Genetic engineering of the β-oxidation pathway in the yeast *Yarrowia lipolytica* to increase the production of aroma compounds

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Abstract

The yeast *Yarrowia lipolytica* possesses five acyl-CoA oxidases (Aox1p to 5), the enzyme catalysing the first reaction of β-oxidation. The understanding of the specific role of each acyl-CoA oxidase is important to construct a yeast strain growing at a good rate and able to produce without degrading the aroma compound γ-decalactone. In this study we observed that Aox4p exhibits a slight activity on a broad spectrum of substrates and that it is involved in lactone degradation. We constructed a strain lacking this activity. Its growth was only slightly altered and it produced 10 times more lactone than the wild type in 48 h.
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1. Introduction

The yeast *Yarrowia lipolytica* is able to transform ricinoleic acid (a hydroxylated C18 fatty acid) into γ-decalactone (Fig. 1), a fruity and creamy aroma compound [1]. As most of the yeast able to carry out this biotransformation, it is also able to degrade the produced lactone. Thanks to its exceptional lipid-degrading enzymatic-machinery, this species, which is also used in several processes [2–7], has been chosen as a model to study this degradation. The pathway of biotransformation involves β-oxidation and requires the lactonisation at the C10 level (when the hydroxy group is in the γ-position) (Fig. 1) [1]. *Y. lipolytica* possesses a five-member family of acyl-CoA oxidases (Aox1p to 5 encoded by *POX1* to 5), the enzymes catalysing the first step of β-oxidation, some of which are long-chain specific (Aox2p) [8] or short-chain specific (Aox3p) [9,10], the specificity resulting from only a small number of amino-acids (see Mlíčková et al. in this volume, [11]). The short-chain spe-

cific enzyme is involved in the degradation of the lactone (Fig. 1) and therefore genetic constructions have been made to remove its activity from yeast strains [12]. It was thus shown that this enzyme was not the only one involved in the degradation and some of the other acyl-CoA oxidases exhibited activity on short-chain substrates [13]. From the evaluation of the acyl-CoA oxidase activity of the *POX*-mutants, it was not easy to appreciate the role of the three other acyl-CoA oxidases (Aox1p, Aox4p and Aox5p): the deletion of *pox1* did not seem to modify the activity as if Aox1p was inactive towards straight-chained acyl-CoA. The deletion of *pox5* decreased activity on the complete chain-length spectrum whereas the deletion of *pox4* exhibited no significant effect. To confirm the activity or inactivity of these enzymes, a construction was made involving the deletion of several acyl-CoA oxidase encoding genes (Δ*pox2*Δ*pox3*Δ*pox5*) and a multicopy insertion of *POX2*, the long-chain specific encoding gene [14] but, as the efficiency of the multicopy insertion was low, the constructed strain exhibited an altered growth resulting in a low production and no degradation of γ-decalactone but whether the absence of detectable degradation was due to a lack of short-chain active acyl-CoA oxidases or to a rate of degradation lower than the rate of production was not determined.
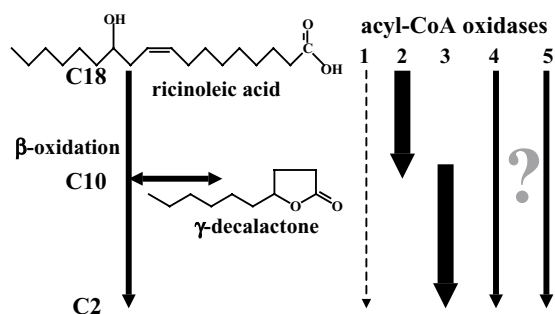
Fig. 1. Schematic representation of the pathway from ricinoleic acid to γ-decalactone and activities of the acyl-CoA oxidases of *Y. lipolytica* (Aox1p to 5).

Table 1
Names, *POX* genotype and growth characteristics of strains of *Y. lipolytica* used in this study

Strains	Genotype	Aox expressed	Growth
W29	Wild type	None	Normal
MTLY36	Δpox2,pox3,pox5	None	Reduced
MTYL37	Δpox2,pox3,pox4,pox5	None	No growth
MTLY40	Δpox2,pox3,pox4,pox5	None	No growth
MTLY36-2P	Δpox3,pox5	Aox2p	Normal
MTLY40-2P	Δpox3,pox4,pox5	Aox2p	Normal

The growth characteristics (in media containing methyl oleate or methyl ricinoleate as the only source of carbon) are given compared to wild type (normal growth).

As the growth rate of mutant strains is very important for industrial application and as this parameter is linked to the number of deletion of genes coding for lipid-degradation-enzymes, it is of fundamental interest to establish the role of each of these enzymes. The goal of the present study was to investigate the effect of Aox4p and Aox5p on the degradation of γ-decalactone and to construct a strain unable to degrade it but able to grow at a rate similar to that of the wild type. Using the same triple disruptant (Δpox2Δpox3Δpox5), a simple copy of *POX2* was added restoring the Ura+ character. With nearly the same genotype as the mutant previously constructed, the phenotype was quite different as the strain was able to grow in a way similar to the wild type but was also able to degrade the lactone produced. These results show that a remaining acyl-CoA oxidase was also able to degrade the lactone and, as Aox1p seems inactive, we did the same *POX2* addition in a Δpox2Δpox3Δpox4Δpox5 mutant. We obtained a mutant able to grow only a little more slowly than the wild type but producing about 10 times more γ-decalactone in only 48 h, and this amount did not significantly decrease in 250 h.

2. Experimental

2.1. Strain constructions

The strain used in this study is *Y. lipolytica* W29 MatA (ATCC 20460, CLIB89) or its derived mutant disrupted for genes coding for acyl-CoA oxidases (*POX*) [8]. To introduce the uracil auxotrophy into the mutant strain MTL37, the *ura3*-41 allele from the H222 *ura3*-41 strain [15] was introduced by transformation and selection of Ura− cells on YNB-5FOA medium as described previously [16] giving rise to strain MTLY40. The various strains are presented in Table 1.

The *POX2* and *POX3* genes were amplified with oligonucleotides pairs *POX2*-ATG/*POX2*-STOP, and *POX3*-ATG/*POX3*-STOP, respectively. Plasmid pYEG1 [17] was digested by BlnI and SfiI and ligated with the PCR fragments digested with the same enzymes, giving rise to plasmid pYEG1-*POX2* and pYEG1-*POX3*. Both

expression cassettes were introduced into MTLY36 and MTLY40 by the lithium acetate method [18]. Prior to integration into yeast cells, plasmids were digested by Not I. Ura+ transformants were selected on YNBcas.

2.2. Culture conditions

Cells were cultured at 27 °C in 500 ml baffled Erlenmeyer flasks containing 200 ml medium and agitated at 140 rpm for the lactone production in different environmental conditions. Cells were precultured in a YPD modified medium (glucose, 20 g/l; trypsic peptone from casein, 20 g/l, yeast extract, 10 g/l) for 19 h to the late logarithmic phase then cells were harvested and inoculated to an $A_{600\,nm}$ of 0.25 (approximately 6.5×10^6 cells/ml) in a medium containing 5 g/l methyl ricinoleate (biotransformation medium: methyl ricinoleate (Stéarinerie Dubois, Boulogne, France), 5 g/l; Tween 80, 1 g/l; yeast nitrogen base, 3.35 g/l).

2.3. Analysis

For lactone quantification, 1.5 ml samples were removed from the biotransformation medium. These samples were centrifuged (10,000 × g, 5 min) and the supernatant (both aqueous and oil phases) mixed. γ-Undecalactone (internal standard) was added to reach a final concentration of 100 mg/l and the mixture was extracted with diethyl ether, in 4 ml glass vials, by shaking for 60 s. The ether phase was analysed in a HP6890 gas chromatograph (Agilent Technologies, Lyon, France) with a HP-INNOWax capillary column (Agilent) (30.0 m × 320 μm × 0.25 μm) with N_2 as a carrier gas at a linear flow rate of 4.3 ml/min. The split injector (split ratio, 7.1:1) temperature was set to 250 °C and that of the FID detector to 300 °C. The oven temperature was programmed to increase from 60 to 145 °C at a rate of 5 °C/min and then to 215 °C at a rate of 2 °C/min.

3. Results and discussion

3.1. Growth of Y. lipolytica wild type and pox mutants

Growth of the various strains in the biotransformation medium is shown in Fig. 2. A 3–4 h lag phase was
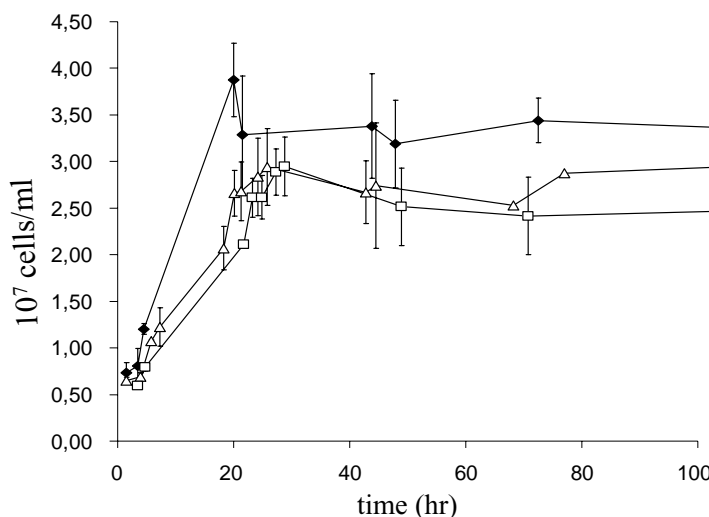
Fig. 2. Growth of *Y. lipolytica* strains in the biotransformation medium (containing methyl ricinoleate as the only carbon source): wild-type W29 (◆), MTLY36-2P (△) and MTLY40-2P (□) strains.

observed for all strains. Prior to inoculation of the biotransformation medium, cells were harvested in log-phase from a preculture medium containing glucose as the carbon source. They required thus the complete induction of the 'lipid-degradation machinery'. This takes time since, in *Y. lipolytica*, β-oxidation occurs in peroxisomes that are inducible compartments [19] and β-oxidation enzymes are induced and produced in the cytoplasm before being targeted to the peroxisome [20].

After this lag-phase, significant differences appeared between the cells. The wild type (W29) grew rapidly to a value of about $3.5-4 \times 10^7$ cells/ml in less than 20 h whereas the MTLY36-2P strain reached its maximum (less than 3×10^7 cells/ml) in more than 25 h and MTLY40-2P in almost 30 h.

The *POX* genotype has thus no effect on the lag-phase, i.e. on the induction time of the enzymatic machinery, but has a significant effect on the growth rate and on the maximal cell concentration. Methyl ricinoleate is the only source of carbon in the biotransformation medium and β-oxidation is thus required for growth. Acyl-CoA oxidases catalyse the first reaction of the β-oxidation cycle and they are generally considered as the rate-limiting enzyme of the sequence [21,22]. The wild type possesses five acyl-CoA oxidases (Aox1p to 5) as presented in Fig. 1. Aox2p seems to play the major role on long-chain acyl-CoA but Aox4p and Aox5p are not negligible. On short-chain substrates, Aox3p is the important enzyme with maybe a role of the two other enzymes (Aox4p and Aox5p).

The MTLY36-2P strain ($\Delta pox3\Delta pox5$) lacks two active acyl-CoA oxidases. The deletion of Aox5p results probably in a decrease in the global activity, and that of the short-chain specific Aox3p, in a decrease in the extent of acyl-CoA degradation. The MTLY40-2P strain ($\Delta pox3\Delta pox4\Delta pox5$) lacks in addition Aox4p which has a small activity according to previous results [8]. The lower growth rate of these two

mutants compared to the wild type can thus be explained by the lack of Aox3p and Aox5p and the observed difference between the two mutant strains suggests a small but significant role for Aox4p. The difference between the maximal cellular concentration reached by both mutants ($2.9-3 \times 10^7$ cells/ml) and by the wild type (about 3.5×10^7 cells/ml) could be due to the non utilisation of short-chain substrates by the mutants lacking Aox3p, the short-chain specific enzyme. These growth kinetics results confirm the importance of acyl-CoA oxidases in the growth on lipids.

The construction of a technologically improved strain able to grow at a good rate requires thus the presence of most of the active acyl-CoA oxidases. Some recent works [20] have shown that Aox2p or Aox3p were required for a good targeting of acyl-CoA oxidases to peroxisomes explaining thus the lower activity of $\Delta pox2\Delta pox3$ mutants previously observed [8]. All the constructions used in the present study possess Aox2p or Aox3p. Another factor that may influence the activity is the role of some of the acyl-CoA oxidases in the regulation of the others: the deletion of *pox1*, *pox4* or *pox5* results in an increase in the acyl-CoA oxidase activity. However, this increase is no more detectable when a second *pox* gene is deleted [8]. As the present constructions have been carried out with the aim of suppressing any activity on short chain substrates, they require the deletion of the short-chain Aoxp encoding gene (*POX3*). It is thus not possible to utilise the regulation capacity of Aox1p, Aox4p or Aox5p. Finally, a recent study (this issue, [11]) reported a role of acyl-CoA oxidases in lipids accumulation by *Y. lipolytica*, role which is probably related to a modification in the lipid metabolism. However, these results are still too partial to be used in the lactone production process.

Concluding this part on the growth capacity of the mutant strains of this study, it can be observed that the growth ability is not dramatically altered but the deletion of both *pox5* and *pox4* decreases the growth rate of *Y. lipolytica*. In
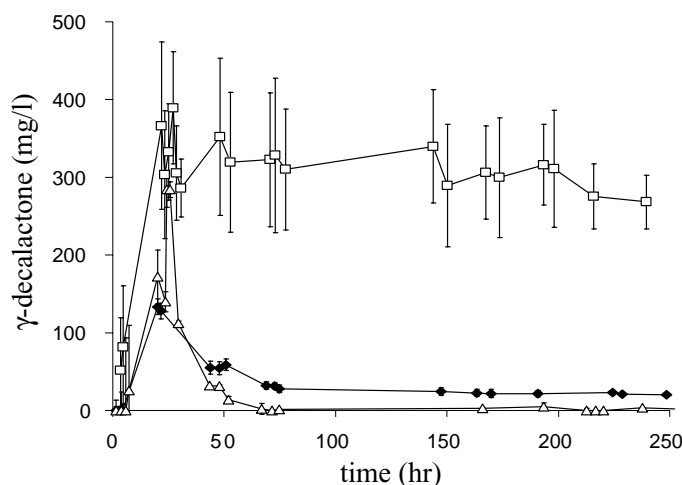
Fig. 3. Production of γ-decalactone by the wild-type W29 (◆), MTLY36-2P (△) and MTLY40-2P (□) strains.

the next part, the interest of both deletions for increasing γ-decalactone production will be investigated.

3.2. Lactone production by the various strains

The production of γ-decalactone by the different strains is shown in Fig. 3. For the wild type, γ-decalactone appeared in the biotransformation medium after 8–10 h. The concentration increased to about 150 mg/l after 24 h and then decreased to 50–60 mg/l after 40 h and eventually stabilised at about 20 mg/l. When compared to growth kinetics, the production occurs with a small delay after growth, corresponding probably to the time necessary for the fatty acids to undergo four β-oxidation cycles, and the degradation (positive difference between the degraded lactone minus the produced lactone) starts right after the end of the growth.

For the MTLY36-2P mutant strain, the production profile (Fig. 3) is very comparable showing without doubt the capacity of this strain to degrade γ-decalactone contrasting with the results obtained with the previous $\Delta pox3 \Delta pox4 \Delta pox5$ construction [14]. A possible explanation for these different results is that the previous construction grew very slowly whereas the new one grows at a rate similar to that of the wild type. As the production of lactone is correlated with the growth phase, a lactone degradation was observed with the new construction after this phase (20 h) whereas, with the previous construction, the growth phase lasted all along the experiment and the 'degradation phase', in which degradation is higher than production, was never reached. According to the present results, it can be concluded that Aox4p contributes to the degradation of γ-decalactone. With our objective to avoid this reaction, it was thus necessary to delete also the Aox4p encoding gene (POX4).

For the corresponding construction, MTLY40-2P, the γ-decalactone production profile is very different (Fig. 3). The production occurs more rapidly, even before the end of the lag growth phase, and increases rapidly reaching values above 300 mg/l at 20 h and exhibiting no significant decrease in the next 230 h. These results show not only that this strain is unable to degrade γ-decalactone in the biotransformation conditions but also that it produces more rapidly, even before growth starts. The absence of degradation can probably not account by itself for this increase and a second mechanism may be implied. The production pathway involves a crossover at the C10 level (Fig. 1). At this stage, a part of the acyl-CoA lactonises before the next β-oxidation step, generating γ-decalactone. But a second part enters the next β-oxidation cycle without forming the lactone. This phenomenon is not a lactone degradation but it constitutes another leakage in the pathway. By decreasing the acyl-CoA oxidase activity at the C10 level, this leakage can be lowered and the production can also be significantly increased. Although this strain grows a little more slowly than the wild type, it is very efficient to produce γ-decalactone in the conditions we used in this study which are not optimised.

4. Conclusion

In this study, the role of the various acyl-CoA oxidases of *Y. lipolytica* was specified. It was shown that Aox4p was not only Implied in the regulation of the other acyl-CoA oxidases, but also that it exhibited a non negligible activity on the entire spectrum of straight-chain acyl-CoA. Our results confirm the importance of the first step of β-oxidation in the degradation of lipids by yeast but other steps are still to be studied [11]. From a biotechnological point of view, a strain with only a slightly altered growth and which exhibited no activity on short-chain substrates was constructed. This type of construction is not only of great interest to produce γ-decalactone but it can also be used for the production of several medium chain-length fatty acids- or alkane-degradation products.

Acknowledgements

References

[1] Y. Waché, M. Aguedo, J.-M. Nicaud, J.-M. Belin, Appl. Microbiol. Biotechnol. 61 (2003) 393.

[2] M. Farbood, B. Willis, Production of γ-decalactone (1985) WO8301072.

[3] R. Cardillo, C. Fuganti, M. Barbeni, P. Cabella, P. Guerda, G. Allegrone, Procédé de production microbiologique des γ- et δ-lactones (1991) EP0412880.

[4] J. Meyer, γ-Decalactone microbial production from alkyl ricinoleate—by hydrolysis, β-oxidation, and chemical cyclisation of 4-hydroxy decanoic acid produced for flavouring and perfume (1993) DE4126997.

[5] J.-M. Nicaud, J.-M. Belin, Y. Pagot, A. Endrizzi-Joran, Bioconversion of substrate with microbe auxotrophic for compound in medium deficient in this compound (1996) FR2734843.

[6] C. Ambid, S. Carle, G. De Billerbeck, Method for producing and extracting aromatic compounds (1999) WO9954432.

[7] J. Rabenhorst, I. Gatfield, Process for the production of γ-decalactone (2000) WO0024920.

[8] H. Wang, M.-T. Le Dall, Y. Waché, C. Laroche, J.-M. Belin, C. Gaillardin, J.-M. Nicaud, J. Bacteriol. 181 (1999) 5140.

[9] Y.S. Luo, H.J. Wang, K.V. Gopalan, D.K. Srivastava, J.-M. Nicaud, T. Chardot, Arch. Biochem. Biophys. 384 (2000) 1.

[10] Y.S. Luo, J.-M. Nicaud, P.P. Van Veldhoven, T. Chardot, Arch. Biochem. Biophys. 407 (2002) 32.

[11] K. Mlíčková, Y. Luo, S. d'Andrea, P. Peè, T. Chardot, J.-M. Nicaud, J. Mol. Catal. B. 28 (2004) 81–85.

[12] Y. Waché, C. Laroche, K. Bergmark, C. Møller-Andersen, M. Aguedo, M.-T. Le Dall, H. Wang, J.-M. Nicaud, J.-M. Belin, Appl. Environ. Microbiol. 66 (2000) 1233.

[13] Y. Waché, M. Aguedo, A. Choquet, I. Gatfield, J.-M. Nicaud, J.-M. Belin, Appl. Environ. Microbiol. 67 (2001) 5700.

[14] Y. Waché, M. Aguedo, M.-T. LeDall, J.-M. Nicaud, J.-M. Belin, J. Mol. Catal. B: Enzym. 19/20 (2002) 347.

[15] S. Mauersberger, H.J. Wang, C. Gaillardin, G. Barth, J.-M. Nicaud, J. Bacteriol. 183 (2001) 5102.

[16] P. Fickers, J.-M. Nicaud, J. Destain, P. Thonart, Appl. Microbiol. Biotechnol. 27 (2003) 27.

[17] J.-M. Nicaud, C. Madzak, P. van den Broek, C. Gysler, P. Duboc, P. Niederberger, C. Gaillardin, FEMS Yeast Res. 2 (2002) 371.

[18] G. Barth, C. Gaillardin, Yarrowia lipolytica: Nonconventional Yeasts in Biotechnology, A Handbook, vol. 1, Springer-Verlag, Berlin, 1996, p. 314.

[19] V.I. Titorenko, R.A. Rachubinski, Nat. Rev. Mol. Cell Biol. 2 (2001) 357.

[20] V.I. Titorenko, J.-M. Nicaud, H. Wang, H. Chan, R.A. Rachubinski, J. Cell Biol. 156 (2002) 481.

[21] N.C. Inestrosa, M. Bronfman, F. Leighton, Biochem. J. 182 (1979) 779.

[22] N.C. Inestrosa, M. Bronfman, F. Leighton, Biochem. Biophys. Res. Commun. 95 (1980) 7.