abstract of Ph.D. thesis

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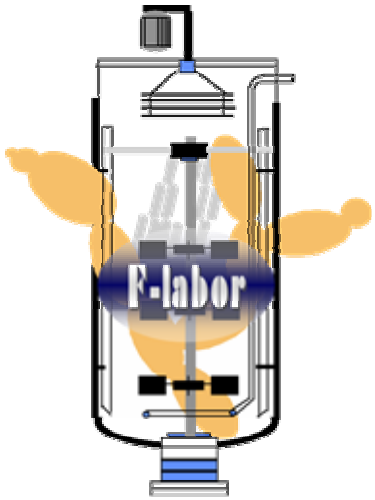
DEVELOPMENT OF RECOMBINANT *PICHIA PASTORIS* FERMENTATION

supervisor:

professor Béla Sevella



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INTRODUCTION

23 years passed by since the first recombinant therapeutic protein, the insulin was registered. The market of recombinant protein based pharmaceuticals exceeds 30 billion USD for 2005, realized on approximately 130 products. Besides the marketed compounds, 500 new recombinant therapeutics are in clinical test phase projecting a further rapid growth of the recombinant protein therapeutics market. The industrialization of the recombinant products had been based on the optimization of the complex production processes starting at the expression system development and finishing with the integration of the technology into the strictly controlled production line. As the production is founded by the successful upstream processes, the biochemical-theoretical and technical-empirical optimization as well as the mathematical description of these techniques are predominant in supporting the control of production. The purpose of my Ph.D. work was to develop the fermentation of the widely used recombinant *Pichia pastoris*, which so far has mainly been used in laboratory experiments. My aim was to gain sufficient result to support large-scale application of this expression tool through the adequate mathematical characterization of the upstream process.

P. pastoris utilizes methanol as sole carbon and energy source by its alcohol-oxidase enzymes (Aox1 and 2; EC 1.1.3.13). The *in vivo* concentration of Aox enzymes is under transcriptional control: glycerol and glucose act through catabolite repression, whereas methanol is inducer of enzyme production. As a result, in the presence of easily fermentable carbon sources, practically zero Aox activity can be measured in the cells, while Aox enzymes accumulate up to the 30 % of total cell proteins in fully activated state. *P. pastoris* has two AOX genes: AOX1 is responsible for the 85-90 % of the activity in induced state, whereas AOX2 supplies the rest. The Aox enzymes show 98 % homology at amino-acid level; however the control regions of the genes are distinct. The promoter of Aox1 is commonly used as expression tool. The recombinant cells may have intact AOX1

and AOX2 genes (Methanol utilization type +, Mut^+) or disrupted AOX1 and functional AOX2 (Methanol utilization type Slow, Mut^S), depending on the integration site of the vector sequence. In spite of that the specific growth rate of Mut^S cells is only 1/7th -1/8th of the wild and Mut^+ type cells, often the Mut^S gives higher functional recombinant protein concentrations. The excellent results by Mut^S are explained by the time requirement of post-translational modifications: the higher-eukaryotic type disulfide-bonds, glycosylation and conformation can often be fully achieved only by Mut^S cells. *P. pastoris* normally recognizes the original leader-sequences; however *Saccharomyces cerevisiae* α-mating factor is most commonly used for exracellular expression. The recombinant *P. pastoris* fermentation is typically composed of two parts: the first is utilizing glycerol while the heterologue product is formed during the second, methanol fed phase. The subtle methanol concentration control is the key factor in the efficient production: above 15 g/L the increased substrate level leads to raised metabolic activity which inhibits both cell growth and product formation due to the accumulation of reactive metabolites such as hydrogen-peroxide and formaldehyde.

AIMS AND SCOPES

My purpose was to support the application of the *P. pastoris* based expression system by setting up statistical and kinetic models of the fermentation process. My Ph.D. work covered the investigation of the influence of pH, temperature, inducer/carbon-source concentration and the methanol concentration control method on the performance of the recombinant *P. pastoris*.

P. pastoris can be cultivated on glycerol in a wide pH and temperature range. As some sensitive recombinant products are degraded by vacuolar neutral and alcalic proteases, I wished to determine the limits of successful products formation in concern of pH and temperature. Lowering the pH can be used to decrease the proteolytic degradation, however it influences the specific product formation rate, as well. Decreasing the temperature decreases the cell lysis this way lowering the protease concentration in the fermentation broth, though the temperature acts through changing the reaction rates in the substrate utilization pathway. Complete kinetic model is available for *P. pastoris* Mut^+ concerning the effect of substrate concentration, whereas for the Mut^S version no such description is known. I whished to gain sufficient data in order to take substrate kinetic statements by keeping different methanol levels in the product forming period of *P. pastoris* Mut^S cultivations. For this purpose I had to design a properly functioning experimental system including the control of methanol concentration in the fermentation broth. Two different strategies were tested for the control of substrate levels: the first relied on a semi-conductor sensor, measuring volatile organic compounds, whereas the second based on the level of dissolved oxygen change for periodic methanol addition. In case of the semi-conductor sensor I had to perform the statistical analysis of function parameters and to set up a mathematical description in order to design the sensor for the desired purposes. The applicability of the generally accepted, however rarely applied dissolved oxygen spike method was investigated by experimentally based statistical analysis as well as setting up a kinetic model followed by *in silico* model runs.

The results of the experimental and kinetic optimization process were applied for the *P. pastoris* Mut^S human serum albumin producing commercially available model strain and for the "in house" developed and selected best propanediol-oxidoreductase (EC 1.1.1.202) producing strains. For this purpose I had to set up a quantitative strain selection strategy as well as a reproducible cell disruption methodology in order to measure intracellular products. The selection and bench-top fermentations were followed by pilot-scale experiments in order to evaluate the applicability of the fermentation strategy and the newly developed recombinant strain. I also wished to test in pilot-scale if the economically more

feasible air-lift reactor can successfully substitute the traditional stirred tank reactor in case of the *P. pastoris* fermentation.

RESULTS

1.1. In spite of the fact that the recombinant *P. pastoris* is capable to grow in a wide temperature and pH range on glycerol, generally 30°C and pH 5.0 is used for heterologue product formation on methanol carbon source. As a great number of product proteins are sensitive for the degradation by neutral and alcalic vacuolar *Pichia* proteases, lowering the pH of the product formation period seemed to be a desirable strategy to increase product yield. Decreasing the temperature is proven to hinder cell lyses, however it may enhance specific product formation as well. I evaluated the effect of pH and temperature on the specific growth rate, specific product formation rate, volumetric productivity and product yield by expanding a linear orthogonal design in the range of pH [3.2; 5.2] T [23; 29]°C with a full second order design for pH [5.2; 7.2] and T [17; 23]°C. *P. pastoris* GS115 Mut^S HSA producing strain was used for the experiments. Statistical analysis showed that specific growth rate is practically independent of pH and T, whereas the other variables are significantly influenced by pH and T. These results suggest that the volumetric productivity is determined mostly by the specific product formation rate not by the specific growth rate in respect of pH and T. By the mathematical description of specific product formation rate both itself and the volumetric productivity can be optimized [4, 7].

1.2. Two methods were used to characterize specific product formation rate in the function of pH and T: the stepwise decomposition of the full second order model and the application of a formal kinetic description:

$$q_P = z_0 + z_1 \cdot pH + z_2 \cdot pH^2 + z_3 \cdot T + z_4 \cdot T^2$$

$$q_P = \nu_P \cdot \frac{K_1 \cdot [H^+]}{K_1 \cdot K_2 + K_1 \cdot [H^+] + [H^+]^2} \cdot \left\{ a \cdot \exp\left(\frac{-\Delta H_1}{RT}\right) - b \cdot \exp\left(\frac{-\Delta H_2}{RT}\right) \right\}$$

where:

q_P – specific product formation rate

z – model constants

T – temperature

ν_P – protein specific product formation rate

K_1, K_2 – constants of Michaelis pH function

a, b – constants of Arrhenius equations

ΔH_1, ΔH_2 – apparent Gibbs energy

R – Regnault constant

The formal kinetic model was more adequate in describing the specific product formation rate (Fig. 1.). The calculated maximum for the specific product formation rate is pH 5.64 and 20.24 °C. The experimental realization of the optimum showed the highest specific product formation value (0,379 mg HSA/g CDW/h) in the whole experimental range [11]. As the experimental value at the optimum showed 8.3 % positive deviation compared to the calculated value, the model can be accepted. The pH and T optima for cell mass production on glycerol and for heterologue protein formation on methanol are different, thus the batch and the fed-batch phase of the *P. pastoris* Mut^S fermentation should be carried out among different circumstances.

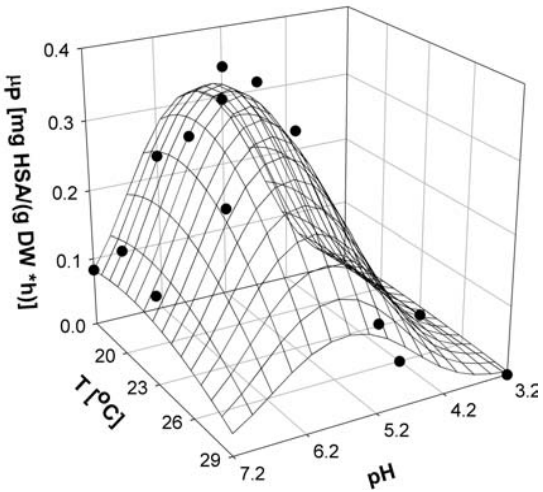

Figure 1. Specific product formation rate [mg HSA / g CDW /h] in the function of pH and T. Experimental data and formal kinetic model.

1.3. As the model product human serum albumin was not the target of significant proteolytic degradation, it is assumed that the model and the optimum point clearly characterize the specific product formation rate. In this case the optimum and the normalized model can be used for any extracellular heterologue protein expressed in *P. pastoris* GS115 Mut^S, unless the product requires extreme post-translational modifications. In case of the proteolysis sensitive proteins, the model equation is a good tool to calculate the production optimum if the product specific ν_P and degradation kinetics are determined.

2. In order to control the methanol concentration in *P. pastoris* fermentations, an SnO_2 semi-conductor based system was developed [1]. The sensor measures the quantity of methanol in a constant air flow, which is directed through a silicon membrane tubing submerged into the fermentation broth. The diffusion rate of methanol into the air flow is determined by the methanol concentration in the fermentation broth thus the sensor signal is proportional to the methanol concentration in the media. According to statistical analysis among the parameters which possibly influence the sensor signal, only the surface of mass transfer (lengths of tubing) proved to be the significant. The sensor signal was described by the following model equation in water-methanol systems:

$$U = \frac{(0{,}0059 * H + 2{,}4604) * C}{-0{,}0078 * H + 0{,}7710 + C}$$

where:

U- sensor signal [V]

C- methanol concentration in the media [(V/V)%]

H- length of tubing [cm]

The baseline of the characteristic curve changes by the application of cell suspension instead of water, however the slope of the response curve remains the same (Figure 2.) [14.].

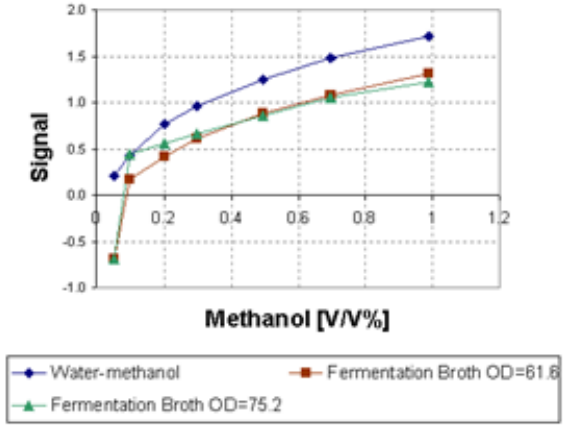

Figure 2. Methanol-sensor calibration in water-methanol model system and in fermentation broth. The sensor signal [V] in the function of methanol concentration.

3. *P. pastoris* GS115 Mut$^+$ shows classical substrate inhibition kinetics in the function of methanol concentration. Generally the accumulation of reactive metabolic products of methanol oxidation, e.g. formaldehyde and hydrogen-peroxide are blamed for the decrease of specific growth rate, however the catabolite repression on AOX promoters by methanol has been also arose as a

possible reason. In case of *P. pastoris* GS115 Mut^S the specific growth rate was independent of the methanol concentration in the range of [0.45; 8.85] g/L in my experiments. This implies that neither metabolic products have inhibitory effect on specific growth rate, nor methanol has catabolite repression on AOX2 promoter. On the other hand, specific product formation rate was strongly influenced by methanol concentration with maximum measured value at 0.45 g/L (Fig. 3.). Moreover, unlike in case of Mut$^+$ cells, for Mut^S variants no linear relation can be set up between the specific growth rate and the specific methanol consumption rate. Whereas volumetric productivity is determined both by the specific growth and product formation rate for Mut$^+$ cells, and the optimum is between the maxima of the two specific rates (at 2.1 g/L methanol), only specific product formation determines the maximum of volumetric productivity for Mut^S cells, and thus the two maxima coincides at 0.45 g/L methanol concentration. If we accept that the specific growth rate is determined at transcription level through the steady-state concentration of the Ax in the cells and no significant metabolic product inhibition is present in Mut^S cells, then the decrease of heterologue product formation under the control of AOX1 promoter for the increase of methanol concentration suggests the catabolic repression of AOX1 promoter by methanol. The methanol concentration independence of specific growth rate in Mut^S cells implies that no such catabolic repression is present on AOX2 promoter. This difference in the control of Aox production explains the so far unknown physiological role of the two parallel AOX genes in *P. pastoris*.

The value of maintenance coefficient was determined (0.026 1/h). As its value is in the range of the measured specific growth rates, strong energy limitation characterizes the Mut^S fermentations, regardless of the methanol concentration [3, 5, 8].

Specific rates in the optimum points were reproducible in scale-up experiments. The reproducibility and the success of a fermentation process are mainly dependent on the proper agitation of the fermentation media. For this reason the air-lift reactor was not suitable for recombinant *P. pastoris* GS115 Mut^S fermentation.

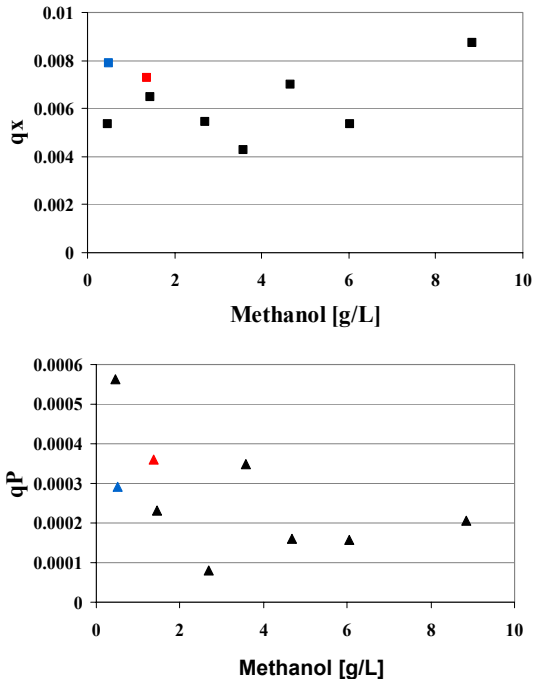

Figure 3. *P. pastoris* GS115 Mut^S HSA steady-state specific growth rate (qx) [1/h] and specific product formation rate (qP)[g HSA/ g CDW/ h] in the function of methanol concentration. Biostat M fermentor (1.2 L); Biostat U stirred tank (19 L); Biostat U air-lift (19 L)

4. *Citrobacter freundii* propanediol-oxidoreductase (EC 1.1.1.202) was expressed in active form in *P. pastoris*. The best producing strain was successfully selected by the application of a self-designed and tested quantitative expression selection system. The selected *P. pastoris* SMD1168 strain showed reproducible product formation in spinning test-tube, shaking-flask and stirred bioreactor cultivations, however required complex media components for successful product formation. In optimized fermentation of the selected *P. pastoris* SMD1168 /49 strain, we reached the same volumetric productivity as in optimized *Klebsiella pneumoniae*

fermentation (10.7 and 10.3 U/ L fermentation broth/ h, respectively), however the value of specific activity was 15 times higher in case of the recombinant yeast (1540 U/L fermentation broth). The fermentation process of the recombinant strain was scaled-up to pilot scale without decrease in volumetric productivity [2, 23].

5. It is generally accepted that methanol concentration during fed-batch recombinant *P. pastoris* Mut$^+$ fermentations can be controlled by the change of dissolved oxygen level for periodic methanol addition. In order to investigate the above statement, I completed the model of Zhang and coworkers by an oxygen balance equation. The model treats the assimilatory and dissimilatory methanol pathways separately, using a partition coefficient "a" to express the share of substrate between the metabolic paths (Fig. 4.). During the model development and parameter fit process, the methanol concentration dependence of the oxygen transfer coefficient was experimentally determined and the original value of cell mass/oxygen yield ($Y_{x/MeOH}$) was altered. The most adequate model uses $Y_{x/MeOH}$=0.104 g CWW/ g methanol and a=0.764 mol O_2/ mol methanol (Fig. 5.). The value of the partition coefficient implies the dominancy of the assimilatory pathway and is between the 0.5 value of the model of Ren and the 1.17 value, suggested by Jahic [8, 14].

The mathematical model based investigation showed that the methanol concentration can not be properly controlled by the dissolved oxygen concentration change for periodic methanol addition. The analysis of variance of the experimental data supplied parallel result with the *in silico* experiments.

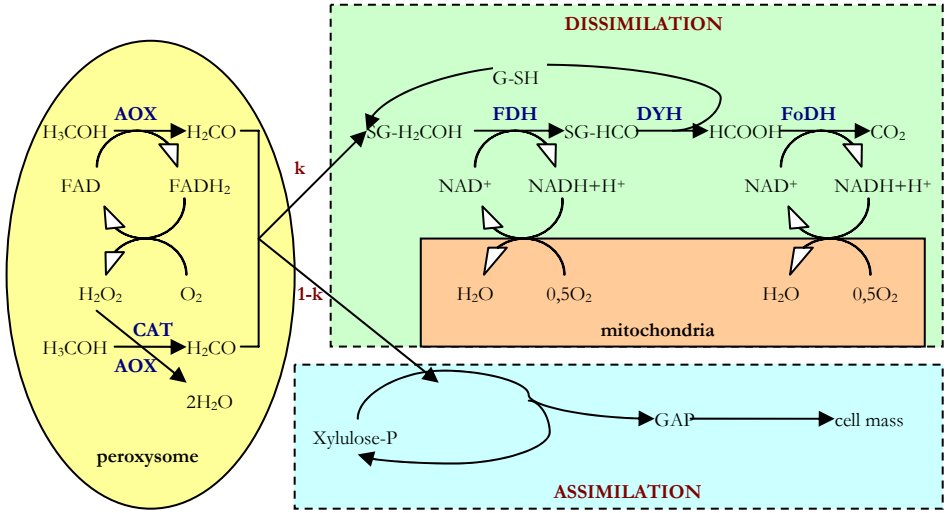

Figure 4. Oxygen metabolism of *P. pastoris* on methanol. GSH - reduced glutathione; AOX – alcohol-oxidase; CAT – catalase; FDH – formaldehyde-dehydrogenase; DYH – S-formyl-glutathion-hydrolase; FoDH – fomate-dehydrogenase; GAP – glyceraldehyde-3-phosphate

The ordinary differential equation system describing the metabolism of *P. pastoris* GS115 Mut$^+$, completed with dissolved oxygen balance:

$$q_x = \frac{0{,}146 \cdot c_{MeOH}}{1{,}5 + c_{MeOH} + c_{MeOH}^2 / 8{,}86}$$

$$\frac{dx}{dt} = q_x \cdot x$$

$$q_{MeOH} = 0{,}84 \cdot q_x + 0{,}0071$$

$$\frac{dc_{MeOH}}{dt} = F \cdot c_{MeOH,be} - q_{MeOH} \cdot x$$

$$\frac{dc}{dt} = K_L a \cdot (c^* - c) - a \cdot q_{MeOH} \cdot x$$

$$K_L a = 75{,}54 + 0{,}1471 \cdot 2{,}9174^{c_{MeOH}}$$

where

q_x – specific growth rate [1/h]

c_{MeOH} – methanol concentration in fermentation broth [g/L]

t – time [h]

x – cell wet weight [g/L]

F – substrate addition rate [g/h]

$c_{MeOH,be}$ – methanol concentration in inlet flux [g/L]

K_La – oxygen adsorption coefficient [1/h]

c* - saturated dissolved oxygen concentration in fermentation broth [g/L]

c - dissolved oxygen concentration in fermentation broth [g/L]

a – partition coefficient for oxygen between the assimilatory and dissimilatory pathways, a=0,5+k

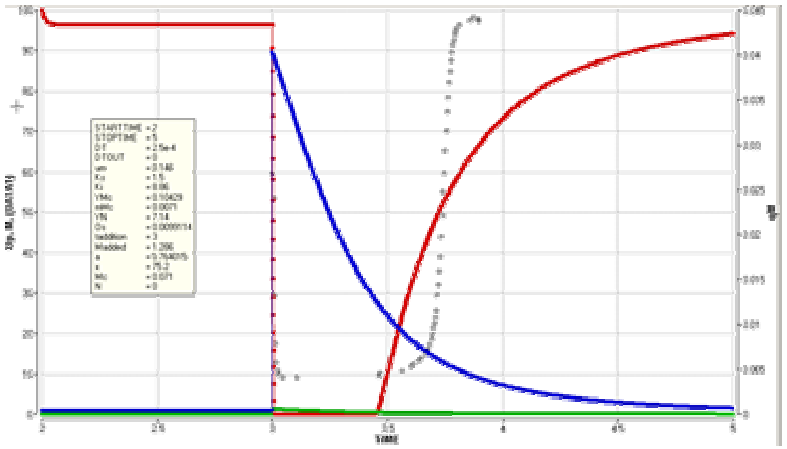

Figure 5. The result of fitting saturated dissolved oxygen concentration, cell/oxygen yield and the partition coefficient "a". — oxygen concentration calculated by the model %; — specific growth rate calculated by the model [1/h]; — specific methanol consumption rate [g methanol / g CWW / h]; ◦ experimental values of dissolved oxygen concentration %

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