Beta-Oxidation of Unsaturated Fatty Acids in Yeast

by

André Guillaume Ntamack

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Prof. Horst Schulz

Date
Signature, Chair of Examining Committee

Prof. Lesley Davenport

Date
Signature, Executive Officer

Prof. David Calhoun

Prof. Gillian M. Small

Prof. Thomas Haines

Prof. Manfred Philipp

Prof. Leslie Kushner

Supervisory Committee

The City University of New York

ABSTRACT

BETA – OXIDATION OF UNSATURATED FATTY ACIDS IN YEAST

by

André Guillaume Ntamack

Advisor: Professor Horst Schulz

The β-oxidation of fatty acids to acetyl-CoA is a central energy-yielding pathway in animals, yeast, and bacteria. This study of the β-oxidation of oleic acid in *Saccharomyces cerevisiae* was initiated by measuring and comparing the growth of wild-type *S. cerevisiae* on oleic acid or palmitic acid with the growth of mutants that have either a deletion in the *DCI1* gene reported to encode $\Delta^{3,5}$, $\Delta^{2,4}$-dienoyl-CoA isomerase (dienoyl-CoA isomerase) or in the thioesterase gene (*TES1*) or in both the *DCI1* and *TES1* genes. Growth of wild-type yeast and the *DCI1* mutant was indistinguishable on either palmitic acid or oleic acid, whereas the *TES1* mutant grew slower and to a lower density on oleic acid but not on palmitic acid. 3,5-Tetradecadienoic acid was detected in the growth medium by gas chromatography/mass spectrometry after growth of wild-type cells on oleic acid, but not when palmitic acid was the carbon source. When the *TES1* mutant was transformed with a plasmid expressing human dienoyl-CoA isomerase to increase the dienoyl-CoA isomerase activity, the growth defect on oleic acid was partially corrected. The purified product of the *DCI1* gene expressed in *E.coli* did not exhibit dienoyl-CoA isomerase activity. Low levels of dienoyl-CoA isomerase activity were detected in wild-type yeast but also in the *DCI1* mutant. However β-oxidation enzymes in the *DCI1* mutant were upregulated and mislocalised to the cytosol. These observations support the conclusion that the *DCI1* gene product is not a β-oxidation enzyme, but rather is involved in protein import into peroxisomes. Hence 3,5-tetradecadienoyl-CoA, an intermediate of oleate β-oxidation, does not seem to be degraded via the reductase-dependent pathway, which does not operate in yeast. Instead the disposal of 3,5-tetradecadienoyl-CoA is achieved by the thioesterase-dependent pathway that involves hydrolysis of acyl-CoA intermediates, including 3,5-tetradecadienoyl-CoA to the corresponding acids that are excreted into the growth medium.

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ABBREVIATIONS

AD	acyl-CoA dehydrogenase
BSA	bovine serum albumin
CoASH	coenzyme A
Dci1p/DI	$\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase
DEPC	diethyl pyrocarbonate
Eci1p/EI	Δ^{3}-Δ^{2}-enoyl-CoA isomerase
EDTA	ethylenediaminetetraacetate
EGTA	[ethylene bis(oxyethylenenitrilo)] tetraacetate
FAO complex	fatty acid oxidation complex
Fox1p/Aox	acyl-CoA oxidase
Fox2p/EH/HAD	enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase
Fox3p/KT	3-ketoacyl-CoA thiolase
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high-performance liquid chromatography
IPTG	isopropyl-1-thio-β-D-galactopyranoside
KPi	potassium phosphate
MBP	maltose binding protein
ML	microsome light
MOPS	3-[N-morpholino]propanesulfonic acid
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride

PNS	post nuclear supernatant
PS	supernatant
SDS	sodium dodecylsulfate
SSC	sodium chloride/sodium citrate
Sps19p/DR	2, 4-dienoyl-CoA reductase
Tes1p/TE	acyl-CoA thioesterase
YPD	yeast extract/peptone/dextrose
YPGO	yeast extract/peptone/glycerol/oleic acid/tween40
YPOLT	yeast ectract/peptone/oleate/tween40
2YT	yeast extract/tryptone/sodium chloride

HISTORIC REVIEW AND BACKGROUND

Introduction

β-Oxidation is the breakdown of fatty acids through the oxidation of carbon atom β (C_β) to a carboxyl group, which occurs in prokaryotic and eukaryotic organisms. In prokaryotic cells, such as *E.coli*, β-oxidation takes place in the cytosol. In yeast, plants, and other lower eukaryotes β-oxidation is exclusively operating in peroxisomes, whereas in mammals this process proceeds in both mitochondria and peroxisomes [1]. It has been demonstrated that saturated fatty acids are completely degraded via the classical four-step of β-oxidation pathway [1,2,3]. Degradation of unsaturated fatty acids with *cis* double bonds at even-numbered positions requires 2,4-dienoyl-CoA reductase (dienoyl-CoA reductase) [EC 1.3.1.34] and Δ^3, Δ^2 enoyl-CoA isomerase (enoyl-CoA isomerase) [EC. 5.3.3.8] besides the enzymes necessary for the breakdown of saturated fatty acids [1,4]. For example, the breakdown of 6-*cis*-octadecadienoyl-CoA (petroselinic acid) with a double bond at carbon atom 6 yields a *cis*-4*, trans*-2-hexadecadienoyl-CoA intermediate, which is metabolized by reduction catalyzed by NADPH-dependent 2,4-dienoyl-CoA reductase. Degradation of *cis* double bonds at odd-numbered positions can occur by several routes (see Scheme 1) [5]. For example, the degradation of oleic acid with a double bond at carbon atom 9 yields 5-*cis*-tetradecenoyl-CoA (II) after two rounds of β-oxidation. The introduction of a *trans*-2 double bond by acyl-CoA dehydrogenase or acyl-CoA oxidase generates 2-*trans*-5-*cis* tetradecenoyl-CoA (III). This intermediate can be degraded by two different routes; it can enter the isomerase-dependent pathway to undergo a third cycle of β-oxidation in which enoyl-CoA hydratase catalyzes its hydration to 3-hydroxy-5-*cis*-tetradecenoyl-CoA (IV), which is dehydrogenated by L-3-hydroxyacyl-CoA dehydrogenase to 3-keto-5-*cis*-tetradecadienoyl-CoA (V). Finally 3-ketoacyl-CoA thiolase cleaves 3-keto-5-*cis*-tetradecenoyl-CoA to produce acetyl-CoA (XVIII) and 3-*cis*-dodecenoyl-CoA (VI). 3-*cis*-Dodecenoyl-CoA is a substrate of enoyl-CoA isomerase that catalyzes a shift of the 3-*cis* double bond to a 2-*trans* double bond. The resultant 2-*trans*-dodecenoyl-CoA is completely degraded via the classical pathway of β-oxidation. 2-*trans*,5-*cis*-Tetradecadienoyl-CoA can also be isomerized by enoyl-CoA isomerase to 3-*trans*-5-*cis*-tetradecadienoyl-CoA (XI), which is a substrate of $\Delta^{3,5}$, $\Delta^{2,4}$ dienoyl-CoA isomerase (dienoyl-CoA isomerase) in the reductase-dependent pathway. 2-*trans*, 4-*trans*-Tetradecadienoyl-CoA (XII) formed by the action of dienoyl-CoA isomerase on 3-*trans*-5-*cis*-dienoyl-CoA intermediates, is reduced by 2,4-dienoyl-CoA reductase to produce 3-*trans*-tetradecenoyl-CoA (XIII). The latter compound is isomerized by enoyl-CoA isomerase to 2-*trans*-tetradecenoyl-CoA (XIV), which reenters the classical β-oxidation cycle for further degradation (Scheme 1).

β-Oxidation of oleic acid in mammals

In mammals, unsaturated fatty acids with odd-numbered double bonds can be degraded in mitochondria and peroxisomes [6-17] either by the isomerase-dependent pathway or the reductase-dependent pathway that requires a novel enzyme, dienoyl-CoA isomerase. It has been determined that more than 80% of oleate β-oxidation occurs via the isomerase-dependent pathway, whereas the more recently discovered reductase-dependent pathway makes a minor contribution to oleate β-oxidation [18] (Scheme.1).

β-Oxidation of oleic acid in *E.coli*

In *E.coli*, the multienzyme complex (FAO complex) of fatty acid β-oxidation in cooperation with acyl-CoA dehydrogenase [19], degrades oleic acid to 2-*trans*,5-*cis*-tetradecadienoyl-CoA. The latter metabolite is degraded to decanoyl-CoA via the isomerase-dependent pathway by the four activities of the FAO complex, named enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase and enoyl-CoA isomerase. Decanoyl-CoA is completely converted to acetyl-CoA by acyl-CoA dehydrogenase and the FAO complex. However a small part of 2-*trans*,5-*cis*-tetradecadienoyl-CoA is converted to 3,5-*cis*-tetradecadienoyl-CoA by the enoyl-CoA isomerase activity of the FAO complex (see Scheme 2). It has been demonstrated that unsaturated fatty acid with even numbered double bonds can be degraded in *E.coli* cells where 2,4-dienoyl-CoA reductase is required to reduce 2-*trans*, 4-*cis* or 2-*trans*, 4-*trans*-dienoyl-CoA intermediates to 2-*trans*-enoyl-CoA [20]. The observation that *E.coli* cells lacking 2,4-dienoyl-CoA reductase do not grow on petroselinic acid but grow on oleic acid [21], suggested that these cells use a pathway other than the reductase-dependent pathway to metabolize 3-*trans*,5-*cis*-tetradecadienoyl-CoA formed during the degradation of oleic acid. A recent study revealed that dienoyl-CoA isomerase is not present in *E. coli* cells and therefore the reductase-dependent pathway for the degradation of oleic acid is not operative [22].

Instead, thioesterases that are present in *E.coli,* hydrolyze 3-*trans*,5-*cis*-tetradecadienoyl-CoA to regenerate free coenzymes A and form 3-*trans*, 5-*cis*-tetradecadienoic acid that is released from the cells into the growth medium [22] (Scheme 2).

β-Oxidation of oleic acid in *Saccharomyces cerevisiae*

Saccharomyces cerevisiae cells offer the opportunity to study fatty acid β-oxidation in an unicellular eukaryotic organism, in which β-oxidation occurs exclusively in peroxisomes [23]. It is well documented that *S. cerevisiae* cells can utilize both *cis*- and *trans*-unsaturated fatty acids as a sole carbon and energy source [24].

During the β-oxidation of oleic acid in *S. cerevisiae*, the 9 double bond becomes a 5 double bond due to chain shortening. The subsequent introduction of a 2-*trans* double bond by acyl-CoA oxidase [25], yields 2-*trans*,5-*cis*-tetradecadienoyl-CoA (III). This 2-*trans*, 5-*cis* intermediate might be degraded by two pathways. It may enter the isomerase-dependent pathway for a third round of β-oxidation where it becomes a substrate of Fox2p, a multifunctional enzyme that has both hydratase and dehydrogenase activities [26,27]. The product of Fox2p is 3-keto-5-*cis*-tetradecenoyl-CoA (V), which is cleaved to acetyl-CoA and 3-*cis*-dodecenoyl-CoA (VI) by the action of 3-ketoacyl-CoA thiolase [28]. 3-*cis*-Dodecenoyl-CoA is converted to 2-*trans*-dodecenoyl-CoA (VII) by the action of enoyl-CoA isomerase (Eci1p) [29,30]. The 2-*trans* intermediate reenters β-oxidation and is reconverted by Fox2p and Fox3p to acetyl-CoA and decanoyl-CoA (X). Decanoyl-CoA is completely degraded by 5 cycles of β-oxidation. The resultant acetyl-CoA is oxidized by the tricarboxylic acid cycle (TCA cycle) to CO_2 and reducing equivalents, which enter the electron transport chain that drives oxidative phosphorylation. Acetyl-CoA is also the precursor of all cellular organic material if oleic acid is the sole carbon source. It is assume that 2-*trans*,5-*cis*-tetradecadienoyl-CoA (III) is also convetred to its to 3-*trans*,5-*cis* isomer by enoyl-CoA isomerase. Yeast cells are thought to contain dienoyl-CoA isomerase that would transform 3-*trans*,5-*cis*-tetradecadienoyl-CoA (XI) to 2-*trans*-4-*trans* tetradecadienoyl-CoA (XII) [31,32]. The 2-*trans*, 4-*trans* intermediate is a potential substrate of yeast dienoyl-CoA reductase (Sps19p) [33,35], which would reduce it to 3-*trans*-tetradecenoyl-CoA (XIII). The latter substrate can be isomerized by enoyl-CoA isomerase to 2-*trans*-tetradecenoyl-CoA (XIV) that is completely degraded by the combined actions of Fox1p, Fox2p and Fox3p.

DCI1 was the gene that was reported to encode yeast peroxisomal dienoyl-CoA isomerase [32]. However, this protein never was purified and shown to be a dienoyl-CoA isomerase. It was observed that a yeast extract could generate 3,5,8,11,14-eicosapentenoyl-CoA from 2,5,8,11,14-eicosapentenoyl-CoA [32] suggesting that Dci1p may act as dienoyl-CoA isomerase. The degradation of oleic acids in *Saccharomyces cerevisiae* was proposed to be similar to oleate β-oxidation in mammals as shown in Scheme 1 [32]. Dienoyl-CoA isomerase is a key enzyme for the isomerization of 3-*trans*,5-*cis* tetradecadienoyl-CoA in the reductase-dependent pathway. Since *Saccharomyces cerevisiae* cells can grow on oleic acid as a sole carbon source, 2-*trans*,5-*cis*-tetradecadienoyl-CoA is formed and might be isomerized to produce 3-*trans*, 5-*cis*-tetradecenoyl-CoA by enoyl-CoA isomerase. Contradictory results have been reported regarding the metabolism of 3-*trans*, 5-*cis*-tetradecadienoyl-CoA in *Saccharomyces cerevisiae* cells. It was observed that during growth of *Saccharomyce cerevisiae* on oleic acid, expression of the *DCI1* gene was induced but this induction was dispensable for the degradation of oleic acid [32]. This finding suggests that all enzymes of the reductase-dependent pathway are present or that this pathway is not operative in *Saccharomyces cerevisiae* cells.

The degradation of oleic acid via the reductase-dependent pathway requires dienoyl-CoA isomerase to act on 3-*trans*, 5-*cis*-tetradecadienoyl-CoA. Even though yeast cells with a deletion in the enoyl-CoA isomerase gene were unable to grow on unsaturated fatty acids [32], yeast cells with a deletion in the *DCI1* gene grew on oleic acid at a rate similar to that of the wild-type strain [34]. This and the observation that yeast cells with a deletion in the *SPS19* gene encoding 2,4-dienoyl-CoA reductase were unable to grow on petroselineate (6-cis-$C_{18:1}$) as the sole carbon source, but grew on oleate [35], support the idea that the reductase-dependent pathway may not be involved in the degradation of unsaturated fatty acids with odd-numbered double bonds.

Perhaps 3-*trans*-5-*cis*-tetradecadienoyl-CoA formed during oleate β-oxidation might undergo other transformations that would facilitate the degradation of oleic acid and support the growth of *Saccharomyces cerevisiae* cells. The observation that *Saccharomyces cerevisiae* cells did not grow on *cis*-9*, trans*-11-octadecadienoyl-CoA (9,11-conjugated linoleic acid, CLA) as the sole carbon source is also consistent with the idea that dienoyl-CoA isomerase activity may not exist or its activity may not be sufficient to metabolize *cis*-9, *trans*-11-octadecadienoic acid in this organism [36]. If

Saccharomyces cerevisiae cells would grow on *cis*-9, *trans*-11-octadecadienoic acid as the sole carbon source, *cis*-3,*trans*-5-dodecadienoyl-CoA should be a major intermediate that ultimately would have to be converted to 2-*trans*,4-*trans*-dodecadienoyl-CoA by the action of dienoyl-CoA isomerase. The resultant 2-*trans*,4-*trans* intermediate would be a substrate for yeast 2,4-dienoyl-CoA reductase, which facilitates its further metabolism. It was shown that during the growth of *Saccharomyces cerevisiae* cells on oleic acid the level of a thioesterase messenger RNA (mRNA) increased compared to yeast cells grown on ethanol [37]. In fact, thioesterase induced during growth on oleate may significantly contribute to the metabolism of acyl-CoA intermediates including 3-*trans*,5-*cis*-tetradecadienoyl-CoA by hydrolyzing the latter to 3-*trans*, 5-*cis*-tetradecadienoic acid, which then may be released from peroxisomes and perhaps from cells into the growth media. The action of a thioesterase may be essential as it may allow the regeneration of free coenzyme A, which is needed for further degradation of oleoyl-CoA to acetyl-CoA. The hydrolysis of 3-*trans*, 5-*cis*-tetradecadienoyl-CoA may also be essential for preventing the inhibition of enzymes that are involved in the β-oxidation of oleic acid. The above observations and considerations justify a re-investigation of the pathway of β-oxidation for unsaturated fatty acids with a focus on thioesterase and the operation of a reductase-dependent pathway involving the *DCI1* gene product in *Saccharomyces cerevisiae* cells growing on oleate (see Scheme 3).

Aim of the thesis project

One goal of my thesis research was to determine if a thioesterase-dependent pathway of β-oxidation is operative and to determine how significant this pathway might be for the metabolism of oleic acid. Another goal was to determine whether the *DCI1* gene product exhibits dienoyl-CoA isomerase activity and if not, what its main function might be.

EXPERIMENTAL PROCEDURES

Materials - CoASH, NADH, acetyl-CoA, butanoyl-CoA, hexanoyl-CoA, octanoyl-CoA, decanoyl-CoA, dodecanoyl-CoA, tetradecanoyl-CoA, palmitoyl-CoA, and oleoyl-CoA were purchased from Life Science Resources. Acyl-CoA oxidase from *Arthrobacter species* was purchased from Sigma. Sep-Pak C_{18} cartridges used for concentrating acyl-CoAs and μBondapak C_{18} columns (30 cm X 3.9 mm) for HPLC were purchased from Waters Associates. Dye reagent for protein assays, polyacrylamide ready gels, and the materials for immunobloting including the goat anti rabit IgG conjugated with alkaline phosphatase were bought from Bio-Rad. Antibodies against maltose binding protein (MBP), all materials for subcloning including restriction enzymes and amylose resin were purchased from New England Biolabs. Rabbit antiserum against yeast Ehd3p was raised by Pocono Rabbit Farms and Laboratory. L-Tryptophan, L-leucine, L-histidine, uracil, adenine, D-(+)-maltose, D-sorbitol and yeast nitrogen base without amino acids were bought from Sigma. Zymolyase-100T from *Arthrobacter luteus* and Tween 40 were obtained from MP Biomedicals, LLC. Oleic acid was bought from Fisher. All primers were purchased from Integrated DNA Technologies, Inc except that oligos for *EHD3* disruption were bought from Sigma Genosys.

Synthesis of Substrates and Metabolites - 2-*trans*-Octenoyl-CoA, 5-*cis*-tetradecenoyl-CoA and 5-*cis*-octenoyl-CoA were synthesized according to the mixed anhydride method as described by Fong and Schulz [38] and purified by HPLC. 3,5-*cis*-Tetradecadienoyl-CoA and 3,5-*cis*-octadienoyl-CoA were synthesized as described [3, 18]. 3-Ketooctanoyl-CoA was synthesized as published [18]. All products were purified by HPLC. The pH values of the acyl-CoA preparations were ajusted to ~3.0-4.0, and the thioester concentrations of these solutions were determined spectrophotometrically by quantification of CoASH with Ellman's reagent [39] after quantitatively cleaving the thioester bond with NH_2OH at pH 7.0 [38].

Plasmids: - pMBP-DCI1 and pMBP-TES1 plasmids were a kind gift from Dr. Stephen J. Gould, The Johns Hopkins University School of Medicine.

In order to express Eci1p and Ehd3p in *E.coli* in the form of MBP-fusion proteins, both *ECI1* and *EHD3* open reading frames (ORFs) were amplified by PCR with *S.cerevisiae* genomic DNA as a template. The complete *EC1I* ORF was amplified by using the following primers

5'- AAAGTCGACAATGTCGCAAGAAATTAGGCAAAATGAG-3' with

5'-TTTGCGGCCGCTCATAAACGATGCTTCCTTTGTTTCGAG-3'

The complete *EHD3* ORF was amplified by using the following primers

5'-AAAGTCGACAATGCTCAGAAATACGCTAAAATGT-3' and

5'-TTTGCGGCCGCTTATTTCCATCTTAAGCCATCGTTAAC-3'. The PCR products from each reaction were then digested with *Sal*I and *Not*I (*Sal*I and *Not*I cleavage sites in the primers are underlined) and cloned into the *Sal*I and *Not*I sites of pMBP-TES1. The resulting plasmids were named pMBP-ECI and pMBP-EHD3, respectively. In order to express His6-tagged Ehd3p, the following pair of oligonucleotides was used to amplify the *EHD3* ORF:

5'-AAAGTCGACAAATGCTCAGAAATACGCTAAAATGT-3' and

5'-TTTGCGGCCGCATTTCCATCTTAAGCCATCGTTAAC-3'. The amplified PCR product was digested with *Sal*I and *Not*I (the cleavage sites in primers are underlined), cloned into the *Sal*I and *Not*I sites of pET-28a (Novagen) and the resultant plasmid was named pET-EHD3.

The nine-amino-acid epitope of influenza virus hemagglutinin (HA) (YPYDVPDYA) was used for C-terminal tagging of the *EHD3* gene product. To create this HA-tagged Ehd3 protein, the pair of oligonucleotides

5'-GGCCCAAGCTTCTCAGAAATACGCTAAAATGTGCCC-3' and

5'-CCACTAGTTTACTAGCGGCCGCCTTTCCATCTTAAGCCATCGTTAAC-3' was designed. The forward primer contains an *Hind*III site, while the reverse oligonucleotide contains a *Not*I site upstream of the stop codon of *EHD3* ORF followed by an additional stop codon and a *Spe*I site (restriction sites are underlined). The product amplified by PCR with this pair of primers and yeast total DNA as a template was digested with both *Hind*III and *Spe*I and cloned into the *Hind*III and *Spe*I sites of pCA21a (Expression Technologies Inc.). The resulting plasmid was named pCA-EHD3. This plasmid was subsequently digested with *Not*I and a triple HA epitope cassette with flanking *Not*I site [40] was then cloned into pCA-EHD3, generating a plasmid named pCA-EHD3-HA.

p6xHis-DCI1 was created as follows. First, a DNA fragment containing the *DCI1* open reading frame was amplified from *S. cerevisiae* genomic DNA using primers

5'-CGCGGATCCAGCAGTCGTGTGTGCTACCATATTAATGG-3' and

5'-GCCGGTACCTTATAACTTGTGGCGCCTGTTTC-3'. This PCR product was then digested with *Bam*HI and *Kpn*I (restriction sites underlined) and cloned into the corresponding restriction sites of pQE-80L to yield a plasmid named p6xHis-DCI1.

PRS306hDCI1POX1 and PRS316hDCI1POX1 were obtained from Dr. Small, City College of New York (CCNY/CUNY).

Gene Disruption - The PCR-based gene targeting approach was used to disrupt the *S.cerevisiae EHD3* gene [42]. For this purpose, the forward primer (5'-CGCCTGTGTACGGGAATCCTCATTTATATAATACTTGGTTCTGTTTAGCCACCAC AAATACGGATCCCCGGGTTAATTAA-3') was designed to contain a region of homology to the 5' end of the *EHD3* gene, while the reverse primer (5'-TTACATGCATAGAAGTGTTGGCAAAATTCTTTACGAGCCACGGGGCTGCTATTTC AGGAATTCGAGCTCGTTTAAAC-3') encoded the 3' non-translated region of the same locus. A DNA fragment amplified by PCR with the pF6a-13Myc-TRP1 template was introduced into W3031A and *dci1Δ*, and the genotype of the resulting TRP+ transformants was verified by diagnostic PCR.

Strains, Media, and Growth Conditions: - Escherichia coli strains DH10B [44] containing the pMBP-TES1 plasmid and BL 21 (DE3) containing plasmid pMBP-DCI, pMBP-ECI, pMBP-EHD3, pET-EHD3, p6HIS-DCI or pCA-EHD3 were used for all bacterial manipulations.

The yeast strains used in the current study are listed in Table 1. pRShDCI1POX1 was used to create yeast transformants.

Bacteria cells were allowed to grow overnight at 37°C in 50 ml LB supplemented with ampicillin, kanamycin, chloramphenicol or the combination of ampicillin/kanamycin or ampicillin/chloramphenicol as required. 40 ml of cultures were diluted into 1 liter of 2YT [45] media supplemented with 0.2% glucose and 100 µg/ml antibiotic. These cultures were grown at 37°C until the A_{600} reached 0.4, at which time isopropyl-thio-β-D-galactopyranoside (IPTG) was added to 0.3 mM to induce protein expression. The induced cultures were incubated at 37°C for 4 hours, 30°C for 16 hours or 18°C for 20 hours. After these periods, cells were harvested at 5000 X g for 10 minutes at 4°C. Cell pellets were washed with 2YT media and stored at –80°C.

Yeast strains were grown overnight at 30°C with agitation in 10 ml YPD (1% yeast extract, 2% peptone, 2% dextrose). The next day, cells were inoculated in fresh YPD solution at 1:10 dilution and allowed to grow at 30°C with agitation for 6 hours. Cells were spun down and resuspended in 1 liter YPGO (1% yeast extract, 2% peptone, 3% glycerol, 0.2% Tween 40, 0.125% oleic acid) or minimum media containing 0.2% yeast extract, 0.67% yeast nitrogen base without amino acid, 0.2% oleic acid, 0.05% Tween 40 and auxotrophic supplements added to 20 μg/ml (40 μg/ml in the case of leucine). Cells were allowed to grow 18 hours (YPGO) or 96 hours (minimum media) at 30°C with vigorous aeration after which time they were harvested by centrifugation at 2000 x g for 10 minutes at 4°C. Cell pellets were washed twice with YPGO or minimum media and stored at –80°C.

Growth Assessment on Plates and in Liquid - Solid media were made of a mixture containing 2% bacto agar, 0.67% yeast nitrogen base w/o amino acids, 0.033% casaminoacid, auxotrophic supplements at a final concentration of 40 μg/ml, 0.2% of Tween 40, and 0.125% of oleic acid (W3031A Yeast strains), or 0.1%Tween 40 and 0.2% of oleic acid (BY4733 Yeast strains). Each component was made separately and autoclaved. 20-25ml were poured on to each petri dish plate and allowed to solidify at room temperature. Single colonies of each yeast strain were inoculated in 5ml YPD and grown overnight with agitation at 30°C. 200μl of each culture were transferred to an Eppendorf tube followed by centrifugation at 14,000 rpm for 10 minutes. Supernatants were discarded. Cell pellets were resuspended in 100µl of deionized water, followed by serial dilutions of 10, 100 and 1000 folds. Cells were allowed to grow on plates containing oleic acid as the sole carbon source at 30°C for 72 hours.

Growth curves in liquid were performed to evaluate and compare the growth of wild-type and mutants. Each yeast strain was inoculated in 5ml YPD followed by overnight growth at 30°C with agitation. Cells were harvested by centrifugation and resuspended in selective media containing 0.2% yeast extract, 0.67% yeast nitrogen base w/o amino acids, 0.1% or 0.2% of Tween 40 as needed, 0.125% or 0.2% of oleic acid as required, supplemented with amino acids and nucleotides appropriate for each yeast strain. Cells were allowed to grow overnight, then washed with water and resuspended in 50 ml selective media. Growth was assessed by removing 1ml aliquots, followed by centrifugation, washing cells with water, and resuspending cells in water before measuring the absorbance at 600nm. Aliquots were taken at 24 hours intervals for 120-144 hours. From the absorbance of each aliquot for each 24 hours was substracted the absorbance measured on the first day corresponding to time 0. A light microscope was used to evaluate the level of growth including contamination of the culture by other organisms such as bateria.

Preparation of Cell Extracts and Protein Purification - Six g of *E.coli* cell paste were resuspended in 12 ml of amylose column binding buffer (20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 1 mM EDTA, and 10 mM 2-mercaptoethanol) or in lysis buffer (pH 8.0) containing 50 mM NaH_2PO_4, 300 mM NaCL, 10 mM imidazole. All cell suspensions were sonicated 6-times for 20 seconds each at intervals of 3 minutes to keep the temperature of the suspensions at 4°C. The soluble extracts were separated from debris by centrifugation at

25,000 x g for 1 hour. The debris was resuspended in the lysis buffer containing 0.5% of TritonX-100, homogenenized and placed on ice for 1-2 hours. The soluble extracts were separated from the remaining debris by centrifugation at 25,000 x g for 30 minutes.

After centrifugation, MBP-DCI1, MBP-TES1 and MBP-EHD3 proteins were purified by one-step affinity chromatography. The soluble extracts containing MBP-DCI1, MBP-TES1 or MBP-EHD3 proteins were diluted to 50 ml with amylose column binding buffer and loaded onto a 20 ml amylose agarose column (New England Biolabs Inc.) at a rate of 1ml/min. The resin was washed with 10 column volumes of binding buffer, and bound proteins were eluted with 1 column volume of elution buffer (column binding buffer with 10 mM maltose). Following SDS-polyacrylamide gel analysis of non-induced homogenate, induced homogenate, soluble extract, insoluble fraction, and remaining pellet after extraction with Triton X-100, Western blotting was performed using an antibody to MBP to detect and determine the level of either MBP-DCI1 or MBP-TES1 induced proteins. Cells containing Ehd3p were resuspended in 20-ml of Ni-NTA resin buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). The suspension was sonicated 15 times for 20 seconds each at 4^oC. A clear soluble extract was generated by centrifugation at 17,000 X g for 1 hour. Proteins were purified by one-step affinity chromatography using a 1m-ml Ni-NTA (QIAGEN) column according to the manufacturer's instruction as follows: 1ml of the 50% Ni-NTA was added to 4 ml cleared lysate and mixed gently by shaking (200 rpm on a rotatory shaker) at 4^oC for 60 minutes. The lysate-Ni-NTA mixture was loaded onto a column with the bottom outlet capped. The bottom cap was then removed and the flow-through was collected. The column was washed twice with 4 ml wash buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH: 8.0, after which proteins were eluted 4 times with 0.5 ml elution buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0 and analyzed by SDS-PAGE. Purified proteins were concentrated and stored at –80°C.

Yeast cell pellets were resuspended in 4ml/g breaking buffer containg 0.6M sorbitol, 5mM MES, 1mM KCL, 0.5 mM EDTA pH 6.0, 1mM PMSF, 1mM benzamidine and homogenized by vortexing with glass beads followed by brief high intensity sonication. A soluble extract was generated by centrifugation at 20,000 x g for 1 hour at 4°C, followed by ultracentrifugation at 109,000 x g for 1 hour at 4°C generating a cleared lysate.

Total RNA purification - This experiment was performed to evaluate the level of thioesterase mRNA in cells grown on oleate as compared to cells grown on glucose or glycerol. The yeast wild-type strain was precultured in YPD to mid-logarithmic phase. Precultures were then used to inoculate YP media containing glucose (38 μl of a cell suspension were resuspended in glucose media), YPG media only or YPG media containing oleic acid as sole carbon source. Yeast cells were collected by centrifugation at 5,000 x g for 5 minutes. Cells were again transferred to 50 ml Sarstedt conical centrifuge tubes followed by a second centrifugation to remove the remaining media. Cells were resuspended in 7.5 ml of preheated extraction buffer (70°C in water bath) containing 50 mM sodium acetate; 10 mM EDTA adjusted to pH 5.0 with acetic acid and, 0.75 ml of 10% SDS were combined, followed by vortexing for about 10 seconds. Nine ml of preheated phenol (70°C in water bath) was added to the mixture. The mixture was placed in a water bath at 70°C and each tube was vortexed 10 times for 10 seconds each, followed by incubation on ice for 10 minutes. The aqueous phase was obtained by centrifugation at 5,000 x g for 10 minutes.

This aqueous phase was transferred to a fresh tube. An equal volume of chloroform: isoamyl alcohol 1:1 was added to the aqueous solution and vortexed for 10 seconds, followed by centrifugation at 5,000 x g for 10 minutes. The aqueous phase was transferred to a fresh tube. One tenth of the volume of 3M sodium acetate and 3 times volumes of 100% ethanol were added to the solution and mixed by inversion. The RNA suspension was incubated at –20°C for 30 minutes, and then warmed up at room temperature for 20 minutes, followed by centrifugation at 5,000 x g for 10 minutes. The ethanol phase was discarded and the remainder was resuspended in 0.5-1 ml of 0.1% diethyl pyrocarbonate (DEPC) in distilled water. The concentration of total RNA was determined by measuring the absorbance at 260 nm.

Oligotex^{TM} Poly(A)$^{+}$ mRNA Purification and Northern Blot Analysis - Nine hundred and twenty µl of total RNA mixed with DEPC-treated water were combined with 200 µl of binding buffer containing 60mM Tris-HCl pH 7.5, 3M NaCl, 6mM EDTA with the addition of 80 µl Oligotex (heat to 37°C and mixed before use). The Eppendorf tube containing the solution was mixed and incubated at 65°C for 10 minutes, followed by incubation at room temperature for 20 minutes. The supernatant was separated from the mixture of Oligotex and mRNA pellet by centrifugation at 14,000 rpm for 2 minutes. The supernatant was removed by aspiration. One ml of wash buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 1 mM EDTA was added to the mixture of Oligotex and mRNA, which was resuspended by vortexing, followed by centrifugation at 14,000 rpm for 3 minutes. The supernatant was discarded. The washing process was repeated for a second time as described above. The mRNA was eluted from Oligotex by adding 1 ml preheated (70-75°C) elution buffer containing 5 mM Tris-HCl (pH 7.5). The mRNA solution was resuspended by vortexing and incubation at 70-75°C for 5 minutes, followed by centrifugation at 14,000 rpm for 3 minutes. The mRNA solution was transferred to another RNAse-free tube. Fifteen µg of mRNA were mixed with 15 µl of loading buffer containing 0.025% (w/v) bromophenol blue, 0.025% (w/v) xylene cyanol, 5 mM EDTA pH 8.0, 0.025% (w/v) SDS, and 95% (v/v) dieonized formamide, heated at 65°C for 15 minutes and loaded on a 1.2% agarose gel containing 2ml formaldehyde, 10ml of 10 times MOPS (0.2M MOPS pH 7.0, 20mM sodium acetate 10 mM EDTA pH 8.0). The gel was allowed to run for about 3 hours, using 1 time MOPS solution as a running buffer at 0.77 V and 0.52 mA. Once the running time was completed, the mRNA gel was resuspended in 500 ml wash solution containing 1.5M NaCl, 0.5M NaOH with gentle shaking for 30 minutes. The gel was rinsed two times (15 minutes each) in deionized water to removed the previous solution and formaldehyde, followed by resuspension in neutralization buffer containing 1M Tris pH 8.0, 1.5M NaCl with gentle shaking for 30min to 1 hour. The mRNA was transferred to a Nylon membrane using 10 times SSC buffer (1.5M NaCl, 0.15M NaCitrate pH: 7.0) as a transfer buffer. The membrane was incubated in crosslinker (UV Crosslinker spectroline, Select™ series) for 20 seconds and introduced in the hybridization bag. The membrane was allowed to pre-hybridize by introducing 4ml of hybridization mixture (6X SSC, 5X Denhardt's reagent, 0.5% (w/v) SDS, 1µg/ml poly(A), 100 µg/ml salmon sperm DNA) followed by incubation at 65°C for 30 minutes.

For Northern blot analysis, the probe labeling was performed according to the Prime-IT® RmT Random Primer Labeling Kit (Single-Use dCTP-Labeling Reactions, STRATAGENE). A solution containing 50 ng of TES1 DNA fragment was mixed with 42 µl of deionized water and boiled for 5 minutes, followed by the addition of 2.5 µl of ^{32}dCTP (6000 Ci/mmol) and 3 µl of Magenta DNA polymerase followed by incubation at 37°C for 1 hour. The solution mixture was briefly centrifuged prior to the addition of 75 µl of phenol and 50 µl of deionized water and centrifugation at 13,000 x rpm for 10 minutes. The aqueous phase was transferred to a new Eppendorf tube to count the radioactivity. Ten µl of 3M sodium acetate (pH 5.9), 2 µl of tRNA (10mg/ml) and 300 µl of ice cold ethanol were added to the remaining pellet followed by centrifugation at 13,000 rpm for 10 minutes. The supernatant was transferred to a new Eppendorf tube to count the radioactivity. The pellet (probe) was resupended in 100 µl of deionized water, boiled for 5 minutes and cooled on ice for 10-20 minutes. The probe was added to 7 ml of hybridization solution, introduced in the bag containing the pre-hybridized membrane. The membrane was sealed and incubated at 65°C for 16 hours. Quantification of signal intensity was performed using Image Quant V1.11 software.

Enzyme and Protein Assays: - For the thioesterase assay, the mixture contained

100 mM KPi, pH 8.0, 10 mM 5,5'-dithiobis(2-nitrobenzoic acid), 30 µM substrate such as; acetyl-CoA, butanoyl-CoA*,* *2*-trans-butenoyl-CoA*,* hexanoyl-CoA, octanoyl-CoA*, 2-trans*-octenenoyl-CoA*, 3-trans-5-cis*-octadienoyl-CoA*,* decanoyl-CoA*, 2-trans*-4-*trans*-decadienoyl-CoA*,* dodecanoyl-CoA*, 3-trans-5-cis*-dodecadienoyl-CoA*,* tetradecanoyl-CoA*, 2-trans*-tetradecenoyl-CoA*, 2-trans-5-cis*-tetradecadienoyl-CoA*, 5-cis*-tetradecenoyl-CoA*, 3-trans-5-cis*-tetradecadienoyl-CoA*, 3-hydroxy-5-cis*-tetradecenoyl-CoA*, 3-trans*-tetradecenoyl-CoA*,* hexadecanoyl-CoA*, 2-trans*-hexadecenoyl-CoA*, 3*-ketohexadecanoyl-CoA*,* octadecanoyl-CoA*,* 9-*cis*-octadecenoyl-CoA. The assays were monitored at 412 nm for

2 minutes. Increases of absorbance were recorded spectrophotometrically at 412 nm [22] with soluble extracts containg thioesterase or 0.67 μg of purified MBP-TES1. An extinction coefficient of 13600 M^{-1} cm^{-1} was used to calculate activities.

Dienoyl-CoA isomerase was assayed spectrophotometrically by measuring the increase in absorbance at 300 nm as described [3, 18]. An extinction coefficient of 28000 M^{-1} cm^{-1} was used to calculate the actvity. Enoyl-CoA hydratase was assayed by following the decrease in absorbance at 263 nm due to the hydration of 2-*trans* double bond of 2-*trans*-octenoyl-CoA [38]. L-3-Hydroxyacyl-CoA dehydrogenase was assayed by measuring the decrease in absorbance at 340 nm due to the dehydrogenation of NADH in the presence of 3-ketooctanoyl-CoA and enzyme [47]. Δ^3-*cis*-Δ^2-*trans*-Enoyl-CoA isomerase was measured by a couple assay based on the increase in absorbances at 340 nm due to the formation of NADH [47]. 3-Ketoacyl-CoA thiolase was assayed by measuring the decrease in absorbance at 303 nm due to the disappearance of the Mg^{2+} - enolate complex of 3-ketooctanoyl-CoA [18]. Extinction coefficients of 6700, 6220 and 6700 M^{-1} cm^{-1} were used to calculate the activities of the hydratase, dehydrogenase, and thiolase, respectively. Catalase assays were performed as described [48]; the ML fraction containing peroxisomes, mitochondria and microsome was sonicated 10 times for 20 seconds each at 4°C to release the soluble enzymes. A clear soluble extract was generated by centrifugation at 20,000 X g for 1 hour. One μl of this soluble extract was incubated in a mixture containing 10 mM imidazole-HCl buffer, pH 7.2, and 1.5 mM hydrogen peroxide; followed by incubation on ice for 15 minutes. The reaction was stopped upon addition of 300 μl of a threefold dilution of a saturated solution of titanium sulphate in 2N H_2SO_4, and the remaining hydrogen peroxide was determine colorimetrically in this mixture as the yellow peroxy titanium sulphate by measuring the absorbance at 410 nm.

One unit of activity is defined as the amount of catalase causing the destruction of 1.5 mM hydrogen peroxide in one minute.

Isolation of Fatty Acids from Yeast Growth Media - Yeast strains were precultured in YPD (1% yeast extract, 2% Bacto peptone, 2% dextrose and 100 μg/ml of ampicilline) medium for 18 hours followed by 5-fold resuspension in YPD for 6 hours to regenerate the cells. Yeast cells were collected by centrifugation at 3,000 x g for 10 minutes, washed twice with water, then resuspended in minimum selective medium containing 0.67% yeast base w/o amino acids, 0.2% yeast extract, 0.2% Tween 40, 0.125-0.2% oleic acid or palmitic acid, 20 μg of amino acid supplements (L-tryptophane, L-histidine, adenine, uracile), 40 μg of L-leucine, and 100 μg/ml of ampicilline. Yeast cells were allowed to grow for 72 hours to reach an absorbance of about 1. Fatty acids were isolated and analyzed as described [22]. 500 ml of supernatant were collected by centrifugation at 2,300 x g for 30 minutes, followed by acidification (pH 1.0-2.0) with 2N H_2SO_4. Fatty acids were extracted four times with 100 ml each of ether. The organic phase was extracted with acqueous sodium bicarbonate. After acidifying the acqueous phase with 2N H_2SO_4, fatty acids were extracted four times with 8 ml of ether each. The combined ether extracts were dried over anhydrous sodium sulfate. After removal of sulfate by filtration, and ether by evaporation, the residual material was methylated by reacting it with 2 ml of BCl_3-methanol (12%, w/w) for 10 minutes at 60°C to 70°C. The reaction mixture was cooled down on ice. 1 ml of H_2O and 1 ml of hexane were added. The organic layer was removed and dried over anhydrous sodium sulfate. After removal of sodium sulfate by filtration and ether by evaporation, the residual material containing methyl esters derived from fatty acids released into the growth medium by yeast cells was dissolved in 50 μl of ethanol. Aliquots of 1 μl of the fatty acid methyl esters were injected at 250°C into a GC/MS instrument (Shimadzu Scientific Instrument) consisting of gas chromatograph (model GC-17A) interphased with with a mass spectrometer (QP-5000) and equipped with a capillary column (30m; inner diameter 0.25 mm; film thickness, 0.25 μm; EC-5; Alltech Associates Inc., Deerfield, IL). The oven temperature was raised from 100 to 230°C at 5°C/min, to 300°C at 20°C/min, and then held constant for 6 min. The mass spectrophotometer served as a detector and was operated at 280°C.

Subcellular Fractionation - Wild type yeast cells and *dci1Δ* cells were grown for 24 h in 5ml YPD. The next day cells were resuspended into fresh YPD at 1:10 dilution and grown at 30°C with agitation for 6 hours. Cell pellets obtained by centrifugation at 3000 x g for 20 minutes were resuspended into induction medium YPOLT (1% yeast extract, 2% bacto-peptone, 0.05% Tween 40, and 0.2% oleic acid) at 1:10 dilution. After growing in YPOLT at 30°C for 72 hours, cells were harvested, washed once with water, and resuspended in 4 ml/g cells (wet weight) of spheroplasting buffer (50 KPi, pH 7.5, 0.5 M KCl, 10 mM Na_2SO_3, 10 mM β-mercaptoethanol, and 1mg of Zymolyase 100T /g cell wet weight. Cell suspensions were incubated at 30°C for 15-20 minutes; the resulting spheroplasts were harvested, washed twice with sorbitol buffer (1.2 M sorbitol, 20 mM KPi. PH 7.4) and resuspended in 10 ml of breaking buffer (0.6 M sorbitol, 5 mM MES, pH 6.0, 1mM KCl, 0.5 mM EDTA, 1 mM PMSF, 1mM benzamidine chloride). The spheroplasts were homogenized by 10 passes in a tight-fitting Potter-Elvehjem homogenizer and then centrifuged at 2, 000 x g for 5 minutes to remove nuclei and cell debris. The supernatant was collected, and the pellet was resuspended in breaking buffer, homogenized and centrifuged a second time. The combine supernatants were centrifuged of 18,000 x g for 30 minutes to produce cytosolic soluble proteins (PS) and an organelle fraction (ML) containing microsomes, peroxisomes, and mitochondria.

Amino Acid Sequence Alignment Analysis - The amino acid sequence of either human dienoyl-CoA isomerase or rat dienoyl-CoA isomerase was blasted with the yeast genome database to identity protein candidates that not only show the highest percentage of identity in an overlap region, but also fit a range of amino acids including Glu^{196} and Asp^{204} of rat dienoyl-CoA isomerase that are responsible for catalyzing the proton transfer at carbon atoms 2 and 6.

SDS-PAGE and Immunoblotting – Samples were treated with equal volume of SDS sample buffer and subjected to electrophoresis on either gradient gels (10%) or 10% ready gels [49]. To check the expression of the yeast *DCI1* gene product, thioesterase, enoyl-CoA isomerase or Ehd3p, 50 μl of each fraction were suspended in 50 μl of SDS sample buffer and boiled for 3-5 minutes. Twenty microliter were taken for SDS-PAGE analysis. For immunoblotting, proteins were transferred to a nitrocellulose membrane by semi-dry blotting [50] using the semi-dry transfer cell from Biorad. Proteins remaining on the gel were visualized by staining with Coomassie blue. The membrane was blocked with 5% non fat dry milk for one hour before incubating it with either 300-fold diluted rabbit antiserum or 10000-fold diluted monoclonal murine anti-Maltose Binding Protein (MBP) for one hour.

After incubating the membrane with goat anti-rabbit IgG conjugated with alkaline phosphatase for one hour, it was developed with a staining mixture containing the alkaline phosphatase substrate until the antigen bands were visualized [51].

HPLC Analysis and Purification of Acyl-CoA Thioesters – Acyl-CoA thioesters were purified, and metabolites were analyzed by reverse – phase HPLC on a Water μBondapak C_{18} column (30 cm X 3.9 mm) attached to a Waters gradient HPLC system. The absorbance of the eluate was monitored at 254 nm. Separation of substrates and metabolites was achieved by linearly increasing the acetonitrile/H_2O (9:1 v/v) content of the 50 mM ammonium phosphate elution buffer (pH 5.5) from 20% to 80% in 20 minutes at a flow rate of 2ml/min. All samples were cleared of particulate matter by passing them through a 0.22-μm pore size membrane before they were injected into HPLC system. Diluted samples were concentrated by passing them through Sep-Pak C_{18} cartridges and eluting them with small amounts of methanol, which subsequently were removed by evaporation under reduced pressure.

RESULTS

Evidence for the existence of a thioesterase-dependent pathway of β-oxidation in *S. cerevisiae*, which facilitates growth on oleate as the sole carbon source.

Yeast peroxisomal thioesterase is repressed when cells are grown in media containing either glucose or ethanol, and is induced by growth on oleate [37]. Growth impairement of a mutant with a deletion in the thioesterase gene promted the suggestion that thioesterase may play an ancillary role in growth on fatty acids [37]. Kinetic data of thioesterase obtained with short straight-chain and branched chain acyl-CoA esters contributed to the idea that this thioesterase is required for the efficient degradation of short straight-chain and branched chain fatty acids [54].

In this part, I will describe the studies I carried out to assess the importance of a thioesterase-dependent pathway of β-oxidation, which involves thioesterase in the metabolism of oleate by *Saccharomyces cerevisiae.*

Comparison of growth of wild-type *S. cerevisiae* and mutants on oleate

The growth of *Saccharomyces cerevisiae* on fatty acids as the carbon source was studies. Cells were viewed under a microscope to detect whether each culture grew well and without contamination by other organisms such as bacteria (see Fig.1).

I confirmed the observation made by Gould et al. [37] that *tes1Δ* and *tes1ΔdciΔ* cells grew poorly on oleate compared to the wild type strain (see Fig.2). Even though the patterns of growth were similar to those reported in the previous report, the growth defect observed here was less prononced than previously reported [37]. Perhaps, the use of different yeast strains may explain these relatively small growth differences. The slower growth of the *tes1Δ* cells and growth to a lower density suggests that in *Saccharomyces cerevisiae* thioesterase is required during β-oxidation of oleic acid. In contrast, when grown on palmitic acid, wild type, *dciΔ* cells, and *tes1Δ* cells showed indistinguishable growth behavior (see Fig.3). This observation supports the idea that thioesterase is most likely not required when palmitic acid serves as a carbon source.

Expression of peroxisomal thioesterase in yeast grown on different media

To investigate whether thioesterase is induced when yeast cells are grown on fatty acids as the sole carbon source, the relative abundance of its mRNA was determined in cells grown on glucose, glycerol or oleate. Poly(A)$^+$ RNAs extracted from log-phase cells were analyzed by Northern blotting using the *TES1* gene as a probe (see Fig.4A and B). The *TES1* mRNA was about 100-fold more abundant in the sample extracted from oleate-grown cells, than in cells grown on either glucose or glycerol. Inductions somilar to that observed with oleate were observed when cells were grown on palmitate or myristate (data not shown). In contrast, the *ACT1*/YFL039c gene is constituvely transcribed, and hence its mRNA level changed little when cells were grown in different media (see Fig.4A). This experiment confirms that growth of yeast on fatty acids causes the induction of thioesterase mRNA expression. When the thioesterase activity was measured in soluble extracts (see Fig.5) of wild-type yeast grown on oleate, glycerol or glucose, it was found to be about 2 to 4 times higher in oleate grown cells than in cells grown on glycerol or glucose. These observations agree with the idea that yeast thioesterase is an auxiliary enzyme required for β-oxidation of fatty acids and perhaps is most important for the β-oxidation of unsaturated fatty acid with odd-numbered double bonds. During the degradation of oleic acid, acyl-CoA intermediates are formed that require the involvement of auxiliary enzymes such as enoyl-CoA isomerase, dienoyl-CoA isomerase, dienoyl-CoA reductase. Such auxiliary enzymes are not required for the β-oxidation of saturated fatty acid such as palmitic acid, myristic acid or stearic acid, because these auxiliary enzymes are only required to act on pre-existing double bond present in unsaturated fatty acids.

In vivo evidence for a thioesterase-dependent pathway of β-oxidation during growth of *S. cerevisiae* on oleate.

The absence of a phenotype of *dciΔ* cells growing on oleic acid as the sole carbon source indicates that dienoyl-CoA isomerase, which catalyzes the isomerization of 3-*trans*-5-*cis*-tetradecadienoyl-CoA to 2-*trans*-4-*trans*-tetradecadienoyl-CoA, might not be required for the β-oxidation of oleate and may be absent from yeast peroxisomes or present at a low level. Such situation could lead to an accumulation of 3-*trans*-5-*cis*-tetradecadienoyl-CoA that might inhibit the flux of metabolites through the isomerase- dependent pathway. If a thioesterase-dependent pathway exists, the main role of thioesterase would be to hydrolyze intermediates that otherwise may accumulate, e.g. 3-*trans*-5-*cis*-tetradecadienoyl-CoA, which would be converted to 3-*trans*-5-*cis*-tetradecadienoic acid and excreted into the growth medium. Such disposition of an intermediate might increase the flux of metabolites during oleate β-oxidation in yeast peroxisomes. If correct, 3-*trans*-5-*cis*-tetradecadienoic acid would accumulate in the medium of oleate grown cells. As suspected, 3-*trans*-5-*cis*-tetradecadienoic acid (see Fig.6) was detected in the medium of yeast cells after 72 hours of growth to mid-log phase on oleate. 3-*trans*-5-*cis*-Tetradecadienoic acid was not detected in the medium of the *tesΔ* mutant or in wild type cells grown on palmitate. This observation demonstrates that yeast thioesterase is responsible for the hydrolysis of acyl-CoAs, specifically 3-*trans*-5-*cis*-tetradecadienoyl-CoA and perhaps other acyl-CoAs.

Attempts to correct the impaired growth of the *S.cerevisiae TES1* mutant on oleate

In an attempt to overcome the growth defect observed when *tes1Δ* cells were grown on oleate, these cells were transformed with a plasmid that carried an insert encoding human dienoyl-CoA isomerase gene. As shown in Fig. 7, the transformation resulted in a correction of the growth defect of *tes1Δ* cells. Cells transformed with a plasmid expressing human dienoyl-CoA isomerase grew at the same rate as wild-type cells but to a slightly lower density. This experiment demonstrated that dienoyl-CoA isomerase can functionally substitute for thioesterase to achieve optimal growth on oleate.

Substrate specificity of purified yeast thioesterase

E.coli strain DH10B containing a plasmid with an insert encoding the maltose binding protein thioesterase fusion protein (MBP-TES1) was grown to a density of 0.4 absorbance unit at 600 nm and then induced by treatment with isopropyl-1-thio-β-D-galactopyranoside (IPTG). Soluble extracts prepared from these *E.coli* cells before and after induction were assayed for thioesterase activity. MBP-TES1 with a molecular mass of about 83 kDa was detected on a SDS-PAGE gel as indicated by the arrow in Fig. 8A. This assignment was confirmed by Western blotting as indicated by the arrow at the lower panel (see Fig. 8B). The purified MBP-TES1 was used to determine substrate specificity. The enzyme has a preference for medium-chain acyl-CoAs but exhibits a lower activity with octanoyl-CoA than either with hexanoyl-CoA or decanoyl-CoA. It is more active with 3,5-tetradecadienoyl-CoA than with tetradecanoyl-CoA and thus would be well suited to hydrolyze the oleate metabolites including 3,5-tetradecadienoyl-CoA. Thioesterase is less active with fatty acyl-CoA intermediates that contain *trans* double bond at position 2, perhaps due to the conjugation of 2-*trans* double bond with the double bond of the carbonyl group.

The *DCI1* gene product is not a dienoyl-CoA isomerase but affects intracellular location of some β-oxidation enzymes

This part of the thesis addresses the question of whether the *DCI1* gene product is a dienoyl-CoA isomerase that is involved in a reductase-dependent pathway of β-oxidation of unsaturated fatty acids in *Saccharomyces cerevisiae* cells. During the β-oxidation of oleic acid, 2-*trans*, 5-*cis*-tetradecadienoyl-CoA is formed, which is isomerized by enoyl-CoA isomerase to 3-*trans*, 5-*cis*-tetradecadienoyl-CoA. The latter compound would be a substrate for dienoyl-CoA isomerase in the reductase dependent pathway of β-oxidation. In higher eukaryotic organisms such as mammals, dienoyl-CoA isomerase was identified as a single hexameric protein located in both mitochondria and peroxisomes [59,60,61]. In *Saccharomyces cerevisiae*, the product of the *DCI1* gene was proposed to be a dienoyl-CoA isomerase [32]. However, the observation that a deletion in the *DCI1* gene does not affect growth on oleate does not seem to agree with the proposed function of *DCI1* gene product as a dienoyl-CoA isomerase. In this part of my thesis, I will describe experiments I carried out to investigate the function of the *DCI1* gene product.

Dienoyl-CoA isomerase activity is not associated with the *DCI1* gene product

Dienoyl-CoA isomerase activity was measured in the soluble extracts of both wild-type yeast cells and mutant cells with a deletion in the *DCI1* gene. Surprisingly, dienoyl-CoA isomerase activity was present at a low level in the soluble extract of the yeast mutant with a deletion in *DCI1* gene. However, the dienoyl-CoA isomerase activity in the mutant was 3-fold higher than the activity in wild-type cells (see Table 3 and Figures 11B-C, 12B-C). In an effort to determine whether the *DCI1* gene product has dienoyl-CoA isomerase activity, the fusion protein of the *DCI1* gene product and maltose binding protein (pMBP-DCI1) was expressed in *E.coli*. The MBP-DCI1 protein was not soluble when cells were grown at 37^oC. However, when the temperature was decreased to 18^oC, the MBP-DCI1 protein was partially soluble as shown by SDS-polyacrylamide gel electrophoresis and Western blotting using an anti- MBP antibody (Figure 13A, B). The MBP-DCI1 protein was purified by amylose binding column chromatography. This protein was assayed for dienoyl-CoA isomerase activity with 3-*trans*, 5-*cis*-octadienoyl-CoA as substrate by measuring the increase in absorbance at 300 nm. But no dienoyl-CoA isomerase activity was detected. This observation together with the fact that the *dci1Δ* cells contained a low dienoyl-CoA isomerase activity, which however was higher than the activity observed in wild-type cells, prompted the conclusion that dienoyl-CoA isomerase activity in *Saccharomyces cerevisiae* is not associated with the *DCI1* gene product. Therefore it is questionable that oleate is degraded by a reductase-dependent pathway of β-oxidation in *Saccharomyces cerevisiae*.

Some β-oxidation enzyme activities are upregulated and mislocalised to the cytosolic compartment in *dci1Δ* cells grown on oleate

The observation that the dienoyl-CoA isomerase activity in soluble extracts from *dci1Δ* cells was 3-fold higher than the activity in wild-type yeast (see Table 3) raised the question as to whether other β-oxidation enzyme activities also are upregulated. To answer this question, the activities of enoyl-CoA hydratase, L-3-dehydroxyacyl-CoA dehydrogenase, enoyl-CoA isomerase, dienoyl-CoA isomerase and enoyl-CoA thiolase activities were determined in subcellular fractions of both wild type and *dci1Δ* cells. For this purpose, spheroplasts obtained after digestion of the cell wall with zymolyase 100T were homogenized with 10 strokes of a Potter-Elvehjem homogenizer, followed by centrifugation at 2,000 x g for five minutes to generate a post nuclear supernatant (PNS). All post-nuclear supernatants were combined and centrifuged at 18,000 for 30 minutes to generate a supernatant (PS) and a pellet containing peroxisomes, mitochondria and microsomes (ML). The activities of dienoyl-CoA isomerase and other β-oxidation enzymes were determined in the PS fraction representing the cytosolic compartment, and in the ML fraction containing mostly peroxisomes and mitochondria. Catalase was assayed as a marker enzyme for peroxisomes in both PS and ML lysates (Table 4). It is known that β-oxidation in yeast occurs only in peroxisomes. Data in Table 4 show that the catalase activity was higher in the ML fraction than the PS fraction, indicating the presence of peroxisomes in the former fraction. A low level of dienoyl-CoA isomerase activity was detected (Table 4) in the ML fraction of wild-type yeast, whereas no dienoyl-CoA isomerase activity was detected in the cytosolic (PS) fraction of wild-type yeast. Thus, dienoyl-CoA isomerase activity is located either in peroxisomes or in mitochondria. In contrast, a relatively large fraction of the dienoyl-CoA isomerase activity was detected in the cytosolic fraction (PS) of the yeast *dci1Δ* strain, and this activity was higher than the activity present in the particulate fraction of wild-type yeast. Only a very low dienoyl-CoA activity was detected in the ML fraction of *dci1Δ* cells (see Table 4). In fact, this activity was so low that it is questionable whether such activity could support the apparently normal growth of the *dci1Δ* strain on oleate if a reductase-dependent pathway would operate. Also, the activities of enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA isomerase and enoyl-CoA thiolase were higher in the cytosolic fraction than in the particulate fraction of *dci1Δ* cells. Further subcellular fractionation to separate peroxisomes from mitochondria was not performed because the dienoyl-CoA isomerase activity present in the ML fraction of wild-type yeast was too low to carry out such experiment. In addition to dienoyl-CoA isomerase, the activities of enoyl-CoA isomerase and 3-ketoacyl-CoA thiolase were upregulated and the major parts of the activities of all tested β-oxidation enzymes was present in the cytosolic compartment (PS) instead of the ML fraction. The accumulation of these enzymes in the cytosol is in agreement with the results of Gould et al. [31] who showed a mislocalization of enoyl-CoA isomerase when the *DCI1* gene was disrupted in yeast. The conclusion is that the *DCI1* gene product is not a dienoyl-CoA isomerase, but it seems to affect the proper localization of β-oxidation enzymes to peroxisomes.

Blast search, sequence alignments analysis and homology

In an effort to determine whether proteins in *S. cerevisiae* have sequences similar to rat dienoyl-CoA isomerase, the full amino acids sequence with 327 amino acids residues of rat dienoyl-CoA isomerase was used to search proteins coded for by the yeast genome. Among seven yeast proteins identified by the Blast search (see Fig. 14), two yeast proteins, namely enoyl-CoA isomerase (Eci1p) and Ehd3p, with 20% identity in an overlap region of 183 amino acids residues, and 21% identity in an overlap region of 181 amino acids residues, respectively, have the highest identity with rat dienoyl-CoA isomerase and were identified as the best protein candidates for this study. Moreover Ehd3p, a protein of unknown function and enoyl-CoA isomerase belong to the isomerase/hydratase family as indicated by the presence of an isomerase/hydratase domain (Fig. 15). It has been shown that amino acid residues Glu^{196} and Asp^{204} facilitated proton transfers at the active site of rat dienoyl-CoA isomerase during the conversion of 3,5-dienoyl-CoA to 2,4-dienoyl-CoA [61]. The amino acid sequence alignment revealed that these two amino acids residues are conserved in Ehd3p and human dienoyl-CoA isomerase but not in yeast enoyl-CoA isomerase (see Fig. 16). The above information prompted me to express both enoyl-CoA isomerase and Ehd3p in *E.coli* cells, to purify the proteins and to measure their dienoyl-CoA isomerase activities.

Expression of Eci1p and Ehd3p in *E.coli*, purification and measurement of enzyme activities

The ECI1 and *EHD3* genes were obtained by PCR, fused with the gene encoding maltose binding protein (MBP), expressed in *E.coli* cells and purified as described under Experimental Procedures. SDS-PAGE analysis of the purified MBP-ECI showed it to be of the expected size (see Fig.17). Similarly MBP-EHD3 was produced and analyzed (see Fig. 18). As both Eci1p and Ehd3p contain isomerase/hydratase domains, they were assayed spectrophotometrically for enoyl-CoA isomerase, dienoyl-CoA isomerase and enoyl-CoA hydratase activities. Only Eci1p expressed enoyl-CoA isomerase activity, but neither had dienoyl-CoA isomerase nor enoyl-CoA hydratase activities. Enoyl-CoA isomerase activity of Eci1p was confirmed by product analysis with HPLC (see Table 5 and Fig. 19). In agreement with Hiltunen's report [62], the recombinant Ehd3p did not exhibit any of the above enzyme activities either as MBP fusion protein (see Fig. 18) or as His-tagged protein.

DISCUSSION

Evidence for the existence of a thioesterase-dependent pathway of β-oxidation in *S. cerevisiae*, which facilitates growth on oleate as the sole carbon source.

It is likely that the thioesterase-dependent pathway of β-oxidation is more important when *S. cerevisiae* cells are grown on unsaturated fatty acid with odd-numbered double bond, such as oleate, than when grown on saturated fatty acid. During the degradation of saturated fatty acids, the first enzyme of the β-oxidation spiral to act on a fatty acyl-CoA is acyl-CoA oxidase, the rate-limiting enzyme, which introduces a *trans* double bond between carbons 2 and 3 [55]. According to the substrate specificity of thioesterase (Fig.9), acyl-CoA intermediates containing 2-*trans* double bond are poor substrates of yeast peroxisomal thioesterase, but are good substrates of the yeast FOX2 gene product, which is a multifunctional enzyme having both enoyl-CoA hydratase activity and 3-hydroxyacyl-CoA dehydrogenase activities [27]. The enoyl-CoA hydratase rapidly catalyzes the hydration of the 2-*trans* double bond of 2-enoyl-CoA to produce 3-hydroxyacyl-CoA that is the substrate of 3-hydroxyacyl-CoA dehydrogenase, the second activity of the bifunctional enzyme. The end product of the dehydrogenation reaction is 3-ketoacyl-CoA, which is the substrate of yeast acy-CoA thiolase, the last enzyme of the β-oxidation cycle that cleaves the starting acyl-CoA to produce acetyl-CoA and an acyl-CoA with two carbon atoms less. The flux through the pathway might be fast because the enzymes may exist as an organized complex organized and therefore act in a coordinated manner. Such situation keeps the levels of intermediates low and therefore gives the peroxisomal thioesterase less of a possibility to hydrolyze acyl-CoA intermediates during the β-oxidation of saturated fatty acids. As a consequence, the *tes1Δ* strain growing on palmitate does not show a phenotype. The peroxisomal thioesterase gene contains a consensus oleate-responsive element (ORE) in its promoter region [56, 57, 58]; therefore it is expected to be induced by oleate, and also by other fatty acids such as myristate and palmitate.

The *TES1* mRNA is about 100-fold more abundant in the sample extract from oleate-grown cells, than in the extract from cells grown on glucose while the thioesterase activity is 2 to 3 fold higher in oleate grown cells than in cells grown on glycerol or glucose. These observations support the idea that the thioesterase activity is important when *S. cerevisiae* cells are growing on oleate. In fact, during the course of oleate degradation in *S. cerevisiae*, oleoyl-CoA undergoes two cycles of classical β-oxidation similar to the degradation of saturated fatty acids to yield 5-*cis*-tetradecadienoyl-CoA. Acyl-CoA oxidase acts on 5-*cis*-tetradecenoyl-CoA by introducing a *trans* double bond at carbon atom 2 to yield 2-*trans*-5-*cis*-tetradecadienoyl-CoA. The metabolism of 2-*trans*-5-*cis*-tetradecadienoyl-CoA not only involves the β-oxidation enzymes required for the degradation of saturated fatty acids such as enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase, but also requires auxiliary enzymes catalyzing the isomerization and reduction of the pre-existing double bond. The action of one of these auxiliary enzymes, enoyl-CoA isomerase, on 2-*trans*-5-*cis*-tetradecadienoyl-CoA creates a second pathway for the degradation of oleate.

Entry into one pathway of degradation is facilitated by enoyl-CoA hydratase that rapidly acts on 2-*trans*-5-*cis*-tetradecadienoyl-CoA to continue the one round of β-oxidation to yield 3-*cis*-dodecenoyl-CoA. Enoyl-CoA isomerase isomerizes 3-*cis*-dodecenoyl-CoA to 2-*trans*-

dodecenoyl-CoA that reenters β-oxidation by the action of enoyl-CoA hydratase and ultimately is completely degraded by β-oxidation. The isomerization catalyzed by enoyl-CoA isomerase is the basis for the name isomerase-dependant pathway (Scheme 1).

In a second pathway of degradation, 2-*trans*-5-*cis*-tetradecadienoyl-CoA is directly isomerized by enoyl-CoA isomerase to 3-*trans*-5-*cis*-tetradecadienoyl-CoA that has to undergo a second isomerization reaction catalyzed by dienoyl-CoA isomerase to yield 2-*trans*-4-*trans*-tetradecadienoyl-CoA. The latter compound can be reduced to 3-*trans*-tetradecenoyl-CoA by yeast 2,4-dienoyl-CoA reductase. Finally, enoyl-CoA isomerase acts on 3-*trans*-tetradecenoyl-CoA to produce 2-*trans*-tetradecenoyl-CoA that can be completely degraded by 5 cycles of β-oxidation. This pathway has been named reductase-dependent pathwa (see Scheme 1).

In yeast, a unicellular eukaryotic organism, β-oxidation exclusively operates in peroxisomes to produce a number of metabolites during oleate degradation. These intermediates undergo various chemical reactions, such as hydration, dehydrogenation, isomerization, reduction and thiolytic cleavage. The biochemical reactions and the resultant flux of acyl-CoA intermediates through the pathway must be efficient to support growth of *Saccharomyces cerevisiae* on oleic acid as the sole source of energy. To prevent the accumulation of intermediates that might impair efficient oleate degradation, an additional pathway has evolved named thioesterase-dependent pathway, which involves peroxisomal thioesterase to dispose of intermediates, including 3-*trans*-5-*cis*-tetradecadienoyl-CoA, by hydrolysis and excretion of the resultant carboxylic acid.

The partial restoration of a growth defect due to the mutation of the *TES1* gene by transformation of *S. cerevisiae* cells with a vector expressing human dienoyl-CoA isomerase demonstrates that yeast does not depend on the reductase-dependent pathway to grow on oleate. Furthermore this study demonstrates that during the β-oxidation of oleic acid, but not of palmitic acid, the thioesterase-dependent pathway operates in yeast peroxisomes.

It is assumed that the thioesterase-pathway prevents the accumulation of acyl-CoAs that might impair fatty acid β-oxidation due to the sequestration of coenzyme A and/or the inhibition of β-oxidation enzymes by acyl-CoAs

The *DCI1* gene product is not a dienoyl-CoA isomerase but affects the translocation of some β-oxidation enzymes into peroxisomes

The *DCI1* gene product named Dci1p was proposed to be dienoyl-CoA isomerase [32] and therefore might be involved in the reductase-dependent pathway of oleate β-oxidation by catalyzing the conversion of 3-*trans*-5-*cis*-tetradecadienoyl-CoA to 2-*trans*-4-*trans*-tetradecadienoyl-CoA. Also, it was shown that Dci1p belongs to the isomerase/hydratase family and shares 50% homology with enoyl-CoA isomerase (Eci1p) [34]. But so far no study has confirmed the proposed dienoyl-CoA isomerase activity of the *DCI1* gene product with purified Dci1p. Here I report the partial purification of Dci1p, which was assayed for dienoyl-CoA isomerase activity. The fact that Dci1p did not exhibit such activity led me to conclude that Dci1p does not function as a dienoyl-CoA isomerase and is not involved in the reductase-dependent pathway of oleate β-oxidation. Furthermore, the detection of a low level of dienoyl-CoA isomerase activity in both wild type cells and *dci1Δ* cells confirms that Dci1p is not reponsible for dienoyl-CoA activity in *Saccharomyces cerevisiae* cells. Further analysis might reveal the function of Dci1p in *S. cerevisiae* cells grown on oleate. An interesting observation related to the function of Dci1p is that activities of some β-oxidation enzymes are increased in the *dci1Δ* strain compared to wild-type cells and that a large fraction of some β-oxidation enzymes is located in the cytosol instead of peroxisomes. These intriguing results indicate that Dci1p may affect the import of some β-oxidation enzymes into peroxisomes.

Furthermore, I investigated whether other proteins such as Eci1p and Ehd3p belonging to the isomerase/hydratase family exhibit dienoyl-CoA isomerase activity that was observed in both wild type and the *dci1Δ* cells. No dienoyl-CoA isomerase activity was detected with purified preparations of Eci1p or Ehd3p. Dienoyl-CoA activity is present in wild-type cells at a very low level compared to other β-oxidation enzymatic activities. In fact the activities of enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, enoyl-CoA isomerase and enoyl-CoA thiolase were ten to several hundred fold higher than the activity of dienoyl-CoA isomerase, which is near the limit of detection in the soluble extracts of the wild-type cells. These data support the conclusion that dienoyl-CoA isomerase activity is not needed for the β-oxidation of oleic acid. Since a dienoyl-CoA isomerase activity was not associated with any purified protein belonging to the isomerase/hydratase family such as Dci1p, Eci1p and Ehd3p, I assume that another protein induced by growth on oleate might exhibit a low level of dienoyl-CoA isomerase activity, perhaps in addition to its main enzymatic activity.

SUMMARY OF THE STUDY

Data presented in this thesis demonstrate the need for a thioesterase-dependent pathway of β-oxidation to efficiently support the growth of *Saccharomyces cerevisiae* cells on oleate and perhaps other fatty acids. One reason for the operation of the thioesterase-dependent pathway of β-oxidation in yeast is the absence of the reductase-dependent pathway. The reason why the reductase-dependent pathway of β-oxidation does not operate in yeast may be the very low dienoyl-CoA isomerase activity.

The conclusion of my thesis is that *Saccharomyces cerevisiae* uses two pathways for the degradation of oleic acid. While the main flux of oleate degradation is facilitated by the isomerase-dependent pathway, the thioesterase-dependent pathway is necessary to dispose of metabolites that otherwise would interfere with β-oxidation via the isomerase-dependent pathway.

Table 1. *S.cerevisiae* strains and plasmids used in this study

Strain	Genotype	Ref.
W3031A	MAT**a** *leu2 ura3 trp1 ade2 his3*	52
eci1Δ	MAT**a** *leu2 ura3 trp1 ade2 his3 eci1::HIS3*	53
dci1Δ	MAT**a** *leu2 ura3 trp1 ade2 his3 dci1::HIS3*	53
eci1Δdci1Δ	MAT**a** *leu2 dci1::LEU2 ura3 trp1 ade2 his3 eci1::HIS3*	53
tes1Δ	MAT**a** *leu2 ura3 tes1::his* G *trp1 ade2 his3*	This study
dci1Δtes1Δ	MAT**a** *leu2 ura3 tes1::his* G *trp1 ade2 his3 dci1::HIS3*	This study
ehd3Δ	MAT**a** *leu2 ura3 trp1 ehd3::*13Myc-T*ADH1-TRP1 ade2 his3*	This study
dci1Δehd3Δ	MAT**a** *leu2 ura3 trp1 ehd3::*13Myc-T*ADH1-TRP1 ade2 his3 dci1::HIS3*	This study
tes1Δ PRS306	MAT**a** *leu2 ura3[*pRS306-*URA3] tes1::his* G *trp1 ade2 his3*	This study
tes1Δ PRS316	MAT**a** *leu2 ura3[*pRS316-*URA3] tes1::his* G *trp1 ade2 his3*	This study
tes1Δ PRS306hDCI1POX1	MAT**a** *leu2 ura3[*pRS306-*POX1*-hDCI-*URA3] tes1::his* G *trp1 ade2 his3*	This study
tes1Δ PRS316hDCI1POX1	MAT**a** *leu2 ura3[*pRS316-*POX1*-hDCI-*URA3] tes1::his* G *trp1 ade2 his3*	This study
BY4733	MAT**a** *his3Δ200 leu2Δ̃0met15Δ0̃ trp1Δ63 ura3Δ0̃*	54
BY4733, *yjr019cΔ::HIS3*	MAT**a** *his3Δ ˜ yjr019c::HIS3 leu2Δ̃ 0met15Δ̃0 trp1Δ63 ura3Δ̃0*	37
Plasmids		
pMBP-TES1		37
pMBP-DCI1		54
pMBP-ECI		This study
pMBP-EHD3		This study
p6xHis-DCI1		This study
pRS306hDCI1POX1		This study
pRS316hDCI1POX1		This study

Table 2. Thioesterase acitvities in soluble extracts from *E.coli* cells expressing thioesterase from *S.cerevisiae*

Soluble extracts	Protein concentration (mg/ml)	Specific activity (mU/mg)
Non induced	6.1	248±2.4
Induced with IPTG	7.7	480±12

Table 3. Dienoyl-CoA isomerase activity in soluble extracts of *S.cerevisiae*

Samples	Specific activities (mU/mg)
Soluble extract of wild-type cells	9.2±0.2
Soluble extract of *DCI1Δ* strain	39.1±0.2
Purified MBP-Dci1p	0*

*The limit of detecting dienoyl-CoA isomerase activity was 0.46 mU/mg

Table 4. Activities of β-oxidation enzymes in supernatants (PS) and particulate fractions (ML) from wild type and *DCI1* mutant cells of *S.cerevisiae*

	Wild type		***DCI1* mutant**	
Enzymes	**PS**	**ML**	**PS**	**ML**
		Specific activities (U/mg)		
Catalase	1.42	12.97	1.02	8.43
Enoyl-CoA hydratase	0.72±0.01	10.18±0.01	6.77±0.08	2.34±0.19
L-3-Hydroxyacyl-CoA dehydrogenase	0.007±0.000	1.08±0.000	0.752±0.005	0.131±0.002
Enoyl-CoA isomerase	0.050±0.002	0.140±0.000	0.408±0.012	0.090±0.010
Dienoyl-CoA isomerase	0.003±0.000	0.014±0.000	0.063±0.002	0.003±0.000
Enoyl-CoA thiolase	0.048±0.017	0.242±0.004	0.537±0.004	0.174±0.006

Table 5. Enzyme activities of purified yeast enoyl-CoA isomerase and Ehd3p from *S.cerevisiae*

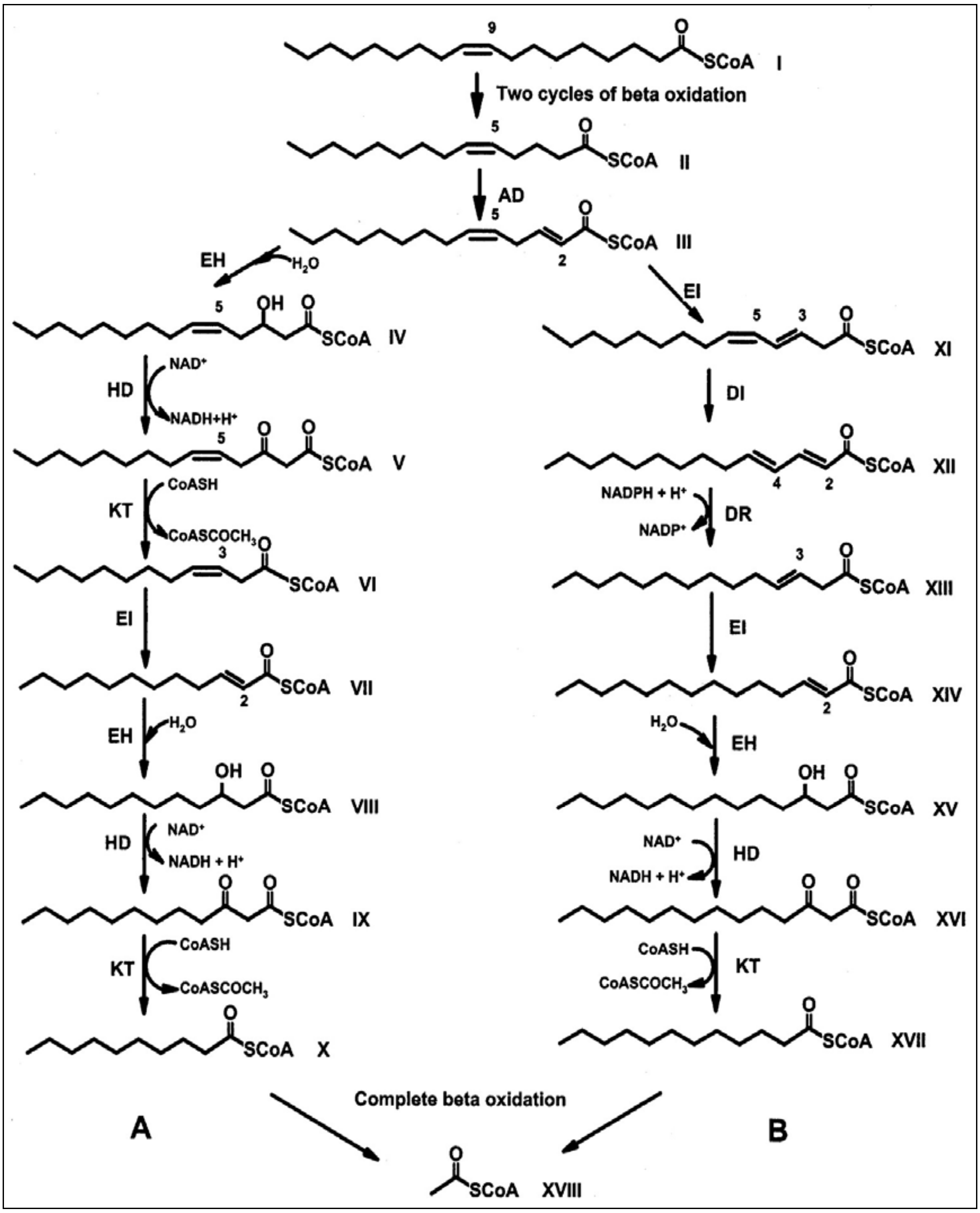
Proteins	Specific activities (U/mg)		
	Enoyl-CoA isomerase	Dienoyl-CoA isomerase	Enoyl-CoA Hydratase
MBP-Eci1p	21.5±0.7	0*	0*
MBP-Ehd3p	0*	0*	0*

*The limit of detecting the activity was 0.8mU/mg for Eci1p and 0.38mU/mg for Ehd3p.

Scheme 1. β-Oxidation of oleoyl-CoA in rat mitochondria**.**

A, isomerase-dependent pathway; **B**, reductase-dependent pathway.

AD, acyl-CoA dehydrogenase; *EH*, enoyl-CoA hydratase; *HD*, L-3-hydroxyacyl-CoA dehydrogenase; *KT*, 3-ketoacyl-CoA thiolase; *EI*, Δ^3,Δ^2-enoyl-CoA isomerase; *DI*, $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA; *DR*, 2,4-dienoyl-CoA-reductase

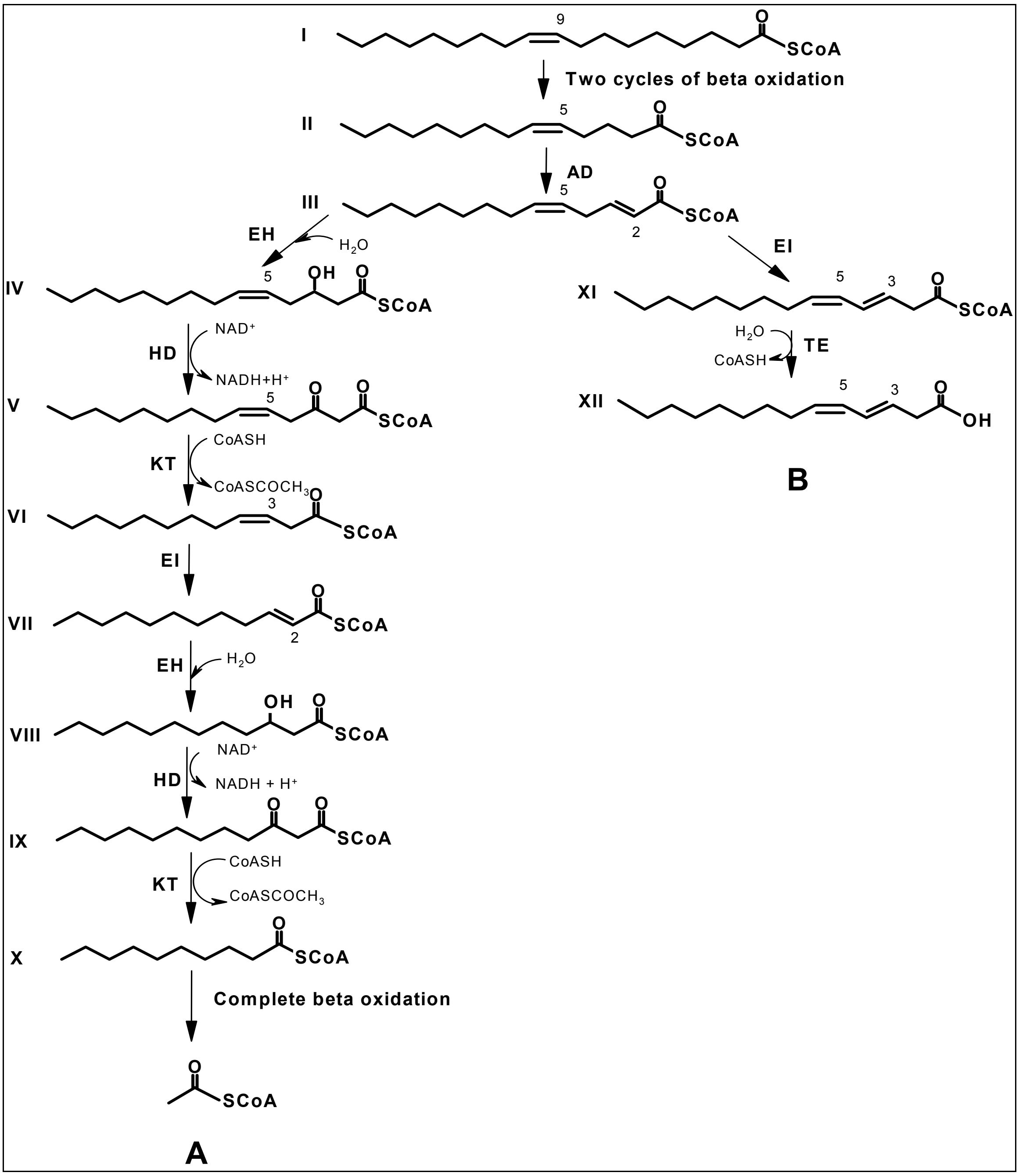

9
SCoA I
Two cycles of beta oxidation
5
SCoA II
AD
5
2
SCoA III
EH
H2O
EI
5
OH
SCoA IV
NAD+
HD
NADH+H+
5
SCoA V
CoASH
KT
CoASCOCH3
3
SCoA VI
EI
2
SCoA VII
H2O
EH
OH
SCoA VIII
NAD+
HD
NADH + H+
SCoA IX
CoASH
KT
CoASCOCH3
SCoA X
5
3
SCoA XI
DI
4
2
SCoA XII
NADPH + H+
DR
NADP+
3
SCoA XIII
EI
2
SCoA XIV
H2O
EH
OH
SCoA XV
NAD+
HD
NADH + H+
SCoA XVI
CoASH
KT
CoASCOCH3
SCoA XVII
Complete beta oxidation
A
B
SCoA XVIII

Scheme 1

Scheme 2. β-Oxidation of oleoyl-CoA in *E. coli*.

A, classical or isomerase-dependent pathway; **B**, alternative pathway.

AD, acyl-CoA dehydrogenase; *EH*, enoyl-CoA hydratase; *HD*, L-3-hydroxyacyl-CoA dehydrogenase; *KT*, 3-ketoacyl-CoA thiolase; *EI*, Δ^3,Δ^2-enoyl-CoA isomerase; *TE*, thioesterase.

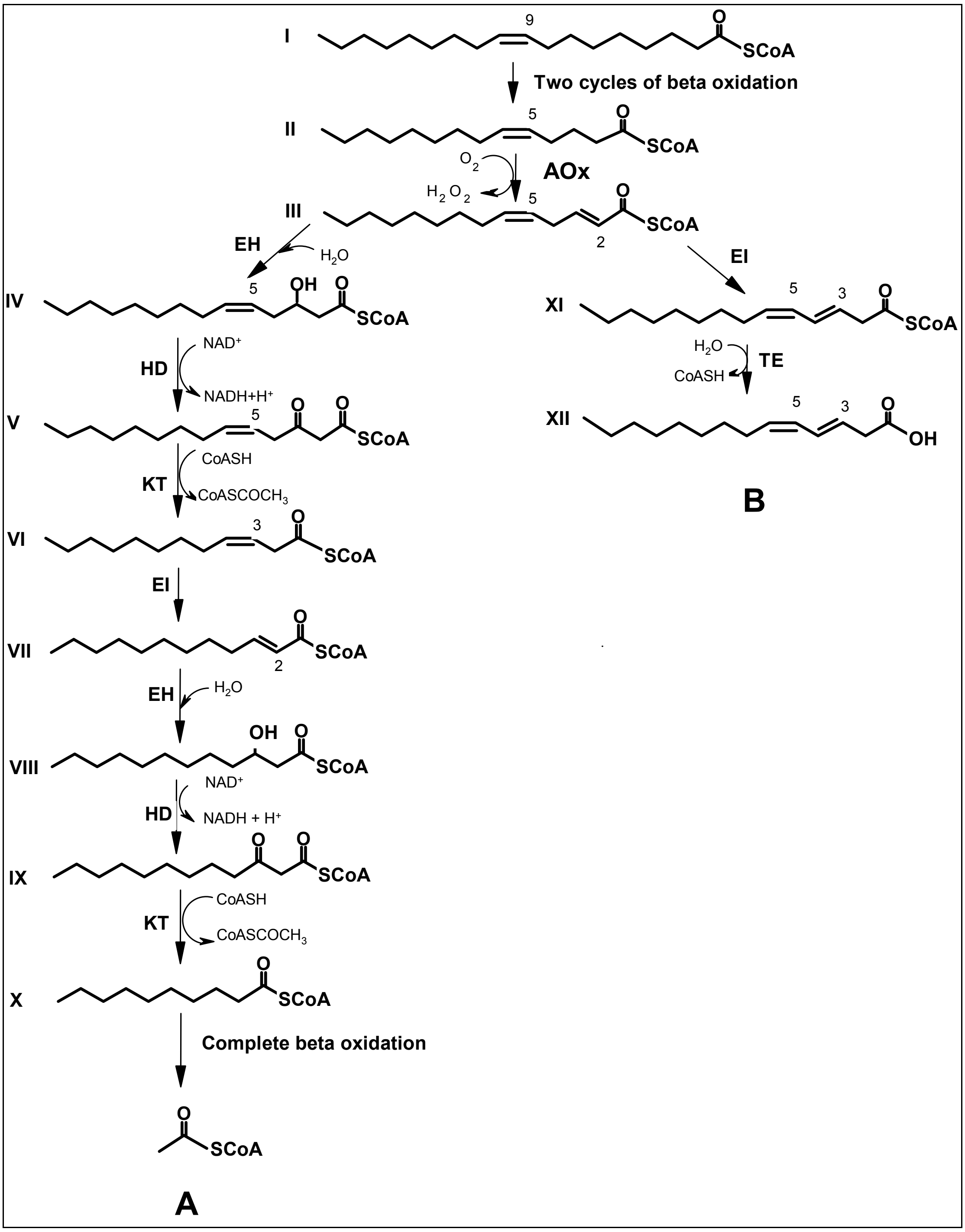

I
9
O
SCoA
Two cycles of beta oxidation
II
5
AD
III
2
EH
H_2O
IV
OH
HD
NAD⁺
NADH+H⁺
V
KT
CoASH
CoASCOCH₃
VI
3
EI
VII
VIII
NADH + H⁺
IX
X
Complete beta oxidation
A
XI
TE
CoASH
XII
OH
B

Scheme 2

Scheme 3. Hypothetical β-oxidation pathway for oleoyl-CoA in *S. cerevisiae*.

A, isomerase-dependent pathway; **B**, thioesterase-dependent pathway

AOx, acyl-CoA oxidase; EH, enoyl-CoA hydratase; HD, L-3-hydroxyacyl-CoA dehydrogenase; KT, 3-ketoacyl-CoA thiolase; EI, Δ^3,Δ^2-enoyl-CoA isomerase; TE, yeast peroxisomal thioesterase.


I
9
O
SCoA
Two cycles of beta oxidation
II
5
AOx
O_2
H_2O_2
III
2
EH
H_2O
IV
OH
V
HD
NAD^+
$NADH+H^+$
KT
CoASH
$CoASCOCH_3$
VI
3
EI
VII
VIII
IX
X
Complete beta oxidation
A
XI
TE
CoASH
XII
OH
B

Scheme 3

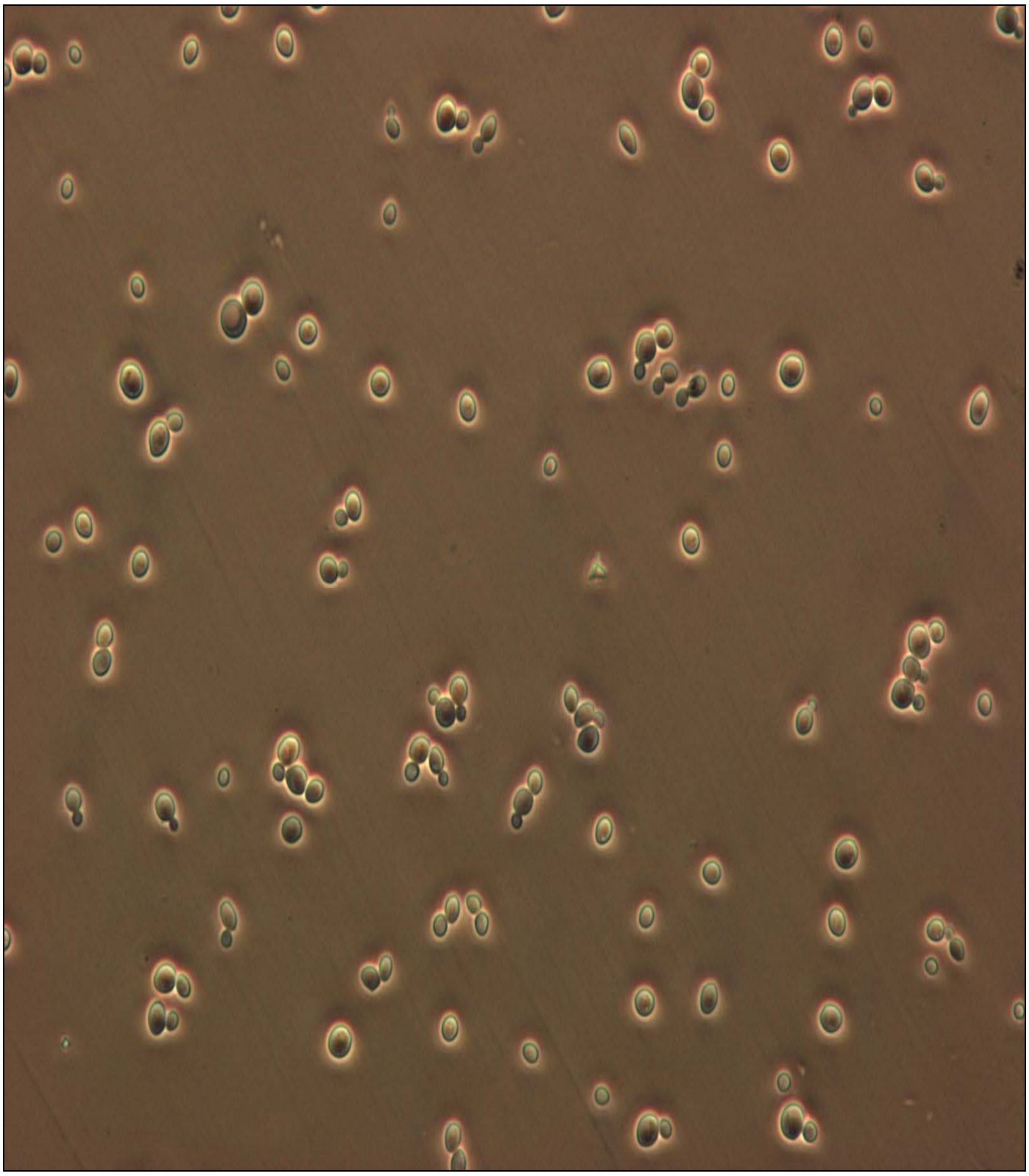


Figure 1. *Saccharomyces cerevisiae* cells under the light microscope.

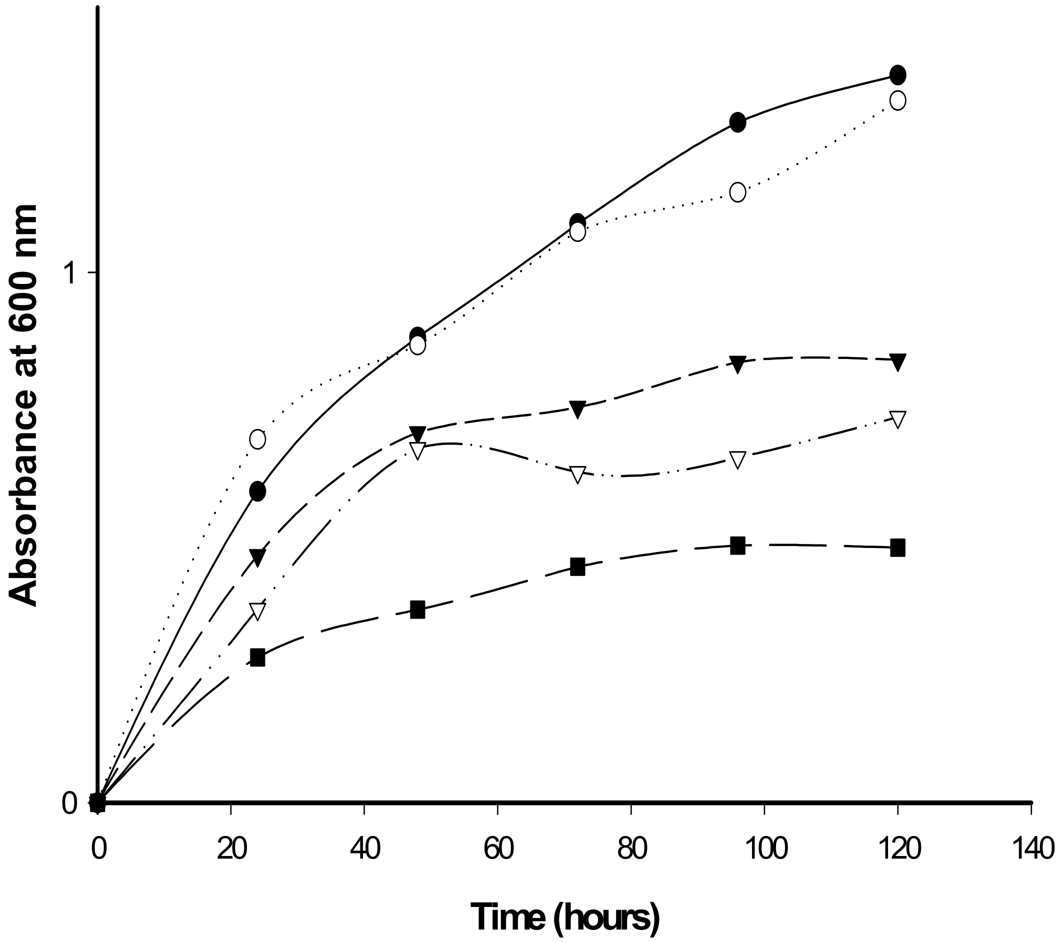


Figure 2. Growth of wild-type and mutant cells of *S. cerevisiae* on oleate

(●) Wild-type; (○) *dci1Δ*; (▼) *tes1Δ*; (▽) *dci1Δ/tes1Δ*; (■) wild-type without oleate.

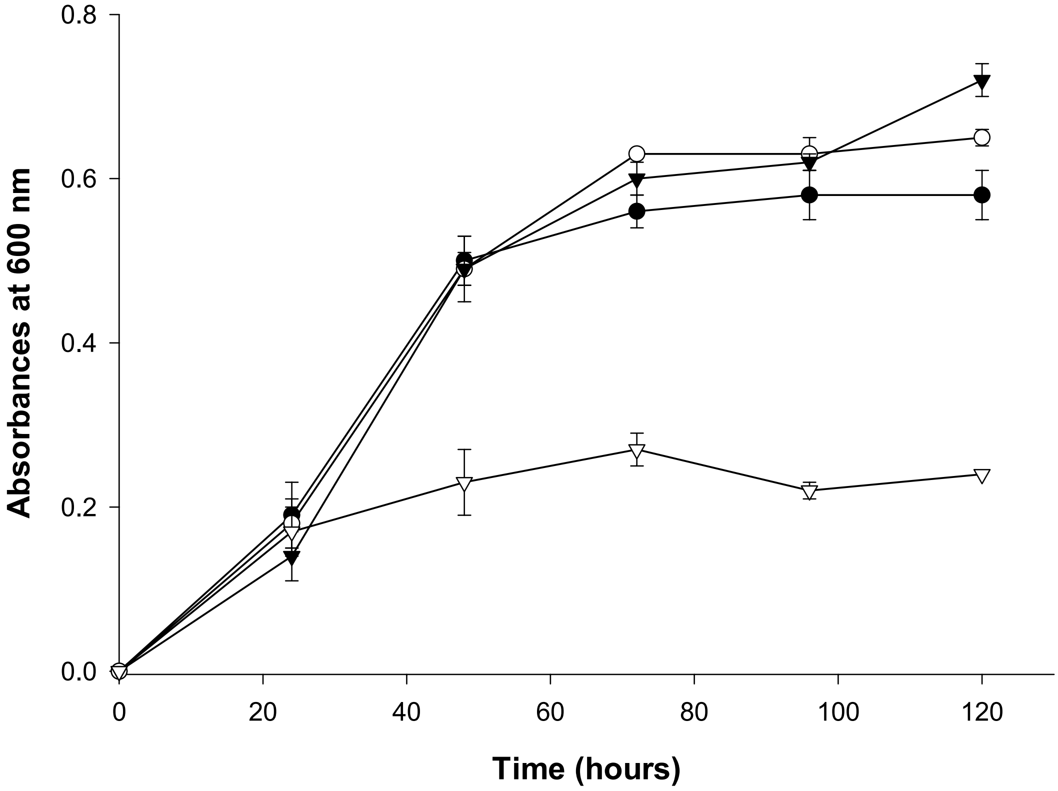


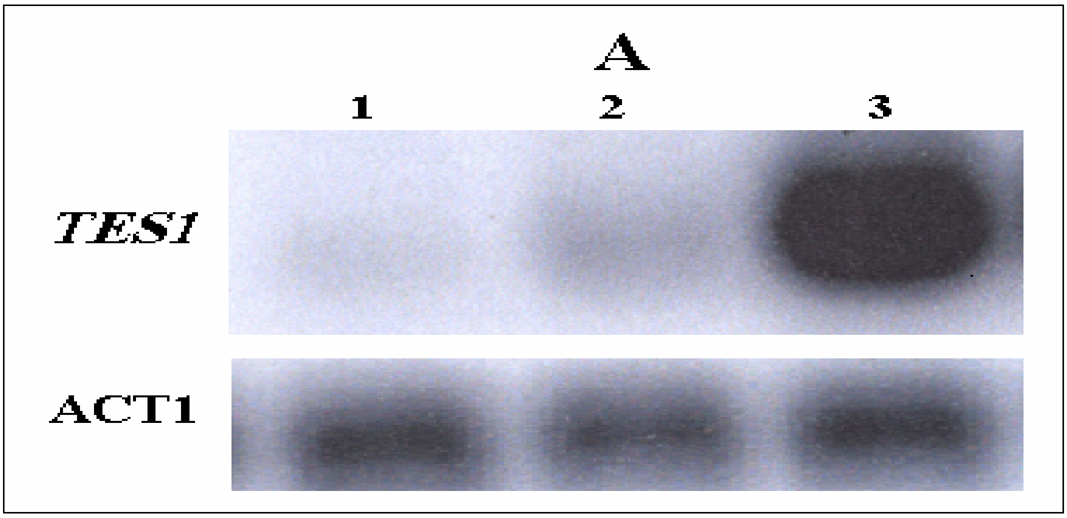
Figure 3. Growth of wild-type and mutant cells of *S. cerevisiae* on palmitic acid

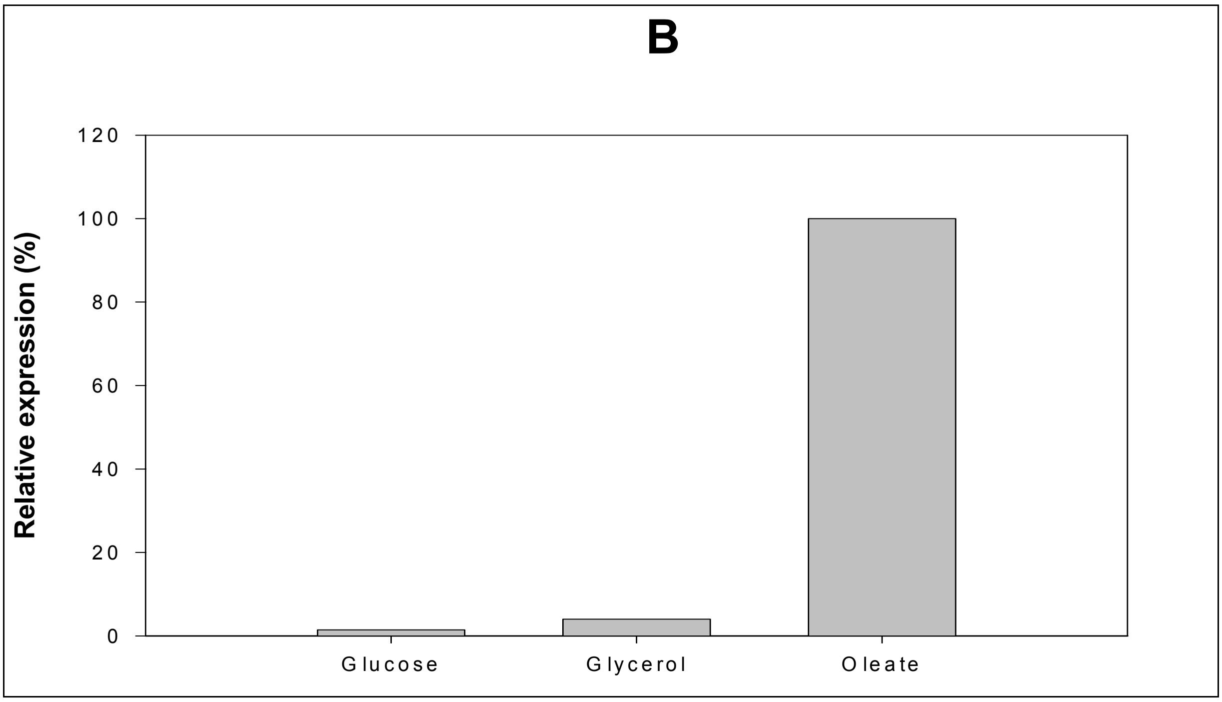
(●) Wild-type; (○) *dci1Δ*; (▼) *tes1Δ*; (▽) wild-type without palmitic acid.

Figure 4. Expression of *TES1* gene under different growth conditions

A: Northern blots of poly(A)$^+$ RNA from yeast cells grown to log phase on glucose (1), glycerol (2) and oleate (3). Upper panel; thioesterase 1 mRNA, lower pane; actin 1 mRNA

B: The data was quantified using molecular dynamics imagequant software. The expression was normalized using actin 1mRNA as a reference. The expression of thioesterase 1 mRNA from cells grown on oleate was taken to be 100%


A
1
2
3
TES1
ACT1


B
Relative expression (%)
120
100
80
60
40
20
0
Glucose
Glycerol
Oleate

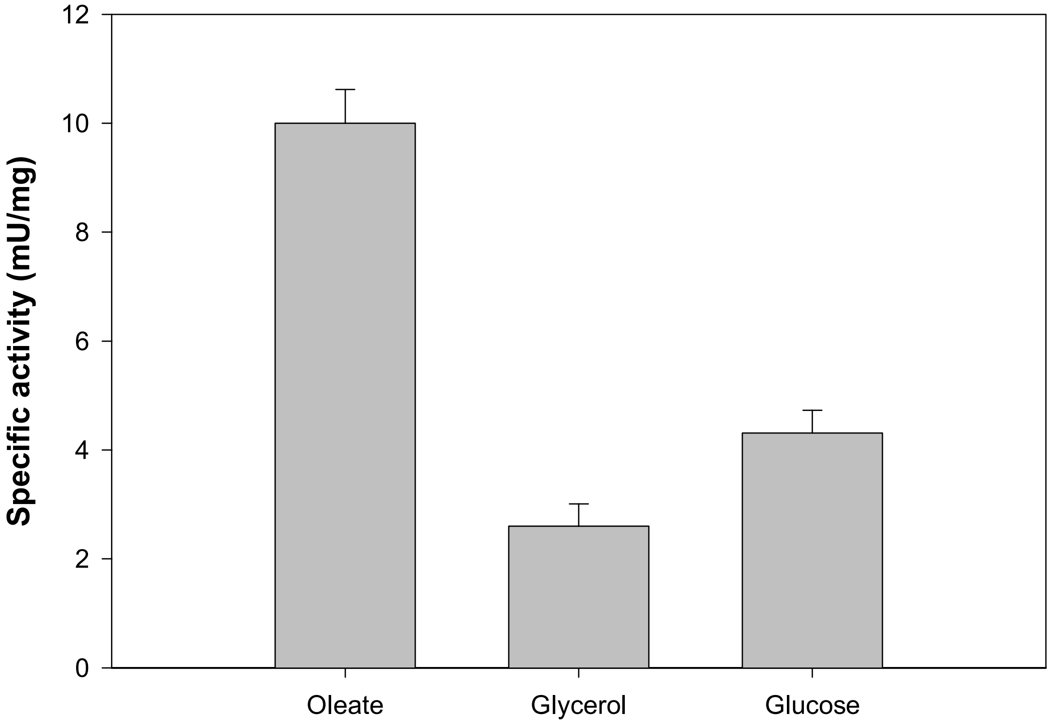


Figure 5. Specific activities of myristoyl-CoA thioesterase in a soluble extract from wild-type *S. cerevisiae* grown on different carbon sources

Figure 6. Identification of 3,5-tetradecadienoic acid in the medium after growth of *S. cerevisiae* on oleate as the sole carbon source. A, gas chromatogram of the methyl esters of the acidic fraction extracted from the growth medium. B, region of the gas chromatogram where methyl methyl 3,5-tetradecadienoate would be eluted. Peak marked P corresponds to material with a molecular ion that has a mass/charge (m/z) ratio of 238. C, mass spectrum of the material that gave rise to peak P in panel B.

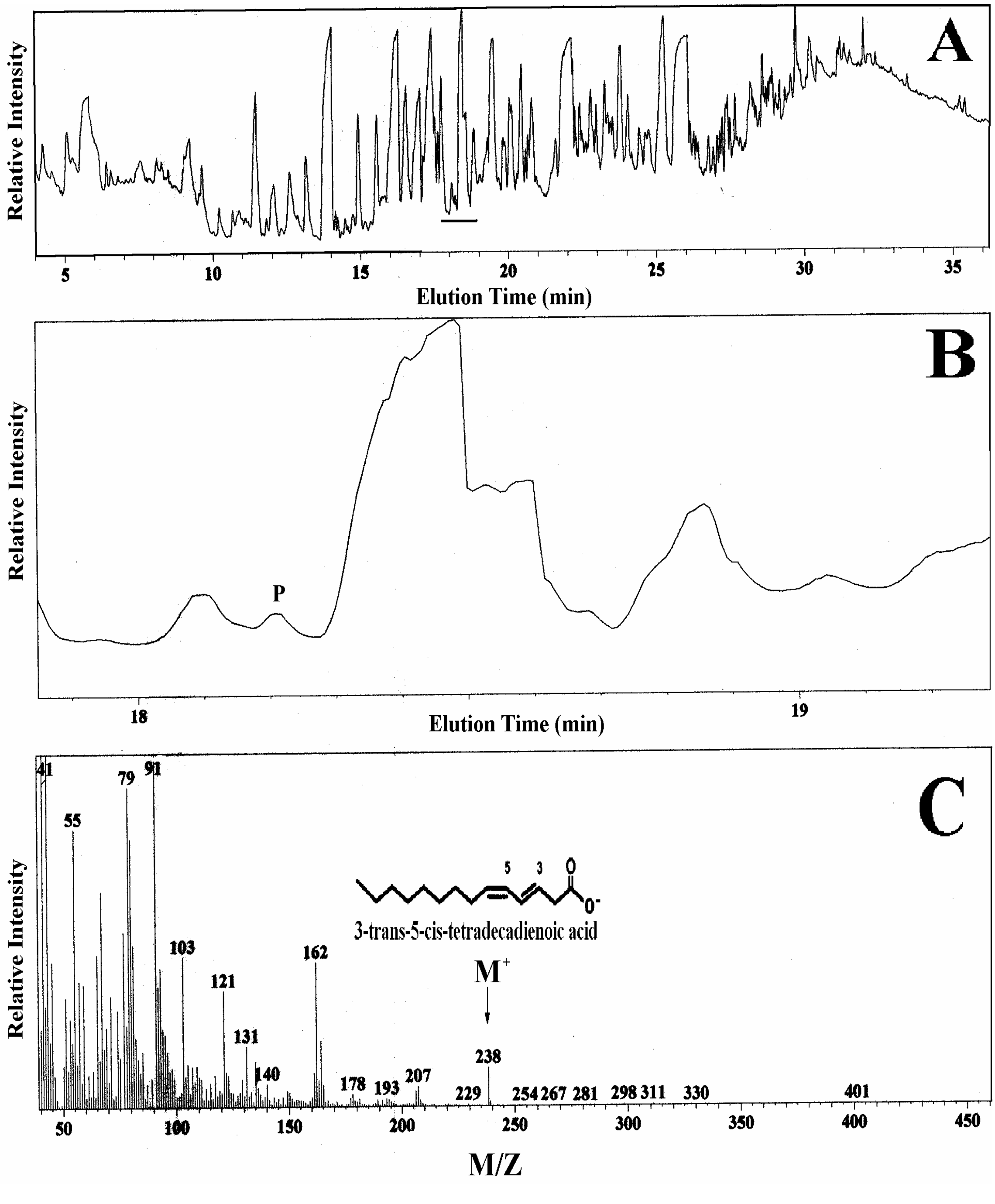

A
Relative Intensity
5
10
15
20
25
30
35
Elution Time (min)
B
Relative Intensity
P
18
19
Elution Time (min)
C
Relative Intensity
41
79
91
55
103
121
131
140
162
178
193
207
229
238
254
267
281
298
311
330
401
5
3
O
O-
3-trans-5-cis-tetradecadienoic acid
M+
50
100
150
200
250
300
350
400
450
M/Z

Figure 6

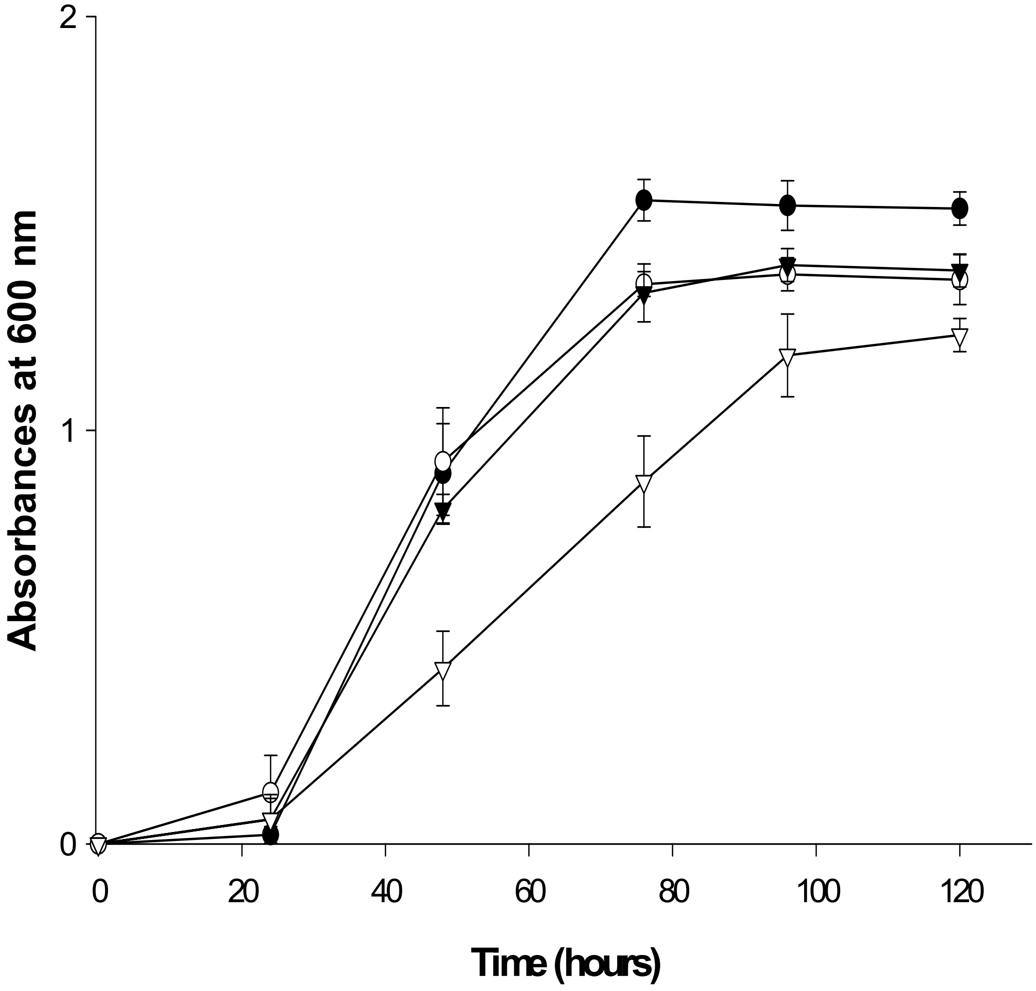


Figure 7. Growth of yeast transformants on oleic acid.

(●) Wild-type cells transformed with vector pRS316; (○) *tes1Δ* cells transformed with plasmid pRS306hDCI1POX1; (▼) *tes1Δ* cells transformed with plasmid pRS316hDCIPOX1; (▽) *tes1Δ* cells transformed with vector pRS306.

Figure 8. SDS-PAGE and Western Blotting of *E.coli* cell extracts expressing the MBP-thioesterase 1 fusion protein (MBP-TES1p).

A, SDS-PAGE after staining with Coomassie blue; lane; 1, molecular mass markers; 2, homogenate of non-induced cells; 3, homogenate IPTG-induced cells; 4, soluble extract from IPTG-induced cells; 5, insoluble fraction from IPTG- induced cells; 6, insoluble pellet from IPTG-induced cells; 7, purified MBP-TES1p protein.

B, Western blot of of the same materials described under A. An antibody to maltose binding protein was used as primary antibody.

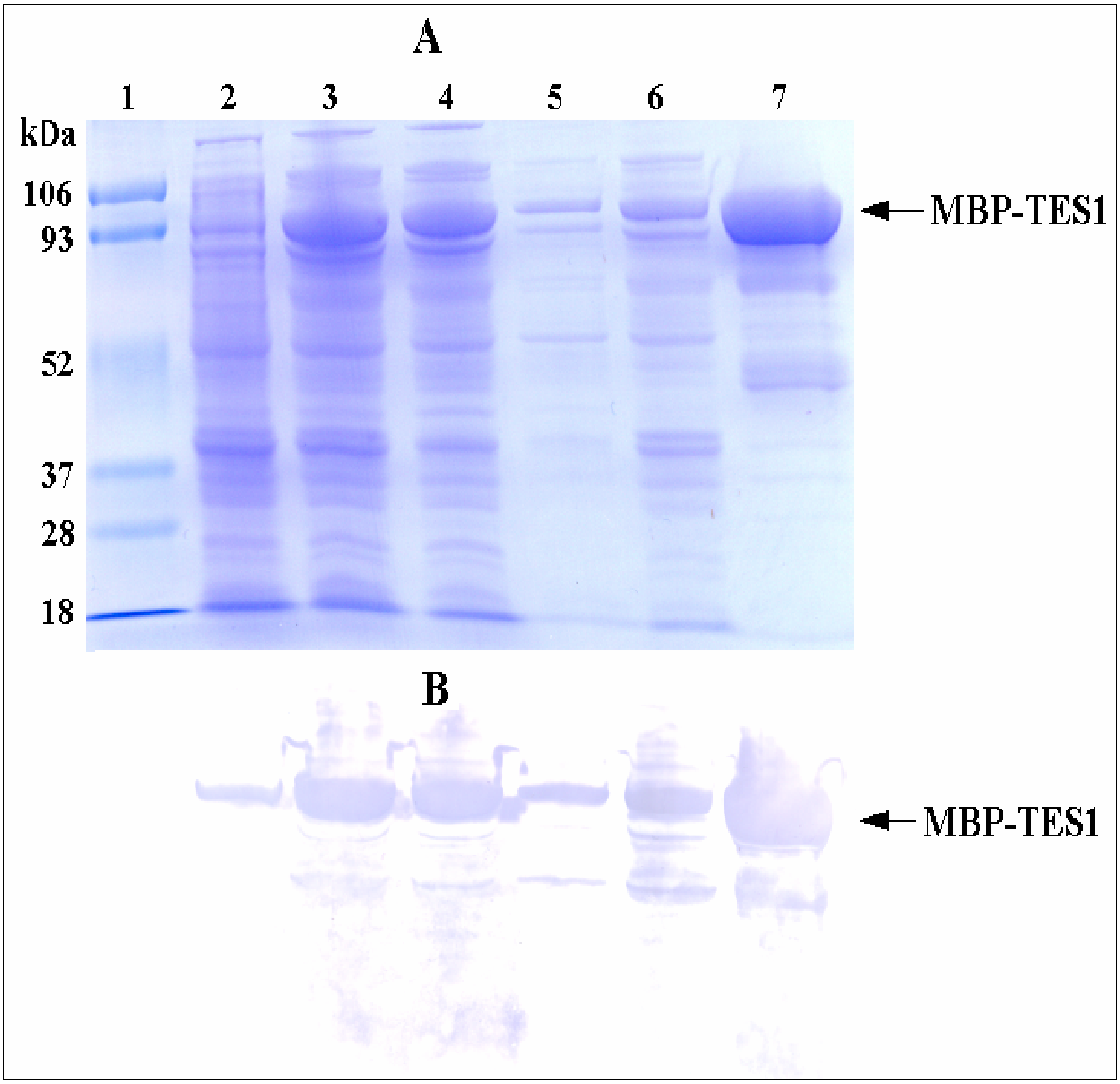


Figure 8

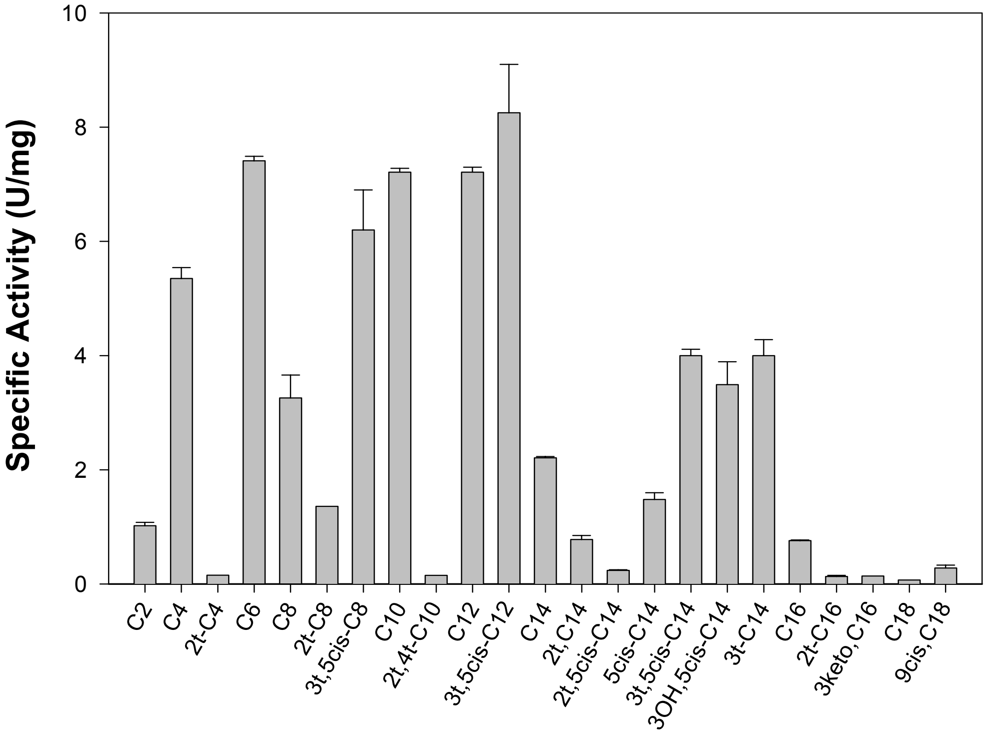


Figure 9. Substrate specificity of purified thioesterase 1:

Substrates used**:** C2, acetyl-CoA; C4, butanoyl-CoA; 2t-C4, 2-*trans*-butenoyl-CoA; C6, hexanoyl-CoA; C8, octanoyl-CoA; 2t-C8, 2-*trans*-octenoyl-CoA; 3t-5cis-C8, 3-*trans*-5-*cis*-octadienoyl-CoA; C10, decanoyl-CoA; 2t-4t-C10, 2-*trans*-4-*trans*-decadienoyl-CoA; C12, dodecanoyl-CoA; 3t-5cis-C12, 3-*trans*-5-*cis*-dodecadienoyl-CoA; C14, tetradecanoyl-CoA; 2t-C14, 2-*trans*-tetradecenoyl-CoA; 2t-5cis-C14, 2-*trans*-5-*cis*-tetradecadienoyl-CoA; 5-cis-C14, 5-*cis*-tetradecenoyl-CoA; 3t-5cis-C14, 3-*trans*-5-*cis*-tetradecadienoyl-CoA; 3-OH-5cis-C14, 3-hydroxy-5-*cis*-tetradecenoyl-CoA; 3t-C14, 3-*trans*-tetradecenoyl-CoA; C16, hexadecanoyl-CoA (palmitoyl-CoA);

2t-C16, 2-*trans*-hexadecenoyl-CoA; 3-keto-C16, 3-*keto*-hexadecanoyl-CoA; C18, octadecanoyl-CoA (stearoyl-CoA); 9-cis-C18, 9-*cis*-octadecenoyl-CoA (oleoyl-CoA).

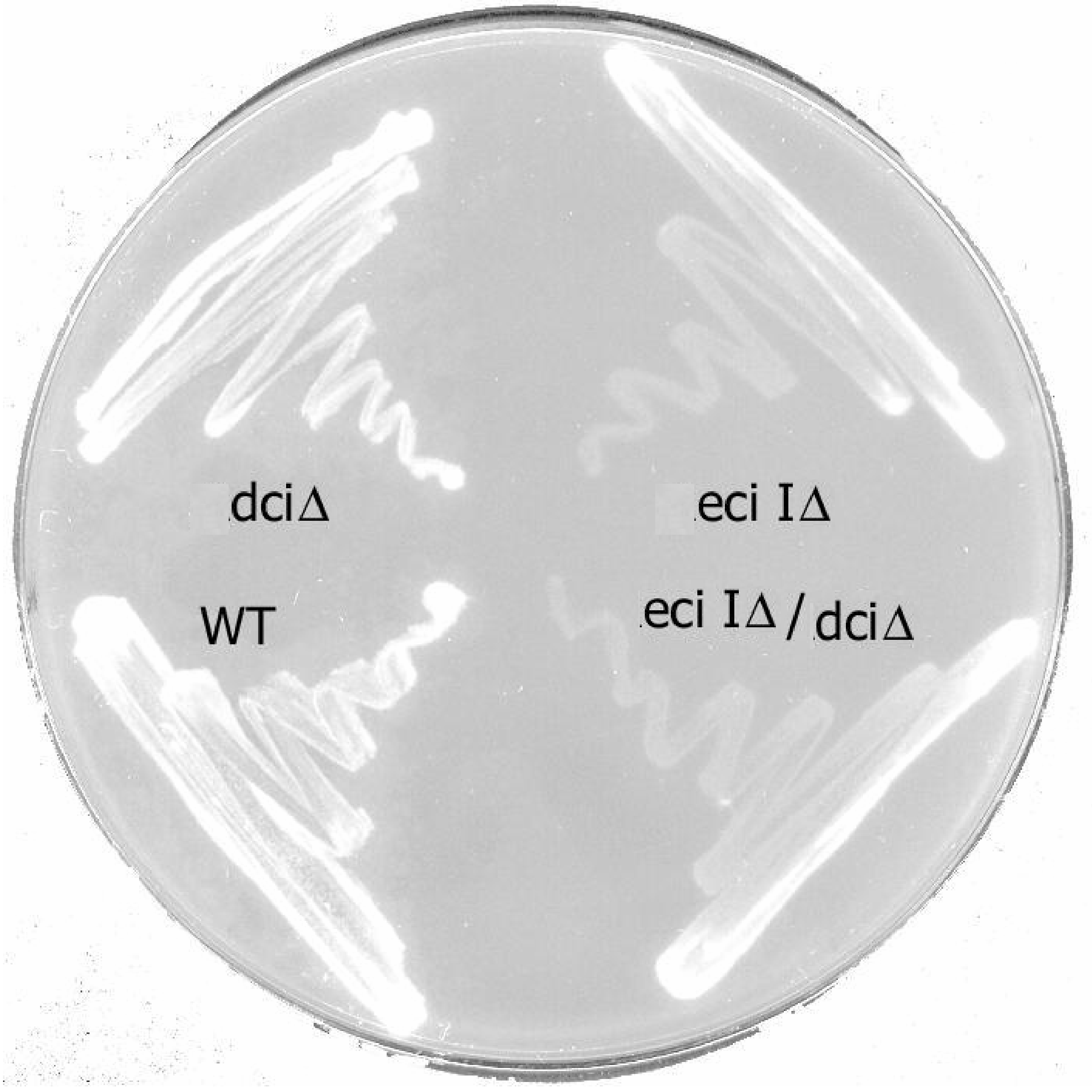


Figure 10. *Saccharomyces cerevisiae* cells growing on an agar plate with oleic acid as the sole carbon source. WT, wild type cells; dciΔ, mutant with a deletion in the *DCI1* gene; eciΔ, mutant with a deletion in the *ECI1* gene

Figure 11: Spectrophotometric analysis of the isomerization of 3,5-octadienoyl-CoA to 2,4-octadienoyl-CoA catalyzed by: **A**, purified rat dienoyl-CoA isomerase; spectrum 1 at time 0; spectra 2-5 were recorded 1 min, 2 min, 4 min, 5 and 10 min after the addition of 20 x 10^{-3} µg of enzyme. **B**, extract of *S. cerevisiae*; spectrum 1 at time 0, spectra 2-4 were recorded 5 min, 10 and 20 min after the addition of 27 µg of the soluble extract. **C**, extract of *dci1Δ* cells; spectrum 1 at time 0, spectra 2-4 were recorded 5 min, 10 and 20 min after the addition of 27 µg of the soluble extract.

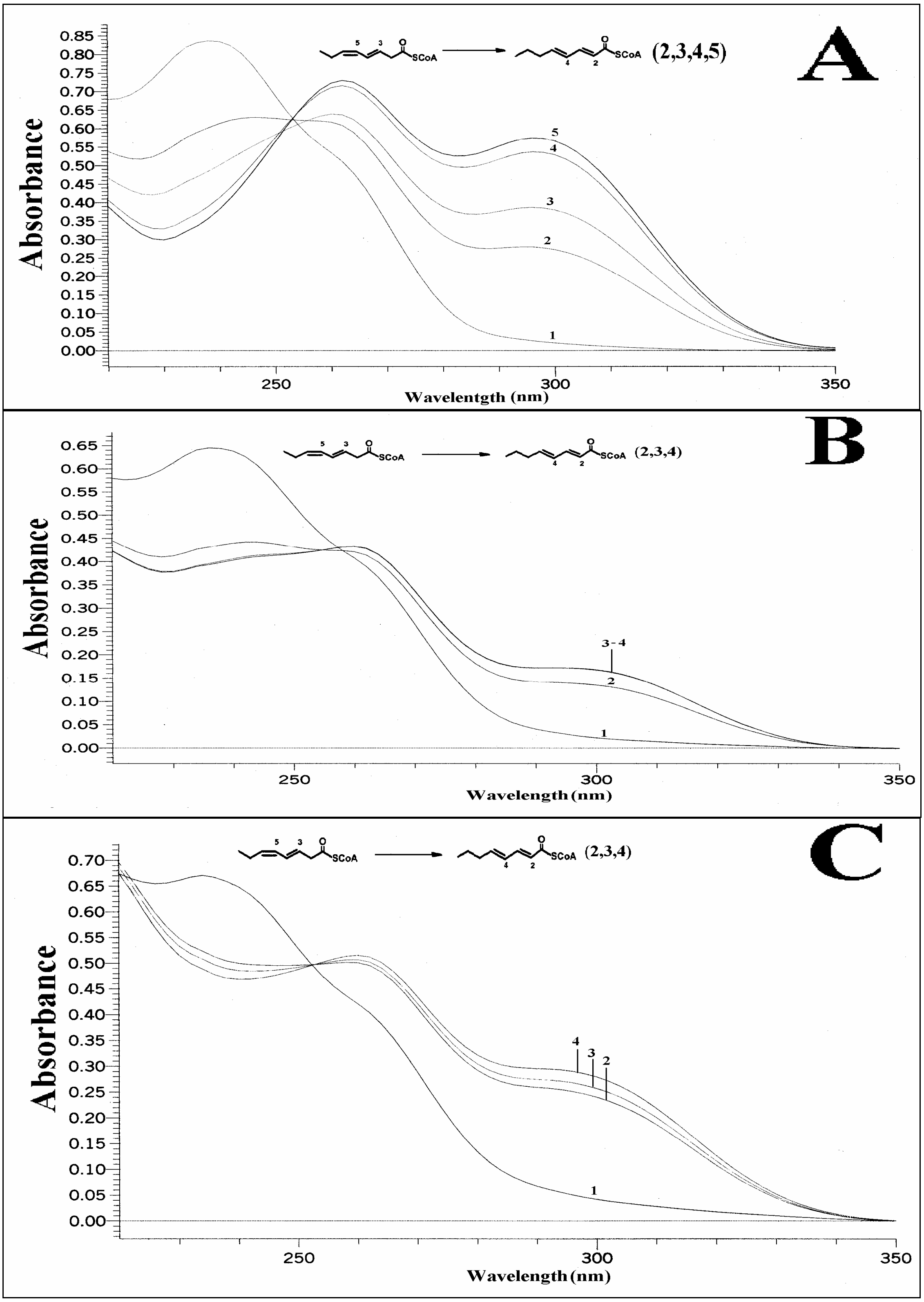

A
5
3
O
SCoA
O
SCoA
4
2
(2,3,4,5)
Absorbance
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
5
4
3
2
1
250
300
350
Wavelentgth (nm)
B
(2,3,4)
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
3-4
2
1
250
300
350
Wavelength (nm)
C
(2,3,4)
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
4
3
2
1
250
300
350
Wavelength (nm)

Figure 11

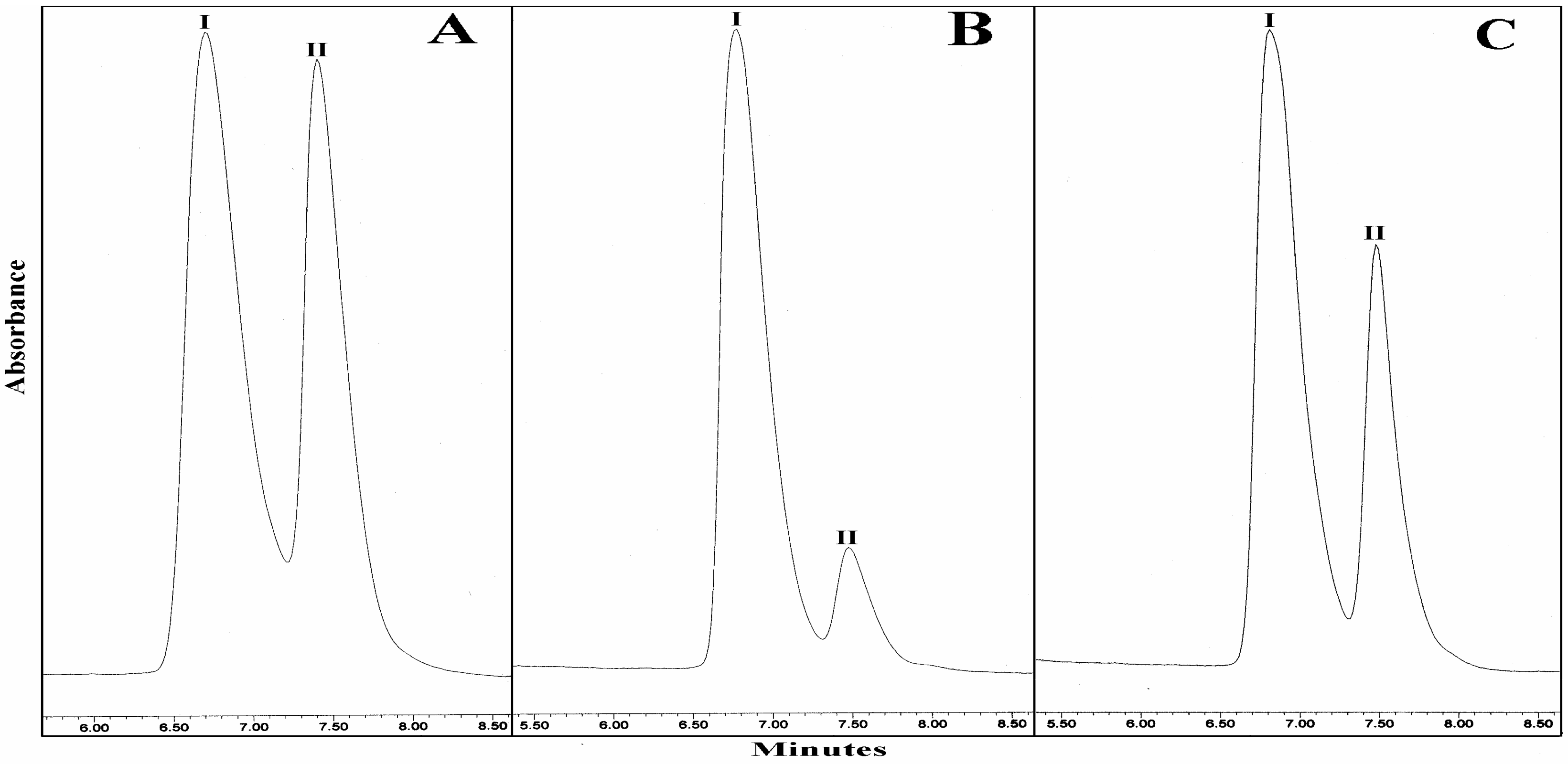


Figure 12. HPLC analysis of the product formed by soluble extracts from *S.cereviae*. **A**, Authentic 3-*trans*-5-*cis*-octadienoyl-CoA (I) and 2-*trans*-4-*trans*-octadienoyl-CoA (II); **B**, conversion of 3-*trans*-5-*cis*-octadienoyl-CoA (I) to 2-*trans*-4-*trans*-octadienoyl-CoA (II) after addition of 27 µg from soluble extract of wild-type cells; **C**, conversion of 3-*trans*-5-*cis*-octadienoyl-CoA (I) to 2-*trans*-4-*trans*-octadienoyl-CoA (II) after addition of 27 µg of soluble extract from *dci1Δ* cells

Figure 13: **A**: SDS-PAGE after staining with Coomassie blus of proteins from *E.coli* cells expressing MBP-Dci1p. cells were grown at 18°C. Lane: 1, proteins markers; 2, homogenate of non-induced cells; 3, homogenate of IPTG-induced cells; 4 soluble extract from IPTG-induced cells; 5, insoluble fraction from IPTG-induced cells; 6, insoluble pellet from IPTG-induced cells; 7, purified MBP-DCI1. **B:** Western blot of the materials described under A. The arrows in panels A and B indicate the position of MBP-DCI1 (72.5 kDa) respectively on the SDS-PAGE gel and on a Western blot.

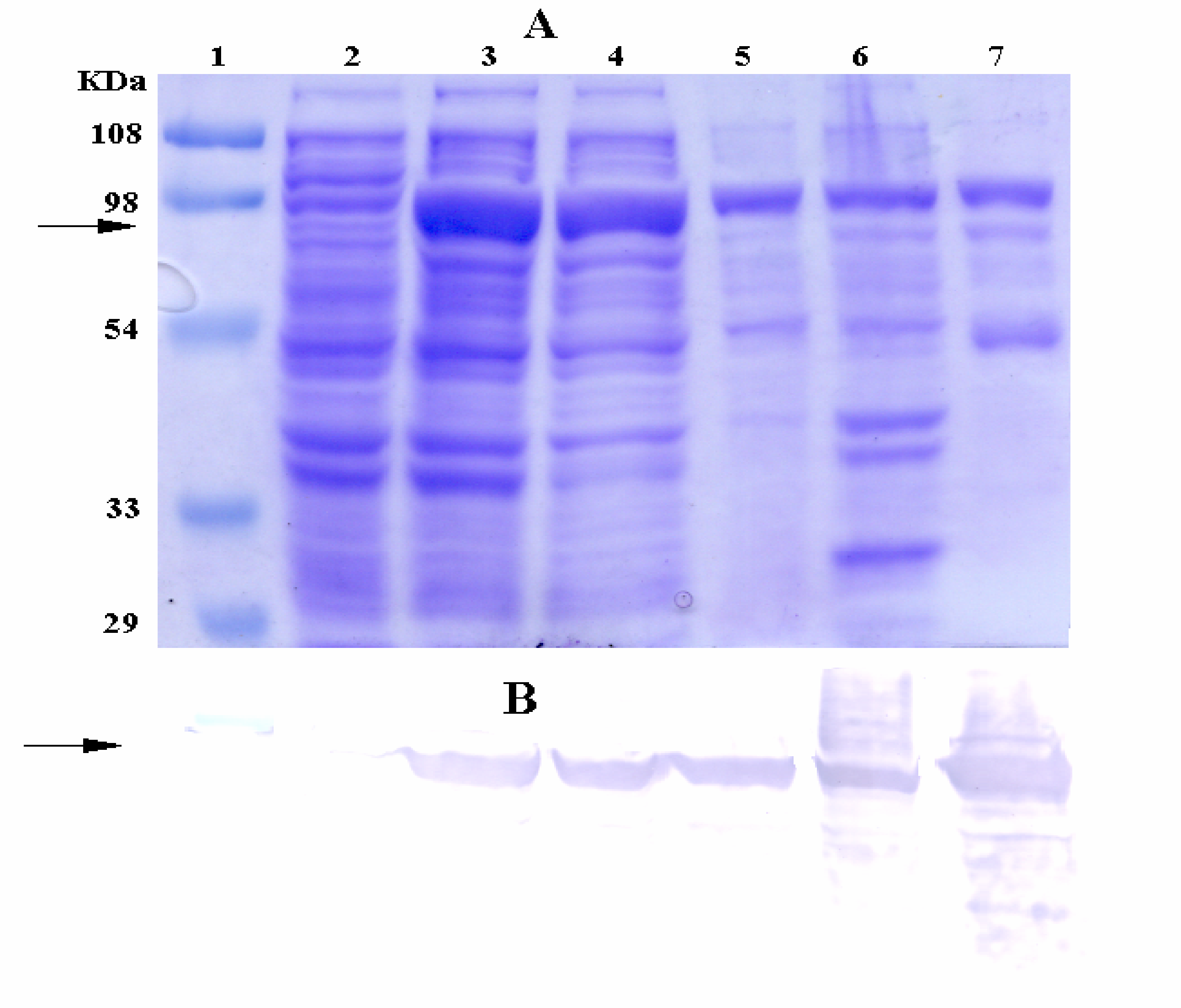


Figure 13

Figure 14

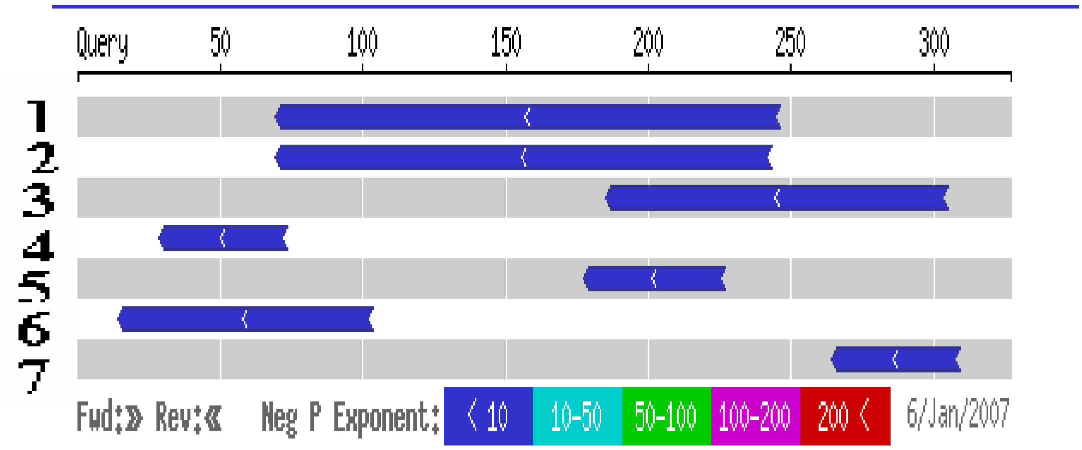


Fig 14. Results of a Blast search when using rat dienoyl-CoA isomerase for searching the yeast protein database: **Query**- rat dienoyl-CoA isomerase; **1**-enoyl-CoA isomerase; **2**-Ehd3p- protein of unknown function; **3**-Cns1p-TPR-contaning co-chaperone; **4**-Hse1p-subunit of the endosomal Vps27p-Hse1p complex; **5**-Dci1p-peroxisomal $\Delta^{3,5}$-$\Delta^{2,4}$-dienoyl-CoA isomerase; **6**-Ykr078w-cytoplasmic protein of unknown function; **7**-Rsr1p-GTP-binding protein of the ras super family.

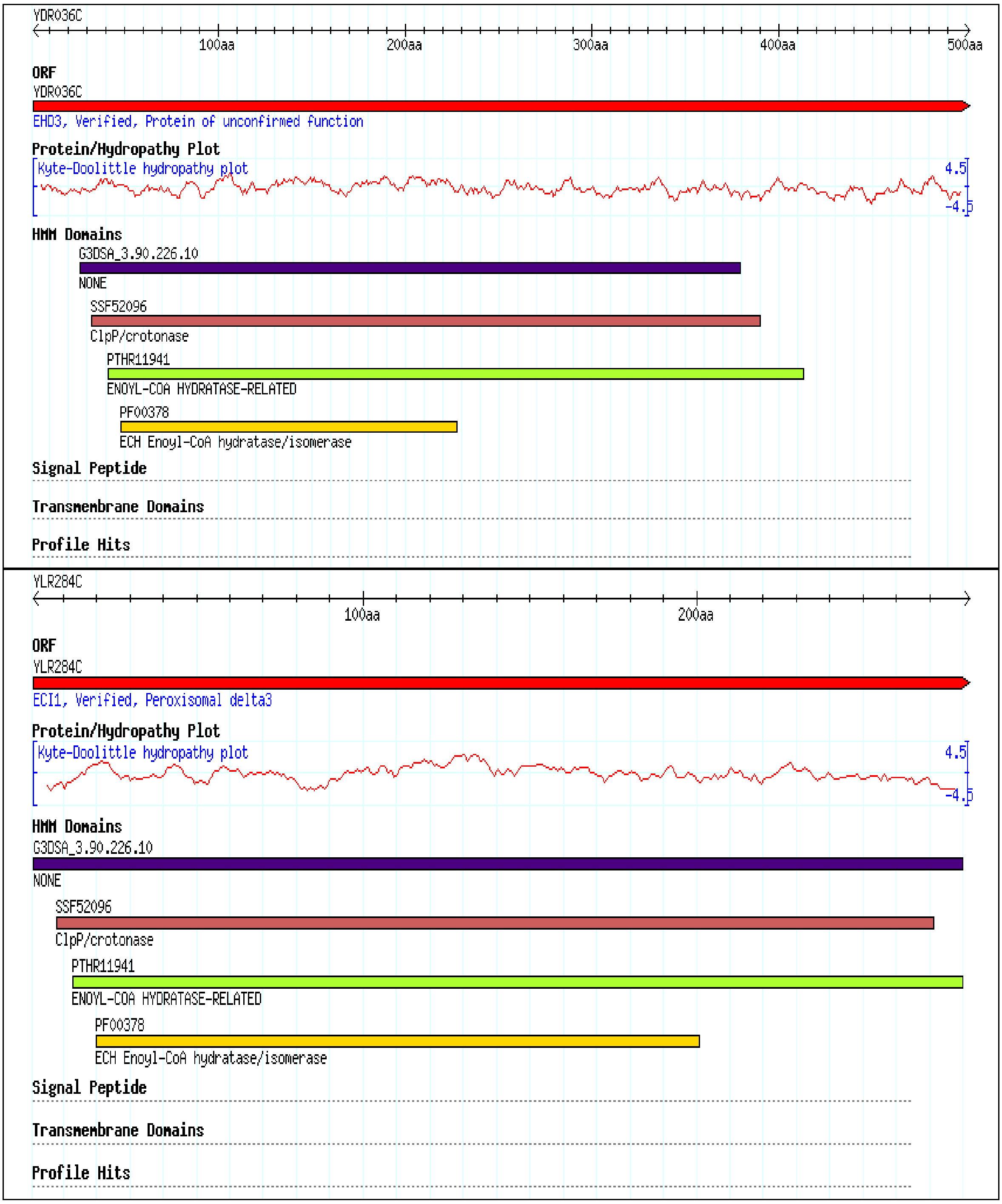
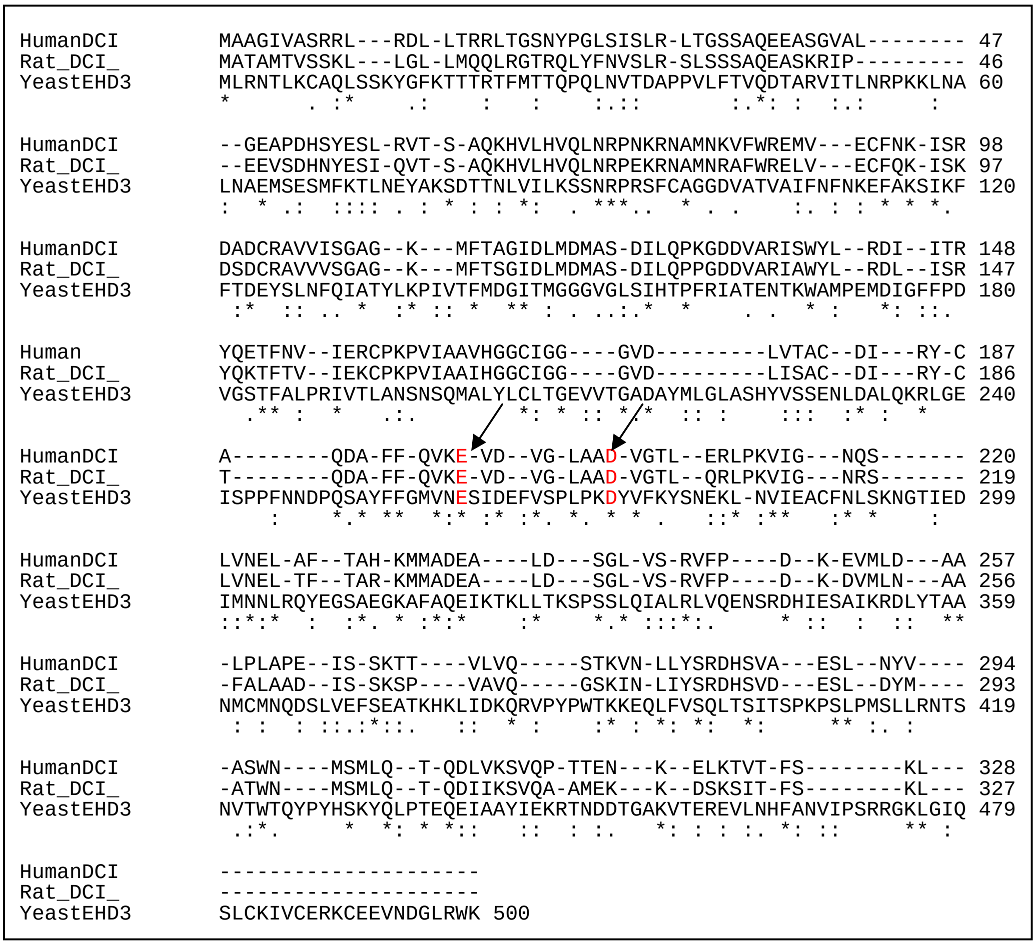


Figure 15. Isomerase/hydratase family domains for YDR036C/Ehd3p and YLR284/Eci1p:

Source: yeast genome database; www.yeastgenome.org



```
HumanDCI        MAAGIVASRRL---RDL-LTRRLTGSNYPGLSISLR-LTGSSAQEEASGVAL-------- 47
Rat_DCI_        MATAMTVSSKL---LGL-LMQQLRGTRQLYFNVSLR-SLSSSAQEASKRIP--------- 46
YeastEHD3       MLRNTLKCAQLSSKYGFKTTTRTFMTTQPQLNVTDAPPVLFTVQDTARVITLNRPKKLNA 60
                *      . :*    .:    :   :    :.::       :.*: :  :.:     :

HumanDCI        --GEAPDHSYESL-RVT-S-AQKHVLHVQLNRPNKRNAMNKVFWREMV---ECFNK-ISR 98
Rat_DCI_        --EEVSDHNYESI-QVT-S-AQKHVLHVQLNRPEKRNAMNRAFWRELV---ECFQK-ISK 97
YeastEHD3       LNAEMSESMFKTLNEYAKSDTTNLVILKSSNRPRSFCAGGDVATVAIFNFNKEFAKSIKF 120
                :  * .:  :::: . : * : : *:  . ***..  * . .    :. : : * * *.

HumanDCI        DADCRAVVISGAG--K---MFTAGIDLMDMAS-DILQPKGDDVARISWYL--RDI--ITR 148
Rat_DCI_        DSDCRAVVVSGAG--K---MFTSGIDLMDMAS-DILQPPGDDVARIAWYL--RDL--ISR 147
YeastEHD3       FTDEYSLNFQIATYLKPIVTFMDGITMGGGVGLSIHTPFRIATENTKWAMPEMDIGFFPD 180
                 :*  :: .. *  :* :: *  ** : . ..:.*  *    . .  * :   *: ::.

Human           YQETFNV--IERCPKPVIAAVHGGCIGG----GVD---------LVTAC--DI---RY-C 187
Rat_DCI_        YQKTFTV--IEKCPKPVIAAIHGGCIGG----GVD---------LISAC--DI---RY-C 186
YeastEHD3       VGSTFALPRIVTLANSNSQMALYLCLTGEVVTGADAYMLGLASHYVSSENLDALQKRLGE 240
                  .** :  *   .:.        *: * :: *.*  :: :    :::  :* :  *

HumanDCI        A--------QDA-FF-QVKE-VD--VG-LAAD-VGTL--ERLPKVIG---NQS------- 220
Rat_DCI_        T--------QDA-FF-QVKE-VD--VG-LAAD-VGTL--QRLPKVIG---NRS------- 219
YeastEHD3       ISPPFNNDPQSAYFFGMVNESIDEFVSPLPKDYVFKYSNEKL-NVIEACFNLSKNGTIED 299
                    :    *.* **  *:* :* :*. *. * * .   ::* :**   :* *    :

HumanDCI        LVNEL-AF--TAH-KMMADEA----LD---SGL-VS-RVFP----D--K-EVMLD---AA 257
Rat_DCI_        LVNEL-TF--TAR-KMMADEA----LD---SGL-VS-RVFP----D--K-DVMLN---AA 256
YeastEHD3       IMNNLRQYEGSAEGKAFAQEIKTKLLTKSPSSLQIALRLVQENSRDHIESAIKRDLYTAA 359
                ::*:*  :  :*. * :*:*    :*    *.* :::*:.     * ::  :  ::  **

HumanDCI        -LPLAPE--IS-SKTT----VLVQ-----STKVN-LLYSRDHSVA---ESL--NYV---- 294
Rat_DCI_        -FALAAD--IS-SKSP----VAVQ-----GSKIN-LIYSRDHSVD---ESL--DYM---- 293
YeastEHD3       NMCMNQDSLVEFSEATKHKLIDKQRVPYPWTKKEQLFVSQLTSITSPKPSLPMSLLRNTS 419
                 : :  : ::.:*::.   ::  * :    :* : *: *:  *:     ** :. :

HumanDCI        -ASWN----MSMLQ--T-QDLVKSVQP-TTEN---K--ELKTVT-FS--------KL--- 328
Rat_DCI_        -ATWN----MSMLQ--T-QDIIKSVQA-AMEK---K--DSKSIT-FS--------KL--- 327
YeastEHD3       NVTWTQYPYHSKYQLPTEQEIAAYIEKRTNDDTGAKVTEREVLNHFANVIPSRRGKLGIQ 479
                 .:*.     *  *: * *::   ::  : :.   *: : : :. *: ::     ** :

HumanDCI        ---------------------
Rat_DCI_        ---------------------
YeastEHD3       SLCKIVCERKCEEVNDGLRWK 500
```

Figure 16**.** Alignment of the deduced amino acid sequence of Ehd3 with human Dci and rat dienoyl-CoA isomerase.

The sequences were aligned using the ClustalW program. Identical amino acids in all three proteins are shown with stars (*), similar amino acids are shown with double dots (:), one dot (.) shows identity or similarity for two proteins only. The amino acid numbers are shown to the right. The two arrows mark the conserved catalytic glutamate (E) and aspartate (D) residues.

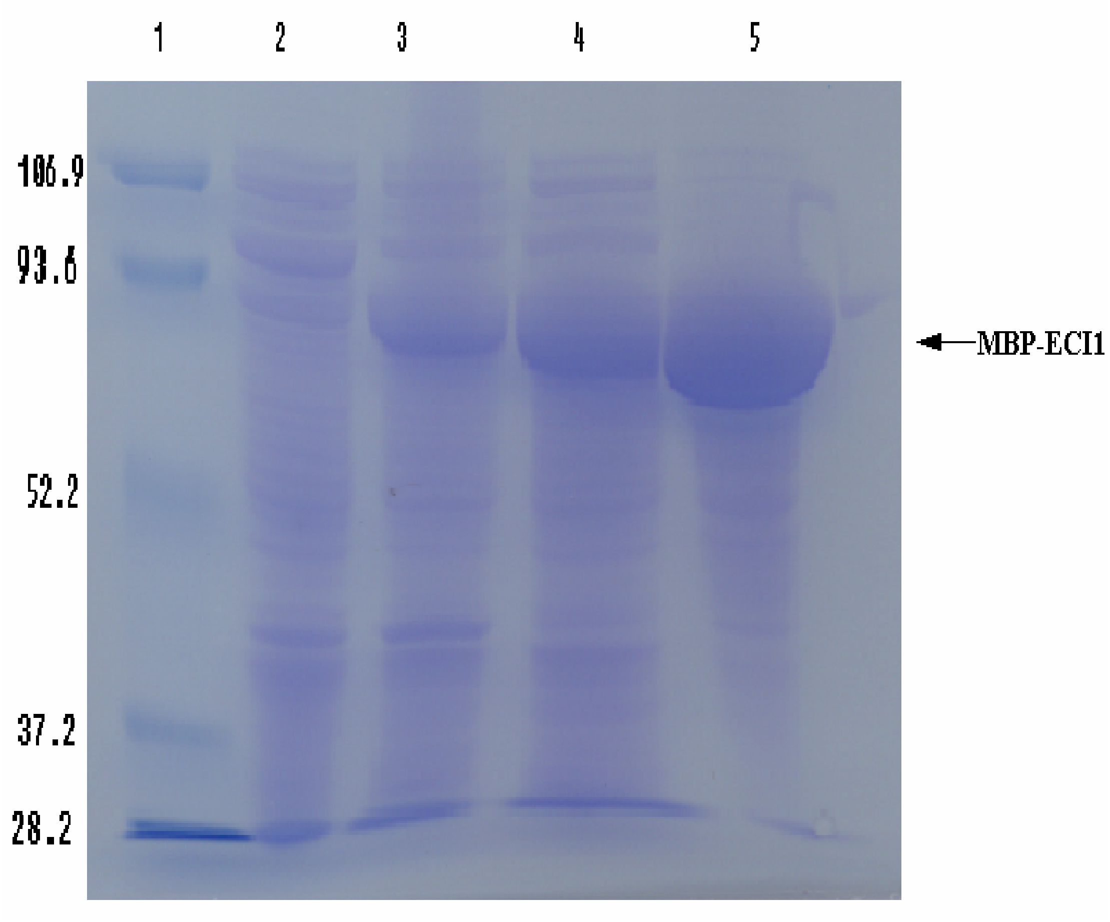


Figure 17. SDS-PAGE of MBP enoyl-CoA isomerase fusion protein after staining with Coomassie blue. Lane: 1, protein markers; 2, homogenate from non-induced cells; 3, homogenate from cells induced with IPTG; 4, soluble extract from cells induced with IPTG; 5, purified MBP enoyl-CoA isemerase fusion protein.

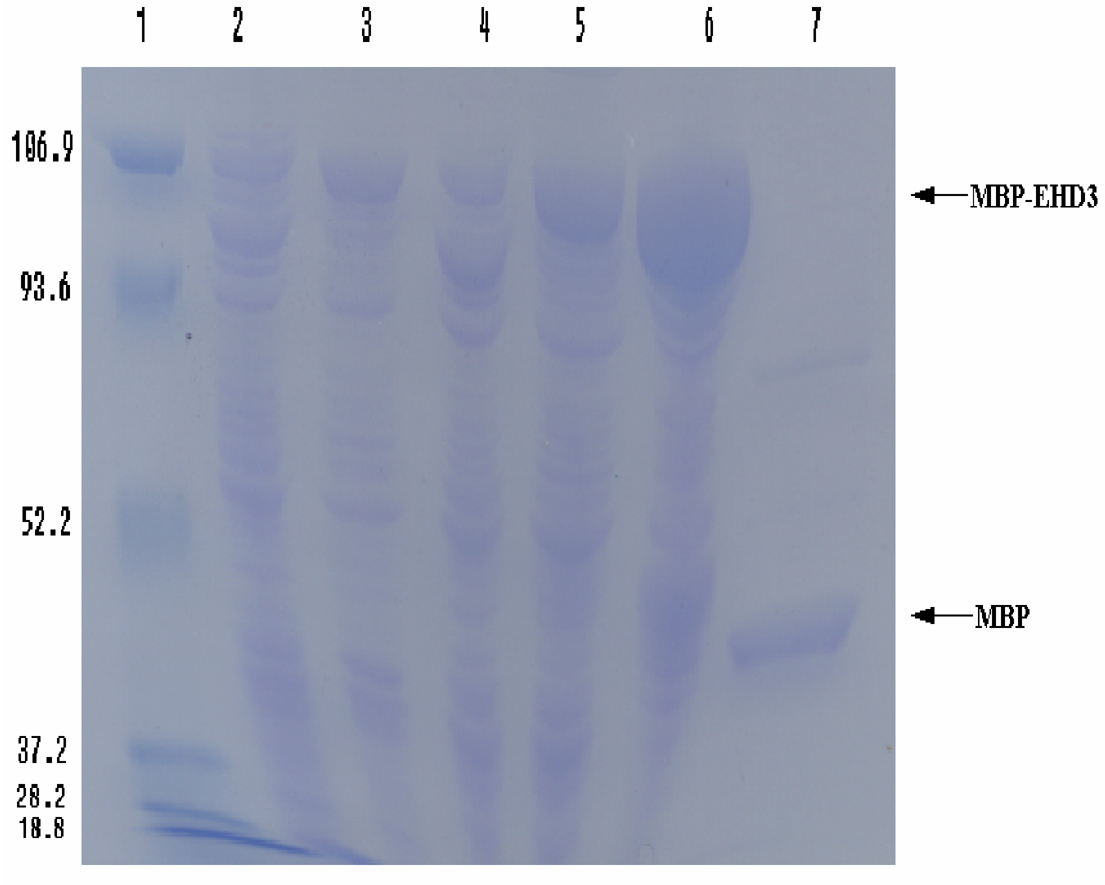


Figure 18. SDS-PAGE of MBP-EHD3p after staining with coomassie blue. Lane: 1- protein markers; 2, homogenate from non-induced cells; 3, homogenate induced with IPTG; 4, soluble extract from non-induced cells; 5, soluble fraction from cells induced with IPTG; 6, purified MBP-EHD3; 7, purified MBP.

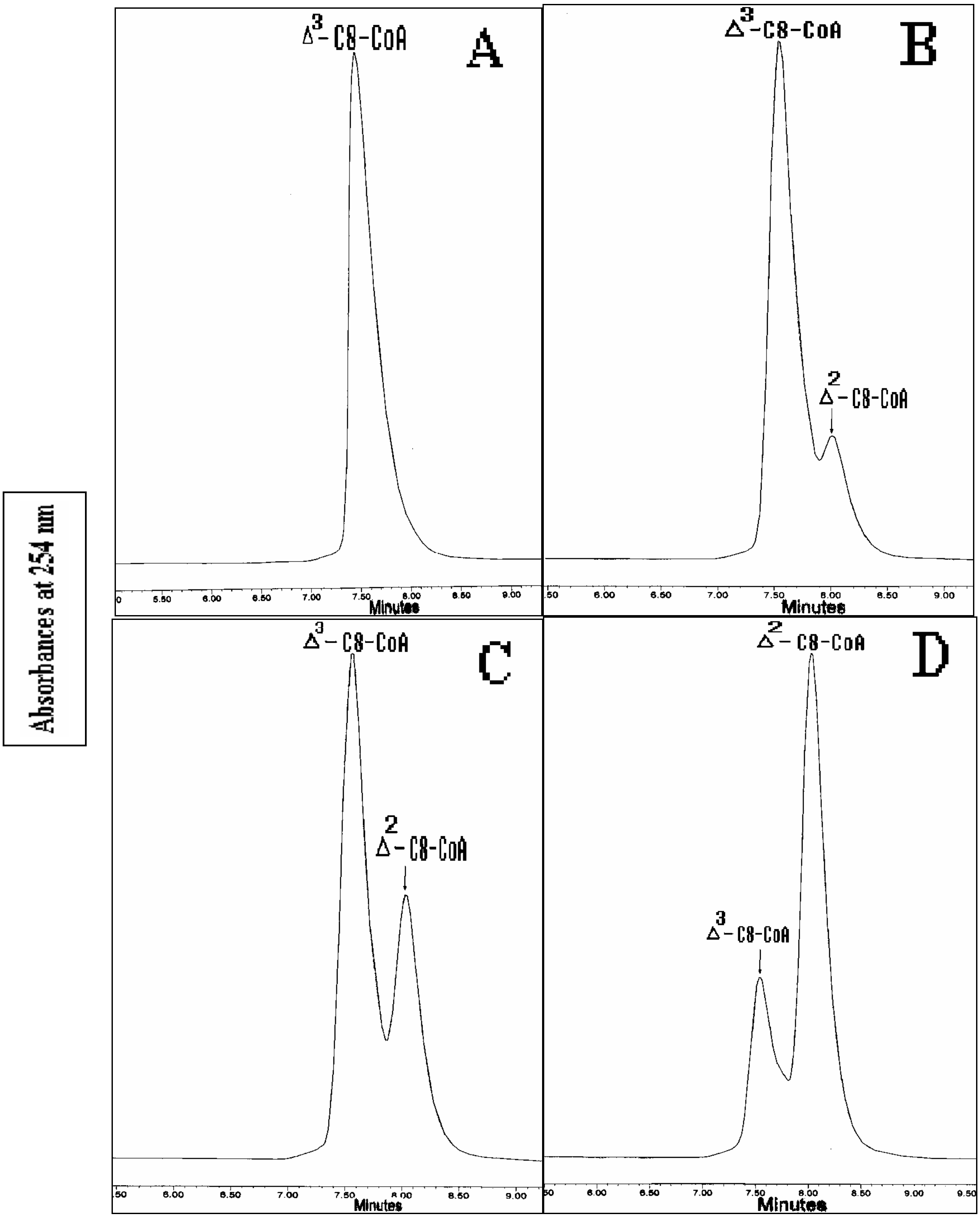


Figure 19. HPLC analysis of the conversion of 3-*trans*-octenoyl-CoA to 2-*trans*-octenoyl-CoA catalyzed by the MBP-enoyl-CoA isomerase fusion protein; **A**-0 minute, **B**- 30 seconds, **C**- 1 minute, **D**- 3 minutes of incubation time.

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