STRATEGY DESIGN FOR THERAPEUTICAL PROTEIN PRODUCTION by *Pichia pastoris*

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Introduction. Yeasts are widely used in production of recombinant proteins (r-proteins) of medical or industrial interest. For the production of a biomolecule in a host a creative metabolic engineering design is achieved, and then optimized both on the molecular genetic and fermentation level, by taking into account the properties of the product, the host organism and the expression cassette. Amongst, the methylotropic yeast *Pichia pastoris* has become a frequently used expression system for r-protein production owing to its strong and tightly regulated promoter *alcohol oxidase I* (*AOX*1). Since methanol is used not only as the carbon and energy source, but also as an inducer of the expression of r-proteins, despite at high concentrations inhibits the growth, fed-batch feeding strategies are employed to increase the product yield [1]. In this work, to improve r-protein productions under strong methanol inducible *alcohol oxidase* I (*AOX*1) promoter, feeding strategies for semi-batch operations were developed in pilot scale bioreactors, for the: i) non-glycosylated protein recombinant human growth hormone (rhGH) production by the constructed *P. pastoris* M13 strain (pPICZαA*::hGH*-Mut$^+$) [2]; ii) the glycoprotein recombinant human erythropoietin (rHuEPO) production by the constructed *P. pastoris* E17 strain (pPICZαA*::epo*-Mut+) [3]. Further, the influences of the co-substrate feeding strategies on the intracellular reaction network of *P.pastoris* will be discussed through metabolic flux analysis.

Methods. *P. pastoris* M13 Mut$^+$ carrying *hGH* cDNA [2] and *P. pastoris* E17 Mut$^+$ carrying *epo* cDNA [3] under the control of *AOX1* promoter were used in the experiments. 3.0 L bioreactors (Braun CT2-2), having a working volume of V=1.0 L was used for the production of r-proteins. After glycerol batch, glycerol fed-batch and methanol transition phases, the influences of the designed feeding strategies were investigated in the fourth phase, which is r-protein production or induction phase. For the simplicity of the calculations and comparisons, the beginning of production phase (Phase IV) was considered as t=0 h. The concentrations of glycerol, methanol, sorbitol, mannitol and the byproducts organic acids were determined by HPLC (Waters HPLC, Milford, MA); rhGH and rHuEPO concentrations were determined by Capillary Electrophoresis (Waters, Quanta 4000E, Milford, MA) [4]. The mass flux balance-based stoichiometric model containing 102 metabolites and 141 reaction fluxes [5] was used to calculate the intracellular reaction fluxes to determine the effects of the co-substrates on the intracellular reaction network of r-protein producing *P.pastoris*.

Results. Among the designed strategies for rhGH production, the highest rhGH and cell concentrations were achieved, respectively, as 0.64 g/L and 105 g/L at t=42 h of the production phase, with the strategy wherein dynamic methanol feeding was employed at a pre-determined feeding rate calculated for constant μ_{M0}=0.03 h^{-1}; where sorbitol concentration was kept at 50 g/L within t=0-15 h with continuous feeding of sorbitol with the pre-determined feeding rate calculated for constant μ_{S0}=0.025 h^{-1}. The overall cell and product yields on the two substrates consumed were, respectively, found as $Y_{X/St}$=0.26 g/g and $Y_{P/St}$=2.26 mg/g.
Related with the rHuEPO production, the highest rHuEPO concentration was achieved as C_{rHuEPO}= 0.65 g/L at t=9 h with the cell concentration C_x=55 g/L in the production phase; wherein methanol was fed to the bioreactor with a pre-determined dynamic feeding rate calculated for constant μ_{M0}=0.03 h^{-1}; where, three consecutive pulses of the co-substrate mannitol was introduced at t=0, 6, and 12 h, and the concentration in the bioreactor increased to 50 g/L. The overall cell and product yields on the substrates methanol and mannitol were found, respectively, as $Y_{X/St}$=0.22 g/g and $Y_{P/St}$=2.23 mg/g. Whereas, with the co-substrate sorbitol, while the highest cell concentration was achieved as C_x=109 g/L at t=48 h, with the strategy where methanol was fed to the bioreactor at a pre-determined feeding rate for constant μ_{M0}=0.03 h^{-1}, and sorbitol concentration was kept at 50 g/L within *t*=0–15 h; the highest rHuEPO production was achieved as C_{rHuEPO}= 0.32 g/L at t=18 h.

Conclusions. This work demonstrates that the design of semi-batch feeding strategy, based on the selection of the substrates and the co-substrates, and their dynamic feeding into the bioreactor, is important to increase the production and productivity in r-protein production by Mut$^+$ strains of *P. pastoris*. Indeed, the choice of the co-substrate is dependent on the target r-protein.

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