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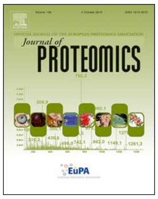

Analysis of the proteins secreted by *Trichoderma harzianum* P49P11 under carbon-limited conditions

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ABSTRACT

The wild type strain *Trichoderma harzianum* was able to synthesize enzymes that can catalyse the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (PNPGase) in glucose-limited chemostat cultures. Fructose/glucose and sucrose conditions provided low levels of PNPGase activity. To investigate whether under these conditions other enzymes were produced, a shotgun proteomics analysis of their supernatants was performed. The analysis has indicated that the different carbon sources used influenced the amounts of proteins secreted including 1,3-beta-glucanosyltransferase, alpha-1,2-mannosidase, alpha-galactosidase and glucan 1,3-beta-glucosidase. The analysis has also suggested the presence of beta-glucosidase, which could also be represented by PNPGase activity. Intracellular metabolites were quantified during PNPGase production for the condition using 20 g/L of glucose in the feed and differences were observed, indicating that intracellular glucose could be inhibiting PNPGase production.

Significance: This work shows that sugars such as glucose, fructose/glucose and sucrose can be used as substrates for the continuous synthesis of different enzymes under carbon-limited conditions by *Trichoderma harzianum*. As far as we know, this is the first work about the continuous synthesis of enzymes under carbon-limited conditions suggesting that different easily assimilated carbon sources can be used to generate different enzymatic cocktails. Each enzyme or uncharacterized protein suggested by shotgun proteomics has the potential to become a promising product for biotechnological applications.

1. Introduction

Proteins are a diverse group of molecules containing different properties such as catalytic activity, molecular weight and solubility [1]. Proteins are also a biotechnological product and their identification and characterization are important for understanding their functionalities and possible applications. In proteomics, a method called shotgun analysis can be used to identify proteins [2]. Proteolytic enzymes such as trypsin are used to digest the proteins, and due to the specificity of this enzyme, it generates peptides of a size that is more readily analysed in the mass spectrometer and facilitates the identification of the protein [1]. Liquid chromatography can be used to separate the peptides, and then peptide sequencing can be performed by tandem mass spectrometry (MS/MS) [1]. In this method, peptides are ionized and selected peptide ions are subjected to sequencing, which is determined by MS/MS spectra using a database search approach [2].

Beta-glucosidase is a group of enzymes responsible for catalysing the final step of the complete hydrolysis of cellulose into glucose [3]. Beta-glucosidase can present a high *p*-nitrophenyl-β-D-glucopyranoside (PNPG) specificity [3], thus the estimation of enzymatic activity using PNPG can be applied to verify the presence of enzymes that can catalyse the hydrolysis of glycosidic bonds like beta-glucosidase.

It was found that *T. harzianum* was able to synthesize extracellular enzymes that can catalyse the hydrolysis of PNPG (PNPGase) during steady-state under carbon-limited chemostat cultivations [4], especially when using glucose as the carbon source. When sucrose or an equimolar mixture of fructose and glucose were used, low levels of PNPGase activity were observed. To investigate whether under these conditions other enzymes were produced, a shotgun proteomics analysis of their supernatants was performed and it is presented in this work.

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Additionally, analysis of PNPGase production was evaluated according to the results of samples from continuous cultures using 20 g/L of glucose in the feed under carbon-limited conditions. Intracellular metabolites were analysed to verify the possible influence of their concentrations on PNPGase production.

2. Materials and methods

2.1. Culture conditions

Trichoderma harzianum P49P11 was isolated from the Amazon forest [5]. Culture conditions are described in Gelain [4]. The microorganism was grown on plates (potato dextrose agar) at 29 °C and after 5–7 days, the spores were harvested with sterilized water, distributed into vials of 1 mL and stored at −80 °C. Each vial was used to inoculate one shake flask (250 mL of medium). Spores were used to inoculate 500 mL shake flasks containing 250 mL of the medium: 10 g/L of glucose (carbon source), 2 g/L of KH_2PO_4, 5 g/L of $(NH_4)_2SO_4$, 0.3 g/L of $MgSO_4.7H_2O$, 0.3 g/L of $CaCl_2.2H_2O$, 1 mL/L of trace elements solution, and 1 g/L of peptone. Trace elements solution: 15 g/L of $Na_2EDTA.2H_2O$, 4.5 g/L of $ZnSO_4.7H_2O$, 1 g/L of $MnCl_2.4H_2O$, 0.3 g/L of $CoCl_2.6H_2O$, 0.3 g/L of $CuSO_4.5H_2O$, 0.4 g/L of $Na_2MoO_4.2H_2O$, 4.5 g/L of $CaCl_2.2H_2O$, 3 g/L of $FeSO_4.7H_2O$, 1 g/L of H_3BO_3, 0.1 g/L of KI. The medium was sterilized at 121 °C for 20 min. The shake flasks were incubated in an orbital shaker for 24–48 h at 29 °C and 200 rpm before inoculating the bioreactor (10% *v*/v).

Different limiting carbon sources were applied in continuous culture: 10 g/L of glucose (G), 10 g/L of sucrose (S), 5.26 and 5.26 g/L of fructose and glucose (FG). The medium composition was the same as described for shake flasks, only peptone was not added to the feed medium. Additionally, 20 g/L of glucose was also tested in the feed with the following modifications to the medium composition: 3 g/L of KH_2PO_4; and 6 g/L of $(NH_4)_2SO_4$. The medium composition used for the batch phase of the condition using 20 g/L of glucose was the same as used for the shake flask cultivation. The experiments G, S and FG were performed in sequence and 20 g/L of sucrose was used as the carbon source in the batch phase. The medium was sterilized by filtration using a filter 0.2 μm.

The experiments were performed using a 7 L bioreactor (Applikon, Delft, the Netherlands) and a constant broth mass of 4 kg. The temperature was controlled by a water bath at 29 °C, and pH 5 was controlled by the addition of 2 M KOH and 2 M H_2SO_4. Sterile air was supplied via a mass flow controller (Brooks 58,505, calibration at 0 °C and 1 bar). The volume fraction of oxygen and carbon dioxide were measured by the NGA 2000 off-gas analyser.

All chemostat experiments were carried out with a dilution rate of $0.05\ h^{-1} \pm 0.003\ h^{-1}$. For the batch phase, the stirring speed was kept between 200 and 400 rpm and for the continuous culture, it was changed to a constant stirring speed of 600 rpm. The airflow of 1 L/min was used, and only for the condition using 20 g/L of glucose, the airflow was 1.5 L/min. A constant antifoam addition (Basildon BC antifoam 86/013) of approximately 7 μL/min was used. The achievement of the steady-state was assumed when the CO_2 production and mycelium concentration were constant for at least 6 residence times.

2.2. Enzymatic activity

The method for the estimation of PNPGase activity was adapted from Zhang et al. [6] using *p*-nitrophenyl-β-D-glucopyranoside (PNPG) as the substrate. The reaction mixture was composed of 80 μL of 1 mM substrate diluted in 50 mM citrate buffer (pH 4.8), and 20 μL of the diluted enzyme extract. After 10 min at 50 °C, the reaction was stopped by adding 100 μL of 1 M sodium carbonate. The measurement was performed at 400 nm.

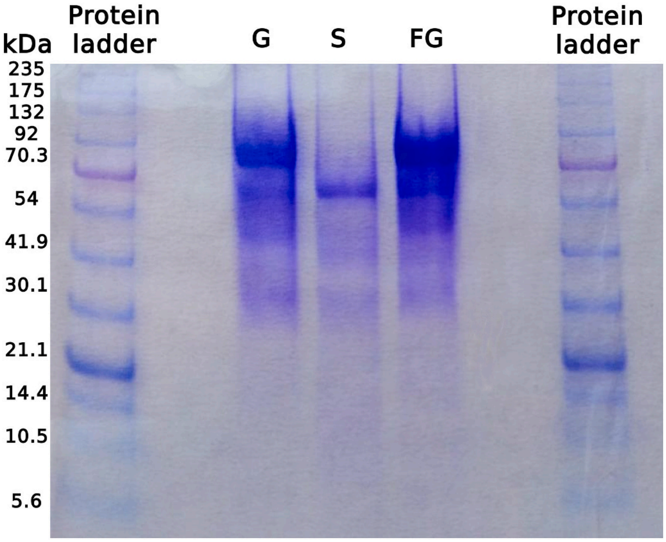

Fig. 1. SDS-PAGE for the conditions using glucose at 10 g/L (G), sucrose (S) and fructose/glucose (FG).

2.3. Quantification of intracellular metabolites

The estimation of the metabolites was performed in duplicate using different samples withdrawn at the same time (10 s difference). Intracellular metabolites were extracted and analysed according to Lameiras et al. [7]. Broth (approx. 1.3 mL) was rapidly withdrawn into 10 mL of pre-cooled 40% (*v*/v) aqueous methanol solution (−20 °C) and after, the samples were weighted for the estimation of cell mass and kept at −20 °C until extraction of the metabolites. Methanol was removed by filtration and the samples were washed thrice with cold methanol solution (−20 °C). Then, boiling ethanol extraction was performed to disrupt the cell and inactivate the enzymes. 25 mL of ethanol solution (75% *v*/v) was first pre-heated at 75 °C, and after the filtration and washing of the samples, they were placed into the ethanol solution along with 100 μL of U-^{13}C-labeled cell extract of *S. cerevisiae* as the internal standard and moved to a water bath at 95 °C for 3 min. After the extraction, the samples were first placed on ice and then stored at −80 °C.

Before the quantification of the metabolites, ethanol was evaporated until almost dryness in a Rapid-Vap under vacuum for 240 min. After evaporation, the residues were suspended in 500 μL of Milli-Q water, and centrifuged at 1000 ×*g* for 5 min in a tube coupled with a filter (0.22 μm) to remove cell debris. The supernatants were stored at −80 °C until analysis. The concentrations of the intracellular metabolites were measured by isotope dilution mass spectrometry (LC-IDMS/MS and GC-IDMS) according to the protocols of Dam et al. [8], Jonge et al. [9] and Cipollina et al. [10].

2.4. SDS-PAGE

The samples were concentrated using Amicon 10 kDa cut-off Eppendorf tube concentrators. Then, they were mixed with NuPAGE LDS sample buffer (4×) (Thermo Fischer Scientific, NP0007) at a ratio of 1 to 4 and heated at 70 °C for 15 min. For the SDS-PAGE analysis, a mini-protean polyacrylamide gel was prepared (10% resolving gel) (BIO-RAD, 1610182), 1 μg protein was loaded from each sample. SDS-PAGE was run using a MES buffer (Thermo Fischer Scientific, NP0002) at constant voltage (200 V) for 25 min. Following a short fixation, the gel was stained using Coomassie Brilliant Blue for 15 min and destained in 10% acetic acid over-night. The gel image was taken using a XY camera and the image was processed using the Microsoft Office Picture Manager, 2010.

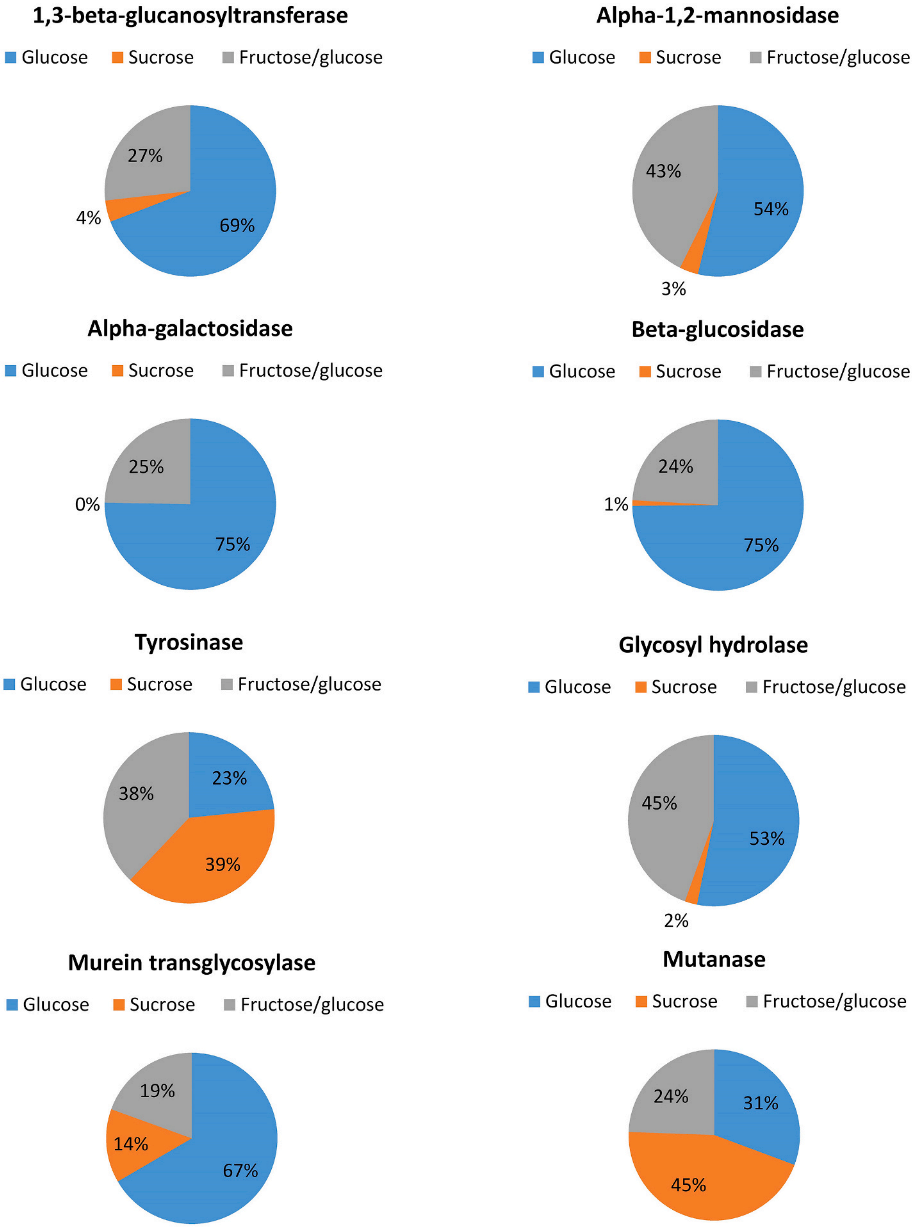

Fig. 2. Spectrum intensity (%), indicating the relative abundance for some groups of enzymes suggested by the shotgun proteomics analysis.

2.5. Shotgun proteomics and label-free quantification

Protein was precipitated from the supernatant using 4 volumes of ice-cold acetone at −20 °C for 20 min, centrifuged at 14.000 rpm for 10 min, and the pellet was washed twice using ice-cold acetone. The protein pellet was reconstituted in 200 mM ammonium bicarbonate containing 6 M urea. The protein solution was further reduced using DTT and then alkylated using iodoacetamide according to the protocol by Herbert et al. [11]. The protein solution was diluted using 200 mM ammonium bicarbonate buffer to approximately 1 M urea and further digested using trypsin in a ratio of protein to trypsin of approximately 50 to 1, at 37 °C over-night [11].

The proteolytic digest was purified using an Oasis HLB solid-phase extraction plate (Waters) and approximately 250 ng of the proteolytic digest was then analysed by a one-dimensional reverse-phase gradient (Acclaim PepMap RSLC 50 μm × 15 cm, 2 μm, 100 A, Thermo) using an EASY-nLC 1200 coupled to a QE plus Orbitrap mass spectrometer operating in top 10 DDA mode. Further details regarding one-dimensional shotgun proteomics approaches are described in Köcher et al. [12]. Tandem-MS data were analysed using PEAKS Studio 8.5 against the TrEMBL *Trichoderma harzianum* protein database (un-reviewed, taxon 5544 http://www.uniprot.org/), and results were filtered for < 1% FDR for both, peptide and protein identification. 3 replicate injections for each condition were finally analysed using the label-free quantification option provided by PEAKS Studio 8.5 [13].

3. Results and discussion

3.1. Secretome analysis

SDS-PAGE analysis was performed in the supernatant of the samples from carbon-limited chemostat cultivations using glucose (G), sucrose (S), and fructose/glucose (FG) (Fig. 1). For the glucose and fructose/glucose-limited chemostat cultures, more intense bands were observed than for the sucrose-limited culture, indicating higher protein concentrations. The molecular weights of the majority of the proteins were between 70.3 and 92 kDa for the glucose and fructose/glucose-limited cultures. For the sucrose-limited culture, the molecular weight of the

Table 1

Description of some enzymes from shotgun proteomics analysis, beta-glucosidase is highlighted in grey frames, G – glucose at 10 g/L, FG – fructose/glucose, and S – sucrose.

Accession	Coverage (%)	Peptides	Unique Peptides	Blank Intensity	G Intensity	S Intensity	FG Intensity	Avg. Mass	Description, GN – gene name, PE – protein existence, SV – sequence version
A0A0G0A6L8|A0A0G0A6L8_TRIHA	43	30	29	5.00E+06	5.51E+08	2.98E+07	6.51E+08	83322	Glycosyl hydrolase, GN=THAR02_00025, PE=4, SV=1
A0A0F9ZTK2|A0A0F9ZTK2_TRIHA	24	17	17	4.13E+05	4.52E+07	0	2.94E+07	104391	Glycosyl hydrolase family 3 N terminal domain-containing protein, GN=THAR02_04349, PE=4, SV=1
A0A0G0AG54|A0A0G0AG54_TRIHA	29	23	23	2.43E+06	3.96E+08	6.78E+06	2.00E+08	104855	Glycosyl hydrolase family 31, GN=THAR02_03951, PE=3, SV=1
A0A0G0A0B1|A0A0G0A0B1_TRIHA	13	9	9	0	7.50E+07	0	3.03E+07	92668	Glycosyl hydrolase family 3 N terminal domain-containing protein, GN=THAR02_02181, PE=4, SV=1
A0A0F9Y0X0|A0A0F9Y0X0_TRIHA	37	26	24	2.35E+06	8.65E+08	1.23E+07	2.77E+08	92791	Beta-glucosidase, GN=THAR02_02132, PE=3, SV=1
A0A0F9X7W2|A0A0F9X7W2_TRIHA	8	8	7	0	1.38E+07	0	5.74E+06	95013	Beta-glucosidase, GN=THAR02_07292, PE=4, SV=1
A0A0F9ZUH3|A0A0F9ZUH3_TRIHA	14	6	6	1.36E+05	5.93E+07	1.03E+07	4.71E+07	52933	1,3-beta-glucanosyltransferase, GN=THAR02_04021, PE=3, SV=1
A0A0F9ZGV2|A0A0F9ZGV2_TRIHA	11	5	5	6.70E+05	5.52E+07	9.15E+07	8.96E+07	60083	Tyrosinase, GN=THAR02_08385, PE=4, SV=1
A0A0F9ZZ00|A0A0F9ZZ00_TRIHA	13	5	5	1.74E+05	5.71E+07	2.73E+08	7.89E+07	46519	Mutanase, GN=THAR02_09460, PE=4, SV=1
A0A0F9ZTK0|A0A0F9ZTK0_TRIHA	12	5	5	4.37E+05	4.45E+07	1.86E+07	1.19E+07	63234	Murein transglycosylase, GN=THAR02_04344, PE=4, SV=1
A0A0F9XN15|A0A0F9XN15_TRIHA	16	7	7	3.21E+05	7.52E+07	0	2.40E+07	48188	Alpha-galactosidase, GN=THAR02_01852, PE=3, SV=1
A0A0F9WYR7|A0A0F9WYR7_TRIHA	13	4	4	6.56E+04	5.74E+07	0	1.69E+07	55601	Alpha-1,2-Mannosidase, GN=THAR02_10337, PE=3, SV=1

majority of the proteins was between 54 and 70.3 kDa.

Shotgun proteomics analysis was performed and indicates the presence of 207 proteins (92 enzymes and 115 other proteins of which 99 are uncharacterized). Fig. 2 highlights spectrum intensity, indicating the relative abundance for specific groups of enzymes. For the glucose-limited condition, higher intensities were observed for most of the proteins, indicating higher protein levels. According to Owen et al. [14], the relative intensity of each spectrum peak is proportional to the peptide concentration; this allows a relative quantification across different experimental conditions. Table 1 shows the result of some enzymes related to Fig. 2. Several enzymes were found including four 1,3-beta-glucanosyltransferase, two alpha-1,2-mannosidase, two alpha-galactosidase, two beta-glucosidase, two glucan 1,3-beta-glucosidase, two glucan endo-1,3-beta-glucosidase, thirteen glycosyl hydrolase and three mutanase. The complete table is presented in the supplementary material. It presents the coverage, the number of peptides and unique peptides identified, and intensities of the samples for each protein. Beta-glucosidase is highlighted in Table 1, the first one had coverage of 37%, 26 peptides, 24 unique peptides, 93 kDa and the second one, coverage of 8%, 8 peptides, 7 unique peptides and 95 kDa.

Vale et al. [15] reported a secretome analysis of *T. harzianum* using batch cultivation on cellulose performed in Erlenmeyer flasks with a liquid volume of 20 mL. They identified 56 proteins based on at least 2 unique peptides. In this project, the proteins with at least 1 unique peptide are presented to visualize the possible enzymatic cocktail secreted. Nevertheless, there are 114 proteins identified based on at least 2 peptides and 93 with only 1 unique peptide. According to Vale et al. [15], nowadays, high MS accuracy, low ppm mass errors, and rich MS/MS data, provided by mass spectrometers, offer excellent identification even based on single peptides.

Fig. 3 indicates through a heat map, the presence of proteins in the supernatant of the three samples analysed (G, S and FG). The B sample corresponds to the blank (no protein present, bright green colour). The samples came from continuous cultures with a dilution rate of 0.05 h^{-1} and the differences observed are mainly due to the carbon sources used since no other variables were changed. Clear differences in protein abundances were observed between the three experimental conditions. If they would have been similar, this would indicate the constitutive production of these enzymes/proteins; however, the difference observed indicates that their production was possibly influenced by different inducers. Qualitative analyses such as ethanol precipitation, followed by hydrolysis of the precipitate confirmed the presence of extracellular polysaccharides in all conditions [4]. The presence of extracellular polysaccharides suggests that they were the source of inducers.

Gómez-Mendoza et al. [16] evaluated the *T. harzianum* secretome using glucose, carboxymethyl cellulose, xylan and an agricultural by-product (sugarcane bagasse) as carbon sources. The experiments were performed in shake flasks containing 1% (*w/v*) of the carbon source with a liquid volume of 30 mL. Glucose was used as the promoter of catabolite repression of enzymes and the condition resulted in the secretion of 107 groups of proteins of which 40 were exclusively identified in this carbon source. The secretion of cellobiohydrolase and beta-glucosidase were detected for this condition and their presence was attributed to the consequence of low constitutive enzyme production. In this project, however, the different colour intensities in Fig. 3 suggest that glucose condition was capable of inducing the production of proteins.

The difference between G and FG conditions is the lack of fructose in the G condition. This change resulted in different protein production as indicated in Fig. 3. It seems that the presence of fructose inhibited/induced the production of different proteins, but the samples also share common ones. The sucrose-limited condition resulted in the lowest protein levels in the culture supernatant. The use of monosaccharides (fructose and glucose – FG) had a positive influence on the diversity of the proteins/enzymes than the disaccharide used. The glucose-limited condition showed the highest diversity of proteins. It seems that each condition provided a distinct enzymatic cocktail, which could be related to the different carbon sources used. However, more research is needed to identify the uncharacterized proteins suggested by shotgun proteomics analysis.

3.2. *Analysis of intracellular metabolites during PNPGase production under glucose-limited conditions*

The intracellular metabolites from the tricarboxylic acid cycle, glycolysis and pentose phosphate pathway, as well as nucleotides, were quantified for samples from glucose-limited chemostat cultures named A and B using 20 g/L in the feed. Both samples (A and B) were withdrawn during the steady-state of cells and CO_2. The difference between them is that sample A was not at steady-state of PNPGase production. The Tukey test with 95% confidence interval (OriginPro 8 software) was applied to analyse the average values for each metabolite. Significant differences among metabolite levels could indicate

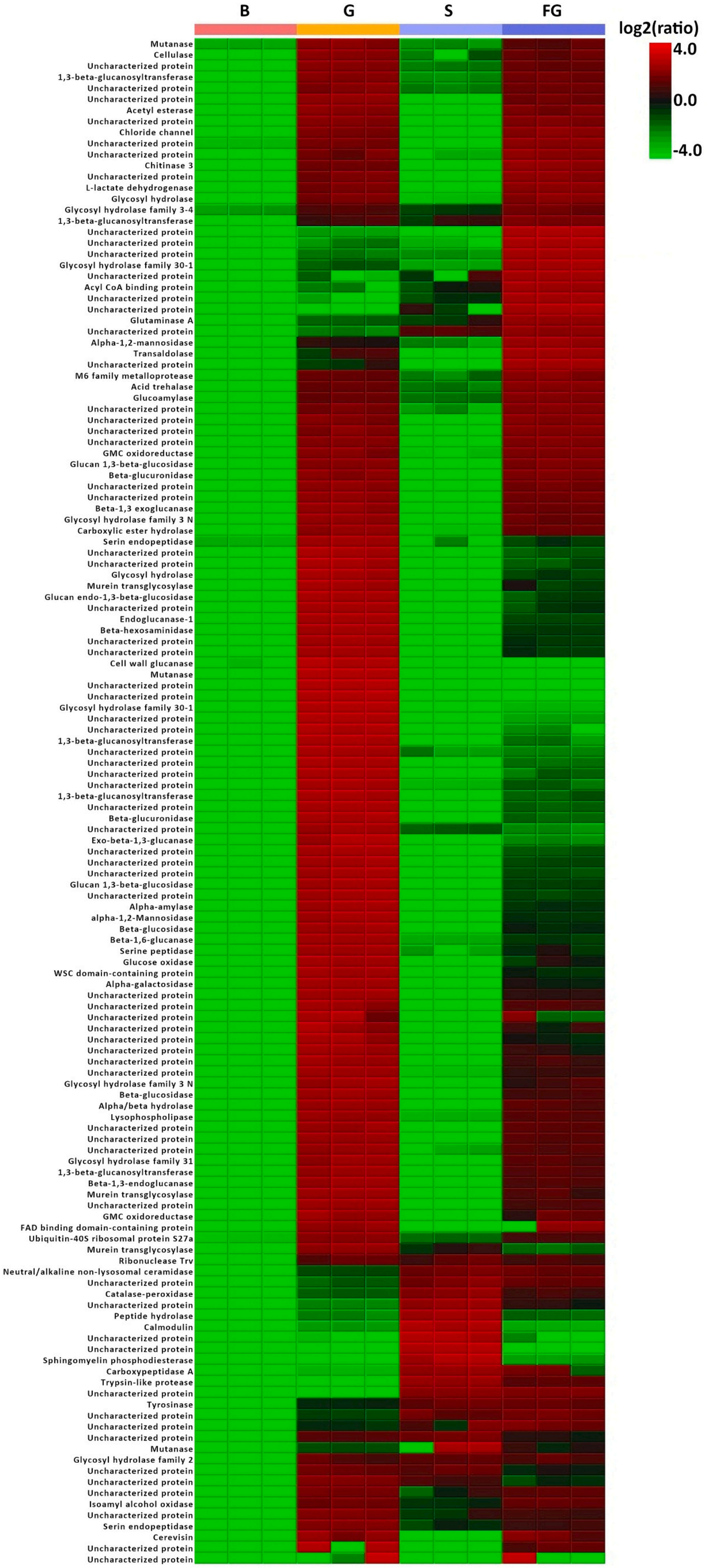

Fig. 3. Heat map from the shotgun proteomics analysis (triplicate), indicating the possible enzymes present in the supernatant of samples at steady-state, B – blank, G – glucose at 10 g/L, FG – fructose/glucose, and S – sucrose.

Table 2
Intracellular metabolite levels analysed for samples from glucose-limited chemostat cultures at non steady-state (A) and at steady state (B) of PNPGase production, metabolites with significantly different levels are indicated in blue (Tukey test with 95% confidence interval).

	A (μmol/g)	B (μmol/g)		A (μmol/g)	B (μmol/g)
Gluc	31.789 ± 2.978	1.559 ± 0.375	**Fum**	1.104 ± 0.016	0.970 ± 0.093
G6P	0.937 ± 0.442	1.417 ± 0.023	**G3P**	0.226 ± 0.060	1.783 ± 0.112
F6P	0.227 ± 0.105	0.298 ± 0.019	**G1P**	0.030 ± 0.012	0.063 ± 0.011
FBP	0.381 ± 0.139	0.410 ± 0.008	**UDPG**	1.256 ± 0.049	1.231 ± 0.003
GAP	0.015 ± 0.001	0.026 ± 0.002	**T6P**	0.025 ± 0.006	0.051 ± 0.007
DHAP	0.108 ± 0.021	0.153 ± 0.014	**Tre**	112.059 ± 16.836	49.693 ± 4.970
3PG	0.396 ± 0.001	0.497 ± 0.020	**M6P**	0.388 ± 0.146	0.478 ± 0.020
2PG	0.041 ± 0.001	0.054 ± 0.004	**AMP**	0.296 ± 0.006	0.533 ± 0.042
PEP	0.011 ± 0.001	0.017 ± 0.0001	**ADP**	1.484 ± 0.055	0.993 ± 0.129
M1P	0.087 ± 0.001	0.097 ± 0.014	**ATP**	2.726 ± 0.083	2.871 ± 0.476
6PG	0.745 ± 0.273	0.618 ± 0.044	**cAMP**	0.018 ± 0.00005	0.021 ± 0.004
Ribu5P	0.283 ± 0.006	0.222 ± 0.038	**UMP**	0.053 ± 0.008	0.065 ± 0.007
Rib5P	0.615 ± 0.033	0.386 ± 0.030	**UDP**	0.129 ± 0.015	0.110 ± 0.010
Xyl5P	0.382 ± 0.004	0.357 ± 0.057	**UTP**	0.715 ± 0.002	0.511 ± 0.096
S7P	0.786 ± 0.021	0.443 ± 0.025	**GMP**	0.213 ± 0.021	0.152 ± 0.012
E4P	0.0046 ± 0.0003	0.0049 ± 0.0004	**GDP**	0.149 ± 0.009	0.222 ± 0.035
Cit	6.656 ± 0.039	13.080 ± 1.005	**GTP**	0.766 ± 0.018	0.679 ± 0.089
iCit	0.098 ± 0.001	0.278 ± 0.014	**CMP**	0.031 ± 0.003	0.058 ± 0.006
αKG	0.525 ± 0.004	0.649 ± 0.043	**CDP**	0.066 ± 0.007	0.061 ± 0.008
Suc	0.590 ± 0.027	1.143 ± 0.242	**CTP**	0.353 ± 0.016	0.180 ± 0.029
Mal	2.441 ± 0.155	3.034 ± 0.223			

2PG 2-phosphoglycerate, **3PG** 3-phosphoglycerate, **6PG** 6-phosphogluconate, **ADP** Adenosine diphosphate, **αKG** α-Ketoglutarate, **AMP** Adenosine monophosphate, **ATP** Adenosine triphosphate, **cAMP** Cyclic AMP, **CDP** Cytidine diphosphate, **Cit** Citrate, **CMP** Cytidine monophosphate, **CTP** Cytidine triphosphate, **DHAP** Dihydroxyacetone phosphate, **E4P** Erythrose-4-phosphate, **F6P** Fructose-6-phosphate, **FBP** Fructose-1,6-bisphosphate, **Fum** Fumarate, **G1P** Glucose-1-phosphate, **G3P** Glycerol-3-phosphate, **G6P** Glucose-6-phosphate, **GAP** Glyceraldehyde-3-phosphate, **GDP** Guanosine diphosphate, **Gluc** Glucose, **GMP** Guanosine monophosphate, **GTP** Guanosine triphosphate, **iCit** Isocitrate, **M1P** Mannitol-1-phosphate, **M6P** Mannose-6-phosphate, **Mal** Malate, **PEP** Phosphoenolpyruvate, **Rib5P** Ribose-5-phosphate, **Ribu5P** Ribulose-5-phosphate, **S7P** Sedoheptulose-7-phosphate, **Suc** Succinate, **T6P** Trehalose-6-phosphate, **Tre** Trehalose, **UDP** Uridine diphosphate, **UDPG** Uridine-5-diphosphoglucose, **UMP** Uridine monophosphate, **UTP** Uridine triphosphate, and **Xyl5P** Xylulose-5-phosphate.

metabolites that might be directly or indirectly related to PNPGase production. Statistically significant differences in concentrations of the metabolites are highlighted in blue (Table 2).

The high experimental errors observed for some metabolites could be attributed to the sample processing and/or analytical errors. Another explanation considered is a possible heterogeneity of cells inside the bioreactor caused by the consumption of fragments of polysaccharides under carbon-limited conditions. Considering that the highest shear rates in a mixing vessel are close to the blades of the impeller [17], there are regions with different shear rates inside the bioreactor. Fragments of polysaccharides from the cell wall can be released due to this shear stress [18], and because of different shear rate regions inside the bioreactor, it could also be expected that there exist regions with different concentrations of fragments. Since the cells can consume these fragments, the cells closer to the regions with a higher concentration of fragments could present different concentrations of some intracellular metabolites than the cells farther from that region.

Although samples A and B were at steady-state of cell concentration and CO_2 production, it seems that there was another parameter, perhaps polysaccharide fragments, influencing the intracellular metabolite concentrations and the changes highlighted in Table 2 were not considered as resulting from sample processing and/or analytical errors due to the low standard experimental errors.

Table 2 shows a significant difference regarding intracellular glucose concentration that decreases from sample A to B and its concentration is inversely proportional to PNPGase activity (A = 1.1 ± 0.1 U/mL, B = 6.2 ± 0.4 U/mL). The higher glucose concentration for sample A could explain the lower enzymatic activity. Perhaps at this stage (A), the process was still unstable and glucose was inhibiting the inducer uptake. Additionally, 1.6 μmol/g (B) seems to be an intracellular glucose concentration that could prevent carbon catabolite repression.

Based on PNPGase activity and intracellular glucose concentration, it can be considered that sample A provides information on an inhibition-state and sample B provides information of an inducer-state for PNPGase synthesis. For sample B, the uptake rate of inducer fragments of PNPGase synthesis was probably higher than for sample A, meanwhile, the uptake rate of hydrolysed fragments was higher for sample A, provoking inhibition. Therefore, significant differences observed between some metabolite levels indicate changes in the metabolic pathway when there is a higher consumption of inducers (sample B) or inhibitors (sample A) of PNPGase production. The higher uptake of inducers resulted in higher concentrations of metabolites from the tricarboxylic acid cycle, part of glycolysis (from glyceraldehyde-3-phosphate to phosphoenolpyruvate) and lower values for metabolites from the pentose phosphate pathway. Nevertheless, more experiments have to be conducted to verify whether the metabolic profiles observed always follow this pattern.

4. Conclusions

Shotgun proteomics and SDS-PAGE analysis were performed for the proteins present in the supernatant of carbon-limited chemostat cultures using glucose, fructose/glucose and sucrose as carbon sources. The shotgun proteomics analysis has indicated that the different carbon sources used greatly influenced the amounts of secreted proteins, of which many of them are enzymes. The possible differences regarding the enzymes secreted could be related to the presence of different inducers. This brings the possibility of creating a hypothesis that different carbon sources easily assimilated by the cells under carbon-limited conditions could lead to the synthesis of different inducers and thus different enzymes. Several uncharacterized proteins were also suggested by the analysis and methods to identify them and their functions could reveal new promising products. Analysis of the intracellular metabolites has suggested that high intracellular glucose concentrations can inhibit the production of PNPGase.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2020.103922.

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