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Expression of *POX2* gene and disruption of *POX3* genes in the industrial *Yarrowia lipolytica* on the γ-decalactone production

Yanqiong Guo[a], Huanlu Song[a,*], Zhaoyue Wang[b], Yongzhi Ding[a]

[a] *Beijing Key Laboratory of Flavor Chemistry, Beijing Technology and Business University, Beijing 100048, PR China*
[b] *The Laboratory of Molecular Genetics and Breeding of Yeasts, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, PR China*

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ABSTRACT

The yeast *Yarrowia lipolytica* growing on methyl ricinoleate can produce γ-decalactone, the worthy aroma compound, which can exhibit fruity and creamy sensorial notes, and recognized internationally as a safe food additive. Unfortunately, the yield is poor because of lactone degradation by enzyme Aox3 (*POX3* gene encoded), which was responsible for continuation of oxidation after C_{10} level and lactone reconsumption. In this paper, we chose the industrial *Y. lipolytica* (CGMCC accession number 2.1405), which is the diploid strain as the starting strain and constructed the recombinant strain Tp-12 by targeting the *POX3* locus of the wild type, one copy of *POX3* was deleted by *CRF1* + *POX2* insertion. The other recombinant strain Tpp-11, which was a null mutant possessing multiple copies of *POX2* and disrupted *POX3* genes on two chromosomes, was constructed by inserting *XPR2* + *hpt* into the other copy of *POX3* of Tp-12. The growth ability of the recombinants was changed after genetic modification in the fermentation medium. The production of γ-decalactone was increased, resulting from blocking β-oxidation at the C_{10} Aox level and *POX2* overexpression. The recombinant strain Tpp-11 was stable. Because there was no reconsumption of γ-decalactone, the mutant strain could be grown in continuous fermentation of methyl ricinoleate to produce γ-decalactone.

1. Introduction

Lactones are widely distributed in foods and beverages as aroma compounds. Among these compounds, γ-decalactone has characteristic peach and apricot flavors and its low detection threshold of 0.088 ppm in the water (Siek et al. 1971). Most industrial processes use the bioconversion of ricinoleic acid by yeasts to produce γ-decalactone (Aguedo et al. 2004; Schrader et al. 2004). The yeast *Yarrowia lipolytica* is one of them which are able to transform a hydroxylated C_{18} fatty acid into γ-decalactone and is also able to degrade the produced lactone (Groguenin et al. 2004). The pathway of biotransformation involves four successive cycles of β-oxidation and ricinoleic acid is degraded into 4-hydroxydecanoic acid which lactonizes to γ-decalactone (Blin-Perrin et al. 2000; Wache et al. 2003). Acyl-CoA oxidases catalyse the first reaction of the β-oxidation cycle and they are generally considered as the rate-limiting enzyme of sequence (Inestrosa et al. 1979; Wache et al. 1998; Wang et al. 1998).

Y. lipolytica possesses a family of five acyl-CoA oxidases (Aox1-5 encoded by *POX1-5*) (Wang et al. 1999a,b; Garcia et al. 2007a; Dujon et al. 2004). Aox1 exhibits no detective activity (Wache et al. 2002), Aox2 and Aox3 are long- (Wang et al. 1999a,b) and short-chain specific (Luo et al. 2000; Luo et al. 2002), respectively and Aox4 and Aox5 exhibit a weak activity on a wide range of substrates (Wache et al. 2002). The long-chain specific enzyme (Aox2) is significant for bioconversion of ricinoleic acid to produce γ-decalactone. The short-chain specific enzyme (Aox3) is involved in the degradation of the lactone and the disruption of the *POX3* gene is helpful to decreases lactone degradation (Wache et al. 2000, 2001). Wang et al. (2009) have been successfully used *CUP1* gene, a copper-resistant gene to screening the recombination strains, and the *CRF1* in *Y. lipolytica* is the same as the *CUP1* gene in *Saccharomyces cerevisiae*.

Splice overlap extension (SOE) PCR allows the fusion of two sequences of DNA at precise junctions irrespective of nucleotide sequences at the recombination site without the use of restriction endonucleases or ligase (Horton et al., 1989). SOE joins two sequences that have regions of short sequence complementarity between the 3′ end of the first sequence and the 5′ end of the other (Jones and Barnard 2005). Complementary oligodeoxyribonucleotide (oligo) primers and the polymerase chain reaction are used to generate two DNA fragments having overlapping ends. These fragments are combined in a subsequent 'fusion' reaction in

* Corresponding author at: College of Food Science and Chemical Engineering, Beijing Technology and Business University, 11 Fucheng Road, Beijing 100048, PR China. Tel.: +86 10 68984025; fax: +86 10 68984025.
E-mail address: songhuanlu@yahoo.com.cn (H. Song).

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Table 1
PCR primers used in this study.

Name	Sequences (5′–3′)	Primer sites
POX3-up-1	acaacaccttcacagagccacc	(2311–2333)
POX3-up-2	gaagacgagttgagacgaagactttcgcacccagtagtcgt	(2685–2705)
CRF1-1	acgactactgggtgcgaaagtcttcgtctcaactcgtcttc	(1425–1446)
CRF1-2	agagccgagggagaataaacggcgaaattggcggttggtat	(2711–2731)
POX2-1	ataccaaccgccaatttcgccgtttattctccctcggctct	(2042–2063)
POX2-2	gattcccgtgcccgtattactctacccgcttgtctattcc	(4268–4286)
POX3-down-1	ggaatagacaagcgggtagagtaatacgggcacgggaatc	(4715–4737)
POX3-down-2	accgaacccatactccac	(4956–4974)
XPR2-L	TGCTCTAGAtgaggtgtctcacaagtgc	(*XbaI*) (1294–1313)
XPR2-R	Actttgagggaatactgcctcggatcgaggtgggcgg	(1380–1396)
Hpt-L	Ccgcccacctcgatccgaggcagtattccctcaaagt	(9535–9555)
Hpt-R	CGAGCTCgatgacgcacaatcccac	(*SacI*) (10,793–10,815)
LH-L	GGAAGATCTaggtcgacaacccttaat	(*BglII*)
LH-R	Gttatcccctgattctgtg	(*NaeI*)
POX3-L	CCGGAATTCgactactgggtgcgaaag	(*EcoRI*) (2689–2707)
POX3-R	CCCAAGCTTcccaaagtttctcgcatc	(*HindIII*) (4573–4591)

which the overlapping ends anneal, allowing the 3′ overlap of each strand to serve as a primer for the 3′ extension of the complementary strand. The resulting fusion product is amplified further by PCR (Ho et al., 1989). This simple and widely applicable approach has significant advantages over standard recombinant DNA techniques.

In the recombinant yeast strains reported by previous investigations, heterologous DNA sequences were introduced or laboratory strain were used. These recombinant yeast strains were generally not suitable for application in food industry. To avoid any potential problem that could be caused by the transfer of heterologous DNA from genetically modified yeasts (Wang et al. 2006; Zhang et al. 2005). It is very important to integrate autologous gene such as *CRF1* gene from yeasts on the chromosome of the industrial *Y. lipolytica* (Garcia et al. 2002) and to use Cre-*loxp* recombination system in selective marker-excision via flanking *loxp* elements (Shigehito et al., 2009).

Currently, the research on the production of γ-decalactone by *Y. lipolytica* yeast focused on the mechanisms of the β-oxidation and the function of the genes encoding five acyl-CoA oxidase isozymes, and most laboratory strains that was be used were haploids strain. To our knowledge, the study of construction of a null mutant that *POX3* genes on two chromosomes were disrupted by gene homologous recombination and SOE PCR was rarely reported. Since there was no reconsumption of γ-decalactone, the mutant strain can be grown in continuous fermentation of methyl ricinoleate to produce γ-decalactone.

In this study, the industrial *Y. lipolytica* which is the diploid strain was chosen as the starting strain and its genetic background is different from that of the laboratory. Firstly, the *POX3* gene on the one chromosome was disrupted by integrating *CRF1* and *POX2* genes from *Y. lipolytica*. And then the second *POX3* gene on the other chromosome was disrupted by integrating *hpt* gene into an industrial *Y. lipolytica.* When the second *POX3* gene was disrupted, the sequence that had been disrupted on the first chromosome was selected. Two rounds of gene disruption were needed in order to obtain a null mutant. Finally, a new safe industrial strain Tpp-11 was constructed, which possessed multiple copies of *POX2* gene, coding for the long-chain specific Aox, and disrupted *POX3* genes on two chromosomes, coding for the short-chain specific Aox, for an increase of γ-decalactone production.

2. Materials and methods

2.1. Strains, plasmids and culture condition

Escherichia coli DH5α (*sup*E44 Δ*lac*U169 (φ80*lac*ZΔM15) *hsd*R17 *rec*Al *end*Al *gyr*A96 *thi*-1 *rel*Al) was used for plasmid construction. *Y. lipolytica* As2·1405, the DNA donor and used as host strain, was obtained from the Center of General Microbiology in China Committee, CGMCC. Plasmids pGwb5 and pUG6 was used to provide the hygromycin-resistance gene (*hpt*) and *loxP* sites.

E. coli strain was grown at 37 °C in Luria–Bertani medium (Sambrook et al. 1989) supplemented with ampicillin (50 mg/ml) when necessary. Yeast strains for transformation were grown in YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose] at 28 °C. Recombinant strains were selected from the YPD [0.17% (w/v) yeast nitrogen base, 1% (w/v) glucose, 0.4% (w/v) ammonium chloride] plate (1.5% agar) using 12 mM copper sulfate ($CuSO_4$) and hygromycin (100 mg/ml) as the selection marker (Cordero Otero and Gaillardin 1996). Methyl ricinoleate (MR) medium was used as fermentation medium for production of γ-decalactone, with the following compositions: 5% (w/v) methyl ricinoleate), 0.5% (w/v) peptone, 0.25% (w/v) yeast extract, 0.4% (w/v) Tween 80, 0.3% (w/v) $MgSO_4 \cdot 7H_2O$ and 0.5% (w/v) NaCl.

2.2. DNA manipulation

Genomic DNA of yeast was prepared as described by Burke et al. (2000). Primers for PCR amplification are listed in Table 1.

2.3. Construction of disruption cassettes

POX3-up (GenBank accession number AJ001301), *CRF1* (GenBank accession number Z23265), *POX2* (GenBank accession number AJ001300), *POX3*-down (GenBank accession number AJ001301) genes were amplified from the *Y. lipolytica*. The four sequences were then fused by splice overlap extension (SOE) PCR (De Muynck et al., 2009) to construct disrupting cassettes for the first chromosomes. Three consecutive PCRs were performed. In the first consecutive PCR the *POX3*-up, *CRF1*, *POX2*, *POX3*-down were amplified respectively using the follow primers: (1) *POX3*-up-1 and *POX3*-up-2; (2) *CRF1*-1 and *CRF1*-2; (3) *POX2*-1 and *POX2*-2; (4) *POX3*-down-1 and *POX3*-down-2. In the second consecutive PCR the *POX3*-up-*CRF1* was performed using *POX3*-up, *CRF1* as template and following pair primer: *POX3*-up-1 and *CRF1*-2. The following cycles were applied: 5 min at 95 °C, 10 cycles of 1 min at 94 °C, 40 s at 45 °C, 1 min 30 s at 72 °C and final extension at 72 °C for 10 min without primers. Then primers were added and the following cycles were applied: 5 min at 95 °C, 30 cycles of 1 min at 94 °C, 40 s at 58 °C, 2 min at 72 °C and final extension at 72 °C for 10 min. The *POX2*-*POX3*-down was performed using *POX2*, *POX3*-down as template and following pair primer: *POX2*-1 and *POX3*-down-2. The following cycles were applied: 5 min at 95 °C, 10 cycles of 1 min at 94 °C, 40 s at 45 °C, 2min20 s at 72 °C and final extension at 72 °C for 10 min without