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High level protein expression in *Pichia pastoris* combining synthetic promoters and synthetic genes

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Introduction

General issues that often hamper successful protein expression at high yields are codon preference, secondary mRNA structure, overall GC content. Gene optimization, therefore, is a powerful tool to overcome initial limitations in protein expression on transcriptional and translational level. Design principles (Figure 1) can be combined to address and overcome the before mentioned issues simultaneously in one gene design approach. However, a basic limitation in such optimizations is the huge number of possible sequence variants that can be obtained for one gene. Consequently, a smart approach to investigate the effects of gene optimization on expression level is required.

In our cooperation with DNA2.0 we combined DNA2.0's expertise in gene design [1] and synthesis with our experience in activity-based micro-scale screening in *Pichia pastoris* and VTU Technology´s expertise in bioreactor cultivations. Using experimental design principles DNA2.0 provided 48 differently optimized gene sequences of the chosen model enzyme CBH2 from *Trichoderma reesei*. Activity landscapes were screened for apparent single copy indication and the resulting data used to generate a model for improved gene design for *P. pastoris*. Fed-batch cultivations were performed to confirm the micro-scale experiments and to test the performance of the synthetic promoter P(De2) [2] under tightly controlled and scalable conditions.

Results and Discussion

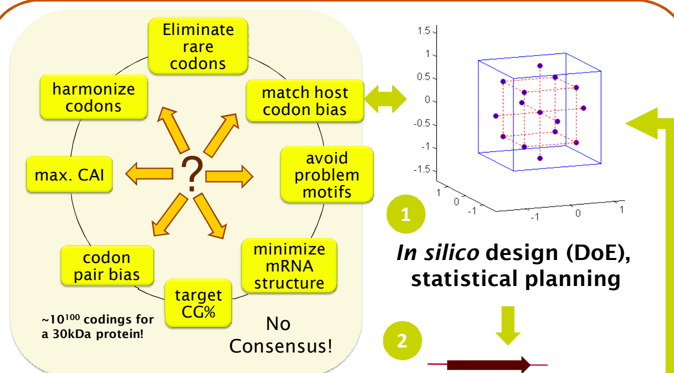
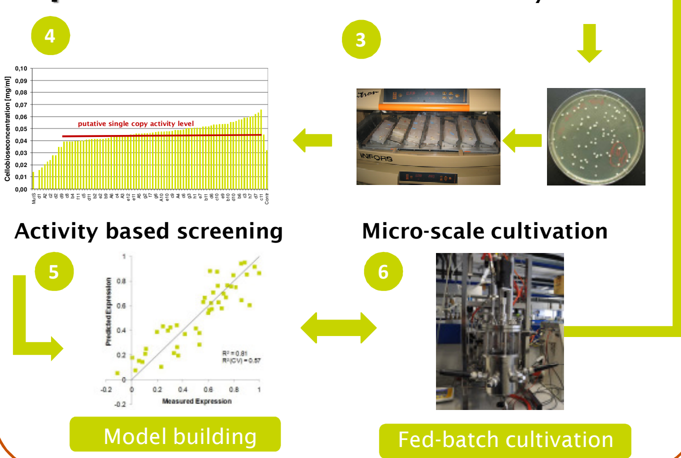


Figure 1: Common design principles for improved gene coding sequences.

Experimental work-flow

Gene synthesis



Promoter performance

The synthetic promoter P(De2) is highly repressed in presence of non-limiting concentrations of glucose but active under derepressed conditions. Additionally, this promoter can also be induced if methanol is used as sole carbon source. To proof this tight regulation fed-batch cultivations of single copy strains expressing *Tr*CBH2 were performed either under the control of the wild- type promoter P(AOX1) or P(De2). Transcript analysis revealed the high performance of P(De2) (Figure 2).

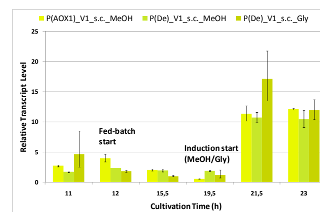


Figure 2: Transcript levels of *Tr*CBH2 gene expression driven either by P(AOX1) or P(De2) using either methanol or glycerol as sole carbon source.

High yield protein production

Strains with defined copy numbers expressing an improved *Tr*CBH2 gene variant under control of P(De2) were employed to confirm the micro-scale results (Figure 3). Within the first 30 hours of induction, protein concentrations of the improved and original *Tr*CBH2 variant were about the same range. Afterwards a significant increase is observed yielding about two-fold more protein at the end of the cultivation process. Furthermore, a different strain under control of P(De2) was cultivated and yielded more than 15 g/l of secreted *Tr*CBH2 although no methanol was fed, but glycerol.

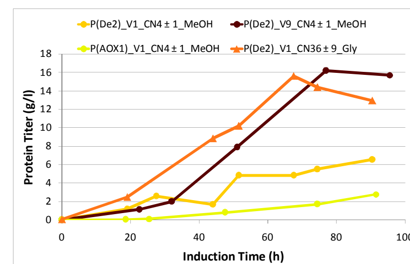


Figure 3: Time- course of *Tr*CBH2 production by *Pichia pastoris* strains harboring a different amount of integrated expression cassettes and gene variants.

Conclusion & Summary

- Out of the 48 active *Tr*CBH2 variants synonymous codon changes caused more than 30-fold differences.
- Fed-batch cultivations were performed and confirmed improved protein production.
- Based on synonymous codon usage a first model for improved heterologous protein production in *P. pastoris* was generated.
- The synthetic promoter P(De2) is repressed by glucose but active under derepressed conditions and in addition can be induced by methanol.
- Fed-batch cultivations with and without methanol yielded more than 15g/l of secreted *Tr*CBH2.

References

[1] Welch M. et al., Design parameters to control synthetic gene expression in Escherichia coli. PLoS One 2009, 4(9):e7002
[2] Hartner, F.S. and Glieder A. (2005), patent AT 501955 owned by VTU Technology GmbH

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