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Cellulomonas fimi secretomes: *In vivo* and *in silico* approaches for the lignocellulose bioconversion

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ABSTRACT

Lignocellulose degradation is a challenging step for value added products and biofuels production. *Cellulomonas fimi* secretes complex mixtures of carbohydrate active enzymes (CAZymes) which synergistically degrade cellulose and hemicelluloses. Their characterization may provide new insights for enzymatic cocktails implementation. Bioinformatic analysis highlighted 1127 secreted proteins, constituting the *in silico* secretome, graphically represented in a 2DE map. According to Blast2GO functional annotation, many of these are involved in carbohydrates metabolism. *In vivo* secretomes were obtained, growing *C. fimi* on glucose, CMC or wheat straw for 24 h. Zymography revealed degradative activity on carbohydrates and proteomic analysis identified some CAZymes, only in secretomes obtained with CMC and wheat straw. An interaction between cellobiohydrolases is proposed as a strategy adopted by soluble multimodular cellulases. Such approach can be crucial for a better characterization and industrial exploitation of the synergism among *C. fimi* enzymes.

1. Introduction

Lignocellulosic biomass (LCB) represents about the 50% of world biomass; since it is a relatively inexpensive and not edible raw-material that holds a high energetic content, LCB is considered an important resource for value-added products production (Sanchez and Cardona, 2008; Zhang, 2008). Nowadays, fossil fuels depletion represents an important issue that can be faced with the widespread consumption of biofuels. Using LCB as feedstock for production of bioethanol, it can be reached a significant reduction of gas emissions (Valdivia et al., 2016; Brehmer et al., 2009) and an increase of economic profits, due to low-cost raw-materials (Balat et al., 2008). In general, LCB consists primarily of cellulose, hemicelluloses and lignin, which are the integral part of cell wall in plant tissues (Li et al., 2014). Cellulose and hemicelluloses are the hydrolysable structural polymers of the cell wall and the main sources of fermentable sugars utilized for bioethanol production (Kang et al., 2014). The conversion of LCB into bioethanol consists basically in the following steps: (1) pretreatment of LCB; (2) hydrolysis of cellulose and hemicelluloses to fermentable sugars; (3) fermentation of hexose and pentose sugars; (4) recovery and purification of ethanol to meet final specifications. The hydrolysis phase (saccharification) is one of the most important steps and it is technically difficult to perform due to the poor accessibility of cellulose caused by many physical, chemical and structural factors, also known as biomass recalcitrance (Himmel et al., 2007). Thus, it is an energy consuming step, contributing substantially to the economic costs of the process and is a subject of many research works (Alvira et al., 2010; Sannigrahi et al., 2010).

Bacteria and fungi have evolved complex enzymatic systems for LCB degradation. Cellulases and hemicellulases hydrolyze glycosidic bonds in polysaccharides generating small oligosaccharides and fermentable monomers (Sharma et al., 2016; Bhattacharya et al., 2015). In addition to hydrolytic enzymes, recently discovered lytic polysaccharide monooxigenases (LPMO) and “amorphogenesis-inducing” or “disrupting” proteins cooperate for cellulose degradation (Vaaje-Kolstad et al., 2017; Georgelis et al., 2015). *Cellulomonas fimi* is a Gram positive mesophilic soil bacterium and its genome has been sequenced (Christopherson et al., 2013). In this study we conducted an *in silico* secretome analysis through the use of two different secretion prediction tools (SignalP and SecretomeP). The proteins identified as secreted were used to elaborate an *in silico* 2DE map and functionally annotated by the bioinformatic tool Blast2GO. Moreover, *in vivo* secretomes were obtained and proteomic and biochemical analysis were performed to evaluate the expression of extracellular proteins by *C. fimi* and their degrading activities on carbohydrates were assayed. In particular, three different secretomes were obtained growing *C. fimi* on glucose, CMC or wheat

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straw as lignocellulosic biomass.

2. Materials and methods

2.1. Bioinformatic analysis

Cellulomonas fimi ATCC 484 protein sequences were downloaded from UNIPROT database (www.uniprot.org) and submitted to two different secretion prediction software: SignalP 4.1 (Petersen et al., 2011) and SecretomeP 2.0 (Bendtsen et al., 2005). Secreted proteins were grouped in three different categories: only predicted by SignalP 4.1, only predicted by SecretomeP 2.0 and commons (predicted by both the tools). Each category was then analyzed with Blast2GO (http://www.blast2go.com/b2ghome) for Gene Ontology (GO) annotation using the standard parameters (Conesa et al., 2005). Data of biological process and molecular function were presented with pie charts. For each predicted protein, theoretical mass weight (Mw) and isoelectric point (pI) were calculated by Compute pI/Mw tool (www.expasy.org). Protein ID of *C. fimi* CAZymes were downloaded from the CAZy database (www.cazy.org) (Levasseur et al., 2013) and finally, *in silico* 2DE maps of the total secretome and of the CAZymes were plotted with Microsoft Excel.

2.2. Bacterial strain, culture conditions and secretomes production

Frozen strains of *C. fimi* ATCC 484 were streaked on Nutrient Agar plates (beef extract 3 g L^{-1}, peptone 5 g L^{-1}, agar 20 g L^{-1}, pH 6.8) at 30 °C for 48 h. Cells were inoculated in flasks containing 100 mL of Broth Medium (BD cat 234000) and incubated at 30 °C with shaking (150 rpm) until reaching OD_{600} ~0.7 (24 h of growth). The pre-culture was then inoculated in Mineral Medium ($NaNO_3$ 1 g L^{-1}, K_2HPO_4 1 g L^{-1}, KCl 0.5 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.5 g L^{-1}, yeast extract 0.5 g L^{-1}, pH 7) obtaining a 2% (v/v, volume of pre-culture/ total volume) final concentration. As unique carbon source was added 0.1% (w/v) of one of the following substrates: glucose (Sigma-Aldrich Co.), medium viscosity carboxymethyl-cellulose (CMC) (Sigma-Aldrich Co.) or steam-explosion pretreated wheat straw. The cultures were then incubated at 30 °C, 150 rpm for 24 h. The samples were centrifuged at 15,000× *g* for 30 min at 4 °C and the supernatants were collected. These secretomes were filtered with 0.22 µm PES filters (Sigma-Aldrich Co.), concentrated using Vivaspin 20 (Sartorius Stedim Biotech GmbH, Goettingen, Germany) with molecular weight cutoff 10 kDa, centrifuging at 5400× *g* at 4 °C in a swinging-bucket rotor (Allegra 25-R, Beckman Coulter Inc.) and used for zymography and proteomic analysis. The protein content of each sample was estimated by FluoroProfile Protein Quantification Kit (Sigma-Aldrich Co.). Further, to evaluate the influence of biomass amount on CAZymes production, different percentages of steam-explosion pretreated wheat straw were tested (0.1%, 1% or 4% (w/v)).

2.3. Zymographic analysis

In-gel activities of cellulases and xylanases were detected separating 10 µg of each protein sample with 10% SDS-PAGE added with 0.4% AZO-CMC (Sigma-Aldrich Co.) or 0.1% Remazol Brilliant Blue R-D-Xylan (Sigma-Aldrich Co.) respectively, as described by Cattaneo et al. (2014).

2.4. Sample preparation for 2DE

Extracellular proteins were precipitated by mixing 10 mL of secretomes with 1 mL of 100% trichloroacetic acid (TCA) and incubated at 4 °C overnight (Sánchez-Herrera et al., 2007). The precipitates were collected by centrifugation at 5400× *g* for 15 min at 4 °C and washed twice with ice-cold 80% (v/v) acetone. The protein pellets were dried for 10 min under vacuum and re-suspended in solubilisation buffer (7 M Urea, 2 M Thiourea, 4% w/v CHAPS, 50 mM DTT). Only the pellet obtained from wheat straw secretome was subjected to phenol extraction (Mangiapane et al., 2014) before being re-suspended in solubilisation buffer.

2.5. 2DE

The bidimensional electrophoresis was performed as previously described by Spertino et al. (2012). Briefly, first dimension isoelectric focusing (IEF) was conducted using 7 cm immobilized linear pH range 4–7 and 3–10 strips (GE Healthcare, Milan, Italy), on an IPG-Phor unit (GE Healthcare Bio-Sciences); the protein samples were mixed with a rehydration buffer (7 M Urea, 2 M Thiourea, 4% w/v CHAPS, 50 mM DTT, 5% Triton X100, 5% 4–7 IPG Buffer or 3–10 IPG Buffer (GE Healthcare), and traces of Bromophenol Blue (BBF)) and the final volume was adjusted to 125 µL with solubilisation buffer. For the second dimension, the focused strips were first reduced in SDS equilibration buffer (50 mM Tris–HCl pH 8.8, 6 M Urea, 30% v/v Glycerol, 2% w/v SDS) containing 10 mg mL^{-1} DTT for 15 min, rinsed with distilled water and then alkylated in SDS equilibration buffer with 45 mg mL^{-1} Iodoacetamide for 15 min. Finally, a 10% SDS-PAGE was performed and 2DE gels were stained with Colloidal Coomassie Brilliant Blue G250 (Bio-Rad Laboratories) in accordance with Neuhoff et al. (1988).

2.6. In-gel digestion and protein identification

The protein spots of interest were manually excised from 2DE gels and trypsin digested as described by Boatti et al. (2012) for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis.

LC-MS/MS analyses were performed by a micro-LC Eksigent Technologies (Dublin, USA) system, as described by Manfredi et al. (2016). The mass spectrometer worked in information dependent acquisition (IDA) mode. MS data were acquired with Analyst TF 1.7 (AB SCIEX, Concord, Canada). The mass spectrometry files were searched using Mascot v. 2.4 (Matrix Science Inc., Boston, MA, USA). Trypsin as digestion enzyme and 2 missed cleavages were specified. The instrument was set to ESI-QUAD-TOF and the following modifications were specified for the search: carbamidomethylated cysteins, oxidized methionines and deamidated asparagines as variable modifications. A search tolerance of 0.6 Da was specified for the peptide mass tolerance, and 100 ppm for the MS/MS tolerance. The charges of the peptides to search for were set to 2 + , 3 + and 4 + , and the search was set on monoisotopic mass. To perform the protein identification using Mascot, we have created an in house database downloading all the protein fasta sequences of *C.fimi* from http://www.ncbi.nlm.nih.gov.

2.7. Reducing sugars evaluation in wheat straw added media

In order to estimate the amount of soluble sugars released from wheat straw in the growth media, reducing sugars assay was conducted according to Nelson-Somogyi method (Nelson, 1944). Briefly, several percentages (0.1%, 1% and 4%) of pretreated wheat straw were added to Mineral Medium and autoclavated. After centrifugation at 15,000× *g* for 30 min, aliquots of surnatant were analyzed.

3. Results and discussion

3.1. In silico secretome and Gene Ontology annotation

The *in silico* analysis permitted to identify the whole secreted proteome of *Cellulomonas fimi* ATCC 484. For a more detailed evaluation, two prediction tools were used as different secretion pathways were considered. In fact, only some proteins are secreted employing signal peptide pathway. *C. fimi* non redundant protein sequences were downloaded from UniProt database and amounted to 3784. Downloaded sequences were submitted to SignalP 4.1 and SecretomeP