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SHORT CONTRIBUTION

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Production of a heterologous proteinase A by *Saccharomyces kluyveri*

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Abstract In order to evaluate the potential of *Saccharomyces kluyveri* for heterologous protein production, *S. kluyveri* Y159 was transformed with a *S. cerevisiae*-based multi-copy plasmid containing the *S. cerevisiae PEP4* gene, which encodes proteinase A, under the control of its native promoter. As a reference, *S. cerevisiae* CEN.PK 113-5D was transformed with the same plasmid and the two strains were characterised in batch cultivations on glucose. The glucose metabolism was found to be less fermentative in *S. kluyveri* than in *S. cerevisiae*. The yield of ethanol on glucose was 0.11 g/g in *S. kluyveri*, compared to a yield of 0.40 g/g in *S. cerevisiae*. Overexpression of *PEP4* led to the secretion of active proteinase A in both *S. kluyveri* and *S. cerevisiae*. The yield of active proteinase A during growth on glucose was found to be 3.6-fold higher in *S. kluyveri* than in the *S. cerevisiae* reference strain.

Introduction

The advantages of using *Saccharomyces cerevisiae* as host for the production of proteins from higher eukaryotes are numerous, including ease of genetic manipulation, ability of performing post-translational modifications, ease of growth and well established industrial processes. The major disadvantages are the low levels of expression of foreign genes and an inefficient secretion apparatus (Romanos et al. 1992). Furthermore, the Crabtree effect exhibited by *S. cerevisiae* necessitates fed-batch cultivation, which has to be operated at low dilution rates to prevent reduction in biomass yield and build-up of toxic levels of metabolites (Hensing et al. 1995; Pronk et al. 1996). Several other yeasts have been employed for heterologous protein production, e.g. *Hansenula polymorpha, Kluyveromyces lactis, Pichia pastoris, Schizosaccharomyces pompe, Schwanniomyces occidentalis* and *Yarrowia lipolytica* (Buckholz and Gleeson 1991). Yeasts, which can be employed for heterologous protein production, are heavily patented; and this is why it is of great interest to find new expression systems. Some of the drawbacks of non-conventional yeasts are the limited number of cloned genes, a limited understanding of the regulation of their metabolism and a limited availability of molecular biological tools (Sudbery 1994; Flores et al. 2000). In order to take advantage of the enormous amount of knowledge available for *S. cerevisiae*, we have searched for new hosts for heterologous protein production among the *Saccharomyces* yeasts. *S. kluyveri* is the most distant *Saccharomyces* relative of *S. cerevisiae* (Kurtzman and Robnett 1998); and recently its genome has been extensively analysed (Langkjær et al. 2000; Neuvéglise et al. 2000). *S. kluyveri* has been shown to be transformable with *S. cerevisiae* plasmids, using the *URA3* marker (Fujimura 1991; Gojkovic et al. 2000).

In this study, *S. kluyveri* is evaluated for its potential for heterologous protein production, using proteinase A (PrA) from *S. cerevisiae* as a model protein. PrA is a vacuolar aspartate protease, encoded by the *PEP4* gene, which is secreted by *S. cerevisiae* if the gene is overexpressed (Rothmann et al. 1986). PrA is synthesised as a zymogen, but can be autoactivated extracellulary in strains that secrete the zymogen as a result of overexpression. Autoactivation of PrA is dependent on pH and ionic strength (van den Hazel et al. 1997). Overexpression of *PEP4* has previously been used to study protein secretion in *S. cerevisiae* (Jochumsen 1995; Carlsen et al. 1997).

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Materials and methods

Strains and plasmid

The following laboratory strains were used: *S. kluyveri* Y159 (*MAT a*, *ura3*), which was obtained from J. Strathern (NCI–Frederick Cancer Research and Development Center, Frederick, USA) as GRY1183, and *S. cerevisiae* CEN.PK113–5D (*MAT a*, *ura3*), which was obtained from P. Kötter (J.W. Goethe Universität, Frankfurt, Germany).

S. kluyveri Y749 was constructed by transformation of *S. kluyveri* Y159 with pJW1103. *S. cerevisiae* Y750 was constructed by transformation of *S. cerevisiae* CEN.PK113–5D with pJW1103. Y numbers refer to the yeast collection of J. Piškur.

Plasmid pJW1103 (Sørensen et al. 1994) is a 2-μ shuttle vector, which contains *S. cerevisiae PEP4*, including the promoter and terminator regions, and *URA3* and *LEU2d* for selection.

Transformation and selection procedures

Yeast strains were transformed using the lithium acetate/polyethylene glycol method (Gietz and Schiestl 1995). After selection of transformants on SD plates [2% (w/v) glucose, 0.67% (w/v) yeast nitrogen base without amino acids (Difco), 1% (w/v) succinic acid, 0.6% (w/v) NaOH], colonies were transferred to YPDM plates [2% (w/v) glucose, 1% (w/v) yeast extract, 0.5% peptone (w/v), 2% (w/v) skimmed milk powder] to test for PrA secretion. A halo around a colony indicated secretion of active proteases, in this case presumably PrA.

Western blotting

Culture supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tris-glycine running buffer) on 12% polyacrylamide gels (Tris-HCl Ready Gels; Bio-Rad) and electro-blotted onto nitrocellulose membranes (Tris-glycine/MeOH transfer buffer), using the Ready Gel system (Bio-Rad) according to the manufacturers recommendations. PrA was immunodetected according to standard procedures, using a primary antibody against *S. cerevisiae* PrA obtained from I. Diers (Novozymes, Copenhagen). The secondary antibody was horseradish peroxidase-coupled goat anti-rabbit immunoglobulins (DAKO, Denmark), which was visualised on the membrane with the Opti-4CN kit (Bio-Rad).

Batch cultivations

Batch cultivations were performed in bioreactors (manufactured in-house) with a working volume of 4 l. The pH was kept at 5.0 (±0.1) by the automatic addition of 2 M NaOH, the stirring rate was 800 rpm, the temperature was maintained (by proportional/integral/derivative controller) at 30±0.2 °C and the air flow was 4.0 l/min (1 vvm). The bioreactors were fitted with condensers cooled to 4 °C. The medium used for batch cultivations was a scaled-up version (to support growth resulting in more than 5 g biomass/l) of the medium given by Verduyn and co-workers (1992). The final medium contained (per litre of demineralised water): 20.0 g glucose, 10.0 g $(NH_4)_2SO_4$, 3.0 g KH_2PO_4, 1.0 g $MgSO_4 \cdot 7H_2O$, 30 mg EDTA, 9 mg $ZnSO_4 \cdot 7H_2O$, 1.7 mg $MnCl_2 \cdot 2H_2O$, 0.6 mg $CoCl_2 \cdot 6H_2O$, 0.6 mg $CuSO_4 \cdot 5H_2O$, 0.8 mg $Na_2MoO_4 \cdot 2H_2O$, 9 mg $CaCl_2 \cdot 2H_2O$, 6 mg $FeSO_4 \cdot 7H_2O$, 2 mg H_3BO_3, 0.2 mg KI, 0.05 mg (D–) biotin, 1 mg Ca (D+) panthotenate, 1 mg nicotinic acid, 25 mg myo-inositol, 1 mg thiamine hydrochloride, 1 mg pyridoxine hydrochloride, 0.2 mg *p*-aminobenzoic acid and 50 μl Antifoam 289 (Sigma). The bioreactors were inoculated with a small amount of exponentially growing preculture, to an initial cell mass concentration of 1 mg dry weight/l. The precultures were grown in baffled shake flasks for 16–24 h at 30 °C and 200 rpm in the same medium as for the batch cultivations, except that the ammonium sulphate concentration was 7.5 g/l, the potassium dihydrogenphosphate concentration was 14.4 g/l, the glucose concentration was 10 g/l and the initial pH was set to 6.5.

Measurements during batch cultivations

Optical density was measured at 600 nm (OD_{600}) in a spectrophotometer (model U-1100, Hitachi) throughout the batch cultivations. At several time points, the dry cell mass concentration was determined by filtration of a known volume of the culture and subsequent drying in a microwave oven, as described in Klein et al. (1999); and a correlation between OD_{600} and dry cell mass concentration was obtained. Concentrations of glucose, succinate, glycerol, ethanol, acetate and pyruvate were determined by HPLC, as described by Klein et al. (1999). PrA activity was determined using an internally quenched fluorescent peptide substrate [2-aminobenzamide-Leu-Phe-Ala-Leu-Glu-Val-Ala-Tyr(NO_2)-Asp] as previously described (van den Hazel et al. 1995). A PrA standard (Sigma P8892), where 1 unit (U) was defined as the amount required to hydrolyse 1 mg insulin chain B/min at pH 6.0 and 25 °C, was used to convert the fluorescent measurements to U/l.

Results

S. cerevisiae CEN.PK 113-5D and *S. kluyveri* Y159 were successfully transformed with pJW1103, containing the *S. cerevisiae PEP4* gene encoding PrA. The *PEP4* gene was under the control of its native promoter. Several of the *URA3*$^+$ transformants secreted proteases, as detected by halos on YPDM plates. Western blot analysis detected PrA in the culture medium of the transformed strains, but not from the untransformed host strains. The apparently best PrA producer of each yeast species were isolated

Table 1 Yield coefficients and PrA production during batch cultivation of *Saccharomyces kluyveri* Y749 and *S. cerevisiae* Y750 on glucose. During batch cultivation on glucose, two distinct phases are observed. In phase I, the yeast consumes glucose and produces ethanol, glycerol and various organic acids. After glucose depletion, a second growth phase (phase II) is observed, where ethanol and the other metabolites are consumed. Yields (*Y*) of PrA were calculated for phase I at the point of glucose depletion, and for phase I + II at the point of ethanol depletion. The yields for acetate, biomass, ethanol, glycerol, pyruvate and succinate are for phase I. μ_{max} is the maximum specific growth rate (per hour) on glucose. *dw* Dry weight, *glc* glucose

	S. kluyveri	*S. cerevisiae*
Biomass (g dw/g glc)	0.27	0.12
Ethanol (g/g glc)	0.11	0.40
Acetate (g/g glc)	0.02	0.01
Glycerol (g/g glc)	0.03	0.06
Pyruvate (g/g glc)	0.02	0.003
Succinate (g/g glc)	0.001	0.001
μ_{max} (h)	0.35	0.37
Yield of PrA after phase I		
Y_{SPrA} (units/g glc)	1.74	0.49
Y_{XPrA} (units/g dw)	6.46	4.08
Yield of PrA after phase I + II		
Y_{SPrA} (units/g glc)	4.05	16.0
Y_{XPrA} (units/g dw)	13.3	64.0

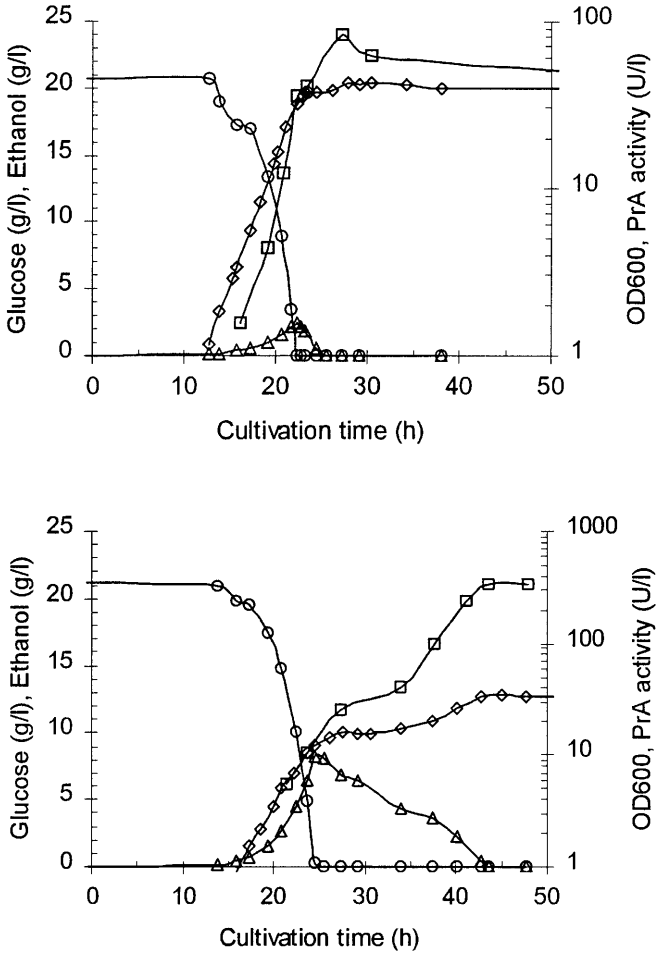

Fig. 1 Batch cultivations on glucose by proteinase A (*PrA*)-producing strains of *Saccharomyces kluyveri* and *S. cerevisiae*. *Upper graph S. kluyveri* Y749, *lower graph*: *S. cerevisiae* Y750. △ Ethanol, ○ glucose, ◇ optical density at 600 nm (*OD600*), □ PrA activity, *U* units of activity. Note that the *right hand axes* are logarithmic

and the growth and PrA production of these recombinant strains, *S. cerevisiae* Y750 and *S. kluyveri* Y749, were analysed in aerobic batch cultivations on glucose minimal medium (Table 1, Fig. 1). The maximum specific growth rate on glucose was similar for the two recombinant strains (0.35/h for *S. kluyveri* Y749 and 0.37/h for *S. cerevisiae* Y750), but the growth pattern of the two yeast species was distinctively different. *S. cerevisiae* showed typical diauxic growth (Fiechter et al. 1981). During the first growth phase, ethanol was produced with a yield of 0.40 g ethanol/g glucose consumed (Table 1). After glucose depletion, a long phase of ethanol consumption followed. In contrast, *S. kluyveri* produced only 0.11 g ethanol/g glucose consumed, which is why the second phase, ethanol consumption, was very short (Fig. 1). As a consequence of the lower ethanol yield in *S. kluyveri*, the biomass yield on glucose was three times higher in *S. kluyveri* than in *S. cerevisiae*. The yields on glucose of biomass, ethanol and the other metabolites measured were constant during the exponential growth on glucose for both *S. cerevisiae* and *S. kluyveri* (Table 1).

PrA production was quantified by measuring PrA activity. PrA was produced both during growth on glucose and subsequently during growth on ethanol by both strains (Fig. 1). The yield of PrA on biomass and on glucose was found to increase with time during the exponential growth on glucose for both *S. cerevisiae* and *S. kluyveri*. The specific PrA productivity was therefore also increasing with time during both batch cultivations. In order to compare the PrA production in the two strains, average yields were calculated at the point of glucose depletion and at the point of ethanol depletion, in relation to the total amount of glucose consumed or biomass produced (Table 1). For the growth phase on glucose, *S. kluyveri* had a 3.6-fold higher yield of PrA on glucose, compared to *S. cerevisiae*, whereas the overall yield of PrA on glucose, including growth on both glucose and ethanol, was 4-fold higher in *S. cerevisiae* than in *S. kluyveri* (Table 1).

Discussion

S. cerevisiae PrA was detected in culture supernatants of *S. kluyveri* Y749 by Western blot analysis and by measuring PrA activity. This means that the *S. cerevisiae PEP4* promoter could drive expression in *S. kluyveri*; and the obtained level of expression was high enough to lead to the secretion of PrA. Overexpression of *PEP4* in *S. cerevisiae* has previously been shown to lead to secretion of PrA, due to saturation of the vacuolar targeting pathway (Rothman et al. 1986; Stevens et al. 1986). The yield of secreted active PrA on glucose was even higher (when *S. cerevisiae PEP4* was overexpressed) in *S. kluyveri* than in *S. cerevisiae*. The higher yield of PrA on glucose in *S. kluyveri* could be due to less glucose-repression of *PEP4* expression, which is moderately glucose-repressed in *S. cerevisiae* (Hansen et al. 1977), or it could be due to a more efficient secretion in *S. kluyveri*. It was indeed observed that most of the PrA production in *S. cerevisiae* took place in the ethanol-consumption phase. The fact that the yield of PrA on biomass was not constant during the glucose-consumption phase has also been reported for another PrA-producing strain of *S. cerevisiae* overexpressing *PEP4* from its native promoter (Jochumsen 1995). It has furthermore been found that the yield of PrA on biomass, by the same PrA-producing strain of *S. cerevisiae*, was constant during glucose-limited continuous cultivation in the respiratory growth regime (Carlsen et al. 1997), which also indicates that glucose-repression is an important regulator of *PEP4* expression. The most important parameter for the evaluation of heterologous protein production is the specific productivity. The average specific productivity of PrA during growth on glucose was 2.3 U/g dry weight/h for *S. kluyveri* Y749 and 1.5 U/g dry weight/h for *S. cerevisiae* Y750. It was surprising that the specific PrA production was 50% higher in *S. kluyveri* Y749 than in

S. cerevisiae Y750, considering that the *PEP4* gene was expressed from a *S. cerevisiae* promoter and a *S. cerevisiae* vector.

The batch cultivations also revealed that *S. kluyveri* had a more oxidative metabolism (with more biomass formation and less ethanol formation) than *S. cerevisiae*, which is a desirable property for heterologous protein production. The fact that *S. kluyveri* produced more PrA during batch cultivation on glucose than *S. cerevisiae* and that *S. kluyveri* also had a higher biomass yield on glucose could indicate a less stringent glucose-repression in this yeast than in *S. cerevisiae*. Therefore, *S. kluyveri* is interesting for the development of a new expression system for heterologous protein production.

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