MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE
KYIV NATIONAL UNIVERSITY OF TECHNOLOGIES AND DESIGN
Faculty of Chemical and Biopharmaceutical Technologies
Department of Biotechnology, Leather and Fur

QUALIFICATION THESIS

on the topic **Synthesis of monoterpene geraniol by peroxisome from *Saccharomyces cerevisiae***
First (Bachelor's) level of higher education
Specialty 162 "Biotechnology and Bioengineering"
Educational and professional program "Biotechnology"

Completed: student of group BEBT-20
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Kyiv 2024

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Faculty: Chemical and Biopharmaceutical Technologies
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Educational and professional program Biotechnology

APPROVE
Head of Department of Biotechnology, Leather and Fur, Professor,
Doctor of Technical Science
Olena MOKROUSOVA
_____
«___»_____2024

ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Xintong Du

1. Thesis topic **Synthesis of monoterpene geraniol by peroxisome from *Saccharomyces cerevisiae***
Scientific supervisor Iryna Voloshyna, Ph.D., As. prof.

approved by the order of KNUTD "_ __ _" _____2024, №__ _____

2. Initial data for work: assignments for qualification thesis, scientific literature on the topic of qualification thesis, materials of Pre-graduation practice

3. Content of the thesis (list of questions to be developed): literature review; object, purpose, and methods of the study; experimental part; conclusions

4. Date of issuance of the assignments_____

EXECUTION SCHEDULE

№	The name of the stages of the qualification thesis	Terms of performance of stage	Note on performance
1	Introduction	From 01 April 2024 to 11 April 2024	
2	Chapter 1. Literature review	From 06 April 2024 to 20 April 2024	
3	Chapter 2. Object, purpose, and methods of the study	From 21 April 2024 to 30 April 2024	
4	Chapter 3. Experimental part	From 01 May 2024 to 10 May 2024	
5	Conclusions	From 07 May 2024 to 12 May 2024	
6	Draw up a bachelor's thesis (final version)	From 20 May 2024 to 25 May 2024	
7	Submission of qualification work to the supervisor for feedback	From 25 May 2024 to 27 May 2024	
8	Submission of bachelor's thesis to the department for review (14 days before the defense)	27 May 2024	
9	Checking the bachelor's thesis for signs of plagiarism (10 days before the defense)	31 May 2024	
10	Submission of bachelor's thesis for approval by the head of the department (from 7 days before the defense)	03 June 2024	

I am familiar with the task:

Student _____ Xintong DU

Scientific supervisor _____ Iryna VOLOSHYNA

SUMMARY

Xintong Du. Synthesis of monoterpene geraniol by peroxisome from *Saccharomyces cerevisiae* – Manuscript.

Qualification thesis on the specialty 162 «Biotechnology and Bioengineering». – Kyiv National University of Technologies and Design, Kyiv, 2024.

Geraniol, an acyclic monoterpenoid compound, is widely used in the production of cosmetics and spices due to its rose-like odor. In addition, its medicinal value has also attracted wide attention. Geraniol, as a precursor for the synthesis of some clinical anticancer drugs, plays a key role in the biosynthesis of monoterpene indole alkaloids such as camptothecin and vincristine. It is also its wide application field and development prospect. By using the microbial cell factory method, this study selected a *Saccharomyces cerevisiae* strain 051H with good upstream and downstream metabolic pathways as a chassis cell to overcome the disadvantages of traditional methods such as low yield and easy to be affected by the environment, and to increase the production of geranyl alcohol. Meet the current high market demand. In this study, by constructing a recombinant plasmid expression frame, the gene encoding peroxisome whole geraniol metabolic pathway was introduced into the genome of S. cerevisiae by homologous recombination technology, so that it could be stably expressed. The recombinant strain P0515 was constructed to avoid the metabolic burden caused by plasmid insertion and improve the production of geraniol without affecting the normal growth of the strain. The research results of this paper are as follows:

(1) Firstly, the efficiency of localization of peroxisome signal peptide was verified. tVoGES was localized into the peroxisome of Xylobacter spp. to construct strain 051P. The yield of geranyl alcohol was 15.46 mg/L, which proved that there was more abundant GPP precursor in peroxisome and this organelles had significant advantages.

Subsequently, in order to realize the synthesis of geraniol in the peroxisomal compartment of S. cerevisiae, other enzymes in the geraniol synthesis pathway

were integrated into S. cerevisiae in turn to complete the reconstruction of the whole geraniol synthesis pathway in peroxisomes. Strain P0515 was obtained, and the yield was increased to 43.96 mg/L, and the growth effect was less than that of the control strain. It is proved that peroxisomes are not essential for cell growth and therefore more suitable for the construction of heterologous compound synthesis pathways.

Keywords: Saccharomyces cerevisiae; Geraniol; Peroxisome; Metabolic pathways; compartmentalization

INTRODUCTION

Monoterpene geraniol is not only a high-quality spice, but also increasingly recognized for its medicinal value, showing a wide range of development prospects and application markets. However, the traditional chemical synthesis and plant extraction processes are complex and laborious with low yield. In recent years, with the development of synthetic biology technology, more and more researches have focused on microbial cell factories for biosynthesis. *Saccharomyces cerevisiae* has gradually become a popular chassis cell because of its easy operation and ability to express complex heterologous proteins. Although introducing exogenous synthetic pathways or optimizing endogenous metabolic pathways still faces multiple challenges, with the in-depth study of the cell structure of S. cerevisiae, especially in recent years, it has been found that organelle engineering plays an important role in the efficient synthesis of complex natural products. The unique physical and chemical properties of peroxisomes lay a solid foundation for transforming peroxisomes into highly efficient product synthesis compartments. In this study, monoterpene geraniol was used as the target product. Based on the peroxisome engineering of S. cerevisiae, the pathway compartment reconstruction was used to construct a platform for the synthesis of geraniol by peroxisomes in S. cerevisiae. Finally, the expression level of geraniol was increased to 43.96mg/L with the help of peroxisome. The results of this study will lay A foundation for the compartment synthesis and extraction of high-value compounds using acetyl-coa as precursors in *S. cerevisiae*.

Purpose of the study – In this study, peroxisome was selected as the reaction site in Saccharomyces cerevisiae, and the signal peptide *ePTS1* was used to target geraniol synthesis pathway to peroxisome by homologous recombination. Purpose is to use the room change strategies in raising production of monoterpenes geraniol, effectively avoid cell internal competition and the toxicity of the intermediates of cells, etc.

Object of study – Geraniol, a monoterpenoid compound, has broad application prospects in cosmetics, medicine and other fields. However, its large-scale synthesis is faced with problems such as insufficient prerequisites and different distribution compartments of enzyme factors, resulting in short supply. Take the cell area chamber strategy, improve geraniol in peroxidase body production.

Subject of study – The disadvantages of traditional methods such as chemical synthesis and plant extraction are the choice of microbial cell factories. The whole genome sequencing of Saccharomyces cerevisiae was first completed. With the development of metabolic engineering, synthetic biology and other technologies, the industrialization of S. cerevisiae has been accelerated.

The mevalonate pathway (MVA pathway) is an endogenous terpenoid biosynthesis pathway in Saccharomyces cerevisiae. By using the complete enzymatic system in Saccharomyces cerevisiae to modify the key enzyme genes in the metabolic pathway, the product can achieve the expected yield.

CHAPTER 1
LITRATURE REVVIEW

1.1 Development of synthetic biology

Synthetic biology refers to the combination of biology with engineering science and other disciplines, using engineering principles to modify or reconstruct biomolecules to construct biological components or artificial biological systems. At present, this technology has been used to endow various organisms with new biological functions and is widely used in various fields. The rise of synthetic biology provides a new direction for microbial synthesis of complex chemicals. Through engineering genes and pathway optimization, high-value compounds such as terpenoids, flavonoids and organic acids have been efficiently synthesized[1]. S. cerevisiae, as a eukaryotic model organism, has attracted much attention due to its high robustness to low pH, high osmolal pressure, ethanol and other inhibitors in harsh industrial environments, thus becoming one of the ideal chassis cells for synthetic biology.

1.2 *Saccharomyces cerevisiae* and its metabolic pathways

1.2.1 Industrialization of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is the earliest microbial strain to achieve industrial manufacturing, and it is also the most widely used strain among yeast species today. The whole genome sequencing was first completed, and it is easy to perform genetic manipulation to express complex heterologous proteins with high biological safety. It lays the foundation for its use as a cell factory to synthesize chemicals. In addition, *Saccharomyces cerevisiae* is rich in proteins, polysaccharides and many other bioactive substances, and there are many complete biological enzyme systems in it. The fermentation process is mature, and it has been widely used in the synthesis of high-value chemicals such as fuel ethanol and terpenoids[2]. With the rapid development of metabolic engineering, synthetic biology and other technologies, S. cerevisiae has gradually exerted its potential to

become a cell factory for various compounds. Yeast cell factories are constructed to compete for two types of traditional manufacturing processes. One is the manufacturing process of some chemicals in the petrochemical industry, such as fumaric acid, α -ketoglutaric acid, and fuel ethanol. The second is the manufacturing process of some natural products in the plant extraction industry, such as terpenoids such as artemisinin and paclitaxel, which are widely used in the pharmaceutical field. At present, some products have been produced on industrial scale in *Saccharomyces cerevisiae*. In the future, with the gradual maturity of CRISPR gene editing tools and synthetic yeast chromosome technology, researchers can further improve the gene editing ability of *Saccharomyces cerevisiae*, and its production potential as a cell factory will also be improved[3].

1.2.2 Endogenous metabolic pathways in *Saccharomyces cerevisiae*

Studies on engineering *Saccharomyces cerevisiae* to produce natural compounds have focused on the synthesis of high value-added terpenoids. *Saccharomyces cerevisiae* has an endogenous Mevalonate Pathway (MVA) for the synthesis of terpenoid synthesis precursors[4]. In the laboratory, metabolic engineering strategies such as increasing the synthesis of precursors, balancing the internal competition, and enhancing the activity of key enzymes are adopted to modify the metabolic pathways and expression levels of monoterpenes in S. cerevisiae. The MVA metabolic pathway can be divided into two parts, upstream and downstream. Acetoacetyl-CoA is converted to isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are common precursors of terpenoids, through A seven-step catalytic reaction. Under the action of isopentenyl pyrophosphate isomerase (IDI),DMAPP and IPP can be interconverted[4]. Further production of the terpenoid precursors Geranyl pyrophosphate (GPP) and Farnesyl pyrophosphate (FPP) based on IPP and DMAPP catalyzed by isoprenyl transferase And Geranylgeranyl pyrophosphate (GGPP). All the above compounds are important precursors for the synthesis of

terpenoids in *Saccharomyces cerevisiae*, and are also direct substrates of terpenoid synthetase[5][6].

1.3 *Saccharomyces cerevisiae* cell factories synthesize monoterpenes

1.3.1 Synthesis of terpenoids

Terpenoids are a kind of natural compounds found to exist in a wide range and diverse species[7]. As a secondary metabolite, terpenoids mainly exist in plants and microorganisms. Many researches have made breakthroughs in their physical and chemical properties and biological activities, for example, they have been widely used in spices, cosmetics, medicine and agriculture[8]. In addition, the chemical structure and properties of some terpenoids are similar to those of fuels, and they can also be used as good biofuels[9]. Therefore, terpenoids have a broad application prospect, and the market demand is increasing. Although they are widely distributed in nature, their content is very low. At present, it is often obtained through plant extraction, chemical synthesis and other methods, which are limited by many problems such as high cost, complicated steps, environmental pollution and low final yield, and there are disadvantages such as low economic benefit and less purification content[10][11]. In contrast, biosynthesis of terpenoids has the advantages of rapid growth and reproduction, short cycle, and little influence from external environment such as climate. Nowadays, the production of natural terpenoids by microbial cell factories (such as *Saccharomyces cerevisiae*) has gradually become a feasible strategy to solve the scarcity of terpenoids[12][13].

1.3.2 Synthesis of monoterpene geraniol by *Saccharomyces cerevisiae*

Monoterpenes have the smallest molecular weight among terpenoids, and their potential value has been explored due to their strong aroma and volatility. They are mainly found in natural plants and have various types of acyclic monoterpenoids, monocyclic monoterpenoids, bicyclic monoterpenoids and tricyclic monoterpenoids. Among them, geraniol is an acyclic monoterpene with rose aroma, which exists in nutmeg, lavender, rose and other plants. It can be

obtained by purifying essential oil or synthesizing from other monoterpenes such as linalool [14]. Geraniol is slightly soluble in water and soluble in organic solvents such as ethanol, acetone and chloroform. On the other hand, geraniol has been shown to play an important role in the treatment of certain diseases due to its pharmacological properties, such as its antitumor activity. In addition, other studies have shown that geraniol can mediate anti-proliferation effects through oxidative stress [15]. Geraniol has good research prospects and economic value both as aroma substance and characteristic drug.

1.4 Regulation strategies of cellular compartmentalization

Cellular compartmentation refers to the existence of organelles with different morphology and functions in cells, which are divided into different functional regions and play their respective roles in maintaining the structural integrity and normal physiological functions of cells [16]. *Saccharomyces cerevisiae* contains a variety of organelles, each of which has a specific environment adapted to different biochemical reactions, such as mitochondrial TCA cycle and peroxisomal β-oxidation. In recent years, compartmentalization strategies have attracted much attention and been widely used in the construction of microbial cell factories. Compartmentalization of metabolic pathways refers to the localization of metabolic pathways in specific organelles or microcompartments to enhance the synthesis of various high value-added compounds [17-18]. Therefore, for efficient production of terpenoids in S. cerevisiae, the optimization of metabolic pathways can inhibit the formation of unwanted by-products and the accumulation of toxic intermediates, which can improve the final yield of target products.

Geraniol metabolic pathway has been successfully reconstructed in the mitochondria of *Saccharomyces cerevisiae* to synthesize geraniol. However, due to the existence of multiple metabolic pathways in mitochondria, adverse competition between endogenous pathways occurs, and the accumulation of metabolic intermediates leads to meaningless consumption of energy. Therefore, to overcome

the above problems, the focus of metabolic pathway modification is focused on a new organelle, peroxisome. Firstly, peroxisome does not adversely affect cell growth, and as the only site of β-oxidation, it can ensure the supply of precursor acetyl-coa. In addition, studies have found that peroxisome can accommodate the synthesis of hydrophobic substances and more useful terpenoids [19]. At the same time, peroxisomes contain abundant catalase, which can degrade and isolate toxic compounds [20]. [21]. Based on these advantages, peroxisomes have become a new choice for the synthesis and improvement of monoterpene geraniol production in S. cerevisiae.

1.5 Significance of this paper

In this study, in order to improve the production of monoterpene geraniol from *Saccharomyces cerevisiae*, a *Saccharomyces cerevisiae* strain with good upstream and downstream metabolic pathways constructed in previous work was selected as the chassis cell. Peroxisome was selected as the new reaction field and ePTS1 was selected to locate the signal peptide of peroxisomes and the geraniol synthesis pathway was targeted to peroxisomes by homologous recombination. The aim of this study is to increase the final yield of monoterpene geraniol by exploiting the abundant acetyl-coa in geraniol, and to overcome the disadvantages such as internal competition and toxic effects of intermediate products.

CHAPTER 2
OBJECT, PURPOSE, AND METHODS OF THE STUDY

2.1 Experimental Materials

2.1.1 Medium

SD-HIS auxotrophic medium: 1.7 g/L Yeast Nitrogen Base (YNB), 5 g/L ammonium sulfate, 0.69 g/L CSM-HIS (an essential amino acid mixture lacking histidine), 20 g/L glucose, pH should be controlled at 6.5-7.0. The pressure was kept at 115 ° C for 30 min.

YEPD medium: 1% yeast extract, 2% peptone, 2% glucose, weigh the above formula 10 g yeast powder, 20 g peptone dissolved in 900 mL of water, stir, if you want to make a solid medium, on the basis of the previous added 2% AGAR powder 20 g, using a sterilization pot, 115 ℃ sterilization 15 min, Twenty g of glucose was added, and the added glucose solution needed to be sterilized in advance.

LB medium: LB was prepared in a total volume of 400mL. 10 g tryptone, 5 g yeast extract, and 10 g sodium chloride were weighed and dissolved in appropriate amount of water. To make a solid medium, 20 g of AGAR powder was added. Sterilization was carried out with a sterilization pot, and sterilization was carried out with an autoclave at 115 ° C for 15 min. Before cooling, antibiotics must be added and the plate inverted.

2.1.2 Main instruments and equipment

Biosafety cabinet, PCR instrument, electrophoresis instrument, electronic balance, autoclave, metal bath, constant temperature incubator, shaking table, high speed freezing centrifuge, Shimadzu gas chromatograph, microplate reader, constant temperature water bath, pH acidity meter, microplate reader.

2.1.3 Experimental reagents

T4 ligase, FastPfu DNA polymerase, EasyTaq DNA polymerase, DNA restriction endonuclease, Plasmid Kit, Gel Extraction Kit, Cycle-Pure Kit), GeneRuler 1 kb DNA Ladder, 15 kb DNA marker, yeast extract, AGAR powder, Ampicillin sodium, dodecane, DNA restriction endonuclease, trypsin, yeast extract, AGAR powder, Genetin, Ampicillin sodium, etc.

2.2 Experimental Methods

2.2.1 Yeast chromosome extraction

(1) Single yeast colonies were selected and cultured in YPD complete medium overnight.

(2) 100 μl of bacterial solution was centrifuged at 13000 rpm for 1 min, and the supernatant was removed. Then 100 μl of 0.2 mol/L lithium acetate containing 1% SDS was added and resuspended.

(3) Heating at 75 ℃ for 10 min.

(4) Add 300 μl absolute ethanol, store under a fume hood, and vortex.

(5) Centrifugation at 12000 rpm for 5 min, and the supernatant was removed.

(6) Add 500 μl 70% ethanol to resuspension and vortex.

(7) Centrifugation at 12000 rpm for 2 min, and the supernatant was removed.

(8) Centrifugation at 12000 rpm for 1 min, removal of the supernatant, and drying.

(9) Add 30 μl double distilled water and vortex.

(10) After centrifugation for 1 min, the supernatant was yeast chromosome.

2.2.2 Synthesis of primers and genes

Primers, gene synthesis, and sequencing were performed by Paisenuo (Qingdao), Jinweizhi Biotechnology (Suzhou), and Nuoweizan Biotechnology (Nanjing).

2.2.3 Ordinary PCR and fusion PCR

Ordinary PCR: is a molecular biology technology that can amplify specific DNA fragments, using this technology to carry out special DNA replication outside the organism, so that a small amount of DNA can be greatly increased.

Fusion PCR: Based on the PCR method, two adjacent DNA sequences are linked together in a specific way. In general, special primers were designed as linker heads for the upper and lower two target fragments. The 5' and 3' ends of the designed primer sequences each contained a template sequence to be fused to link the two DNA sequences together.

Table 2-1 Reaction temperature and time for each step of PCR

	Temperature	Time
predenaturation	95 ℃	10 min
Change of sex	95 ℃	15 s
annealing	56 ℃	15 s
extension	72 ℃	1 kb/min
Terminal extension	72 ℃	10 min
save	4 ℃	10 min

Table 2-2 PCR amplification system

The system	Volume(50 μl)
ddH2O	18
Buffer	25
dNTP	1
Primers	2+2
template	1

2.2.4 DNA fragment gel recovery

(1) The target genes obtained by PCR were subjected to agarose gel electrophoresis. Under ultraviolet light, the DNA fragments containing the target bands were cut out of the gel, transferred to a centrifuge tube, and weighed.

(2) An equal volume (mass $\times 1000\,\mu$ l) of XP Buffer was added and soled for 10 min at 60 ° C.

(3) Absorb 800 μ l on BL to equilibrate, centrifuged at 12000 rpm for 1 min, and pour out the bottom solution.

(4) 600 μ l PW (with ethanol) was added and centrifuged at 12000 rpm for 1 min.

(5) Repeat step (4).

(6) Centrifugation at 12000 rpm for 2 min, and drying on EP tube for 3 min.

(7) Add 30 μ l double distilled water and centrifuge at 12000 rpm for 2 min.

(8) The water in the EP tube was sucked up to the center of the column and centrifuged at 12000 rpm for 2 min.

(9) Measure the concentration.

2.2.5 Enzyme digestion reaction

(1) If the DNA concentration is 600 ng/ μ l, add the following ingredients:

Table 2-3 Digestion reaction system

DNA	10 μl
BamHI	6 μl
ApaI	6 μl
FD Buffer (10×Buffer)	3 μl
Water	5 μl

(2) Water bath at 37 ℃ for more than 2 hours.

(3) Agarose gel electrophoresis verification.

2.2.6 Plasmid construction

(1) 2×MultiF Seamless Assembly Mix kit was used to connect the plasmid and the target fragment. The total system consisted of 20 μl: 1 μl of target fragment, 2 μl of vector, 7 μl of double distilled water, and 10 μl of 2×MultiF Seamless Assembly Mix.

(2) Metal bath at 50 ℃ for 15 min.

2.2.7 E. coli transformation

(1) Add 5 μl plasmid to 50 μl competent cells, blow and suction once, knock several times to mix.

(2) The plasmid was adsorbed on the competent cells by ice bath for 30 min.

(3) The plasmids were induced into competent cells by heat shock at 42 ℃ for 90 s.

(4) Ice bath for 3 min.

(5) Add 1 ml LB liquid to EP tube and incubate at 37℃ for 1 hour.

(6) Plates were coated on LB+Amp solid medium.

(7) Single colonies were selected and expanded into 8 ml LB plus 4 μl ampicillin medium with a final concentration of 100 μl/ml for plasmid extraction.

2.2.8 Plasmid extraction

(1) The bacteria were collected and centrifuged at 4000 rpm for 5 min, and the supernatant was discarded.

(2) Add 250 μl Solution 1 to resuspend the bacteria and move them to a 1.5 ml EP tube.

(3) Add 250 μl Solution 2 to mix the mixture upside down, and let it stand for 2 min to make the cells fully lysed.

(4) 350 μl Solution 3 was added and gently mixed upside down to precipitate intracellular proteins.

(5) Centrifugation at 13000 rpm for 10 min.

(6) The supernatant was absorbed on the adsorption column and centrifuged at 13000 rpm for 1 min.

(7) 500 μl HBC Buffer was added and centrifuged at 13000 rpm for 1 min to remove the supernatant.

(8) Add 600 μl DNA Wash Buffer, centrifuge at 13000 rpm for 1 min, and pour out the bottom solution.

(9) Repeat step (8).

(10) Centrifugation with an empty column at 13000 rpm for 2 min.

(11) Rack to 1.5 ml EP tube and let dry for 5 min.

(12) 40 μl of double distilled water was added to the center of the column and centrifuged at 13000 rpm for 2 min.

(13) The water in the EP tube was sucked up to the center of the column and centrifuged at 13000 rpm for 2 min.

(14) The concentration of plasmid was measured.

2.2.9 Validation of positive transformants

(1) Pour plate: Add 25 mL LB culture medium and 25 mL Amp.

(2) Draw a square on the bottom of the plate and mark it as 1,2,3...

(3) The PCR system was prepared according to mix PCR system except template.

(4) After packing, PCR tubes were labeled as 1,2,3...

(5) 20 single colonies were selected from the coated long plates.

(6) Pick out the colonies, dip them into area 1 of plate 1, gently point them on the surface of the medium, insert them into the PCR tube and blow for several times, and then pick out other colonies.

(7) The PCR tube was used for PCR, and the gel was run for verification.

(8) Two colony labels with correct size bands were selected.

(9) Pick the correct colony number from the plate and shake it in a small white bottle.

(10) After growth to a certain concentration, the plasmid of E. coli was extracted and sequenced.

2.2.10 Yeast transformation

(1) After activation, yeast was transferred to 20 ml YEPD medium, and the initial OD was adjusted to 0.2.

(2) The cells were cultured at 30 ℃ for 3-4 h to make the OD between 0.7-1.0.

(3) The bacterial solution was transferred into a sterile centrifuge tube and centrifuged at 4000 rpm for 5 min to remove the supernatant.

(4) The cells were resuspended in 1 ml of 100 mM lithium acetate, mixed gently with a micropipettor, transferred the suspension into a sterile 1.5 ml EP tube, centrifuged at 8000 rpm for 15 seconds, and repeated twice.

(5) Add 240 μl 50% PEG and mix well.

(6) Add 36 μl 1 mol/L lithium acetate.

(7) 10 μl of 10 mg/ml single-stranded protamine DNA was added.

(8) 10 μl DNA and 60 μl double distilled water were added to the total system of 70 μl.

(9) Keep at 30 ℃ for 30 min, and then heat shock at 42 ℃ for 25 min.

(10) After centrifugation at 8000 rpm for 15 s, the supernatant was discarded. Then 1 ml sterile water was added and centrifuged at 8000 rpm for 15 s, and the supernatant was discarded. 600 μl sterile water was added, shaken, and 60 μl was sucked to coat the plate.

2.2.11 Microbiological techniques

(1) Body culture: When Escherichia coli is cultured on solid plate, firstly, the LB solid medium which is prepared in advance and sterilized by high pressure steam is put into the microwave oven for melting. When there is no suspended solid substance, it is put into the ultra-clean table for cooling and reserve (turn on

the UV lamp and put two sterile petri dishes). After the medium was allowed to room temperature, the light and ventilation were turned on on the ultra-clean table. In the ultra-clean table, 50mL of unsolidified LB medium was taken using a 50mL sterile centrifuge tube, and it was poured into two Petri dishes, about 25mL each. At the same time, observe whether there is a layer of medium with appropriate thickness at the bottom of the Petri dish, wait for a period of time, let it solidify, inoculated Escherichia coli into the solid medium by streeting way for culture, put it into a constant temperature incubator, set the temperature at 37 ℃, cultured for 12 h, the growth of the colony was observed and recorded. In the case of liquid culture, pre-prepared and sterilized liquid LB medium was poured into a triangular flask, and then cultured on a shaker at 37 ℃ and 200 rpm for 12-14 hours. In the case of yeast, the medium was changed to YEPD and the incubation temperature was changed to 30 ° C, and all incubation times could be appropriately adjusted as required.

(2) Preservation of strains: temporary preservation: The obtained strains were inoculated on solid medium by streaking or coating, incubated at a suitable temperature for a certain period of time, and then removed from the incubator and placed in a refrigerator at 4 ° C to preserve the strains for later use. Long-term preservation: E. coli preservation. Single colonies were selected and cultured in 2mL LB liquid medium in small white bottles overnight. After the yeast solution was turbid, 700uL Escherichia coli seed preservation solution was added to the sterile seed preservation tube, 700uL bacterial solution was added to the seed preservation solution, and put into the cold storage refrigerator for seed preservation. The yeast preserves the seed. The bacteria were collected and centrifuged at 13000rpm for 1min to remove the supernatant. Then 1 mL sterile seed preservation solution was added and mixed by blowing and suction, and then transferred to a sterile seed preservation tube. The refrigerator can be stored at -20 ℃ for one to two years, and at -70 ℃ for more than 5 years.

(3) Shake flask fermentation: Single colonies were incubated in 2 mL SC-His liquid medium for 24 h at 30 ° C and 200 rpm under sterile conditions, then transferred to 2 mL SC-His liquid medium for secondary activation for 12 h, and then transferred to 100 mL triangular flasks with 2 mL SC-His liquid medium. The initial OD was 0.2, and when the OD value rose to 1, dodecane was added.

(4) GC-MS detection: Preparation of standard curve: First, 1 mg/mL geraniol standard was prepared, Methods: Weight 5 mg geraniol, add dodecane to dissolve it, then volume to 5 mL, divide it into six parts, dilute it with dodecane to prepare density gradient, Standard geraniol solution of 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.0625 mg/mL and 0.03125 mg/mL, respectively. The peak area response value of each geraniol concentration was detected by GC-MS, and then the peak area was taken as the vertical axis. Geraniol concentration (mg/mL) was the abscissa, and the standard curve was made. Agilent 7890B-5977 GC-MS qualitative standards and experimental samples were used, and GC-MS qualitative standards and experimental samples were used.

(5) GC/FID detection method: Shimadzu GC-2030 system used geraniol standard for qualitative analysis and drawing the standard curve; GC chromatographic conditions: DB-FFAP column (30 m×0.25 mm×0.25 μm) was used. The injection volume was 1 μL, the flow rate was 0.78 mL/min, and the split ratio was 10:1. The initial temperature of the column temperature chamber was 60 ℃, the sample temperature was set at 260℃, and the detector temperature was set at 280℃. Programmed heating conditions: The instrument was maintained at an initial temperature of 60 ° C for 2 min and then heated to 150 ° C at a rate of 10 ° C /min for 5 min. The analysis time was 30 min. Helium was used as the carrier gas

2.2.12 Molecular biology technology

(1) Construction of geraniol metabolic pathway directed into peroxisome plasmid: In this study, geraniol synthesis pathway (MVA pathway) was targeted

into peroxisomes with the help of ePTS1 signal peptide, and monoterpene geraniol was produced in peroxisomes using a compartment strategy to overcome the endogenous metabolic pathway and the accumulation of toxic intermediates and provide geraniol production.

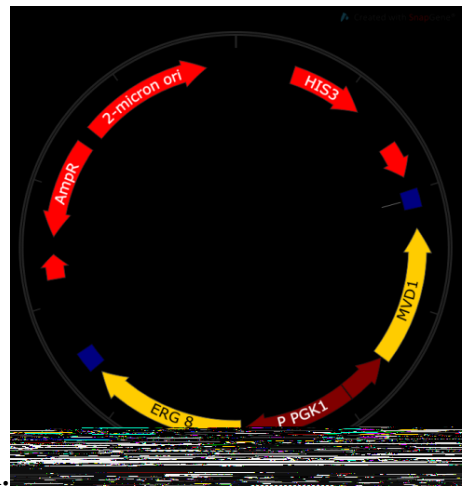
Take PIYC04e8M as an example. MVD1 and ERG8 genes were inserted between the strong promoter and the terminator of PIYC04 plasmid. The target fragment was amplified together with the strong promoter, the key enzyme gene and the terminator with the designed primers as the knockin cassette. Since PTS1 is a small peptide, for ease of manipulation, we chose to insert PTS1 directly into the end of the primer during primer design, so that it carries a targeting tag for the subsequent chromosome integration process. The same results were obtained for PIYC04etI, PIYC04e12t and PIYC04e1013. Using the above plasmids, geraniol synthesis pathway of *Saccharomyces cerevisiae* was integrated into peroxisome chromosome by homologous recombination technology, and the peroxisome chromosome was stably expressed.

(2) Amplification of gene fragments: The primers were designed based on the whole genome, plasmid or gene fragment of S. cerevisiae, and the target gene fragments were amplified by high-fidelity DNA polymerase PCR according to the instructions.

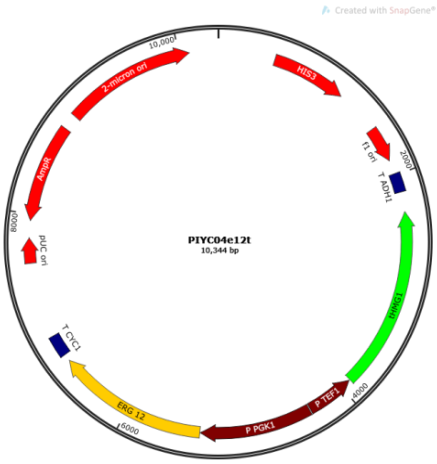
(3) Validation of gene fragments: the PCR products were verified by agarose gel electrophoresis. The electrophoresis conditions were 200 V and 18 min. Then, the gel imager was used to observe whether the target gene fragment length was correct.

(4) DNA purification and concentration determination: PCR products were purified according to the instructions of Omega Cycle-Pure Kit.

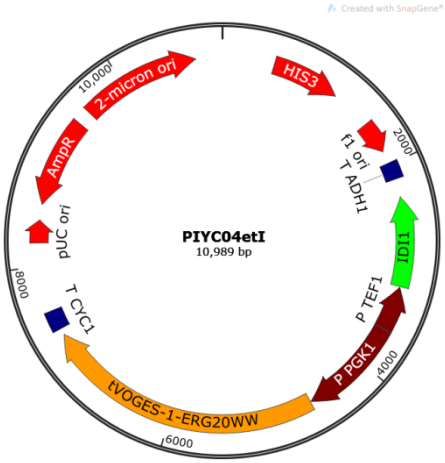
Operation: The product purification method by agarose Gel electrophoresis was carried out according to the instructions of Omega Gel Extraction Kit. The purified DNA fragments were used for concentration determination using a microplate reader.



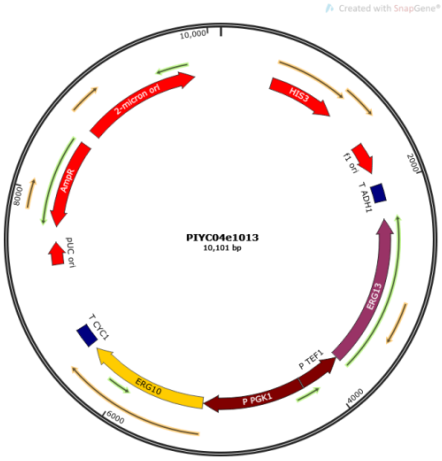
A.



B.



C.



D.

Figure 2-1 Construction of recombinant plasmids (Panel A: PIYC04e8M, panel B: PIYC04e12t, panel C: PIYC04etI, panel D: PIYC04e1013)

(5) Restriction endonuclease reaction: The DNA restriction enzyme reaction was completed according to the instructions. The amount of DNA restriction enzyme and the time of digestion can be adjusted according to the specific experiment. After the completion of the reaction, the product was purified and verified according to the purification and verification method of DNA fragments. The purified vector was used for concentration determination by microplate reader.

CHAPTER 3
EXPERIMENTAL PART

3.1 Recombinant plasmids and strains

3.1.1 Primers

Name	sequence (5 '-3')	Use
eMVD1-Sac1-R	GGCGAAGAATTGTTAATTAAGAGCTCTCACAATTTGGATCTTCTACCTCTTCCCAATTCCTTTGGTAGACCAGTCTTTGC	Construction of the p48Me plasmid
eMVD1-Not1-F	GTTTTAATTACAAGCGGCCGCATGACCGTTTACACAGCATCCG	
eERG8-Banmh1-F	CTACTTTTTACAACAAATATAAAACAAGGATCCATGTCAGAGTTGAGAGCCTTCAG	
eERG8-Xho1-R	GCGGTACCAAGCTTACTCGAGTCACAATTTGGATCTTCTACCTCTTCCCAATTTATCAAGATAAGTTTCCGGATCTTTTTCTTTC	
etHMG1-SacI-R	GAAGAATTGTTAATTAAGAGCTCTCACA	Construction of the p412te plasmid

	ATTTGGATCTTCTA CCTCTTCCCAAGGA TTTAATGCAGGTGA CGGACC	
etHMG1-Not1-F	GTTTTAATTACAAG CGGCCGCATGGCTG CAGACCAATTGGTG	
eERG12-Bamh1-F	CTTTTTACAACAAA TATAAAACAAGGA TCCATGTCATTACC GTTCTTAACTTCTG CAC	
eERG12-Xho1-R	GCGGTACCAAGCTT ACTCGAGTCACAAT TTGGATCTTCTACC TCTTCCCAATGAAG TCCATGGTAAATTC GTGTTTCC	
eERG10-Bmh1-F	CAACAAATATAAA ACAAGGATCCATGT CTCAGAACGTTTAC ATTGTATCGAC	Construction of the p41013e plasmid
eERG10-Xho1-R	CGGTACCAAGCTTA CTCGAGTCACAATT TGGATCTTCTACCT CTTCCCAATATCTT TTCAATGACAATAG AGGAAGCACC	
eERG13-SacI-R	GGCGAAGAATTGTT AATTAAGAGCTCTC	

	ACAATTTGGATCTT CTACCTCTTCCCAA TTTTTTAACATCGT AAGATCTTCTAAAT TTGTCATCG	
eERG13-Not1-F	GTTTTAATTACAAG CGGCCGCATGAAA CTCTCAACTAAACT TTGTTGGTGTG	
eIDI1-SacI-R	GGCGAAGAATTGTT AATTAAGAGCTCTC ACAATTTGGATCTT CTACCTCTTCCCAA TAGCATTCTATGAA TTTGCCTGTCATTT TCC	Construction of the p4Ite plasmid
eIDI1-Not1-F	GTTTTAATTACAAG CGGCCGCATGACTG CCGACAACAATAG TATGC	
eGES20-Bamh1-F	CTTTTTACAACAAA TATAAAACAAGGA TCCATGTCAACAGC TTCATCAATTGCTG	
eGES20-Xho1-R	CGGTACCAAGCTTA CTCGAGTCACAATT TGGATCTTCTACCT CTTCCCAATTTACT TCTCTTGTAAACCT TGTTCAAAAACG	

HO-U-F	GAAGCATGATGAAGCGTTCTAAACG	HO upstream homology arm
HO-U-R	GGTTGTCGACCTGCAGCGGGGATCTAACCTACCAGGTTCAC	
HO-D-F	GTTTTGGGACGCTCGAAGGGCAAAAGACAAAGGCGAAAAATTG	HO downstream homology arm
HO-D-R	CTGAGGAAAGTTGATCAAGACCCAATAATAATC	
Pc-IDI1-F	CGCTTAAACACTATATCAATAATGACTGCCGACAACAATAGTATG	HO knockout
e8M-R	GAGCGACCTCATGCTATACCTG	
OYE2-U-F	CGTGCTTTCTCTCGAGGTAACC	The upstream homology arm of OYE2
OYE2-U-R	GTTGTCGACCTGCAGCGCGGCCCAGTCTCTGTTTG	
OYE2-D-F	GGTATAGCATGAGGTCGCTCCTCTAATCCAGATTTGGTTGATCGTTTG	Downstream homology arm of OYE2
OYE2-D-R	GAAAGCCCAGTTTACTCCTTTCCAC	

e12-F	GACCTCATGCTATACCTGAGAAAGCCTTCGAGCGTCCCAAAACC	OYE2 knockout
Tadh1-R	GAGCGACCTCATGCTATACCTG	
OYE3-UP-F	CGTGTAGCCGCTCAAGGTTTATTTC	The upstream homology arm of OYE3
OYE3-UP-R	CTTTTATTTTGCTTTGCCCTGGTTCTTCTAAATTTAAACTTCGCTATACTGAACTACAG	
OYE3-DOWN-F	GTTTAATAACTCGAAAATTCTGCGTTGGAACAAGAACTGAAATCATGAATAAAGAC	OYE3 downstream homology arm
OYE3-DOWN-R	GGAATATTAATCACGCCCGAATCACG	
TDH3p-F	TCGAGTTTATCATTATCAATACTGCCATTTC	OYE3, DPP1 knockout
CCW12p-F	AACCAGGGCAAAGCAAAATAAAAGAAAC	
DPP1-UP-F	GATAGAAACCCAACGTTGGATAACCTC	Upstream homology arm of DPP1
DPP1-UP-R	GTTATATTAAGGGT	

	TGTCGACCTGCAGCGCGAAACTCTGTTCATTTTGGTCGTTTG	
DPP1-DOWN-F	GTATTTTAAGTTTAATAACTCGAAAATTCTGCGTTCGGATGAGGAATTACATCCTTTG	DPP1 downstream homology arm
DPP1-DOWN-R	CACCAGGTTCTTATCTCAAAAATCAGCTAC	
LPP1-up-F	ATATCAAAACATAGTAAAAAATTACAATGC	Theupstream homology arm of LPP1
LPP1-R	ATATTAAGGGTTGTCGACCTGCAGCGCCCACGAGTTCGTTAGGTACATAACG	
LPP1-down-F	CGCAATACAAGAAGTTGCATTTGGT	The downstream homology arm of LPP1
LPP1-down-R	CAATGGTAACCCTTCTGGAGTTTCGTCTGT	
KanMX-F	CGCTGCAGGTCGACAAC	KanMX amplification
KANMX-R	GCCACTAGTGGATCTGATATCACC	

3.1.2 plasmid

Plasmids	Description	Sources
pIYC04	*2μ, Ampr, HIS3,* P_{TEF1}-T_{ADH1}, P_{PGK1}-T_{CYC1}	[145]
p48Me	pIYC04, T_{ADH1}-*ePTS1-MVD1*-T_{ADH1}, P_{PGK1}-*ERG8-ePTS1*-T_{CYC1}	This study
p41013e	pIYC04, T_{ADH1}-*ePTS1-ERG10*-T_{ADH1}, P_{PGK1}-*ERG13-ePTS1*-T_{CYC1}	This study
p412te	pIYC04, T_{ADH1}-*ePTS1-ERG12*-T_{ADH1}, P_{PGK1}-*tHMG1-ePTS1*-T_{CYC1}	This study
p4Ite	pIYC04, T_{ADH1}-*ePTS1-IDI1*-T_{ADH1}, P_{PGK1}-*tVoGES-GGGS-ERG20*^{WW}-*ePTS1*-T_{CYC1}	This study

3.1.3 strain

Strain	Parent strain, Genotype/Plasmid	Resource
051H	BSPX051, *his3Δ*	[57]
P0515	051H, pJte, *HO::*T_{ADH1}-*ePTS1-IDI1*-T_{ADH1}, P_{PGK1}-*tVoGES-GGGS-ERG20*^{WW}-*ePTS1*-T_{CYC1}, *OYE2::*T_{ADH1}-*ePTS1-MVD1*-T_{ADH1}, P_{PGK1}-*ERG8-ePTS1*-T_{CYC1}, *LPP1::*T_{ADH1}-*ePTS1-ERG12*-T_{ADH1}, P_{PGK1}-*tHMG1-ePTS1*-T_{CYC1}, *DPP1::*T_{ADH1}-*ePTS1-MVD1*-T_{ADH1}, P_{PGK1}-*ERG8-ePTS1*-T_{CYC1}	This study

3.2 Localization and expression analysis of peroxisome proteins in *Saccharomyces cerevisiae*

A *Saccharomyces cerevisiae* strain 051H, which has a good suitability for upstream and downstream metabolic pathways in our previous work, was used as a

chassis cell. Firstly, a plasmid containing geranyl alcohol synthetase gene localized to peroxisomes was transferred into 051H to construct a new strain 051P. In this study, the two reconstituted strains were fermented in YPD medium at 30℃, and the OD value was measured at an interval of 6 or 12 hours. Compared with the OD value of the 051H control strain, the growth curves of the three strains were drawn to analyze the effects of the modification on the growth of the strains. In addition, geraniol's physical and chemical properties make it less soluble in water and more soluble in organic solvents. In this experiment, in light of the above properties, 20% of the fermentation volume of dodecane was added to the shaker during the fermentation process to extract geraniol in the fermentation broth. After 48 h, 60 h and 72 h of culture, the dodecane layer was taken for GC test and analysis. As shown in Figure 3-2, compared with the blank control group 051H, the geraniol standard showed a maximum area peak at 15.887 min, which was confirmed as geraniol by qualitative mass spectrometry analysis. At the same time, the recombinant strain also showed obvious peaks at 15.345 min. The peak position of the recombinant strain was compared with the mass spectrometry data of geraniol standard obtained by mass spectrometry, and the peak position was consistent with the value. It could be judged that geraniol synthase had been successfully functionally expressed in the recombinant strain.

3.3 Geraniol synthesis pathway in *Saccharomyces cerevisiae* peroxidase

We then chose to reconstruct the whole geraniol synthesis pathway in the peroxisome (as shown in Figure 3-3) by homologous recombination of the knock-in cassette amplified from the plasmid containing the geraniol synthesis gene expression cassette. DPP1, LPP1, HO and OYE2 were selected as the chromosomal integration sites for the peroxisomal geraniol synthesis pathway in S. cerevisiae. Finally, the recombinant strain P0515X, which can synthesize monoterpene geraniol in peroxisomes, was obtained.

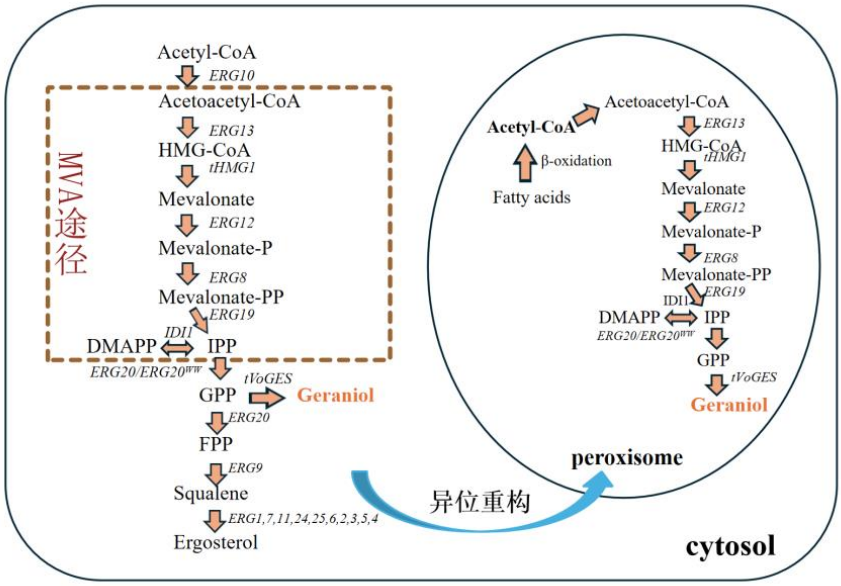


Figure 3-3 Flow chart of peroxisomal geraniol pathway construction

The control strains 051H and 051P were incubated with the recombinant strain P0515, which stably expressed geraniol metabolic pathway in *Saccharomyces cerevisiae* peroxisome, by shaking flask fermentation. Dodecane was added to extract geraniol at about 1OD, and the content of geraniol was analyzed and calculated. According to the measured data, the bar charts of geraniol production of the control strain 051H and the recombinant strains 051P and P0515 were drawn to analyze the difference in geraniol production between the modified strain and the control strain, and to discuss the feasibility of synthesis of geraniol by peroxisome compartment in S. cerevisiae. Results As shown in Figure 3-1, the production of monoterpene geraniol reached 43.96mg/L when the geraniol synthesis pathway was targeted into the peroxisome chromosome of *Saccharomyces cerevisiae*, which was nearly six times higher than that of 6.17mg/L produced by mitochondria in the previous laboratory. These results indicated that geraniol synthesis pathway could be transferred to peroxisome to reduce the metabolic burden on the growth of strain and the yield of geraniol, so as to improve the yield of geraniol. In addition, the growth of recombinant strain P0515 was not significantly different from that of the control strain 051H, indicating that the modification of peroxisome did not affect the normal growth of

the strain. These results further demonstrated that peroxisome can be transformed into an organelle for the efficient and specific production of monoterpene geranyl alcohol and other compounds with high hydrophobic value, and the production potential should not be overlooked.

CONCLUTION

(1) optimize metabolic pathways. To explore and screen more efficient peroxisome localization proteins and geraniol synthetase to improve the efficiency of enzyme transport and product synthesis. Improve the activity of rate-limiting enzymes in geraniol synthesis pathway, increase the flux of the pathway, and reduce the accumulation of intermediate toxic metabolites. Enhance precursor supply and regulate the metabolic pathways of competing precursor acetyl-coa, accurately allocate the flow of acetyl-coa, and balance product synthesis and growth demand.

(2) development of highly tolerant strains. The strains were further optimized on the existing chassis to achieve higher environmental tolerance and product toxicity tolerance.

(3) optimizing the culture conditions. Choosing a nutrient rich medium that is suitable for the growth of the target strain can shorten the growth time and increase the growth density.

(4) Try the two-compartment strategy. The feasibility of introducing geraniol synthesis pathway into *Saccharomyces cerevisiae* peroxisomes has been proved, and the yield of geraniol has been improved. By optimizing metabolic conditions, finding new organelles and using their physiological and biochemical functions to take part of the consumption of peroxisomes can further improve the ability of S. cerevisiae to synthesize geraniol and continuously increase the yield of geraniol.

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