Optimization Strategy of Specific Knockout of Key POX3 Gene in Γ-Decalactone Metabolism of Yarrowia Lipolytica

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Abstract. As an important spice raw material, γ-decalactone is widely used in the food industry. Six peroxisome acyl-CoA oxidases (POX) are rate-limiting enzymes for γ-decalactone metabolism in Yarrowia lipolytica. However, there are many unsolved problems in the function of each member of this family, which limits the strain optimization and the industrial production capacity efficiency of γ-decalactone. In this study, based on the conservation analysis of the ORF and Flanking sequences of POX1-POX6, the gene knockout verification primers with high specificity were designed based on the Cre / LoxP system. Based on the CRISPR / Cas9 system, two target sites with high specificity were screened to achieve specific knockout of the POX3 gene which is in Yarrowia lipolytica, aiming to explore the function of POX3 and subsequently refine the production process of γ-decalactone. The results of this study provide a new genetic engineering design idea for the microbial production capacity of γ-decalactone, deeply reveal the function of the POX gene family, help optimize the production efficiency of γ-decalactone, and lay a theoretical foundation for industrial application.

1 Introduction

Use The compound γ-decalactone embodies a potent, fruity, and creamy aroma. It is widely found in fruits and fermented products such as peaches, coconuts, and rum. It is an important raw material for the perfume industry. At present, the main production method of γ-decalactone is through microbial fermentation, among which Yarrowia sp. showed better production effect. In its metabolic process, Acyl-coenzyme A oxidase (ACO) is considered to be the rate-limiting enzyme, and its activity directly affects the yield of γ-decalactone [1, 2]. ACO has six isozymes ACO1-ACO6, which pass through six peroxisomal acyl-CoA oxidase (POX) genes: POX1 (YALI0 _ E32835g), POX2 (YALI0 _ F10857g), POX3 (YALI0 _ D24750g), POX4 (YALI0 _ E27654g), POX5 (YALI0 _ C23859g), POX6 (YALI0 _ E06567g).

There have been many studies on the function of POX1-POX6 through genetic engineering at home and abroad. Zhang et al. [3] knocked out the POX1, POX3, POX4, POX5, and POX6 genes and found that the lactone production decreased to only 0.27 g / L, which was 26 % lower than that of the original strain. Malajowicz et al [4] constructed POX3, POX4, POX5 gene deletion strains, and found that the yield of γ-decalactone increased by 2.53 times to 5.50 g / L, and the yield reached 0.032 g / L / h. By knocking out one or more POX1-POX5 genes at the same time, Wache et al.preliminarily studied the effect of isozymes expressed by five genes on the production of γ-decalactone [5]. Wache et al. [4] found that long-chain specific acyl-CoA oxidase ACO2 can specifically catalyze ricinoleic acid to produce γ-decalactone, while the presence of short-chain specific acyl-CoA oxidase ACO3 will promote the decomposition of γ-decalactone produced by Yarrowia lipolytica (Fig. 1). Since then, Groguenin, Wache et al. [5, 6] knocked out the POX3 gene and found that the strain γ-decalactone increased by 4.4 times.

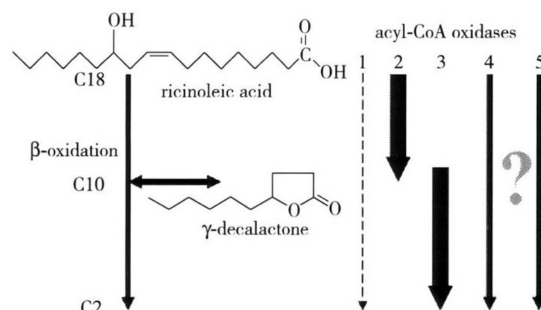

Fig. 1. Role of isozyme AOX1-AOX5 on the γ-decalactone production [7].

Braga [8] et al constructed POX2-POX5 gene deletion and ectopic overexpression of POX2 gene in Yarrowia lipolytica MTLY40-2p engineering strains, which greatly reduced the number of POX2-POX5 gene deletion and ectopic overexpression of POX2 gene in Yarrowia lipolytica.Guo [9] constructed an engineering strain of Y.lipolytica MTLY40-2p with POX3 gene deletion and integration of copper resistance gene CRF1 by homologous recombination technology, and the investigation revealed a substantial escalation in the

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yield of γ-decalactone, which progressed from an initial output of 0.19 g / L to 0.53 g / L, an increase of 2.8 times. Subsequently, the strain was further modified and recombined to obtain a recombinant strain Tpp-11. The ACO2 gene was overexpressed in the chromosome of the strain, and the POX3 gene was knocked out, so that the yield of γ-decalactone increased from 0.90 g / L to 3.30 g / L [10]. According to the existing research shows that [11], only ACO3 has inhibitory effect on the production of γ-decalactone, so the specific knockout of POX3 gene is considered to be the key way to improve the production of γ-decalactone.

Yarrowia lipolytica has six chromosomes with a genome of 20.5 Mb. At present, there are two main types of gene editing techniques: CRISPR system is used to delete, integrate and edit genes; the Cre-loxP system is used for the recovery and multi-round integration of screening markers. Although a number of studies have explored the function of POX1-POX6 through genetic engineering, off-target phenomenon is still a potential problem in the process of gene editing due to the high similarity of the coding region sequences of ACO isozymes. For example, Zhang et al. [12] knocked out multiple ACO genes, the production of γ-decalactone decreased, which was inconsistent with other research results. Therefore, precise knockout of ACO3 and further study of its function are of great significance for improving the production capacity of γ-decalactone.

In summary, there are many unsolved mechanism problems in the microbial production capacity of γ-decalactone, such as the specific function of POX1-POX6 genes, the domain function of ACO protein and the interaction effect between multiple genes. The aforementioned challenges impede the optimization process of strains, subsequently hindering the enhancement of industrial-scale production efficiency for γ-decalactone. In addition, the production capacity of existing fermentation strains has not yet reached the requirements of industrial applications, and the primary emphasis of research and developmental endeavors has progressively transitioned towards refining and optimizing the conditions under which fermentation processes occur, but the efficiency of gene modification still needs to be improved. At the same time, through the above content, it can be found that the POX3 gene has a significant inhibitory effect on the production of γ-decalactone, while other genes in the POX1-POX6 gene have no such effect. Therefore, the POX3 gene was selected as the research object in this study，aiming to specifically knock out POX3 through two sets of schemes : Cre / loxP and CRISPR system，explore the function of POX3 gene, and to optimize the production efficiency of γ-decalactone in Yarrowia lipolytica. This study provides a new genetic engineering design idea for the microbial production of γ-decalactone, helps to understand the metabolic mechanism in depth, and lays a foundation for improving industrial production efficiency in the future.

2 Method

2.1 Identification of POX genes

Six POX genes (Table 1.) were obtained by searching the keyword ' Acyl-coenzyme A oxidase ' in the Yarrowia sp.genome database (Ensembl Fungi, https://fungi.ensembl.org/Yarrowia_lipolytica/Info/Index) and NCBI (https://www.ncbi.nlm.nih.gov/).

Table 1. Information of POX gene family.

Gene name	Gene ID	Accession number
POX1	2912003	YALI0_E32835g, , YALI2 F00431g
POX2	2907872	YALI0_F10857g, YALI2 F00214g
POX3	2910308	YALI0_D24750g, YALI2 D00649g
POX4	2911586	YALI0_E27654g, YALI2 E01209g
POX5	2909505	YALI0_C23859g, YALI2 B00346g
POX6	2912402	YALI0 E06567g, YALI2 E01629g

2.2 Sequence conservation analysis of POX genes and Flanking sequences

Six POX genes and their Flanking sequences were collected from the database. Starting from the first base A of ATG (initiation codon) and the last base A / G of TAA / TAG (termination codon), 50 nt were taken from the ORF region and 60 nt were taken from the Flanking region, which were organized into Fasta format. Analyzed and plotted using the Sequence Logo tool (https://weblogo.berkeley.edu/logo.cgi).

2.3 Screening and design of specific target sites of CRISPR system

By selecting Yarrowia lipolytica (GCF _ 000002525.2) species and POX3 gene in CHOPCHOP (http://chopchop.cbu.uib.no/), in the output results, first of all, according to the position of the target sequence in the POX3 gene, the target sequence near the actual codon and the termination codon was selected. The target sites with higher ranking were screened near these two positions, and the off-target situation could be evaluated by detailed point opening. Considering the target sequence with relatively complete knockout ORF, high efficiency and good specificity as the candidate target sequence of this study, the construction of CRISPR specific knockout system was carried out.

3 Result

3.1 Used the Cre-loxP system to specifically knock out POX3

The Cre-loxP system consists of Cre recombinase (Cyclization Recombination Enzyme) and loxP (locus of X-overP1) sequences. The Cre recombinase possesses the capability to uniquely identify the loxP sequence located within cellular genes or DNA strands,enabling it to orchestrate diverse, specific recombination events

contingent upon the location and orientation of the loxP sequence: When two loxP sites are located on the same chromosome and the direction is opposite, the sequence between the sites will be reversed under the action of the Cre enzyme ; when two loxP sites are on the same chromosome and in the same direction, the sequence between the sites will be deleted under the action of Cre enzyme. When two loxP sites are located on different chromosomes and in the same direction, they will lead to DNA strand exchange or chromosome translocation under the action of Cre enzyme [13] (Fig. 2).

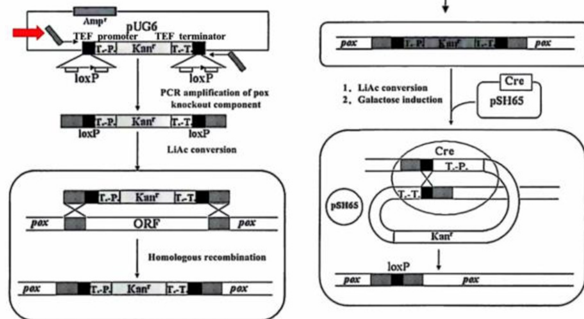

Fig. 2. Gene knockout process by Cre/loxP.

Including primer design, PCR, construction of knockout fragments, transformation into yeast to achieve gene recombination.

In this experiment, the sequence between the two loxP sites in the Cre-loxP system was knocked out by using the two loxP sites on the same chromosome and the same direction. In order to ensure the specificity of homologous recombination, i.e. the accuracy of knocking out the POX3 gene, it was necessary to ensure that the homologous recombination arm sequence at the red arrow in Fig. 2 was specific, so as to dock the target gene. Conservative analysis of the ORF and Flanking sequence of POX1-POX6 was performed on the WebLogo website. The 60 nt Flanking sequence-50 nt ORF sequence was taken at the 3 ' end, and the 50 nt ORF sequence-60 nt Flanking sequence was taken at the 5 ' end. The results showed that the difference between the 5 ' Flanking and 3 ' Flanking regions was obvious among several genes, while the ORF part was highly similar. Since the sequences between POX1 and POX6 are highly similar, the required sequences should be searched from the whole sequence of ORF (open reading frame) and Flanking.

primer	5 '→3 ' (the underline part is the homologous region with the ORF of the gene to be knocked out)	nt
L1	ATCTCCCCCAACCTCACAGCTAACGTCGAGATTGACGGCAAGCAGTACAACACCTTCACAAACGCGGCCGCCAGCTGAAG	80
R1	AAAGTAATGCTTGTAAACGTCACCATCGTAGTTTCCAATGGGAGCGTTGATGAACCAGTCCTATAGGGAGACCGGCAGAT	80
DL1	TTCCTCATCTTCTGCCTAGGTTCC	24
DR1	TATTTCTCTACAGGGGCGCGGCGT	24
DL2	AAGAACTTGTCATTTGTATAGTTT	24
DR2	ACTCTACTAATTTGATCTTGGAAC	24

Fig. 3. Primer sequence information of POX3 gene knockout component.

In order to verify whether POX3 was specifically knocked out, specific verification primers needed to be designed. L1 and L2 are a pair of long primers for amplification, and DL1, DR1, DL2 and DR2 were two pairs of short primers for verifying whether the specific knockout was completed (Fig. 3). Since several genes of POX are highly similar, the key to the success of the experiment is whether the specificity of the long fragment primers L1 and R1 is sufficient to maintain the target specificity. In addition, DL1 and DR2 are not easy to achieve high specificity. If other POX genes are deleted, the same verification effect will be obtained. Based on the above results, the design of this subject was carried out: short primers DR1 and DL2 had high specificity and continued to be used; in order to improve the specificity of primers, the long primers L1 and L2 were redesigned. In this way, Cre-loxP system-specific verification primer design can be achieved (Fig. 4).

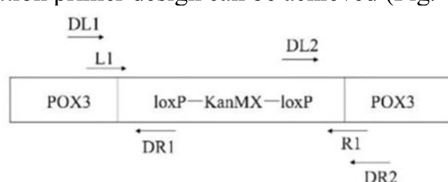

Fig. 4. Gene knockout process by Cre/loxP.

Therefore, when designing 80 nt long primers for homologous recombination, the 60 nt base part could be designed from the Flanking part, or Flanking and part of the ORF sequence. By comparing the results of conservative analysis, the design scheme of POX3 was : because the design principle of forward amplification primer L1 was ' 5 ' -upstream vector homologous sequence - restriction site (can be retained or deleted) - gene-specific forward amplification primer-3 ', ', the long primer L1 is ' 5'Flanking (50nt) -ORF (10nt) -loxP (20nt) ' ; similarly, due to the high similarity of ORF sequences adjacent to 3'Flanking, the long primer R1 can be ' 3'Flanking (60nt) -loxP (20nt) '.

Through the above experimental results, the improved long primer L1 was : 5 ' -GGACACAGTCGCCCTGGACAACGTCACTACCTC TACGATACACAATGATCTCCCTAACTTCGTATAA TGTATGC-3 '.Similarly, the improved long primer R1: 5 ' -ATCATGTCTACTAACACTCACAACTCCATAGAA AACATCGACTCAGAACACACGCTCCAT ATAACTTCGTATAGCATACA-3 '.

3.2 Used CRISPR / cas9 system to specifically knock out POX3

The CRISPR / Cas (Clustered regularly interspaced short palindromi repeats / CRISPR-associated) system is widely distributed in the genomes of bacteria and archaea. It is an adaptive immune system formed during evolution and can degrade invading viruses or plasmids [14-16]. CRISPR / Cas can be divided into type I, type II and type III systems according to the core element sequence of Cas gene. The type II system is relatively simple, with Cas9 protein and guide RNA (gRNA) as the core components. The chimeric RNA formed by the connection of Cas9 protein and a crRNA-tracrRNA constitutes a new genome directed editing system CRISPR / Cas9 [17]. In modern gene editing technology, CRISPR/cas9 system has become the mainstream gene editing system widely used due to the advantages of simple vector construction process and high editing efficiency.

Through genetic engineering methods, crRNA (CRISPR RNA) and tracrRNA (trans-activiting crRNA) were modified and connected together to obtain sgRNA (single guide RNA). The plasmid that can express both sgRNA and Cas9 was obtained by connecting the original expression of sgRNA with the original expression of Cas9 (Fig. 5). By transfecting it into cells, it is possible to perform gene manipulation on the target gene and cut ~ 3bp upstream of PAM (5 ' -NGG).

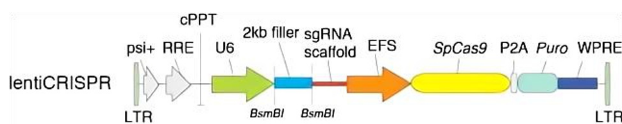

Fig. 5. The filler sequence of POX3 gene was designed with lentiCRISPR plasmid as an example.

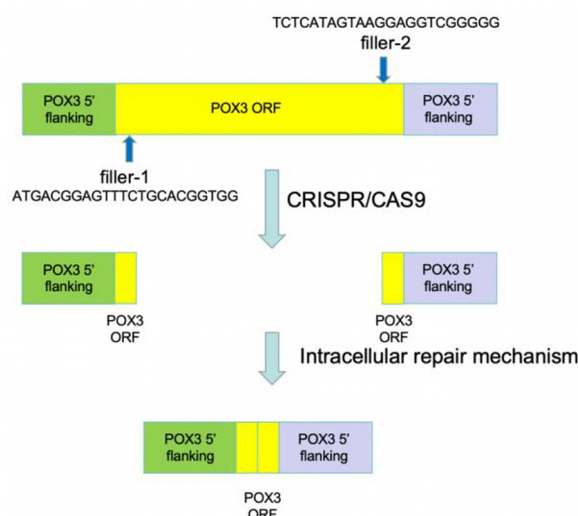

Fig. 6. Gene editing design of AOX3.

The CRISPR system for knocking out POX3 was designed by selecting Yarrowia lipolytica (GCF _ 000002525.2) species and POX3 gene in CHOPCHOP. In the target sequence with high score, the target sites filler-1 (ATGACGGAGTTTCTGCACGGTGG) and filler-2 (TCTCATAGTAAGGAGGTCGGGG) near the two ends of the gene were considered to achieve the maximum knockout of POX3. After the filler-1 located in the negative strand of the sequence and the filler-2 located in the positive strand of the sequence were specifically cleaved by the CRISPR / cas9 system, the ORF sequences at both ends of POX3 still had a small amount of residue, and then the two were connected under the action of intracellular repair mechanism, thereby completing the specific excision of the POX3 gene (Fig. 6).

4 Discussion

By analyzing the conservation of genes and Flanking sequences between ACO1-ACO6 isoenzymes, especially the sequence similarity of 5'Flanking and 3'Flanking in the coding region is much smaller within about 60 bases (Fig 2), when designing a gene-specific knockout system, the discovery that the sequence similarity of the Flanking region is small can be used to design homologous recombination primers with better specificity and PCR detection primers to be used after recombination. In addition, this study also considered the use of CRISPR / Cas9 to specifically knock out the POX3 gene to ensure the acquisition of POX3 single mutant strains.

Through the specific knockout of the POX3 gene by the above method, the degradation of γ-decalactone in yeast cells will be greatly reduced. At the same time, other genes control the amount of ricinoleic acid catalyzed to γ-decalactone unchanged, which can greatly improve the production efficiency of γ-decalactone in industrial production. In addition, through the innovation of genetic engineering design, more resources can be tilted in production research and development to improve production efficiency in all aspects of downstream projects such as optimizing substrate properties and fermentation conditions.

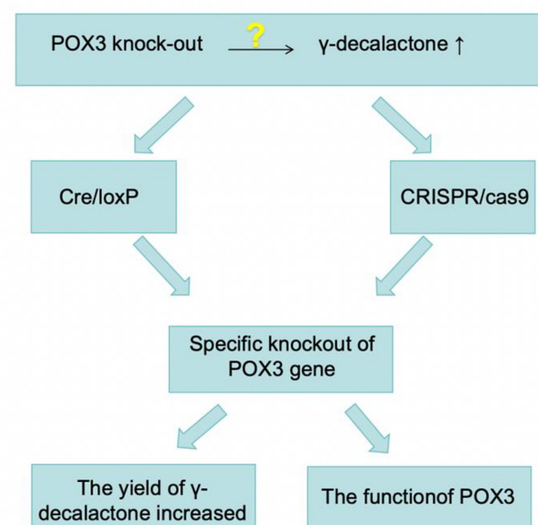

Fig. 7. Summary of research design.

This study lays a foundation for further exploring the function of POX gene family and POX3 gene, helping to understand the metabolic mechanism, optimizing the production efficiency of γ-decalactone in Yarrowia lipolytica, and improving the industrial production efficiency in the future (Fig. 7).

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