

## Optimization of *Yarrowia lipolytica*'s $\beta$ -oxidation pathway for $\gamma$ -decalactone production

Yves Waché<sup>a,\*</sup>, Mario Aguedo<sup>a</sup>, Marie-Thérèse LeDall<sup>b</sup>,  
Jean-Marc Nicaud<sup>b</sup>, Jean-Marc Belin<sup>a</sup>

<sup>a</sup> Laboratoire de Biotechnologie (IMSA) de l'ENSBANA, Université de Bourgogne, 1 esplanade Erasme, 21000 Dijon, France

<sup>b</sup> Laboratoire de Génétique des Microorganismes, INRA-CNRS, URA1925, 78850 Thiverval-Grignon, France

Received 31 August 2001; received in revised form 15 May 2002; accepted 29 May 2002

### Abstract

The yeast *Yarrowia lipolytica* growing on methyl ricinoleate produces various lactones,  $\gamma$ -decalactone, the worthy aroma compound, 3-hydroxy- $\gamma$ -decalactone without sensorial properties and two decenolides of various interest. Unfortunately, these three latter lactones are produced at high levels by this yeast, decreasing yields and complicating the extraction of  $\gamma$ -decalactone. In this study, the production of  $\gamma$ -decalactone was increased through a genetic engineering of the strain and the accumulation of the three other lactones was lowered. These results show that it is possible to improve the mastering of the complex  $\beta$ -oxidation pathway (the metabolic pathway involved in these bioconversions) by playing on genetic factors.

© 2002 Elsevier Science B.V. All rights reserved.

**Keywords:**  $\gamma$ -Decalactone; 3-Hydroxy- $\gamma$ -decalactone; Decenolide; Methyl ricinoleate; *Yarrowia lipolytica*

### 1. Introduction

Interest in the catabolism of hydroxy fatty acids arose in the 1960s when Okui et al. [1] observed the accumulation of  $\gamma$ -decalactone (DECA) during growth of a *Candida* species on methyl ricinoleate. Since then, this lactone exhibiting fruity and creamy sensorial notes and present in many fruits and fermented products has become one of the most biotechnologically produced aroma compounds [2]. The metabolic pathway involves peroxisomal  $\beta$ -oxidation,

but, from ricinoleyl-CoA to acetyl-CoA, other intermediates can accumulate decreasing yields. Among them, *Y. lipolytica* accumulates other lactones: 3-hydroxy- $\gamma$ -decalactone (3-OHDEC), dec-2-en-4-olide (2-DECEN) and dec-3-en-4-olide (3-DECEN) (Fig. 1) [3]. The first one has no sensorial properties whereas 2-DECEN exhibits mushroom notes and 3-DECEN has been described as having fruity notes [3]. As for unsaturated  $\delta$ -lactones [4,5], decenolides can be reduced to decalactone in the presence of *S. cerevisiae* or other yeasts [6].

The lactonization process takes place spontaneously at the C10 level, when the product of ricinoleic acid degradation has its hydroxy brought by the carbon in  $\gamma$ . This esterification occurs competitively to  $\beta$ -oxidation and can happen between the four reactions depending on kinetics data. An exit of the acyl-CoA from the  $\beta$ -oxidation loop before the oxidation step, yielding

**Abbreviations:** DECA,  $\gamma$ -decalactone; 3-OHDEC, 3-hydroxy- $\gamma$ -decalactone; 2-DECEN, dec-2-en-4-olide; 3-DECEN, dec-3-en-4-olide; Aox, acyl-CoA oxidase; POX, gene coding for Aox

\* Corresponding author. Tel.: +33-3-80-39-66-80;

fax: +33-3-80-39-66-41.

E-mail address: ywache@u-bourgogne.fr (Y. Waché).

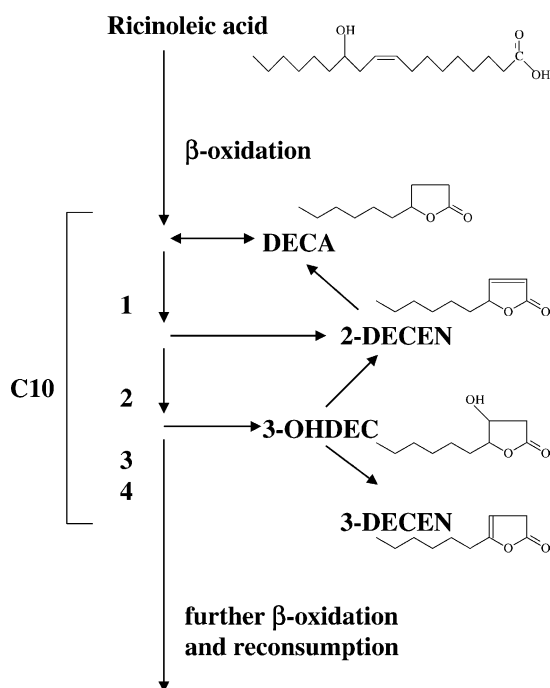


Fig. 1. Pathway from methyl ricinoleate to the various lactones (inspired from [3,10]). The substrate enters  $\beta$ -oxidation and, after four oxidation loops gives rise to C10 intermediates: decanoyl-CoA can be hydrolyzed and lactonized to  $\gamma$ -decalactone (DECA, horizontal arrow) or be oxidized by 1 (acyl-CoA oxidase) to 2-decenoyl-CoA, being itself lactonized to dec-2-en-4-olide (2-DECEN) or hydrated by 2 (2-enoyl-CoA hydratase) to 3-hydroxyacyl-CoA. This latter compound can also lactonize to 3-hydroxy- $\gamma$ -decalactone (3-OHDEC) or be dehydrogenated by 3 (3-hydroxyacyl-CoA dehydrogenase) and cleaved by 4 (thiolase) to enter the next  $\beta$ -oxidation loop. 3-OHDEC can be dehydrated to 2- and dec-3-en-4-olide (3-DECEN) which can be reduced to DECA.

DECA, will attest a rate-limiting role for Aox whereas the exit of the 3-hydroxyacyl-CoA before the dehydrogenase step giving 3-OHDEC and its two dehydration products, 2-DECEN and 3-DECEN will show the control role of 3-hydroxyacyl-CoA dehydrogenase.

The yeast *Y. lipolytica* possesses a five-gene family (POX1 to POX5) coding for acyl-CoA oxidase (Aox1 to Aox5) the first enzyme of the pathway which is generally considered as catalyzing the rate-limiting step [7]. Using mutant strains disrupted for one or several *pox* genes enabled us to evaluate the activity of the different enzymes. Aox1 exhibits no detectable activity in the conditions we tested, Aox2 and Aox3 are long- and short-chain specific, respectively and Aox4 and

Aox5 exhibit a weak activity on a wide range of substrates [8]. We have observed in previous works the important role of the acyl-CoA oxidase in the pathway, Aox3 (short-chain specific) being responsible for continuation of oxidation after the C10 level [9] and for lactone reconsumption [10]. Mutants with decreased Aox activity accumulated more DECA as the remaining Aox activity was the rate-limiting step of the pathway [10].

Our aim in this work was to optimize the production of DECA by modifying the genotype of the yeast to block  $\beta$ -oxidation at the C10 Aox level. To achieve this goal, we constructed a strain possessing multiple copies of *POX2*, coding for the long-chain specific Aox, and disrupted for the main Aox active on short-chain acyl-CoA. Such a strain was theoretically not able to degrade C10- or shorter CoA and was thus supposed to accumulate the precursor of  $\gamma$ -decalactone.

## 2. Materials and methods

### 2.1. 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 comparison of the various strains. Cells were precultured in a glucose medium for 19 h to the late logarithmic phase as described previously [9] then cells were harvested and inoculated to an  $OD_{600\text{ nm}}$  of 0.25 (approximately  $6.5 \times 10^6$  cells/ml) in a medium containing 3.5 g/l methyl ricinoleate (MR medium) [9]. The methyl ester form was used to avoid the possible toxicity exhibited by the free acid form and to enable a gradual liberation of the free ricinoleic acid in the medium as the yeast *Y. lipolytica* used in this study possesses active extracellular lipases [11].

### 2.2. Strains construction

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]: strain MTLY35, MatA *pox2* $\Delta$  *pox3* $\Delta$  *pox5* $\Delta$  (disrupted for the genes coding for Aox2, Aox3 and Aox5); strain JMY185, MatA *pox2* $\Delta$  *pox3* $\Delta$  *pox5* $\Delta$  *POX2* amplified, corresponds to an

Ura + transformant of strain MTLY35 with the plasmid JMP3POX2. The plasmid JMP3POX2 was constructed by insertion of the *POX2* gene into the defective vector JMP3 (this vector contained the *ura3d4* defective allele which allows multicopy integration of the plasmid into the genome upon Ura selection) [11].

### 2.3. Analysis

After acidification to pH 2 with concentrated HCl in order to favor lactonization, the internal standard,  $\gamma$ -undecalactone, was added and samples were extracted with diethyl ether. The analysis was performed with a HP6890 gas chromatograph with a HP-INNOWax capillary column as described previously [9].

## 3. Results and discussion

### 3.1. Production of lactones by the wild type

Production of  $\gamma$ -decalactone was investigated in conditions of growth on methyl ricinoleate as the only carbon source. As shown in Fig. 2, the wild type produced high amounts of 3-OHDEC and 2- and 3-DECEN, and lower amounts of DECA. Furthermore, this latter lactone was reconsumed rapidly contrary to the other lactones for which the total

concentration reached 433 mg/l and only slightly decreased to 380 mg/l after 192 h. In contrast, DECA attained 71 mg/l after 10 h but decreased to less than 14 mg/l after 24 h. Although the accumulation of the various lactones begins concomitantly, the accumulation of DECA is stopped between 10 and 24 h, when the concentration of methyl ricinoleate decreases below 100 mg/l (not shown).

Different hypotheses can be proposed to explain this difference in reconsumption. The regulation of the enzymes of the  $\beta$ -oxidation loop could be differently linked to the substrate concentration: the presence of long-chain acyl-CoA could decrease the Aox activity for short-chain acyl-CoA. In that case, the completion of the consumption of methyl ricinoleate could correspond to an increase in the C10-Aox activity, enhancing  $\gamma$ -decalactone reconsumption. This might happen through a different regulation of the different *POX* genes: the presence of long-chain substrate could stimulate *POX2* (coding for the long-chain Aox) and repress *POX3* (coding for the short-chain Aox). In contrast, the other  $\beta$ -oxidation enzymes could be insensitive to this parameter and thus could not accept more substrate at this stage. A second hypothesis could be the absence of hydrolases accepting hydroxy- or unsaturated-lactones contrasting with the presence of a  $\gamma$ -decalactone delactonase. Eventually, the differential reconsumption could be linked to the environmental conditions. One of the environmental differences between the conditions for DECA reconsumption,

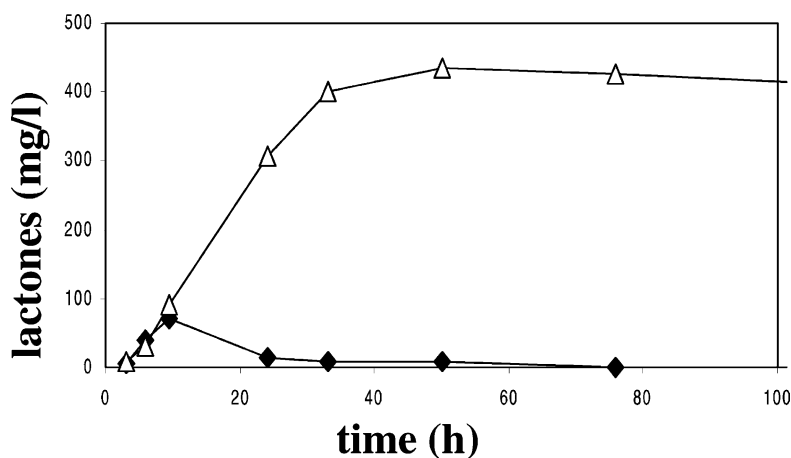


Fig. 2. Accumulation of DECA (◆) and 3-OHDEC + 2-DECEN + 3-DECEN (△) during growth of wild type on 200 ml methyl ricinoleate (3.5 g/l) in 500 ml baffled Erlenmeyer flasks.

which occurs relatively early in the culture, and for the absence of reconsumption of the other lactones that attain their maximal concentration later in the culture, is the pH conditions. At the end of the culture, the pH is 1 or 2 pH unit lower (around 3) than when DECA is consumed. Although the reconsumption pathway is not precisely known, it requires an entry of the lactone into the cell and prior to or after this step, a hydrolysis of the lactone cycle. The difference of pH may have an impact on the reconsumption as, at pH 3, some surface transport proteins can be less active than at a higher pH earlier in the culture. The activity of intracellular enzymes can also be decreased as the intracellular pH is 0.5 pH unit lower at pH 3 than at pH 5.5 [12]. This may result in lowered enzymatic activities decreasing the entry into the cell or reconsumption of lactones.

In order to avoid lactone reconsumption and the accumulation of other lactones resulting in low yields, we constructed a strain modified for its Aox activity with a decreased activity for short-chain acyl-CoA.

### 3.2. Production of DECA by the optimized strain

The production for the Aox engineered strain compared to the wild type is shown in Fig. 3. The wild type produced rapidly DECA in the first 12 h, reaching concentrations of 71 mg/l, and then reconsumed it. The constructed strain produced slowly but

steadily DECA during 4 days to a concentration of 150 mg/l DECA. However, after 4 days, this strain had not consumed all the methyl ricinoleate present contrasting with the wild type for which there was almost no more detectable methyl ricinoleate after 80 h.

We have noticed, interestingly and contrasting with the wild type, that the concentrations of other lactones were very low in the biotransformation medium for the mutant strain used in this study (results not shown) confirming results obtained previously with other strains [10] showing that, for mutants with several *pox* genes disrupted, Aox possessed a high control on the pathway. No other metabolic pathways for methyl ricinoleate degradation were detected: the substrate was degraded very slowly and we did not detect any accumulation of new products, such as  $\alpha$ - $\omega$ -dicarboxylic acids, compared to the wild type. However, other studies describe the utilization of strains disrupted for all Aox encoding genes to favor the cytochrome P450 pathway in the purpose of producing dicarboxylic acids [13].

There was no reconsumption of DECA for the mutant strain, but whether this point was due to the absence of short-chain Aox in the strain or to the presence of methyl ricinoleate, long-chain substrate, until the end of the culture is not known. The simultaneous disruption of *POX3* (short-chain specific Aox) and *POX5* (Aox exhibiting also a weak activity on short-chain acyl-CoA) does not enable to differentiate

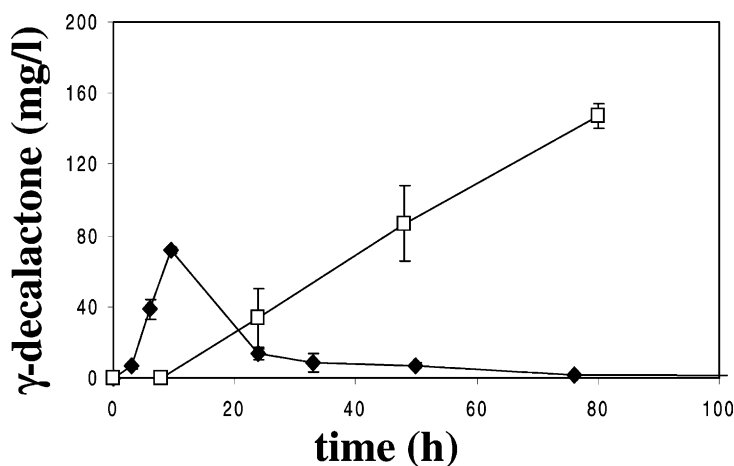


Fig. 3. Accumulation of DECA during growth of wild type (◆) and mutant strain (□) on 200 ml methyl ricinoleate (3.5 g/l) in 500 ml baffled Erlenmeyer flasks.

between the two hypothesis and we will now study the regulation of the various *POX* genes with *lacZ* reporter constructions.

Such a result, although more than twice better than for the wild type, is not fully satisfying as the biotransformation took place very slowly. This shows that the metabolic pathway of the modified strain is altered by the disruption of several Aox encoding genes even if, in a previous study [8] we have shown that Aox2 (the remaining and long-chain specific enzyme) was very active. The low Aox activity in the mutant strain could result from the lack of several Aox important in the enzyme structure formation. The Aox of *Y. lipolytica* has been recently shown to consist in a heteropentamer composed of the five Aox [14]. This polymeric structure is formed in the cytoplasm and targeted to the peroxisomes. However, when Aox2 or Aox3 are lacking, the polymerization and subsequent targeting to the organelle is weak.

#### 4. Conclusion

The optimized strain of *Y. lipolytica* with almost no remaining Aox activity on short-chain acyl-CoA, although efficient for lactone production, was not growing as rapidly as the wild type on fatty acids. This problem can have many sources, among them the enzyme may not form its complex inside the peroxisome. This point is currently investigated.

In the production of lactones, the lactonization step remains to be investigated. In particular, it would be interesting to understand which reaction occurs between

the spontaneous lactonization in acidic media and the enzymatic catalyzed reaction. The rate of lactonization for the various C10-lactones is also an important point which could be determinant for the production of one or the other lactone.

#### References

- [1] S. Okui, M. Uchiyama, M. Mizugaki, J. Biochem. 54 (1963) 536.
- [2] I.L. Gatfield, in: R. Teranishi, E.L. Wick, I. Hornstein (Eds.), *Biotechnological Production of Natural Flavor Materials*, Kluwer Academic Publisher, New York, 1999, p. 211.
- [3] I.L. Gatfield, M. Güntert, H. Sommer, P. Werkhoff, Chem. Mikrobiol. Technol. Lebensm. 15 (1993) 165.
- [4] G. Fronza, C. Fuganti, P. Grasselli, M. Barbeni, Tetrahedron Lett. 33 (1992) 6375.
- [5] M. Farbood, G. Harris, L.E. Kizer, L.B. McLean, J. Morris, European Patent EP0,952,226 (1999).
- [6] I. Gatfield, German Patent DE4,401,388 (1995).
- [7] N.C. Inestrosa, M. Bronfman, F. Leighton, Biochem. J. 182 (1979) 779.
- [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. 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.
- [10] Y. Waché, M. Aguedo, A. Choquet, I. Gatfield, J.-M. Nicaud, J.-M. Belin, Appl. Environ. Microbiol. 67 (2001) 5700.
- [11] G. Pignède, H. Wang, F. Fudalej, M. Seman, C. Gaillardin, J.-M. Nicaud, Appl. Environ. Microbiol. 66 (2000) 3283.
- [12] M. Aguedo, Y. Waché, J.-M. Belin, FEMS Microbiol. Lett. 200 (2001) 185.
- [13] S. Picataggio, T. Rohrer, K. Deanda, D. Lanning, R. Reynolds, J. Mielenz, L.D. Eirich, Biotechnology 10 (1992) 894.
- [14] V.I. Titorenko, J.-M. Nicaud, H. Wang, H. Chan, R.A. Rachubinski, J. Cell. Biol. 156 (2002) 481.