Mutant *Hansenula polymorpha* Strain with Constitutive Alcohol Oxidase and Peroxisome Biosynthesis

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A mutant of the methylotrophic yeast *Hansenula polymorpha* with constitutive alcohol oxidase (AOX) and peroxisome biosynthesis was obtained after UV treatment followed by cell plating on a medium containing methanol and 2-deoxy-D-glucose (DOG). DOG-resistant colonies of mutants were insensitive to catabolic repression by glucose and methanol. A selection procedure is described that allows the isolation of a mutant exhibiting a constitutive phenotype of AOX involved in methanol utilization. Furthermore, additional features of the constitutive presence of peroxisomes are demonstrated. 562 DOG-resistant colonies were tested, 24 of them demonstrating constitutive AOX formation. Based on quantitative analysis, one of the strains – DOG-13 was selected and its growth, biochemical and ultrastructural characteristics were examined. Its specific enzyme activity when cultivated on a yeast nitrogen base + 1% glucose (YNB + 1% Glucose) was found to reach 145 $nmol.min^{-1}.mg^{-1}$protein (compared to zero of the parent strain) after he 20^{th} hour of cultivation. This was confirmed by fine-structure analysis, showing typical peroxisomes, which number and size increased with the enzyme activity. This study demonstrates a constitutive AOX and peroxisome biosynthesis by the mutant strain *H. polymorpha* DOG-13 obtained.

Introduction

Growth of yeast on methanol as a sole carbon source depends on the synthesis and function of enzymes involved in C^1 metabolism, as well as the presence of cellular organelles called peroxisomes (Harder, 1990; van Dijk *et al.*, 2000; Gellissen and Veenhuis, 2001). Under these conditions, peroxisome matrix contains mainly the key enzymes alcohol oxidase (AOX) and catalase (Keizer *et al.*, 1992; Gleeson and Sudbery, 1988; Veenhuis and Goodman, 1990; Gunkel *et al.*, 2001). AOX (EC 1.1.3.13) is the first enzyme of methanol utilization pathway, catalyzing the oxidation of methanol to formaldehyde. It is a major component of peroxizomal protein and it is considered a model protein for studying the mechanisms of protein translocation across peroxisomal membrane (Van der Klei *et al.*, 1991; Baerends *et al.*, 2000; van der Klei *et al.*, 1995; Hansen and Hollenberg, 1996). On the other hand, the changes in peroxisome number and size are closely related to the level of AOX in the cells. These are predetermined by the cultivating conditions, in particular by the nature of the carbon source and the culture growth phase (Veenhuis *et al.*, 1978). It is known that the addition of glucose or ethanol to methanol-grown cells causes rapid degradation of AOX and peroxisomes called catabolic inactivation (Veenhuis *et al.*, 1983; Kulachkovsky *et al.*, 1998; Egli *et al.*, 1980). This process involves a proteolytic breakdown of the organelles and matrix enzymes. The peroxisomes are degraded individually by an autophagic process. The signals initiating catabolic inactivation are not directed against the matrix proteins but, instead, to the peroxisomal membrane (Stasyk *et al.*, 1999; Sulter *et al.*, 1993). Thus, AOX and peroxisome synthesis are regulated by repression/derepression and induction mechanisms (Eggeling and Sahm, 1978). Based on this, a common regulatory mechanism(s) for organelle proliferation and synthesis of peroxisomal enzymes can be assumed. The isolation of constitutive AOX synthesis mutants and identification

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of constitutive peroxisome formation mutants amongst them, is a useful approach toward understanding this controlling mechanism (Roggenkamp, 1988; van der Bremt *et al.*, 1999).

The main objective of this study was to obtain a *H. polymorpha* mutant strain with constitutive AOX and peroxisomes biosynthesis.

Materials and Methods

Strain

Haploid strain *H. polymorpha* CBS 4732–6C (ade-2 ade-3) from the collection of Laboratory of Genetics, Institute of Microbiology, BAS was used a as parental strain for mutant isolation.

Media and growth conditions

The following media were used: YEPD (1% yeast extract, 2% peptone, 2% glucose); YEPD5 (1% yeast extract, 2% peptone, 5% glucose); SC (0.67% yeast nitrogen base without amino acids (YNB), 1% glucose supplemented with the following amino acids and bases: 30 mg adenine, 20 mg uracil, 20 mg L-methionine, 30 mg L-leucine, 30 mg L-arginine); DS (SC without one of the listed amino acid and bases) SCM (0,67% YNB, 1% methanol); SC5M (0.67% YNB, 5% glucose, 1% methanol); SCMD (0.67% YNB, 3% methanol, 0.1% 2-deoxy-D-glucose).

Mutants isolated were cultivated on YEPD-agar at 37 °C and kept at 4 °C. They were periodically checked for keeping the parental auxotrophic markers on SC and DS solid media.

Induction and isolation of mutant strains

Cells of the parental strains were cultured on solid YEPD and after 48-h cultivation were harvested and suspended in sterile water to concentration 5×10^8 CFU/ml. Aliquots of 0.1 ml were plated on solid SCMD and were subjected to UV-irradiation with 1–10% cells to survive. After 5–7 days cultivation the colonies were transferred on solid YEPD, SCMD and YEPD5. The mutants thus isolated were resistant to DOG and their ability to synthesize AOX was further tested.

Enzyme activity determination

AOX synthesis was detected by the method of Eggeling and Sahm (1980) and Sibirny and Titorenko (1986). The strains tested were transferred from YEPD5 on solid SCMD to induce the enzymes involved in the methanol metabolism. After an overnight incubation, yeast colonies were covered by semi-solid agar supplemented with digitonin (1 mg/l), *o*-dianizidin (0.025%), peroxidase (3 U/ml) and 2% methanol. The colour of the colonies was observed after an overnight incubation. The strains synthesizing AOX were distinguished by their brown colour.

The specific enzyme activity determination was performed as follows: Cells of the strains tested were cultivated in liquid SCM until reaching the stationary growth phase. The biomass was then washed, inoculated in liquid SC5M with 1% methanol and incubated for 24 h. Cell-free extracts were then prepared and AOX activity was determined as described in a previous work (Hristozova *et al.*, 2002).

Protein concentration was determined according to Lowry *et al.* (1951) with bovine serum albumin as a standard.

Methods for transmission electron microscopy (TEM)

Cells of *H. polymorpha* DOG-13 cultivated in 1% glucose medium after AOX repression with 10% glucose were harvested in exponential growth phase – (20-th hour) of cultivation. Ultrathin sections of the cells were obtained after appropriate processing and observations were carried out on a Zeiss EM 10C electron microscope at 60–80 kV.

Results and Discussion

Isolation of mutant strains with constitutive AOX synthesis

The parent strain *H. polymorpha* 4732 –6C (ade-2 ade-3) does not grow on methanol-containing media with 0.1% 2-deoxy-D-glucose (DOG). This suggests that the mutant strains able to grow on this medium could be expected to possess constitutive AOX synthesis. 3896 DOG colonies were obtained after UV-irradiation. They were checked for keeping the parental auxotrophic markers, plated on solid YEPD5 for complete repression of AOX and tested for AOX activity by plating on SC5M. At this stage, 169 DOG mutant strains

Table I. Specific AOX activity of mutant strains cultivated on SC5M liquid medium.

Strain	5–6C	12–6C	13–6C	60–6C	84–6C	92–6C	103–6C	140–6C
Specific AOX activity [μmol.min^{-1}.mg^{-1}*]	0.6 ± 0.05	0.8 ± 0.02	3.9 ± 0.12	1.2 ± 0.06	1.0 ± 0.04	0.9 ± 0.02	0.2 ± 0.03	0.3 ± 0.03

* Values represent the mean ± standard deviation from assays of three samples.

demonstrating AOX activity in the presence of glucose were selected. Eight strains with a well expressed AOX phenotype were chosen to be tested for AOX activity when cultivated in liquid SC5M medium (Table I).

Results of the AOX activity test indicated that the mutant strain DOG-13–6C (further referred as DOG-13) had the highest enzyme activity. The strain was found to keep its parent auxotrophic markers as well. Based on this, the strain was chosen for further testing its ability to grow on SC liquid medium with 1% glucose as a sole carbon source.

For the purpose, both the parental strain and DOG-13 were cultivated on YNB with 1% methanol for growth curves and AOX production (Figs. 1 and 2). Maximum activity for both strains was observed at the 20th hour of cultivation. The cells were harvested at this point, washed and transferred to YNB supplemented with glucose and YNB supplemented with ethanol, both repressors added in increasing concentrations to examine the appropriate type and concentration of the repressor. Glucose and ethanol cause inactivation

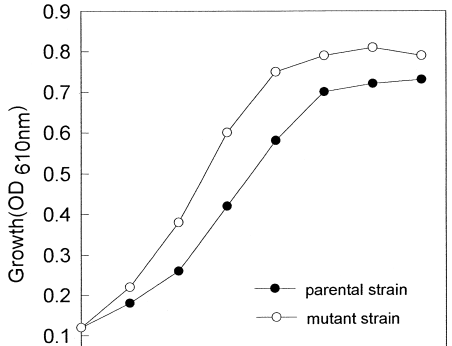

Fig 1. Growth curves of the parental strain and mutant strain DOG-13 on SCM medium.

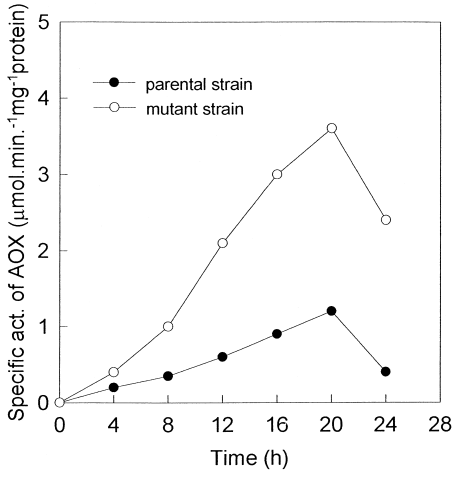

Fig 2. AOX activity of the parental strain and mutant strain DOG-13 on SCM medium.

of AOX for both the parental and the mutant strain. A complete repression of AOX (zero activity) was observed at the 20th h. at 4% ethanol and 10% glucose in the medium, respectively (Fig. 3).- The inactivation was slower for the mutant strain and, taking in account the slower cell growth after being transferred in the glucose-supplemented medium (data not shown), it can be assumed that this is due to a partial blocking of glucose assimilation of the mutant strain.

Cells of both strains were then washed and transferred to YNB medium with 1% glucose. The enzyme activities were determined at the 16th, 20th and 24th hour of incubation. The maximum AOX activity of 146 nmol;min^{-1};mg^{-1}protein was registered at the 20th hour when glucose was prior used as a repressor while, when ethanol was prior used as repressor, the maximum AOX activity was 60 nmol;min^{-1};mg^{-1}protein at the same time point (Fig. 4). Òhese results correlate with the studies of Titorenko *et al.* (1991) with *P. pinus* where the AOX activity of isolated mutants are between 20–125 nmol;min^{-1};mg^{-1} protein.

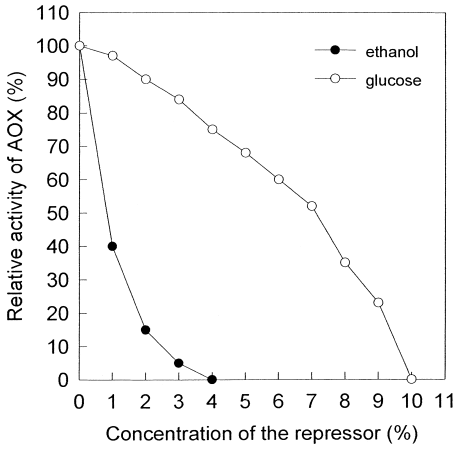

Fig 3. Inactivation of AOX activity of mutant strain DOG-13 after ethanol and glucose repression.

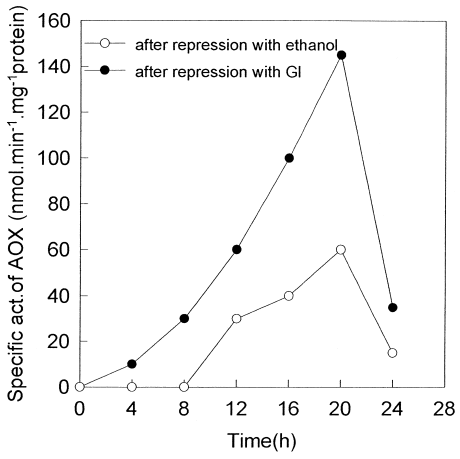

Fig 4. AOX activity of mutant strain DOG-13 on SC medium with 1% glucose.

When strain DOG-13 was cultivated on YNB with 1% glucose after 10% glucose repression typical peroxisomes with spherical shape were formed. The constitutive AOX synthesis included the constitutive expression of peroxisomes, although proliferation of the organelles obviously underwent repression of glucose metabolism. The constitutive peroxisome formation observed in mutant strain DOG-13 may be eventually based on the growth of a limited number of small organelles as a result of constitutive expression of the AOX activity. The enzyme activity demonstrated throughout the study, as well as the typical for methylotrophic yeasts cell organelles registered, confirm the constitutive AOX synthesis of the mutant strain *H. polymorpha* DOG-13 obtained.

Conclusions

In this study, a mutant strain DOG-13 was obtained by UV-treatment and its biosynthetic properties were explored. The strain demonstrated constitutive AOX and peroxisome biosynthesis when glucose was used as a sole carbon source. This could contribute to a better understanding of metabolite – directed regulation and will form the basis of further genetic studies on mutants of methylotrophic yeasts with increased biotechnological potential.

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