). In the current work, the extracellular extracts from Cellulomonas sp. B6 retained 80% of xylanase activity after lyophilisation and further re-suspension, without the need of stabilising additives. This is a promising result since freeze-drying is a simple method for enzyme preservation but is usually associated with protein denaturation (Berghout et al. 2016).
Analysis of different glycoside hydrolase activities in the lyophilised samples showed that the extracellular enzyme fraction contained activities for polymeric hydrolysis in much higher quantities than activities for side-chain sugar hydrolysis. However, side activities were abundant in the intracellular enzyme fraction. Similarly, Rajoka (2005) reported that the cell-free supernatants of *Cellulomonas flavigena* NIAB 441 culture exhibited greater endo-xylanase activity than cell-associated β-xylanosidase activity.

Lyophilisation allowed us to concentrate the xylanase activity up to 10-fold and to use the extracts for hydrolysis of extruded barley straw (EBS). In order to improve the process, we supplemented the extracellular extract with the intracellular enzymes released by sonication. Using the enzyme cocktails containing different amount of LEE and IE fractions of *Cellulomonas* sp. B6 for enzymatic hydrolysis experiments of EBS resulted in the release of xylose and xylooligosaccharides as the main soluble products. Supplementing LEE samples with appropriate amounts of IE fraction significantly enhanced xylose release from EBS. Addition of appropriate xylanases during the enzymatic deconstruction of pre-treated barley straw is of great importance, since xylanase supplementation can significantly improve the efficiency of cellulose-hydrolysis (García-Aparicio et al. 2007). Moreover, hydrolysis by xylanases alone can result in promising bio-products like prebiotic xylo-oligomers or monomer xylose which may be then converted into high-value products (e.g. xylitol). Hence, LEE and IE from *Cellulomonas* sp. B6 may be interesting biotechnological candidates for biomass hydrolysis to produce xylo-oligomers, xylose or enhance enzymatic cellulolytic deconstruction.

We have concluded that the xylanolytic activity of *Cellulomonas* sp. B6 and *C. fimi* was differentially induced by varying the substrates utilised for its growth. Low-cost lignocellulosic residues were the most suitable inducers of secreted hemicellulases, which were then efficient for biomass decomposition into xylose and xylooligosaccharides. Fermentation in a bench-scale bioreactor followed by supernatant lyophilisation was a successful tool to increase enzyme production and concentrate enzymatic activity. The larger scale-up parameterisation and the design of xylanolytic cocktails are some of the further objectives that emerge from this research. Thorough study of the molecular mechanisms involved in the activity could also be useful strategy to understand and improve the (hemi)cellulolytic capacity of the strains.

EC and OO are Research Career members from CONICET, JT and MG hold Doctoral fellowships from CONICET and are PhD students from the FBMC and QB Departments of FCEN, University of Buenos Aires (UBA).

**Availability of data and materials** The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD022718 (available upon publication). Strain *Cellulomonas* sp. B6 has been deposited in public collections DSMZ and NCIMB, as DSM 107934 and NCIMB 15124, respectively.

**Code availability** Not applicable

**Authors’ contributions** OMO and SB conducted experiments, analysed the data and wrote the manuscript. SG, MMG, JT, DJ and AF conducted experiments. PV was responsible for proteomic analysis. EC and CF conceived and designed the research, were responsible for funding acquisition and corrected the manuscript. All authors read, corrected and approved the manuscript.

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**Declarations**

**Ethics approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Consent to participate** Not applicable

**Consent for publication** Not applicable

**Conflict of interest** The authors declare they have no conflict of interest.

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