



US 20040014198A1

(19) **United States**

(12) **Patent Application Publication**

Craft

(10) **Pub. No.: US 2004/0014198 A1**

(43) **Pub. Date: Jan. 22, 2004**

(54) **NON-REVERTIBLE BETA-OXIDATION
BLOCKED CANDIDA TROPICALIS**

Publication Classification

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(51) **Int. Cl.⁷** **C12N 1/14**; C12N 1/16
(52) **U.S. Cl.** **435/254.22**; 424/93.51

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(57) **ABSTRACT**

(21) Appl. No.: **10/444,467**
(22) Filed: **May 22, 2003**

Related U.S. Application Data

(60) Provisional application No. 60/383,332, filed on May 23, 2002.

Genetically modified strains of *C. tropicalis*, which will not revert to wild-type activity at the POX 4 and/or POX 5 locus, are disclosed. The strains are β -oxidation blocked and have been transformed through homologous recombination with a construct which deletes a portion of the POX 4 and/or POX 5 gene. The modified strains may be used to increase yields of dicarboxylic acids produced in host cells of the strains. Methods for blocking the β -oxidation pathway in a *C. tropicalis* host cell are also provided.

FIG. 1

POX 4 Sequence

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      10      20      30      40      50      60      70      80
GAGCTCCAATTGTAATATTTCGGGAGAAATATCGTTGGGGTAAACACAGAGAGAGAGAGAGATGGTTCTGGTAG

      90     100     110     120     130     140     150     160
AATTATAATCTGGTTGTTGCAAAATGCTACTGATCGACTCTGGCAATGTCTGTAGCTCGCTAGTTGTATGCAACTTAGGTG

      170     180     190     200     210     220     230     240
TTATGCATACACACGGTTATTTCGGTTGAATTGTGGAGTAAAAATTTGCTGAGTTGTGCTTAGCTACTGGCTGGCCCCC

      250     260     270     280     290     300     310     320
GCGAAAGATAATCAAAATTACACTTGTGAATTTTTTGCACACACACCGATTAAACATTTCCCTTTTTTGTCCACCGATACAC

      330     340     350     360     370     380     390     400
GCTTGCCTCTTCTTTTTTTTCTCTGTGCTTCCCCCTCCTGTGACTTTTTCCACCATTGATATAAAATCAACTCCATTTC

      410     420     430     440     450     460     470     480
CTAAAACTCTCCCAGATTCTAAAACAACTTCTTCTCTCTGCTTTTCTTTTTTTTGTATATTTTACCATCCCTT

      490     500     510     520     530     540     550
TTTTTTTGAATAGTTATTCCCCACTAACATTGTTCAAATCTTCACGACATA ATG ACT TTT ACA AAG AAA AAC
                      M T F T K K N>

      560     570     580     590     600     610
GTT AGT GTA TCA CAA GGT CCT GAC CCT AGA TCA TCC ATC CAA AAG GAA AGA GAC AGC TCC
V S V S Q G P D P R S S I Q K E R D S S>

      620     630     640     650     660     670
AAA TGG AAC CCT CAA CAA ATG AAC TAC TTC TTG GAA GGC TCC GTC GAA AGA AGT GAG TTG
K W N P Q Q M N Y F L E G S V E R S E L>

      680     690     700     710     720     730
ATG AAG GCT TTG GCC CAA CAA ATG GAA AGA GAC CCA ATC TTG TTC ACA GAC GGC TCC TAC
M K A L A Q Q M E R D P I L F T D G S Y>

      740     750     760     770     780     790
TAC GAC TTG ACC AAG GAC CAA CAA AGA GAA TTG ACC GCC GTC AAG ATC AAC AGA ATC GCC
Y D L T K D Q Q R E L T A V K I N R I A>

      800     810     820     830     840     850
AGA TAC AGA GAA CAA GAA TCC ATC GAC ACT TTC AAC AAG AGA TTG TCC TTG ATT GGT ATC
R Y R E Q E S I D T F N K R L S L I G I>

      860     870     880     890     900     910
TTT GAC CCA CAG GTC GGT ACC AGA ATT GGT GTC AAC CTC GGT TTG TTC CTT TCT TGT ATC
F D P Q V G T R I G V N L G L F L S C I>

      920     930     940     950     960     970
AGA GGT AAC GGT ACC ACT TCC CAA TTG AAC TAC TGG GCT AAC GAA AAG GAA ACC GCT GAC
R G N G T T S Q L N Y W A N E K E T A D>

      980     990     1000     1010     1020     1030
GTT AAA GGT ATC TAC GGT TGT TTC GGT ATG ACC GAA TTG GCC CAC GGT TCC AAC GTT GCT
V K G I Y G C F G M T E L A H G S N V A>

      1040     1050     1060     1070     1080     1090
GGT TTG GAA ACC ACC GCC ACA TTT GAC AAG GAA TCT GAC GAG TTT GTC ATC AAC ACC CCA
G L E T T A T F D K E S D E F V I N T P>

      1100     1110     1120     1130     1140     1150
CAC ATT GGT GCC ACC AAG TGG TGG ATT GGT GGT GCT GCT CAC TCC GCC ACC CAC TGT TCT
H I G A T K W W I G G A A H S A T H C S>
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FIG. 1 (cont'd.)

1160	1170	1180	1190	1200	1210
GTC TAC GCC AGA TTG ATT GTT GAC GGT CAA GAT TAC GGT GTC AAG ACT TTT GTT GTC CCA					
V Y A R L I V D G Q D Y G V K T F V V P>					
1220	1230	1240	1250	1260	1270
TTG AGA GAC TCC AAC CAC GAC CTC ATG CCA GGT GTC ACT GTT GGT GAC ATT GGT GCC AAG					
L R D S N H D L M P G V T V G D I G A K>					
1280	1290	1300	1310	1320	1330
ATG GGT AGA GAT GGT ATC GAT AAC GGT TGG ATC CAA TTC TCC AAC GTC AGA ATC CCA AGA					
M G R D G I D N G W I Q F S N V R I P R>					
1340	1350	1360	1370	1380	1390
TTC TTT ATG TTG CAA AAG TTC TGT AAG GTT TCT GCT GAA GGT GAA GTC ACC TTG CCA CCT					
F F M L Q K F C K V S A E G E V T L P P>					
1400	1410	1420	1430	1440	1450
TTG GAA CAA TTG TCT TAC TCC GCC TTG TTG GGT GGT AGA GTC ATG ATG GTT TTG GAC TCC					
L E Q L S Y S A L L G G R V M M V L D S>					
1460	1470	1480	1490	1500	1510
TAC AGA ATG TTG GCT AGA ATG TCC ACC ATT GCC TTG AGA TAC GCC ATT GGT AGA AGA CAA					
Y R M L A R M S T I A L R Y A I G R R Q>					
1520	1530	1540	1550	1560	1570
TTC AAG GGT GAC AAT GTC GAT CCA AAA GAT CCA AAC GCT TTG GAA ACC CAA TTG ATA GAT					
F K G D N V D P K D P N A L E T Q L I D>					
1580	1590	1600	1610	1620	1630
TAC CCA TTG CAC CAA AAG AGA TTG TTC CCA TAC TTG GCT GCT GCC TAC GTC ATC TCC GCT					
Y P L H Q K R L F P Y L A A A Y V I S A>					
1640	1650	1660	1670	1680	1690
GGT GCC CTC AAG GTT GAA GAC ACC ATC CAT AAC ACC TTG GCT GAA TTG GAC GCT GCC GTT					
G A L K V E D T I H N T L A E L D A A V>					
1700	1710	1720	1730	1740	1750
GAA AAG AAC GAC ACC AAG GCT ATC TTT AAG TCT ATT GAC GAC ATG AAG TCA TTG TTT GTT					
E K N D T K A I F K S I D D M K S L F V>					
1760	1770	1780	1790	1800	1810
GAC TCT GGT TCC TTG AAG TCC ACT GCC ACT TGG TTG GGT GCT GAA GCC ATT GAC CAA TGT					
D S G S L K S T A T W L G A E A I D Q C>					
1820	1830	1840	1850	1860	1870
AGA CAA GCC TGT GGT GGT CAC GGT TAC TCG TCC TAC AAC GGC TTC GGT AAA GCC TAC AAC					
R Q A C G G H G Y S S Y N G F G K A Y N>					
1880	1890	1900	1910	1920	1930
GAT TGG GTT GTC CAA TGT ACT TGG GAA GGT GAC AAC AAT GTC TTG GCC ATG AGT GTT GGT					
D W V V Q C T W E G D N N V L A M S V G>					
1940	1950	1960	1970	1980	1990
AAG CCA ATT GTC AAG CAA GTT ATC AGC ATT GAA GAT GCC GGC AAG ACC GTC AGA GGT TCC					
K P I V K Q V I S I E D A G K T V R G S>					
2000	2010	2020	2030	2040	2050
ACC GCT TTC TTG AAC CAA TTG AAG GAC TAC ACT GGT TCC AAC AGC TCC AAG GTT GTT TTG					
T A F L N Q L K D Y T G S N S S K V V L>					
2060	2070	2080	2090	2100	2110
AAC ACT GTT GCT GAC TTG GAC GAC ATC AAG ACT GTC ATC AAG GCT ATT GAA GTT GCC ATC					
N T V A D L D D I K T V I K A I E V A I>					
2120	2130	2140	2150	2160	2170
ATC AGA TTG TCC CAA GAA GCT GCT TCT ATT GTC AAG AAG GAA TCT TTC GAC TAT GTC GGC					
I R L S Q E A A S I V K K E S F D Y V G>					

FIG. 1 (cont'd.)

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      2180      2190      2200      2210      2220      2230
GCT GAA TTG GTT CAA CTC TCC AAG TTG AAG GCT CAC CAC TAC TTG TTG ACT GAA TAC ATC
A E L V Q L S K L K A H H Y L L T E Y I>

      2240      2250      2260      2270      2280      2290
AGA AGA ATT GAC ACC TTT GAC CAA AAG GAC TTG GTT CCA TAC TTG ATC ACC CTC GGT AAG
R R I D T F D Q K D L V P Y L I T L G K>

      2300      2310      2320      2330      2340      2350
TTG TAC GCT GCC ACT ATT GTC TTG GAC AGA TTT GCC GGT GTC TTC TTG ACT TTC AAC GTT
L Y A A T I V L D R F A G V F L T F N V>

      2360      2370      2380      2390      2400      2410
GCC TCC ACC GAA GCC ATC ACT GCT TTG GCC TCT GTG CAA ATT CCA AAG TTG TGT GCT GAA
A S T E A I T A L A S V Q I P K L C A E>

      2420      2430      2440      2450      2460      2470
GTC AGA CCA AAC GTT GTT GCT TAC ACC GAC TCC TTC CAA CAA TCC GAC ATG ATT GTC AAT
V R P N V V A Y T D S F Q Q S D M I V N>

      2480      2490      2500      2510      2520      2530
TCT GCT ATT GGT AGA TAC GAT GGT GAC ATC TAT GAG AAC TAC TTT GAC TTG GTC AAG TTG
S A I G R Y D G D I Y E N Y F D L V K L>

      2540      2550      2560      2570      2580      2590
CAG AAC CCA CCA TCC AAG ACC AAG GCT CCT TAC TCT GAT GCT TTG GAA GCC ATG TTG AAC
Q N P P S K T K A P Y S D A L E A M L N>

      2600      2610      2620      2630      2640      2650
AGA CCA ACC TTG GAC GAA AGA GAA AGA TTT GAA AAG TCT GAT GAA ACC GCT GCT ATC TTG
R P T L D E R E R F E K S D E T A A I L>

      2660      2670      2680      2690      2700      2710      2720
TCC AAG TAA GAATAGAAGAGAGTGACTCTTTTGATAAGAGTCGCAAATTTGATTTCATAAGTATATAT
S K *>

      2730      2740      2750      2760      2770      2780      2790      2800
TCATTATGTAAAGTAGTAAATGGAAAAATTCATTAAAAAAAAGCAAATTTCCGTTGTATGCATACTCCGAACACAAAAC

      2810      2820      2830      2840
AGCCCCGGAAAAACCCCTTAGTTGATAGTTGCGAATTTAGGTCGAC
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Sequence Range: 1 to 1712

10	20	30	40	50	60	70	80
GGTACCGAGC	TCACGAGTTT	TGGGATTTTC	GAGTTTGGAT	TGTTTCCCTT	GTTGATTGAA	TTGACGAAAC	CAGAGGTTTT
90	100	110	120	130	140	150	160
CAAGACAGAT	AAGATTGGGT	TTATCAAAAC	GCAGTTTGAA	ATATTCCAGT	TGGTTTCCAA	GATATCTTGA	AGAAGATTGA
170	180	190	200	210	220	230	240
CGATTTGAAA	TTTGAAGAAG	TGCAGAAGAT	CTGGTTTGGG	TTGTTGGAGA	ATTTCAGAA	TCTCAAGATT	TACTCTAACG
250	260	270	280	290	300	310	320
ACGGGTACAA	CGAGAAATTGT	ATTGAATTGA	TCAAGAACAT	GATCTTGGTG	TTACAGAAAC	TCAAGTTCTT	GGACCAGACT
330	340	350	360	370	380	390	400
GAGAAATGCCA	CAGATATACA	AGGCGTCATG	TGATAAAATG	GATGAGATTT	ATCCCACAAT	TGAAGAAAAG	GTTTATGGAA
410	420	430	440	450	460	470	480
AGTGGTCAAC	CAGAAAGCTAA	ACAGGAAGAA	GCAAAACGAAG	AGGTGAAACA	AGAAAGAAGAA	GGTAAATAAG	TATTTTGTAT
490	500	510	520	530	540	550	560
TATATNACAA	ACAAAAGTAAG	GAATACAGAT	TTATACAATA	AATTGCCATA	CTAGTCACGT	GAGATATCTC	ATCCATTCCC
570	580	590	600	610	620	630	640
CAACTCCCAA	GAAAAAANA	AAGTGAAAAA	AAAAATCAA	CCCAAGATC	AACCTCCCCA	TCATCATCGT	CATCAAAACC

FIG. 2

650 CCAGCTCAAT TCGCAATGGT TAGCACAAAA ACATACACAG AAAGGGCATC AGCACACCCC TCCAAGGTTG CCCAACGTTT
 660 M V S I K T Y T E R A S A H P S K V A Q R L>
 670
 680
 690
 700
 710
 720
 730
 740
 750
 760
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 810
 820
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 900
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 920
 930
 940
 950
 960
 970
 980
 990
 1000
 1010
 1020
 1030
 1040
 1050
 1060
 1070
 1080
 1090
 1100
 1110
 1120
 1130
 1140
 1150
 1160
 1170
 1180
 1190
 1200

ATTCGCTTA ATGGAGTCCA AAAAGACCAA CCTCTGGCC TCGATCGACG TGACCACAAC CGCCGAGTTC CTTTCGCTCA
 F R L M E S K K T N L C A S I D V T T T A E F L S L>
 TCGACAAGCT CGGTCCCCAC ATCTGTCTCG TGAAGACGCA CATCGATATC ATCTCAGACT TCAGCTACGA GGCACGATT
 I D K L G P H I C L V K T H I D I I S D F S Y E G T I>
 GAGCCGTTGC TTGTGCTTGC AGAGGCCAC GGGTTCTTGA TATTCGAGGA CAGGAAGTTT GCTGATATCG GAAACACCCGT
 E P L L V L A E R H G F L I F E D R K F A D I G N T V>
 GATGTTGCAG TACACCTCGG GGTATACCG GATCGCGCGG TGGAGTGACA TCACGAACGC GCACGGAGTG ACTGGGAAGG
 M L Q Y T S G V Y R I A A W S D I T N A H G V T G K>
 GCGTCGTTGA AGGGTTGAAA CGCGGTGCGG AGGGGGTAGA AAAGGAAAGG GCGGTGTGA TGTTCGCGGA GTTGTCCAGT
 G V V E G L K R G A E G V E K E R G V L M L A E L S S>
 AAAGGCTCGT TGGCGCATGG TGAATATACC CGTGAGACGA TCGAGATTGC GAAGAGTGTAT CGGGAGTTTCG TGATTGGGTT
 K G S L A H G E Y T R E T I E I A K S D R E F V I G F>

FIG. 2 (cont'd.)

1210 1220 1230 1240 1250 1260 1270 1280
CATCGCGCAG CCGGACATGG GGGGTAGAGA AGAAGGGTTT GATTGGATCA TCATGACGCC TGGTGTGGG TTGGATGATA
I A Q R D M G G R E E G F D W I I M T P G V G L D D>
1290 1300 1310 1320 1330 1340 1350 1360
AAGGCGATGC GTTGGGCCAG CAGTATAGGA CTGTTGATGA GGTGTTCTG ACTGGTACCG ATGTGATTAT TGTCGGGAGA
K G D A L G Q Q Y R T V D E V V L T G T D V I I V G R>
1370 1380 1390 1400 1410 1420 1430 1440
GGGTTGTTG GAAAAGGAAG AGACCCCTGAG GTGGAGGGAA AGAGATACAG GGATGCTGGA TGGAAAGGCAT ACTTGAAGAG
G L F G K G R D P E V E G K R Y R D A G W K A Y L K R>
1450 1460 1470 1480 1490 1500 1510 1520
AACTGGTCAG TTAGAATAAA TATTGTAATA AATAGGTCTA TATACATACA CTAAGCTTCT AGGACGTCAT TGTAGTCTTC
T G Q L E *>
1530 1540 1550 1560 1570 1580 1590 1600
GAAGTTGTCT GCTAGTTTAG TTCTCATGAT TTCGAAAACC AATAACGCAA TGGATGTAGC AGGATGGTG GTTAGTCCGT
1610 1620 1630 1640 1650 1660 1670 1680
TCCTGACAAA CCCAGAGTAC GCCGCCTCAA ACCACGTCAC ATTCGCCCTT TGCTTCATCC GCATCACTTG CTTGAAGGTA
1690 1700 1710
TCCACGTACG AGTTGTAATA CACCTTGAAG AA

FIG. 2 (cont'd.)

NON-REVERTIBLE BETA-OXIDATION BLOCKED CANDIDA TROPICALIS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims benefit under 35 U.S.C. §119(c) of earlier filed and copending U.S. Provisional Application Serial No. 60/383,332 filed May 23, 2002, the contents of which are incorporated by reference herein.

STATEMENT REGARDING FEDERAL SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was funded, at least in part, under grants from the Department of Commerce, NIST-ATP Cooperative Agreement Number 70NANB8H4033. The Government may therefore have certain rights in the invention.

BACKGROUND OF THE INVENTION

[0003] Aliphatic dioic acids are versatile chemical intermediates useful as raw materials for the preparation of perfumes, polymers, adhesives and macrolid antibiotics. While several chemical routes for the synthesis of long-chain α , ω -dicarboxylic acids are available, the synthesis is not easy and most methods result in mixtures containing shorter chain lengths. As a result, extensive purification steps are necessary. While it is known that long-chain dioic acids can also be produced by microbial transformation of alkanes, fatty acids or esters thereof, chemical synthesis has remained the most commercially viable route, due to limitations with the current biological approaches.

[0004] Several strains of yeast are known to excrete α , Ω -dicarboxylic acids as a byproduct when cultured on alkanes or fatty acids as the carbon source. In particular, yeast belonging to the Genus *Candida*, such as *C. albicans*, *C. cloacae*, *C. guilliermondii*, *C. intermedia*, *C. lipolytica*, *C. maltosa*, *C. parapsilosis* and *C. zeylenoides* are known to produce such dicarboxylic acids (*Agr. Biol. Chem.* 35: 2033-2042 (1971)). Also, various strains of *C. tropicalis* are known to produce dicarboxylic acids ranging in chain lengths from C_{11} through C_{18} (Okino et al., "Production of Macrocyclic Musk Compounds via Alkanedioic Acids Produced From N-Alkanes", from Lawrence, et al. (eds.), *Flavors and Fragrances: A World Perspective*. Proceedings of the 10th International Conference of Essential Oils, Flavors and Fragrances, Elsevier Science Publishers BV Amsterdam, pp.753-760 (1988)), and are the basis of several patents as reviewed by Buhler and Schindler, in *Aliphatic Hydrocarbons in Biotechnology*, H. J. Rehm and G. Reed (eds.), Vol. 169, Verlag Chemie, Weinheim (1984).

[0005] Studies of the biochemical processes by which yeasts metabolize alkanes and fatty acids have revealed three types of oxidation reactions: α -oxidation of alkanes to alcohols, Ω -oxidation of fatty acids to α , Ω -dicarboxylic acids, and the degradative β -oxidation of fatty acids to CO_2 and water. The first two types of oxidations are catalyzed by microsomal enzymes while the last type takes place in the peroxisomes. In *C. tropicalis*, the first step in the Ω -oxidation pathway is catalyzed by a membrane-bound enzyme complex (Ω -hydroxylase complex) including a cytochrome P450 monooxygenase and a NADPH cytochrome reductase. This hydroxylase complex is responsible for the primary

oxidation of the terminal methyl group in alkanes and fatty acids as described, e.g., in Gilewicz et al., *Can. J. Microbiol.* 25:201 (1979), incorporated herein by reference. The genes which encode the cytochrome P450 and NADPH reductase components of the complex have previously been identified as P450ALK and P450RED respectively, and have also been cloned and sequenced as described, e.g., in Sanglard et al., *Gene* 76:121-136 (1989), incorporated herein by reference. P450ALK has also been designated P450ALK1. More recently, ALK genes have been designated by the symbol CYP and RED genes have been designated by the symbol CPR. See, e.g., Nelson, *Pharmacogenetics* 6(1):1-42 (1996), which is incorporated herein by reference. See also Ohkuma et al., *DNA and Cell Biology* 14:163-173 (1995), Seghezzi et al., *DNA and Cell Biology*, 11:767-780 (1992) and Kargel et al., *Yeast* 12:333-348 (1996), each incorporated herein by reference. In addition, CPR genes are now also referred to as NCP genes. See, e.g., De Backer et al., *Antimicrobial Agents and Chemotherapy*, 45:1660 (2001). For example, P450ALK is also designated CYP52 according to the nomenclature of Nelson, supra.

[0006] Fatty acids are ultimately formed from alkanes after two additional oxidation steps, catalyzed by alcohol oxidase as described, e.g., in Kemp et al., *Appl. Microbiol. and Biotechnol.* 28: 370-374 (1988), incorporated herein by reference, and aldehyde dehydrogenase. The fatty acids can be further oxidized through the same or similar pathway to the corresponding dicarboxylic acid. The Ω -oxidation of fatty acids proceeds via the Ω -hydroxy fatty acid and its aldehyde derivative, to the corresponding dicarboxylic acid without the requirement for CoA activation. However, both fatty acids and dicarboxylic acids can be degraded, after activation to the corresponding acyl-CoA ester through the β -oxidation pathway in the peroxisomes, leading to chain shortening. In mammalian systems, both fatty acid and dicarboxylic acid products of β -oxidation are activated to their CoA-esters at equal rates and are substrates for both mitochondrial and peroxisomal β -oxidation (*J. Biochem.*, 102:225-234 (1987)). In yeast, β -oxidation takes place solely in the peroxisomes (*Agr.Biol.Chem.* 49:1821-1828 (1985)).

[0007] The dicarboxylic acids produced through fermentation by most yeasts, including *C. tropicalis*, are most often shorter than the original substrate by one or more pairs of carbon atoms and mixtures are common (Ogino et al., "Studies of Utilization of Hydrocarbons by Yeasts Part II. Diterminal Oxidation of Alkanes by Yeast", *Agr. Biol. Chem.*, Vol. 29, No. 11, pp. 1009-1015 (1965); Shiiro et al., "Microbial Production of Long-chain Dicarboxylic Acids from n-Alkanes, Part I. Screening and Properties of Micro-organism Producing Dicarboxylic Acids", *Agr. Biol. Chem.*, Vol. 35, No. 13, pp. 2033-2012 (1971); Rehm et al., "Mechanisms and Occurrence of Microbial Oxidation of Long-chain Alkanes", *Institute for Microbiology*, pp. 176-217 (1980); Hill et al., "Studies on the Formation of Long-chain Dicarboxylic Acids from Pure n-alkanes by a Mutant of *Candida tropicalis*", *Appl. Microbiol. Biotechnol.*, 24:168-174 (1986). This is due to the degradation of the substrate and product by the peroxisomal β -oxidation pathway. This series of enzymatic reactions leads to the progressive shortening of the activated acyl-CoA through the cleavage of 2 carbon acetyl-CoA moieties in a cyclic manner. The initial step in the pathway, involving oxidation of the acyl-CoA to its enoyl-CoA derivative, is catalyzed by acyl-CoA oxidase.

The enoyl-CoA is further metabolized to the β -keto acid by the action of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase as a prerequisite to the cleavage between the α and β carbons by 3-ketoacyl-CoA thiolase. Mutations causing partial blockage of these latter reactions result in the formation of unsaturated or 3-hydroxy-monocarboxylic or 3-hydroxy-dicarboxylic acids (Meussdoeffler, 1988). These undesirable by-products are often associated with biological production of dicarboxylic acids.

[0008] It is known that the formation of dioic acids can be substantially increased by the use of suitable mutants (Shiio et al., supra; Furukawa et al., "Selection of High Brassylic Acid Producing Strains of *Torulopsis candida* by Single-Cell Cloning and by Mutation", *J. Ferment. Technol.*, Vol. 64, No. 2, pp. 97-101 (1986); Hill et al., supra; Okino et al., supra). While the wild-type yeasts produce little if any dicarboxylic acids, mutants partially defective in their ability to grow on alkane, fatty acid or dicarboxylic acid substrates often demonstrate enhanced dicarboxylic acid yields. However, these mutants have not been characterized beyond their reduced ability to utilize these compounds as a carbon source for growth. In all likelihood, their ability to produce dicarboxylic acids is enhanced by a partial blockage of the β -oxidation pathway. It is also known that compounds known to inhibit β -oxidation (i.e. acrylate) also result in increased dicarboxylic acid yields. Jianlong et al., "The Regulation of Alanine on the Fermentation of Long-Chain Dicarboxylic Acids in *Candida tropicalis* NPcoN22", p.4 (1988).

[0009] β -oxidation blocked *C. tropicalis* strains, such as H5343 (ATCC No. 20962) are disclosed in U.S. Pat. No. 5,254,466, the entire contents of which are incorporated herein by reference. These *C. tropicalis* strains have disrupted chromosomal POX 4A, POX 4B and both POX 5 genes. The disruption of the POX genes was performed by insertional disruption in which the URA3 nutritional marker was inserted into a construct containing the gene(s) encoding the acyl CoA oxidase, POX 4 or POX 5, and transformed into the target organism. Homologous recombination yielded organisms that were disrupted for POX 4 and POX 5.

[0010] The POX 4 and POX 5 genes encode distinct subunits of long chain acyl-CoA oxidase, which are the peroxisomal polypeptides (PXPs) designated PXP-4 and PXP-5, respectively. The disruption of the POX 4 and POX 5 genes encoding these PXPs effectively blocks the β -oxidation pathway at its first reaction (which is catalyzed by acyl-CoA oxidase), thereby redirecting the substrate toward the Ω -oxidation pathway while preventing the degradation and/or reutilization of the dicarboxylic acid products by the β -oxidation pathway. Therefore, a *C. tropicalis* strain in which all four POX genes are disrupted will synthesize substantially pure α , Ω -dicarboxylic acids with increased quantitative yield because the biosynthetic pathway which produces undesirable chain modifications associated with passage through the β -oxidation pathway, such as unsaturation, hydroxylation, or chain shortening, is no longer functional.

[0011] *C. tropicalis* strains may also have one or more cytochrome P450 (P450ALK) genes and/or reductase (P450RED) genes amplified, which results in an increase in the amount of rate-limiting Ω -hydroxylase through P450

gene amplification and increases the rate of substrate flow through the Ω -oxidation pathway. Specific examples of CPR (reductase) genes include the CPRA and CPRB genes of *C. tropicalis* 20336 as described, e.g., in U.S. Pat. No. 6,331,420 and International Application No. PCT/US99/20797, the contents of each of which are incorporated herein by reference. Other known *C. tropicalis* strains include AR40, an amplified H5343 strain wherein all four POX genes are disrupted by a URA3 selectable marker, which also contains 3 additional copies of the cytochrome P450 gene and 2 additional copies of the reductase gene, the P450RED gene. Strain R24 is an amplified H5343 strain in which all four POX genes are disrupted by a URA3 selectable marker and which also contains multiple copies of the reductase gene. Strains AR40 and R24 are described in U.S. Pat. Nos. 5,620,878 and 5,648,247, the contents of each of which are incorporated by reference herein.

[0012] These strains have been the basis for newly modified strains created and employed for use in the dicarboxylic acid production process for a number of years. However, upon fermentation of fatty acid and/or alkane substrates using β -oxidation blocked yeast strains, such strains have shown reversion at the POX 4 locus. Upon reversion to a wild-type POX 4 gene, the β -oxidation pathway is no longer blocked and a decrease in dicarboxylic acid production results. Therefore, a need exists for improved yeast strains comprising a stable POX 4 disruption that will not revert to wild-type activity.

SUMMARY OF THE INVENTION

[0013] Novel yeast strains, having their β -oxidation pathway blocked and a portion of a chromosomal target gene deleted, are disclosed. The deletion of the portion of the chromosomal target gene is of sufficient size that it prevents the strain from reverting to wild-type activity.

[0014] In a preferred embodiment, the yeast strain is *C. tropicalis* and the chromosomal target gene is the POX 4 gene or the POX 5 gene. The deletion of a portion of the POX 4 or POX 5 genes results in a non-revertible β -oxidation blocked *C. tropicalis* strain. More preferred embodiments also have disruptions of other genes of the β -oxidation pathway and amplifications of the cytochrome P450 genes and/or reductase genes. The resulting strains thus have both a blocked β -oxidation pathway as well as an increase in the amount of rate-limiting Ω -hydroxylase, which increases the rate of substrate flow through the Ω -oxidation pathway and results in the production of substantially pure α , Ω -dicarboxylic acids.

[0015] The POX 4 gene may be POX 4A, POX 4B, or both POX 4A and POX 4B. The POX 5 gene may, in some embodiments, be both copies of the chromosomal POX 5 gene. In some embodiments, the deletion may be to POX 4A, POX 4B, POX 5, both copies of the chromosomal POX 5 gene, or any combination thereof.

[0016] Preferably, the deletion of the portion of the chromosomal target gene, i.e., POX 4 or POX 5, occurs by homologous recombination of said POX 4 or POX 5 gene with a DNA fragment comprised of a selectable marker gene flanked on both ends by DNA sequences having homology to said POX 4 or POX 5 gene, but said DNA sequences are not contiguous. The resulting vector is referred to herein as a POX 4-deleted disruption cassette or a POX 5-deleted

disruption cassette. The selectable marker is one which confers a particular phenotype to the cell into which the DNA fragment is transformed. More preferably, the selectable marker confers a prototrophic phenotype to transformed cells, such as for a particular pyrimidine, whereby the transformed cells may then be selected by their ability to grow in a medium deficient in the pyrimidine. In a most preferred embodiment the selectable marker gene is URA 3.

[0017] In accordance with the present invention, a yeast host cell transformed with a POX 4-deleted disruption cassette has its chromosomal POX 4 gene replaced with a partially deleted POX 4 gene. Likewise, a yeast host cell transformed with a POX 5-deleted disruption cassette has its chromosomal POX 5 gene replaced with a partially deleted POX 5 gene. In either case, the resulting yeast strain has its β -oxidation pathway blocked and will not revert to wild-type activity.

[0018] In a particularly preferred embodiment, *C. tropicalis* H53 is transformed with a POX 4-deleted disruption cassette comprised of a 1676 bp URA 3 selectable marker gene which contains a 647 bp promoter, 803 bp ORF and 226 bp UTR flanked by two non-contiguous flanking sequences from POX 4. When integrated into the *C. tropicalis* genome, the POX 4-deleted disruption cassette deletes 653 bp of the POX 4 ORF and inserts the 1676 bp URA 3 selectable marker. The resulting transformed *C. tropicalis* strains containing the deleted POX 4 alleles in the H53 (pox5/pox5) are called HDC100.

[0019] Another aspect of the present invention provides methods for producing transformed yeast strains having their β -oxidation pathway by having a portion of one or more genes of the β -oxidation pathway deleted. Preferably, these strains are produced by transforming yeast host cells with a vector which, after homologous recombination with the yeast genome, results in a deletion of a portion of a target gene in the host cell. More preferably, the target gene is POX 4 or POX 5, and the vectors are referred to as a POX 4-deleted disruption cassette or a POX 5-deleted disruption cassette, respectively. A POX 4-deleted disruption cassette comprises a DNA fragment having homology to a portion of the POX 4 gene, a selectable marker gene, and a DNA fragment having homology to a different portion of the POX 4 gene. A POX 5-deleted disruption cassette comprises a DNA fragment having homology to a portion of the POX 5 gene, a selectable marker gene, and a DNA fragment having homology to a different portion of the POX 5 gene. A yeast host cell transformed with a POX 4-deleted disruption cassette has its chromosomal POX 4 gene replaced with a partially deleted POX 4 gene and the selectable marker gene. Likewise, a yeast host cell transformed with a POX 5-deleted disruption cassette as described has its chromosomal POX 5 gene replaced with a partially deleted POX 5 gene and the selectable marker gene.

[0020] Yet another aspect of the present invention provides a method of increasing the specific productivity of substantially pure α , β -dicarboxylic acids in increased quantitative yield by fermenting culture media with a transformed yeast strain having its β -oxidation pathway blocked and a portion of a chromosomal target gene deleted. In a preferred embodiment, the transformed yeast strain comprises a *C. tropicalis* host having a portion of its POX 4 gene deleted. In some embodiments, the *C. tropicalis* strain may

have all of its POX 4 and POX 5 genes disrupted, including the deletion of a portion of the POX 4 gene, and may also have one or more cytochrome P450 (P450ALK) genes and/or reductase (P450RED) genes amplified. Preferably, the culture medium contains a nitrogen source, an organic substrate and a cosubstrate. In a more preferred embodiment, the nitrogen source of the culture medium is an inorganic or organic source of nitrogen normally used in processes for culturing microorganisms; the organic substrate is an aliphatic compound wherein at least one terminal carbon of the aliphatic compound is a methyl group and the aliphatic compound has from about 4 to about 22 carbon atoms; and the cosubstrate is selected from the group consisting of glucose, fructose, maltose, glycerol and sodium acetate.

BRIEF DESCRIPTION OF THE FIGURES

[0021] FIG. 1 shows the complete DNA sequence including regulatory and coding regions for the POX 4 gene of *C. tropicalis*.

[0022] FIG. 2 is the complete DNA sequence encoding URA3A from *C. tropicalis* ATCC 20336.

DETAILED DESCRIPTION OF THE INVENTION

[0023] The present invention provides improved yeast strains possessing blocked β -oxidation pathways. The subject yeast strains have a portion of one or more genes of the β -oxidation pathway deleted and are an improvement in the art since reversion to wild-type activity is avoided. Preferably, the yeast strains belong to the Genus *Candida*. Examples of *Candida* species that may be used in accordance with the present invention include, but are not limited to, *C. albicans*, *C. cloacae*, *C. guillermoidii*, *C. intermedia*, *C. lipolytica*, *C. maltosa*, *C. parapsilosis* and *C. zeylanoides*. In a more preferred embodiment, the yeast is *C. tropicalis*.

[0024] In one embodiment, the present invention comprises yeast strains possessing a deletion of the chromosomal POX 4 gene. The size of the deletion may vary, but is sufficient to prevent reversion to wild-type activity at the POX 4 locus once the yeast strain is subjected to the stresses of fermentation. While the deletion could comprise as little as 1 (one) nucleotide, preferably the yeast strain has from about 400 to about 900 nucleotides, or base pairs (bp), of the POX 4 gene deleted. More preferably, a subject yeast strain possesses a deletion of the POX 4 gene of about 500 to about 800 bp. Even more preferably, the deletion comprises about 650 bp of the POX 4 gene. In a most preferred embodiment, the deletion is a 653 bp sequence from the POX 4 gene corresponding to nucleotides 1178-1829 of the POX 4 gene sequence (See FIG. 1).

[0025] The improved yeast strains of the present invention are produced by transforming yeast host cells with a vector which, after homologous recombination with the yeast genome, results in a deletion of a portion of a target gene in the host cell. For example, a POX 4-deleted disruption cassette may be used to replace a yeast chromosomal POX 4 gene. Likewise, a POX 5-deleted disruption cassette may be used to replace a yeast chromosomal POX 5 gene.

[0026] A vector is defined as any linear or circular nucleic acid molecule capable of introducing a foreign nucleic acid

molecule into a host cell. Preferably, the vector is an integrating vector. An integrating vector is one which has a nucleic acid sequence with homology to a target site in the genome of the host cell so that vector nucleic acids may be integrated into the nuclear DNA of the host cell.

[0027] Linear DNA vectors may be advantageous when it is desirable to avoid introduction of non-native (foreign) DNA into the cell. For example, DNA consisting of a desired target gene(s) flanked by DNA sequences which are native to the cell may be introduced into the cell by electroporation, lithium acetate transformation, spheroplasting, and the like. Flanking DNA sequences may include selectable markers and/or other tools for genetic engineering.

[0028] In order to produce a non-revertible β -oxidation blocked yeast strain, an integrating vector with homology to a target site in the genome of the host cell may be used. Preferably, the nucleic acid sequence contained in the vector will include a selectable marker gene, a non-functional copy, or copies, of which may also be present in the nuclear DNA of the host. The fact that the nucleic acid sequence in the vector has homology to some portion of the host's genome makes it integratable into the genome in the region of homology. More preferably, the nucleic acid sequence is DNA. As used herein, the term "integratable" means capable of being inserted into a host genome as a region covalently linked on either side to the host sequences. The region of the host genome into which the nucleic acid sequence from the vector is integrated is defined as the target site. In accordance with the present invention, the target site is a gene which encodes an enzyme involved in the β -oxidation pathway. Preferably the target gene is POX 4 or POX 5. Most preferably, the target gene is POX 4.

[0029] The vector used to produce a non-revertible β -oxidation blocked yeast strain comprises a target gene with a deletion contained therein. Such vectors may include serially arranged linear DNA fragments comprised of a DNA fragment which has homology to a gene encoding an enzyme of the β -oxidation pathway in the native yeast genome, a selectable marker gene, and a DNA fragment which has homology to a different portion of the gene encoding an enzyme of the β -oxidation pathway in the native yeast genome. The selectable marker is, therefore, flanked on both ends by DNA sequences which are homologous to a gene in the native yeast genome which encodes an enzyme of the β -oxidation pathway, but which sequences are not contiguous.

[0030] For example, a vector may comprise a POX 4-deleted disruption cassette or a POX 5-deleted disruption cassette. A POX 4-deleted disruption cassette comprises a DNA fragment having homology to a portion of a POX 4 gene, a selectable marker gene, and a DNA fragment having homology to a different portion of the POX 4 gene. A POX 5-deleted disruption cassette comprises a DNA fragment having homology to a portion of a POX 5 gene, a selectable marker gene, and a DNA fragment having homology to a different portion of the POX 5 gene. A yeast host cell transformed with a POX 4-deleted disruption cassette as described has its chromosomal POX 4 gene replaced with a partially deleted POX 4 gene. Likewise, a yeast host cell transformed with a POX 5-deleted disruption cassette as described has its chromosomal POX 5 gene replaced with a partially deleted POX 5 gene.

[0031] A disruption cassette can be constructed by sub-cloning a selectable marker into DNA sequences corresponding to a target gene having a deletion. Any type of selectable marker which is extraneous to the target gene may be used. Preferably, the selectable marker is one which confers a particular phenotype to the cell into which the disruption cassette is transformed. Most preferably, the selectable marker confers a prototrophic phenotype to transformed cells which can be reversibly changed to auxotrophy, if desired, so that the same selectable marker may be subsequently used in multiple gene disruptions in the same strain.

[0032] For example, a *C. tropicalis* transformation host which is auxotrophic for a particular pyrimidine may be transformed to prototrophy by a POX 4 or POX 5-deletion disruption cassette containing a functional selectable marker gene required for the synthesis of the particular pyrimidine. The resulting transformants, which have been made prototrophic for said particular pyrimidine, are selected by their ability to grow in a medium deficient in the pyrimidine. These transformants contain a targeted gene deletion as the result of the replacement of a functional target gene with a nonfunctional target gene which includes said deletion.

[0033] Where the yeast is *C. tropicalis*, suitable marker genes include, but are not limited to, URA 3A, URA 3B, or HIS 4 genes. In the case where the selectable marker is a URA gene, a host cell is auxotrophic for uracil; where the selectable marker is a HIS 4 gene, a host cell is auxotrophic for histidine.

[0034] In a preferred embodiment, a *C. tropicalis* transformation host, auxotrophic for uracil (Ura⁻), is transformed to uracil prototrophy with a disruption cassette containing a URA 3 functional gene as the selectable marker. The transformed cells are selected by their ability to grow in the absence of uracil.

[0035] In those cases where the yeast transformation host cell is already prototrophic for a specific phenotype, e.g., uracil, confirmation of the pox- phenotype in the resulting transformed strain may be confirmed by the inability of the strain to grow on media containing alkanes, such as decane and dodecane, as the sole carbon source.

[0036] Preferably, a significant portion of the open reading frame (ORF) of the target gene, i.e., POX 4, is removed prior to or during the insertion of the selectable marker gene, i.e., URA 3. The selectable marker gene is flanked on one end by a 5'-POX 4 sequence and on the other end by a 3'-POX 4 sequence. The 5'-POX 4 sequence and the 3'-POX 4 sequence are not contiguous and correspond to different portions of the POX 4 gene. Accordingly, transformed cells lack the nucleotides of the POX 4 gene located in the region of the genome between the two POX 4 flanking sequences. By having deleted a portion of the target gene's ORF, removal of the selectable marker gene will not result in reversion to wild-type function, i.e., β -oxidation.

[0037] In a particularly preferred embodiment, *C. tropicalis* H53 may be transformed with a POX 4 disruption cassette comprised of a 1676 bp URA 3 selectable marker gene which contains a 647 bp promoter, 803 bp ORF and 226 bp UTR flanked by two non-contiguous flanking sequences from POX 4. When recombined into the *C. tropicalis* genome, the POX 4 disruption cassette results in

a yeast having a 653 bp deletion in the coding region of the POX 4 ORF, and an insertion of the 1676 bp URA 3 selectable marker. The resulting transformed *C. tropicalis* strains containing the deleted POX 4 alleles in the H53 (pox5/pox5) are called HDC100. HDC100 is a non-revertible (pox4Δ:: URA3/pox4A:: URA3 pox5/pox5) strain. Since the transformed cells have all four POX 4 and POX 5 genes disrupted, they are β -oxidation blocked as there is little or no production of acyl-CoA oxidase, an enzyme required in the initial step of the β -oxidation pathway. The deletion of the portion of the POX 4 gene prevents further recombination to a POX 4 gene encoding a functional protein. Reversion to wild-type activity and reutilization of the dicarboxylic acid products through the β -oxidation pathway is therefore avoided. Accordingly, the resulting HDC 100 strains produce little if any unwanted side products such as β -hydroxy acids, unsaturated acids, or shorter chain acids.

[0038] The resulting β -oxidation blocked *C. tropicalis* cell is a genetically modified *C. tropicalis* strain wherein a portion of one or more genes of the β -oxidation pathway has been deleted. Preferably, the deletion is to a portion of the chromosomal POX 4 or POX 5 genes, and in some embodiments other genes of the β -oxidation pathway have also been disrupted. While the process of the present invention may be used with a wild-type strain, it is especially suitable for use with strains in which the β -oxidation pathway is already blocked or partially blocked. The following strains may be transformed using the methods described herein: H53, in which both POX 5 genes are disrupted; H5343, in which all four POX 4 and POX 5 genes are disrupted; H41, in which the POX 4A gene is disrupted; H41B, in which the POX 4B gene is disrupted; H43, in which both POX 4 genes are disrupted; H435, in which one of the POX 5 genes and both POX 4 genes are disrupted; H51, in which one POX 5 gene is disrupted; H45, in which one POX 5 and the POX 4A genes are disrupted; H534, in which both copies of POX 5 and the POX 4A genes are disrupted; and H534B, in which both copies of POX 5 and the POX 4B genes are disrupted. Following the teachings of the present invention, any of the strains listed hereinabove may be transformed so that one or more alleles at the POX 4 or POX 5 locus is not only disrupted but also contains a deletion. The deleted portion of the POX 4 or POX 5 genes prevents the strain from reverting to wild-type activity.

[0039] In another embodiment, the *C. tropicalis* strain may also have one or more cytochrome P450 (P450ALK) genes and/or reductase (P450RED) genes amplified, which results in an increase in the amount of rate-limiting Ω -hydroxylase through P450 gene amplification and an increase in the rate of substrate flow through the Ω -oxidation pathway. In a preferred embodiment, CPR (reductase) genes, including the CPRA and CPRB genes of *C. tropicalis* 20336 as described in U.S. Pat. No. 6,331,420 and International Application No. PCT/US99/20797, are included.

[0040] Another aspect of the present invention involves using these transformed yeast strains to produce dicarboxylic acids. In a preferred embodiment, the substrate flow in transformed *C. tropicalis* strains is redirected to the Ω -oxidation pathway as the result of functional inactivation of the competing β -oxidation pathway by POX gene disruption and deletion. Most preferred embodiments utilize the yeast strain HDC100, which is β -oxidation blocked and will not revert to wild-type activity as a result of a deletion of a

portion of the POX 4 gene. These strains, when cultured on a suitable medium, demonstrate an increase in the specific production of substantially pure α , Ω -dicarboxylic acids.

[0041] While suitable media conducive to the production of α , Ω -dicarboxylic acids may readily be determined by those skilled in the art, in a most preferred embodiment the medium contains a nitrogen source, an organic substrate and a cosubstrate.

[0042] The nitrogen source of the culture medium may be any inorganic or organic source of nitrogen normally used in processes for culturing microorganisms. Inorganic nitrogen sources include alkali metal nitrates such as sodium or potassium nitrate, ammonium salts such as ammonium sulfate, ammonium chloride, ammonium nitrate, ammonium acetate, etc. Organic nitrogen sources include urea, corn steep liquor, yeast extracts, and other organic nitrogen sources known to those skilled in the art.

[0043] The organic substrate of the culture medium can be any aliphatic compound having from about 4 to about 22 carbon atoms wherein at least one of the terminal carbons is a methyl group, a terminal carboxyl group and/or a terminal functional group which is oxidizable to a carboxyl group by biooxidation. Such compounds include alkanes, alkenes, alkynes, carboxylic acids and their esters, and arenes. Preferred substrates are alkanes having from about 4 to about 22 carbon atoms and fatty acids and their methyl or ethyl esters wherein the acyl portion contains from about 4 to about 22 carbon atoms. The most preferred substrates are dodecane, tridecane, tetradecane, oleic acid, methyl oleate, methyl palmitate, methyl palmitoleate and methyl myristate.

[0044] The cosubstrate may include glucose, fructose, maltose, glycerol, sodium acetate and combinations thereof. The preferred cosubstrate is glucose. A cosubstrate is necessary because the β -oxidation pathway of *C. tropicalis* strains used in the methods of the invention are totally or partially blocked, and energy is not available from the oxidation of the substrate. Adding glucose at a defined rate along with the substrate strikes a balance between providing an energy source for the cells while allowing the partial oxidation of the substrate to an α , Ω -dicarboxylic acid.

[0045] The following examples will serve to illustrate but not limit the invention.

EXAMPLES

[0046] A POX 4 disruption construct was designed to delete 653 bp of the POX 4 ORF while inserting a 1676 bp (647 bp promoter, 803 bp ORF and 226 bp UTR) URA 3 selectable marker gene. PCR primers were designed so as to amplify the two flanking regions (590 bp and 815 bp) of the POX 4 ORF. These primers included restriction sensitive cloning sites so as to facilitate cloning of the URA 3 gene. Upon successful PCR of the flanking POX 4 regions, the DNA was restricted, gel-isolated and used in a ligation to generate the desired POX 4 disruption construct. This construct was used to transform *C. tropicalis* H53 (pox5/pox5) ura- to a pox4/pox4 URA+phenotype. Four *C. tropicalis* pox5/pox5 pox4/pox4 homozygotes were confirmed by Southern analysis. Moreover, these strains were unable to grow on dodecane/decane indicating their pox-phenotype. These strains were labeled HDC100-1,-2,-3, and -4.

Example 1

Generation of POX 4 Flanking Sequences

[0047] In order to generate the POX 4 flanking sequences to direct integration of the disruption cassette, two sets of PCR primers were designed.

```
Set #1 (5' to 3')
218-27a-AGGCGCGCCCATCCAAAAGGAAAGACAGC (SEQ ID NO: 1)
218-27b-CCTTAATTAACGTCACAATCAATCTGGCGTAGAC (SEQ ID NO: 2)
Set #2 (5' to 3')
218-27c-CCTTAATTAATCAGCGTTACTCGTCTACAACGG (SEQ. ID NO. 3)
218-27d-AGCTTTGTTTAAACGACGCGGTTTCATCAGACTTTTC (SEQ. ID NO. 4)
```

[0048] PCR fragments of these two flanking sequences were purified, restricted with *Asc*I, *Pac*I and *Pme*I restriction enzymes (*Asc*I and *Pac*I for the Set #1 fragment, and *Pac*I and *Pme*I for the Set #2 fragment) and ligated to a gel purified, QiaexII cleaned *Asc*I-*Pme*I digest of plasmid pNEB193 purchased from New England Biolabs (Beverly, Mass.). The ligation was performed with an equimolar number of DNA termini at 16° C. for 16 hours using T4 DNA ligase (New England Biolabs). Ligations were transformed into *E. coli* XL1-Blue cells (Stratagene, LaJolla, Calif.) according to manufacturers recommendations. White colonies were isolated, grown, plasmid DNA isolated and digested with *Asc*I-*Pme*I to confirm insertion of the flanking sequences into pNEB193.

[0049] Restriction enzyme-sensitive cloning sites were incorporated into each primer as shown in italics. Primer set #1 was used to amplify a region of DNA from base pair 587-1177. Likewise, primer set #2 was used to amplify a region of DNA from base pair 1830-2645.

[0050] These fragments, when ligated to a nutritional marker (URA 3) produced in accordance with Example 2 below and upon integration into the *Candida tropicalis* genome, yielded a deletion of 653 base pairs of the POX 4 ORF from nucleotides 1178-1829 (see **FIG. 1**).

Example 2

Generation of a URA 3 Selectable Marker

[0051] A primer set was made to amplify a 1676 base pair URA 3 containing *Pac*I restriction sensitive cloning sites.

```
(5' TO 3')
Primer 179a-CCTTAATTAAGCTCAGAGTTTGGGATTTTC (SEQ. ID. NO: 5)
Primer 179b-CCTTAATTAATGGATACCTTCAAGCAAGTG (SEQ. ID. NO. 6)
```

[0052] Primers were designed and synthesized based on the 1712 bp sequence of the URA 3A gene of *C. tropicalis* 20336 (see **FIG. 2**). Primers 179a and 179b described above were used in PCR with *C. tropicalis* 20336 genomic DNA to amplify URA 3A sequences between nucleotides 9 and 1685

as shown in **FIG. 2**. These primers were also designed to introduce a unique *Pac*I restriction site into the resulting amplified URA 3A fragment. PCR fragments of the URA 3A gene were purified, and restricted with *Pac*I restriction enzymes. The resulting vector possessed a 1676 bp (647 bp promoter, 803 bp ORF and 226 bp UTR) URA 3 selectable marker gene with *Pac*I restriction-sensitive cloning sites.

EXAMPLE 3

Creation of a pox4-Deleted Disruption Construct

[0053] In order to make this disruption construct, the 1676 bp URA 3 selectable marker gene with *Pac*I restriction-sensitive cloning sites produced in accordance with Example 2 was cloned into the *Pac*I restricted and dephosphorylated pNEB193 vector containing the POX 4 flanking fragments produced in accordance with Example 1. In a preferred aspect of the present invention, no foreign DNA other than that specifically provided by synthetic restriction site sequences was incorporated into the DNA which was cloned into the genome of *C. tropicalis*, i.e., with the exception of restriction site DNA only native *C. tropicalis* DNA sequences were incorporated into the genome.

[0054] The *Pac*I restricted pNEB193 vector containing the POX 4 flanking fragments produced in accordance with Example 1 was digested with *Pac*I, Qiaex II cleaned, and dephosphorylated with Shrimp Alkaline Phosphatase (SAP) (United States Biochemical, Cleveland, Ohio) according to the manufacturer's recommendations. Approximately 500 ng of *Pac*I linearized pNEB193 vector containing the POX 4 flanking fragments was dephosphorylated for 1 hour at 37° C. using SAP at a concentration of 0.2 Units of enzyme per 1 pmol of DNA termini. The reaction was stopped by heat inactivation at 65° C. for 20 minutes.

[0055] The *Pac*I URA 3A fragment derived from the procedures described in Example 2 above was then ligated to the dephosphorylated pNEB 193 vector containing the POX 4 flanking fragments which had also been digested with *Pac*I. The ligation procedure followed the same steps described above in Example 1. The ligation mixture was transformed into *E. coli* XL1 Blue MRF' (Stratagene). Transformants were selected and screened for correct constructs containing the vector sequence, the POX 4 flanking sequences, the URA 3A selectable marker gene, and missing the region between the POX 4 flanking sequences (653 base pairs of the POX 4 ORF from nucleotides 1178-1829 (see **FIG. 1**)). The constructs identified as being correct were sequenced and compared to the URA 3A sequence to confirm that PCR did not introduce DNA base changes that would result in an amino acid change.

Example 4

Transformation of *C. tropicalis* Using Lithium Acetate

[0056] Following ligation and confirmation of the construct described above in Example 3, *Asc*I and *Pme*I liberated a 3083 base pair fragment which was used to transform, by a lithium acetate transformation protocol, a *Candida tropicalis* strain (H53) in which the POX 5 gene had previously been disrupted to uracil prototrophy.

[0057] The lithium acetate transformation protocol used to transform the *C. tropicalis* was in accordance with the

general procedures described in Current Protocols in Molecular Biology, Supplement 5, 13.7.1 (1989), incorporated herein by reference. 5 ml of YEPD was inoculated with *C. tropicalis* H53 (previously disrupted to uracil prototrophy) from a frozen stock and incubated overnight on a New Brunswick shaker at 30° C. and 170 rpm. The next day, 10 μ l of the overnight culture was inoculated into 50 ml YEPD and growth was continued at 30° C., 170 rpm. The following day the cells were harvested at an OD₆₀₀ of 1.0. The culture was transferred to a 50 ml polypropylene tube and centrifuged at 1000X g for 10 min. The cell pellet was resuspended in 10 ml sterile TE (10 mM Tris-Cl and 1 mM EDTA, pH 8.0). The cells were again centrifuged at 1000Xg for 10 min and the cell pellet was resuspended in 10 ml of a sterile lithium acetate solution [LiAc (0.1 M lithium acetate, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA)]. Following centrifugation at 1000Xg for 10 min., the pellet was resuspended in 0.5 ml LiAc. This solution was incubated for one hour at 30° C. while shaking gently at 50 rpm. A 0.1 ml aliquot of this suspension was incubated with 5 μ g of transforming DNA at 30° C. with no shaking for 30 min. A 0.7 ml PEG solution (40% wt/vol polyethylene glycol 3340, 0.1 M lithium acetate, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA) was added and incubated at 30° C. for 45 min. The tubes were then placed at 42° C. for 5 min. A 0.2 ml aliquot was plated on synthetic complete media minus uracil (SC-uracil) (Kaiser et al. Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, USA, 1994, incorporated herein by reference). Growth of transformants was monitored for 5 days. After three days, several transformants were picked and transferred to SC-uracil plates for genomic DNA preparation and screening. The transformants were identified as strain HDC100.

Example 5

Transformant Analysis

[0058] The transformants prepared in accordance with Example 4 were then analyzed. Transformants were initially plated onto media containing dodecane and decane as the sole carbon sources. Transformants that failed to grow or grew slowly on this media (indicating that one or both of the POX 4 genes were disrupted) while retaining uracil prototrophy were subjected to Southern analysis. Intensity of the band signal from the Southern analysis was used as a measure of the integration events (i.e. the more copies of the URA 3A selectable marker gene which are present, the stronger the hybridization signal).

[0059] Transformants were grown at 30° C., 170 rpm, in 10 ml SC-uracil media for preparation of genomic DNA. Following the isolation of genomic DNA from the transformants, the DNA was digested with *PacI* or *EcoRI* and the digests were processed according to the standard Southern method. A 0.95% agarose gel was used to prepare a Southern hybridization blot. The DNA from the gel was transferred to a MagnaCharge nylon filter membrane (MSI Technologies, Westboro, Mass.) according to the alkaline transfer method of Sambrook et al, Molecular Cloning: A Laboratory Manual, 2ed. Cold Spring Harbor Press, USA (1989). For the Southern hybridization, a 1.7 kb URA 3 DNA fragment was used as a hybridization probe of the *PacI* digests and a 6.6 Kb POX 4 probe was used for the *EcoRI* digests. 300 ng of URA 3A DNA was labeled using a ECL Direct labeling

and detection system (Amersham) and the Southern were processed according to the ECL kit specifications. The blot was processed in a volume of 30 ml of hybridization fluid corresponding to 0.125 ml/cm². Following a prehybridization at 42° C. for 1 hour, 300 ng of URA 3A probe was added and the hybridization continued for 16 hours at 42° C. Following hybridization, the blots were washed two times for 20 minutes each at 42° C. in primary wash containing urea. Two 5 minute secondary washes at RT were conducted, followed by detection according to directions. The blots were exposed for 16 hours as recommended.

[0060] Only those strains receiving the disruption/deletion construct yielded the anticipated 1.676 Kb band. The presence of an 11.8 Kb fragment indicated that the POX 4 gene was disrupted. The presence of a 10.8 Kb fragment indicated that the POX 4 gene was wild-type. In those cases where both a wild-type allele and a disrupted allele were present, those strains were subjected to 5-fluoroorotic acid (5-FOA) treatment to regenerate the URA 3 selectable marker.

[0061] Strains were tested for growth in media containing various concentrations of 5-FOA, an analogue of a uracil pathway intermediate which is toxic to Ura⁺ cells. Both strains were grown to mid-log phase in YEPD medium (2% Bacto-peptone, 2% glucose, 1% Bacto-Yeast Extract) and were plated at various dilutions onto FOA medium (Boeke et al., [1984] Molec. Gen. Genet 197; p345-346) or YEPD medium.

[0062] Strains that were identified as uracil auxotrophs were transformed again with the disruption construct so as to disrupt the non-disrupted POX 4 allele. The presence of a single 11.8 Kb band indicated the disruption of both alleles of the POX 4 gene. Four *C. tropicalis* *pox5/pox5 pox4/pox4* homozygotes were confirmed by Southern analysis. These strains were HDC100-1,-2,-3, and -4.

Example 6

Fermentation Analysis of Non-Reversion

[0063] Under fermentation conditions using strain H5343 or its progeny, reversion to β -oxidation typically occurred around 72 hours post substrate induction. This condition can be facilitated by glucose limitation. When the same conditions of fermentation that induce β -oxidation in H5343 were applied to HDC100, no reversion to β -oxidation was observed. This indicated that β -oxidation was still disrupted in HDC100. Moreover, when samples of these fermentation cultures were plated onto media containing dodecane and decane as the sole carbon sources, no growth of HDC100 was observed, indicating their *pox*- phenotype.

[0064] It will be understood that various modifications may be made to the embodiments disclosed herein. Therefore, the above description should not be construed as limiting, but merely as exemplifications of preferred embodiments. Those skilled in the art will envision other modifications within the scope and spirit of the claims appended hereto.

What is claimed is:

1. A *Candida tropicalis* cell having a disrupted chromosomal POX 4 gene wherein a portion of said POX 4 gene has been deleted through homologous recombination with a DNA fragment comprising a selectable marker gene, wherein said selectable marker gene is flanked on both ends

by DNA sequences having homology to non-contiguous portions of said POX 4 gene and wherein the presence of the deleted portion of the POX 4 gene prevents recombination to a functional POX 4 gene.

2. The *Candida tropicalis* cell of claim 1 wherein the POX 4 gene is POX 4A.

3. The *Candida tropicalis* cell of claim 1 wherein the POX 4 gene is POX 4B.

4. The *Candida tropicalis* cell of claim 1 wherein the POX 4 gene comprises both POX 4A and POX 4B.

5. The *Candida tropicalis* cell of claim 1 wherein the DNA fragment comprises a URA 3 selectable marker gene.

6. The *Candida tropicalis* cell of claim 1 comprising the strain H53.

7. A process for producing a substantially pure α , Ω -dicarboxylic acid which comprises culturing the *Candida tropicalis* cell of claim 1 in a culture medium containing a nitrogen source, an organic substrate and a cosubstrate.

8. A *Candida tropicalis* cell having a disrupted chromosomal POX 5 gene wherein a portion of said POX 5 gene has been deleted through homologous recombination with a DNA fragment comprising a selectable marker gene, wherein said selectable marker gene is flanked on both ends by DNA sequences having homology to non-contiguous portions of said POX 5 gene and wherein the presence of the deleted portion of the POX 5 gene prevents recombination to a functional POX 4 gene.

9. The *Candida tropicalis* cell of claim 8 comprising both copies of a chromosomal POX 5 gene.

10. The *Candida tropicalis* cell of claim 8 wherein the DNA fragment comprises a URA 3 selectable marker gene.

11. The *Candida tropicalis* cell of claim 8 comprising the strain H53.

12. A process for producing a substantially pure α , Ω -dicarboxylic acid comprising culturing the *Candida tropicalis* cell of claim 8 in a culture medium containing a nitrogen source, an organic substrate and a cosubstrate.

13. A *Candida tropicalis* cell having disrupted chromosomal POX 4 and POX 5 genes wherein a portion of both of said POX 4 and POX 5 genes has been deleted through homologous recombination with a DNA fragment comprising a selectable marker gene wherein said selectable marker gene is flanked on both ends by DNA sequences having homology to non-contiguous portions of both of said POX 4 and POX 5 genes and wherein the presence of the deleted portions of both of the POX 4 and POX 5 genes prevent recombination to functional POX 4 and POX 5 genes.

14. The *Candida tropicalis* cell of claim 13 wherein the POX 4 gene is POX 4A.

15. The *Candida tropicalis* cell of claim 13 wherein the POX 4 gene is POX 4B.

16. The *Candida tropicalis* cell of claim 13 wherein the POX 4 gene comprises both POX 4A and POX 4B.

17. The *Candida tropicalis* cell of claim 13 comprising both copies of a chromosomal POX 5 gene.

18. The *Candida tropicalis* cell of claim 13 wherein the DNA fragment comprises a URA 3 selectable marker gene.

19. The *Candida tropicalis* cell of claim 13 comprising the strain H53.

20. The *Candida tropicalis* cell of claim 17 wherein the POX 4 gene is POX 4A.

21. The *Candida tropicalis* cell of claim 17 wherein the POX 4 gene is POX 4B.

22. The *Candida tropicalis* cell of claim 17 wherein the POX 4 gene is both POX 4A and POX 4B.

23. A process for producing a substantially pure α , Ω -dicarboxylic acid which comprises culturing the *Candida*

tropicalis cell of claim 13 in a culture medium containing a nitrogen source, an organic substrate and a cosubstrate.

24. A process for completely blocking the β -oxidation pathway in a *Candida tropicalis* host cell which comprises disrupting through homologous recombination a portion of a POX 4 gene with a DNA fragment comprising a selectable marker gene wherein said selectable marker gene is flanked on both ends by DNA sequences having homology to non-contiguous portions of said POX 4 gene and wherein the presence of the deleted portion of the POX 4 gene prevents recombination to a functional POX 4 gene.

25. The process of claim 24 wherein the POX 4 gene is POX 4A.

26. The process of claim 24 wherein the POX 4 gene is POX 4B.

27. The process of claim 24 wherein the POX 4 gene comprises both POX 4A and POX 4B.

28. The process of claim 24 wherein the DNA fragment comprises a URA 3 selectable marker gene.

29. The process of claim 24 wherein the *Candida tropicalis* host cell comprises strain H53.

30. A process for completely blocking the β -oxidation pathway in a *Candida tropicalis* host cell which comprises disrupting through homologous recombination a portion of a POX 5 gene with a DNA fragment comprising a selectable marker gene wherein said selectable marker gene is flanked on both ends by DNA sequences having homology to non-contiguous parts of said POX 5 gene and wherein the presence of the deleted portion of the POX 5 gene prevents recombination to a functional POX 5 gene.

31. The process of claim 30 wherein the host cell comprises both copies of a chromosomal POX 5 gene.

32. The process of claim 30 wherein the DNA fragment comprises a URA 3 selectable marker gene.

33. The process of claim 30 wherein the *Candida tropicalis* host cell comprises strain H53.

34. A process for completely blocking the β -oxidation pathway in a *Candida tropicalis* host cell which comprises disrupting through homologous recombination a portion of both of POX 4 and POX 5 genes with a DNA fragment comprising a selectable marker gene wherein said selectable marker gene is flanked on both ends by DNA sequences having homology to non-contiguous portions of both of said POX 4 and POX 5 genes and wherein the deleted portions of both of the POX 4 and POX 5 genes prevent recombination to functional POX 4 and POX 5 genes.

35. The process of claim 34 wherein the POX 4 gene is POX 4A.

36. The process of claim 34 wherein the POX 4 gene is POX 4B.

37. The process of claim 34 wherein the POX 4 gene comprises both POX 4A and POX 4B.

38. The process of claim 34 wherein the host cell comprises both copies of the chromosomal POX 5 gene.

39. The process of claim 34 wherein the DNA fragment comprises a URA 3 selectable marker gene.

40. The process of claim 34 wherein the *Candida tropicalis* host cell comprises strain H53.

41. The process of claim 38 wherein the POX 4 gene is POX 4A.

42. The process of claim 38 wherein the POX 4 gene is POX 4B.

43. The process of claim 38 wherein the POX 4 gene comprises both POX 4A and POX 4B.

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