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Continuous production of enzymes under carbon-limited conditions by *Trichoderma harzianum* P49P11

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ABSTRACT

Carbon-limited chemostat cultures were performed using different carbon sources (glucose, 10 and 20 g/L; sucrose, 10 g/L; fructose/glucose, 5.26/5.26 g/L; carboxymethyl cellulose, 10 g/L; and carboxymethyl cellulose/glucose, 5/5 g/L) to verify the capability of the wild type strain *Trichoderma harzianum* to produce extracellular enzymes. All chemostat cultures were carried out at a fixed dilution rate of 0.05 h^{-1}. Experiments using glucose, fructose/glucose and sucrose were performed in duplicate. Glucose condition was found to induce the production of enzymes that can catalyse the hydrolysis of p-nitrophenyl-β-D-glucopyranoside (PNPGase). A concentration of 20 g/L of glucose in the feed provided the highest productivity (1048 ± 16 U/mol h). Extracellular polysaccharides were considered the source of inducers. Based on the obtained results, a new PNPGase production process was developed using mainly glucose. This process raises interesting possibilities of synthesizing the inducer substrate and the induced enzymes in a single step using an easily assimilated carbon source under carbon-limited conditions.

1. Introduction

Continuous processes provide possibilities for attaining higher productivity in comparison with batch cultures. According to Peebo and Neubauer (2018), experiments in continuous cultures result in a steady-state operation that is easier to characterize and can also have economic benefits. A major advantage of chemostat cultures is the option of maintaining cells in a producing state for extended periods, which can lead to high volumetric productivities (Peebo and Neubauer, 2018).

Carbon catabolite repression (CCR) is one of the mechanisms that prevent the unnecessary synthesis of cellulase. In this mechanism, the presence of an easily assimilated substrate (e.g. glucose) activates the repression of the production of enzymes responsible for the degradation of complex substrates (Suto and Tomita, 2001). During growth under carbon-limited conditions, it is expected that CCR does not occur due to low substrate concentration. Xiong et al. (2014) reported that a transcription factor (*vib-1*) could repress CCR under carbon-limited conditions in *Neurospora crassa*. Apart from CCR, other mechanisms can be responsible for controlling the production of enzymes. Catabolite inhibition is also a mechanism reported to control enzyme production by inhibiting the transport of the inducer (McGinnis and Paigen, 1969). The latter mechanism could be more active under carbon-limited conditions.

Beta-glucosidase can be divided into cellobiase, with a high cellobiose specificity, and aryl-beta-glucosidase, with a high p-nitrophenyl-β-D-glucopyranoside (PNPG) specificity (Eyzaguirre et al., 2005). There are also beta-glucosidases that can act on several substrates and these enzymes are the most commonly observed (Bhatia et al., 2002). Due to the hydrolytic and synthetic activities of beta-glucosidases, they can have several biotechnological applications (Bhatia et al., 2002). For efficient saccharification of lignocellulosic materials by cellulase, high activity of beta-glucosidase is needed to prevent inhibition by cellobiose (Sørensen et al., 2013). Reverse hydrolysis and transglycosylation reactions performed by beta-glucosidases can be applied for the synthesis of oligosaccharides aiming at the manufacture of

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pharmaceuticals, fine chemicals, and food ingredients (Bhatia et al., 2002).

Glucose, usually reported as a repressor of the synthesis of cellulase, can be used as the substrate for the production of enzymes that can catalyse the hydrolysis of p-nitrophenyl-β-D-glucopyranoside (PNPGase) in batch cultivation (Jäger et al., 2001) as well as in carbon-limited continuous cultures (Strobel and Russell, 1987). The presence of PNPGase during the growth on glucose as the sole carbon source could be due to a constitutive production. Another possibility could be the presence of extracellular polysaccharides whose fragments, under carbon-limited conditions, could stimulate the production of enzymes, thus the cells could use the polysaccharides as another carbon source. Pessoni et al. (2015) reported that fructose could induce β-fructofuranosidase activity in *Penicillium janczewskii*, which could be related to structural changes in the cell wall (a decrease in the cell wall thickness) when the carbon source was depleted, indicating that polysaccharides released from the cell wall could be inducing enzymes.

In this work, the production of cellulase and PNPGase were measured in carbon-limited chemostat cultures employing the wild strain *Trichoderma harzianum* using different carbon sources. This study shows the potential of this microorganism to produce PNPGase continuously and suggests that the optimization of the operating conditions as well as the strain could lead to a promising enzyme production process using mainly glucose.

2. Materials and methods

2.1. Microorganism

A wild type strain, *T. harzianum* P49P11, was used in this study. The strain was isolated from the Amazon forest (Delabona et al., 2012). The microorganism was grown on plates (potato dextrose agar) at 29 °C and after 5–7 d, 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).

2.2. Culture conditions

Spores from *T. harzianum* were used to inoculate 500 mL shake flasks containing 250 mL of 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 to evaluate the production of the enzymes in continuous cultures: 10 g/L of glucose (G101 and G102), 10 g/L of sucrose (S1 and S2), 5.26 and 5.26 g/L of fructose and glucose (FG1 and FG2), 10 g/L of carboxymethyl cellulose (CMC), 5 and 5 g/L of carboxymethyl cellulose and glucose (CMCG). 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 (G201 and G202) with the following modifications to the medium composition: 3 g/L of KH_2PO_4; and 6 g/L of $(NH_4)_2SO_4$. These alterations were made to keep the same residual concentrations of these compounds in the effluent as for the condition using 10 g/L of glucose. All chemostat cultivations with glucose, sucrose and fructose/glucose as the carbon sources were performed in duplicate. The medium composition used for the batch phase, preceding the chemostat phase, was the same as used for the shake flasks cultivations, except for the first batch experiment in which 20 g/L of sucrose was used as the carbon source. Glucose consumption was faster than sucrose in the batch phase, thus sucrose was replaced by glucose for the next experiments. The media for the batch and chemostat phase were sterilized by filtration using a filter 0.2 μm.

2.3. Chemostat system

The experiments were performed using a 7 L bioreactor (Applikon, Delft, the Netherlands). The mass of the cultivation medium was controlled through a pneumatic system connected to a control unit, which opened the valve at the bottom of the bioreactor and started an effluent pump when it was needed to maintain 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 58505, calibration at 0 °C and 1 bar). The gas outflow was passed through a condenser at 4 °C and a Nafion dryer before the volume fraction of oxygen and carbon dioxide were measured by the NGA 2000 off-gas analyser. An overpressure of 0.2–0.3 bar was applied.

2.4. Operating conditions

All chemostat experiments were started in batch mode and after a sharp drop of the CO_2 production, observed after 22–24 h, the process was switched to carbon-limited continuous cultures with a dilution rate of 0.05 ± 0.003 h^{-1}.

For the batch phase, the stirring speed was kept between 200 and 400 rpm. The stirring speed was dependent on the values of dissolved oxygen to guarantee an excess of oxygen in the growth medium. For the continuous culture, it was changed to a constant stirring speed of 600 rpm. 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 as well as a constant PNPGase activity for at least 4 residence times.

Samples for quantification of the mycelium concentration and enzyme activities were taken before the steady-state for some conditions to observe the profiles, and at steady-state for all the conditions to also estimate the concentration of residual sugars, total organic carbon (TOC) and total nitrogen (TN). For sugar analysis, the samples were frozen in liquid nitrogen right after the filtration by 0.45 μm filter (Millex-HV durapore PVDF membrane) and stored at −80 °C. The experimental errors calculated correspond to the population standard deviation.

2.5. Enzymatic activity

The enzymatic activity corresponds to enzyme units per volume of medium and was performed according to Gelain et al. (2015). Enzymatic activity of cellulase was determined using the filter paper activity (FPA) assay (Ghose, 1987). The scale was 10 times reduced to minimize time and reagents. Filter paper activity was assayed by incubating the diluted enzymes (50 μL) with 100 μL of 50 mM sodium citrate buffer (pH 4.8) containing the filter paper Whatman No. 1 (5 mg). The reaction mixture was incubated at 50 °C for 60 min and stopped by adding 300 μL of DNS reagent. Reducing sugars were measured by the DNS method (Miller, 1959). The solution was placed in a water bath at 95 °C for 5 min and, after

cooling, 2 mL of water was added and the measurement was made at 540 nm in the spectrophotometer.

The method for the estimation of the PNPGase activity was adapted from Zhang et al. (2009). The activity was measured 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 the addition of 100 μL of 1 M sodium carbonate. The measurement was made at 400 nm.

2.6. Sugar analysis

Glucose, sucrose and fructose were measured. For this analysis, the samples were diluted with 1 M NaOH to precipitate the proteins that could interfere with the analysis. Subsequently, the precipitate was removed by centrifugation (2000×*g*, 10 min). The samples were analysed using high-performance anion-exchange (HPAE) Dionex ICS-5000 with PAD detector (Rohrer et al., 2013).

2.7. Total organic carbon (TOC) and total nitrogen (TN)

For the determination of the TOC and TN, 3 mL of supernatant was filtered by 0.45 μm membrane (Millex-HV durapore PVDF membrane) and analysed with a Shimadzu TOC-L analyser using the difference method for the TOC estimation and the total nitrogen unit (TNM-L) for TN estimation.

2.8. Dry cell weight concentration

For the estimation of dry cell weight, 5 mL of sample was withdrawn from the bioreactor. The weight of the samples was measured and then they were poured on a 0.45 μm filter of known mass (Pall membrane filter, Supor). Subsequently, the cells were filtered and washed with Milli-Q water. The filter was placed in a 70 °C oven for one day and cooled down in a desiccator before the measurement of the weight.

2.9. Qualitative analysis of polysaccharides

From supernatant samples filtered through 0.45 μm pore size membrane filters (Millex-HV durapore PVDF membrane), qualitative analysis of polysaccharides was performed using ethanol precipitation whereby 1 mL of sample was mixed with 3 mL of pure ethanol. Then, after centrifuging at 2000×*g* for 5 min, the precipitate was solubilized with water (1 mL) and precipitated again with ethanol (3 mL), followed by a second centrifugation step, whereafter the precipitate was freeze-dried. Fourier-transform infrared spectroscopy (FT-IR) was performed placing 2–5 mg of precipitate on a universal attenuated total reflectance accessory (PerkinElmer spectrum 100).

2.10. Carbon recovery, by-products and specific rates

For all chemostat experiments, the carbon balances were calculated to verify the production of by-products. Carbon recovery (C_r, %) is described by Equation (1), considering the carbon dioxide production rate (R_{CO_2}, Cmol/h), cell production rate (R_X, Cmol/h), carbon from by-product formation (R_{byp}, Cmol/h) and carbon consumption rate (R_C, Cmol/h).

$$C_r(\%) = \left(\frac{R_{CO_2} + R_X + R_{byp}}{R_C}\right)100 \quad (1)$$

By-products were estimated using the total organic carbon analysis (C_{TOC}, Cmol/kg) by Equation (2). For the carbon balance, all the carbon from the feed was considered to be consumed due to the low concentration of residual sugars (C_{sugars}, Cmol/kg) in the liquid outflow (F_l^{out}, kg/h). Carbon present in the proteins was not considered due to the low concentration and unknown composition.

$$R_{byp} = F_l^{out}(C_{TOC} - C_{sugars}) \quad (2)$$

Because no elemental composition analysis of this microorganism was performed, the carbon fraction in the cells was assumed as being the same as *Trichoderma reesei* QM9414 (Ross et al., 1983): $CH_{1.8}O_{0.71}N_{0.143}$ + 9.4 % of ashes.

Specific production and consumption rates (q_i, mol/mol h) were defined as described by Equation (3), where i is the component analysed, R_i (mol/h) is the production or consumption rate, C_X (mol/kg) is the cell concentration and M_l (kg) is the mass of liquid.

$$q_i = \frac{R_i}{C_X M_l} \quad (3)$$

3. Results and discussion

3.1. Enzyme production using different carbon sources

Previous experiments in batch mode with different initial concentrations of sucrose (Fig. 1) demonstrated that *T. harzianum* was able to synthesize PNPGase. The culture and operating conditions used were similar as reported in section 2.2 for batch mode, with the addition of 0.15 g/L of urea. For this reason, sucrose was used as the carbon source in carbon-limited chemostat cultures to verify whether the synthesis of PNPGase would also occur in this condition. Cellulase activity was also analysed for all the conditions studied in this project, however, these enzymes were not present in the supernatant according to the analytical methods employed.

The chemostat cultivation of *T. harzianum* was initiated by first performing batch cultivation using 20 g/L of sucrose as the sole carbon source. After the batch phase was finished, the culture was switched to chemostat mode, using 10 g/L of sucrose in the feeding medium (S1) (Fig. 2). During the first six d of chemostat cultivation, corresponding to approximately seven volume changes, the dry cell weight concentration decreased from close to 10 g/L to a steady-state concentration of 4.5 ± 0.2 g/L. Thereafter, there was a decrease in the measured PNPGase activity in the supernatant until achieving a steady-state value of around 0.1 U/mL, which corresponded to specific productivity of about 1 U/g (cells) h. This value was significantly lower than the specific productivity calculated from the batch culture data (Fig. 1), which was approximately 6.7 U/g (cells) h from 12 to 48 h during the carbon excess phase with an initial sucrose concentration of 20 g/L. This shows that the sucrose-limited condition was not beneficial for PNPGase production.

After maintaining the steady-state on sucrose for another ten volume changes, the chemostat feed medium was replaced by a medium containing an equimolar mixture of fructose and glucose (FG1), whereby the amounts of glucose and fructose were the same as for the condition using only sucrose, but in this case, the sugars were available as the monomers of the disaccharide. As can be observed in Fig. 2, the cell concentration remained the same, as was expected, however, the enzymatic activity increased roughly by a factor of two. After 15 days (18 volume changes) the feed was switched to 10 g/L of glucose as the sole carbon source (G101). Again, the cell concentration remained the same but a sharp

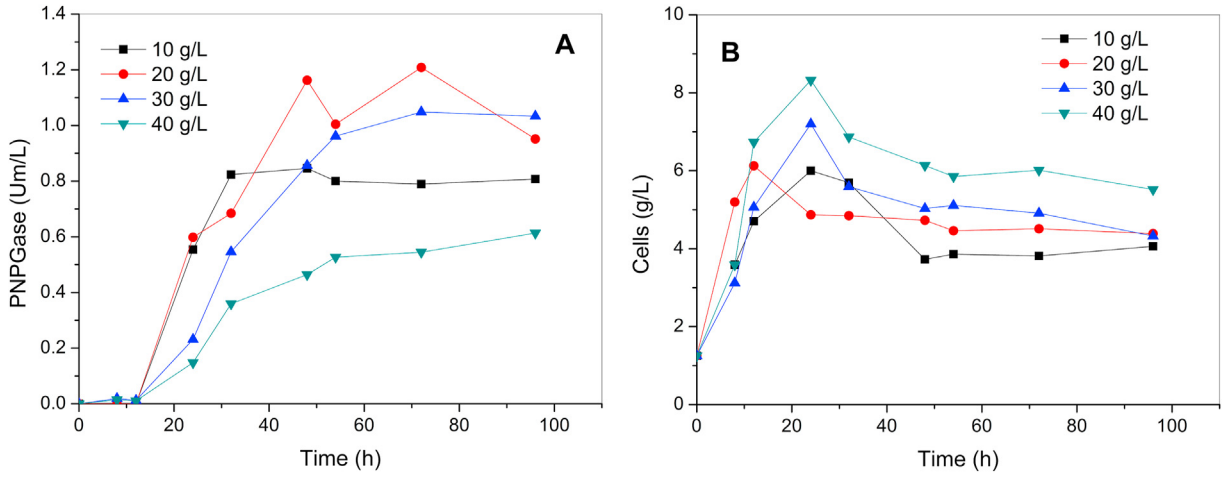

Fig. 1. PNPGase activity (A) and cell concentration (B) during batch cultivations using different initial concentrations of sucrose.

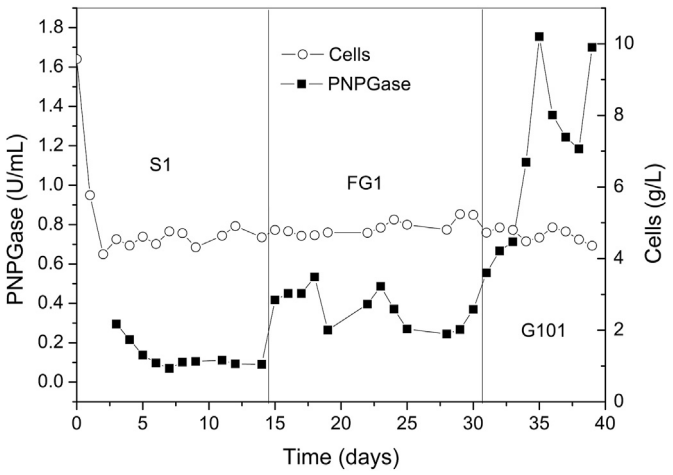

Fig. 2. Continuous PNPGase production using sucrose (S1), fructose/glucose (FG1) and glucose (G101) in sequence.

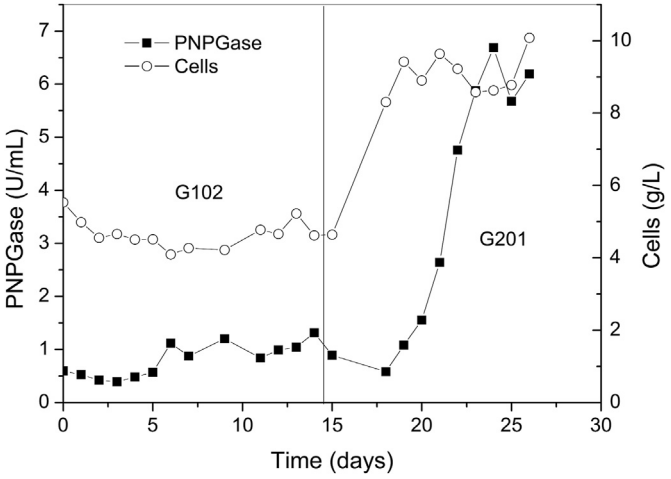

Fig. 3. Continuous PNPGase production using glucose at 10 (G102) and 20 g/L (G201) in sequence.

increase in PNPGase activity was observed. The activity achieved an increase of around 17 and 5 times when compared to the sucrose and fructose/glucose conditions, respectively.

This first continuous culture in sequence indicated that under carbon-limited conditions, the presence of sucrose and fructose might have inhibited PNPGase synthesis, however, glucose fed as monomer could stimulate the production.

Although glucose as the sole carbon source provided the highest activity, this was achieved after 36 volume changes in the carbon-limited chemostat cultivation. Therefore, a second chemostat experiment was carried out starting with glucose at 10 g/L (G102), to verify the reproducibility of the measured enzyme activity during the glucose-limited condition. After the steady-state was obtained, the glucose concentration in the feed medium was increased to 20 g/L (G201) to verify whether an increase in the cell concentration at steady-state would result in a proportional increase in PNPGase production.

Fig. 3 shows the result of this sequential chemostat experiment. The batch phase was performed with an initial glucose concentration of 10 g/L. The PNPGase activity after the batch phase was comparable to what was observed in the previous experiment (Fig. 2). After the start of the chemostat phase with 10 g/L of glucose in the feed, the activity of the enzymes remained at a low level and increased after the 5th d (Fig. 3), to reach a more or less stable value of 1 U/mL. On the 14th d, after 17 volume changes, the feed was switched to 20 g/L of glucose. After another 4 volume changes, the enzymatic activity sharply increased to 6 U/mL, 6 times higher than the condition using 10 g/L of glucose, while a doubling was expected if the specific productivity (U/g (cells) h) would have remained the same. These data indicate that an increase in cell concentration leads to a significant increase in specific PNPGase productivity.

A third glucose-limited chemostat experiment was carried out with a glucose concentration of 20 g/L in the feed medium (G202). Initially, the measured PNPGase activity in the supernatant was low but sharply increased to a value of 7 U/mL after 7 d of chemostat cultivation (Fig. 4), thus reaching a similar value as in the first 20 g/L of glucose chemostat (G201). The delay observed in Fig. 4 for enzyme production could be related to the transition phase (phase between the batch and steady-state of PNPGase production), where an inducer substrate could be absent or at an inhibiting concentration. After day 12 (Fig. 4), the enzymatic activity seems to be decreasing or oscillating, thus longer experiments should be performed to obtain more information on the stability of this enzyme production process.

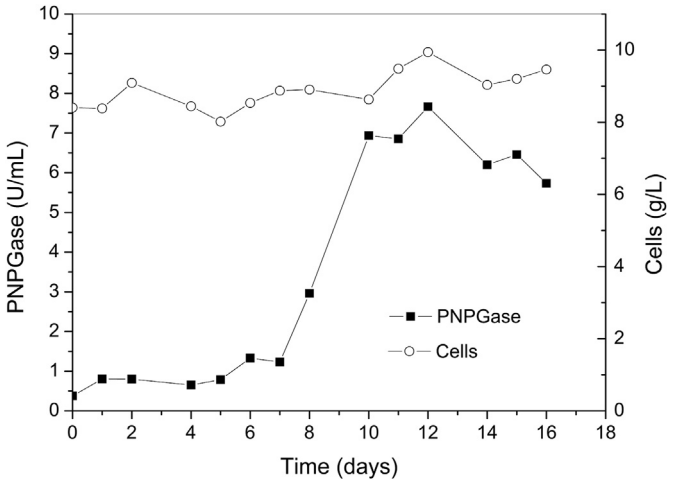

Fig. 4. Continuous PNPGase production using glucose at 20 g/L (G202).

Table 1
Results for the conditions using sucrose (S2), fructose/glucose (FG2), carboxymethyl cellulose (CMC) and carboxymethyl cellulose/glucose (CMCG) at steady-state.

Condition	PNPGase (U/mL)			Cells (g/L)		
S2	0.054	±	0.0072	4.40	±	0.24
FG2	0.93	±	0.18	4.90	±	0.28
CMCG	0.53	±	0.051	2.16	±	0.32
CMC	0.077	±	0.0031	0.82	±	0.28

Table 1 summarizes the results for the duplicate of sucrose (S2) and fructose/glucose (FG2) conditions. It also shows the conditions using carboxymethyl cellulose without and with an equimolar amount of glucose (CMC and CMCG). The glucose polysaccharide carboxymethyl cellulose (CMC) was used as the sole carbon source (10 g/L) and in combination with glucose (5 g/L CMC + 5 g/L glucose) to verify whether CMC would induce cellulase production. However, from a filter paper activity test, it was observed that cellulase activity was not present under those conditions. The combination of CMC with glucose (CMCG) resulted in a steady-state of cell concentration of around 2 g/L, indicating that CMC was not fully consumed. CMC alone provided the lowest cell concentration and consequently a low enzymatic activity. Even though *T. harzianum* can consume complex substrates such as sugarcane bagasse pretreated in batch mode (Gelain et al., 2015), it was not able to consume a soluble glucose polymer in chemostat culture, confirming that cellulase activity was not induced by CMC. The chemostat cultivation on CMC as the sole carbon source was the only experiment where the cell concentration was not very stable after 6 residence times, probably due to the low concentration.

Duplicates of the sucrose and fructose/glucose chemostat cultivations showed similar cell concentrations compared to the previous experiments (Fig. 2). The PNPGase activity for the duplicate of the sucrose cultivation (S2) was again very low. In the fructose/glucose duplicate chemostat (FG2), the activity was higher than for FG1, and this could have been caused by the fact that experiment FG2 was performed after a glucose-limited chemostat in the same bioreactor (feed concentration of 20 g/L). Therefore, cells from the previous experiment had accumulated on the walls of the bioreactor, and when part of these cells fell back into the liquid phase, this could have influenced PNPGase production. FG2 was the only experiment for which the cells on the walls of the bioreactor could have influenced the enzymatic activity since other conditions were very stable. Nevertheless, this could indicate that components from the cell wall when the experiments are performed with glucose could act as inducers of PNPGase and this should be better exploited in future works.

3.2. *Quantitative physiology of* T. harzianum

Using the material balance equations with the data from each steady-state chemostat, the specific consumption and production rates of substrates and products can be calculated from the measured gas and liquid in- and outflows and the concentrations of compounds in the gas and liquid phases. The thus obtained cell specific rates provide quantitative information and can be used to compare conditions aiming at the increase in the productivity and product yield, verify the presence of by-products and also to detect possible problems during the chemostat cultivation. If the specific rates are similar among different conditions, this indicates that there is low or no influence of the cultivation conditions on the culture behaviour. Table 2 shows the calculated average for cell specific conversion rates during steady-state for all conditions applied, except for the chemostat cultivations on CMCG and CMC as carbon sources. The table shows the specific cell growth rate (μ_X), specific carbon consumption rate (q_C), specific PNPGase production rate (q_B), specific oxygen consumption rate (q_{O_2}), specific carbon dioxide production rate (q_{CO_2}), specific nitrogen consumption rate (q_N), specific by-product formation rate (q_{byp}) and the carbon recovery (C_r).

It is assumed that the chemostat experiments would result in similar specific conversion rates during the steady-state. Firstly, because the sugars used as substrates were highly similar (glucose, fructose and sucrose) and all enter the central metabolism via glycolysis. Secondly, because the cell growth rate was the same in all experiments, 0.05 ± 0.003 $h.^{-1}$

Considering all the conditions, the analysis provided similar values for the specific growth rate (0.05 h^{-1} ±5.7 %), specific carbon consumption rate (0.105 C mol/mol h ±8.6 %), specific oxygen consumption rate (0.0421 mol/mol h ±13 %), specific carbon dioxide production rate (0.0470 mol/mol h ±12 %), specific nitrogen consumption rate (0.0094 mol/mol h ±1.6 %) and carbon recovery (106 % ± 5.6 %). However, the conditions provided large differences in the specific PNPGase production rate (407 U/mol h ±97 %) and

Table 2
Specific conversion rates during carbon-limited chemostat cultivations on different carbon sources: 10 g/L glucose (G101 and G102); 20 g/L glucose (G201 and G202); fructose/glucose (FG1 and FG2); and sucrose (S1 and S2). All rates are expressed per mol of cell per hour, whereby one mol of cell is defined as the amount containing one mol of carbon.

	G101	G102	G201	G202
Feed **(g/L)**	10	10	20	20
μ_X **(h^{-1})**	0.0475	0.0550	0.0538	0.05
q_C **(Cmol/mol h)**	0.0971	0.111	0.120	0.103
q_B **(U/mol h)**	414.7	347.9	1064.4	1032.4
q_{O_2} **(mol/mol h)**	0.039	0.041	0.048	0.038
q_{CO_2} **(mol/mol h)**	0.043	0.048	0.054	0.043
q_N **(mol/mol h)**	0.0092	0.0094	0.00955	0.0096
q_{byp} **(Cmol/mol h)**	0.0105	0.0225	0.0167	0.0084
C_r **(%)**	103.94	113.08	103.25	98.78
	FG1	**FG2**	**S1**	**S2**
Feed **(g/L)**	5.26/5.26	5.26/5.26	10	10
μ_X **(h^{-1})**	0.0475	0.0475	0.0475	0.05
q_C **(Cmol/mol h)**	0.0936	0.0975	0.104	0.117
q_B **(U/mol h)**	80.3	267.07	30.5	18.4
q_{O_2} **(mol/mol h)**	0.036	0.042	0.039	0.054
q_{CO_2} **(mol/mol h)**	0.040	0.047	0.043	0.058
q_{byp} **(Cmol/mol h)**	0.00921	0.0176	0.0128	0.0229
C_r **(%)**	102.92	115.21	99.51	111.99

specific by-product production rate (0.0151 C mol/mol h ±36 %). This indicates that the carbon sources used influenced the specific production of PNPGase and by-products. The rates of by-product formation fluctuated from 0.0084 to 0.0229 C mol/mol h, and these by-products are probably related to the presence of extracellular polysaccharides and proteins. Extracellular polysaccharides were qualitatively analysed by ethanol precipitation and their presence was confirmed in the supernatant of all samples from chemostat cultures using glucose, fructose/glucose and sucrose. The FT-IR analysis was applied for some samples from glucose-limited chemostat cultures and is presented in Fig. 5. The samples show a clear peak in the region of polysaccharides (1200-900 cm^{-1}) (Thumanu et al., 2015). Extracellular polysaccharides can be loosely attached to the cell wall and fragments can be released due to shear stress (Rau, 1999). Thus, the concentration of these fragments could be influencing the enzyme induction under carbon-limited conditions.

Fig. 6 highlights the average of specific PNPGase production rates (U/mol (cells) h) for all the conditions at steady-state in ascending order of PNPGase productivity, starting with sucrose, followed by CMC, fructose/glucose, CMCG, glucose at 10 g/L, and the highest, 20 g/L of glucose. Doubling the cell concentration resulted in an increase of 6 fold in PNPGase activity for the experiments using glucose, and this result can be further exploited by working with higher cell concentrations than applied in this project.

3.3. Sugar analysis

Samples were taken for quantification of the residual concentrations of fructose, glucose and sucrose during the last d of the continuous cultures. All the conditions presented low concentrations of the sugars analysed. Due to low concentrations, oscillations for the same conditions were observed. Fructose concentration was lower than 25 mg/L. The highest concentration of glucose remaining in the bioreactor was for the condition using CMC (16–19 mg/L). For the other conditions, the concentration was lower than 7 mg/L. In the first experiment with sucrose (S1) the residual concentration was 17 mg/L and in the second sucrose experiment (S2) the concentration of this sugar dropped from 465 to 77 mg/L. From the residual sugar analysis, it seems that the low sugar concentrations in the glucose experiments could have prevented carbon catabolite repression, which indicates that another mechanism could be controlling extracellular enzyme synthesis.

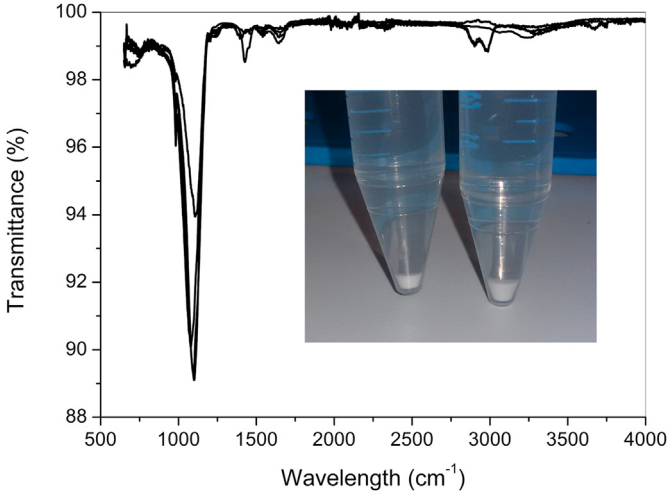

Fig. 5. FT-IR analysis of precipitates from glucose-limited chemostat cultures and a picture of the precipitates.

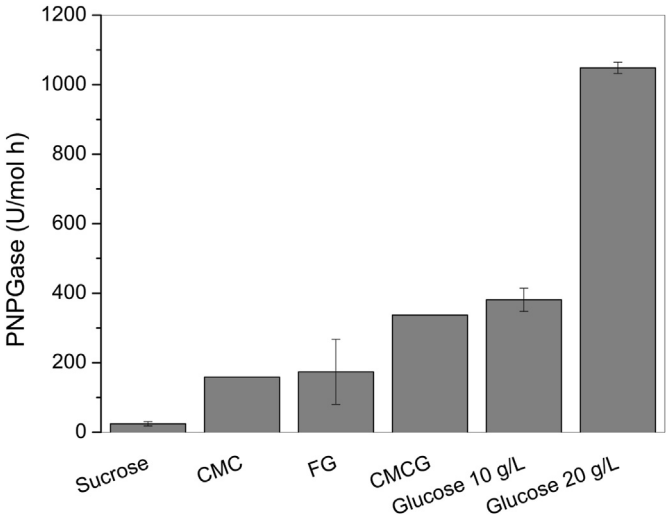

Fig. 6. Average of specific PNPGase production rates at steady-state, CMC – carboxymethyl cellulose, FG – fructose/glucose, CMCG - carboxymethyl cellulose/glucose.

Carbon-limited continuous cultures were achieved due to the low concentration of sugars and the excess of nitrogen and phosphorus present in the effluent (data not presented) for the experiments using glucose, sucrose and fructose/glucose.

3.4. Comparison of beta-glucosidase productivity

To compare the productivities obtained in this study with works present in the literature, it was assumed that the PNPGase activity has resulted mainly due to the presence of beta-glucosidase. The presence of beta-glucosidase was confirmed through a shotgun proteomics analysis for the experiments using mainly glucose as the carbon source (Gelain et al., 2020). The highest productivities achieved by this work were 325 U/L h and 35 U/g h using 20 g/L of glucose in the feed at steady-state of PNPGase synthesis. The productivities calculated do not include the time during the batch phase and transition phase (phase preceding the steady-state of PNPGase synthesis).

Li et al. (2016) reported the production of beta-glucosidase in batch mode by *T. reesei* TRB1 that achieved 19 U/mL on day 7th (113 U/L h) using microcrystalline cellulose as the inducer substrate in 50 mL flask containing 10 mL of medium. PNPG was used as the substrate to measure the beta-glucosidase activity.

Xia et al. (2018) presented a high-level production of beta-glucosidase in fed-batch mode that achieved 129 U/mL at 96 h (1344 U/L h) in a 1 m^3 fermenter using mixed-feed strategy, in which glycerol/methanol at the ratio of 1:5 and 30 g/L of corn steep powder were fed to maintain the methanol concentration at 0.7 % (v/v). The final cell concentration (*Pichia pastoris*) was approximately 112 g/L, which resulted in specific productivity of 12 U/g h at the end of the process. The substrate for the estimation of beta-glucosidase activity was not clearly described.

Delabona et al. (2016) reported a cellulase and beta-glucosidase production strategy using the same strain employed in this work. The microorganism was first grown in flasks using glycerol and then transferred to a bioreactor (the working volume of the fermenter was 1.1 L). The production in batch mode started with 4.5 g/L of cell and 30 g/L of sugarcane bagasse pretreated in batch mode. This condition achieved 9.04 U/mL at 72 h (126 U/L h) of beta-glucosidase. PNPG was used as the substrate to measure the beta-glucosidase activity.

Khisti et al. (2011) reported that the highest beta-glucosidase activity of 19 U/mL in 14 d (57 U/L h) of culture was detected when *Aspergillus niger* was grown at 30 °C for the first five days followed by further incubation at 36 °C in batch mode. The enzyme production was performed in flasks with 75 mL of the medium containing 3 % of xylan, 0.5 % of urea and 2.5 % of glycerol. PNPG was used as the substrate to measure the beta-glucosidase activity.

The strategy proposed by Xia et al. (2018) was performed in a large fermenter, in fed-batch mode and resulted in the highest productivity (U/L h) evaluated in this work. For the obtaining of that productivity, a high cell concentration was required. In a process using a high cell density, operating parameters such as pH, temperature and the concentration of components could be difficult to maintain stable and homogenous throughout the bioreactor. Therefore, it could require precise control.

The highest specific productivity calculated in this work (U/g h) is higher than the value calculated for the work of Xia et al. (2018). However, the highest activity for the condition G202, for example, was only observed after 10 d of cultivation and this time was not included to calculate the productivities. Therefore, more research is needed to understand the induction mechanism to optimize the time required to achieve a high production stage.

Based on the analysis of the productivities evaluated from the literature, the productivity achieved by this work at steady-state using 20 g/L of glucose in the feed demonstrates the high potential of this strain for the continuous production of enzymes that can catalyse the hydrolysis of glycosidic bonds. The next challenge includes the optimization of the production by keeping a high PNPGase synthesis during the steady-state without large variations in the enzymatic activity.

4. Conclusions

This is the first time that *T. harzianum* P49P11 was used in continuous culture under carbon-limited conditions. This microorganism showed an interesting potential to produce enzymes that can catalyse the hydrolysis of glycosidic bonds using glucose as the carbon source. In this project, the hydrolysis of p-nitrophenyl-β-D-glucopyranoside was analysed to verify the efficiency of the enzymes produced. PNPGase production under glucose-limited conditions can be improved through optimization of the operating conditions as well as through genetic engineering.

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References

Bhatia, Y., Mishra, S., Bisaria, V.S., 2002. Microbial beta-glucosidases: cloning, properties, and applications. Crit. Rev. Biotechnol. 22 (4), 375−407. https://doi.org/10.1080/07388550290789568.

Delabona, P.S., Farinas, C.S., da Silva, M.R., Azzoni, S.F., Pradella, J.G., 2012. Use of a new *Trichoderma harzianum* strain isolated from the Amazon rainforest with pretreated sugar cane bagasse for on-site cellulase production. Bioresour. Technol. 107, 517−521. https://doi.org/10.1016/j.biortech.2011.12.048.

Delabona, P.S., Lima, D.J., Robl, D., Rabelo, S.C., Farinas, C.S., Pradella, J.G., 2016. Enhanced cellulase production by *Trichoderma harzianum* by cultivation on glycerol followed by induction on cellulosic substrates. J. Ind. Microbiol. Biotechnol. 43, 617−626. https://doi.org/10.1007/s10295-016-1744-8.

Eyzaguirre, J., Hidalgo, M., Leschot, A., 2005. β-Glucosidases from filamentous fungi: properties, structure, and applications. In: Yarema, K.J. (Ed.), Handbook of Carbohydrate Engineering. CRC Taylor and Francis group, pp. 645−685.

Gelain, L., Pabst, M., Pradella, J.G.C., Costa, A.C., van der Wielen, L., van Gulik, W.M., 2020. Analysis of the proteins secreted by *Trichoderma harzianum* P49P11 under carbon-limited conditions. J Proteomics 227, 103922. https://doi.org/10.1016/j.jprot.2020.103922.

Gelain, L., Pradella, J.G.C., Costa, A.C., 2015. Mathematical modeling of enzyme production using *Trichoderma harzianum* P49P11 and sugarcane bagasse as carbon source. Bioresour. Technol. 198, 101−107. https://doi.org/10.1016/j.biortech.2015.08.148.

Ghose, T.K., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59, 257−268. https://doi.org/10.1351/pac198759020257.

Jäger, S., Brumbauer, A., Fehér, E., Réczey, K., Kiss, L., 2001. Production and characterization of β-glucosidases from different *Aspergillus* strains. World J. Microbiol. Biotechnol. 17, 455−461. https://doi.org/10.1023/A:1011948405581.

Khisti, U., Bastawde, K.B., Gokhale, D.V., 2011. Hyper-production of beta-glucosidase and beta-xylosidase by *Aspergillus niger* NCIM 1207 in xylan-containing media. Bio 6, 2066−2076.

Li, C., Lin, F., Li, Y., et al., 2016. A β-glucosidase hyper-production *Trichoderma reesei* mutant reveals a potential role of cel3D in cellulase production. Microb. Cell Factories 15, 151. https://doi.org/10.1186/s12934-016-0550-3.

McGinnis, J.F., Paigen, K., 1969. Catabolite inhibition: a general phenomenon in the control of carbohydrate utilization. J. Bacteriol. 100, 902−913.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426−428. https://doi.org/10.1021/ac60147a030.

Peebo, K., Neubauer, P., 2018. Application of continuous culture methods to recombinant protein production in microorganisms. Microorganisms 6, 56. https://doi.org/10.3390/microorganisms6030056.

Pessoni, R.A., Tersarotto, C.C., Mateus, C.A., Zerlin, J.K., Simões, K., Figueiredo-Ribeiro, R.D., Braga, M.R., 2015. Fructose affecting morphology and inducing β-fructofuranosidases in *Penicillium janczewskii*. SpringerPlus 4, 487. https://doi.org/10.1186/s40064-015-1298-7.

Rau, U., 1999. Production of schizophyllan. In: Bucke, C. (Ed.), Carbohydrate Biotechnology Protocols. Humana Press Inc, London, pp. 48−55. https://doi.org/10.1007/978-1-59259-261-6.

Rohrer, J.S., Basumallick, L., Hurum, D., 2013. High-performance anion-exchange chromatography with pulsed amperometric detection for carbohydrate analysis of glycoproteins. Biochemist 78, 697−709. https://doi.org/10.1134/S000629791307002X.

Ross, A., Schfigerl, K., Scheiding, W., 1983. Cellulase production by *Trichoderma reesei*. Eur. J. Appl. Microbiol. Biotechnol. 18, 29−37. https://doi.org/10.1007/BF00508126.

Sørensen, A., Lübeck, M., Lübeck, P., Ahring, B., 2013. Fungal beta-glucosidase: a bottleneck in industrial use of lignocellulosic materials. Biomolecules 3, 612−631. https://doi.org/10.3390/biom3030612.

Strobel, H.J., Russell, J.B., 1987. Regulation of beta-glucosidase in *Bacteroides ruminicola* by a different mechanism: growth rate-dependent derepression. Appl. Environ. Microbiol. 53, 2505−2510.

Suto, M., Tomita, F., 2001. Induction and catabolite repression mechanisms of cellulase in fungi. J. Biosci. Bioeng. 92, 305−311. https://doi.org/10.1016/S1389-1723(01)80231-0.

Thumanu, K., Sompong, M., Phansak, P., Nontapot, K., Buensanteai, N., 2015. Use of infrared microspectroscopy to determine leaf biochemical composition of cassava in response *to Bacillus subtilis* CaSUT007. J. Plant Interact. 10, 270−279. https://doi.org/10.1080/17429145.2015.1059957.

Xia, Y., Yang, L., Xia, L., 2018. High-level production of a fungal β-glucosidase with application potentials in the cost-effective production of *Trichoderma reesei* cellulase. Process Biochem. 70, 55−60. https://doi.org/10.1016/j.procbio.2018.03.031.

Xiong, Y., Sun, J., Glass, N.L., 2014. VIB1, a link between glucose signaling and carbon catabolite repression, is essential for plant cell wall degradation by *Neurospora crassa*. PLoS Genet. 10 https://doi.org/10.1371/journal.pgen.1004500.

Zhang, Y.H., Hong, J., Ye, X., 2009. Cellulase assays. In: Mielenz, J. (Ed.), Biofuels. Methods in Molecular Biology (Methods and Protocols). Humana Press, Totowa, NJ, pp. 213−231. https://doi.org/10.1007/978-1-60761-214-8_14.