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Adaptation of *Saccharomyces cerevisiae* expressing a heterologous protein

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ABSTRACT

Production of the heterologous protein, bovine aprotinin, in *Saccharomyces cerevisiae* was shown to affect the metabolism of the host cell to various extent depending on the strain genotype. Strains with different genotypes, industrial and laboroatory, respectively, were investigated. The maximal specific growth rate of the strains was reduced by 54% and 33%, respectively, upon the introduction of the gene encoding aprotinin. Growing the strains in sequential shake flask cultivations for 250 generations led to an increased maximal specific growth rate and a decrease in the yield of aprotinin as a result of the adaptation. Determination of the level of mRNA encoding aprotinin and the plasmid copy number pointed to different mechanisms responsible for the decline in aprotinin yield in the different strains.

1. Introduction

Saccharomyces cerevisiae is used both as a eukaryotic model system and in production of various biotech products. Some industrial products are natural products of *S. cerevisiae* metabolism, e.g. ethanol, whereas others require insertion of foreign DNA in the cell, e.g. active pharmaceutical proteins. The foreign DNA is often inserted into *S. cerevisiae* on a plasmid, an approach that allows the use of high copy plasmids and therefore also in principle high expression of the desired product. In academia and industry many yeast strains with different genotypes and ploidy are investigated and applied. The performance of different strains has been the focus of many research projects; however, lack of comparative studies between the genotypes precludes interlaboratory interpolation of results. A number of studies dealing with plasmid stability and protein production in strains with different ploidy have been published (Takagi et al., 1985; Spalding and Tuite, 1989; Fu et al., 1996). However, this did not give any clear correlation between plasmid stability and ploidy. The dissimilar outcome of the investigations of the influence of ploidy on plasmid stability might be a consequence of the different genotypes of the hosts investigated, but also be due to the different complexity of the expressed heterologous protein.

Heterologous protein production in industry is highly dependent on stable production strains. Stability is especially an issue in continuous cultivation since a high number of generations is reached. However, even during fed-batch cultivations around 35 generations are required from the stock culture to the final stage and consequently, even low instability of the expression cassette will have a detrimental impact on the overall productivity (Hensing et al., 1995). Changes in the phenotype of different microorganisms have earlier been reported after prolonged cultivation under the same conditions (Sauer, 2001). Kuyper et al. (2004) studied directed evolution in relation to xylose metabolism in *S. cerevisiae*, and in the initial yeast strain, the gene necessary for xylose metabolism (a xylose isomerase) was inserted allowing aerobic growth on xylose. By repeated cultivations with increased selection pressure a strain was found that could grow on xylose under anaerobic conditions. In some cases adaptation can be beneficial in the development of new strains; nevertheless phenotypic changes can also be undesired, and during heterologous protein production adaptations can lead to reduced productivity. The exact cause of the phenotypic changes is unknown in most cases, but it is often speculated to be correlated to a reduction of the copies of the gene encoding the heterologous protein. In the current study, parameters affecting the yield of a heterologous protein were investigated in order to elucidate the observed phenotypic changes.

In the current study, heterologous protein production in *S. cerevisiae* strains with different genotype (laboratory and industrial) and ploidy (haploid and diploid) was characterised. The

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production of the heterologous protein bovine aprotinin was investigated. Aprotinin is a small protein (6.5 kDa) with three disulfide bridges present and an isoelectric point of 10.5. It is a serine protease inhibitor and production of the protein is thus troublesome for the strains. The gene encoding aprotinin was transformed into *S. cerevisiae* on a 2-μ high copy number plasmid, a strong selection for the plasmid was achieved by transforming strains carrying deletions in the *TPI1* gene with a plasmid encoding the *POT* gene from *Schizosaccharomyces pombe* (Thim et al., 1986). *TPI1* and *POT* encode triose phosphate isomerase from *S. cerevisiae* and *S. pombe*, respectively. Triose phosphate isomerase is an essential enzyme in the glycolysis used for growth on glucose as sole carbon source (glucose concentrations above 0.1% inhibit growth in *TPI1*-deleted strains (Compagno et al., 1996)). The study also included the following controls, a strain transformed with a plasmid with an empty expression cassette and a wild type strain. The aprotinin-producing strains and a strain with an empty expression cassette were grown at their maximal specific growth rate for a large number of generations to test the influence of strain genotype and the presence of an expression cassette on adaptation. After the adaptation period, the strains were compared with the unadapted strains in batch cultivations.

2. Materials and methods

2.1. Strains

Five different *S. cerevisiae* strains with two different strain genotypes were investigated in the present study (Table 1). The laboratory strain, CEN.PK 530-1C was constructed from the CEN.PK 113-7D (Overkamp et al., 2002). The construction of the industrial strain is described in Bjørn et al. (1996). All strains except the laboratory reference strain carry a deletion in the chromosomal *TPI1* gene (encoding triose phosphate isomerase) and have been transformed with a 2-μ-derived plasmid. The *POT* gene is transcribed at relatively low levels in *S. cerevisiae* leading to a high copy number of the plasmid (Kawasaki and Bell, 1999). The diploid aprotinin-producing strain with laboratory genotype was constructed by mating the haploid aprotinin-producing laboratory strain (CEN.PK 530-1C, MATa) with a laboratory strain carrying a deletion in the chromosomal *TPI1* gene as well, but not containing any plasmid (CEN.PK 530-1B, MATα) (Overkamp et al., 2002). The mating was performed in YPD medium. The occurrence of mating was confirmed by the ability to sporulate. The strains were plated on a presporulation medium followed a sporulation medium (Kaiser et al., 1994). All the investigated strains were kept at −80 °C in a 15% (v/v) glycerol solution. One vial of cells (1 mL) was used for the inoculation of one shake flask.

2.2. Adaptation

The investigated strains were adapted to the minimal medium by growth in sequential shake flasks as described below.

2.2.1. Inoculation conditions

The shake flasks were inoculated with a small volume from the preceding shake flask. The first shake flask was inoculated with one vial of cells from a −80 °C stock. The volume and OD of the cells in the new shake flask varied between the strains and during the time course of the adaptation experiment, as the cells were kept in the exponential growth phase at all times. The cells grew in each shake flask for 24 h after which they were transferred to a shake flask containing fresh media and it was checked that the preceding shake flask contained residual glucose (as there only is selection for the plasmid when the cells grow on glucose).

2.2.2. Medium and growth conditions

The aerobic shake flask cultures were performed in 500-mL shake flasks with two baffles and 100 mL of medium. A defined minimal medium containing vitamin and trace metal solution was used (Verduyn et al., 1992). The medium had the following composition: 7.5 g/L $(NH_4)_2SO_4$, 14.4 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 2 mL/L trace metal solution, 50 μL/L antifoam and 1 mL/L vitamin solution with an initial glucose concentration of 10 g/L. Initial pH was 6.5, the temperature was kept at 30 °C and aeration was performed by incubating the shake flasks on an orbital shaker at 150 rpm.

2.3. Batch cultivations

2.3.1. Inoculation conditions

One shake flask, with the same medium composition as used in the adaptation series, was inoculated with one vial (1 mL) from a −80 °C stock. Prior to each cultivation the yeast grew at 30 °C for 24–48 h (depending on the strain) and the fermentor was inoculated with exponentially growing cells. The fermentor was inoculated with a volume of the inoculation culture resulting in an initial OD in the fermentor between 0.001 and 0.020.

2.3.2. Medium and growth conditions

Batch cultivations with a starting volume of 4 L were carried out using in-house-built 5-L fermentors. A defined minimal medium containing vitamin and trace metal solution was used in the cultivations (Verduyn et al., 1992). The medium had the following composition: 10 g/L $(NH_4)_2SO_4$, 3 g/L KH_2PO_4, 1 g/L $MgSO_4 \cdot 7H_2O$, 2 mL/L trace metal solution, 50 μL/L antifoam and 1 mL/L vitamin solution with an initial glucose concentration of 20 g/L. Agitation, aeration rate and temperature were kept constant at 800 rpm, 1 vvm, and 30 °C, respectively. The pH was maintained at 5.0 by automatic addition of 2 M NaOH.

2.4. Analytical methods for biomass and metabolite measurements

During the cultivations the concentration of biomass was followed by measurements of the optical density at 600 nm. The samples were diluted in distilled water to ensure an OD measurement below 0.3. The dry weight of biomass was determined by the use of a Gelman Supor®-450 membrane, 0.45 μm. A known volume

Table 1
Strains used in this study

Strain description	Construction of the strains	Genotype
Industrial aprotinin diploid	Transformed with plasmid A	*a/α leu2-3,112/leu2 pep4-3/pep4-3 $^+$his4-580 tpi::LEU2/tpi::LEU2 [cir$^+$]*
Laboratory aprotinin haploid	Transformed with plasmid A	*MATa tpi1(41-707)::loxP-kanMX4-loxP*
Laboratory aprotinin diploid	Transformed with plasmid A	*a/α tpi1(41-707)::loxP-kanMX4-loxP*
Industrial dummy diploid	Transformed with plasmid B	*a/α leu2-3,112/leu2 pep4-3/pep4-3 $^+$his4-580 tpi::LEU2/tpi::LEU2 [cir$^+$]*
Laboratory reference haploid	Wild type	*MAT*a

Plasmid A contains the *POT* gene from *Schizosaccharomyces pombe* and the gene for aprotinin (BPTI, Swiss-prot P00974). Plasmid B contains the *POT* gene from *Schizosaccharomyces pombe*.