Notice

DESCRIPTION CN115595277A

Methods for enriching and isolating endophytic nitrogen-fixing bacteria from duckweed

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富集分离浮萍中内生固氮菌的方法

[0001]

Technical Field

技术领域

[n0001]

This invention belongs to the field of bacterial function research technology, and more specifically, this invention relates to a method for enriching and isolating endophytic nitrogen-fixing bacteria from duckweed tissue.

本发明属于细菌功能研究技术领域，更具体地说，本发明涉及一种富集分离浮萍组织中的内生固氮菌的方法。

[0003]

Background Technology

背景技术

[n0002]

Plant endophytic bacteria are natural resource bacteria that promote plant growth and resist stress and disease. They have broad theoretical research value and development and application prospects, and have become a research hotspot in multiple disciplines such as botany, microbiology, and plant protection.

植物内生细菌是植物生长促进与抗逆防病的天然资源菌，具有广阔的理论研究价值和开发应用前景，已成为植物学、微生物学、植物保护学等多学科的研究热点。

Endophytic bacteria colonize within plants, forming a symbiotic relationship with the host plant and playing a vital role in the plant's growth and health.

内生菌定植于植物体内，与宿主植物形成共生关系，对植物的生长和健康起到至关重要的作用。

Therefore, research on plant endophytic bacteria is becoming a focus in environmental, biological, and chemical fields both domestically and internationally.

因此，有关植物内生菌的研究正成为国内外环境、生物、化学等领域的焦点。

[n0003]

Plant endophytic fungi are ubiquitous in all parts of plants and come in a wide variety of species, but we still know very little about their habitats and species diversity.

植物内生菌普遍存在于植物体的各个部位且种类繁多，而人们对这些内生菌的栖息部位和种类多样性仍然知之甚少。

Therefore, in order to fully develop and utilize plant endophytic bacteria and their metabolite resources, it is essential to first achieve complete and accurate isolation of plant endophytic bacteria.

因而，为了充分开发利用植物内生菌及其代谢产物资源，首先必须实现植物内生菌的完全、准确的分离。

However, current research on plant endophytic bacteria is mostly focused on terrestrial plants, with relatively few studies on the enrichment and isolation of endophytic bacteria in aquatic plants.

然而目前对植物内生菌的研究多集中于陆生植物，对水生植物的内生菌富集与分离研究相对较少。

[n0004]

With the rapid development of industry and agriculture, the overuse of nitrogen and phosphorus resources has led to an increase in the eutrophication of rivers, lakes and other water bodies.

随着工业和农业的飞速发展，氮磷资源的过度使用，导致河、湖等水体富营养化程度加剧。

Duckweed is an aquatic plant with strong environmental adaptability, characterized by its fast growth rate and strong nitrogen and phosphorus absorption capacity. Recent studies have found that multiple endophytic bacteria isolated from duckweed can improve the growth rate of duckweed, accelerate the absorption of nitrogen and phosphorus in eutrophic waters, and degrade heavy metals. Therefore, exploring the endophytic functional bacteria of duckweed, including nitrogen-fixing bacteria and phosphorus-solubilizing bacteria, is of great significance for solving problems such as nitrogen absorption and degradation of heavy metals in eutrophic water bodies, and for enabling the aquatic ecosystem to develop towards a higher level of biodiversity and a more stable self-regulating mechanism.

浮萍(Duckweed)是一种环境适应能力强的水生植物，具有生长速率快、氮磷吸收能力强等特点。 近年来研究发现，从浮萍分离的多株内生菌够提高浮萍生长速率、加快富营养化水体中氮磷的吸收和降解重金属等。 因此，挖掘浮萍内生功能菌包括固氮菌、溶磷菌等对解决富营养化水体中氮素的吸收和降解重金属等水环境污染等问题，使水环境生态体系向更高水平的生物多样性和更稳定的自我调节机制方向发展提供重要意义。

[n0005]

The traditional method for isolating and purifying endophytic nitrogen-fixing bacteria is the streak plate method, which involves repeatedly diluting the microbial sample "from point to line" on the surface of a solid culture medium to achieve separation.

传统的分离纯化内生固氮菌的方法是平板划线分离法，其原理是将微生物样品在固体培养基表面多次作"由点到线"稀释而达到分离的目的。

The specific method is as follows: First, sterilize the surface of the material to remove the epiphytic bacteria on the surface, and then place it in a nitrogen-fixing medium for culture. Use an inoculation loop to dilute the mixed microorganisms or different cells in the same microbial community on the surface of the plate medium by streaking to obtain a large number of independently distributed single cells. After culture, they grow and multiply into single colonies. Usually, such single colonies are regarded as the pure culture of the microorganism to be isolated. Sometimes, these single colonies do not all originate from a single cell, so repeated isolation is necessary to obtain a pure culture.

具体的方法是：先是将材料表面进行灭菌，清除表面的附生菌，再置于固氮培养基中培养，用接种环将混杂在一起的微生物或同一微生物群体中的不同细胞在平板培养基表面通过分区划线稀释而得到较多独立分布的单个细胞，经培养后生长繁殖成单菌落，通常把这种单菌落当作待分离微生物的纯种。 有时这种单菌落并非都由单个细胞繁殖而来的，故必须反复分离多次才可得到纯种。

[n0006]

Traditional methods for isolating and purifying endophytic nitrogen-fixing bacteria typically involve surface sterilization using disinfectants such as hydrogen peroxide, ethanol, and

mercuric chloride. However, these disinfectants can be harmful to the plant itself, causing the production of reactive oxygen species within the plant, which in turn can negatively impact the endophytic bacteria.

传统的分离纯化内生固氮菌的方法，通常使用过氧化氢，乙醇，升汞等消毒剂进行表面灭菌，这样的消毒剂对植物体本身是有伤害的，会导致植物体内产生活性氧，进而也会对植物内生菌造成不良影响。

There are also reports of using formulated chemical detergents in conjunction with sterilizing solutions for surface sterilization. However, chemical detergents alone cannot remove epiphytic bacteria, and if the concentration of the matching sterilizing solution is not appropriate, it will also remove the target endophytic bacteria. Although the combination of the two can achieve the effect of removing epiphytic bacteria, the solution has too many components, is very complicated to prepare, and the treatment steps are cumbersome and time-consuming. It is crucial to find a simple tissue surface sterilization solution that can thoroughly eliminate epiphytic bacteria without affecting endophytic bacteria.

也有使用配制的化学洗涤剂配合灭菌液进行表面灭菌的报道，然而，单独使用化学洗涤液不能清除附生菌，单独使用配套的灭菌液，如果浓度不合适，会将目标内生菌一起清除掉，两者配合使用，虽然能够达到清除附生菌的效果，但是溶液成分过多，配制起来很复杂，处理步骤繁琐耗时。 找到一种简单的、能彻底清除附生菌，而又不影响内生菌的组织表面灭菌液非常重要。

[n0007]

More importantly, the inventors of this application have discovered through long-term research that there are far fewer microorganisms in water than in the soil environment where terrestrial plants live. As a result, the number and types of microorganisms adhering to the roots or leaves of aquatic plants are not large, and endophytic bacteria are even fewer.

更为重要的一点是：本申请的发明人经过长期的研究中发现，相对于陆生植物生活的土壤环境，水体中的微生物少很多，因而黏附在水生植物根系或叶片的微生物种群和含量并不多，内生菌则更少。 Therefore, the traditional method of screening endophytic nitrogen-fixing bacteria by streak plating is difficult to enrich endophytic nitrogen-fixing bacteria and cannot fully obtain the target bacteria. New methods and strategies are needed to enrich and isolate endophytic nitrogen-fixing bacteria from duckweed.

因此，采用传统的平板划线分离筛选内生固氮菌的方法，难以富集内生固氮菌，不能充分获得目标菌。 如何能够富集分离到浮萍中内生固氮菌，需要新的方法和策略。

[0010]

Summary of the Invention

发明内容

[n0008]

Based on this, one of the objectives of the present invention is to provide a method for enriching and separating endophytic nitrogen-fixing bacteria from duckweed, which can rapidly, completely, and accurately enrich and separate endophytic nitrogen-fixing bacteria from duckweed.

基于此，本发明的目的之一是提供一种富集分离浮萍中内生固氮菌的方法，该方法可以快速且完全、准确地富集并分离浮萍中的内生固氮菌。

[n0009]

The specific technical solution to achieve the above-mentioned objectives is as follows:

实现上述发明目的的具体技术方案如下：

[n0010]

A method for enriching and isolating endophytic nitrogen-fixing bacteria from duckweed includes the following steps:

一种富集分离浮萍中内生固氮菌的方法，包括以下步骤：

[n0011]

(1) After surface sterilization of duckweed with tissue surface sterilization solution, mechanically grind it in 0.1% to 1% PBS, filter it, and obtain the filtrate;

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(1)、使用组织表面灭菌液对浮萍进行表面灭菌后，于0.1%～1%PBS中机械研磨，过滤，得到滤液；

[n0012]

(2) Place the filtrate in a nitrogen-fixing culture medium for enrichment culture for 5 to 7 days, centrifuge, discard the supernatant, and obtain the enriched nitrogen-fixing bacteria.

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(2)、将滤液置于固氮培养液中富集培养5天～7天，离心，弃上清，得到富集的固氮菌群；

[n0013]

(3) Add nitrogen-fixing culture medium to a 96-well culture plate, and add the culture medium enriched in step (2) according to the gradient. Culture for 5 to 7 days to obtain bacterial culture. Then, take out the bacterial culture as reaction templates and add it to a 96-well PCR plate for PCR amplification and sequencing.

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(3)、在96孔培养板中加入固氮培养液，按梯度分别添加步骤(2)中经富集培养的培养液，培养5天～7天得到菌液，再分别吸取菌液作为反应模板，添加至96孔PCR板，进行PCR扩增，测序；

[n0014]

(4) Bacterial solutions with no extraneous bands in PCR sequencing results are single endophytic nitrogen-fixing bacteria; bacterial solutions with extraneous bands in PCR sequencing results are streaked on nitrogen-fixing medium to separate and purify until single endophytic nitrogen-fixing bacteria are obtained.

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(4)、PCR测序结果无杂带的菌液，即为单一内生固氮菌；PCR测序结果有杂带的菌液，在固氮培养基上划线分离纯化至得到单一内生固氮菌。

[n0015]

In some embodiments, the gradient in step (3) is: 50 μL, 20 μL, 10 μL, 5 μL, 1 μL and 0.5 μL of culture medium are added to 100 μL of nitrogen-fixing culture medium, respectively.

在其中一些实施例中，步骤(3)中所述梯度为：100μL固氮培养液中分别添加50μL、20μL、10μL、5μL、1μL和0.5μL的培养液。

[n0016]

In some embodiments, the primers for PCR amplification in step (3) are shown in SEQ ID No. 1 and SEQ ID No. 2.

在其中一些实施例中，步骤(3)中所述PCR扩增的引物如SEQ ID No.1和SEQ IDNo.2所示。

[n0017]

In some embodiments, the PCR amplification reaction system comprises: 2 μL–4 μL bacterial culture, 5 μL bacterial cell lysis buffer, 2 μL–3 μL 10x PCR buffer, 0.3 mM–0.6 mM MgClNER, 0.1 mM–0.3 mM dNTPs, 0.5 μM–1.0 μM primers, 0.3 μL–0.6 μL 5 U/μL Taq polymerase, and sterile ddHNERO to a final volume of 25 μL.

在其中一些实施例中，所述PCR扩增的反应体系为：2μL～4μL菌液、5μL细菌细胞裂解液、2μL～3μL 10x PCR buffer缓冲液、0.3mM～0.6mM MgCl₂、0.1mM～0.3mM dNTPs、引物各0.5μM～1.0μM、0.3μL～0.6μL 5U/μLTaq聚合酶，加无菌ddH₂O至25μL。

[n0018]

In some embodiments, the tissue surface sterilization solution in step (1) comprises the following components: 0.5× to 1.5×PBS, Triton-X100 with a volume percentage of 0.2% to 0.4%, sodium hypochlorite with a mass-volume concentration of 3% to 6%, and pH 6.5 to 7.0.

在其中一些实施例中，步骤(1)中所述组织表面灭菌液包括以下组分：0.5×～1.5×PBS、体积百分浓度0.2%～0.4%的Triton-X100、质量体积浓度3%～6%的次氯酸钠，pH6.5～7.0。

[n0019]

In some embodiments, the tissue surface sterilization solution in step (1) comprises the following components: 1× to 1.5×PBS, Triton-X100 with a volume percentage of 0.3% to 0.4%, sodium hypochlorite with a mass-volume concentration of 4% to 6%, and pH 6.8 to 7.0.

在其中一些实施例中，步骤(1)中所述组织表面灭菌液包括以下组分：1×～1.5×PBS，体积百分浓度0.3%～0.4%的Triton-X100、质量体积浓度4%～6%的次氯酸钠，pH6.8～7.0。

[n0020]

In some embodiments, the surface sterilization method in step (1) is as follows: add duckweed to the tissue surface sterilization solution, shake and wash at 1500 rpm to 2500 rpm for 3 min to 5 min, pour off the tissue surface sterilization solution, add sterile water, shake and wash at 1500 rpm to 2500 rpm for 1 min to 2 min, and repeat 2 to 3 times.

在其中一些实施例中，步骤(1)中所述表面灭菌的方法为：在组织表面灭菌液中加入浮萍，1500rpm～2500rpm振荡清洗3min～5min，倒去组织表面灭菌液，再加入无菌水，1500rpm～2500rpm振荡清洗1min～2min，重复2～3次。

[n0021]

In some embodiments, the method for determining whether the duckweed surface is thoroughly sterilized in step (1) is as follows: the cleaning solution after washing the duckweed with sterile water is dripped into the bacterial culture medium LB. If no colonies are formed, it indicates that the duckweed surface has been thoroughly sterilized.

在其中一些实施例中，步骤(1)中确定所述浮萍表面灭菌是否彻底的方法为：将无菌水清洗浮萍后的清洗液，滴至细菌培养基LB中，若无菌落形成，则表示浮萍表面已彻底灭菌。

[n0022]

In some embodiments, the grinding time in step (1) is 30s to 60s.

在其中一些实施例中，步骤(1)中所述研磨的时间为30s～60s。

[n0023]

In some embodiments, the culture temperature in steps (2) and (3) is 28°C to 32°C.

在其中一些实施例中，步骤(2)和步骤(3)中所述培养温度为28°C～32°C。

[n0024]

This invention aims to provide a method for rapidly enriching and separating endophytic nitrogen-fixing bacteria from the aquatic plant duckweed. Compared with the prior art, this invention has the following advantages and beneficial effects:

本发明旨在提供一种快速富集与分离水生植物浮萍中的内生固氮菌的方法，与现有技术相比，本发明具有以下优点和有益效果：

[n0025]

1. In response to the difficulty in enriching endophytic nitrogen-fixing bacteria in duckweed using traditional methods, the inventors have proposed a new approach and strategy to rapidly and accurately enrich and isolate endophytic nitrogen-fixing bacteria from duckweed. Based on the functional research system of duckweed as an "aquatic model plant," the method first enriches endophytic nitrogen-fixing bacteria in duckweed, which are originally present in small populations and quantities. Then, a gradient dilution method is used, combined with high-throughput separation and sequencing analysis. The presence of heterogeneous bands in the sequencing results determines whether the endophytic nitrogen-fixing bacteria are a single pure species. Compared with the traditional streak plate method for isolating and purifying endophytic nitrogen-fixing bacteria, the method of this invention can isolate endophytic nitrogen-fixing bacteria from duckweed more quickly, completely, and accurately.

1、针对发明人发现的难以用传统方法来富集浮萍中的内生固氮菌，发明人从新的思路和策略提供了一种能够快速和准确的富集分离浮萍中内生固氮菌的方法：基于紫萍“水生模式植物”的功能研究体系，先富集浮萍中原本种群和含量都很少的内生固氮菌，再采用梯度稀释法，并结合高通量分离、测序解析，通过测序结果是否含有杂带来判断内生固氮菌是否为单一的纯种，相比起传统的平板划线法分离纯化内生固氮菌，本发明的方法可以更为快速地、完全地、准确地使浮萍中的内生固氮菌得以分离；

[n0026]

2. This invention uses a tissue surface sterilization solution to sterilize duckweed tissue. Sodium hypochlorite in the sterilization solution is an oxidizing bactericide; a concentration of 4% to 6% can quickly kill epiphytic bacteria with weak adhesion to the plant. When combined with PBS, which maintains cell osmotic pressure and protects cell structure, and Triton-X100, which can dissociate membrane proteins from the cell membrane and wash away tissue surface deposits but has no killing effect on bacteria or other microorganisms, this invention achieves thorough tissue surface sterilization while protecting plant and endophytic bacterial cells from damage. This greatly simplifies the required solution components and makes preparation simple.

2、本发明通过使用组织表面灭菌液对浮萍组织进行表面灭菌，灭菌液中的次氯酸钠是一种氧化性杀菌剂，4%～6%的浓度就可以快速杀灭植物体上黏附能力较弱的附生菌，再恰到好处地配合使用可以维持细胞渗透压，保护细胞结构PBS，以及可将膜蛋白从细胞膜上解离下来，清洗掉组织表面附着物，但对细菌等微生物没有杀伤作用的Triton-X100；从而可以在保护植物和内生菌细胞不受破坏的同时，又达到组织表面彻底灭菌的目的，极大地简化了所需溶液成分，配制简单；

[n0027]

3. The method of enriching and separating endophytic nitrogen-fixing bacteria in duckweed of the present invention helps to discover more functional nitrogen-fixing bacteria, improve the

growth rate of duckweed, and accelerate the absorption of nitrogen in eutrophic water bodies and the degradation of heavy metals and other water pollution problems. It is of great significance for duckweed water body restoration and water environment purification, enabling the water environment ecosystem to develop towards a higher level of biodiversity and a more stable self-regulation mechanism.

3、本发明的富集分离浮萍中内生固氮菌的方法有助于挖掘更多功能固氮菌提高浮萍生长速率，以及加快富营养化水体中氮素的吸收和降解重金属等水环境污染等问题，对浮萍水体修复和水环境净化具有重要意义，使水环境生态体系向更高水平的生物多样性和更稳定的自我调节机制方向发展。

[0031]

Attached Figure Description

附图说明

[n0028]

Figure 1 is a structural diagram of the endophytic nitrogen-fixing bacteria community enriched from surface-sterilized duckweed in Example 1 of the present invention. DW is the

experimental group that sterilizes the surface of duckweed using the tissue surface sterilization solution of the present invention; CK is the control group that sterilizes the surface of duckweed using the salt solution (SD+B) of the prior art.

图1是本发明实施例1中从表面灭菌后的浮萍中富集的内生固氮菌群的组成结构图，其中，DW为采用本发明的组织表面灭菌液对浮萍表面灭菌的实验组；CK为采用现有技术的盐溶液(SD+B)对浮萍表面灭菌的对照组。

[0033]

Detailed Implementation

具体实施方式

[n0029]

To facilitate understanding of the present invention, a more comprehensive description of the present invention will be provided below.

为了便于理解本发明，下面将对本发明进行更全面的描述。

This invention can be implemented in many different forms and is not limited to the embodiments described herein.

本发明可以以许多不同的形式来实现，并不限于本文所描述的实施例。

Conversely, these embodiments are provided to enable a more thorough and complete understanding of the disclosure of this invention.

相反地，提供这些实施例的目的是使对本发明公开内容的理解更加透彻全面。

[n0030]

Experimental methods not specifically described in the following examples are generally performed under standard conditions, such as those described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989), or as recommended by the manufacturer.

下列实施例中未注明具体条件的实验方法，通常按照常规条件，例如Sambrook等人，分子克隆：实验室手册(NewYork:Cold Spring Harbor Laboratory Press,1989)中所述的条件，或按照制造厂商所建议的条件。

All commonly used chemical reagents used in the examples are commercially available products.

实施例中所用到的各种常用化学试剂，均为市售产品。

[n0031]

Unless otherwise defined, all technical and scientific terms used in this invention have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains.

除非另有定义，本发明所使用的所有的技术和科学术语与属于本发明的技术领域的技术人员通常理解的含义相同。

The terminology used in this specification is for the purpose of describing particular embodiments only and is not intended to limit the invention.

本发明的说明书中所使用的术语只是为了描述具体的实施例的目的，不用于限制本发明。

The term "and/or" as used in this invention includes any and all combinations of one or more of the associated listed items.

本发明所使用的术语“和/或”包括一个或多个相关的所列项目的任意的和所有的组合。

[n0032]

The present invention will be further described in detail below with reference to specific embodiments and accompanying drawings.

以下结合具体实施例和附图对本发明作进一步详细的说明。

[n0033]

Example 1: A method for enriching and isolating endophytic nitrogen-fixing bacteria from duckweed

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实施例1一种富集分离浮萍中内生固氮菌的方法

[n0034]

This embodiment of a method for enriching and isolating endophytic nitrogen-fixing bacteria from duckweed includes the following steps:

本实施例的一种富集分离浮萍中内生固氮菌的方法，包括以下步骤：

[n0035]

1 Surface sterilization of duckweed

1、浮萍表面灭菌

[n0036]

Pour 20 mL of sterile water into a 50 mL centrifuge tube, add a small amount of collected duckweed (from Guangzhou, Guangdong) into the centrifuge tube, and shake and wash it at 2000 rpm for 3 minutes. Repeat 3 times.

在50mL离心管中倒入20mL无菌水，取少量采集的浮萍(广东广州)放入离心管中，以2000rpm的速率在振荡器上进行振荡清洗3min，重复3次。

[n0037]

The following methods were used to sterilize the surface of the cleaned duckweed.

分别采取以下方法对清洗后的浮萍进行表面灭菌。

[n0038]

a. The tissue surface sterilization solution of the present invention (labeled as DW)

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a、本发明的组织表面灭菌液(标记为DW)

[n0039]

Pour 20 mL of sterile salt solution into a 50 mL centrifuge tube, then place the washed duckweed into the centrifuge tube and shake it at 2000 rpm for 5 minutes.

在50mL离心管中倒入20mL无菌盐溶液，再将清洗后的浮萍放入离心管中，以2000rpm的速率在振荡器上进行振荡清洗5min。

Discard the sterile salt solution from the centrifuge tube, add sterile water, and shake at 2000 rpm for 1 minute on a shaker to rinse away any residual sterile solution on the surface of the duckweed.

倒去离心管中的无菌盐溶液，加入无菌水，以2000rpm的速率在振荡器上进行振荡清洗1min，以冲洗掉浮萍表面残留的灭菌液。

[n0040]

In this embodiment, the tissue surface sterilization solution comprises the following components: 1×PBS, 0.3% (v/v) Triton-X100, 5% (g/100ml) sodium hypochlorite, pH 7.0.

在该实施例中，组织表面灭菌液包括以下组分：1×PBS、0.3%(v/v)Triton-X100，5%(g/100ml)次氯酸钠，pH7.0。

[n0041]

b. Control group consisting of chemical detergent and sterilizing solution (labeled CK)

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b、化学洗涤液+灭菌液对照组(标记为CK)

[n0042]

Pour 20 mL of chemical washing solution SD into a 50 mL centrifuge tube, then place the washed duckweed into the centrifuge tube and shake it at 2000 rpm for 5 minutes.

在50mL离心管中倒入20mL化学洗涤液SD，再将清洗后的浮萍放入离心管中，以2000rpm的速率在振荡器上进行振荡清洗5min。

Discard the salt solution from the existing method in the centrifuge tube, add sterile water, and shake and wash at 2000 rpm for 1 min on a shaker to rinse away the residual chemical

detergent SD on the surface of the duckweed; then wash with 5% sodium hypochlorite for 1 min to 2 min, and finally rinse twice with Na₂S₂O₃ solution, and label it as CK;

倒去离心管中的现有方法的盐溶液，加入无菌水，以2000rpm的速率在振荡器上进行振荡清洗1min，以冲洗掉浮萍表面残留的化学洗涤液SD；再用5%的次氯酸钠洗涤1min～2min，最后用Na₂S₂O₃溶液冲洗2次，标记为CK；

[n0043]

The SD contains the following components: 140 mM NaCl, 2.5 mM KCl, 10 mM NaNER6HPONER7, 2 mM KHNER8PONER9, 0.5 mM MgSONER10, 1 mM CaClNER11, 0.1% (v/v) Triton-X100, pH 7.5.

其中，SD包括以下组分：140mM NaCl，2.5mM KCl，10mM Na₂HPO₄，2mM KH₂PO₄，0.5mMMgSO₄，1mM CaCl₂，0.1%(v/v)Triton-X100，pH7.5。

[n0044]

2. Place sterilized duckweed in a nitrogen-fixing medium for cultivation to enrich endophytic nitrogen-fixing bacteria.

2、将灭菌浮萍置于固氮培养基中培养，富集内生固氮菌

[n0045]

Place 10-20 surface-sterilized duckweed pieces from step 1 into 50mL beakers, add 10-20mL of sterilized 1×PBS buffer, and grind them on a tissue homogenizer for 45-60s. Filter the homogenate with sterile filter paper and transfer the filtrate to nitrogen-fixing culture medium HB8541 (purchased from Qingdao Haibo Biotechnology). Incubate at 28°C-32°C for 5-7 days.

将步骤1中表面灭菌后的10～20片浮萍分别置于50mL烧杯中，加入10mL～20mL灭菌后的1×PBS缓冲液，并在组织研磨仪上研磨45s～60s，无菌滤纸过滤研磨液，将滤液转移至固氮培养液HB8541(购买厂家：青岛海博生物)中，于28°C～32°C培养5～7天。

[n0046]

Place the filtrate in a centrifuge and centrifuge at 6000 rpm to 8000 rpm for 10 to 15 minutes. Discard the supernatant. The obtained product is the enriched endophytic nitrogen-fixing bacteria community of duckweed. Store it frozen at -80°C. It can be used for subsequent high-throughput isolation and sequencing to identify the types of endophytic nitrogen-fixing bacteria in the duckweed endophytic nitrogen-fixing bacteria community.

将滤液置于离心机中，6000rpm～8000rpm离心10min～15min，弃上清；获得的即为富集的浮萍内生固氮菌群，于-80°C冷冻保存，可用于接下来的高通量分离、测序，解析浮萍内生固氮菌群中的内生固氮菌种类。

[n0047]

3. High-throughput isolation of enriched endophytic nitrogen-fixing bacteria in duckweed.

3、对富集的浮萍内生固氮菌群进行高通量分离

[n0048]

Using a multichannel pipette, add 100 μL of nitrogen-fixing culture medium HB8541 (purchased from Qingdao Haibo Biotechnology) to a sterile square 96-well cell culture plate in advance. Take the culture medium enriched with duckweed endophytic nitrogen-fixing bacteria in step 2 and add 50 μL, 20 μL, 10 μL, 5 μL, 1 μL and 0.5 μL to each well, respectively. Incubate at 28°C～32°C for 5 to 7 days. Repeat each gradient 3 to 5 times.

用多通道移液枪在无菌方形96孔细胞培养板中提前加入100μL固氮培养液HB8541(购买厂家：青岛海博生物)，吸取步骤2中富集了浮萍内生固氮菌群的培养液，每个孔中分别加样50μL、20μL、10μL、5μL、1μL和0.5μL，于28°C～32°C恒温培养箱培养5天～7天，每个梯度重复3～5次。

After serial dilution culture, it can be seen that the color in the 96-well cell culture plate becomes uneven. Bacterial solution is taken from the culture wells with different color intensities for PCR identification, so as to screen different species of single endophytic nitrogen-fixing bacteria from the endophytic nitrogen-fixing bacteria community.

经梯度稀释培养，可以看出，96孔细胞培养板中的颜色变得深浅不一，从颜色深浅不一的培养孔中吸取菌液用于PCR鉴定，以便从内生固氮菌群中筛选不同种类的单一内生固氮菌；

[n0049]

4 PCR amplification and sequencing were used to identify the types of endophytic nitrogen-fixing bacteria in duckweed.

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4 、PCR扩增、测序鉴定浮萍内生固氮菌的种类

[n0050]

The depth of the colony was visually determined based on the transparency of the bacterial culture. 2–4 μL of culture medium was aspirated from each of the 96-well cell culture plates and placed into new 96-well PCR plates. PCR reaction reagents were added to each well, including: 5 μL bacterial cell lysis buffer, 2.5 μL 10x PCR buffer, 0.2 mM dNTPs, 0.8 μM each of the nitrogen fixation gene nifH primers (forward primer polF-SEQ ID No:1:

TGCGAYCCSAARGCBGACTC, reverse primer polR-SEQ ID No:2: ATSGCCATCATYTCRCCGGA, where Y = C/T, R = A/G, S = G/C, B = G/T/C), 0.5 μL 5 U/μL Taq polymerase, and finally, sterile ddH₂O was added to a final volume of 25 μL per well.

依据菌液透明度直观判断菌落的深浅，从96孔细胞培养板分别吸取2～4μL培养液，置于新的96孔PCR板中，在每个孔中分别加入PCR反应试剂，包括：5μL细菌细胞裂解液、2.5μL 10x PCRbuffer缓冲液，0.2mM dNTPs，固氮基因nifH引物(正向引物polF-SEQ IDNo:1：TGCGAYCCSAARGCBGACTC、反向引物polR-SEQ ID No:2：ATSGCCATCATYTCRCCGGA，其中Y＝C/T，R＝A/G，S＝G/C，B＝G/T/C)各0.8μM，0.5μL 5U/μL Taq聚合酶，最后加无菌ddH₂O至每孔25μL。

Perform PCR amplification reaction. PCR reaction conditions: 95°C for 5 min; 95°C for 1 min, 50°C for 1 min, 72°C for 1 min, 25 cycles; 72°C for 5 min.

进行PCR扩增反应，PCR反应条件：95°C5min；95°C1min，50°C1min，72°C1min，25次循环；72°C5min。

[n0051]

After the PCR reaction was completed, the PCR products were stained with ethidium olfactate and then subjected to 1% agarose gel electrophoresis for about half an hour. The PCR amplification results were then detected and photographed using a gel imaging system.

PCR反应结束，经嗅化乙锭染色后，PCR产物通过1%的琼脂糖凝胶电泳半小时左右，再经过凝胶成像仪检测PCR扩增结果并拍照。

PCR products showing bands in gel imaging were purified using ExoSAP-IT (ThermoFisher, USA) reagent and then sent to a sequencing company for gene sequencing.

对凝胶成像有条带的PCR产物，用ExoSAP-IT(ThermoFisher,USA)试剂对其纯化，然后送至测序公司进行基因测序；

[n0052]

The raw sequences obtained from sequencing are processed using software such as Geneious (https://www.geneious.com/) and Serial Cloner (http://serialbasics.free.fr/Serial_Cloner.html), and then compared with the NCBI gene database using BLAST to identify the species of bacteria.

测序获得的原始序列通过相关软件如Geneious(https://www.geneious.com/)和Serial Cloner (http://serialbasics.free.fr/Serial_Cloner.html)处理后，在NCBI的基因数据库中通过BLAST进行比对，进而鉴定细菌所属种类。

[n0053]

The presence or absence of impurities in the PCR sequencing peaks can be used to determine whether the bacterial culture needs further separation and purification on nitrogen-fixing medium or can be directly frozen for preservation.

基于PCR测序峰图有无杂带，来判断菌液是否需要在固氮培养基上继续分离纯化，还是可以直接进行冷冻保存。

If the sequencing results show impurities, the bacterial culture is plated on nitrogen-fixing solid medium, streaked to separate and purify it, and the single endophytic nitrogen-fixing bacteria are screened for further culture, sequencing, identification, and cryopreservation. If the sequencing results do not show impurities, it means that the bacterial culture is a purified single endophytic nitrogen-fixing bacterium, and it can be directly cryopreserved.

测序结果若出现有杂带，则将其菌液，在固氮固体培养基上涂板，划线分离纯化，筛选得到单一内生固氮菌进行培养后再进行测序、鉴定，冷冻保存；测序结果若没有出现杂带，说明菌液已经是纯化的单一内生固氮菌，则可以直接冷冻保存。

[n0054]

The results are shown in Figure 1.

结果如图1所示。

[n0055]

As shown in Figure 1, a total of 12 endophytic nitrogen-fixing bacteria were identified in the duckweed tissue treated with the surface tissue sterilization solution (DW) of the present invention. Among them, 6 belonged to Azotobacters sp. and the remaining 6 were Rhizobium sp.

从图1可以看出，经本发明的表面组织灭菌液(DW)表面灭菌处理的浮萍组织中，总共鉴定得到12种内生固氮菌，其中6个均属于Azotobactersp(固氮菌属)，剩余6个分别为Rhizobium sp.

(Rhizobium), Dechloromonas sp.

(根瘤菌属)，Dechloromonas sp.

Thaurera sp. (Deoxymonas)

(脱氧单胞菌属)，Thauera sp.

(Flavobacterium sp.)

(陶厄氏菌属)，Flavobacterium sp.

(Flavobacterium), Hydrogenophaga sp.

(黄杆菌属)，Hydrogenophaga sp.

(Thiobacillus sp.)

(嗜氢菌属)，Thiobacillussp.

(Thiobacillus).

(硫杆菌属)。

[n0056]

A total of 7 endophytic nitrogen-fixing bacteria were identified in the CK group (chemical detergent + sterilization solution control group), of which 4 belonged to Azotobacters sp. and the remaining 3 were Rhizobium sp.

CK组(化学洗涤液+灭菌液对照组)中总共鉴定得到7种内生固氮菌，其中4个均属于Azotobactersp(固氮菌属)，剩余3个分别为Rhizobium sp.

(Rhizobium), Dechloromonassp.

(根瘤菌属)，Dechloromonassp.

(Deoxymonas) Hydrogenophaga sp.

(脱氧单胞菌属)，Hydrogenophaga sp.

(Genus of hydrogenophilic bacteria).

(嗜氢菌属)。

[n0057]

The results in Table 1 show that, compared with the control group (CK), the surface sterilization of duckweed using chemical washing solution + sterilization solution, the surface tissue sterilization solution of the present invention can sterilize the surface of duckweed without damaging the plant tissue or destroying the symbiotic nitrogen-fixing bacteria in the tissue, while obtaining a richer variety of endophytic nitrogen-fixing bacteria genera, and isolating a greater number of endophytic nitrogen-fixing bacteria from a single genera.

表1的结果表明：相较于CK对照组中，采用化学洗涤液+灭菌液对浮萍进行表面灭菌，采用本发明的表面组织灭菌液对浮萍表面进行灭菌，在不损害植物组织和破坏组织内共生固氮菌的同时，能够获取种类更丰富的内生固氮菌属，且单一菌属中分离出数量更多的内生固氮菌。

[n0058]

Comparative Example

对比例

[n0059]

The difference between the comparative example and Example 1 is that the traditional streak plate method is used in step 3 to separate the endophytic nitrogen-fixing bacteria in duckweed.

对比例与实施例1的区别在于：步骤3中采用传统平板划线的方法分离浮萍中的内生固氮菌。

All other steps are the same as in Example 1.

其他步骤均与实施例1相同。

[n0060]

First, use a sterile loop to pick up the filtrate and streak it repeatedly on a nitrogen-fixing solid medium, repeating 3 to 5 times. Incubate at a constant temperature of 28°C to 32°C for 5 to 7 days. After colonies grow on the plate, use a sterile loop to pick out different colonies of different shapes and sizes, and streak them repeatedly on a nitrogen-fixing solid medium. Incubate at a constant temperature of 28°C to 32°C for 5 to 7 days. Repeat this process multiple times until a single colony is selected from the plate.

首先，用灭菌环蘸取滤液，在固氮固体培养基上反复划线，重复3次～5次，于28°C～32°C恒温培养箱培养5天～7天；待平板上菌落长出后，再用灭菌环将形状大小不一的不同菌落挑出，分别在固氮固体培养基上反复划线，于28°C～32°C恒温培养箱培养5天～7天，反复重复多次，直至平板上筛选出单一菌落。

[n0061]

Endophytic nitrogen-fixing bacteria in duckweed were isolated using the method of Example 1 and the traditional streak plate method. The number and names of the isolated bacterial species are shown in Table 1.

采用实施例1的方法与传统的平板划线法分离浮萍中的内生固氮菌，分离得到的菌种数量和名称结果如表1所示。

[n0062]

Table 1 Comparison of the number and names of bacterial strains isolated in Example 1 and the comparative example.

表1实施例1和对比例中分离得到的菌种数量和名称比较

[n0065]

As can be seen from Table 1, the number of nitrogen-fixing bacteria isolated by traditional streak plating is relatively small. Only five endophytic nitrogen-fixing bacteria with high content in duckweed filtrate can be isolated, which belong to Azotobacters sp (two species of nitrogen-fixing bacteria) and Hydrogenophaga sp.

从表1可以看出，用传统平板划线分离到的固氮菌种类较少，只能分离出浮萍滤液中含量较高的5种内生固氮菌，分别属于Azotobactersp(固氮菌属，2种)、Hydrogenophagasp.

(Genus Dechloromonassp., 1 species)

(嗜氢菌属，1种)、Dechloromonassp.

(Deoxymonas genus, 1 species) and Rhizobium sp.

(脱氧单胞菌属，1种)和Rhizobiumsp.

(Rhizobium genus, 1 species) Four genera.

(根瘤菌属，1种)四个属。

The high-throughput separation and screening method in Embodiment 1 of the present invention isolated a total of 12 endophytic nitrogen-fixing bacteria, belonging to Azotobacters sp. and Rhizobium sp., respectively.

而本发明实施例1中的高通量分离筛选方法，总共分离得到12种内生固氮菌，分别属于

Azotobactersp(固氮菌属)，Rhizobium sp.

(Rhizobium), Dechloromonassp.

(根瘤菌属)，Dechloromonassp.

Thaurera sp. (Deoxymonas)

(脱氧单胞菌属)，Thauera sp.

(Flavobacterium sp.)

(陶厄氏菌属)，Flavobacterium sp.

(Flavobacterium), Hydrogenophaga sp.

(黄杆菌属)，Hydrogenophaga sp.

(Thiobacillus sp.)

(嗜氢菌属)，Thiobacillus sp.

(Thiobacterium) Six genera.

(硫杆菌属)六个属。

[n0066]

This is because: through the high-throughput separation and screening of the present invention, the filtrate is added to different culture wells after multiple dilutions, which gradually separates the bacterial species with different characteristics in the community, allowing them to grow without restriction. This is conducive to the growth of bacteria with lower content or slower growth rate, and thus these bacteria with lower content or slower growth rate can also be screened out.

这是因为：通过本发明的高通量分离筛选，滤液经多次稀释后添加至不同培养孔，将群落中不同特性的菌种逐渐分开，彼此生长不受限制，有利于含量较低或者生长速率偏慢的菌生长，因而这些含量较低或者生长速率偏慢的菌也可以筛选出来。

When using the traditional streak plate separation method to separate endophytic nitrogen-fixing bacteria from duckweed, bacteria with low content or slow growth rate in the nitrogen-fixing bacteria community contained in the filtrate are not the dominant bacteria. The growth of bacteria will restrict each other. Therefore, when dominant and non-dominant bacteria are streaked onto the plate at the same time, the dominant bacteria will grow first, limiting or even inhibiting the growth of other non-dominant bacteria. As a result, they cannot be screened out even after repeated screening.

而采用传统的平板划线分离法对浮萍中的内生固氮菌进行分离时，滤液所含的固氮菌群中，含量较低或者生长速率偏慢的菌并不是处于优势菌，菌与菌之间的生长会相互制约，因而当优势菌和非优势菌同时划线到平板上时，优势菌则优先长出，限制甚至抑制了其他非优势菌种的生长，故经多次反复筛选也未能筛出。

The comparative results show that the traditional streak plate separation method is not suitable for the isolation and screening of endophytic nitrogen-fixing bacteria in aquatic plants with low bacterial populations.

对比例的结果说明，传统的平板划线分离法并不适合菌群含量少的水生植物中内生固氮菌的分离筛选。

[n0067]

The technical features of the above embodiments can be combined in any way. For the sake of brevity, not all possible combinations of the technical features in the above embodiments are described. However, as long as there is no contradiction in the combination of these technical features, they should be considered to be within the scope of this specification.

以上所述实施例的各技术特征可以进行任意的组合，为使描述简洁，未对上述实施例中的各个技术特征所有可能的组合都进行描述，然而，只要这些技术特征的组合不存在矛盾，都应当认为是本说明书记载的范围。

[n0068]

The above embodiments are merely examples of several implementation methods of the present invention, and their descriptions are relatively specific and detailed, but they should not be construed as limiting the scope of the invention patent.

以上所述实施例仅表达了本发明的几种实施方式，其描述较为具体和详细，但并不能因此而理解为对发明专利范围的限制。

It should be noted that those skilled in the art can make various modifications and improvements without departing from the concept of this invention, and these modifications and improvements are all within the scope of protection of this invention.

应当指出的是，对于本领域的普通技术人员来说，在不脱离本发明构思的前提下，还可以做出若干变形和改进，这些都属于本发明的保护范围。

Therefore, the scope of protection of this invention patent shall be determined by the appended claims.

因此，本发明专利的保护范围应以所附权利要求为准。

[0074]

sequence list

序列表

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<110> Guangdong Academy of Sciences Institute of Biotechnology

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<110> 广东省科学院生物工程研究所

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<120> Methods for enriching and isolating endophytic nitrogen-fixing bacteria from duckweed

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