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## Clostridium cellulovorans metabolism of cellulose as studied by comparative proteomic approach

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Abstract: Clostridium cellulovorans is among the most promising candidates for consolidated bioprocessing (CBP) of cellulosic biomass to liquid biofuels (ethanol, butanol). C. cellulovorans can metabolize all the main plant polysaccharides and its main catabolite is butyrate. This makes this strain a potential butanol producer since most reactions of butyrate and butanol biosynthesis from acetyl-CoA are common. Recent studies demonstrated that introduction of a single heterologous alcohol/aldehyde dehydrogenase diverts the branching-point intermediate, i.e. butyryl-CoA, towards butanol production in this strain. Despite C. cellulovorans potential for CBP of plant biomass, engineering its metabolic pathways towards industrial utilization requires better understanding of its metabolism. The present study aimed at improving comprehension of cellulose metabolism in C. cellulovorans by comparing growth kinetics, substrate consumption/product accumulation and whole-cell soluble proteome (data available via ProteomeXchange, identifier PXD015487) with those of the same strain grown on a soluble carbohydrate, glucose, as the main carbon source. Modulations of the central carbon metabolism in response to different growth substrate were detected, including regulation of glycolytic enzymes, fermentation pathways and nitrogen assimilation possibly affecting the redox balance. Higher energy expenditure seems to occur in cellulose-grown C. cellulovorans, which induces up-regulation of ATP synthetic pathways, e.g. acetate production and ATP synthase.

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## Cover letter

Dear Editor,

please find attached the manuscript entitled “*Clostridium cellulovorans* metabolism of cellulose as studied by comparative proteomic approach”. *C. cellulovorans* is among the most attractive candidates for direct fermentation of lignocellulosic biomass to industrially relevant chemicals, particularly, biofuels. Recently, metabolic engineering has enabled butanol production in this strain, which is a milestone in one-step production of this biofuel from plant biomass. However, currently scarce information on the central carbon metabolism of *C. cellulovorans* hampers further implementation of metabolic engineering strategies towards application of this strain in industrial processes. The present study aimed at improving understanding of cellulose metabolism in *C. cellulovorans*. Growth kinetics, substrate consumption, catabolite accumulation and whole-cell soluble proteome of cellulose-grown cells were compared with those of glucose-grown cells. This analysis showed specific modulations of the central carbon metabolism in response to changes in the growth substrate, including modifications in the redox and energy balance. We think that the results of the present study will help better understanding of *C. cellulovorans* physiology. Furthermore, these data could be useful to determine key genes and possible metabolic bottlenecks to be addressed towards improved metabolic engineering of *C. cellulovorans*.

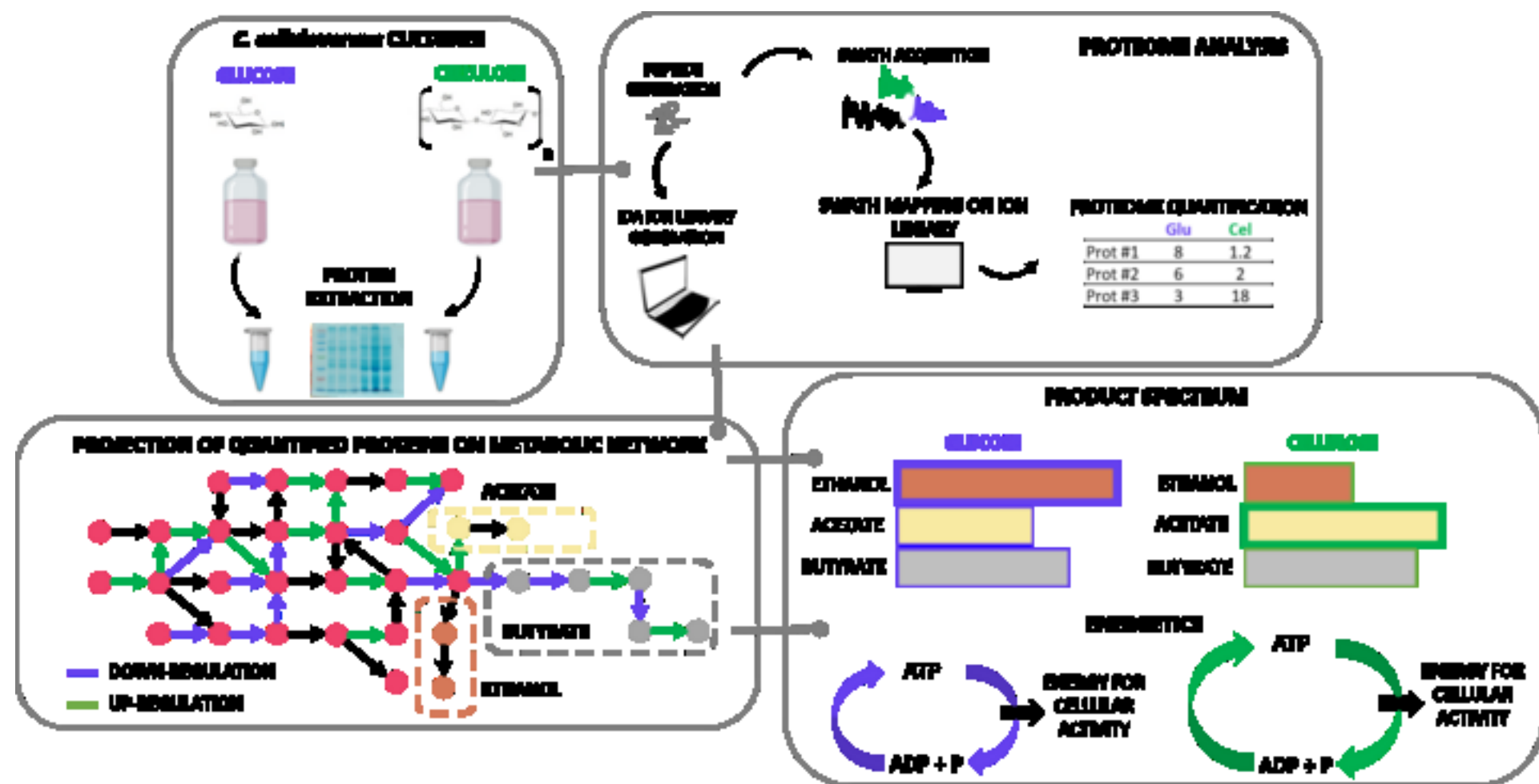
We hope that the subject and of the present manuscript will be of interest for publication in Journal of Proteomics. We look forward to receive your answer and we send you our best regards.

Sincerely yours,

Roberto Mazzoli

## Significance

*C. cellulovorans* can metabolize all the main plant polysaccharides (cellulose, hemicelluloses and pectins) and, unlike other well established cellulolytic microorganisms, can produce butyrate. *C. cellulovorans* is therefore among the most attractive candidates for direct fermentation of lignocellulose to high-value chemicals and, especially, n-butanol, i.e. one of the most promising liquid biofuels for the future. Recent studies aimed at engineering n-butanol production in *C. cellulovorans* represent milestones towards production of biofuels through one-step fermentation of lignocellulose but also indicated that more detailed understanding of the *C. cellulovorans* central carbon metabolism is essential to refine metabolic engineering strategies towards improved n-butanol production in this strain. The present study helped identifying key genes associated with specific catabolic reactions and indicated modulations of central carbon metabolism (including redox and energy balance) associated with cellulose consumption. This information will be useful to determine key enzymes and possible metabolic bottlenecks to be addressed towards improved metabolic engineering of this strain.



## Highlights

- Whole-cell soluble proteome of cellulose- and glucose-grown *C. cellulovorans*
- Cellulose-grown cells produce higher amount of acetate and lower amount of ethanol
- Modulation of glycolysis, fermentative pathways, nitrogen assimilation was detected
- Cellulose induces up-regulation of ATP biosynthetic pathways
- Cellulose-grown cells show lower intracellular ATP content

1 ***Clostridium cellulovorans* metabolism of cellulose as studied by**  
2 **comparative proteomic approach**

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25

26 **Key words:** ATP, acetate, ethanol, Alcohol dehydrogenase, pyruvate phosphate dikinase, glucose

27

28 **Running title:** Cellulose metabolism in *C. cellulovorans*

29 **Abbreviations:**

**ABC**                      ATP binding cassette

**ACAT**                    Acetyl-CoA acetyltransferase

**ACK**                     Acetate kinase

**ADH**                     Alcohol dehydrogenase

**BCD**                     Butyryl-CoA dehydrogenase

**BUK**                     Butyrate kinase

**CAZY**                   Carbohydrate active enzyme

**CBP**                     Consolidated bioprocessing

**CoA**                     Coenzyme A

<b>COGs</b>	Cluster of Orthologous Genes
<b>DSMZ</b>	German Collection of Microorganisms and Cell Cultures
<b>DTT</b>	Dithiothreitol
<b>ECH</b>	Enoyl-CoA hydratase
<b>F1,6BP</b>	Fructose 1,6-bisphosphate
<b>F6P</b>	Fructose-6-phosphate
<b>FDR</b>	False discovery rate
<b>GDH</b>	Glutamate dehydrogenase
<b>GlnS</b>	Glutamine synthase
<b>GluS</b>	Glutamate synthase
<b>HBD</b>	Hydroxybutyryl-CoA dehydrogenase
<b>MDH</b>	Malate dehydrogenase
<b>ME</b>	Malic enzyme
<b>PA</b>	Pyruvic acid

<b>PEP</b>	Phosphoenolpyruvate
<b>PEPC</b>	Phosphoenolpyruvate carboxylase
<b>PEPCK</b>	Phosphoenolpyruvate carboxykinase
<b>PEPS</b>	Phosphoenolpyruvate synthetase
<b>PFL</b>	Pyruvate formate lyase
<b>PFOR</b>	Pyruvate ferredoxin oxidoreductase
<b>PK</b>	Pyruvate kinase
<b>PP<sub>i</sub></b>	Pyrophosphate
<b>PPDK</b>	Pyruvate phosphate dikinase
<b>PTA</b>	Phosphate acetyltransferase
<b>PTB</b>	Phosphate butyryltransferase
<b>PTS</b>	Phosphotransferase system
<b>SWATH-MS</b>	Sequential Window Acquisition of All Theoretical Mass Spectra
<b>TCA</b>	Tricarboxylic acid



## 31    **Abstract**

32    *Clostridium cellulovorans* is among the most promising candidates for consolidated bioprocessing  
33    (CBP) of cellulosic biomass to liquid biofuels (ethanol, butanol). *C. cellulovorans* can metabolize all  
34    the main plant polysaccharides and its main catabolite is butyrate. This makes this strain a potential  
35    butanol producer since most reactions of butyrate and butanol biosynthesis from acetyl-CoA are  
36    common. Recent studies demonstrated that introduction of a single heterologous alcohol/aldehyde  
37    dehydrogenase diverts the branching-point intermediate, i.e. butyryl-CoA, towards butanol production  
38    in this strain. Despite *C. cellulovorans* potential for CBP of plant biomass, engineering its metabolic  
39    pathways towards industrial utilization requires better understanding of its metabolism. The present  
40    study aimed at improving comprehension of cellulose metabolism in *C. cellulovorans* by comparing  
41    growth kinetics, substrate consumption/product accumulation and whole-cell soluble proteome (data  
42    available via ProteomeXchange, identifier PXD015487) with those of the same strain grown on a  
43    soluble carbohydrate, glucose, as the main carbon source. Modulations of the central carbon  
44    metabolism in response to different growth substrate were detected, including regulation of glycolytic  
45    enzymes, fermentation pathways and nitrogen assimilation possibly affecting the redox balance. Higher  
46    energy expenditure seems to occur in cellulose-grown *C. cellulovorans*, which induces up-regulation of  
47    ATP synthetic pathways, e.g. acetate production and ATP synthase.

48

## 49    **Significance**

50    *C. cellulovorans* can metabolize all the main plant polysaccharides (cellulose, hemicelluloses and  
51    pectins) and, unlike other well established cellulolytic microorganisms, can produce butyrate. *C.*  
52    *cellulovorans* is therefore among the most attractive candidates for direct fermentation of lignocellulose  
53    to high-value chemicals and, especially, n-butanol, i.e. one of the most promising liquid biofuels for the  
54    future. Recent studies aimed at engineering n-butanol production in *C. cellulovorans* represent  
55    milestones towards production of biofuels through one-step fermentation of lignocellulose but also

56 indicated that more detailed understanding of the *C. cellulovorans* central carbon metabolism is  
57 essential to refine metabolic engineering strategies towards improved n-butanol production in this  
58 strain. The present study helped identifying key genes associated with specific catabolic reactions and  
59 indicated modulations of central carbon metabolism (including redox and energy balance) associated  
60 with cellulose consumption. This information will be useful to determine key enzymes and possible  
61 metabolic bottlenecks to be addressed towards improved metabolic engineering of this strain.

62

63

## 64    **Introduction**

65    Current awareness of the effects that fossil fuel exploitation brings about on global warming and  
66    climate changes has prompted search for alternative energy sources with lower environmental footprint  
67    [1]. Bio-based processes, such as biorefineries, have been especially promoted because of their  
68    potential significant benefits to environmental, economic and societal sustainability issues [2].  
69    Lignocellulosic biomass is the most abundant raw material on the Earth, hence, it is among the most  
70    promising feedstock for the so-called 2<sup>nd</sup> generation biorefineries. Unlike 1<sup>st</sup> generation biorefineries,  
71    these processes rely on non-food biomass, such as agricultural/land by-products (e.g. cereal straw,  
72    forest residues), municipal or industrial wastes (e.g. paper mill sludge) [3]. However, lignocellulose is  
73    very recalcitrant to biodegradation and its bioconversion currently requires physical/chemical or  
74    enzymatic pre-treatment to improve its accessibility to enzymes and fermenting microorganisms [4,5].  
75    Moreover, multiple bioreactors in series are necessary for enzyme production and/or polysaccharide  
76    hydrolysis and/or fermentation of soluble sugar(s) [2]. Development of consolidated bioprocessing  
77    (CBP) of lignocellulose, that is single-pot fermentation to high-value chemicals, could significantly  
78    reduce current costs of lignocellulose fermentation [6,7]. Since natural microorganisms isolated so far  
79    do not possess the biochemical features enabling CBP, metabolic engineering has been employed to  
80    develop improved microbial strains [7–10]. Candidates for CBP include both cellulolytic  
81    microorganisms such as *Clostridium thermocellum* and *Clostridium cellulolyticum* [9] and high-value  
82    compound producing yeasts and bacteria (e.g. *Saccharomyces cerevisiae*, lactic acid bacteria) [7,8].

83        *C. cellulovorans* is a strict anaerobic, mesophilic bacterium [11] among the most interesting  
84    candidates for CBP of plant biomass. *C. cellulovorans* shows some advantageous metabolic features  
85    with respect to other well established cellulolytic microorganisms such as *C. thermocellum*, *C.*  
86    *cellulolyticum* or *Thermoanaerobacterium saccharolyticum*: i) it ferments a larger panel of substrates  
87    that include all the main plant polysaccharides, namely cellulose, hemicelluloses and pectins [12,13];  
88    ii) it produces butyryl-CoA, which is a key intermediate for n-butanol production [11]. n-butanol is

89 considered the most promising liquid biofuel for future use in transportation [14]. With respect to  
90 ethanol, butanol has higher combustion energy and can be used in pure form in engines (while ethanol  
91 must be blended with gasoline) [15].

92         However, so far, research on *C. cellulovorans* has been mainly focused on its enzyme system  
93 for depolymerizing plant polysaccharides. The latter features dozens of carbohydrate active enzymes  
94 (CAZys) including both glycosyl hydrolases, carbohydrate esterases and polysaccharide lyases [16].  
95 Remarkably, the number of CAZys encoded by *C. cellulovorans* genome is 37% higher than that found  
96 in *C. thermocellum* (one of the most efficient cellulolytic microorganisms isolated so far) [8]. Several  
97 of these enzymes, that is those containing a dockerin domain, are physically associated to form huge  
98 protein complexes called cellulosomes, which are tethered to the *C. cellulovorans* cell surface [17].  
99 Several studies have shown that the expression of *C. cellulovorans* cellulosomal and non-cellulosomal  
100 CAZYs is modulated by the available growth substrate(s) [18–21].

101         Recently, gene tools for the manipulation of *C. cellulovorans* have been developed [22–24].  
102 This led to improvement of production of liquid biofuels, namely ethanol and n-butanol, in *C.*  
103 *cellulovorans* by metabolic engineering [25,26]. These studies have been milestones towards n-butanol  
104 production by CBP of plant biomass, using a single microorganism. However, the n-butanol titer  
105 obtained by these investigations (i.e. 3.47 g/L) is insufficient for industrial application and further  
106 optimization of these strains is necessary [25,27]. Improvements of gene systems for *C. cellulovorans*  
107 manipulation are desirable to increase transformation efficiency and success rate of gene modification  
108 attempts [24,27]. Moreover, detailed understanding of *C. cellulovorans* central carbon metabolism is  
109 essential to identify key genes and possible metabolic bottlenecks that should be addressed by  
110 metabolic engineering strategies. As far as we know, only two previous studies have focused on  
111 intracellular proteins involved in the metabolism of cellulose or other plant polysaccharides in *C.*  
112 *cellulovorans* [12,13] which seem insufficient for the aforementioned purposes.



113 In the present study, a comparative approach was applied to *C. cellulovorans* cells grown with  
114 either crystalline cellulose (i.e. avicel) or a soluble carbohydrate, namely glucose, as the main carbon  
115 source. Previous studies on other cellulolytic clostridia have shown that overall metabolism on  
116 cellulose (and other polysaccharides) may highly differ from that observed on soluble mono- /di-  
117 saccharides, because of different kinetics and energetics of complex versus simple carbohydrate  
118 metabolism [2]. To gauge the global effects caused by cellulose utilization, we analyzed *C.*  
119 *cellulovorans* growth kinetics and substrate consumption/metabolite accumulation in conjunction with  
120 label-free quantitative proteomics.

## 121 **Materials and Methods**

### 122 GROWTH CONDITIONS

123 *Clostridium cellulovorans* was grown anaerobically in the DSMZ medium 320  
124 ([https://www.dsmz.de/microorganisms/medium/pdf/DSMZ\\_Medium320.pdf](https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium320.pdf)), with some  
125 modifications. Trypticase peptone and rumen fluid were not supplemented because not required for  
126 growth [11]. Na<sub>2</sub>CO<sub>3</sub> was replaced by PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid)] (15.12 g/l)  
127 as pH buffering agent [11]. Media were purged with pure N<sub>2</sub> (instead of 80:20 N<sub>2</sub>-CO<sub>2</sub> mixture) and  
128 sterilized by autoclaving (20 min 121°C). Either 5 g/l D-glucose or 10 g/l avicel® PH-101  
129 microcrystalline cellulose (50 µm particle size; Sigma-Aldrich Inc., St. Louis, MO, USA) were  
130 supplemented as the main carbon source. Inocula were grown in glucose-supplemented medium until  
131 exponential growth phase and then transferred into 500 ml butyl-stoppered bottles containing glucose-  
132 or avicel-supplemented medium. Cultures were incubated at 37°C without agitation. For each growth  
133 condition three independent cultures were performed.

134

### 135 ESTIMATION OF BACTERIAL GROWTH

136 Microbial growth was monitored at regular time intervals though estimation of total protein content.  
137 For protein extraction, 15 ml of culture was collected by centrifugation (4000 xg, 4°C, 20 min) and  
138 washed twice with 0.9% (w/v) NaCl. The cell pellet was re-suspended in 1 ml of 0.2 M NaOH and  
139 incubated 10 min at 100°C. Protein samples were quantified by using the Bradford reagent (Sigma-  
140 Aldrich Inc., St. Louis, MO, USA) following the manufacturer's instruction. Bovine serum albumin  
141 was used as the standard.

142

#### 143 SUBSTRATE CONSUMPTION DETERMINATION

144 Glucose concentration in cell free supernatants was measured hourly using the glucose oxidase-  
145 peroxidase kit (K-GLUC, gopod format, Megazyme International, Bray, Ireland), following the  
146 manufacturer's instructions.

147 To determinate cellulose consumption, the cellulose concentration was measured from cell pellet every  
148 three days. Briefly, after two washing steps with 0.9% (w/v) NaCl, the pellet was re-suspended in 67%  
149 (v/v) H<sub>2</sub>SO<sub>4</sub> and incubated for one hour under stirring at room temperature, to promote cellulose  
150 hydrolysis. The total carbohydrate quantification was performed with the phenol-sulfuric acid method  
151 [28], using glucose for the standard curve.

152

#### 153 END-PRODUCT AND INTRACELLULAR ATP QUANTIFICATION

154 Accumulation of acetic acid, lactic acid, formic acid and butyric acid in the growth medium was  
155 quantified by high-pressure liquid chromatography (HPLC; Agilent Technologies 1200 series),  
156 equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA), a Micro-guard (cation H;  
157 Bio-Rad, Hercules, CA, USA) and a UV-Vis detector set at 210 nm. The mobile phase was 5 mM  
158 H<sub>2</sub>SO<sub>4</sub> at a flux of 0.5 ml/min and a temperature of 50°C.

159 Ethanol in the growth medium was quantified by the Ethanol assay kit (K-EtOH, Megazyme  
160 International, Bray, Ireland), following the manufacturer's instructions.

161 For quantification of total intracellular ATP content, 1 ml of culture was collected and centrifuged at  
162 10000 xg for 10 min. Pellets were resuspended in 200 µl of PBS, lysed with CellTiter-Glo® One  
163 Solution (Promega Corporation, Madison, WI, USA), and ATP content was measured by following the  
164 manufacturer's instructions.

165

## 166 PROTEOMIC ANALYSIS

### 167 *Cytosolic protein extraction*

168 Biomass samples were harvested (4000 xg, 4°C, 20 min) 5 hours and 7 days after inoculum from  
169 glucose- and avicel-supplemented cultures, respectively, and washed twice with 0.9% (w/v) NaCl.  
170 Protein were extracted according to Munir *et al.*, 2015 [29]. Briefly, the pellets were resuspended in 6  
171 ml of 2% (v/v) SDT-lysis buffer (2 mM Tris-HCl, 0.4% (w/v) SDS, pH 7.6), with 100 mM DTT. The  
172 samples were incubated 10 min at 95°C and centrifuged (4000 xg, 20 min). The supernatant was  
173 centrifuged again (10 min, 10000 xg) to discard unlysed cells and cell debris. Proteins were  
174 precipitated from supernatants by methanol-chloroform method [30]. The protein pellets were  
175 resuspended in 25 mM NH<sub>4</sub>HCO<sub>3</sub> with 0.1% (w/v) SDS and the protein concentration was measured by  
176 the 2-D Quant kit (GE Healthcare, Chicago, IL, USA), following the manufacturer's instructions.

### 177 *In-solution protein digestion*

178 Prior to SWATH-MS (Sequential Window Acquisition of all Theoretical fragment ion spectra Mass  
179 Spectrometry) [31,32] analysis, proteins were digested with trypsin. Briefly, the samples were prepared  
180 to have 100 µg of proteins in 25 µl of 100 mM NH<sub>4</sub>HCO<sub>3</sub>. The proteins were reduced by adding 2.5 µl  
181 of 200 mM DTT and incubating them at 90°C for 20 min, and alkylated with 10 µl of 200 mM  
182 iodoacetamide for 1 h at room temperature in the dark. Excess of iodoacetamide was finally removed  
183 by 200 mM DTT. After dilution with 300 µl of water and 100 µl of NH<sub>4</sub>HCO<sub>3</sub> to raise the pH to 7.5-  
184 8.0, 5 µg of trypsin (Sequence Grade, Promega Corporation, Madison, WI, USA) was added and

185 digestion was performed overnight at 37 °C. Trypsin activity was stopped by adding 2 µl of neat formic  
186 acid and digests were dried by Speed Vacuum [33]. The samples were desalted on the Discovery®  
187 DSC-18 solid phase extraction (SPE) 96-well Plate (25 mg/well) (Sigma-Aldrich Inc., St. Louis, MO,  
188 USA) and then analyzed as previously described [34].

#### 189 *SWATH-MS analysis*

190 LC–MS/MS analyses were performed using a micro-LC Eksigent Technologies (Dublin, OH, USA)  
191 system with a stationary phase of a Halo Fused C18 column (0.5 × 100 mm, 2.7 µm; Eksigent  
192 Technologies, Dublin, OH, USA). The mobile phase was a mixture of 0.1% (v/v) formic acid in water  
193 (A) and 0.1% (v/v) formic acid in acetonitrile (B), eluting at a flow-rate of 15.0 µl/min at an increasing  
194 concentration of solvent B from 2% to 40% in 30 min. The LC system was interfaced with a 5600+  
195 TripleTOF system (SCIEX, Concord, Canada). Samples used to generate the SWATH-MS spectral  
196 library were subjected to the traditional data-dependent acquisition (DDA) and to cyclic data  
197 independent analysis (DIA) of the mass spectra, using a 25-Da window as reported elsewhere [35]. The  
198 MS data were acquired with Analyst TF 1.7 (SCIEX, Concord, Canada). Three instrumental replicates  
199 for each sample were subjected to the DIA analysis [36].

#### 200 *Protein data search*

201 The MS files were searched using Protein Pilot v. 4.2 (SCIEX, Concord, Canada) with the following  
202 parameters: cysteine alkylation, digestion by trypsin, no special factors and False Discovery Rate at  
203 1%; The files were search also with Mascot v. 2.4 (Matrix Science Inc., Boston, USA) using trypsin as  
204 enzyme, with 2 missed cleavages and a search tolerance of 50 ppm was specified for the peptide mass  
205 tolerance, and 0.1 Da for the MS/MS tolerance, charges of the peptides to search for were set to 2 +, 3  
206 + and 4 +, and the search was set on monoisotopic mass. The instrument was set to ESI-QUAD-TOF  
207 and the following modifications were specified for the search: carbamidomethyl cysteines as fixed  
208 modification and oxidized methionine as variable modification. The UniProt/Swiss-Prot reviewed

209 database containing *C. cellulovorans* proteins (NCBI\_Clostridium\_cellulovorans743B, version  
210 30102017, 4278 sequence entries) was used.

#### 211 *Protein quantification*

212 The label-free quantification was performed by integrating the extracted ion chromatogram of all the  
213 unique ions for a given peptide using PeakView 2.0 and MarkerView 1.2. (Sciex, Concord, ON,  
214 Canada). SwathXtend was employed to build an integrated assay library, built with the DDA  
215 acquisitions, using a protein FDR threshold of 1 %. Six peptides per protein and six transitions per  
216 peptide were extracted from the SWATH files. Shared peptides were excluded as well as peptides with  
217 modifications. Peptides with FDR lower than 1 % were exported in MarkerView for the t-test. The up-  
218 and down-regulated proteins were selected using p-value < 0.05 and fold change > 1.5.

219 The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via  
220 the PRIDE [37] partner repository (<https://www.ebi.ac.uk/pride/archive/>) with the dataset identifier  
221 PXD015487.

#### 222 *Protein classification and statistical analysis*

223 For each primary annotations of proteins, files were downloaded from the National Center for  
224 Biotechnology Information resource  
225 ([ftp://ftp.ncbi.nlm.nih.gov/genomes/refseq/bacteria/Clostridium\\_cellulovorans/](ftp://ftp.ncbi.nlm.nih.gov/genomes/refseq/bacteria/Clostridium_cellulovorans/)), the PATRIC Bacterial  
226 Bioinformatics Resource Center (<https://www.patricbrc.org>) and the eggNOG database of orthologous  
227 groups and functional annotation (<http://eggnogdb.embl.de/#/app/home>). The eggNOG resource  
228 provides Clusters of Orthologous Groups (COGs) of proteins  
229 (<https://www.ncbi.nlm.nih.gov/pubmed/9381173>), which represent a framework for functional protein  
230 classification on the basis of accurately deciphered evolutionary relationships  
231 (<https://www.ncbi.nlm.nih.gov/pubmed/26582926>). Protein-level annotations overall *C. cellulovorans*  
232 proteins are detailed in the **Supplementary File 1**. Fisher's exact test and fold enrichment were used to

233 identify significantly overrepresented COG categories in the up- and down-regulated proteins  
234 compared to the overall quantified proteins. Multiple testing adjustment for p-values derived from the  
235 Fisher's exact test was carried out using the Benjamini-Hochberg method.  
236 For predicting both signal peptides and subcellular localization of the proteins involved in  
237 lignocellulose depolymerization Signal-P 4.1 (cut-off > 0.45) [38]  
238 (<http://www.cbs.dtu.dk/services/SignalP-4.1/>) and PSORTb v.3.0 [39] (<https://www.psorth.org/psorth/>)  
239 were used, respectively.

240

## 241 Results and discussion

### 242 GROWTH CHARACTERISTICS AND FERMENTATION PRODUCTS

243 *C. cellulovorans* growth and substrate consumption in cultures with 5 g/l D-glucose and 10 g/l  
244 avicel (microcrystalline cellulose) are shown in **Fig. 1A, B**. Glucose supported around 16-fold higher  
245 growth rate ( $\mu = 0.39 \text{ h}^{-1}$ ) with respect to cellulose ( $\mu = 0.025 \text{ h}^{-1}$ ). These observations essentially  
246 confirm previous reports on *C. cellulovorans* [13,22]. The supplied substrates were only partially  
247 consumed, that is 3.1 g/l of cellulose (in 28 days) and 1.4 g/l of glucose (in 9 hours) were consumed in  
248 avicel- and glucose-supplemented cultures, respectively. Therefore, factors other than carbon substrate  
249 depletion (*e.g.* metabolite accumulation, pH) determined growth arrest. Actually, complete  
250 consumption of glucose (about 10 g/l in about 4 days) and higher consumption of cellulose (about 7 g/l  
251 in 10 days) by *C. cellulovorans* was previously reported by maintaining the pH of the culture between  
252 6.0 and 7.0 [22] while no pH regulation was used in the present study.

253 All the main fermentation products, that is acetic acid, butyric acid, ethanol, formic acid and  
254 lactic acid (**Fig. 1C, Supplementary Figure 1**) were produced at higher yield in glucose-grown  
255 cultures. The sum of their final concentrations indicates that about 98% of the carbon derived from  
256 glucose was converted into these products. However, only 27% of the cellulose consumed by avicel-

grown *C. cellulovorans* was converted into these catabolites. Since the final bacterial biomass was similar in the two growth conditions studied, the rest of consumed cellulose should have taken other metabolic fates. Other cellulolytic bacteria, such as *Ruminiclostridium cellulolyticum* (previously *Clostridium cellulolyticum*) and *C. thermocellum*, may accumulate glycogen and/or exopolysaccharides and/or extracellular cellodextrins and/or amino acids [40,41]. This has never been reported in *C. cellulovorans*, but it could not be excluded, because of the scarce number of available studies on the metabolism of this strain. Further investigations necessary to test this hypothesis were beyond the scope of the present study. Butyric, formic and lactic acid were accumulated in similar amounts in the two growth conditions tested (**Fig. 1C**). Butyric and formic acid were the most abundant end-products while low amounts of lactic acid were detected. However, cellulose- and glucose-grown *C. cellulovorans* produced different amounts of acetate and ethanol (**Fig. 1C**). More in detail, cellulose-grown cultures accumulated higher amounts of acetate (t-test, p-value = 0.01) and lower amounts of ethanol (t-test, p-value = 0.02). Previous investigations reported no ethanol accumulation by glucose- or cellulose-grown *C. cellulovorans* [22,25]. However, growth media with significantly different composition and different pH regulation of bacterial cultures in these studies may justify the different catabolite profiles observed. Changes in metabolite production profiles between different growth conditions have been reported for other cellulolytic bacteria. In *Clostridium termitidis* CT1112, reduction in ethanol (and formate) production and increase in acetate accumulation was observed when it was grown on cellulose instead of a soluble sugar (*i.e.* cellobiose) [42]. In *R. cellulolyticum*, the carbon flux partition between the main catabolic products (*i.e.* acetate, ethanol and lactate) is greatly affected by pH and entering carbon flows, with higher acetate production from substrates which are more slowly metabolized [43]. Lower acetate production and higher ethanol accumulation as the growth rate increases has also been observed in *C. thermocellum* [44]. The results obtained in the present study are therefore consistent with previous observations on other cellulolytic clostridia. Interestingly, this study shows that cellulose promotes a pathway, *i.e.* acetate production, involved in

282 ATP synthesis, while it down-regulates ethanol production which is involved in NAD(P)H-  
283 consumption in *C. cellulovorans*.

284

## 285 **COMPARATIVE PROTEOMIC STUDY**

### 286 *Overall findings*

287 The present study was focused on soluble whole-cell extracts in order to identify proteins specifically  
288 associated with glucose or crystalline cellulose metabolism. Most previous proteomic studies on  
289 cellulose metabolism by *C. cellulovorans* were interested in extracellular proteins [19–21,42]. Only  
290 very recently, whole-cell proteomes of *C. cellulovorans* grown on either glucose- or cellulose-  
291 supplemented medium were compared for a total of 1016 identified proteins in both conditions [13].  
292 Samples for the present investigation were harvested in the late exponential phase (**Fig. 1A, B**).  
293 Quantitative proteomic analysis was performed by the data independent acquisition-based Sequential  
294 Window Acquisition of All Theoretical Mass Spectra (SWATH-MS) approach [31,32] combining deep  
295 proteome coverage capabilities with quantitative consistency and accuracy. In this study, 621 proteins  
296 were quantified (**Supplementary File 1**) corresponding to about 15 % of the *C. cellulovorans*  
297 annotated proteins [16]. To analyze the distribution of their biological functions, the quantified proteins  
298 were annotated by means of the Cluster of Orthologous Genes (COGs) categories  
299 (<http://eggnogdb.embl.de/#/app/home>). The large majority (522 proteins, 84 %) of quantified proteins  
300 were associated with at least a known function grasped by a COG category whereas the remaining 16  
301 % consisted of proteins with unknown function (**Fig. 2A**). Of the total quantified proteins, 319 were  
302 found as differentially expressed when comparing the two growth conditions, that is they were at least  
303 1.5-fold more abundant (p-value < 0.05) in one growth condition compared to the other.  
304 Most (258 proteins, 81 %) differentially expressed proteins were classified into the variety of COG  
305 categories shown in **Fig. 2A** while the remaining 19% are still functionally uncharacterized. This  
306 suggests that proteins among those with yet unknown biological function could be involved in



cellulose/glucose metabolism thus confirming previous reports [19]. We noticed that 124 proteins corresponding to 39 % of the differentially expressed proteins were annotated as having a transport function. Actually, differentially expressed proteins were not fairly distributed across the COG categories (**Fig. 2B**). The proteins involved in cell duplication and protein translation were the largest COG categories shared by the proteins resulting down-regulated in cellulose-grown cells. This latter observation is likely related to the higher growth rate shown by glucose-grown cells. The proteins involved in carbohydrate transport and metabolism were instead the largest COG category in up-regulated proteins in cellulose-grown cultures. The latter observation was corroborated by the analysis of the functional enrichment with respect of all quantified proteins (Fisher's exact test, FDR < 0.05) whereby overexpressed proteins in cellulose-grown cultures were functionally enriched in the category of metabolism and transport of carbohydrates (**Fig. 2C**) and especially (74% of them) in plant polysaccharide depolymerization. In the following sections, differentially expressed proteins will be thoroughly discussed according to their functional classification.

### ***Proteins involved in plant polysaccharide depolymerization***

Many proteins that are more abundant in avicel-grown cultures are involved in plant polysaccharide depolymerization (**Table 1**). They include 24 out of 57 cellulosomal subunits encoded by the *C. cellulovorans* genome [45]. Cellulosomal subunits represent 24 out of 39 (62%) over-expressed proteins belonging to the class of carbohydrate transport and metabolism. Exoglucanase S (ExgS, Clocel\_2823), *i.e.* a cellulase, Mannanase A (ManA, Clocel\_2818), *i.e.* a hemicellulase, and CbpA (Clocel\_2824), *i.e.* the main scaffolding protein of the *C. cellulovorans* cellulosome, showed the largest differential expression, since they were 44-fold, 36-fold and 33-fold more abundant in cellulose-grown bacteria, respectively. These proteins, together with additional up-regulated proteins identified in the current study (*i.e.*, EngH, Clocel\_2822; EngK, Clocel\_2821; EngL, Clocel\_2819; EngM, Clocel\_2816; HbpA, Clocel\_2820), are encoded by a large cellulosomal gene cluster on the *C. cellulovorans* chromosome [45]. Endoglucanase Z (EngZ, Clocel\_2741) and Endoglucanase E

333 (Clocel\_2576) were also among the most strongly up-regulated (19- and 17-fold, respectively) proteins,  
334 consistently with their key role in hydrolysis of crystalline cellulose [46] and for the function and  
335 architecture of the *C. cellulovorans* cellulosome [47,48], respectively.

336 Over-expressed non-cellulosomal proteins include three cellulases (EngD, Clocel\_3242; EngO,  
337 Clocel\_1478; Clocel\_2606) and two hemicellulases ( $\beta$ -mannanase, Clocel\_1134;  $\beta$ -xylosidase,  
338 Clocel\_2595).

339 The present analysis showed that hemicellulases were expressed in cellulose-grown cells and  
340 the same cellulases and hemicellulases up-regulated in cellulose-grown cells were detected also in  
341 glucose-grown cells, although at a lower abundance. These data agree with most results obtained by  
342 previous transcriptomic/proteomic studies on *C. cellulovorans* which reported that several cellulosome  
343 components (e.g. CbpA, EngE, EngL, EngY, ExgS, ManA, and the products of genes Clocel\_0619,  
344 Clocel\_2575, Clocel\_2576, Clocel\_2607, Clocel\_4119), including hemicellulases, are constitutively  
345 biosynthesized [19,49]. On the other hand, overexpression of some of these proteins (e.g. CbpA, EngB,  
346 EngE, and EngZ) is induced by crystalline cellulose [46,49].

347

#### 348 ***Proteins involved in protein secretion***

349 Three components of the Sec protein-translocation complex (SecF, Clocel\_2074; SecD, Clocel\_2075;  
350 SecY, Clocel\_3713) were shown to be more abundant (1.9-, 1.8- and 1.7-fold, respectively) in  
351 cellulose-grown cells. This finding could be related to increased need to secrete CAZYS involved in  
352 cellulose depolymerization. Almost no information is available on mechanisms of secretion of  
353 cellulases in native cellulolytic microorganisms. For most (91 %) cellulases reported in UniProtKB  
354 (<https://www.uniprot.org/help/uniprotkb>) no signal peptide is annotated [50,51]. Among cellulases  
355 from Gram-positive bacteria, 10 % contained twin-arginine translocation (Tat)-like signal peptides,  
356 while 11 % featured amino acid patterns ascribable to the Sec secretory system [50,51]. The main  
357 components of the Sec system are a protein-conducting channel SecYEG, and an ATP-dependent  
358 motor protein SecA [52]. The auxiliary SecDF membrane protein-complex seems to enhance

translocation efficiency in a proton motive force-powered manner [52]. A signal peptide was predicted at the N-terminus of all the over-expressed proteins involved in cellulose/hemicellulose depolymerization identified in this study by Signal-P 4.1 (<http://www.cbs.dtu.dk/services/SignalP-4.1/>), except for Clocel\_2595. More in detail, the N-terminus of 72 % of these proteins shows the typical structure of Sec-type signal peptides, that is a positively charged N-terminal (N-region), a hydrophobic core (H-region) and a negative charged C-region with alanine-rich cleavage site [53]. The variability occurring at the C-terminus of the cleavage site, with 67% of proteins displaying a VXA motif instead of the most typical AXA motif of the Gram-positive bacteria (**Fig. 3**) [51], may indicate that a different consensus sequence is used by *C. cellulovorans* for secreting many of its cellulases. Future investigations will be necessary to confirm this hypothesis and the possible role of the Sec machinery proteins (SecD, Clocel\_2074; SecF, Clocel\_2075; SecY, Clocel\_3713) that were over-expressed in this study in cellulase secretion in *C. cellulovorans*. Understanding cellulase secretion in native cellulolytic microorganisms is crucial for improving comprehension of plant polysaccharide degradation and its exploitation in biotechnological applications such as the construction of recombinant cellulolytic microorganisms, which are frequently hampered by inefficient expression of heterologous cellulases [7].

375

### 376 *Substrate uptake*

It is generally considered that most cellulose is extracellularly converted to different length cellodextrins (rather than glucose) by anaerobic cellulolytic bacteria [40]. Cellodextrins are then transported into the cytoplasm and depolymerized mainly through phosphorolysis [40]. Seven proteins related to ATP binding cassette-type (ABC) transporters (Clocel\_0040; Clocel\_1357; Clocel\_4050; Clocel\_3636; Clocel\_3461; Clocel\_3460; Clocel\_3857) were more abundant in cellulose-grown cells. Protein sequence alignments by the protein BLAST(<https://www.uniprot.org/blast/>) highlighted sequence identity ranging from 54 % to 86 % with proteins involved in the transport of a variety of

384 substrates in other clostridia. In addition, four soluble-binding proteins (Clocel\_0038; Clocel\_3201;  
385 Clocel\_0435; Clocel\_1358), that is proteins which present soluble substrates to the transport channel,  
386 were overexpressed in cellulose-grown cultures [54]. Amino acid sequence analysis of these proteins  
387 strongly indicated that some of them could be involved in the uptake of mono-/oligo-saccharides  
388 derived from extracellular cellulose depolymerization. More in detail Clocel\_0040 (5-fold over-  
389 expressed in cellulose-grown cells) featured 64 % amino acid sequence identity with a carbohydrate  
390 ABC transporter membrane protein from *Clostridium* sp. DMS 8431 (SAMN04487886\_10195).  
391 Similarly, the ABC transporter encoded by Clocel\_3857 (1.6-fold more abundant in cellulose-grown  
392 cells) has 72 % sequence identity with the sugar ABC transporter ATP-binding protein of *Clostridium*  
393 *pasteurianum* (C1I91\_02605). However, the proteins encoded by Clocel\_1357, Clocel\_3460 and  
394 Clocel\_3461 are likely involved in other functions since they show higher sequence identity (50-70 %)  
395 with Clostridial peptide- and sodium-transport systems. The protein products of Clocel\_4050 and  
396 Clocel\_3636 lacked conserved residues required for the propagation of feature annotation.  
397 Furthermore, a modulation of the expression of sugar phosphotransferase systems (PTS) was observed.  
398 PTS are multicomponent transporters that couple sugar transport with phosphorylation using  
399 phosphoenolpyruvate (PEP) as energy source [55]. Our analysis showed that some PTS components,  
400 namely the phosphotransferase system lactose/cellobiose-specific IIB subunit (Clocel\_2881) and the  
401 permease IIC component (Clocel\_2880), were overexpressed while other subunits, such as the  
402 phosphoryl carrier protein (Clocel\_2058) and the phosphoenolpyruvate-protein phosphotransferase  
403 (Clocel\_3686), were down-regulated in cellulose-grown cultures. A cytoplasmic cellodextrin  
404 phosphorylase (Clocel\_2717) was also identified in this study, but its expression level did not  
405 significantly change between the two growth conditions considered.

406

## 407 ***Central carbon metabolism***

### 408 *Glycolysis*

409 *C. cellulovorans* metabolizes glucose through the Embden-Meyerhof-Parnas pathway [16] (**Fig. 4A**).

410 Our analysis revealed that the expression of several *C. cellulovorans* glycolytic enzymes was affected

411 by carbon source change, albeit at different extent (**Fig. 4A, B**). Phosphofructokinases generally control

412 glycolysis and are allosterically activated by ADP and inhibited by fructose-6-P (F6P) and ATP in

413 Clostridia [13,56]. The *C. cellulovorans* genome encodes two ATP-dependent 6-phosphofructokinases

414 (namely Clocel\_2901 and Clocel\_0388), *i.e.* enzymes that catalyze the phosphorylation of F6P to

415 fructose 1,6-bisphosphate (F1,6BP) by consuming ATP. The present study revealed that Clocel\_0388

416 was not differentially expressed, whereas Clocel\_2901 was 5-fold more abundant in cellulose-grown

417 cells. Overexpression of Clocel\_2901 in cellulose-grown *C. cellulovorans* (fold change, FC, comprised

418 between 4 and 16) was also reported by another recent study [13]. *C. cellulovorans* genome also

419 encodes a pyrophosphate (PP<sub>i</sub>)-fructose 6-phosphate 1-phosphotransferase (Clocel\_1603) (that

420 catalyzes the phosphorylation of F6P using PP<sub>i</sub>) but this enzyme was not found as differentially

421 expressed in the current study. This observation could indicate a preferential ATP-dependent

422 conversion of F6P in the cells grown on cellulose. Furthermore, four glycolytic enzymes were present

423 in lower abundance in cellulose-grown cells: triosephosphate isomerase (Clocel\_0721, FC = 0.03),

424 glyceraldehyde-3-phosphate dehydrogenase (Clocel\_0719, FC = 0.23), glucose-6-phosphate isomerase

425 (Clocel\_1364, FC = 0.25), and phosphoglycerate kinase (Clocel\_0720, FC = 0.6). In general, these

426 results do not seem to differ from those previously reported by Aburaya et al. [13], since most of these

427 enzymes were slightly down-regulated in cellulose- versus glucose grown *C. cellulovorans* (with the

428 only exception of the late stationary phase). Glyceraldehyde-3-phosphate dehydrogenase is among the

429 most important glycolytic enzymes since it catalyzes NADH production through oxidation of

430 glyceraldehyde-3-phosphate and has previously been identified as a probable bottleneck in glycolysis

431 [57] and towards alcoholic biofuel production [58]. Glyceraldehyde-3-phosphate dehydrogenase up-

432 regulation in glucose-grown *C. cellulovorans* agrees with previous studies on *C. termitidis* [42] and *C.*

433 *thermocellum* [59], that reported that the same enzyme was one of the most abundantly expressed

434 proteins during growth on cellobiose, a soluble  $\beta$ -glucose disaccharide.

435

## 436 *Pyruvate metabolism*

437 Interconversion between pyruvate (PA) and phosphoenolpyruvate (PEP) is most frequently mediated  
438 by the antagonistic enzymatic couple consisting of ADP-dependent pyruvate kinase (PK) (catabolic  
439 role) and phosphoenolpyruvate synthetase (PEPS) (anabolic role). In *C. cellulovorans* PK is encoded  
440 by Clocel\_0389 whereas no PEPS is annotated. However, *C. cellulovorans* genome encodes a pyruvate  
441 phosphate dikinase (PPDK, Clocel\_1454) that may catalyze the reversible production of PA, ATP and  
442  $P_i$  from PEP, AMP and  $PP_i$  [60]. In some organisms such as *Acetobacter xylinum*, *Propionibacterium*  
443 *shermanii* and *Microbispora rosea* [61–63], PPDK seems to exert its activity in the anabolic (PEP)  
444 direction [64,65], whereas it fulfils a catabolic role in others, such as *Clostridium symbiosum* [66]. In  
445 *Thermoproteus tenax*, the combined action of PPDK, PK and PEPS was shown to control the  
446 interconversion between PEP and PA [67]. In *C. thermocellum*, which does not possess PK, PPDK can  
447 substantially support the production of PA from PEP [68]. In the present study, PK did not show any  
448 differential expression between the two growth conditions, while PPDK showed 11-fold higher  
449 abundance in cellulose-grown cells. Consistently, a 3.5-fold up-regulation was observed also for a  
450 PPDK putative regulatory protein (Clocel\_4349) [69]. Up-regulation of PPDK (Cter\_0809) in  
451 cellulose-grown cells was reported also in *Clostridium termitidis* CT1112 [42]. Previous  
452 characterization of PK and PPDK from different microorganisms has shown that their activity is  
453 generally allosterically regulated. More in details, PPDK is regulated by the ATP/AMP ratio in  
454 *Trypanosoma brucei* and *Acetobacter xylinum* [70,71] whereas PK activity by intracellular  
455 concentration of ADP, AMP and phosphate sugars [72–74]. The present study points at PPDK as a  
456 possible key enzyme regulating carbon flux during cellulose metabolism by *C. cellulovorans*. Further  
457 investigations on this enzyme, such as understanding potential regulation by allosteric effectors, appear  
458 essential to better understand its role in *C. cellulovorans* metabolism.

459 In some bacteria, malic enzyme (ME), malate dehydrogenase (MDH) and phosphoenolpyruvate  
460 carboxykinase (PEPCK) are involved in an alternative three-step pathway converting PEP to pyruvate

that is called malate shunt [75] (**Fig. 4A**). In *C. thermocellum*, the malate shunt was proposed as a strategy to transfer electrons from NADH to NADP<sup>+</sup>, thus supplying most of the NADPH necessary for biosynthetic routes [68]. A putative ME (Clocel\_0393) was down-expressed, *i.e.* 3-fold less abundant, in cellulose-grown *C. cellulovorans*. We were therefore interested to understand if a malate shunt could be present in *C. cellulovorans* also. In its genome, two lactate/malate dehydrogenase are annotated (Clocel\_1533; Clocel\_2700). The protein product of Clocel\_1533 shares an amino acid sequence identity of 40% with *C. thermocellum* MDH (Cthe\_0345) which includes key residues in the active site [76]. Although a confirmation by enzyme activity assay is necessary, these findings strongly suggest that Clocel\_1533 encodes a MDH. No PEP carboxykinase is annotated in the *C. cellulovorans* genome, but PEP carboxylase (PEPC, Clocel\_1149), that catalyzes the conversion of PEP to oxaloacetate [77], could functionally replace it. PEPC was identified in the present study, although in similar amounts in the two growth conditions. Hence, a malate shunt could be hypothesized in *C. cellulovorans*, especially to supply NADPH for biosynthetic reactions in fast growth conditions such as in glucose-supplemented cultures. Further investigations by enzyme activity assay of the products of Clocel\_1533 and Clocel\_1149 will be necessary to confirm this hypothesis. Since previous studies reported that ME is allosterically inhibited by PP<sub>i</sub> [78] and PPDK activity controls the intracellular PP<sub>i</sub> concentration, a possible regulatory interconnection between the two pathways could exist.

478

#### 479 ***Tricarboxylic acid cycle and nitrogen assimilation***

*C. cellulovorans* can partially operate the tricarboxylic acid (TCA) cycle in a reductive manner [77]. Clostridia use TCA cycle mainly to produce intermediates for biosynthetic routes and regulate the redox balance inside the cells. According to the present study, biosynthetic levels of aconitase (Clocel\_1405), citrate synthase (Clocel\_3688), isocitrate dehydrogenase (Clocel\_2469) and fumarate hydratase (Clocel\_0392) were not affected by the carbon sources used. However, glutamate dehydrogenase (GDH, Clocel\_1284) was 10-fold more abundant in glucose-grown bacteria. Furthermore, glutamine synthetase (GlnS, Clocel\_3873) and glutamate synthase (GluS, Clocel\_2665)

487 were found as 3- and 1.5-fold more abundant in glucose- versus cellulose-grown *C. cellulovorans*,  
488 respectively. All these proteins are typically involved in nitrogen assimilation and synthesis of  
489 components of cell biomass in bacteria. GDH catalyzes the reversible NAD(P)H-dependent reductive  
490 amination of 2-ketoglutarate to glutamate [79]; GlnS aminates glutamate to glutamine in ATP-  
491 dependent manner; GluS catalyzes the reversible transfer of an amino group from glutamine to 2-  
492 ketoglutarate with the consumption of NAD(P)H (or reduced ferredoxin) and the production of 2  
493 glutamate molecules and NAD(P)<sup>+</sup> (or oxidized ferredoxin) [41]. In addition, intracellular  
494 accumulation of glutamate and secretion of amino acids (up to 15-17 % of the total substrate  
495 consumed) have been reported in other cellulolytic clostridia (e.g. *C. thermocellum* and *R.*  
496 *cellulolyticum*) [41,80]. However, overexpression of GDH in *C. thermocellum* was reported for cells  
497 growing on cellulose with respect to cultures growing on a soluble carbohydrate (*i.e.* cellobiose) [81].  
498 Higher abundance of these enzymes in glucose-grown *C. cellulovorans* could be related with higher  
499 growth rates measured in this condition. In addition, activities of these proteins affect the redox balance  
500 of the cell. A recent study on *C. thermocellum* demonstrated that deletion of the gene *glnA* encoding its  
501 main glutamine synthetase significantly decreases amino acid secretion and increases ethanol yield  
502 likely by increasing NADH availability in the cell [41]. Finally, GDH, GlnS and GluS have been  
503 referred as involved in pH homeostasis in *C. thermocellum* [80]. However, no significant difference in  
504 extracellular pH between glucose- and cellulose grown *C. cellulovorans* cultures has been detected in  
505 the present study (data not shown).

506

### 507 ***End-product synthesis pathways***

508 The main products of *C. cellulovorans* metabolism are H<sub>2</sub>, CO<sub>2</sub>, acetic acid, lactic acid, butyric acid  
509 and ethanol [11]. The proteins encoded by three out of the four hydrogenase genes annotated in the *C.*  
510 *cellulovorans* genome [82] were not identified in this study while the Fe-only hydrogenase  
511 (Clocel\_4097) resulted 1.5-fold more abundant in glucose-grown cells. Therefore, the protein encoded  
512 by Clocel\_4097 may be the main responsible for H<sub>2</sub> production in this strain. Hydrogen production is



513 the fastest way to dispose of excess of reduced cofactors generated through carbohydrate metabolism  
 514 [83]. Up-regulation of Clocel\_4097 in glucose-grown cells, appears as a strategy to maximize  
 515 glycolytic turnover in this condition. This Fe-only hydrogenase probably receives electrons from the  
 516 oxidative activity of pyruvate ferredoxin oxidoreductase PFOR (Clocel\_1684), which converts  
 517 pyruvate to acetyl-CoA, yielding CO<sub>2</sub> and reduced ferredoxin (**Fig. 4A**) Consistently, PFOR was found  
 518 1.6-fold more abundant in glucose-grown cells. According to gene annotation, *C. cellulovorans*  
 519 genome encodes another PFOR (Clocel\_2840) and an indole pyruvate oxidoreductase subunit  
 520 (Clocel\_4184), that were not detected in the present study. These data therefore point at Clocel\_1684 as  
 521 the main PFOR in *C. cellulovorans*.

522 Another enzyme that was more abundant (2-fold) in glucose-grown cells is pyruvate formate lyase  
 523 (PFL, Clocel\_1811). This enzyme catalyzes pyruvate conversion to formate and acetyl-CoA production  
 524 from pyruvate (**Fig. 4A**). Consistently, a pyruvate-formate lyase activating enzyme (Clocel\_1812) [84]  
 525 was also found as 5-fold more abundant in the same growth condition.

526 *C. cellulovorans* genome encodes seven alcohol dehydrogenases (ADHs), four of which were  
 527 overexpressed in cellulose-grown bacteria: Clocel\_2402 (2.5-fold), Clocel\_1990 (8-fold), Clocel\_3817  
 528 (1.6-fold), Clocel\_1140 (2.2-fold) (**Supplementary File 1**). The presence of multiple ADHs is  
 529 common in solvent-producing clostridia and other microorganisms [85,86]. ADHs can differ for their  
 530 substrate and coenzyme specificity, but the physiological significance of multiple ADHs in the same  
 531 strain has not always been elucidated. Clocel\_2402, Clocel\_3817 and Clocel\_1140 encode bifunctional  
 532 aldehyde/alcohol dehydrogenases, possibly involved in 2-step reduction of acetyl-CoA to acetaldehyde  
 533 and finally to ethanol with consumption of two NADH (**Fig. 4A**). The amino acid sequence of ADH  
 534 encoded by Clocel\_1990 shows 89% identity with glycerol dehydrogenases from other Clostridia, *i.e.*  
 535 enzymes that catalyze the oxidation of glycerol [87]. However, owing to the scarce substrate specificity  
 536 generally shown by ADHs, it is difficult to definitely assess their role in ethanol or other alcohol  
 537 production without enzyme activity assays. Up-regulation of these ADHs in cellulose-grown *C.*  
 538 *cellulovorans* is not reflected by increased ethanol production since a lower amount of ethanol is

539 accumulated in this growth condition (**Fig. 1C**). Apart from possible involvement of these up-regulated  
 540 enzymes in other alcohol synthesizing pathways, a reasonable explanation for this observation is that  
 541 ADHs may be reversible enzymes and also catalyze alcohol oxidation. Most studies on characterization  
 542 of ADH from clostridia focused on their substrate and coenzyme specificity but few papers reported  
 543 clostridial ADHs able to oxidize alcohols, although their activity was mainly in the aldehyde-reducing  
 544 direction [88,89]. Previous studies showed that relative protein abundances do not always correlate  
 545 with end-product distribution profiles. In particular, higher abundance of ADHs with respect to lactate  
 546 dehydrogenase was observed in some growth conditions in *Thermoanaerobacter*  
 547 *thermohydrosulfuricus* WC1, however, lactate was always the major end-product of this strain [90].  
 548 The present study confirms that protein abundance is not the only player determining carbon flux  
 549 distribution in cells, which is also influenced by other parameters such as catabolic bottlenecks,  
 550 allosteric regulation or cofactor availability.

551 Phosphate acetyltransferase (PTA, Clocel\_1891), which catalyzes the conversion of acetyl-CoA to  
 552 acetyl-phosphate, was found up-regulated (1.9-fold) in cellulose-grown cells whereas acetate kinase  
 553 (ACK, Clocel\_1892), which catalyzes the subsequent conversion of acetyl-phosphate to acetate and  
 554 ATP did not result as differentially expressed in the present study (**Fig. 4B**). Genes encoding PTA and  
 555 ACK form an operon in *C. cellulovorans*, so, our results indicate that post-transcriptional events may  
 556 differentially regulate the expression of these genes. Changes in proteomic levels of PTA are consistent  
 557 with increased production of acetate by cellulose-grown *C. cellulovorans* (**Fig. 1C**). These data also  
 558 suggest that the level of PTA activity could be a bottleneck for acetate production in *C. cellulovorans*,  
 559 while ACK could have a higher specific activity.

560 Butyric acid derives from acetyl-CoA through a multi-step pathway as shown in **Fig. 4A**. Some of the  
 561 enzymes involved in this pathway are also implicated in other metabolic processes, such as fatty acid  
 562 and amino acid metabolism. Maybe for this reason, some of them, that is acetyl-CoA acetyltransferase  
 563 (ACAT, Clocel\_3058), hydroxybutyryl-CoA dehydrogenase (HBD, Clocel\_2972), enoyl-CoA  
 564 hydratase (ECH, Clocel\_2976) and phosphate butyryltransferase (PTB, Clocel\_3675), were down-

regulated while butyryl-CoA dehydrogenase (BCD, Clocel\_2975) and butyrate kinase (BUK, Clocel\_3674) were up-regulated in cellulose-grown cells. Actually, no significant changes in butyrate amounts accumulated by cellulose- or glucose-grown *C. cellulovorans* was observed in this study.

## ENERGY PRODUCTION

Three components of the F<sub>0</sub>F<sub>1</sub>-ATP synthase, i.e. ATP synthase F<sub>1</sub> sector subunit alpha (Clocel\_3051) and subunit beta (Clocel\_3049) and F<sub>0</sub> portion subunit c (Clocel\_3054), were 2-fold more abundant in cellulose-grown *C. cellulovorans*. ATP synthase F<sub>0</sub> subunit b (Clocel\_3053) and F<sub>1</sub> gamma chain (Clocel\_3050) did not show any differential expression between the two growth conditions. The genes encoding these proteins together with Clocel\_3048, Clocel\_3052 and Clocel\_3055 form an operon which codes for the entire ATP synthase complex [16]. Some F<sub>0</sub>F<sub>1</sub>-ATP synthases of strict anaerobes mainly hydrolyze ATP to pump H<sup>+</sup> out of the cell, such as in *C. thermocellum* where this function has been related to pH homeostasis [80,91,92]. However, no significant difference in extracellular pH between glucose- and cellulose-grown *C. cellulovorans* cultures has been detected in the present study (data not shown). Other strict anaerobic bacteria have ATP synthases which mainly function towards ATP synthesis by using either Na<sup>+</sup>, such as *Propionigenium modestum* [93] and *Acetobacterium woodii* [94], or H<sup>+</sup>, e.g. *Moorella thermoacetica* [95], transmembrane gradient as energy source. In anaerobes, Na<sup>+</sup>/H<sup>+</sup> gradient can be generated by several mechanisms such as anaerobic electron chains, H<sub>2</sub> oxidation [91] and membrane bound proton-translocating pyrophosphatases [59]. It can therefore be hypothesized that up-regulation of F<sub>0</sub>F<sub>1</sub>-ATP synthase could provide additional ATP to that generated by substrate-level phosphorylation in *C. cellulovorans* (**Fig. 4A**).

## ATP QUANTIFICATION

Metabolite and proteomic analyses indicated an up-regulation of acetate biosynthetic pathway in cellulose-grown *C. cellulovorans*. Acetate, as well as butyrate, production is involved in ATP synthesis through substrate-level phosphorylation. Furthermore, over-expression of some F<sub>0</sub>F<sub>1</sub> ATP synthase subunits was detected in cellulose-grown *C. cellulovorans*. These observations suggest possible differences in ATP-content of glucose- or cellulose-grown cells. In most growth phases, the ATP content of cellulose-grown bacteria ranged between 0 and 3 ng/mg, that is much lower (about a 100 times) than that measured in glucose-grown cells (**Fig. 5**). Interestingly, ATP content of cellulose-grown cultures at the time of inoculum is of the same order of magnitude of that observed in glucose-grown cells. It is worth reminding that both glucose- and cellulose-grown cultures were inoculated with glucose-grown bacteria, hence, just after inoculum, *C. cellulovorans* cells likely still show most characteristics associated with glucose metabolism. Our findings seem to contradict previous studies claiming bioenergetic benefits for anaerobic bacteria grown on cellulose instead of simple carbohydrates [40,96]. Increased acetate production and up-regulation of ATP synthase subunits by cellulose-grown *C. cellulovorans* can be interpreted as a mean to improve ATP cell supply in conditions requiring high ATP consumption (e.g. for cellulase biosynthesis and secretion) [97].

604

## 605 **Conclusions**

The present comparative analysis indicated that the metabolism of cellulose-grown *C. cellulovorans* significantly differs from that of the same strain grown on simple carbohydrates, i.e. glucose, thus confirming previous reports on other cellulolytic microorganisms [40]. Apart from modulation of the expression of cellulosomal and non-cellulosomal enzymes directly involved in cellulose depolymerization, the present investigation revealed that other modifications of the metabolic network are induced by the growth substrate. The up-regulation of some subunits of the Sec-machinery in cellulose-grown *C. cellulovorans* point at them as interesting candidates possibly involved in cellulase secretion in this strain. This could be a starting point for filling the gaps in understanding the

614 mechanisms of cellulase secretion in cellulolytic microorganisms. The most original findings brought  
615 by the present study concern modifications of the central metabolism and fermentative pathways. It is  
616 worth noting that previous studies did not report any significant modification in fermentation product  
617 profile [22] or any major change in the expression of enzymes involved in the central metabolism  
618 between glucose- and cellulose-grown *C. cellulovorans* [13]. This is most probably caused by different  
619 composition [22] and/or different pH regulation of the growth medium [13]. In some cases, the present  
620 study helped to identify the key genes associated with specific catabolic reactions or pathways among  
621 multiple paralogs with the same annotation in *C. cellulovorans* based on their expression levels (e.g.  
622 H<sub>2</sub>ase, PFOR). In other cases (e.g. ADHs, MDH), our results clearly indicated that further studies (e.g.  
623 substrate and cofactor specificity, catalytic activity regulation) are essential to understand the  
624 physiological role of certain gene products. The main findings of the present investigation can be  
625 summarized as follows:

- 626 • Proteomic data indicate that a re-distribution of the central carbon flow occurs between glucose-  
627 and cellulose-grown *C. cellulovorans*, through a modulation of the biosynthesis of different key  
628 enzymes that include ATP-dependent 6-phosphofructokinase (Clocel\_2901), PPKK  
629 (Clocel\_1454), GDH (Clocel\_1284), PTA (Clocel\_1891), PFL (Clocel\_1811) and different  
630 ADHs.
- 631 • A macroscopic consequence of this re-arranged metabolic network is that cellulose promotes  
632 acetate accumulation, while glucose induces higher ethanol production. Based on previous  
633 reports, this could depend on the different substrate used, or the different growth rate/carbon  
634 flux supported by these substrates [42–44].
- 635 • Cellulose-grown cells have significantly lower ATP content, possibly related to higher energy  
636 expenditure for cellulase biosynthesis and secretion. Up-regulation of acetate pathway and ATP  
637 synthase subunits may help cope with this high energy demanding condition. Therefore, these  
638 observations seem to contradict previous studies claiming bioenergetic benefits of anaerobic  
639 bacteria growing on cellulose instead of simple carbohydrates [40,96].

640 In conclusion, this study pointed out some aspects of glucose and cellulose metabolism in *C.*  
641 *cellulovorans*, which could be useful for better understanding the physiology of this strain and also  
642 towards engineering of its metabolic pathways for application in processes for biorefining plant  
643 biomass.

644

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650

## 651 **Conflict of interest**

652 The authors declare no conflict of interest

653

## 654 **Author contributions**

655 RM designed research and supervised experiments. GU, SC, AR and MM performed experiments and  
656 analyzed proteomic data. All the Authors contributed in discussing experimental data and writing the  
657 manuscript.

658

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936

## Figure captions

938

939 **Figure 1.** *C. cellulovorans* growth kinetics in a medium containing 5 g/l glucose (A) or 10 g/l cellulose  
940 (B) as the main carbon source. Growth curves are represented by solid lines, while dashed lines  
941 represent substrate concentration. Bacterial biomass was measured by total cell protein determination.  
942 Syringe symbols indicate the cell sampling points for proteomic analysis, i.e. 5 hours and 7 days after  
943 inoculum for glucose- and cellulose-supplemented media, respectively. End-product final concentration  
944 (g/l) measured in glucose- (purple) or cellulose- (green) supplemented *C. cellulovorans* cultures (C).  
945 Data are displayed as mean  $\pm$  SD across triplicate cultures. Asterisks indicate statistically significant  
946 differences (t-test, p-value  $\leq 0.05$ ) between the two growth conditions.

947

948 **Figure 2.** Functional categorization of quantified proteins. (A) Cluster of Orthologous Genes (COG)  
949 functional classification of the quantified proteins along with the differentially expressed proteins  
950 (DEP) resulting from the comparison between *C. cellulovorans* cultures grown on cellulose or glucose  
951 as the main carbon source. (B) COG-based functional classification of the proteins resulting up- or  
952 down-regulated when comparing *C. cellulovorans* cultures grown on cellulose or glucose as the main  
953 carbon source. (C) Functional enrichment analysis of up- and down-regulated proteins according to  
954 COG annotation. The dashed line corresponds to a fold enrichment equal to 1.5. Statistically significant  
955 over-representation of a COG category in the up- or down-regulated proteins (Fisher's exact test, FDR  
956  $< 0.05$ ) is labelled with an asterisk.

957

958 **Figure 3.** Consensus sequence of N-terminal signal peptide of the *C. cellulovorans* proteins involved in  
959 plant polysaccharide depolymerization that were up-regulated in cellulose-grown cultures.

960

961 **Figure 4.** (A) Scheme representing the metabolic changes observed in both *C. cellulovorans* central  
962 carbon metabolism and end-product synthetic pathways, based on protein differential expression. (B)  
963 Function, gene locus and fold change of the proteins involved in the central carbon metabolism and  
964 end-product synthesis in *C. cellulovorans*. Up- and down-regulated proteins are displayed in green and  
965 purple, respectively. Proteins that are not differentially expressed or not quantified in the present study  
966 are indicated in grey. GPI, glucose 6-phosphate isomerase; PFK, phosphofructokinase; ALD, aldolase;  
967 TPI, triosephosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK,

968 phosphoglycerate kinase; PMG, phosphoglycerate mutase; ENO, enolase; PK, pyruvate kinase; PPK, pyruvate  
969 pyruvate phosphate dikinase; PEPC, phosphoenolpyruvate carboxylase; PFOR, pyruvate ferredoxin  
970 oxidoreductase; PFL, pyruvate formate lyase; ME, malic enzyme; MDH, malate dehydrogenase. Fd-  
971 H2ase, ferredoxin-hydrogenase; ADH, bifunctional aldehyde/alcohol dehydrogenase; PTA, phosphate  
972 acetyltransferase; ACK, acetate kinase; ACAT, acetyl-CoA acetyltransferase; HBD, hydroxybutyryl-  
973 CoA dehydrogenase; ECH, enoyl-CoA hydratase; BCD, butyryl-CoA dehydrogenase; PTB, phosphate  
974 butyryltransferase; BUK, butyrate kinase.

975

976 **Figure 5.** Intracellular ATP concentration measured in glucose- (A) and cellulose-grown *C.*  
977 *cellulovorans* (B). Intracellular ATP concentration is expressed as Log<sub>2</sub> ng of ATP per mg of proteins  
978 extracted from cell biomass. Data are displayed as mean ± SD of triplicate cultures.

979

980 **Supplementary File 1.** List of the quantified proteins in *C. cellulovorans* grown on avicel and glucose.  
981 The fold-change is given by the ratio between the average of the protein abundances (based on  
982 SWATH-MS data acquisition) for the three replicates of *C. cellulovorans* grown on avicel and the  
983 average of the protein abundances (based on SWATH-MS data acquisition) of the three replicates in *C.*  
984 *cellulovorans* grown on glucose. The proteins with fold-change > 1.5 and p-value > 0.05 are considered  
985 as up-regulated (green), while the proteins with fold-change < 0.67 and p-value > 0.05 are considered  
986 down-regulated (purple).

987

988 **Supplementary Figure 1.** Kinetics of accumulation of end-product measured in glucose- (A) or  
989 cellulose- (B) supplemented *C. cellulovorans* cultures. Data are displayed as mean ± SD across  
990 triplicate cultures.

**Table 1.** Up-regulated proteins involved in plant polysaccharide depolymerization. Protein function, gene locus, CAZy category, fold change, signal peptide prediction (Signal-P 4.1, D-value > 0.5) and cellular localization prediction (PsortB v3.0) are shown. GH, glycoside hydrolase; CBM, carbohydrate binding module; PL, pectate lyase; SLH, S-layer homology; NA, not annotated.

Function	Gene locus	CAZy	FC	Signal-P	Psortb
<i>Cellulosomal proteins</i>					
Exoglucanase S	Clocel_2823	GH48	43.62	0.88	Extracell
Mannanase A	Clocel_2818	GH5	35.86	0.78	Unknown
Cellulose-binding protein A	Clocel_2824	CBM3, SLH, HBD	33.49	0.89	Extracell
Endoglucanase	Clocel_2576	GH9, CBM3	19.41	0.74	Extracell
Endoglucanase Z	Clocel_2741	GH9, CBM3	18.64	0.55	Extracell
Dockerin type 1	Clocel_3193	NA	17.38	0.57	Extracell
Endoglucanase E	Clocel_3359	GH5, CBM65, SLH	16.93	0.82	Unknown
Endoglucanase L	Clocel_2819	GH9	14.49	0.80	Membrane
β-xylanase	Clocel_2900	GH10, CBM22	11.29	0.80	Extracell
Endoglucanase H	Clocel_2822	GH9, CBM3	10.97	0.57	Extracell
Mannanase	Clocel_4119	GH26, CBM35	9.83	0.73	Unknown
Endoglucanase	Clocel_0983	GH5	8.48	0.85	Unknown
Mannanase	Clocel_2575	GH26, CBM35	7.81	0.61	Unknown
Mannanase	Clocel_2607	GH26, CBM35	6.83	0.84	Extracell
Endoglucanase Y	Clocel_1624	GH9, CBM30	6.71	0.75	Extracell
Endoglucanase	Clocel_2600	GH5, CBM32	5.97	0.78	Extracell
Endoglucanase M	Clocel_2816	GH9, CBM4	5.94	0.54	Extracell
Endoglucanase B	Clocel_1150	GH5	5.03	0.68	Membrane
Endoglucanase	Clocel_3111	GH5	5.00	0.61	Extracell
Hydrophobic protein A	Clocel_2820	SLH, HBD	4.97	0.66	Extracell
Endoglucanase	Clocel_0619	GH5	4.69	0.86	Extracell
Endoglucanase	Clocel_0930	GH9, CBM3	4.50	0.78	Extracell
Endoglucanase K	Clocel_2821	GH9, CBM4	4.38	0.80	Extracell
Pectate lyase A	Clocel_1623	PL1, PL9	4.35	0.57	Extracell
<i>Non-cellulosomal proteins</i>					
Endoglucanase O	Clocel_1478	GH9, CBM4	6.06	0.60	Extracell
B-mannanase	Clocel_1134	GH26, CBM23	4.22	0.66	Cell wall
Endoglucanase D	Clocel_3242	GH5, CBM2	3.35	0.59	Membrane
β-xylosidase	Clocel_2595	GH43	3.16	-	Unknown
Endoglucanase	Clocel_2606	GH5, CBM46	2.64	0.64	Cell wall

Figure 1

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Figure 1

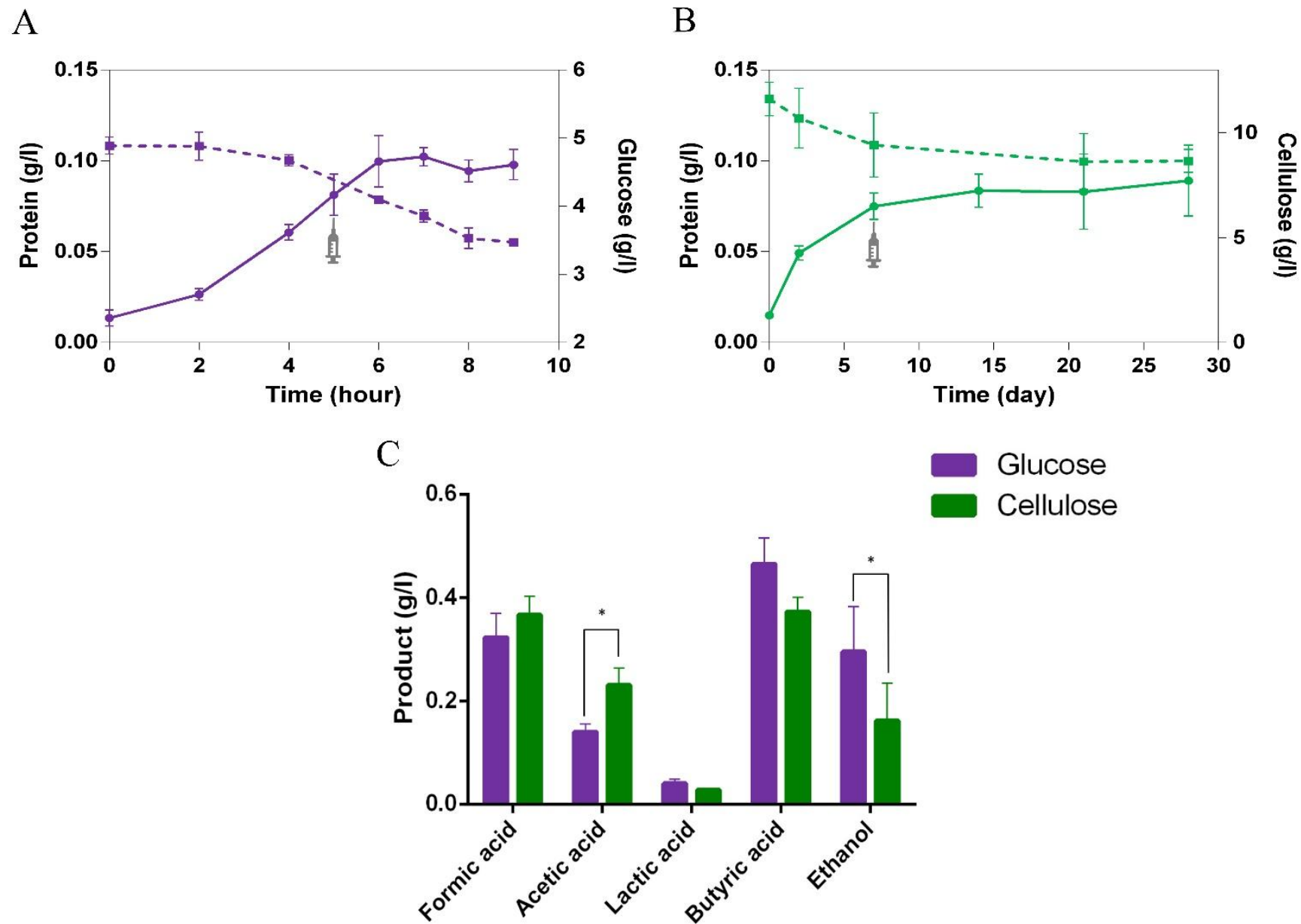


Figure 2

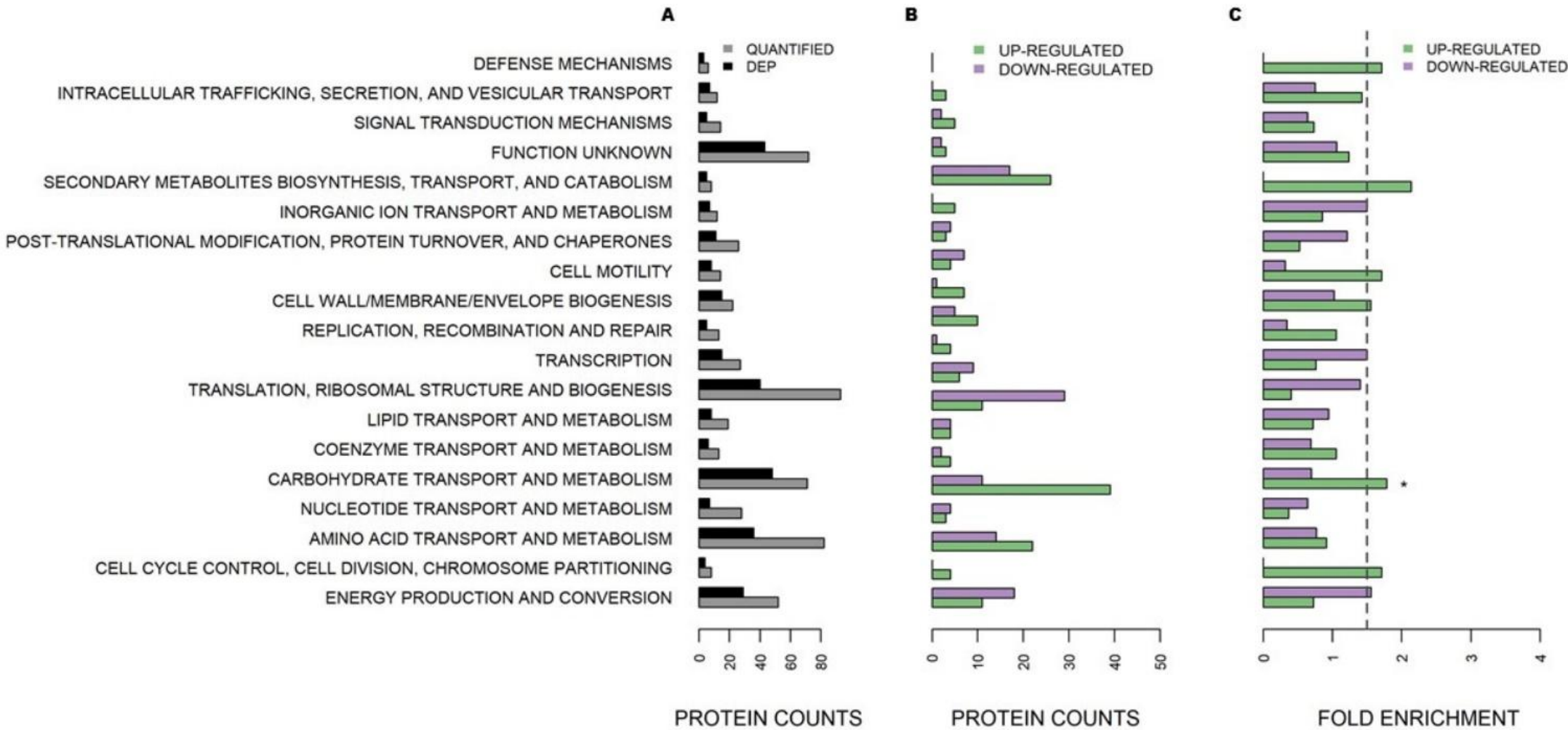


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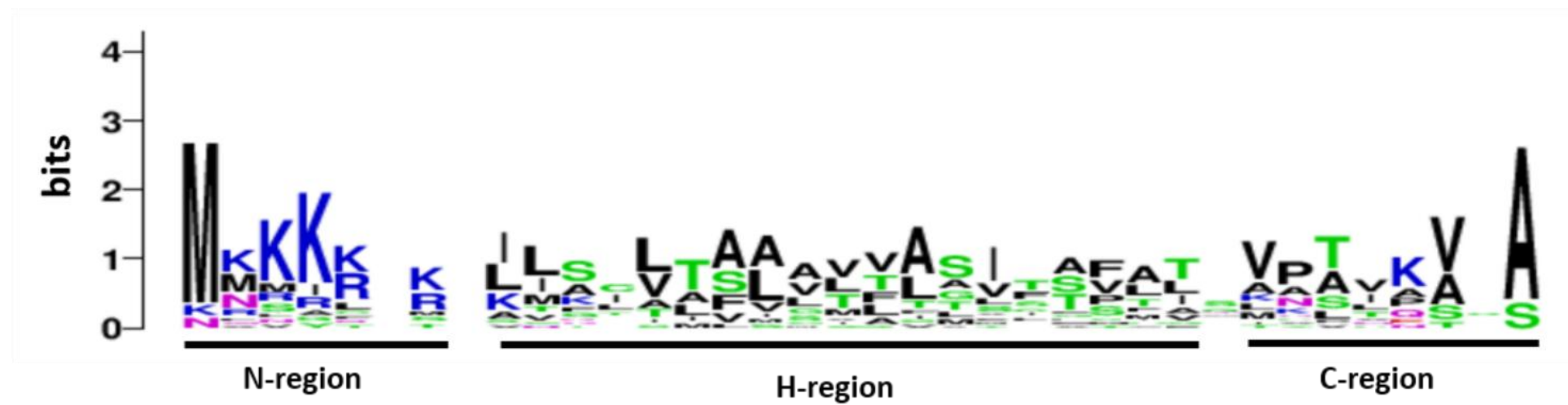
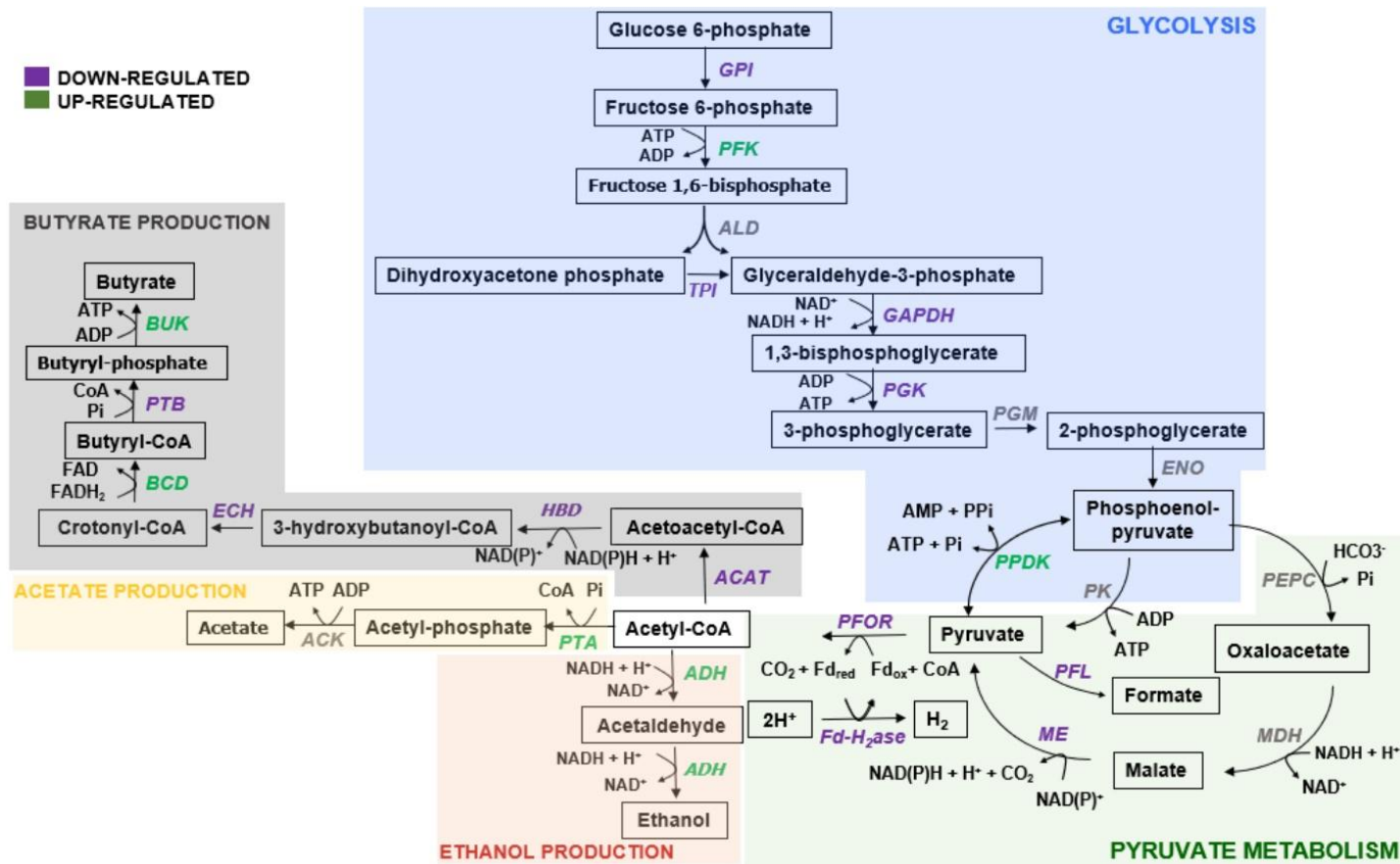


Figure 4  
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Figure 4

A



B

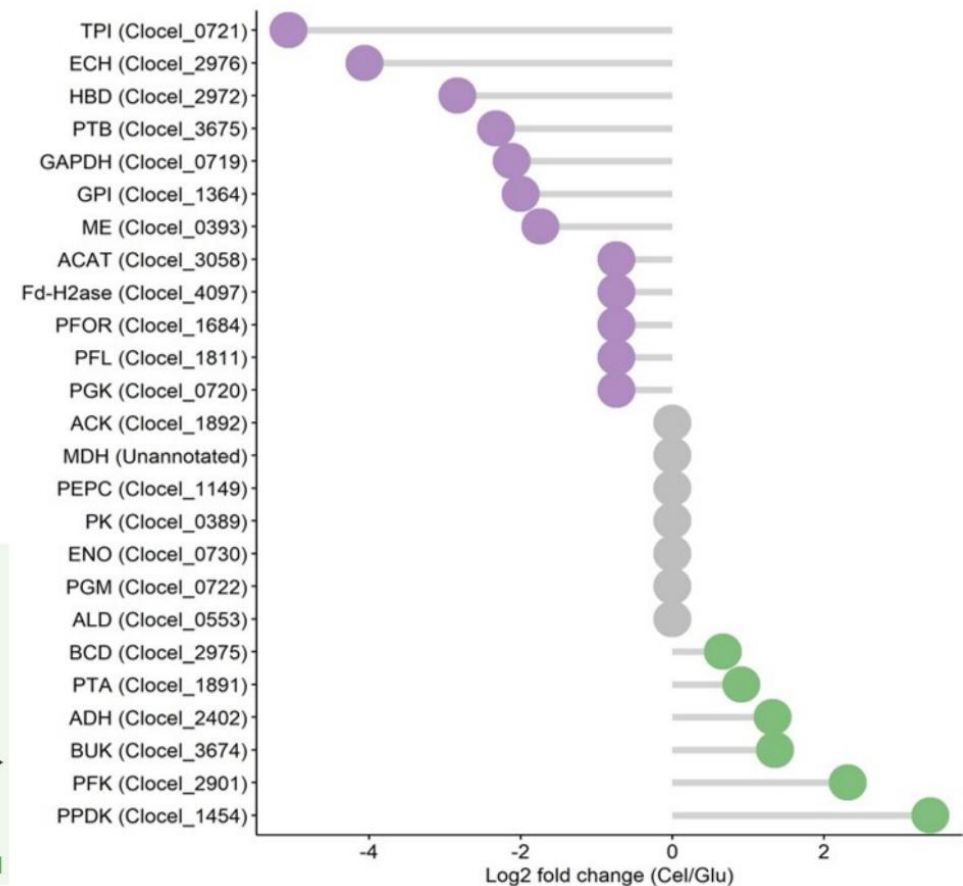
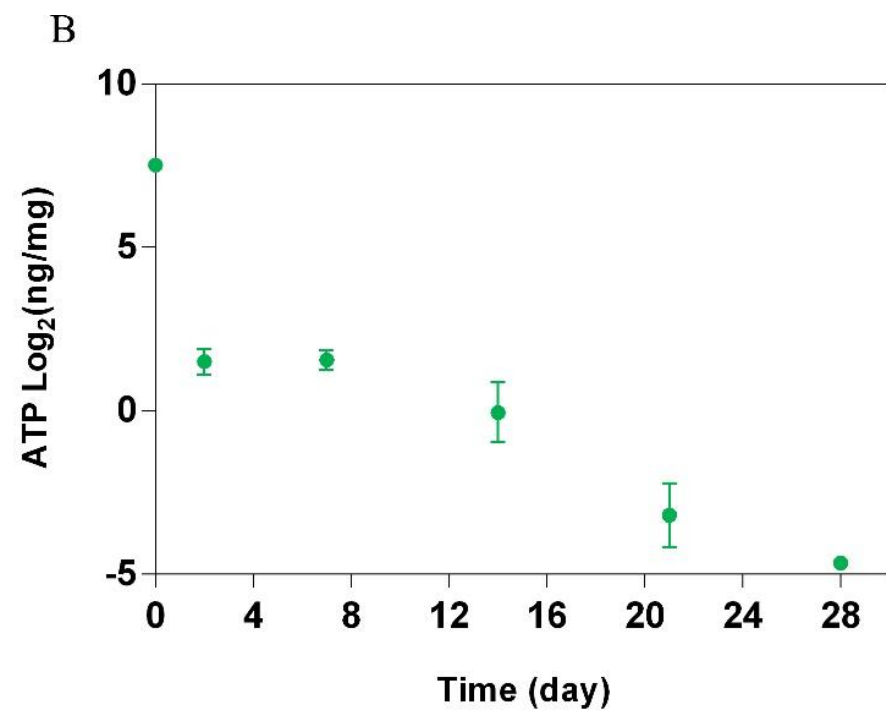
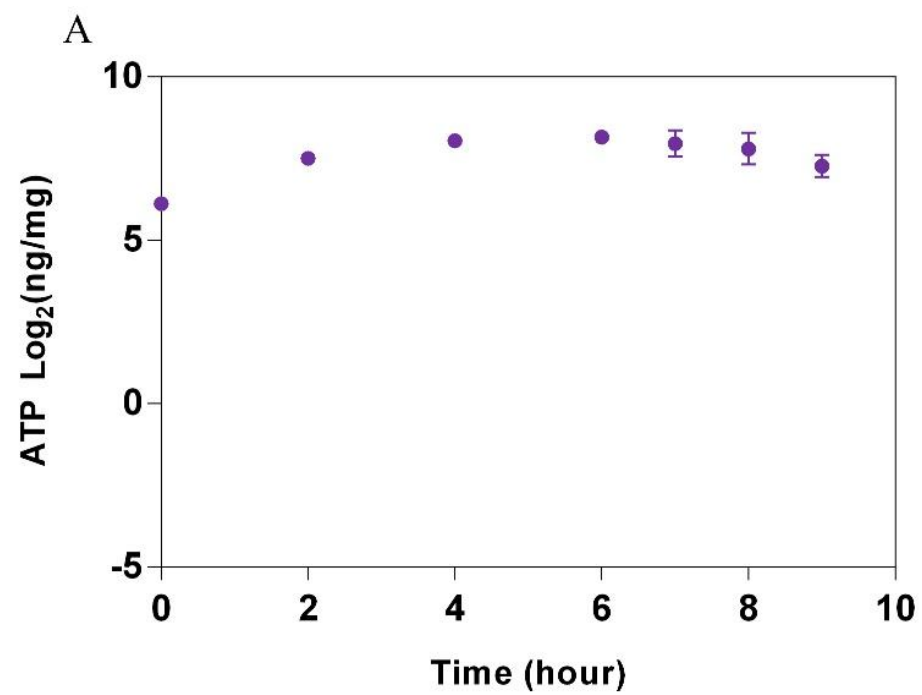




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Figure 5



## Supplementary figure 1

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