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## DESCRIPTION CN112342151A

An anaerobic culture method for Akkermansia myxophilus

**[0001]**

Technical Field

**[0002]**

This invention relates to the field of microbial culture technology, and specifically to an anaerobic culture method for *Akkermansia myxophilus*.

**[0003]**

Background Technology

[0004]

*Akkermansia muciniphila* (hereinafter referred to as Akk) was first successfully isolated by Derrien M et al. in 2004. It belongs to the phylum Verruciformis, is Gram-negative, strictly anaerobic, and can use mucin as its sole carbon, nitrogen and energy source.

Recent studies have shown that Akk is associated with many metabolic diseases in humans, and its content is one of the important markers of gut microbiota balance. Akk abundance is negatively correlated with human weight, type 1 diabetes and type 2 diabetes, and also negatively correlated with waist circumference, weight and body mass index. Many scholars call it the next generation of probiotics.

Given that Akk is a novel candidate probiotic, its cultivation is of particular importance.

However, the cultivation of Akk bacteria is not easy due to its high nutritional requirements for growth, its sensitivity to oxygen, and its low redox potential.

[0005]

Derrien M et al (Derrien M. *Akkermansia muciniphila* gen. nov. sp. nov. a human intestinal mucin-degrading bacterium [J].

Int. J. Syst. Evol. Microbiol. 2004, 54. The invention discloses that *Akkia myxophilus* can grow on a basic anaerobic medium containing porcine gastric mucin as the sole carbon and nitrogen source, and the culture atmosphere is  $N_2/CO_2$  (80:20, v/v). When the inventors used the above culture conditions and conventional anaerobic chamber culture conditions (95%  $N_2$ , 5%  $H_2$ ) for shake flask culture, they found that *Akk* growth was limited by the liquid level, and the initial culture liquid level could not be lower than 2 cm. Another Chinese patent, CN201910265701.0, discloses a culture medium for culturing *Akkermansia myxophilus* and its method of use. The components of the culture medium include soybean peptone, threonine, glucose, N-acetylglucosamine, sodium chloride, and disodium hydrogen phosphate. The medium is cultured under anaerobic conditions of 85%  $CO_2$ , 10%  $N_2$ , and 5%  $H_2$ . However, under the culture conditions of the above patent, *Akkermansia myxophilus* grows slowly, has low bacterial

activity, and requires 3-4 days or even longer to culture. The bacterial abundance is also relatively low, which makes in-depth research on it difficult and hinders the development of research work.

**[0006]**

Summary of the Invention

**[0007]**

In view of the above-mentioned shortcomings of the existing technology, the present invention provides an anaerobic culture method for *Akkermansia myxophilus*.

[0008]

A method for anaerobic culture of *Akkermansia myxophilus*, wherein the anaerobic culture uses a mixed gas ratio of 85-94% N<sub>2</sub>, 5% H<sub>2</sub>, and 1-10% CO<sub>2</sub>.

In addition to promoting the growth and reproduction of Akk, CO<sub>2</sub> can also dissolve in the culture medium to form carbonic acid, increasing the concentration of inorganic carbon and maintaining a suitable pH value in the culture medium. Therefore, the concentration of CO<sub>2</sub> should not be too high or too low. If it is too high, the pH value will drop too quickly, and if it is too low, the pH value will be too high, neither of which is suitable for the growth and reproduction of Akk.

[0009]

Furthermore, the anaerobic culture uses a mixed gas ratio of 88-92% N<sub>2</sub>, 5% H<sub>2</sub>, and 3-7% CO<sub>2</sub>.

## **[0010]**

Furthermore, the anaerobic culture uses a mixed gas ratio of 90% N<sub>2</sub>, 5% H<sub>2</sub>, and 5% CO<sub>2</sub>.

## **[0011]**

Furthermore, the anaerobic culture uses a modified BHI medium, which contains added mucin and yeast extract.

**[0012]**

Furthermore, the modified BHI culture medium formula (g/L) is as follows: Brain-heart Infusion Extract 37g/L, Yeast Extract 5g/L, Mucin 2.5g/L, Resazurin 1mg/L, and L-cysteine hydrochloride 100mg/L.

**[0013]**

Furthermore, the method for preparing the modified BHI culture medium is as follows:

**[0014]**

S1. Preparation of Mucin solution: Dissolve Mucin in PBS buffer to a concentration of 1% (W

/V), sterilize, let stand overnight, centrifuge at 12000 rpm for 5 min, and collect the supernatant for later use.

## **[0015]**

S2, Preparation of L-cysteine hydrochloride solution: Dissolve L-cysteine hydrochloride in ultrapure water to obtain a 10% (W/V) L-cysteine hydrochloride solution, filter and sterilize for later use;

## **[0016]**

S3. Add 37g Brain-heart Infusion Extract, 5g Yeast Extract, and 1mL 0.1% (W/V) Resazurin to 1L of water. Mix well and autoclave. Then, take 375mL of the sterilized solution and add 125mL of the Mucin solution from step S1. Mix well and add 10mL of the L-cysteine hydrochloride solution from step S2 to obtain the modified BHI medium.

[0017]

Furthermore, the anaerobic culture is conducted at a temperature of 37°C.

[0018]

A method for anaerobic culture of *Akkermansia myxophilus*, wherein the anaerobic culture uses a mixed gas ratio of 95% N<sub>2</sub> and 5% H<sub>2</sub>; the culture medium used is a modified BHI medium, wherein the modified BHI medium is supplemented with Mucin, Yeast Extract and 0.7-7mM NaHCO<sub>3</sub>.

NaHCO<sub>2</sub>, as a source of CO<sub>2</sub>, can supplement CO<sub>2</sub> in anaerobic culture.

**[0019]**

Furthermore, the modified BHI culture medium formula (g/L) is as follows: Brain-heart Infusion Extract 37g/L, Yeast Extract 5g/L, Mucin 2.5g/L, Resazurin 1mg/L, L-cysteine hydrochloride 100mg/L, NaHCO<sub>3</sub>-NER25 0.7~7mM.

**[0020]**

Furthermore, the concentration of NaHCO<sub>3</sub>-NER26 is 3.5 mM.

**[0021]**

The technical solution provided by this invention has the following beneficial effects:

**[0022]**

1. The combination of culture medium and culture conditions in this invention enables Akk bacteria to grow and reproduce without being limited by the liquid level. It can grow well in both shake flasks and plate surfaces, reducing the difficulty of Akk bacteria cultivation. At the same time, it reduces the growth rate of Akk bacteria from 3-4 days in the prior art to 24-36 hours, greatly shortening the cultivation time, improving the activity of Akk bacteria, and promoting Akk bacteria growth. This is of great significance for the large-scale cultivation and in-depth research of Akk bacteria.

**[0023]**

2. The culture method of the present invention eliminates the need for pouring or puncture culture to form a certain liquid level. Instead, it allows the formation of typical colony

morphologies on the surface of solid culture medium using streak plating or spreading methods. This method is very convenient for screening Akk bacteria and is suitable for the screening, isolation, and culture of bacterial strains.

[0024]

Attached Figure Description

[0025]

Figure 1 is a shake flask culture diagram of the anaerobic culture mixed gas optimization in Example 3; where A: anaerobic system A, 95% N<sub>2</sub>, 5% H<sub>2</sub>; B: anaerobic system B, 80% N<sub>2</sub>, 20% CO<sub>2</sub>; C: anaerobic system C, 90% N<sub>2</sub>, 5% H<sub>2</sub>, 5% CO<sub>2</sub>;

[0026]

Figure 2 is a plate culture diagram of the anaerobic culture mixed gas optimization in Example 3; where A: anaerobic system A, 95% N<sub>2</sub>, 5% H<sub>2</sub>; B: anaerobic system B, 80% N<sub>2</sub>, 20% CO<sub>2</sub>; C: anaerobic system C, 90% N<sub>2</sub>, 5% H<sub>2</sub>, 5% CO<sub>2</sub>;

[0027]

Figure 3 is a colony morphology diagram of Example 6;

[0028]

Figure 4 is a crystal violet staining diagram of Example 6;

**[0029]**

Figure 5 is a PCR amplification diagram of Example 6; where CK1: blank control, CK2: positive control, Akk1: bacterial culture as PCR template, Akk2: single colony as PCR template, M: marker;

**[0030]**

Figure 6 is a growth curve diagram of Example 6.

**[0031]**

Detailed Implementation

**[0032]**

The technical solution of the present invention will be clearly and completely described below with reference to the accompanying drawings. Obviously, the described embodiments are only some embodiments of the present invention, and not all embodiments.

Based on the embodiments of the present invention, all other embodiments obtained by those skilled in the art without inventive effort are within the scope of protection of the present invention.

**[0033]**

### Example 1 Anaerobic culture method of Akk bacteria

[0034]

An anaerobic culture method for *Akkermansia myxophilus* involves activating a single Akk colony overnight, inoculating it into a modified BHI medium at a 1% inoculum, and incubating at a constant temperature of 37°C under anaerobic conditions of 85–94% N<sub>2</sub>, 5% H<sub>2</sub>, and 1–10% CO<sub>2</sub>.

[0035]

The modified BHI medium was formulated as follows: Brain-heart Infusion Extract 37 g/L, Yeast Extract 5 g/L, Mucin 2.5 g/L, Resazurin 1 mg/L, and L-cysteine hydrochloride 100 mg/L.

[0036]

The method for preparing the modified BHI culture medium is as follows:

[0037]

S1. Preparation of Mucin solution: Dissolve Mucin in PBS buffer to a concentration of 1% (W/V), sterilize, let stand overnight, centrifuge at 12000 rpm for 5 min, and collect the supernatant for later use; In this example, specifically: weigh 1.25 g Mucin, dissolve in 125 mL PBS (pH 7.4), sterilize, let stand overnight, centrifuge at 12000 rpm for 5 min, and collect the supernatant for later use;

**[0038]**

S2, Preparation of L-cysteine hydrochloride solution: Dissolve L-cysteine hydrochloride in ultrapure water to obtain a 10% (W/V) L-cysteine hydrochloride solution, filter and sterilize for later use;

**[0039]**

S3. Add 37g Brain-heart Infusion Extract, 5g Yeast Extract, and 1mL 0.1% (W/V) Resazurin to 1L of water. Mix well and autoclave. Then, take 375mL of the sterilized solution and add 125mL of the Mucin solution from step S1. Mix well and add 10mL of the L-cysteine hydrochloride solution from step S2 to obtain the modified BHI medium.

**[0040]**

The *Akkermansia muciniphila* strain used in this invention is *Akkermansia muciniphila* ATCC BAA-835, purchased from the China Center for Type Culture Collection.

**[0041]**

Example 2: Culture medium optimization

**[0042]**

Single colonies of Akk bacteria were activated overnight and inoculated into 1 mL of different culture media (medium A and B) at an inoculum rate of 1%. The cultures were incubated at 37° C for 24 h under anaerobic conditions of 90% N<sub>2</sub>, 5% H<sub>2</sub>, and 5% CO<sub>2</sub>. After centrifugation at 6000 r/min for 5 min, the supernatant was discarded,

and the precipitated bacterial cells were washed twice with sterile PBS. Finally, the bacterial cells were resuspended in PBS, and the bacterial concentration  $OD_{600nm}$  was determined by spectrophotometer at a wavelength of 600 nm. The specific results are shown in Table 1.

#### [0043]

Culture medium A: Brain-heart Infusion Extract 37g/L, Mucin 2.5g/L, Resazurin 1mg/L, L-cysteine hydrochloride 100mg/L;

#### [0044]

Culture medium B (modified BHI medium): Brain-heart Infusion Extract 37g/L, Yeast Extract 5g/L, Mucin 2.5g/L, Resazurin 1mg/L, L-cysteine hydrochloride 100mg/L.

[0045]

Table 1 Effects of different culture media on the growth of Akk bacteria

[0046]

Culture conditions: Bacterial concentration: Medium A 0.80; Medium B 1.345

[0047]

As shown in Table 1, Akk bacteria grew faster in BHI medium with added Yeast Extract than in

the group without it, and the turbidity of the bacterial solution was also higher, indicating that Yeast Extract promoted the growth and reproduction of the strain.

Therefore, unless otherwise specified, this invention uses culture medium B as the optimal culture medium for *Akkermansia myxophilus*.

**[0048]**

Example 3: Optimization of Anaerobic Culture Mixed Gas

**[0049]**

Single colonies of Akk bacteria were activated overnight and inoculated into 1 mL of modified BHI medium at a 1% inoculum. The culture was then placed in anaerobic systems with the following mixed gases (anaerobic system A: 95% N<sub>2</sub>, 5% H<sub>2</sub>).

/sub>ER49; anaerobic system B: 80% N<sub>N</sub>ER50, 20% CO<sub>N</sub>ER51; anaerobic system C: 90% N<sub>N</sub>ER52, 5% H<sub>N</sub>ER53, 5% CO<sub>N</sub>ER54) and incubated at 37°C for 24 h. Separately, the activated bacterial solution was streaked onto modified BHI solid medium and incubated upside down at 37°C for 24-48 h, and the growth was recorded. The results of shake-flask and plate cultures are shown in Figures 1 and 2, respectively.

[0050]

As can be seen from Figures 1 and 2, under the conditions of anaerobic system A and anaerobic system B, when the liquid level of the anaerobic bottle culture medium is 1 cm, Akk bacteria basically do not grow. Only when the liquid level is not less than 2 cm can Akk bacteria be cultured in the anaerobic bottle, and the growth rate and concentration of bacteria are positively correlated with the liquid level. When cultured on plates, Akk bacteria can only grow inside the culture medium, but cannot grow on the surface of the culture medium.

Under the culture system of this invention, Akk bacteria are not limited by the liquid level.

They can grow and reproduce well in anaerobic bottles or on plate surfaces, which greatly improves the survival rate of bacteria and reduces the difficulty of bacterial culture, making it suitable for the screening, isolation and culture of bacterial strains.

**[0051]**

Example 4: Effect of CO<sub>2</sub> concentration on Akk bacteria growth

**[0052]**

Single colonies of Akk bacteria were activated overnight and inoculated into 1 mL of modified BHI medium at an inoculum rate of 1%. The culture was carried out in anaerobic systems with different CO<sub>2</sub> concentrations (1-5) as described below, and incubated at 37°C for 30 h. Samples were taken periodically and centrifuged at 6000 r/min for 5 min. The supernatant was discarded, and the precipitated bacterial cells were washed twice with

sterile PBS. Finally, the bacterial cells were resuspended in PBS, and the bacterial concentration ( $OD_{600nm}$ ) was measured at 600 nm using a spectrophotometer. The specific results are shown in Table 2.

[0053]

Table 2. Effects of different CO<sub>2</sub> concentrations on Akk bacteria growth.

[0056]

As shown in Table 2, the growth rate of Akk bacteria increased significantly with the increase of CO<sub>2</sub> concentration, and the turbidity of the bacterial solution also increased accordingly. However, when the CO<sub>2</sub> concentration increased from 5% to 10%, the growth rate of bacteria was relatively fast in the early stage, but slowed down after 24

hours. The bacterial concentration at 30 hours was actually lower than that at 5% CO<sub>2</sub>. Therefore, 5% CO<sub>2</sub> was the most suitable culture condition.

[0057]

Example 5: Effect of NaHCO<sub>3</sub>-NER63 concentration on the growth of Akk bacteria

[0058]

Single colonies of Akk bacteria were activated overnight and inoculated at a rate of 1% into 3 mL of modified BHI medium containing 0.7 mM, 1.4 mM, 3.5 mM, and 7 mM NaHCO<sub>3</sub>-NER64-, respectively. The medium was placed in an anaerobic system of 95% N<sub>2</sub>-NER65- and 5% H<sub>2</sub>-NER66- and incubated at 37°C for 30 h. Samples were taken periodically and centrifuged at 6000 r/min for 5 min. The supernatant was discarded, and the precipitated bacterial cells were washed twice with sterile PBS. Finally, the bacterial cells were resuspended in PBS, and the

bacterial concentration OD- $\text{NER}_{67}$ - was determined by spectrophotometer at a wavelength of 600 nm. The specific results are shown in Table 3.

[0059]

Table 3. Effects of different  $\text{NaHCO}_3$ - $\text{NER}_{68}$  concentrations on the growth of Akk bacteria.

[0061]

1-7 represent a 3mL culture system, with approximately 3mL of liquid remaining on the surface.

[0062]

As shown in Table 3, when 0.7 mM to 7 mM  $\text{NaHCO}_3$  was added to the modified BHI medium, the growth rate of Akk bacteria increased significantly compared to the group without addition (culture system 6), indicating that  $\text{NaHCO}_3$  promoted the growth and reproduction of the strain. Furthermore, as the concentration of  $\text{NaHCO}_3$  increased, the turbidity of the bacterial solution also increased accordingly. However, when the concentration of  $\text{NaHCO}_3$  increased from 3.5 mM to 7 mM, the difference in bacterial growth rate was not significant, and the bacterial concentration even decreased after 30 h. Therefore, 3.5 mM  $\text{NaHCO}_3$  was the most suitable culture condition.

**[0063]**

Example 6 Effect Verification

[0064]

#### 1) Bacterial purity

[0065]

Akkella colonies were activated overnight and inoculated into 1 mL of modified BHI medium at a 1% inoculum. The culture was incubated at 37°C for 24 h under anaerobic conditions of 90% N<sub>2</sub>, 5% H<sub>2</sub>, and 5% CO<sub>2</sub>. The colonies were then streaked onto modified BHI solid medium and incubated upside down for 2–3 days. Colony morphology was observed (Figure 3). Simultaneously, samples were taken for crystal violet staining and microscopic examination of bacterial morphology (Figure 4). Finally, bacterial suspension and single colonies were boiled at 100°C for 10 min, and 1 µL of the supernatant was used as a PCR template. PCR amplification was performed using Akkella myxophilus primers (primer sequences: forward primer: CAGCACGTGAAGGTGGGGAC; reverse primer: CCTTGCGGTTGGCTTCAGAT). The amplified products were subjected to agarose gel

electrophoresis, and the molecular weight of the original template DNA was determined using an automated gel imaging system (Figure 5).

[0066]

As shown in Figures 3-5, the colonies on the plates are small, white and transparent, which is consistent with the colony morphology of *Akkermansia myxophilus*. Moreover, there are no other bacteria growing on the plates. Under microscopic examination, Akk is Gram-negative and short rod-shaped, which is consistent with the microscopic morphology of *Akkermansia myxophilus*. Furthermore, no other obvious bacteria were observed under the microscope, proving that the bacteria in the bacterial solution are pure bacteria, and proving that the bacteria in the shake flask culture medium are pure bacteria.

After PCR amplification using bacterial culture and single colonies as templates, gel electrophoresis revealed a distinct band in the 250–500 bp range, consistent with the size of the 327 bp target gene, which is in line with previous reports.

In conclusion, the bacteria in the bacterial solution are pure *Akkermansia myxophila*.

[0067]

## 2) Growth curve of bacteria

[0068]

A bacterial culture with an OD<sub>600</sub> of 0.6 was inoculated into 200 mL of modified BHI medium at an inoculum size of 2%. The culture was incubated at 37 °C under anaerobic conditions of 90% N<sub>2</sub>, 5% H<sub>2</sub>, and 5% CO<sub>2</sub>. The bacterial concentration OD<sub>600nm</sub> was measured at regular intervals. The growth curve is shown in Figure 6, with 3 replicates per group.

[0069]

The combination of culture medium and culture conditions in this invention allows Akk bacteria to grow and reproduce without being limited by the liquid level. It can grow well in both shake flasks and plate surfaces, reducing the difficulty of Akk bacteria cultivation. At the same time, it reduces the growth rate of Akk bacteria from 3-4 days in the prior art to 24-36 hours, greatly shortening the cultivation time, improving the activity of Akk bacteria, and promoting Akk bacteria growth. This is of great significance for the large-scale cultivation and in-depth research of Akk bacteria.

[0070]

As shown in Figure 6, Akk grows rapidly with no obvious lag phase, meaning it enters the logarithmic growth phase quickly and exhibits high bacterial activity. The time from the logarithmic phase to the stationary phase is 8–28 hours, and the bacterial concentration reaches its maximum after 32 hours of culture, with an OD<sub>600nm</sub> of 2.109. Viable

cell counts yielded a maximum viable cell concentration of  $2.4 \times 10^{12}$  cfu/mL, significantly shortening the culture time and promoting Akk growth. Furthermore, compared to existing technologies, this invention yields a higher viable cell count, indicating that the culture conditions provided by this invention are fully capable of enabling the in vitro expansion of Akk culture.

**[0071]**

sequence list

**[0072]**

<110> Junwei'an (Wuhan) Life Science Technology Co., Ltd.

**[0073]**

<120> An anaerobic culture method for *Akkermansia myxophilus*

**[0074]**

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**[0076]**

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