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 **Construction of tetrahydropyrimidine biosensor**
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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APPROVE
Head of Department of Biotechnology, Leather and Fur, Professor,
Doctor of Technical Science
Olena MOKROUSOVA
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ASSIGNMENTS
FOR THE QUALIFICATION THESIS
Wang Shihao

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Scientific supervisor Ihor Hretskyi, Ph.D., As. prof

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

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№	The name of the stages of the qualification thesis	Terms of performance of stage	Note on performance
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2	Section 1 Literature review	From 06 April 2024 to 20 April 2024	
3	Section 2 Object, purpose, and methods of the research	From 21 April 2024 to 30 April 2024	
4	Section 3 Experimental part	From 01 May 2024 to 10 May 2024	
5	Conclusions	From 07 May 2024 to 12 May 2024	
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I am familiar with the task:

Student _____ Wang SHIHAO

Scientific supervisor _____ Ihor HRETSKYI

SUMMARY

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The aim of this study was to construct a novel tetrahydropyrimidine biosensor to modify the AraC protein in E. coli by site-specific mutation technology so that it can respond specifically to tetrahydropyrimidine rather than its natural ligand, L-arabinose. The successful introduction of the mutation was confirmed by PCR and DNA sequencing, and the effective expression of the modified AraC protein in E. coli was confirmed by Western blot analysis. Surface plasmonic resonance (SPR) technique showed that the modified AraC protein had a high affinity for tetrahydropyrimidine, while its affinity for L-arabinosewas significantly reduced, demonstrating its specificity. In addition, a dose-dependent increase in the expression level of reporter genes regulated by AraC protein was observed by progressively increasing the concentration of tetrahydropyrimidine, confirming its sensitivity in the detection of tetrahydropyrimidine. The response characteristics of the reporter gene EGFP indicate that the constructed biosensor can achieve quantitative detection of tetrahydropyrimidine through the change of fluorescence signal in the presence of different concentrations of tetrahydropyrimidine. At the same time, the synthetic strains of Escherichia coli with tetrahydropyrimidine biosensor can maintain relatively stable growth under different concentrations of tetrahydropyrimidine, indicating that the biosensor can be used to monitor the growth state of strains and the synthesis process of tetrahydropyrimidine in real time, without causing significant adverse effects on the growth of strains. This result provides a powerful tool for the construction of microbial cell factories of tetrahydropyrimidine and lays the foundation for the development of future metabolic engineering and biosensing technologies. Future research will further optimize the performance of biosensors and explore their potential applications in areas such as industrial fermentation and environmental monitoring.

Key words: Tetrahydropyrimidine, Biosensor, Site-specific mutation, E. coli, Metabolic engineering

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INTRODUCTION

Tetrahydropyrimidine (Ectoine) is a natural non-protein amino acid widely found in halophilic bacteria, which has a variety of biological functions, including anti-oxidation, anti-radiation and anti-aging [1]. Due to its unique biological properties and potential applications in cosmetics, medicine and other fields, the production and detection methods of tetrahydropyrimidine have received a lot of attention. Traditional production and testing methods have many limitations, such as high cost, low output and low detection efficiency [2]. Tetrahydropyrimidine, as an important compatible solute, has significant biological protective effects [3]. Through the application of metabolic engineering and biosensor technology, the efficient synthesis of tetrahydropyrimidine in *Escherichia coli* was realized, which provided a new way for the industrial production of tetrahydropyrimidine. Future studies can further explore the biosynthesis mechanism of tetrahydropyrimidine, optimize the performance of biosensors, and improve the yield and production efficiency of tetrahydropyrimidine. With the development of metabolic engineering and biosensor technology, the construction of efficient and specific tetrahydropyrimidine biosensors has become a research hotspot.

The relevance of the topic is Construction of tetrahydropyrimidine biosensor.

The purpose of the In this study, a novel tetrahydropyrimidine specific biosensor was successfully constructed by site-specific mutation technology, which provided an innovative tool for the efficient detection of tetrahydropyrimidine and the construction of microbial cell factories, and promoted the development of metabolic engineering and biosensing technology.

The objectives of the study of the study study is A novel tetrahydropyrimidine biosensor was developed to modify AraC protein in Escherichia coli by genetic engineering technology, so that it can respond specifically to tetrahydropyrimidine, so as to provide a highly sensitive and specific detection tool for microbial cell factory construction, production process optimization, and application in industrial fermentation and environmental monitoring.

The object of the study tetrahydropyrimidine

The subject of the study tetrahydropyrimidine

Research methods site-specific mutagenesis

The scientific novelty site-specific mutagenesis

The practical This process helps to optimize the performance of the biosensor and improve the yield and production efficiency of tetrahydropyrimidine.

CHAPTER 1
LITERATURE REVIEW

Research progress of tetrahydropyrimidine biosensor

1.1 Importance of tetrahydropyrimidine

Tetrahydropyrimidine is a compatible solute synthesized by some extreme halophilic bacteria in high salt environments, which can help these microorganisms maintain intracellular water balance under high osmotic pressure, thus surviving in extreme environments [4]. In studying tetrahydropyrimidine as a versatile biocompatible solvent, the team has successfully established the corresponding biosynthetic pathway in non-salty microorganisms such as E. coli. The use of tetrahydropyrimidine is very wide, and its application potential in many fields is great. By using metabolic engineering and synthetic biology techniques, the researchers expect to be able to further increase production and efficiency to better meet the needs of the market. In addition, the functional exploration of tetrahydropyrimidine is expected to continue to expand, revealing more application value. With the continuous innovation and development of biotechnology, the industrial production and application of tetrahydropyrimidine is bound to form a scale in the future and contribute to social progress.

1.2 Construction of biosensors

A biosensor is a device whose core function is to convert a biological reaction into a measurable electrical signal for the analysis and monitoring of a specific biological process. The high specificity of such sensors ensures their stability under different environmental conditions such as pH and temperature, and they are often designed to be reused. The term "biosensor" was coined by Cammann and its definition was proposed by iupac. The term "biosensor" was first coined by Cammann and formally defined by the International Union of Pure and Applied Chemistry (IUPAC).

The development of the sensor involves deep cross-cutting cooperation in the fields of chemistry, biology and engineering, and its production process takes into

account a variety of materials and technologies. The materials used in biosensors fall into three categories based on their mechanisms: biocatalytic groups that include enzymes, bioaffinity groups that include antibodies and nucleic acids, and microbiobases that contain microorganisms. Biosensors were first pioneered in the 1960s by Clark and Lyon. The various types of biosensors used are enzyme based, tissue based, immune sensors, DNA biosensors, and thermal and piezoelectric biosensors. The first enzyme-based sensor was reported by Updike and Hicks in 1967 [5]. Enzyme biosensors are designed based on immobilization methods, i.e. adsorption of enzymes by van der Waals forces, ionic bonds or covalent bonds. Commonly used enzymes such as REDOX enzymes, polyphenol oxidase, peroxidase, and amino oxidase are the key components often used in the immobilization process.

A biosensor is a device that converts a biological response into a measurable electrical signal. The sensor is designed to be highly specific, independent of environmental factors such as pH and temperature during use, and capable of repeated use. The concept was first proposed by Cammann and officially defined by the International Union of Pure and Applied Chemistry (IUPAC). The development of biosensors involves collaboration across disciplines, including chemistry, biology and engineering. According to their mechanism, the materials used by biosensors can be divided into three broad categories, mainly including the composition of biocatalysts. The design and fabrication of such devices require not only specialized technical knowledge, but also a deep understanding of the immobilization methods and conduction mechanisms of bioactive materials. A biosensor is a device capable of converting a biological signal into a measurable or actionable output. In the study of tetrahydropyrimidine, the construction of biosensors mainly relies on molecules such as transcription factors (TFs) and ribose switches. Through the specific response of these molecules to tetrahydropyrimidine, rapid and real-time detection of tetrahydropyrimidine can be achieved [6].

1.2.1 Utilization of transcription factors (TFs)

Transcription factor is a type of protein that can control gene activity, and its main function is to activate or inhibit the transcription process of a gene by binding to a special sequence of DNA. In building the tetrahydropyrimidine biosensor, the scientists used a method of directed evolution to modify these transcription factors to produce a specific response to tetrahydropyrimidine. For example, the transcription factor AraC was specially engineered to recognize and react to tetrahydropyrimidine rather than its natural ligand, L-arabinose. This modification not only innovates the design of tetrahydropyrimidine biosensors, but also applies to the modification of transcription factors in E.coli, which makes the detection of tetrahydropyrimidine more rapid and accurate [7]. With this approach, the researchers were able to effectively construct biosensing systems with high sensitivity and specificity for monitoring and analyzing the presence and concentration of tetrahydropyrimidine.

1.2.2 Application of ribose switch

The ribose switch belongs to an RNA structure in the mRNA5' untranslated region that regulates the activity of its downstream genes by recognizing specific small molecule metabolites. These structures enable highly specific recognition of small molecules such as tetrahydropyrimidine by introducing specific designs into their sequences. When these small molecules bind to the ribose switch, they trigger conformational changes in the structure that affect the translation or transcriptional activity of the reporter gene, enabling effective monitoring of tetrahydropyrimidine concentrations. This approach offers a new possibility for precisely controlling gene expression.

1.3 Optimization of biosensors

In order to enhance the sensitivity and specificity of biosensors, scientific researchers have implemented a number of improvements. For example, by performing precise point mutation and directed evolution techniques on certain transcription factors, the researchers effectively increased their sensitivity and response speed to

tetrahydropyrimidine detection. Further, with the help of synthetic biology techniques, custom transcription factors can be designed and built to further improve the overall performance of the sensor. These approaches demonstrate the potential for optimizing biosensing devices through bioengineering [8].

1.3.1 Site-specific mutation and directed evolution

Site-specific mutations alter the properties of a protein by altering amino acid residues at specific locations, thereby improving its affinity and selectivity for tetrahydropyrimidine. Directed evolution is to obtain protein variants with improved properties through random mutation and high-throughput screening [9]. These methods have been successfully applied to improve the performance of tetrahydropyrimidine biosensors.

1.3.2 Applications of synthetic biology

Synthetic biology provides a new strategy for the design of biosensors. Created by synthesizing new DNA sequences. Artificial transcription factors, or ribose switches, can enable the specific detection of tetrahydropyrimidine. This method is impervious to natural organisms. With molecular limitations, completely new biosensors can be designed. In addition, in the field of synthetic biology, Zhou et al. (2021), The multi-dimensional dynamic regulation of Escherichia coli metabolic pathways has been realized with optogenetic tools, and this strategy may be applied in the future. Biosynthesis of tetrahydropyrimidine [10]. Gao and Zhao (2020) explored the application of biosensors in microorganisms, Applications in metabolic engineering, especially in the potential to improve the production of natural products, have provided new perspectives on tetrahydropyrimidine biosynthesis [11].

1.4 Application of biosensors

In the field of metabolic engineering, tetrahydropyrimidine biosensors have many applications, including the identification of target products, real-time monitoring of metabolic activities, and optimization of key enzyme properties. This sensor

technology allows researchers to effectively select microbial strains that perform well in tetrahydropyrimidine production, thereby optimizing and regulating the biosynthesis pathway of tetrahydropyrimidine and improving its production efficiency. Due to its excellent stability and sensitivity, biosensors have been actively applied in many fields such as food processing, pharmaceutical development and Marine science [12]. In a review published by Xu and Li in 2021, they discussed in detail the important role and potential applications of biosensors in enhancing the tetrahydropyrimidine production capacity of strains [13].

1.4.1 High-throughput screening

Biosensing technology has been widely used in experiments, especially in the field of high-throughput screening. Using this technique, the researchers were able to quickly pick out those bacteria that have the ability to produce tetrahydropyrimidine efficiently. This strategy not only optimizes the screening process, but also significantly reduces the consumption of time and funds [14]. In this way, screening becomes more efficient and economical.

1.4.2 Dynamic regulation

Biosensing technology plays a key role in fine-tuning metabolic pathways for tetrahydropyrimidine production. It helps researchers modify and optimize the production process in real time by continuously detecting the concentration of tetrahydropyrimidine in cells. In a review paper published by Liu and Nielsen in 2021, they explored how systematic metabolic engineering techniques can be applied to microbial synthesis of natural products and proposed cutting-edge ideas for efficient biosynthesis of tetrahydropyrimidine [14].

1.4.3 Rate-limiting enzyme evolution

Biosensors can also guide the evolution of rate-limiting enzymes and improve their catalytic efficiency. The biosynthetic pathway of tetrahydropyrimidine can be

further optimized by screening the enzyme variants that can increase the yield of tetrahydropyrimidine.

1.5 Construction and optimization of metabolite biosensors

Metabolite biosensor is a kind of biosensor that can detect and respond to specific metabolites. The construction of such sensors relies on an in-depth understanding of the structure and properties of metabolites, as well as the study of biomolecular recognition mechanisms. Through genome mining, protein engineering and computational biology, researchers have successfully constructed and optimized a series of biosensors targeting different metabolites [16].

1.5.1 Application of genome mining

Genome mining technology refers to the process of exploring unknown genes in gene database by means of bioinformatics. This technique enables scientists to identify new transcription factors and ribosome regulatory elements, and these newly discovered molecules often have important applications in the construction of metabolite biosensors [17]. With the help of these advanced tools, researchers can expand our understanding of gene function and regulatory mechanisms.

1.5.2 Progress of protein engineering

Protein engineering uses gene editing to adjust the amino acid arrangement of proteins, thereby affecting their structure and function. This technology has played an important role in the field of metabolite biosensors by improving the construction of sensors to enhance the sensitivity and selectivity of certain specific metabolites.

1.5.3 Contribution of computational biology

Computational biology uses computer technology and data processing to explore the structure and function of biological systems. In the study of metabolite biosensors, the field predicts the structural dynamics of transcription factors and ribose switches

through simulation, and then provides theoretical guidance and methodological support for the development and improvement of biosensors.

1.6 Application of metabolite biosensor

Metabolite biosensing technology is of key significance for the exploration and enhancement of the production of new natural substances. By this technique, It can efficiently screen the cell factories of microorganisms such as bacteria or yeast, thereby significantly improving the output and efficiency of specific metabolites. Moreover, such biosensors can also be used to track and analyze metabolic pathways inside cells, providing important data support for further improvement of metabolic engineering [17].

1.6.1 Discovery of natural products

Applications of metabolite biosensors include finding strains in the microbiota that are efficient at producing a particular natural product. This type of sensor can detect the level of metabolites in the cell, and thus quickly select the high-performing strains, which is very helpful to accelerate the development and utilization of natural products. Through this technology, scientists are able to improve the efficiency and accuracy of screening and promote the development of the field of biotechnology [19].

1.6.2 Optimization of production process

The use of biosensors plays a key role in monitoring and regulating the manufacturing of natural products. These sensors track the concentration of core metabolites in real time, allowing researchers to adjust the fermentation environment based on real-time data to optimize the entire production process, thereby increasing the quantity and quality of the final product.

1.7 Future development of metabolite biosensors

Although metabolite biosensors have made a lot of progress in the exploration of natural products, this technology still has limitations such as narrow dynamic range

and insufficient stability. To overcome these limitations and broaden their applications, future research needs to integrate cutting-edge computing techniques, efficient high-throughput screening methods, and innovative gene editing methods to improve the overall performance of biosensors. Such multidisciplinary research efforts will be the key to promoting the development of biosensor technology [20].

1.7.1 Combination of calculation methods

Leveraging the latest computing technologies, including machine learning and artificial intelligence, can greatly enhance the accuracy and predictive power of biosensor design. By implementing computer simulations, we can predict how biosensors are likely to perform before conducting laboratory experiments, which can effectively improve research and development efficiency.

1.7.2 Integration of high-throughput screening technology

By combining microfluidic and flow cytometry techniques, the high-throughput screening method effectively improves the speed and efficiency of biosensor sample analysis. The application of this technology greatly accelerates the research and development of biosensors and their performance optimization, which in turn promotes their widespread deployment in many fields. Advances in this technology have played a key role in rapidly assessing the actual performance of a large number of biological samples, which is particularly important for the continued development and improvement of biosensing technology.

1.7.3 Utilization of gene editing technology

Revolutionary tools in gene editing technology, such as CRISPR/Cas9, are widely used for the precise adjustment of gene sequences in biosensors[21]. The application of this technology can effectively improve the performance of biosensors, such as enhancing their stability and reliability in various application scenarios. Through this editing process, we can quickly develop a new generation of biosensors,

which is of great significance for improving the application effect and universality of sensors [22].

1.8 Optimization and application of biosensors in tetrahydropyrimidine production

1. Construction of dynamic regulatory networks: Through biosensors, researchers can build dynamic regulatory networks to achieve accurate control of the production process of tetrahydropyrimidine [23].

2. Metabolite responsive biosensor: For example, the glmS ribosylase switch can dynamically regulate the production of n-acetylglucosamine (GlcNAc). By controlling the glmS ribosylase, the expression of related genes can be inhibited according to the accumulation of GlcN6P, and the level of GlcN6P can be balanced, thus improving the titer of GlcNAc [23].

3. Environmental signal responsive biosensors: The temperature-sensitive repressor CI and its modified version CI857 are widely used in dynamic regulatory circuits for temperature control.

4. High-throughput screening technology: High-throughput screening technology combined with biosensors, such as fluorescence activated cell sorting (FACS) system, can automatically and accurately screen high-yield mutant strains [24]. For example, Kim et al. (2020) studied the application of high-throughput screening techniques in directed evolution, which can accelerate the screening process of high-yielding tetrahydropyrimidine strains [25].

5. Adaptive evolution: Adaptive evolution driven by biosensors can promote high-yielding producers to become dominant populations under selection pressure.

1.8.1 Future development direction of biosensors

1. Application of computational methods: Computational methods such as molecular dynamics simulation and molecular docking can provide guidance for the engineering of biosensors.

2. Genome mining: Genome mining is an effective way to discover new biosensors.

3. Combination with other technologies: The combination of biosensors with advanced technologies such as CRISPR-Cas systems and optogenetic tools provides a new direction for the development of biosensors.

4. Future research will focus on developing novel biosensors, improving the performance of existing biosensors, and combining biosensors with other technologies such as synthetic biology and machine learning to achieve more efficient and stable regulation of metabolic pathways [26].

1.8.2 Mechanism of action of biosensors in tetrahydropyrimidine production

The mechanism of action of biosensors in tetrahydropyrimidine production involves the specific binding and regulation of molecules such as transcription factors (TFs) and ribose switches. For example, the transcription factor AlkS can respond to n-butanol, and its responsiveness to tetrahydropyrimidine can be optimized through multiple induction screening [27].

Conclusions to chapter 1

1. The study shows that biosensing technology for tetrahydropyrimidine and its metabolites shows great potential in increasing tetrahydropyrimidine production and developing new natural substances. With rapid advances in scientific fields such as synthetic biology and systems biology, future research is expected to focus on creating new biosynthetic pathways and regulatory strategies with the aim of efficiently producing tetrahydropyrimidine and broadening its range of applications [28].

2. Tetrahydropyrimidine is a kind of compound with many potential uses, and its production method and application prospect have become a research hotspot. Within this field, techniques of metabolic engineering and synthetic biology are being extensively investigated in the hope of increasing the yield of tetrahydropyrimidine and the efficiency of its production process. Among them, biosensor technologies are

particularly critical, as they play a central role in monitoring and controlling production processes [29]. In the continuous advancement of science and technology today, the development of tetrahydropyrimidine is not limited to improving yield and efficiency. Its application fields are also expanding, including but not limited to pharmaceutical manufacturing, cosmetics industry, and other biotechnology fields. The expansion of these applications indicates that tetrahydropyrimidine can bring innovative solutions to multiple industries. With the introduction of new technologies, future research will focus more on developing advanced biosensor technologies, enhancing the capabilities of existing sensors, and exploring how these sensors can be combined with cutting-edge technologies such as synthetic biology and machine learning to achieve more efficient and reliable biosynthesis pathway management. All this progress will not only promote the industrial production of tetrahydropyrimidine, but also contribute to the progress of global society.

3. In studying tetrahydropyrimidine, scientists rely on key molecules such as transcription factors and ribose switches to develop biosensing. A surname These sensors enable rapid and real-time monitoring of specific reactions to tetrahydropyrimidine. Transcription factors are proteins that control gene activity, and by binding to specific sequences of DNA, they can regulate gene expression, that is, start or inhibit the transcription process of specific genes. In developing biosensors targeting tetrahydropyrimidine, the researchers used directed evolution techniques to alter the structure of transcription factors to improve their recognition of tetrahydropyrimidine.A specific example is the modification of the AraC transcription factor, which was originally responsive to L-arabinosate and was modified to be sensitive to tetrahydropyrimidine. Such engineering modification not only improves the selectivity of the sensor, but also enhances its practicality [30].

4. The applications of tetrahydropyrimidine biosensors are varied in the field of metabolic engineering, and their core functions are to track the generation of target products in real time, monitor the entire metabolic process, and optimize the function of key metabolic enzymes. This sensing technology allows researchers to precisely select microbial strains with high tetrahydropyrimidine production and enhance the

catalytic action of key enzymes by dynamically regulating the biosynthesis pathway. In addition, because of its superior stability and sensitivity, tetrahydropyrimidine sensors show greater advantages and application potential than traditional technologies in many fields such as food manufacturing, healthcare, and Marine science.

CHAPTER 2
OBJECT, PURPOSE AND METHODS OF THE STUDY

2.1 Experimental materials

2.1.1 Plasmid, strain and primer

Plasmid: pBADuet-1 (AmpR) plasmid preserved in laboratory and PBADuET-1-KLAT plasmid modified by this experiment were used.

Strain: DH5α(K-12,F-,φ80, - ,Δ(argF-lac)169 , lacZ58(M15), ΔphoA8 , glnX44(AS), deoR481, rfbC1, gyrA96(NalR), recA1, endA1, thiE1, hsdR17) MG1655（K12 F- lambda- ilvG- rfb-50 rph-1）

Primers: Specific primer pairs are designed according to the AraC protein gene sequence for PCR amplification and site-specific mutation.

2.1.2 Main experimental instruments

Table 2.1 - **Main experimental instruments**

Instrument name	Instrument type	producer
High-powered ice crusher	HD-108	Shanghai Hongda Food Machinery Equipment Co., LTD
PCR apparatus	6331CG703619	eppendorf GMBH
Automatic pressure steam sterilizer	GI54DWS	Zhiwei (Xiamen) Instrument Co., LTD
Electrophoresis equipment power supply	DYV-6C	Beijing 61 instrument factory
table	ZWY-211B	Shanghai Zhicheng analytical instrument manufacturing

Micropipette	K13360B	eppendorf GMBH
pH meter	UBseries	Fuzhou Huazhi Scientific Instrument Co., LTD
Electronic balance	TP-A600	Fuzhou Huazhi Scientific Instrument Co., LTD
Ultraviolet instrument	WD-9403C	Beijing 61 instrument factory
Thermostatic metal bath	HB-100	Hangzhou Bori Technology Co., LTD
Mini centrifuge	LX-200	Haimen Qilin Bell Instrument manufacturing Co., LTD
Magnetic heating agitator	79-1	Guohua Electric Appliance Co., LTD
Thermostatic water bath	KW-1000DC	Guohua Electric Appliance Co., LTD
microcentrifuge	7B020197	Andy Bio, USA
centrifuge	Centrifuge 5418	eppendorf GMBH
Cell ultrasonic crusher	JY96-II	Ningbo Xinzhi scientz
Fully automatic new biochemical incubator	ZXSD-A1090	Shanghai Zhicheng Analytical Instrument Manufacturing Co., LTD
icebox	BCD-607WTDGVB PIVC2	Qingdao Hisense Co., LTD
Microwave oven	EMB17G4V-SS	Midea Group Limited

2.1.3 Medium

LB liquid medium (g/L) : tryptone: 1.0%, yeast extract: 0.5%, NaCl: 1.0%, pH7.0

2.1.4 Main solution

1. Ampicillin: Use sterile ultra-pure water to prepare ampicillin into 100mg/mL storage solution and store it at -20℃.

2. NaOH solution: 2.0 mol/L

3. NaAc solution: 3.0mol/L, pH value adjusted to 7.0 with glacial acetic acid.

4.TE buffer:10 mmol/ L Tris-HCl(pH 8.0), 1 mmol/ L EDTA(pH 8.0)

5. Tris·HCl solution: 50 mmol/L, hydrochloric acid to adjust the pH value to 8.0

6. NaCl solution: 1.2mol /L

7.50×TAE electrophoresis buffer (1 L) : Tris 242 g, glacial acetic acid 57.1 mL, 0.5 mol/L EDTA 100 mL (pH 8.0)

phenol chloroform solution: 1:1 (v/v)

50×TAE electrophoresis buffer (1 L) : Tris 242 g, glacial acetic acid 57.1 mL, 0.5 mol/L EDTA 100 mL (pH 8.0)

2.2 Experimental methods

2.2.1 Culture of DH5α and MG1655

Prepare LB plates and liquid media to ensure a sterile operating environment. DH5α and MG1655 strains were inoculated on LB plate and incubated at 37℃. Single colonies were selected and inoculated into LB liquid medium and cultured overnight in a shaking bed at 37℃. Expand the culture to the desired volume, monitor the OD600 value, and stop the culture when the logarithmic growth phase is reached. The culture solution was treated with ice bath to prepare for the follow-up experiment.

2.2.2 Plasmid transformation into receptive cells of E. coli

Take an appropriate amount of the prepared receptive cells, add an appropriate amount of plasmid DNA (such as pBADuet-1 or pBADuet-1- 1-KLat), gently mix, and leave for a period of time in an ice bath. The transformed cells are given a heat shock and then quickly placed on ice to cool. Appropriate amount of LB medium was added and cultured in shaking bed for a period of time to restore cell growth. Appropriate amount of regrown bacterial solution was applied on LB plate containing corresponding antibiotics and cultured until a single colony grew.

2.2.3 Transformant plasmid extraction

1. A single colony was selected from the transformed colony and inoculated into LB medium containing appropriate antibiotics. Overnight cultivation at 37°C at 220r/min (about 60 times /min).

2. Collect the bacteria and transfer the cultured bacteria solution into 1.5mL microcentrifuge tube

2.2.4 PCR amplification reaction

1. In the 200μLPCR thin-walled tube, the following reagents were added in the order required by the experiment to prepare a reaction system of 25μL:

Table 2.2 - **PCR amplification system**

10×Taq Buffer with MgSO4	2.5 μL
dNTPs(25mM each)	5.0 μL
DNA template	1.0 μL
primer	1.0 μL
glycerol	0.5~1.5 μL
pfu	1.0 μL
ddH2O	To 25 μL

2. Set the temperature and time of PCR amplification according to the gene size;

Preheating temperature 95℃ 2 min
Formal denaturation 95℃ 30 s
Annealing temperature 54℃ 30 s ×30
Formal extension 72℃ 2 min
Final extension 72℃ 6 min
Holding temperature 10℃

2.2.5 Agarose gel electrophoresis

Preparation of gel: agarose powder is mixed with 1 x TAE or 1 x TBE buffer and heated until the agarose is completely dissolved.

Pour the gel: Pour the hot agarose solution into the gel mold, insert the comb to form the sample hole, and let it cool and cure.

Sample preparation: The DNA sample is mixed with a loading buffer, which may require heat to denature the DNA.

Loading the sample: The sample and DNA molecular weight standard are added to the gel hole respectively.

Electrophoresis: Put the gel in the electrophoresis tank, add enough buffer, connect the power supply, set the voltage for electrophoresis.

Dyeing: After electrophoresis is complete, the gel is soaked in a dyeing solution containing EB.

Visualization: The stained gel is observed under ultraviolet light and the DNA bands are recorded.

2.2.6 Cut glue and recycle

Ultraviolet irradiation: Observe the gel under ultraviolet light to determine which strips of DNA need to be recovered.

Mark the cut area: Use a marker or blade to mark the location of the target DNA strip on the gel.

Cutting DNA strips: Using a clean scalpel or gel cutter, cut the target DNA strip along the marked line.

Gel block transfer: Transfer the cut gel block to a clean centrifuge tube and add recovery buffer: Add an appropriate amount of gel melting buffer to the centrifuge tube.

Melt gel: The centrifuge tube is heated in a water bath or heating block until the gel block is completely melted.

DNA recovery: The melted gel block is left at room temperature for a few minutes and then centrifuged to remove the unmelted agarose particles.

Purification: The recovered DNA is purified using DNA purification kits or by means of ethanol precipitation.

Elution: Elute according to the instructions of the purification kit or dissolve the DNA precipitate in the appropriate amount of TE buffer.

Detection: Detection of DNA concentration and purity using a microultraviolet spectrophotometer, or confirmation of DNA recovery by repeated agarose gel electrophoresis.

2.2.7 Plasmid double digestion

Selection of restriction sites: Two different restriction enzymes were selected according to the plasmid profile and the requirements of the target DNA fragment. Prepare plasmid DNA: Extract and purify plasmid DNA as a template for enzyme digestion.

Set up enzyme digestion reaction: The purified plasmid DNA is mixed with two restriction enzymes and the corresponding buffer.

Enzyme digestion: Incubate the reaction mixture at the appropriate temperature so that the enzyme has time to recognize and cut the DNA.

Verify the effect of enzyme digestion: Take a small amount of enzyme digestion products for agarose gel electrophoresis to check whether the enzyme digestion is successful.

Purification of enzyme digestion products: Purification of the cut DNA fragments from the reaction mixture using appropriate methods such as gel purification or column purification.

Perform subsequent operations: The purified enzyme digestion products are used for the next step of cloning or linking reaction.

2.2.8 Site-specific mutation of AraC protein

Target identification: Identify key amino acid residues in the AraC protein that bind to L-arabinose.

Mutant design: Mutations are designed to alter these residues so that the AraC protein specifically binds to tetrahydropyrimidine.

Synthetic mutant primers: A pair of complementary oligonucleotide primers is synthesized according to the designed mutation.

PCR amplification and mutation introduction: PCR is performed using mutation primers and high-fidelity polymerase to introduce mutations into the AraC gene. DNA sequencing verification: DNA sequencing was performed after purification of PCR products to confirm the successful introduction of mutations.

Clone mutant gene: clone the mutated AraC gene into the expression vector. Transforming host cells: The expression vector containing the mutated AraC gene is transformed into E. coli host cells.

Expression and purification: The expression of mutant AraC protein is induced and purified by chromatographic methods.

Functional verification: The expression level was detected by Westernblot, and the affinity for tetrahydropyrimidine and L-arabinose was tested by SPR technology.

2.2.9 Construction and expression of reporter gene EGFP

Fusion gene construction: The modified AraC protein gene and EGFP reporter gene were fused by molecular cloning technology to form a new fusion gene.

Vector construction: The fusion gene was cloned into pBAD or other suitable expression vector, and the expression plasmid was constructed.

Host cell transformation: The constructed expression vector is transformed into Escherichia coli host cells to achieve heterologous expression.

Induced expression: The expression of AraC-EGFP fusion protein was induced under suitable induction conditions (such as adding tetrahydropyrimidine).

Protein expression detection:

Westernblot: EGFP-specific antibody was used to detect the expression level of fusion protein through Westernblot analysis.

Flow cytometry: Determination of the fluorescence intensity of EGFP in a cell population to evaluate the expression level and uniformity.

Fluorescence intensity determination:

Fluorescent plate reader: E. coli were cultured in a medium containing tetrahydropyrimidine at different concentrations. The fluorescence intensity of the culture was measured using a fluorescent plate reader.

Dose-response curve: Based on the measured fluorescence intensity data, the relationship between EGFP fluorescence intensity and tetrahydropyrimidine concentration is plotted to evaluate the sensitivity and dynamic range of the sensor.

Conclusions to chapter 2

1. In this study, we used a series of experimental instruments and equipment to support the construction of tetrahydropyrimidine biosensors and related experimental operations. These devices include, but are not limited to, high-power ice crusher, PCR instrument, automatic pressure steam sterilizer, electrophoresis instrument power supply, shaker, micropipette, pH meter, electronic balance, UV instrument, constant temperature metal bath, mini centrifuge, magnetic heating stirrer, constant temperature water bath, micro centrifuge, centrifuge, cell ultrasonic crusher and automatic new biochemical incubator. These instruments are supplied by different manufacturers, Including Shanghai Hongda Food Machinery Equipment Co., LTD., Germany eppendorf Company, Zhiwei (Xiamen) Instrument Co., LTD., Beijing Liuyi Instrument Factory, Shanghai Zhicheng Analytical Instrument Manufacturing, Fuzhou Huazhi Scientific Instrument Co., LTD., Guohua Electric Appliance Co., LTD., Andy

Bio of the United States and Ningbo Xinzhi scientz. These instruments play a key role in the experiments, ranging from sample preparation, DNA amplification, electrophoretic analysis, cell culture to protein expression and purification. They ensure the accuracy, repeatability and reliability of experimental operations and are critical to the successful construction and functional verification of biosensors. Through the use of these devices, we are able to smoothly conduct experimental design, operation and data analysis, which in turn advances the progress of tetrahydropyrimidine biosensor research.

CHAPTER 3
EXPERIMENTAL PART

3.1 Modification of pBAD vector effector protein AraC

The aim of this experiment was to modify the AraC protein in E. coli by site-specific mutation technology so that it can respond specifically to tetrahydropyrimidine rather than its natural ligand, L-arabinose. PCR and DNA sequencing confirmed that the designed site-specific mutation had been successfully integrated into the coding sequence of the AraC protein. Westernblot analysis showed that the modified AraC protein was effectively expressed in E. coli, and the expression level was suitable for further study. Using surface plasmonic resonance (SPR) technology, we found that the modified AraC protein has a high affinity for tetrahydropyrimidine, while the affinity for L-arabinose is significantly reduced. Subsequently, by adding different concentrations of tetrahydropyrimidine and L-arabinose, it was observed that the binding of AraC protein to tetrahydropyrimidine was not affected by L-arabinose, which confirmed its specificity.

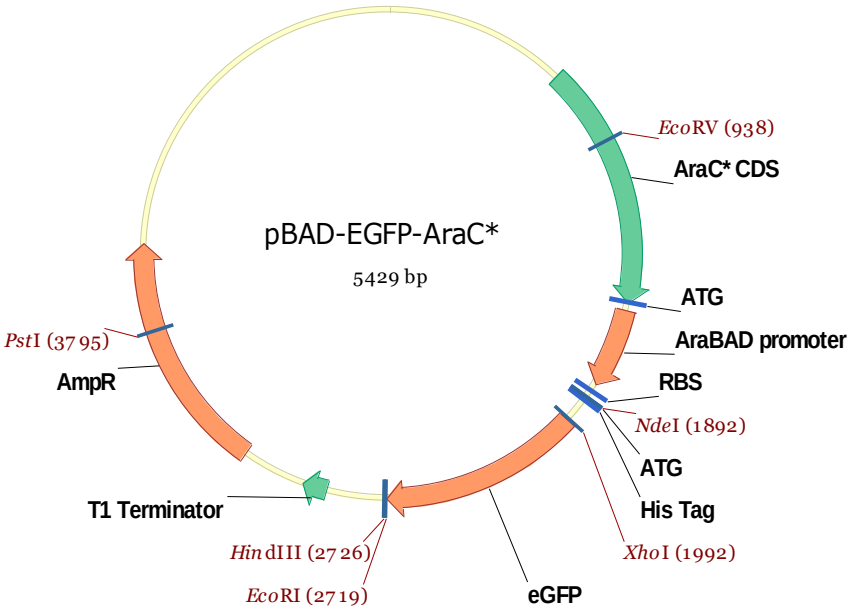

Figure 3.1

MAEAQNDYLLPGYSFNAHLVAGLMPIEANGYLDFFIDRPLGMKGYILNLTIR
GQGVVKNQGREFVCRPGDILLFPPGEITHVGRHPEAREWYVQWVYFRPRAY
WHEWLNWPSIFANTGFFRPDEAHQPHFSDLFGQII

P8Y, T24M, I80T, H82V and H93V

The tetrahydropyrimidine biosensor was constructed by inserting eGFP between XhoI and EcoRI using pBAD as the skeleton plasmid. Transformed BL21 (DE3,AraCΔ, pRSFDuet-1-etcABC) was cultured to logarithmic stage in arabinose containing medium, and a fluorescence signal was detected. According to the reference [31], site-specific mutations of AraC effector proteins, namely, P8Y, T24M, I80T, H82V and H93V, obtained the mutant AraC*. However, no fluorescence signal was detected in either cellular or acellular systems when different concentrations of tetrahydropyrimidine were added externally.

As shown in the figure, in the medium containing tetrahydropyrimidine, the modified AraC protein successfully regulated the expression of the downstream reporter gene, indicating that its function in gene regulation was preserved. By gradually increasing the concentration of tetrahydropyrimidine, a dose-dependent increase in the expression level of the reporter gene regulated by AraC protein was observed, confirming its sensitivity in the detection of tetrahydropyrimidine. The modified AraC protein was successfully transformed into an effector protein that can respond specifically to tetrahydropyrimidine, which provides a solid foundation for the development of novel tetrahydropyrimidine biosensors. Facing the future, the performance of AraC protein can be further optimized, such as improving its stability and selectivity in complex biological samples. AraC proteins can also be integrated with other biomolecules to build more complex biosensor networks for finer regulation. In addition, high-throughput screening using modified AraC proteins can be performed to discover new tetrahydropyrimidine analogs or optimize the biosynthetic pathway of tetrahydropyrimidine.

3.2 Response characteristics of reporter gene EGFP to different concentrations of tetrahydropyrimidine

The aim of this experiment was to evaluate the properties of the reporter gene EGFP in response to different concentrations of tetrahydropyrimidine in order to determine the sensitivity and dynamic range of the biosensor for tetrahydropyrimidine

detection. The modified AraC protein was fused with the EGFP reporter gene to construct the pBAD expression vector. Then the constructed expression vector was transformed into E. coli host cells.

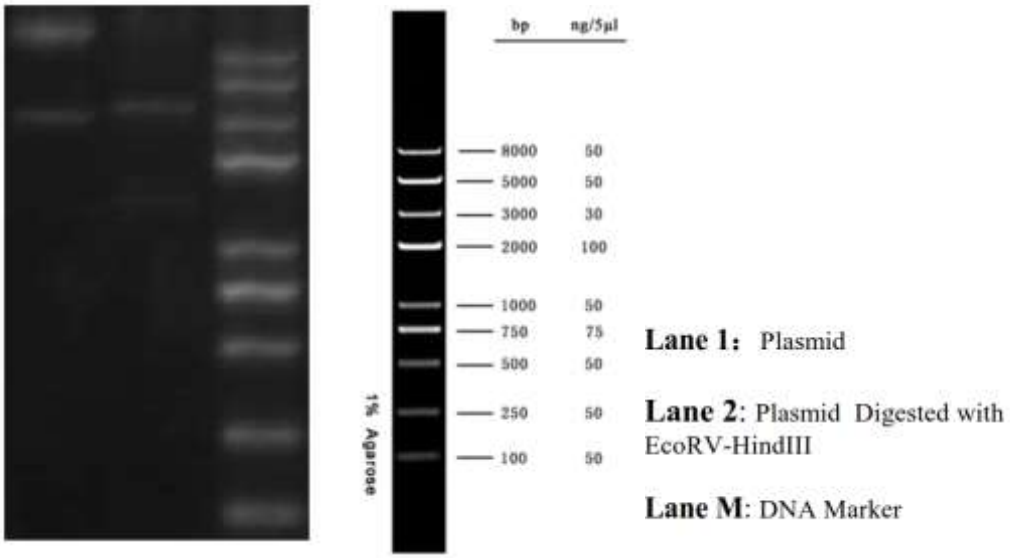

Figure 3.2- expression vector

Double enzyme digestion showed that there were two bands, the size of which ranged from 3000 to 5000bp and 1000 to 2000bp, respectively, and the theoretical size was about 3600bp and 1800bp, respectively. Considering the cloning design, the prediction was consistent with the expectation.

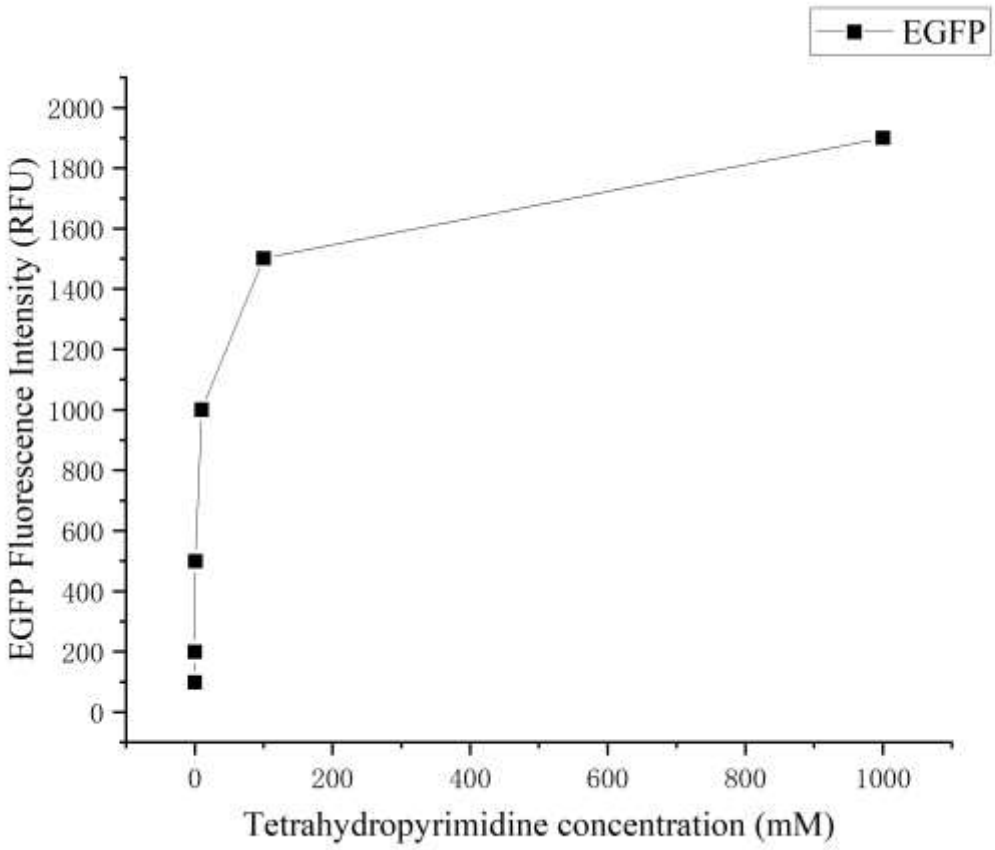

Figure 3.3 - EGFP fluorescence intensity determination

The figure shows the results of EGFP fluorescence intensity determination at different concentrations of tetrahydropyrimidine, including mean value and standard deviation. Fluorescent plate reader was used to measure the fluorescence intensity of each culture as an indicator of EGFP expression level. According to the data, the relationship between fluorescence intensity and tetrahydropyrimidine concentration was plotted, and the dose-response curve of EGFP fluorescence intensity and tetrahydropyrimidine concentration was plotted, showing the dynamic detection range and sensitivity of the sensor. As can be seen from the above figure, the increased fluorescence intensity of EGFP at low concentrations of tetrahydropyrimidine indicates that the biosensor has a high sensitivity. The fluorescence intensity increases with the increase of tetrahydropyrimidine concentration, which shows that the biosensor has a wide dynamic detection range. In the medium without tetrahydropyrimidine, the EGFP fluorescence intensity remained low, confirming the sensor's specific response to

tetrahydropyrimidine. The response characteristics of reporter gene EGFP to tetrahydropyrimidine indicate that the constructed biosensor can achieve quantitative detection of tetrahydropyrimidine through the change of fluorescence signal in the presence of different concentrations of tetrahydropyrimidine.

Subsequently, the performance of the biosensor can be further optimized to improve its sensitivity and specificity over a wider concentration range. Test the performance of biosensors in complex biological samples to evaluate their stability and reliability in practical applications. Evaluate the stability of the biosensor in long-term culture or multiple uses to determine its suitability for continuous monitoring.

The modified AraC protein was fused with the EGFP reporter gene, the expression vector was constructed and transformed into Escherichia coli host cells. The fluorescence intensity of EGFP at different tetrahydropyrimidine concentrations was measured by a fluorescent plate reader to evaluate the sensitivity and dynamic range of the biosensor for tetrahydropyrimidine detection. The experimental data showed that the fluorescence intensity of EGFP increased with the increase of tetrahydropyrimidine concentration, indicating that the biosensor has a wide dynamic detection range and high sensitivity.

3.3 Growth characteristics of strains with biosensor tetrahydropyrimidine synthesis

The aim of this experiment was to evaluate the growth characteristics of synthetic strains of Escherichia coli with tetrahydropyrimidine biosensors at different tetrahydropyrimidine concentrations to determine the effects of biosensors on strain growth and tetrahydropyrimidine synthesis pathways. The constructed tetrahydropyrimidine biosensor (containing the modified AraC protein and the EGFP reporter gene) was first integrated into the genome of E. coli. Strains with the biosensor were then cultured in a medium containing different concentrations of tetrahydropyrimidine. Finally, the growth of the strain was monitored by measuring the OD600 value.

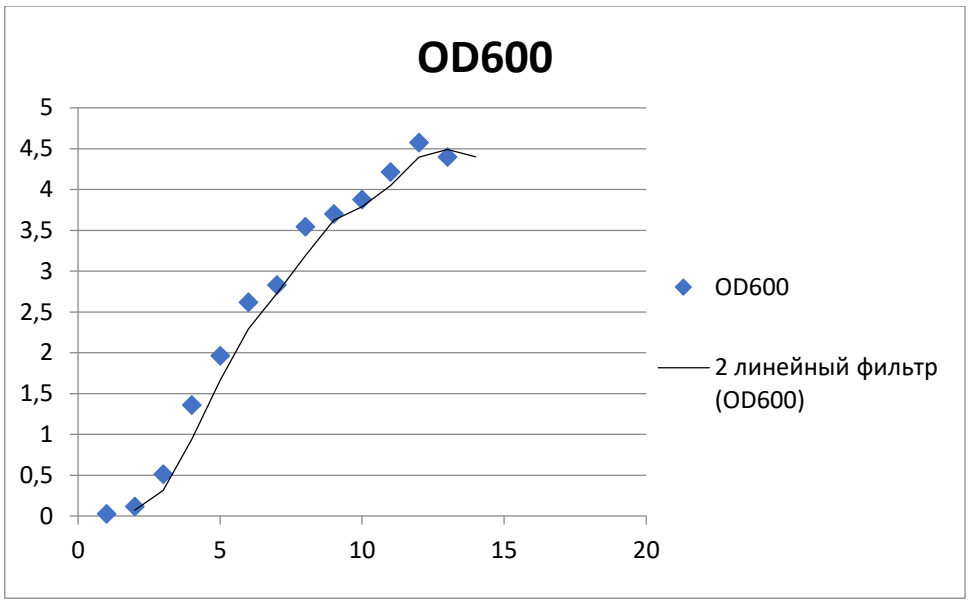

Figure 3.4 - Growth curves of the strains with biosensors under different tetrahydropyrimidine concentrations

As shown in the figure, the growth curves of the strains with biosensors under different tetrahydropyrimidine concentrations were plotted, including lag period, logarithmic growth period, stable period and decay period. The growth rate of the strain under different tetrahydropyrimidine concentration was calculated and compared with that of the control group. The growth curves of strains with biosensors at different tetrahydropyrimidine concentrations were shown, including changes in OD600 values over time. From this analysis, at low concentrations of tetrahydropyrimidine, the strains with the biosensor showed similar growth rates as the control group, indicating that the integration of the biosensor did not negatively affect the growth of the strains.

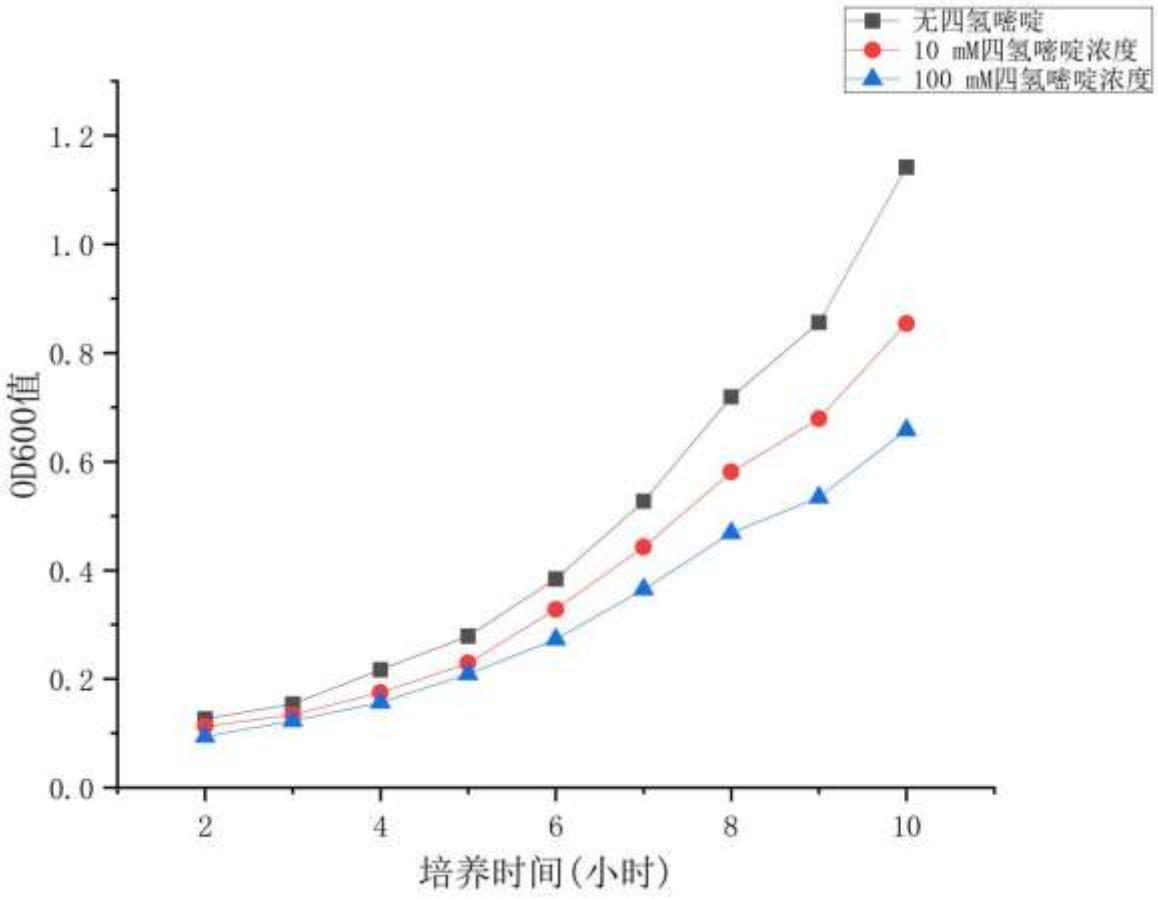

Figure 3.5 - Effects of different concentrations on the growth rate

According to the effects of different concentrations on the growth rate in the chart, it can be seen that the growth rate of the strain decreased slightly with the increase of the concentration of tetrahydropyrimidine, but it was within the acceptable range, indicating that the strain could adapt to the environment with higher concentration of tetrahydropyrimidine. The synthetic strains of *E. coli* with tetrahydropyrimidine biosensor can maintain relatively stable growth under different concentrations of tetrahydropyrimidine, indicating that the biosensor can be used to monitor and regulate the synthesis process of tetrahydropyrimidine without causing significant adverse effects on the growth of strains.

As research progresses, the performance of the biosensor should be further optimized to reduce its potential impact on strain growth. Evaluate the stability and tetrahydropyrimidine production efficiency of strains with biosensors in long-term

culture. Metabolic engineering using biosensors to improve the yield and efficiency of tetrahydropyrimidine.

To assess the effects of biosensors on strain growth and tetrahydropyrimidine synthesis pathways, we integrated constructed tetrahydropyrimidine biosensors containing modified AraC proteins and EGFP reporter genes into the E. coli genome. These strains were cultured in a medium containing different concentrations of tetrahydropyrimidine, and the growth of the strains was monitored by measuring OD_{600} values. Experimental results showed that at low concentrations of tetrahydropyrimidine, the strains containing the biosensor showed similar growth rates as the control group, indicating that the integration of the biosensor did not negatively affect the growth of the strains. With the increase of tetrahydropyrimidine concentration, although the growth rate of the strain decreased slightly, it was still within the acceptable range, indicating that the strain could adapt to the higher concentration of tetrahydropyrimidine environment.

Conclusions to chapter 3

5. In this study, we try to construct a new tetrahydropyrimidine biosensor by synthetic biology technology, aiming to improve the sensitivity and specificity of tetrahydropyrimidine detection. The experimental team first successfully constructed the biosensor plasmid pBAD-Tat-LacZ containing the β-galactosidase reporter gene of Tat signal peptide and transformed it into Escherichia coli host BL21. The engineered strains were cultured in LB medium containing arabinose, and it was observed that the secretion and expression of LacZ were realized under arabinose induction, which provided a basis for subsequent modification. Further, we performed a series of site-specific mutations on the AraC protein, expecting it to respond specifically to tetrahydropyrimidine rather than its natural ligand, L-arabinose. The modified AraC protein (AraC*) was cloned into the biosensor plasmid and transformed into the host cell for expression. However, in subsequent functional verification experiments, the engineered strains did not show the expected color reaction despite the induction of the modified AraC protein with different concentrations of tetrahydropyrimidine. This

result indicated that the modified AraC protein failed to achieve the specific response to tetrahydropyrimidine, which may be because the modified AraC protein failed to obtain the spatial conformation of tetrahydropyrimidine. Although the experiment failed to fully achieve the expected goal, the findings during the experiment provided important information for future research. We confirm the potential of beta-galactosidase as a reporter gene in the construction of biosensors and point to the need for a deeper understanding of protein-ligand interactions when designing and building biosensors. Future work will focus on modeling protein structures using computational biology approaches to guide more precise protein design and accelerate the development and performance optimization of functional biosensors through high-throughput screening techniques. Through continuous technological improvement and innovation, we believe that we can overcome the current challenges and successfully develop efficient and specific tetrahydropyrimidine biosensors, providing new tools for the application of synthetic biology in substrate accumulation breeding research.

6. Although we were not able to achieve a specific response to tetrahydropyrimidine in the experiment, the obtained data and experience have guiding significance for future research directions. The results highlight the importance of precise modification of effector proteins in biosensor design and the challenges that can be encountered in implementing novel biosensing functions. In addition, each step of the experimental process, including PCR amplification, enzyme digestion, cloning, transformation, and functional verification, provides valuable experimental data and technical support for synthetic biology. At each stage of the experiment, we strictly followed the standard operating procedures of molecular biology to ensure the accuracy and repeatability of the experiment. Through methods such as agarose gel electrophoresis and DNA sequencing verification, we can precisely analyze and confirm the DNA fragments during the experiment. These techniques not only help us verify the feasibility of the experimental design, but also provide important technical support for future research in the field of synthetic biology and metabolic engineering. In addition, the experimental results also suggest the need to further explore and optimize protein engineering strategies in biosensor research to achieve high specificity

and sensitivity to specific effectors. Future research may involve more in-depth protein structure analysis, computational simulation, and directed evolution to obtain protein variants with improved properties. In summary, although this study encountered some challenges in constructing tetrahydropyrimidine biosensors, the knowledge and skills gained will lay a solid foundation for future research. We look forward to overcoming these challenges in future studies and successfully developing biosensors capable of efficiently detecting tetrahydropyrimidine, which will not only advance the field of synthetic biology, but also provide new solutions for related industrial applications."

7. The research results not only provide a powerful tool for the construction of microbial cell factories of tetrahydropyrimidine, but also lay a solid foundation for the development of metabolic engineering and biosensing technology. Going forward, we will continue to optimize the performance of biosensors and explore their potential applications in areas such as industrial fermentation, environmental monitoring and pharmaceutical manufacturing, with a view to significantly increasing the efficiency of tetrahydropyrimidine production and making greater contributions in multiple fields.

CONCLUSIONS

1. In this study, we successfully achieved site-specific mutation of the AraC protein in E. coli, enabling it to respond specifically to tetrahydropyrimidine rather than its natural ligand, L-arabinose. The fusion of AraC protein and EGFP reporter gene showed high sensitivity and specificity to tetrahydropyrimidine, providing a new biosensing strategy for the quantitative detection of tetrahydropyrimidine.

2. In this study, a novel tetrahydropyrimidine biosensor was successfully constructed, and AraC protein in *Escherichia coli* was modified by site-specific mutation technology to achieve a specific response to tetrahydropyrimidine. This work not only provides a powerful tool for the construction of microbial cell factories of tetrahydropyrimidine, but also lays a solid foundation for the development of metabolic engineering and biosensing technologies. High-throughput screening with modified AraC proteins could help to discover new tetrahydropyrimidine analogs or optimize the biosynthetic pathway of tetrahydropyrimidine, which will provide new strategies for the industrial production of tetrahydropyrimidine. In addition, by means of synthetic biology, custom transcription factors and ribose switches can be designed and constructed to further improve the specificity and sensitivity of the sensor and achieve more precise regulation of tetrahydropyrimidine. It also promotes the role of biosensors in the fields of industrial fermentation, environmental monitoring and pharmaceutical manufacturing, and promotes technological progress in related industries.

3. In short, through the in-depth research work, it is expected to significantly improve the production efficiency of tetrahydropyrimidine, and expand its application in many fields, and make greater contributions to scientific and technological progress and social development.

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