Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 28 (2004) 81–85
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Acyl-CoA oxidase, a key step for lipid accumulation in the yeast *Yarrowia lipolytica*

Kateřina Mlíčková[a,c], Yishan Luo[c], Sabine d'Andrea[c], Pavel Peč[a], Thierry Chardot[c], Jean-Marc Nicaud[b,*]

[a] *Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 11, 78371 Olomouc, Czech Republic*
[b] *Laboratoire de Microbiologie et Génétique Moléculaire, CNRS-INRA-INAPG UMR 2585, Centre de Biotechnologie Agro-Industrielle, URA 1925, 78850 Thiverval-Grignon, France*
[c] *Laboratoire de Chimie Biologique, INRA-INAPG UMR 206, Centre de Biotechnologie Agro-Industrielle, 78850 Thiverval-Grignon, France*

Received 5 August 2003; received in revised form 8 December 2003; accepted 15 January 2004

Abstract

The yeast *Yarrowia lipolytica* is able to use fatty acids as carbon source via β-oxidation. This yeast contains five acyl-CoA oxidases (Aoxps) encoded by *POX* genes. Two Aoxps are of particular interest: Aox2p, a medium chain oxidase, and Aox3p, an oxidase specific for short chain substrates. To date, no 3D structure of these enzymes is available. To better understand their specificity, we have expressed 10 enzymes in *Escherichia coli*; Aox2p, Aox3p, a mutated Aox3 (Aox3mp), and seven chimera obtained by shuffling fragments of Aox2p and Aox3p (M1 to M4 and M6 to M8). Aox3mp and one chimeric protein (M6) were active, Aox3mp exhibited broad chain length specificity.

We tested complementation of growth for strain MTLY36 (Δpox2,3,5; reduced growth on oleic acid media) and MTLY40 (Δpox2,3,4,5; not growing on oleic acid media) by expressing either Aox2p, Aox3p, Aox3mp, and M6 chimera encoding genes. We demonstrate that the *POX* genomic content affects both γ-lactone production [J. Mol. Catal. B, this issue] and lipid storage. Strain MTLY36 having Aox4p could still use oleic acid but grew slower than the wild type strain and did not accumulate lipids. The strains containing Aox3p, Aox3mp (alone or with Aox4p) grew on oleic acid and stored lipids as well as did the wild type. Strains expressing Aox2p (MTLY40-2P) and Aox2p, Aox4p (MTL36-2P) stored more lipids than the wild type. Mastering *POX* genotype allowed us to construct strains, able to accumulate high lipid content, which could be used for single cell oil (SCO) production.

Keywords: Acyl-CoA oxidase; γ-Lactone; β-Oxidation; Lipid accumulation; *Yarrowia lipolytica*

1. Introduction

The yeast *Yarrowia lipolytica* is able to utilize fatty acids or oil as carbon source. During growth of *Y. lipolytica* on castor oil, ricinoleic acid or methyl ricinoleate, γ-decalactone was shown to be accumulated [2]. This lactone is used in the food industry due to its fruity peach flavor. We have previously shown that β-oxidation was involved both in the bioconversion and in the lactone reconsumption [3–5]. In this pathway, which consists of four main reactions, acyl-CoA oxidase was shown to control a key step [6]. *Yarrowia lipolytica* possesses five acyl-CoA oxidases (Aoxs) encoded by the genes *POX1* through *POX5* [7,8]. Two of them present chain length specificity; Aox2p being a medium chain oxidase [9] and Aox3p an oxidase specific towards short chain substrates [10,11].

Single cell oil (SCO) production has potential industrial application [12]. SCO production by *Y. lipolytica* has been shown to be dependent on growth conditions and substrates [13–16] and could be enhanced by *Teucrium polium* L. aqueous extract [17]. Lipids accumulated inside the yeast cells represent 0.44–0.54 g/g of biomass. These lipids are principally composed of triacylglycerols (55%) and free fatty acids (35%) [16].

Our aim in this work was to identify the region of the protein involved in chain length specificity and to determine whether the enzyme specificity profile could be modified. We wished to assess the importance of the Aoxp specificity for lactone production and for lipid utilization and storage.

* Corresponding author. Tel.: +33-1-3081-5450; fax: +33-1-3081-5457.
E-mail address: jean-marc.nicaud@grignon.inra.fr (J.-M. Nicaud).

1381-1177/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2004.01.007

2. Materials and methods

2.1. Media and strains

Yarrowia lipolytica strains used in this study, derived from the wild-type *Y. lipolytica* strain W29 (ATCC 20460), were MTLY36 and MTLY40 (an uracil auxotroph of MTLY37) with multiple disruptions of *POX* genes coding for acyl-CoA oxidase (Aoxp) [8]. The uracil auxotrophe MTLY40 was obtained by transformation of MTLY37 with the PCR fragment carrying the *ura3-41* allele followed by selection on 5FOA plates according to Fickers et al. [18]. *Escherichia coli* DH5α and M15 strains were used for gene manipulation and Aoxp expression in *E. coli*, respectively. *Yarrowia lipolytica* was handled and grown according to Barth and Gaillardin [19] and *E. coli* according to Maniatis et al. [20]. Minimal oleic acid medium (YNBO) contained yeast nitrogen base without amino acids and ammonium sulfate, YNBww 0.17% (Difco, Paris France), NH4Cl 0.5%, yeast extract 0.1%, and oleic acid 5% (Merck, Fontenay-sous-Bois Cedex, France), buffered with 50 mM phosphate buffer (pH 6.8). Oleic acid was emulsified by sonication in the presence of 0.02% Tween 40. Uracile (0.1 g/l) was added when required.

2.2. Plasmid and strain constructions

Plasmids coding for mutated and chimeric oxidases were constructed as described in Fig. 1. Briefly, fragments 2A, 2B, 2C, 2AB, and 2BC were obtained from pQE32-Aox2p [9] and fragments 3A, 3BC from pQE32-Aox3p [10]. Fragment 3b*, 3c*, and 3bc* containing the *Bgl*I restriction site were obtained by PCR using pQE32-Aox3p as template and the primers a: 5′ACGGCTGGATCCAGTTCAA3′; b: 5′CCTCTCTGCCGACCAGGCCTTTGA; c: 5′CAAAGGCCTGGTCGGCAGAGAGGC3′; d: 5′CGCCAAGCTAGCTTGGATTC3′ containing the restriction sites *BamH*I, *Bgl*I, and *Nhe*I (underlined and Fig. 1). The pQE32-M1 through pQE32-M8 plasmids for *POX2*/*POX3* shuffled gene expression in *E. coli* were obtained by ligation of pQE32

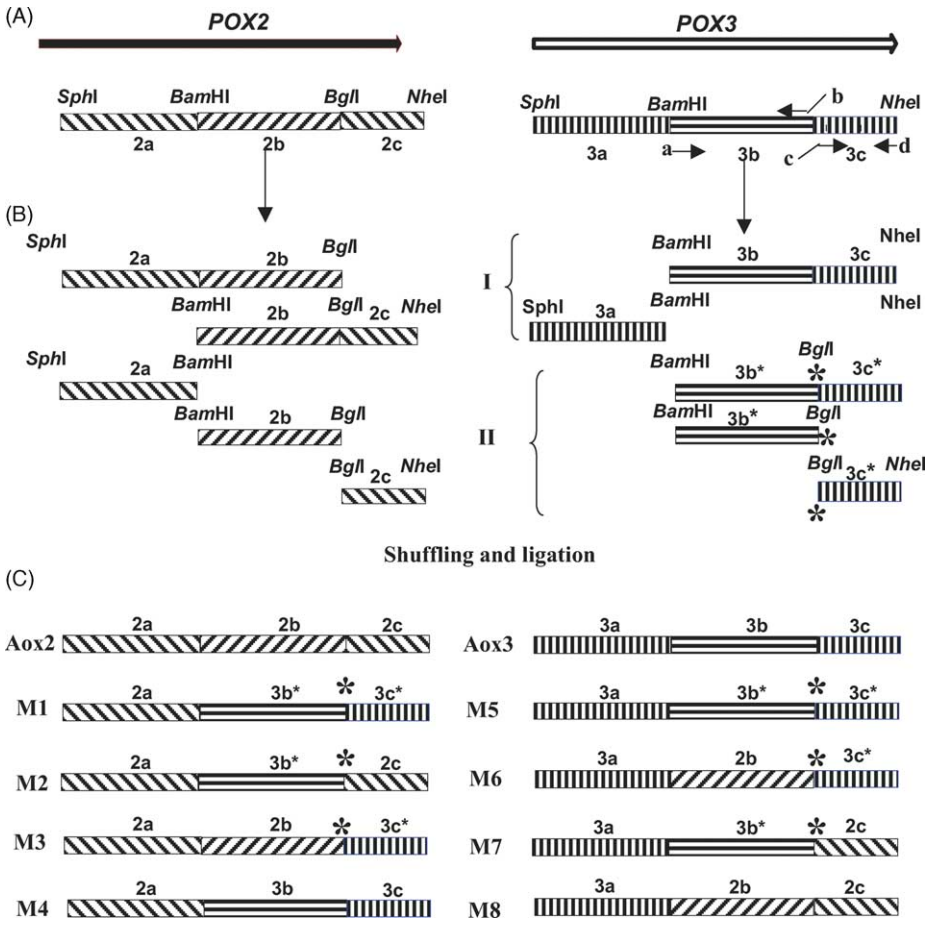

Fig. 1. Construction of *POX2*, *POX3*, mutated and chimeric *POX* genes by *POX2*/*POX3* DNA shuffling. (A) Schematic representation of *POX2* and *POX3* genes with the restriction sites used for the construction of shuffled genes which determined three domains named 2a, 2b, and 2c for *POX2* and 3a, 3b, and 3c for *POX3*. The localization of primers used to introduce a *Bgl*I site into *POX3* are indicated by arrows. (B) Fragments obtained after digestion of pQE32-POX2 and pQE32-POX3 or by PCR with primer a, b, c, and d. (C) Schematic representation of the 10 genes expressed in *E. coli*; the wild type *POX2* (coding for Aox2) and *POX3* (coding for Aox3), and the mutated (M5) and chimeric genes (M1 to M8) resulting from *POX2*/*POX3* domain shuffling. *POX3* fragments containing the *Bgl*I site is indicated with a star (*).

digested by *Sph*I and *Nhe*I together with the corresponding fragments as indicated in Fig. 1B. For expression of Aox2p, Aox3p, and the mutated Aox3p (M5; Aox3mp) and M6 in *Y. lipolytica*, coding sequences were amplified with the appropriate pairs from the oligonucleotides POX2-ATG, POX2-STOP, POX3-ATG, and POX3-STOP. The PCR fragments were digested with *Bln*I and *Sfi*I and ligated into plasmid pYEG1 digested by the same enzymes [21] giving rise to plasmid pYEG1-*POX2*, pYEG1-*POX3*, pYEG1-M5, and pYEG1-M6.

Escherichia coli expression plasmids were transformed into *E. coli* M15 competent cells. Transformant were selected on LB ampicilin medium. Aox induction was performed at 25 °C as described previously for Aox2 and Aox3 [9–11]. *Yarrowia lipolytica* expression plasmids were selected in *E. coli* on LB kanamycin and introduced into *Y. lipolytica* strains MTLY36 and MTLY40 by the lithium acetate method [19]. Prior to integration into yeast cells, plasmids were digested by *Not*I. Ura^+ transformants were selected on YNBcas. The resulting transformants were designated MTLY36-2P, MTLY36-3P, MTLY36-M5, and MTLY36-M6 expressing *POX2*, *POX3*, *POX-M5*, and *POX-M6*, respectively. Similarly, MTLY40 transformants were designated MTLY40-2P, MTLY40-3P, MTLY40-M5, and MTLY40-M6, respectively.

2.3. Acyl-CoA activity assays

Cell extract preparation, acyl-CoA oxidase purification and activity measurement were carried out as described previously [11].

2.4. Light and electron microscopy

Cells were grown in YNBO and fixed with formaldehyde (5 h at room temperature). For light microscopy, lipid bodies (LB) were stained with Nile Red (Molecular Bioprobe, Montluçon France; 1 mg/ml in acetone). Nile Red was added to the cell suspension (1/10 (v/v)) and incubated for 1 h at room temperature. After centrifugation, cells were washed twice with distilled water and re-suspended in 50 mM K–P_i buffer (pH 6.8). Cells were observed with Olympus BX 51 light microscope equipped with 100× oil immersion objective. Images were recorded using the Photometrics CoolSNAP software. For electron microscopy cells were fixed with glutaraldehyde (2%) for 1 h, 28 °C. Cell were harvested by centrifugation and resuspended in 50 mM phosphate buffer (pH 6.8) containing 3% glutaraldehyde and fixed for further 24 h at room temperature. Lipid bodies were stained with 2% osmium-tetraoxide in 0.2 M imidazole buffer (pH 7.5; 1 h) according to Angermüller and Fahimi [22]. Cells were dehydrated in graded acetone series: 30, 40, 70, 100% (v/v), embedded in LRwhite resin, cut with an ultramicrotome Ultracut E (Leica, Rueil Malmaison, France) and observed with a Phillips 420 microscope (Phillips electron optics, Eindhoven, Netherlands).

3. Results

3.1. Modification of acyl-CoA oxidase chain length specificity

Acyl-CoA oxidases can present chain length specificity. A short chain and a medium chain Aox were found in *C. tropicalis* and *Y. lipolytica* [7]. It is not possible to predict Aoxp chain length specificity from protein sequence comparison. In order to determine if chain length specificity could be modified and which part of the protein was involved, we decided to build chimeric enzymes containing part of Aox2p and Aox3p coding sequences. Seven different chimeric genes and one containing a point mutation were assembled as described in Fig. 1 and expressed in *E. coli*. Induction of chimeric Aoxs was performed at 25 °C as previously described for Aox2p and Aox3p [9–11]. Aox activity was measured on *E. coli* extracts. Among the mutated and chimeric proteins, only Aox3mp (M5) and M6 were found to be active. For M6, activity with C6, C10, and C14 substrates was observed, however, the protein was found to be very unstable and its activity decreased on a timescale of minutes, which did not allow us to perform a complete chain length activity profile. Aox3mp (M5) protein was also not very stable and lost its activity on a timescale of hours. Nevertheless, we could demonstrate that this mutant enzyme was able to oxidize substrates with chain lengths comprised between C8 and C16 (Fig. 2). This indicates that a single mutation could result in a change of chain length specificity. Such minor amino acid changes could result in chain length differences as observed between butyryl CoA deshydrogenase (BCAD) and medium chain acyl CoA deshydrogenase (MCAD). Indeed, the overall peptide fold of BCAD and MCAD are similar, but they exhibit contrasted substrate specificity, essentially due to a single amino acid insertion and difference in the side chains of the helices making the substrate bind-

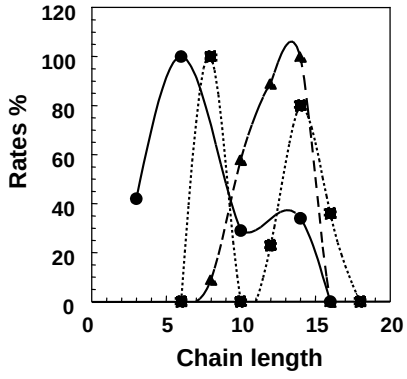

Fig. 2. Acyl-CoA oxidase activity profiles of Aox2p, Aox3p, and chimeras as a function of substrate chain length. Activity profile of purified Aox2p (▲, dashed line) and Aox3p (●, continuous line) and the mutant enzyme Aox M5 (■, dotted line). C14 activity for Aox2p and Aox-M5, and C6 activity for Aox3p were used as 100%. For the sake of clarity, experimental points corresponding to other chimera were omitted.

ing cavity of BCAD relatively shallow compared to MCAD [23].

3.2. Expression of Aox2p, Aox3p, Aox-M5, and Aox-M6 in Y. lipolytica and growth complementation

We have previously shown that strain MTLY36 deleted for *POX2*, *POX3*, and *POX5* presents a growth defect on oleic acid media and that strain MTYL37 deleted for *POX2*, *POX3*, *POX4*, and *POX5* could not grow at all on this substrate [8]. A MTLY37 uracil auxotroph designated MTLY40 was obtained by transformation as described in Section 2. This strain was shown to be unable to grow on oleic acid as MTLY37 (data not shown). To determine whether Aox2p, Aox3p, and the mutant enzyme could restore normal growth of MTLY36 or sustain growth of strain MTLY40, we constructed the expression plasmids pYEG1-*POX2*, pYEG1-*POX3*, pYEG1-M5, and pYEG1-M6 as described in Section 2 and introduced them into the two strains. The resulting strains MTLY36-2P, MTLY36-3P, and MTLY36-M5 present a normal growth on YNBO similar to that of the wild-type strain W29 (data not shown). Furthermore, MTLY40-2P, MTLY40-3P, and MTLY40-M5 presented a wild-type growth, indicating that Aox2p, Aox3p, and Aox-M5 were separately sufficient to restore full growth on oleic acid medium. The Aox-M6 was unable to restore normal growth of MTLY36, nor growth of MTLY40. This suggested that this protein was too unstable to complement this deficit. This also indicates that even though Aox2p presents undetectable activity toward short chain fatty acids and Aox3p very little activity toward long chains, they both confer full complementation. Though we could not observe changes by growth rate analysis, we may expect to see changes in the β-oxidation rate and modification of lactone production. Increase in lactone production without lactone reconsumption has been reported by Groguenin et al. (this issue [1]). We also observed changes in lipid accumulation as shown below.

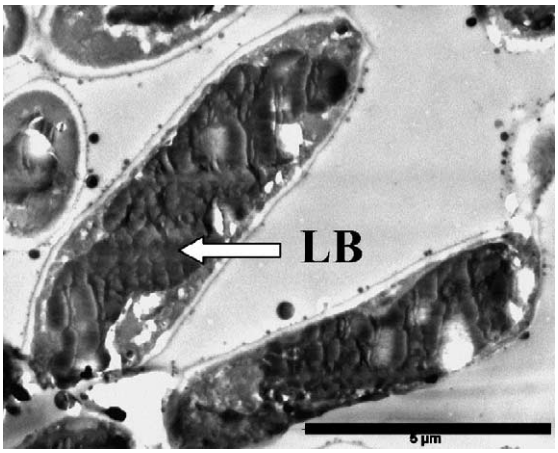

Fig. 3. Lipid accumulation into lipid bodies in MTLY36-2P. Cells were grown in YNBO medium at 28 °C. At different time points, cells were harvested, fixed and prepared for transmission electron microscopy. Electron micrograph of strain MTLY36-2P 40 h after transfer from glucose media. Scale bar = 5 μm, magnification was 8200×. Lipid bodies (LB) are seen as black intracellular vesicles in cytoplasm.

3.3. Lipid accumulation in Y. lipolytica strains modified for their POX genotype

Yarrowia lipolytica has been shown to be able to accumulate lipids [13–17]. When yeasts were grown in lipid media, they could either degrade the lipid by β-oxidation or store it in lipid bodies. Since we demonstrate that modifications of the *POX* genotype affected β-oxidation, we decided to test these effects on lipid storage. Therefore, we analyzed lipid accumulation using Nile red staining of MTLY36, MTLY40, and their derivatives. We observed an increase in lipid accumulation for the wild type when grown on YNBO (5% oleic acid). First several lipid bodies are observed 6 h after transfer from glucose to YNBO. At 18 h, LB number decreased but their size increased. And finally at 40 h, we observed 1–3 large LB representing roughly half of the cell volume (data not shown). All strains present similar lipid accumula-

Table 1
Growth and lipid accumulation characteristics of *Y. lipolytica* strains used in this study

Strains	Genotype/auxotrophy	Aox expressed	Growth	Lipid accumulation
MTLY36	Δ2,3,5/prototrophe	None	Reduced	Reduced
MTYL37	Δ2,3,4,5/prototrophe	None	No growth	NS
MTLY40	Δ2,3,4,5/auxototrophe	None	No growth	NS
MTLY36-2P	Δ3,4/prototrophe	Aox2p	Normal	High
MTLY36-3P	Δ2,4/prototrophe	Aox3p	Normal	Normal
MTLY36-M5	Δ2,3,4-3 m/prototrophe	Aox3mp	Normal	Normal
MTLY36-M6	Δ2,3,4-M6/prototrophe	Aox-M6	Reduced	Reduced
MTLY40-2P	Δ3,4,5/auxototrophe	Aox2p	Normal	High
MTLY40-3P	Δ2,4,5/auxototrophe	Aox3p	Normal	Normal
MTLY40-M5	Δ2,3,4,5-3 m/auxototrophe	Aox3mp	Normal	Normal
MTLY40-M6	Δ2,3,4,5-M6/auxototrophe	Aox-M6	No growth	Reduced

Strain names, *POX* genotype and altered *POX* gene introduced; mutated *POX3* (Aox3m) and chimeric M6, with the Aox expressed in the transformants, the growth characteristics: growth as the mutant strain MTLY36; reduced, no growth as the mutant MTLY40; no growth, or as the wild-type strain; normal. Symbols for lipid accumulation as determined by fluorescent microscopy and electron microscopy are: NS, no significant accumulation observed; reduced, lowed accumulation than the wild-type strain; normal, similar accumulation that of the wild type; high, observation of huge oil bodies as shown in Fig. 3.

tion, except MTL36, MTLY36-2P, and MTL40-2P. MTL36 accumulated small amounts of lipids, while MTLY36-2P and MTL40-2P accumulated large quantities of lipids, having LB filling the full cell volume. These results were confirmed by electron microscopy as shown in Fig. 3 where we observed in the case of MTLY36-2P after 40 h of growth in YNBO, that the LB represents the major part of the cell volume.

4. Conclusion

Using gene shuffling, we have demonstrated that we could change Aoxp chain length specificity, even with limited sequence modifications of Aoxps. A single mutation P540A into Aox3p results in an Aoxp presenting a broad substrate specificity. This indicates that it could be possible by error prone mutagenesis or DNA shuffling to generate an Aox2p unable to utilize a C10 substrate or new Aoxp which could accommodate branched fatty acid (*Y. lipolytica* is unable to grow on pristane). This study also demonstrates that acyl-CoA oxidases play a key role in lipid accumulation by *Y. lipolytica*. This suggests that an intermediate of the β-oxidation is implicated in either induction of lipid storage or in repression of lipid mobilization. Interestingly, these results provide us with strains which present high lipid content and which could be used for SCO production (Table 1).

Acknowledgements

Authors wish to thank F. Jaunet and J. Laperonnie (Service de Microscopie électronique, INRA Versailles) for expert sample preparations and microscopic observations. K. Mlíčková was a recipient of a Marie Curie training fellowship during her stay at the Yeasts of Technological Interest (YETI) INRA training site in Grignon. Authors are thankful to F. Jagic for technical help and Aoxp purification. Thanks to Tinsley Colin for proofreading this manuscript.

References

[1] A. Groguenin, Y. Waché, E. Escamilla Garcia, M. Aguedo, F. Husson, M.-T. Le Dall, J.-M. Nicaud, J.-M. Belin, J. Mol. Catal. B. 28 (2004) 75–79.
[2] S. Okui, M. Uchiyama, M. Mizugaki, J. Biochem. 54 (1963) 536.
[3] 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.
[4] Y. Waché, M. Aguedo, A. Choquet, I. Gatfield, J.-M. Nicaud, J.-M. Belin, Appl. Environ. Microbiol. 67 (2001) 5700.
[5] Y. Waché, M. Aguedo, J.-M. Nicaud, J.-M. Belin, Appl. Microbiol. Biotechnol. 61 (2003) 393.
[6] Y. Waché, M. Aguedo, M.-T. Le Dall, J.-M. Nicaud, J.-M. Belin, J. Mol. Catal. B: Enzym. 19–20 (2002) 347.
[7] H. Wang, M.-T. Le Dall, Y. Waché, P. Laroche, J.-M. Belin, C. Gaillardin, J.-M. Nicaud, Cell. Biochem. Biophys. 31 (1999) 165.
[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, J.M. Nicaud, P.P. Van Veldhoven, T. Chardot, Arch. Biochem. Biophys. 407 (2002) 32.
[10] H. Wang, A. Le Clainche, M.-T. Le Dall, Y. Waché, Y. Pagot, J.-M. Belin, C. Gaillardin, J.-M. Nicaud, Yeast 14 (1998) 1373.
[11] Y.S. Luo, H.J. Wang, K.V. Gopalan, D.K. Srivastava, J.-M. Nicaud, T. Chardot, Arch. Biochem. Biophys. 384 (2000) 1.
[12] C. Ratledge, in: B.S. Kamel, Y. Kakuda (Eds.), Technological Advances in Improved and Alternative Sources of Lipids, Blackie, London, 1994, p. 235.
[13] D. Montet, R. Ratomahenina, P. Galzy, M. Pina, J. Graille, Biotechnol. Lett. 7 (1985) 733.
[14] S. Papanikolaou, G. Aggelis, Bioresour. Technol. 82 (2002) 43.
[15] S. Papanikolaou, I. Chevalot, M. Komaitis, G. Aggelis, I. Marc, Antonie Van Leeuwenhoek 80 (2001) 215.
[16] S. Papanikolaou, I. Chevalot, M. Komaitis, I. Marc, G. Aggelis, Appl. Microbiol. Biotechnol. 58 (2002) 308.
[17] G. Aggelis, M. Komaitis, Biotechnol. Lett. 21 (1999) 747.
[18] P. Fickers, J.-M. Nicaud, J. Destain, P. Thonart, Appl. Microbiol. Biotechnol. 63 (2003) 136.
[19] G. Barth, C. Gaillardin, *Yarrowia lipolytica*, Nonconventional Yeast in Biotechnology, a Handbook, vol. 1, Springer-Verlag, Berlin/Heidelberg/New York, 1996, p. 314.
[20] E. Maniatis, J. Fritsch, J. Sambrook, Molecular Cloning: a Laboratory Manual, second ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.
[21] J.M. Nicaud, C. Madzak, P. van den Broek, C. Gysler, P. Duboc, P. Niederberger, C. Gaillardin, FEM Yeast Res. 2 (2002) 371.
[22] S. Angermuller, D.H. Fahimi, Histochem. J. 14 (1982) 823.
[23] S. Djordjevic, C.P. Pace, M.T. Stankovich, J.J. Kim, Biochemistry 34 (1995) 2163–2171.