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Comparison of two expression platforms in respect to protein yield and quality: *Pichia pastoris* versus *Pichia angusta*

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ABSTRACT

The methylotrophic yeasts *Pichia pastoris* and *Pichia angusta* (*Hansenula polymorpha*) were used for the comparative heterologous production of two model mammalian proteins of pharmaceutical interest, the NK1-fragment (22 kDa) of human hepatocyte growth factor and the extracellular domain (28 kDa) of mouse tissue factor (MTF). Both recombinant proteins were engineered to contain an N-terminal *Strep*-(WSHPQFEK) and a C-terminal His_6-tag. In addition, both proteins contained the pre-pro-sequence of *Saccharomyces cerevisiae* mating factor alpha to allow secretion. Following vector construction, transformation and zeocin amplification, the best *Pichia* producers were identified in a screening procedure using Western blot and a Luminex xMAP™ based high-throughput method. Recombinant NK1-fragment and MTF were purified from culture supernatants of the best producers by affinity chromatography (Ni–nitrilotriacetic acid columns). Using *P. pastoris* as a host for the synthesis of NK1-fragment a protein yield of 5.7 mg/l was achieved. In comparable expression experiments *P. angusta* yielded 1.6 mg/l of NK1-fragment. NK1-fragment apparently was not glycosylated in either system. For the production of MTF, *P. pastoris* was also the superior host yielding 1.2 mg/l glycosylated recombinant protein whereas *P. angusta* was clearly less efficient (<0.2 mg/l MTF). For both expression systems no correlation between the amount of recombinant protein and the copy number of the chromosomally integrated heterologous genes was found. In *P. pastoris* strains less degradation of the two model recombinant proteins was observed. Altogether, this paper provides a structured protocol for rapidly identifying productive *Pichia* strains for the synthesis of full-length recombinant proteins.

Introduction

The yeast genus *Pichia* is characterized by multilateral budding, presence or absence of pseudohyphae/septate hyphae, and by ascospores that may be hat-shaped, hemispheroidal, or spherical with or without a ledge. The genus *Hansenula* was characterized by the same phenotypic traits with the exception that *Hansenula* species assimilated nitrate whereas *Pichia* species did not. The demonstration that strains of some species of *Pichia* could assimilate nitrate whereas strains of some *Hansenula* species could not removed the main character that separated the two genera, which prompted reassignment of *Hansenula* species to *Pichia* [1,2].

Yeasts of the genus *Pichia* have a strong preference for respiratory growth, consequently do not produce significant amounts of toxic ethanol (as compared to many species of the genus *Saccharomyces*) [3] and may grow to very high cell densities on inexpensive media. *Pichia pastoris* (9.8 Mbp genome) and *Pichia angusta* (9–10 Mbp genome) both perform posttranslational modifications such as glycosylation and proteolytic processing and are efficient hosts for the synthesis of active/soluble e.g. mammalian proteins [4]. *Pichia pastoris* has become a popular academic tool: as of 2007 more than 600 genes have been cloned and expressed using this microorganism [5]. In the meantime *P. angusta* has been used for industrial production of proteins e.g. for the synthesis of hepatitis B surface antigen, human interferon IFNα-2a, insulin, hexose oxidase (EC 1.1.3.5) and phytase (EC 3.1.3.8) [6].

Only a few studies exist that describe the comparative expression (and secretion) of defined identical model proteins in several yeasts [4,7]. As model proteins for our experiments two pharmaceutically relevant proteins were chosen, NK1-fragment of human hepatocyte growth factor (HGF) (22 kDa) and the extracellular domain of mouse tissue factor (MTF) (28 kDa). Fig. 1 shows the amino acid sequences of the two native proteins including their native signal peptides, and the amino acid sequences of both proteins as they were produced in yeast. HGF is a multifunctional secreted 90 kDa protein that acts as a mitogen, motogen, and/or morphogen depending on the cellular target and context [8]. Two alternatively spliced, truncated variants of HGF exist naturally. They are designated NK1 (25 kDa) and NK2 (35 kDa), respectively.

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A NK1-fragment native sequence (GenBank M73240)

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1      MWVTKLLPAL LLQHVLLHLL LLPIAIPYAE GQRKRRNTIH EFKKSAKTTL IKIDPALKIK
61     TKKVNTADQC ANRCTRNKGL PFTCKAFVFD KARKQCLWFP FNSMSSGVKK EFGHEFDLYE
121    NKDYIRNCII GKGRSYKGTV SITKSGIKCQ PWSSMIPHEH SYRGKDLQEN YCRNPRGEEG
181    GPWCFTSNPE VRYEVCDIPQ CSEVE
```

B Recombinant NK1 fragment synthesized in *Pichia pastoris* and *Pichia angusta*

```
1      MRFPSIFTAV LFAASSALAA PVNTTTEDET AQIPAEAVIG YSDLEGDFDV AVLPFSNSTN
61     NGLLFINTTI ASIAAKEEGV SLEKRWSHPQ▼FEKQRKRRNT IHEFKKSAKT TLIKIDPALK
121    IKTKKVNTAD QCANRCTRNK GLPFTCKAFV FDKARKQCLW FPFNSMSSGV KKEFGHEFDL
181    YENKDYIRNC IIGKGRSYKG TVSITKSGIK CQPWSSMIPH EHSYRGKDLQ ENYCRNPRGE
241    EGGPWCFTSN PEVRYEVCDI PQCSEVEHHH HHH
```

C MTF native sequence (GenBank P20352)

```
1      MAILVRPRLL AALAPTFLGC LLLQVIAGAG IPEKAFNLTW ISTDFKTILE WQPKPTNYTY
61     TVQISDRSRN WKNKCFSTTD TECDLTDEIV KDVTWAYEAK VLSVPRRNSV HGDGDQLVIH
121    GEEPPFTNAP KFLPYRDTNL GQPVIQQFEQ DGRKLNVVVK DSLTLVRKNG TFLTLRQVFG
181    KDLGYIITYR KGSSTGKKTN ITNTNEFSID VEEGVSYCFF VQAMIFSRKT NQNSPGSSTV
241    CTEQWKSFLG ETLIIVGAVV LLATIFIILL SISLCKRRKN RAGQKGKNTP SRLA
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D Recombinant MTF synthesized in *Pichia pastoris* and *Pichia angusta*

```
1      MRFPSIFTAV LFAASSALAA PVNTTTEDET AQIPAEAVIG YSDLEGDFDV AVLPFSNSTN
61     NGLLFINTTI ASIAAKEEGV SLEKRW▼SHPQFEKAGIPEKA FNLTWISTDF KTILEWQPKP
121    TNYTYTVQIS DRSRNWKNKC FSTTDTECDL TDEIVKDVTW AYEAKVLSVP RRNSVHGDGD
181    QLVIHGEEPP FTNAPKFLPY RDTNLGQPVI QQFEQNGRKL NVVVKDSLTL VRKNGTFLTL
241    RQVFGKDLGY IITYRKGSST GKKTNITNTN EFSIDVEEGV SYCFFVQAMI FSRKTNQNSP
301    GSSTVCTEQW KSFLGEHHHH HH
```

Fig. 1. Amino acid sequences of NK1-fragment of human hepatocyte growth factor and of the extracellular domain of mouse tissue factor (MTF). The primary structures of the native proteins (A, NK1-fragment, and C, MTF) and their N-terminal native signal peptides (single underlined) are shown. The amino acid sequences of recombinant NK1-fragment (B) and MTF (D) as they were synthesized in *P. pastoris* and *P. angusta* (the alpha mating factor signal peptide of *Saccharomyces cerevisiae* is single underlined) and the expected (after R) and observed (▼) cleavage sites that removed the mating factor signal peptides are shown as well. The *Strep*-tag is double underlined and the C-terminal His_6-tag is single underlined (B and D). In the case of MTF (D) one domain (dotted line in C) was removed.

Production of NK1-fragment was achieved earlier using *Escherichia coli* [9] and insect cells [8]. MTF (33 kDa) is a 264-amino acid transmembrane glycoprotein that serves as the cellular receptor and cofactor for plasma FVII/VIIa [10]. Highly similar proteins to MTF were successfully synthesized using *P. pastoris* [11], *S. cerevisiae* [12] and *E. coli* [13]. In the present work the fully functional extracellular domain (28 kDa) was produced.

To our knowledge, this is the first report directly comparing the performance of the two expression systems *P. pastoris* and *P. angusta*. The promoter of the *P. pastoris* gene *AOX1* was used to drive heterologous gene expression in *P. pastoris*, and the *P. angusta FMD* (*FDH*) promoter was used in *P. angusta*. The gene *AOX1* codes for alcohol oxidase (EC 1.1.3.13) and *FMD* codes for formate dehydrogenase (EC 1.2.1.2). Both enzymes are active in the cytosolic methanol utilization pathway of methylotrophic yeasts [14]. This report provides the researcher with an efficient routine for the rapid identification of high-performance protein overproducing *Pichia* strains using a combination of Western blot analysis, a novel Luminex based screening method [15] and qPCR.

Materials and methods

Yeast strains, growth conditions, overexpression experiments and processing of extracellular protein

Pichia pastoris X33 purchased from Invitrogen and *P. angusta* DSM 70277 (German Collection of Microorganisms and Cell Cultures) were used as hosts for expression of the genes for NK1-fragment and MTF. Both yeast species were cultured on YPD (1% yeast extract, 2% peptone, 2% glucose) at 30 °C. For gene expression, recombinant yeast strains were cultured on BMGY (1% glycerol, 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6, 1.34% yeast nitrogen base and 4×10^{-5}% biotin) to generate biomass and BMMY (0.5% methanol, 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6, 1.34% yeast nitrogen base and 4×10^{-5}% biotin) for induction experiments. Small scale (5 ml) expression experiments were carried out in glass reaction tubes (total volume 20 ml) as follows. The inoculum was prepared by transfer of a single colony to 5 ml BMGY and growth for 16 h at 250 rpm. Cells were harvested by centrifugation and resuspended in 5 ml BMMY to an OD_{600} of 1.0. The cells were grown for 3 days at 250 rpm. Methanol was added once a day to a final concentration of 1%. Cells were harvested and the supernatant was treated with trichloroacetic acid (TCA) to extract total protein (see "protein purification and analysis"). The inoculum for large scale (500 ml) expression experiments was prepared by transfer of a single colony to 100 ml BMGY. Cells (grown in shake flasks with baffles, without anti-foam, to an OD_{600} of 5.0) were harvested and resuspended in 500 ml BMMY to an OD_{600} of 1.0. Cells were grown in shake flasks with baffles (without anti-foam), treated with methanol and harvested as described for small scale cultures. For protein purification the volume of the supernatant was reduced by cross-flow filtration carried out using a LabscaleTFF-System (Millipore AB) supplied with a 10 kDa Pellicon XL filter module (Millipore AB). During cross-flow filtration the buffer was changed to PBS (10 mM $Na_2HPO_4 \times 2H_2O$, 1.8 mM KH_2PO_4, 135 mM NaCl, 2.7 mM KCl, pH 7.5).