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ISOLATION AND IDENTIFICATION OF LIGNIN-DEGRADING BACTERIAL STRAINS

Abstract: *Screening bacterial strains with lignin-degrading ability is very necessary for processing agricultural and forestry waste. In this experiment, we collected soil samples from the Qinling Mountains in China and isolated 99 bacterial strains. Further, 18 strains of bacteria were screened out using alkaline lignin solid medium and cultured at 30°C for 48 hours, and they were molecularly identified. After that, lignin liquid culture medium was used for shake flask culture, and a microplate reader was used to measure the absorbance changes of lignin at visible light at 280 nm before and after culture. A total of 12 strains of bacteria with lignin degradation ability were screened out, respectively. For QL-D3, QL-D5, QL-D6, QL -D7, QL-D8, QL-D9, QL-D11, QL-D13, QL-D14, QL-D16, QL-D17, QL -D18. Their lignin degradation rates after culturing in lignin 1,5 g/L liquid medium for 7 days were 13,01%, 16,76%, 10,48%, 23,3%, 6,88%, 12,48%, 22,07%, 11,84%, 18,62%, 17,88%, 13,95%, 16,28% respectively. Scanning electron microscopy was used to observe the morphology of the two bacterial strains QL-D7 and QL-D11 with the highest lignin degradation rate.*

Key words: *Screening bacterial strains, lignin-degradation, medium culture, lignin-degrading rate, morphology observation.*

Introduction

Lignocellulose is a polymer compound present in plants, mainly composed of three substances: cellulose, hemicellulose and lignin [7]. Cellulose is composed of microfibers that form the network skeleton of fiber cell walls, while lignin and hemicellulose play the role of filling and binding [23]. Lignin has functions such as moisture transport, mechanical support and resistance to biological decomposition. It is estimated that approximately 25% of the biomass fixed by plants through photosynthesis each year is ultimately converted into lignin through secondary metabolism. It is the most abundant aromatic compound in nature and the most abundant renewable carbon source on earth after cellulose [19].

Under natural conditions, lignin is difficult to degrade, which has become the main factor limiting the utilization of lignin. Traditional methods attempt to break the polymeric structure of lignin using high-temperature and high-pressure steam treatment, but this is a highly energy-consuming method. It was noted that in China, 216 million tons of the above-mentioned biomass are produced annually, but the fact that more than half of the remainder is not utilized indicates the need to implement advanced treatment strategies [1]. Therefore, it is necessary to find environmentally friendly ways to process lignin. In the natural environment, there are microorganisms that can degrade lignin. Common fungi that can degrade lignin mainly include brown rot fungi, soft rot fungi, white rot fungi, litter decomposing fungi and microfungi. These fungi can effectively degrade lignin components and convert them into small molecular organic matter to enter the energy cycle. Therefore they are often used as model microorganisms for lignin degradation [4, 5, 8]. However, studies have shown that bacteria that can degrade lignin also exist in nature. Masai et al. completed the whole genome sequencing of the bacterium *Sphingomonas paucimobilis* SYK-6 isolated from the soil, cloned more than 20 lignin degradation-related genes, and constructed a relatively complete lignin degradation metabolic pathway [14]. Compared with eukaryotes, prokaryotes have the advantages of shorter growth cycles, easier growth control, and more convenient molecular manipulation, making bacteria more suitable for industrial production. Therefore, finding bacteria that can degrade lignin in nature for the utilization of lignin is very meaningful.

Various microorganisms live together in nature. Soil is the home base of microbial life, and many valuable strains can be isolated from it [25]. To study the characteristics of a certain microorganism, the microorganism must first be kept in a pure culture state. The process of obtaining only a certain strain of microorganisms from a mixed population of microorganisms is called the separation and purification of microorganisms [6].

This study is dedicated to finding bacteria that can degrade lignin in the natural environment, and to detect their growth and lignin degradation efficiency when cultured in liquid shake flasks, and molecular identification by 16SrDNA comparison. The strains with higher degradation efficiency were observed under a scanning electron microscope (SEM). A batch of lignin-degrading strains were obtained through the above method, which provides more options for bacterial degradation of lignin and creates a basis for further research.

Methodology

1. Isolation and purification of microorganisms

Soil samples were collected from Qinling Mountains, China. Add 10 g of soil sample to 90 mL of deionized water, shake and mix, and record it as 10^{-1} g/mL. Add 1 mL of the above soil dilution to 10 mL of deionized water and record it as 10^{-2} g/mL. Repeat this step until the dilution reaches 10^{-8} g/mL. Take 0.5 mL of the soil suspension with dilutions of 10^{-6} g/mL, 10^{7} g/mL, and 10^{-8} g/mL and spread it evenly on the LB medium with a spreader [2]. Cultivate in a constant temperature incubator at 30°C for 48 hours. Use an inoculation loop to pick single colonies of different shapes, inoculate them into liquid LB culture medium, place them in a shaker (200 rpm/s), and culture at 30°C to the logarithmic growth phase (OD_{600}=0,8). Pipette 0.5 mL of bacterial suspension and mix 1:1 with 50% glycerol, and store in a -80°C refrigerator for later use [18]. All the reagents and tools used above were sterilized by high temperature and high pressure with 121℃, 20 mins [3].

2. Identification of lignin degradation ability

Culturing bacteria on a single carbon source to obtain bacterial strains that can utilize it is a common method to obtain bacteria that can degrade that carbon source [9]. Here, we used alkaline lignin as a single carbon source configuration solid medium to screen bacterial strains that can degrade lignin. Afterwards, alkaline lignin was used to prepare a liquid medium for culture, and its ability to degrade lignin was measured quantitatively.

Lignin has strong absorption near 280 nm, followed by 210 nm, and weak absorption near 230 nm and 320 nm. Among them, 210 nm is the absorption band of the conjugated olefin bond, and 280 nm is the absorption band caused by the unsubstitutable m-position in the aromatic ring. This absorption value basically follows Lambert-Beer's law, so 280 nm can be selected as the wavelength for quantitative determination of lignin [11].

In order to understand the relationship between absorbance and unit lignin concentration, we configured a standard concentration lignin solution and fitted it linearly. By fitting the results, we can determine that the lignin concentration is proportional to the absorbance under visible light at 280 nm. All samples were measured after dilution tenfold. The lignin concentration in the culture medium under initial conditions is marked as A, and after culture, it is marked as B. The lignin degradation rate is calculated using the following equation:

lignin degradation rate (%) = $\frac{(A-B)}{A} \times 100\%$

Under initial conditions, the lignin concentration in the culture medium was 1,5g/L, 100mL. After the bacteria were cultured to the logarithmic growth phase (OD600=0,8), they were inoculated into the lignin medium, and the bacterial inoculation amount was 1% of the volume, Culture for 7 days. All samples were tested after being diluted ten times. Mix well before detection, pipette 200μL and drop into the sample well. All samples repeated at least 3 times.

All the above absorbance values were measured by one microplate reader (TECAN. Infinite M 200PRO).

The culture media used in this experiment are as follows:

(1) Strain activation medium: LB medium (peptone 10 g/L, yeast powder 5 g/L, NaCl 10 g/L pH 7,0-7,2).

(2) Liquid lignin culture medium (add 1,5% agar (m/V) to solid): 1,5 g/L alkaline lignin, K_2HPO_4 0,1 g/L, $MgSO_4 \cdot 7H_2O$ 0,01 g/L, $CaCl_2$ 0,008 g/L, $FeSO_4 \cdot 7H_2O$ 0,005 g/L, $MnCl_2$ 0,002 g/L, peptone 0,2g/L, pH 7,0.

3. Strain species identification

Colony PCR does not require extraction of target gene DNA or enzyme digestion for identification. Instead, it directly uses the DNA exposed after pyrolysis of the bacteria as a template for PCR amplification, saving time and effort. It is recommended to use universal primers on the vector to screen for positive clones. This PCR method is usually used to screen the inserted target gene or DNA sequencing analysis. The final PCR product size is the fragment size between the universal primers of the vector [15].

The target gene (16SrDNA) was amplified by colony PCR, and universal primers (27 F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492 R: 5'-GGTTACCTTCTTACGACTT-3') were used to amplify 16SrDNA [16, 24], Primers were synthesized by Sangon Bioengineering (Shanghai) Co., Ltd. sent to the company (Sangon Bioengineering (Shanghai) Co., Ltd.) for sequencing, and the sequencing results were compared in the GenBank(https://www.ncbi.nlm.nih.gov/) database and EzBioCloud (https://www.ezbiocloud.net/).

4. Morphological observation of bacterial strains

Scanning electron microscopy (SEM) is often used to observe the surface morphology of objects. Culture the bacteria in liquid LB medium to logarithmic growth phase (OD_{600}=0.8), and collect the cells by centrifugation (6000 rpm, 3 min). After adding physiological saline to resuspend, centrifuge again and repeat three times. The collected bacteria were dehydrated step by step using 30%, 50%, 70%, 80%, 90%, and 100% ethanol. Then they were fixed with glutaraldehyde for 2 h. The prepared samples were evenly spread on silicon wafers and freeze-dried overnight. After gold spraying, observing with a scanning electron microscope (FEI. Nova Nano SEM-450).

Result:

1. Lignin-degrading strains

We isolated 99 strains from soil samples, inoculated these strains into alkaline lignin medium, and cultured them in a 30°C constant-temperature incubator for 48 hours. The growth of some strains was observed. Summarize the strains that can grow, perform colony PCR on these strains to amplify their 16SrDNA fragments, and compare them in database. The results are shown in the table below:

Table 1 – Lignin-degrading strains species information

Strain number	NCBI comparison results		EzBioCloud comparison results		Strain name
	Generic name	Similarity	Generic name	Similarity	
QL-D1	Stenotrophomonas	99,79%	Stenotrophomonas	99,45%	*Stenotrophomonas rhizophila* QL-D1
QL-D2	Pseudomonas	99,93%	Pseudomonas	99,16%	*Pseudomonas sp.* QL-D2
QL-D3	Arthrobacter	98,37%	Arthrobacter	99,71%	*Arthrobacter sp.* QL-D3
QL-D4	Pseudomonas	99,50%	Pseudomonas	99,44%	*Pseudomonas sp.* QL-D4
QL-D5	Pseudomonas	99,72%	Pseudomonas	99,68%	*Pseudomonas sp.* QL-D5
QL-D6	Pseudomonas	99,86%	Pseudomonas	99,51%	*Pseudomonas sp.* QL-D6
QL-D7	Pseudomonas	99,65%	Pseudomonas	99,44%	*Pseudomonas sp.* QL-D7
QL-D8	Providencia	99,23%	Providencia	98,82%	*Providencia sp.* QL-D8
QL-D9	Pseudomonas	99,51%	Pseudomonas	99,68%	*Pseudomonas sp.* QL-D9
QL-D10	Pseudomonas	99,58%	Pseudomonas	99,68%	*Pseudomonas sp.* QL-D10
QL-D11	Raoultella	99,86%	Raoultella	99,16%	*Raoultella sp.* QL-D11
QL-D12	Pseudomonas	99,44%	Pseudomonas	99,24%	*Pseudomonas sp.* QL-D12
QL-D13	Pseudomonas	99,79%	Pseudomonas	99,65%	*Pseudomonas sp.* QL-D13
QL-D14	Pseudomonas	99,65%	Pseudomonas	99,44%	*Pseudomonas sp.* QL-D14
QL-D15	Peribacillus	99,58%	Peribacillus	99,24%	*Peribacillus sp.* QL-D15
QL-D16	Priestia	99,86%	Priestia	99,72%	*Priestia sp.* QL-D16
QL-D17	Bacillus	99,66%	Bacillus	99,45%	*Bacillus sp.* QL-D17
QL-D18	Staphylococcus	99,72%	Staphylococcus	99,17%	*Staphylococcus sp.* QL-18

The above strain species information has been uploaded to the NCBI 16SrDNA database, and the accession number is as shown below

SUB13852066 QL-D1 OR574220, SUB13852066 QL-D2 OR574221, SUB13852066 QL-D3 OR574222, SUB13852066 QL-D4 OR574223, SUB13852066 QL-D5 OR574224, SUB13852066 QL-D6 OR574225, SUB13852066 QL-D7 OR574226, SUB13852066 QL-D8 OR574227, SUB13852066 QL-D9 OR574228, SUB13852066 QL-D10 OR574229, SUB13852066 QL-D11 OR574230, SUB13852066 QL-D12 OR574231, SUB13852066 QL-D13 OR574232, SUB13852066 QL-D14 OR574233, SUB13852066 QL-D15 OR574234, SUB13852066 QL-D16 OR574235, SUB13852066 QL-D17 OR574236, SUB13852066 QL-D18 OR574237.

1. Lignin degradation rate

1.1 Lignin concentration standard curve

The lignin concentration standard curve is shown in the Figure 1:

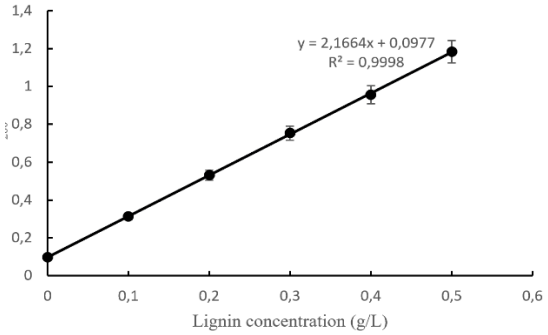


Figure 1 – Lignin concentration standard curve

1.2 Different strain's lignin-degrading rate

The lignin degradation rates of different strains are shown in the Table 2.

Table 2 – Strain's lignin-degrading rate

Strain name	lignin-degrading rate
Stenotrophomonas rhizophila QL-D1	0,62%
Pseudomonas sp. QL-D2	0,59%
Arthrobacter sp. QL-D3	13,01%
Pseudomonas sp. QL-D4	4,76%
Pseudomonas sp. QL-D5	16,76%
Pseudomonas sp. QL-D6	10,48%
Pseudomonas sp. QL-D7	23,3%
Providencia sp. QL-D8	6,88%
Pseudomonas sp. QL-D9	12,48%
Pseudomonas sp. QL-D10	3,17%
Raoultella sp. QL-D11	22,07%
Pseudomonas sp. QL-D12	0,57%
Pseudomonas sp. QL-D13	11,84%
Pseudomonas sp. QL-D14	18,62%
Peribacillus sp. QL-D15	4,47%
Priestia sp. QL-D16	17,88%
Bacillus sp. QL-D17	13,95%
Staphylococcus sp. QL-18	16,28%

2. Morphological observation of bacterial strains

As it is shown in table 2., We believe that QL-D11 and QL-D7 have application potential, so we conducted morphological observations on these two strains (Figure 2).

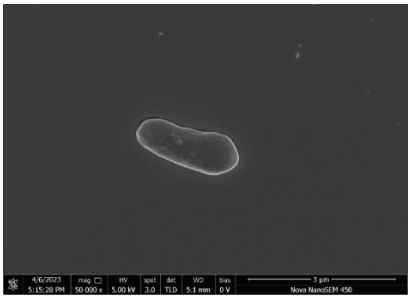
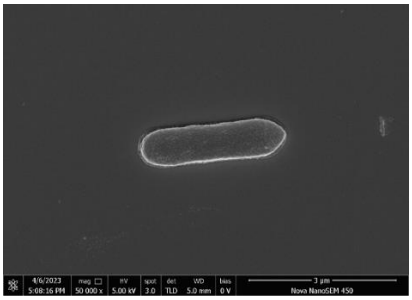
a.  b. 

Figure 2 – Morphological observation of bacterial strains a. QL-D11 and b. QL-D7

Discussion:

In this experiment, a total of 18 strains that could grow using lignin as the sole carbon source were screened out using a lignin solid medium coating experiment. However, in subsequent shake flask culture experiments, we observed that strains with strain numbers QL-D1, QL-D2, QL-D4, QL-D10, QL-12 and QL-D15 had almost no lignin degradation ability. Their lignin degradation rates in averages were all less than 5%, we can almost consider them to be in the same interval. It can be considered that this difference in readings is due to the fluctuation of the measuring instrument itself. It is speculated that the reason for this phenomenon is that the bacterial cells themselves store a

small amount of nutrients when expanding the culture using LB liquid medium [21, 22]. As a result, bacterial strains can grow in a short time and in a small area after being inoculated on lignin solid medium. The authors included these bacteria in the initial 18 strains out of caution, but in subsequent experiments, we determined that these strains did not have lignin-degrading ability. Therefore, liquid shake flask culture is very meaningful for screening lignin-degrading strains.

Among the bacterial strains possessing lignin-degrading ability, we identified *Pseudomonas* sp. There have been many reports showing the lignin degradation ability of *Pseudomonas* [12, 13, 17], which is consistent with our research results. At the same time, we rarely observed the degradation of lignin by *Raoultella* species, and there are few reports on lignin degradation by *Raoultella* species. At the same time, we also observed *Bacillus*, which is also a bacterium that is often reported to have lignin-degrading ability [20].

Among the existing lignin degradation model strains, many have a lignin degradation rate greater than 20% [10, 17]. Therefore, we believe that the lignin degradation rate should be greater than 20% when screening bacteria with application potential. In this experiment, only QL-D7 and QL-D11 met this condition, so we believe that it is necessary to continue studying these two strains of bacteria.

Conclusion:

This experiment screened out QL-D3, QL-D5, QL-D6, QL-D7, QL-D8, QL-D9, QL-D11, QL-D13, QL-D14, QL-D16, QL-D17, QL -D18 has a total of 12 lignin-degrading strains. Among them, QL-D7 and QL-D11 have the highest degradation rates, 23.3% and 22.07% respectively. We performed morphological observations under a scanning electron microscope. Further research on these two strains of bacteria is planned.

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ЛИГНИНДІ ЫДЫРАТАТЫН БАКТЕРИЯЛАРДЫҢ ШТАМДАРЫН ОҚШАУЛАУ ЖӘНЕ АНЫҚТАУ

Лигнинді ыдырататын бактериялардың штамдарын таңдау ауыл шаруашылығы мен орман шаруашылығы қалдықтарын өңдеу үшін өте қажет. Осы эксперимент барысында біз Қытайдың Цинлин тауларынан топырақ үлгілерін жинап, бактериялардың 99 штаммын бөліп алдық. Әрі қарай, бактериялардың 18 штаммы сілтілі лигнинді қатты ортаны пайдаланып таңдалды және 30°C температурада 48 сағат бойы өсірілді, содан кейін олар молекулалық түрде анықталды. Осыдан

кейін шайқалған колбада өсіру үшін лигнині бар сұйық культура ортасы пайдаланылды, ал өсіруден бұрын және кейін 280 нм-де көрінетін жарықта лигниннің сіңуінің өзгеруін өлшеу үшін микропластинаны оқу құралы қолданылды. Лигниннің ыдырау қабілеті бар бактериялардың жалпы 12 штаммы таңдалды. QL-D3, QL-D5, QL-D6, QL-D7, QL-D8, QL-D9, QL-D11, QL-D13, QL-D14, QL-D16, QL-D17, QL-D18 үшін. 7 күн ішінде құрамында 1,5 г/л лигнин бар сұйық ортада өсіргеннен кейін олардың лигниннің ыдырау жылдамдығы 13,01%, 16,76%, 10,48%, 23,3%, 6,88%, 12,48%, 22,07%, 11,84%, 18,62%, 17,88%, 13,95%, 16,28% тиісінше. Сканерлеуші электронды микроскопия арқылы QL-D7 және QL-D11 бактерияларының екі штаммының морфологиясы лигниннің ең жоғары ыдырау жылдамдығымен зерттелді.

Түйін сөздер: *бактерия штаммдарының скринингі, лигниннің деградациясы, ортада өсіру, лигниннің деградация жылдамдығы, морфологияны бақылау.*

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ВЫДЕЛЕНИЕ И ИДЕНТИФИКАЦИЯ ШТАММОВ БАКТЕРИЙ, РАЗЛАГАЮЩИХ ЛИГНИН

Отбор штаммов бактерий, способных расщеплять лигнин, крайне необходим для переработки отходов сельского и лесного хозяйства. В ходе этого эксперимента мы собрали образцы почвы в горах Циньлин в Китае и выделили 99 штаммов бактерий. Далее, 18 штаммов бактерий были отобраны с использованием щелочной лигниновой твердой среды и культивировались при 30°C в течение 48 часов, после чего они были молекулярно идентифицированы. После этого для культивирования во встряхиваемой колбе использовали жидкую культуральную среду с лигнином, а для измерения изменений поглощения лигнина в видимом свете при 280 нм до и после культивирования использовали считыватель микропланшетов. В общей сложности было отобрано 12 штаммов бактерий, обладающих способностью к разложению лигнина, соответственно. Для QL-D3, QL-D5, QL-D6, QL-D7, QL-D8, QL-D9, QL-D11, QL-D13, QL-D14, QL-D16, QL-D17, QL -D18. Их скорость разложения лигнина после культивирования в жидкой среде с содержанием лигнина 1,5 г/л в течение 7 дней составила 13,01%, 16,76%, 10,48%, 23,3%, 6,88%, 12,48%, 22,07%, 11,84%, 18,62%, 17,88%, 13,95%, 16,28% соответственно. С помощью сканирующей электронной микроскопии была изучена морфология двух штаммов бактерий QL-D7 и QL-D11 с наибольшей скоростью разложения лигнина.

Ключевые слова: *скрининг штаммов бактерий, деградация лигнина, культивирование в среде, скорость деградации лигнина, наблюдение за морфологией.*

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РАЗРАБОТКА СУШИЛЬНОЙ УСТАНОВКИ ДЛЯ ПРОИЗВОДСТВА КУРТА

Аннотация: *Статья посвящена разработке сушильной установки для производства курта, традиционного кисломолочного продукта, пользующегося большой популярностью в Казахстане. Курт является не только национальным продуктом, но и обладает высокой пищевой ценностью, содержанием витаминов и минералов. Регулярное употребление курта способствует восстановлению полезной микрофлоры кишечника и особенно полезно для беременных женщин, детей и пожилых людей. Традиционный способ сушки курта на открытом воздухе имеет ряд недостатков, таких как зависимость от погодных условий и низкая производительность. Разработанная конвективная сушильная установка, позволяет решить эти проблемы. Она обеспечивает равномерную и быструю сушку курта в контролируемых условиях, что повышает качество и производительность. Установку спроектирована с учетом принципов простоты в обслуживании, удобства в эксплуатации и рентабельности. Она является доступной и экономически выгодной для индивидуальных предпринимателей и малых предприятий. Доступность, цена, качество, производительность, ремонтопригодность, надежность, малый вес и мобильность оборудования делают его актуальным для развития малого бизнеса в Казахстане. В ходе работы были проведены ряд испытании по работоспособности сушильной установки. Также был разработан эскизный чертеж, разработана сушильная установка и подобраны оптимальные рабочие параметры конструкции. Полученная сушильная установка является перспективной для внедрения в производство курта, в условиях индивидуальных и фермерских хозяйств. Она позволит повысить качество и производительность продукта, а также создать новые возможности для малого и среднего бизнеса в Казахстане. Также, такое оборудование может дать толчок местному гастрономическому туризму, который становится все более популярным видом туризма, поскольку туристы ищут уникальные и аутентичные впечатления. Предлагая туристам возможность познакомиться с местной культурой через ее кухню.*

Ключевые слова: *национальная кухня, курт, сушильная установка, кисломолочные продукты, развитие малого и среднего бизнеса, сушильное оборудование, производство курта, сушка пищевых продуктов.*

Введение

Казахстан, страна с субконтинентальным климатом [1], характеризуется значительными температурными колебаниями, особенно в северных, центральных, восточных и западных регионах. Традиционно производство национального продукта курта [2, 3], ограничивалось летними месяцами из-за невозможности сушки в зимний период. В связи с этим возникла необходимость создания рентабельного сушильного оборудования для малых и средних предприятий, которое позволило бы производить курт круглогодично и сделать его доступным