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Metabolic engineering of CHO cells to alter lactate metabolism during fed-batch cultures

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ABSTRACT

Recombinant yeast pyruvate carboxylase (PYC2) expression was previously shown to be an effective metabolic engineering strategy for reducing lactate formation in a number of relevant mammalian cell lines, but, in the case of CHO cells, did not consistently lead to significant improvement in terms of cell growth, product titer and energy metabolism efficiency. In the present study, we report on the establishment of a PYC2-expressing CHO cell line producing a monoclonal antibody and displaying a significantly altered lactate metabolism compared to its parental line. All clones exhibiting strong PYC2 expression were shown to experience a significant and systematic metabolic shift toward lactate consumption, as well as a prolonged exponential growth phase leading to an increased maximum cell concentration and volumetric product titer. Of salient interest, PYC2-expressing CHO cells were shown to maintain a highly efficient metabolism in fed-batch cultures, even when exposed to high glucose levels, thereby alleviating the need of controlling nutrient at low levels and the potential negative impact of such strategy on product glycosylation. In bioreactor operated in fed-batch mode, the higher maximum cell density achieved with the PYC2 clone led to a net gain (20%) in final volumetric productivity.

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1. Introduction

Whether to support new therapeutic antibody development or to respond to market needs, the ability to cost-efficiently generate high yield and high quality of biotherapeutics is critical for the biopharmaceutical industry, owing to the great pressure to lower the cost and shorten the period between drug discovery and product manufacturing. The development and implementation of high-yield fed-batch strategies has contributed greatly at increasing the productivity of mammalian cell cultures (Bibila and Robinson, 1995; Wlaschin and Hu, 2006; Wurm, 2004). In fed-batch processes, the prolonged culture time is often associated with significant lactate and ammonia accumulation in the culture medium over time, which can have detrimental impacts on cell growth and product quality. Continuous cell lines, like CHO cells, show a deregulated glucose metabolism associated with high lactate production that can cause medium acidification or undesired osmolality changes due to alkali addition for culture pH control (Cruz et al., 2000; Lao and Toth, 1997; Omasa et al., 1991; Ozturk et al., 1992). Ammonia is well-known to alter the glycosylation patterns of monoclonal antibodies and other glycoproteins (Andersen and Goochee, 1995; Borys et al., 1994; Chen and Harcum, 2006; Gawlitzek et al., 2000; Yang and Butler, 2002). Appropriate glycosylation of monoclonal antibodies and recombinant proteins is necessary to ensure their biological function and their use as therapeutics. Thus, maintaining consistent and comparable product quality in high cell density cultures remains a critical challenge.

Several waste reduction strategies have been proposed using either metabolic or process engineering approaches. This includes the use of nutrients replacement strategies, for example the substitution of glucose with galactose (Altamirano et al., 2006, 2000, 2004) or pyruvate (Genzel et al., 2005) or substituting glutamine with asparagine (Kurano et al., 1990) or glutamate (Altamirano et al., 2000; Hassel and Butler, 1990). While effective at reducing waste metabolite formation, these substitutions are often associated with a concomitant reduction in cellular growth. Reduced lactate and ammonia production during fed-batch can be achieved by maintaining glucose and glutamine at very low levels

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(Omasa et al., 1992; Zhou et al., 1995). Under low nutrient availability, the cell metabolism is shifted toward a more efficient metabolic state characterized by a lower molar ratio of lactate/glucose (Chee Furng Wong et al., 2005; Maranga and Goochee, 2006). However, this requires the constant monitoring of nutrient levels and feed rate adjustments since non-limiting concentrations must be maintained at all time during the culture. Even short period of glucose starvation were shown to alter the glycosylation of the product (Xie et al., 1997). Moreover, it has been demonstrated that low glucose concentrations (<15 mM) lead to reduced site occupancy and decreased galactosylation and sialylation of antibodies (Liu et al., 2014). A dynamic fed-batch strategy to maintain low glutamine (0.1–0.3 mM) and glucose (0.35–0.70 mM) concentrations was also shown to decrease complex-type glycan formation and sialic acid content (Chee Furng Wong et al., 2005). These results emphasize the need to optimize both product titer and quality concurrently.

Different metabolic engineering approaches have also been investigated to generate cell lines with improved metabolic characteristics. The amplification of the glutamine synthetase gene in CHO and myeloma cells allowed growth in glutamine-free medium, thereby significantly reducing ammonia formation (Paredes et al., 1999; Zhang et al., 2006). Partial disruption of the gene encoding lactate dehydrogenase A by the means of homologous recombination in hybridoma cells (Chen et al., 2001) or siRNA in CHO cells (Jeong et al., 2006; Kim and Lee, 2007a) was shown to greatly lower the production of lactate, as well as glucose consumption. Studies by Chen et al. (2001) and Kim and Lee (2007a) on the down regulation of LDHA led to increased final product concentrations, although cell growth remained unaffected. In contrast, Jeong et al. (2006) observed an increased cell growth rate, but no significant impact on tPA production unless downregulation of LDH was combined with the overexpression of glycerol-3-phosphate dehydrogenase. A recent study demonstrated that the simultaneous downregulation of LDH and pyruvate dehydrogenase kinase can significantly reduce lactate accumulation and increase the volumetric antibody production, without impairing cell growth (Zhou et al., 2011). The expression of anti-apoptotic genes was also found to significantly alter the lactate metabolism of CHO cells, inducing a metabolic shift toward lactate consumption and increasing the final antibody titer by 40% (Dorai et al., 2009). Another approach consisted in the expression of the cytosolic yeast pyruvate carboxylase 2 (PYC2) gene in BHK (Irani et al., 1999) and HEK293 cells (Elias et al., 2003; Henry and Durocher, 2011; Vallée et al., 2013). In all cases, lactate formation was significantly decreased and, depending on the cell type and prevailing culture conditions, translated into improvement of either productivity or cell growth, but not both. In the case of CHO cells, two strategies were explored. First, the insertion of the human pyruvate carboxylase (hPC) gene in DG44 cells, which led to a slight decrease in lactate production (Kim and Lee, 2007b), as this enzyme is expressed in the mitochondria and is therefore not competing directly with LDH for pyruvate conversion. Second, one study reported on the expression of PYC2 in CHO cells, which allowed to decrease the specific lactate production (Fogolín et al., 2004). However, the molar ratio of lactate/glucose remained high (∼1.5) and relatively unchanged compared to the untransformed cells (∼1.7), indicating that the reduction in lactate accumulation was mostly due to a decrease in glucose uptake rate. Moreover, while prolonged cell viability was observed, this was at the expense of the maximum cell density, which exhibited a 2-fold reduction in batch culture. Recently, PYC2 overexpression in CHO cells was shown to prolong culture longevity and reduce the lactate/glucose ratio by 25%, but at the expense of a 50% reduction in the cell specific antibody production rate and an overall decrease in antibody titer (Wilkens and Gerdtzen, 2015).

In the present work, we show the establishment of a PYC2-expressing CHO cell line with a greatly improved metabolic efficiency that translated into reduced lactate accumulation without impairing the cells' specific productivity. All the PYC2-expressing clones that were generated exhibited a prolonged exponential growth phase leading to increases in maximum cell concentration and volumetric productivity. We also demonstrate that the PYC2-expressing cells can maintain their improved phenotype during fed-batch cultures employing concentrated nutrient solutions. In fed-batch bioreactor cultures, this resulted in further significant increases in terms of maximum cell density and final product titer.

2. Materials and methods

2.1. Stable cell line development

The CHO-EG2 clone 1A7 stably expressing a chimeric human-llama heavy chain monoclonal antibody (EG2) against epidermal growth factor receptor (EGFR) (Bell et al., 2010) was established from the CHO-DUXB11 cell line (Agrawal et al., 2012). The CHO-EG2-PYC2 clone was obtained by transfection of the CHO-EG2 cell line with the pTT18 vector encoding the yeast pyruvate carboxylase 2 gene. After transfection, hygromicin B was added to the cell culture at a final concentration of 900 μg/mL. After two weeks of selection, the pool of hygromycin-resistant cells was cloned by limiting dilution in 96-well plate. After 2 weeks, colonies were expanded and screened for their PYC2 expression level by western blot. Since the PYC2 enzyme is naturally biotinylated, it allows its detection using HRP-conjugated streptavidin (Sigma). Briefly, cells pellets were incubated on ice 20 min in a lysis buffer containing 50 mM HEPES pH 7.4, 150 mM NaCl, 1% Thesit® (Roche), 0.5% Na Deoxycholate, 1× complete-protease inhibitor cocktail (Roche). Cell lysates were centrifuged for 20 min at 12,000 × *g* at 4 °C. Supernatants were incubated 5 min at 90 °C in SDS sample buffer containing 5 mM dithiothreitol. Samples were electrophoresed on an SDS polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane (Schleicher & Schuell). Membranes were probed with HRP-conjugated streptavidin (Sigma). An anti-GAPDH rabbit polyclonal antibody (Sigma) was utilized for detection of the GAPDH to control total protein load. As a secondary antibody, an anti-rabbit polyclonal antibody HRP-conjugated (sigma) was employed. The blots were developed using a chemiluminescence kit (Boehringer Mannheim, Germany) and visualized with the Kodak imager system 440CF.

2.2. Fed-batch culture in shake flasks for clone evaluation

Eleven PYC2-positive clones and 4 PYC2-negatives clones were cultivated in 250 mL shake-flasks. After 4 days of cultivation, glucose was added daily in order to maintain a concentration of 20 mM. Lactate formation was measured daily for phenotypic screening to confirm that the change in lactate metabolism was the results of PYC2 overexpression rather than clonal effect.

2.3. PYC2 cytosolic expression

Three clones were selected according to their stable expression of PYC2. In order to confirm cytosolic PYC2 expression, the cytosols of these clones were isolated using mitochondria isolation kit for cultured cells (Thermo Scientific- cat#89874). Their PYC2 expression level was assessed by western blot using HRP-conjugated streptavidin (Sigma) as described before.

2.4. Medium and culture conditions

PowerCHO2 chemically defined medium (Lonza) supplemented with 8 mM glutamine, 0.1% Kolliphor® (Sigma) and 2% (v/v) anti

clumping agent (Gibco) was used for stable cell line development and batch shake-flasks experiments. Cultures were inoculated at a cell concentration of 0.2×10^6 cells/mL and were grown at 37 °C and 5% CO_2 under constant agitation (120 rpm).

2.5. pH monitoring in 6-well plates

Cell cultures were inoculated at a cell concentration of 0.2×10^6 cells/mL and grown at 37 °C and 5% CO_2 under constant agitation (120 rpm) in 6-well plates with integrated pH sensors (HydroDish®, PreSens-Precision Sensing GmbH, Germany).

2.6. Fed-batch cultivation

For fed-batch cultivation in shake flask or bioreactor, cells were seeded at a target cell concentration of 0.2×10^6 cells/mL in BalanCD (Irvine) chemically defined medium supplemented with 8 mM glutamine, 0.1% Kolliphor® and 2% (v/v) anti clumping agent (Gibco). Samples were taken daily to measure the cell density and nutrient/metabolite concentrations. Once the analyses completed, addition of CHO CD EfficientFeed™ B (Gibco) supplemented with 40 mM of glutamine was performed on a daily basis. The daily feeding volume is calculated according to the cell specific consumption of glucose:

$$\text{FeedVolume} = \frac{\left(R \times \text{IVCD}_{\text{predicted}}\right) - \left(C_t \times V - C_{\text{target}} \times V\right)}{C_{\text{feed}}}$$

where R denotes the specific nutrient consumption rate. C_t and C_{target} are the nutrient concentration and the desired nutrient concentration, respectively. V denotes the volume of the vessel and C_{feed} represents the nutrient concentration in the feed.

2.7. Bioreactor

Bioreactor experiments were performed in 2 L stirred bioreactor (Labfors 4 cell, Infors HT, Switzerland) operated at a 0.6 L starting volume. Dissolved oxygen was maintained at 50% of air saturation by surface aeration using a gas mixture of oxygen/nitrogen/air with a gas flow set at 100 mL/min. When necessary, pure oxygen was sparged at 2 mL/min with an on/off controller. The temperature was maintained at 37 °C with a double-wall water-jacketed. The stirring speed started at 80 rpm and was gradually increased during culture to reduce cell clumping and optimize gas transfer, until a maximum of 120 rpm. Culture pH was controlled at 7.0 by a surface gassing of CO_2 or 1 M sodium hydroxide addition. Addition of feeding medium was operated with a digital tubing pump delivering the desired feed volume at a flow rate of 280 μL/min.

2.8. Analytical methods

2.8.1. Cell count and viability, glucose, lactate, ammonium, glutamine quantification

Cell number and viability were assessed by trypan blue exclusion and counting on a hemocytometer. Samples were centrifuged and the supernatants were frozen at −80 °C for subsequent analysis. The glucose, lactate and glutamine concentrations in supernatants were measured with YSI 2700 Select biochemistry analyzer (Yellow Spring, USA). Ammonia concentration was measured by the VITROS 350 (Orthoclinical Diagnostics, USA).

2.8.2. Antibody quantification

Antibody quantification in supernatants was determined by high performance liquid chromatography (WATERS Corporation, Milford, MA) using a protein A cartridge (POROS® A20 column, 2.1 mmD × 30 mmH, 104 μL, Invitrogen, Grand Island, NY). Samples were filtered by centrifugation at 8000–11,000 × g for 3 min using NANOSEP MF GHP 0.45 μm centrifugal devices (PALL Life Sciences) prior to being injected on the column at a flow rate of 2 mL/min (PBS) and elution was performed using 0.15 M NaCl, pH 2.0. UV detection was done at 280 nm.

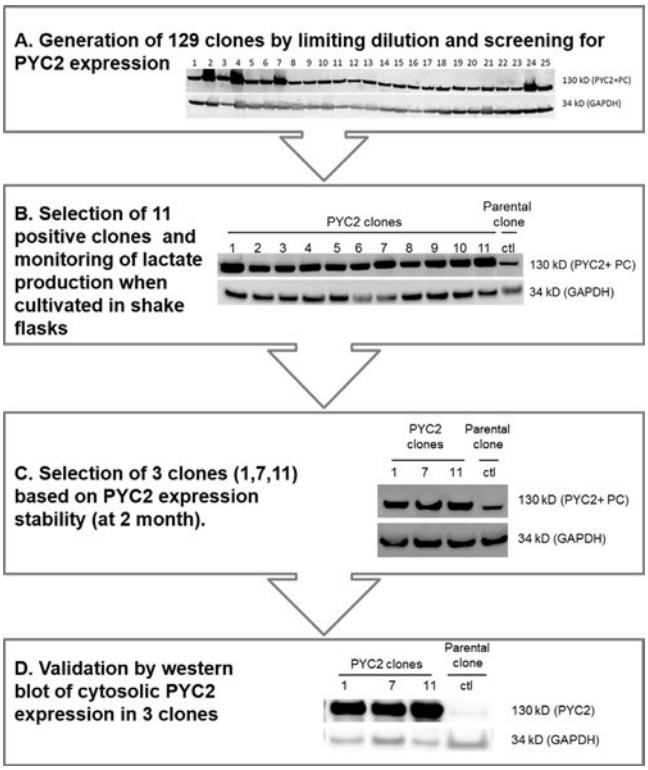

Fig. 1. Generation of a stable cell line expressing PYC2. (A) Example of western blot analysis of CHO cells transfected with the PYC2 gene. PYC2 and PC expression levels are detected using HRP-conjugated streptavidin. Lane 1: parental cell line (untransfected), lanes: 2, 4, 7, 24: PYC2 positive clones. Lanes: 3, 5, 6–23, 25: PYC2 negative clones. (B) Western blot of the 11 PYC2 positive clones chosen for batch cultivation. (C) Western blot of the 3 PYC2 clones exhibiting overexpression of PYC2 after 2 month in culture (D) Western blot analysis of PYC2 expression in the cytosolic fraction. Lanes 1–3: PYC2 positive clones. Lane 4: parental cell line (untransfected).

3. Results

3.1. Generation of CHO clones overexpressing PYC2

The pTT18 vector encoding the yeast pyruvate carboxylase 2 gene was transfected in CHO cells stably expressing a chimeric human-llama heavy chain monoclonal antibody (EG2). 129 individual clones were assessed for their level of PYC2 expression by western blot (Fig. 1A). For the parental cell line (Lane 1), the observed signal corresponds to the presence of the endogenous pyruvate carboxylase (PC) naturally expressed in the mitochondria of cells and which bears the same apparent molecular weight. PYC2-expressing clones (e.g., lanes 2, 4, 7 and 24 in Fig. 1A) all displayed a stronger signal reflecting the combined expression of PC and PYC2. Clones transfected with the pTT18 vector but showing a signal similar to the parental cells (e.g., lanes 3, 5, 6, 8–23 and 25 in Fig. 1A) were considered as negative. Finally, 11 clones that exhibited significant PYC2 expression were selected and used for further analysis (Fig. 1B). Given that PYC2 is expected to be expressed in the cytosol (in contrast to endogenous PC that is localized in the mitochondria), a subcellular fractionation was performed on three PYC2-positive clones (clones 1, 7 and 11 from Fig. 1C) that were selected on the basis of their stable PYC2 expression for over 2 months in culture. As shown in Fig. 1D, western blot analysis

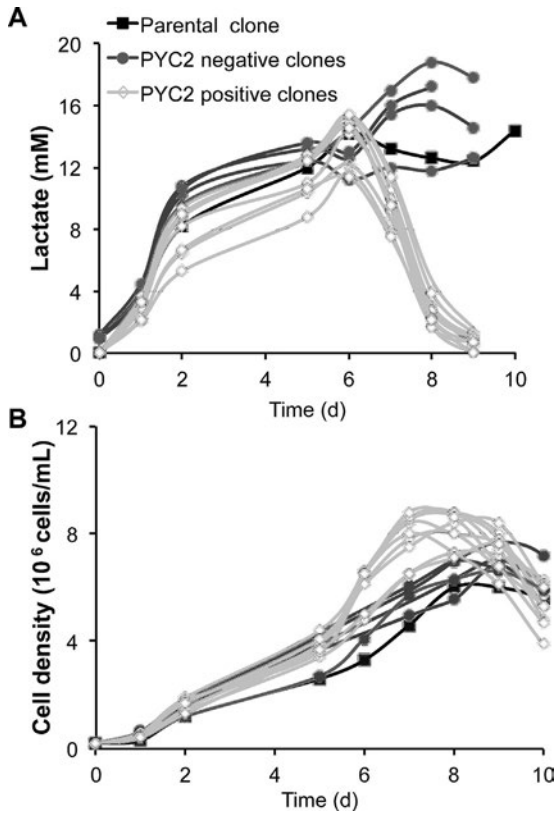

Fig. 2. (A) Lactate and (B) viable cell concentration profiles for PYC2 clones compared to PYC2 negative clones and the parental cell line cultivated in shake-flask cultures.

revealed a signal corresponding to PYC2 that was indeed specific to the transfected clones and confirmed its proper expression in the cytosol. As expected, GAPDH expression was observed in all the cytosolic fractions.

3.2. Phenotypic evaluation of PYC2 clones

To rapidly assess the impact of PYC2 expression on cell growth and metabolism, 11 PYC2-positive clones were cultivated in shake flasks operated in fed-batch mode, whereby glucose was added on a daily basis to maintain a target concentration of 20 mM. Maintaining glucose in excess was used to create conditions favoring an overflow metabolism (i.e., lactate production) and to avoid limitations that could alter lactate metabolism as reported in previous studies (Altamirano et al., 2006; Martínez et al., 2013). To rule out potential effects attributable to clonal variation, the kinetics of cell growth and lactate production of the 11 PYC2-positive clones were evaluated and compared to that of the parental cell line and to 4 PYC2-negative clones that were randomly chosen to serve as negative controls. As shown in Fig. 2A and in striking contrast with the negative clones and the parental cells, all the PYC2-expressing clones exhibited the same altered metabolism characterized by a significant shift towards lactate consumption around day 6. Noteworthy, cell growth was not negatively impacted and most of the positive clones even reached slightly higher maximum viable cell concentrations (Fig. 2B).

Several studies have previously shown that the culture medium composition can strongly influence the metabolism of a given cell line, including its pattern of lactate production/consumption (Altamirano et al., 2006; Luo et al., 2012; Ma et al., 2009; Yuk et al., 2014; Zagari et al., 2013a). In order to confirm the consistency of the metabolic shift displayed by the PYC2 clones, cells

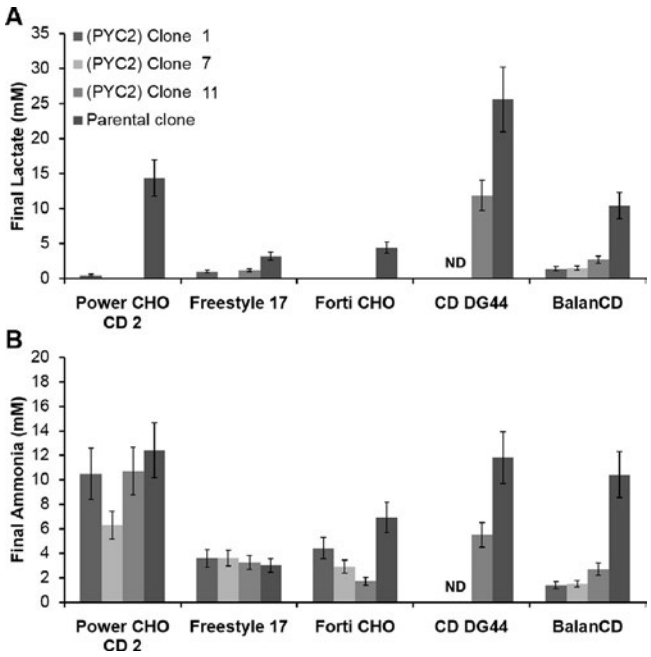

Fig. 3. (A) Final lactate and (B) ammonia concentrations for PYC2 and parental cell lines cultivated in 4 different media in shake-flask cultures. Error bars depict standard deviations of the measurements. ND: medium not tested for clones 1 and 2.

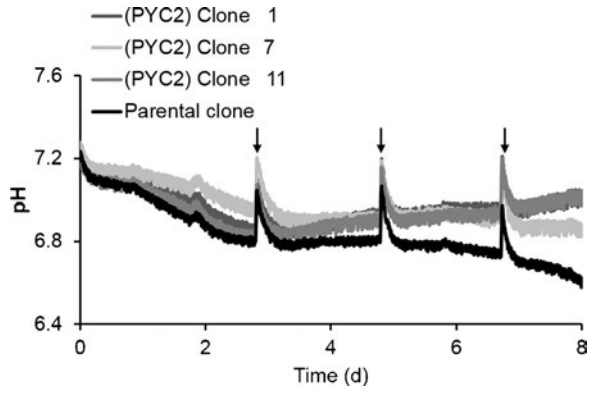

Fig. 4. Evolution of pH for the parental cell line and 3 PYC2 positive clones cultivated in 6 well plates equipped with pH sensors (HydroDish®). The 3 pH peaks indicated by arrows correspond to sampling of the cultures.

were cultivated in 5 commonly used commercial media. As shown in Fig. 3A, the final lactate concentrations varied depending on the culture medium, but were always consistently lower in the case of the PYC2-expressing clones compared to the parental cells. It should be emphasized that the differences observed were not the result of significant changes in cell densities, as the calculated cell specific lactate production rates were also consistently lower for PYC2 clones compared to the parental cell line (data not shown). Of salient interest, significant reductions in final ammonia concentrations (up to 50%) were also observed for PYC2 clones in three of the five commercial media formulation tested (Fig. 3B).

Lactate accumulation and the consequent medium acidification in uncontrolled flask cultures can be detrimental to the cells and/or the product quality. We therefore investigated the impact of PYC2 expression on the evolution of pH. To that end, cells were cultivated in batch mode in a 6-well plates and pH was monitored using a sensor dish reader (SensorDish®). The recorded pH signals are presented in Fig. 4 and clearly demonstrate that the acidification of the

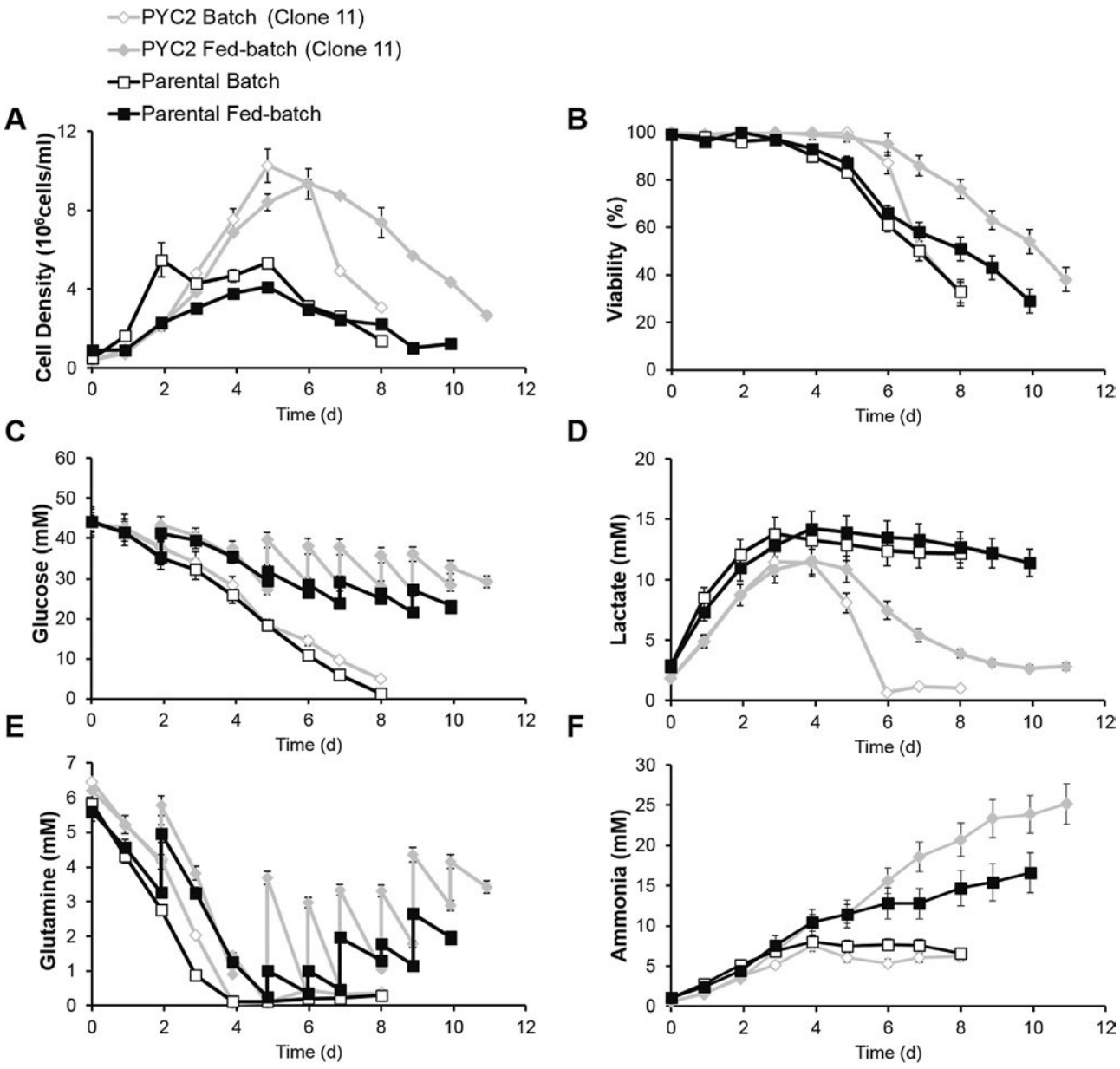

Fig. 5. (A) Cell density, (B) cell viability, (C) glucose, (D) lactate, (E) glutamine and (F) ammonia concentration profiles during batch and fed-batch cultivations of parental and PYC2-expressing cells in shake-flask cultures. Error bars depict standard deviations of the measurements.

medium is significantly reduced in cultures performed with PYC2 clones.

3.3. Batch and fed batch cultivations in shake flasks

In order to assess the potential of PYC2 clones for fed-batch applications, cells were cultivated in shake flasks under batch and fed-batch modes using the BalanCD medium, which yielded the highest cell density in shake flask batch cultures. Clone #11 (Fig. 1) was used in all the subsequent experiments in shake flask and bioreactor cultures. The resulting cell density profiles are shown in Fig. 5A for both the parental and PYC2-expressing cells. When cultivated in batch mode, the growth curves differed markedly between both cell lines. The PYC2 cell line reached a maximum cell concentration of about 1×10^7 cells/mL, a nearly two-fold increase compared with the parental cells. The maximum cell specific growth rates were also fairly different for the two cell lines (0.033 and 0.023 h^{-1} for the PYC2 and the parental clones, respectively) and the exponential phase of the PYC2 clone was extended by more than 24 h. Upon reaching their maximum cell density on day 6, the PYC2 cells exhibited a sharp decrease in viable cell concentration thereafter (Fig. 5B). This sudden drop in cell viability was most likely due to a key nutrient depletion given the high cell density reached in these batch cultures. When grown in fed-batch, the PYC2 cells had a similar cell specific growth rate (0.032 h^{-1}), but the feeding strategy allowed to maintain high cell densities over a prolonged period of time, as the cell viability decreased below 85% only after day 7. In contrast, the same feeding regime had only marginal impact on the growth and viability of the parental cells (Fig. 5A & B) compared with the batch mode.

Batch cultures were characterized by a nearly total depletion of glucose by day 8 for both PYC2 and parental cells (Fig. 5C), while the fed-batch strategy allowed to maintain glucose around the target concentration of 35 mM. Noteworthy, despite those elevated glucose concentrations, the PYC2 clone exhibited the same altered lactate metabolism witnessed in batch (Fig. 5D). A comparison of

Table 1
Cell specific consumption/production rates in shake flask cultures.

	Batch		Fed-batch	
Exponential phase	PYC2	Parental	PYC2	Parental
$q_{glucose}$ (pmol/cell.d)	1.00 ± 0.17	1.24 ± 0.21	1.18 ± 0.19	1.65 ± 0.30
$q_{glutamine}$ (pmol/cell.d)	0.35 ± 0.06	0.37 ± 0.07	0.47 ± 0.08	0.53 ± 0.09
$q_{lactate}$ (pmol/cell.d)	0.46 ± 0.06	0.98 ± 0.18	0.54 ± 0.09	1.18 ± 0.21
$q_{ammonia}$ (pmol/cell.d)	0.57 ± 0.10	0.86 ± 0.18	0.93 ± 0.20	1.31 ± 0.28
Lactate/glucose ratio	0.46	0.79	0.46	0.72
Ammonia/glutamine ratio	1.63	2.32	1.98	2.47
Stationary phase				
$q_{glucose}$ (pmol/cell.d)	0.69 ± 0.10	1.19 ± 0.21	1.42 ± 0.24	1.73 ± 0.29
$q_{glutamine}$ (pmol/cell.d)	–	–	0.27 ± 0.05	0.31 ± 0.05
$q_{lactate}$ (pmol/cell.d)	−0.36 ± 0.06	−0.05 ± 0.01	−0.15 ± 0.03	−0.03 ± 0.01
$q_{ammonia}$ (pmol/cell.d)	–	–	0.41 ± 0.09	0.36 ± 0.08
Lactate/glucose ratio	−0.52	−0.04	−0.11	−0.02
Ammonia/glutamine ratio	–	–	1.52	1.16

the main cell specific consumption and production rates evaluated during the exponential and the stationary phases is presented in Table 1. In batch and fed-batch cultures, only slight reduction in the glucose specific consumption rates were observed for PYC2 cells, but marked differences were noted for lactate as the specific production was reduced by 50% during the exponential phase. The differences were even greater during the stationary phase, as lactate was significantly consumed by PYC2 cells, unlike in the parental cell line (Table 1). As evident from the calculated lactate/glucose ratios, the PYC2 clone exhibited a much more efficient glucose utilization.

In batch cultures, glutamine depletion occurred rapidly (by day 4), but the average cell specific glutamine consumption rates were not significantly different for the two cell lines (Fig. 5E & Table 1). The supplementation of glutamine in fed-batch cultures led to an increased consumption rate and greater ammonia

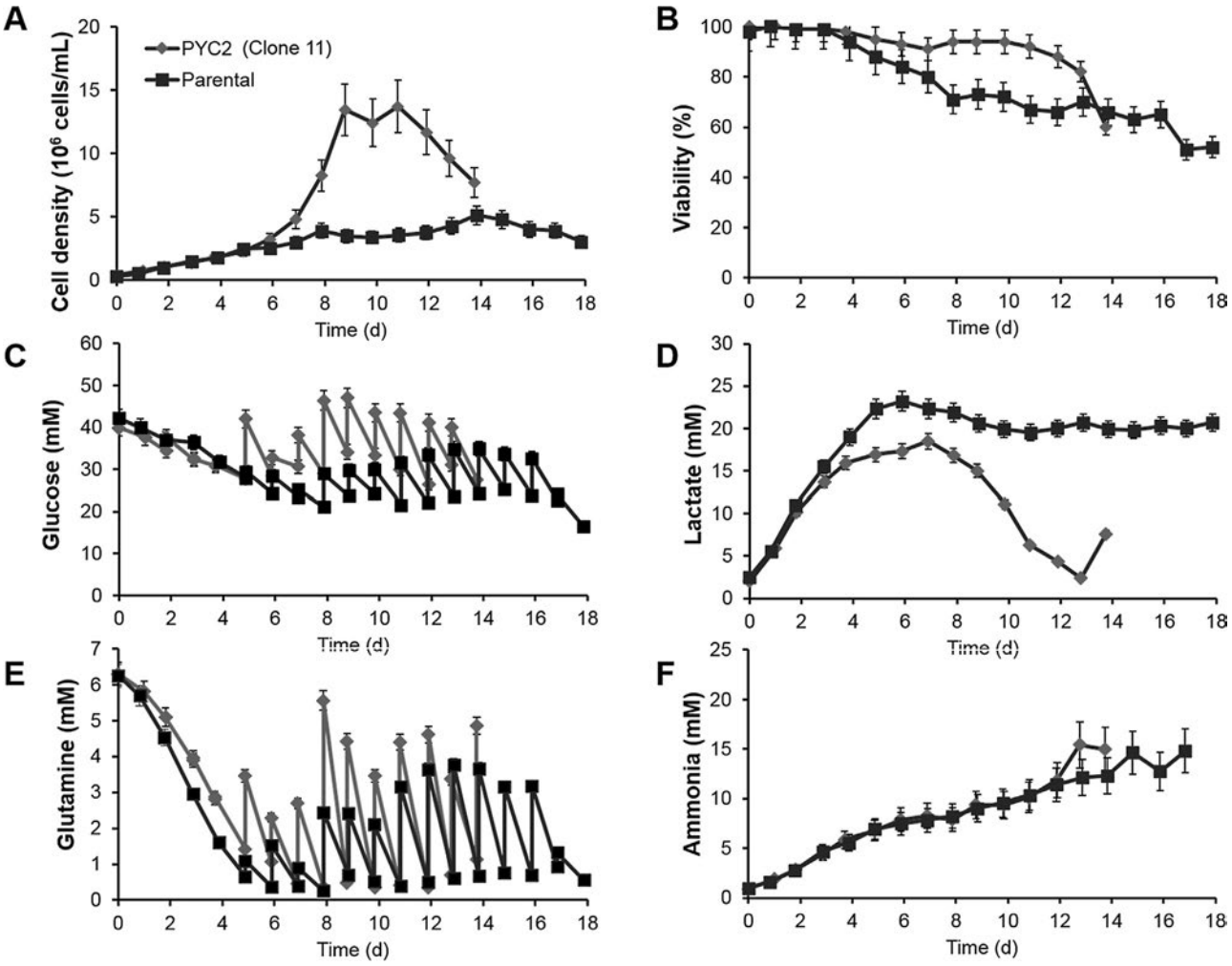

Fig. 6. (A) Cell viability, (B) cell density, (C) glucose, (D) lactate (E) glutamine and (F) ammonia concentration profiles fed-batch cultivations of parental and PYC2 expressing clones in bioreactor cultures. Error bars depict standard deviations of the measurements.

Table 2
Cell specific consumption/production rates in bioreactor cultures.

	Exponential phase		Stationary phase	
	PYC2	Parental	PYC2	Parental
$q_{glucose}$ (pmol/cell.d)	2.46 ± 0.42	2.49 ± 0.44	1.57 ± 0.27	2.06 ± 0.33
$q_{glutamine}$ (pmol/cell.d)	0.75 ± 0.12	1.18 ± 0.20	0.44 ± 0.08	0.56 ± 0.09
$q_{lactate}$ (pmol/cell.d)	0.43 ± 0.07	2.34 ± 0.41	−0.17 ± 0.03	0.24 ± 0.04
$q_{ammonia}$ (pmol/cell.d)	0.31 ± 0.05	1.11 ± 0.18	0.19 ± 0.03	0.24 ± 0.02
Lactate/glucose ratio	0.17	0.79	-0.11	0.11
Ammonia/glutamine ratio	0.41	0.94	0.43	0.82

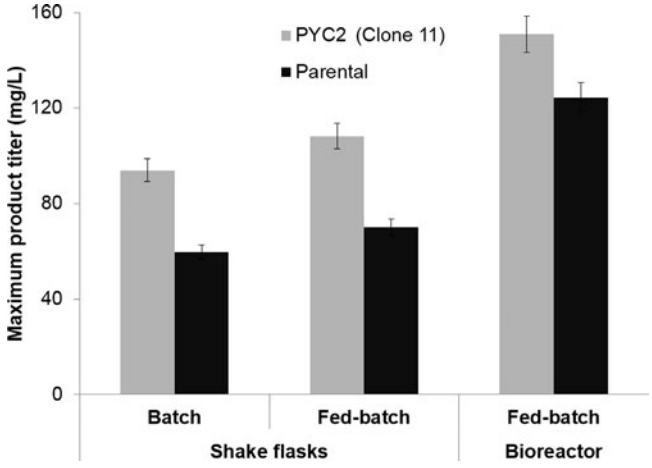

Fig. 7. Comparison of maximum antibody titers between parental and PYC2-expressing cells cultivated in shake flasks and bioreactors. Error bars depict standard deviations of the measurements.

accumulation, particularly for the PYC2 clone due to the greater cell density and the prolonged culture. While the specific productions were quite similar during the exponential phase, both cell lines showed a decreased production of ammonia per cell during the stationary phase (Table 1). Whether the cultures were performed in batch or fed-batch modes, the final product titer in cultures with PYC2-expressing cells was 35% greater compared to the parental cell line (Fig. 7). This augmentation can be attributed to both the increased cell density and the prolonged culture longevity associated with PYC2 expression. The cell specific productivity of PYC2-expressing cells was comparable in batch and fed-batch modes (1.91 ± 0.40 and 2.02 ± 0.44 pg/cell day, respectively), while that of the parental clone increased from 1.69 ± 0.35 pg/cell day in batch to 2.69 ± 0.54 pg/cell day in fed-batch. Compared to the parental cell line, the slightly lower cell specific productivity of the PYC2 clone is largely compensated by the increased cell growth.

3.4. Fed-batch cultivation in bioreactor

The sharp and sudden viability decrease witnessed in shake flasks suggested that these uninstrumented culture conditions could not support the high cell densities reached by PYC2 cells (around 1×10^7 cells/mL). Cells were thus cultivated under fed-batch mode using fully controlled bioreactors so as to provide better regulated conditions in terms of pH and oxygen. The feeding strategy consisted in the daily addition of a concentrated solution (CHO CD EfficientFeed B) supplemented with 40 mM of glutamine. The resulting cell density profiles are shown in Fig. 6A. The PYC2 cell line reached a maximum cell concentration of about 1.3×10^7 cells/mL, a more than 2-fold increase compared with the parental cells. The maximum cell specific growth rates were fairly similar for the two cell lines (0.023 and 0.024 h^{-1} for the PYC2 and the parental clones, respectively), but the exponential growth phase was extended by 3 days in the case of the PYC2 clone. Upon reaching their maximum in concentration between days 9 and 12 (13×10^6 cells/mL), there was a sharp decrease in viable cell density, similar to the trend observed in small-scale cultures (Fig. 6B). Since the dissolved oxygen set-point was successfully maintained at around 50% throughout the culture, this was likely due to some other nutrient limitation.

As was observed in shake flask cultures, the expression of the PYC2 gene led to a significant alteration of the cell metabolism, with lactate being consumed after 7 days of cultivation despite elevated glucose concentration (Fig. 6C & D). The greater nutrient utilization efficiency of the PYC2 cell line was even observed during the exponential phase, as the cell specific lactate production rate was reduced by 80% for a comparable glucose uptake rate (Table 2). PYC2 expression did not significantly impact the glutamine metabolism, and, as a result, the cultures achieved similar final levels of ammonia (Fig. 6E & F), although the specific ammonia production rate of the PYC2 clone was lower (Table 2).

Consistent with small-scale fed-batch results, the average cell specific antibody production rates was slightly lower for the PYC2 clone (2.21 ± 0.45 and 2.86 ± 0.58 pg/cell day for PYC2 and parental cells), but the final product titer was augmented by 28% due to the higher cell density achieved (Fig. 7). Of interest, the maximum volumetric titer was reached 4 days earlier in cultures with the PYC2 cells. While the fed-batch strategy brought only a slight antibody volumetric productivity improvement in shake flasks, cultures in bioreactor improved by around 30–40% the volumetric production of PYC2 and parental cell lines, respectively.

4. Discussion

The aim of this work was to generate a PYC2-expressing CHO cell line with an improved metabolic efficiency and demonstrate its potential for the development of a fed-batch process for antibody production. In any metabolic engineering strategy, clonal variations should be considered when trying to assess the real impact of gene overexpression. Clone-based studies often fail to establish a relationship between transgene expression and the desired trait. In the specific case of lactate reduction strategies, a variety of other factors may cause differences in metabolic phenotypes, such as changes in the medium composition (Kishishita et al., 2015; Luo et al., 2012; Zagari et al., 2013a). In our study, we have initially compared the kinetics of growth and metabolite formation of 11 PYC2-positive, 4 PYC2-negative and the parental clones. The fact that all 11 PYC2-positive clones exhibited comparable phenotypes when tested in different media formulation provided strong evidence that the differences observed were due to PYC2 expression and not to clonal variations.

The PYC2 expressing CHO cells that were generated displayed a much more efficient metabolism characterized by a lower lactate/glucose ratio, especially in fed-batch cultures using an enriched nutrient solution. More importantly, the slightly lower cell specific antibody production of the PYC2 clone is largely compensated by the increase in maximum cell density, translating into a net gain in volumetric productivity. PYC2 expressing cells

maintained their distinctive phenotype, even when exposed to high glucose levels during fed-batch cultures. In addition, the cell specific productivity is not significantly altered by the metabolic modification, as was previously observed when the same gene was overexpressed in HEK293 cells producing interferon in batch (Henry and Durocher, 2011) or fed-batch (Vallée et al., 2013) cultures. In contrast with our results, an apparent two-fold increase in specific productivity was observed in PYC2 transfected BHK-21 cells producing recombinant EPO, although these cells were cultivated in perfusion mode under limiting glucose conditions (Irani et al., 2002). However, no positive impacts were observed in terms of maximum cell density and cell specific growth rate. Similarly, a two-fold increase of specific hGM-CSF productivity was noted for CHO cells expressing PYC2 cultivated in batch and following a temperature switch (Fogolín et al., 2004). The cell specific growth rate and maximum cell density were reduced, but culture longevity was extended leading to a rise in final product titer. In an earlier study, HEK293 expressing PYC2 showed no significant increase in SEAP titer in spite of increase cell longevity and reduced waste production (Elias et al., 2003). Similarly, a decrease in the specific antibody productivity was found for CHO cells expressing PYC2, even though the cell growth rate and glucose/lactate ratio were improved (Wilkens and Gerdtzen, 2015). Taken together, these results indicate that the effects of PYC2 expression are dependent on the cell type and the culture conditions. In the present study, the greatest impact of PYC2 expression in CHO cells was on the maximum cell density attained during the cultures. Results from our shake flask and bioreactor cultures in fed-batch mode showed that the increase in cumulative viable cell concentration, combined with the maintenance of antibody specific productivity, resulted into a net increase in the final volumetric titer.

In the transition from small-scale to bioreactor, the metabolism and growth of the PYC2 clone remained mostly similar, but the product titer increased substantially. This titer improvement can likely be attributed, at least in part, to tightly regulated conditions in the bioreactor. In the case of PYC2 cells, regardless of the culture mode (batch or fed-batch) or culture device (shake flask or bioreactor), the lactate shift seemed to occur systematically 24 h before reaching the maximal cell density, as the cells entered the deceleration phase. For the parental CHO cell line, the net accumulation of lactate ceased when cell growth stopped. In either of the two cell lines, these results suggest a link between a certain lactate level, growth rate reduction and lactate production shutdown. In the case of cells expressing PYC2, the enzyme could have an additive effect leading to a net lactate consumption due to the competition for pyruvate between this enzyme and lactate dehydrogenase (Irani et al., 1999). Lactate consumption has been reported to occur under low glucose concentrations (Altamirano et al., 2004; Cruz et al., 2000; Ozturk et al., 1992) or under non-limiting levels (Luo et al., 2012; Ma et al., 2009; Mulukutla et al., 2012; Ozturk et al., 1997; Pascoe et al., 2007). The co-consumption of lactate and glucose was identified as a highly desirable trait for industrial cell lines, since it is typically associated with increased culture longevity and greater product yields (Le et al., 2012). This phenomenon and its underlying physiological mechanisms are still not fully understood and, consequently, not yet well-controlled. Recently, some studies have suggested that the copper level in the culture medium could be a trigger of lactate consumption (Luo et al., 2012; Yuk et al., 2014), but other factors have also been associated to this metabolic shift, including an increase in oxygen consumption, a reduction of glycolysis and glutaminolysis, as well as cell growth slowdown (Luo et al., 2012; Ma et al., 2009; Mulukutla et al., 2012; Pascoe et al., 2007).

Studies on BHK-21 expressing PYC2 have reported increases in oxygen uptake and intracellular ATP pool, pointing to an enhanced TCA activity resulting from this genetic modification (Irani et al., 1999). More recently, ^{13}C metabolic flux analysis conducted on HEK293-PYC2 expressing cells confirmed an increase in the pyruvate flux to oxaloacetate (Henry and Durocher, 2011), although this intermediate metabolite can subsequently have multiple fates. First, in the cytosol, the latter can be accumulated or could be converted into malate through cytosolic malate dehydrogenase (MDH) with the concomitant release of NAD^+. Then the malate can be recycled into pyruvate via cytosolic malic enzyme or enter the mitochondria via the malate/aspartate shuttle to be completely oxidized in the TCA. A previous study on CHO cells expressing mitochondrial MDH II reported enhanced glycolysis, reduced lactate secretion and elevated intracellular ATP/NADH levels along with antibody titer improvement (Chong et al., 2010). These findings suggest a critical role of the redox balance in the regulation of the glycolytic flux and the proportion of pyruvate entering the TCA. The capacity of the LDH reaction to regenerate NAD^+ allows to maintain high glycolytic rates, decreasing the availability of cytosolic pyruvate as a substrate for the TCA cycle. The malate-aspartate shuttle plays a pivotal role in maintaining the redox balance by allowing the regeneration of NAD^+ in the cytosol. In CHO cells, overexpression of aralar1, an aspartate/glutamate carrier and critical component of the malate-aspartate shuttle, was shown to promote a metabolic switch to lactate consumption without any reduction of the glycolytic rate (Zagari et al., 2013b). For PYC2 overexpression, it can be speculated that the reduction in the LDH flux is likely compensated by an increase in the malate dehydrogenase flux to maintain the redox balance.

The distinctive phenotype of PYC2-expressing CHO cells can provide additional benefits in the context of the development of a cell culture manufacturing process. First, it is well known that glycosylation pattern of glycoproteins can be negatively altered by elevated waste product accumulation (Andersen and Goochee, 1995; Gawlitzek et al., 2000; Yang and Butler, 2002). Since significant lactate/ammonia accumulation is likely to occur in standard high cell density fed-batch cultures, the lower waste metabolite formation obtained in PYC2 cells could be expected to enhance final product quality and stability in the culture medium, but further work is required to assess the impact of PYC2 overexpression on product quality attributes. Furthermore, this phenotypic change eliminates the need to control glutamine/glucose at low levels, strategies which make process operation more complex and also have adverse effects on the glycosylation profile of the protein of interest (Liu et al., 2014). Second, it is generally recognized that small-scale studies performed in shake flasks during process development may not translate well to bioreactor during scale-up, particularly due to lactate accumulation. Given that the pH changes are damped with PYC2-expressing cells, this represents another significant advantage to streamline the optimization of culture conditions.

5. Conclusion

We have demonstrated that PYC2 expression in CHO cells can efficiently alter their metabolism and induce a desired shift towards lactate consumption that was shown to occur consistently in all culture modes and commercial media tested. Given the marked reduction in waste metabolite formation, the results presented here demonstrate that PYC2 cells are well suitable for fed-batch process employing concentrated feed solutions. Indeed, a simple feeding strategy based on glucose consumption was able to substantially increase culture growth and longevity and lead to a significant improvement of the volumetric titer. Since the cell specific productivity of PYC2 clones was marginally reduced compared to the parental cells, this enhancement was attributable to the significant increase in maximum cell density. Further improvement could

potentially be achieved by screening key culture supplements or process conditions leading to increased culture longevity and/or stimulating the cell specific productivity (e.g. mild hypothermia conditions).

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