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DESCRIPTION CN111304133A

A culture medium for culturing Akkermansia myxophilus, its usage and application

[0001]

Technical Field

[0002]

This invention belongs to the field of microbial culture medium technology, specifically relating to a culture medium for culturing *Akkermansia myxophilus*, its usage method, and its application.

[0003]

Background Technology

[0004]

Akkermansia muciniphila, or Akk for short, is a Gram-negative, strictly anaerobic coccus.

In 2004, Professor Drian and her team isolated Akk bacteria from the human gut. Akk bacteria are ubiquitous in the human digestive tract, accounting for about 3-5% of the total. They can degrade mucin in the human gut and are negatively correlated with obesity, diabetes, inflammation, and metabolic disorders. They also have the effects of delaying aging, inhibiting neurodegenerative diseases, and reducing lipids and weight.

[0005]

Low levels of Akk bacteria in the gut may lead to a thinning of the mucosal layer, resulting in a

weakened intestinal barrier function and making it easier for toxins in the gut to invade the body.

Therefore, Akk bacteria have become star bacteria in gut microbiota research in recent years.

At the same time, Akk bacteria have some effects in improving various physical ailments, and many scholars regard them as the next generation of probiotics. Preparations with Akk bacteria as the main ingredient are bound to have broad development prospects and market potential.

[0006]

Optimization of Akk bacteria synthesis media is a necessary and crucial step in increasing Akk bacteria yield.

Currently, the cultivation of Akk both domestically and internationally largely relies on a culture medium prepared from bovine brain heart infusion agar powder and porcine gastric mucin. Although the cultivation effect is acceptable, the cost is relatively high.

[0007]

CN109810931A discloses a culture medium for culturing *Akkermansia myxophilus* and its usage method, belonging to the field of microbial culture medium technology.

The components of the culture medium include soybean peptone, threonine, glucose, N-acetylglucosamine, sodium chloride, and disodium hydrogen phosphate.

The culture medium described above can be used to cultivate the amount of bacteria required for functional studies of *Akkermansia myxophilus*. The components of the culture medium are from safe sources and there are no potential biohazards. However, even without adding bovine brain heart extract agar powder and porcine gastric mucin, *Akkermansia mucin* can still be cultured using the above-mentioned culture medium, but the OD₆₀₀ value of Akk bacteria obtained is still low.

[0008]

Therefore, there are few reports on whether adding other reagents is more conducive to the cultivation of Akk bacteria, and whether reducing the amount of bovine brain heart extract powder and porcine gastric mucin can achieve better cultivation results.

Therefore, developing a culture medium that is conducive to the growth of Akk bacteria and has a low cost is an urgent problem to be solved in this field.

[0009]

Summary of the Invention

[0010]

In view of the problems existing in the prior art, the present invention provides a culture medium for culturing *Akkermansia myxophilus*, its method of use and application. The culture medium is composed of multiple components, which reduces the amount of bovine brain heart extract powder and porcine gastric mucin used. By screening other components and scientifically proportioning them, the resulting culture medium can achieve better culture results.

[0011]

To achieve this objective, the present invention adopts the following technical solution:

[0012]

In a first aspect, the present invention provides a culture medium for culturing *Akkermansia myxophilus*, the culture medium comprising brain and heart extract, porcine gastric mucin, L-cysteine hydrochloride, threonine, N-acetyl-D-glucosamine, and vitamins.

[0013]

In addition to brain and heart extract and porcine gastric mucoprotein, the culture medium also includes L-cysteine hydrochloride, threonine, N-acetyl-D-glucosamine, and vitamins.

Cysteine is a reducing agent that can consume oxygen in the culture medium, which is beneficial to the anaerobic growth of *Akkermansia myxophilus*.

Threonine, N-acetyl-D-glucosamine, and vitamins can also increase the carbon and nitrogen content in the culture medium. N-acetyl-D-glucosamine is a basic building block of many important polysaccharides in organisms and can provide a carbon source. Vitamins, as a growth factor, can promote bacterial growth. All of these are nutrients required to maintain the normal growth and reproduction of bacterial strains.

[0014]

As a preferred embodiment of the present invention, the culture medium comprises 25-40 g/L brain and heart extract, 1.5-6 g/L porcine gastric mucoprotein, 0.1-5 g/L L-cysteine hydrochloride, 1-10 g/L threonine, 1-10 g/L N-acetyl-D-glucosamine, and vitamins with a mass fraction of 0.01-0.05%, wherein the mass fraction represents the ratio of vitamins to the total mass of the culture medium.

[0015]

When the concentrations of each component in the culture medium are within the above-mentioned range, it is effective for culturing Akk bacteria. If the concentration is lower than the minimum concentration, it may lead to insufficient nutrients in the culture medium. If the

concentration is higher than the maximum concentration, it may lead to an imbalance between the growth and reproduction of Akk bacteria, resulting in poor growth.

In order for the strain to grow and develop normally, the culture medium must not only have sufficient carbon and nitrogen nutrients, but also pay attention to the carbon-to-nitrogen ratio. This can ensure the nutritional needs of the vegetative growth stage as well as the smooth progress of reproductive growth.

[0016]

In this invention, the mass concentration of the brain and heart extract in the culture medium is 25-40 g/L, for example, it can be 25 g/L, 26 g/L, 28 g/L, 30 g/L, 32 g/L, 35 g/L, 36 g/L, 38 g/L or 40 g/L, etc.

[0017]

The concentration of porcine gastric mucoprotein in the culture medium is 0.5-2 g/L, for example, it can be 0.5 g/L, 0.6 g/L, 0.8 g/L, 1 g/L, 1.2 g/L, 1.4 g/L, 1.5 g/L, 1.6 g/L, 1.8 g/L or 2 g/L, etc.

[0018]

The mass concentration of L-cysteine hydrochloride in the culture medium is 0.1-5 g/L, for example, it can be 0.1 g/L, 0.5 g/L, 1 g/L, 1.2 g/L, 1.5 g/L, 1.8 g/L, 2 g/L, 2.4 g/L, 3 g/L, 3.5 g/L, 4 g/L, 5.5 g/L or 5 g/L, etc.

[0019]

The mass concentration of threonine in the culture medium is 1-10 g/L, for example, it can be 1 g/L, 2 g/L, 3 g/L, 4 g/L, 5 g/L, 6 g/L, 7 g/L, 8 g/L, 9 g/L or 10 g/L, etc.

[0020]

The mass concentration of N-acetyl-D-glucosamine in the culture medium is 1-10 g/L, for example, it can be 1 g/L, 2 g/L, 3 g/L, 4 g/L, 5 g/L, 6 g/L, 7 g/L, 8 g/L, 9 g/L or 10 g/L, etc.

[0021]

The vitamin content in the culture medium is 0.01-0.05% by mass, for example, it can be 0.01%, 0.012%, 0.015%, 0.02%, 0.025%, 0.03%, 0.035%, 0.04%, 0.045%, or 0.05%, etc.

[0022]

Preferably, the vitamin includes vitamin K₁.

[0023]

Preferably, the culture medium uses water as a solvent.

[0024]

As a preferred embodiment of the present invention, the culture medium also includes trace elements.

[0025]

Preferably, the trace elements include any one or a combination of two or more of zinc, boron, copper, iron, molybdenum, nickel, manganese or cobalt.

[0026]

Preferably, the content of trace elements in the culture medium is 0.05-0.15 g/L, for example, it can be 0.06 g/L, 0.07 g/L, 0.08 g/L, 0.09 g/L, 0.1 g/L, 0.12 g/L, 0.14 g/L or 0.15 g/L, etc.

[0027]

By adding the aforementioned trace elements, the survival viability of Akk bacteria can be further enhanced, giving it a greater advantage in growth during culture.

[0028]

As a preferred technical solution of the present invention, the solution containing the trace elements includes any one or a combination of two or more of the following: zinc chloride ($\text{ZnCl}_{2/2}$), boric acid ($\text{H}_{3/3}\text{BO}_{3/3}$), copper chloride dihydrate ($\text{CuCl}_{2/2} \cdot 2\text{H}_{2/2}\text{O}$), ferrous chloride tetrahydrate ($\text{FeCl}_{2/2} \cdot 4\text{H}_{2/2}\text{O}$), sodium molybdate dihydrate ($\text{Na}_{2/2}\text{MoO}_{4/4} \cdot 2\text{H}_{2/2}\text{O}$), nickel chloride hexahydrate ($\text{NiCl}_{2/2} \cdot 6\text{H}_{2/2}\text{O}$), manganese chloride tetrahydrate ($\text{MnCl}_{2/2} \cdot 4\text{H}_{2/2}\text{O}$), or cobalt chloride hexahydrate ($\text{CoCl}_{2/2} \cdot 6\text{H}_{2/2}\text{O}$).

[0029]

Preferably, the solution containing the trace elements is formulated as follows: 0.05-0.1 g/L (e.

g., 0.05 g/L, 0.06 g/L, 0.07 g/L, 0.08 g/L, 0.09 g/L, or 0.1 g/L, etc.) zinc chloride, 0.05-0.1 g/L (e.g., 0.05 g/L, 0.06 g/L, 0.07 g/L, 0.08 g/L, etc.). Boric acid at concentrations of 0.09 g/L, 0.1 g/L, etc.; copper chloride dihydrate at concentrations of 0.01-0.02 g/L (e.g., 0.01 g/L, 0.012 g/L, 0.014 g/L, 0.016 g/L, 0.018 g/L, or 0.02 g/L); and copper chloride dihydrate at concentrations of 1-2 g/L (e.g., 1 g/L, 1.2 g/L, 1.4 g/L, 1.6 g/L). Ferrous chloride tetrahydrate, 0.02-0.03 g/L (e.g., 0.02 g/L, 0.022 g/L, 0.024 g/L, 0.026 g/L, 0.028 g/L, or 0.03 g/L, etc.); Sodium molybdate dihydrate, 0.02-0.03 g/L (e.g., 0.02 g/L, 0.022 g/L, etc.). Nickel chloride hexahydrate (0.024 g/L, 0.026 g/L, 0.028 g/L or 0.03 g/L, etc.), manganese chloride tetrahydrate (0.05-0.15 g/L, for example, 0.05 g/L, 0.07 g/L, 0.08 g/L, 0.1 g/L, 0.12 g/L or 0.15 g/L, etc.), and cobalt chloride hexahydrate (0.1-0.15 g/L).

[0030]

Preferably, the formulation of the solution containing the trace elements is: 0.07 g/L zinc chloride, 0.06 g/L boric acid, 0.015 g/L copper chloride dihydrate, 1.5 g/L ferrous chloride tetrahydrate, 0.025 g/L sodium molybdate dihydrate, 0.025 g/L nickel chloride hexahydrate, 0.1 g/L manganese chloride tetrahydrate, and 0.12 g/L cobalt chloride hexahydrate.

[0031]

As a preferred embodiment of the present invention, the culture medium further includes a surfactant.

[0032]

Preferably, the surfactant includes Tween-80.

Tween-80 is an excellent emulsifier and dispersant that can make the nutrients in the culture medium more uniform.

[0033]

Preferably, the mass fraction of the surfactant (the ratio of surfactant to the total weight of the culture medium) is 0.01-0.02%, for example, it can be 0.01%, 0.012%, 0.014%, 0.015%, 0.016%, 0.017%, 0.018%, 0.019%, or 0.02%, etc.

[0034]

As a preferred embodiment of the present invention, the content of each component in the culture medium is as follows:

[0035]

25-40 g/L brain and heart extract, 1.5-5 g/L porcine gastric mucoprotein, 0.5-2 g/L L-cysteine hydrochloride, 3-10 g/L threonine, 3-10 g/L N-acetyl-D-glucosamine, 0.01-0.02% vitamin K (NER19), 0.05-0.15 g/L trace elements, and 0.01-0.02% Tween-80.

[0036]

Preferably, the content of each component in the culture medium is as follows: 27-30 g/L brain and heart extract, 2-2.5 g/L porcine gastric mucoprotein, 1.4-1.6 g/L L-cysteine hydrochloride, 4-6 g/L threonine, 0.014-0.016% vitamin K (NER20), 4-6 g/L N-acetyl-D-glucosamine, 0.08-0.12 g/L trace elements, and 0.014-0.016% Tween-80.

[0037]

For example, the content of each component in the culture medium may be 29.6 g/L brain

heart extract, 1.152 g/L porcine gastric mucoprotein, 1.5 g/L L-cysteine hydrochloride, 5 g/L threonine, 0.015% vitamin K (NER21), 5 g/L N-acetyl-D-glucosamine, 0.1 g/L trace elements, and 0.015% Tween-80.

[0038]

Secondly, the present invention provides a method of using the culture medium as described in the first aspect, the method comprising the following steps:

[0039]

The bacterial culture of *Akkermansia myxophilus* was inoculated into the culture medium as described in the first aspect and subjected to anaerobic culture.

[0040]

In this invention, the *Ackermania pseudomallei* strain is extracted and isolated from feces, and the specific steps are as follows:

[0041]

(1) Sample preparation: Take a 250mL Erlenmeyer flask, put in 150mL of physiological saline solution, seal the flask, and put it together with the culture medium described in this invention into an autoclave. Sterilize at 121°C for 20 minutes, take it out and let it cool.

Add 25g of normal healthy human feces, shake well, filter to remove insoluble matter, perform serial dilution, select seven gradients from 10^{-3} to 10^{-9} , centrifuge at 3000r/min, wait for the culture medium to cool to 55°C, pour 15-20mL of culture medium into the plate, and then use a pipette to take 0.2mL and inject it into the plate

containing the culture medium, spread it evenly, and after solidification, invert it into a sealed container containing an anaerobic gas-generating bag at 37°C for incubation.

[0042]

(2) Isolation and culture: After culturing at 37°C for 2 weeks, colony morphology was observed and Gram staining was performed. Colonies with no impurities around them, a diameter of 1 mm, and milky white color were selected from the solid culture medium plate. Then, 0.2 mL of bacterial solution was taken and plated again. The screening continued until only one type of bacteria was found on the plate. The obtained strain is *Akkermansia myxophilus*.

[0043]

As a preferred embodiment of the present invention, the concentration of the bacterial solution is $(0.8-1.2) \times 10^4$ cfu/mL, for example, it can be 0.8×10^4 cfu/mL, 0.85×10^4 cfu/mL, 0.9

$\times 10$ cfu/mL, 0.95×10 cfu/mL, 1×10 cfu/mL, 1.05×10 cfu/mL, 1.1×10 cfu/mL, or 1.2×10 cfu/mL, etc.

[0044]

Preferably, the inoculation volume of the bacterial solution is 100-200 μ L, for example, it can be 110 μ L, 120 μ L, 130 μ L, 140 μ L, 150 μ L, 160 μ L, 170 μ L, 180 μ L, 190 μ L or 200 μ L.

[0045]

Preferably, the anaerobic culture temperature is 35-40°C, for example, it can be 35°C, 36°C, 37°C, 38°C, 39°C or 40°C, etc.

[0046]

Preferably, the anaerobic culture time is 72-96 hours, for example, it can be 72 hours, 75 hours, 78 hours, 80 hours, 82 hours, 85 hours, 88 hours, 90 hours, 92 hours, 94 hours or 96 hours.

[0047]

As a preferred technical solution of the present invention, the method of use includes the following steps: inoculating the culture medium described in the first aspect with a bacterial suspension of *Akkermansia myxophilus*, wherein the concentration of the bacterial suspension is $(0.8-1.2) \times 10^{8\text{cfu/mL}}$ and the inoculation amount is 100-200 μL , and then anaerobic culture is carried out at 35-40°C for 72-96h.

[0048]

Thirdly, the present invention also provides the use of *Akkermansia myxophilus* cultured using the culture medium described in the first aspect in the preparation of a medicament for treating metabolic diseases.

[0049]

The numerical range described in this invention includes not only the point values listed above, but also any point values within the numerical ranges not listed above. Due to space limitations and for the sake of brevity, this invention will not exhaustively list all the specific point values included in the range.

[0050]

Compared with the prior art, the present invention has at least the following beneficial effects:

[0051]

(1) The culture medium provided by the present invention includes brain heart extract, porcine gastric mucin, L-cysteine hydrochloride, vitamin K₁, threonine and N-acetyl-D-glucosamine. The culture medium can provide the necessary nutrients for the growth and reproduction of Akk bacteria. At the same time, by further adding trace elements and Tween-80 reagent, a higher number of viable bacteria can be obtained than when cultured in mucin culture medium. This shows that the culture medium provided by the present invention can fully realize the in vitro amplification culture of Akk.

[0052]

(2) After Akk bacteria are cultured in the culture medium provided by the present invention using a suitable culture method, the OD value of the obtained strain is 0.8211-0.8741.

Furthermore, the present invention has confirmed that the strain in the culture medium is indeed Akk bacteria through PCR technology. Therefore, the culture medium provided by the present invention is beneficial for the culture of Akk bacteria, which facilitates the subsequent preparation of Akk bacteria into formulations and enables subsequent functional research and industrialization.

[0053]

Attached Figure Description

[0054]

Figure 1 is a comparison of the DNA sequences of Akk bacteria cultured in Example 1 and the standard Akk bacteria in the database.

[0055]

Detailed Implementation

[0056]

The technical solution of the present invention will be further described below with reference to the accompanying drawings and specific embodiments. However, the following examples are merely simple examples of the present invention and do not represent or limit the scope of protection of the present invention. The scope of protection of the present invention shall be determined by the claims.

[0057]

In the following examples, all bacterial strains and experimental reagents were purchased from conventional manufacturers, as detailed in Table 1 below:

[0058]

Table 1

[0059]

Experimental Strains or Reagents Company Physiological Saline Guangzhou Ruishu Bio-Reagent Co., Ltd. Brain and Heart Infusion Solarbio Porcine Gastric Mucin SIGMA Threonine Shanghai Sangon Biotech Co., Ltd. N-Acetyl-D-Glucosamine Shanghai Sangon Biotech Co., Ltd. Ethanol Shanghai Guoyao Group Tween-80 Shanghai Sangon Biotech Co., Ltd. Fast PFU DNA Polymerase Kit Beijing TransGen Biotech Co., Ltd. Primers Shanghai Sangon Biotech Co., Ltd. ZnCl_2 Tianjin Zhiyuan Chemical Reagent Co., Ltd. H_3BO_3 Tianjin Zhiyuan Chemical Reagent Co., Ltd. $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ Tianjin Zhiyuan Chemical Reagent Co., Ltd. $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ Tianjin Zhiyuan Chemical Reagent Co., Ltd. $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ Tianjin Zhiyuan Chemical Reagent Co., Ltd. $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ Shanghai Guoyao Group $\text{MnCl}_4 \cdot 4\text{H}_2\text{O}$ (Tianjin Zhiyuan Chemical Reagent Co., Ltd.) $\text{CoCl}_4 \cdot 6\text{H}_2\text{O}$ (Tianjin Zhiyuan Chemical Reagent Co., Ltd.)

[0060]

In the following embodiments, the formulation of the trace elements is shown in Table 2 below:

[0061]

Table 2

[0062]

Chemical Composition and Content (g/L): ZnCl 0.07, HO 0.06, CuCl • 2HO 0.015, FeCl • 4HO
1.5, NaO • MoO 0.025, NiCl • 6HO 0.025, MnCl • 4HO 0.1, CoCl • 6HO 0.12

[0063]

In the following embodiments, the sources of the experimental instruments are shown in Table 3 below:

[0064]

Table 3

[0065]

Laboratory Instruments Company: Magnetic Stirrer (Hangzhou Mio Instruments Co., Ltd.), Electronic Balance (Shanghai Shunyu Hengping Scientific Instruments Co., Ltd.), High-Speed Centrifuge (ThermoFisher), Vertical Autoclave (Shanghai Shenan Medical Instrument Factory), Microplate Reader (Zhengzhou Antu Bioengineering Co., Ltd.)

[0066]

Example 1

[0067]

This embodiment provides a culture medium, the culture medium comprising:

[0068]

7.4g brain and heart extract, 0.8g porcine gastric mucoprotein, 0.3g L-cysteine hydrochloride,

1.0g threonine, 0.015% vitamin K (NER67), and 1.0g N-acetyl-D-glucosamine were added to a final volume of 200mL with water. The mixture was stirred for 30 minutes and then transferred to an Erlenmeyer flask for sterilization.

[0069]

Example 2

[0070]

This embodiment provides a culture medium, the culture medium comprising:

[0071]

7.4g brain and heart extract, 0.8g porcine gastric mucoprotein, 0.3g L-cysteine hydrochloride, 1.0g threonine, 0.015% vitamin K (NER68), 1.0g N-acetyl-D-glucosamine, and 0.1mL trace elements were added to a final volume of 200mL with water. The mixture was stirred for 30 minutes to ensure homogeneity, then transferred to an Erlenmeyer flask and sterilized.

[0072]

Example 3

[0073]

This embodiment provides a culture medium, the culture medium comprising:

[0074]

7.4g brain and heart extract, 0.8g porcine gastric mucoprotein, 0.3g L-cysteine hydrochloride, 1.0g threonine, 0.015% vitamin K (NER69), 1.0g N-acetyl-D-glucosamine, 0.1mL trace elements, and 0.015% Tween-80 were added to a final volume of 200mL with water. The mixture was stirred for 30 minutes to ensure homogeneity, then transferred to an Erlenmeyer flask and sterilized.

[0075]

Example 4

[0076]

This embodiment provides a culture medium, which differs from Example 3 only in that the mass of porcine gastric mucoprotein is 0.48g.

[0077]

Example 5

[0078]

This embodiment provides a culture medium, which differs from Example 3 only in that the mass of porcine gastric mucoprotein is 0.4g.

[0079]

Example 6

[0080]

This embodiment provides a culture medium, the culture medium comprising:

[0081]

7.4g brain and heart extract, 0.4g porcine gastric mucoprotein, 0.45g L-cysteine

hydrochloride, 1.5g threonine, 0.02% vitamin K (NER70), 1.5g N-acetyl-D-glucosamine, 0.15mL trace elements, and 0.02% Tween-80 were added to a final volume of 200mL with water. The mixture was stirred for 30 minutes to ensure homogeneity, then transferred to an Erlenmeyer flask and sterilized.

[0082]

Example 7

[0083]

This embodiment provides a culture medium that differs from that in Example 3 in that the mass of the brain and heart extract is 5.92g and the mass of porcine gastric mucoprotein is 0.48g.

[0084]

Example 8

[0085]

This embodiment provides a culture medium that differs from that in Example 3 in that the mass of the brain and heart extract is 5.5g and the mass of porcine gastric mucoprotein is 0.48 g.

[0086]

Example 9

[0087]

The difference from Example 3 is that the mass of L-cysteine hydrochloride in the culture medium is 1g.

[0088]

Example 10

[0089]

The difference from Example 3 is that the mass of L-cysteine hydrochloride in the culture medium is 0.1 g.

[0090]

Example 11

[0091]

The difference from Example 3 is that the mass of threonine in the culture medium is 2g.

[0092]

Example 12

[0093]

The difference from Example 3 is that the mass of threonine in the culture medium is 0.5g.

[0094]

Example 13

[0095]

The difference from Example 3 is that the mass fraction of vitamin K₁ in the culture medium is 0.05%.

[0096]

Example 14

[0097]

The difference from Example 3 is that the mass of N-acetyl-D-glucosamine in the culture medium is 2g.

[0098]

Comparative Example 1

[0099]

This comparative example provides a culture medium, the culture medium comprising:

[0100]

7.4g brain and heart extract and 0.8g porcine gastric mucoprotein were added to a final

volume of 200mL with water. After stirring for 30 minutes to mix thoroughly, the mixture was transferred to an Erlenmeyer flask and sterilized.

[0101]

Comparative Example 2

[0102]

This comparative example provides a culture medium, the culture medium comprising:

[0103]

7.4g brain and heart extract, 0.8g porcine gastric mucoprotein, and 0.3g L-cysteine hydrochloride were added to a final volume of 200mL with water. After stirring for 30 minutes to mix thoroughly, the mixture was transferred to an Erlenmeyer flask and sterilized.

[0104]

Comparative Example 3

[0105]

This comparative example provides a culture medium, the culture medium comprising:

[0106]

7.4g brain and heart extract, 0.8g porcine gastric mucoprotein, 0.3g L-cysteine hydrochloride, and 1.0g threonine were added to a final volume of 200mL with water. The mixture was stirred for 30 minutes to mix thoroughly, then transferred to an Erlenmeyer flask and sterilized.

[0107]

Comparative Example 4

[0108]

This comparative example provides a culture medium, the culture medium comprising:

[0109]

7.4g brain and heart extract, 0.8g porcine gastric mucoprotein, 0.3g L-cysteine hydrochloride, 1.0g threonine, and 0.2mL vitamin K₁ were added to a final volume of 200mL with water. After stirring for 30 minutes to mix thoroughly, the mixture was transferred to an Erlenmeyer flask and sterilized.

[0110]

Comparative Example 5

[0111]

The difference from Example 3 is that L-cysteine hydrochloride is replaced with lysine hydrochloride in the culture medium.

[0112]

Comparative Example 6

[0113]

The difference from Example 3 is that N-acetyl-D-glucosamine is replaced with glucose in the culture medium.

[0114]

Comparative Example 7

[0115]

The difference from Example 3 is that the trace elements in the culture medium are replaced with a single element, iron.

[0116]

Performance testing

[0117]

Akkermansia myxophilus was cultured using the culture media provided in Examples 1-16 and Comparative Examples 1-7.

[0118]

1.

OD value detection

[0119]

After sterilizing and cooling the culture medium, *Akkermansia myxophilus* suspension (1×10^8 cfu/mL, 200 μ L) was inoculated into the culture medium. After inoculation, the medium was placed in a closed container with two anaerobic gas-generating bags. The medium was cultured at 37°C for 4 days. After 4 days, the medium was removed, centrifuged at 3000rpm for 10min, and the precipitate was resuspended in 2mL of sterile PBS. The precipitate was then added to 96-well plates at 200 μ L per well. PBS served as a blank control.

[0120]

The OD value of the bacterial culture was measured at 630 nm using an ELISA reader. The measurement was repeated three times, and the average value was taken. The results are shown in Table 4.

[0121]

Table 4

[0124]

As shown in the table above, the Akk bacteria culture medium provided by this invention can all enable the strains to grow normally, and their OD values are all greater than 0.82.

Furthermore, as shown in Examples 3, 4, and 5, when the content of porcine gastric mucin is changed alone, the culture effect is best when the mass of porcine gastric mucin is 0.48g. As shown in Examples 4, 7, and 8, when the content of brain and heart extract is changed alone, the culture effect is best when the content of brain and heart extract is 5.92g.

[0125]

As shown in the table above, the optimal culture medium prepared in this invention is: 5.92g brain heart extract, 0.48g porcine gastric mucoprotein, 0.3g L-cysteine hydrochloride, 1.0g threonine, 0.2mL vitamin K₁, 1.0g N-acetyl-D-glucosamine, 0.1mL trace elements, 0.1mL Tween-80, and water to a final volume of 200mL.

[0126]

2.

Purification of mucin

[0127]

Mucin contains many impurities. If the culture medium is not purified, it will be cloudy and opaque. However, purification can remove the impurities, making the culture medium clear and not cloudy.

[0128]

Dissolve mucin in water and stir at 30-35°C for 5 hours to obtain a mixture. Centrifuge the mixture at 3000 rpm for 10 minutes, collect the supernatant, add ethanol, let stand for 4 hours, remove the supernatant, dissolve the precipitate in water, wash and purify twice with ethanol, and then dissolve the precipitate in water to obtain the purified mucin.

[0129]

Identification of bacterial strains in the culture medium

[0130]

DNA was extracted from the cultured AKK bacteria, and the DNA concentration was detected using a nanodrop 2000. 5 µL of DNA was then used to amplify the AKK bacterial DNA sequence using the following primers:

[0131]

Upstream primer (SEQ ID NO.1): CAAGTCGAACGAGAGAATTGCTAGC

[0132]

Downstream primer (SEQ ID NO.2): CATCCCAGTTACCAGTCTCACCTT.

[0133]

The PCR reaction system is shown in Table 5 below:

[0134]

Table 5

[0135]

Reagents Content (μL) Fast PFU DNA Polymerase 1 5X Fast PFU Buffer 10 dNTPs (2.5mM) 2
AKK Upstream Primer (10μM) 2 AKK Downstream Primer (10μM) 2 AKK Bacterial DNA (34ng
/μL) 5 3dH₂O 28 Total Volume 50

[0136]

The PCR reaction conditions are shown in Table 6 below:

[0137]

Table 6

[0140]

5 μ L of total PCR product was added to 1 μ L of DNA loading buffer, and electrophoresis was performed on a 1% agarose gel. Images were collected using a Chemi-Doc XRS+ imager. The remaining PCR product was sent to Shanghai Sangon Biotech Co., Ltd. for sequencing using upstream primers. The obtained sequence is SEQ ID NO.3. The results were compared with the BLAST program of NCBI. The sequencing results showed that the data similarity with the Akk bacteria data in NCBI was 100%, as shown in Figure 1, proving that the cultured strains were all Akk bacteria.

[0141]

In summary, the culture medium provided by this invention can provide the necessary nutrients for the growth and reproduction of Akk bacteria, and can obtain a higher number of

viable bacteria than when cultured in mucin medium. It fully realizes the in vitro expansion culture of Akk bacteria, and has good culture effect and low culture cost.

[0142]

The applicant declares that the present invention is illustrated by the above embodiments to illustrate the detailed structural features of the present invention, but the present invention is not limited to the above detailed structural features, that is, it does not mean that the present invention must rely on the above detailed structural features to be implemented.

Those skilled in the art should understand that any improvements to the present invention, equivalent substitutions for the components used in the present invention, additions of auxiliary components, and selection of specific methods all fall within the protection and disclosure scope of the present invention.

[0143]

SEQUENCE LISTING

[0144]

<110> Suzhou Purison Gene Technology Co., Ltd.

[0145]

<120> A culture medium for culturing *Akkermansia myxophilus*, its usage and application.

[0146]

<130> 20200313

[0147]

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[0148]

<170> PatentIn version 3.3

[0149]

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[0151]

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[0152]

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[0165]

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[0173]

tcactgggcg taaagcgtgc gtaggctggt tcgtaagtcg tgtgtgaaag gcgcgggctc 480