**Doubling humanized L-asparaginase expression by *Pichia pastoris* through DO-stat controlling induction strategy in bench-bioreactor**

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**Highlights**

* The engineered *Pichia pastoris* expressed humanized L-asparaginase (ASNase)
* Three induction strategies were studied
* DO-stat strategy resulted in, approximately, two-fold more maximum ASNase activity

**1. Introduction**

During the last ten years, the yeast *Pichia pastoris (Komagataella phaffi)* has been consolidated as a platform for biopharmaceutical production. Moreover, the development of engineered strains capable to perform ‘humanized’ glycosylation increased the chances to reach the requirements of the regulatory agencies (Jacobs et al, 2009). Facing this potential, a strain of *P. pastoris* Glycoswitch® (Biogrammatics Inc.) was transformed to express the enzyme L-asparaginase (ASNase), which is applied in acute lymphoblastic leukaemia treatment, with ‘humanized’ glycosylation. The objective of this work is to obtain the highest ASNase activity through studying different induction strategies aiming the scaling up production of this innovative biopharmaceutical.

**2. Methods**

2.1 Cell strain and expression vector

*Pichia pastoris* GS115 Glycoswitch® SuperMan5 his+ (Biogrammatics Inc.) transformed by electroporation with the plasmid pJAG\_s1 (Biogrammatics Inc.) containing the gene asn*B* (GenScript) of the bacteria *Erwinia chrysanthemi (Dickeya chrysanthemi)* for ASNase expression, the factor αMF for extracellular expression and the gene for G418 resistance for selection (Effer et al, 2019). The plasmid was linearized and integrated to the promoter AOX1.

2.2 Cell storage and reactivation

Cells storage and seeding were performed according to Invitrogen’s protocol (Invitrogen, 2002). The pre-inoculum was prepared to inoculate the bioreactor with 1 gdrycell/L.

2.3 Bioreactor cultures

Bioreactor cultures began with 1 litre of BSM (Basal Salt Medium) and 4.35mL of PTM1 (metal trace *Pichia* solution) formulated according to Invitrogen (2002) in a 2L-BIOSTAT B (Sartorius, Germany). The glycerol batch phase was performed according to the same protocol and equal in the three experiments with dissolved oxygen (DO) kept over 20% air saturation with control cascade of the agitation between 700 and 1000 rpm and aeration kept at 1 litre per minute. Following, there was a period of starving for, approximately, 2 hours and then induction was started. In the experiment A, the induction was performed with the addition of 10mL-pulses at every 24 hours; in experiment B, methanol was linearly fed with constant rate of approximately 18mL/h and DO was controlled over 20% with pure oxygen feeding; and, in C, methanol was fed to keep DO at 20% in a control mesh (DO-stat).

2.4 Analytic Methods

The dry mass concentration was related to optical density at 600nm measured in a spectrophotometer (Spectra Max®, Molecular Devices); glycerol was evaluated using a colorimetric kit (Triglicerides Liquiform®, Labtest); ASNase activity, modified protocol of Drainas, Kinghorn and Pateman (1977) and protein concentration, BCA method (Bicinchoninic Acid Kit, Sigma®).

**3. Results and discussion**

The three cultures provided similar performances during the growth phase on glycerol, with a specific maximum growth rate of 0.18±0.02 h-1 and cell productivity of 1.1±0.1 gdrycell/L/h after 24 hours of cultivation. However, during the expression phase, the ASNase production was different according to the induction strategy. The maximum activities were 1674 U/L (100h), 1501 U/L (76h) and 3068 U/L (97h) in A, B and C, respectively. Moreover, the population grew and produced protein significantly only in culture C. Therefore, the induction strategy based on controlling methanol-feeding by O2 concentration (C) was the most suitable, since the metabolism was permanently stimulated to produce ASNase and consume methanol differently from pulses strategy (A), that was intermittent, and did not achieve toxic levels as in the constant feeding (B). This result agrees with previously reported by Lim et al. (2003) that achieved 40% higher rGuamerin secretion using DO-stat strategy than manual control of methanol-feeding. This strategy avoids methanol accumulation and lack of oxygen, improving cell growth and heterologous protein production (Lim et al, 2003).

**4. Conclusions**

As described in the literature (Liu et al, 2019), the induction strategy influenced significantly the heterologous protein production. The maximum ASNase activity approximately doubled with methanol-feeding controlled by DO-stat. This strategy kept methanol constant in a range that stimulated ASNase production, but at a level that was not toxic for the cells during the culture. Therefore, the induction strategy that considered aspects of cell metabolism was the most suitable for humanized ASNase production.

**References**

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