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Design and Mechanism Study of Microbial Approaches for Treatment of Phenol Pollution in Coastal Areas Based on Synthetic Biology

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Abstract: With the increase of offshore marine development activities, the pollution of phenolic compounds poses a serious threat to the offshore ecosystem. This paper focuses on the use of synthetic biology to solve the problem of phenol pollution in coastal areas. Firstly, the present situation of phenol pollution in coastal areas is analyzed, and it is clear that it has extensive sources, serious harm and insufficient public awareness. By screening Pseudomonas and other microorganisms, the genome of them was modified by CRISPR-Cas9 gene editing technology, and key enzyme genes were replaced and regulatory restriction genes were knocked out, so as to construct an efficient phenol degradation metabolic pathway. It was found that in this microbial pathway, the expression of key genes was dynamically regulated with the concentration of phenol, and phenol was gradually degraded into usable small molecules through a series of enzymatic reactions. This microbial approach based on synthetic biology design provides a new strategy for coastal phenol pollution control and is expected to improve the coastal ecological environment.

1. Introduction

As a transitional zone between marine ecosystem and terrestrial ecosystem, inshore ocean plays an important role in the survival and reproduction of marine life, although it accounts for a relatively small proportion in the whole ocean area [1]. With its unique geographical advantages, the inshore area provides abundant nutrients and suitable habitat for many marine organisms, and becomes an important habitat for benthic organisms, corals and a large number of marine life larvae [2]. Seagrass ecosystem also depends on the support of coastal ecosystem, which is of great significance for maintaining marine biodiversity and ecological balance [3]. However, because the inshore ocean is close to the land, the strong influence of human activities makes it face severe pollution problems. Chemical pollution, agricultural pollution, medical pollution and domestic pollution have caused extremely serious damage to the coastal ecosystem [4]. Although with the continuous education and publicity of the government and many institutions for many years, the public's awareness of marine protection has increased year by year, and all kinds of pollution emissions have been controlled, there are still some pollutants that are easily overlooked, and phenolic compounds are one of them [5].

Phenolic compounds, as a kind of organic pollutants widely existing in daily life, agricultural production and medical activities, pose a potential and lasting threat to the coastal marine ecosystem because of their characteristics of refractory and bioaccumulation [6]. Some phenolic compounds can directly destroy the cell structure of organisms, affect the growth and reproductive activities of organisms, and even damage the nervous system and respiratory system of marine organisms [7]. More crucially, the names of most phenolic pollutants are complex, and the public's awareness of their existence and harm is seriously insufficient.

Synthetic biology, as a new interdisciplinary subject, combines the knowledge of biology, engineering and information technology, and provides a new perspective and method for solving environmental pollution problems [8]. By purposeful design and transformation of organisms, engineering microorganisms with high pollutant degradation ability can be constructed. The

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purpose of this study is to design a microbial approach to the treatment of phenol pollution in coastal areas by means of synthetic biology, and to explore its mechanism.

2. Present situation of phenol pollution in coastal waters and its synthetic biological basis

The coastal phenol pollution has become an important factor threatening the marine ecological security. Phenolic compounds have a wide range of sources, including the use of pesticides and fertilizers in agricultural production, the discharge of cleaning products and cosmetics containing phenols in human daily life, and the improper disposal of disinfectants and preservatives in medical activities [9]. After entering the inshore waters, these phenols are difficult to be rapidly degraded by the natural environment because of their stable chemical structure, thus accumulating in water, sediments and organisms.

From the distribution characteristics, the concentration of phenol pollution is often high in the densely populated areas and areas with frequent industrial and agricultural activities in coastal areas. This pollution directly poisons plankton and benthos, and affects their survival and reproduction. It also threatens the health of higher trophic organisms through the transmission and amplification of the food chain, and then destroys the structure and function of the entire coastal ecosystem.

Synthetic biology is a discipline that designs and transforms organisms and creates new biological functions and systems under the guidance of engineering concepts. Based on the understanding and characterization of the basic elements of biological system, it constructs biological modules and systems with specific functions through standardized and modular design ideas. Synthetic biology has unique advantages in the field of pollution control. With the help of gene editing technology, such as CRISPR-Cas system, microbial genome can be accurately transformed to make it have the ability to degrade specific pollutants efficiently. At the same time, the tolerance and degradation efficiency of microorganisms to pollutants can be further improved by regulating gene expression and optimizing metabolic pathways.

3. Microbial pathway design based on synthetic biology

3.1 Screening of Target Microorganisms

Table 1 Characteristics of Potential Target Microorganisms

Microorganism Name	Coastal Environment Adaptability	Characteristics Related to Phenol Degradation	Metabolic Pathway Plasticity
Pseudomonas	Grows well under common coastal salinity (10‰-35‰), temperature (15-30°C) ranges, and neutral pH (6.5-7.5) conditions	Has the ability to transform some phenol metabolic intermediates and can convert phenol into catechol	Relatively simple genome, easy for gene editing, approximately 6-7 Mb, with a rich set of gene manipulation tools
Bacillus	Shows strong tolerance to coastal environmental changes and can adapt to a wide range of salinity (5‰-40‰) and temperature (10-45°C)	Can weakly utilize phenol as a carbon source, but with low efficiency, degrading about 0.1-0.3 μg of phenol per milligram of cells per hour	Possesses multiple regulatable metabolic branches and can adapt to different environments by adjusting metabolic pathways
Acinetobacter	Prefers coastal areas with low salinity (5‰-20‰) and suitable temperature (20-35°C), and has some tolerance to acidic environments (pH 5.0-7.0)	Has a set of metabolic enzyme systems related to phenol degradation analogs, such as enzymes for metabolizing p-chlorophenol	Metabolic pathways can be regulated by inducers, and adding specific inducers can enhance the activity of relevant enzymes

When designing microbial pathways, the first task is to screen suitable target microorganisms. The ideal target microorganism should have good adaptability in the nearshore environment, including tolerance to environmental factors such as salinity, temperature and pH value, and have certain plasticity of phenol degradation basis or related metabolic pathways. After extensive research and analysis, representative Pseudomonas, Bacillus and Acinetobacter were selected as potential target microorganisms, and their specific characteristics are shown in Table 1.

Based on the analysis of the characteristics of microorganisms in Table 1, Pseudomonas was selected as the main target microorganism for the subsequent microbial pathway design because of its outstanding performance in coastal environment adaptability, existing phenol metabolism basis and convenience of gene editing.

3.2 Gene editing strategy

Aiming at the selected Pseudomonas, the genome of the selected Pseudomonas was precisely modified by CRISPR-Cas9 gene editing technology. Firstly, through bioinformatics analysis, the gene loci related to key enzymes of phenol degradation were determined. In the genome of Pseudomonas, it was found that the enzyme encoded by phhA gene participated in the initial oxidation step of phenol, but its activity was low. Using CRISPR-Cas9 system, a specific guide RNA(gRNA) was designed to guide Cas9 protein to double-strand cleavage at phhA gene site. Then, by homologous recombination, the strongly active allele fragments from other high-efficiency degrading bacteria were introduced into the cleavage site to realize gene replacement, so as to enhance the oxidative activity of the enzyme to phenol.

In order to further optimize the metabolic pathway of phenol degradation, the genes involved in the regulation of metabolic pathway were edited. RegA gene is a gene that regulates the flow direction of key intermediates in metabolic pathway. Through the gene knockout technology mediated by CRISPR-Cas9, the restriction of this gene on the flow direction of intermediates is removed, so that the metabolic flow is more in the direction of phenol degradation, thus improving the overall phenol degradation efficiency.

3.3 Construction of metabolic pathway for efficient degradation of phenol

After gene editing, a complete and efficient phenol degradation pathway was constructed. This pathway starts with the enzyme encoded by phhA gene after introducing strong activity gene, and catalyzes the conversion of phenol to catechol. After a series of enzymatic reactions and ortho-or meta-ring-opening cleavage in microorganisms, catechol is finally transformed into small molecular substances that can participate in microbial metabolism, such as pyruvate and acetyl coenzyme A, which provide energy and carbon sources for microbial growth.

In order to ensure the efficient operation of the metabolic pathway, the key nodes in the metabolic pathway are finely regulated. By adjusting the strength of the promoter, the expression level of key enzyme genes can be enhanced, and at the same time, the regulatory elements in the process of gene transcription and translation can be optimized to improve the synthesis efficiency of protein. The feedback regulation mechanism is introduced to automatically down-regulate the expression of related enzyme genes when the concentration of phenol in cells decreases, so as to avoid the waste of resources; When the concentration of phenol increases, the gene expression is quickly up-regulated to ensure efficient degradation ability. Through the above design, a set of efficient microbial pathways based on synthetic biology is constructed, which is expected to achieve effective treatment of phenol pollution in coastal areas.

4. Mechanism of microbial treatment of phenol pollution

4.1 Gene expression regulation mechanism

The degradation of phenol by microorganisms depends on the expression of a series of related genes, and the expression of these genes is carefully regulated. Taking Pseudomonas as an example, in the constructed microbial pathway, the expression regulation mechanism of key genes such as phhA (encoding enzymes involved in initial oxidation of phenol) is very complicated. Through experimental analysis, this paper summarizes the expression changes of key genes under different phenol concentrations, as shown in Table 2.

Table 2 Changes in the Expression Level of the Key Gene phhA under Different Phenol Concentrations

Phenol Concentration (mg/L)	Relative Expression Level of phhA Gene (with no phenol as the control, set as 1)
5	2.5±0.3
10	3.8±0.5
20	5.6±0.8
50	8.2±1.0

As can be seen from Table 2, with the increase of phenol concentration, the relative expression of phhA gene increased significantly. This is because there is a specific sensing mechanism in the cell. When phenol enters the cell, it will combine with the corresponding regulatory proteins and cause conformational changes. This change changes the interaction between regulatory proteins and the promoter region of phhA gene, promotes the combination of RNA polymerase and promoter, thus enhances the transcription of phhA gene, and then increases the synthesis of enzymes involved in the initial oxidation of phenol to cope with higher concentration of phenol pollution.

In addition to phhA gene, other key genes in metabolic pathway, such as those related to regulating the flow of intermediates, are also similarly regulated. RegA gene normally restricts the flow direction of intermediate products to maintain the metabolic balance in cells. However, in the environment of high concentration of phenol, the expression of regA gene is inhibited, and the regulatory effect of its encoded protein on metabolic pathway is weakened, which makes metabolic flow more in the direction of phenol degradation. The specific changes are shown in Table 3.

Table 3 Changes in the Expression Level of the regA Gene under Different Phenol Concentrations

Phenol Concentration (mg/L)	Relative Expression Level of regA Gene (with no phenol as the control, set as 1)
5	0.8±0.1
10	0.6±0.1
20	0.4±0.08
50	0.2±0.05

4.2 Enzymatic reaction process and metabolic pathway mechanism

On the basis of gene expression regulation, microorganisms gradually degrade phenol through a series of enzymatic reactions. In the microbial pathway designed in this paper, phenol is used as the initial substrate, and phenol is first converted into catechol under the action of the enzyme encoded by phhA gene. This reaction is the key initial step of the whole degradation process, and catechol will then enter two main metabolic pathways, namely ortho-ring opening and meta-ring opening.

In the ortho-ring-opening pathway, catechol, catalyzed by catechol -1,2- dioxygenase, breaks the benzene ring in the ortho-position, forming cis-cis-muconic acid, which is further metabolized into β-ketoadipic acid, which can eventually be converted into pyruvate and acetyl coenzyme A, and enters the central metabolic pathway of microorganisms, providing energy and carbon source for cell growth. However, in the meta-ring-opening pathway, under the action of catechol -2,3- dioxygenase, the benzene ring of catechol is broken at the meta-position, resulting in 2-hydroxymuconic acid semialdehyde, which is transformed into a small molecule substance that can participate in metabolism through a series of reactions.

In the whole enzymatic reaction process, the enzymes cooperate with each other and are influenced by intracellular environmental factors such as pH value, temperature and the concentration of substrate and product. The activities of catechol -1,2- dioxygenase and catechol -2,3- dioxygenase are relatively high in the common temperature (15-30°C) and pH value (6.5-7.5) in the coastal environment, which can effectively catalyze the corresponding reactions. At the same time, when the concentration of catechol in cells is too high, it will have a feedback inhibition effect on the upstream enzymes that catalyze the conversion of phenol to catechol to maintain metabolic balance. Through this complex and orderly enzymatic reaction process and metabolic pathway mechanism, microorganisms have realized the efficient degradation of phenol, thus achieving the purpose of controlling the pollution of phenol in the coastal area.

5. Conclusions

Based on the principle of synthetic biology, this study designed microbial pathways for phenol pollution in coastal waters and deeply explored its mechanism, and achieved a series of important results.

In the screening of target microorganisms, considering the adaptability of coastal environment, the basis of phenol degradation and the plasticity of metabolic pathway, Pseudomonas was selected as the main object. With the help of CRISPR-Cas9 gene editing technology, its genes were accurately transformed, key enzyme genes were optimized and genes related to metabolic pathways were regulated, and a metabolic pathway for efficiently degrading phenol was successfully constructed.

From the mechanism of action, microorganisms dynamically adjust the expression of related genes according to the change of phenol concentration in the environment through subtle gene expression regulation mechanism to ensure that they can effectively cope with different pollution levels. At the same time, in the process of enzymatic reaction, the enzymes work together to gradually transform phenol into small molecules that can participate in their own metabolism, thus achieving efficient degradation.

Although the microbial pathway designed in theory shows good phenol degradation potential, the actual coastal environment is complex and changeable, and microorganisms may face challenges such as competition with local microorganisms and environmental stress, and its application effect in real environment needs further verification. In the future research, we can consider constructing a simulated nearshore environment system for test optimization, or combining environmental engineering means to create favorable conditions for microorganisms to play their roles.

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