

## Applied Microbiology 2016- Pichia process optimization by methanol/sorbitol co-feeding – Patrick Fickers - University of Liege

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### Abstract

Recombinant protein production driven by AOX1 promoter is challenged by a high oxygen demand and warmth production, especially in large-scale bioreactor. A reliable solution depends on a methanol/sorbitol co-feeding strategy during the induction phase. During this work, transient continuous cultures were first performed to quantitatively assess the advantages of a methanol/sorbitol co-feeding process with a *P. pastoris* Mut<sup>+</sup> strain bearing a pAOX1-lacZ construct served as a reporter gene. Our results demonstrated that cell-specific oxygen consumption (qO<sub>2</sub>) might be reduced by decreasing the methanol fraction within the feeding media. Optimal pAOX1 induction was achieved and maintained within the range of 0.45~0.75 C-mol/C-mol of methanol fraction. Additionally, the qO<sub>2</sub> was reduced by 30% at the most in those conditions. By support of a simplified metabolic network, metabolic flux analysis (MFA) was performed to quantify intracellular metabolic flux distributions during the transient continuous cultures, which further shed light on the advantages of methanol/sorbitol co-feeding process. Secondly, chemo stat cultures were performed to research the cell growth, metabolism and regulation of the AOX1 promoter (pAOX1) regarding co-feeding rate of optimized methanol/sorbitol mixture. Our results highlight that methanol/sorbitol co-feeding allowed cells to adapt to oxygen transient limitation that always occur at industrial scale with reduced effect on pAOX1 induction and cell viability. The optimal feeding rate tested here was 6.6 mmolC.(DCW.h)<sup>-1</sup> at an oxygen transfer rate (OTR) of 8.28 gO<sub>2</sub> (l.h)<sup>-1</sup> with over five-fold pAOX1 induction (probably directly related to target protein productivity) compared with previous work.

### Introduction

The methylotrophic yeast *Pichia pastoris* may be a well-established expression host which is usually utilized in the assembly of protein pharmaceuticals. It can produce high levels of recombinant proteins using a strong and tightly regulated methanol-inducible alcohol oxidase promoter (AOX1). The most widely utilized fermentation process of *P. pastoris* consists of a three steps approach (6). During the first step, cells are cultured in batches using a defined medium with glycerol as the carbon source to rapidly achieve high cell densities. As the AOX1 promoter is repressed by unlimited growth on glycerol recombinant protein expression is repressed

at this stage. During the second step, so as to extend biomass production and de-repress the methanol metabolic machinery, a glycerol-limited fed-batch procedure is initiated. This phase leads to gradual de-repression of the enzymes necessary for the dissimilation of methanol and reduces the time necessary for the cells to adapt to growth on methanol. Finally, recombinant protein expression is typically induced by feeding methanol because the sole carbon source.

A fermentation guideline for both Mut<sup>+</sup> and Muts strains of *P. pastoris* is available from Invitrogen. Stratton et al. devised a protocol for *P. pastoris* high cell-density fermentation, during which detailed procedures are provided. Using this protocol, in our laboratory, recombinant human somatotropin (rhGH) was produced with *P. pastoris* Mut<sup>+</sup> during which AOX1 promoter was induced with methanol for 30 h.

However, the methanol feed rate profiles described by Stratton et al. are fixed and may be inapplicable for those strains whose ability to utilize methanol has changed as a result of the expression of heterologous genes. For a fed-batch fermentation process, the substrate feed rate usually must be optimized to maximise productivity. Various methods for such optimization are reported in other production systems.

Methanol can act as both the carbon source and therefore the inducer of the expression of recombinant proteins. However, in the presence of high concentrations of methanol, which has a high substrate affinity for alcohol oxidase-*P. pastoris* leads to intoxicate with metabolites (formic acid, formaldehyde). Hence, at high concentrations, methanol inhibits the organism's growth. Therefore, so as to stay methanol concentration below the toxic limit, fed-batch operation has been utilized because the standard protocol for recombinant protein production by *P. pastoris* through AOX1 promoter.

The gathering of methanol leads to cytotoxic effect, so methanol feeding strategies are studied to partially replace or a minimum of complement methanol with other carbon sources like glycerol glucose or sorbitol. Moreover, the use of a less repressing carbon source may result in higher specific production rates, improving overall productivity and eliminating the need for the tight control of residual substrate levels. Among them, sorbitol may be a widely accepted non-

repressive carbon source for *P. pastoris*. The advantages of mixed feeds of sorbitol and methanol for the assembly of various recombinant proteins with *P. pastoris* have been reported.

As a follow-up to other studies, this work aimed to compare the effect of various concentrations of sorbitol in mixed feeding strategy with stepwise addition of methanol to develop a new scientific method that might maximize the assembly of hGH in

*P. pastoris*. In this study sorbitol was added batch-wise to the medium at the start of the induction phase, continuing with methanol feeding for 30 h. In order to know the consequences of varied feeding strategies, supported different co-substrate concentrations, on production of hGH in *P. pastoris*, cell density, total protein, hGH expression level and hGH concentration were analyzed and therefore the results compared with the essential protocol of methanol feeding using one-way analysis of variance (ANOVA).