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DESCRIPTION CN111763653A

A novel electron acceptor-based culture method for *Akkermansia muciniphila*

[0001]

Technical Field

[0002]

This invention belongs to the field of microbial culture technology, specifically relating to a method for culturing *Akkermansia myxophilus* based on a novel electron acceptor.

[0003]

Background Technology

[0004]

Akkermansia muciniphila (hereinafter referred to as Akk bacteria) belongs to the phylum Verrucomicrobia and is a Gram-negative bacterium.

Akk bacteria are widely distributed in the intestinal mucus layer of humans and animals and are one of the most abundant symbiotic bacteria in the gut.

Akk bacteria are a type of bacteria in the human gut that can degrade mucin. They can use mucin as their sole source of carbon, nitrogen, and energy, and produce acetate, ethanol, propionate, and sulfate during fermentation.

Studies have found that Akk bacteria are associated with various systemic diseases such as obesity, diabetes, immune regulation, alcoholic fatty liver, and coronary artery disease. Its content is one of the important biomarkers for measuring the balance of the gut microbiota. As a novel candidate probiotic, the cultivation of Akk bacteria is particularly important.

[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. Chinese patent CN110551658A discloses a method for low-oxygen culture of *Akkmanella myxophilus*, which involves culturing *Akkmanella* in a basal anaerobic medium containing porcine gastric mucin as the sole carbon and nitrogen source, with a culture atmosphere of N_2/CO_2 (80:20, v/v). It is generally believed that CO_2 is used as the electron acceptor in the culture of *Akk* bacteria. However, using CO_2 is more expensive, and *Akk* bacteria will produce a small amount of CO_2 during the culture process, which will cause some fluctuations in the gas environment and affect the normal growth of *Akk* bacteria.

[0006]

Summary of the Invention

[0007]

The purpose of this invention is to address the problems existing in the prior art by adding nitrate or TMAO solution to the culture medium to replace the traditional CO₂ as the electron acceptor of Akk bacteria, thereby obtaining a lower-cost and more controllable method for culturing Akkmanophilus.

[0008]

To achieve the above objectives, the technical solution adopted by the present invention is as follows:

[0009]

A method for culturing *Akkermansia myxophilus* based on a novel electron acceptor involves adding nitrate or TMAO solution to the culture medium and culturing under mixed gas ratios of 80–95% N₂, 5–20% H₂, and 0–3% O₂.

[0010]

To avoid the impact of fluctuations in CO₂ concentration on Akk bacteria growth during cultivation, this invention breaks with conventional thinking in the field by using nitrate or TMAO (trimethylamine oxide) solution instead of the traditionally used CO₂ as the electron acceptor for Akk bacteria. This allows Akk bacteria to grow normally in the absence of CO₂, resulting in lower production costs and greater controllability of liquid solution concentration compared to gaseous electron acceptors.

Meanwhile, nitrate or TMAO solution can enhance Akk bacteria's tolerance to oxygen, allowing Akk bacteria to grow normally in the presence of a certain concentration of oxygen. Furthermore, under conditions with an appropriate concentration of oxygen, the growth of Akk bacteria can be appropriately promoted. Compared to the traditional absolute anaerobic environment, the culture conditions are more relaxed.

[0011]

Preferably, the concentration of nitrate or TMAO in the culture medium is 1–10 mM.

Nitrate or TMAO acts as an electron acceptor in the anaerobic respiration of Akk bacteria. If the concentration is too low, it will affect the normal physiological metabolism of Akk bacteria, while if the concentration is too high, it will affect cell osmotic pressure and enzyme activity, thereby affecting the normal growth of Akk bacteria.

Therefore, the optimal concentration of nitrate or TMAO for Akk bacteria growth is 1–10 mM.

[0012]

Preferably, the concentration of nitrate or TMAO in the culture medium is 3–8 mM.

[0013]

Preferably, the concentration of the nitrate solution in the culture medium is 5 mM, or the concentration of the TMAO solution in the culture medium is 8 mM.

[0014]

Preferably, the nitrate is one or more of sodium nitrate, potassium nitrate, ammonium nitrate, and calcium nitrate.

[0015]

Preferably, the mixture of gases is 90% N₂ and 10% H₂.

In other words, after adding nitrate or TMAO solution to the culture medium, there is no need to introduce CO₂ separately to ensure the normal growth of Akk bacteria.

[0016]

Preferably, the mixture of gases is in the following proportions: 87-90% N₂, 8-12% H₂, and 1-2% O₂.

Nitrate or TMAO solution enhances Akk bacteria's tolerance to oxygen, and at an oxygen concentration of 1-2%, it can also promote Akk bacteria growth to some extent.

[0017]

Preferably, the mixture of gases is composed of 88.5% N₂, 10% H₂, and 1.5% O₂.

[0018]

Preferably, the culture medium is BHI culture medium, and the formula of the BHI culture medium is: Brain-heart Infusion Extract 37g/L, Yeast Extract 5g/L, Mucin 2.5g/L, Resazurin 1mg/L, L-cysteine hydrochloride 100mg/L.

[0019]

Compared with the prior art, the beneficial effects of the present invention are:

[0020]

(1) Nitrate or TMAO solution was used instead of the traditional CO₂ as the electron acceptor of Akk bacteria. The optimal concentration of nitrate or TMAO solution for Akk bacteria growth was creatively selected. This not only avoids the impact of CO₂ concentration fluctuations on Akk bacteria growth, but also reduces costs, increases controllability, and facilitates large-scale culture, providing a new approach to Akk bacteria culture.

[0021]

(2) Nitrate or TMAO solution can also enhance the oxygen tolerance of Akk bacteria, not only avoiding the inhibitory effect of oxygen on the growth of Akk bacteria, but also promoting the growth of Akk bacteria within a certain oxygen concentration range. Compared with the traditional absolute anaerobic environment, the culture conditions are more relaxed, and a culture method for large-scale Akk bacteria culture has been obtained.

[0022]

Detailed Implementation

[0023]

The technical solution of the present invention will be clearly and completely described below with reference to the embodiments thereof. 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.

[0024]

The *Akkermansia muciniphila* strain used in this embodiment of the invention is *Akkermansia muciniphila* ATCCBAA-835, which was purchased from the China Center for Type Culture Collection.

[0025]

In all embodiments of this invention, BHI medium was used to culture Akk bacteria. The preparation method of the BHI medium is as follows:

[0026]

S1. Add 37g of Brain-heart Infusion Extract, 5g of Yeast Extract, and 1mL of 0.1% (W/V) Resazurin to 1L of water. Mix well and stir to dissolve. Boil to remove oxygen for 30min. Then dispense into anaerobic vials, seal, and autoclave at 121°C for 15min.

[0027]

S2. Under anaerobic conditions, dissolve Mucin in anaerobically-free PBS buffer at a

concentration of 1% (W/V), autoclave and let stand overnight, then centrifuge at 12000 rpm for 5 min and take the supernatant to obtain the Mucin solution.

[0028]

S3. Dissolve L-cysteine hydrochloride in ultrapure water to obtain a 10% (W/V) L-cysteine hydrochloride solution, filter and sterilize to obtain a sterile L-cysteine hydrochloride solution.

[0029]

S4. Take 375 mL of the mixture obtained in step S1, then add 125 mL of the Mucin solution from step S2, mix well, and then add 10 mL of the L-cysteine hydrochloride solution from step S3 to obtain BHI culture medium.

[0030]

Example 1: Optimization and Selection of Electron Acceptors

[0031]

Experimental group: Akk bacteria in the logarithmic growth phase were inoculated into the BHI medium at a ratio of 1:1000 and dispensed into 3 mL/tube of anaerobic test tubes. Then, the following electron acceptor solutions with a concentration of 5 mM were added respectively: NaNO₃ (nitrate), TMAO (trimethylamine oxide), NaNO₂ (nitrite), Na₂SO₃ (sulfite), Na₂SO₄ (sulfate), Na₂S₂O₃ (thiosulfate), FeCl₃ (ferric ion), NaClO (hypochlorite), DMSO (dimethyl sulfoxide), and disodium fumarate (fumarate).

[0032]

Blank control group: Akk bacteria in the logarithmic growth phase were inoculated into the BHI medium at a ratio of 1:1000 and dispensed into 3 mL/tube of anaerobic test tubes without electron acceptors and with an equal volume of sterile water.

[0033]

The experimental group and the blank control group were simultaneously cultured under conditions of no CO₂ (mixed gas ratio of 90% N₂, 10% H₂) or with CO₂ (mixed gas ratio of 80% N₂, 10% H₂, 10% CO₂) at a culture temperature of 37°C.

Then, samples were taken every 24 hours, and the OD value (denoted as OD₆₀₀) of each sample at a wavelength of 600 nm was measured using an ELISA reader. At the same time, the total number of bacterial communities (denoted as CFU value) in each milliliter of sample was determined using the plate count method.

The results are shown in Table 1-2.

Table 1 shows the effect of different electron acceptors on the growth of Akk bacteria without CO₂; Table 2 shows the effect of each electron acceptor on the growth of Akk bacteria with CO₂.

[0034]

Table 1. Effects of different electron acceptors on the growth of Akk bacteria (without CO₂)

[0037]

Table 2. Effects of different electron acceptors on the growth of Akk bacteria (with CO₂)

[0039]

Since the initial inoculum of Akk bacteria was the same, the initial Akk bacteria concentration in each sample was the same.

Due to the influence of reagent color and reagent oxidation during the detection process, the OD₅₀₀ value of some samples was higher than expected (such as samples with added FeCl₃ and NaClO₂). In addition, Akk bacteria have different growth rates in the presence of different electron acceptors, and some bacteria died during the measurement process. The spectrophotometer could not accurately distinguish the survival status of the bacteria, resulting in a higher measured OD₅₀₀ value. Therefore, the total bacterial community (i.e., CFU value) is used as the main indicator to determine the growth status of Akk bacteria.

[0040]

Based on the above results, it can be found that in the absence of CO_2 , the blank control group gradually died due to the lack of electron acceptors. Compared with the blank control group, the CFU data of NaNO_2 , FeCl_3 , and NaClO_2 measured at 24h were significantly lower than those of the blank control group, meaning that NaNO_2 , FeCl_3 , and NaClO_2 all significantly inhibited the normal growth of Akk bacteria; Na_NER61 Although SO_2 NER62, NaNER63, SO_2 NER64, SO_2 NER65, DMSO, and disodium fumarate appropriately prolonged the survival time of Akk bacteria, the CFU values showed that these electron acceptors were still insufficient to maintain the normal growth and proliferation of Akk bacteria, meaning that Akk bacteria could not utilize these electron acceptors. In contrast, NaNO_2 NER66 and TMAO enabled Akk bacteria to proliferate normally, indicating that Akk bacteria could utilize NaNO_2 NER67 and TMAO as electron acceptors for growth and proliferation.

In the presence of CO_2 , Akk bacteria in the blank control group can utilize CO_2 as an electron acceptor for normal growth and proliferation. Furthermore,

CO_2 can restore the growth of Akk bacteria supplemented with Na_2SO_4 , $\text{Na}_2\text{S}_2\text{O}_3$, DMSO, and disodium fumarate to normal levels, but it cannot compensate for the inhibitory effects of NaNO_2 , FeCl_3 , and NaClO_2 . Meanwhile, CO_2 does not affect the growth of Akk bacteria in NaNO_3 and TMAO. Therefore, even if some CO_2 is mixed in the culture environment, it will not affect the growth of Akk bacteria, making the culture environment more relaxed.

Meanwhile, compared to using CO_2 as an electron donor, using NaNO_3 or TMAO as an electron donor can promote the proliferation of Akk bacteria to a certain extent.

[0041]

Example 2: Optimization of electron acceptor concentration

[0042]

Using NaNO_3 or TMAO as electron donors, Akk bacteria in the logarithmic growth phase were inoculated into the BHI medium at a ratio of 1:1000 and dispensed into 3 mL anaerobic test tubes. Then, NaNO_3 and TMAO were added to each test tube sequentially at concentration gradients of 0.5 mM, 1 mM, 3 mM, 5 mM, 8 mM, 10 mM, and 12 mM, respectively, as electron acceptors. The culture was carried out under the conditions of 90% N_2 and 10% H_2 mixed gas and a culture temperature of 37°C.

Samples were taken every 24 hours, and the OD value of each sample was measured at a wavelength of 600 nm using an ELISA reader (denoted as OD_{600}). At the same time, the total number of bacterial communities per milliliter of sample was measured using the plate count method (denoted as CFU value).

The results are shown in Table 3-4.

Table 3 shows the effect of NaNO_3 -NER89 concentration on the growth of Akk bacteria; Table 4 shows the effect of TMAO concentration on the growth of Akk bacteria.

[0043]

Table 3 Effects of NaNO₃ concentration on Akk bacteria growth

[0046]

Table 4. Effect of TMAO concentration on Akk bacteria growth

[0048]

The above results show that the concentrations of NaNO₃ and TMAO in the

range of 1 to 10 mM can maintain the normal growth and proliferation of Akk bacteria. When the concentration is below 1 mM, the normal physiological metabolism of Akk bacteria will be affected due to the decrease in electron acceptor content. When the concentration is above 10 mM, it will affect cell osmotic pressure and enzyme activity, thereby affecting the normal growth of Akk bacteria.

Furthermore, concentrations of NaNO_3 and TMAO at 3–8 mM are more conducive to the proliferation of Akk bacteria, and 5 mM of NaNO_3 or 8 mM of TMAO can maximize the growth and proliferation of Akk bacteria.

[0049]

Example 3: Optimization of Mixed Gas Ratio

[0050]

Akk bacteria in the logarithmic growth phase were inoculated into the BHI medium at a ratio of 1:1000 and dispensed into 3 mL anaerobic test tubes. 5 mM NaNO₃-NER94 (nitrate) and 8 mM TMAO were added to each test tube as electron acceptors. The test tubes were then divided into 7 groups and cultured in a mixed gas with the following ratio at 37°C.

[0053]

Samples were taken every 24 hours, and the OD value (denoted as OD₆₀₀) of each sample was measured at a wavelength of 600 nm using a spectrophotometer. At the same time, the total number of bacterial communities per milliliter of sample (denoted as CFU value) was determined using the plate count method.

The results are shown in Table 5-6.

Table 5 shows the effect of different gas mixture ratios on the growth of Akk bacteria when NaNO₃ is used as an electron donor; Table 6 shows the effect of different gas mixture ratios on the growth of Akk bacteria when TMAO is used as an electron donor.

[0054]

Table 5. Effects of different gas mixture ratios on Akk bacteria growth (NaNO₃)

[0056]

Table 6. Effects of different gas mixture ratios on Akk bacteria growth (TMAO)

[0059]

The above test results show that using NaNO₃ and TMAO as electron donors can

enhance the oxygen tolerance of anaerobic bacteria Akk, enabling Akk to grow and proliferate normally at oxygen concentrations of 0-3%. However, when the oxygen concentration is higher than 3%, the inhibitory effect of oxygen on Akk increases, thereby inhibiting the growth of Akk.

Furthermore, oxygen concentrations of 1%–2% significantly promote the growth of Akk bacteria, with the strongest promoting effect observed at an oxygen concentration of 1.5%.

[0060]

Although embodiments of the invention have been shown and described, it will be understood by those skilled in the art that various changes, modifications, substitutions and alterations can be made to these embodiments without departing from the principles and spirit of the invention, the scope of which is defined by the appended claims and their equivalents.